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LETTER TO THE EDITOR

Design and functional characterization of a tetravalent NK cell-engaging bispecific antibody with enhanced half-life for CD30$^+$ lymphoma

KEY WORDS

Bispecific antibody;
IgAb−ScFv;
In vitro activity;
In vivo characterization

To the Editor:

Immunotherapy has become an important approach to combat hematological malignancies such as lymphoma. Bispecific antibodies can redirect immune cells to fight against tumors, utilizing either adaptive or innate immune systems. AFM13, a tetravalent bispecific antibody against CD30/CD16a, is dependent on natural killer (NK) cells to treat CD30$^+$ lymphoma[1]. CD30 is a member of the tumor necrosis factor (TNF) receptor superfamily and is highly expressed in classical Hodgkin's lymphoma (cHL) and anaplastic large cell lymphoma (ALCL)[2]. Several therapeutics are currently in clinical development for relapsed/refractory cHL, including small molecules that affect signaling pathways and specific/non-specific immunotherapies. However, despite the favorable response rate in most patients, median progression-free survival remains less than six months[3].

The circulating half-life of AFM13 is less than 20 h, while it displayed good safety and efficacy profiles in phase I trials, indicating that the weekly dosing could not achieve maximum efficacy[1]. Thus, we designed a bispecific antibody format (IgAb−ScFv) by introducing a functionally silenced Fc conjugated with the variable region of AFM13. While production and purification were improved, affinity, NK cell activation and tumor cell lysis remained comparable to that of AFM13. *In vivo* pharmacokinetics analysis revealed that IgAb−ScFv ($t_{1/2}$ = 203 h) is superior to AFM13. No observable difference was noted in terms of non-discriminatory antitumor activity and safety profile when evaluated in immunodeficient NSG mice.

With the rapid clinical progress of AFM13, we chose this molecule as a template to design a new tetravalent bispecific antibody (2 + 2, IgAb−ScFv) with human IgG_1 Fc to balance high affinity and antibody-dependent cell-mediated cytotoxicity (ADCC, Fig. 1A). Since the Fc also binds to FcγRIIIb (CD16b) and FcγRIIa/b/c, we silenced it to reduce non-specific interaction with other Fc receptors, thereby allowing neonatal FcR (FcRn) function and extended half-life[4]. It was reported that Fab and ScFv in VH-VL orientation of anti-CD16a led to NK cell autophagy. This problem was solved by the introduction of short peptides at the C-terminal of Fc to ensure effective cytotoxicity induced by high-affinity anti-CD16a[5].

IgAb−ScFv was transiently expressed in Expi293 cells and purified by one-step protein A affinity chromatography (Supporting Information Fig. S1A). The purity (91.4%) and integrity of the IgAb−ScFv polypeptide chains were confirmed by SEC-HPLC and SDS-PAGE analysis. We also prepared the tetravalent bispecific antibody AFM13 (TandAb) and ADCC-enhanced CD30 monoclonal antibody (Fc-enhanced mAb). SEC-HPLC and SDS-PAGE showed that both were of high purity (Fig. S1B and S1C). It was worth noting that TandAb without Fc needs two-step purification to achieve a purity of more than 90%, whereas IgAb−ScFv only required one-step of purification.

ELISA analysis confirmed that anti-CD16a in IgAb−ScFv specifically bound to biotin-labeled CD16a^{158V} and CD16a^{158F} proteins, but not CD16b (Fig. 1B). The binding capacity of IgAb−ScFv to both variants of CD16a was much higher than that of Fc-enhanced mAb (Supporting Information Table S1), suggesting that the ScFv for CD16a is located at the C-terminal of Fc without compromising its binding to the target and is functionally intact.

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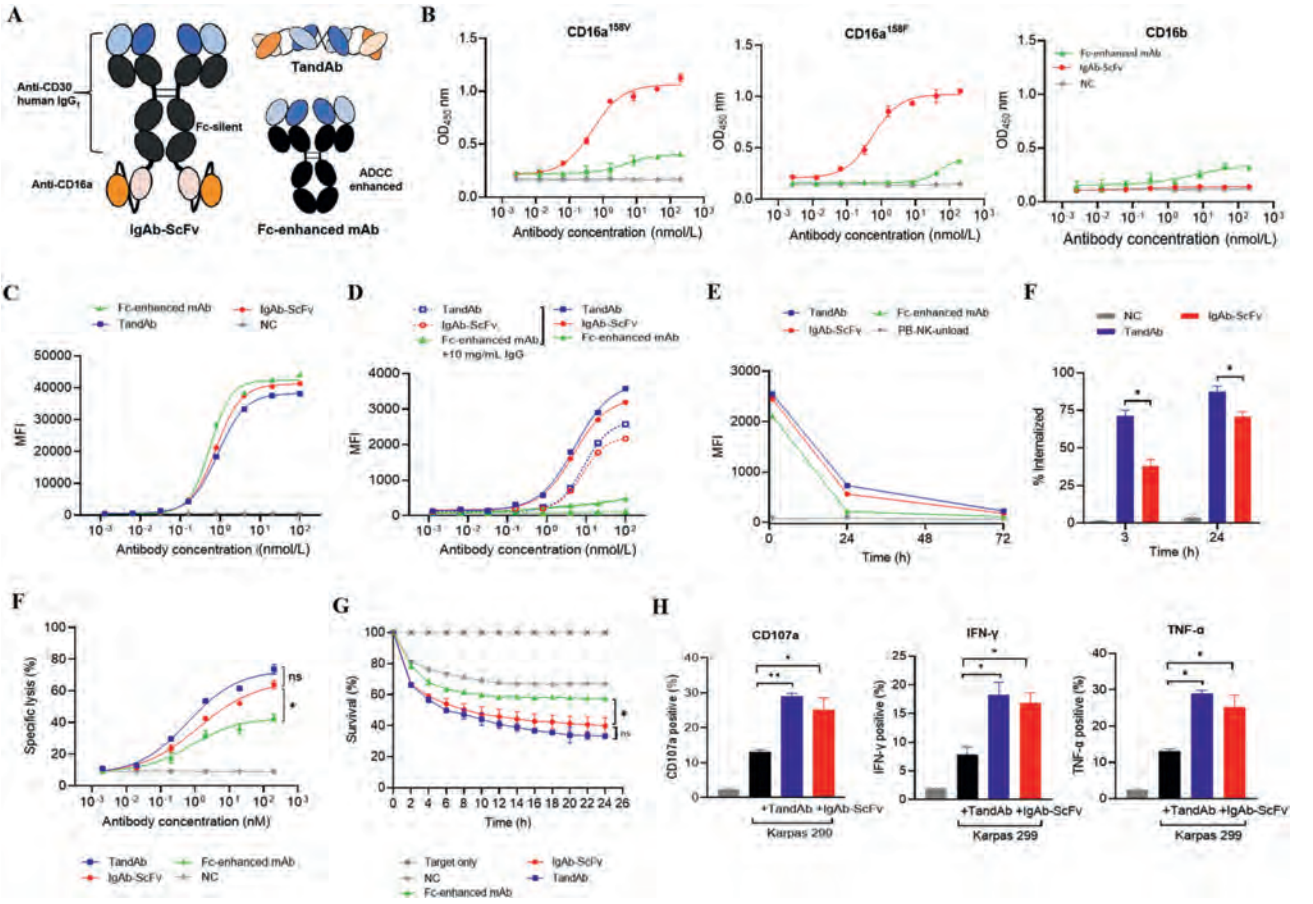

Figure 1 Structural and *in vitro* activity analysis of IgAb−ScFv. (A) Structures of IgAb−ScFv and other control antibodies. IgAb−ScFv is a tetravalent bispecific antibody with silenced human IgG1 Fc (L234F/L235E/D265A). Structures of the tetravalent bispecific antibody AFM13 and ADCC-enhanced CD30 monoclonal antibody are shown. The blue and yellow ovals represent the variable regions of CD30 and CD16a, respectively. (B) ELISA analysis of anti-CD16a antibody (IgAb−ScFv) and Fc-enhanced mAb (anti-CD30 monoclonal antibody) incubated with different antigens (CD16a^{158V}, CD16a^{158F} and CD16b). Experiment with duplicate. Error bars represent the SD. (C) IgAb−ScFv has a similar antigen-binding ability as that of the TandAb on Karpas 299 tumor cells. (D) Binding of IgAb−ScFv, biotinylated anti-CD30 to enriched primary human NK cells was assessed by flow cytometry in the presence (open symbols) or absence (filled symbols) of 10 mg/mL anti-RSV IgG1 palivizumab (as a surrogate for polyclonal human serum IgG) at 37 °C. (E) Cell surface retention assay. Normalized median fluorescence intensity values were plotted. (F) Flow cytometry detection of the internalization percentage of CD30$^+$ Karpas 299 cells to IgAb−ScFv and TandAb at different time points. The percentage of internalization of antibody was determined using the median fluorescence intensity (MFI). Error bars represent SD, paired *t*-test was used for statistical analysis. $^*P < 0.05$. (G) *In vitro* cytotoxicity of CD30$^+$ Karpas 299 cells, which were co-cultured with pre-loaded antibodies (0−200 nmol/L) human NK cell complex for 4 h at E:T ratio 5:1. (H) Killing of CD30$^+$ Karpas 299 over a 24 h period by loaded IgAb−ScFv (solid circular), TandAb (solid square) or Fc-enhanced mAb (solid triangle), along with unloaded (target only) NK cells (E:T = 1:1, antibodies concentration was 2 nmol/L) as measured by IncuCyte. (I) IFN-γ and TNF-α production and CD107a degranulation by IgA-ScFv/TandAb loaded (200 nmol/L) NK cells *vs* unloaded NK cells co-cultured with Karpas 299 cells for 6 h (E:T = 1:1). Error bars represent SD. Statistical test used: paired *t*-test. ns, not significant; $^*P < 0.05$, $^{**}P < 0.01$.

IgAb−ScFv showed a similar affinity to TandAb in CD30$^+$ tumor (Karpas 299) cells by flow cytometry (Fig. 1C). To demonstrate if IgAb−ScFv binds to a specific epitope on CD16a different from Fc[6], it was studied in the presence or absence of human IgG (Fig. 1D). The results showed that IgAb−ScFv bound to NK cells with high affinity (EC_{50} = 4.8 nmol/L) in the absence of competitive IgG; addition of IgG only led to a slightly reduced binding (EC_{50} = 8.1 nmol/L), like TandAb (EC_{50} = 8.8 nmol/L) (Supporting Information Table S2). This observation strongly suggests that IgAb−ScFv recognizes a unique region of CD16a in NK cells that does not overlap with Fc. It follows that the dissociation rate of bivalent IgAb−ScFv on NK cell surface was found to be significantly slower than that of Fc-enhanced mAb but equivalent to that of TandAb (Fig. 1E). In this assay, the maximum concentration of antibody used was 200 nmol/L, because co-incubation of 1 μmol/L TandAb with NK cells for 72 h caused cell death (Supporting Information Fig. S2).

Residual data of TandAb on the surface of CD30$^+$ Karpas 299 cells showed that its retention rate was lower than that of natural and Fc-enhanced IgGs[7]. To determine whether Fc-antibody could reduce internalization by target cells, the percentages of Karpas 299 internalized IgAb−ScFv and TandAb at different time points were investigated. After incubation at 37 °C for 3 h, the internalization rate of TandAb was 71%, and that of

IgAb−ScFv was 38%. With the extension of time, the internalization rate of TandAb gradually increased, and the introduction of Fc did reduce internalization (Fig. 1F).

Lysis of $CD30^+$ tumor cells by IgAb−ScFv was assessed using a range of antibody concentrations. IgAb−ScFv induced ADCC was similar to that of TandAb but more effective than Fc-enhanced mAb (Fig. 1G). We also evaluated the cytotoxicity of NK cell loaded IgAb−ScFv complex against $CD30^+$ tumor cells (24 h) using a low antibody concentration (2 nmol/L) and an effector to target ratio (E:T) of 1:1, IgAb−ScFv remained efficacious (Fig. 1H).

Specificity of IgAb−ScFv mediated NK cell activation was subsequently examined by co-culturing $CD30^+$ and $CD30^-$ tumor cells with NK cells at increasing concentrations of IgAb−ScFv. When CFSE (carboxyfluorescein diacetate succinimidyl ester) labeled $CD30^+/CD20^-$ Karpas 299 cells were mixed with unlabeled $CD30^-/CD20^+$ Raji cells, IgAb−ScFv induced dose-dependent lysis while rituximab only induced marginal lysis of Karpas 299 (Supporting Information Fig. S3A). In contrast, in CFSE labeled Raji cells and unlabeled Karpas 299 cells, only rituximab mediated lysis of $CD20^+$ Raji cells (Fig. S3B). The data suggest that bivalent IgAb−ScFv binding to NK cells does not induce nonspecific cytotoxicity and that CD30 bystander cells are not affected by IgAb−ScFv mediated target cell lysis.

To determine whether bivalent CD16a binding of IgAb−ScFv leads to NK cell activation, cytokine releases by NK cells were measured in the presence or absence of $CD30^+$ Karpas 299 cells. Fig. 1I shows that after 6 h incubation, releases of interferon-γ and TNF-α as well as surface CD107a (marker of NK-cell degranulation) were significantly higher than the control, indicating that IgAb−ScFv activated NK cells in the presence of $CD30^+$ cells. Analysis of functional markers on the surface of NK cells showed that such an activation was accompanied by upregulation of CD25, NKp44 and death associated ligand (FASL), although the increase of CD69 expression was not significant (Supporting Information Fig. S4A and S4B). Our results are consistent with previous observation that NK cells were activated by AFM13 through CD16[8].

Regarding the question whether IgG in blood circulation competes with CD16a on NK cells thereby reducing the potency and efficacy of IgAb−ScFv, we used 10 mg/mL of monoclonal anti-respiratory syncytial virus (RSV) IgG_1 (palivizumab) instead of polyclonal IgGs that interfere with ADCC[9] as a substitute to study ADCC against Karpas 299. It was found that the efficacy (top lysis) of Fc-enhanced mAb in ADCC was reduced more obviously (by approximately 1.8-fold) in the presence of competitive IgG than that of IgAb−ScFv and TandAb (by about 1.3-fold) (Supporting Information Table S3), suggesting that NK cells bound by IgAb−ScFv can mediate effective tumor cell clearance in the presence of IgG.

The pharmacokinetics profiles of IgAb−ScFv and TandAb were evaluated in mice by ELISA. The half-life of IgAb−ScFv was 203 h, comparable to that of standard IgG molecules, while that of TandAb (AFM13 equivalent in this study) lacking Fc was only 17 h (Fig. 2A). We also analyzed the AUC, which defines the maximum exposure of a molecule in an organism over time. As expected, IgAb−ScFv with Fc displayed a higher AUC value, whereas that of TandAb was only 12% of its counterpart (Supporting Information Table S4). These data indicate that the newly designed IgAb−ScFv is indeed superior to TandAb in terms of pharmacokinetics behavior.

In addition to serum half-life, the antitumor activity of IgAb−ScFv was preliminarily studied *in vivo* by intravenous

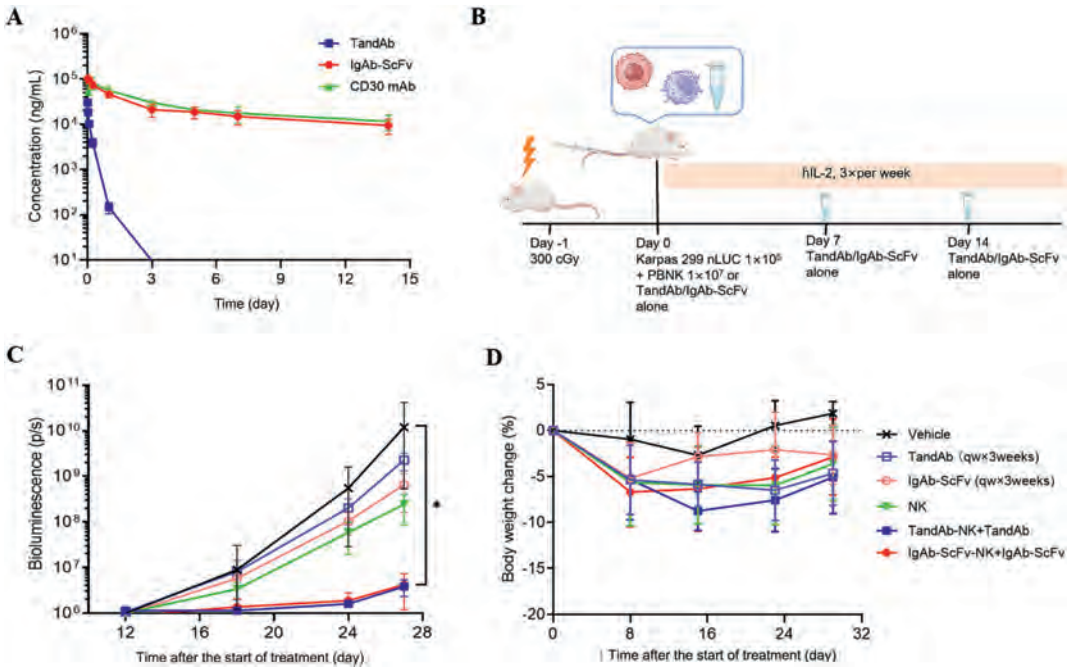

Figure 2 *In vivo* characterization of IgAb−ScFv. (A) Comparison of pharmacokinetics profiles of different antibodies for six mice per group, with CD30 used as an experimental control. (B) Experimental protocol for evaluating the *in vivo* antitumor activity of IgAb−ScFv. (C) nLuc-labeled $CD30^+$ Karpas 299 tumor cells in NSG mice were monitored by bioluminescence imaging (BLI). The graph summarizes bioluminescence data from six groups of mice ($n = 4-6$ mice per group). (D) The broken line graph summarizes the change of animal weight over time as a measure of toxicity. Error bars represent SD. Paired *t*-test was used for statistical analysis. $^*P < 0.05$; ns, not significant.

injection of NK cells (1×10^7) pre-loaded with IgAb−ScFv to NSG mice. General conditions of the animals including body weight over a period of one month were monitored (Fig. 2D). The bispecific antibody was assessed in lymphoma bearing mice (Fig. 2B). The experiment was divided into six groups: 1) Karpas 299 cells alone; 2) Karpas 299 cells plus one dose of unloaded NK cells (1×10^7); 3) and 4) Karpas 299 cells plus one dose of IgAb−ScFv or TandAb (200 nmol/L) pre-loaded with NK cells (1×10^7) with subsequent two weekly injection of 200 nmol/L antibody; and 5) and 6) Karpas 299 cells plus three weekly intravenous injection of 200 nmol/L IgAb−ScFv or TandAb. All mice received intraperitoneally three injections/week of recombinant human IL-2 (10,000 units/mouse) to support the survival of human NK cells in NSG mice. Compared to the control, both IgAb−ScFv−NK and TandAb−NK complexes controlled tumor growth and improved survival with comparable efficacy as demonstrated by BLI (Fig. 2C). No statistically significant difference in weight changes was noted between experimental and control groups. In addition, no graft versus host disease (GVHD) was seen in these mice (Fig. 2D). All animal protocols were approved by the Ethical Committee for Animal Care and Use of China State Institute of Pharmaceutical Industry.

The success of the tetravalent bispecific antibody AFM13 has encouraged more studies on multi-specific agents targeting NK cell activating receptors and tumor-associated antigens to enhance cytotoxicity of tumor infiltration such as trispecific killer engager (TriKE) and NK cell engager (NKCE)[10]. RO7297089, a molecule similar to IgAb−ScFv but targeting BCMA and CD16a, was described[6] as a template of bispecific or trispecific antibodies that link NK cells to treat patients with cHL and acute myeloid leukemia rather than multiple myeloma[10]. Therefore, the design of IgAb−ScFv may have therapeutic potential for CD30$^+$ lymphoma. Compared to TandAb, IgAb−ScFv simplifies production at laboratory scale. It does not interact with CD16b, thus preventing NK cell non-specific binding. CD16a is not only an activating receptor of NK cells, but also highly expressed in macrophages, expanding its antitumor scope beyond NK cell specific activating receptors NKp46 and NKp30[10]. Although the binding affinity of NK cells is slightly reduced in the presence of soluble IgG, compared with Fc-enhanced mAb, IgAb−ScFv can still induce strong cytotoxicity and redirect NK cells without the need of cell−cell contact.

The residual amount of IgAb−ScFv on the NK cell surface was found similar to that of TandAb, implying that IgAb−ScFv does not affect the stable binding with NK cells despite its large molecular weight and a complex structure. Meanwhile, IgAb−ScFv displayed a longer half-life and less internalization by CD30$^+$ tumor cells than TandAb, consistent with the phenomenon reported previously that the retention of TandAb was lower than that of natural and Fc-enhanced IgGs[7]. In the cytokine and NK cell surface functional marker analyses, IgAb−ScFv was more potent than TandAb in stimulating the expression of death ligand (FASL), which may produce better clinical responses in treating CD30$^+$ tumors by improving the recruitment of targeted immune effector cells.

However, our experiments have limitations in assessing *in vivo* efficacy. First, repeated injections of IgAb−ScFv or TandAb on Day 14 after the first administration may not fully reflect the NK cytotoxicity mediated by them because of decreased human NK cells in NGS mice. Our previous study showed that when PB-NK cells (1×10^7) were introduced to NGS mice on Day 0, their presence in the peripheral blood declined to a low level on Day 14 (unpublished data). Second, AFM13 has the murine CD30 domain, and its sequence can be optimized for humanization. Although IL-2 could drive NK cell proliferation in our experiment, IL-15 was required to obtain full effector function[11]. Therefore, the introduction of IL-15 to IgAb−ScFv formulation may provide better efficacy.

In conclusion, our data suggest that IgAb−ScFv is an effective NK cell engager, not only prolonging the half-life but also inducing a stronger ADCC in CD30$^+$ tumor cells. It is not affected by CD16a polymorphism and soluble IgG has negligible impact on its binding. *In vivo* studies showed that its antitumor activity is similar to that of TandAb. Notably, the modular design of IgAb−ScFv with the tumor-associated antigen (TAA) positioned at the N-terminal of Fc allows for adaptation to other Fab or VHH structures (Supporting Information Fig. S5), making it a versatile candidate for therapeutic development in CD30$^+$ lymphomas and beyond.

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Author contributions

Caizhi Zhao and Youjia Hu designed the research; Caizhi Zhao guided the expression and purification of antibodies; Caizhi Zhao conducted *in vitro* and *in vivo* assays; Caizhi Zhao and Liping Xie analyzed the data; Caizhi Zhao, Ming-Wei Wang and Youjia Hu drafted and edited the manuscript with inputs of all co-authors.

Conflicts of interest

The authors declare that there is no conflict of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2025.03.012.

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