CD137/OX40 Bispecific Antibody Induces Potent Antitumor Activity that Is Dependent on Target Coengagement

Miguel Gaspar, John Pravin, Leonor Rodrigues, Sandra Uhlenbroich, Katy L. Everett, Francisca Wollerton, Michelle Morrow, Mihriban Tuna, and Neil Brewis

ABSTRACT

Following the success of immune checkpoint blockade therapy against cancer, agonistic antibodies targeting T-cell costimulatory pathways are in clinical trials. The TNF superfamily of receptors (TNFRSF) members CD137 and OX40 are costimulatory receptors that stimulate T-cell proliferation and activation upon interaction with their cognate ligands. Activating CD137 and OX40 with agonistic mAbs stimulates the immune system due to their broad expression on CD4+ and CD8+ T cells and natural killer cells and has antitumor effects in preclinical models. Most TNFRSF agonist antibodies require crosslinking via FcγRs and, therefore, may not induce T-cell activation and proliferation in vivo. CD137/OX40 bispecific antibody targeting CD137 and OX40, activated both CD4+ and CD8+ T cells in an FcγR-independent mechanism, dependent on concurrent binding. A mouse surrogate version of the bispecific antibody displayed antitumor activity in syngeneic tumor models, independent of T regulatory cell depletion and FcγR interaction, but associated with peripheral T-cell activation and proliferation. These data support initiation of clinical development of FS120, a first-in-class dual agonist bispecific antibody for the treatment of human cancer.

Introduction

OX40 and CD137 costimulatory receptors belong to the TNF receptor superfamily (TNFRSF; ref. 1). Both are expressed on activated T cells and natural killer cells, and are attractive targets for cancer immunotherapy as stimulation of these receptors results in increased T-cell activation, proliferation, and survival in vitro and in vivo (1). The expression patterns of OX40 and CD137 are overlapping, but distinct with expression of OX40 higher on CD4+ T cells and that of CD137 higher on CD8+ T cells (2). CD137 stimulation preferentially stimulates CD8+ T cells when compared with CD4+ T cells and CD40 stimulation preferentially stimulates CD4+ T cells when compared with CD8+ T cells (3). However, coexpression of these receptors is demonstrated in both CD4+ and CD8+ T cells and both are expressed in tumor-infiltrating lymphocytes (TIL; refs. 4, 5). Antibodies stimulating these targets show activity in a variety of murine tumor models by both depleting regulatory T cells (Treg) and activating CD8+ and CD4+ T cells (6, 7). The combination of OX40 and CD137 agonist antibodies stimulates both CD4+ and CD8+ T cells and induce the cytotoxic function of both antigen-experienced and antigen-inexperienced bystander CD4+ T cells (8, 9).

Several clinical trials are underway to test agonist antibodies to OX40 or CD137 either as monotherapies or in combination with other agents to treat various cancers (10). Clinical trials with OX40 agonist antibodies demonstrate peripheral T-cell activation and proliferation without associated toxicity (11) but show limited clinical efficacy (12). Two CD137 agonist antibodies have different clinical outcomes. Urelumab (BMS-663513, clone 20H4.9) induces severe transaminitis at doses higher than 1 mg/kg resulting in two hepatotoxicity-related deaths (13) and utomilumab (PF-05082566, clone MOR7480.1), which does not induce severe adverse events, has modest clinical activity (14). A combination trial with utomilumab and PF-04518600 (an OX40 agonist antibody) is underway (NCT02315066).

TNFRSF antibodies typically have no or low intrinsic agonist activity and require secondary crosslinking of antibody-receptor complexes to induce sufficient receptor clustering and activation, thereby mimicking the TNFRSF ligand superclusters (15). In vivo, this secondary crosslinking requires the interaction with Fcγ receptors (FcγR; ref. 16). The availability of FcγR-expressing cells in the tumor microenvironment and the low affinity interaction between FcγRs and the Fc-region of IgG antibodies may limit the agonist activity of TNFRSF antibodies and, consequently, their antitumor activity (17). In addition, interaction with FcγRs mediates antibody effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) and could lead to the depletion of the tumor-specific T cells that would be activated by these antibodies (18). Consequently, the clinical activity seen with OX40 and CD137 antibodies may not represent the full potential of activating these receptors.

An alternative to FcγR-mediated crosslinking of TNFRSF agonist antibodies is the use of bispecific antibodies, the dual binding of which results in the clustering of the TNFRSF target, independent of FcγR engagement (19). This study described FS120 mAb2, a dual agonist bispecific antibody targeting CD137 and OX40 that activated both CD4+ and CD8+ T cells, whereas OX40 or CD137 monospecific antibodies only activated CD4+ or CD8+ T cells, respectively. FcγR-disabling mutations (LALA mutations; ref. 20) were introduced to enable antibody crosslinking from the coengagement of the two different receptors when coexpressed and to potentially avoid depletion of OX40- or CD137-expressing cells. A mouse-specific surrogate version of FS120 showed antitumor activity in the absence of FcγR engagement.
interaction or after Treg depletion. When compared with a cross-linkin
de-CD137 agonist mAb, FS120 surrogate showed reduced liver T-cell infiltration, which decreased over time.

These results indicate that targeting coexpressed receptors with bisppecific antibodies may be a potent and safe mechanism to cluster and activate TNFRSF costimulatory receptors and induce antitumor immunity.

Materials and Methods
Antibody reagents
Antibodies were cloned by replacing the V\textsubscript{H} or V\textsubscript{L} domain sequences in human IgG1 with identified sequences from patents or literature using methods described previously (21). The LALA (L334A-L235A, Eu numbering; ref. 22) mutations are denoted by the suf
ded sequences from patents or
mation. Cell lines were selected by incubation with 5 \mu M puromycin (BioLegend, 100202 clone 17A2 at 0.1 ppL/minute). The following cell lines were tested by coating anti-mouse CD3 antibody (BioLegend, 895285). Human and mouse OX40 and CD137 cDNA constructs were synthesized (GenScript) with flanking 5’ EcoRI and 3’ Not I restriction sites and cloned into the lentivirus vector pLVX-EF1-IRE-PRO (Clontech 631988). Lentiviruses were produced using the LentiX Expression system (Clontech 631253) and used to transduce DO11.10 cells according to the manufacturer’s instructions. Cell lines were selected by incubation with 5 \mu M puromycin (InvivoGen, anti-pr-1) and individual cell lines were cloned by serial dilution. DO11.10 cells expressing human or mouse CD137 were stimulated with coated anti-mouse CD3 antibody (BioLegend, 100202 clone 17A2 at 0.1 ppL/minute) and mouse IL-2 concentration in the supernatants was measured by ELISA (Thermo Fisher Scientific, 88-7024-88).

Flow cytometry
For cell binding assays, cells were incubated with primary antibodies or mAb\textsuperscript{b} followed by detection with an anti-human Fc-488 secondary antibody (Jackson ImmunoResearch). Excised tissues were dissociated using relevant Miltenyi Biotec Dissociation Kits (tumors, 130-096-730; livers, 130-105-807; and spleens, 130-095-926) using a Miltenyi gen
tleMACS Octo Dissociator and C-tubes according to the manufactu
er's instructions. Resulting cell suspensions were strained (70 \mu m cell strainer (Corning CLS41751)), washed, and resuspended in PBS. Collected blood samples were treated twice with Red Blood Cell Lysis Buffer (eBioscience 00-430054) according to the manufacturer’s instruction. Cells isolated from tissues and blood were stained for flow cytometry with fluorochrome-conjugated antibodies including those against CD4, Ki67, FoxP3, CD69, CD3, and CD8 (Thermo Fisher Scientific), CD45 (BD Biosciences), and fixable live/dead dye (Thermo Fisher Scientific) in the presence of Fc Block (Thermo Fisher Scientific) according to the manufacturer’s recommendations. Cells were

Surface plasmon resonance analysis
Data were acquired using a BIACore 3000 or BIACore T200. Dilution mixtures prepared in HBS-P or HBS-EP Buffer (GE Healthcare). For K\textsubscript{D} determination, FS120 was captured either via the Fc region using a Human Antibody Capture Kit (GE Healthcare) and antibodies (100 nmol/L) were coinjected with either human OX40 or CD137 (100 nmol/L) or HBS-EP buffer. Affinity for FcyRs (R&D Systems, hFcyR1i (1257-CF-050), hFcyR2a (1330-CF-050), hFcyR2b (1875-CF-050), and hFcyR3a (4325-CF-050) was tested by coating biotinylated human OX40 (BPS Bioscience 71310-1) or CD137 (in-house) his-tagged antigens onto an Streptavidin Chip (GE Healthcare) and coinjecting antibodies (100 nmol/L) and human FcyRs (500 nmol/L) at 20 \mu L/minute flow rate and the dissociation was followed for 5 minutes. For specificity assessment, human TNFRSF members [R&D Systems, TNFR1 (372-RI-050/CF), TNFRII (726-R2-050), GTR1 (689-GR-100), NGFR1 (367-NR-050/CF), CD44 (1493-CD-050), and DR6 (144-DR-100)] were immobilized on CM5 Chips (GE Healthcare) to approximately 1,000 RU and FS120 (1 \mu M), isotype control (anti-FITC (Ctrl(4420) WT mAb; clone 4420 in human IgG1 backbone; ref. 23) or positive control antibodies [R&D Systems, anti-TNFR1 (MAB225R-100); anti-TNFRII (MAB726-100); anti-GTR1 (MAB689-100); anti-NGFR1 (MAB367); anti-CD40 (MAB6321-100); and anti-DR6 (AF144)] were flowed over at a flow rate of 30 \mu L/minutes. Data were analyzed using BIA Evaluation Software (GE Healthcare).

Cell line creation and reporter T-cell assay
All cells used in the experiments described in this article were kept in culture for a maximum of 2 months before starting new cultures from master vials. CT26 (CRL-2638) and B16-F10 (CRL-6475) cell lines were purchased from ATCC in 2015. DO11.10 cells were purchased from Public Health England (8508201) in 2014 and used under license from National Jewish Health. Expi293F cells were purchased from Thermo Fisher Scientific (A14528) in 2015. No reauthentication tests were performed. Mycoplasma testing was performed on all cell lines monthly using R&D MycoProbe Mycoplasma Detection Kit (R&D Systems, 895285). Human and mouse OX40 and CD137 cDNA constructs were synthesized (GenScript) with flanking 5’ EcoRI and 3’ Not I restriction sites and cloned into the lentivirus vector pLVX-EF1-IRE-puro (Clontech 631988). Lentiviruses were produced using the LentivX Expression system (Clontech 631253) and used to transduce DO11.10 cells according to the manufacturer’s instructions. Cell lines were selected by incubation with 5 \mu g/mL Puromycin (InvivoGen, anti-pr-1) and individual cell lines were cloned by serial dilution. DO11.10 cells expressing human or mouse CD137 were stimulated with coated anti-mouse CD3 antibody (BioLegend, 100202 clone 17A2 at 0.1 \mu g/mL) and mouse IL-2 concentration in the supernatants was measured by ELISA (Thermo Fisher Scientific, 88-7024-88).
Peripheral blood mononuclear cells and T-cell stimulation assays

Ficoll-purified human peripheral blood mononuclear cells (PBMC) were stimulated with 100 ng/mL staphylococcal enterotoxin A (SEA, Sigma) in the presence of FS120 or control antibodies for 5 days at 37°C, 5% CO₂ in T-cell media [RPMI1640 (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific), 1% penicillin–streptomycin (Thermo Fisher Scientific), 1 mmol/L Sodium Pyruvate (Gibco), 10 mmol/L Hepes (Gibco), 2 mmol/L l-Glutamine (Gibco), and 50/μmol/L 2-mercaptoethanol (Gibco)]. T cells were isolated from PBMCs using Miltenyi Enrichment Kits (Human CD3+, 109-096-533; Human CD4+, 130-096-533; Human CD8+, 130-096-495; and Mouse CD3+, 130-095-130) according to the manufacturer's instructions and activated overnight using CD3/CD28 Dynabeads (Life Technologies catalog numbers: Human, 11131D and Mouse, 11453D) at a 1:1 cell to bead ratio. CD3/CD28 beads were removed using a DynaMag-15 magnet and activated T cells were washed with T-cell media and stimulated with coated CD3 antibody [Human: R&D Systems (MAB100) clone UHCT1 at 2.5 μg/mL (total and CD4+ T cells) or 5 μg/mL (CD8+ T cells) or Mouse: BioLegend (815) according to the manufacturer's instructions]. OX40 and CD137 receptors quantified using Miltenyi Enrichment Kits (Human OX40, 130-096-495; and Mouse OX40, 130-095-130), and anti-mouse IgG (Bangs Laboratories 815) according to the manufacturer's recommendations.

Results

FS120 simultaneously bound to CD137 and OX40

Phage and yeast libraries were used with directed evolution methods described previously (21, 26) to identify and improve OX40-binding Fc antigen binders, termed Fcabs (Fc-region with antigen binding), as well as CD137-binding Fab regions. The Fcab (OX40 Fcab, clone FS20) and Fab (CD137 Fab, clone FS30) with the overall highest activity in T-cell stimulation assays and affinity in cell binding assays were combined to generate the bispecific FS120 mAb² (or FS120, Fig. 1A). Affinity determination by surface plasmon resonance (SPR) showed that FS120 has subnanomolar binding to both human and cynomolgus monkey OX40 (Kᵢₐ: Human, 0.2 nmol/L and Cyno, 0.9 nmol/L) and CD137 (Kᵢₐ: Human, 0.2 nmol/L and Cyno, 0.2 nmol/L; Fig. 1B) and FS120 bound both OX40 and CD137 simultaneously (Fig. 1C). FS120 did not bind to other related members of the TNFR superfAMILY (CD40, GITR, NGRF, DR6, TNFRI, and TNFRII, Supplementary Fig. S1). FS120, which contains the LALA mutations, had reduced FcγR binding as compared with WT IgG1 (non-LALA containing) OX40 and CD137 antibodies (Supplementary Fig. S2). FS120 bound to cell surface expressed human and cynomolgus OX40 and CD137 receptors on engineered DO11.10 T cell lines but not to the non-transduced parental DO11.10 cell line (Fig. 1D).
FS120 agonistic activity was dependent on the dual binding of CD137 and OX40

To test the agonist activity of FS120, PBMCs were stimulated with SEA superantigen, which crosslinks MHC class II molecules at the surface of antigen-presenting cells and the T-cell receptor (TCR) of T cells (27), in the presence or absence of secondary crosslinking agents to mimic the effect of FcR-mediated crosslinking. The amount of T-cell activation resulting from OX40 or CD137 stimulation was then measured by the production of IL2. All antibodies were tested in the same isotype background as FS120, human IgG1 with the LALA mutations, to minimize interference from FcR-mediated crosslinking.

Agonistic activity of OX40 or CD137 monospecific antibodies was only observed in the presence of crosslinking (Fig. 2A). In contrast, FS120 mAb showed activity in the absence of secondary crosslinking agent suggesting the coengagement of OX40 and CD137 resulted in effective receptor clustering and activation (Fig. 2A). Similar results were observed when isolated T cells were stimulated with plate-bound CD3 antibody and costimulated with OX40- or CD137-specific antibodies or FS120 mAb (Fig. 2B). The activity of FS120 was not increased by the secondary crosslinking agent, either in maximum response or in a decrease in EC50 (Fig. 2C and D), indicating that the dual binding to OX40 and CD137 resulted in the maximum stimulation induced by FS120.

When CD137 agonist antibodies were crosslinked, the 20H4.9 Fab clone (Fab present in urelumab) was observed to have a higher activity as compared with clones MOR7480.1 (Fab present in utomilumab) or FS30 (Fab present in FS120; Fig. 2A and B). The crosslinked OX40-targeting antibodies induced higher IL2 production than the crosslinked CD137-targeting antibodies, and the combination of the OX40 Fcab and the CD137 Fab components of FS120 did not show a synergistic effect as compared with the OX40 Fcab alone (Fig. 2A).
and B). This result indicated that these assays were more sensitive to OX40 stimulation and that only potent CD137 stimulation resulted in substantial T-cell activation. The higher response to OX40 agonism could have been explained by the higher proportion of CD4\(^+\) T cells in human PBMCs and the higher expression of OX40 on activated CD4\(^+\) T cells (Supplementary Fig. S3).

Titrations of these antibodies in both the PBMC SEA stimulation and T-cell CD3 stimulation assays were performed and the concentration at which these antibodies induced the highest IL2 production was chosen for this analysis. FS120, and crosslinked OX40 Fcab, induced the production of additional proinflammatory cytokines (IL6, IL12p70, IL13, and TNFa) by T cells and reduced the levels of IL10, a typical anti-inflammatory cytokine (Supplementary Fig. S4).

To test whether the activity of FS120 required simultaneous binding to the two receptors, the ability of FS120 to coengage OX40 and CD137 was blocked using 100-fold molar excess of either the OX40 Fcab or the CD137 Fab components of FS120 or both. The results showed that the FS120-induced T-cell activation was reduced when the mAb2 component parts were present either individually or in combination (Fig. 2E), indicating that FS120 required dual binding to OX40 and CD137 to induce the clustering and activation of these receptors. FS120 did not induce T-cell activation in a cytokine release assay (28) in the absence of TCR stimulation, unlike the two positive

**Figure 2.**

FS120 stimulated T cells in the absence of anti-Fc crosslinking. A and B, Human PBMCs stimulated with SEA (100 ng/mL; A) or human T cells stimulated with coated CD3-antibody (clone UCHT-1 at 2.5 \mu g/mL; B) and costimulated with FS120 or control antibodies (3.7 nmol/L) in the presence or absence of crosslinking reagents at 1:1 molar ratio [FITC dextran for OX40/Ctrl(4420) mAb2 and anti-Fc antibody (clone MK1A6 in mouse IgG1 format) for other antibodies]. C and D, FS120 titration in the presence or absence of anti-Fc crosslinking in SEA-stimulated PBMCs (C) or CD3-stimulated T cells (D). E, CD3-stimulated T cells costimulated with FS120 (1 nmol/L) and isotype control antibody or component parts of FS120 and their combination (100 nmol/L). A–E, Data from duplicates is shown as mean ± SD (representative results of three independent experiments). Statistical testing by two-way ANOVA and Tukey multiple comparison test (A and B) or one-way ANOVA and Dunnett multiple comparisons test (E). Asterisks on top of error bars represent the significant difference to Ctrl(4420) mAb–treated samples (*, \(P < 0.05\); **, \(P < 0.002\); ***, \(P < 0.0002\); ****, \(P < 0.00001\)). For EC\(_{50}\) determinations, antibody concentration data were log transformed before analysis and fit using the log agonist versus response using Prism Software (GraphPad; C and D). See also Supplementary Figs. S2 and S3.
control antibodies used, a CD28 antibody shown to induce cytokine storm in the clinic (TGN1412; ref. 29) and a CD3 antibody (Supplementary Fig. S5).

**FS120** stimulated both CD4\(^+\) and CD8\(^+\) T cells

OX40 and CD137 receptors were coexpressed on CD4\(^+\) and CD8\(^+\) T cells, with OX40 expressed in higher percentages and at a higher receptor number in CD4\(^+\) T cells than CD8\(^+\) T cells and, conversely, more CD137 receptors were expressed on CD8\(^+\) T cells (Supplementary Fig. S1). The differential expressions correlated with the activity of OX40- or CD137-targeting antibodies. On CD4\(^+\) T cells stimulated with plate-coated CD3 antibody, both the crosslinked OX40 mAb and the OX40 Fcab induced IL2 production, but CD137 antibodies did not show activity (Fig. 3A and B). On CD8\(^+\) T cells, the crosslinked CD137 antibodies (Fab clones 20H4.9 and FS30) induced IL2 production and OX40 antibodies did not (Fig. 3A and B). When tested in the absence of secondary crosslinking agent, FS120 increased IL2 production on both CD4\(^+\) and CD8\(^+\) T cells (Fig. 3A). The crosslinked activity of clone FS30 [CD137(FS30) mAb] demonstrated that the mAb\(^2\) could activate the CD137 receptor when crosslinked by binding of the Fcab to OX40 on CD8\(^+\) T cells. The activity of the crosslinked OX40 Fcab [OX40/Ctrl(4420) mAb\(^2\)] showed that the mAb\(^2\) could activate the OX40 receptor when crosslinked by binding of the Fab arms to CD137 on CD4\(^+\) T cells.

The different CD137 antibodies tested showed varying activity on CD8\(^+\) T cells. Fab clone MOR7480.1 did not show activity in the absence of crosslinking and only a moderate, nonsignificant, increase when crosslinked (Fig. 3A). Clone FS30 displayed higher activity when crosslinked, but no activity in the absence of crosslinking (Fig. 3A). However, Fab clone 20H4.9 induced higher levels of IL2 production in the absence of crosslinking, which was increased with crosslinking (Fig. 3A). When these antibodies were tested in a model DO11.10 T cell line expressing human CD137 and stimulated with coated anti-mouse CD3 antibody, Fab clone 20H4.9 also showed crosslink-independent activity, whereas Fab clones FS30 and MOR7480.1 did not (Supplementary Fig. S6A).

**FS120 surrogate bound mouse OX40 and CD137 and activates T cells**

As the FS120 mAb\(^2\) does not bind to mouse OX40 or CD137, a surrogate molecule was generated for in vivo testing by pairing a mouse-specific OX40 Fcab with a CD137 mAb (clone Lob12.3). The Fc-modifying technology used to create Fcabs is based on human IgG1 therefore the FS120 surrogate has a human IgG1 domain. All in vivo experiments were performed using molecules with the same human IgG1 backbone. The FS120 surrogate bound to cell surface expressed mouse OX40 and CD137 receptors on engineered DO11.10 T cell lines (Supplementary Fig. S7A) and...
concurrent binding to mouse OX40 and CD137 was demonstrated by SPR (Supplementary Fig. S7B).

FS120 surrogate was functionally characterized by testing its ability to costimulate primary mouse T cells in the presence of coated anti-mouse OX4 and FS120 surrogate induced the production of IL-2 in the absence of secondary crosslinking agent, unlike the OX4 monospecific antibody, which required anti-Fe antibody for crosslinking (Supplementary Fig. S8A). No activity of the CD137 antibody was detected in this assay, but in DO11.10 T cell line expressing mouse CD137 and stimulated with coated anti-mouse CD3 antibody, clone Lob12.3 increased IL2 production in the presence of crosslinking (Supplementary Fig. S6B). A different anti-mouse CD137 (clone 3H3) was also tested in this assay and showed crosslink-independent activity, similar to that seen with Fab clone 20H4.9 (Fig. 3A; Supplementary Fig. S6A and S6B). This result indicated that these two molecules could be surrogates for each other, whereas clone Lob12.3 only showed activity when crosslinked and was therefore a surrogate for Fab clones MOR7480.1 and FS30 mAb.

The activity of FS120 surrogate in the absence of secondary crosslinking agent was also dependent on dual binding to OX40 and CD137; when either of these receptors was blocked with excess mouse OX40 Fab or mouse CD137 mAb (clone Lob12.3), the activity of FS120 surrogate was reduced, similar to that observed with FS120 in human T cells (Supplementary Fig. S8B).

**Antitumor activity of the FS120 surrogate**

OX40 and CD137 antibodies demonstrate antitumor activity in a variety of syngeneic models with responses depending on dose, time of treatment initiation, antibody isotype, and clones used (3, 30–32). Intratumoral Treg depletion is part of the mechanism of antitumor activity of OX40 (clone OX86; ref. 6) and CD137 antibodies (clone Lob12.0; ref. 7).

To test the antitumor activity of FS120 and to understand the in vivo mechanism of action, FS120 surrogate or control antibodies were injected intraperitoneally at 1 mg/kg on days 11, 13, and 15 post-CT26 tumor cells inoculation (Fig. 4A). The OX40 (clone OX86) or CD137 (clone Lob12.3) control antibodies with human IgG1 isotype showed no antitumor activity (Fig. 4B), unlike previously published results using the original rat versions of these antibodies (3). This could be explained by the later start time of antibody treatment, the lower dose, or the human IgG1 isotype used. FS120 surrogate showed significant antitumor activity as compared with the isotype control antibody and the antitumor activity observed for FS120 surrogate was similar to a WT IgG1 surrogate (WT mAb2); Fig. 4B). This indicated the mechanism of antitumor activity of FS120 surrogate was independent of FcR interaction, either FcR-mediated crosslinking or FcR-mediated effector functions such as ADCC or ADCP. FS120 surrogate–treated mice with no tumors at day 60 were rechallenged with CT26 tumors and did not show tumor growth (Supplementary Fig. S9).

When intratumoral Tregs were analyzed, CT26 tumors treated with CD137 antibody or the combination of OX40 and CD137 antibodies had fewer Tregs compared with the isotype control–treated tumors (Fig. 4C). CT26 tumors treated with the FS120 surrogate (WT mAb2) also had fewer intratumoral Tregs, similar to the percentages observed with the combination of OX40 and CD137 antibodies (Fig. 4C). However, due to the presence of the LALA mutations, the FS120 surrogate–treated tumors did not have fewer intratumoral Tregs compared with the isotype control–treated tumors (Fig. 4C). None of the treatments induced any significant changes in the frequency of CD4+ or CD8+ T cells or T-cell proliferation (Fig. 4C). The antitumor activity of the FS120 surrogate was therefore not associated with intratumoral Treg depletion.

When the FS120 surrogate was tested in the B16-F10 model, a poorly immunogenic model and thus harder to treat with immunotherapies (33), FS120 surrogate also had antitumor activity as compared with an isotype control antibody (Supplementary Fig. S10).

**Peripheral T-cell activation and proliferation mediated by FS120 surrogate**

The induction of T-cell proliferation by OX40 and CD137 agonist antibodies in vitro and in vivo is described in both preclinical models and in the clinic (11, 34). Comparing T-cell proliferation and activation in the blood of CT26 tumor–bearing mice treated with FS120 surrogate or control antibodies overtime, it was observed that FS120 surrogate induced more CD4+ and CD8+ T-cell proliferation, as measured by the expression of Ki67 (Fig. 5A and B) than the OX40 and CD137 antibodies or their combination. The effect of FS120 surrogate on T-cell activation, as measured by the expression of CD69, was delayed as compared with the proliferative effect with the highest frequencies observed 3 days after the third dose (Fig. 5B). Similar effects on T-cell activation were observed with the combination of OX40 and CD137 antibodies (Fig. 5B).

**FS120 surrogate did not induce liver inflammation**

Increased liver T-cell infiltration was observed with various CD137 agonist antibodies in mice, suggesting a similar mechanism of liver associated with CD137 agonist antibodies (30, 31). To understand the potential hepatotoxic risk of FS120, T cells and their proliferation and activation in the liver, spleen, and blood induced by FS120 surrogate was compared with that induced by OX40 and CD137 agonist antibodies or their combination (Fig. 6A). The results showed a clear difference between the two CD137 agonist antibodies tested, clone 3H3 induced a sustained increase in T-cell infiltration, proliferation, and activation in the liver and spleen, whereas clone Lob12.3 did not (Fig. 6B). OX40 stimulation did not show an increase in T cells in the liver, spleen, or blood, but induced transient T-cell proliferation in all tissues studied and T-cell activation in the liver at 14 days post-dose (Fig. 6B). Combination of OX40 and CD137 (clone Lob12.3) agonism induced a transient increase in T cells in the liver, which was associated with increased T-cell proliferation. FS120 surrogate also showed a moderate, but not statistically significant, increase in liver T-cell infiltration, proliferation, and activation at 7 days post-dose, which returned to normal at 14 days post-dose (Fig. 6B). This transient increase in T cells and proliferation was also observed in the blood of these naive mice, as expected from studies in CT26 tumor–bearing mice (Fig. 5B). In the spleen, FS120 surrogate also induced transient T-cell proliferation (Fig. 6A and B). The increased liver T-cell infiltration observed with the crosslink-independent CD137 agonist antibody (clone 3H3) as compared with the crosslink-dependent CD137 agonist antibody (clone Lob12.3; Fig. 6B) correlated with observations that uredulamab (crosslink-independent clone 20H4.9) induces hepatotoxicity at doses above 1 mg/kg and utoxulumab (crosslink-dependent clone MOR7480.1) is well-tolerated up to 10 mg/kg (13, 14). Both FS120 and the FS120 surrogate molecules had crosslink-dependent CD137 agonist Fab arms and were only able to induce CD137 agonism via binding to OX40 as shown by the competition experiments in Fig. 2E and Supplementary Fig. S8B. This dependency on OX40 binding for CD137 stimulation resulted in decreased liver T-cell infiltration in this preclinical study.
Figure 4.
Antitumor activity of the FS120 surrogate. A and B, Balb/c mice (n = 15) inoculated with 10^6 CT26 cells subcutaneously (s.c.) and treated with 1 mg/kg FS120 surrogate or controls every 2 days (Q2D) starting on day 13 post-tumor inoculation for three doses injected intraperitoneally (IP). Tumor volume measured every other day. Data shown are mean ± SEM. Statistical testing of tumor volume over time by mixed model analysis. C, TIL analysis of day 21 CT26 tumors (n = 5) treated with 1 mg/kg FS120 surrogate or controls Q2D starting on day 10 for three doses injected intraperitoneally by flow cytometry. Individual sample data are shown as well as mean ± SD (representative data from two independent experiments). Statistical testing by one-way ANOVA and Tukey multiple comparisons test. Asterisks on top of error bars represent the significant difference to Ctr(4420) mAb–treated mice (*, P < 0.032; ***, P < 0.0002). See also Supplementary Figs. S5, S6, and S7.
Peripheral T-cell activation and proliferation induced by the FS120 surrogate.

A, Schematic representation of experimental design. B, Balb/c mice (n = 5) inoculated with 10^6 CT26 cells subcutaneously (s.c.) and treated with 1 mg/kg FS120 surrogate or controls every 2 days starting on day 10 post-tumor inoculation for three doses injected intraperitoneally (IP). Tail vein blood collected on days 10 (predose), 11, 15, 17, and 24 for flow cytometric analysis. Data shown are mean ± SD. Statistical testing by two-way ANOVA and Tukey multiple comparisons test. Asterisks on top of error bars represent the significant difference to Ctrl(4420) mAb–treated mice (***, P < 0.0001).
Figure 6.
Increased inflammation induced by the crosslink-independent CD137 agonist antibody. **A**, Schematic representation of experimental design. **B**, Balb/c mice (n = 6) treated with 10 mg/kg FS120 surrogate or controls every 2 days starting on day 1 for three doses injected intraperitoneally (IP). Livers, spleens, and blood from 3 mice collected on days 7 and 14 post-last dose (experiment days 11 and 18) and processed for flow cytometric analysis. Individual sample data are shown as well as mean ± SD. Statistical testing by two-way ANOVA and Tukey multiple comparisons test. Asterisks on top of error bars represent the significant difference to Ctrl(4420) mAb–treated mice (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
Discussion

TILs express various checkpoint receptors and costimulatory receptors, including OX40 and CD137 (4, 5). These receptors, in the absence of ligand interaction, are likely to contribute to the dysfunctional phenotype of tumor reactive TILs (37, 38). Activating TILs with agonist antibodies against OX40 and CD137 has the potential to unleash existing antitumor immune responses and reduces tumor growth and increases survival in several syngeneic tumor models (39, 40). In clinical trials, however, despite inducing peripheral T-cell activation, neither OX40 antibodies nor CD137 antibodies induce complete responses unlike the results observed in preclinical studies (12).

The lack of translation between the preclinical models and the clinical results is likely due to various factors. Limited availability of FcyR-expressing cells in the tumor microenvironment of human cancers and low affinity interaction between FcyRs and the Fc region of IgG antibodies (41) could result in suboptimal crosslinking of these agonist antibodies (42). The depletion of intratumoral Tregs, described as the mechanism of action of OX40 and CD137 antibodies in mouse syngeneic tumor models, may not be as effective in human cancers (6, 7, 43) and may also result in the depletion of the very same cells the OX40 and CD137 antibodies aim to stimulate (15).

CD137 agonist antibodies are associated with liver inflammation in preclinical models and urelumab induces lethal hepatic inflammation in preclinical trials at doses above 1 mg/kg (13). Although the mechanism of toxicity in the clinic is unclear, in preclinical models this is associated with activation of liver myeloid cells, which express CD137 and the production of IL27, which then recruits CD8\(^+\) T cells, and T cell lines expressing cells and clone Lob12.3 requiring crosslinking. The liver inflammation observed in these antibodies was markedly different, with clone 3H3 showing sustained increased T cells in the liver. Increased alanine aminotransferase and liver T-cell infiltration is observed in mice treated with rat versions of the same clones (46). Because urelumab and clone 3H3 are associated with increased liver inflammation and both clones (20H4.9 and 3H3) have the ability to stimulate CD137 in the absence of crosslinking, it is possible that this may contribute to the hepatotoxicity risk presented by CD137-targeting antibodies.

In conclusion, FS120 is a bispecific antibody targeting OX40 and CD137 that simultaneously bound these receptors and induced FcyR-independent T-cell activation in vitro; the FS120 surrogate induced T-cell activation in vivo. This in vitro and in vivo activity, which was dependent on coengagement with both receptors, suggested that the dual binding resulted in efficient receptor clustering and activation. The existence of two OX40-binding sites in the Fc region of FS120 raised the possibility of tetraivalent binding, which was likely to be involved in the clustering mechanism. In addition, the binding to these two separate TNFRSF members with a single molecule could potentially create receptor superclusters resulting in increased signaling via these receptors as they share intracellular signaling intermediates such as TRAF2 but also have unique adapters (TRAF1 for CD137 and TRAF5 for OX40) and stimulate distinct pathways (47). The crosslinking of OX40 and CD137 receptors by FS120 could also lead to increased internalization, as is required for CD137 signaling (48). The increased T-cell activation by FS120 surrogate which resulted in FcyR-independent antitumor activity was independent of Treg depletion. Furthermore, due to its crosslink-dependent CD137-targeting Fab, which required Fcab binding to OX40 for activity, FS120 may potentially provide a potent and safe way of stimulating CD137. These data support initiation of clinical development of FS120, a first-in-class dual agonist bispecific antibody for the treatment of human cancer.

Disclosure of Potential Conflicts of Interest

M. Gaspar is a principal scientist at and has ownership interest (including patents) in F-star Therapeutics Ltd. S. Uhlenbroich is a senior scientist at and has ownership interest (including patents) in F-star Therapeutics Ltd. K.L. Everett is a senior scientist at F-star Therapeutics Ltd. N. Brewis is Chief Scientific Officer at, reports receiving a commercial research grant from, and has ownership interest (including patents) in F-star Therapeutics Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Gaspar, M. Morrow, M. Tuna, N. Brewis Development of methodology: M. Gaspar, J. Pravin, L. Rodrigues, S. Uhlenbroich, K.L. Everett Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Gaspar, J. Pravin, L. Rodrigues, S. Uhlenbroich, F. Wollerton Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Gaspar, J. Pravin, L. Rodrigues, F. Wollerton, K.L. Everett Writing, review, and/or revision of the manuscript: M. Gaspar, J. Pravin, F. Wollerton, M. Morrow, N. Brewis Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Gaspar, J. Pravin Study supervision: M. Gaspar, M. Tuna

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