Myeloid precursors and acute myeloid leukemia cells express multiple CD33-related Siglecs

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Objectives. CD33 is a cell surface marker of committed myelomonocytic precursors and circulating monocytes, and is also found on acute myeloid leukemia (AML) cells. CD33 belongs to a family of sialic acid–binding cell surface proteins named Siglecs, among which there are 7 other functional CD33-related Siglecs (CD33rSiglecs). We sought to characterize the spectrum of expression of the other CD33rSiglecs on bone marrow precursors and AML cells and asked if they can potentially serve as targets for therapy.

Methods. Cell surface CD33rSiglecs were analyzed by flow cytometry. The ability of certain anti-Siglec antibodies to target toxin-mediated cell killing of Siglec-expressing cell lines was characterized and compared.

Results. We demonstrate that Siglecs-3, -5, -6, -7, and -9 are expressed on subsets of normal bone marrow precursors, including promonocytes and myelocytes. Furthermore, most AML (but not ALL) cells express these Siglecs. There is substantial variability in Siglec type and expression level between cases, with each having a unique “CD33rSiglec fingerprint.” Individual anti-Siglec antibodies along with a saporin toxin–conjugated secondary antibody can target myelomonocytic leukemia cells for death, and targeting of multiple Siglecs improves cell killing. Cytotoxicity was further enhanced by sialidase treatment of target cells, which improves antibody binding. We also confirmed that antibody binding induced rapid internalization of Siglecs from the cell surface, which is a requirement for cell killing via saporin.

Conclusions. Multiple CD33rSiglecs are expressed on normal and malignant myelomonocytic cells. Targeting these Siglecs, possibly in combinations, could improve anti-CD33 antibody therapy or be used as an alternative to anti-CD33.

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human acute myeloid leukemias (AMLs) are arrested at various stages of myeloid development, CD33 has been used as a target for diagnosis and therapy of AML [6,7]. Antibody cross-linking of CD33 can induce apoptosis and inhibition of proliferation in normal myeloid cells, as well as in leukemia cells from AML and chronic myeloid leukemia patients [8]. The protein kinase Syk has been demonstrated to be involved in these CD33 signaling effects [9]. While the exact pathways for death signaling have yet to be fully elucidated, anti-CD33 activates death signals that are similar to the chemotherapeutic agents cytosine arabinoside and idarubicin [10]. Gemtuzumab ozogamycin (GO, Mylotarg) is a humanized anti-CD33 Ab linked to a toxin (N-acetyl-gamma calicheamicin dimethyl hydrazide), and is approved for use in patients 60 years or older with relapsed AML [11]. Although this drug causes some reversible toxicity on normal myelomonocytic precursors, it is currently effective at inducing remissions in some 25 to 30% of relapsed AML patients [12–14]. Studies have found that GO is also effective for untreated AMLs, and in some pediatric AML patients [13,14].

In addition to CD33, Siglec-5 and Siglec-7 (p75/AIRM-1) are also known to be expressed on AML cells [8,15]. Siglec-5 was found to be negative on CD34+ bone marrow and cord blood stem cells, but upregulated on differentiating cord blood cells, at a later stage than CD33 [15]. Here, we determine the expression profiles of all CD33rSiglecs (Siglecs-3, -5 through -10) of AML cells from peripheral blood samples. Interestingly, we uncovered unique Siglec expression patterns for individual AML patients. We also show that these profiles could potentially be utilized to improve therapy in combination with anti-CD33 or as an alternative to anti-CD33 in order to individualize therapy.

Materials and methods

Cells and reagents

U937 human histiocytic lymphoma cells (established from pleural effusion malignant cells of a patient with histiocytic lymphoma) [16] and THP-1 human acute monocytic leukemia cells were propagated in RPMI-1640 (Gibco/Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS) (hereafter referred to as cRPMI). All blood and bone marrow samples were collected under full compliance with protocols approved by the UCSD Institutional Review Board (Human Subjects Committee). Cryopreserved circulating acute leukemia cells from human patients were thawed, washed in RPMI-1640, and resuspended in phosphate-buffered saline (PBS) for flow cytometry studies. The following antibodies were generously provided by Dr. Paul Crocker, University of Dundee, Scotland: anti-Siglec-5 (clone 1A5), anti-Siglec-7 (clones 7.5A and 7.7A), anti-Siglec-8 (clone 7C9), anti-Siglec-10 (clone 5G6), and nonspecific mouse IgG hybridoma supernatant (X63). Purified anti-CD33 (clone HIM3-4), anti-Siglec-6 (clone E20-205), anti-Siglec-9 (clone E10-286), and PE-anti-CD34 (clone 581) were from BD Pharmingen (San Diego, CA, USA). Mab-ZAP (goat anti-mouse IgG–saporin conjugate) was purchased from Advanced Targeting Systems (San Diego, CA, USA). Phycoerythrin (PE) anti-human CD14 (clone M5E2) and PE anti-human CD16 (clone 3G8) were purchased from BioLegend (San Diego, CA, USA). Allophycocyanin (APC) anti-CD13 (clone TuK 1) was purchased from Caltag Laboratories (Burlingame, CA, USA). Goat anti-mouse IgG Alexa Fluor 488 conjugate and annexin V Alexa Fluor 488 conjugate were purchased from Invitrogen (Carlsbad, CA, USA).

Flow cytometry

Flow cytometry was used to detect cell surface Siglecs by antibody staining. Cells (1–5 × 10⁶) were incubated with 0.5 to 2 µg of anti-Siglec mAb (purified or in hybridoma supernatant) in 100 µL of PBS for 30 minutes on ice. Cells were then washed and incubated with goat anti-mouse IgG Alexa Fluor 488 conjugate (GAM IgG-AF488) in PBS for 30 minutes on ice. Cells were resuspended in PBS and analyzed immediately. Cellular fluorescence was quantitated on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using CellQuest software. For bone marrow cell triple labeling, cells were blocked with PBS containing 10% normal rabbit serum, 2.5% normal goat serum, and 1% bovine serum albumin. Cells were then stained with anti-Siglec antibodies and GAM IgG-AF488 as above, washed, blocked with nonspecific mouse IgG, and then incubated with APC anti-CD13 and PE anti-CD14 or PE anti-CD16 concurrently.

Siglec internalization

Siglec internalization was analyzed based on the loss of cell surface Siglecs upon mAb binding at 37°C. Briefly, cells were incubated with saturating amounts of mAb on ice, washed with PBS, resuspended in cRPMI, and then incubated at 37°C for the indicated amounts of time between 0 and 2 hours. Cells were then chilled on ice and mixed with GAM IgG-AF488 (10 µg/mL final concentration) to detect remaining cell surface mAb. Fluorescence was quantitated by flow cytometry.

Toxin-mediated anti-Siglec Ab cell killing

U937 and THP-1 cells were utilized as leukemia cell models for cell killing. Cells in cRPMI were mixed with anti-Siglec mAbs and Mab-ZAP in triplicate wells of 96-well tissue culture plates. Cells were cultured for 48 hours and then analyzed for death, either by incorporation of propidium iodide or lack of esterase activity, which is measured by the conversion of nonfluorescent carboxyfluorescein diacetate, succinimidyl ester (CFSE) into its fluorescent derivative. Both methods gave comparable results for cell killing.

Statistical analysis

Results were analyzed using a paired Student’s t-test, with p < 0.05 considered as significant.

Results

AMLs express Siglecs-3, -5, -6, -7, -9, and -10

We examined the expression of CD33rSiglecs on peripheral blood cells from patients with AML and acute lymphocytic leukemia (ALL) using previously characterized mAbs. We found that while AML cells express Siglecs-3, -5, -6, -7, -9, and -10 to varying degrees, ALL cells expressed little to none of these molecules (Fig. 1A). While high expression
of Siglec-3 was found on various samples from all AML French-American-British (FAB) classification subtypes, high expression of Siglecs-5, -6, -7, and -9 (defined as greater than 25% of cells being positive) was found on only M4 and M5 subtypes. Similarly, previous studies demonstrated that Siglec-5 is generally present on FAB subtypes M3, M4, and M5, while Siglec-7 is more often present on M4 and M5 subtypes [8,15]. We did not test any M3 subtypes. Siglec-10, which is known to be present on monocytes and eosinophils [17,18], was less than 10% positive on any of the samples tested. Siglec-8 (normally found only on eosinophils, mast cells, and basophils) [19] was not present on the first 6 AML and 4 ALL samples (data not shown), and was not further analyzed. Furthermore, our samples demonstrated no clear correlation between FAB subtype and CD33 expression, as evidenced by high CD33 expression in 4 of 6 M1 and/or M2 AMLs, 1 of 3 M4 AMLs, and 7 of 10 M5 AMLs (Fig. 1A). Gates were defined based on established side- and forward-scatter positions for myeloid and monocytic cells [5], and

**Normal bone marrow cells express multiple Siglecs**

It has been demonstrated that CD34+ bone marrow precursors do not express Siglec-5, but often upregulate CD33 early in the maturation process [15]. The expression of other Siglecs on normal developing bone marrow cells has not been well characterized. We found by flow cytometry that several subsets of bone marrow cells were positive for Siglec-3, -5, -6, -7, and -9 (Fig. 2). Double-label experiments revealed that the majority of CD34+ myeloblasts and monoblasts were mostly negative for Siglec-3, except for low levels of Siglec-3 and -6 (both less than 8% positive, data not shown). Triple-labeling experiments including anti-CD13 and anti-CD14 or anti-CD16 as cell development markers [5] revealed that while myeloid precursor cells (gate A/CD13+/CD16-) expressed Siglec-3, -5, -6, and -9 (Fig. 2A), monocytic precursors (gate B/CD13+/CD14-) expressed Siglec-3, -5, -6, -7, and -9 (Fig. 2B). Gates were defined based on established side- and forward-scatter positions for myeloid and monocytic cells [5], and
based on bright CD16 staining (metamyelocyte/neutrophil marker) preferentially in gate A and bright CD14 staining (monocyte marker) preferentially in gate B (data not shown). Despite extensive blocking, high background staining was observed with cells in gate A. Regardless, positive staining of Siglecs was easily identified (Fig. 2A). Lymphocytic and erythrocytic cell populations did not express Siglecs above the background mouse IgG control (data not shown). These data indicate that developing myeloid and monocytic cells in the bone marrow do upregulate CD33rSiglecs before final maturation and entry into the bloodstream.

Antibodies against Siglecs induce rapid internalization

Previous studies have demonstrated that antibodies against CD33 induce rapid internalization upon cross-linking [20,21]. This mechanism is thought to be important for the intracellular delivery of the calicheamicin toxin in GO, and for the toxin present in Mab-ZAP (see below), which is used for killing in our subsequent experiments [22]. We sought to determine if antibodies against Siglec-5 and -9 would induce similar internalization from the cell surface. Using mAb binding at 37°C, we found that antibodies against Siglec-5 and -9 induced internalization comparable to that observed with an anti-CD33 mAb (Fig. 3). Within 2 hours, 40 to 60% of cell surface Siglecs were internalized by mAb binding. Thus, other Siglecs in addition to CD33 could potentially be utilized for mAb-directed intracellular delivery of cell toxins.

Toxin-mediated anti-Siglec Ab cell killing correlates with Siglec cell expression

Leukemia cells isolated directly from patients show high levels of spontaneous death, and are not well suited for accurate in vitro killing studies. We therefore used cultured U937 and THP-1 cells as models for Siglec-expressing leukemias, to examine the in vitro effects of targeting Siglecs. For toxicity studies, we used Mab-ZAP, which is a goat anti-mouse IgG conjugated to saporin, a ribosome-inactivating protein from the seeds of Saponaria officinalis [22]. The Mab-ZAP alone in the absence of anti-Siglec Abs showed very low toxicity in U937 cells (Fig. 4A). However, when mAbs against Siglecs-3, -5, -6, -7, -9, or -10 were added, they induced significant levels of cell death (Fig. 4A). The level of cell killing corresponded with the expression level of each Siglec on U937 cells as determined by flow cytometry (Fig. 4B). Antibody binding alone did not induce detectable cell toxicity (data not shown).

Combination anti-Siglec targeting improves cell killing

Based on the above toxicity studies using saporin, we predicted that targeting multiple Siglecs would improve cell killing. Using a dose-response curve to evaluate killing at various concentrations of anti-Siglec/Mab-ZAP, we compared anti-CD33 alone with a combination of anti-CD33, anti-Siglec-5, and anti-Siglec-9. For this experiment, Mab-ZAP was kept at a constant concentration ratio to antibody (2 Mab-ZAP : 1 Ab). We observed significantly increased killing of U937 and THP-1 cells treated with
**Discussion**

Here we demonstrate that human AML cells can express multiple CD33rSiglec molecules to varying degrees. We also demonstrate that normal bone marrow monocytic precursor cells express Siglecs-3, -5, -6, -7, and -9, and myeloid precursor cells express Siglecs-3, -5, -6, and -9. Using anti-Siglec Abs and Mab-ZAP, we characterize leukemic cell killing by targeting single or multiple Siglecs. The levels of cell killing correlated with the relative expression of each Siglec. Importantly, we observed that a 10-fold reduction in total toxin produced equivalent or better killing using a combination of anti-Siglecs-3, -5, and -9 compared to anti-Siglec-3 alone (Fig. 5). We conclude that the targeting of multiple Siglecs improves killing above anti-CD33 Ab alone on cells that express multiple Siglecs.

GO is currently the only anti-Siglec-targeted Ab approved for therapy of relapsed AML and, potentially, untreated AML. Our findings demonstrating the improvement in cell killing with multiple anti-Siglec Abs is meaningful for the combination compared to anti-CD33 alone at Ab concentrations above 20 ng/mL (Fig. 5A). Remarkably, for U937 cells, the amount of killing at 200 ng/mL with the combination was as effective as anti-CD33 alone at 2 µg/mL, despite a 10-fold difference in anti-Siglec Ab and Mab-ZAP concentration. These data suggest that targeting multiple Siglecs could improve killing and lower the required therapeutic dosing by as much as 10-fold for AMLs that express multiple Siglecs.

We also wanted to characterize if killing by Mab-ZAP occurred through apoptosis or necrosis. Following a 2-day incubation with Ab/Mab-ZAP, we stained U937 and THP-1 cells with annexin V–Alexa Fluor 488 conjugate and propidium iodide (PI). We found that the majority of annexin V+ cells were also PI+ (approximately two-thirds), indicative of necrotic killing that allows the entry of annexin V into cells (Fig. 5B). The remaining third of annexin V+ cells were negative for PI, suggesting that apoptosis does occur in some cells prior to necrotic death. A smaller proportion of cells were PI+ but annexin V−. In comparing anti-CD33 alone, anti-Siglec-5 alone, anti-CD33 + anti-Siglec-5, or anti-CD33 + anti-Siglec-5 + anti-Siglec-9, there was increased overall death with Ab combinations, but the proportions of annexin V and PI staining remained consistent.

**Sialidase treatment of cells improves antibody binding and cell killing**

Sialidase treatment to remove cell surface sialic acids has been demonstrated to improve the binding of anti-CD15 Abs against cancer cells [23]. We wanted to determine if sialidase treatment might increase the binding of anti-Siglec Abs and increase the effectiveness of saporin-mediated killing. Sialidase treatment alone had no effect on U937 cell viability. However, we found that after sialidase treatment, the amount of Ab binding dramatically increased for all Abs tested (Fig. 6A). This was not likely to be a Siglec-specific effect, since the binding of Abs against other proteins such as CD45 and CD71 also increased (data not shown), suggesting that perhaps Ab access, in general, is increased upon sialidase treatment. As expected, the increase in mAb binding also resulted in increased cell death upon treatment with identical amounts of mAb plus Mab-ZAP (Fig. 6B).

Once again, Ab combinations were more effective at killing than anti-CD33 alone for both normal cells and sialidase-treated cells. While we did observe more background death with Mab-ZAP in the absence of primary Ab on sialidase-treated cells, the increases in killing with anti-Siglec Abs were significantly greater. Thus, the sialidase effect was not simply additive. This increased background death is likely due to increased nonspecific Mab-ZAP binding resulting from the removal of negatively charged sialic acid molecules from the cell surface.
several reasons. Firstly, improved cell killing should increase the percentage of complete remissions, increase remission time, and decrease the likelihood of relapse. Secondly, improved cell killing should decrease the total requirement for toxin therapy, resulting in decreased nonspecific cell killing and side effects. Thirdly, for those AML patients who have relatively low expression of CD33, either in the natural state or due to outgrowth of CD33<sup>+</sup> cells after undergoing GO therapy, alternative Siglecs may be valuable targets for therapy. Finally, high CD33 antigen loads in the periphery have been demonstrated to reduce GO saturation and toxicity on bone marrow AML cells [24]. Therefore, targeting of other CD33r-Siglecs would improve the delivery of toxin to these AML cells. Of course, these improvements would only prove true for AMLs that do express multiple Siglecs. In our studies, we did not compare GO to the Mab-ZAP saporin toxin because GO requires less time and less dosage to kill cells, and the two toxins cause cytotoxicity by different methods. Therefore, our optimal control was to use anti-CD33 alone with Mab-ZAP in order to extrapolate the effectiveness of combination targets.

We realize of course that the presence of these Siglecs on some normal bone marrow myelomonocytic precursors could result in some bystander killing by an anti-Siglec-toxin conjugate. However, even if a new anti-Siglec toxin had a negative impact on some normal precursor cells, this would not be different from the current situation with toxicity of CD33-toxin conjugates. The important point is that CD34<sup>+</sup> cells are not Siglec<sup>+</sup>. Thus, combination therapy with multiple Siglec-toxin conjugates would not be expected to cause any permanent damage to more primitive precursors.

The uniqueness of Siglec expression patterns on AML cells from different patients raises some interesting points. We feel that the determination of this profile would be necessary to evaluate a course for therapy, even for considering GO therapy alone. We found that anti-Siglec cell killing directly correlated with expression levels and antibody binding on U937 cells, which is corroborated by previous studies on GO cytotoxicity [21,25]. Several of our AML samples were less than 10% positive for CD33, and GO therapy would not likely be effective in these cases. In support of this, a recent study has identified the role of CD33 expression levels and ITIM-dependent internalization for the cytotoxicity of GO [25]. On the other hand, others have observed complete remissions in CD33<sup>−</sup> AMLs, which was attributed to CD33-independent endocytosis of GO [26]. Regardless, our data support the concept that Abs against other CD33r-Siglecs can be used to target AMLs and provide information to allow clinicians to
choose the appropriate combination of Abs for therapy based on an individual patient’s Siglec profile, as presented in Figure 1. Clinical trials are needed to determine if such combinations are truly more effective in patients.

The improved targeting of antibodies against sialidase-treated cells may also have implications for anti-Siglec-targeted therapy. Here, we demonstrated that antibody binding was dramatically improved by pretreatment of cells with sialidase. We predict that ex vivo purging of AML bone marrow cells for autologous bone marrow transplants would be improved by pretreatment with sialidases [27]. In addition, we speculate that in vivo targeting of sialidase activity using a noninternalizing anti-Siglec Fab might improve the binding and delivery of anti-Siglec-toxin conjugates. Such targeting of sialidase activity would remove sialic acids only on the cell of interest, thus increasing therapeutic Ab binding. Future studies would be needed to determine if this might be a feasible method to improve AML therapy. Our data further support the concept that up-regulation of sialic acids on cancer cells may play a role in the evasion of cancer cells from the immune system, particularly in this case, antibody binding [28–32].

In summary, we present new data demonstrating unique CD33rSiglec expression profiles in normal human bone marrow and human AML cells. We propose that individualized therapy targeting multiple Siglecs could potentially improve outcomes by providing better leukemia killing with lower toxin dosages.

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References


