Single cell-produced and in vitro-assembled anti-FcRH5/CD3 T-cell dependent bispecific antibodies have similar in vitro and in vivo properties

Ayse Meric Ovacik, Ji Li, Marie Lemper, Dimitry Danilenko, Nicola Stagg, Mary Mathieu, Diego Ellerman, Vinita Gupta, Navdeep Kalia, Trung Nguy, Vicki Plaks, Clarissa David Johnson, Weiru Wang, Jochen Brumm, Bernard Fine, Teemu Junttila, Kedan Lin, Paul J. Carter, Saileta Prabhu, Christoph Spiess, and Amrita V. Kamath

Genentech Research and Early Development, Genentech, Inc., South San Francisco, CA, USA

ABSTRACT

Bispecific antibody production using single host cells has been a new advancement in the antibody engineering field. We previously showed comparable in vitro biological activity and in vivo mouse pharmacokinetics (PK) for two novel single cell variants (v10 and v11) and one traditional dual cell in vitro-assembled anti-human epidermal growth factor receptor 2/CD3 T-cell dependent bispecific (TDB) antibodies. Here, we extended our previous work to assess single cell-produced bispecific variants of a novel TDB against FcRH5, a B-cell lineage marker expressed on multiple myeloma (MM) tumor cells. An in vitro-assembled anti-FcRH5/CD3 TDB antibody was previously developed as a potential treatment option for MM. Two bispecific antibody variants (designs v10 and v11) for manufacturing anti-FcRH5/CD3 TDB in single cells were compared to in vitro-assembled TDB in a dual-cell process to understand whether differences in antibody design and production led to any major differences in their in vitro biological activity, in vivo mouse PK, and PK/pharmacodynamics (PD) or immunogenicity in cynomolgus monkeys (cynos). The binding, in vitro potencies, in vitro pharmacological activities and in vivo PK in mice and cynos of these single cell TDBs were comparable to those of the in vitro-assembled TDB. In addition, the single cell and in vitro-assembled TDBs exhibited robust PD activity and comparable immunogenicity in cynos. Overall, these studies demonstrate that single cell-produced and in vitro-assembled anti-FcRH5/CD3 T-cell dependent bispecific antibodies have similar in vitro and in vivo properties, and support further development of single-cell production method for anti-FcRH5/CD3 TDBs and other single-cell bispecifics.

Introduction

Multiple myeloma (MM) is characterized by abnormal growth of plasma cells (PCs) in the bone marrow (BM) and the overproduction of immunoglobulin by these abnormal PCs, leading to hypercalcemia, anemia, renal impairment or bone pain. While the life expectancy of MM patients is increasing due to the availability of novel agents, the disease remains incurable in most patients owing to repeated relapses. Modalities that recruit T cells, including chimeric antigen receptor therapies, to kill tumors have shown promise in patients with B cell malignancies. There is also growing interest on developing bispecifics and T-cell recruiting therapeutics for solid tumors.

Previously, we described the design, mechanism of action (MOA) and pharmacokinetics (PK)/pharmacodynamics (PD) of an in vitro-assembled anti-FcRH5/CD3 TDB. The anti-CD 3 arm of this T-cell dependent bispecific (TDB) engages T cells and the anti-FcRH5 arm engages FcRH5-expressing cells such as B cells, PCs and MM cells to form an immunological synapse. When the synapse is formed, T cells are activated, resulting in FcRH5-dependent cell killing either through cytokine release caused by T-cell activation or through direct killing by granymes and perforin-induced cell lysis. The anti-FcRH5/CD3 TDB exhibited cytotoxic activity against normal human PCs and patient-derived primary MM cells. Additionally, the anti-FcRH5/CD3 TDB showed robust PD activity, including complete depletion of B cells and PCs in cynomolgus (cyno) BM.

In vitro-assembled anti-FcRH5/CD3 TDB is produced by a two-cell process, where the two component antibodies (anti-FcRH5 ‘knob’ and anti-CD3 ’hole’) are first expressed in separate host cells. These half-antibodies containing knobs-into-holes (KIH) mutations are then purified, and the anti-FcRH5/CD3 TDB is subsequently assembled in vitro. This design includes mutations in the C\text{H}3 domain of the Fc region for preferential heavy chain heterodimerization to ensure efficient bispecific antibody production (Figure 1). In vitro-assembly is well established and has been used to produce a large number of bispecific IgGs at Genentech. In recent years, several different groups, including ours, have developed novel methods to efficiently produce bispecific IgGs in single host cells. Single-cell production of bispecific IgGs offers simpler, faster, and more cost efficient manufacturing. We previously reported two novel
designs for single cell bispecific antibodies (v10 and v11) that included modifications in the antigen-binding fragment (Fab) arms to promote selective pairing of cognate heavy and light chains in addition to the KIH mutations in the antibody Fc. Specifically, the charge-pair modifications of the single cell design v10 are located at the V_H-V_L interface, outside of the complementarity-determining regions (CDRs) and at the C_H1-C_L interface (Figure 1). These charge-pairs do not perturb the structure of the molecule and have a minimal solvent accessible surface area. Design v11 differs from v10 by utilizing a remodeled C_H1-C_L interface instead of a charge pair in one of the C_H1-C_L interfaces. We produced single cell variants (v10 and v11) of another TDB, namely anti-human epidermal growth factor receptor 2 (HER2)/CD3 TDB, using a different anti-CD3 antibody than the anti-FcRH5/CD3 TDB. The designs did not affect binding of the HER2 antigen, and they had comparable in vitro biological activities and similar PK in mice compared to in vitro-assembled anti-HER2/CD3 TDB.

Here, we extended our previous work to assess single cell produced bispecific variants of a novel TDB against FcRH5. In addition to the in vitro and in vivo characterization conducted previously for the anti-HER2/CD3 TDBs,18 we included assessment of cyno PK/PD and immunogenicity. The single cell anti-FcRH5/CD3 TDBs have the same CDRs as the in vitro-assembled anti-FcRH5/CD3 TDB. The designs did not affect binding of the HER2 antigen, and they had comparable in vitro biological activities and similar PK in mice compared to in vitro-assembled anti-HER2/CD3 TDB.

Results

Single cell-produced and in vitro-assembled anti-FcRH5/CD3 TDBs have comparable binding properties

The binding affinities of the single cell-produced TDBs (termed single cell TDBs) to human and cyno FcRH5 and CD3ε were comparable to the corresponding binding affinities of in vitro-assembled anti-FcRH5/CD3 TDB as measured by surface plasmon resonance (SPR) or radioligand cell-binding assay (Table 1). In addition, no differences in affinities for FcRH5 or CD3ε were observed between the two single cell TDBs, v10 and v11. The affinities of the anti-CD3 arm in all 3 variants were generally lower for the human CD3ε compared to the cyno CD3ε, whereas the affinities of the FcRH5 arm for all 3
variants were lower for cyno FcRH5 compared to human FcRH5.

**Single cell and in vitro-assembled anti-FcRH5/CD3 TDB showed robust in vitro pharmacological activity**

We evaluated the in vitro pharmacological activities of two single cell TDBs along with the in vitro-assembled TDB in two different assays: 1) cytotoxicity of FcRH5-expressing PCs, and 2) cytotoxicity and T-cell activation of the FcRH5-expressing MM cell line MOLP-2. The concentration-cell killing percentage and $E_{\text{MAX}} - E_{\text{EC50}}$ model fits are shown in Figure 2. The summary of estimated parameters is provided in Table 2 (see Fig. S2 for each estimated $E_{\text{MAX}} - E_{\text{EC50}}$ value).

The capacity of the anti-FcRH5/CD3 TDBs to kill PCs was analyzed by targeting BM mononuclear cells (BMMCs) isolated from BM aspirates of one cyno (Figure 2(a)) and two healthy human donors (Figure 2(b), representative profile). The expression patterns of FcRH5 were previously shown to be similar in humans and cynos. The single cell and in vitro-assembled TDBs had robust and comparable PC cytotoxicity profiles for cyno and human cells. In addition, the maximum cell killing values of all the TDBs were slightly higher, although not substantially, for human PC (~80%) compared to cyno PC (~60%).

The capacity of the anti-FcRH5/CD3 TDBs to kill MOLP-2 cells (Figure 2(c)) and induce T-cell activation (Figure 2(d)) were analyzed by cytotoxic activity of MOLP-2 cells co-cultured with isolated human CD8+ T cells from three human donors. The single cell and in vitro-assembled TDBs exhibited robust and comparable MOLP-2 cell killing profiles, although high variability was observed across donors (Fig. S1B and E). In addition, single cell TDBs exhibited marginally

**Table 1.** Single cell- and in vitro-assembled anti-FcRH5/CD3 TDBs had comparable antigen binding affinities for human and cyno FcRH5 and CD3ε. Radio ligand cell-binding assays were used to determine the $K_D$ of the 3 anti-FcRH5/CD3 TDBs to human and cyno FcRH5. Surface plasmon resonance was used to determine $K_D$ of the three anti-FcRH5/CD3 TDBs to human and cyno CD3ε. Measurements were taken at 25°C and pH 7.4 for both methods. The affinities reported here were monovalent affinities of each arm.

|                | FcRH5, $K_D$ (nM) | CD3ε, $K_D$ (nM) |
|----------------|-------------------|------------------|
| **Anti-FcRH5/CD3 TDB** |                  |                  |
| **Human**     |                   |                  |
| **Cyno**      |                   |                  |
| **Human/Cyno**|                   |                  |
| In vitro-assembled | 3.1              | 8.2              |
| Single cell v10 | 2.6              | 7.1              |
| Single cell v11 | 3.3              | 7.9              |

* Ratio of human to cyno binding, dimensionless

|                | FcRH5, $K_D$ (nM) | CD3ε, $K_D$ (nM) |
|----------------|-------------------|------------------|
| **Anti-FcRH5/CD3 TDB** |                  |                  |
| **Human**     |                   |                  |
| **Cyno**      |                   |                  |
| **Human/Cyno**|                   |                  |
| In vitro-assembled | 1.8              | 0.7              |
| Single cell v10 | 2.2              | 0.4              |
| Single cell v11 | 2.5              | 0.6              |

|                | FcRH5, $K_D$ (nM) | CD3ε, $K_D$ (nM) |
|----------------|-------------------|------------------|
| **Anti-FcRH5/CD3 TDB** |                  |                  |
| **Human**     |                   |                  |
| **Cyno**      |                   |                  |
| **Human/Cyno**|                   |                  |
| In vitro-assembled | 3.1              | 8.2              |
| Single cell v10 | 2.6              | 7.1              |
| Single cell v11 | 3.3              | 7.9              |

* Ratio of human to cyno binding, dimensionless

**Figure 2.** Single cell and in vitro-assembled TDBs have robust in vitro activities. Concentration-response (in vitro activity) data were determined in independent duplicates of sample size, n. Mean and standard deviation of the duplicate measurements are presented as symbols (blue circles denote in vitro-assembled TDB, green reverse triangles denote single cell TDB v10, red squares denote single cell TDB v11). Sigmoidal $E_{\text{MAX}} - E_{\text{EC50}}$ model fits were shown as blue solid (in vitro-assembled TDB), green dashed (single cell TDB v10), and red dotted line (single cell TDB v11). Estimated $E_{\text{MAX}}$ and $E_{\text{EC50}}$ values are summarized in Table 2 and are also provided in Fig. S2 for concentration-response (in vitro activity) data. (a) Cytotoxicity data for single cell and in vitro-assembled TDBs mediated killing of cyno PCs. The data were obtained from one cyno BM aspirate. (b) Cytotoxicity data for single cell and in vitro-assembled TDBs mediated killing of human PCs. The data were obtained from two donors (n = 2). One representative profile is shown. (c) Cytotoxicity data for single cell and in vitro-assembled TDBs mediated killing of MOLP-2 cells co-cultured with isolated human CD8+ T cells. The data were obtained from 3 donors (n = 3). One representative profile is shown. (d) Anti-FcRH5/CD3 TDB dependent T cell (CD8+) activation when co-cultured with the MOLP-2 cell line for single cell and in vitro-assembled TDBs. The data were obtained from the same 3 donors as in MOLP-2 cell cytotoxic activity (n = 3). One representative profile is shown.
higher (~10–20%) maximal T-cell activation (E_max) (Fig. S1C), and slightly lower, albeit not meaningful, (~1.5 fold) potency (EC50) (Fig. S1F), when compared to in vitro-assembled TDB. No difference was observed between the single cell TDBs in T-cell activation.

### Single cell and in vitro-assembled anti-FcRH5/CD3 TDB had similar PK properties in SCID.bg mice

The anti-FcRH5/CD3 TDBs do not cross-react with mouse FcRH5 or CD3ε. Hence, the mouse PK profiles of the anti-FcRH5/CD3 TDBs provided an opportunity to compare the PK of the two single cell TDBs to in vitro-assembled TDB in the absence of target binding. Serum concentration-time profiles of the three anti-FcRH5/CD3 TDBs were assessed along with two control antibodies: anti-gD (a non-binding control IgG, targeting the glycoprotein D epitope of herpes simplex virus) and anti-gD/CD3 TDB (IgG1 framework, KIH bispecific antibody with the same anti-CD3 arm as the in vitro-assembled anti-FcRH5/CD3 TDB). The PK data of each group were characterized by non-compartmental analysis (NCA) (Table 3) and in a two-compartment (2-C) model (Table S1). The PK data of all groups exhibited biphasic profiles (See Fig. S2 for the two-compartment model fits).

The systemic exposures of the single cell TDBs were generally comparable to the exposure of the in vitro-assembled TDB (Table 2 and Figure 3). Moreover, simulations of compartmental model parameters for each group (single cell v10, v11 and in vitro-assembled TDB) at 5 mg/kg dosing showed that exposures of the 3 TDBs overlapped (Fig. S3).

The anti-gD administered group had higher exposure compared to all anti-FcRH5/CD3 TDB administered groups, as well as the anti-gD/CD3 administered group. Given the lower exposure of the CD3-containing TDBs compared to the bivalent anti-gD control, we asked if there were characteristics of the CD3 arm that could have contributed non-specifically to the observed lower exposure. We estimated the antibody variable region (Fv) charge and hydrophobicity of anti-CD3 using iCAT (in silico clearance assessment tool, see Material and Methods). The calculated Fv charge was +7.6, which is outside the range for acceptable in vivo clearance (Fv charge ≤ 0 or ≥ +6.2). In addition, the structure of the anti-CD3 arm Fab region (Figure 4) showed a positively charged region that was surface exposed.

### Single cell and in vitro-assembled TDB had similar PK properties in cynos

The anti-FcRH5/CD3 TDBs cross-reacted with cyno FcRH5 and CD3ε. Hence, the cyno PK profiles provided an opportunity to compare the PK of the single cell TDBs to the in vitro-assembled TDB in the presence of both targets. Serum concentration-time profiles of the anti-FcRH5/CD3 TDBs following a single intravenous (IV) bolus dose of 2 mg/kg in cyno are shown in Figure 5. All the animals that were given TDBs tested positive for anti-drug antibodies (ADA) (Fig. S4), whereas the vehicle-administered control animals and pre-dose samples from all animals were negative. ADA was observed as early as day 7 and was apparent in all the cynos administered TDBs by day 13. Therefore, the observed C_max and AUC0-4 were used to compare the different variants.

### Table 2. Single cell and in vitro-assembled anti-FcRH5/CD3 TDBs had similar in vitro cytotoxic activities. Data show estimated E_max and EC50 values of cytotoxic activity and T-cell activation for single cell and in vitro-assembled TDBs. CD8+ cells were used as effectors in a 3:1 effector:target ratio in MOLP-2 cell line cytotoxicity assay. Data are presented as mean and standard deviation where applicable. Individual E_max and EC50 values were provided in Fig. S1.

| Anti-FcRH5/CD3 TDB | In vitro activity | Anti-FcRH5/CD3 TDB |
|--------------------|------------------|-------------------|
| Hill equation parameter | Cyno PC cytotoxicity n=1 | Human PC cytotoxicity n=2 | MOLP-2 cytotoxicity n=3 | T-cell activation n=3 |
| In vitro-assembled | E_max (ng/mL) | 57.5 | 79.3 ± 1.6 | 75.3 ± 15.3 | 32.9 ± 6.0 |
| | EC50 (ng/mL) | 8.8 | 16.5 ± 6.9 | 21.7 ± 12.3 | 27.2 ± 6.3 |
| Single cell v10 | E_max (ng/mL) | 60.6 | 78.2 ± 5.9 | 86.4 ± 11.8 | 47.7 ± 11.7 |
| | EC50 (ng/mL) | 13.5 | 19.7 ± 12.9 | 21.1 ± 11.2 | 38.2 ± 6.65 |
| Single cell v11 | E_max (ng/mL) | 59.3 | 78.3 ± 0.7 | 86.9 ± 11.1 | 47.9 ± 12.8 |
| | EC50 (ng/mL) | 7.7 | 15.6 ± 10.9 | 18.8 ± 11.3 | 44.0 ± 10.1 |

### Table 3. Similar PK parameters of single cell- and in vitro-assembled TDBs in SCID mice. PK parameters for control antibodies anti-gD and anti-gD/CD3 are shown. The PK data were characterized with NCA. Sparse sampling was used to generate PK profiles and actual dose concentrations were used in data analysis (see Material and Methods). Standard error values are provided where applicable. C_max = Maximum observed serum concentration, AUC0-last = Area under the serum-concentration time curve from time 0 to last measured time points, day 21. Parameters from compartmental analysis and associated model fits are provided in Table S1 and Fig. S2, respectively.

| PK parameter | Control groups | Anti-FcRH5/CD3 TDB |
|--------------|----------------|-------------------|
| Actual dose (mg/kg) | Anti-gD | 4.4 | 57.5 | 60.6 | 60.6 |
| E_max (ng/mL) | gD | 87.3 ± 6.3 | 82.9 ± 3.6 | 74.5 ± 2.7 | 65.9 ± 0.5 | 85.9 ± 4.0 |
| EC50 (ng/mL) | 19.8 | 14.5 | 14.7 | 14.1 | 13.1 |
| AUC0-last (day·μg/mL) | 633 ± 34 | 409 ± 12 | 318 ± 7 | 273 ± 4.5 | 358 ± 19 |
| AUC0-last/Dose (day·μg/mL)/(mg/kg) | 144 | 72.8 | 63.0 | 58.7 | 54.5 |
Table 4. Similar PK parameters of single cell- and in vitro-assembled TDBs in cynos. The PK data were characterized with NCA. Actual dose concentrations were used in data analysis (see Material and Methods). Standard error values for each parameter are provided. $C_{\text{max}}$ = Maximum observed serum concentration, $AUC_{0-4}$ = Area under the serum-concentration time curve from time 0 to day 4.

| PK parameter          | In vitro-assembled (n = 4) | Single cell v10 (n = 4) | Single cell v11 (n = 4) |
|-----------------------|---------------------------|-------------------------|-------------------------|
| Actual dose (mg/kg)   | 2.12                      | 1.96                    | 1.94                    |
| $C_{\text{max}}$ (µg/mL) | 46.6 ± 4.31              | 41.8 ± 3.66             | 36.6 ± 4.02             |
| $C_{\text{max}}$/Dose (µg/mL/mg/kg) | 22.0 ± 2.03               | 18.7 ± 2.05             | 21.6 ± 1.88             |
| $AUC_{0-4}$ (day·µg/mL) | 43.7 ± 9.65              | 41.2 ± 4.70             | 37.2 ± 9.43             |
| $AUC_{0-4}$/Dose (day·µg/mL)/(mg/kg) | 20.7 ± 4.35              | 19.0 ± 4.78             | 21.2 ± 2.42             |

Figure 3. Similar PK profiles of single cell and in vitro-assembled TDBs following single dose (5 mg/kg) IV administration to C.B-17 SCID mice ($n = 3$ at each time point). Individual data points (symbols) are shown together with mean values connected (solid lines). Blue circles denote in vitro-assembled TDBs, green reverse triangles denote single cell TDB v10, red squares denote single cell TDB v11. The serum-concentration time profiles are limited to the first 13 days, after which values were below LLOQ. Associated ADA data are shown in Fig. S5.

Figure 4. The structure of the anti-CD3 arm Fab from the side and the top show the exposed positive charges on the Fab. The molecular surface rendering is color coded by electrostatic potential: positively charged (blue), negatively charged (red) or neutral (white). The structure on the left shows the Fab fragment from the side, and the structure on the right shows the Fab arm from the top. The curved arrow points in the direction of the rotation of the structure from the side to the top. The green dashed circle denotes the antigen-binding region on the anti-CD3 arm Fab and the exposed positively charged surface (blue) on the anti-CD3 Fab on both.

Figure 5. Similar PK profiles of single cell and in vitro-assembled TDBs following single dose (2 mg/kg) IV infusion administered to cynos ($n = 4$). Individual data points (symbols) are shown together with mean values connected (solid lines). Blue circles denote in vitro-assembled TDBs, green reverse triangles denote single cell TDB v10, red squares denote single cell TDB v11. The serum-concentration time profiles are limited to the first 13 days, after which values were below LLOQ. Associated ADA data are shown in Fig. S5.

The PK profiles of the single cell TDBs overlapped with the PK profile of the in vitro-assembled TDB (Figure 5). All the concentrations were below the limit of quantification by day 13 for animals administered anti-FcRH5 TDBs, which could be due to ADA or antigen-mediated clearance of the antibodies. The nominal and dose-normalized mean $C_{\text{max}}$ and $AUC_{0-4}$ values were comparable across the 3 TDB groups (Table 4).

In addition, we conducted two-compartment model fitting for cyno PK. Because ADA was detected as early as day 7, we analyzed the PK profiles until day 4. While we obtained relatively good fits for individual profiles, the standard error values for parameters $V_p$ and $CL_D$ were rather high for some of the subjects (data not shown). The two-compartment model analysis could not accurately estimate these parameters and resulted in high standard error values due to limited data points up to only day 4.

**Single cell and in vitro-assembled anti-FcRH5/CD3 TDBs showed consistent PD activity in cynos**

A decrease of FcRH5-expressing cells in cynos is the primary PD endpoint for the anti-FcRH5/CD3 TDBs. Therefore, we measured PCs and B cells in BM, as well as B cells in blood, to evaluate the PD activity of the three TDBs in cynos administered a single dose of 2 mg/kg (Figure 6 and Fig. S5). In addition, we measured serum IgG as a secondary endpoint resulting from PC depletion. We also measured T-cell activation (Figure 7(a,b)), relevant cytokine levels (i.e., interleukin (IL)-6 and interferon (IFN)-γ) (Figure 7(c,d)), and T cell counts in circulation (Fig. S6), which are all relevant PD endpoints associated with anti-FcRH5/CD3 TDB MOA.

All TDB-administered groups exhibited robust PC and B cell decreases in cyno BM on day 7 (Figure 6(a,b)) that was followed by a trend towards recovery on day 22 and full recovery by day 55 (Fig. S6A-D). Serum B cell counts decreased immediately in all animals following TDB administration (Figure 6(d)). B-cell counts remained at minimal...
levels up to day 7 and exhibited a gradual increase starting at day 14 until the end of the study (Figure 6(d)) for all TDB-administered groups.

We observed an initial decrease in serum IgG levels up to day 7 in all TDB-administered groups (Figure 6(c)). After day 7 serum IgG levels increased up to day 22 (Figure 6(c)), before returning to baseline in all TDB-administered animals by the end of the study.

All TDB-administered groups exhibited transient T-cell activation (Figure 7(a,b)) and cytokine level changes (IL-6 and IFN-γ) (Figure 7(c,d)) returning to baseline within 24 hours. Lastly, all the groups treated with TDB exhibited an immediate and transient decrease in T cell counts following TDB administration (Fig. S6). T cell counts increased up to 14 days above their baseline and returned to baseline at the end of the study.

Discussion

In this study, we compared single cell anti-FcRH5/CD3 TDBs (v10 and v11) to in vitro-assembled anti-FcRH5/CD3 TDB using comprehensive in vitro and in vivo PK/PD assessments. Pharmacological performance of two single cell bispecific designs was tested in a binding animal species (cyno). This study showed for the first time that cyno PK/PD behaviors of the single cell TDBs were pharmacologically comparable to in vitro-assembled TDB. In addition, we evaluated recovery profiles of anti-FcRH5/CD3 TDB-dependent PD changes, such as PC and B cell decreases and serum IgG levels in cynos beyond 7 days. Our results showed that PD changes mediated by anti-FcRH5/CD3 TDB were reversible, and that the recovery profiles agreed with the expected MOA of the TDB.

Single cell TDBs and in vitro-assembled TDB had comparable binding to both FcRH5 and CD3 antigens. In addition, all three TDBs showed robust and consistent in vitro cytotoxic activity of cyno and human PCs, MOLP-2 cell line, as well as T-cell activation. We observed slightly higher T-cell activation (Figure 2(d)) for the single cell TDBs compared to in vitro-assembled TDB, but this difference did not translate to any differences in MOLP-2 cell line cytotoxic activity. We did not observe 100% cell killing in in vitro experiments, consistent with observations for other TDBs. While the exact cause of incomplete in vitro cell killing is unknown, one possible explanation is the variability in the relative numbers of effector cells and expression levels of target cells from different donors since the killing activity of TDBs depend on both of these parameters. One limitation of these results was the small
sample size and the low number of donors used in the in vitro studies. However, we still confirmed in vitro activity of the single cell TDBs and that there were no major differences between single cell TDBs and the in vitro-assembled TDB before proceeding to in vivo PK/PD studies.

The mouse (non-binding species) PK study was conducted to identify any issues with target-independent PK of single cell TDBs before evaluating the PK/PD in cynos (binding species). We selected an immune-deficient mouse strain, SCID.bg mice, to avoid any potential ADA impact on the exposure of the TDBs. The observed variability in the PK profiles was likely due to sparse sampling, where the samples were collected from different mice at different time points and the samples were pooled to get the full PK profile for each group. The exposures of the single cell TDBs were comparable to the exposure of the in vitro-assembled TDB in SCID.bg mice. Furthermore, all anti-FcRH5/CD3 TDBs showed lower exposure compared to anti-gD in SCID.CD17 mice. Interestingly, the anti-gD/CD3 bispecific antibody also appeared to clear more rapidly than the anti-gD antibody, suggesting that the lower exposure of the three TDBs was likely due to the anti-CD3 arm. Therefore, we explored whether physiochemical properties of the CD3 arm contributed, non-specifically, to the observed lower exposure. iCAT evaluation suggested that anti-CD3 had a higher than acceptable Fv charge. One limitation of using iCAT to estimate Fv charge is that this tool was developed for bivalent, rather than bispecific, molecules to assess their risk for fast clearance in cynos. We also examined the structure of the anti-CD3 arm Fab region. A positive charge patch has been shown to increase the non-specific clearance of bivalent antibodies. Together these findings suggest that positive charge patches on the anti-CD3 arm could have potentially contributed to the lower exposures of both FcRH5/CD3 and anti-gD/CD3 compared to the bivalent anti-gD without a CD3 arm.

It is generally assumed that exposure drives biological activity for protein therapeutics; hence, comparison of PK alone has been considered an adequate measure for evaluating the differences in in vivo behavior of a molecule. In vitro-assembled anti-FcRH5/CD3 TDB exhibited similar PK behavior when compared with the previous cyno study. We included the PD endpoint evaluation in this cyno study due to the steep dose response observed in PC depletion with the anti-FcRH5/CD3 TDB in the previous cyno study. In that study, depletion of PCs following anti-FcRH5/CD3 TDB treatment was observed in cynos treated with doses of 2 mg/
kg or higher, while doses of 1 mg/kg resulted in incomplete PC depletion.\textsuperscript{11} Since anti-FcRH5/CD3 TDB was well tolerated at 1, 2 and 4 mg/kg in the cynos,\textsuperscript{11} we initially considered conducting the study at 4 mg/kg, which was the highest tested dose. However, conducting the study at 4 mg/kg would mean that the comparison would have been on the plateau of the dose-response curve, and any potential differences would not be captured. Hence, we chose a 2 mg/kg dose so that PC depletion information could identify pharmacological differences, if any, between single cell TDBs and in vitro-assembled TDB. Single cell TDBs and in vitro-assembled TDB exhibited robust PC and B cell killing, T-cell activation and cytokine level increases in cynos. In addition to PK/PD of single cell TDBs, immunogenicity of the single cell TDBs was also assessed, and no differences were observed when compared to immunogenicity of the in vitro-assembled TDB.

In addition, we evaluated the recovery of anti-FcRH5/CD3-dependent PD changes. After being produced in BM, B cells migrate to other secondary lymphoid tissues through circulation where they mature and differentiate. Our results showed that B cells recovered fully in BM within 55 days after a single dose of the TDBs. Similarly, B cell counts in peripheral blood exhibited a comparable recovery rate to B cells in BM. The observed recovery rate of B cells in BM and blood is consistent with the B cell proliferation rate of \(-2\%\) per day, which corresponds to \(\sim 50\) day lifespan in cynos.\textsuperscript{31}

We expected a similar correlation between PCs and IgG levels such that PCs in BM and IgG levels in blood would follow similar recovery patterns.\textsuperscript{32} However, our results showed IgG levels decreased for the first 7 days, and then increased above baseline levels until day 22 before returning to the baseline. The PCs in the BM in TDB-treated groups were not higher than the PCs in the control group at day 22. The mechanism underlying the substantial rebound of IgG levels around day 22 is not entirely understood. We may have missed a rebound effect in BM PCs due to constraints with BM aspirate sampling (i.e., collections can only be done weekly in cynos), making it difficult to show correlations between BM PCs and IgG levels in circulation. An alternative hypothesis is that available B cells in circulation (B cell numbers in circulation increased after day 7) and plasmablasts may overproduce IgGs to reach homeostasis and to compensate for IgG depletion as quickly as possible. The presence of ADA may have also contributed to total IgG levels, as likely ADA-producing B cells increased in number after day 7.

Both IgG levels and B cells in circulation started increasing after day 7 when the concentration of anti-FcRH5 TDB was below \(\sim 1\) \(\mu\)g/mL. We have insufficient data to draw any quantitative conclusions on the relationship between anti-FcRH5/CD3 TDB concentration and IgG levels or PCs in BM. However, it is possible that maintaining a certain level of exposure of anti-FcRH5/CD3 TDB in circulation might prevent the increase of IgG levels and/or PCs. Further PK/PD studies combined with a computational modeling analysis will be needed to fully understand these relationships.

TDBs have the potential of a longer half-life in human compared to smaller size formats such as blinatumomab due to the presence of the Fc region, which confers the advantage of FcRn recycling similar to monoclonal antibodies.\textsuperscript{33}

In summary, we compared single cell TDBs to in vitro-assembled TDBs qualitatively using comprehensive in vitro and in vivo PK/PD analyses. In addition to similar in vitro properties and mouse PK, the single cell TDBs exhibited similar PK/PD behavior and immunogenicity to in vitro-assembled TDB in cynos, which is the relevant preclinical species for anti-FcRH5/CD3 TDBs. We also showed that anti-FcRH5/CD3 TDB-mediated PD changes were reversible, and that the recovery profiles agreed with the TDB MOA. Overall, our study supports further development of the single cell anti-FcRH5/CD3 TDBs and other future single cell bispecific programs. Single cell production of single cell bispecifics will facilitate a faster and simpler production process in addition to being more cost efficient. As a result, this offers an overall effective production process for bispecific antibodies.

Materials and methods

Ethics statement

The authors confirm that they have obtained appropriate institutional review board, in Genentech and in Covance, approval for all animal experimental investigations.

Production of test materials

Anti-CD3 and anti-FcR5H antibodies are humanized hybridoma antibodies obtained from mice immunized with their respective antigens. In vitro-assembled anti-FcR5H/CD3 was produced as previously described.\textsuperscript{11} In brief, the knob (T366W) and hole (T366S, L368A, Y407V) half-antibodies were expressed in separate Chinese hamster ovary cells to ensure cognate light chain pairing and purified via Protein A affinity chromatography.\textsuperscript{11,13-15,34} Equal amounts of the two half-antibodies were incubated with a 200-fold molar excess of reduced glutathione at pH 8.5 overnight at 32°C to facilitate formation of the inter-heavy chain disulfide bonds. The assembled bispecific antibody was purified from contaminants through hydrophobic interaction chromatography.

Single cell bispecific designs included amino acid changes in the Fab variable region, outside of the CDRs and the constant domains in conjunction with previously described KIH mutations.\textsuperscript{14} Anti-FcRH5/CD3 single cell design v10 and anti-FcR5H/CD3 single cell design v11 were produced as previously described.\textsuperscript{18} The control IgG was anti-gd, targeting the glycoprotein D epitope of herpes simplex virus, and was produced in Genentech.

Radioligand cell binding assay for human and cyno FcR5H affinity measurement

The three anti-FcRH5/CD3 variants were iodinated using the iodogen method.\textsuperscript{35} In a competition reaction, serially-diluted unlabeled antibody was mixed with a fixed concentration of corresponding iodinated antibody in 96-well plates. The final concentration of the unlabeled antibody started at 500 nM, followed by eleven 3-fold dilutions. One reaction was performed without the addition of any unlabeled antibody. Stably transfected SVT2 (SV40-transformed mouse fibroblast cells) cell lines expressing human FcR5H or cyno FcR5H were detached from the flasks using cell dissociation solution (Sigma-Aldrich, C5914), washed with binding buffer, and added to the competition reaction in the 96-well plates. The competition reactions with cells were assayed
in triplicate for each concentration of unlabeled antibody and incubated for 2 h at room temperature. After the 2 h incubation at room temperature, the competition reactions were washed 4 times with binding buffer to remove the unbound iodinated antibody. The air-dried filters were counted on a Wallac Wizard 2470 gamma counter (PerkinElmer Life and Analytical Sciences) and the binding data were evaluated using NewLigand software (Genentech), which uses the fitting algorithm of Munson and Rodbard to determine the $K_D$ of the antibody.\(^{36}\)

**Affinity measurement by SPR for human and cyno CD3ε antigen**

Kinetic interactions were measured by SPR on a Biacore T200 instrument. Using a Series S CM5 chip, soluble recombinant CD3ε antigen was immobilized by means of amine coupling. Immobilization levels were between 200 and 250 RU for each of the 3 test flow cells (2, 3 and 4). Flow cell 1 was activated and blocked as for active flow cells without antigen present to be used as control. Each antibody construct flowed over the chip in a concentration series ranging from 0.05–50 nM. Measurements were taken at 25°C at a flow rate of 30 µL/min in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20 (HBSP) running buffer. Kinetic information was calculated by fitting data to a 1:1 binding model. Reference subtraction and data fitting were performed using BIAevaluation software (GE Life Sciences).

**Antibody variable region (Fv) charge and hydrophobicity**

**In silico** clearance assessment tool (iCAT) is a sequence-based calculation tool that provides a theoretical risk assessment of antibody clearance in cynos.\(^{25}\) This assessment is based on theoretical parameters extracted from the Fv domain sequence. The iCAT score was evaluated for the anti-CD3 bivalent antibody.

**In vitro cytotoxicity assay for cyno PCs and human donor PCs**

A cyno BM aspirate sample (n = 1, male) was procured from BioreclamationIVT. BMMCs were isolated from the cyno BM aspirate by lysing the red blood cells twice with ACK red cell lysis buffer for 10 min at room temperature upon arrival at Genentech. Cyno BMMCs were incubated for 72 h at 37°C with various concentrations of the 3 anti-FcRH5/CD3 TDB variants. The cyno PC were classified by flow cytometry as CD45$^+$CD20$^+$CD38$^+$PC$.^+^{37}$ The killing activity was calculated as follows: 100 (number of live target cells without TDB – number of live target cells with TDB)/number of live target cells without TDB.

Human BM aspirates from healthy donors (n = 2, male) were chosen to collect 3 samples per time point with a sparse sampling so that basic descriptive statistics could be obtained.

**PK of single cell and in vitro-assembled anti-FcRH5/CD3 TDBs in CB-17. SCID mice**

This study was designed to evaluate and compare the PK of three anti-FcRH5/CD3 TDB variants. Anti-gD and anti-gD/CD3 were also included as control antibodies. Five groups of female CB-17.SCID mice ($n = 9$ per group; Charles River Laboratories, 251) were administered a single IV dose of each antibody at a dose of 5 mg/kg. The number of animals was chosen to collect 3 samples per time point with a sparse sampling so that basic descriptive statistics could be obtained.

Female mice were used for convenience. Historically, we have not observed any differences in PK studies that used male or female mice. Blood samples were collected via the femoral vein at selected time points (3 replicates for each time point) for up to 21 days. Total antibody concentrations in serum were determined by a GRIP ELISA (plate coated with anti-human IgG and detected with anti-human IgG) with the limit of detection of 15.6 ng/mL and used for PK evaluations. The dosing solution recoveries were 88%, 114%, 101%, 93%
and 130%, for anti-gD, anti-gD/CD3, anti-FcRH5/CD3 in vitro-assembled, anti-FcRH5/CD3 v10 and anti-FcRH5/CD3 v11, respectively. The PK profiles were pooled from different mice at different time points. Nominal sample collection times and actual dose solution concentrations were used in data analysis. NCA parameters were estimated using Phoenix WinNonlin® 64 with sparse sampling and IV bolus input. Two-compartment model parameters were estimated using Simbiology® in MATLAB® 2016a with the combined error model described in the software. Standard error values were provided for both analyses. Mice were euthanized after being anesthetized with isoflurane (5% isoflurane with 2 L/min of O2.). All procedures were approved by and conformed to the guidelines and principles set by the Institutional Animal Care and Use Committee (IACUC) of Genentech and were performed in a facility accredited by Association for Assessment and Accreditation of Laboratory Animal Care International.

**PK/PD of single cell and in vitro-assembled anti-FcRH5/CD3 tdb's in cynos**

The PK and PD properties of three anti-FcRH5/CD3 TDB variants were evaluated in naive, male cynos at Covance, Madison. Cynos were administered a single-dose, 2 mg/kg IV infusion (1 h) of each test article (single cell v10, single cell v11 and in vitro-assembled TDB) and vehicle (n = 4 per group). The vehicle control article was 20 mM histidine chloride, 240 mM sucrose, 0.02% v/v Tween 20 in sterile water. Male cynos were used for consistency with the previous published cyo study.11 Blood samples were collected by venipuncture via the femoral vein pre-study and at pre-dose and selected time points for 55 days after dosing for analyses of hematology, serum chemistry, coagulation, and PK and PD endpoints (cytokines, flow cytometry of T lymphocytes, B lymphocytes, activated T lymphocytes and cyno serum IgG).

BM was collected from anesthetized animals by aspiration from the humerus pre-study and on days 7, 22 and 55 for evaluation of B lymphocytes and PC by flow cytometry. The study was terminated at day 55. All the surviving animals were returned to the colony. All procedures were approved by the Covance IACUC and were performed in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal welfare.

The number of animals chosen for this study was limited by ethical concerns. Previously, we used 3 cynos per group and demonstrated clear PC depletion compared to a control group at 2 mg/kg when dosed with in vitro-assembled TDB.11 In this cyo study, we used 4 cynos per group and dosed the animals with 2 mg/kg of single cell or in vitro-assembled TDBs. Our expectation was that, if the single cell TDBs were pharmacologically comparable to in vitro-assembled TDB, we would observe clear PC depletion in the single cell TDB dosed groups compared to the control group at 2 mg/kg.

**PK assay evaluation in cyno**

High-performance liquid chromatography with mass spectrometry (MS)/MS detection (multiple reaction monitoring) was used to quantify the total form of the three anti-FcRH5/CD3 variants in cyo sera. An affinity capture approach using streptavidin magnetic beads coated with biotinylated anti-HuIgG antibody (Clone R10Z, Genentech) was used to enrich anti-FcRH5/CD3 in serum. Three characteristic tryptic peptides, derived from the heavy chain region and light chain region, were selected for monitoring in the assay as surrogates of the total antibody concentration originating from anti-FcRH5/CD3. The method is applicable to the quantitation of anti-FcRH5/CD3 within a nominal range of 100–25,000 ng/mL. The lower limit of quantification (LLOQ) is nominally 100 ng/mL.

NCA parameters were estimated using Simbiology®, in MATLAB® 2016b with IV infusion input. The dosing solution recoveries were 106%, 97% and 98% for anti-FcRH5/CD3 in vitro-assembled, anti-FcRH5/CD3 v10 and anti-FcRH5/CD3 v11, respectively. Nominal sample collection times and actual dose solution concentrations were used in data analysis. All PK analysis was based on individual animal data.

**Flow cytometric assessment of cyno BM PCs and B cells**

Cyno BM aspirates were diluted (1:10) into ammonium chloride-potassium chloride buffer to lyse the red blood cells. All antibodies for cell staining were from BD Biosciences unless otherwise indicated. Cyno BM cells were stained with anti-CD 45-V500 (sc-374,650, Santa Cruz Biotechnology), anti-CD20-PE (sc-7733, Santa Cruz Biotechnology), and anti-CD38-AF 647 (sc-374,650, Santa Cruz Biotechnology) using the manufacturer’s recommended volumes for the antibodies. The cyo PC were identified by flow cytometry as those cells that were CD45 CD20 CD38 PC*. Depletion of B cells in BM was evaluated by the percentage of CD45 CD20+ in CD45 cells.

**ADA detection in cyno**

A generic immune-complex immunoassay was used to screen cyo serum samples for anti-drug antibodies as described elsewhere.38 In this assay, baseline and post-baseline samples from each animal were diluted and incubated with anti-FcRH5/CD3 variants, to allow formation of any ADA-drug complex. A mouse anti-human Fc- antibody (R10Z8E9, Genentech) immobilized on 96-well microtiter plates captured the ADA/therapeutic immune-complexes. A horseradish peroxidase (HRP)-labeled goat anti-monkey IgG (H + L) antibody (sc-2458, Santa Cruz Biotechnology) was used for detection. The relative sensitivity of the assay in neat serum, estimated using the positive control (human IgG and cyno IgG fusion) was approximately 40.7 ng/mL. The assay could detect 1,000 ng/mL of affinity-purified cyo anti-HuIgG antibodies in the presence of 440 μg/mL of a monoclonal antibody-derived molecule.

**IgG levels in cyino**

Samples and QCs containing IgG were bound to the immobile anti-monkey IgG, and detected using goat anti-monkey IgG conjugated to HRP (sc-2458, Santa Cruz Biotechnology). A standard curve was manually prepared fresh on the day of use in sample diluent using protein A purified cyo IgG. The calibration standards ranged from 6.25 to 800 ng/mL (in-well concentrations). Study samples were diluted to the minimum
required dilution (MRD) of 1/150,000 in sample diluent. The sensitivity of the assay (LLOQ) was 6.25 ng/mL. SoftMax Pro 6.3 with 4PL curve fit with 1/y weighting was used for regression of calibration curve and data analysis.

**Flow cytometric assessment of cyno blood**

The relative percentages of each phenotype obtained from the flow cytometer were multiplied by the absolute lymphocyte count from the hematology analysis in order to enumerate absolute cell counts. The following lymphocyte subsets were quantitated using flow cytometry: B cells (CD4+CD8−CD20+), CD4 cells (CD4+CD8−), CD8 cells (CD4+CD8−CD16−), CD25+CD69+ expressing CD4+ T cells (CD4+CD8−CD25+CD69+), CD25+CD69− expressing CD8+ T cells (CD4+CD8−CD16−CD25+CD69−), and CD25+CD69+ expressing CD8+ T cells (CD4+CD8−CD16−CD25+CD69+). The percentages of CD8+ and CD4+ T cells that were CD69+CD25+ were reported as T-cell activation.

**Cytokine levels in cynos**

Plasma samples were analyzed for selected cytokines on the Milliplex MAP NHP Cytokine Magnetic Bead Panel Kit in Plasma using a method validated at Covance Greenfield. Cytokine levels were similar to values reported previously. Therefore, only IL-6 and INF-γ levels are presented here.

**Abbreviations**

ADA anti-drug antibodies  
AUC area-under-the-curve  
BM bone marrow  
BMMC bone marrow mononuclear cell  
CDR complementarity-determining region  
cynos cynomolgus monkeys  
Fc crystallographic fragment  
FcrRH5 Fc receptor-like protein 5  
Fv antibody variable fragment  
iCAT in silico clearance assessment tool  
IV Intravenous  
KIH knobs-into-holes  
LLOQ lower limit of quantification  
MM multiple myeloma  
MOA mechanism of action  
NCA non-compartmental analysis  
PBMPC peripheral blood mononuclear cell  
PC plasma cells  
PD pharmacodynamics  
PK pharmacokinetics  
SPR surface plasmon resonance  
TDB T cell-dependent bispecific

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**Disclosure statement**

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**ORCID**

Nicola Stagg http://orcid.org/0000-0002-1082-6768  
Diego Ellerman http://orcid.org/0000-0002-3056-3600  
Trung Nguy http://orcid.org/0000-0002-0171-2873  
Weiru Wang http://orcid.org/0000-0001-8845-301X  
Jochen Brumm http://orcid.org/0000-0001-9518-038X  
Kedan Lin http://orcid.org/0000-0003-0144-6883  
Paul J. Carter http://orcid.org/0000-0001-7854-062X  
Christoph Spiess http://orcid.org/0000-0002-0570-9700

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