FcγRIIB engagement drives agonistic activity of Fc-engineered αOX40 antibody to stimulate human tumor-infiltrating T cells

Lucia Campos Carrascosa [ORCID],[1] Adriaan A van Beek [ORCID],[1] Valeska de Ruiter,[1] Michail Doukas,[2] Jie Wei,[3] Timothy S Fisher,[3] Keith Ching,[3] Wenjing Yang,[3] Karlijn van Loon,[1] Patrick P C Boor,[1] Yannick S Rakké,[1] Lisanne Noordam,[1] Pascal Doornebosch,[4] Dirk Grünhagen,[5] Kees Verhoef,[5] Wojciech G Polak,[5] Jan N M IJzermans,[5] Irene Ni,[3] Yik Andy Yeung,[3] Shahram Salek-Ardakani [ORCID],[3] Dave Sprengers,[1] Jaap Kwekkeboom[1]

ABSTRACT

Background OX40 (CD134) is a costimulatory molecule of the tumor necrosis factor receptor superfamily that is currently being investigated as a target for cancer immunotherapy. However, despite promising results in murine tumor models, the clinical efficacy of agonistic αOX40 antibodies in the treatment of patients with cancer has fallen short of the high expectation in earlier-stage trials.

Methods Using lymphocytes from resected tumor, tumor-free (TF) tissue and peripheral blood mononuclear cells (PBMC) of 96 patients with hepatocellular and colorectal cancers, we determined OX40 expression and the in vitro T-cell agonistic activity of OX40-targeting compounds. RNA-Seq was used to evaluate OX40-mediated transcriptional changes in CD4+ and CD8+ human tumor-infiltrating lymphocytes (TILs).

Results Here, we show that OX40 was overexpressed on tumor-infiltrating CD4+ T cells compared with blood and TF tissue-derived T cells. In contrast to a clinical candidate αOX40 antibody, treatment with an Fc-engineered αOX40 antibody (αOX40_v12) with selectively enhanced FcγRIIB affinity, stimulated in vitro CD4+ and CD8+ TIL expansion, as well as cytokine and chemokine secretions. The activity of αOX40_v12 was dependent on FcγRIIB engagement and intrinsic CD3/CD28 signals. The transcriptional landscape of CD4+ and CD8+ TILs shifted toward a prosurvival, inflammatory and chemotactic profile on treatment with αOX40_v12.

Conclusions OX40 is overexpressed on CD4+ TILs and thus represents a promising target for immunotherapy. Targeting OX40 with currently used agonistic antibodies may be inefficient due to lack of OX40 multimerization. Thus, Fc engineering is a powerful tool in enhancing the agonistic activity of αOX40 antibody and may shape the future design of antibody-mediated αOX40 immunotherapy.

INTRODUCTION

Checkpoint inhibition has revolutionized cancer treatment in the past decade. However, only a minority of patients suffering from solid tumors like hepatocellular carcinoma (HCC) and colorectal cancer (CRC) show sustained responses on blockade of PD1 and CTLA4 pathways.[1–4] Therefore, additional strategies are required to optimize cancer immunotherapy. Agonistic antibodies targeting costimulatory receptors, particularly tumor necrosis factor receptor (TNFR) superfamily members, may represent an additional treatment avenue in boosting anti-tumor immunity.

TNFR member OX40 (TNFRSF4, CD134) is transiently expressed on human T helper (Th) cells, regulatory T cells (Tregs) and CD8+ T cells on T-cell receptor (TCR) activation, while CD28-mediated costimulation, as well as interleukin (IL)-2 augment and sustain TCR-induced OX40 expression.[5 6] Accordingly, CD4+ and CD8+ tumor-infiltrating lymphocytes (TILs) were shown to upregulate OX40 on coculture with autologous tumor cells.[7] Although tumor-infiltrating (TI) CD4+ T cells derived from several cancers express higher OX40 levels than peripheral T cells,[8–12] in-depth OX40 expression patterns on colorectal and HCC cancer-derived CD4+ and CD8+ TILs remain largely unknown.

In response to antigen encounter, OX40 ligation boosts effector T-cell expansion, survival, cytokine secretion and memory formation in synergy with CD28 costimulation.[6] In contrast, OX40 ligation in Tregs leads to reduced suppressive activity[13–16] by suppressing the 'master regulator' Foxp3.[17] Efficient signal transduction requires OX40 multimerization, which could be achieved by Fc engineering an αOX40 monoclonal antibody to enhance the affinity for inhibitory

receptor FcγRIIB in a reporter cell line assay.[18] This is in line with previous work on other TNFR members, in which FcγRIIB-mediated multimerization drives agonistic antibody activity, including αCD40,[19 20] αDR5[21] and αCD95.[22]

Due to its expression patterns as well as distinct roles on effector T cells and Tregs, OX40 treatment was used in several preclinical mouse models to reinvigorate antitumor immunity. OX40 costimulation in mouse models was achieved by treatment with agonistic αOX40 antibodies, OX40L.Ig fusion proteins, OX40 RNA aptamers and OX40L-expressing tumor cells, which leads to reduced tumor burden.[23 24] While some studies point toward a direct prosurvival effect on CD8+ TILs or CD4-mediated CD8 help, other studies emphasize reduced suppressive capacities or depletion of Tregs to be the main driver of OX40 therapy efficacy in mouse tumor models.[5]

Due to the promising antitumor effects, OX40 antibody-mediated immunotherapy either alone or in combination with antibodies against other immune checkpoint molecules, chemotherapy or radiation is already tested in several clinical trials.[25] Preliminary results of a phase I study (NCT02315066) with clinical αOX40 IgG2 antibody (PF-8600) confirmed the antitumor properties of OX40 targeting as inflammatory and interferon (IFN)-γ gene sets were upregulated in tumors of patients under OX40 treatment.[26] However, only a minority of patients (2/48) showed partial clinical responses.[27] Besides, more phase I studies have been performed in which agonistic αOX40 antibody treatment showed low clinical efficacy in advanced solid tumors.[28 29]

Hence, we wondered whether the reason for the limited clinical efficacy was due to (1) low OX40 expression on human TILs, (2) minor effects of OX40 costimulation on TIL functions and expansion, or (3) suboptimal performance of the currently used αOX40 antibodies. Therefore, we measured OX40 levels on distinct TIL subsets of colorectal and HCC tumor tissue, thus demonstrating that TI activated T-helper (aTh) and activated regulatory T cell (aTreg) do express high OX40 levels. Then, we analyzed the agonistic activity of several OX40-targeting compounds, including the clinical candidate αOX40 and Fc-engineered αOX40_v12 antibody with selectively enhanced FcγRIIB affinity on expansion and activation of TILs by transcriptomic analyses, flow cytometry and multiplex assay. In contrast to the currently used αOX40 antibody, Fc-engineered αOX40_v12 induced CD4+ as well as CD8+ TIL expansion, increased proinflammatory cytokine and chemokine secretion, and shifted the transcriptional landscape toward prosurvival and chemotactic pathways. Thus, our study might contribute to shaping next-generation OX40 immunotherapy for patients with cancer.

METHODS
Patients
In this study, 96 patients and 4 healthy donors were enrolled between August 2016 and May 2020.

Twenty-seven patients with HCC who were eligible for resection (n=26) or liver transplantation (n=1) were enrolled. Sixty-nine patients with CRC who were eligible for resection of primary colorectal cancer (pCRC) (n=43) or liver metastasis (LM-CRC) (n=26) were included in this study. Matched fresh tissue samples from tumor tissue and surrounding tumor-free (TF) tissue (>2 cm from tumor margin) were collected and subsequently, intrahepatic or intracolorectal as well as TILs were isolated. Peripheral blood was obtained perioperatively. Enrolled patients did not receive chemotherapy or immunosuppressive therapy at least 4 weeks prior to surgery. Online supplementary tables 1 and 2 shows clinical patient characteristics. The majority of enrolled patients was from Caucasian origin.

Cell preparation
Tumor as well as surrounding TF tissue were either stored in University of Wisconsin (UW) solution at 4°C overnight or immediately processed on receipt. Single-cell suspensions were obtained by enzymatic tissue digestion followed by ficoll density centrifugation, as described previously.[30] Details can be found in the online supplementary Methods.

Ex vivo polyclonal T-cell expansion and activation assay
Isolated total TIL and peripheral blood mononuclear cell (PBMC) fractions were either used directly for in vitro culture or purified using Magnetic Cell Separation (MACS). TILs were positively selected using human CD3 MicroBeads to obtain CD3+ T cells (Miltenyi Biotec, 130-050-101). CD25-depleted TIL fractions were obtained by negatively selecting CD25− TILs with human CD25 MicroBeads II (Miltenyi Biotec, 130-092-983). Efficiency of MACS separation was set to a minimum of 85% purity of CD3+ or CD25− TIL, respectively. TILs were cultured in Roswell Park Memorial Institute (RPMI) 1640 solution supplemented with 10% human AB serum 2 mM L-glutamine (Invitrogen), 50 mM Hepes Buffer (Lonza), 1% penicillin–streptomycin (Life Technologies), 5 mM sodium pyruvate (Gibco) and 1% minimum essential medium nonessential amino acids at 37°C in 96-well round-bottom plates (5×10^5 CD45+ cells per well) in the presence of either αCD3/CD28 activation Dynabeads (cell:bead ratio 100-10:1) or 10–100 ng/mL plate-bound αCD3 antibody (clone OKT3, BioLegend, 317326). Usually, experiments in the range of 15%–90% baseline Ki67 expression on T cells after day 8–10 of culture were included, unless mentioned otherwise. For indicated experiments, TILs were preincubated with 10 µg/mL αFcγRIIB-blocking antibody (ch2B6N279Q,[31] kind gift of MacroGenics) for 60 min at 37°C. Cells were treated with 2–20 µg/mL hexameric human OX40L (Pfizer), 10 µg/mL soluble human αOX40 IgG1, human isotype HA-IgG1, human αOX40 IgG2 (PF-04518600), human isotype HA-IgG2 or human αOX40IgG1_v12 (all Pfizer). αOX40 IgG2 and isotype IgG2 antibody coupling to Dynabeads was performed by using Dynabeads Antibody coupling Kit according to the manufacturer's instructions

(14 311D), whereby 6 µg antibody was coupled per 1 mg of beads. Subsequently, cells were treated with 100 ng/mL coupled αOX40IgG2- or IgG2 isotype-coupled beads. After 8–10 days, culture supernatants were collected and quantified using LegendPlex 'Human T helper Cytokine Panel' or 'Human Pro-Inflammatory Chemokine Panel' (BioLegend, 740 722 or 740003). Cells were harvested and stained with antibodies as indicated in online supplementary table 3. Before flow cytometric measurements on FACS Canto or FACSAria II (BD Biosciences), counting beads (Thermo Fisher, 01-1234-42) were added to allow ratiometric determination of absolute cell counts.

RNA-SEQ AND BIOINFORMATICAL ANALYSES

CD25+ TILs were positively isolated using human CD25 MicroBeads II (Miltenyi Biotec, 130-092-983) directly after TIL isolation. Selected CD25+ TILs were stained with 5 µM CellTrace Violet (CTV, Thermo Fisher) according to the manufacturer's instructions. Afterwards, CTV-stained CD25+ TILs were cocultured with non-stained CD25– TILs for 3–5 days in the presence of αCD3/CD28 activation beads (ctrl) or additionally 10 µg/mL αOX40_v12. Then, Th (CD45+CD3+CD56 CD4+CTV−), CD8+ (CD45 +CD3+CD56 CD8+CTV−) and CD25+ (CD45 +CD3+CD56 CD4+CTV+) TILs were sorted into 500 µL RLTplus buffer. Experimental details can be retrieved from the online supplementary Methods.

STATISTICAL ANALYSES

Differences between two paired groups of data were analyzed by Wilcoxon matched test. For more than two groups, Kruskal-Wallis test was applied. Correlation analysis according to Spearman was performed. The statistical analysis was performed using GraphPad Prism Software V.8.0. For DESeq analysis, a cut-off of $p < 0.05$ and fold change (FC) of >0.3 was used (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

RESULTS

### OX40 is predominantly expressed on TI aTh and aTreg

In this study, resected tumor and surrounding TF tissue as well as PBMC of patients with HCC and LM-CRC were included, which showed at least some CD3+ immune cell infiltration in tumor tissue or at tumor margin (>100 000 TILs/1 g tissue). CD8 and CD4+ T-cell subsets were present in all tissues; however, frequencies were highly variable between patients (online supplementary figure S1A). CD4+ T-cell subsets can be distinguished into regulatory and effector subsets according to Foxp3 and CD45RA expression.[32] While Th (Th=CD45RA$^{+/−}$ Foxp3$^−$) cells do not express transcription factor Foxp3, aTh (aTh=CD45RA-Foxp3^{low}) cells express low levels of Foxp3 (figure 1A). The highest Foxp3 expression can be observed in aTreg (aTreg=CD45RA-Foxp3^{high}), which exerts regulatory functions.[32] In all tested tumor types,

CD4+ TILs contained higher frequencies of aTreg as compared with CD4+ lymphocytes from either PBMC or TF tissue, while resting (r)Treg constituted only a small fraction of CD4+ lymphocytes in tumor, TF tissue and blood (online supplementary figure S1B). In order to evaluate the potential of targeting OX40 in antitumor immunotherapy, OX40 expression was measured ex vivo on TILs, TF liver-resident and colon-resident lymphocytes, as well as PBMC of patients with LM-CRC and HCC. Thereby, tumor-derived CD8 cells displayed higher OX40 expression compared with PBMC, while Th, aTh and aTreg demonstrated higher OX40 levels than PBMC, as well as TF tissue-derived CD4+ T cells (online supplementary figure S1D). Throughout all tumor types, aTh and aTreg contained significantly higher numbers of OX40-expressing cells than Th and CD8 cells, while no apparent differences between tumor types were observed (figure 1E).

Taken together, CD4+ TILs derived from HCC and LM-CRC tissues expressed increased levels of the costimulatory molecule OX40 compared with blood- and TF-derived T cells with highest expression on TI aTh and aTreg.

OX40 crosslinking is essential for OX40-mediated TIL expansion and cytokine secretion

We then investigated the effect of the clinical antibody αOX40 (IgG2, PF-04518600), as well as an hexameric OX40L on freshly isolated HCC-derived, LM-CRC-derived and pCRC-derived TILs in an in vitro model (figure 2A). Primary TILs were cultured in the presence of αCD3/CD28 activation beads to provide initial TCR as well as CD28 signaling, which is required for efficient OX40-mediated costimulation.[5] After 8–10 days of in vitro culture, CD4+ and CD8+ TIL expansions were determined by ratiometric evaluation of cell numbers by flow cytometry (online supplementary figure S2A). On costimulation with the monomeric αOX40 antibody, we failed to detect expansion of either CD4+ or CD8+ TILs over control TILs stimulated in the presence of αCD3/CD28 alone (figure 2B). In striking contrast, treatment with hexameric OX40L led to significant expansion of CD4+ and CD8+ TIL numbers and upregulation of proliferation marker Ki67 (figure 2B,C and online supplementary figure S2B–D). Correlating with OX40 expression data, CD4+ TILs proliferated to a higher extent than CD8+ TILs in response to OX40L treatment (online supplementary figure 2SC,D). Furthermore, elevated levels of proinflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-22 were secreted by OX40L-treated TILs (online supplementary figure S2E), whereas IFN-γ and TNF-α levels were not affected by αOX40 IgG2 treatment (figure 2D). OX40 is well known to require receptor trimerization for efficient signal transduction.[33] Hence, we wondered whether multimerizing αOX40 IgG2 antibody to magnetic beads might improve its agonistic activity in our in vitro assay. Accordingly, we cultured TILs in the presence of either monomeric αOX40 antibody or multimerized antibody, as well as the respective isotype antibodies. Stimulation
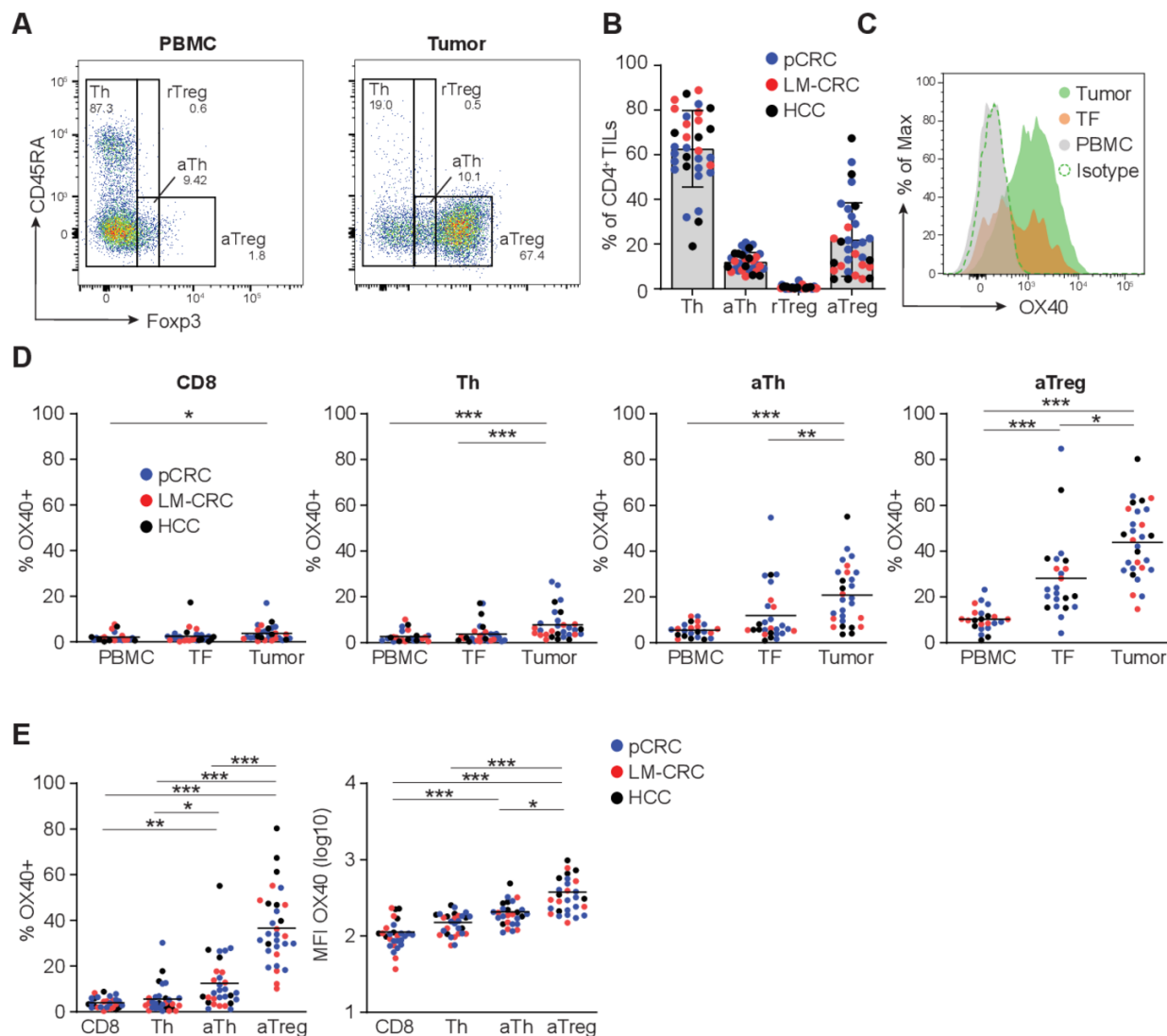
Figure 1 OX40 is highly expressed on aTreg and aTh TILs. PBMC, TF and tumor tissue from patients with LM-CRC and HCC were collected, enzymatically digested and immediately stained. (A) Representative dot plot depicting Treg gating strategy using distinctive CD45RA and Foxp3 expression in CD4+ T cells of one patient with HCC (Th=CD45RA±Foxp3−, aTh=CD45RA-Foxp3low, aTreg=CD45RA-Foxp3high). (B) Frequencies of Th, aTh and aTreg within CD4+ TILs. (C) Representative plot depicting OX40 levels in aTreg of one patient with pCRC. (D,E) OX40 expression was acquired by flow cytometry (CD8=CD45+CD3+CD4−). (D,E) One-way non-parametric Kruskal-Wallis, including Dunn's post-testing, was performed (pCRC n=13, LM-CRC n=9, HCC=9); *p≤0.05, **p≤0.01, ***p≤0.001. aTh, activated T helper; aTreg, activated regulatory T cell; HCC, hepatocellular carcinoma; LM-CRC, liver metastasis colorectal cancer; PBMC, peripheral blood mononuclear cell; pCRC, primary colorectal cancer; TF, tumor-free; Th, T helper; TIL, tumor-infiltrating lymphocyte.

with multimerized αOX40 IgG2 significantly upregulated CD4+ TIL expansion and showed tendencies to enhance CD8+ TIL proliferation (p=0.13) compared with monomeric αOX40 (figure 2F).

Thus, OX40-mediated TIL expansion was dependent on efficient OX40 crosslinking.

FcγRIIB engagement drives agonistic activity of Fc-engineered αOX40 antibody

Receptor trimerization of TNFR members can be mediated by antibody binding to inhibitory receptor FcγRIIB, as shown for αCD40, αCD95 and αDR5 antibodies.[19 21 22] Thus, we wondered if the poor efficacy of αOX40 IgG2 in our in vitro assay might be attributed

to insufficient FcγRIIB engagement and therefore investigated if Fc engineering can enhance crosslinking capacities of αOX40 antibodies. For this purpose, we Fc-engineered a human αOX40 antibody (αOX40_v12) that contains an IgG1 Fc region comprising six mutations, which resulted in enhanced binding affinity to FcγRIIB[18] while sharing an identical paratope with clinical candidate αOX40 antibody (figure 3A). We then treated TILs with wild-type IgG1 or Fc-engineered αOX40_v12 antibody and measured TIL expansion at the end of the in vitro culture. Compared with its wild-type counterpart, agonistic αOX40_v12 led to significantly increased CD4+ and CD8+ TIL proliferation
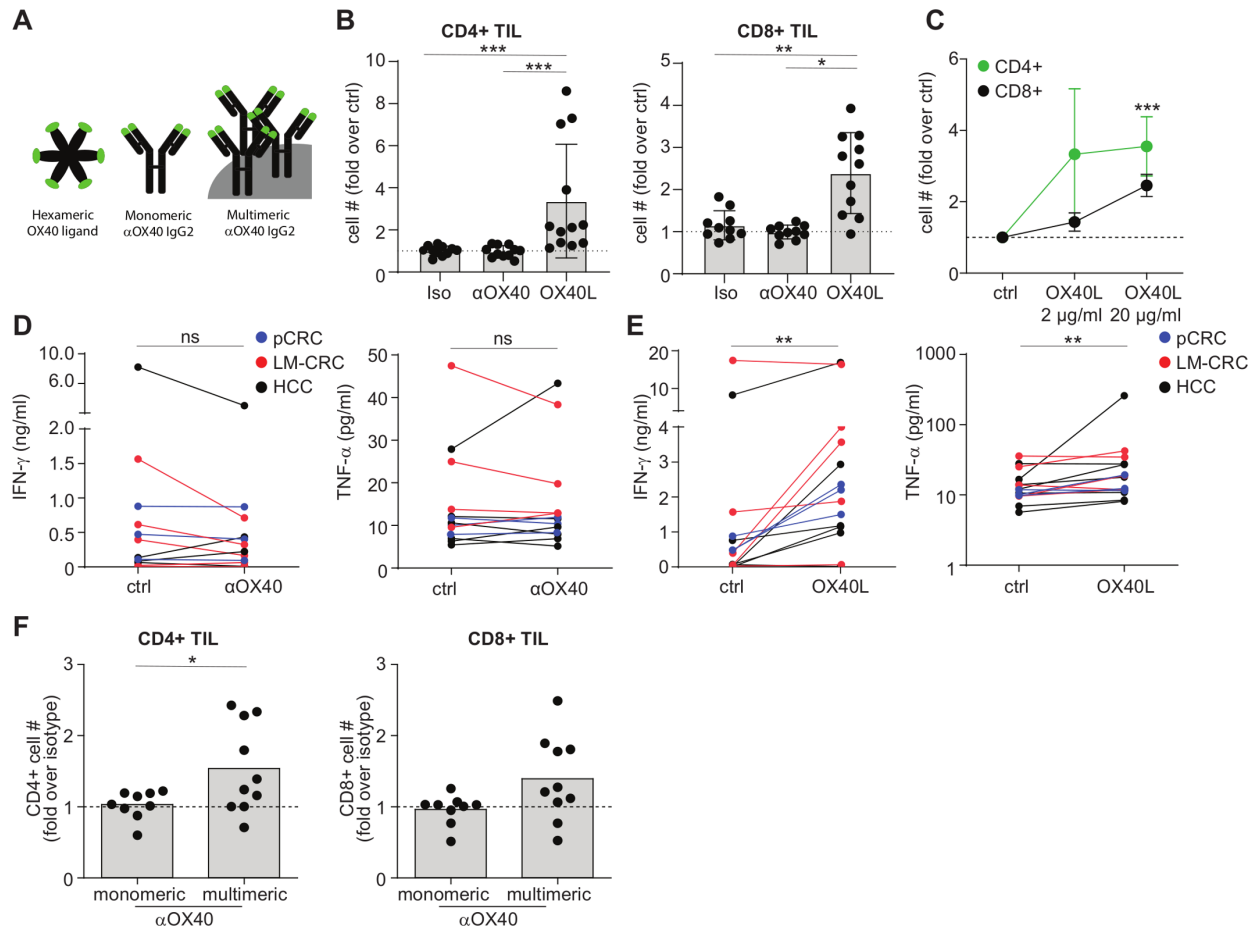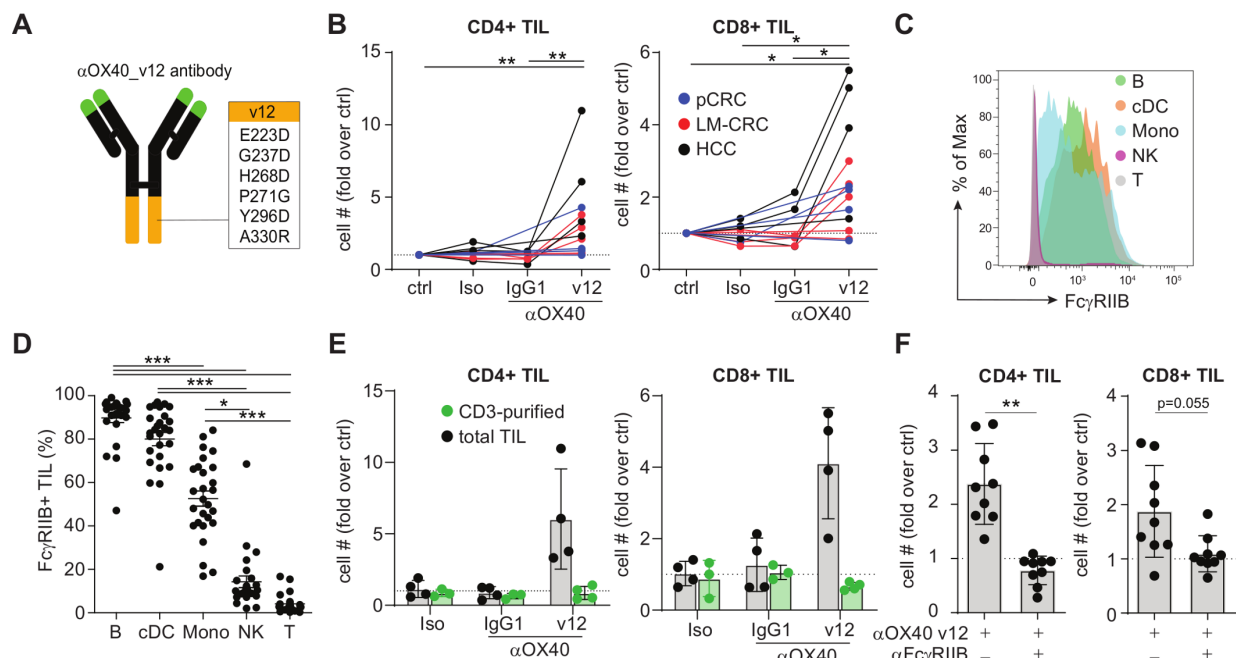
Figure 2 Multimeric OX40 ligation enhances TIL expansion, and activation. Tumor tissues from patients with LM-CRC and HCC were collected; immune cells were isolated and cultured in vitro in the presence of αCD3/CD28 activation beads (ctrl) and additionally (B,C,E) 20 µg/mL or (C) 2 µg/mL OX40L or (B,D,E) 10 µg/mL monomeric αOX40 IgG2 or (F) multimeric αOX40 IgG2 for a period of 8–10 days. (A) Schematic outline of hexameric OX40L, monomeric αOX40 IgG2 antibody and αOX40 IgG2 antibodies conjugated to magnetic beads. (B,C,F) TIL numbers after in vitro cultures were acquired by flow cytometry and normalized to counting beads. (B,C) Relative changes over cell numbers in ctrl are shown (n=11). (D,E) Cytokine levels in culture supernatants were acquired by cytokine multiplex assay; (D) n=12, (E) n=15. (F) Cell numbers are depicted as relative to soluble and bead-conjugated IgG2 isotypes, respectively (n=9). (B) One-way non-parametric Kruskal-Wallis, including Dunn's post-testing and (C–F) non-parametric Wilcoxon test, was performed. *P≤0.05, **P≤0.01, ***P≤0.001. HCC, hepatocellular carcinoma; IFN, interferon; LM-CRC, liver metastasis colorectal cancer; ns, not significant; OX40L, OX40 ligand; pCRC, primary colorectal cancer; TIL, tumor-infiltrating lymphocyte; TNF-α, tumor necrosis factor alpha.

(figure 3B,C) pointing toward an important role of FcγRIIB engagement in OX40 costimulation.

To study the αOX40_v12-induced proliferative effect of different CD4+ subsets, we compared cell numbers of Foxp3– and Foxp3^{hi} CD4+ TILs after in vitro culture. In the majority of samples, Foxp3– CD4+ TILs, responded to a higher degree than Foxp3^{hi} TILs (online supplementary figure S3A). To verify the potential of Th TILs to respond to OX40 costimulation, we cultured CD25-depleted TILs for 8–10 days. We observed a similar expansion of CD4+ TILs in the presence or absence of CD25+CD4+ Treg TILs on day 8–10, suggesting that CD25–CD4+ Th TILs respond to OX40 treatment (online supplementary figure S3B).

We then assessed the frequencies of B cells, conventional dendritic cells (cDC), monocytes/macrophages and natural killer (NK) cells within the CD45+ immune cells (online supplementary figure S4A) and determined expression of FcγRIIB on those cell types as well as T cells (figure 3C,D and online supplementary figure S4C). The majority of B cells and cDC expressed FcγRIIB in tumor, TF tissue and PBMC, while monocytes and NK cells in tumor as well as TF tissue expressed FcγRIIB to a smaller extent (figure 3C,D and online supplementary figure S4B,C). Since T cells were almost devoid of any FcγRIIB expression (figure 3), we compared the effects of αOX40_v12 between the total TIL fraction and CD3-purified TILs, which were activated with equal ratios of αCD3/28 activation beads. While the total TIL fraction showed an increase of CD4+ and CD8+ TIL proliferation on αOX40_v12 costimulation, CD3-purified TILs failed to do so in the absence of FcγRIIB-expressing non-CD3 TILs (figure 3E). Lastly, to verify whether FcγRIIB was the main mediator of OX40 crosslinking, we antagonized FcγRIIB

Figure 3 Fc-engineered αOX40 antibody triggers OX40 costimulation by facilitating FcγRIIB-mediated crosslinking. (A) schematic outline of αOX40 v12 depicting six mutations in the Fc region, resulting in selectively enhanced FcγRIIB affinity. (B–F) Tumor tissues from patients with LM-CRC and HCC were collected; immune cells were isolated and either (D) directly stained or (B,D,F) cultured in the presence of αCD3/CD28 activation beads (ctrl) and additionally αOX40 antibodies or IgG1 isotype for a period of 8–10 days. Parts of the TILs were either (E) purified for CD3+ TILs using Magnetic Cell Separation (MACS) or (F) pretreated with αFcγRIIB blocking antibody. (B,E,F) TIL numbers after in vitro culture were acquired by flow cytometry and normalized to counting beads. Indicated is the FC relative to ctrl-treated cells (B, n=13; E, n=4; F, n=9). (C) Histogram representing FcγRIIB expression on TI B (CD45+ CD14 CD19+), conventional dendritic cells (cDC; CD45+CD3−CD14−BDCA1+), monocytes (mono=CD45+CD3 CD14+), natural killer (NK; CD45+CD3 CD56+) and T (CD45+CD56 CD3+) cells. (D) FcγRIIB expression on TI B, NK and T cells, as well as monocytes and cDC, was measured (n=27). (B,D) One-way non-parametric Kruskal-Wallis, including Dunn's post-testing and (F) non-parametric Wilcoxon test, was performed. *P≤0.05, **P≤0.01, ***P≤0.001. FC, fold change; HCC, hepatocellular carcinoma; LM-CRC, liver metastasis colorectal cancer; pCRC, primary colorectal cancer; TI, tumor-infiltrating; TIL, tumor-infiltrating lymphocyte.

by preincubation with a blocking antibody (humanized clone ch2B6N279Q) prior to culture with αOX40_v12. Blocking FcγRIIB abrogated the αOX40_v12-mediated increase in CD4+ and CD8+ TIL expansion and Ki67 expression (supplementary figures S3F and S4D,E).

Collectively, our data suggest that treatment with Fc-engineered antibody αOX40_v12 induced TIL expansion via FcγRIIB engagement.

OX40 costimulation skews the cytokine and chemokine profile of CD3+ TILs

OX40 engagement on murine and human PBMC-derived T cells has been shown to stimulate production of several cytokines and chemokines, including IFN-γ, IL-4, as well as CCL20.[5 34] To test the impact of OX40 costimulation on the cytokine and chemokine profiles of patient-derived TILs, we analyzed transcriptomic data of sorted CD25−CD4+ TILs, CD25+CD4+ TILs and CD8+ TILs of three patients that were cultured in the absence or presence of agonistic αOX40_v12 antibody. Prior to in vitro culture, CD25+ TILs were purified using MACS and labeled with CellTrace Violet to enable identification after coculture with non-stained CD25− TILs (online supplementary figure S5A,B). Enrichment of Foxp3^{hi} Treg within

CD25+CD4+ TILs at the time of sorting was verified by RNA and protein level (online supplementary figure S5C,D).

Treg-enriched CD25+CD4+ TILs acquired a specific cytokine profile on OX40 engagement (figure 4A). Thereby, *IFNG*, *GZMB*, and *IL21R* were significantly upregulated in CD25+CD4+ TILs, while expression of several cytokines (*IL21*, *IL6*, *IL1B*, and *IL5*) was enhanced but did not reach statistical significance (as determined by p<0.05 and Log2FC>0.3, figure 4A). In accordance with enhanced *IFNG* expression in CD25+CD4+TILs (as well as CD25−CD4+ and CD8+ TILs) on OX40 engagement, IFN-γ protein levels in the culture supernatant were highly enriched (figure 4A,B), in line with a recent report demonstrating the induction of Th1 cytokines in Tregs on OX40 ligation.[35] Gene set enrichment analysis (GSEA) revealed a significant enrichment of gene sets associated with 'IFN-γ response' in CD25+CD4+ TILs (figure 4C) and to a lower extent in CD25−CD4+ and CD8+ TILs (online supplementary figure S6B). Besides, *IL21R* mRNA was significantly upregulated in CD25+CD4+ TILs, while IL-21 protein levels were elevated in supernatants of OX40-treated TIL cultures, although not significantly (p=0.06, figure 4D). Furthermore, we detected tendencies for
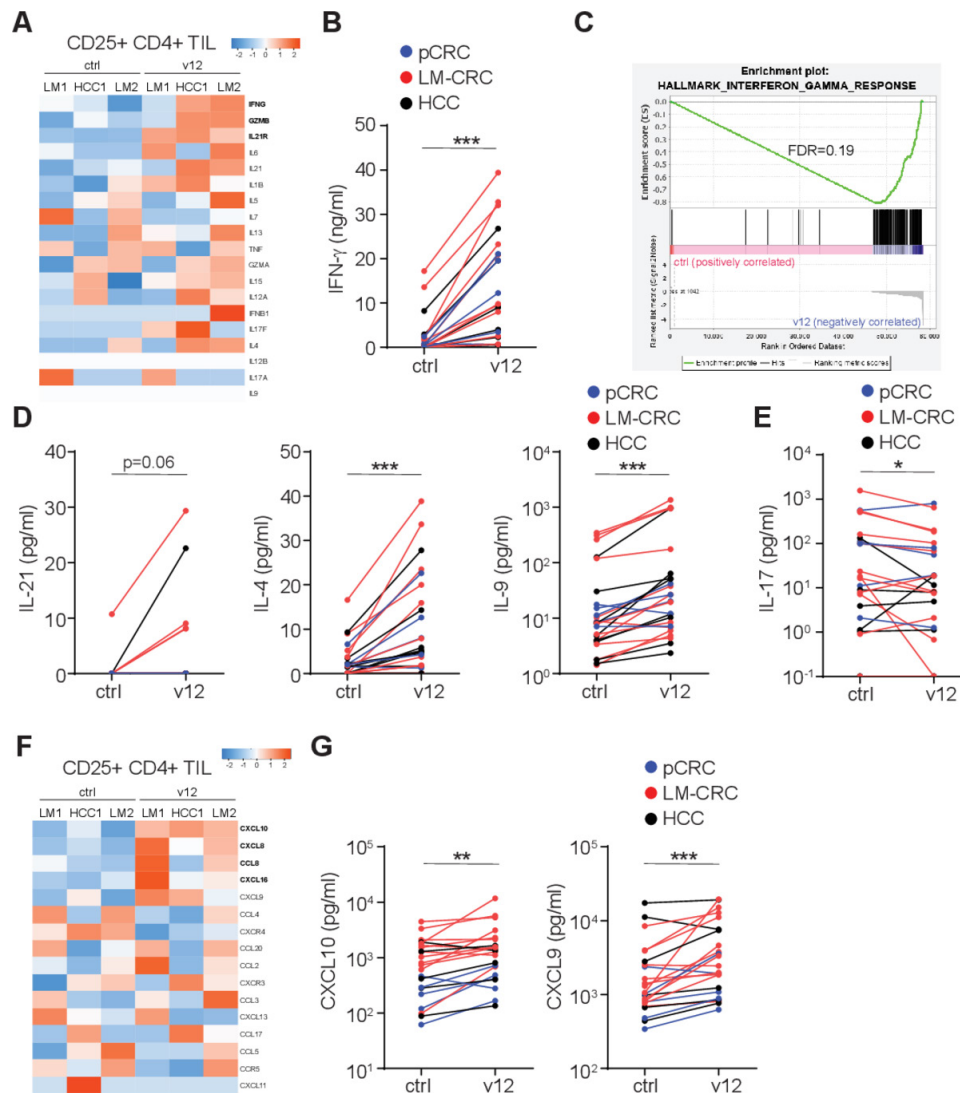
Figure 4 OX40 costimulation reprograms cytokine and chemokine landscape of CD4+ TILs. Tumor tissues from patients with LM-CRC and HCC were collected and immune cells were isolated. (A,F,C) CD25+ TILs were positively selected, labeled with CellTrace Violet (CTV) and cocultured with non-stained CD25− TILs in the presence of αCD3/CD28 activation beads and 10 µg/mL αOX40_v12 antibodies for or left untreated (ctrl). CD25+ CTV+ TILs were sorted, and RNA was isolated after 3–5 days of in vitro culture. (A,F) Heatmap shows color-coded z-scored transcripts per million fragments mapped. Indicated in bold are differentially expressed genes (p<0.05 and log2FC>0.3, n=3). (B,D,E,G) TILs were cultured for 8–10 days in the presence of αCD3/CD28 activation beads (ctrl) or additionally with αOX40_v12 (v12). Cytokine and chemokine levels in culture supernatants were acquired by multiplex assays (B,D,E, n=25; G, n=23). (C) Gene set enrichment analysis (GSEA) was performed according to the Broad Institute with the hallmark 'IFNG response'; indicated is false discovery rate (FDR). (B,D,E,G) Non-parametric Wilcoxon test was performed. *P≤0.05, **P≤0.01, ***P≤0.001. HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; LM-CRC, liver metastasis colorectal cancer; pCRC, primary colorectal cancer; TIL, tumor-infiltrating lymphocyte; FC, fold change.

higher *IL21* mRNA levels in CD25+ and CD25−CD4+ TILs (online supplementary figure S6A), pointing towards an involvement of OX40 costimulation in follicular helper T cell (Tfh) differentiation as previously reported.[36 37]

Moreover, αOX40 treatment led to an increase in secretion of Th2/9 family cytokines, like IL-4 and IL-9 in culture supernatants after 8–10 days, while in some patients, elevated mRNA levels of Th2 cytokines were already detected at early stages of in vitro culture in CD25+CD4+ TILs (days 3–5; figure 4A,D) in accordance with previous reports.[5] In contrast, IL-17 secretion was suppressed in response to OX40 treatment (figure 4E),

pointing towards a distinctive role of OX40 stimulation in Th polarization. OX40 engagement also elevated gene expression of several chemokines, including *CXCL10* (figure 4F) as well as CXCL10 and CXCL9 protein levels in the culture supernatants. These IFN-γ-inducible chemokines were recently demonstrated to be crucial for CD8+ T-cell infiltration in tumors[38 39] (figure 4G).

Lastly, OX40 signaling was described to be implicated in suppression of Treg functions[15 17]; however, Foxp3 protein remained unchanged, while mRNA levels were upregulated in response to OX40 costimulation in CD25+CD4+ TILs (online supplementary figure S7A,B). Furthermore,

in line with a recent report,[35] expression of genes associated with regulatory functions of Treg, including *CTLA4*, *IL10*, and *TGFB1*, were not significantly affected by OX40 treatment in CD25+CD4+ TIL (online supplementary figure S7A), while IL-10 secretion was upregulated in response to OX40 costimulation in culture supernatants (online supplementary figure S7C).

Taken together, OX40 costimulation altered the immunological profile of CD4+ and, to a lower extent, CD8+

TILs by boosting chemokine production as well as Tfh-related, Th1-related and Th2-related cytokines.

OX40 shapes the transcriptional landscape of TILs by inducing prosurvival and chemotaxis pathways

Transcriptomic analyses of TILs cultured in the absence or presence of OX40 costimulation showed a profound transcriptional shift particularly in the CD25+CD4+ TILs as visualized by principal component analysis (figure 5A).
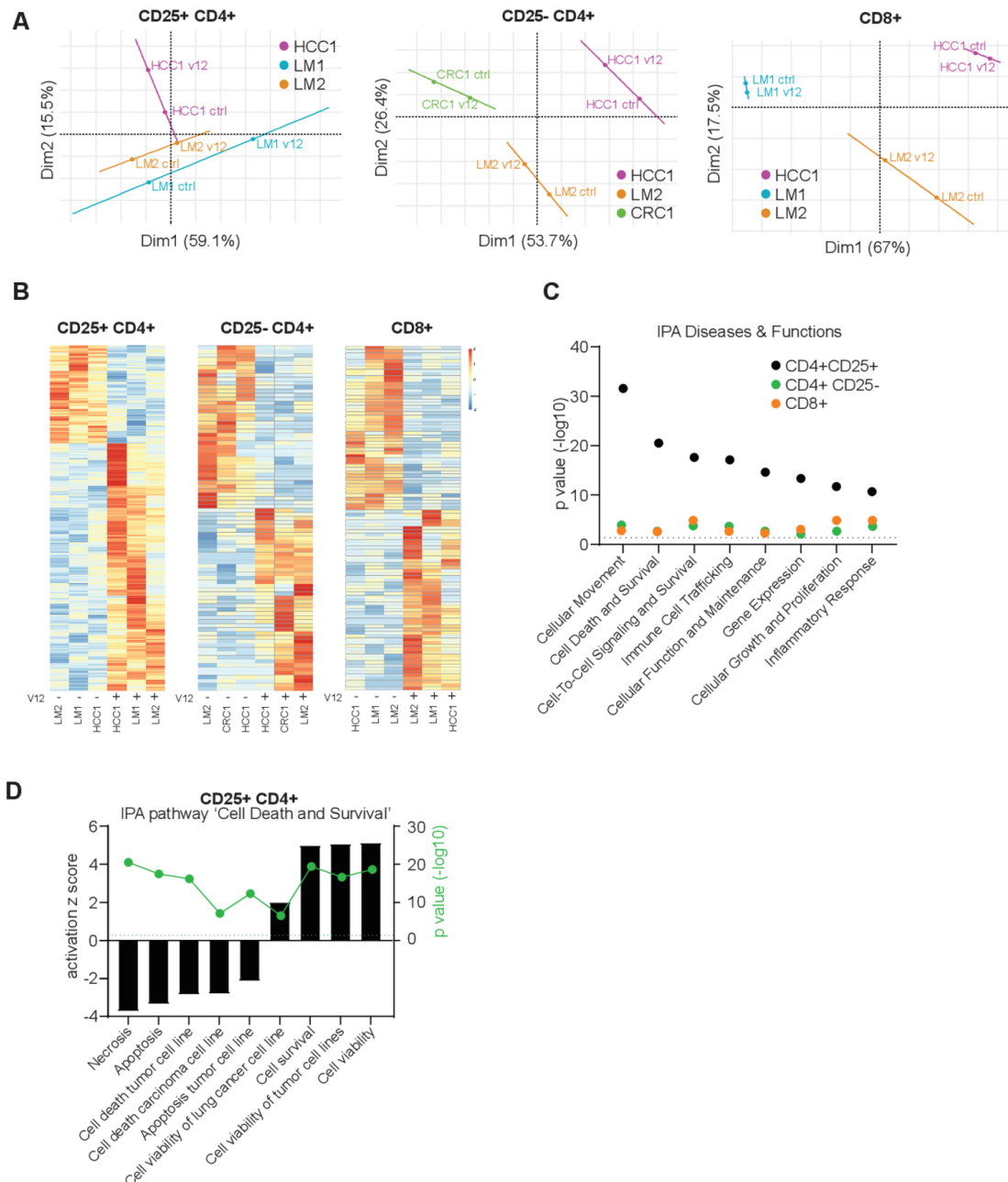
Figure 5 OX40 skews TILs toward a prosurvival and inflammatory transcriptional profile. Tumor tissues from patients with liver metastasis colorectal cancer and HCC were collected and immune cells were isolated. CD25+ TILs were positively selected, labeled with CellTrace Violet (CTV) and cocultured with non-stained CD25– TILs in the presence of αCD3/CD28 activation beads and 10 µg/mL αOX40_v12 antibodies or left untreated (ctrl). CD25+CTV+ TILs, CTV–CD4+ TILs and CD8+ TILs were sorted and RNA was isolated after 3–5 days of in vitro culture. (A) Principal component analysis was performed. (B) Heatmap shows color-coded z-scored transcripts per million fragments mapped. (C,D) Ingenuity pathway analysis (IPA) for DE genes of disease and function pathways. Dotted line indicates threshold for p value of 0.05. (D) Activation z-score and corresponding p values are shown. CRC, colorectal cancer; HCC, hepatocellular carcinoma; LM-CRC, TIL, tumor-infiltrating lymphocyte.

A total of 891 differentially expressed (DE) annotated genes were detected in αOX40_v12-treated versus non-treated CD25+CD4+ TILs (p<0.05 and log2FC>0.3). Thereby, 630 genes were upregulated, while 261 genes were significantly downregulated in response to OX40 engagement (figure 5B). In CD25−CD4+ or CD8+ TILs, 46 or 50 genes were upregulated, whereas 41 or 55 genes were significantly downregulated, respectively (figure 5B). We used Ingenuity pathway analysis (IPA)'s knowledge base to identify over-represented functional pathways in OX40-treated TILs using DE genes. Cellular movement and immune cell trafficking were identified among the top predicted pathways pointing toward an involvement of OX40 in chemotaxis in CD25+CD4+ and, to a lower extent, also in CD25−CD4+ and CD8+ TILs (figure 5C). Furthermore, OX40 costimulation led to a downregulation of apoptosis and cell death pathways, while prosurvival pathways were highly enriched within the DE genes of CD25+CD4+ TILs (figure 5D).

In conclusion, OX40 signaling particularly affected the transcriptional landscape of CD25+CD4+ TILs by inducing prosurvival and cellular movement pathways.

TCR/CD28-mediated TIL preactivation is crucial for responsiveness to OX40 costimulation

Since the observed TIL expansion in response to αOX40_v12 showed high interpatient variability (figure 3B), we tried to identify factors that predicted responsiveness to OX40 costimulation. First, we plotted the ex vivo expression of OX40 on freshly isolated TILs against the respective 'proliferative response' to OX40 costimulation on days 8–10 of culture. Proliferative response was measured as cell number FC of αOX40_v12-treated TILs over non-treated TILs. Surprisingly, there was no correlation between ex vivo OX40 expression on either total CD4+ or CD4+ Foxp3^{hi} TILs to αOX40_v12-triggered proliferative response in CD4+ TILs (online supplementary figure S8A). Nor did ex vivo OX40 expression on CD8 TILs correlate with CD8+ TIL expansion on αOX40_v12 treatment after culture (online supplementary figure S8A). Next, we investigated the impact of underlying CD3/CD28-induced T-cell activation to OX40 responsiveness since OX40 signaling has been shown to maintain initial CD3/CD28-induced prosurvival and proliferative signals in T cells.[5] We treated patient TILs with different αCD3/CD28 cell:bead ratios to achieve a diverse array of baseline in vitro activation levels. Then, we plotted the baseline in vitro activation (depicted as Ki67 expression in control) against the proliferative TIL response after in vitro OX40 treatment. Particularly, CD4+ TILs showed a robust correlation between baseline in vitro activation and OX40-triggered proliferation (figure 6A). Furthermore, using plate-bound αCD3 antibody, we demonstrated that CD3 signals alone were sufficient to render T cells responsive to OX40 costimulation (online supplementary figure S8B).

Since the baseline activation in our assay is mainly derived from αCD3/CD28 activation beads, we evaluated the ex vivo Ki67 expression levels in CD3+ TILs immediately after isolation. In tumor tissue, Ki67 expression levels were variable but increased compared with lymphocytes in TF tissue in both CD4+ and CD8+ TILs (figure 6B). We hypothesized that double expression of Ki67 and OX40 might be a prerequisite of responsiveness to OX40. In fact, OX40 expression was enriched in Ki67+CD4+ and Ki67+CD8+ TILs (figure 6C). Accordingly, ex vivo OX40 and Ki67 expression correlated significantly in CD4+ and CD8+ TILs (figure 6D). To analyze whether pretreatments like radiation or chemotherapy might influence ex vivo Ki67 expression, patients were grouped according to whether or not radiotherapy or chemotherapy was received within 12 months prior to resection or transplantation. CD4+ and CD8+ TILs of patients who underwent previous pretreatment expressed higher levels of Ki67 than those of untreated patients (online supplementary figure S8C).

Taken together, Ki67 expression served as a predictive marker for responsiveness to OX40 costimulation in vitro and might be enhanced by previous radiotherapy and/or chemotherapy.

DISCUSSION

OX40 costimulation represents a promising target for cancer immunotherapy as demonstrated in several murine models.[5] However, the clinical responses to agonistic OX40 immunotherapy observed in clinical trials did not meet the expectations that were built by the success of αOX40 therapy in murine tumor models. In a phase I study (NCT02315066), only 2 out of 45 patients developed a partial response.[27] Thus, we applied ex vivo and in vitro assays with human TILs to determine (1) OX40 expression on TIL subsets, (2) the impact of OX40 costimulation on TIL functions and proliferation, and (3) the agonistic activities of a current clinical candidate αOX40 antibody, as well as a potential next-generation antibody.

In a cohort of 96 patients with HCC and CRC, we first demonstrated that OX40 expression was increased on tumor-derived, proliferating CD4+ and CD8+ TILs. Particularly, aTh and aTreg overexpressed OX40 compared with their counterparts in surrounding tumor-free tissue and blood. Thus, we expanded the previous knowledge of OX40 expression on human TILs,[9–12] further highlighting the role of OX40 as a promising target for selectively targeting TILs.

In accordance with disappointing clinical trial results,[27–29] the clinical candidate human αOX40 IgG2 antibody failed to stimulate TIL proliferation or cytokine secretion in our in vitro expansion assay, while treatment with OX40L resulted in TIL expansion and increased secretion of proinflammatory cytokines. The clinical αOX40 antibody and OX40L differ with regard to their structure: αOX40 antibody is monomeric, whereas OX40L is of hexameric shape. Thus, when we mimicked a multimeric form by conjugating αOX40 antibodies to
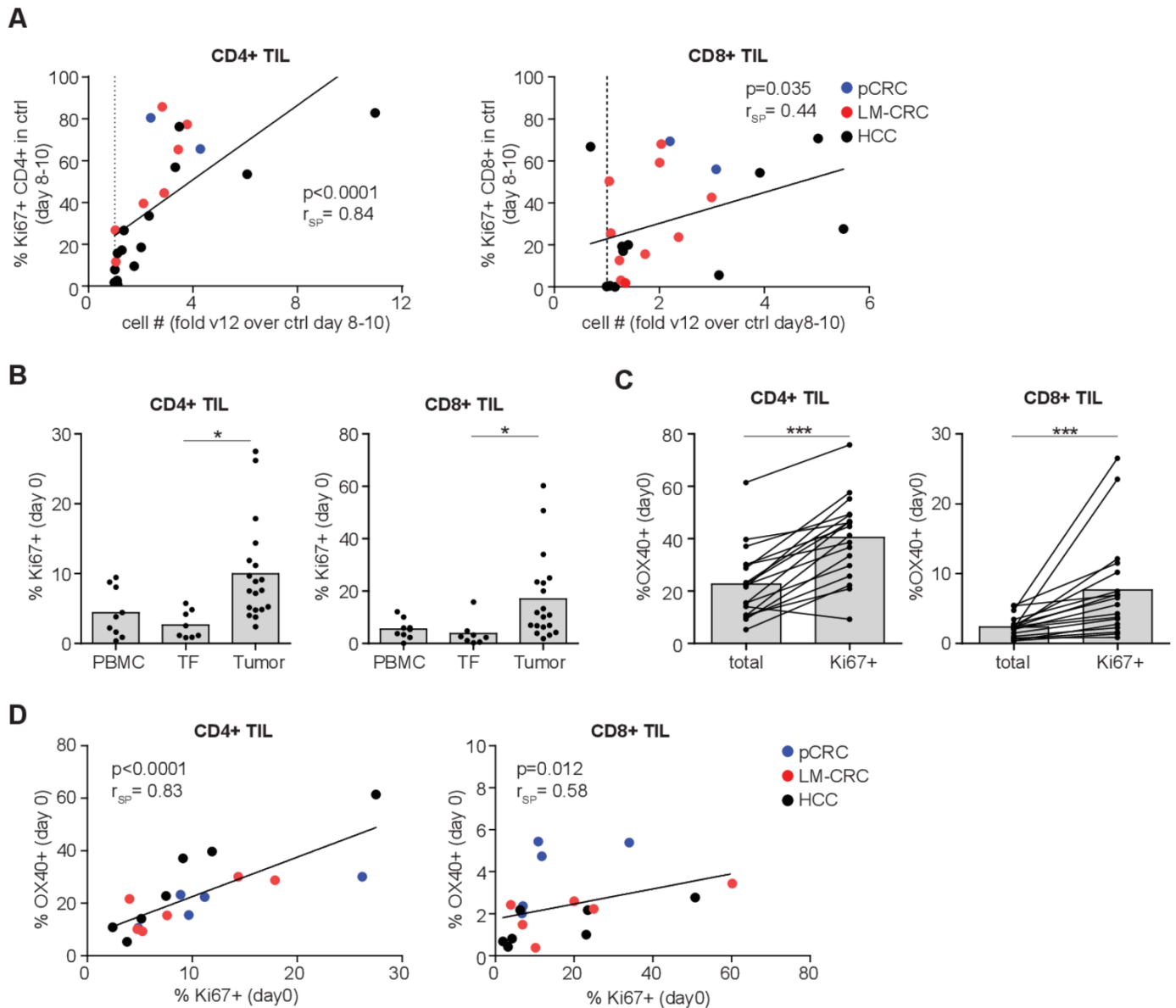
Figure 6 Ki67 expression predicts TIL responsiveness to OX40 stimulation. Tumor tissues from patients with LM-CRC and HCC were collected; immune cells were isolated and (B–D) either immediately stained or (A) cultured in vitro in the presence of αCD3/CD28 activation beads (ctrl) with or without αOX40_v12 antibody for a period of 8–10 days. (A) X-axis shows fold change in cell numbers of αOX40_v12-treated TILs relative to ctrl-treated TILs after in vitro stimulation. Y-axis represents Ki67 levels in ctrl-treated TILs after 8–10 days of in vitro culture (n=24). (B) Flow cytometric evaluation of Ki67 expression in freshly isolated lymphocytes (n=19). (C) OX40 expression on freshly isolated TILs was acquired by flow cytometry (n=19). (D) Correlation of OX40 and Ki67 expression in freshly isolated TILs (n=19). (A,D) Correlation analysis according to Spearman and (B) one-way non-parametric Kruskal-Wallis, including Dunn's post-testing and (C) non-parametric Wilcoxon test were performed. *P≤0.05, ***P0.001. HCC, hepatocellular carcinoma; LM-CRC, liver metastasis colorectal cancer; TIL, tumor-infiltrating lymphocyte.

magnetic beads, αOX40 antibody treatment successfully enhanced TIL expansion.

Physiologically, antibody multimerization is driven by FcγRIIB engagement.[40] Hence, we treated TILs with an Fc-engineered αOX40 human IgG1 antibody, termed αOX40_v12, that contains six mutations leading to increased affinity to FcγRIIB.[18] In contrast to the paratope-sharing wild-type counterpart, Fc-engineered αOX40_v12 potently stimulated TIL proliferation and activation in this study. Blocking FcγRIIB (which was abundantly expressed on TI myeloid and B cells) by a neutralizing antibody abrogated the αOX40_v12-induced effects, demonstrating the dependence on FcγRIIB engagement. Agonistic human αCD40_v2141-11 antibody is another example of an Fc-engineered αTNFR antibody with selectively enhanced affinity to FcγRIIB leading to therapeutic efficacy in mouse models.[41] Fc-engineered agonistic antibodies with improved affinity to FcγRIIB need to undergo rigorous toxicity testing since FcγRIIB-triggered systemic immune toxicity contributed to the severe cytokine release syndrome during the phase I trial of αCD28 IgG4 antibody.[42] Therefore, fully humanized FcR mouse model

might support the identification of non-toxic dosing regimens as demonstrated for αCD40 antibodies.[43]

OX40 costimulation has been implicated regulating a wide array of cellular functions ranging from the induction of Th1 and Th2 family cytokines, to the decrease of suppressive capacity in Tregs.[17 44 45] We observed high expression levels of OX40 on TI Foxp3^{hi} CD45RA- aTreg which represent a major contributor of tumor immune evasion. Therefore, we used transcriptome analyzes to reveal the role of OX40 costimulation on the transcriptional network of CD4+ subsets, as well as CD8+ TILs. However, the isolation of aTreg after in vitro culture posed an irreconcilable challenge due to αCD3/CD28 signal-mediated upregulation of CD25 expression on Th cells.[32] Therefore, we isolated CD25+ TILs ex vivo, stained them with a cell tracker dye and cocultured them together with non-stained CD25– TILs (including FcγRIIB+ accessory cells) in the presence or absence of αOX40_v12 antibody. We acknowledge that CD25+ TILs are a hetergenous population, that is enriched for Treg (60-70%) but also contains Foxp3^{int} aTh and Foxp3– Th cells. Nevertheless, we observed a shift in the transcriptional landscape of immune mediators early after OX40 treatment (days 3–5), represented by increased expression of Th1/2, as well as Tfh cytokines and proinflammatory chemokines, including CXCL8 and CXCL10 in the Treg-enriched CD25+CD4+ TIL fraction and to a lower extent in Th and CD8+ TILs. The Tfh cytokine IL-21 has been associated with increased survival by contributing to the formation of tertiary lymphoid structures,[46] while CXCL9 and CXCL10 are driver chemokines for Th and CD8+ infiltration into tumors.[39 47] While a shift toward a proinflammatory/Th1-like profile was observed, suppressive signatures, including Foxp3 protein, and *TGFB* and *CTLA4* gene expression, remained stable in OX40-treated CD25+CD4+ TILs, in line with a recent report.[35] Thus, we hypothesize that human TI Tregs are shifted toward a Th1-like profile but maintain the expression of key regulatory molecules on OX40 costimulation, suggesting that combination therapies with agonistic αOX40 antibodies and TGF-β inhibitors or CTLA-4 blockade might be a promising therapeutic option.

Due to high heterogeneity of immunological responses, the following question prevails in the immunotherapy field: how are responders versus non-responders identified prior to therapy? Even though the implementation of PD1 and CTLA4 checkpoint blockade profoundly changed antitumor treatment, the response rates especially in most solid tumor types are considerably low.[3 48] A multitude of factors have been identified that contribute in distinguishing responders from non-responders, including PDL1 expression, tumor mutational load, presence of additional immune-inhibitory pathways as well as T-cell infiltration, yet the predictive accuracy is low.[48] We investigated the predictive potential of αCD3/CD28-mediated T-cell activation for responsiveness to OX40 treatment. We demonstrated that OX40 engagement only costimulated preactivated, Ki67-expressing TILs but was unable to stimulate TILs that are devoid of underlying CD3/CD28 signaling.

Of note, ex vivo Ki67 expression in TILs was highly variable but significantly correlated with OX40 expression suggesting that αOX40 immunotherapy mainly targets cells that might be sensitive for OX40 costimulation. When studying the patients' prior treatment regimen, it became clear that a previous chemotherapy or radiotherapy significantly correlated with enhanced Ki67 expression in TILs. Thus, our data support the concept of combining OX40 immunotherapy with chemotherapy or radiotherapy to render tumor antigens accessible to immune surveillance and boost T-cell priming and activation at the tumor site. Currently, several clinical studies are under investigation,[49] including a phase I clinical trial combining αOX40 antibody with radiation in the treatment of metastatic breast cancer (NCT018629000).

The strength of our study is the use of patient-derived TILs, but this entails several limitations. First, we obtained TILs from surgically resected tumors, while only a minority of patients with HCC are eligible for resection.[50] Thus, the cohort of patients with HCC included in this study does not represent the majority of patients with HCC. Second, purification of TILs partially revokes their access to antigen, which is substituted by addition of low amounts of polyclonal αCD3/CD28 activation beads in our study, thereby altering the TIL activation status during the in vitro culture. This may contribute to the observation that ex vivo OX40 and ex vivo Ki67 (data not shown) do not correlate with in vitro OX40-mediated responses. Lastly, considering the low expression of OX40 on CD8+ TILs ex vivo, it remains unclear if CD8+ TILs responded directly or indirectly via CD4+ help to OX40 costimulation, as suggested by previous studies.[5]

Here we demonstrate for the first time that an Fc-engineered agonistic αOX40 antibody reinvigorates CD4+ and CD8+ TIL expansions, as well as cytokine and chemokine secretions of human TILs. By comparing conventional wild-type with an Fc-engineered antibody, we show that OX40 multimerization plays a key role in efficient signal transduction, which is driven by FcγRIIB engagement. OX40 costimulation is thereby dependent on CD3/CD28 signals and skews the transcriptional profile of TI T cells toward a proinflammatory pattern. Thus, this study reveals important insights in the requirements and mechanisms of agonistic αOX40 treatment and may therefore contribute to harnessing the full potential of OX40 cancer immunotherapy.

Author affiliations
[1]Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands
[2]Pathology, Erasmus MC-University Medical Center, Rotterdam, Netherlands
[3]Pfizer Cancer Immunology Discovery, Pfizer Inc, San Diego, California, USA
[4]Surgery, IJsselland Hospital, Capelle aan den IJssel, Netherlands
[5]Surgery, Erasmus MC-University Medical Center, Rotterdam, Netherlands

ORCID iDs
Lucia Campos Carrascosa http://orcid.org/0000-0003-3321-7153
Adriaan A van Beek http://orcid.org/0000-0003-0786-1682
Shahram Salek-Ardakani http://orcid.org/0000-0002-2336-8452

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