Targeting CD19 for the treatment of B-cell related autoimmune diseases with a novel T cell engager

Andy Qingan Yuan[1,4], Isabella Yang[2], Ray Qirui Yuan[3], Likun Zhao[1], Lili Bai1[1], Qingwu Meng[1]

1. Excyte LLC, 15601 Crabbs Branch Way, Rockville MD 20855 and Excyte Biopharma Ltd, NO.5 Kaituo Rd, Haidian Distr., Beijing China
2. Blair high school, Magnetic program, MD USA
3. Richard Montgomery high school, IB program, MD USA
4. Corresponding author, andyyuan@iexcyte.com

Abstract

Autoimmune disorder, affecting more than twenty million in United States and billions of people worldwide, occurs when human immune system mistakenly attacks its own body. Autoreactive B cells, particularly the autoantibodies they produce, play a critical role in the etiology, progress, prognosis, and pathogenesis of multiple autoimmune diseases. While targeting B cells medicines, especially anti-CD19 or anti-CD20 monoclonal antibodies showed promise in both pre-clinic models and some clinic practices, many patients failed to respond or relapsed to monoclonal antibody therapies. Novel therapy is desperately needed to fight this challenge. In this study, a novel designed anti-CD19xCD3 T cell engager (TCE), named 19K3, developed to cure precursor pre-B acute lymphatic leukemia and non-Hodgkin's lymphoma, was repurposed to bring T cells to CD19 positive B cells for treatment of autoimmune diseases. 19K3 has so significantly reduced binding affinity to CD3 compared to its parental clone that even as bivalent binding to TCR, free 19K3 alone does activate T cells. In contrast, in the presence of CD19 positive Raji target cells and mediated by 19K3, human T cells can be activated in a dose-dependent manner measured by upregulation of CD25, IFN-γ and granzyme B expression. As an engager, crosslinking of B cell with T cell by 19K3, the conjugation elicited T cell-mediated cytotoxicity only to CD19-positive Raji cells but not CD19-negative MV-4-11 cells. More interestingly, 19K3 induced much reduced *in vitro* cytokines release compared to that of Blincyto biosimilar, implying to be a potentially me-better therapeutics replacement in clinics for B-cell related diseases. In summary, this study demonstrated that by novel designing, an anti-CD3xCD19 TCE, 19K3 harnesses cytolytic T cells to eradicate B cells has the potential to treat B cell-mediated autoimmune diseases with better efficacy and less side effects.

Abbreviations:

ADCC: antibody-dependent cellular cytotoxicity
ALL: acute lymphatic leukemia
BiTE: bi-specific T cell engager
CD: cluster of differentiation
CAR T: chimeric antigen receptor T cells
Fc: fragment crystallizable
MS: multiple sclerosis

NMO: neuromyelitis optica

NHL: non-Hodgkin's lymphoma

TCE: T cell engager

RA: rheumatoid arthritis

SLE: systemic lupus erythematosus

scFv: single chain variable fragment

Introduction

An autoimmune disorder occurs when a person's immune system mistakenly attacks its own body due to the failure of self-tolerance[1]. There are more than 80 types of autoimmune diseases, which affect 23.5 million Americans and have devastating impacts on health, well-being, and quality of life. B cell is a critical component of adaptive immune system and has the ability to generate antibodies, which help for the elimination of the invaded pathogens[2]. However, autoreactive B cells play a key role in the pathogenesis of a wide variety of autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis (RA), multiple sclerosis (MS), and neuromyelitis optica (NMO). The pathogenic roles for B cells vary across autoimmune diseases, which can be autoantibody dependent or independent [1, 3].

Depletion of autoreactive B cells is efficacious for multiple autoimmune diseases in both preclinical models and clinical settings[3]. B cell targeted therapies have been approved for the treatment of RA, SLE, MS and the indications of autoimmune diseases that can be treated with such strategy are still expanding [3, 4]. One major treatment approach to target B cells is to directly deplete B cells with monoclonal antibodies against CD20, such as rituximab and ocrelizumab to induce Fc-mediated ADCC. Despite its approval, the responses to rituximab vary in a wide range and a substantial proportion of patients do not show improvement of disease severity[3, 5, 6]. Rituximab can deplete B cells efficiently in the periphery; however, it is less efficient to eradicate the autoreactive B cells in the lymphatic organ and inflamed tissues [6]. The mechanisms underlying the impaired depletion by anti-CD20 in lymphoid and inflamed tissues are not yet well understood. Novel strategies to target autoreactive B cells are needed to improve the treatment outcomes on autoimmune patients.

While CD20 is expressed by mature B cells, CD19 is expressed throughout B cell development and maturation including mature B cells, and some pre-B cells, plasma blast cells, and plasma cells [7]. The polyreactive and thus autoreactive B cells are predominantly selective in the early B cell development stage, and they do not express CD20[3]. In addition, the long-lived plasma cells have high CD19 and low CD20 expression and are resistant to B-cell depletion therapy[3]. Overexpression of human CD19 in transgenic mice results in hyperactivation of B cells with elevated humoral response, disrupted tolerance and spontaneous induction of autoimmunity[8]. CD19 expression is 20% higher on B cells from some patients with MS compared with healthy controls and the levels of expression are correlated with disease susceptibility[9]. Thus, targeting B cells via CD19 may allow broader targeting of B cells, including $CD19^{+}CD20^{lo}$ plasmablasts and $CD19^{+}$ pre-B cells, which could provide better outcomes in comparison to anti-CD20 for autoimmune disease patients.

Bispecific T cell engager (BiTE) is a class of T cell engager (TCE) molecules that simultaneously binds an antigen on a target cell and CD3 on a T-cell. BiTE brings T cells to the proximity of the target cells and activate T cells that can subsequently kill the target cells[10]. The first-in-class BiTE, blinatumomab (Blincyto®), is a fusion protein composed of two single-chain antibodies (scFVs) and has been approved for the treatment of patients with relapsed and/or refractory B cell-precursor acute lymphoblastic leukemia[11]. Owing to its small size and strong affinity to CD3, blinatumomab has very short half-life and strong toxicity[11]. So far, TCEs have been extensively studied for the treatment of various cancers and have not been widely investigated for the treatment of autoimmune diseases. We hypothesized that an engineered novel CD3xCD19 TCE, named 19K3, with significant weak binding to CD3 and reduced potency to activated T cells, would be able to kill autoreactive $CD19^{+}$ T cells and have better safety and efficacy than anti-CD20 or Blincyto®, besides its original intention for treatment of blood cancers. In this study, we investigated the potential of 19K3 for the treatment of autoimmune diseases by killing the autoreactive B cells.

19K3 is composed of a Fc-end-fused anti-CD3 scFv to a full anti-CD19 IgG4. It has much weakened binding affinity to CD3, compared to its parental antibody. In addition, 19K3 only induced activation of T cells in the presence of $CD19^{+}$ Raji B cells and initiated T cell-mediated killing of $CD19^{+}$ Raji B cells but not MV-4-11 $CD19^{-}$ monocytic leukemia cells. 19K3 induced much reduced *in vitro* cytokines release compared to that of Blincyto® biosimilar. The study

revealed the potential of this novel anti-CD3xCD19 TCE for the treatment of autoimmune diseases by inducing T cells-mediated killing of the autoreactive B cells.

Materials & Methods

Cells and culture medium

Raji cells, MV-4-11 cells, and Jurkat T cells were purchased from ATCC and cultured in RPMI Medium 1640 (Gibco, 21870) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin (Gibco, 15070063).

19K3 design, expression, and purification

The variable sequence of the anti-CD19 and anti-CD3 were derived from patent-expired clones, HD37 and OKT3, respectively. The tetravalent bispecific antibody 19K3 was built by FIST (fusion of IgG and scFv technology) platform optimized in Excyte Biopharma Ltd. It is composed of anti-CD19 IgG4P and anti-CD3 scFv, assembled in an in-house vector pG4HK by standard molecular cloning methods. The plasmid was stably transfected into CHO-K1 (Shanghai Biochemical Institute, China Academia of Sciences) suspension cells. High expression clones were selected, and fermentation supernatants were purified by Protein-A affinity chromatography using MabSelect™ column (GE Healthcare, Princeton, NJ). The bispecific antibody was further polished by cation exchange chromatography using Capto MMC (GE Healthcare, Princeton, NJ) and ion exchange chromatography using Q HP (GE Healthcare, Princeton, NJ) columns. The purified protein was concentrated and stored in 50mM PBS containing 0.15M NaCl buffer. The purity was analyzed with intact mass spectrometry, SDS-PAGE and size-exclusion chromatography (HPLC-SEC). Blinatumumab (blincyto®) was generated and prepared in mammalian cells in house according to its published amino-acid sequence.

Determination of CD19 expression in Raji B cells and MV-4-11 cells

The expression of CD19 in Raji B cells and MV-4-11 cells was determined by flow cytometry. Cultured Raji cells and MV-4-11 cells were washed twice with PBS and stained with FITC-conjugated mouse anti-human CD19 (Biolegend, 302205) for 30 mins. Cells were washed and analyzed with a Guava Technologies flow cytometer.

Binding analysis of 19K3 bispecific antibodies to CD3 and CD19

The binding affinities of 19K3 to CD3 and CD19 were determined by flow cytometry to $CD3^+$ Jurkat T cells and $CD19^+$ Raji B cells respectively. In brief, Jurkat T cells and Raji cells were washed twice with PBS and incubated with FACS buffer (1×PBS with 0.5% BSA) in a 96-well U bottom plate containing various concentrations of 19K3 for 30 minutes at room temperature. Subsequently, the cells were washed twice with FACS buffer and stained with Alexa Fluor 488-conjugated goat-anti human IgG Fc antibody (Jackson Laboratory, 109-545-098). The binding of mouse anti-human CD3 (clone: OKT-3) to Jurkat T cells, Alexa Fluor 488-conjugated goat-anti mouse IgG Fc antibody (Jackson Laboratory, 115-545-003) was used for the second antibody. Samples were analyzed by flow cytometry with a Guava Technologies flow cytometer.

To measure the apparent binding affinity of 19K3 to human T and Raji cells, saturated method[22] was applied with titrated 19K3 added to the two cells, respectively.

Peripheral blood mononuclear cell (PBMC) isolation and activation of T cells

PBMCs were isolated from healthy donor buffy coat (purchased from StemCell, Rockville) and isolated by Ficoll density gradient centrifugation. PBMCs were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, 16000044), 100 U/ml penicillin and streptomycin (Gibco, 15070063), 1 mM pyruvate (Gibco, 11360070), and 55 μM β-mercaptoethanol (Sigma, m3148). Cells were activated by TCR stimulation using plate-bound human anti-CD3 and soluble CD28 (clone OKT-3 and clone 9.3 Bio X Cell, respectively, 5 μg/mL of each) for 3 days, followed by interleukin 2 (IL-2) (20 ng/mL, Gibco) stimulation for 5 days.

Determination of T cell activation by flow cytometry

For measurements of T cell activation and cytokine expression, PBMCs from human donors were cocultured with Raji cells or MV-4-11 cells at an E:T ratio of 3:1 in the presence or absence of 1 μg/ml anti-CD3xCD19 for 72 hours. T cell activation was assessed by incubating cells with fluorescein isothiocyanate-conjugated anti-human CD8 (Biolegend, 100705) and phycoerythrin-conjugated anti-human CD25 (Biolegend, 356103).

For cytokine expression, cocultured cells were stimulated with PMA, ionomycin and GolgiPlug for 4 hours, and antibodies against phycoerythrin-conjugated anti-IFN-γ (Biolegend, 383303) and fluorescein isothiocyanate-conjugated anti-granzyme B (Biolegend, 372205) were

used for intracellular staining. Samples were analyzed by flow cytometry with a Guava Technologies flow cytometer.

Cytokine release induced by 19K3 or Blinatumumab, with or without dexamethasone

CRS (cytokine release storm) is a frequent side effect associated with the activation of human immune cells. Administration of Dexamethasone is prescribed as a preventive approach for CRS in the clinical usage of Blinatumumab (Blincyto®). To investigate the CRS levels released *in vitro* by activated human PBMC mediated by 19k3 or Blincyto (biosimilar), with and without dexamethasone, liquid-nitrogen-stored human PBMC was immediately thawed at a 37°C-water bath and washed with PBS twice to remove storage medium. The cells were resuspended in X-VIVO to 2×10^6 cells/ml and dispensed at 50 µl/well to a 96-well culture plate. Raji cells at 2×10^5 cells/ml were added to the each well at 50 µl/well to reach PBMC: Raji =100000:10000（10：1). A blank control well without Raji was set. Stock 19K3 or Blincyto, adjusted to 8 nm, was diluted at 4-fold down and added to each well at 100 µl, in a duplicate manner. A constant 100nM dexamethasone was applied to each well for the effect on cytokine release in a separate plate. The plates were incubated at a cell culture incubator for 48 hours after gentle mixing. An ELISA method was applied to measure the concentrations of individual IL-2, TNF-α, IFN-γ, IL-6 released in each well. Plots of each cytokine level verse 19K3 or Blincyto concentration, with and without dexamethasone, were generated by Microsoft Excel.

Cell cytotoxicity determination with LDH release assay

For cell cytotoxicity, activated T cells were co-cultured with Raji or MV-4-11 tumor cells at a 5:1 ratio in the presence of different concentrations of 19K3 for 24 hours. Afterwards, T cell cytotoxicity was assessed by quantification of LDH levels in the supernatants using the LDH-Glo cytotoxicity kit (Promega, J2380) according to the manufacturer's instructions.

Results

Schematic representation and characterization of the 19K3 T cell engager

To develop highly potent anti-CD3xCD19 bispecific antibodies for autoimmune diseases, we fused the scFv of anti-CD3 with a full anti-human CD19 IgG4 at its Fc region, as depicted in Figure 1A. The designed molecule 19K3 processes two antigen binding sites for CD19 at the N-terminal and two binding sites for CD3 at the C-terminal. Next, we used flow cytometry to

determine the binding activities of 19K3 to $CD3^+$ Jurkat T cells and $CD19^+$ Raji cells (Figure 1B-D). 19K3 effectively bound Jurkat T cells and Raji cells in a dose-dependent manner (Figure 1C and D). By saturated measuring method, the apparent binding affinity (Kd) for 19K3 binding to Jurkat T cells was 24.85 µg/ml (Figure 1, E), while the parental OKT-3 anti-CD3 binding to Jurkat T cells was 0.36 µg/ml (Figure 1, B), indicating significant reduced binding affinity by the molecular design. The apparent binding affinity (Kd) of the 19K3 to CD19+ cells (Raji) was 0.2667 µg/ml (Figure 1, F).

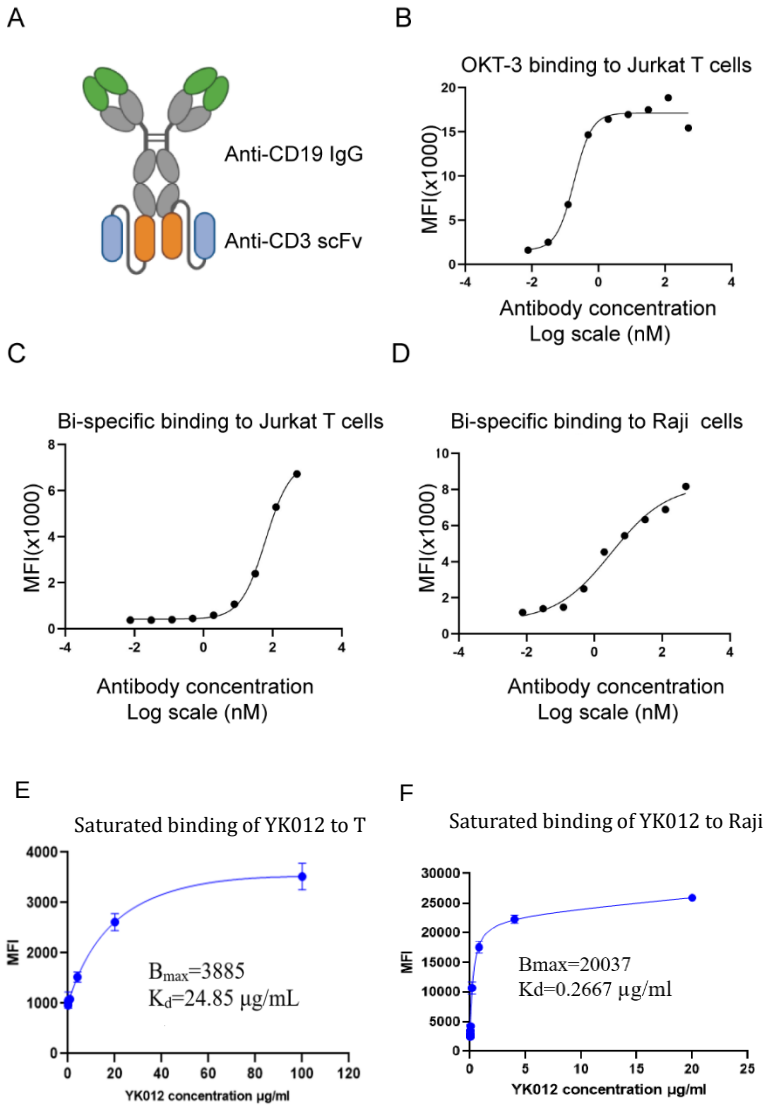

Figure 1. Schematic representation and characterization of the anti-CD3xCD19 T cell engager

(A) Schematic of bi-specific anti-CD3xCD19 19K3 that contains two scFv of anti-CD3 fused with the anti-CD19 IgG4 at the C-terminal. (B) Flow cytometry analysis of binding of increasing amounts of OKT3 to Jurkat T cells (B), bi-specific anti-CD3xCD19 to Jurkat T cells (C) and Raji cells (D). Saturated binding and Kd of YK012 to human T cells (E) and Raji cells (F) were measured respectively.

19K3 activates T cells requiring the presence of CD19$^+$ Raji cells

Next, we investigated the ability of 19K3 to activate T cells in the absence or presence of CD19$^+$ Raji cells. Activated T cells express higher amounts of CD25. Using CD25 as a marker of T cell activation, 19K3 alone could not activate CD8$^+$ T cells (Figure 2A-C). In contrast, in the presence of CD19$^+$ Raji cells, 19K3 induced more CD25 expression, with 56% of the T cells expressing CD25 compared to 8% in the antibody alone group (Figure 2A and B), among them 10.9% were CD8$^+$ T cells in the bi-specific stimulated group in the presence of CD19$^+$ Raji cells versus 0.3% without CD19$^+$ B cells (Figure 2A and C).

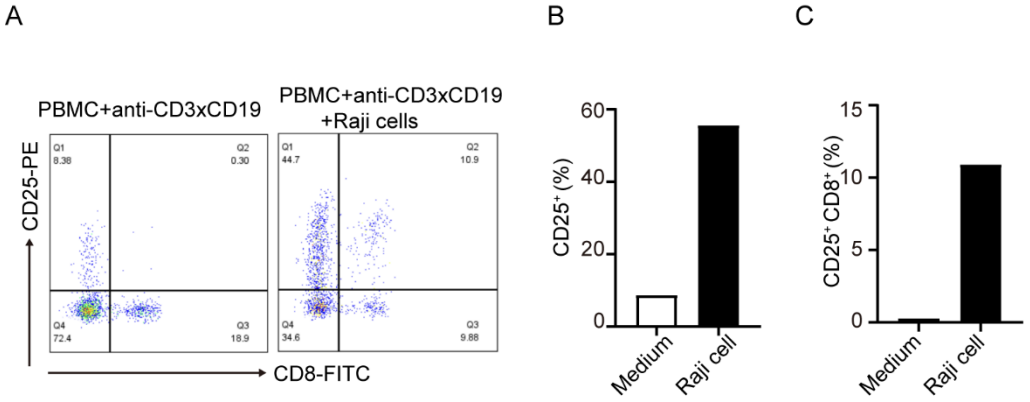

Figure 2. CD3xCD19 activates T cells and requires the presence of CD19$^+$ Raji cells

(A-C) Isolated PBMCs from healthy donors were cultured with 1 μg/ml bi-specific anti-19K3 in the presence or absence of Raji cells for 72 hours. Afterwards, surface expression of CD25 on CD8$^+$ T cells were determined by flow cytometry.

19K3 activates T cells requires the presence of CD19$^+$ Raji cells

Activated CD8$^+$ T cells express IFN-γ and granzyme B and the levels were associated with their cytolytic function. Subsequently, we compared the expression of IFN-γ and granzyme B in CD8$^+$ T cells activated by 19K3 alone or in the presence of CD19$^+$ Raji B cells. We found little

production of cytokines by T cells when PBMCs were stimulated with 19K3 alone (0.46% IFN-γ^+CD8$^+$ and 1.16% GZMB$^+$CD8$^+$) (Figure 3A-F). However, in the presence of CD19$^+$ Raji cells, 19K3 induced 13.3-fold and 6.5-fold enhanced IFN-γ- and granzyme B-positive CD8$^+$ T cells respectively (6.10% IFN-γ^+CD8$^+$ and 7.56% GZMB$^+$CD8$^+$) (Figure 3A-F). Taken together, these data suggest that 19K3 only activated T cells in the presence of CD19-positive cells.

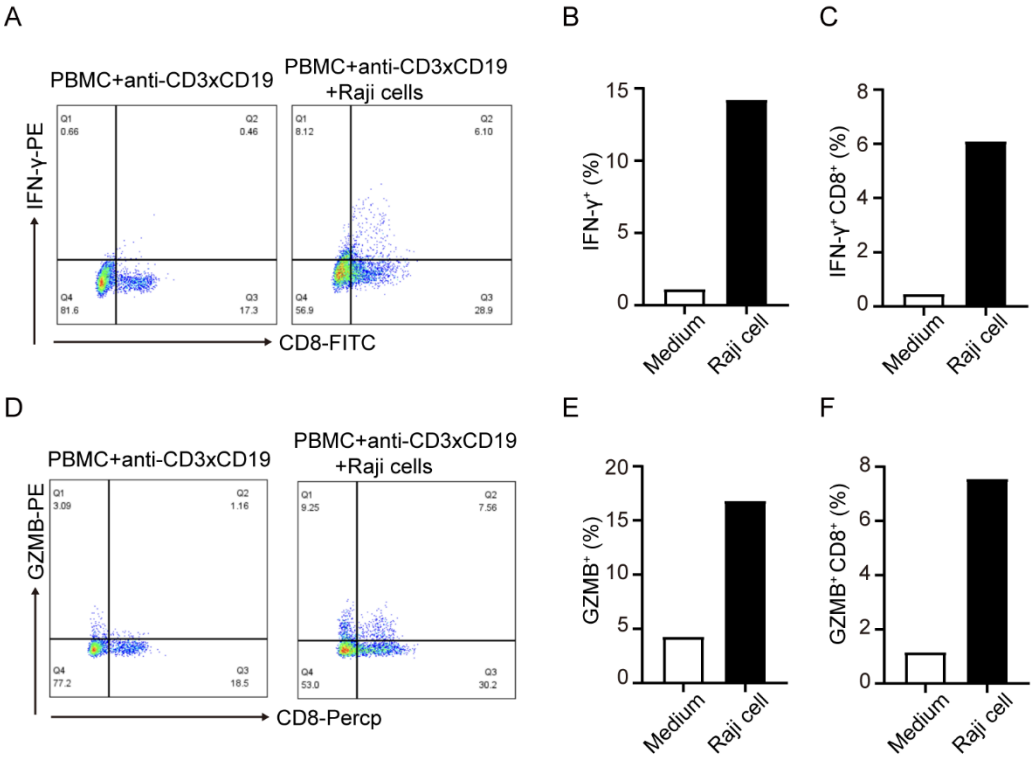

Figure 3. 19K3 activates T cells and requires the presence of CD19$^+$ Raji cells

(A-F) Isolated PBMCs from healthy donors were cultured with 1 μg/ml 19K3 in the presence or absence of Raji cells for 72 hours. Afterwards, co-cultured cells were stimulated with PMA, ionomycin and GolgiPlug for another 4 hours. Cytokines production of IFN-γ (A-C) and Granzyme B (D-F) in CD8$^+$ T cells were determined by intracellular staining.

19K3 induced less cytokine release *in vitro* than Blincyto

To measure and compare the cytokine level release of activated PBMC cells conjugated by 19K3 or blinatumomab with Raji cells, with and without dexamethasone, supernatant from the incubated cell mixture of human PBMC and Raji cells, in the presence of diluted 19K3 or Blinatumab, was collected and measured by an ELISA method. The major cytokines, including

IL-2, TNF-α, IFN-γ, IL-6, were individually quantitated and plotted against antibody concentrations (Figure.4).

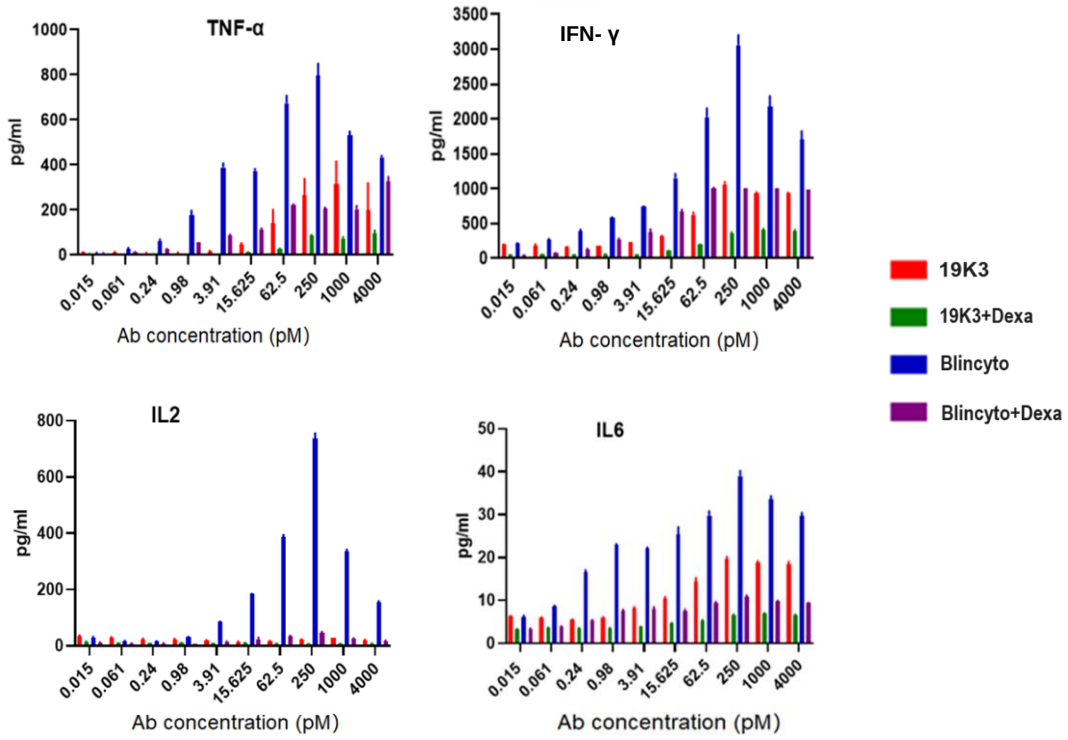

Figure 4. CRS released by PBMC mediated by 19K3 or Blincyto

$CD19^+$ Raji cells were co-cultured with activated human PBMC cells at an E:T ratio of 10:1 in the presence of varying concentrations of 19K3 or Blincyto, with or without dexamethasone, for 48 hours. Cytokines of TNF-α, IFN-γ, IL-2, IL-6 were individually quantitated by ELISA method and plotted against antibody concentration.

While the cytokines induced in 19K3 group were generally significantly lower than those of Blincyto (biosimilar), the application of dexamethasone demonstrated huge reduction in both situations across the wide range of antibody concentrations. This implies that the future mitigation of clinical severe CRS risk by the preventive prescription of dexamethasone for the usage of 19K3 to treat B-cell related abnormalities.

19K3 mediates T cell killing of $CD19^+$ Raji cells but not MV-4-11 cells

To test if 19K3 kills target cells in a CD19-dependent manner, we first compared the expression of CD19 in Raji B cells and MV-4-11 cells. As depicted in Figure 5, Raji cells expressed high amount of CD19 while MV-4-11 had minimal CD19 expression (Figure 5, A). Next, we compared the T cell cytotoxicity induced by 19K3 between $CD19^+$ Raji cells and $CD19^-$ MV-4-11 cells. We found that 1 μM 19K3 induced maximum killing of $CD19^+$ Raji cells, and the cytotoxic potency was approximately 70% (Figure 5, B). As MV4-11 cells do not express CD19, 19K3 did not elicit any T cell: target cell crosslinking or cytotoxicity, as no/little clustering was observed (Figure 5, C).

Taken together, these data demonstrated that 19K3 elicited T cell-mediated cytotoxicity only to CD19-positive cells but not to CD19-negative MV-4-11 cells.

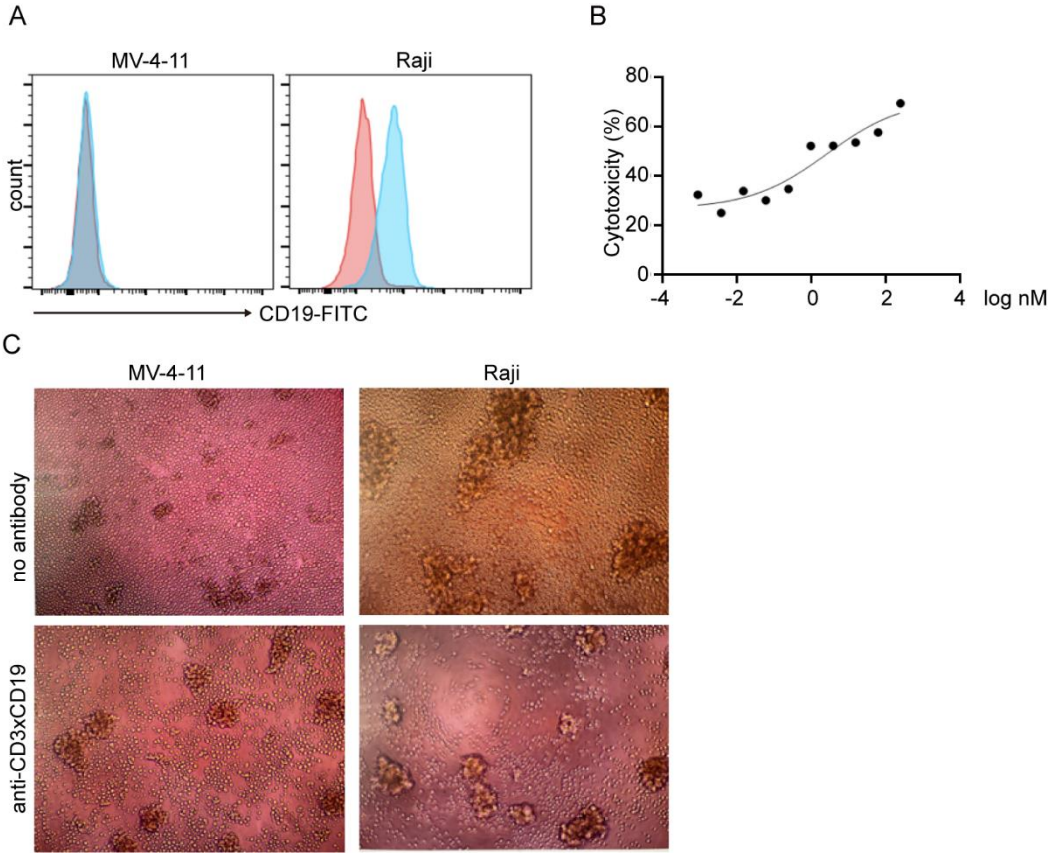

Figure 5. 19K3 mediates T cell killing of $CD19^+$ Raji cells but not MV-4-11 cells

(A) CD19 expressions in MV-4-11 and Raji cells were determined by flow cytometry. (B) $CD19^+$ Raji cells were co-cultured with activated T cells at an E:T ratio of 5:1 in the presence of varying concentrations of 19K3 for 24 hours. The cytotoxicity was measured by the amounts of released LDH relative to a control containing 3% Trion X-100. (C) Microscope images of $CD19^+$

Raji cells or MV-4-11 cells co-cultured with activated T cells at an E:T ratio of 5:1 in the presence of 5 μg/ml 19K3 for 24 hours.

The mechanism of action of 19K3 can be depicted in Figure 6. 19K3 can bind simultaneously to T cells and $CD19^+$ autoreactive B cells, which leads to the activation of T cells. Activated T cells will upregulate CD25 expression and secret IFN-γ, perforin, and granzyme B. Both perforin and granzyme B have cytotoxic effect on target cells to induce apoptosis and lysis of target cells, thus leading to the elimination of the autoreactive B cells to cure the autoimmune diseases.

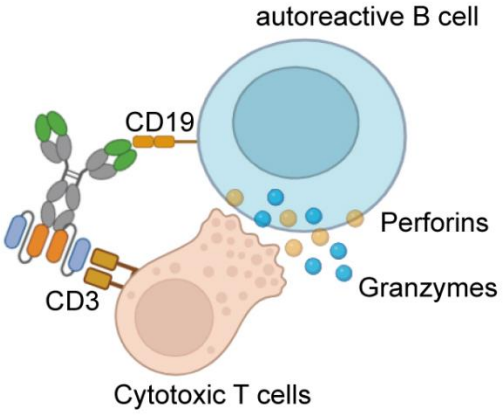

Figure 6. Mechanism of action of 19K3-mediated killing of autoreactive B cells

19K3 recruit cytotoxic T cells to autoreactive B cell and secret perforins and Granzymes resulting in cell lysis.

Discussion

Targeting autoreactive B cells showed promise for the treatment of multiple autoimmune diseases. However, a large proportion of patients do not respond to the therapies[3]. In this study, we proposed that using a novel tetravalent anti-CD3xCD19 (19K3) could be more efficient for the killing of autoreactive B cells and provide benefit for the autoimmune patients compared to monoclonal antibody therapy. In this study, we confirmed that while 19K3 has much reduced affinity to CD3 compared to parent antibody OKT-3, it activated T cells in the presence of $CD19^+$ target cells, and effectively elicited T cell-mediated cytotoxicity only to $CD19^+$ cells. Meanwhile the *in vitro*

released major cytokines associated with CRS were minimized compared to Blincyto biosimilar, additional reduction was possible when accompanied by dexamethasone. These data demonstrated that the engineered 19K3 has the potential to be more effective but less toxicity for the treatment of autoimmune diseases by killing $CD19^+$ autoreactive B cells.

Nowadays the approved approach for B cell targeting is using anti-CD20 monoclonal antibody to deplete B cells[12, 13, 14]. Although it is highly efficient to deplete B cells in the periphery, it is less efficient to deplete autoreactive B cells in the lymphoid organs and inflamed tissues[6]. Currently, it is not well understood why the Fc-mediated ADCC of B cells is impaired in those tissues. Autoreactive pre-B cells and plasma cells have been shown to mediate the pathogenesis of autoimmune diseases and manifestations of symptom and autoimmunity[6, 15, 16]. The lack of or low CD20 expression in pre-B cells and plasma cells could be another reason for patients not responding to anti-CD20 treatment[6, 15, 16]. In contrast, CD19 is broadly expressed throughout B cell linage including pre-B cells and plasma cells and acts as a critical regulator for B cell activation and function[7, 8, 17]. This suggests targeting CD19 may have more advantages than targeting CD20. In line with this, a non-depleting antibody LY3541860 potently inhibited B cell function and showed superior activity in the inhibition of disease severity in a TD1 mouse model, compared to anti-CD20[18].

The TCE targeting CD19 approach has been approved for leukemia. Blinatumomab is the first BiTE approved by the FDA for the treatment B-ALL[11]. It is composed of two scFV connected with a flexible linker with small size (55 kDa), which leads to quick kidney clearance and requires continuous infusion. But it has limitations owing to its extremely short half of slightly over 2 hours and strong adverse events including CRS and neurotoxicity. The short space between anti-CD3 scFv and anti-CD19 scFV was attributed for its strong activation ability on T cells[19]. The distance between the two antigen binding sites has been proposed to be a strength regulator for the anti-CD3 arm to induce storm-like T cell activation[19]. Here, we engineered a TCE that contains a full anti-CD19 IgG4 with fusion of anti-CD3 scFV at the C-terminal. Compared to the parent anti-CD3, the molecule we designed has less binding affinity to CD3 as well as longer distance between the two binding sites, suggesting that it may induce weak T cell activation and has less side effect. Further, the engineered 19K3 only elicited killing on $CD19^+$ Raji B cells but not $CD19^-$MV-4-11. This suggest 19K3 would not induce non-specific killing, thus further limits its potential systematic toxicity.

Immunotherapy modalities that harness the cytolytic potential of T cells to target and kill tumor cells, such as Chimeric antigen receptors (CAR) -T cells or BiTE, have been intensively studied for the treatment of a variety of cancers [10, 20]. Only recently have CAR-T cells been investigated for autoimmune diseases. In 2021, a woman with severe systemic lupus erythematosus was treated with CD19-targeting CAR-T cells[21]. The infusion of CAR-T cells significantly reduced the disease severity and showed acceptable safety[21]. These data demonstrate it is feasible to harness T cells to kill autoreactive B cells in clinic. However, preparation of autologous CAR-T cells requires intensive labor and is time consuming. In contrast, bi-specific antibody is convenient to manufacture and can be easily administrated to patients. Currently it has not been reported that using BiTE or alike to harness the T cytotoxicity to kill the autoreactive B cells. Our candidate 19K3 has strong potential to further improve the treatment outcomes for autoimmune diseases.

This study has several limitations. Raji B cells were used here to demonstrate the induced cytotoxicity of 19K3 on autoreactive B cells. Due to time limitation, regulation, and logistic reasons, no pharmacokinetics of the anti-CD3xCD19 *in vivo* was conducted. It remains unclear whether 19K3 can inhibit pathogenesis and ameliorate the autoimmune manifestations in animal models. The *in vivo* comparison between 19K3 and anti-CD20 is also warranted.

In summary, the engineered 19K3 bispecific antibody has the potential to provide better effective treatment options for patients with autoimmune diseases.

Reference

1. Barnas, J.L., Looney, R.J. & Anolik, J.H. B cell targeted therapies in autoimmune disease. *Curr Opin Immunol* **61**, 92-99 (2019).
2. Dorner, T. & Radbruch, A. Antibodies and B cell memory in viral immunity. *Immunity* **27**, 384-392 (2007).
3. Lee, D.S.W., Rojas, O.L. & Gommerman, J.L. B cell depletion therapies in autoimmune disease: advances and mechanistic insights. *Nat Rev Drug Discov* **20**, 179-199 (2021).
4. Wemlinger, S.M. & Cambier, J.C. Therapeutic tactics for targeting B lymphocytes in autoimmunity and cancer. *Eur J Immunol*, e2249947 (2023).
5. Md Yusof, M.Y. *et al.* Predicting and managing primary and secondary non-response to rituximab using B-cell biomarkers in systemic lupus erythematosus. *Ann Rheum Dis* **76**, 1829-1836 (2017).
6. Ramwadhdoebe, T.H. *et al.* Effect of rituximab treatment on T and B cell subsets in lymph node biopsies of patients with rheumatoid arthritis. *Rheumatology (Oxford)* **58**, 1075-1085 (2019).
7. Anderson, K.C. *et al.* Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood* **63**, 1424-1433 (1984).
8. Inaoki, M., Sato, S., Weintraub, B.C., Goodnow, C.C. & Tedder, T.F. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *J Exp Med* **186**, 1923-1931 (1997).
9. Tsuchiya, N. *et al.* Association of a functional CD19 polymorphism with susceptibility to systemic sclerosis. *Arthritis Rheum* **50**, 4002-4007 (2004).
10. Goebeler, M.E. & Bargou, R.C. T cell-engaging therapies - BiTEs and beyond. *Nat Rev Clin Oncol* **17**, 418-434 (2020).
11. Topp, M.S. *et al.* Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol* **29**, 2493-2498 (2011).
12. Furie, R. *et al.* Two-Year, Randomized, Controlled Trial of Belimumab in Lupus Nephritis. *N Engl J Med* **383**, 1117-1128 (2020).
13. Furie, R. *et al.* Safety and Efficacy of Belimumab in Patients with Lupus Nephritis: Open-Label Extension of BLISS-LN Study. *Clin J Am Soc Nephrol* **17**, 1620-1630 (2022).
14. Merrill, J.T. *et al.* Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum* **62**, 222-233 (2010).
15. Chen, D. *et al.* Autoreactive CD19+CD20- Plasma Cells Contribute to Disease Severity of Experimental Autoimmune Encephalomyelitis. *J Immunol* **196**, 1541-1549 (2016).
16. Vital, E.M. *et al.* B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis Rheum* **63**, 3038-3047 (2011).
17. Rigley, K.P. & Callard, R.E. Inhibition of B cell proliferation with anti-CD19 monoclonal antibodies: anti-CD19 antibodies do not interfere with early signaling events triggered by anti-IgM or interleukin 4. *Eur J Immunol* **21**, 535-540 (1991).
18. Boyles, J.S. *et al.* A nondepleting anti-CD19 antibody impairs B cell function and inhibits autoimmune diseases. *JCI Insight* **8** (2023).

19. Nagorsen, D., Kufer, P., Baeuerle, P.A. & Bargou, R. Blinatumomab: a historical perspective. *Pharmacol Ther* **136**, 334-342 (2012).
20. Sterner, R.C. & Sterner, R.M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* **11**, 69 (2021).
21. Mougiakakos, D. *et al.* CD19-Targeted CAR T Cells in Refractory Systemic Lupus Erythematosus. *N Engl J Med* **385**, 567-569 (2021).
22. Hunter SA, Cochran JR. Cell-Binding Assays for Determining the Affinity of Protein-Protein Interactions: Technologies and Considerations. Methods Enzymol. 2016;580:21-44.