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 Fcγ receptors (FcγR), which can limit their clinical activity. FS120 mAb1, a dual agonist 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 of FcγR interaction, but associated with peripheral T-cell activation and proliferation. When compared with a crosslink-independent CD137 agonist mAb, the FS120 surrogate induced lower liver T-cell infiltration. 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 OX40 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 stimulate 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 TNFSF 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 mAb1, 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 (21).
interaction or after Treg depletion. When compared with a crosslink-independent CD137 agonist mAb, FSi120 surrogate showed reduced liver T-cell infiltration, which decreased over time. These results indicate that targeting coexpressed receptors with bispecific 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 VH or VK domain sequences in human IgG1 with identified sequences from patents or literature using methods described previously (21). The LALA (L33A4-L235A, Eu numbering; ref. 22) mutations were introduced via site-directed mutagenesis when indicated. Absence of LALA mutations is denoted by the suffix WT (wild-type) after the clone name. For antibody production, pTT5 expression vectors (National Research Council of Canada) containing the mAb or mAb² sequences were transfected into Exp293f cells (Thermo Fisher Scientific, A14528) using PEpro Transfection Reagent (Polyplus, PPLU115) according to the manufacturer’s instructions. Antibodies were purified using a 5 mL MabSelect SuRe Column (GE Healthcare, 11003494) on an AKTA Explorer (GE Healthcare) according to the manufacturer’s instructions. The following antibodies were used in the experiments described in this article: anti-FcTC (Ctrl[4420] WT mAb; clone 4420 used as isotype control; ref. 23); anti-human OX40 (OX40[11D4] mAb; clone 11D4 from patent EP2242771B1); anti-human CD137 (InvivoGen, ant-pr-1) and individual cell lines were cloned by serial dilution. Cell lines were selected by incubation with 5 μg/mL anti-CD40 (MAB6321-100); and anti-DR6 (AF144) were used in the experiments described in this article.

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 Kd determination, FSi120 was captured either via the Fc region using a Human Antibody Capture Kit (GE Healthcare) and human or cynomolgus CD137 was flowed over at a range of concentrations; or via the Fab region using a Human Fab Capture Kit (GE Healthcare) and human or cynomolgus OX40 was flowed over at a range of concentrations. For dual binding determination of FSi120, biotinylated human CD137 or OX40 was immobilized on a Streptavidin Chip (GE Healthcare) and coinjecting antibodies (100 nmol/L) were coinjected with either mouse OX40 or CD137 (100 nmol/L) or HBS-EP buffer. Affinity for FcRy1 (R&D Systems, hFcRy1 (1257-CF-050), hFcRy2a (1330-CF-050), hFcRy2b (1875-CF-050), and hFcRy3a (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 FcRy1s (500 nmol/L) at 20 μL/minute flow rate and the dissociation was followed for 5 minutes. For specificity assessment, human TNFRSF members [R&D Systems, TNFRI (372-RI-050/CF), TNFRII (726-R2-050), GITR (689-GR-100), NGRF1 (367-NR-050/CF), CD40 (1493-CD-050), and DR6 (144-DR-100)] were immobilized on CM5 Chips (GE Healthcare) to approximately 1,000 RU and FSi120 (1 μmol/L) was flowed over at a flow rate of 30 μL/min. 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 (85082301) in 2014 and used under license from National Jewish Health. Exp293f 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 restriction sites and cloned into the lentivirus vector pLVX-EF1-IRESPuro (Clontech 631988). Lentiviruses were produced using the LentIX Expression system EI1tX Version (Clontech 631253) and used to transduce DO11.10 cells according to the manufacturer’s instructions. Cell lines were selected by incubation with 5 μg/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 μg/mL) and mouse IL2 concentration in the presence of Fc Block (Thermo Fisher Scientific, 88-7044-88).

Flow cytometry

For cell binding assays, cells were incubated with primary antibodies or mAb² 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 gentleMACS Octo Dissociator and C-tubes according to the manufacturer's instructions. Resulting cell suspensions were strained (70 μm cell strainer (Corning CLS417511)), 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, K67, 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...
Peripheral blood mononuclear cells and T-cell stimulation assays

Ficol-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-i-GLutamine (Gibco), and 50 μmol/L 2-mercaptoethanol (Gibco)]. T cells were isolated from PBMCs using Miltenyi Enrichment Kits (Human CD3+, 130-096-533; 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 (100002) clone 145-2C11 at 2.5 μg/mL] in the presence of FS120 or control antibodies for 3 days at 37°C, 5% CO2. Anti-human Fc (clone MKI1A6, produced in-house) was used as crosslinking agent at a 1:1 molar ratio with test antibodies. FITC dextran (70 kDa, Sigma 46945) was used as crosslinking agent at a 1:1 molar ratio with OX40 Fabs paired with FITC-binding Fab (clone 4420). IL2 concentration in the supernatants was determined by 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 (Kd: Human, 0.2 nmol/L and Cyno, 0.9 nmol/L) and CD137 (Kd: 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, NGFR, DR6, TNFRI, and TNFRII; Supplementary Fig. S2). FS120 bound to monkey OX40 (AfD: Human, 0.2 nmol/L and Cyno, 0.9 nmol/L) and CD137 (AfD: 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, NGFR, DR6, TNFRI, and TNFRII; 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 FcγR-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 FcγR-mediated crosslinking.

Agonistic activity of OX40 or CD137 monospecific antibodies was only observed in the presence of crosslinking (Fig. 2A). In contrast, FS120 mAb2 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 mAb2 (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 TNFα) 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 mAb 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
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 CD4⁺ 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 cross-linked activity of clone FS30 [CD137(FS30) mAb] demonstrated that the mAb² could activate the CD137 receptor when crosslinked by binding of the Fcab to CD8⁺ T cells. The activity of the cross-linked OX40 Fcab [OX40(Ctr(4420) mAb²)] showed that the mAb² could activate the OX40 receptor when crosslinked by binding of the Fab arms to 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 mAb² 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 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

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**Figure 3.**

FS120 mAb² stimulated both CD4⁺ and CD8⁺ T cells. **A**, Human CD4⁺ and CD8⁺ T cells stimulated with coated CD3 antibody (clone UCHT-1 at 2.5 μg/mL for CD4⁺ and 5 μg/mL for CD8⁺ T cells) and costimulated with FS120 or OX40 and CD137 antibody controls (3.7 nmol/L) in the presence or absence of crosslinking reagents at 1:1 molar ratio [FITC dextran for OX40/Ctr(4420) mAb² and anti-Fc (clone MK1A6 in mouse IgG1 format) for other antibodies]. Data from duplicates are shown as mean ± SD (representative results of three independent experiments). Statistical testing by two-way ANOVA and Dunnett multiple comparison test. Asterisks on top of error bars represent the significant difference to Ctrl(4420) mAb-treated samples (*, P < 0.032; **, P < 0.0021; ***, P < 0.0002; ****, P < 0.0001). **B**, OX40 and CD137 receptor quantification in CD4⁺ and CD8⁺ T cells from 3 PBMC donors, activated overnight with CD3/CD28 beads (1:1 ratio), by flow cytometric fluorescence quantification with beads (Bangs Laboratories). See also Supplementary Figs. S1 and S4.
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 (3, 30). To understand the potential hepatotoxic risk of FS120, T cells and their infiltration 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-last 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-last dose, which returned to normal at 14 days post-last dose (Fig 6B). This transient increase in T cells and proliferation was also observed in the blood of these naïve mice, as expected from other 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 uredolumab (crosslink-independent clone 20H4.9) induces hepatotoxicity at doses above 1 mg/kg and utomolumab (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 model.
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 Ctrl(4420) mAb–treated mice (*, P < 0.032; ***, P < 0.0002). See also Supplementary Figs. S5, S6, and S7.
Figure 5.
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 < 0.052; **, P < 0.002; ***, P < 0.0002; ****, P < 0.00001).
and suggested that FS120 may have a lower hepatotoxicity risk than crosslink-independent CD137 agonist antibodies.

**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 FcγR-expressing cells in the tumor microenvironment of human cancers and low affinity interaction between FcγRs 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 clinical 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 CD137 ligand (48). The increased T-cell activation by FS120 surrogate which resulted in FcR-independent antitumor activity was independent of Treg depletion. Furthermore, due to its crosslink-dependent CD137-targeting Fab, which required FcαR 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**


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


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