 DOI: 10.1002/eji.201142298 Eur. J. Immunol. 2012. 42: 2176–2186

European Journal of Immunology

Siglec-7 tetramers characterize B-cell subpopulations and leukemic blasts

Friederike Gieseke[*1], *Philippa Mang*[*2], *Susanne Viebahn*[2], *Inga Sonntag*[2], *Anne Kruchen*[1], *Annika Erbacher*[2], *Matthias Pfeiffer*[2], *Rupert Handgretinger*[2] *and Ingo Müller*[3]

[1] Research Institute Children's Cancer Center, Hamburg, Germany
[2] University Children's Hospital, Department of General Pediatrics, Hematology and Oncology, Tübingen, Germany
[3] Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Cell surface glycosylation has important regulatory functions in the maturation, activation, and homeostasis of lymphocytes. The family of human sialic acid-binding immunoglobulin-like lectins (siglecs) comprises inhibitory as well as activating receptors intimately involved in the regulation of immune responses. Analyses of the interaction between siglecs and glycans are hampered by the low affinity of this interaction. Therefore, we expressed siglec-7 in eukaryotic cells, allowing for glycosylation, and oligomerized the protein in analogy to MHC tetramers. Using this tool, flow cytometric analysis of lymphocytes became possible. Sialic acid-dependent binding of siglec-7 tetramers was confirmed by glycan array analysis and loss of siglec tetramer binding after neuraminidase treatment of lymphocytes. In contrast to most lymphocyte subpopulations, which showed high siglec-7 ligand expression, B-cell subpopulations could be further subdivided according to different siglec-7 ligand expression levels. We also analyzed blasts from acute lymphoblastic leukemias of the B-cell lineage as well as the T-cell lineage, since malignant transformation is often associated with aberrant cell surface glycosylation. While pediatric T-ALL blasts highly expressed siglec-7 ligands, siglec-7 ligands were barely detectable on cALL blasts. Taken together, oligomerization of recombinant soluble siglec-7 enabled flow cytometric identification of physiologic lymphocyte subpopulations and malignant blasts.

Keywords: B lymphocytes · Cell surface glycosylation · Sialic acid · Siglec (sialic acid-binding immunoglobulin-like lectins)

Supporting Information available online

Introduction

The cell surface is not only comprised of proteins and lipids, but is also further diversified by glycosylation of these macromolecules. In the immune system, cell surface glycosylation, and in particular sialylation, characterizes different lymphocyte lineages and differentiation stages as well as activation-dependent changes [1]. Sialic acids are most often located at the reducing ends on N- and O-linked glycans or glycosphingolipids. About 20 different sialyltransferases conjugate sialic acids in the α2,3-, α2,6-, or α2,8 position to the subterminal carbohydrate. These sialic acids

Correspondence: Dr. Friederike Gieseke
e-mail: gieseke@kinderkrebs-forschung.de

*These authors contributed equally to this work.

are ligands for sialic acid-binding immunoglobulin-like receptors (siglecs), which exhibit a certain selectivity depending on the glycosidic linkage of the sialic acid as well as the underlying glycan structure [2]. Siglecs often interact with their sialic acid binding partners on the same cell, masking their binding site [3, 4]. The human siglec family comprises 14 members, most of them expressed on human leukocytes. Siglecs are expressed in distinct patterns on the cells of the immune system. The majority of CD33-related siglecs feature intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and ITIM-like motifs. Siglec engagement by their ligands mostly results in inhibitory signals [5]. It has been shown that engagement of CD33-related receptors inhibits cellular proliferation [6, 7], raises the cellular activation threshold [8], and induces apoptosis [9, 10] as well as induces the release of anti-inflammatory cytokines [11]. The importance of siglecs in setting the threshold for effector cell activation has been demonstrated for both B cells and T cells [8, 12]. Additionally, CD33-related siglecs can act as endocytosis receptors, which could play an important role in the clearance of sialylated antigens and may be involved in antigen presentation [2].

One of the most widely studied siglecs is siglec-7, which was described as a NK cell inhibitory receptor, but was also found to be expressed on a subset of $CD8^+$ T cells, on monocytes, and also on granulocytes [13, 14]. Siglec-7 binds to α2,8-linked sialic acid found in Neu5Acα2,8Neu5Ac-contaning glycans [15], but also to α2,6- and α2,3-linked sialic acids. Expression analysis of sialic acids is commonly done using plant lectins, because of their high affinity. However, it is known that there are considerable differences in the selectivity of siglecs and plant lectins. Approaches using recombinant soluble siglecs themselves for detection of ligands by flow cytometry or immune histochemistry are hampered by the low affinity interaction between glycan and protein in this case. Therefore, we oligomerized the extracellular domains of siglecs in analogy to MHC tetramers [16]. Here, we report for the first time the flow cytometric detection of siglec-7 ligands on human peripheral blood mononuclear cells (PBMCs) by using oligomerized siglec-7 as a prototype for the CD33-related siglecs. This tool facilitated the identification of a B-cell subpopulation expressing ligands for siglec-7.

Results

Oligomerized siglec-7 proteins as new tool to analyze cell surface glycosylation

Siglecs are important regulators of immune responses. However, the presence of their cognate ligands is less clear. We focused on the expression of ligands for siglec-7, which is expressed on a subset of T cells and NK cells. The N-terminal V-set domain and the two C2-set domains of siglec-7 were cloned using mRNA of dendritic cells (Fig. 1A). Plasmid sequencing of the amplicon and flanking regions verified correct insertion and sequence identity. The recombinant siglec-7 fusion protein was expressed in eukaryotic 293T cells to ensure mammalian glycosylation. After purification by affinity chromatography, the specific siglec-7 protein band was identified by immunoblot with anti-siglec-7 antibody (Fig. 1B). The calculated, unglycosylated molecular weight of siglec-7 is 40 kDa, but due to glycosylation, the band in the immunoblot analysis migrated at 60 kDa. After treatment with PNGase F, the deglycosylated siglec-7 protein was detected between 35 and 45 kDa. The purified siglec-7 protein was enzymatically biotinylated and subsequently detected by immunoblot using streptavidin-(R)-phycoerythrine (SA-PE). In order to analyze siglec-7 ligand expression, freshly isolated PBMCs were stained with biotinylated monomeric siglec-7 protein followed by incubation with SA-PE and flow cytometric analysis, but no binding of siglec-7 was detectable (Fig. 1C). This could be ascribed to the low affinity of monovalent siglecs for their ligands. In analogy to MHC tetramers, the siglec-7 protein was tetramerized with SA-PE ($(Siglec\text{-}7)_4$:SA-PE) to enhance the avidity (Fig. 1A). The oligomerized siglec-7 protein showed a stable and reproducible binding signal in flow cytometry (Fig. 1D). The specificity of the oligomerized fusion protein was demonstrated by treatment of the target cells with neuraminidase, which abolished binding completely due to cleavage of α2,3-, α2,6-, and α2,8-linked sialic acids on the cell surface (Fig. 1E).

Characterization of siglec-7 ligands by glycan array analysis

First binding studies on lymphocytes showed a sialic acid-dependent binding of $(Siglec\text{-}7)_4$:SA-PE (Fig. 1). In order to characterize the binding specificity of $(Siglec\text{-}7)_4$:SA-PE in more detail, we submitted $(Siglec\text{-}7)_4$:SA-PE to the Consortium for Functional Glycomics (CFG) for glycan array analysis. The analysis was performed using the protocol for a direct binding assay as the $(Siglec\text{-}7)_4$:SA-PE is a fluorescently-labeled sample. For comparison also, a two-step protocol was performed, in which monomeric siglec-7 was used, incubated on the slide followed by a second incubation step with SA-PE. Analogous to the flow cytometric analysis with the two-step incubation using monomeric siglec-7 and SA-PE (Fig. 1C), only extremely weak fluorescent signals were detectable, which indicated that monomeric siglec-7 did not bind specifically, or rather the affinity of the monomeric siglec-7 was much too low. However, the $(Siglec\text{-}7)_4$:SA-PE showed high fluorescent signals preferring short sialic acid-containing glycans. The three glycan structures with the highest fluorescent signals were the monovalent sialic acid (Neu5Acα), Neu5Acα2-8Neu5Acα, and Neu5Acα2-3Galβ1-4[6OSO3]GlcNAcβ, which is the main branch of the 6Gal-S-Sia-Lewis^x. Overall, the highest signals arose from glycans that have a motif containing sialic acid and sulfate (Fig. 2 and Supporting Information Table 1).

$(Siglec\text{-}7)_4$: SA-PE analysis of lymphocyte subpopulations

In order to analyze binding specificities of the tetramerized siglec-7 to different lymphocyte subpopulations, PBMCs were

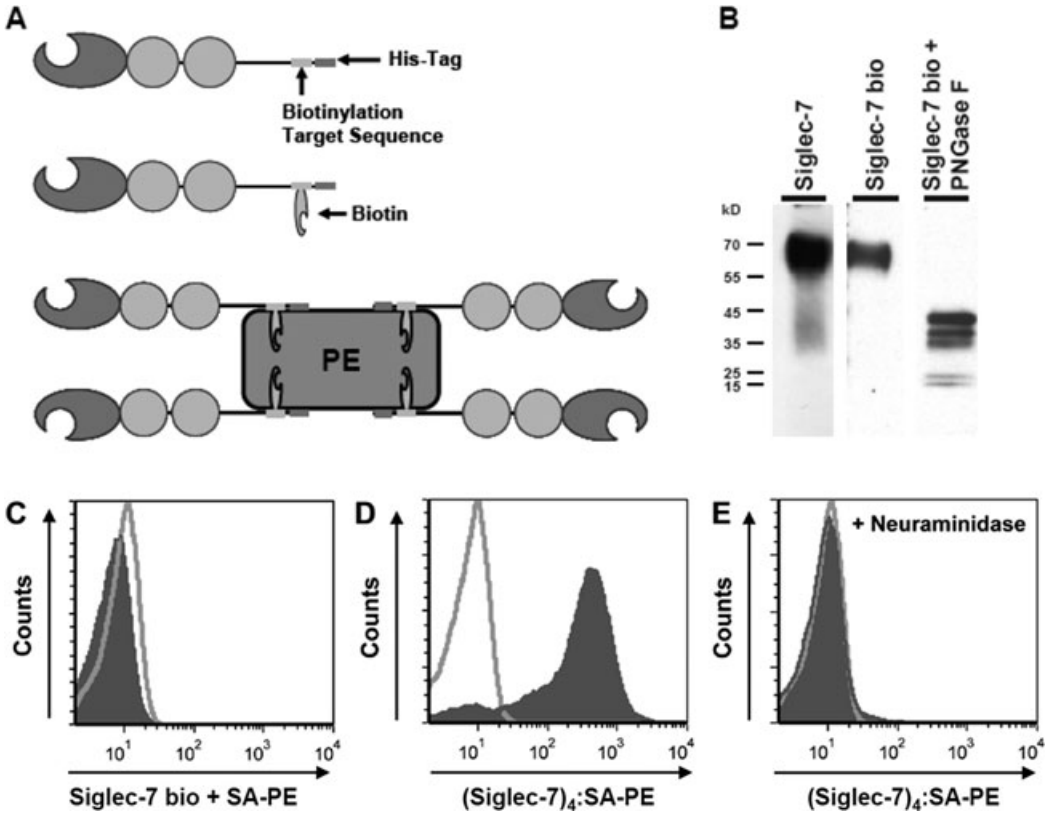

Figure 1. Generation of the siglec-7 fusion protein (Siglec-7)$_4$:SA-PE. (A) Extracellular Ig-like domains of human siglec-7 with an additional biotinylation sequence and a six-histidine-tag were expressed in 293T cells and isolated from the supernatant by affinity chromatography followed by enzymatic biotinylation with BirA ligase. To increase avidity, tetramers were formed by incubation with SA-PE-conjugates. (B) Immunoblot analysis. Siglec-7 fusion protein (left lane) and the biotinylated siglec-7 fusion protein (siglec-7 bio, middle lane) were detected by biotinylated anti-siglec-7 antibody or SA-HRP, respectively. After treatment with PNGase F, the deglycosylated forms of siglec-7 were detected (right lane). (C) Flow cytometric analysis. Monomeric siglec-7 has low affinity for its ligands. PBMCs were stained with monomeric biotinylated siglec-7 followed by detection with SA-PE (gray line histogram: SA-PE only for control). (D) Oligomerization of siglec-7 increases avidity significantly. PBMCs were stained with (Siglec-7)$_4$:SA-PE. (E) PBMCs were stained with (Siglec-7)$_4$:SA-PE after neuraminidase treatment, which reduced binding of the oligomerized siglec-7 strongly, indicating the specificity of (Siglec-7)$_4$:SA-PE for sialic acids. (Data show representatives of at least three independent experiments.)

freshly isolated and stained with anti-CD4, anti-CD8, anti-CD14, anti-CD56, and anti-CD19 antibodies respectively (Fig. 3). CD4$^+$ T cells, CD8$^+$ T cells, and CD56$^+$ NK cells stained positive to similar extent for siglec-7 ligands, which were nearly completely eliminated after neuraminidase treatment of the cells. Monocytes showed also a high affinity for (Siglec-7)$_4$:SA-PE, though the binding was only partially reduced after neuraminidase treatment (Fig. 3B). Interestingly, the staining with (Siglec-7)$_4$:SA-PE showed that the CD19$^+$ B-cell population did not express siglec-7 ligands homogeneously. B cells could be subdivided into a siglec-7 ligand positive and a negative subpopulation (Fig. 3A).

Siglec-7 ligand expression characterizes B-cell subpopulations

We asked if the siglec-7 ligand positive and negative B-cell subpopulations reflect defined B-cell subpopulations. Therefore, freshly isolated PBMCs were stained with an antibody cocktail for determination of the B-cell subpopulations including anti-CD19, anti-IgD, anti-CD38, anti-CD27, anti-IgM, and anti-CD23 according to previous classification of peripheral B cells [17, 18]; for a detailed antibody panel, see the Supporting Information. CD19$^+$ lymphocytes were determined as total B-cell population. To further analyze the B-cell subpopulations, we applied different B-cell classifications. The first approach was based on the IgD/CD38 expression [17, 19] and a subsequent analysis of CD23 and CD27 [18]. According to the mature B cell (Bm) classification, we defined the following B-cell subsets: IgD$^+$CD38$^-$CD23$^-$CD27$^-$ (Bm1, naïve), IgD$^+$CD38$^+$CD23$^+$CD27$^-$ (Bm2, activated naïve), IgD$^+$CD38^{++}CD27$^-$ (Bm2′, germinal center founder), IgD$^-$CD38$^+$CD27$^+$ (eBm5, early memory), IgD$^-$CD38$^-$CD27$^+$ (Bm5, memory), and IgD$^-$CD38^{++} (Bm3+4 + PB, plasma blasts) [17, 18]. Representative density and histogram plots show the gating strategy for the B-cell subsets and the siglec-7 ligand expression from one healthy donor accordingly (Fig. 4A and B). All together, we analyzed B-cell subsets from 16 different healthy donors, seven males and nine females, who were between 22 and 51 years of age (average age 35 years). Figure 4C summarizes the analyses of all donors showing the percentages of siglec-7

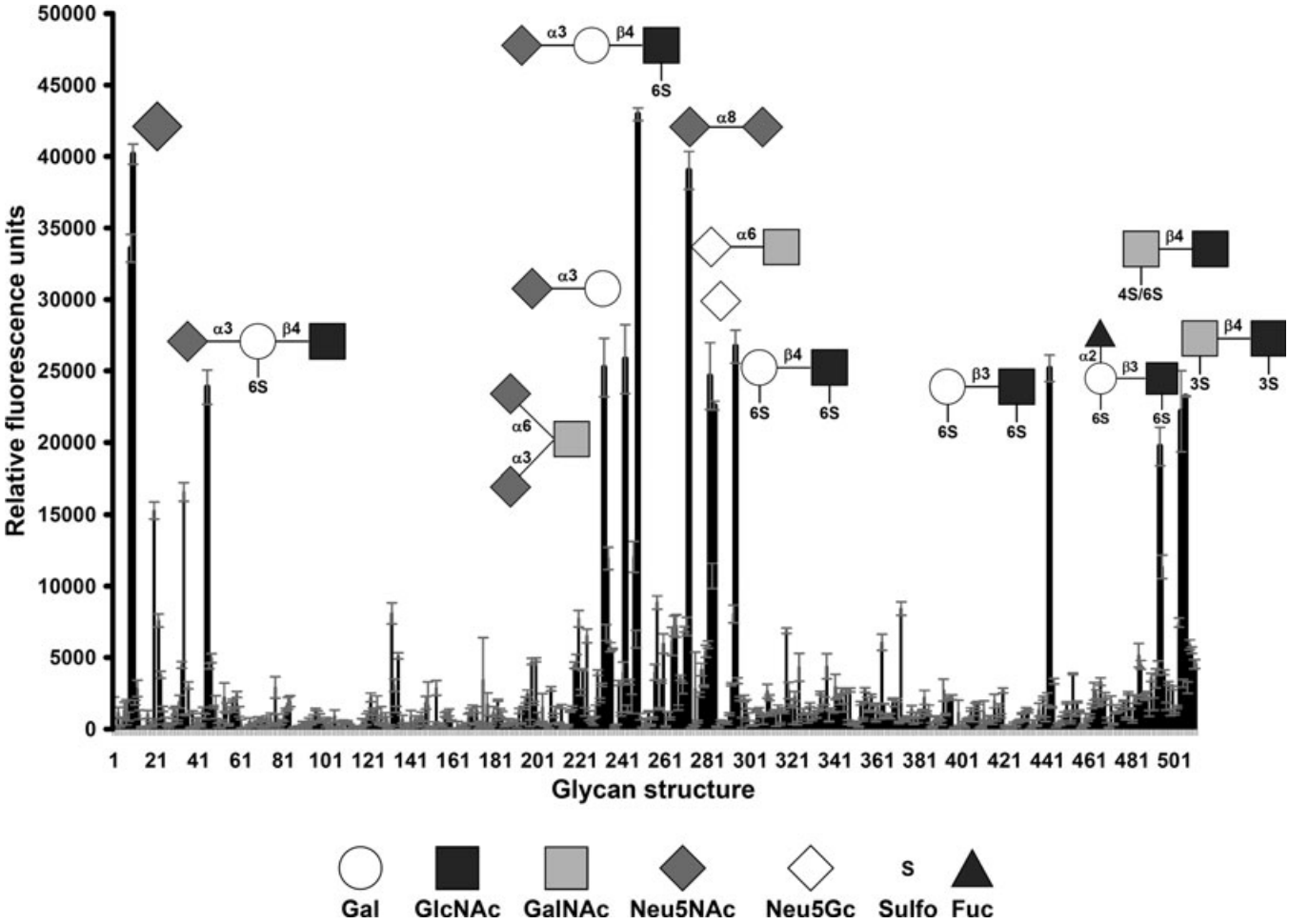

Figure 2. Glycan array analysis. Binding profile of $(Siglec\text{-}7)_4$:SA-PE assayed on the CFG-printed glycan array with glycans printed at 100 μM. $(Siglec\text{-}7)_4$:SA-PE was applied at a concentration at 200 μg/mL (representative of two independent experiments).

ligand positive cells within the different B-cells subsets. Most of the Bm2 and Bm2′ cells were negative for siglec-7 ligands; Bm1 cells showed an intermediate expression of siglec-7 ligands, but most of the early and late memory cells were positive for siglec-7 ligands and especially the $IgD^{-}CD38^{++}$ B cells were nearly completely positive for siglec-7 ligands. Additionally, we also analyzed siglec-7 ligands applying another classification based on the expression of IgD/CD27 and furthermore on the expression of IgM, CD38, and CD24. The results of this analysis are shown in the Supporting Information Fig. 1 and 2. Considering IgD/CD27 expression only, naïve B cells ($IgD^{+}CD27^{-}$) showed the lowest percentage of siglec-7 ligand positive cells, but the expression was still rather heterogeneous. Further analysis of these naïve B cells using anti-CD24 and anti-CD38 allowed a better distinction between siglec-7 ligand negative and positive naïve B cells. Within the naïve B cells, two populations were identified; $CD24^{++}CD38^{-/low}$ naïve B cells comprised mostly of the siglec-7 positive naïve B-cells, whereas $CD24^{low/+}CD38^{low/+}$ naïve B cells comprised most of the siglec-7 negative naïve B cells (Supporting Information Fig. 1 and 2). Another subset showing low expression of siglec-7 ligands was transitional B cells, identified by IgM^{high} and $CD38^{high}$. In line with the analysis by the Bm classification, memory B cells, both unswitched and switched, showed a very high percentage of siglec-7 ligand expression. And over 90% of plasma blasts expressed siglec-7 ligands (Supporting Information Fig. 2). All marker combinations, the percentage of the according B-cells subsets and the siglec-7 expression is summarized in the Supporting Information Tables 2, 3, and 4.

Cytokine responses to B-cell activation differs between siglec-7 ligand negative and positive B cells

In order to analyze functional differences between siglec-7 ligand negative and positive B-cell subsets, we determined their cytokine response to different stimuli. Therefore, we isolated B cells by MACS technology, stained them with $(Sigelc\text{-}7)_4$:SA-PE, and sorted them into siglec-7 ligand negative and positive subfractions. These subfractions were either activated through B-cell receptor (BCR) cross-linking using anti-human IgG and IgM or activated through CD40 engagement using MegaCD40L. After 48 h of incubation, supernatants were collected and

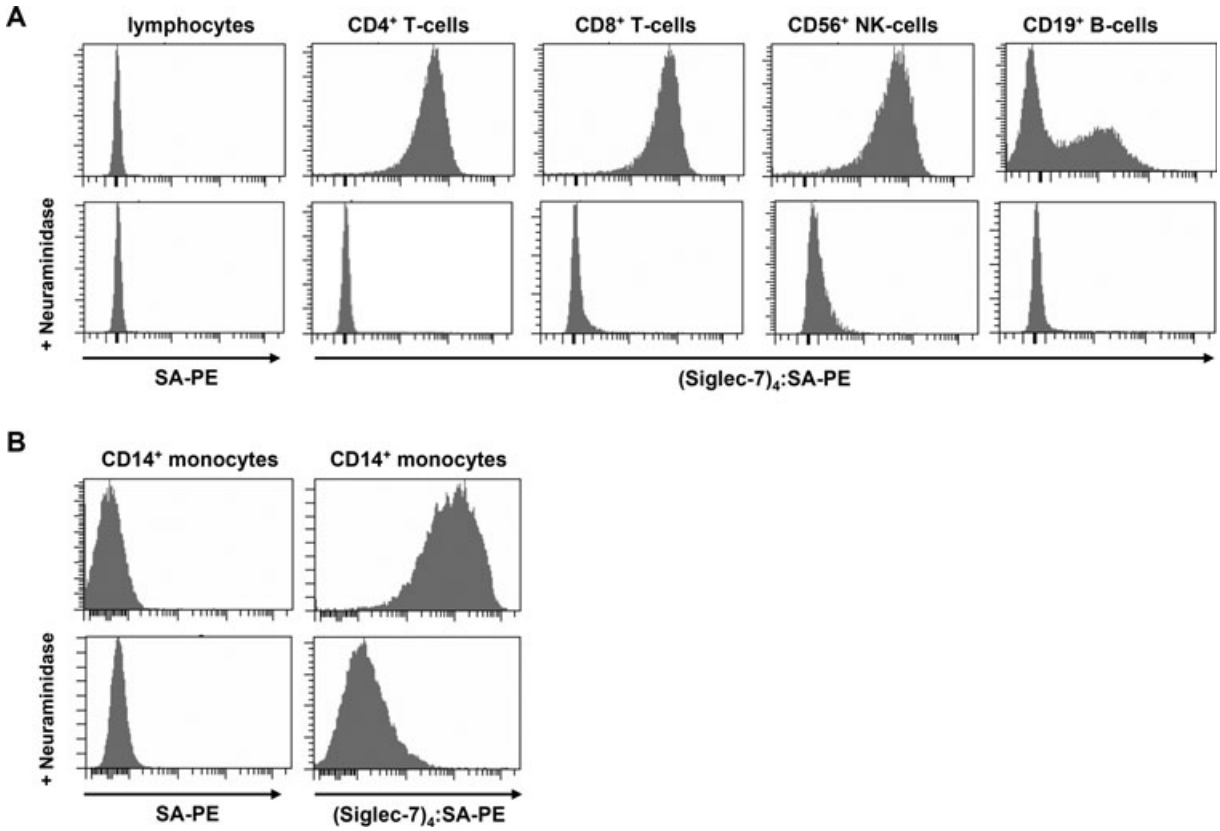

Figure 3. Expression of siglec-7 ligands on lymphocyte subpopulations. Freshly isolated PBMCs were stained with (A) anti-CD4, anti-CD8, anti-CD19, anti-CD56, and (B) anti-CD14 and $(Siglec\text{-}7)_4$:SA-PE, respectively (top panels). Every subpopulation showed a nearly homogenous binding of the tetrameric siglec-7 with the exception of the B cells detected by anti-CD19. To determine sialic acid-dependent binding for control reasons, PBMCs were treated with neuraminidase prior to incubation with $(Siglec\text{-}7)_4$:SA-PE (bottom panels). The analysis of one representative donor of at least five donors is shown.

concentrations of tumor necrosis factor (TNF)-α, TNF-β, IL-6, IL-10, and IL-12 were determined. Upon BCR activation, we observed no significant differences in the release of these cytokines (Fig. 5A). However, upon CD40 engagement, concentrations of TNF-α and TNF-β were significantly higher in supernatants of the siglec-7 ligand positive subfraction (Fig. 5B). In addition, there was a tendency toward higher amounts of IL-6 and IL-10 in supernatants of the positive fraction. The concentrations of IL-12 did not differ between the two subsets. Thus, in the absence of antigen engagement through anti-IgG and anti-IgM, siglec-7 ligand negative B cells stimulated by CD40L alone failed to produce marked amounts of cytokines in contrast to the siglec-7 ligand positive B cells. Taken together, these assays showed significant differences in the cytokine pattern produced by siglec-7 ligand negative B cells as compared with siglec-7 ligand positive B cells.

Pediatric T-ALL blasts express high levels of siglec-7 ligands in contrast to B-ALL blasts

Expression of ligands for inhibitory siglecs may serve as an immune escape mechanism for malignant cells. Thus, we analyzed the cell surface sialylation of blasts of childhood acute lymphoblastic leukemias (ALLs) using the oligomerized siglec-7. Leukemic blasts from nine patients with T-ALL and nine patients with B-ALL (with cALL phenotype, the common subtype of B lineage ALL blasts) were analyzed by flow cytometry (Fig. 6A). On the surface of cALL blasts, siglec-7 ligands were barely detectable, but on T-ALL blasts, siglec-7 ligands were expressed at high densities (Fig. 6). However, within the T-ALL samples, the binding of the siglec-7 was quite variable. Based on the guideline of the European Group for Immunological Classification of Leukemias (EGIL), four of these nine T-ALL samples were classified as pre-T-ALL, three as cortical-T-ALL, and two as mature T-ALL [20]. There was no correlation between these subtypes and the MFI.

Discussion

Cell surface glycans are products of glycosyltransferases and glycosidases representing important posttranslational modifications with key effector functions in the regulation of immune responses. Remodeling of surface glycans are hallmarks of activation, differentiation, and homing. In the present study, we developed a new technique to detect ligands for siglecs by flow cytometry; these

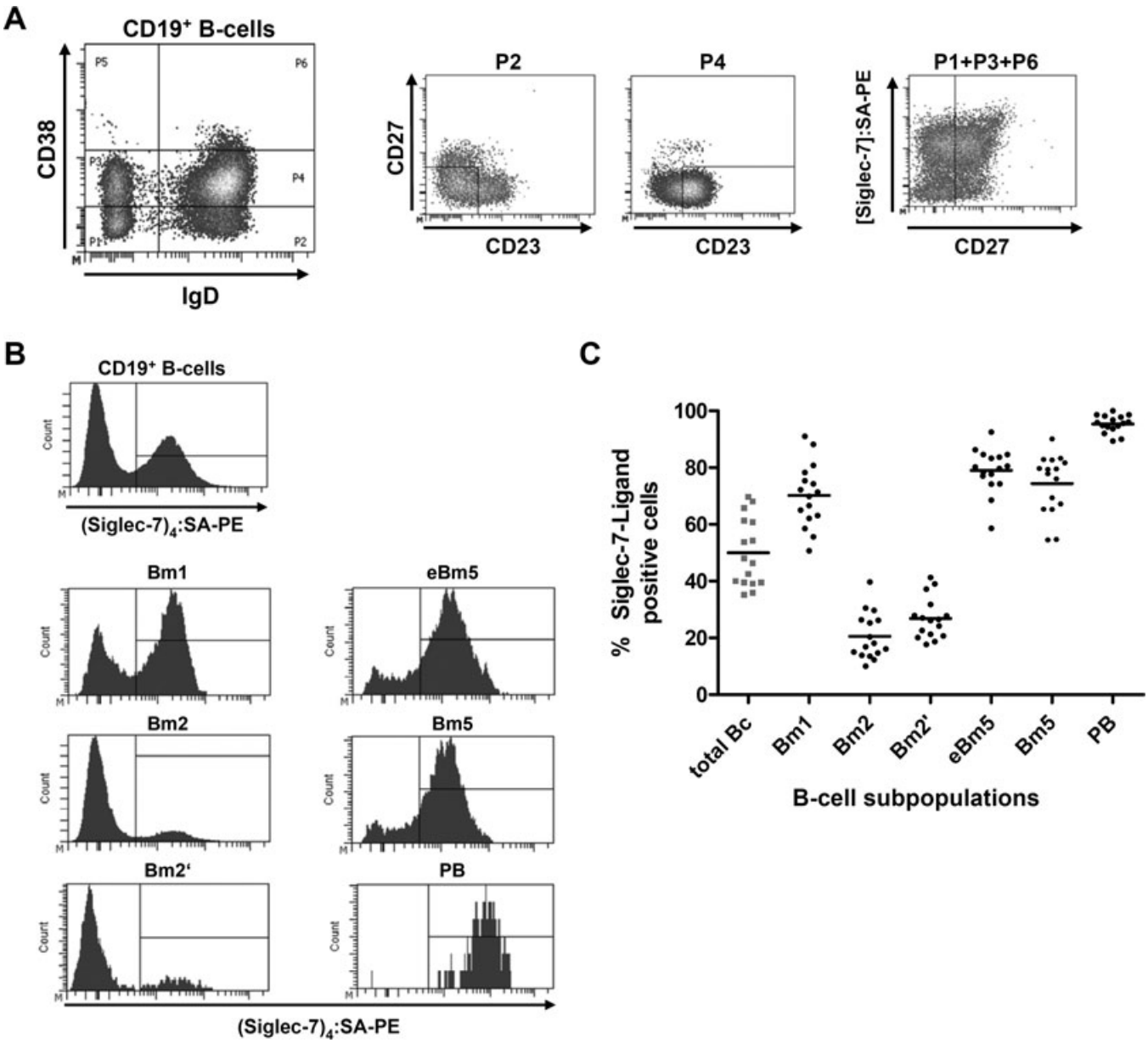

Figure 4. Diverse expression of siglec-7 ligands within different B-cell subpopulations. PBMCs were freshly isolated and stained with anti-CD19-, anti-CD23-, anti-CD27, anti-CD38, anti-IgD-antibodies, and $(Siglec\text{-}7)_4$:SA-PE. The total B-cell population ($CD19^+$) divided into a siglec-7 ligand negative and a positive subfraction. (A) Classification of human B-cell subsets was performed based on the expression of IgD/CD38 in the first gating step. In the second gating step, expressions of CD23 and CD27 were analyzed. IgD^+CD38^- B cells (P2), which were additionally $CD27^-CD23^-$, were classified as Bm1. IgD^+CD38^+ B cells (P4), which were additionally $CD27^-CD23^+$, were classified as Bm2. Bm2' were IgD^+CD38^{++} (P6) and $CD27^+$; eBm5 were IgD^-CD38^+ (P3) and $CD27^+$; Bm5 were IgD^-CD38^- (P1) and $CD27^+$. Plasmablasts (PB) were defined as IgD^-CD38^{++} (P5). (B) Representative histogram blots of B-cell subsets from one healthy donor showing siglec-7 ligand expression. (C) Summary of the analyses of B cells from 16 healthy donors showing the percentage of siglec-7 ligand positive cells within the defined B-cell subsets; bars represent means. The percentages of B-cell subsets are listed in Supporting Information Table 3. Dot plots and histogram plots show the analysis of one donor representative of 16 healthy donors.

ligands contain sialic acids that are the most frequent terminating carbohydrates.

Siglecs display relatively weak interactions with terminal saccharides; affinity constants are typically in the millimolar range for an individual monosaccharide [21]. This may be the reason why detection of siglec ligands on PBMCs by flow cytometry was not successful, when monomeric recombinant siglec-7 was used (Fig. 1C). To overcome the problems associated with the weak interaction, we oligomerized recombinant siglec-7 protein in order to generate tetramers in analogy to MHC tetramers [16]. This tool allowed for the first time a reproducible detection of siglec-7 ligands by flow cytometry. Staining of cell surfaces with oligomerized

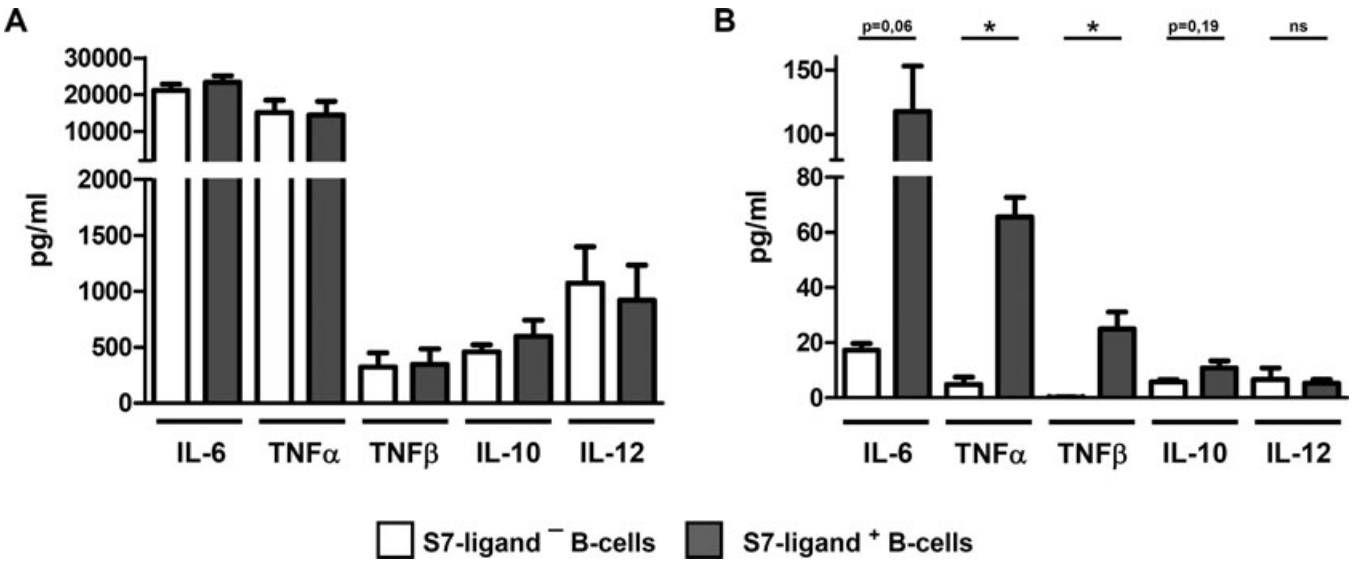

Figure 5. Cytokine responses of siglec-7 ligand positive and negative B cells upon B-cell activation. B cells were isolated by MACS technology and labeled with $(Siglec\text{-}7)_4$:SA-PE. The cells were then sorted by FACS into a siglec-7 ligand negative (S7-ligand$^-$ B cells, white bars) and a positive fraction (S7-ligand$^+$ B cells, gray bars). The subfractions were either activated with (A) anti-human IgM and IgG or (B) CD40L; after 48 h, supernatants were collected and cytokines were measured. Data show means + SEM from three independent experiments. ns.: not significant. $^*p < 0.05$, Student's *t*-test.

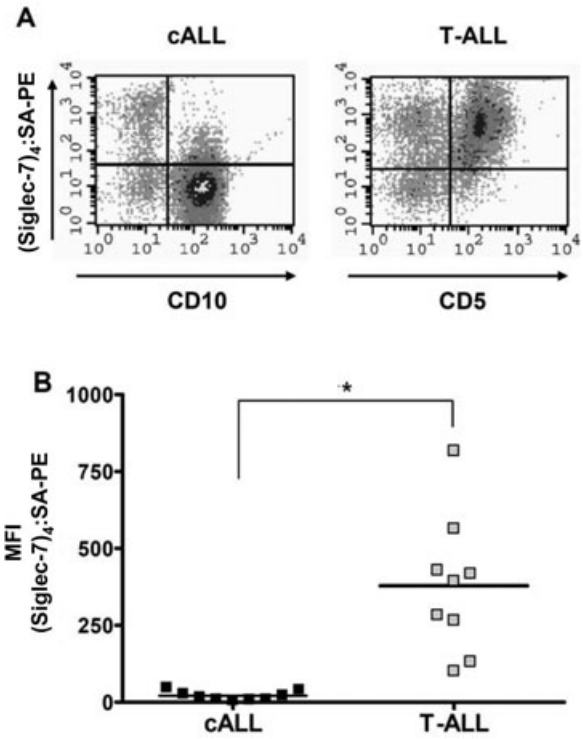

Figure 6. Pediatric T-ALL blasts express high density of siglec-7 ligands whereas B-ALL blasts lack siglec-7 ligands. (A) Representative flow cytometry plots of PBMCs from pediatric patients at first presentation showing presence of siglec-7 ligands on T-ALL blasts, but not on cALL blasts (representative of nine different patients). (B) Statistical analysis of siglec-7 ligand expression from nine different cALL (■) or T-ALL (□) patients each; bars represent means. $^*p < 0.05$, Student's *t*-test.

siglec-7 was not due to increased nonspecific protein–protein interactions, as neuraminidase treatment completely abrogated binding of oligomerized siglec-7 (Fig. 1D). This sialic acid-dependent binding of siglec-7 tetramer was confirmed by the glyan array data of the CFG. Previous glycan array analyses identified preferential binding of siglec-7 to gangliosides, for example GD2 and GD3, and to sialosides with Neu5Acα2-8Neu5Ac structures [15, 22]. In the current version of the glycan array, similarities to previous studies were found, although ligands with higher affinity than GD2 and GD3 were identified. The siglec-7 tetramer bound to the terminal sugar moieties of Sialyl-Lewis^x, which were additionally sulfated. These affinities and, especially, the impact of sulfation has been described before [23]. Moreover, our data indicated a strong affinity of the siglec-7 tetramer to disulfated oligosaccharide probes. However, there are several possible explanations for the minor differences in the binding affinities detected in our study compared to previous studies. In previous studies, the siglec protein was expressed as a recombinant soluble IgG F_c chimera in Chinese hamster ovary cells [15, 23], whereas we expressed the extracellular Ig-like domains without the additional F_c part in 293T cells, which is a human cell line and may cause different glycosylation of the recombinant protein.

Using the tetramerized siglec-7 construct for flow cytometric analysis, we interestingly observed considerable differences among B cells and other lymphocyte subpopulations regarding siglec-7 ligand expression. Sialylation of B cells and the role of siglecs in this population had been studied in detail. Sialylation-dependent cell–cell interactions regulate effector functions of B cells predominantly via siglec-2 (CD22) [24]. On resting B cells, siglec-2 binds to neighboring ligands on the same B cell.

However, upon cellular activation in vitro, siglec-2 appears to be unmasked [25]. This indicates that changes in glycosylation occur during B-cell activation processes, which are balanced by sialyltransferases and sialidases. These findings are in line with Koethe et al., who showed that during human peripheral B-cell development, the glycosylation of CD45RB is strictly regulated with low expression in naïve and high expression in memory peripheral blood B cells [26]. These observations were made by using an antibody that detects a glycosylation-dependent epitope on CD45RB. So far, sialylation patterns on different B-cell stages could not be detected by recombinant human receptor molecules directly. Using the siglec-7 tetramer, we were now able to detect striking differences in sialylation within the $CD19^+$ B lymphocytes. Staining for different surface antigens in order to distinguish the diverse B-cell stages revealed that the siglec-7 ligand negative cell fraction was mainly associated with activated naïve B cells (Bm2), characterized by $IgD^+CD27^-CD38^+CD23^+$, with GC founder (Bm2', $IgD^+CD27^-CD38^{++}$) and with transitional B cells (IgM^{high}, $CD38^{high}$). In contrast, memory B cells (IgD^-CD27^+) and especially plasmablasts ($CD27^+CD38^{++}$) were mainly positive for siglec-7 ligands. This expression profile resembles the expression of the glycosylated CD45RB observed by Koethe et al. [26]. Thus, maybe the siglec-7 ligand includes or overlaps with the glycosylated CD45RB epitope.

In addition to distinct expression of siglec-7 ligands on the different B-cell subsets, we also observed functional differences between siglec-7 ligand negative and positive B-cell subsets (Fig. 5). In contrast to the positive subfraction, the negative fraction showed a marked cytokine response only upon BCR stimulation and not upon CD40 engagement alone. Duddy et al. had analyzed the effector cytokine profiles of memory and naïve B-cell subsets in a similar way [27], and discussed in [28]. They found an almost exclusive IL-10 production by $CD27^-$ naïve B-cells, while on the other hand TNF-α and -β were largely produced by $CD27^+$ memory B cells. Our data showed that siglec-7 ligand expression seems to be associated with a functional stage within either subpopulation.

Furthermore, siglec-7 expressing cells, for example, NK cells, might interact in different ways with activated naïve B cells or memory B cells. Since siglecs have been suggested to play a role in self-recognition and also in B-cell tolerance [29], the different sialylation patterns on the B-cell subpopulations may play a role in pathological conditions such as autoimmunity, organ graft rejection, and graft-versus-host disease following bone marrow transplantation, which has to be analyzed in more detail.

Altered glycosylation of cell surface proteins can be an early feature of malignant transformation [30]. These modifications may contribute to the loss of contact inhibition, metastasis, and immune evasion [31]. Regarding immune evasion, siglec-7 is of interest as an inhibitory receptor in cell contact-dependent cytotoxicity. α2,8-linked disialic acids, which are found for example on melanoma cells, serve as ligands for the inhibitory siglec-7 receptor on NK cells [32]. Consequently, high expression of siglec-7 ligands may contribute to the immune escape mechanism of malignant cells. In the present study, we analyzed blasts from pediatric acute lymphoblastic leukemia, which is the most frequent hematological malignancy of childhood, with T-ALL having a poorer outcome than cALL in most studies [33]. Interestingly, we detected siglec-7 ligands on the surface of T-ALL blasts, but not on the common subtype of B lineage ALL blasts (cALL) (Fig. 6). These distinctions between T-ALL and B-ALL blasts might resemble the differences of peripheral B and T cells from healthy donors (Fig. 3). Considering the siglec-7 ligand expression pattern on B cells, the lack of siglec-7 ligand expression on cALL blasts raises the question, if these blasts are derived from cells with a lower developmental stage. However, the higher expression of siglec-7 ligands on T-ALL blasts than on cALL blasts may facilitate immune evasion and contribute to the poor prognosis of T-ALL in contrast to cALL. The analyzed T-ALL blasts could be assigned to three different subtypes, pre-, cortical, and mature T-ALL. There was no correlation between the MFI of the siglec-7 ligand expression and these three subtypes of T-ALL (data not shown). However, the sample numbers of the different subtypes are much too small to draw any conclusions. Though, a more detailed analysis of siglec-7 ligand expression on T-ALL subclasses and additionally on other malignant cells could help to study these malignancies in the future.

In conclusion, we established siglec tetramers as a new tool to reliably detect cell surface sialylation by flow cytometry. Cloning, expression, and oligomerization of other siglec family members will additionally reveal new information about siglec ligands as part of the immunophenotype in lymphocyte subpopulations, about siglec-dependent cell–cell interactions, and about the role of siglecs in immune modulation.

Materials and methods

Cloning and expression of siglec-7 fusion protein

The N-terminal V-set domain and the following two C2-set domains of human siglec-7 (amino acids 1–338) were cloned using the following primers: TCTAGAATGCTGCTGCTGCTGCTGCT, and TCTAGAGTTGCAGGGAGAGGTTCAGG. The biotinylation target sequence was introduced by primer extension using the sequences ATAAGGGCCCGGATCCCTGCATCATATTCTGGATGCACAGAAAAT and GGTTACCGGTACGATGATTCCACACCATTTTCTGTGCATCCAGAA. Products were cloned 3′ of the siglec into the standard backbone of the plasmid pcDNA3.1 purchased from Invitrogen (Carlsbad, CA, USA). The recombinant protein was expressed in 293T cells to allow mammalian glycosylation. 293T cells were transfected with calcium phosphate precipitation. By positive selection employing G418 (Geneticin; Biochrom, Berlin, Germany), we obtained a stably transfected cell line overexpressing the fusion protein siglec-7 consisting of the V-set domain and the two adjacent C2-set domains of the human siglec-7 (aa 1–338) followed by a biotinylation target sequence and a his tag.

Protein purification, enzymatic biotinylation, and oligomerization

The six-histidine-tagged protein was purified from cell culture supernatants by affinity chromatography using Ni-NTA-agarose beads (Qiagen). Proteins were eluted using 250 mM imidazole in buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween 20, pH 6.8. Eluates were concentrated by ultra filtration using U-Tube Concentrators 15H (Novagen, Nottingham, UK) with a 30-kDa cut off. SDS-PAGE and Coomassie staining were performed to determine protein concentration. Siglec-7 fusion protein was enzymatically biotinylated using the bacterial ligase BirA. To this end, the recombinant siglec-7 was incubated in a buffer composed of 80 mM Tris (pH 8), 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM biotin, and 2 μg/mL BirA at 28°C for 3 h. The protein was filtered in a 0.22 μM filter (Spin-X, Corning, Schiphol-Rijk, Netherlands) and dialyzed against Hank's buffered saline (Biochrom) using Slide-A-Lyzer® (Pierce, Cramlington, UK) dialysis systems (cut off of 30 kDa) at 4°C overnight. Oligomerization was performed using SA-PE (Invitrogen), which was titrated to the biotinylated siglec-7 protein at a molar ratio of 1:4 over a time period of 8 h at 4°C in order to establish tetramers.

Western blot analysis

Detection of the protein was performed by immunoblot. Briefly, biotinylated siglec-7 was separated by gradient SDS-PAGE (4–12%, Pierce, Rockford, IL, USA) and transferred to a nitrocellulose membrane (0.45 μm, Invitrogen). After blocking with TBS (pH 7.6) containing 2% BSA, the siglec-7 fusion protein was detected by a biotinylated anti-siglec-7 antibody (0.1 μg/mL, clone 194212, R&D Systems, Minneapolis, MN, USA) and horseradish peroxidase (HRP)-conjugated streptavidin (1 μg/mL SA-HRP, Pierce); biotinylated siglec-7 protein was detected with SA-HRP directly. Detection was performed with Super Signal West Pico Chemiluminescent Substrate (Pierce).

Glycan array

The glycan binding specificities of the $(Siglec\text{-}7)_4$:SA-PE were analyzed by the CFG (http://www.functionalglycomics.org/glycomics/) using Glycan Array v4.2 carried out according to the direct binding assay protocol as described previously [34]. Briefly, the biotin-labeled siglec-7 was precomplexed with phycoerythrine-labeled streptavidin. The sample at 200 μg/mL was diluted with 10% BSA (final concentration 1% BSA) and added to the glycan printed slide for 1 h at room temperature. For negative control, binding of SA-PE was analyzed.

Deglycosylation

For PNGase treatment before blotting, 20 μg of protein were denatured at 100°C for 10 min and incubated with NP-40, G7 buffer, and 25 units PNGase F (all from New England BioLabs, Ipswich, MA, USA) in a 20-μL reaction for 1 h at 37°C according to the manufacturer's instructions.

Primary leukemic cells

Leukemic blasts of pediatric patients had been cryopreserved for scientific use after obtaining parental informed consent and approval by the local Institutional Review Board (IRB). Aliquots of leukemic cells were thawed, resuspended in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 I.E./mL penicillin, and 100 μg/mL streptomycin and immediately used for experiments.

Flow cytometry

Peripheral whole blood was obtained from healthy donors (IRB approval 279/2003V). PBMCs were isolated from whole blood by density centrifugation over Ficoll (Biochrom) and stained using the following monoclonal antibodies: CD4 (clone RPA-T4)-PerCP, CD8 (HIT8a)-allophycocyanin, CD14 (HCD14)-FITC, CD19 (HIB19)-allophycocyanin, CD23 (EBVS-5)-PerCP/Cy5.5, CD24 (ML5)-PerCP/Cy5.5, CD27 (O323)-allophycocyanin-Cy7, CD38 (HIT2)-PE/Cy7, CD56 (HCD56)-FITC IgM (MHM-88)-PerCP/Cy5.5, and matching isotype controls were purchased from Biolegend (Munich, Germany). CD5 (L17F12)-FITC and CD10 (W8E7)-FITC were obtained from BD Pharmingen (Heidelberg, Germany) and goat $F(ab')_2$ anti-human IgD (δ chain specific) was obtained from Southern Biotech. Hank's buffered saline (Biochrom) containing 2% FCS was used for staining. Flow cytometric analyses were performed on a FACSCanto flow cytometer (BD Biosciences) using FACSDiva 6.1 software. Analysis of leukemic blasts was performed on a FACS Calibur and data were analyzed with CellQuest software (BD Biosciences).

B-cell stimulation and cytokine analyses

Human PBMCs were isolated from whole blood by density centrifugation and subsequently B cells were isolated by depletion of non-B cells using MACS technology (Miltenyi Biotech). For fluorescence activating cell sorting, the cells were labeled with $(Siglec\text{-}7)_4$:SA-PE and anti-CD3-allophycocyanin (Biolegend). Cell sorting was performed on a FACSAria™ cell sorter from BD Biosciences. To further avoid contaminating T cells after B-cell isolation, the CD3-positive cells were excluded within the sorting. The CD3-negative B-cell fraction was then sorted into a siglec-7 ligand negative fraction and a siglec-7 ligand positive fraction. The two cell fractions were resuspended in RPMI 1640 supplemented with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin, and 2 mM L-glutamine. 150,000 cells were plated in a total volume of 200 μL in U-bottom 96 well plates.

The two B-cell fractions were either stimulated with 200 μg/mL MegaCD40L™ (Enzo Life Sciences) or with 0.5 μg/mL goat anti-human IgG and IgM (Jackson ImmunoResearch Laboratories) to induce BCR cross-linking. After 48 h at 37°C in 5% CO_2, supernatants were collected. TNF-α, TNF-β, IL-6, IL-10, and IL-12p70 were measured using Ready-Set-Go ELISA kits from eBioscience according to the manufacturer's protocol.

Neuraminidase treatment

Up to 3×10^7/mL cells were treated with 0.5 U/mL neuraminidase from *Vibrio cholerae* (Fluka—Sigma, Darmstadt, Germany) and incubated for 90 min at 37°C, in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 I.E./mL penicillin and 100 μg/mL streptomycin. After incubation, the cells were washed twice before staining for flow cytometry analysis. Neuraminidase of *Vibrio cholerae* is an enzyme specifically cleaving α2,3-, α2,6-, and α2,8-linked sialic acids on cell surfaces [35].

Statistics

Statistical analysis was performed with Student's *t*-test, using GraphPad Prism 4.0 (GraphPad software, San Diego, CA, USA), $p \leq 0.05$ was considered significant (*).

Acknowledgments: The authors would like to acknowledge The Consortium for Functional Glycomics funded by the NIGMS GM62116 for resources provided by the Glycan Array Synthesis Core (Core D) that produced the mammalian glycan microarray and the Protein–Glycan Interaction Core (H) that assisted with analysis of samples on the array. We thank A. Düsedau, M. Pechan, and C. Treuner for perfect technical assistance and S. Stevanovic (University of Tübingen) for providing the enzyme BirA. This work was supported by the German Ministry of Education and Research (Project No. 0315144) and by the Förderverein für krebskranke Kinder e. V. PM was a scholar of the DFG Graduiertenkolleg 794. FG was a scholar of the Deutsche José Carreras Leukämie-Stiftung e. V (F 07/06).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Crocker, P. R.**, Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling. *Curr. Opin. Struct. Biol.* 2002. **12**: 609–615.

2 **Crocker, P. R., Paulson, J. C. and Varki, A.**, Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 2007. **7**: 255–266.

3 **Freeman, S. D., Kelm, S., Barber, E. K. and Crocker, P. R.**, Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. *Blood* 1995. **85**: 2005–2012.

4 **Hanasaki, K., Varki, A. and Powell, L. D.**, CD22-mediated cell adhesion to cytokine-activated human endothelial cells. Positive and negative regulation by alpha 2–6-sialylation of cellular glycoproteins. *J. Biol. Chem.* 1995. **270**: 7533–7542.

5 **Varki, A. and Angata, T.**, Siglecs–the major subfamily of I-type lectins. *Glycobiology* 2006. **16**: 1R–27R.

6 **Balaian, L., Zhong, R. K. and Ball, E. D.**, The inhibitory effect of anti-CD33 monoclonal antibodies on AML cell growth correlates with Syk and/or ZAP-70 expression. *Exp. Hematol.* 2003. **31**: 363–371.

7 **Vitale, C., Romagnani, C., Falco, M., Ponte, M., Vitale, M., Moretta, A., Bacigalupo, A. et al.**, Engagement of p75/AIRM1or CD33 inhibits the proliferation of normal or leukemic myeloid cells. *Proc. Natl. Acad. Sci. USA* 1999. **96**: 15091–15096.

8 **Ikehara, Y., Ikehara, S. K. and Paulson, J. C.**, Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J. Biol. Chem.* 2004. **279**: 43117–43125.

9 **Nutku, E., Aizawa, H., Hudson, S. A. and Bochner, B. S.**, Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 2003. **101**: 5014–5020.

10 **von Gunten, S., Yousefi, S., Seitz, M., Jakob, S. M., Schaffner, T., Seger, R., Takala, J. et al.**, Siglec-9 transduces apoptotic and nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment. *Blood* 2005. **106**: 1423–1431.

11 **Ando, M., Tu, W., Nishijima, K. and Iijima, S.**, Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs. *Biochem. Biophys. Res. Commun.* 2008. **369**: 878–883.

12 **Kelm, S., Gerlach, J., Brossmer, R., Danzer, C. P. and Nitschke, L.**, The ligand-binding domain of CD22 is needed for inhibition of the B cell receptor signal, as demonstrated by a novel human CD22-specific inhibitor compound. *J. Exp. Med.* 2002. **195**: 1207–1213.

13 **Falco, M., Biassoni, R., Bottino, C., Vitale, M., Sivori, S., Augugliaro, R., Moretta, L. et al.**, Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells. *J. Exp. Med.* 1999. **190**: 793–802.

14 **Nicoll, G., Ni, J., Liu, D., Klenerman, P., Munday, J., Dubock, S., Mattei, M. G. et al.**, Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes. *J. Biol. Chem.* 1999. **274**: 34089–34095.

15 **Avril, T., North, S. J., Haslam, S. M., Willison, H. J. and Crocker, P. R.**, Probing the cis interactions of the inhibitory receptor Siglec-7 with alpha2,8-disialylated ligands on natural killer cells and other leukocytes using glycan-specific antibodies and by analysis of alpha2,8-sialyltransferase gene expression. *J. Leukoc. Biol.* 2006. **80**: 787–796.

16 **Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J. et al.**, Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996. **274**: 94–96.

17 **Bohnhorst, J. O., Bjorgan, M. B., Thoen, J. E., Natvig, J. B. and Thompson, K. M.**, Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. *J. Immunol.* 2001. **167**: 3610–3618.

18 **Potter, K. N., Mockridge, C. I., Rahman, A., Buchan, S., Hamblin, T., Davidson, B., Isenberg, D. A. et al.**, Disturbances in peripheral blood B cell subpopulations in autoimmune patients. *Lupus* 2002. **11**: 872–877.

19 **Sanz, I., Wei, C., Lee, F. E. and Anolik, J.**, Phenotypic and functional heterogeneity of human memory B cells. *Semin. Immunol.* 2008. **20**: 67–82.

20 **Bene, M. C., Castoldi, G., Knapp, W., Ludwig, W. D., Matutes, E., Orfao, A. and van't Veer, M. B.**, Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia.* 1995. **9**: 1783–1786.

21 **Crocker, P. R., Vinson, M., Kelm, S. and Drickamer, K.**, Molecular analysis of sialoside binding to sialoadhesin by NMR and site-directed mutagenesis. *Biochem. J.* 1999. **341**: 355–361.

22 **McMillan, S. J. and Crocker, P. R.**, CD33-related sialic-acid-binding immunoglobulin-like lectins in health and disease. *Carbohydr. Res.* 2008. **343**: 2050–2056.

23 **Campanero-Rhodes, M. A., Childs, R. A., Kiso, M., Komba, S., Le Narvor, C., Warren, J., Otto, D. et al.,** Carbohydrate microarrays reveal sulphation as a modulator of siglec binding. *Biochem. Biophys. Res. Commun.* 2006. **344**: 1141–1146.

24 **Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I. and Varki, A.**, Natural ligands of the B cell adhesion molecule CD22 beta carry N-linked oligosaccharides with alpha-2,6-linked sialic acids that are required for recognition. *J. Biol. Chem.* 1993. **268**: 7019–7027.

25 **Razi, N. and Varki, A.**, Masking and unmasking of the sialic acid-binding lectin activity of CD22 (Siglec-2) on B lymphocytes. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 7469–7474.

26 **Koethe, S., Zander, L., Koster, S., Annan, A., Ebenfelt, A., Spencer, J. and Bemark, M.**, Pivotal advance: CD45RB glycosylation is specifically regulated during human peripheral B cell differentiation. *J. Leukoc. Biol.* **90**: 5–19.

27 **Duddy, M., Niino, M., Adatia, F., Hebert, S., Freedman, M., Atkins, H., Kim, H. J. et al.,** Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J. Immunol.* 2007. **178**: 6092–6099.

28 **Lund, F. E.**, Cytokine-producing B lymphocytes-key regulators of immunity. *Curr. Opin. Immunol.* 2008. **20**: 332–338.

29 **Duong, B. H., Tian, H., Ota, T., Completo, G., Han, S., Vela, J. L., Ota, M. et al.,** Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. *J. Exp. Med.* **207**: 173–187.

30 **Hakomori, S. and Handa, K.**, Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: essential basis to define tumor malignancy. *FEBS Lett.* 2002. **531**: 88–92.

31 **Dall'Olio, F.** and **Chiricolo, M.**, Sialyltransferases in cancer. *Glycoconj. J.* 2001. **18**: 841–850.

32 **Nicoll, G., Avril, T., Lock, K., Furukawa, K., Bovin, N. and Crocker, P. R.**, Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. *Eur. J. Immunol.* 2003. **33**: 1642–1648.

33 **Schrauder, A., Reiter, A., Gadner, H., Niethammer, D., Klingebiel, T., Kremens, B., Peters, C. et al.,** Superiority of allogeneic hematopoietic stem-cell transplantation compared with chemotherapy alone in high-risk childhood T-cell acute lymphoblastic leukemia: results from ALL-BFM 90 and 95. *J. Clin. Oncol.* 2006. **24**: 5742–5749.

34 **Alvarez, R. A. and Blixt, O.**, Identification of ligand specificities for glycan-binding proteins using glycan arrays. *Methods Enzymol.* 2006. **415**: 292–310.

35 **Drzeniek, R.**, Substrate specificity of neuraminidases. *Histochem. J.* 1973. **5**: 271–290.

Abbreviations: **Bm**: mature B cell · **cALL**: common acute lymphoblastic leukemia · **CFG**: Consortium for Functional Glycomics · **DN**: double negative · **PB**: plasmablast · **RFU**: relative fluorescence unit · **S7**: siglec-7 · **SA-PE**: streptavidin-(R)-phycoerythrine · **Siglec**: sialic acid-binding immunoglobulin-like lectin · **(Siglec-7)$_4$:SA-PE**: oligomerized siglec-7 conjugated to SA-PE · **T-ALL**: acute T lymphoblastic leukemia

Full correspondence: Dr. Friederike Gieseke, Research Institute Children's Cancer Center Hamburg, Germany, Martinistr. 52, 20251 Hamburg, Germany
Fax: +49-40-42605-1219
e-mail: gieseke@kinderkrebs-forschung.de

Received: 1/12/2011
Revised: 29/3/2012
Accepted: 27/4/2012
Accepted article online: 15/5/2012

