Crosslink-independent CD137 Agonism is Associated with Liver Inflammation

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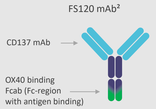
Stimulating existing anti-cancer immune responses by activating T cell costimulatory receptors of the Tumour Necrosis Factor Receptor Superfamily (TNFRSF) is potentially the next significant stage of cancer immunotherapy. Clustering of TNFRSF members is required to initiate signal transduction and crosslinking of TNFRSF-specific antibodies via Fcγ receptor (FcγR) interaction is typically required to mimic this effect. This is likely to limit their clinical activity due to the relatively low affinity of Fc:FcγR interactions, off target (non-tumour) cell activation due to the expression pattern of these receptors and the potential to induce ADCC-mediated depletion of the cells intended for activation. To avoid the limitations of FcγR-mediated crosslinking, we developed FS120, a bispecific antibody of proprietary format (mAb^{2tm}) targeting OX40 and CD137. These two TNFRSF members are co-expressed on activated T cells and enriched in the tumour microenvironment. FS120 circumvents the requirement for FcγR-mediated crosslinking by engaging OX40 and CD137 simultaneously thereby crosslinking and clustering the two receptors. Activating immune costimulatory receptors has the potential to induce unchecked immune stimulation which can be toxic. Certain agonist antibodies targeting CD137 have been shown to induce liver inflammation either in the clinic or in preclinical models, with the latter being associated with increased liver T cell infiltration, although there is currently no understanding of why only certain antibodies induce liver inflammation and not others. To better understand the hepatotoxicity risk presented by FS120's novel crosslinking mechanism, we compared it to different CD137 agonist antibodies *in vitro* and *in vivo*.

Our results show varying levels of *in vitro* potency when testing CD137-targeting antibodies, with some of them being able to induce crosslinking-independent T cell activation. The CD137 antibody clone present in urelumab, which induced hepatotoxicity in the clinic, showed crosslink-independent activity and was more potent as compared to the clone present in utomilumab, which was well tolerated in the clinic. The data indicate that a mouse CD137 antibody (clone 3H3 on human IgG1 backbone) had crosslink-independent activity and led to increased sustained liver T cell infiltration, activation and proliferation as compared to a crosslink-dependent clone (Lob12.3 on human IgG1 backbone) suggesting that the crosslink-independent activity of CD137 agonist antibodies may contribute to their hepatotoxicity risk observed in the clinic. The CD137 antibody in FS120 is crosslink-dependent, and FS120 requires binding to both target receptors for optimum crosslinking and activity. The activity of a mouse specific surrogate of FS120, containing a crosslink-dependent CD137 clone (Lob12.3), was not associated *in vivo* with increased liver T cell infiltration suggesting targeting co-expressed receptors with a bispecific antibody has the potential to be a well-tolerated and effective mechanism of crosslinking and agonising CD137, as well as OX40, and is independent of FcγR-mediated crosslinking.



1. FS120's activity does not rely on external crosslinking agents but on simultaneously binding to both OX40 and CD137

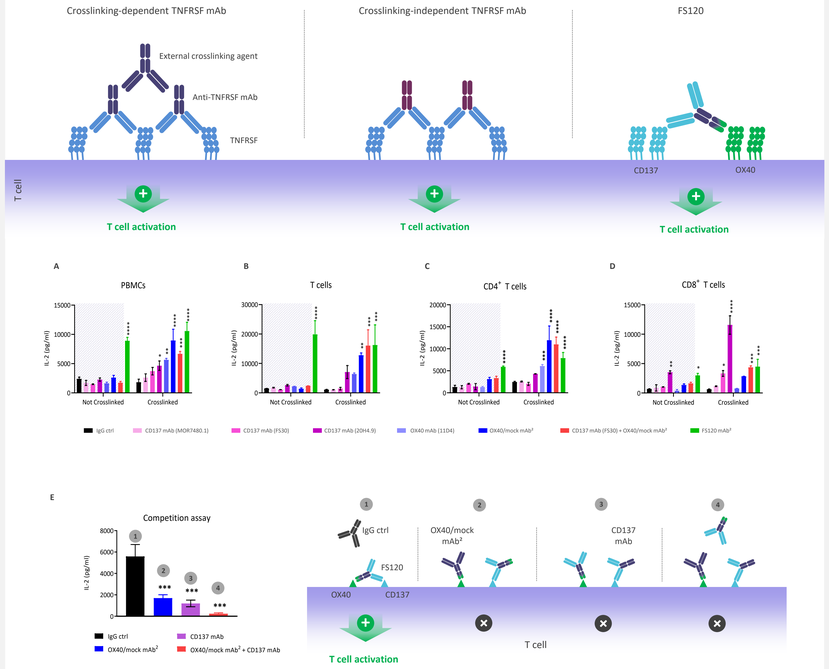


Figure 1. Most OX40 and CD137 agonist antibodies require crosslinking to mediate T cell stimulation and FS120 mAb^2 is crosslinked by simultaneously engaging OX40 and CD137. **A -** Human PBMCs stimulated with SEA (100 ng/ml) **B -** human T cells stimulated with coated CD3-antibody (clone UCHT-1 at 2.5 mg/ml) **C -** human CD4$^+$ **D -** or 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 co-stimulated with FS120 mAb^2 or control antibodies (3.7 nM) in the presence or absence of external crosslinking agents at 1:1 molar ratio. **E -** CD3-stimulated T cells co-stimulated with FS120 (1nM) and isotype control antibody or component parts of FS120 mAb^2 and their combination (100nM). **A - E** Data from duplicates is shown as mean ± standard deviation (SD) (representative results of 3 independent experiments). Statistical testing by two-way ANOVA and Tukey's multiple comparison test (A-B) or one-way ANOVA and Dunnett's multiple comparisons test (**C-E**). Asterisks on top of error bars represent the significant difference to IgG ctrl treated samples (* p<0.032, ** p<0.0021, *** p<0.0002, **** p<0.0001).

2. Some CD137 agonist antibodies can activate T cells in the absence of external crosslinking agents

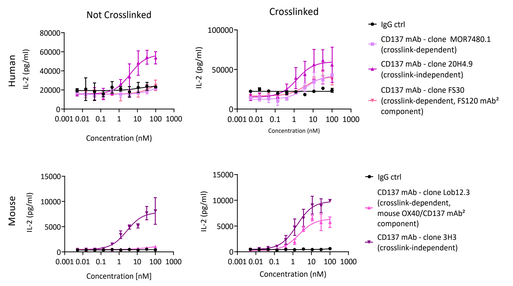


Figure 2. Crosslink-dependent and crosslink-independent agonism of CD137 by different agonist antibodies. (A) IL-2 production by DO11.10 cells expressing **A-** human or **B-** mouse CD137 stimulated with coated CD3 antibody (clone 17A2 at 0.1 mg/ml) and co-stimulated with CD137 antibodies or isotype control in the presence or absence of anti-Fc crosslinking antibody.
Representative results from 3 independent experiments. Data from duplicates is shown as mean ± standard deviation (SD).

3. Mouse OX40/CD137 mAb² features a favourable safety profile while crosslink-independent CD137 agonist antibody induces increased liver T cell infiltration

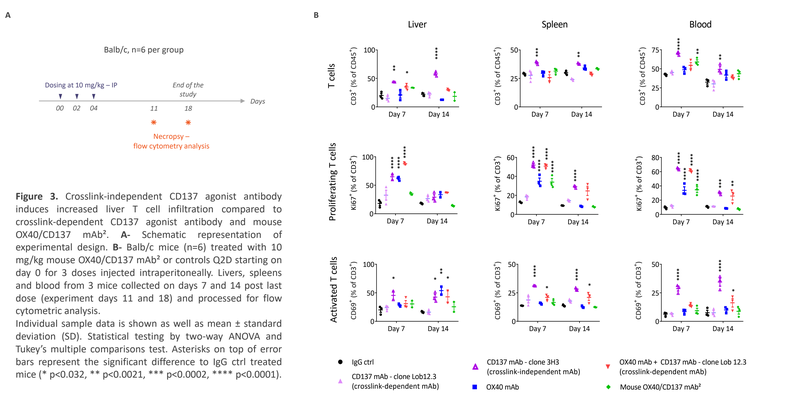


Figure 3. Crosslink-independent CD137 agonist antibody induces increased liver T cell infiltration compared to crosslink-dependent CD137 agonist antibody and mouse OX40/CD137 mAb^2. **A-** Schematic representation of experimental design. **B-** Balb/c mice (n=6) treated with 10 mg/kg mouse OX40/CD137 mAb^2 or controls Q2D starting on day 0 for 3 doses injected intraperitoneally. 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 is shown as well as mean ± standard deviation (SD). Statistical testing by two-way ANOVA and Tukey's multiple comparisons test. Asterisks on top of error bars represent the significant difference to IgG ctrl treated mice (* p<0.032, ** p<0.0021, *** p<0.0002, **** p<0.0001).

4. Crosslink-dependent mouse OX40/CD137 mAb² shows similar anti-tumour activity and peripheral T cell activation than a crosslink-independent mAb² construct

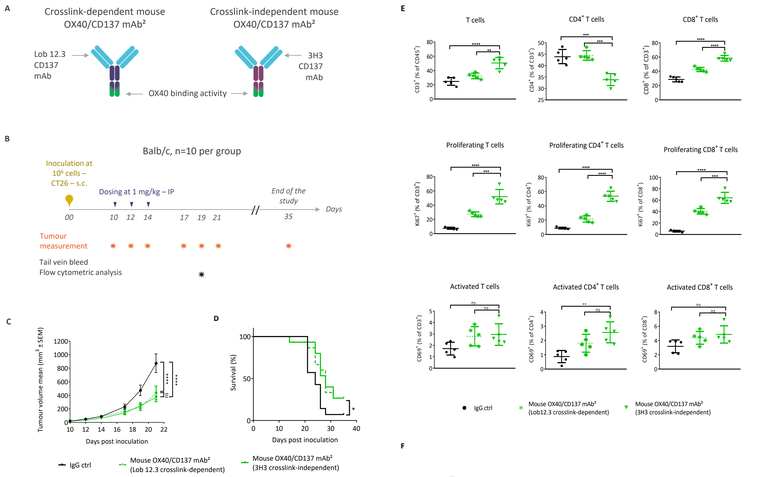


Figure 4. OX40/CD137 mAb^2 containing crosslink-independent CD137 agonist antibody has similar anti-tumour efficacy to FS120 surrogate despite inducing increased peripheral T cell proliferation. **B -** Schematic representation of experimental design. **C -** Balb/c mice (n=10) inoculated with 10^6 CT26 cells subcutaneously and treated with 1 mg/kg FS120 surrogate or controls Q2D starting on day 10 post-tumour inoculation, for 3 doses injected intraperitoneally. Tumour volume measured every other day. Data shown is mean ± standard error mean (SEM). Statistical testing of tumour volume on day 21 by two-way ANOVA and Tukey's multiple comparison test. **D -** Kaplan-Meier plot of survival (percentage), statistical testing by log rank test (Mantel-Cox). **E -** Tail vein blood collected on day 5 post last dose (experiment day 19) for cytometric analysis. Individual sample data is shown as well as mean ± standard deviation (SD). Statistical testing by one-way ANOVA and Tukey's multiple comparisons test. Asterisks represent the significant difference (* p<0.032, ** p<0.0021, *** p<0.0002, **** p<0.0001). **F –** Proposed mechanism of action

Conclusion

Varying levels of *in vitro* potency were observed when testing CD137-targeting antibodies, with some of them being able to induce crosslinking-independent T cell activation. The CD137 antibody clone present in urelumab, which induced hepatotoxicity in the clinic, showed crosslink-independent activity and was more potent as compared to the clone present in utomilumab, which was well tolerated in the clinic. A mouse CD137 antibody (clone 3H3) also showed crosslink-independent activity and increased sustained liver T cell infiltration, activation and proliferation as compared to a crosslink-dependent clone (Lob12.3) suggesting that the crosslink-independent activity of CD137 agonist antibodies may contribute to their hepatotoxicity risk. The CD137 antibody in FS120 is crosslink-dependent, FS120 requires binding to both receptors for crosslinking and activity. Mouse OX40/CD137 mAb^2 activity was not associated *in vivo* with increased liver T cell infiltration suggesting targeting co-expressed receptors with a bispecific antibody has the potential to be a well-tolerated and effective mechanism of crosslinking and agonising CD137, as well as OX40, and is independent of FcγR-mediated crosslinking.

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