Surrogate target cells expressing surface anti-idiotype antibody for the clinical evaluation of an internalizing CD22-specific antibody

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Abbreviations: CMC, complement mediated cytotoxicity; MOA, mechanism of action; NHL, non-Hodgkins lymphoma; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; mAb, monoclonal antibody; ADCC, antibody dependent cell cytotoxicity; PBMC, peripheral blood mononuclear cell; PK, pharmacokinetic; HACA, human anti-chimeric antibody

SM03, a chimeric antibody that targets the B-cell restricted antigen CD22, is currently being clinically evaluated for the treatment of lymphomas and other autoimmune diseases in China. SM03 binding to surface CD22 leads to rapid internalization, making the development of an appropriate cell-based bioassay for monitoring changes in SM03 bioactivities during production, purification, storage, and clinical trials difficult. We report herein the development of an anti-idiotype antibody against SM03. Apart from its being used as a surrogate antigen for monitoring SM03 binding affinities, the anti-idiotype antibody was engineered to express as fusion proteins on cell surfaces in a non-internalizing manner, and the engineered cells were used as novel “surrogate target cells” for SM03. SM03-induced complement-mediated cytotoxicity (CMC) against these “surrogate target cells” proved to be an effective bioassay for monitoring changes in Fc functions, including those resulting from minor structural modifications borne within the Fc-appended carbohydrates. The approach can be generally applied for antibodies that target rapidly internalizing or non-surface bound antigens. The combined use of the anti-idiotype antibody and the surrogate target cells could help evaluate clinical parameters associated with safety and efficacies, and possibly the mechanisms of action of SM03.

Introduction

There are a number of anti-CD22 antibodies in different stages of clinical trials for treating lymphomas and other autoimmune diseases.1-3 SM03 is one such antibody developed in China, where it is being evaluated clinically for treating non-Hodgkin’s lymphoma (NHL), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). CD22 expression is restricted to lymphocytes of the B-cell lineage and found in the cytoplasm of pro- and pre-B cells. Surface expression is detected on matured B cells, but is subsequently lost in plasma cells and activated B cells.4-6 Antibodies that bind to surface CD22 on lymphoma cells are rapidly internalized,7 suggesting an as yet unknown mechanism of action (MOA) different from that of other B-cell specific antibodies. In the absence of a clear MOA and an associated bioassay, as in the case of SM03, assays that separately monitor the binding and functional moieties of the antibody should therefore be developed.

The clinical applications of monoclonal antibodies (mAbs) primarily lie in their specificity and strong affinity for a target antigen, and their ability to mediate immune effector functions such as complement-mediated cytotoxicity (CMC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Changes in the affinity and specificity of SM03 could be monitored by competitive flow cytometry or binding studies against human Burkitt’s lymphoma cell, exogenous CD22,8,9 or surrogate antigens; however, standard CMC or ADCC assays for monitoring effector functions were not applicable because CD22 antigens are rapidly internalizing.9 The current bioassay for SM03 relies on cytotoxicity induced by artificially hyper-
crosslinking surface CD22 on lymphoma cells and bears little relevance to the MOA of the antibody.8 Importantly, the assay is independent of a functional Fc, and could not be used for monitoring the Fc functionality and intactness, especially on microheterogeneity arising from the manufacturing process and upon storage.10-12

In an attempt to develop assays to measure blood levels of residual SM03 in patients treated with SM03, an anti-idiotype single-chain variable fragment (anti-Id scFv) antibody that binds specifically to the idiotope of SM03 was developed.15 By genetically fusing the anti-Id Fab to non-inneralizing surface anchoring proteins/structures, cell lines could be engineered to express these structures on their surfaces and be used as the surrogate target cells for CMC and ADCC interactions with SM03. The surrogate target cells proved to carry the sensitivity that can differentiate subtle glycoform variations within the Fc region of SM03, and could potentially be used to correlate between residual SM03 Fc potency and clinical efficacies in patients treated with the antibody.

Results

Hc5 anti-Id mIgG as the surrogate antigen for CD22

The Hc5 scFv was used successfully for Phase 1 clinical pharmacokinetic (PK) analysis of lymphoma patients treated with SM03.1 The Hc5 scFv was converted into a full immunoglobulin with murine IgG2a/kappa isotype (Hc5 anti-Id mIgG). The bindings of Hc5 scFv and anti-Id mIgG toward binders (SM03 and SM06) and non-binders (SM09 humanized anti-CD20 and N009 chimeric anti-TNF) were compared; the dose response curves of anti-Id mIgG were distinctively more sensitive and clear cut (Fig. 1A). SM03 with different binding affinity toward CD22 were generated by heat inactivation at 70°C for 0, 24, 48, and 72 hrs. Competitive and direct binding studies of the progressively heat-inactivated SM03 against Raji cells or recombinant CD22 containing domains 2-49 indicated corresponding affinity reduction proportional to the time of SM03 heat inactivation (Fig. 1B(i & ii)). A similar pattern in affinity change was also observed with the heat-inactivated SM03 against the Hc5 anti-Id mIgG, suggesting that the Hc5 anti-Id mIgG could be used as a surrogate antigen for the evaluation of SM03 binding activities (Fig. 1B(iii)).

Surrogate target cells expressing surface Hc5 for monitoring SM03 Fc bioactivities

Fusion proteins containing the Hc5 anti-Id Fab were genetically constructed, either as membrane bound murine IgD, or as Fab’ fused with the transmembrane portion of glycoporphin A14 or as Fab’ fused with the glycoporphatinolysinotil (GPI) signal sequence isolated from decay accelerating factor (DAF) protein15 (Fig. 2A). SP2/0 murine myeloma cells that express endogenous Igβ16 were transfected with vectors containing sequences encoding the individual constructs, and surface expression of SM03 binding moiety was examined by flow cytometry (Fig. 2B).

No surface expression was detected when only the IgD construct was expressed (data not shown), although marginal surface IgD expression was observed when Igκ was co-expressed. On the contrary, cells transfected with the anti-Id Fab’-glycoporphin A (Fab’-GlycoA) or anti-Id Fab’-DAF (Fab’-GPI) fusion protein constructs were shown to exhibit strong surface expression, manifested as dose-dependent binding of SM03 in flow cytometry studies.

Although cell cytotoxicity against Ramos cell line could be induced by immobilized SM03,8 cytotoxicity was not detected until the immobilizing SM03 was at a concentration of 0.13 µg/ml or above, yet with no dose-responsive cytotoxicity observed (Fig. 2C(i)). SM03 failed to induce CMC against lymphoma cells, whereas a humanized anti-CD20 antibody (SM09) achieved typical dose-dependent CMC activities (Fig. 2C(ii)). When both Fab-GlycoA and Fab-GPI cells were used as the surrogate target cells for SM03, significant dose-dependent CMC activities with maximum killing at over 90% were observed, with EC50 determined to be at 0.135 and 0.216 µg/ml, respectively; no CMC activities, however, were detected with the Fab-IgD cells (Fig. 2C(iii)). The results were in agreement with the levels of surface expression of the respective fusion proteins, and suggested Fab-GPI and Fab-GlycoA could be used as the surrogate target cells for SM03 CMC evaluation.

IgG CH2-appended N-glycosylation is required for CMC activities. SM03 glycan was enzymatically cleaved by treatment with PNGase, as evident by corresponding SM03 molecular shifts in CE-SDS under reducing (heavy chain) and non-reducing (whole IgG) conditions (Fig. 3A(i)). The loss of glycan in SM03 was further confirmed with an altered IEF profile and glyco-staining (Fig. 3A(ii)).

BLAcore analyses (average K_D of 0.164 nM and 0.091 nM before and after deglycosylation, respectively) indicated that enzymatic treatment did not affect the binding affinity of SM03; the results were in agreement with that of direct competitive binding studies against Hc5 anti-Id mIgG (Fig. 3B(i)), and competitive flow cytometry studies against Ramos cells (Fig. 3B(ii)). The immobilized SM03, before or after enzymatic treatments, were equally effective in inducing Ramos cell death, with or without IgM augmentation (Fig. 3B(iii)). By contrast, when the Fab-GlycoA cells were used as the surrogate target cells, deglycosylated SM03 showed dramatic reduction in CMC activities, while the glycosylated SM03 achieved distinct dose-dependent killing (Fig. 3C(i)). Similarly, when human peripheral blood mononuclear cells (PBMCs) were used as the effector cells, the ADCC activities of SM03 were abrogated when the CH2-appended glycan was enzymatically removed (Fig. 3C(ii)).

We have expressed humanized anti-CD22 (SM06) in murine myeloma SP2/0 and Chinese hamster ovary (CHO) cells; conceivably, SM06 expressed in these different hosts would have identical structure, except at the CH2-appended N-glycan, and possibly other post-translational modifications. SM06 isolated from the different host cells exhibited different IEF profiles (Fig. 4A). These structural variations did not affect the binding properties of SM06 in competitive binding studies against the
surrogate antigen or competitive flow cytometry studies against Raji cells (Fig. 4B). CMC studies against the Fab-GlycoA cells revealed that SM06 from SP2/0 is more potent than that from CHO, regardless of the source of complements (guinea pig vs human) (Fig. 4C).

The major species of N-linked glycans carried by SM06 from the different hosts were essentially the same, and the different structures comprising mostly G0-N, G0F-N, G0, G0F, Man5, G1F/G1F', and G2F glycans are shown in Figure 5. Antibodies expressed in SP2/0, when compared to that from CHO cells, contained a higher proportion of galactosylated glycans (26.1% vs 13.1%), but lower proportion of fucosylated glycans (9.2% vs 11.1%) (pie chart, Figure 5).

Use of the Hc5 anti-Id mIgG and the surrogate cell line for the evaluation of clinical parameters in patients treated with SM03

Using the Hc5 anti-Id mIgG as the surrogate antigen, the PK profile of SLE patients in a Phase 1 dose-escalation study was monitored. Figure 6A illustrated a typical PK profile of a SLE patient treated with SM03 at a dose of 240 mg/m², once a week, for four consecutive weeks, and the levels of residual SM03 in circulation increased gradually over the weekly injection cycle. Average data taken from four patients (N = 4) treated with four doses of SM03 at 240 mg/m² revealed a mean Cmax and a mean AUC₀₋₄ value of 381.39 ± 117.34 µg/mL and 113285.79 ± 62789.65 µg h/mL, respectively.

In addition, the relative amount of anti-SM03 antibodies in the sera of two RA and two SLE patients collected at different time points were compared using Hc5 anti-Id mlg with a standard sandwich-type ELISA assay (Fig. 6B). The human anti-chimeric antibody (HACA) responses were detected on day 14 for both RA patients, but not with SLE patients. Although the method is incapable of delineating the exact amount of HACA, a standard curve using the Hc5a anti-Id mIgG as a standard reference for gauging HACA responses for subsequent comparison. In view of the role played by Fcγ receptor-mediated phagocytosis of IgG immune complex in antigen presentation, the distinct HACA responses observed might be the result of differential Fc processing in different immunological environments of SLE and RA patients.

Blood from SLE patients treated with SM03 were collected on days 8, 15 and 22; and the CMC activities of the SM03 retrieved from patient’s blood determined using the Fab-GlycoA cells. Intriguingly, the CMC response from SLE patient #1 was consistently higher than that of SLE patient #2, and the differences in CMC responses of SM03 retrieved from the two patients increased with time (Fig. 6C). Preliminary clinical evaluation suggested a positive clinical response was observed in SLE patient #1, but not SLE patient #2. Patient’s blood collected prior to SM03 administration did not exhibit noticeable CMC activities.
Discussion

The current bioassay is not sufficient for monitoring structural and functional changes of SM03

Although the Hc5 anti-Id mIgG helps in assessing the identity and affinity of SM03, it is insufficient in revealing subtle changes in tertiary/quaternary structure resulting from the manufacturing processes and upon storage, such as alternative disulfide pairings, isoaspartyl residues, glycation, methionine oxidation, deamidation, pyro-glutamate formation, partial enzymatic cleavage of C-terminal lysines. Those structural changes could have the potential to affect the safety or efficacy of SM03. Cell-based assays or bioassays are therefore needed to circumvent the problem and to reflect attributes relating to the clinical response, potency, or MOA.

The current bioassay for SM03 relies on cytotoxicity induction against lymphoma cell lines by cross-linked or immobilized SM03, which is not quantitative and more of a manifestation of the binding between SM03 and the CD22 antigen, and the assay does not address possible effects of post-translational modification or structural changes in the antibody Fc.

Although the rapidly internalizing SM03 fails to induce CMC against lymphoma cell lines in vitro, it is still possible that SM03 Fc can play a role in tumor regression in vivo. In tumor sites where cell density and geometry become permissible, SM03 can simultaneously bind to CD22 on its target cells and via its Fc to the Fcγ receptors on B cells, dendritic cells or macrophages, preventing internalization while clustering CD22. The ligation of the exposed SM03 Fc could reach a density that allows reaction with C1q to initiate the complement activation cascade. Moreover, it was recently suggested that an anti-CD22 antibody exerted its immunomodulatory effect via trogocytosis, which is a FcγR-dependent mechanism that would result in the reduction of surface expressed CD22, CD19, and CD79b, and possibly other BCR modulators and cell-adhesion molecules, leading to
the incapacitation of B cells and rendering them unresponsive to activation by T-cell-dependent antigens. These findings have highlighted the importance of developing bioassays that address Fc functions of SM03.

Surrogate target cells could mediate SM03 induced immune responses

The barely detectable levels of transmembrane IgD expression on cells transfected with the IgD construct might be related to its dependency on the levels of the concomitantly expressed Igx and IgB, which were difficult to control in the transfected cell line. The problem was aggravated by the fact that binding on surface IgD by SM03 might induce internalization, defeating the purpose of the original design. In contrast, high levels of surface anti-Id expression were detected in the Fab-GlycoA and Fab-GPI cells, with corresponding effectiveness for the induction of dose-response CMC activities by SM03. Fab-GlycoA or Fab-GPI cells as surrogate target cells for SM03 CMC had proved to be superior in terms of relevance, sensitivity, and reproducibility for the evaluation of SM03 Fc-functions. Experiments performed with CMC assays using the Fab-GlycoA and Fab-GPI cells throughout this study were found to be robust and consistent when the EC50 of each assay was determined (refer to the corresponding figures). The assays were established as a routine QC standard for the release of manufactured SM03 for clinical purposes.

Figure 3. Bioassays employing the surrogate target cells could be used to monitor the presence or absence of Fc-appended glycan on SM03. (A) The carbohydrate attached to the CH2 domain of SM03 was successfully removed by treatment with PNGase as confirmed by (i) CE-SDS which revealed corresponding shifts (indicated by arrows) in the molecular sizes of the enzymatically treated SM03 IgG (non-reducing) and SM03 heavy chain (SM03 HC) (reducing), and (ii) IEF and glycol-staining which showed a change in the IEF profile and reduction of the intensity of glyco-staining in the heavy chain of SM03 under reducing conditions; (B) Deglycosylation of SM03 would not affect the resultant binding affinities or the cytotoxicities induced by immobi-lized SM03: The binding affinities of untreated SM03 (□), SM03 treated with buffer only (○), and PNGase treated (deglycosylated) SM03 (■) were compared in (i) competitive binding assay against Hc5 anti-Id mIgG, and (ii) competitive flow cytometry assay against Raji cells. Where applicable, N009, a chimeric anti-TNF antibody, was used as the negative control (▼); and (iii) the cytotoxicity induced by the immobilized antibodies, including untreated SM03 (□), SM03 treated with buffer only (■), and PNGase treated (deglycosylated) SM03 (●) were compared in the absence (No IgM) or presence (+ IgM) of IgM (IgM can enhance SM03-induced cytotoxicity). No significant differences in the cytotoxicity activity of the differently treated SM03 against Ramos lymphoma cells were observed. (C) when the Fab-GlycoA cells were used as the surrogate target cells, whether it was in a standard (i) CMC assay, or (ii) ADCC assay using human peripheral blood mononuclear cells (PBMCs) as the effector cells, the deglycosylated SM03 (■) exhibited substantially reduced activities compared to the untreated (□) or buffer-treated (○) SM03. The EC50 in the CMC assay against the Fab-GlycoA cells for the untreated and buffer-treated SM03 were determined to be 0.09 and 0.11 µg/ml, respectively. Anti-CD20 antibody (Rituximab) against Raji target cells were used as the controls, similarly, significant ADCC activities were observed with the glycosylated (untreated) antibody, whereas such activities were abrogated when the antibody was deglycosylated (data not shown).
Establish bioassays with the surrogate target cells to monitor Fc-functions

SM03 is of the human IgG1/kappa isotype, with N-linked glycosylation occurring at position 297 (Asn297) of the CH2 domain; the CH2-appended glycan is critical for antibody-mediated immune responses such as ADCC,23,24 and is known to have important effects on product consistency, stability, safety, potency, efficacy, immunogenicity, and circulation half-life.25 It is therefore important that factors affecting the final glycosylation profile and glycan structure can be monitored and controlled. Minor structural variations in carbohydrate structure could occur with changes in the manufacturing bioprocess, such as feeding strategy,26,27 or the use of different host cells. In fact, other than E. coli-produced non-glycosylated Fab fragments (7%), CHO (48%) and mouse myeloma cells (NS0, SP2/0, hybridoma) (45%) are the most commonly used mammalian host cells for the production of market-approved therapeutic antibodies or Fc-fusion proteins.28-30 The presence of the CH2-appended glycan is a prerequisite for the elicitation of CMC activities,31 and the strength of which is also dependent on the structures of the attached carbohydrates.32,33

Consistent with previous observations that the biological activity of therapeutic antibody CAMPATH-1H varied with the host cell lines and culture conditions used,34 SM06 (a humanized counterpart of SM03) produced in the two different hosts were found to carry similar numbers of species of glycoforms, but there were approximately 2-fold more galactosylated glycoform (G1F-G1F’+G2F) on SP2/0-expressed SM06 than on the CHO-expressed counterpart (Fig. 5). Indeed, a higher degree of galactosylation could promote activation of C1q and lead to enhanced CMC.35 Degalactosylation of alemtuzumab, for example, was shown to reduce CMC killing.24,36 The G1F-G1F’ (symmetric monogalactosylated) glycoform of rituximab also triggered a 2-fold enhanced CMC killing than its agalactosylated counterpart (G0F-G0F).24 The higher CMC killing by SP2/0-SM06 against the surrogate target cells is in agreement with the higher percentage/content of terminal galactose in SP2/0-expressed SM06, and further corroborates the sensitivity of the assay.

Antibody glycosylation might also affect ADCC activity, although the role of galactosylation remains controversial. There were reports suggesting the presence of terminal galactose residue could enhance ADCC,32,33 while others failed to find any correlations.37 In general, it is now more acceptable to link ADCC to fucosylation, and a number of studies had already shown that defucosylation led to pronounced enhancement in ADCC activity.24,35,37 The similar overall content of fucosylation in SP2/0-SM06 and CHO-SM06 (~90%) suggested they should have comparable ADCC activity, but further experiments are needed.

Nevertheless, these results confirmed that cell lines engineered to express non-internalizing anti-Id binding moieties could be

Figure 4. Bioassay employing Fab-GlycoA cell as surrogate target cell in CMC assay could differentiate the Fc-functionalities of SM06 (humanized SM03) expressed in SP2/0 (○) and CHO (●) host cells. (A) SM06 produced by the different host cells demonstrated different post-translational modifications, as revealed by their distinct isoelectric focusing (IEF) profiles suggesting the presence of charge variants; (B) the antigen binding affinities of the SM06 charge variants against either (i) the Hc5 anti-Id mIgG surrogate antigen, assessed in a standard competition ELISA assay, or (ii) surface CD22 expressed on Raji cells, assessed in a standard competitive flow cytometry assay, were comparable; (C) using the surrogate Fab-GlycoA line as the target cell, SM06 expressed in SP2/0 (○) consistently demonstrated stronger CMC activities than that expressed in CHO (●) host cells, reacting to complements sources either from (i) guinea pig serum (EC50 for SP2/0 and CHO were determined to be 0.13 and 0.21 μg/ml, respectively), or (ii) human serum (EC50 for SP2/0 and CHO were determined to be 0.07 and 0.12 μg/ml, respectively).
used to reveal subtle variations in the glycoforms or structures of an antibody. Given that an anti-Id against a corresponding therapeutic antibody is routinely developed for purposes of monitoring serum PK and quantitating any immune responses to the antibody, the example described herein has illustrated an expanded application of the anti-Id antibody, and, as a general approach, for the development of bioassays that were previously either non-existent, or lacking robustness. This is particularly useful for rapidly internalizing antibodies, such as antibodies targeting the invariant chain (Ii), Lewis Y antigen, and CD33.\textsuperscript{38-40} For antibodies that do not involve the Fc for biological activities, such as cytokine-neutralizing antibodies (e.g., infliximab) or antiviral antibodies (e.g., palivizumab), a surrogate target cell artificially made to express the corresponding anti-Id Fab’ on the cell

\begin{figure}
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\caption{N-linked glycan analysis of SM06 produced from SP2/0 and CHO. Glycan release was performed by PNGase. Glycoforms were separated by UPLC-FLR system equipped with a BEH Glycan column and were verified by Q-TOF mass spectrometry. Inserted pie charts show the relative amount of galactosylation and fucosylation. SP2/0-SM06 demonstrated a \sim2-fold higher amount of galactosylated glycoforms than CHO-SM06 while their degrees of fucosylation were similar.}
\end{figure}
surface could be established so that similar cell-based assays can be developed to reflect structural changes within the Fc moiety, as a measure to ensure product safety and consistency.

**Use of the anti-Id mIgG and the surrogate target cell for the clinical evaluation of SM03**

Clinically, both the Hc5 anti-Id mIgG and the surrogate target cells have proved useful for the evaluation of PK, HACA, and residual SM03 bioactivity in the blood of patients treated with SM03. Finding an appropriate positive HACA control has always been difficult. While no HACA response was detected in the SLE patients tested, RA patients were HACA positive (day 14 post SM03 administration), precluding the possibility of false negatives while confirming the validity of the assay method. The standard curve established with the Hc5 anti-Id mIgG offered an objective reference to help quantify and compare HACA responses from patients of different disease indications, and draw clinical conclusions. In fact, Baert and co-workers were able to correlate the extent of HACA in Crohn’s disease patients treated with infliximab to the risk of infusion reactions and duration of response to treatment with the availability of a positive reference.

Of particular interest is the demonstration that the surrogate target cell might be able to differentiate CMC potency of SM03 retrieved from the blood of SM03 responders and non-responders. These clinical parameters, when used in conjunction with PK and HACA data, might help correlate patients’ responses with the MOA of the antibody. Nevertheless, further experimentation with statistically meaningful patient sample size will be needed before meaningful clinical correlations could be established. The clinical trials and related studies are currently underway.

**Materials and Methods:**

**Transfections and screening**

DNA vectors encoding the different fusion constructs containing the Fab moieties of the Hc5 anti-Id antibody were constructed using standard techniques in molecular biology. SP2/0 cells were transfected with the different constructs using the method of electroporation as described previously. Presence of secreted immunoglobulin in the culture media was examined by standard ELISA assay using goat anti-mouse Fab immunoglobulin as the capture antibody, and HRP-conjugated goat anti-mouse IgG Fc-specific immunoglobulin as the detecting
antibody (Jackson ImmunoResearch Laboratories, Inc.). For the detection of cell clones expressing surface Hc5 anti-Id binding moiety, cell-based ELISA were used for preliminary screening. Positive clones were further analyzed by flow cytometry, first by reacting with SM03, and later revealed by FITC-conjugated goat anti-human Fc specific immunoglobulins (Jackson ImmunoResearch) using a FACScan (Becton Dickinson, San Jose, CA).

**Binding Studies**

Hc5 scFv and the domain 2-4 of CD22 (CD22d2-4) were prepared as previously described. For direct binding studies, increasing concentrations of SM03 or other control antibodies were added into the wells of ELISA plates coated with 5 - 10 μg/ml of either CD22d2-4, Hc5 anti-Id scFv or mlgG, and the extent of binding of these antibodies were revealed with HRP-conjugated goat anti-human Fc-specific antibodies (Jackson ImmunoResearch). For competition binding assay, increasing concentrations of SM03 or other control antibodies were mixed with fixed amount of HRP-conjugated SM03 (1:1000 dilution), and the mixtures were added into the Hc5 anti-Id scFv or mlgG coated wells. The extent of binding of HRP-conjugated SM03 on the coated anti-Id scFv or mlgG in the presence of other competitors was revealed at OD450 after the addition of TMB substrates (Life Technologies). SM03 binding to the CD22 antigen on either Raji or Ramos cells was performed by flow cytometry studies (direct binding or competitive binding) as previously described.

For affinity determination using BLAcore (GE Healthcare Life Sciences, Piscataway, NJ), Hc5 anti-Id mlgG was immobilized on carboxymethylated dextran-coated CM5 sensor chip following standard procedures. A control surface was likewise prepared but with chimeric anti-TNF mAb (N009) (SinoMab BioScience Limited, Hong Kong). The kinetic analysis was performed by injecting serially-diluted SM03 in running buffer over the Hc5 anti-Id mlgG and control surfaces for 3 min at a flow rate of 10 μL/min. The dissociation was studied by washing with running buffer for 15 min. Kinetic parameters including $k_a$ (association rate constant) and $k_d$ (dissociation rate constant) were estimated using the BLAevaluation 3.1 software (GE Healthcare Life Sciences). The specific binding curves were normalized by subtracting the control curve.

**Cytotoxicity Assays**

Cell cytotoxicity against Ramos and Raji cells could be observed with immobilized SM03, and was previously described. Briefly, varying concentrations of SM03 in carbonate buffer (pH 9.6) were allowed to be immobilized into triplicate wells of a 96-well ELISA plate. Log phase Ramos (human Burkitt’s lymphoma) cells at a concentration of 2 x 10^7/mL in RPMI1640 (Life Technologies) were prepared, and 100 μL of the cells were added into each well containing immobilized SM03. After incubation at 37°C for 4 to 24 hours, the viabilities of Ramos cells were determined by trypan blue exclusion assays or using cell counting kit-8 (CCK-8) as described below. CMC assays were performed using either guinea pig serum (GPS; Cedarlane Laboratories Ltd, Ontario, Canada) or human complement serum (HCS; Sigma-Aldrich, St. Louis, MO) as the complement sources. Briefly, target cells at 2 x 10^5 cells/well were mixed with various concentrations of reacting antibodies, including SM03, SM06, or other control antibodies, in the presence of GPS or HCS (1:10 dilution). After the mixture was incubated at 37°C for 2 hours, CCK-8 solution was added, and the % killing was determined by OD450 measurement following the manufacturer’s specifications (Dojindo Molecular Technologies, Inc. Rockville, MD) using the following equation: Killing (%) = (OD450 control – OD450 Sample) / (OD450 Control – OD450 Blank) x 100%.

For ADCC assay, PBMCs were prepared from freshly collected whole blood from human. Washed PBMC were resuspended in RPMI 1640 media containing 10% FBS, and was being used as the effector cells. Surrogate target cells (Fab-GlycoA cells) (50 μL of 3 x 10^5 cells/ml) and PBMC effector cells (50 μL of 1.2 x 10^5 cells/ml) were mixed at a target-to-effector cell ratio of 1:40 in the presence of varying concentrations of SM03 (PNGase treated or untreated). The mixture was incubated at 37°C in a humidified incubator, 5% CO2, for 5 hrs, before 50 μL of supernatant was evaluated for the level LDH released by LDH assay kit (Promega, Madison, WI) following the manufacturer’s specifications. The extent of killing by ADCC was revealed at OD490nm.

**Capillary-electrophoresis sodium dodecyl sulfate**

The removal of the CH2-appended glycan group on SM03 treated with PNGase was revealed by capillary-electrophoresis sodium dodecyl sulfate (CE-SDS). Briefly, PNGase treated and untreated SM03 were mixed with loading buffer containing iodoacetamide (Sigma, St. Louis, MO) with (reducing conditions) or without (non-reducing conditions) β-mercaptoethanol (2-ME). Samples were heated in boiling water for 3 min before they were loaded into the PA 800 plus Pharmaceutical Analysis System (Beckman Coulter Inc., Indianapolis, IN) according to the manufacturer’s specifications.

**Glycoprotein Staining and Glycoprofiling**

Glycoprotein staining was performed with Pierce Glycoprotein Staining Kit according to manufacturer’s specifications (Thermo Fischer Scientific Inc., Rockford, IL).

Glycoprofiling by UPLC-FLR/MS analysis of 2-aminobenzamide (2-AB)-labeled N-glycans

N-glycans of SM06 were first released enzymatically and labeled before chromatographic analysis. The released N-glycans were labeled by reductive amination with 2-aminobenzamide (2-AB). The labeled glycoforms (2 μL) were separated by an
ULPC system equipped with a BEH Glycan column (bead size: 1.7 μm, dimensions: 2.1 mm × 150 mm; Waters Corp., Milford, MA) thermostated at 60°C. Resolved glycoforms were detected by fluorescence spectrometry with an excitation wavelength at 350 nm and an emission wavelength at 420 nm. Peaks were further analyzed by an on-line Q-TOF mass spectrometer (Waters Corp.) via electrospray ionization (ESI). Analyte mass between 650 and 1500 was detected. Chromatogram analyses and deconvolution of mass spectra were performed by Empower 3 and UNIFI software, respectively (Waters Corp.).

Phase 1 Clinical Trials

The study design and drug administration for SLE and RA patients were approved by the China Food and Drug Administration. The Phase 1 study was conducted at Peking Union Medical College Hospital, Beijing, China, and all subjects gave written informed consent. Blood samples of SM03-treated patients were collected by the clinical team in accordance with the ethical principles of the Declaration of Helsinki.

Disclosure of Potential Conflicts of Interest

Authors KG, GYW, KWC, KYL, QZ, WTC and JZW declare no competing financial interests. Author SOL is one of the co-founders of SinoMab Bioscience Limited.

Authors’ Contributions

SOL, KG, GYW, KWC, KYL, QZ performed the research; JZW, SOL, KG, WTC designed the research, discussed and interpreted the experimental results; SOL, KG, JZW provided the research materials; SOL, WTC, JZW provided the financial support; SOL, KG, JZW wrote and edited the paper.

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