BRIEF CONCLUSIVE REPORT

Optimizing TNFR2 antagonism for immunotherapy with tumor microenvironment specificity

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Abstract
Most approved cancer immunotherapies lack T-regulatory (Treg) or tumor specificity. TNF receptor 2 (TNFR2) antibody antagonism is emerging as an attractive immunotherapy due to its tumor microenvironment (TME) specificity. Here we show that the human TNFR2 receptor is overexpressed on both human tumor cells and on human tumor-residing Tregs, but negligibly expressed on beneficial T effectors (Teffs). Further, we found widespread, if variable, TNFR2 expression on 788 human tumor cell lines from diverse cancer tissues. These findings provided strong rationale for developing a targeted immunotherapy using a TNFR2 antibody antagonist. We designed a novel, human-directed TNFR2 antibody antagonist and tested it for function using three cell-based TME assays. The antagonist showed TME specificity by killing of TNFR2-expressing tumor cells and Tregs, but sparing Teffs, which proliferated. However, the antagonist shuffled between five isoforms, only one of which showed the desirable function. We designed and tested several new chimeric human versions of the antagonist, finding that the IgG2 isotype functioned better than the IgG1 isotype. To further improve function, we introduced targeted mutations to its amino acid sequence to stabilize the natural variability of the IgG2 isotype's hinge. Altogether, our findings suggest that optimal TNFR2 antagonists are of the human IgG2 isotype, have hinge stabilization, and have wide separation of antibody arms to bind to newly synthesized TNFR2 on rapidly growing tumor cells. Antagonistic antibodies with these characteristics, when bound to TNFR2, can form a nonsignaling cell surface dimer that functions with high TME specificity.

KEYWORDS
3 cell-based assays, hinge stabilization, IgG2 isoform

1 | INTRODUCTION

The TNFR2 receptor on T regulatory (Treg) cells is a master control switch with broad therapeutic implications for diseases of immune dysregulation.1 Agonism of TNFR2 is advantageous for treating autoimmunity because it stimulates adult Treg expansion into potent immunosuppressor cells and reciprocally kills CD8 cytotoxic T cells.2,3 Antagonism of TNFR2, on the other hand, is advantageous for treating cancer because it inactivates or kills Tregs, thereby removing tumor environment immunosuppression.4–6 TNFR2 is also overexpressed in the tumor microenvironment (TME), which consists of infiltrating TNFR2+ Treg cells, myeloid suppressor cells, and tumor cells.7,8 Agonism of TNFR2 sets off downstream proliferation through transcription factor NF-kB for preferential survival.1 Antagonism of TNFR2, by contrast, kills target cells through inactivation of NF-kB signaling and the stabilization of strong hexagonal networks of receptors with poor signaling activity.5,9,10 In most human tumors, every Treg in the TME expresses newly made and high-density TNFR2.7 Human cancers with high TNFR2 expression in the TME have poor prognosis and secrete into the serum soluble TNF (sTNFR2), a prognostic marker.11–15 The most suppressive subset of Tregs highly express TNFR2 in the TME of both humans and rodents.5–7 Therefore,
the central role of TNFR2 in diseases of immune dysregulation, such as autoimmunity and cancer, is established.1-5,10

The most suppressive human-directed TNFR2 antagonistic antibodies appear to have specificity for the TME due in part to differential expression of TNFR2 on key target cells. In peripheral blood, only a subpopulation of Tregs expresses TNFR2 as a stable trimer with bound TNF for effective agonistic signaling and most lymphoid cell populations are negative for TNFR2.4 Antagonist antibodies preferentially kill human tumor cells expressing the TNFR2 oncogene, and they kill infiltrating TNFR2-expressing Tregs, which, in turn, lifts suppression of tumor-fighting T effectors (Teffs).4,10 Human TNFR2 antagonistic antibodies have little activity against normal circulating human T cells.4 Antagonistic antibodies cannot disrupt preformed trimers, but can bind to newly synthesized TNFR2 and stabilize it by forming an anti-parallel dimer that creates a nonsignaling complex.4,9 The antiparallel dimer may be a preferred structure for the TNF superfamily receptor antagonism; recent mechanistic and structural data find that the CD40 receptor can also be inhibited by antibodies that stabilize surface receptor dimers.16 The stabilization of TNFR2 as antiparallel dimers prior to trimerization with TNF may allow an open stabilized hexagonal complex to form on the cell surface for inhibited signaling (Graphical Abstract).4,9

Our previous experience with a TNFR2 antagonist similar to the one studied here found that (i) it was not dependent on Fc receptor binding or antibody-dependent cellular cytotoxicity (ADCC) mechanism, (ii) it maintained its antagonism in the presence of natural TNF, and (iii) it did not require cross-linking for activity.4,10 These features suggest that antibody regions other than the Fc region are crucial for functional impact, and they suggest lower toxicity when translated into human clinical trials.

Here we first examine differential TNFR2 antigen density on nearly 800 tumor cell lines followed by a study of the impact of density on TNFR2 antibody killing in dose–response experiments. Our additional focus using these refined cell-based killing assays is the testing and refinement of a new set of human-directed TNFR2 antagonistic antibodies to optimize activity. Using three cell-based TME assays (tumor cells, Treg cells, Teff cells), we test the antibody’s capacity to kill TNFR2-expressing tumor cells and Tregs, and spare Teffs, which can proliferate because of removal of Tregs. Teff expansion is crucial for immune system destruction of tumor cells. We test several IgG2 human chimeric isofoms of our antagonistic antibody for their potency and TME specificity. Finally, we introduce targeted mutations to the antibody antagonist’s amino acid structure to determine what factors optimize its TME specificity.

2 | MATERIALS AND METHODS

2.1 | Human samples

This study was conducted with full local institutional review board approval and involved written and informed consent of all human subjects. Human blood samples were collected from cutaneous T cell lymphoma (Sézary syndrome; SS) patients (n = 28 new samples from 10 subjects) and healthy controls (n = 11 subjects; 11 subjects) according to a human studies protocol approved by both the Massachusetts General Hospital (MGH) Human Studies Committee (MGH-2001P001379) and Stanford University Human Studies Committee (study# 2001P001379 IRB 5538 and IRB 13844). For additional human Treg and Teff experiments, blood samples were collected from 23 non cancer subjects according to the same MGH human studies protocol (MGH-2001P001379). All donors provided written informed consent. For SS lymphoma subjects, almost all were in stage IV disease and by the requirements of this study were not taking antiimitotic drugs; this drug class interferes with effectiveness of TNFR2 antagonism, which requires rapidly proliferating cells.4 Blood was collected into BD Vacutainer EDTA Tubes (BD Diagnostics, Franklin Lakes, NJ, USA) and processed within 24 h of phlebotomy.

2.2 | Isolation and culture of cells

Fresh human blood was processed within 24 h of venipuncture. Blood was washed twice with 1x HBSS (Invitrogen, Carlsbad, CA, USA) plus 2% FBS (Sigma-Aldrich, St. Louis, MO, USA), and CD4+ cells were isolated using Direct Human CD4+TCell Isolation Kit (Stem Cell Technologies, Vancouver, Canada). Isolated CD4+ cells were resuspended in Roswell Park Memorial Institute (RPMI) GlutaMAX (Life Technologies, Carlsbad, CA, USA) plus 10% FBS (Sigma-Aldrich Corporation, Natick, MA) and 1% penicillin-streptomycin (Life Technologies). Because isolated and cultured human T cells die without IL-2 in the media, all culture conditions in all experiments contained a low amount of IL-2 (200 U/ml) to prevent IL-2 withdrawal from influencing the data. CD4+ cell cultures were incubated at 37°C with 5% CO2. Colorectal adenocarcinoma cell line SW480 [SW-480] (ATCC CCL-228) was cultured in Leibovitz’s L-15 Medium (ATCC 30-2008) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies). SW480 cell cultures were incubated at 37°C with no CO2 exchange. Both the T cell leukemia cell line MOTN-1 (DSMZ ACC 559) and T cell lymphoma Jeko-1 (ATCC CRL-3006) were cultured in RPMI GlutaMAX (Life Technologies), 20% FBS (Sigma-Aldrich), and 1% penicillin-streptomycin (Life Technologies) plus additional IL-2 (100U/ml) for MOTN-1.

We tested 788 different human tumor cells lines, and cultured the cells according to the vendor’s instructions (ATCC Manassas, VA, USA and DSMZ, Braunschweig, Germany). From approximately 5 × 10^6 cells, we isolated RNA using an mRNA isolation kit (Sigma-Aldrich). After isolation the RNA was stored in the freezer. Quantitative PCR (qPCR) was applied using PCR technology (Bio-Rad, Hercules, CA, USA) and commercial primers to the human TNFR2 gene (TNFRSF1B). The known amplification primer was forward CGTTTCTCCAACACGACCTCATCC and the reverse primer was AGCGGCAACCTGCATCATGC. qPCR also used SYBR Green ER Super Mix (Invitrogen), 200 nM primers, and 200 ng cDNA followed the recommended testing cycling conditions. The Invitrogen Superscript IV kit and the ProtoScript kits were used for the cDNA synthesis with oligo(dT) (deoxythymidine) primers. PCR primers were designed as constructs
with the assistance of either GeneFisher or Primer3.16,17 Real-time quantitative PCR (qPCR) was performed using the Bio-Rad PCR machinery, and all reactions were performed according to the manufacturers’ instructions. For these quantitative experiments of relative mRNA expression between different cell lines, our endogenous control gene was 18S ribosomal RNA (Amplifluro Human 18S rRNA Primer Set, Sigma-Aldrich).

2.3 | Treg assays

For all Treg assays, freshly isolated CD4+ cells were seeded in 96-well round-bottom plates at a concentration of 0.2 × 10^5 to 1 × 10^6 cells per well, treated with TNFR2 antagonists and various reagents, and incubated for 48 to 72 h. After incubation, cells were collected and stained for FACS analysis.

2.4 | Direct cancer-killing assays

SW480 colon cancer cells were cultured in 96-well flat-bottom plates at a concentration of 1 × 10^5 cells per well in 200 µl of media. Cells were treated directly with TNFR2 antagonistic antibodies and incubated for various time points but optimally for 7 d. MOTN-1 cells were cultured in 96-well U-bottom plates at a concentration of 1 × 10^5 cells per well in 200 µl of media. Cells were treated directly with TNFR2 antagonistic antibodies and incubated for 72 h or longer but optimally for 72 h. JEKO-1 cells were also cultured in 96-well U-bottom plates at a concentration of 1 × 10^5 cells per well in 200 µl of media. After incubation, cells were detached from the plate and stained with trypan blue (Sigma-Aldrich) to count viable cells. The past, full dose–response curves were performed too see if killing increased with the dose or if killing reach a plateau followed by a decline, a trait of many antibodies dependent of Fc receptor binding or antibodies that agonize death receptors.18

2.5 | Reagents and flow cytometry

Murine monoclonal antibody (mAb) directed against human TNFR2 was produced, as previously described.4,10 In this paper, we moved Murine monoclonal antibody (mAb) directed against human TNFR2 2.5 at a concentration of 1 µl per well in 200 µl of media. SW480 colon cancer cells were cultured in 96-well flat-bottom plates at a concentration of 0.2 × 10^6 to 1 × 10^6 cells per well, treated with TNFR2 antagonists and various reagents, and incubated for 48 to 72 h. After incubation, cells were collected and stained for FACS analysis.

intracellular staining of FOXP3 and human CD25 (clone BC96; BioLegend) for cell surface staining of CD25. This is a standard kit and method for identifying the Treg population within isolated CD4 T cells. Treg populations, which were assessed by FACS with FL2 (red) versus FL1 (green); Tregs were defined as CD4+CD25+FoxP3-positive, whereas T eff populations were defined as CD4+CD25low and FoxP3-negative. Antibody MAB2261 (R&D Systems, Minneapolis, MN, USA) was used for measuring TNFR2 cell surface expression. All unlabeled antibodies at our lab are labeled with Allophycocyanin (APC) using the Lightning Link APC Conjugation Kit from Novus Biologicals (Littleton, CO, USA). FACS data were processed using FlowJo software (version 10.1).

2.6 | Construction of human chimeric variants

We designed several new human chimeric constructs to improve TNFR2 antibody efficacy. These constructs were based on the literature or from direct protein sequencing of the antibody from the Kabat database of antibody heavy and light chain amino acid sequences.19 The asterisk (*) is the continuation of the conserved region of the sequence. The cDNAs of the light and heavy chains of the antibodies were chemically synthesized with optimization for mammalian expression. The cDNA were cloned into expression vector pATX2. The heavy and light chains were expressed in 500 µl in cultures of 293F cells. Purification was done with affinity chromatography on protein G resin. The media was TBS with pH 7.5/8.0 buffer and elution with 0.1 M glycine at pH 3.0. Neutralization was with 1 M Tris-HCL, pH 8.5. All purified antibody variants were run on SDS-PAGE and all endotoxin was removed so final levels were <1EU/ml. Bacterial endotoxin levels were determined using the Endosafe-PTS system and Endosafe PTS cartridges (Charles River Laboratories, Willington, MA). For removal the Toxin Eraser Endotoxin Removal System was used (Genescript). The regions of the humanized variants for the chimeric antibodies are indicated below. All other antibody regions were of mouse origins.

IgG2 consensus

ASTKGSFPLAPCSRSTSESTAAAGLCLKDFPEPVTWVNSWNGALT SGVHTFPAVLQSGLYSLSVVTQPSNFGTQTYTNCVNDHKPS C232S, C233S

ASTKGSFPLAPCSRSTSESTAAAGLCLKDFPEPVTWVNSWNGALT SGVHTFPAVLQSGLYSLSVVTQPSNFGTQTYTNCVNDHKPS C232S

ASTKGSFPLAPCSRSTSESTAAAGLCLKDFPEPVTWVNSWNGALT SGVHTFPAVLQSGLYSLSVVTQPSNFGTQTYTNCVNDHKPS C233S

ASTKGSFPLAPCSRSTSESTAAAGLCLKDFPEPVTWVNSWNGALT SGVHTFPAVLQSGLYSLSVVTQPSNFGTQTYTNCVNDHKPS C127S

ASTKGSFPLAPCSRSTSESTAAAGLCLKDFPEPVTWVNSWNGALT SGVHTFPAVLQSGLYSLSVVTQPSNFGTQTYTNCVNDHKPS C127S

ntKVKDFTV7KMEQPCPPAPVAPVQPSFLLPFPKDTLKMLISRTPE EVTCVVDVHSDEPVQFNVWVDGEVHNAKTPREEQFN C232S, C233S
NTKVDKTVERKSSVECPCAPPVAGPSVFLFPKPKDTLIMISRTPE VTCVVDVSHEDPEVQFNWYVGDVEHVKAKKPKREEQFNC232S
NTKVDKTVERKSCVECPAPPVAGPSVFLFPKPKDTLIMISRTPE VTCVVDVSHEDPEVQFNWYVGDVEHVKAKKPKREEQFNC233S
NTKVDKTVERKCSVECPAPPVAGPSVFLFPKPKDTLIMISRTPE VTCVVDVSHEDPEVQFNWYVGDVEHVKAKKPKREEQFNC127S
NTKVDKTVERKCCVECPAPPVAGPSVFLFPKPKDTLIMISRTPE VTCVVDVSHEDPEVQFNWYVGDVEHVKAKKPKREEQFNC127S

The Kabat numbering scheme was used for all antibody sequences. The Kabat numbering system is the numbering of amino acid residues in antibodies based upon variable regions.

2.7 | Protein gel electrophoresis

The mAb protein samples were run on NuPAGE 3–8% Tris-Acetate gels with Tris-Glycine Native Running Buffer (Life Technologies) at 225V for 2.5 h. Gels were stained for 45 min with SimplyBlue SafeStain (Invitrogen) under nonreducing conditions.

2.8 | SCX fractionation with high-pressure liquid chromatography (HPLC)

SCX fractionation (strong cation-exchange chromatography) was performed on an Agilent 1260 model HPLC. Key components included Quat Pump model number: G1311B, Multiwavelength detector model number: G1365C, Fraction Collector model number G1330B. Data analysis was performed on Agilent Chemstation. The column used was a Proteomix SCXNP5 4.6 × 250 mm column from Sepax (catalogue number 401NP5-4625PK). Running conditions were as follows: flow rate: 1 mL/min; detector: UV 280 nm; and column temperature: 25°C. A total of 20 μL of the antibody was directly injected on to the column in each run. The sample was kept at 4°C in a temperature controlled autosampler. The mobile phase was composed of 2-morpholinoethanesulfonic acid (MES), MOPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 3-Cyclehexylamino 2-hydroxy-1-propanesulfonic acid (CAPSO), NaCl, and HPLC-grade water was used throughout. Mobile phase A was composed of 16 mM MES, 10 mM MOPS, 10 mM TAPS, and 10 mM CAPSO pH 4.5. Mobile phase B was composed of 10 mM MES, 12 mM MOPS, 14 mM TAPS, 16 mM CAPSO, and 30 mM NaCl pH. Mobile phase C was composed of 10 mM MES, 12 mM MOPS, 14 mM TAPS, 16 mM CAPSO, and 30 mM NaCl pH 10.2 + 1 M NaCl. Each run had a gradient of mobile phase A of 5% mobile and this was compared to phase B of 35% mobile during a 30 min period followed by 2 min of isocratic flow. A 4.5 min wash in mobile phase C was performed at the end of each gradient to clean the column. Fractions were collected and pooled together after all the fractions were collected. Purity of each fraction was verified by HPLC, run on the same column and same instrument. The recovery yield was calculated by comparing peak area between the crude and purified sample after normalizing for injection volume and concentration. Pooled fractions were stored at 4°C.

2.9 | Statistical analysis

Data analysis was performed by Student’s t-test using Excel (Microsoft) or SPSS (IBM). Significance was determined by P < 0.05.

3 | RESULTS AND DISCUSSION

3.1 | TNFR2 is differentially expressed on cells of the TME; TNFR2 oncogene expression on tumor cell lines is widespread: TNFR2 density on tumor cells determines rate of killing

Log mean fluorescent intensity (MFI) by FACS analysis of TNFR2 in lymphoid cell populations of humans with end-stage cutaneous T cell lymphoma (SS) demonstrates the following: (i) Treg cells (CD4+CD25hiFoxp3hi) from SS samples had a significantly higher TNFR2 MFI level than that of Treg cells from control lymphocytes (from normal blood donors) (P = 0.04); (ii) Teff cells (CD4+CD25−Foxp3−) from SS subjects had a TNFR2 MFI level similar to control, both of which were close to background level of fluorescence (dashed line); and (iii) the CD4+ lymphoma cells within the CD26− cellular subset had a higher TNFR2 MFI level than controls, that is, donors or noncancer cells from the same patient. MFI, was below background (P = 0.03) (Supporting Information Fig. S1). Similar findings were reported previously with a similar TNFR2 antibody antagonist, but with fewer samples. A survey of 788 commercially available human cancer cell lines by qPCR showed widespread, if variable, expression of the TNFR2 oncogene. The greatest expression was on hematopoietic and lymphoid cell lines, as well as lung and breast tumor lines (Fig. 1A). The SW480 colon cancer cell line has low TNFR2 oncogene expression, with an MFI of 44; TNFR2 antagonistic antibody killing of this tumor cell line was slow, taking 7 d (Fig. 1B). In contrast, MOTN-1 (leukemia cell line) and JEKO-1 (lymphoma cell line) had high TNFR2 density and rapid TNFR2 antagonistic killing—within only 48–72 h of incubation time. These data suggest that the antagonist worked most rapidly against tumor cell lines with the highest density of TNFR2. The total elimination of tumor cells with either TNFR2 antagonistic antibody at high doses or with extended culture was consistent with antibody killing not cell conversions or loss of phenotype.

3.2 | IgG2b TNFR2 antagonist shuffles between five isoforms

For years, the important antibody region for target specificity was the variable region, but here the focus is on antibody isotypes and isoforms. Antibody isotypes are known to have an impact on function. Antibody shuffling between isoforms is defined as the process of creating variants through covalent intrachain interactions in the hinge made naturally in vivo and influenced by manufacturing
FIGURE 1  Widespread aberrant TNFR2 expression on tumor cell lines; TNFR2 antagonist appears to kill faster-growing tumors more rapidly. (A) Analysis of TNFR2 mRNA expression in 788 human tumor cell lines shows widespread, if variable, expression across body and tissue sites of the cancer. Background expression of negative cells with control primers from normal tissue donors was set at 4. (B) Left panels show histogram of TNFR2 expression. Right panels indicate in vitro culture of the cell line with TNFR2 antagonistic antibodies. Colorectal adenocarcinoma cell line, SW480, for 24–48 h or for 7 d with various doses of the TNFR2 antagonist antibody (0 to 50 µg/ml) demonstrated killing by 7 d. Data are means ± SEM from \(n = 23\) independent experiments; all \(P < 0.05\) at 0.5, 2.5, 5, 12.5, 25, and 50 µg/ml; paired \(t\)-test. A significant linear downward trend in SW480 viability occurred with increasing dose, \(P < 0.0001\) with extended 7 d culture on this low expressing cell line (MRI = 42.2). In contrast, killing of the leukemia cell line MOTN-1 occurred rapidly (within 48–72 h) in a dose–response manner with TNFR2 antagonist antibody (0 to 50 µg/ml) (all \(P < 0.05\)), as did killing of the lymphoma cell line JEKO-1 (all \(P < 0.05\)). Both MOTN-1 and JEKO-1 have high TNFR2 surface density of the TNFR2 growth receptor (MFI = 246; MFI = 775). The pink histogram shows tumor cells; the blue background histogram shows isotype controls. The full TNFR2 antagonist antibody dosing scale is: 0, 0.05, 0.1, 0.5, 2.5, 5, 25, 50 but due to a limitation of space, not all doses are represented.

conditions.\(^{22–29}\) Our murine IgG2b anti-human TNFR2 antagonistic antibody, using standard production, showed on nonreducing gels, 4–5 IgG2 isoforms attributable to various types of hinge region disulfide bonds (Fig. 2A). Reducing conditions always generated on gels one heavy and one light chain band (Supporting Information Fig. S2), confirming that the nonreducing gel band variability was due to antibody hinge sulfhydryl bonds. Despite consisting of various hinge isoforms, the parent monoclonal displayed the desired antitumor functional activity in our three cell-based TME assays: Treg killing, Teff proliferation, and SW480 colon cancer cell killing (Fig. 2B). Using HPLC, the single mAb separated into 5 discrete bands, labeled F1–F5, for testing in functional assays (Fig. 2C).

3.3 Only one of the five IgG2 isoforms has desired antitumor activity on cell-based TME assays

Of the five visible IgG2b bands on HPLC, only fraction F1 had the desired functional activity compared to the other hinge isoforms of the
Native mouse IgG2b anti-human TNFR2 has desired TNFR2 antagonistic “shuffled” isoforms. (A) The parent mouse antibody, an IgG2b isoform, has the five bands expected on nonreducing gels. (B) When this mixture of IgG2b isoforms of this single monoclonal TNFR2 antagonistic antibody is tested in fresh peripheral blood, the mixture performs as desired: it kills T-regulatory cells (Tregs), induces proliferation of T effectors (Teffs), and kills SW480 tumor cells. For all three test parameters, the P-value was <0.05 using the mixed effects model. This experiment on fresh CD4 T cells is a representative sample but has been performed over 100× on normal blood donors. The p value is from a mixed effects model of antibody dose compared to baseline on 100 donor samples; the experiment on SW480 tumor cells was performed >10 times with identical results and the figure shown is pooled data from 10 separate experiments (C). Strong cation-exchange chromatography (SCX) fractionation was performed on an Agilent 1260 model HPLC to separate the various IgG2b isoforms for further functional testing on an individual basis. Using this method of charge separation, at least 5 antibody peaks could be physically separated for further studies.

same mAb (Figs. 2 and 3). For these screening assays, noncancerous blood samples were utilized due to lack of availability of fresh peripheral blood from SS donors on a daily basis, so the magnitude of the effect is not as great as that obtained with TME infiltrates. Peripheral blood from a liquid tumor, such as SS, constitutes the TME.

3.4 | Human chimeric IgG2 is functionally superior to the human chimeric IgG1 antibody variant

Having established on the murine IgG2b antibody framework that the function of the antagonistic antibody varies with isoform shuffling, we tested the functional impact of chimeric antibody structure (Fig. 4). We created two chimeric antibodies by replacing the mouse IgG2b heavy chain constant region with that of either human IgG1 or IgG2. The human IgG1 native framework hinge yielded, as expected, one band on nonreducing gel; the human IgG2 native framework yielded, as expected, two bands on nonreducing gels (Fig. 4C, right panels). The human chimeric IgG1 antibody had no or almost undetectable functional activity in relation to Treg killing and Teff proliferation (Fig. 4B). It had minimal activity against SW480 direct tumor cell killing. The human chimeric IgG2 antibody showed a paradoxical inverted V-shaped Treg killing curve with a downward V-shaped Teff proliferation...
HPLC separations of the mouse anti-human IgG2b antibody shows that only one isoform fraction has the desired antitumor activity. HPLC separation of the mouse IgG2b antibody into the various isoforms revealed 5 physical protein peaks (Fig. 2) that were tested separately in functional assays in this experiment, HPLC fractions F1-F5. Each fraction was tested in functional assays of the rate of T-regulatory (Treg) killing (left panels), T effectors (Teff) expansion (middle panels), and killing of the SW480 tumor cell line (right panel). Data involve the culture of fresh peripheral human CD4+ cells in response to the TNFR2 antagonist (0–50 µg/ml) for 48–72 h. The functional assay was the killing of SW480 colorectal adenocarcinoma cultured for 7 d with the TNFR2 antagonist (0–50 µg/ml). The percentage Tregs or Teff is in comparison to total CD4 T cells. Data are mean ± SEM. Statistical analysis by 1-way ANOVA, Tukey’s multiple comparison test, and for the slope compared to baseline the mixed effects model **P < 0.01. All fractions were tested on fresh CD4 cells for the Tregs and Teff or on SW480 tumor cells at least 5 times; a representative experiment is shown for the Tregs and Teff experiments. For the SW480 cells, the error bars represent 8 separate experiments. Data are mean ± SEM.

As an additional experiment to verify that the 4–5 bands of the murine IgG2b were due to the murine hinge region shuffling, the murine hinge of the native IgG2b antibody was placed onto the human chimeric IgG2 construct (Supporting Information Fig. S3). As expected, the human chimeric IgG2 with the murine hinge once again on nonreducing gels had 4–5 bands. The functional consequences were mixed. This isoform construction, like the parent human IgG2, had an inverted V pattern of killing on Tregs and a V curve on Teffs, with modest SW480 tumor cell killing. This suggested that antibody hinge drives antitumor function.

3.5 | A double IgG2 mutation stabilizes the antibody hinge, enlarges the arms, and has desired functional activity

For the IgG2 isotype, known mutations in the hinge region of cysteine (C) to serine (S) prevent shuffling, and past reports confirm that these stabilized mutants have distinct functions.22–29 The hinge forms are shown in Supporting Information Figure S4. We mutated the human IgG2 chimeric antibody into three variants to test their functional impact in the TME assays: double mutant C232S C233S, single mutant C232S, and single mutant C233S. Figure 5 shows that the double mutant was the only one to successfully reproduce the function of the parent murine IgG2a antibody (Treg killing, Teff proliferation, and SW480 tumor killing in a dose-dependent manner). The data also shows the single hinge mutants were only partially successful. The enlarged arm width of the double mutant is likely necessary to bind to the wide size of TNFR2 on rapidly growing tumor cells. The nonreducing gel confirmed the presence of the desired single IgG2 antibody isoform (Fig. 5, left).

The importance of the IgG2 hinge on function has been recently confirmed by a study of the anti-CD40 antibody agonist for cancer.
FIGURE 4  Native IgG2 shows superior performance than IgG1 TNFR2 antagonist. Chimeric anti-human TNFR2 antibodies were constructed using a human IgG1 heavy chain (A) Antibody activity assays with their indicated desired properties for tumor microenvironment specificity. (B) Human chimeric IgG1 showed a single band on a nonreducing gel, but no activity in the T-regulatory (Treg) or T effector (Teff) assay and minimal activity in the SW480 tumor cell killing assay. (C) Human chimeric IgG2 showed two bands on a nonreducing gel with bi-modal Treg, Teff activity but excellent SW480 tumor killing. All fractions were tested on fresh CD4 cells for the Tregs and Teff or on SW480 tumor cells. A representative experiment is shown for the Tregs and Teff experiments but the experiment was performed on >5 normal donors. The percentage Tregs or Teff is in comparison to total CD4 T cells. For the SW480 cells, the error bars represent 8 separate experiments. Data are mean ± SEM. Statistical analysis by 1-way ANOVA and Tukey’s multiple comparison test, **P < 0.01.

FIGURE 5  Mutated hinge variants of IgG2 produce single and stable isoforms showing different functional activity in cell-based tumor microenvironment (TME) assays. Chimeric human IgG2 antibodies were constructed with hinge mutations at C232S and C233S (A), C232C only (B), and C233S only (C). (A) Human IgG2 isoform with the hinge stabilization mutants C232S, C233S showed a single band on a nonreducing gel and restored T-regulatory (Treg) killing, T effector (Teff) proliferation and SW480 tumor cell killing. (B-C) Human IgG2 isoform with the hinge stabilization mutant C232S or C233S showed a single band on a nonreducing gel, but no Treg killing, good Teff activity, and sluggish SW480 tumor killing. A representative experiment is shown for the Tregs and Teff experiments, performed >5 times on different normal blood donors. For the SW480 cells, the error bars represent 8 separate experiments. Data are mean ± SEM. Statistical analysis by 1-way ANOVA and Tukey’s multiple comparison test, **P < 0.01.
Another point mutation was made in the amino acid sequence at residue C127S to bring the arms of the antibody closer together. We hypothesized that this would convert the antibody from an antagonist into an agonist as past data has shown. This hypothesis did not hold true, as there was some weak killing of the T-regulatory (Treg) cells, slight proliferation of the T effector (Teff) cells, but no distinct pattern for the SW480 cells. A representative experiment is shown for the Tregs and Teff experiments; overall the experiment was performed 5 times on different normal blood donors. For the SW480 cells, the error bars represent 7 separate experiments. Data are mean ± SEM. Statistical analysis by 1-way ANOVA and Tukey’s multiple comparison test, **P < 0.01.

immunotherapy.20,22,24 The structurally rigid IgG2 isoforms with the C127S mutation optimized agonism. The CD40 receptor is expressed on the surface of APCs greatly increases their antigen presentation and costimulatory capacity, resulting in a more effective activation of cytotoxic T cells if this receptor was agonized. The natural ligand for the CD40 receptor is CD154; this interaction also facilitates effector T cell dependent B cell activation.30

3.6 Another mutation that brings the IgG2 antibody arms closer together reduces its desired functional activity

Unmodified IgG2 isotype, compared to other isotypes, has a considerably broader arm span.27,28,30,31 It is known that if the heavy chain C127S mutation is introduced, it will stabilize the human IgG2 hinge, prevent disulfide shuffling, and tighten the arm distance. This mutation (C127S) succeeded in preventing the shuffling in the hinge, according to the single band on nonreducing gel (Fig. 6, right). Compared to the parent IgG2b monoclonal, its Treg killing, Teff proliferation, and SW480 tumor killing was modest to nonexistent (Fig. 6). This mutation, based on previous reports, converts antibodies into agonists. We did not observe this, but we did observe poor recovery of the desired antagonistic response.

4 CONCLUSION

This study took advantage of the differential expression of TNFR2 on lymphoid cells of the TME to develop a novel, human-directed TNFR2 antagonist with potential as a targeted cancer immunotherapy. The IgG2 isotype of the antibody antagonist functioned best against target cells in the TME. It is known to have hinge variants that confer widely spaced and symmetrical arms for antigen binding. Stabilization of those open IgG2 structures with hinge disulfide double mutations at C232S and C233S optimized the antibody’s antagonism. It has been proposed by us that the antibody locks in the nonsignaling antiparallel dimer state to inhibit NF-kB signaling, leading to cell death. This finding, combined with the knowledge that this form of antagonism is independent of the Fc region of the antibody, should encourage development of therapeutic TNFR2 antagonistic antibodies with greater TME specificity and improved safety.

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AUTHORSHIP

D.L.F. conceived the experiments. M.Y. and H. Takahashi performed the tumor cell line experiments; M.Y. and K.C. organized the Sepax experiments, prepared antibody, and performed gels; L.T., H. Torrey, and Y.S. performed the fresh lymphocyte experiments; H.P., M.Y., and K.C. recruited subjects for the fresh blood studies; W.M.K. analyzed the results; M.Y. and K.C. prepared figures with subsequent writing and figure preparation undertaken by D.L.F.; and H.Z. performed the statistics on the represented data.
DISCLOSURES

None of the authors consult nor have board seats on the commercialization of this technology. All technology is owned by the Massachusetts General Hospital.

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REFERENCES


SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.