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Glycovariant anti-CD37 monospecific protein therapeutic exhibits enhanced effector cell-mediated cytotoxicity against chronic and acute B cell malignancies

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Abbreviations: ADCC, antibody dependent cellular cytotoxicity; ADCP, antibody dependent cellular phagocytosis; ALL, acute lymphocytic leukemia; CDC, complement dependent cytototoxicity; CLL, chronic lymphocytic leukemia; FcγR, Fc gamma receptor; MDM, monocyte derived macrophages

TRU-016 is a SMIP™ (monospecific protein therapeutic) molecule against the tetraspanin transmembrane family protein CD37 that is currently in Phase 2 trials in Chronic Lymphocytic Leukemia (CLL) and Non-Hodgkin Lymphoma (NHL). In an attempt to enhance the ADCC function of SMIP-016, the chimeric version of TRU-016, SMIP-016^{GV} was engineered with a modification in a glycosylation site in the Fc domain. The wild-type and glycovariant SMIP proteins mediate comparable Type I antibody-like direct cytotoxicity in the presence of anti-human Fc crosslinker and show a similar tyrosine phosphorylation pattern post-treatment. However, NK cells stimulated with the SMIP-016^{GV} exhibit enhanced activation and release 3-fold more interferon-γ compared with SMIP-016. SMIP-016^{GV} shows enhanced ADCC function against cells expressing CD37 with NK cell effectors derived from both normal and CLL-affected individuals. Enhanced ADCC is observed against CLL cells and is sustained at concentrations of SMIP-016^{GV} as low at 5E^{-6} μg/mL on cells expressing minimal CD37 antigen. In support of the biological relevance of this, SMIP-016^{GV} mediates effective ADCC against primary acute lymphoblastic leukemia (ALL) cells with low surface expression of CD37. Collectively, these data suggest potential use of the novel therapeutic agent SMIP-016^{GV} with enhanced effector function for B cell malignancies, including CLL and ALL therapy.

Introduction

CD37 is a tetraspanin transmembrane family protein that is expressed on the surface of mature, immunoglobulin-producing B cells[1] but not in CD10$^+$, CD34$^+$ and CD34$^-$ B cell precursors found in the bone marrow. Surface CD37 expression becomes strong in CD10$^-$ mature B-lymphocytes and its expression further increases as the B-lymphocytes continue to mature and move into the lymph nodes and peripheral blood. Finally, surface CD37 expression is lost in terminally differentiated plasma B cells.[2,3] CD37 is also highly expressed on the surface of transformed mature B cell leukemia and lymphoma cells but not on myeloma cells.[3] CD37 is dimly expressed on T cells, monocytes and granulocytes and is not expressed on the surface of natural killer (NK) cells, platelet and erythrocytes.[1,2] This limited expression makes it an ideal therapeutic target in B cell malignancies[2] such as chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL).

CD37 was first examined as a potential therapeutic target in the late 1980s. Radio-labeled mouse monoclonal antibodies

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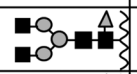
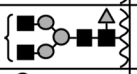
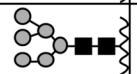
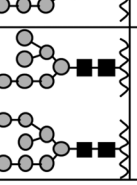
Table 1. Comparison of the N-linked Glycan Composition of SMIP-016 vs. SMIP-016^{GV}

Glycoform Mass by LC-MS	Probable Glycoform Composition[a]	Likely Structure[a]	Glycoform Type	Relative Abundance (%)	
				SMIP-016	SMIP-016^{GV}
1445	$(HexNAc)_2(DeoxyHex)_1 + (Man)_3(GlcNAc)_2$ = G0F		Fucosylated Complex	75	5
1607	$(Hex)_1(HexNAc)_2(DeoxyHex)_1 + (Man)_3(GlcNAc)_2$ = G1F		Fucosylated Complex	19	0
1378	$(Hex)_3 + (Man)_3(GlcNAc)_2$		Non-Fucosylated High Hexose	6	0
1703	$(Hex)_5 + (Man)_3(GlcNAc)_2$		Non-Fucosylated High Hexose	0	7
2027	$(Hex)_7 + (Man)_3(GlcNAc)_2$		Non-Fucosylated High Hexose	0	88

[a] GlycoMod (http://www.expasy.ch/tools/glycomod/)

○ galactose ● glucose ● mannose ■ N-acetylglucosamine △ fucose ≋ protein backbone

against CD37 were studied in B cell lymphoma patients and were shown to produce anti-tumor responses.[4-6] However, due to the perceived targeting potential of CD20, CD37 as a therapeutic target was not further developed until recently with an engineered monoclonal antibody mAb 37.1 that has been shown to be effective in preclinical models of B cell malignancies.[7] Furthermore, our laboratory has shown that a novel protein therapeutic directed against CD37, SMIP-016 induces more apoptosis in CLL B cells than rituximab[8] in vitro, when it is used alongside an anti-human Fc crosslinking antibody. Its mechanism of action is through a caspase independent pathway, which suggests it can be used in combination therapy with other caspase activation-dependent cytotoxic antibody therapies or chemotherapeutic agents, such as fludarabine. The direct cytotoxic effect of SMIP-016 on CLL B cells is proportional to the amount of CD37 present on the cell surface, making it a highly selective therapy toward malignant B cells. Furthermore, SMIP-016 showed potent anti-lymphoma activity in a Raji/SCID xenograft mouse model. TRU-016, a humanized anti-CD37 SMIP molecule derived from SMIP-016, is currently in Phase 2 clinical trials and showing single agent activity in CLL.[9]

In addition to direct killing, a major potential mechanism involved in TRU-016 tumor elimination is ADCC. SMIP-016 induced NK cells mediated antibody-dependent cellular cytotoxicity (ADCC) both in vitro and in vivo.[8] Monoclonal antibodies with bisected, complex, non-fucosylated oligosaccharides attached to the asparagine 297 residue in the CH2 region, bind with increased affinity to FcγRIIIa.[10] This glycoform engineering has been shown to enhance ADCC[11] through cells bearing FcγRIIIa, an important component in how monoclonal antibodies are clinically effective.[12] For example, afucosylated anti-CD20 antibodies show higher B cell depletion than their fucosylated counterpart by reaching saturated ADCC levels at lower concentrations and through improved FcγRIIIa binding.[13] In addition, it has been reported that antibodies lacking the core fucose in Fc oligosaccharides elicit high ADCC responses by two mechanisms.[14] On the effector cell side, afucosylated anti-CD20 antibodies were less inhibited by human plasma IgG. On the target cells, cells treated with non-fucosylated anti-CD20 antibodies showed markedly stronger binding to NK cells than fucosylated anti-CD20.[14]

Due to the success of the parent compound SMIP-016, we sought to determine if modifying the Fc oligosaccharides of a SMIP protein would enhance its activity. Herein, we describe a second generation anti-CD37 SMIP molecule, SMIP-016^{GV}, with an afucosylated Fc receptor binding region designed for enhanced effector function. Our data demonstrates SMIP-016^{GV} has enhanced effector function with NK cells and monocyte derived macrophages (MDM), making it an exciting novel CD37-targeted peptide therapeutic for B cell malignancies.

Results

Engineering and characterization of glycovariant SMIP protein. SMIP-016^{GV} was generated by treating a DG44 CHO cell line transfected with SMIP-016 cDNA with castanospermine (CS), a potent plant alkaloid inhibitor of glucosidases involved in N-linked processing of glycoproteins such as glucosidase I.[15] LC/MS glycoprofiling analysis of the products demonstrated that the observed mass species in both CS treated (SMIP-016^{GV}) and untreated (SMIP-016) preparations were consistent with the expected amino acid sequence with a typical, heterogeneous mammalian glycosylation pattern. A slight difference in amino acid composition was noted between the two preparations with a greater proportion of the SMIP-016^{GV} monomers found to lack the C-terminal lysine compared with the SMIP-016 monomers (89% vs. 72%). With respect to the N-linked glycoforms, there was a near total reversal in the percentage of fucosylated and non-fucosylated glycan species between the two preparations (**Table 1**). For SMIP-016, the fucosylated complex glycoforms

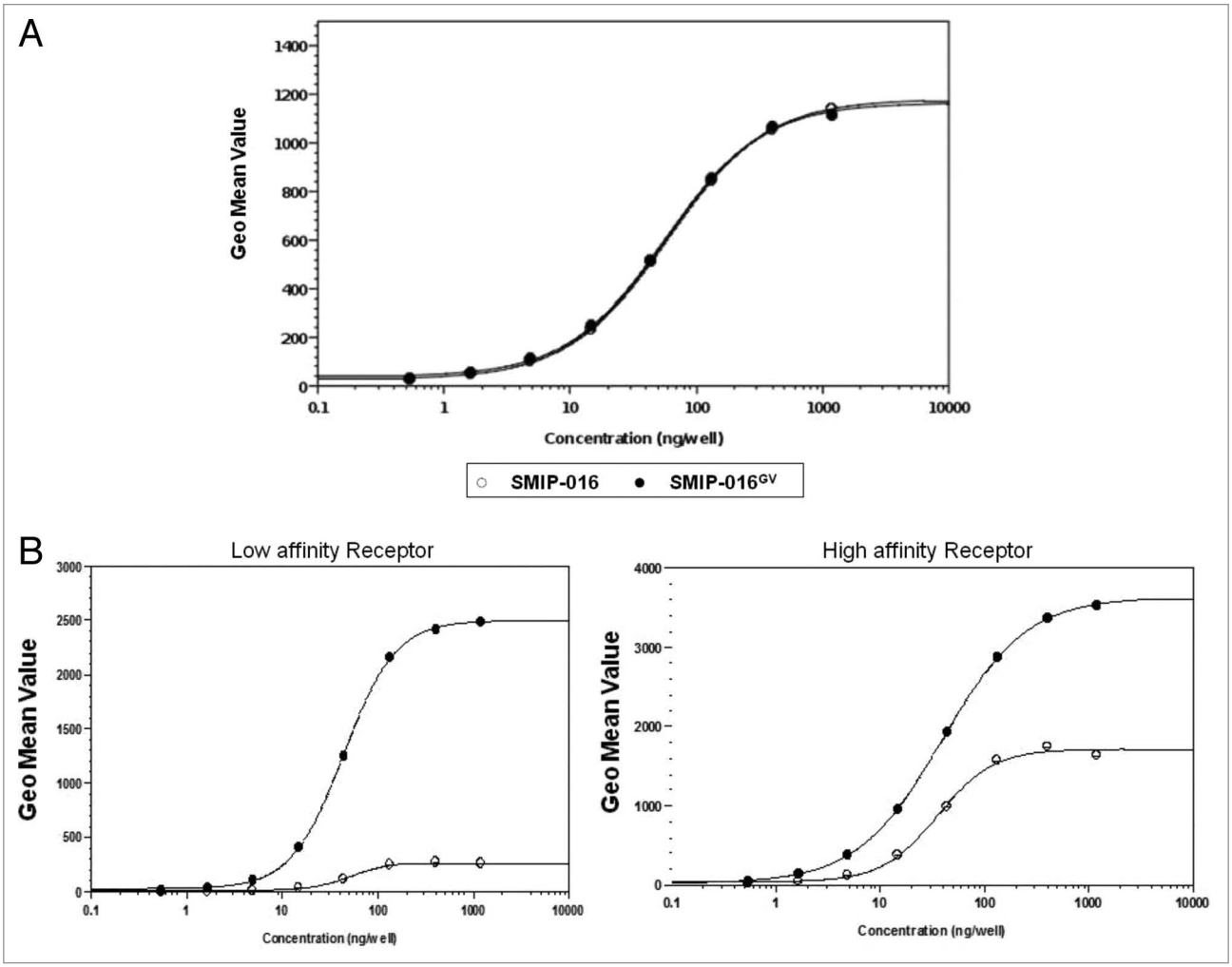


Figure 1. Characterization of SMIP-016^{GV} molecule. (**A**) Binding affinity of SMIP-016 and SMIP-016^{GV} to target antigen CD37. (**B**) Enhanced binding of SMIP-016^{GV} with both high and low affinity soluble FcγRIII.

(94% of total) were composed of 80% G0F and 20% G1F species, with the remaining 6% of glycans being represented by non-fucosylated high hexose forms of $(Man)_3$ + $(Man)_3(GlcNAc)_2$ composition. In the case of SMIP-016^{GV}, only 5% of the glycans were of fucosylated complex form (all G0F), while the remaining (95%) were of non-fucosylated high hexose form comprised mostly of $(Hex)_7$ + $(Man)_3(GlcNAc)_2$ glycans with the remainder of $(Man)_5$ + $(Man)_3(GlcNAc)_2$ composition.

SMIP-016^{GV} and SMIP-016 have equal binding affinity for CD37. To test whether the glycovariant form of SMIP-016 had similar antigen binding affinity as the parent compound, both SMIP proteins were bound to Daudi cells, a human B-lymphoblastoid cell line derived from Burkitt lymphoma that expresses high levels of CD37. A FITC-labeled anti-huIgG antibody was added to bind to the SMIP proteins and the bound fluorescence was measured by flow cytometry. As shown in the **Figure 1A**, SMIP-016 and its CS generated glycovariant SMIP-016^{GV} yielded virtually identical binding profiles on CD37$^+$ Daudi cells, demonstrating that alteration of the N-linked glycosylation of SMIP-016^{GV} had no effect on target antigen binding of the protein.

The Fc portion of SMIP-016^{GV} has enhanced affinity for low and high affinity soluble FcγRIII. The single nucleotide polymorphisms (SNP) in FcγRIIIa resulting in either a valine (V) or phenylalanine (F) at position 158 results in low or high affinity receptors in humans, and this has been implicated to be indicative of response to rituximab immunotherapy.[16-18] This SNP, however, has shown no correlation to response in CLL.[19,20] To test the binding affinities to these receptors, SMIP-016 and SMIP-016^{GV} were bound to CD37+ Daudi cells and their ability to bind to soluble versions of low and high affinity FcγRIIIa was tested by flow cytometry. SMIP-016^{GV} demonstrated a 9.3-fold increase in binding over that observed with SMIP-016 for the FcγRIII low affinity receptor and a 2.1-fold increase in affinity for the high affinity FcγRIII receptor, respectively (**Fig. 1B**).

SMIP-016 and SMIP-016^{GV} show comparable levels of cytotoxicity through similar mechanisms. Direct cytotoxicity is one of the mechanisms of killing mediated by SMIP-016. To test whether the changes in the glycosylation pattern on this SMIP protein influenced its potential for direct cytotoxicity, we tested SMIP-016 and SMIP016^{GV} on isolated primary CLL cells.

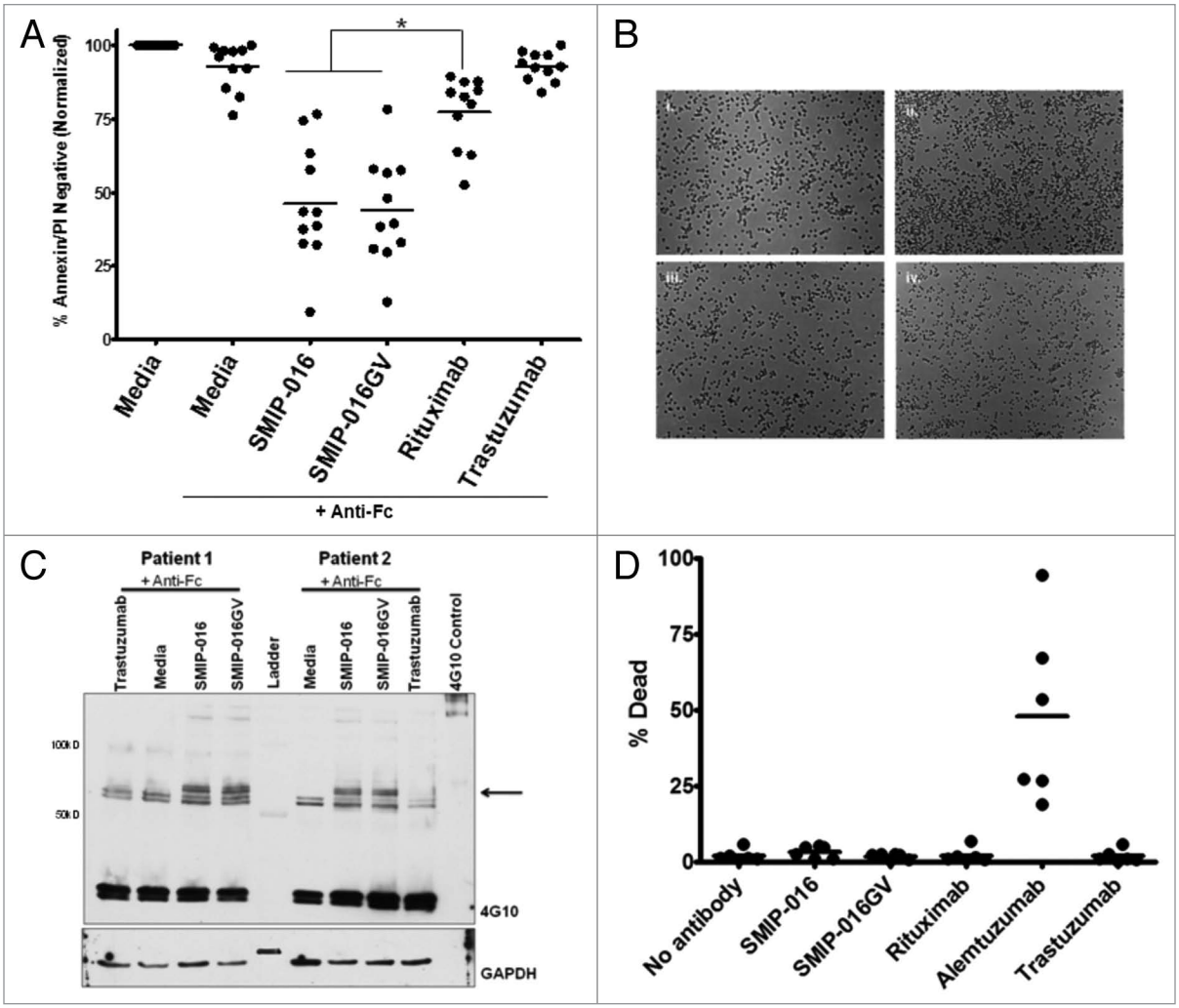


Figure 2. SMIP-016 and SMIP-016^{GV} show similar methods of direct cytotoxicity. (**A**) SMIP-016 and SMIP-016^{GV} show comparable levels of direct cytotoxicity in primary CLL B cells (n = 11) (p = 0.0708). (**B**) SMIP-016 and SMIP-016^{GV} kills cells in a Type I antibody-like manner. Microscopy images of CLL B cells (i) untreated (ii) Type II antibody (iii) SMIP-016 or (iv) SMIP-016^{GV} for 16 h (n = 3). (**C**) Both SMIP-016 and SMIP-016^{GV} induced a similar pattern of tyrosine phosphorylation of cellular proteins at 65 kDa. CLL cells were treated with 5 μg/mL of SMIP-016 or SMIP-016^{GV} with anti-Fc cross-linking antibody in PBS for 10 min and phospho tyrosine proteins were detected by western blot analysis using anti-phosphotyrosine antibody 4G10 (n = 6). (**D**) SMIP-016 and SMIP-016^{GV} do not initiate complement dependent cytotoxicity in CLL B cells (n = 6).

SMIP-016^{GV} mediates direct cytotoxicity in primary CLL B cells with goat anti-human Fc crosslinker in comparable levels to those seen with the parent (unmodified) protein (**Fig. 2A**). When cells are drugged for 48 h along with anti-human Fc crosslinker, SMIP-016^{GV} decreased cell viability by about 60%, which was significantly higher than rituximab ($p < 0.0001$) but comparable to SMIP-016 ($p = 0.708$; **Figure 2A**). Therefore, both SMIP-016 and its glycovariant SMIP-016^{GV} show similar levels of direct cytotoxicity at the same concentration and time points, suggesting they have the similar potential for signaling of direct cytotoxicity in cells expressing CD37 on their surface. Furthermore, the SMIP proteins initiate apoptosis in a Type I antibody-like manner (**Fig. 2B**) compared with a Type II anti-CD20 antibody.

Zhao et al. previously reported that SMIP-016 works in a novel caspase-independent pathway and that in vitro stimulation of CLL B cells with SMIP-016 along with an anti-Fc crosslinker showed activation of protein tyrosine phosphorylation.[8] This was subsequently shown to involve SHP1 and a complex array of signaling via the ITIM motif of CD37.[21] To test whether the SMIP-016^{GV} was signaling within the cells via the same molecular pathways, we stimulated CLL cells with SMIP-016 or SMIP-016^{GV} along with anti-human Fc crosslinker. Both SMIP-016 and SMIP-016^{GV} show similar patterns of increased tyrosine phosphorylation after 10 min of in vitro stimulation of CLL cells (**Fig. 2C**). In particular, western blot analysis of cellular lysates with an anti-phosphotyrosine antibody revealed a predominant protein at ~65 kD in cells treated with SMIP-016 or SMIP-016^{GV}, an observation previously described by our group[8,21] and determined to be SHP-1 and Lyn phosphorylation. The identical binding affinity to the target antigen CD37 along with the equivalent amount of cytotoxicity against CLL cells via identical phosphorylation of

downstream proteins collectively suggests that the parent compound SMIP-016 and the glycovariant SMIP-016^{GV} are working through similar molecular pathways in CLL cells to cause direct cytotoxicity, as previously described.[21]

Next, we tested if SMIP-016^{GV} had any complement initiating capabilities. We have shown that the SMIP-016 is unable to mediate complement-dependent cytotoxicity.[8] This is also seen with the SMIP-016^{GV} (**Fig. 2D**). The alteration of the Fc glycosylation pattern does not affect the SMIP's inability to initiate complement.

SMIP-016^{GV} shows enhanced phagocytosis by monocyte-derived macrophages. We reported previously that NK cells, and not monocytes, were the major effector population mediating cytotoxicity with SMIP-016,[8] but the importance of the role of monocytes and macrophages in the function of therapeutic antibodies is emerging,[22-25] especially in anti-CD20 therapy. Similar to what we had seen previously, we saw low ADCC function with SMIP-016 and SMIP-016^{GV} in monocytes against CLL B cells (data not shown).

In addition, plate bound SMIP proteins were not efficient at inducing cytokines in peripheral blood monocytes. TNF production by monocytes stimulated with SMIP-016 and SMIP-016^{GV} for 24 h was decreased compared with non-specific IgG or rituximab (**Fig. 3A**). Furthermore, monocyte-derived macrophages (MDM) stimulated similarly for 24 h also had lower TNF production (data not shown).

To study if SMIP-016^{GV} could elicit a cytotoxic response from MDM, which express FcγRIIIa,[22,26] we tested it in antibody-dependent cellular phagocytosis (ADCP) assays using MDM from normal donors. Primary CLL samples were coated with either SMIP-016 or SMIP-016^{GV} and subsequently co-incubated with MDM. The glycovariant SMIP protein mediates significantly more ADCP than the parent SMIP protein ($p < 0.05$; **Fig. 3B**).

SMIP-016^{GV} enhances NK cell activation and ADCC toward CD37-expressing targets. Previous studies have shown that changing the glycosylation pattern of the Fc portion of antibodies can influence their ability to elicit effector function by enhancing their binding affinity to FcγRIIIa on human immune cells.[14,27] We saw similar enhanced affinity to FcγRIIIa with SMIP-016^{GV} (**Fig. 1C**). In addition, compared with SMIP-016, SMIP-016^{GV} mediated enhanced activation of NK cells as revealed by the increased expression of surface CD107a following stimulation with the SMIP proteins or IgG control antibodies by 8-fold ($p = 0.012$) (**Fig. 3C**) and by the 16-fold increase in interferon (IFN)-γ production ($p = 0.009$, **Fig. 3D**).

To determine whether the afucosylation of glycoproteins on the SMIP-016^{GV} engineered using TRU-ADhanCe™, a glycovariant optimization technology, influence the molecule's ability to elicit NK cell ADCC, we performed ADCC assays with NK cells from healthy donors against CD37 positive (Raji) and negative (697) malignant B cell lines targets. As shown in **Figure 3E**, SMIP-016^{GV} mediated significantly more ($p = 0.0013$ in all E:T ratios > 0) ADCC against Raji cells than SMIP-016. In contrast, using 697 cells, a human B cell precursor leukemia cell line that does not express CD37, SMIP-016 and SMIP-016^{GV} did not mediate any ADCC effects (**Fig. 3F**), validating the antigen specificity of the two SMIP proteins.

NK cells from CLL patients show enhanced ADCC with SMIP-016^{GV}. Ziegler et al. showed in a landmark paper that the NK cells from CLL patients are deficient in activity. The NK cell activity was not detectable in patients with advanced disease and six times lower than control in patients with early disease.[28] This finding, however, has been challenged by others.[29] To test whether CLL NK cells would be capable of mediating an effective response with SMIP-016^{GV}, we used NK cells from patients with early stage disease as effectors in ADCC assays against Raji cells targets. Their response was compared with the response from NK cells isolated from healthy donors. The SMIP-016^{GV} showed increased levels of cytotoxicity than SMIP-016 with normal and CLL NK cells ($p < 0.0001$ for both) (**Fig. 4A**). The trend in response to the SMIP-016^{GV} from primary NK cells from CLL patients as in NK cells from healthy donors was comparable ($p = 0.0154$). Another statistical test showed that the difference between the healthy vs. CLL NK cells and the SMIP-016^{GV} is not significant ($p = 0.86$), indicating that the superiority of SMIP-016^{GV} over SMIP-016 is consistent for CLL and normal NK cells. The enhanced cytotoxicity achieved by SMIP-016^{GV} with CLL NK cells validates our hypothesis that the glycovariant SMIP-016 may be an excellent therapeutic in CLL patients.

SMIP-016^{GV} shows enhanced ADCC against primary CLL B cells. Given the documented clinical activity of TRU-016, the clinical version of SMIP-016, in CLL, we next compared the ability of SMIP-016 with the SMIP-016^{GV} to elicit effector function against primary CLL B cells. CLL B cells have a wide range of CD37 on their surface.[8] SMIP-016^{GV} was able to induce greater cytotoxicity in CLL cell targets compared with SMIP-016 ($p < 0.0001$; **Fig. 4B**). SMIP-016^{GV} mediates significantly greater ADCC than rituximab ($p < 0.0001$) and is comparable to alemtuzumab ($p = 0.691$); however, SMIP-016^{GV} is more specific to malignant B cells than the anti-CD52 antibody. The superiority of SMIP-016^{GV} over SMIP-016 for ADCC was observed for all of the effector:target ratios tested (average across E:T ratios > 0 vs. E:t = 0 for SMIP-016^{GV} vs. SMIP-016, $p < 0.0001$) (**Fig. 4C**).

SMIP-016^{GV} at low concentrations is effective in enhancing NK cell ADCC. Our initial ADCC experiments were done using 5 μg/mL of both SMIP proteins, which was the optimal dose found to be effective for direct cytotoxicity in CLL B cells.[8] Given the enhanced ADCC by SMIP-016^{GV}, we hypothesized that this may maintain the enhanced ADCC at lower SMIP protein concentrations. It has been suggested that serum plasma and IgG can affect the concentrations of therapeutic monoclonal antibodies within the body,[12] and, therefore, efficacy at low concentrations is desirable for therapy. SMIP-016, SMIP-016^{GV} and rituximab were used at decreasing log concentrations in an ADCC assay using normal donor NK cells as effectors and CLL B cells as targets. The SMIP-016^{GV} showed enhanced ADCC compared with the SMIP-016 across a wide range of concentrations, even as low as 5×10^{-6} μg/mL ($p < 0.0001$ compared with both SMIP-016 and rituximab, **Fig. 4D**). The cytotoxicity with SMIP-016 reaches a plateau after the 0.005 μg/mL concentration while SMIP-016^{GV} continues to increase. The trend in cytotoxicity for SMIP-016^{GV}

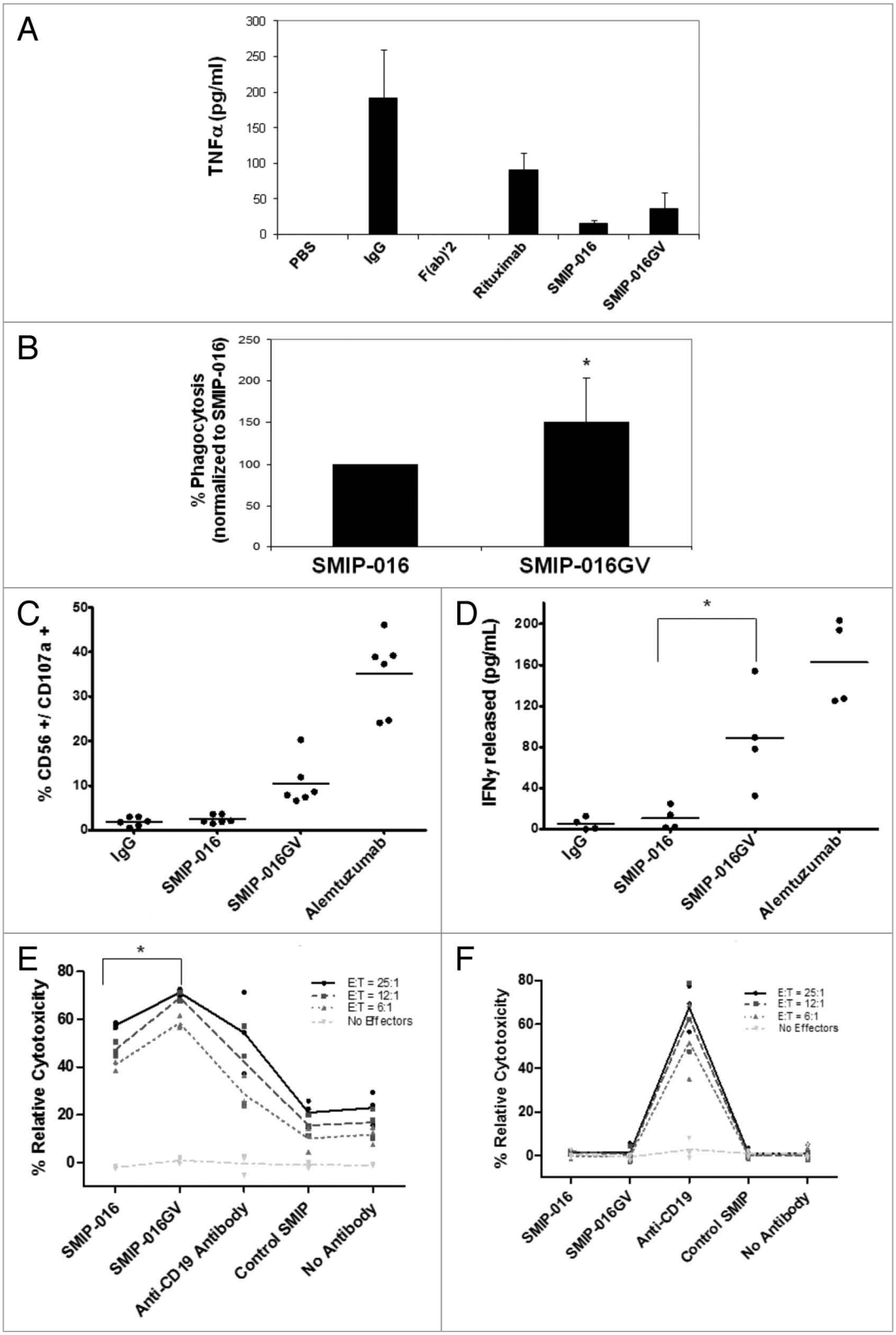


Figure 3. SMIP-016^{GV} can mediate cytotoxicity through effector cells. (**A**) Intact antibodies, F(ab)$_2$, or SMIP bound to plates induced TNF from peripheral blood monocytes (n = 4). (**B**) SMIP-016^{GV} mediates enhanced antibody-dependent cellular phagocytosis (ADCP) by monocyte-derived macrophages (MDM) of primary CLL cells compared with SMIP-016, as measured by flow cytometry (n = 3) ($p < 0.05$). (**C**) Induction of CD107a on the surface of CD56$^+$ NK cells by SMIP-016^{GV}, as measured by flow cytometry (n = 6). (**D**) Enhanced induction of interferon gamma from NK cells by SMIP-016^{GV} compared with SMIP-016 ($p = 0.009$) (n = 4). (**E, F**) ADCC with normal donor NK cell effectors and Raji (**E**) or 697 (**F**) targets. SMIP-016^{GV} shows enhanced ADCC function in the CD37 expressing Raji cells compared with SMIP-016 ($p = 0.0013$ of average of all E:T ratio > 0), but no activity in the 697 cells (n = 3).

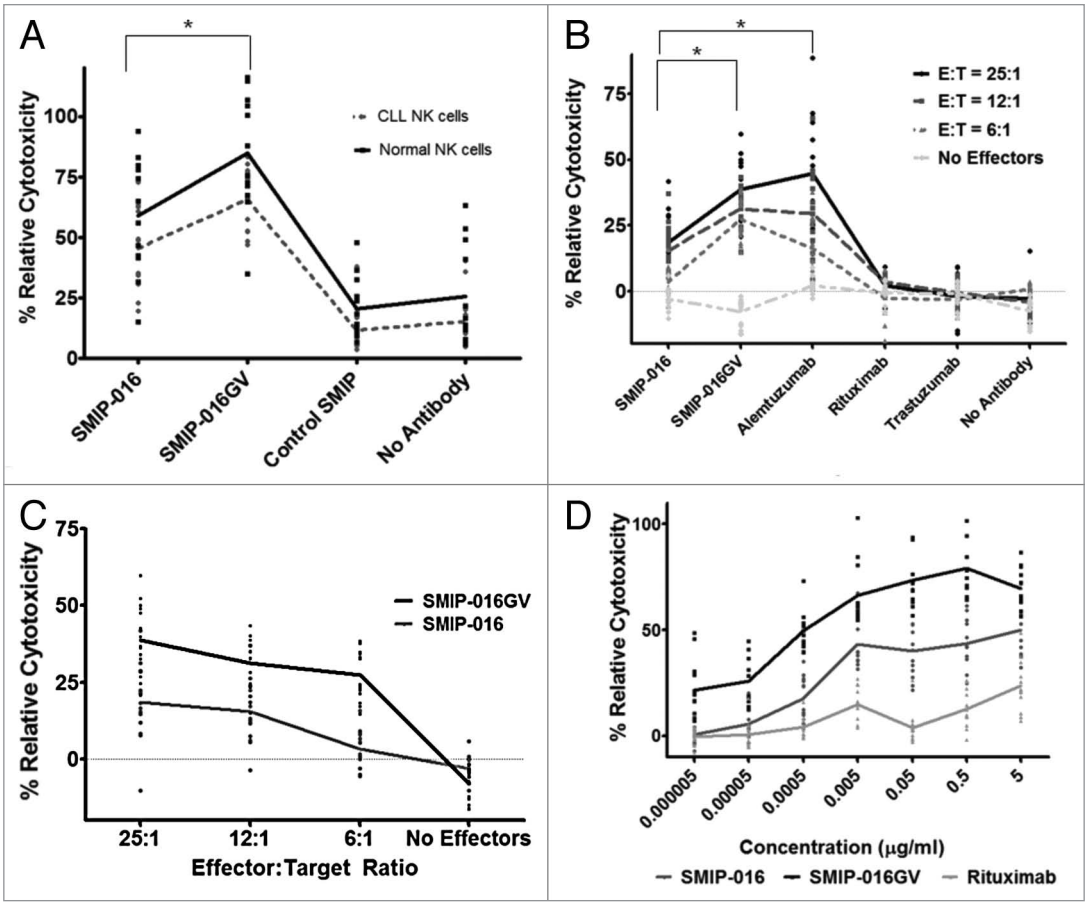


Figure 4. SMIP-016^{GV} is effective against CLL B cells. (**A**) SMIP-016^{GV} shows comparable enhanced cytotoxicity trends in ADCC assays with primary normal donor or CLL NK cells used as effectors against Raji cell targets at an 25:1 E:T ratio (n = 12) (p = 0.01). The levels of cytotoxicity achieved with the CLL NK cells were significantly lower than what is seen with comparable treatments in the normal donor NK cells. (p = 0.0154 for SMIP-16^{GV} and p = 0.0104 for SMIP-016). (**B**) ADCC with normal donor NK cells against primary CLL B cell targets shows enhanced ADCC function with SMIP-016^{GV} compared with SMIP-016 (p < 0.0001). Each patient at each E:T ratio is represented by a dot (n = 15). (**C**) The enhanced ADCC function seen in (**B**) is sustained over all effector to target ratios tested (average SMIP-016^{GV} vs. SMIP-016 at E:T ratio > 0, p < 0.0001). (**D**) Dose dependent ADCC function of SMIP-016^{GV} against CLL B cells. SMIP-016^{GV} shows significantly enhanced ADCC compared with SMIP-016 (p < 0.0001) or rituximab (p < 0.0001) even at the lowest concentration (n = 12). The trend showed significantly increased ADCC with SMIP-016^{GV} when compared with SMIP-016 (p < 0.0001) or rituximab (p < 0.0001). The non-specific Control SMIP showed less than 5% cytotoxicity at all concentrations tested (data not shown).

was significantly steeper than for SMIP-016 (p < 0.0001) and for rituximab (p < 0.0001).

SMIP-016^{GV} enhances ADCC in cells expressing low levels of surface CD37. SMIP-016-mediated direct cytotoxicity has been shown to be dependent on antigen density.[8,21] We wanted to test whether antigen density played a role in ADCC with the SMIP-016 and whether the glycovariant could overcome this. To test this, we created a model system by retrovirally transducing 697 cells (CD37 negative cell line) with a pBABE-CD37 vector construct and isolated clones with differing levels of surface CD37 by limiting dilution cloning. We validated that the clones were producing CD37 mRNA by RT-PCR (data not shown) and quantified the amount of surface CD37 protein by flow cytometry (**Fig. 5A**). These clones expressed between 5000 and 80000 molecules of CD37 on their surface, substantially less than Raji cells, which have hundreds of thousands of CD37 molecules. To test a range of CD37 antigen levels, low, medium and high CD37 antigen density clones were chosen for our assays.

Our experimental system allowed us to determine if antigen density played a role in the level of NK cell-mediated ADCC by SMIP-016^{GV}. Using normal donor NK cells as effectors and the CD37-expressing 697 clones as targets, ADCC assays were performed to test varied amounts of antigen on the target cells against decreasing SMIP protein concentrations (**Fig. 5B**). As shown previously (**Fig. 3E**), the parent 697 cells were not susceptible to ADCC by the CD37 specific SMIP protein, but despite a relatively low level of CD37 expression in the 697-CD37 V1 clone (under 20,000 molecules/cell), SMIP-016^{GV} elicited excellent NK-mediated ADCC in these cells, up to ~80% relative cytotoxicity (**Fig. 5B**). In addition, the results showed that with SMIP-016^{GV}, cytotoxicity increased with increasing CD37 surface expression (p = 0.002). Enhanced ADCC with increasing CD37 antigen was also seen in SMIP-016 treated cells (p = 0.0005).

Interestingly, the expression of CD37 amplifies the response difference between SMIP-016^{GV} and SMIP-016 in the low and

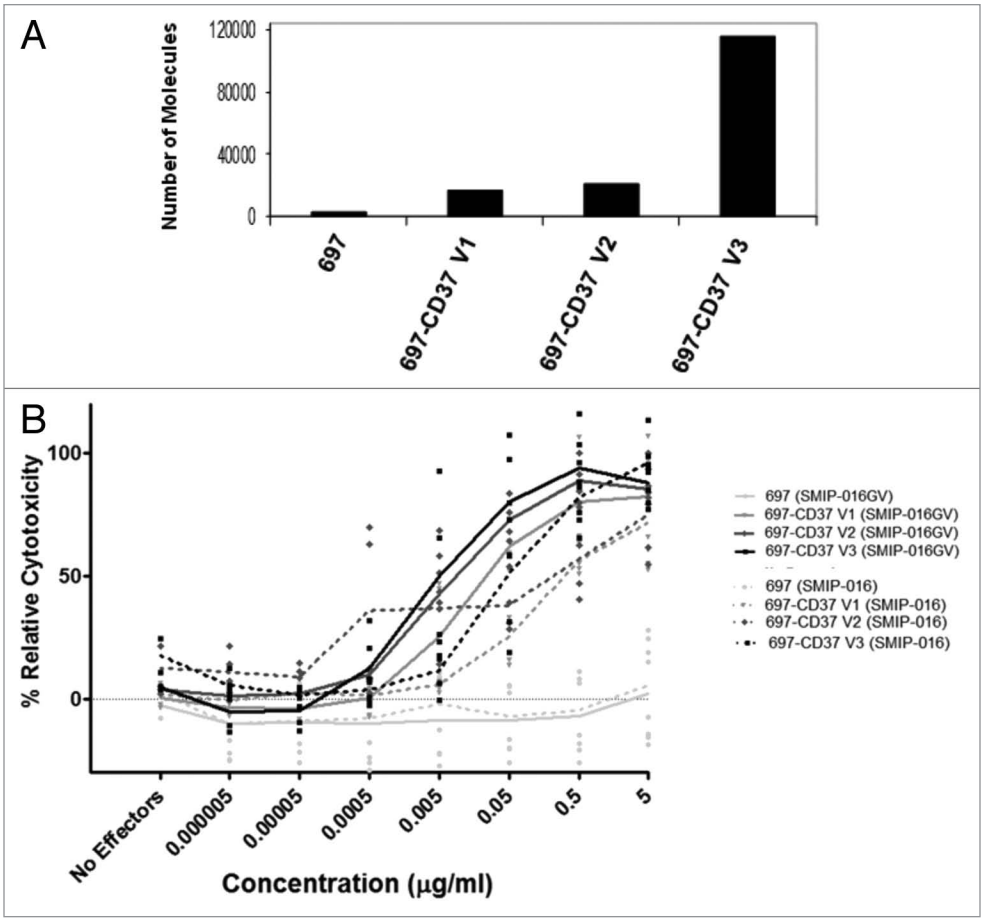


Figure 5. SMIP-016^{GV} mediates enhanced ADCC in cells expressing a range of surface CD37. (**A**) Quantification of surface CD37 on parental 697 (697), 697-CD37 clones V1, V2 and V3 by flow cytometry. (**B**) ADCC assay with normal donor NK cell effectors against the different 697 cell clones as targets at a 25:1 E:T ratio. SMIP-016 and SMIP-016^{GV} were used in decreasing concentrations (n = 4 NK donors). Increasing surface CD37 levels enhanced ADCC with both the SMIP-016^{GV} (p = 0.002) and SMIP-016 (p = 0.0005).

medium CD37-expressing 697 cell clones, 697-CD37 V1 and V2. In 697-CD37 V3, the trend difference between the SMIP-016 and the glycovariant was not significant. This result may be due to the high amount of cytotoxicity already obtained with SMIP-016. The SMIP-016^{GV} mediates greater killing but reaches 100%, thus reaching the maximal limit of the assay. Therefore, the SMIP-016^{GV} could be beneficial for B cell malignancies with a low level of surface CD37.

SMIP-016^{GV} shows enhanced ADCC function against acute lymphoblastic leukemia cells. Acute lymphoblastic leukemia (ALL) cells have been reported as having low surface expression levels of CD37.[3] From our results in **Figure 5**, we hypothesized that the SMIP-016^{GV} would be capable of enhanced NK cell mediated ADCC against these leukemic cells. First, we quantified the levels of CD37 on the surface of primary ALL bone marrow cells, CLL cells and normal B cells (**Fig. 6A**). With an average of ~12,000 molecules of surface CD37, the surface levels of CD37 on ALL cells are 3-fold lower than CLL cells (p = 0.0051), which have an average of ~36,000 molecules, and 5-fold lower than normal peripheral B cells (p < 0.0001), which have an average of about 63,000 molecules on their surface. As shown previously,[8] CLL cells have a wide range of CD37 expression.

Direct cytotoxicity was tested with SMIP-016, SMIP-016^{GV} and alemtuzumab in primary ALL samples. Viability analysis by flow cytometry at 24 h revealed that both SMIP proteins exhibited comparable cytotoxicity of 20% (**Fig. 6B**) (p = 0.7425), while alemtuzumab mediated about 40% cytotoxicity in these cells.

Finally, the primary ALL samples were used as targets in ADCC experiments with normal donor NK cell effectors. We saw a minimal response with SMIP-016 compared with the control SMIP protein. The SMIP-016^{GV} was able to elicit an enhanced ADCC response against ALL samples, despite the low levels of surface CD37 (**Fig. 6C**). This is significantly enhanced cytotoxicity compared with SMIP-016 (p < 0.0036 for all effector to target ratios tested). Collectively, this suggests SMIP-016^{GV} might serve as a potential therapeutic for ALL.

Discussion

Herein we report successful modification of the Fc binding region of a SMIP protein to effectively remove fucosylation in a manner that still allows efficient production of the protein for clinical use. Production of SMIP-016 in CHO cultures that contain CS results in a final product that is afucosylated. This

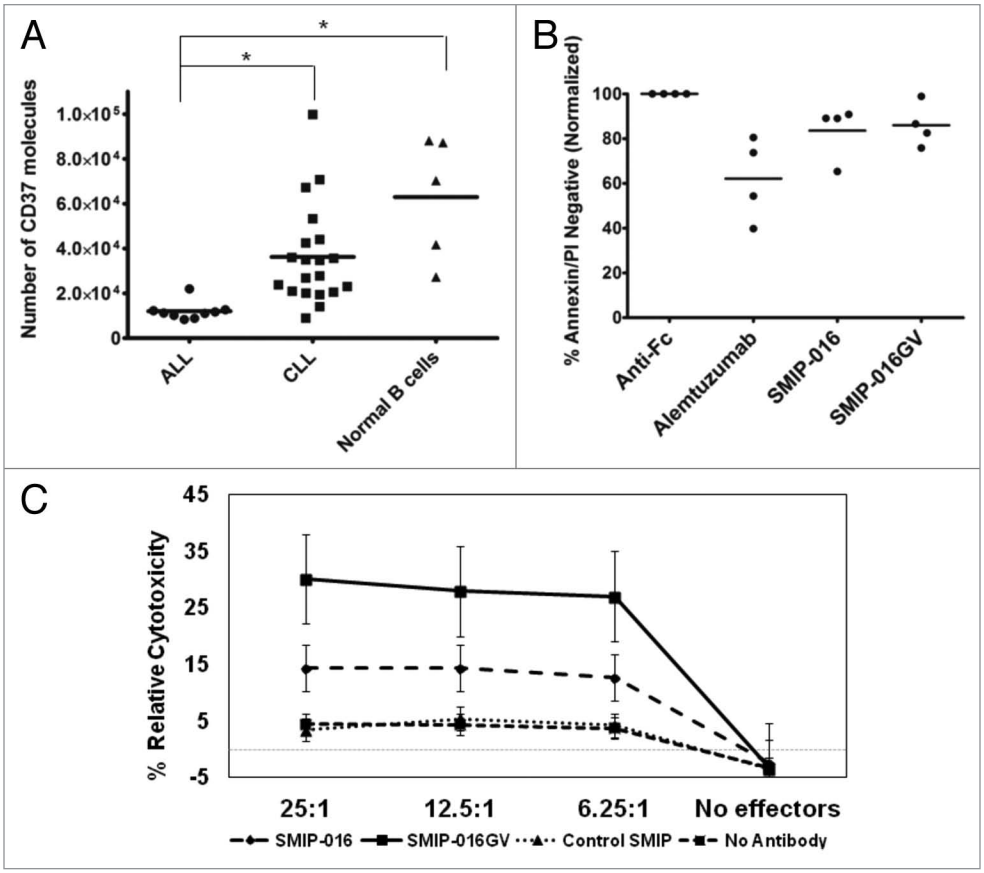


Figure 6. SMIP-016^{GV} mediates effective NK cell ADCC against primary Acute Lymphoblastic Leukemia cell. (**A**) Quantification of surface CD37 by flow cytometry shows ALL cells (n = 9) have significantly lower levels of surface CD37 than CLL B cells (n = 20) ($p = 0.0051$) and normal B cells (n = 5) ($p < 0.0001$). (**B**) SMIP-016 and SMIP-016^{GV} show comparable levels of modest direct cytotoxicity against primary ALL bone marrow samples (n = 4) ($p = 0.74$). (**C**) ADCC using ALL bone marrow samples as targets and normal donor NK cell effectors shows enhanced ADCC with SMIP-016^{GV} compared with SMIP-016. (n = 8 ALL samples × 3 NK cell effectors each) ($p < 0.0036$ for all E:T ratios tested).

modification of the molecule does not affect its binding affinity to CD37 and subsequent direct cytotoxicity, nor does it alter SMIP-016^{GV} from signaling through mechanisms previously described by our group.[8,21] From these data, we hypothesized that SMIP-016^{GV}'s direct cytotoxicity is dependent on surface levels of CD37 on CLL cells and is irrespective of prognostic factors such as IgVH mutational status, Rai stage and common cytogenetic abnormalities, as described with SMIP-016.[21] SMIP-016^{GV}, however, shows enhanced NK cell-mediated ADCC against primary CLL B cells compared with its parent compound SMIP-016. We demonstrated that NK cell effectors from CLL patients are not as effective in SMIP-016-mediated ADCC as normal NK cell effectors. The level of ADCC obtained with SMIP-016^{GV} and CLL NK cell effectors, however, were similar to that achieved with the parent compound SMIP-016 and normal NK cell effectors, indicating that the SMIP-016^{GV} can overcome these deficiencies. Finally, we demonstrated that SMIP-016^{GV} mediates superior ADCC against both low surface level expressing 697 cell lines and also primary ALL cells, whereas SMIP-016 lacks activity against these. Collectively, these data provide a strong rationale for clinical development of SMIP-016^{GV} in B cell malignancies expressing high, as well as low, amounts of surface CD37.

With the success of antibody therapy, substantial effort has gone into modifying the Fc domain to enhance effector cell recruitment, ability to fix complement and also increased affinity for FcRn to enhance serum half life. These manipulations of function can occur through amino acid engineering via substitutions in the constant region of antibodies and have been extensively described.[30-32] An alternative way to modify antibody effector function with enhanced recruitment of FcγRIIIa binding is to reduce Fc region fucosylation through genetic manipulation of the antibody-producing cell line.[33,34] This has been described for CD20-directed antibody therapeutics where studies have shown enhanced NK cell ADCC function at lower antibody concentrations.[13,35,36] It provides the opportunity for using less therapeutic antibody, thereby diminishing production and ultimately treatment cost.

Modification of Fc glycosylation of clinically-relevant alternative peptide therapies such as SMIP proteins has not been previously described. Afucosylation of the FcγR binding region had potential to affect stability or other properties important for commercial development. In this paper, we demonstrate that the production process yielded functional afucosylated SMIP-016, which suggests that it may also enhance effector cell function

of other immunoglycoprotein therapeutics. Given SMIP-016 has already demonstrated single agent activity against CLL and low grade B cell lymphoma,[9] further pursuit of SMIP-016^{GV} with enhanced NK cell effector cell function seems worthwhile.

The Phase 1 study of TRU-016 in CLL demonstrated that this agent has significant single agent activity in both symptomatic untreated CLL and also those patients having received 1 or 2 prior therapies.[9] Exploration of TRU-016 in ALL was not warranted based upon very modest expression of this antigen in less mature B cells.[3] Our in vitro data showing that SMIP-016 lacks ADCC against ALL cells provide further justification for this decision but studies done with low-expressing CD37 transfected 697 cell lines suggest that SMIP-016^{GV} was more effective at mediating ADCC at low copy number of CD37 antigen. Outside of kinase inhibitors targeting the Philidelphia chromosome positive ALL, little therapeutic progress has been made over the past two decades in the treatment of B cell ALL. A small proportion of patients are cured with intensive chemotherapy-based approaches or allogeneic stem cell transplant, but many ultimately die from their disease. This is particularly true for elderly patients who do not tolerate current therapy well. Immunotherapy for adult ALL is being studied in clinical trials; with rituximab and alemtuzumab, very modest benefit has been observed.[37-40] This in part relates to low antigen density of CD20 and populations of cells not expressing CD52 in ALL.[40-42] Herein, we demonstrate that SMIP-016^{GV} is effective at NK cell-mediated ADCC against primary ALL cells, which have lower CD37 surface expression. This provides support for use of SMIP-016^{GV} as a potential immune therapy of ALL.

In summary, our data suggest the potential use of the SMIP-016^{GV} with enhanced ADCC function as a new alternative for therapy in B cell malignancies, including CLL and ALL. SMIP-016^{GV} mediated cytotoxicity mainly through FcγR mechanisms. The capacity of monoclonal antibodies to interact with FcγRs in humans has been shown to be vital for therapeutic efficacy.[11] This same concept seems to apply to SMIP proteins, with SMIP-016^{GV} being highly effective against CLL and ALL cells due to interactions with FcγRs on effector cells.

Materials and Methods

Production and structural analysis of SMIP-016 and SMIP-016^{GV}. SMIP-016^{GV} was generated by culturing SMIP-016, clone 8g5, a DG44 CHO cell line transfected with SMIP-016 cDNA with 400 μM castanospermine (CS). After purification, SMIP-016 and SMIP-016^{GV} were analyzed by LC/MS via an ESI-TOF (Agilent Technologies) mass spectrometer detector.

Patient sample processing and cell culture. Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board (IRB) of The Ohio State University (Columbus, OH).[43] All patients had immunophenotypically defined CLL and had been without prior therapy for a minimum of 30 d at the time of collection. CLL cells were isolated from freshly donated blood with ficoll density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, catalog # 17–1440–03). Enriched CLL B cells were prepared with the use of the "Rosette-Sep" kit from Stem Cell Technologies (Catolog # 15024) according to the manufacturer's instructions. Isolated cells were incubated in RPMI 1640 media (Life Technologies, catalog # 12633–012) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, catalog # F4135–500 ml), 2 mM L-glutamine (Life Technologies, catalog # 25030–149) and 56 U/mL penicillin/56 μg/mL streptomycin (Life Technologies, catalog # 15140–122) at 37°C in an atmosphere of 5% CO_2. Normal cells were obtained from either Red Cross partial leukocyte preparations or donors as part of a second approved exemption protocol. Natural killer (NK) cells were negatively selected with Rosette-Sep kits (StemCell Technologies, catalog # 15025) according to the manufacturer's instructions. Monocytes were positively selected using MACS system (Miltenyi Biotec, catalog # 130–049–601). The purity of enriched populations of normal cells was routinely checked with the use of PE labeled CD19, CD14 and CD56 staining by flow cytometry. The Daudi and Raji cell lines were obtained from ATCC (ATCC# CCL-213 and CCL-86 respectively) and the 697 cell line was obtained from DSMZ, (catalog # ACC 42) and the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Assessment of apoptosis by flow cytometry. Cell viability was measured using Annexin V-FITC/PI staining followed by FACS analysis according to the manufacturer's protocol (BD PharMingen, catalog # 556422 and 556463) as described previously.[8] Data were analyzed with CXP software package (Beckman-Coulter). At least 10,000 cells were collected for each sample and data were acquired in list mode. Results were expressed as the percentage of total Annexin V/PI negative cells over untreated control.

In vitro treatment of cells with antibodies. Primary CLL or ALL cells were suspended in complete media at a density of 1 x 10^7 cells/mL. SMIP-016 and SMIP-016^{GV} were used a 5 μg/mL concentration unless otherwise noted. All antibodies (trastuzumab, rituximab and alemtuzumab) were used at a concentration of 10 μg/mL. The cross-linker, goat anti-human IgG (Fc specific) (Jackson Immunoresearch, catalog # 109–005–008) was added to the cell suspension 5 min after adding the primary antibodies at a concentration 5 times that of the therapeutic protein or antibody (i.e., 25 μg/mL for 5 μg/mL of SMIP-016). A group of samples with no treatment was collected as media control.

Immunoblot analysis. Whole cell extracts were prepared as previously described by our group.[44] Equivalent amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Following antibody incubations, proteins were detected with chemiluminescent substrate (Pierce SuperSignal, catalog # 34077). The following antibodies were used for detection: anti-phosphotyrosine (4G10) and anti-GAPDH (both from Millipore, catalog # 05–321 and #MAB374).

Antibody-dependent cellular cytotoxicity (ADCC) assay. ADCC activity was determined by standard 4-h ^{51}Cr-release assay using methods previously reported by our group.[45]

Antibody-dependent cellular phagocytosis (ADCP) assay. Monocyte-derived macrophages (MDMs) were derived from

peripheral blood monocytes using monocyte-colony stimulating factor (R and D Systems, catalog #216-MC) for seven days. They were fluorescently labeled with Min-Claret dye (Sigma, catalog # PKH26GL). CLL cells were fluorescently labeled with PKH-67 (Sigma, catalog # MIDI67) and coated with antibody for 1 h at 4°C. MDM and CLL cells were co-incubated for 30 min at a 1:5 E:T ratio, then colocalization of CLL with MDM was scored using flow cytometry and verified using microscopy.

Complement dependent cytotoxicity (CDC) assay. This assay was performed on primary CLL cells as previously reported by our group.[8]

Retroviral transductions. A CD37 construct was designed with the human CD37 gene inserted onto a pBABE (Promega) backbone. The huCD37-pBABE vector, an empty pBABE vector and a no vector 697 cell control were independently transfected into separate flasks of Phoenix Ampho™ cells (Orbigen (now Allele), catalog #ABP-RVC-10001) using a calcium phosphate-mediated transfection kit from Promega. The supernatant containing the retrovirus was collected from the Phoenix Ampho™ cells after two days and the 697 cells were resuspended in the viral supernatant along with polybrene (hexadimethrine bromide) for 8 h and then resuspended in fresh media. Puromycin was added as a selection agent after two days. The cells were grown under selection and then separated out in limited dilution cloning.

Assessment of antibody-binding and antigen surface density. Quantitative analysis of CD37 surface density was done using the Quantum Simply Cellular kit (Bangs Laboratories, catalog # 816), according to the manufacturer's instructions.

NK cell and monocyte in vitro stimulation and cytokine release assays. For in vitro NK-cell stimulation experiments, SMIP protein or antibody were immobilized on a plate. Normal donor NK cells were plated at 2×10^5 NK cells/well. CD107a-FITC or IgG1-FITC control was added to the suspension at the start of the 4 h incubation at 37°C. NK cells were harvested at the end of incubation period, stained with CD56-PE and analyzed by FACS for CD107a surface expression. For supernatant experiments, cell-free culture supernatants were harvested after 4 h and analyzed for levels of IFNγ by a Quantikine Human IFNγ ELISA performed according to the manufacturer's instructions (R&D Systems, catalog # DIF50).

Primary monocytes were incubated on plated antibodies or SMIP proteins for 24 h and supernatant was harvested. TNF levels were measured using a Quantikine Human TNFα ELISA, performed according to the manufacturer's instructions (R&D Systems, catalog # DTA00C).

Live cell imaging. CLL cells were treated with 5 μg/mL of SMIP protein or a Type II antibody for 1–16 h. Images were captured every hour using a Zeiss CCD camera (AxioCam Mrm) in ApoTome Microscope (Axio Observer.Z1; Carl Zeiss MicroImaging GmbH) and were analyzed with Zeiss Axiovision (Vs40) image acquisition software.

Statistical methods. Since each patient's cells were under all conditions of each experiment, linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests.[46] Holm's method was used to adjust for multiplicity.[47]

Disclosure of Potential Conflicts of Interest

JM, MM, RL, BS, AS and PA are employees of Emergent Biosolutions and have financial interests in TRU-016 and TRU-016^{GV} development.

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Author's Contributions

SR designed and performed experiments, wrote the first draft of the manuscript, contributed to revisions of the paper and approved the final submitted version. CC, JPB, GL, NKJ, RL provided input into experimental design, performed experiments and reviewed and approved the final version of the manuscript. DJ and XM assisted in design of experiments, performed the statistical analysis reported, reviewed and approved the final version of the manuscript. ST, JJ, JMF, LA and SD provided input into experimental design, patient samples and reviewed drafts of the manuscript and approved the final submitted version. JM, MM, RL, BS, AS and PA generated and provided reagents and reviewed and approved the final version of the manuscript. NM and JCB obtained funding to perform the research, designed the experiments, participated in the analysis of the data, review of multiple drafts of the manuscript and approved the final version for submission.

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