

CD15 expression in human myeloid cell differentiation is regulated by sialidase activity

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The glycan determinant CD15 (also known as Lewis x, or Le^x) is a distinguishing marker for human myeloid cells and mediates neutrophil adhesion to dendritic cells. Despite broad interest in this structure, the mechanisms underlying CD15 expression remain relatively uncharacterized. Accordingly, we investigated the molecular basis of increasing CD15 expression associated with human myeloid cell differentiation. Flow cytometric analysis of differentiating cells together with biochemical studies using inhibitors of glycan synthesis and of sialidases showed that increased CD15 expression is not due to *de novo* biosynthesis of CD15, but results predominantly from induction of $\alpha(2-3)$ -sialidase activity, which yields CD15 from cell-surface sialyl-CD15 (also known as sialyl-Lewis x, sLe^x or CD15s). This differentiation-associated conversion of surface CD15s to CD15 occurs mainly on glycoproteins. Until now, modulation of post-translational glycan modifications has been attributed solely to dynamic variations in glycosyltransferase expression. Our results unveil a new paradigm by demonstrating a critical role for post-Golgi membrane glycosidase activity in the 'biosynthesis' of a key glycan determinant.

The CD15 antigen, or Lewis x (Le^x), is a cell-surface glycan consisting of a trisaccharide with the structure Gal- $\beta(1-4)$ -(Fuc- $\alpha(1-3)$)-GlcNAc. It was initially identified by monoclonal antibodies (mAbs) in the early 1980s and was quickly appreciated as a useful marker for human myeloid differentiation^{1,2}—in particular, in identifying granulocyte-series cells. Otherwise known as the stage-specific embryonic antigen-1 (SSEA-1 antigen), CD15 also serves as a marker of mouse embryonic stem cells³ and of mouse mesenchymal stem cells⁴. CD15 is related to another structure, sialyl-CD15 (NeuNAc- $\alpha(2-3)$ -Gal- $\beta(1-4)$ -(Fuc- $\alpha(1-3)$)-GlcNAc; CD15s or sLe^x, where "s" refers to "sialylated"), which differs only by the addition of a sialic acid (*N*-acetylneuraminic acid, NeuNAc) in $\alpha(2-3)$ linkage to the galactose in the core Le^x trisaccharide^{5,6}. Though apparently subtle, this sialylation has profound implications for immunoreactivity and biological functions. Although bearing a common trisaccharide core, antibodies to CD15s do not recognize CD15 and vice versa. Identification of CD15s with mAbs such as HECA-452 has been useful in defining subsets of cells that bind E-selectin and display specialized tissue migration patterns, such as dermatotropic lymphocytes^{7,8} and osteotropic stem cells^{9,10}. Early studies of hematopoietic differentiation showed that expression of the sLe^x determinant is associated with the most primitive subset of the resident bone marrow cells in humans and that myeloid maturation is accompanied by relative loss of CD15s and gain of CD15 expression^{11,12}. These results suggested that, within the bone marrow microenvironment, partitioning of CD15s and CD15 expression on immature cells may also be important in the creation of "hematopoietic niches." Similarly, upregulation of CD15 expression on

neutrophils has been implicated in modulating innate and/or adoptive immune responses via engagement to the dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN)^{13,14}.

Despite keen interest in the CD15 determinant, the molecular regulation of its expression has not been fully elucidated. For essentially all cell-surface glycans described so far, expression has been shown to be secondary to induction of specific glycosyltransferases within the endoplasmic reticulum and/or Golgi apparatus¹⁵⁻¹⁷. Although surface display of CD15 has been attributed to transcriptional upregulation of pertinent glycosyltransferases¹⁷, the reciprocal variations in CD15s and CD15 expression observed in myeloid cell differentiation^{18,19} prompted us to examine the mechanisms regulating membrane expression of these glycans (Fig. 1). For this purpose, we exploited two models of differentiation: one based on the capacity of anti-CD44 mAbs to induce maturation of myeloid leukemic cells^{20,21}, and the other on granulocyte colony-stimulating factor (G-CSF)-induced differentiation of native hematopoietic progenitor cells. In both models, our studies revealed that the maturation-associated increases in CD15 expression are conferred predominantly by induction of cell-surface sialidase activity with resultant cleavage of $\alpha(2-3)$ -linked sialic acid, which yields CD15 from CD15s. This transformation occurs predominantly on glycoproteins, including two sialomucins serving as selectin ligands, P-selectin glycoprotein ligand-1 (PSGL-1) (refs. 8,22) and CD43 (refs. 23,24). These findings offer new perspectives on the molecular basis of glycan expression by revealing that stage-specific cropping of 'mature' membrane glycans yields 'new' epitopes, thus highlighting a

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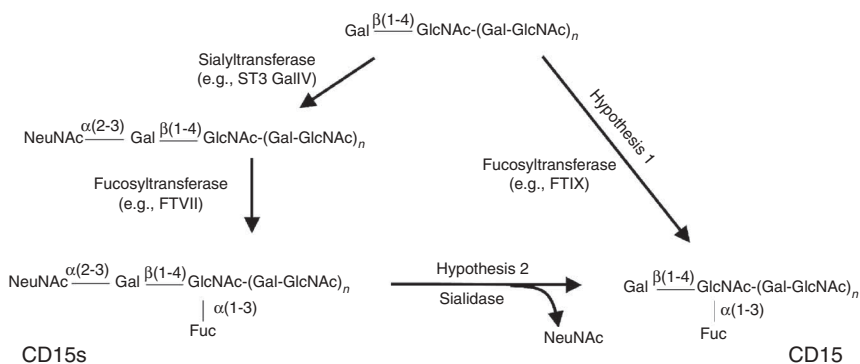


Figure 1 Hypotheses for increased CD15 expression during myeloid differentiation. Structures are shown in schematic, with relevant chemical steps (arrows) for synthesis of CD15s and CD15. Note that the two hypotheses are not mutually exclusive.

key role for dynamic induction of post-Golgi glycosidases in the regulation of cell-surface carbohydrate decorations.

RESULTS

CD44 ligation increases CD15 and decreases CD15s expression

Previous studies have shown that anti-CD44 mAbs induce differentiation of leukemic cell lines and primary acute myeloid leukemia (AML) blasts^{20,21}. Using this model of induced maturation to investigate CD15 expression, we cultured HL60 cells and primary AML blasts in the presence of the anti-CD44 mAb Hermes-1, without mAb, or with isotype control mAb for 72 h. CD44 mAb treatment resulted in morphologic changes characteristic of granulocytic differentiation: nuclear condensation and lobulation, increased cytoplasmic granules and increased cytoplasm-to-nuclear ratio (Supplementary Fig. 1 online). Anti-CD44 mAb treatment significantly increased CD15 expression (consistently >40% increase in mean fluorescence intensity (MFI); $P \leq 0.05$) in both HL60 (Fig. 2a, groups 1 and 2; Supplementary Fig. 2 online) and primary AML cells (Fig. 2b, groups 1 and 2). In all experiments, no changes in morphology or in CD15 or CD15s expression levels were observed between precultured cells (on day 0), compared with cultured untreated or isotype mAb-treated cells on day 3.

Using the CD15s-specific mAb CSLEX-1 (ref. 25) to quantify expression of this determinant, we found that increased expression of CD15 following anti-CD44 mAb-induced myeloid differentiation is accompanied by a decrease in CD15s levels (Fig. 2, groups 9 and 10; Supplementary Fig. 2); similar results were obtained using mAb HECA-452 (Fig. 2, groups 17 and 18), which recognizes a sLe^x-like epitope. As with CD15, incubation of cells with isotype control mAb did not change CD15s expression (data not shown). The reciprocal changes in CD15 and CD15s expression associated with myeloid differentiation prompted us to test two nonmutually exclusive processes that could account for this observation: (i) preferential neosynthesis of the Le^x epitope with

concurrent decreased sLe^x production, and/or (ii) desialylation of cell-surface sLe^x determinants (Fig. 1).

Lactosamine synthesis is not required for increased CD15

To assess whether increased CD15 expression during CD44-induced differentiation results from *de novo* synthesis, cells were treated with anti-CD44 mAb in the presence of 2-acetamido-1,3,6-tri-*O*-acetyl-4-fluoro-D-glucopyranose (4-F-GlcNAc, 1). 4-F-GlcNAc incorporates into poly-*N*-acetyllactosamine chains and blocks the formation of neo-synthesized Le^x and sLe^x epitopes: the non-nucleophilic fluorine substitution at the 4 position of GlcNAc terminates poly-lactosamine synthesis without interfering with homeostatic pathways of protein synthesis and cell growth⁷. Insofar as all $\alpha(2-3)$ -sialyltransferase enzymes are confined to the Golgi apparatus²⁶, $\alpha(2-3)$ -sialyllactosamine-bearing membrane molecules require intracellular assembly of nascent lactosamine scaffolds. We therefore first assessed the inhibitory effect of 4-F-GlcNAc on poly-lactosamine synthesis on HL60 and AML cells by measuring the recovery of CD15s expression following sialidase treatment of these cells cultured in the presence or absence of this agent. As previously observed in nonmyeloid cells following sialidase treatment⁷, CD15s re-expression was markedly diminished in the presence of 4-F-GlcNAc compared with control cells (return of CD15s consistently <15% of medium control), which indicates that 4-F-GlcNAc blunts *de novo* poly-lactosamine synthesis required for expression of CD15s. HL60 and AML blasts were thus cultured with or without anti-CD44 mAbs for 3 d, in the presence or absence of

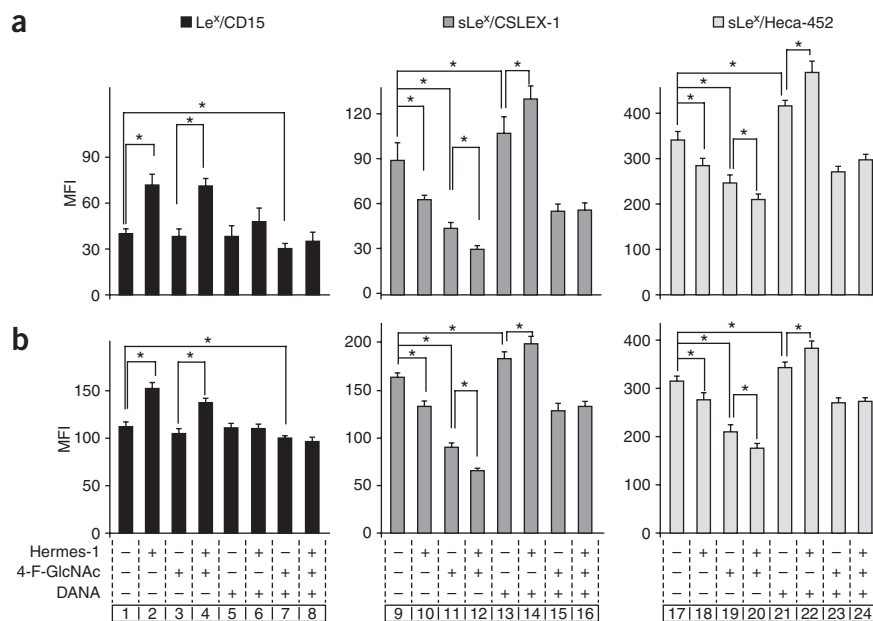


Figure 2 CD44 ligation-induced changes in expression of CD15s and CD15. (a,b) HL60 cells (a) and primary AML blasts (b) ($n = 5$) were treated with Hermes-1 for 72 h in the presence (+) or absence (-) of 4-F-GlcNAc, and/or DANA. Expression of CD15 and CD15s was determined by flow cytometry. b is one representative experiment out of five AML specimens with analysis in triplicate cultures. Values are mean \pm s.d. Statistical significance ($P \leq 0.05$) for respective comparison groups is shown by brackets and asterisk.

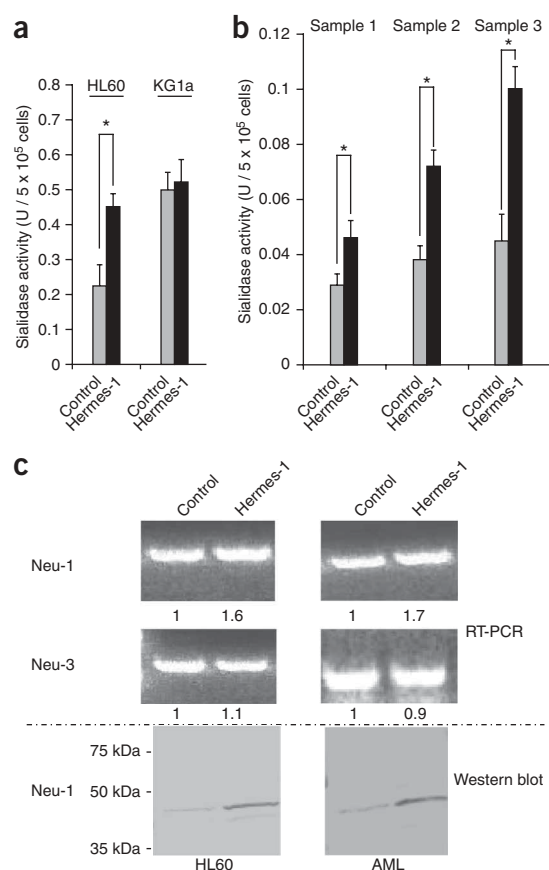


Figure 3 CD44 ligation increases sialidase activity on myeloid cells. (a,b) Cell-surface sialidase activity was measured using 4-MU-NANA as substrate on HL60 and KG1a cells (a) and human AML cells (b) (samples 1, 2 and 3), cultured in the presence or absence of Hermes-1 for 48 h. Values are mean \pm s.d. Statistical significance ($P \leq 0.05$) for respective comparison groups in a and b is shown by brackets and asterisks. (c) Top, representative ethidium bromide-stained gels of PCR-amplified RNA encoding sialidases Neu-1 and Neu-3 from HL60 cells and AML blasts treated with isotype-matched mAb (control) or Hermes-1 (48 h treatment). Numbers indicate the relative expression of RT-PCR product normalized against GAPDH control. Bottom, western blot analysis of Neu-1 protein expression in HL60 cells and AML blasts treated with isotype mAb (control) or Hermes-1 (48 h treatment).

4-F-GlcNAc. Incubation with 4-F-GlcNAc alone (that is, in the absence of anti-CD44 mAb treatment) significantly ($P \leq 0.05$) diminished the expression of CD15s on all cells (Fig. 2, groups 11 and 19; Supplementary Fig. 2) without appreciable effects on CD15 levels (Fig. 2, group 3; Supplementary Fig. 2), which indicates that steady state synthesis and surface turnover of CD15s is greater than that of CD15. Consistent with these results, Hermes-1-associated decreased expression of CD15s was greater in the presence of 4-F-GlcNAc than that observed with Hermes-1 alone (Fig. 2, groups 11 and 12, and groups 19 and 20; Supplementary Fig. 2). However, when used in combination with Hermes-1, 4-F-GlcNAc did not dampen the increase of CD15 induced by the anti-CD44 mAb (Fig. 2, compare groups 2, 3 and 4; Supplementary Fig. 2), which indicates that the CD44-mediated increase of CD15 does not require *de novo* poly-lactosamine synthesis. Notably, semiquantitative RT-PCR analysis of glycosyltransferases creating sLe^x-specific modifications (ST3GalIV and FTVII) showed no decrease in transcripts following anti-CD44

treatment, which indicates that the observed decrease in CD15s display was not a consequence of diminished gene expression for such enzymes. Importantly, there was also no appreciable increase in transcripts encoding fucosyltransferases directing CD15 expression (FTIV and FTIX¹⁶) during differentiation induced by anti-CD44 mAbs (Supplementary Fig. 3 online). Thus, the observed differences in CD15 and CD15s were not accompanied by altered gene expression of relevant enzymes that fucosylate terminal lactosamines critical for synthesis of respective glycans¹⁷.

Sialidase inhibition blunts increased Le^x expression

To assess the contribution of $\alpha(2-3)$ -sialidases to the observed increase in CD15 expression, we inhibited the activity of these enzymes using the potent sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA, 2)²⁷. HL60 cells and AML blasts were treated with anti-CD44 mAbs in the absence or presence of 100 μ M DANA, and expression of CD15 and CD15s was analyzed by flow cytometry. Treatment of cells with DANA significantly ($P \leq 0.05$) abrogated the anti-CD44-induced changes in CD15 (Fig. 2, compare group 2 to group 6; Supplementary Fig. 2) and CD15s (Fig. 2, compare group 10 to group 14, Supplementary Fig. 2 (for CSLEX-1) and group 18 to group 22 (for HECA-452)), which indicates that the increased CD15 and decreased CD15s expression during myeloid differentiation is sialidase dependent. Importantly, in the presence of DANA, Hermes-1 treatment induced a slight increase in CD15s (Fig. 2, compare groups 13 and 14, and groups 21 and 22; Supplementary Fig. 2). Taken together, these results indicate that both CD15s synthesis and sialidase activity are induced by CD44 ligation, but the increase in sialidase activity dominates, such that the overall expression pattern upon CD44 ligation is a decrease in CD15s expression with an accompanying increase in CD15. Consistent with these findings, the CD44 mAb-induced increase in CD15s in the presence of DANA (Fig. 2, group 14, Supplementary Fig. 2 for CSLEX-1; and group 22 for HECA-452) was abrogated when 4-F-GlcNAc was used simultaneously with anti-CD44 treatment (group 16 for CSLEX-1, and group 24 for HECA-452). Collectively, these data indicate that CD15 does not undergo heightened synthesis or degradation coincident with differentiation, and they exclude a role for the addition of sialic acid onto the core trisaccharide (by action of sialyltransferases) in the creation of CD15s.

CD44 ligation increases sialidase activity

To directly analyze whether CD44 ligation modulates sialidase activity in AML cells, we measured degradation of the exogenous sialidase substrate 2'-*(4*-methylumbelliferyl-*N*-acetyl- α -*D*-neuraminic acid) (4MU-NANA, 3)²⁸⁻³⁰. Cells were treated with Hermes-1 for 48 h before measurement of sialidase activity. Cell viability (measured by propidium iodide incorporation and trypan blue exclusion) was preserved. Hermes-1 induced a significant ($P \leq 0.05$) increase in the surface sialidase activity of HL60 cells and of AML blasts obtained from three different donors (Fig. 3a,b). These findings provide direct evidence that CD44-induced differentiation causes the elaboration of sialidases that are responsible for the modulation of CD15 level by converting CD15s into CD15. Consistent with this result, treatment of KG1a cells, a human leukemia cell line that does not differentiate with CD44 ligation²⁰, does not induce CD15 expression or morphologic changes, and there is no associated increase in sialidase activity (Fig. 3a). Importantly, increased sialidase expression seems to be a consequence of differentiation and not a precipitant for differentiation, as direct addition of $\alpha(2-3)$ -sialidases (from either *Vibrio cholerae* or *Streptococcus pneumoniae*) to cultures of leukemia cells and of mobilized hematopoietic progenitors results in (expected) marked

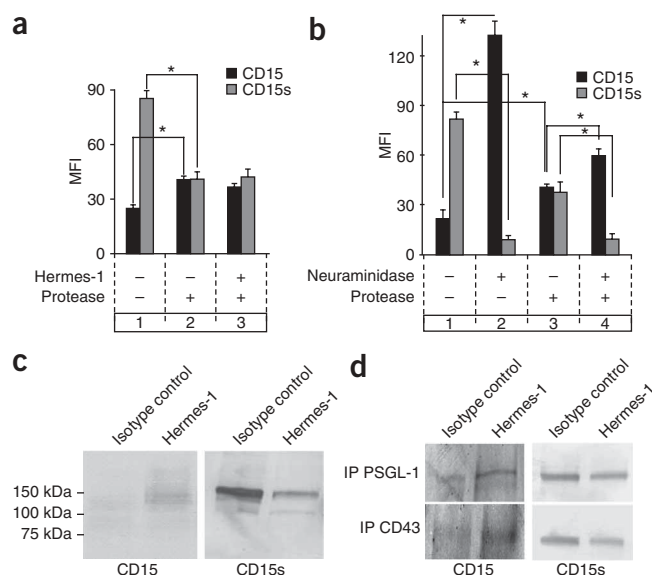


Figure 4 CD44 ligation increases CD15 and decreases CD15s expression on glycoproteins of myeloid cells. **(a)** HL60 cells were first cultured with Hermes-1 (+) or isotype control mAb (-) for 72 h, then treated with protease (bromelain) (+) or buffer alone (-) immediately before flow cytometric analysis of CD15 and CD15s expression. **(b)** HL60 cells were treated with neuraminidase (+) and/or protease (+) or buffer (-), respectively, before flow cytometric analysis of CD15 and CD15s expression. Values are mean \pm s.d. Statistical significance ($P \leq 0.05$) for comparison groups in **a** and **b** is shown by brackets and asterisks. **(c)** Western blot analysis of CD15 and CD15s expression on HL60 cells treated with isotype control mAb or Hermes-1. **(d)** Western blot analysis of CD15 and CD15s expression on PSGL-1 and CD43 immunoprecipitated from HL60 cells treated with isotype control mAb or Hermes-1. For each experiment, one representative of three is shown.

increases in CD15 and decreases in CD15s expression (by MFI, consistently >five-fold increase in CD15 expression and >80% decrease in CD15s), without inducing any morphologic changes (data not shown). Though four distinct sialidases have been described in mammalian cells (Neu-1 to Neu-4)^{28–33}, only Neu-1 and Neu-3 can hydrolyze 4MU-NANA (refs. 28–31,33). To assess whether the increased sialidase activity reflected changes in expression of these enzymes, semiquantitative RT-PCR analysis of transcripts encoding these products was performed. Treatment of HL60 cells with Hermes-1 for 48 h was associated with increased Neu-1 transcript levels and no change in Neu-3 (Fig. 3c). Consistent with these findings, western blot analysis showed increased levels of Neu-1 protein for both HL60 and AML blasts (Fig. 3c), but no changes in Neu-3 were observed.

CD44-induced CD15 expression occurs on glycoproteins

Because CD15 and CD15s determinants are present on both glycoproteins and glycolipids³⁴, and both are potential targets of human sialidases, we sought to determine their relative contribution to the increase of CD15 and the decrease of CD15s induced by CD44 mAbs. Two overlapping experimental approaches were taken. In the first set of experiments, HL60 cells untreated or treated with Hermes-1 for 72 h were digested with bromelain to cleave surface proteins immediately before flow cytometric analysis of CD15 and CD15s levels (Fig. 4a). The efficacy of protein digestion was confirmed by a lack of CD44 expression in the bromelain-treated cells compared with control cells. We observed that bromelain treatment on HL60 cells, in the absence of Hermes-1, resulted in paradoxically increased MFI for CD15 (compare groups 1 and 2), which indicates that membrane proteins natively shield display of prominent glycolipid expression of CD15; the contrary is true for CD15s, which is markedly diminished following bromelain digestion, which indicates dominance of glycoprotein expression of CD15s (compare groups 1 and 2). To exclude the possibility that these results reflect sialidase contamination of bromelain, we treated cells first with a different protease, proteinase K, and measured CD15 and CD15s levels. Subsequent treatment with bromelain showed no further changes in either CD15 or CD15s levels. Following protease digestion in the absence of (group 2) or following (group 3) anti-CD44 mAb treatment, there was no difference in membrane CD15 levels or in CD15s levels. Thus, anti-CD44 mAb-induced differentiation does not alter CD15s displayed on glycolipids

and does not increase CD15 displayed on glycolipids as measured by flow cytometry.

In the second series of experiments, HL60 cells were treated with exogenous sialidase (*V. cholera* neuraminidase at 0.1 U ml⁻¹). As expected, this treatment resulted in an almost complete abrogation of CD15s expression on intact cells, with a commensurate profound increase in CD15 expression (almost six-fold) (Fig. 4b, groups 1 and 2). To analyze the contribution of membrane glycolipids to the observed increase in CD15 expression, HL60 cells were digested with bromelain following sialidase treatment; as shown in Figure 4b (group 2 and 4), CD15s expression on bromelain-resistant scaffolds was diminished, similar to that of intact cells, which indicates that native membrane glycolipids are accessible to exogenous sialidase digestion. However, the associated increase of CD15 levels on glycolipids is modest (1.4-fold) compared with that observed on intact cells (~six-fold). Collectively, these results show that although both membrane glycoproteins and glycolipids are substrates of exogenous sialidase, the observed increase in CD15 results predominantly from conversion of CD15s to CD15 on glycoproteins.

To examine this issue further, western blot analysis of CD15 and CD15s expression was performed on cell lysates from cells treated with Hermes-1 or isotype mAb (control). Anti-CD44 treatment increases CD15 expression on multiple glycoproteins, prominently at 130 and 150 kDa, thus confirming the results of protease studies (Fig. 4c). This increase of CD15 was accompanied by a decrease in CD15s on glycoproteins of similar molecular weight (Fig. 4c). Two glycoproteins known to bear CD15s substitutions, PSGL-1 (refs. 8,22) and CD43 (refs. 23,24), migrate on SDS/PAGE within a 130–150 kDa range. To examine whether these membrane proteins are targets of sialidase digestion, PSGL-1 and CD43 were immunoprecipitated from cell lysates of HL60 before and after anti-CD44 mAb treatment, and western blot analysis was performed using Heca-452 and anti-CD15 mAb. CD15 expression increases on both PSGL-1 and CD43 after anti-CD44 treatment (Fig. 4d). Concomitant with this increase, there was a decrease of CD15s expression on each of these proteins.

G-CSF increases sialidase activity in myeloid progenitors

To determine whether conversion of CD15s into CD15 by sialidases occurs during differentiation of native myeloid progenitors, immature myeloid cells were obtained from human bone marrow. We compared sialidase activity on myeloid cells before and after treatment with G-CSF. As shown in Figure 5a, myeloid cells express a ~two-fold increase in sialidase activity after 72 h of treatment with G-CSF, coincident with an increase in CD15 expression (Fig. 5b) and with morphological changes of differentiation (data not shown). Moreover, increased CD15 expression was blunted by the use of DANA (Fig. 5b), showing the important role played by sialidases in the creation of

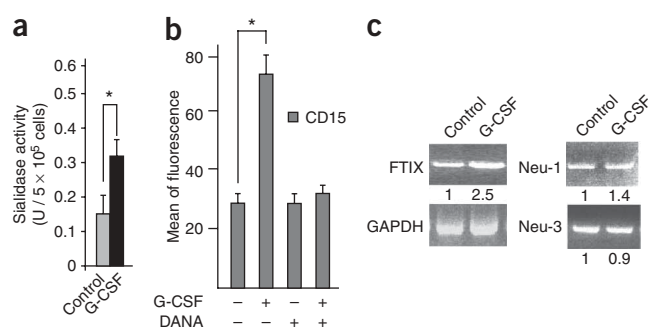


Figure 5 G-CSF treatment increases sialidase activity on myeloid cells. **(a)** Sialidase activity was measured from myeloid progenitor cells, before (control) and after treatment *ex vivo* with G-CSF for 72 h. **(b)** Expression of CD15 of native progenitor cells untreated (–) or treated (+) with G-CSF for 72 h and in the presence (+) or absence (–) of DANA was determined by flow cytometry using anti-CD15 mAb. One representative experiment out of three specimens with analysis in triplicate cultures is shown. Values are mean \pm s.d. Statistical significance ($P \leq 0.05$) for comparison groups in **a** and **b** is shown by brackets and asterisks. **(c)** Typical ethidium bromide-stained gels of PCR-amplified RNA from human hematopoietic progenitor cells with or without (control) G-CSF treatment. Numbers indicate the relative expression of RT-PCR product normalized against GAPDH control.

CD15 displayed during native myeloid differentiation. Altogether, these data indicate that conversion of immature to mature phenotypes among native myeloid cells is dynamically associated with induction of surface sialidase activity. In keeping with these findings, we observed an increase in transcripts encoding Neu-1 coincident with myeloid differentiation (Fig. 5c).

DISCUSSION

In this study, we sought to determine the molecular mechanisms regulating increased expression of CD15 associated with human myeloid cell differentiation. So far, augmented expression of membrane carbohydrate determinants has been shown to be secondary to induction of specific glycosyltransferases within the endoplasmic reticulum and Golgi apparatus^{15–17}. Our data indicate, for the first time (to our knowledge), that post-Golgi enzymatic glycoside hydrolysis is the predominant mechanism for the enhanced expression of a key cell-surface glycan determinant.

The sLe^x and Le^x structures consist of monosaccharide substitutions on a common lactosamine core. We used a specific inhibitor of lactosamine synthesis (4-F-GlcNAc) to define the contribution of *de novo* glycan synthesis to expression of both CD15 and CD15s during myeloid differentiation. CD44 ligation had no effect on CD15 and CD15s levels when sialidase and lactosamine synthesis were inhibited simultaneously, which demonstrates the importance of lactosamine synthesis for creation of CD15s. This result also excludes a role for addition of sialic acid onto the core trisaccharide in the synthesis of CD15s. Additionally, there was no significant change in the expression of fucosyltransferases FucTIV and FucTIX, which indicates that the increase in CD15 associated with myeloid differentiation did not result from augmented levels of these fucosyltransferases directing CD15 synthesis¹⁷. Collectively, our data show that during anti-CD44-induced myeloid differentiation, both CD15s synthesis and sialidase activity are increased; however, the increase in sialidase activity dominates, such that the overall expression pattern is an increase in CD15 and a decrease in CD15s expression.

We have previously shown that G-CSF increases CD15s expression of native myeloid cells³⁵, and we report here concomitant increased

expression of CD15. Importantly, G-CSF-induced maturation of native hematopoietic progenitors was also accompanied by increased cell-surface sialidase activity. Notably, despite the fact that G-CSF treatment of native progenitor cells increases FucTIV (ref. 35) and FucTIX, inhibition of sialidase by DANA abrogated $\sim 90\%$ of the CD15 increase induced by G-CSF treatment. Thus, differentiation-associated CD15 expression is dominantly regulated by sialidase digestion of CD15s in both leukemic blasts and native progenitors. Conversely, anti-CD44 treatment did not induce surface sialidase activity or changes in expression of either CD15s or CD15 levels on the primitive human leukemic cell line KG1a, which does not differentiate following CD44 ligation. Notably, neither an increase in sialidase activity nor an increase in CD15 levels drives myeloid cell maturation, as exogenous sialidase treatment strongly increased CD15 expression and decreased CD15s expression, yet with no differentiation-specific morphologic changes.

Four distinct sialidases have been described in mammalian cells (Neu-1, Neu-2, Neu-3 and Neu-4)^{28–33}. Changes in their expression have been detected in cells that were induced to differentiate or that were activated^{28–31,33}. The sialidases can be cytosolic or membrane-associated, and they can have different substrate specificities. Only Neu-1 and Neu-3 can hydrolyze 4MU-NANA (refs. 28–31,33). Both enzymes are inhibited by DANA. Neu-3 is surface associated, and although it is best recognized for cleaving sialic acid expressed on glycolipids (gangliosides), it can also serve as a sialidase for glycoproteins³². Neu-1 is predominantly lysosomal and preferentially cleaves glycoproteins, but it can be translocated to the membrane or secreted by the cell²⁷. The absence of changes in expression for Neu-3 by RT-PCR and western blot is consistent with the observed relative absence of desialylation of glycolipids during myeloid differentiation, whereas the finding of increased Neu-1 expression by RT-PCR and western blot suggests that myeloid differentiation is associated with induction of this enzyme.

We show here that the bulk of CD15s-to-CD15 transformation on myeloid cells occurs predominantly on glycoproteins, despite the fact that glycolipids express relatively more CD15 than glycoproteins. Among these glycoproteins was PSGL-1, a sialomucin expressed on most leukocytes and well-recognized as playing a critical role in cellular trafficking^{7,36} and in hematopoiesis³⁷. These functions are mediated by PSGL-1 engagement with E-selectin, P-selectin and L-selectin, via the presentation of CD15s on the PSGL-1 protein backbone. We found that increased CD15 expression, coincident with a decrease of CD15s, occurs also on CD43, another sialomucin that binds E-selectin²⁴.

There is increasing evidence that modulations in sialic acid display impact normal and pathologic hematopoietic cell development. Aberrantly enhanced sialylation, with concomitant CD15 “masking,” plays an important role in leukemogenesis and cancer cell metastasis³⁸, and it has also been shown that abnormal sialylation of myeloid cells in chronic myelogenous leukemia inhibits binding to hematopoietic growth factors, which contributes to the block in differentiation³⁹. Consistent with these observations, treatment of HL60 cells with sialidase increases their binding to G-CSF (ref. 39) and their proliferation in response to GM-CSF (ref. 40). Accordingly, by increasing sialidase activity in AML cells, CD44 ligation may not only promote their binding to growth factors necessary for their terminal differentiation, but may also prevent their seeding distant sites by decreasing expression of CD15s, the canonical selectin binding determinant. These notions support future studies for use of anti-CD44 mAbs in differentiation therapy of AML. Moreover, our studies indicate a role for sialidase in regulating CD15 expression on nonmalignant hematopoietic cells, raising the possibility that microenvironment-specific

expression of sialidase activity could impact cell adhesion events during cell development. Beyond implications for engagement of PSGL-1 in hematopoiesis, sialylation regulates binding of several myeloid antigens, such as the well-recognized myeloid-specific marker CD33, a member of the siglec family^{41,42}. By modulating binding to siglecs and other lectins, the discrete changes in expression of $\alpha(2-3)$ -linked sialyl residues could significantly impact hematopoiesis by directing localization of progenitors to distinct bone marrow micro-environmental 'niches'. Similarly, sialidase expression may regulate CD15 expression on nonhematopoietic cells and among cells of nonhuman mammals, with implications for the elaboration of the well-recognized SSEA-1 antigen (CD15¹), an important marker of embryonic stem cells and neural progenitors in mice^{43,44}. Our results thus support a general model whereby dynamic induction of sialidase activity offers biological versatility to existing cell-surface glycan display. Further studies are warranted to elucidate how localized variations in sialidase activity may direct critical cell-cell and cytokine-cell interactions within specialized growth microdomains.

METHODS

Antibodies, reagents and sources of human cells. See **Supplementary Methods** online. All human samples were obtained with procedures approved by the Human Experimentation and Ethics Committees of the Partners Cancer Care Institutions (MGH, BWH and DFCI).

Induction of differentiation of AML blasts. AML blasts were seeded in triplicate at $3 \times 10^5 \text{ ml}^{-1}$ and treated with Hermes-1 ($20 \mu\text{g ml}^{-1}$) or with rat IgG2a ($20 \mu\text{g ml}^{-1}$) for up to 3 d, then analyzed for differentiation as described below.

Treatment with G-CSF. Cells ($1 \times 10^6 \text{ cells ml}^{-1}$) were cultured for 72 h in the presence of recombinant human G-CSF (Amgen; 10 ng ml^{-1}). In all experiments, L-selectin expression of cells was determined by flow cytometry to test the efficacy of G-CSF treatment (confirmed by loss of L-selectin expression, as previously described)³⁵. After 72 h of treatment, cells were used for flow cytometry analysis and sialidase activity assay.

Treatment with sialidase, DANA and 4-F-GlcNAc. Cells were cultured at $5 \times 10^6 \text{ ml}^{-1}$ in the presence or absence of neuraminidase (0.1 U ml^{-1} , *V. cholerae* neuraminidase) at 37°C for 1 h and up to 3 d (for differentiation studies, where neuraminidase was added every 24 h). For neuraminidase inhibition, cells were treated with DANA (0.1 mM), which was added every 24 h to cell cultures. For metabolic inhibitor treatment, cells were incubated with 4-F-GlcNAc ($100 \mu\text{M}$).

Analysis of myeloid differentiation. Myeloid differentiation was evaluated by cell morphology analysis and by measurement of changes in CD15 expression. To assess morphological changes, cytopspins of treated and untreated cells were stained with May-Grunwald Giemsa (Sigma-Aldrich) for analysis by light microscopy. For all experiments, expression of CD15 and of CD15s was measured by flow cytometry before cell culture ($t = 0$) and following 3 d of culture; because HL60 cells and all native human myeloid cells express variable levels of CD15, the degree of cell differentiation was measured by the relative increase in mean channel fluorescence of CD15-positive cells. Dead cells, labeled by propidium iodide staining, were excluded from the analysis.

Cell extract, immunoprecipitation and western blot analysis. Cells (5×10^5 to 1×10^7) were solubilized at 4°C for 1 h in 50 to 200 μl lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton, 150 mM NaCl, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl, $10 \mu\text{g ml}^{-1}$ pepstatin, $10 \mu\text{g ml}^{-1}$ leupeptin and $10 \mu\text{g ml}^{-1}$ aprotinin). The protein content was determined using the Bradford assay (Sigma-Aldrich). PSGL-1 and CD43 were immunoprecipitated using protein-G agarose (Invitrogen). For western blots, all lanes were normalized for total protein ($30 \mu\text{g sample}^{-1}$); samples were diluted in reducing sample buffer and separated in 4–20% gradient SDS/PAGE gels. Resolved proteins were transferred to Sequi-blot polyvinylidene difluoride (PVDF) membrane

(Bio-Rad). Blots were blocked with fetal bovine serum (FBS) for 2 h at room temperature (25°C) and incubated with anti-CD15 or HECA-452 antibodies ($1 \mu\text{g ml}^{-1}$) overnight at 4°C . The membranes were washed and incubated for 1 h with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (for CD15) or goat anti-rat IgM (for Heca-452) at 1:2,000 dilution and developed by using western blue stabilized substrate for alkaline phosphatase (Promega). For western blot analysis of Neu-1 and Neu-3, blots were probed respectively with polyclonal anti-Neu-1 antibody (Santa Cruz Biotechnology) and anti-Neu-3 mAb (MBL Japan).

Measurement of sialidase activity. Sialidase activity was assessed using 4-MU-NANA as substrate. Cells (2×10^6) were resuspended in 200 μl of a solution containing 0.05 M sodium acetate pH 4.4 and 0.125 mM 4-MU-NANA at pH 4.5 and incubated at 37°C for 2 h. The reaction was terminated by adding 1 ml of a solution containing 0.133 M glycine, 0.06 M NaCl and 0.083 M Na_2CO_3 pH 10.7, just before the fluorometric determination of released 4-methylumbelliferone (4-MU) at excitation wavelength 365 nm and emission wavelength 450 nm using a Photon Technologies International fluorometer. The concentration of 4-MU generated was measured by subtracting the fluorescence reading of the blank from the fluorescence reading of the samples and comparing the result to a standard curve generated from solutions of 4-MU (Sigma Aldrich). One unit of sialidase is the amount required to release one nmol of 4-MU per 10^6 cells.

Protease digestion. Cells ($5 \times 10^6 \text{ ml}^{-1}$) were treated for 1 h at 37°C with 0.1% bromelain in RPMI 1640 medium or with proteinase K ($200 \mu\text{g ml}^{-1}$) in Hanks' balanced salt solution containing 150 mM NaCl, then washed in $1 \times$ phosphate-buffered saline, and Le^x and sLe^x expression levels were analyzed by flow cytometry. The efficacy of protein digestion by bromelain was verified by observed decreased expression of CD44.

Reverse transcription (RT)-PCR. Equal amounts of RNA were used as templates for RT-PCR with Titan One Tube RT-PCR system (Roche Molecular Biochemicals) and the primers described in **Supplementary Table 1** online. Optimal PCR conditions were 94°C for 2 min, 60°C for 45 s, and 72°C for 1 min on a PTC-200 Peltier Thermal cycler (MJ Research). Amplified bands were visualized after 1% agarose gel electrophoresis of the PCR products.

Statistical analysis. Results are presented as mean \pm s.d. for the indicated number of experiments. Statistical analyses were performed using one-way ANOVA and Student's *t*-test. A value of $P \leq 0.05$ was considered to be statistically significant. Brackets in figure legends are shown only for relevant data displaying statistically significant differences.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

S.Z.G. designed the research, performed the experiments, analyzed the data and wrote the paper. R.S. conceived the study, designed the research, analyzed the data, wrote the paper, provided funding for the research and supervised all experimentation.

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