Development of Anti-CD32b Antibodies with Enhanced Fc Function for the Treatment of B and Plasma Cell Malignancies

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ABSTRACT

The sole inhibitory Fcγ receptor CD32b (FcγRIIb) is expressed throughout B and plasma cell development and on their malignant counterparts. CD32b expression on malignant B cells is known to provide a mechanism of resistance to rituximab that can be ameliorated with a CD32b-blocking antibody. CD32b, therefore, represents an attractive tumor antigen for targeting with a monoclonal antibody (mAb). To this end, two anti-CD32b mAbs, NVS32b1 and NVS32b2, were developed. Their complementarity-determining regions (CDR) bind the CD32b Fc binding domain with high specificity and affinity while the Fc region is afcylated to enhance activation of FcγRIIAs on immune effector cells. The NVS32b mAbs selectively target CD32b+ malignant cells and healthy B cells but not myeloid cells. They mediate potent killing of opsonized CD32b+ cells via antibody-dependent cellular cytotoxicity and phagocytosis (ADCC and ADCP) as well as complement-dependent cytotoxicity (CDC). In addition, NVS32b CDRs block the CD32b Fc-binding domain, thereby minimizing CD32b-mediated resistance to therapeutic mAbs including rituximab, obinutuzumab, and daratumumab. NVS32b mAbs demonstrate robust antitumor activity against CD32b+ xenografts in vivo and immunomodulatory activity including recruitment of macrophages to the tumor and enhancement of dendritic cell maturation in response to immune complexes. Finally, the activity of NVS32b mAbs on CD32b+ primary malignant B and plasma cells was confirmed using samples from patients with B-cell chronic lymphocytic leukemia (CLL) and multiple myeloma. The findings indicate the promising potential of NVS32b mAbs as a single agent or in combination with other mAb therapeutics for patients with CD32b+ malignant cells.

Introduction

Multiple myeloma is a complex disease characterized by the presence of profound intratumoral heterogeneity that increases progressively from the stages of monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic/smoldering multiple myeloma to symptomatic or clinical multiple myeloma. Despite the approval of novel mAb medicines targeting CD38 (daratumumab; ref. 1) and SLAMF7 (elotuzumab; ref. 2) as well as small-molecule classes including proteasome inhibitors and thalidomide analogue immunomodulatory drugs, the majority of patients relapse. Likewise, non-Hodgkin Lymphoma (NHL) and CLL remain incurable diseases despite the advent of medicines including mAbs and BTK inhibitors that have revolutionized the treatment paradigm. As such, there remains a need for highly effective novel therapeutics.

We report herein that malignant B cells including CLL and NHL (diffuse large B-cell lymphoma, DLBCL; mantle cell lymphoma, MCL; and follicular lymphoma), frequently express CD32b, the sole inhibitory FcγR. These findings are largely consistent with prior observations by others (3–5). Importantly, we also demonstrate that CD32b is expressed on primary multiple myeloma cells collected by bone marrow (BM) aspiration. While it is not apparent that increased CD32b expression affords malignant cells a survival advantage, data suggest that such upregulation may be a mechanism of resistance to therapeutic antibodies with an Fc-dependent mode of action (MoA) such as rituximab. Specifically, expression of CD32b on tumor cells is associated with reduced clinical benefit from rituximab-containing regimens (5). Consistent with this hypothesis, increased CD32b expression is a bona fide mechanism of resistance to alemtuzumab in vivo, and shRNA-mediated knockdown of CD32b sensitized leukemic cells to alemtuzumab-mediated ADCC (6). Furthermore, Roghanian and colleagues (7) have described a novel Fc wild-type (WT) hIgG1 mAb, which binds the CD32b Fc-binding domain via its CDR, thereby preventing CD32b from driving resistance to therapeutic antibodies with an Fc-dependent MoA, for example, anti-CD20–targeted mAb therapy. Taken together, these data support a functional role for CD32b as a mechanism of resistance to antibodies with Fc-dependent antitumor activity. Furthermore, Clynes and colleagues demonstrated that tumor-targeting therapeutic antibodies...
with Fc-dependent activity were more active in CD32b knockout mice (8). These authors hypothesized that in the absence of CD32b inhibitory signaling, CD32b−/− macrophages were able to mount a more robust ADCC/ADCP response. These seminal observations provided an initial rational for the therapeutic inhibition of CD32b signaling.

While an Fc WT anti-CD32b mAb has been proposed as a therapeutic for B-cell malignancies (7, 9), we hypothesized that a CD32b-targeted mAb with enhanced ability to engage activatory Fc receptors and to activate innate immune cells would be a superior therapeutic for patients with treatment-naïve or relapsed/refractory multiple myeloma and B-cell malignancies. Herein we describe the discovery and characterization of two glycoengineered fully human anti-CD32b mAbs, NVS32b1_Fc_afuc and NVS32b2_Fc_afuc, with enhanced effector function achieved via the increased affinity of their Fc to the activatory FcR family expressed on effector immune cells.

Materials and Methods

Cell lines used in these experiments were Mycoplasma-free based on monthly testing and their identity was confirmed by Affymetrix SNP 6.0.

CD32b expression analysis

The anti-CD32b mAb, clone 2B6, was conjugated with AlexaFluor 647 (Molecular Probes Alexa Fluor Antibody Labeling Kit, Thermo Fisher Scientific) and used to detect CD32b expression on primary cells and cell lines. Patient samples for CD32b expression analysis were purchased from Conversant Bio, now part of Discovery Life Science. NVS32b Antibody characterization

Chinese hamster ovary (CHO) cells stably expressing WT cynomolgus CD32b, huCD32a, huCD32b, or huCD32b encompassing the indicated amino acid mutations were generated using the Flp-In technology and were cultured in DMEM (Lonza) containing 10% FBS (Seradigm) and 600 μg/mL Hygromycin B (Life Tech) following Hygromycin B selection. Cells were harvested using 0.25% Trypsin (Gibco), washed with PBS, and resuspended at 2 × 10⁷ cells/mL before addition to 96-well U-bottom plates (Falcon; 100 μL/well). Cells were then stained in FACS Buffer containing Alexa Fluor 647-conjugated (Molecular Probes) huCD32b-reactive antibodies (all in a human IgG1 [N297A] scaffold) for 30 minutes at 4°C. Samples were then washed and acquired on a FACSCanto II. Binding intensities were measured based on AF647 MFI values.

ADCC assay

A primary natural killer (NK) cell–based ADCC assay was utilized to assess the Fc-dependent activity of NVS32b antibodies against CD32b−/− Jeko1, Dauidi and Karpass422 target cell lines. PBMCs were isolated from a Leukopak (HemaCare) via a ficoll gradient. NK cells were then negatively selected using an NK Cell Isolation Kit (Miltenyi Biotech) and then incubated in basic media overnight (RPMI/10%FBS/15 mmol/L HEPES/1% l-glutamine/1% penicillin–streptomycin) containing 0.1 ng/mL rhIL2 (recombiant human IL2; PeproTech). The following day, CD32b−/− target cells were labeled with Calcein acetoxyl–methyl ester (Calcein-AM; Molecular Probes) and transferred to a 96-well U-bottom plate at a concentration of 10,000 cells per well. The cells were then preincubated for 20 minutes with the indicated antibodies before NK-cell addition, followed by 2–4 hours of incubation in a 37°C incubator with 5% CO₂. Supernatants were then collected and free Calcein levels therein were measured with a fluorescence counter (Envision, Perkin Elmer). Target cells only and target cells with 1% Triton X-100 (Sigma) were included as controls to measure spontaneous and maximal Calcein release, respectively. The percent-specific target cell lysis was calculated using the following formula: [(sample – spontaneous release)/(maximal release–spontaneous release)] × 100%.

ADCP assay

For this assay, luciferase-expressing Dauidi or Karpass422 cells were cocultured with macrophages differentiated from primary human monocytes. A Monocyte Isolation Kit II (Miltenyi Biotech) was used to enrich monocytes from PBMCs, and cells were grown for 7 days in X-VIVO15 medium (Lonza) containing 10% FBS and 10 ng/mL macrophage–colony stimulating factor. The differentiated macrophages were then allowed to adhere to 96-well flat-bottom plates (200,000 cells/well), after which 10,000 target cells were added per well and cocultured for 18 hours in a 37°C incubator with 5% CO₂. Bright Glo (Promega) was used to quantify target cell lysis relative to maximal luminescence observed in antibody-free wells. For imaging-based ADCP assays, CellTracker Red CMTPX and CellTracker Green CMFDA dyes were used to label target cells and macrophages, respectively, as per manufacturer’s recommendation 1 hour before imaging. Macrophages had been allowed to adhere in 8-well Ibidi chambers for 3 days prior to imaging. Target cells and AF647-conjugated antibodies were added to the macrophages immediately before imaging with a Zeiss spinning disk confocal microscope (Axio Observer.Z1). The full imaging protocol is included in the Supplementary Methods.
CDC assay
Karpas422 cells were plated in white flat-bottomed 96 well plates (8,500 cell/well) to which the indicated antibodies were added in triplicates, and then rabbit complement (diluted 1:8 in assay buffer in 50 μL) was added to a total of 150 μL. Blank (no sample or complement), negative (no Ab), and positive (no Ab + 1% Triton X-100) controls were prepared in opunticulate. After a 2-hour incubation at 37°C cell killing was quantified using a CellTiterGlo Kit (Promega) based on provided directions.

Macrophage- and monocyte-derived dendritic cell ADCC
Macrophages were prepared as in ADCC assays. Monocyte-derived dendritic cell (moDC) were differentiated from monocytes for seven days in RPMI containing 1% r-glutamine, 10% FBS, 50 ng/mL GM-CSF, and 50 ng/mL IL4 (PeproTech). These cells were either used directly (immature moDCs) or matured via treatment with 100 ng/mL IFNγ and 200 ng/mL LPS overnight (mDCs). Macrophages, DCs, and Daudi cells were labeled with Calcein and seeded at 60,000 cells/well. NK cells from a different healthy donor were added at 120,000 cells/well (effector-to-target ratio [E:T] = 2). Antibodies were then added as indicated and Calcein release was measured after 2-hour coculture. Target cell lysis were then calculated as described above for ADCC assays.

FcγR activation reporter assay
Jurkat cells expressing an NFAT promoter-driven luciferase reporter gene were transduced to overexpress the high activity V158 variant of human FcγRIIa (CD16a). A similar Jurkat reporter cell line over-expressed using the FcγRIV (F(Fc)-gamma) was used to measure receptor FcγR activation. NVS32b antibody–opsonized target cells induce cross-linking of FcγRIIa via the Fc-FcγR interaction, leading to the activation of downstream signaling factors and NFAT binding to the promoter, driving luciferase expression. A total of 1.5 × 10⁴ target cells/well were cocultured with Jurkat reporter cells at a 6:1 E:T ratio in 120 μL RPMI + 10% FBS in a 96-well white plate. NVS32b mAbs, rituximab, or daratumumab were added at indicated concentrations. Control wells included reporter cells only, reporter cells and antibody, or reporter cells and target cells. After 4 hours, Bright Glo (Promega) was used to quantify luciferase activity based on provided directions.

For secondary MoA studies using the FcγR activation reporter assay, Ramos and MM1.s cells stably expressing huCD32b were expressed on B cells, that is, receptor occupancy (RO), was determined from the residual binding of NVS32b2. CD32b RO was determined from the residual binding of NVS32b2_Fc_WT to viable CD45lowSSChigh granulocytes were determined on an Attune platform with a LEAP autosampler, a nanoACQUITY UPLC System, similar to those described previously (11), using a Waters HDx-MS spectrometry platform with a LEAP autosampler, a nanoACQUITY UPLC System, and a Synapt G2 mass spectrometer. All measurements were carried out using a minimum of three analytic triplicates.

Whole blood depletion, NK-cell activation, CD32b expression, and cytokine release
The antibodies as indicated were incubated in lithium-heparinized whole blood from healthy human donors (recruited through an internal Novartis donation program after providing informed consent in compliance with national guidelines) at 37°C and 5% CO2, in a humidified incubator for 24 hours. Absolute counts of viable CD4⁺CD19⁻CD3⁻ B cells, viable CD4⁺CD14⁻ monocytes, and viable CD45lowSSChigh granulocytes were determined on an Attune NxT flow cytometer (Thermo Fisher Scientific) after immunophenotyping of stimulated whole blood with respective fluorochrome-conjugated marker antibodies following the exclusion of dead cells using a viability dye. The percentage of depletion was calculated on the basis of the change in absolute counts induced by the test antibody in comparison with the absolute counts measured with the buffer control: 100 – [absolute counts (test condition × 100/absolute counts (buffer))].

NK-cell degranulation after 24 hours of whole blood stimulation with the WT and afucosylated NVS32b2. CD32b RO was determined from the residual binding of AF488-conjugated NVS32b2_Fc_WT to viable CD45⁺CD19⁻CD3⁻ B
cells in relation to the buffer control: 100 – [AF488 MFI (NVS32b2) × 100/AF488 MFI (buffer)]. Cytokine concentrations (IL6, IL8, IL1β, IFNγ, TNFα, and MCP-1) in plasma supernatants after 24-hour whole blood incubation with the test and control antibodies were measured using a Milliplex MAP Kit (Millipore) on a Luminex MapPix instrument.

CD32b expression on viable CD45+CD19+CD3− B cells, viable CD45−CD14+ monocytes, and viable CD45lowSSChigh granulocytes in fresh and 24-hour buffer control–cultured whole blood was determined by flow cytometry using NVS32b2 Fc WT-AF488 and appropriate fluorochrome-conjugated surface marker antibodies.

**Human immune complex and treatment of huCD32b CHO cells and moDCs**

Human polyclonal serum IgG (1,000 μg/mL in PBS; Millipore Sigma) was heat aggregated at 63°C for 30 minutes. Resultant immune complex (IC) were labeled with goat F(ab’)2 anti-human IgG - (Fab’)2 DyLight 550 preadsorbed (1:100; Abcam) on ice for 20 minutes, protected from light.

CHO cells stably expressing huCD32b were plated in a 96-well round-bottom plate (100,000 cells/well) with NVS32b2 or isotype control antibodies for 4 hours at 37°C, followed by the addition of 10 μg of fluorescently labeled ICs per well. Cells were then incubated on ice for 60 minutes, washed twice in FACS buffer, and resuspended in FACS buffer prior to analysis by flow cytometry.

Immature moDCs were prepared as above and plated (100,000 cells/well) in AIM-V Media containing 200 IU/mL rhIL4 and 400 IU/mL rh GM-CSF. Cells were pretreated with 10 μg/mL of IgG control or NVS32b1 for 30 minutes on ice, after which ICs (200 μg/mL) were added, and cells were cultured overnight at 37°C. Cells were then washed twice with FACS buffer, resuspended in 30 μL of FACS buffer containing Human TruStain FcX (BioLegend), and stained using fluorescently conjugated antibodies specific for human CD14 and CD86 (BioLegend). Following washing, cells were analyzed by flow cytometry.

**Ex vivo functional assay using patient samples**

The Fc-dependent activity of NVS32b antibodies against primary CD32b+ CLL tumor cells was assessed via a modified ADCC assay. Briefly, CLL patient PBMCs and healthy allogeneic donor NK cells (used due to the very low NK-cell frequencies in patients with CLL) were processed as above. These cell types were then combined at a 1:1 ratio.

Figure 1.

CD32b is a promising tumor antigen for targeting with an Fc-competent mAb in B and plasma cell malignancies. A, CD32b mRNA expression was found across B and plasma cell malignancies. B and C, CD32b protein abundance on primary cells from patients with multiple myeloma (MM) or CLL was determined by flow cytometry and presented as MFI. D, Novel anti-CD32b–targeted highGMab mediate primary NK-cell lysis of CD32b+ Jeko1 cells via ADCC. E, CD32b-targeted NVS32b1(Fc_WT) displayed antitumor activity in mice harboring established, BM disseminated Jeko1 xenografts as monitored by whole body luciferase signal. F, NVS32b1_Fc_WT also demonstrated activity against subcutaneously implanted Daudi xenografts. This in vivo activity is Fc dependent as the N297A variant, which lacks binding to mouse FcγR, is inactive. *p < 0.01, **p < 0.001; statistically significant difference in change in tumor volume or luciferase signal with treatment relative to vehicle or isotype control.
Phage-display antibody campaign yielded anti-CD32b antibodies that exhibited efficient antitumor activity in mouse models of B-cell malignancies

Therapeutic antibodies against human CD32b extracellular domain (ECD) were generated by the selection of clones that bound to human CD32b protein with the highly homologous human CD32a as a counter screen. The Morphosys HuCAL PLATINUM phage display library was used as a source of antibody variants. The phagemid library is based on the HuCAL concept (15, 16) and employs the CysDisplay technology for displaying the Fab on the phage surface (17).

Two antibody variants were discovered using ELISA screening and two were discovered using next-generation sequencing screening and subsequently subcloned into a human IgG1 expression vector. Given the limited epitope space differentiating CD32b ECD from CD32a ECD, antibodies with specificity for CD32b over CD32a lacked binding to cynomolgus monkey, rat, or mouse CD32b. These Fab WT antibodies were tested for the ability to kill CD32b-positive JeKo-1 cells in an ADCC assay with primary NK cells. In this assay, the cytotoxic activity of antibody NVS32b1 far exceeded that of all other antibodies profiled (Fig. 1D) suggesting that it had a unique ability to opsonize CD32b-positive cells and to subsequently recruit and activate NK cells. To determine whether this activity translated in vivo, the Fab WT hIgG1 version (NVS32b1_Fc_WT) was administered to female scid.beige mice harboring established, BM-homing JeKo-1 tumor xenografts. A single 5 mg/kg i.v. dose of NVS32b1_Fc_WT resulted in marked tumor growth inhibition as demonstrated by reduced luciferase signal relative to the irrelevant isotype control–treated mice (P < 0.001, Fig. 1E). These data demonstrate that the CD32b-targeted antibody NVS32b1_Fc_WT is capable of suppressing the in vivo growth of an aggressive orthotopic xenograft intravenously inoculated into immunocompromised mice.

To explore the Fc-dependent activity of NVS32b1_Fc_WT, its Fc region was engineered to prevent the interactions with mouse FcγR. Specifically, NVS32b1 was generated as an Fc-silent version (NVS32b1_N297A), which lacks or has reduced binding to FcγRs (18). The CDR regions which recognize CD32b were not altered. Female nude mice harboring established Daudi xenografts were randomly assigned to receive a weekly intravenous dose of PBS, NVS32b1 in the Fc WT (NVS32b1_Fc_WT, 20 mg/kg) or Fc-silenced (NVS32b1_N297A, 20 mg/kg) format. While NVS32b1_Fc_WT demonstrated robust tumor regression (P < 0.01), the Fc-silent NVS32b1_N297A was largely inactive (Fig. 1F). The data demonstrated that the in vivo antitumor activity of the CD32b-targeted NVS32b1 is dependent upon engagement of mouse immune effector cell via Fc–FcγR interaction.

In light of the promising antitumor activity demonstrated by NVS32b1_Fc_WT, further protein engineering was performed in the heavy chain CDR to optimize the manufacturing process. This effort yielded additional anti-CD32b antibodies, among which NVS32b2 was selected as a close homolog of NVS32b1 with one amino acid difference in the HCDR3 region (Supplementary Table S1). This modification had no impact on binding or activity as NVS32b1 and NVS32b2 were functionally indistinguishable (Supplementary Fig. S2). We first confirmed the specificity of NVS32b1 and NVS32b2 by assessing their binding to CHO cells overexpressing human CD32b or CD32a (CHO_CD32b or CHO_CD32a). The Fc-silenced versions (N297A) of both mAbs were used to prevent any Fc-mediated binding such that binding was exclusively via mAb CDR. The antibodies were individually conjugated with AlexaFluor 647 to allow for the detection of binding using flow cytometry. Both NVS32b1_N297A and NVS32b2_N297A bound strongly to CHO_CD32b cells with similar
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were produced by CHO cells transfected with GDP-6-deoxy-D-lyxo-4-NVS32b1 and NVS32b2, which lack the (fucose synthesis (20). This glycoengineering of the mAb Fc domain increases its affinity to the activatory Fcy receptor IIIa (FcyRIIIa, CD16a), which translates into enhanced Fc-dependent cytotoxic activity (20).

Relative to the Fc WT versions, the afucosylated NVS32b2 and an isotype control resulted in approximately 3 and 9 times improved affinity to human FcyRIIIa V158 and F158, respectively, as assessed by surface plasmon resonance. As anticipated, the N297A-mutant lacked binding to FcyRIIIa (Supplementary Table S2 and S3). The isotype controls showed very low binding affinity to CD32b (Supplementary Table S2) highlighting its relatively low affinity for monomorphic IgG. As NVS32b binding to CD32b is dominated by the CDR, its Kd on CD32b (approximately 40 nmol/L) was not influenced by the Fc backbone (Supplementary Table S3).

Because CD32b expression levels among patient samples are heterogeneous we chose Daudi, (derived from Burkitt’s lymphoma) to represent the low-to-intermediate level and Karpas422 (derived from B-NHL) to represent high level of CD32b expression seen in patients. NVS32b2 mediated potent ADCG against Daudi and Karpas422 by primary NK cells (Fig. 2A); similar results were observed with NVS32b1 (Supplementary Fig. S2D). Consistent with surface plasmon resonance binding data in Supplementary Table S3, a 6- or 23-fold left shift in EC50 in the ADCG assays was observed for afucosylated NVS32b2 relative to its Fc-WT version, indicating a significant increase in the potency. The NVS32b2 mAbs alone do not result in tumor cell lysis as the effector NK cells are required for the ADCG (Supplementary Fig. S2E and S2F). The lack of ADCG activity in the presence of a nontargeting IgG control or the Fc-silent NVS32b2_N297A indicated that target cell lysis required both CDR-mediated binding to tumor cell surface CD32b and Fc/FcyR-mediated engagement with effector cells. In addition, the activation of FcyRIIIa was measured using a reporter cell line (JNL-FcγRIIIa), which reads out NFAT-promoter driven luciferase in engineered Jurkat cells that overexpress FcyRIIIa. Afucosylated NVS32b2 (Fc_a-fuc) demonstrated the highest extent of FcγRIIIa activation, the Fc WT version showed moderate FcγRIIIa activation, whereas the Fc-silent version (NVS32b2_N297A) did not show any activation at all (Supplementary Fig. S2G and S2H). As expected, the three Fc variants of NVS32b1 showed similar extent of activity to NVS32b2 in this assay.

mAbs including rituximab, obinutuzumab, and daratumumab provide significant clinical benefit to patients with B and plasma cell malignancies and have revolutionized the treatment of these diseases. In an effort to understand the therapeutic potential of our Fc enhanced anti-CD32b mAbs, we sought to benchmark their activity to that of these approved mAb therapies. Specifically, we compared the ADCC activity mediated by NVS32b2_Fc_a-fuc, daratumumab, obinutuzumab, and rituximab using Daudi (CD32b+CD38−CD20+) and Karpas422 (CD32b+CD38−CD20+) as target cells (Supplementary Fig. S3A and S3B). NVS32b2 demonstrated robust ADCC activity compared with clinically approved mAbs, highlighting its therapeutic potential.

Subsequently, ADCP and CDC activities of NVS32b against CD32b-positive target cells were characterized. With primary human macrophages as effector cells, afucosylated NVS32b2 mAbs demonstrated more robust killing of Daudi and Karpas422 relative to the Fc WT format (Fig. 2B; Supplementary Fig. S2I and S2J). The lack of target cell death in the presence of an afucosylated IgG control or the Fc-silent version of NVS32b (N297A) confirmed that target cell lysis required both CDR-CD32b and Fc/FcyR binding. Visualized with time-lapse confocal imaging, Daudi cells were rapidly phagocytosed by macrophages in the presence of NVS32b2 (Fig. 2C and D), which supported that target cell killing by macrophages occurred via ADCP. The ability of NVS32b2 to engage complement and subsequently kill CD32b-positive cells was also assessed, where NVS32b2_Fc_a-fuc demonstrated efficient dose-dependent CDC at a higher level than rituximab on CD32b+CD20+ Karpas422 cells (Fig. 2E). This assay measured tumor cell survival rather than plasma membrane integrity, and therefore is not a direct readout for CDC. However, the tumor cell lysis shown here was indeed dependent on the addition of complement-containing serum as the antibodies do not have direct growth inhibition effect on target cells (Supplementary Fig. S2E and S2F).

Next, we determined the ADC activity of NVS32b2 on primary malignant cells from patients with CLL. PBMCs obtained from patients with CLL contain very few NK cells and previous attempts at demonstrating autologous ADCG by mAbs including rituximab have been very challenging. To measure NK-cell-mediated ADC activity ex vivo, we elected to coculture CLL patient-derived PBMCs with NK cells from a healthy allogeneic donor in the presence of NVS32b2, rituximab or control antibodies for 20 hours. NVS32b2_Fc_WT demonstrated efficient dose-dependent CDC at a higher level than rituximab on CD32b+CD20+ Karpas422 cells (Fig. 2E). This assay measured tumor cell survival rather than plasma membrane integrity, and therefore is not a direct readout for CDC. However, the tumor cell lysis shown here was indeed dependent on the addition of complement-containing serum as the antibodies do not have direct growth inhibition effect on target cells (Supplementary Fig. S2E and S2F).

Fce engineered anti-CD32b demonstrates enhanced antitumor activity in vivo

The therapeutic potential of NVS32b1_Fc_WT was demonstrated in initial studies conducted with mice harboring intravenously implanted, bone homing Jeko1 cells (Fig. 1E). To further profile the antitumor activity of Fc-engineered CD32b targeted mAbs, additional in vivo efficacy studies were conducted in higher throughput,
subcutaneous xenograft models. Consistent with its robust Fc-dependent in vitro activity, NVS32b2 demonstrated marked antitumor activity in vivo. Specifically, when administered intravenously at three different dose levels (3, 10, or 30 mg/kg i.v. weekly), afucosylated NVS32b2 achieved dose-dependent exposure and antitumor activity in mice bearing established Daudi xenografts (Supplementary Table S4; Fig. 3A). NVS32b2_Fc_afuc dosed at 10 or 30 mg/kg qw (once weekly) yielded significant (P < 0.01) tumor growth inhibition relative to IgG-treated controls while a dose of 3 mg/kg qw was inactive. The antitumor activity translated into dose-dependent differences in time to endpoint curves (P < 0.01) with a median time to endpoint of 21 days and 38 days for IgG control and NVS32b2_Fc_afuc dosed 10 mg/kg qw, respectively. Median time to endpoint was not reached for mice receiving NVS32b2_Fc_afuc dosed 30 mg/kg qw. Consistent with the in vitro findings described above, the in vivo activity of afucosylated NVS32b1 and NVS32b2 was indistinguishable in mice harboring Daudi xenografts (Supplementary Fig. S2K).

We demonstrated above that malignant plasma cells from a significant portion of patients with multiple myeloma overexpress CD32b. To explore the therapeutic potential of a CD32b-targeted antibody in this setting, NSG mice harboring established Karpas620 multiple myeloma xenografts were treated with NVS32b2 afuc intravenously at 10 mg/kg once weekly. NVS32b2 afuc administered at this dose and schedule resulted in regression of the established Karpas620 xenografts (P < 0.01, Fig. 3B).

Differences in Fc/FcR interactions between mouse and human complicate the assessment of human mAb Fc-dependent activity in mice. Specifically, Overdijk and colleagues (21) demonstrated that therapeutic mAbs in a hlIgG1 scaffold demonstrated reduced Fc-dependent antitumor activity in vivo relative to the same antibody in a mlG2a scaffold; an effect that has the potential to underpredict the Fc-dependent therapeutic activity of a hlIgG1 mAb in mice. Consistent with these reports, we demonstrated species-specific differences in the ability of two Fc enhancement strategies, that is, Fc glycoengineering or sequence engineering, to activate hlFcRIIIa in JNL reporter assays. Specifically, with Daudi target cells, NVS32b1 produced as an afucosylated hlIgG1 (20) or S239D/J332E/A330L (DEL; ref. 22) demonstrated a similar capacity to crosslink and activate hlFcRIIIa (Supplementary Fig. S4A). However, in an mFcRIV reporter assay, afucosylated NVS32b1 was markedly less capable of activating mFcRIV relative to the NVS32b1_DEL hlIgG1 (Supplementary Fig. S4B). The reduced ability of an afucosylated mAb, relative to the Fc-engineered DEL version, to activate mFcRIV is anticipated to be due to the fact that while afucosylation increases the af difference in the overall systemic exposure (Supplementary Table S5) and yielded significant tumor growth inhibition relative to control PBS-treated mice (P < 0.01), with the mlG2a_DE format yielding numerically greater antitumor activity (Fig. 3C). Both antibodies yielded time to endpoint curves that were statistically different from those of the
control mice ($P < 0.01$). Furthermore, NVS32b2_mIgG2a_DE yielded a greater response than NVS32b2_Fc_afuc ($P < 0.01$). Median times to endpoint were 26 and 49 days for control and NVS32b2_Fc_afuc–treated mice, respectively. Median time to endpoint was not reached for mice administered NVS32b2_mIgG2a_DE, highlighting its greater in vivo antitumor activity and the therapeutic potential of a CD32b-targeted mAb with an engineered Fc to optimally engage and activate host effector immune cell FcγR. Several types of immune cells (24) as well as liver sinusoidal endothelial cells (25) are known to express CD32b. Because of the fact that NVS32b1 and NVS32b2 do not bind mouse CD32b, these experiments do not allow for the assessment of how normal tissue target mediated disposition could alter the pharmacokinetic parameters of the NVS32b mAbs. Furthermore, these studies do not enable the preclinical assessment of tolerability associated with CD32b expression on normal cells including liver sinusoidal endothelial cells.

The data described above establish a role for immune effector cells in the cytotoxic activity of NVS32b1 and NVS32b2. We next explored how intravenous administration of NVS32b2 impacted the tumor microenvironment. The DEL mutation was utilized in this experiment for its inability to engage complement (22), thereby allowing us to specifically probe Fc/FcγR–dependent immunomodulatory effects of NVS32b in vivo. Specifically, the impact of NVS32b2_hlgG1_DEL on the abundance of intratumoral macrophages, an important immune effector cell in mice, was assessed. Nude mice bearing established Daudi xenografts were administered a 10 mg/kg i.v. dose of NVS32b2_Fc_DEL or a nontargeted isotype control hIgG1_DEL. After either a single dose or two weekly doses, NVS32b2_Fc_DEL yielded increases in intratumor F4/80 positivity, indicating an increased mouse macrophage infiltration. Following a single intravenous dose, increases in tumor macrophage infiltration were observed at 7 days postdose followed by a time-dependent return to pretreatment levels through 21 days postdose (Fig. 3D). No change in intratumoral F4/80 positivity was observed in the tumors of mice administered the nonbinding hIgG control antibody, demonstrating that macrophage infiltration requires CDR-mediated binding of NVS32b2_Fc_DEL to CD32b expressed on tumor cells. Because the CDR of NVS32b2 does not bind to murine CD32b, the mouse macrophages can only be engaged via the interaction between mouse FcγRs and the Fc of NVS32b2. These data provide direct evidence that the antitumor activity of an Fc enhanced NVS32b mAb is associated with the intratumoral infiltration of mouse macrophages.
The unique epitopes of NVS32b mAbs enable them to reverse CD32b-mediated resistance

CD32b expression on malignant B cells is a proposed mechanism of resistance to rituximab in patients with NHL (3) and alemtuzumab in preclinical models (4). It is hypothesized that CD32b expressed on tumor cells can bind the Fc of rituximab, which in turn causes the internalization of rituximab into the target cells or masks the rituximab Fc from activating effector cells such as NK cells and macrophages. To model this CD32b-mediated resistance to rituximab in vitro, we exogenously expressed human CD32b in CD20⁺CD32b⁻/C0 Ramos (a Burkitt’s lymphoma cell line; Supplementary Fig. S5) and measured FcRIIa activation by anti-CD20 mAbs using the JNL-FcRIIa reporter assay. Indeed, exogenous CD32b expression in Ramos (Ramos-hCD32b) resulted in reduced FcRIIa activation by rituximab and obinutuzumab individually (Fig. 4A) relative to parental Ramos cells. Similar experiments were conducted with the CD38⁺CD32b⁻/+ MM1.s, and its CD32b-overexpressing variant (Supplementary Fig. S5). Consistent with Ramos cells, overexpression of CD32b in MM1.s (MM1.s-hCD32b) decreased FcRIIa activation mediated by the anti-CD38 ADCC-competent antibody daratumumab (Fig. 4B). Vaughan and colleagues (27) previously demonstrated that an anti-CD32b antibody specific for the CD32b Fc-binding domain could block the interaction between CD32b and the Fc of a coadministered ADCC-competent therapeutic antibody, such as rituximab, thereby increasing the activity of such coadministered antibodies. To determine the major epitope of the NVS32b1 and NVS32b2 CDRs on CD32b, we assessed their binding to cells engineered to express human CD32b with point mutations known to disrupt Fc binding. The Fc-silenced (N297A) version of these antibodies were used to enable assessment of their binding to CD32b via CDR. Stable CHO cell lines expressing WT human CD32b or CD32b mutants with amino acid mutations that disrupt either the Fc binding pocket or the N terminal domain (not involved with Fc binding) were generated (Fig. 4C). In EDI103 (Q15P, R30Q, T32A, H33R, N138T) and EDI105 (R30Q, T32A, H33R, N138T) mutants, the CD32b N-terminal domain was...
disrupted. Both NVS32b1 and NVS32b2 demonstrated robust, concentration-dependent binding to these mutants similar to WT CD32b (Fig. 4D). In contrast, the CD32b Fc binding domain was disrupted in ED106 (Q15P, V107M, K130Q, S135L, N138T) and ED107 (V107M, K130Q, S135L, N138T) mutants, which resulted in severely impaired binding by NVS32b1 and NVS32b2. These data indicate that the major epitope recognized by both antibodies overlaps with the CD32b Fc-binding domain. To support findings from these cell binding experiments, we performed epitope mapping experiments via hydrogen-deuterium exchange and mass spectrometry. Consistent with the CD32b-mutant binding data, we mapped the amino acid positions blocked by NVS32b2 (107V-123F; Fig. 4E) to be partially overlapping with the known Fc-binding domain 112S-119V, 135S-138T (28) and the key residues identified in FACS experiments in Fig. 4D.

We then sought to determine whether blockade of the CD32b Fc-binding domain by NVS32b1 or NVS32b2 can alleviate CD32b-mediated resistance to antibodies with Fc-dependent activity. The Fc-silent (N297A) versions of these mAbs were used to specifically enable the assessment of CDR-mediated binding and blockade of the CD32b Fc-binding domain.

As anticipated, the Fc-silent NVS32b2 on its own did not activate FcγRIIa in the reporter JNL cell line when cocultured with Ramos.hCD32b. When combined with Fc-competent rituximab or Fc-enhanced obinutuzumab, Fc-silenced NVS32b1 enhanced FcγRIIa activation by both of these antibodies (Fig. 4F). Similarly, in \textit{in vitro} combination studies using the JNL reporter cell line and MM1.s-hCD32b cells in coculture, Fc-silenced NVS32b2_N297A combined with Fc-competent daratumumab yielded enhanced FcγRIIa activation compared with daratumumab alone (Fig. 4G). As anticipated, NVS32b2_N297A alone did not activate FcγRIIa in the reporter cell line. These data demonstrate that NVS32b1 and NVS32b2 have a shared, CDR-mediated ability to resensitize tumor cells to antibodies with Fc-dependent MoA in the setting of CD32b-mediated resistance. These findings are consistent with the known binding epitope of the NVS32b antibodies, that is, the CD32b Fc-binding domain. The genetically engineered Ramos.hCD32b and MM1.s-hCD32b cells express high levels of CD32b, and therefore the impact of CD32b on the patient response to rituximab, obinutuzumab, or daratumumab may vary based on the actual level of CD32b expression on the malignant cells.

To explore this biology \textit{in vivo}, we modeled the combination treatment of NVS32b mAbs and approved mAbs rituximab, obinutuzumab, or daratumumab in nude mice bearing established Daudi subcutaneous xenografts which express relatively low levels of CD32b. Mice were randomly assigned to receive 10 mg/kg weekly intravenous doses of NVS32b1_Fc_DEL, rituximab, obinutuzumab, NVS32b1_Fc_DEL + rituximab, or NVS32b1_Fc_DEL + obinutuzumab. Mice receiving combination treatment of NVS32b1_Fc_DEL with either rituximab or obinutuzumab exhibited decreased tumor volume and increased time to endpoint compared with control mice and mice receiving CD20-targeted monotherapy \textit{(P < 0.01; Fig. 4I)}.

Both combination treatments also yielded numerical increases in median time to endpoint relative to mice administered CD32b targeted monotherapy, (43 days, 84 days, and not reached for NVS32b2_Fc_DEL, NVS32b2_Fc_DEL + rituximab, and NVS32b2_Fc_DEL + obinutuzumab, respectively). However, the numerical differences between these three groups were not statistically significant, perhaps, in part, due to the relatively low number of mice per group \textit{(n = 6/group)}. Daudi cells express low levels of CD32b and, therefore, do not fully recapitulate the CD32b-mediated interference on the activity of other mAbs. A more robust combination response might be achievable with a tumor model that features higher CD32b expression and therefore greater CD32b-mediated resistance to rituximab or obinutuzumab.

To determine the combination effect of NVS32b2_N297A or NVS32b2_DE with daratumumab, we tested the combination treatment in nude mice bearing subcutaneous xenografts of MM1.s-hCD32b cells with high CD32b expression (Fig. 4I). In this model, the single-agent NVS32b2_DE moderately delayed tumor growth while NVS32b2_N297A had no impact due to the silenced Fc. Daratumumab mediated higher antitumor activity than did NVS32b2 and achieved tumor stasis. Combination of daratumumab with either NVS32b2_N297A or NVS32b2_DE achieved a synergistic antitumor effect that led to rapid tumor regression, providing evidence that NVS32b2 can reverse CD32b-mediated resistance, particularly when tumor cells express high levels of CD32b.

Together, these data demonstrate that the unique binding epitopes of NVS32b1 and NVS32b2 on CD32b enable them to disrupt the interaction between CD32b and its ligand, hIgG1 Fc. This enables the antibodies to resensitize tumor cells to therapeutic mAbs with an Fc-dependent MoA, such as obinutuzumab, rituximab, and daratumumab, in the setting of CD32b-mediated resistance. As large proportions of multiple myeloma and B-cell malignancies express CD32b, the above data suggest that combining NVS32b1 or NVS32b2 with these approved antibodies will likely have the potential to achieve meaningful benefit to patients.

\textbf{Fc enhanced anti-CD32b antibodies selectively depletes healthy donor B cells but not myeloid cells.} In humans, CD32b is highly expressed on normal B cells. In contrast, myeloid cells including monocytes, granulocytes, macrophages, and DCs express low levels of CD32b protein and high levels of its highly homologous protein CD32a (Supplementary Fig. S6; and reviewed in ref. 12). To assess the activity of NVS32b on normal immune cells, a series of \textit{ex vivo} assays were carried out using human whole blood or differentiated primary myeloid cells.

To assess whether NVS32b selectively targets B cells in physiologic conditions and in the context of immune cells expressing lower levels of CD32b with high CD32a, human whole blood from healthy donors was stimulated with NVS32b2_Fc_αfuc, NVS32b2_Fc_WT, and with the respective control IgGs for 24 hours. NVS32b2 induced dose-dependent NK-cell activation as demonstrated by upregulation of CD107α, cytokine release, and B-cell depletion (Fig. 5A–C). B-cell depletion occurred in an exponential relationship to B-cell receptor occupancy (RO; Fig. 5D). As RO is a function of CDR binding to antigen, NVS32b2_Fc_αfuc and NVS32b2_Fc_WT demonstrated identical RO as a function of antibody concentration (Supplementary Fig. S7). The afucosylated NVS32b2 triggered NK-cell activation, B-cell depletion, and cytokine release at an average 10-fold lower concentration and lower RO relative to the NVS32b2 Fc WT while simultaneously yielding a greater magnitude of response. Specifically, at maximum RO (~95%) NVS32b2_Fc_αfuc elicited higher maximum B-cell depletion than NVS32b2_Fc_WT at a matching antibody concentration and RO. These data highlight the improved therapeutic potential of an Fc enhanced anti-CD32b-targeted mAb. The number of monocytes was not affected by NVS32b2 (Fig. 5A) despite these cells retaining CD32b expression during a 24-hour whole blood culture (Supplementary Fig. S6). In addition, NVS32b2 did not deplete CD32a+ granulocytes either, providing orthogonal confirmation of specificity against the highly homologous CD32a.
Fc Enhanced Anti-CD32b mAbs for B and Plasma Cell Tumors

Figure 5.
NVS32b2 mAbs mediate depletion of CD32b⁺ B cells but not myeloid cells. A-D, NVS32b2 demonstrated dose-dependent NK-cell activation, cytokine release and B-cell depletion in healthy donor whole blood. Consistent with prior observations, the Fc-glycoengineered NVS32b2 is more potent than NVS32b2_Fc_WT. Viability of monocytes and granulocytes was not affected. Whole blood from 10 different healthy subjects was incubated with 0.001–600 μg/mL of the afucosylated anti-CD32b NVS32b2 (red circle), NVS32b2_Fc_WT (green square), and the respective Fc-matched negative control antibodies afuc IgG control (black circle) and Fc WT IgG control (black square) for 24 hours to determine percent of B cell, monocyte, and granulocyte depletion (A). Cytokine concentration in plasma supernatants (B), NK-cell activation as CD107a upregulation (C) and IFN-γ and TNF-α release (D). E, ADCC by NK cells. Target cells: Daudi vs. monocytes. F, ADCC by NK cells. Target cell: Daudi vs. DCs. Mφ, macrophage; Mo, monocyte; B, B cell; M, myeloid cell.

In summary, Fc engineering of NVS32b2 facilitates the selective and effective crosslinking of CD32b on B cells with FcyRIIa on NK cells leading to robust B-cell depletion in whole blood from healthy humans. The observation that NVS32b2_Fc_afuc did not deplete peripheral human immune cells that express low levels of CD32b, or its highly homologous CD32a suggests that this Fc-engineered anti-CD32b exhibits an optimal potency and selectivity profile. Whole blood depletion assays were performed for NVS32b1 mAbs as well and similar results were observed (Supplementary Fig. S8).

In addition to circulating myeloid cells, differentiated macrophages and DCs are known to express CD32b as well. We compared the NVS32b-mediated ADCC by NK cells against Daudi cells to ADCC against human macrophages or moDCs differentiated from healthy donor monocytes. While Daudi was efficiently lysed by NK cells in the presence of NVS32b2_Fc_afuc, we did not observe significant lysis of macrophages or moDCs by NK cells (Fig. 5E and F). In addition, no phagocytosis was observed between autologous macrophages labeled with CellTrace Green and CellTrace Red separately and then cocultured in the presence of NVS32b2_afuc control IgG. Viability of peripheral human immune cells that express low levels of CD32b, or its highly homologous CD32a suggests that this Fc-engineered anti-CD32b exhibits an optimal potency and selectivity profile.

Taken together, these data demonstrate a potential therapeutic window of afucosylated NVS32b mAbs between B cells (healthy and...
Anti-CD32b antibody treatment enhances IC-mediated maturation of human moDCs

In addition to their ability to mediate direct tumor cell lysis, previous studies have shown that anti-CD32b antibodies can enhance immune effector cell responses to Fc-dependent stimuli owing to their ability to block inhibitory signaling downstream of CD32b activation (7). To explore the ability of NVS32b2 to modulate Fc-dependent immune cell activation, we generated human moDCs, which expressed both CD32a and CD32b at baseline (Fig. 6A). Heat-aggregated ICs was utilized as an Fc-dependent stimulus, whose binding to CD32b were blocked by antibody pretreatment, both the N297A and afucosylated formats of NVS32b2 facilitated a significant increase in the frequency of mature DCs in response to NVS32b2 pretreatment and subsequent IC treatment, suggesting that CD32a expression is necessary to facilitate DC stimulation in response to ICs and NVS32b2 treatment (Fig. 6F). These results are consistent with a model wherein NVS32b2 mAbs are able to bind to cell surface CD32b on DCs, blocking this receptor and associated immunosuppressive signaling and leading to a net enhancement of maturation in response to Fc-dependent cross-linking of activating Fc receptors.

Taken together, these data suggest that ICs can induce moDC maturation in a manner that may partially depend on CD32a expression, and that NVS32b2 can enhance this maturation by blocking the inhibitory CD32b receptor, allowing ICs to bind exclusively to activating FcγRs, thereby inducing signaling pathways that promote maturation without engaging the negative feedback pathways downstream of CD32b activation. These results are consistent with previous studies of CD32b-specific antibodies, and highlight the potential immunomodulatory activity of such constructs.

**NVS32b mAbs are effective at targeting myeloma cells from patients with multiple myeloma**

Finally, we determined the activity of NVS32b mAbs on primary myeloma cells using a total of 16 BM samples. Depletion of multiple malignant and myeloid cells including monocytes, granulocytes, macrophages, and DCs.

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Finally, we determined the activity of NVS32b mAbs on primary myeloma cells using a total of 16 BM samples. Depletion of multiple
myeloma cells in the BMMCs and CD32b expression were measured by flow cytometry (Supplementary Fig. S9A). CD32b expression on multiple myeloma cells was heterogeneous (Fig. 7A), and the maximum lysis of multiple myeloma cells correlated with the percentage of CD32b-positive cells (Fig. 7B). For samples with higher expression of CD32b (defined as ≥ 50% CD32b+ multiple myeloma cells), ADCC activity driven by NVS32b2_Fc_afuc was observed at similar levels to daratumumab, albeit with high variability between samples (Fig. 7C and D, left). For samples with less than 50% CD32b+ multiple myeloma cells, NVS32b2_afuc mediated no to low levels of multiple myeloma cell lysis (Fig. 7C and D, right). NVS32b1_afuc has also been tested in a fraction of the samples in parallel and showed highly similar activity profile as NVS32b2_afuc (Supplementary Fig. S9B and S9C).

We compared the ADCC activity mediated by NVS32b antibodies between samples from daratumumab relapsed/refractory (R/R) patients and those from patients at diagnosis. Only one out of six daratumumab R/R patient samples was sensitive to NVS32b_Fc_afuc (Supplementary Fig. S9B and S9C). The ADCC activity pro-

![Figure 7](https://mct.aacrjournals.org/article/19/10/2101/fig7)

**Figure 7.**

The ADCC activity of CD32b-specific antibody against myeloma cells from patients with multiple myeloma. A, CD32b expression on CD38+CD138+ malignant plasma cells in multiple myeloma patient BMMCs. For staining controls isotype Ab and FMO (full minus one) were included. B, Correlation between %CD32b+ multiple myeloma cells and maximum lysis of multiple myeloma cells. Samples from daratumumab relapsed/refractory patients were labeled in red. C and D, The ADCC activity of the CD32b antibody against malignant plasma cells. Patient samples were categorized on the basis of CD32b expression, with higher levels of expression on the left (CD32b+ multiple myeloma cells ≥50%) and lower levels of CD32b expression on the right (CD32b+ cells <50%) for CD32b. Individual patient sample response was shown in spider plots in (C) with daratumumab-unexposed patients as black lines and daratumumab relapsed/refractory patient samples as red lines. In (D), the data is presented as the mean ± SEM of all samples tested. Daratumumab (10 μg/mL) and isotype IgG (10 or 100 μg/mL, matching the top concentration of NVS32b2 for each sample) were used as controls.
Discussion

NVS32b antibodies (NVS32b1 and NVS32b2) are fully human IgG1 monoclonal antibodies that selectively bind the inhibitory FcγRIIb, CD32b, by their CDRs with high affinity. While targeting CD32b with a therapeutic mAb has been previously considered (7, 9), we sought to explore Fc engineering as a means of enhancing Fc-dependent antitumor activity. Specifically, NVS32b1 and NVS32b2 were glycoengineered to yield afucosylated antibodies, which lack the (α1,6)-linked core fucose, resulting in the increased ability of the Fc to engage, crosslink, and activate activatory FcγRs on innate immune effector cells. These afucosylated anti-CD32b mAbs mediate potent target cell lysis by three distinct mechanisms: ADCC, ADCP, and CDC. This enables these antibodies to mediate robust, Fc-dependent antitumor activity in vitro and in vivo.

On the basis of our findings described herein, NVS32b1 and NVS32b2 have the potential to benefit patients with CD32b+ malignancies via two distinct mechanisms. First, they can bind to the malignant cells and subsequently recruit and activate innate immune effector cells via the afucosylated Fc domain, resulting in potent tumor cell killing. Consistent with prior reports (3–5), CD32b is expressed on the cell surface of B and plasma cells and their malignant counterparts in a significant portion of patients with CLL, NHL, and multiple myeloma. Expression of CD32b on these malignant cells renders them sensitive to direct targeting by NVS32b. Importantly, NVS32b1 demonstrated marked antitumor activity in the bone-homing, orthotopic Jeko1 xenograft model, providing important proof of the therapeutic concept in this disease relevant model. Further in vivo profiling of Fc-enhanced NVS32b1 or NVS32b2 demonstrated that these mAbs achieve robust antitumor activity in immunocompromised mice bearing B-cell or plasma cell subcutaneous xenografts. These xenograft models feature a level of CD32b expression that is generally lower than that observed in B and plasma cell malignant cells collected from patients. Furthermore, preclinical studies benchmarking our novel CD32b mAbs to approved mAb therapies, that is, obinutuzumab, rituximab, and daratumumab, highlight their therapeutic potential. While the in vitro and in vivo experiments described above enable direct comparison of the relative potencies of these antibodies, limitations of these experiments exist. For example, as NVS32b1 and NVS32b2 do not bind to murine CD32b, the in vivo experiments described above do not enable the assessment of how normal tissue CD32b expression–driven antibody clearance would alter pharmacokinetic properties of these antibodies. Despite this limitation, the data do inform the relationship between systemic exposure and the antitumor response and, importantly, provide insight into how Fc engineering alters the exposure/response relationship. A previous report by Williams and colleagues (30) concluded that anti-CD32b mAbs would have limited therapeutic potential due to normal tissue CD32b expression driving antibody clearance. More recently, however, disclosures describing clinical activity of the Fc WT CD32b-targeted mAb BI-1206 suggests that therapeutically relevant exposures are being achieved in patients (31).

It is additionally important to note that subcutaneous xenograft tumor models used in this study do not accurately recapitulate the biology of hematologic malignancies in BM, with distinct microenvironmental signaling and ligand availability having the potential to influence therapeutic efficacy. Despite this limitation, these models allowed for the effective interrogation of the relationship between systemic exposure and the biological relevance of different Fc-modified variants. Coupled with our use of the Jeko1 model to demonstrate therapeutic proof of concept, we believe that these data make a compelling case for the in vivo utility of NVS32b mAbs. NVS32b2_Fc_afuc robustly depleted CD32b positive B cells while sparing CD32a+ or CD32b-positive myeloid cells in healthy donor whole blood depletion assays. In addition, macrophages and moDCs were resistant to NVS32b2-mediated ADCC and ADCP compared with malignant B cells. Together, these data suggest that a glycoengineered mAb that engages the CD32b Fc-binding domain by its CDR has the potential to bring clinical benefit to patients with B and plasma cell malignancies. The potential impact of NVS32b1 or NVS32b2 binding to other normal tissues, for example, liver sinusoidal endothelial cells, on tolerability is not addressed in the in vivo studies described herein as NVS32b1 and NVS32b2 do not bind mouse CD32b. Because of the lack of appropriate cell-based models, the in vitro sensitivity of LSECs to NVS32b mAbs was not examined either.

The second mechanism of action for the anti-CD32b NVS32b antibodies is via the enhancement of coadministered antibodies that target other therapeutically relevant tumor antigens, for example, CD38 (daratumumab), SLAMF7 (elotuzumab), or CD20 (rituximab and obinutuzumab). High levels of CD32b expression on malignant B cells and multiple myeloma cells is likely a mechanism of resistance to therapeutic antibodies with an Fc-dependent MoA (5, 6). By blocking the Fc-binding pocket on CD32b via their CDRs, NVS32b1 and NVS32b2 were able to reduce the impact of CD32b-mediated resistance to mAbs whose function requires Fc–FcγR interaction. Finally, we demonstrated the ADC activity of NVS32b mAbs on CD32b+ primary tumor cells from patients with CLL and multiple myeloma. CD32b expression levels correlated with ex vivo response to NVS32b mAbs among the 16 patients with multiple myeloma samples tested. Of note, BMMCs from patients at diagnosis or relapsed/refractory on daratumumab showed differential ex vivo sensitivity to NVS32b_afuc antibody. The low ADC activity observed in samples from daratumumab-treated patients may be attributed, in part, to low CD32b expression as well as NK-cell depletion by daratumumab. Taken together, these data suggest that CD32b expression analysis as well as a thoughtfully designed combination strategy are needed to achieve maximal benefit in patients with multiple myeloma.

CD32b is the sole inhibitory FcγR and contains an ITIM. In addition to B cells, it is expressed on myeloid cells including DCs and macrophages. Whereas B cells only express the inhibitory FcγRIIb, DCs and macrophages express several activating FcγRs (FcγRI, FcγRIIa, FcγRIIIa), whose downstream signaling is modulated by the inhibitory CD32b_FcγRIIB (24). Seminal work by Clynes and colleagues (8), using CD32b knockout mice suggests that inhibition of CD32b on immune cells may enable a more robust antitumor immune response. Here, we demonstrated that treatment with NVS32b2 promoted maturation of moDCs in the context of FcγR crosslinking by immune complexes. Therefore, blockade of CD32b by NVS32b antibodies may suppress CD32b-mediated inhibitory signaling, thereby tipping the balance towards a net activatory signal in DCs and macrophages and thus resulting in their activation and maturation. Such an MoA has the potential to yield favorable immunomodulatory effects locally or systemically and is the subject of ongoing further investigation.

Disclosure of Potential Conflicts of Interest

E. Choi reports other from Novartis Institutes for Biomedical Research [employer (former)] and Unum Therapeutics [employer (current)] outside the submitted work. P. L. Lindenbergh reports grants and nonfinancial support from Novartis (Novartis financially supported our contribution to this project and provided the antibodies evaluated here) during the conduct of the study. N W. C. van de Donk reports grants from Novartis (research support) during the conduct of the study; grants and other
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Authors’ Contributions

H. Lu: Conceptualization, resources, data curation, formal analysis, supervision, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing. R.D. Molony: Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing-review and editing. D. Chen: Resources, data curation, investigation, methodology. S. Jang: Data curation, validation, investigation, methodology. B. Wolf: Resources, data curation, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. S. Ewert: Conceptualization, resources, supervision, methodology, writing-original draft, writing-review and editing. M. Flaherty: Conceptualization, resources, supervision, validation, methodology. F. Xu: Data curation, formal analysis, validation, investigation, methodology. S. Isim: Data curation, validation, investigation, methodology. Y. Shim: Resources, data curation, validation, investigation, methodology. C. Dorneles: Resources, data curation, validation, investigation, methodology. N. Balke: Data curation, validation, investigation, methodology. E. Choi: Resources, data curation, formal analysis, validation, investigation, methodology. S. Jang: Resources, data curation, validation, investigation, methodology. J. Koelln: Resources, data curation, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. E. Choi: Resources, data curation, formal analysis, validation, investigation, methodology. X.C. Leber: Conceptualization, resources, data curation, formal analysis, validation, investigation, methodology, writing-review and editing. N.W.C.J. van de Donk: Resources, supervision, funding acquisition, validation, investigation, methodology, writing-review and editing. T. Mutis: Resources, data curation, supervision, funding acquisition, validation, investigation, methodology, writing-review and editing. H. Huet: Conceptualization, resources, data curation, formal analysis, validation, investigation, methodology, writing-original draft, project administration. E. Lees: Resources, supervision, project administration. M.I. Meyer: Conceptualization, resources, data curation, formal analysis, supervision, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing.

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