bjh short report

Sialic acid-binding immunoglobulin-like lectin (Siglec)-15 is a rapidly internalised cell-surface antigen expressed by acute myeloid leukaemia cells

Huan Cao,[1] Andreas Neerincx,[2] Bernard de Bono,[3] Ursula Lakner,[4] Catherine Huntington,[5] John Elvin,[5] Emma Gudgin,[6] Clare Pridans,[6] Mark A. Vickers,[1] Brian Huntly,[6] John Trowsdale[2] and Alexander D. Barrow[7]

[1]School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, [2]Immunology Division, Pathology Department, University of Cambridge, Cambridge, UK, [3]Auckland Bioengineering Institute, University of Auckland, Auckland, New Zealand, [4]Medical Faculty, University of Tübingen, Tübingen, Germany, [5]MedImmune, Cambridge, [6]University of Edinburgh Centre for Inflammation Research, Edinburgh, UK, and [7]Department of Microbiology and Immunology (DMI), The University of Melbourne, Melbourne, Australia

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Correspondence: Huan Cao, School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK.
E-mail: h.cao@abdn.ac.uk

Summary

Sialic acid-binding immunoglobulin-like lectin (Siglec)-15 has recently been identified as a critical tumour checkpoint, augmenting the expression and function of programmed death-ligand 1. We raised a monoclonal antibody, A9E8, specific for Siglec-15 using phage display. A9E8 stained myeloid leukaemia cell lines and peripheral cluster of differentiation (CD)33$^+$ blasts and CD34$^+$ leukaemia stem cells from patients with acute myeloid leukaemia (AML). By contrast, there was minimal expression on healthy donor leucocytes or CD34$^+$ stem cells from non-AML donors, suggesting targeting Siglec-15 may have significant therapeutic advantages over its fellow Siglec CD33. After binding, A9E8 was rapidly internalised (half-life of 180 s) into K562 cells. Antibodies to Siglec-15 therefore hold therapeutic potential for AML treatment.

Keywords: Siglec-15, acute myeloid leukaemia, antibody, phage display, endocytosis.

Introduction

Acute myeloid leukaemia (AML) is a significant health burden with an incidence of around four cases/100 000/year[1] with ~3000 cases/year in the UK. Curative treatment is intensive, arduous for the patient, and requires long hospital admissions. In contrast to other malignancies, immunotherapeutic agents are yet to play a major role in treatment.

Identifying suitable tumour-specific antigens is a major barrier in developing immunotherapies.[2] In AML, the most promising target has been the sialic acid-binding immunoglobulin-like lectin (Siglec), cluster of differentiation (CD)33, which is targeted by gemtuzumab ozogamicin (GO, Mylotarg) a toxin-(calicheamicin) conjugated antibody.[3] Despite setbacks, a randomised trial of 237 patients aged ≥60 years and ineligible for intensive chemotherapy showed an improved overall survival (hazard ratio 0·69) in those assigned to GO induction and consolidation compared with best supportive care.[4]

Sialic acids are nine carbon-based sugars found at the termini of most mammalian glycan structures. The Siglecs are immunoglobulin superfamily receptors that bind to sialic acids and are commonly expressed on immune cells, particularly of the myeloid lineage.[5] Most Siglecs are inhibitory, but some are activating, namely Siglecs-14, -15 and -16.[6,7] A rapidly evolving sub-family of Siglecs, known as the CD33-related (CD33r) Siglecs, demonstrate potential as AML targets.[8]

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Siglec-15 is a new member of the Siglec family primarily expressed on a subset of myeloid cells.[6] Siglec-15 has also been shown to bind the tumour antigen sialyl-Thomsen-nouvelle antigen (sTn).[6] Siglec-15 is unusual in that it is equipped with both negative and positive signalling motifs.[6] Siglec-15 contains a lysine residue in the transmembrane that potentially mediates association with signalling adaptor molecules, such as DNAX-activating protein 10 (DAP10), DAP12 and the Fc receptor common γ (FcRγ) chain, to promote cellular activation, yet also contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM)-like motif known as immunoreceptor-tyrosine based switch motif (ITSM) that generally mediates inhibitory signals.[9] In support of this, Siglec-15 has recently been identified as a novel check point inhibitor with mutually exclusive expression to the classical checkpoint inhibitor, programmed death-ligand 1 (PD-L1), suggesting antibodies to Siglec-15 may have a role as immune checkpoints that can complement PD-L1 inhibitors.[10]

In the present study, we developed a monoclonal antibody (A9E8) to Siglec-15 using phage display. We show that A9E8 stains Siglec-15 on $CD33^+$ peripheral blood monocytic cells from patients with AML but not from healthy donors. A9E8 also stains $CD34^+$ leukaemic stem cells (LSCs) in AML, but not equivalent $CD34^+$ cells from non-AML donors. Finally, A9E8 induces the rapid internalisation of Siglec-15 on myeloid leukaemia cells, such as K562, suggesting therapeutic potential for AML treatment.

Results

Generation of Siglec-15 specific antibody, A9E8

Phage display was employed to select a specific antibody, A9E8, against Siglec-15 (Figure S1), which binds to overexpressed cell surface Siglec-15 as well as endogenous Siglec-15 from leukaemia cell lines such as K562 (Figure S2; Figure S3). Clustered regularly interspaced short palindromic repeats (CRISPR) deletion of *SIGLEC15* from the cell-line K562 resulted in loss of A9E8 staining (Figure S1D). Enhanced surface expression of full length Siglec-15 (N-terminal FLAG-tagged) was observed when paired with clones stably expressing DAP10, DAP12 and FcRγ (Figure S4).

Absence of Siglec-15 surface expression on peripheral blood leucocytes from healthy donors

Siglec-15 is absent on the surface of most mature leucocytes from healthy donors: T cells (0·2% $CD3^+$), B cells (0·2% $CD19^+$) and natural killer cells (0·3% $CD3^-CD56^+$), but a small (~0·8%) proportion of monocytes ($CD14^+$) showed Siglec-15 surface expression (Figure S5A). The overall negative staining of mature circulating leucocytes was confirmed on nine healthy donors (Fig 1D). We report very weak Siglec-15 surface expression on 7% of cultured macrophages derived from peripheral blood monocytes and extremely low Siglec-15 surface expression on ~4% of dendritic cells before lipopolysaccharide (LPS) stimulation (Figure S5B), both of which were lost following 24 h of LPS stimulation (Figure S5B). These results show that in healthy donors, expression of Siglec-15 is low or absent on the cell surfaces of most mature circulating leucocytes as well as *in vitro* cultured macrophages and dendritic cells.

Siglec-15 is a prominent surface antigen on circulating myeloid blasts

In contrast, significantly higher Siglec-15 surface expression was found on peripheral blood cells from nine of the 12 patients with AML tested (Fig 1A–D), with an average of ~18% of the subpopulation being Siglec-15 positive as compared to only 0·6% for circulating peripheral blood mononuclear cells (PBMCs) from healthy donors (Fig 1D). CD33 and Siglec-15 show comparable level of percentage expression on circulating leucocytes (Fig 1E), giving a strong correlation (Pearson's correlation $r = 0{\cdot}9823$, $n = 12$) (Fig 1E). Three of the patients with AML were profiled in detail, where we see consistent co-expression of Siglec-15 with CD14 (Fig 1A–C). CD33 has been reported to be expressed on 90% of AML blasts.[11] Surprisingly, we identified one AML patient amongst the 12, patient B (Fig 1B), who was CD33-negative, expressed a significant level of Siglec-15 on 20% of the peripheral cells. The strong correlation between Siglec-15 and CD33, as well as the existence of CD33-Siglec-15^+ phenotypes is supported by public data found in Figure S6. The overall A9E8 binding specificity to circulating AML leucocytes over healthy cells is also reflected in the fluorescence intensities (Fig 1F). These data indicate that Siglec-15 is expressed on significantly higher percentage of circulating leucocytes in patients with AML than in healthy donors.

A9E8 induces a rapid internalisation of Siglec-15 from the cell surface

A fast rate of Siglec-15 internalisation would be advantageous for toxin-conjugated antibody targeting of Siglec-15-positive AML blasts. Rapid internalisation of Siglec-15 is expected from its cytoplasmic ITSM motif (sequence: SNYENL), conforming to the classical endocytosis YxxΦ motif (X, any amino acid; Φ, hydrophobic residue).[12] Extremely rapid endocytosis of Siglec-15 was noted on K562 cell line through cross-linking of A9E8 with a half-life of only 174 s (Fig 2A). A9E8 does not induce endocytosis if Siglec-15 lacking its cytoplasmic tail (Fig 2B), indicating the observed phenomenon cannot be explained by dissociation of the A9E8 antibody from the cell surface. Rapid endocytosis is also observed in AML peripheral blood samples (Fig 2C), consistent with K562 cell line and an AML cell line, U937 (Figure S7). Z-stack confocal microscopy showed the presence of A9E8 antibody in permeabilised K562 cells (Fig 2D, Ei-v).

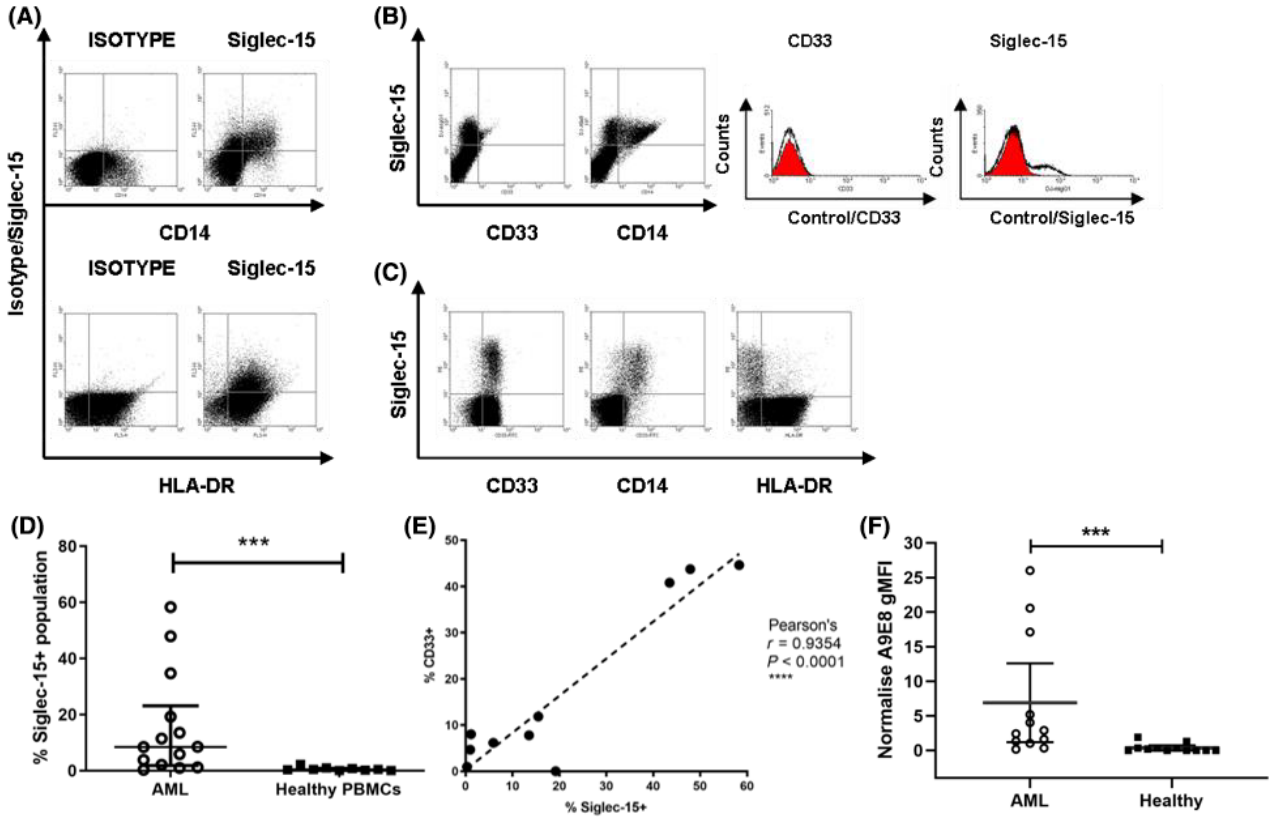

Fig 1. High sialic acid-binding immunoglobulin-like lectin (Siglec)-15 surface expression on circulating blood cells from patients with acute myeloid leukaemia (AML). Cell surface expression of Siglec-15 on peripheral blood leucocyte preparations from the peripheral blood of patients with AML. Nine out of 12 patients with AML showed significant cell surface expression of Siglec-15 compared to healthy peripheral blood leucocytes. (A) Representative plots from 'Patient A' showing high levels of cell-surface Siglec-15 expression on AML blasts [36% of all peripheral blood mononuclear cells (PBMCs)]. Siglec-15 is co-expressed with CD14 and human leucocyte antigen-DR isotype (HLA-DR). Negative control: mouse immunoglobulin G1 (IgG1) isotype antibody. (B) Representative plots (left panels) from 'Patient B' showing negative cell surface staining for CD33 on AML blasts, although 20% of blasts are positive for Siglec-15. Histograms (right panels) comparing cell surface expression of CD33 and Siglec-15 on AML blasts from 'Patient B' (isotype control staining, filled histograms; anti-CD33/Siglec-15 antibody staining, open histograms). (C) Representative plots from 'Patient C' showing 10% of AML blasts express Siglec-15 on the cell surface, most of which were $CD33^{+}CD14^{+}HLA\text{-}DR^{-}$. (D) Percentage of total circulating PBMCs expressing cell-surface Siglec-15 from 14 patients with AML (open circles) and nine healthy donors (solid squares). Normalised percentage positive expression shown. Mann–Whitney two-tailed *t*-test, median and interquartile ranges are indicated (***$P = 0{\cdot}0002$). (E) Correlation between normalised percentage $Siglec\text{-}15^{+}$ and $CD33^{+}$ populations from 10 patients with AML. Pearson's correlation: $r = 0{\cdot}9354$, $P < 0{\cdot}0001$, two tailed test. (F) Geometric mean of fluorescence intensity (gMFI) values of A9E8 binding normalised against isotype binding for PBMCs expressing cell-surface Siglec-15 from 12 patients with AML (open circles) and 12 healthy donors (solid squares). Mann–Whitney two-tailed *t*-test, median and interquartile ranges are indicated (***$P = 0{\cdot}0002$). [Colour figure can be viewed at wileyonlinelibrary.com]

Circulating peripheral cells from non-AML patients but who have had granulocyte-colony stimulating factor (G-CSF) mobilisation showed no A9E8 staining (Figure S8A,B). These peripheral cells offer a higher representation of progenitor cells, including $CD34^{+}$ stem cells (Figure S8A). These data provide support for the specificity of the A9E8 antibody in AML bone marrows, as healthy $CD34^{+}$ stem cells would not be targeted. Furthermore, $CD34^{+}CD38^{-}$ subpopulations of circulating peripheral cells from four AML and three non-AML G-CSF-treated patients were compared (Figure S8C–E). This so-called leukaemic stem cells (LSCs) compartment appears to have high levels of Siglec-15 expression in AML, but not in non-AML equivalents (Figure S8C–E).

Discussion

We used phage display to develop an antibody, A9E8, specific for Siglec-15 and observed expression on circulating cells in patients with AML with contrasting low/negligible expression on healthy cells. The antibody also induced rapid endocytosis, with a half-life of 3 min compared to >100 min for Siglec-5 and -9.[8] These properties support A9E8 as a potential novel therapeutic agent for the treatment of AML.

CD33 is expressed on monocytes and macrophages of healthy individuals. Due to the relatively low expression of Siglec-15 on mature myeloid cells, A9E8 has the potential to be associated with less myelosuppression than GO.[5,13] We

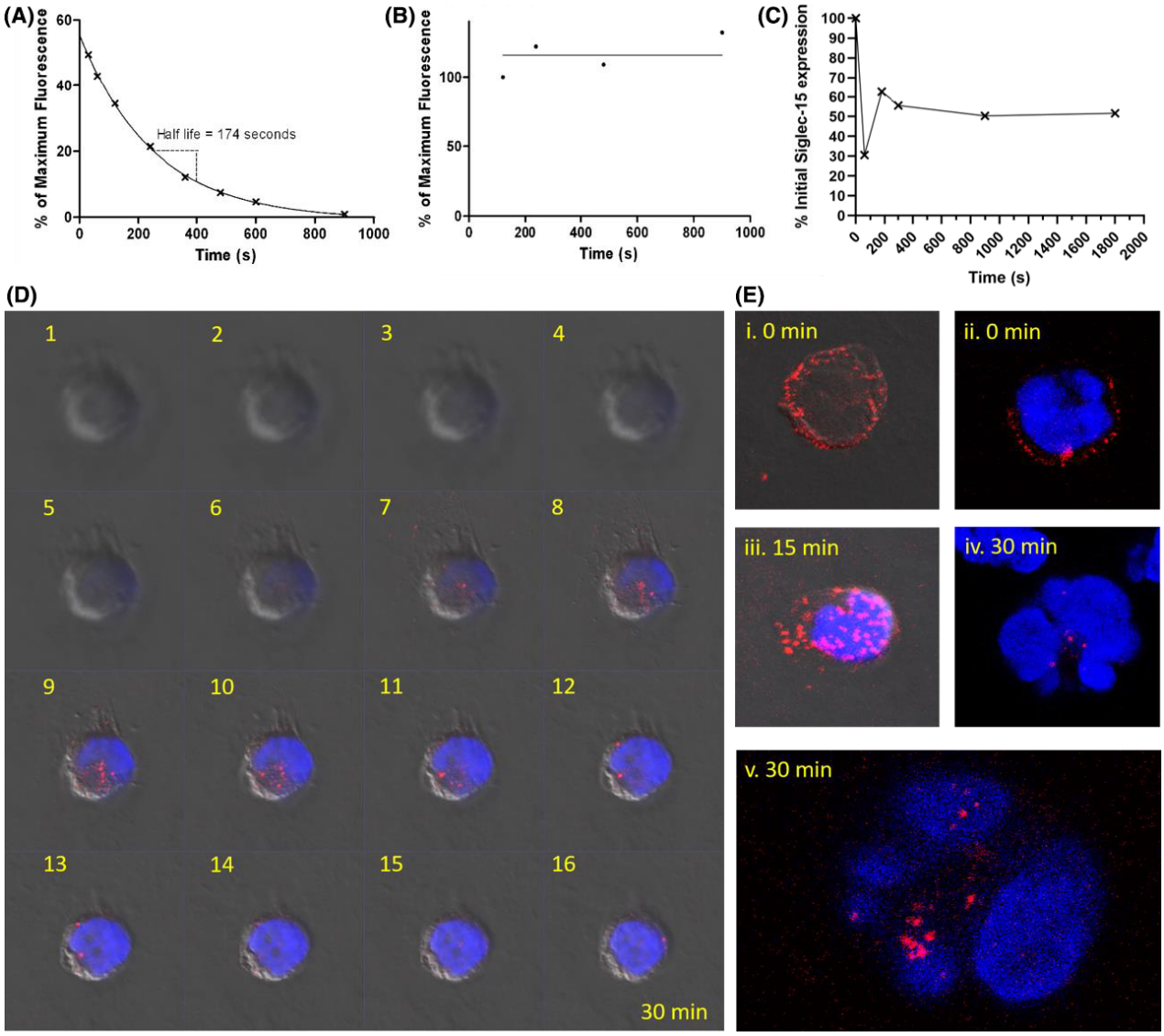

Fig 2. Internalisation of sialic acid-binding immunoglobulin-like lectin (Siglec-15) from the cell surface. Following endocytosis, mean fluorescence of A9E8 binding above isotype control antibody staining was calculated for the time-points indicated, expressed as percentage of the maximum surface staining without incubation at 37°C. (A) K562 endocytosis: one phase decay curve is fitted to the time-points where 37°C incubation was applied (≥30 s) to derive a half-life of 174 s, r^2 = 0·995. Data are representative of two independent experiments. (B) A parallel experiment using Chinese hamster ovary (CHO) cells stably expressing cell surface Siglec-15 from a pDisplay construct indicating that the A9E8 antibody does not dissociate from the cell surface during the first 15 min of incubation at 37°C. (C) Endocytosis of A9E8 in an acute myeloid leukaemia (AML) sample. Siglec-15 positive subpopulation percentage is tracked over 30 min following A9E8 staining and 37°C incubation. Percentage of the initial expression is shown. Data representative of two AML samples. (D, 1–16) K562 endocytosis: Z-stack cross-sections using confocal microscopy obtained following 30 min of antibody-mediated cross-linking of cell-surface Siglec-15 using the A9E8 antibody. Following fixation and permeabilisation, internalised A9E8 antibody was detected using goat anti-mouse (GaM) Alexa 555-conjugated secondary antibodies (GaM, red); 4',6-diamidino-2-phenylindole (DAPI) nuclear DNA, blue; differential interference contrast (DIC), greyscale. (E, i-v) Images at various time-points following A9E8 antibody cross-linking demonstrates the dynamic internalisation of Siglec-15 molecules from the cell surface of K562 cells: time = 0 min (ii, DIC and GaM; iii, DAPI and GaM); time = 15 min (iv, DIC and GaM) and time = 30 min (v, DAPI and GaM). [Colour figure can be viewed at wileyonlinelibrary.com]

found that in one case of $CD33^-$ AML, there was significant expression of Siglec-15, suggesting that Siglec-15 may be a viable target on blasts that lack CD33.[5,13,14] While a potential toxin-conjugated A9E8 may target some osteoclasts that express Siglec-15, depletion of Siglec-15^+ osteoclasts may provide the benefit of preventing bone-loss in AML disease and AML metastasis to bone.[15]

Collectively, our present data demonstrate A9E8 is specific for AML blasts and LSCs, but not healthy cells, and induces rapid Siglec-15 internalisation on the K562 myeloid

leukaemia cell line. A9E8 is therefore a promising antibody for targeting conjugated toxins to AML blasts and the LSC compartment. Further characterisation of the expression pattern of Siglec-15 on haematopoietic cells from patients with AML and healthy donors should be undertaken.

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Ethical approval

Ethical approval was obtained for the study 'The causes of clonal blood cell disorders' Research Ethics Committee reference number is 07/MRE05/44. All patients used for the study were consented.

Author contributions

Huan Cao performed the research, helped to design the research and drafted the manuscript. Andreas Neerincx helped to design the CRISPR work. Bernard de Bono, Ursula Lakner helped performed the research. Catherine Huntington and John Elvin helped to design the research and draft the manuscript. Emma Gudgin and Claire Pridans contributed essential reagents and samples. Mark A. Vickers helped to write the manuscript. Alexander D. Barrow, John Trowsdale and Brian Huntly all helped to design the research and write the manuscript.

Conflict of interest

MedImmune has filed the following patent: 'Anti-Siglec-15 antibodies and uses thereof'. United States Patent 9447192. This patent has been reviewed in 2015.[16]

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Assessment of monoclonal antibody A9E8 specific for Siglec-15.

Fig S2. Siglec-15 transcript expression.

Fig S3. Siglec-15 surface expression on myeloid leukaemic cell lines.

Fig S4. Adaptor association of Siglec-15.

Fig S5. Siglec-15 is not expressed by most healthy peripheral blood leucocytes.

Fig S6. Expression of *CD33* and *SIGLEC15* are positively correlated in AML.

Fig S7. Endocytosis of A9E8 antibody on AML cell line U937 and AML peripheral blood cells.

Fig S8. Granulocyte-colony stimulating factor (G-CSF) mobilised peripheral blood staining with A9E8.

Fig S9. Amino acid alignment of splice variants of SIGLEC15.

Data S1. Materials and methods.

References

1. De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. *Blood Cancer J*. 2016;**6**:e441.
2. Goswami M, Hourigan CS. Novel antigen targets for immunotherapy of acute myeloid leukemia. *Curr Drug Targets*. 2017;**18**:296–303.
3. Bross PF, Beitz J, Chen G, Chen XH, Duffy E, Kieffer L, et al. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin Cancer Res*. 2001;**7**:1490–6.
4. Amadori S, Suciu S, Selleslag D, Aversa F, Gaidano G, Musso M, et al. Gemtuzumab ozogamicin versus best supportive care in older patients with newly diagnosed acute myeloid leukemia unsuitable for intensive chemotherapy: results of the randomized phase III EORTC-GIMEMA AML-19 trial. *J Clin Oncol*. 2016;**34**:972–9.
5. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol*. 2014;**14**:653–66.
6. Angata T, Tabuchi Y, Nakamura K, Nakamura M. Siglec-15: an immune system Siglec conserved throughout vertebrate evolution. *Glycobiology*. 2007;**17**:838–46.
7. Cao H, Lakner U, de Bono B, Traherne JA, Trowsdale J, Barrow AD. SIGLEC16 encodes a DAP12-associated receptor expressed in macrophages that evolved from its inhibitory counterpart SIGLEC11 and has functional and non-functional alleles in humans. *Eur J Immunol*. 2008;**38**:2303–15.
8. Nguyen DH, Ball ED, Varki A. Myeloid precursors and acute myeloid leukemia cells express multiple CD33-related Siglecs. *Exp Hematol*. 2006;**34**:728–35.
9. Kikuchi-Maki A, Catina TL, Campbell KS. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. *J Immunol*. 2005;**174**:3859–63.
10. Wang J, Sun J, Liu LN, Flies DB, Nie X, Toki M, et al. Siglec-15 as an immune suppressor and potential target for normalization cancer immunotherapy. *Nat Med*. 2019;**25**:656–66.
11. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID. Expression of normal myeloid-associated antigens by acute leukemia cells. *Blood*. 1986;**67**:1048–53.
12. Collawn JF, Stangel M, Kuhn LA, Esekogwu V, Jing S, Trowbridge IS, et al. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell*. 1990;**63**:1061–72.
13. Mulford D. Antibody therapy for acute myeloid leukemia. *Semin Hematol*. 2008;**45**:104–9.
14. Taussig DC, Pearce DJ, Simpson C, Rohatiner AZ, Lister TA, Kelly G, et al. Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood*. 2005;**106**:4086–92.
15. Frisch BJ, Ashton JM, Xing L, Becker MW, Jordan CT, Calvi LM. Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. *Blood*. 2012;**119**:540–50.
16. Horne GA, Kinstrie R, Copland M. Novel drug therapies in myeloid leukemia. *Pharm Pat Anal*. 2015;**4**:187–205.

