Aus der Abteilung für Klinische Pharmakologie
Direktor: Prof. Dr. med. Stefan Endres

Medizinischen Klinik und Poliklinik IV
Klinik der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Martin Reincke

The role of the inducible T cell co-stimulator in T cell bispecific antibody-mediated anti-tumor efficacy

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Ramona Murr
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Berichterstatter:	Prof. Dr. med. Sebastian Kobold
Mitberichterstatter:	PD Dr. med. Johanna Tischer
	Prof. Dr. med. Martina Rudelius

Mitbetreuung durch die promovierten Mitarbeiter:
Marina Bacac, PhD,
Dr. rer. physiol. Tanja Fauti
Prof. Dr. med. Stefan Endres

Dekan: Prof. Dr. med. dent. Reinhard Hickel

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1 Abstract

Cancer immunotherapy is transforming the way cancer is treated. Different from the other, more traditional cancer therapies that are targeting tumor cells, cancer immunotherapy engages with the immune system to enable a better recognition and therefore killing of tumor cells. Treatment with T cell bispecific antibodies (TCB) has the potential to improve the survival of late stage cancer patients. However, many tumors escape by resistance mechanisms that prevent immune surveillance, for instance upon expression of co-inhibitory ligands on the tumor cell surface. Blocking the interaction of these ligands with inhibitory T cell proteins, such as CTLA-4 or PD-1, showed promising anti-tumor activity. The activation of T cell co-stimulatory receptors serves as another approach to boost a patient's immune response.

ICOS, a member of the Ig-like receptor family, is one such T cell co- stimulatory receptor expressed on Treg, Tfh and Th17 at baseline, as well as on literally all T cell subsets upon activation. In the present study a systematic assessment of ICOS expression on different healthy and tumor-derived immune subsets, on resting and activated T cells, activated in presence of different stimuli was performed. It could be shown that ICOS is comparably upregulated upon stimulation by anti-CD3 – anti-CD28 or by TCB. Moreover, for the first time, the potential therapeutic application of targeting ICOS with an agonistic anti-ICOS antibody in combination with TCB treatment was investigated. Several formats of a human, targeted ICOS antibody were generated and demonstrated an increased T cell activation, cytokine secretion and central memory T cell differentiation upon ICOS co-stimulation in the context of *in vitro* TCB activation. Single cell transcriptome analysis supported these findings and moreover revealed that the underlying signalling pathways of TCB and ICOS co-stimulation seem to be very similar, hence ICOS co-stimulation increases the magnitude of TCB-mediated T cell activation.

Further investigations into the possible combination of ICOS signalling with checkpoint inhibition is recommended to better address the flexibility and dynamics of the adaptive anti-tumor immune system.

2 Zusammenfassung

Die Immuntherapie gewinnt in der Krebsbehandlung zunehmend an Bedeutung. Bei dieser Art von Behandlung ist es das Ziel, das Immunsystem des Krebspatienten zu (re-) aktivieren und somit die Tumorzellen zu bekämpfen.

Der Einsatz T-Zell bispezifischer Antikörper (TZB) hat Potenzial zur Behandlung fortgeschrittener Tumorerkrankungen. Allerdings entwickeln viele Tumore Resistenzmechanismen, die eine Erkennung durch das Immunsystem verhindern oder die Immunantwort durch die Sekretion inhibierender Botenstoffe und die Expression spezifischer Liganden supprimieren. Präklinische Daten weisen darauf hin, dass die Kombination mit Antikörpern, welche T-Zell inhibierende Rezeptoren blockieren, die Therapieeffizienz verbessern können. Ein weiterer therapeutischer Ansatz ist die Aktivierung stimulierender T-Zell-Rezeptoren, um die Immunantwort zu verstärken. Dazu zählt der induzierbare T-Zell-Kostimulator (ICOS), der auf allen aktivierten T-Zellen exprimiert wird und nach Bindung an seinen Liganden die Proliferation und Reifung der T-Zelle induziert.

In dieser Arbeit wurde die ICOS-Expression auf verschiedenen Immunzellen gesunder Donoren oder Tumorpatientenproben bestimmt. Es konnte gezeigt werden, dass ICOS auf Tumor infiltrierenden T-Zellen hochreguliert und gemeinsam mit CTLA-4 und PD-1 exprimiert wird. Desweiteren ist ICOS nach T-Zell Aktivierung durch anti-CD3 -anti-CD28 Antikörper oder TZB gleichermassen exprimiert. Um den therapeutischen Nutzen des ICOS Signalweges im TZB Kontext besser verstehen zu können, wurden verschiedene, neuartige ICOS Antikörper im monovalenten und bivalenten Format generiert und in Kombination mit TZB auf ihre immunstimulierende Aktivität getestet. Die *in vitro* Kombination von TZB und ICOS Kostimulierung durch neuartige, humane ICOS Antikörper resultierte in erhöhter T-Zell Aktivierung, gesteigerter Zytokinsekretion und T-Zell Differenzierung. Gensignaturen auf Einzelzellebene deuteten darauf hin, dass besonders Gedächtnis-T-Zellen nach Stimulierung durch ICOS-Antikörper aktiviert werden, die durch TZB und ICOS Kostimulierung aktivierten Signalwege prinzipiell vermutlich aber ähnlich sein müssen.

Der nächste Schritt wäre die Evaluierung der Kombination im Mausmodell. Neueste Ergebnisse der klinischen und präklinischen Forschung deuten desweiteren auf eine

Kombination des ICOS-Signalweges mit T-Zell-Inhibitoren hin, um noch besser die Komplexität und Anpassungsfähigkeit des adaptiven Immunsystems zu adressieren.

3 Introduction

3.1 Cancer immunotherapy

Cancer is the second leading cause of death globally and every year more cases are diagnosed (World Health Organization, 2018). For many years, cancer treatment was tumor-centric, focusing on approaches such as surgery, radiotherapy, chemotherapy and targeted therapy (Chabner *et al.*, 2005, Kobold *et al.*, 2015). However, during the last decades the immune system has emerged as a promising, novel therapeutic target for cancer treatment (Hanahan *et al.*, 2011, Kobold *et al.*, 2015, Leach *et al.*, 1996).

Our immune system`s main function is the continuous recognition and control of foreign agents. These can be pathogens such as bacteria or viruses, but also cancer cells that arise from tumor-specific mutations distinguishing a malignant cell from the body`s own healthy tissues. The fact that cancerous cells can be recognized and eliminated by the host's immune system is termed immune surveillance. The first proof of the immune system`s role in fighting cancer was presented in 1890 by William Coley. He observed that patients suffering from bacterial infections, having a pre-activated immune system, were superior in rejecting tumor cells (Ai *et al.*, 2015). Moreover, a higher tumor burden can be observed in individuals whose immune system is suppressed (Delves *et al.*, 2012). Various mechanisms are known that allow the tumor to evade the immune system`s control. This so called immune escape can occur as a consequence of several factors: (i) deficient tumor antigen recognition by effector immune cells due to down-modulation of tumor antigen; poor priming and activation of T cells in the secondary lymphoid organs due to up-regulation of immune checkpoint receptor ligands; (ii) deficient trafficking or infiltration of T cells into the tumor; recruitment of suppressive tumor-associated cells (as regulatory T cells (Treg cells), myeloid suppressor cells or tumor-associated macrophages); and (iii) the secretion of soluble factors such as cytokines and chemokines (Beatty *et al.*, 2015, Dunn *et al.*, 2004, Ventola, 2017a).

Consequently, the main goal of cancer immunotherapy is to revive the suppressed immune system to eliminate cancer cells. This can be achieved by various approaches reaching from activation of adaptive and innate immune system components to neutralizing suppressive immune mechanisms (Suzuki *et al.*, 2016, Ventola, 2017a, Ventola, 2017b, Ventola, 2017c).

Strategies to enhance the patient's immune response to fight cancer comprise the administration of cytokines, chemokines or growth factors that regulate immunity and inflammation. This non-specific approach can be interferon alpha (IFN-α) or interleukin-2 (IL-2) (Berraondo *et al.*, 2018, Fisher *et al.*, 2000). Cellular approaches include cancer vaccines displaying cancer antigens and thereby stimulating the immune system`s attack to recognize cancer (van Dodewaard-de Jong *et al.*, 2016); cell-based therapies such as allogenic human stem cell transfer and adoptive T cell transfer. Neutralization of suppressive immune mechanism include the use of monoclonal antibodies that bind a tumor antigen and directly kill the tumor cell via mechanisms as antibody derived cellular cytotoxicity (e.g. rituximab (Maloney, 2001) or by being conjugated to toxic particles (Beck *et al.*, 2017, Suzuki *et al.*, 2016, Ventola, 2017a). During the last decade special attention was given to checkpoint inhibitors. Immunoinhibitory T cell receptors, referred to as immune checkpoint receptors, such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1) guard from unwanted, self-directed immune responses by downregulation of T cell activation. Tumor cells make use of this mechanism by the up-regulation of immune checkpoint receptor ligands. This can turn the patient`s immune system off and promote tumor progression (Korman *et al.*, 2006, Mahoney *et al.*, 2015). Antibodies binding CTLA-4 or PD-1 and thereby blocking the inhibitory axis between tumor and T cells resulted in durable responses in patients with advanced cancer (Brahmer *et al.*, 2012, Schadendorf *et al.*, 2015). Checkpoint inhibitors targeting CTLA-4 and PD-1 have been designated as important breakthroughs in the evolution of cancer immunotherapy (Couzin-Frankel, 2013) and honoured with the Nobel prize in physiology or medicine 2018.

3.2 Relevance of T cells for cancer therapy

For long-term protection against pathogens, adaptive immunity mediated by B and T lymphocytes is needed. The adaptive immune response is based on re-arrangement of B and T cell receptors to allow specific recognition of surface protein structures, so called antigenic epitopes. Maturation of T cells occurs in the thymus by positive and negative selection. As a result, naïve T cells arise, carrying T cell receptors (TCR) that allows specific recognition of ingested antigens presented in the grooves of the major histocompatibility complex (MHC) on antigen-presenting myeloid cells. T cells can be classified into CD8+ cytotoxic T cells (Tcyt) or CD4+ helper T cells (Th). Moreover, differentiated lineages of Th cells can be

distinguished based on their cytokine secretion pattern: Th1 cells (IFN-γ, IL-2), Th2 cells (IL-4, IL-5, IL-10), Th9 cells (IL-9, IL-10) or Th17 cells (IL-17A, IL-17F, IL-21, IL-22) (Dong *et al.*, 2015, Geginat *et al.*, 2014).

Naïve T cells circulate through the body until they recognize MHC-presented peptide on antigen presenting cells (Fujimi *et al.*) matching their TCR. Following this initial T cell activation, a network of co-stimulatory as well as co-inhibitory receptors regulate the T cell response (Figure 1).

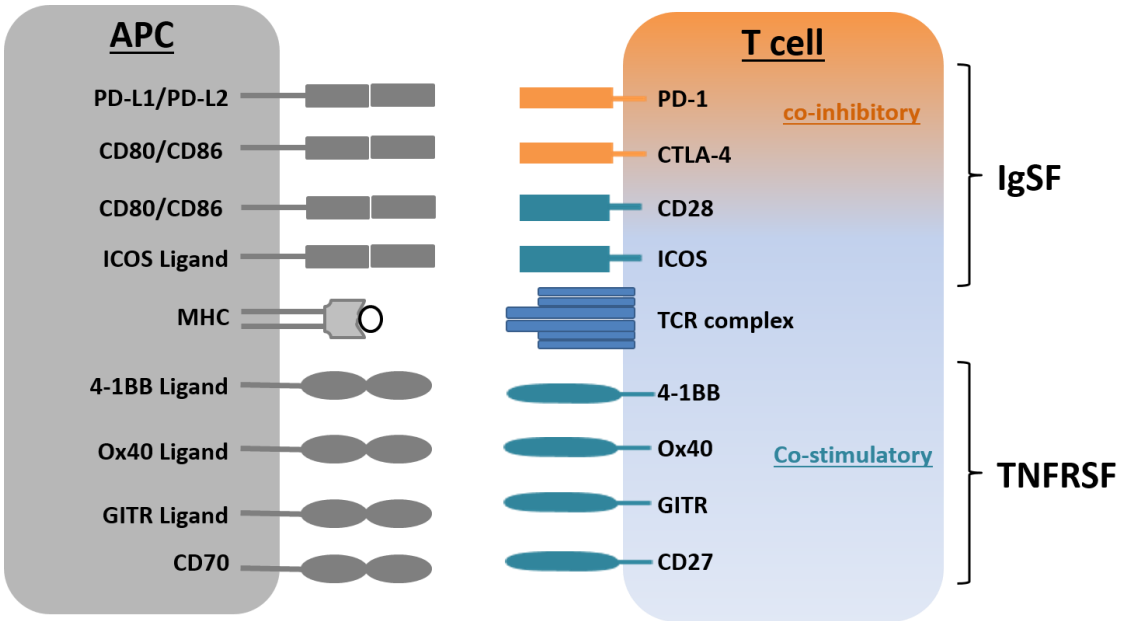

Figure 1: Schematic overview of T cell co-stimulatory and co-inhibitory receptors Upon T cell receptor (TCR) engagement with major histocompatibility complex (MHC) presented peptides on antigen-presenting cells (APC) a network of immunomodulatory, secondary receptors further regulating T cell activation and tolerance. Co-stimulatory (blue) as well as co-inhibitory (orange) receptors expressed on T cells and their corresponding ligand on APC are indicated. The shape indicates members of the tumor necrosis factor receptor superfamily (TNFRSF) or the immunoglobulin superfamily (IgSF). Figure has been adapted from an illustration published by (Mahoney *et al.*, 2015).

To ensure safe stimulation of the adaptive immune system, T cell activation requires a secondary, co-stimulatory signal. Co-signalling molecules can be subdivided into the tumor necrosis factor receptor (TNFR) or the the immunoglobulin (IgG)-like superfamily. Numerous members of the TNFR superfamily such as 4-1BB, Ox40, glucocorticoid-induced TNFR-related protein (GITR) and CD27 deliver stimulatory signals. However, the most prominent co-stimulatory receptor is the constitutively expressed cell-surface receptor CD28.

Binding of the IgG-like superfamily member CD28 to its ligand CD80 or CD86 expressed on APC augments survival and proliferation of T cells. In order to control CD28-mediated T cell expansion, its inhibitory counter receptor CTLA-4 competes with CD28 for binding to CD80 and CD86 and thereby prevents overstimulation of the immune system (Esensten *et al.*, 2016, Rudd *et al.*, 2009). Besides CTLA-4, engagement of the co-inhibitory receptor PD-1 with its ligand PD-L1 or PD-L2 expressed on APC or tumor cells can also transduce a signal that inhibits T cell activation and proliferation. In addition to the prototypic, co-stimulatory role of CD28 for T cell activation, the inducible T cell co-stimulator (ICOS) is another co-stimulatory receptor belonging to the immunoglobulin (IgG)-like superfamily. Given the important role of IgG-like superfamily members as targets for cancer immunotherapy, ICOS serves as an interesting, novel target for modulation of immune response by T cell co-stimulation (Mahoney *et al.*, 2015).

3.3 Inducible T cell co-stimulator (ICOS)

ICOS is a co-stimulatory cell surface receptor that is upregulated upon TCR engagement. Belonging to the IgG-like superfamily, ICOS is structurally related to CD28 and CTLA-4. ICOS is expressed as a disulfide-linked homodimer consisting of two single extracellular immunoglobulin variable-like (IgV) domains coupled to a transmembrane domain and a cytoplasmic tail (Hutloff *et al.*, 1999). The expression of ICOS is described to be restricted to T cells (Ogasawara *et al.*, 2002). Low levels of ICOS can be found on resting naïve Th1 and Th2 effector T cell populations (Paulos *et al.*, 2010) while higher levels are expressed on resting Th17, follicular helper T cells (Tfh) and Treg cells. However, unlike CD28, which is constitutively expressed on naïve T cells, ICOS is strongly upregulated on all T cell subsets by stimulation of the TCR complex (Hutloff *et al.*, 1999, Yoshinaga *et al.*, 1999).

ICOS binds exclusively to its ligand, the so-called ICOS ligand (ICOSL, B7H2, B7RP-1), which is expressed on B cells, macrophages, dendritic cells (DC), and non-immune cells treated with TNF-α (Simpson *et al.*, 2010, Swallow *et al.*, 1999). Even though structurally related, ICOS cannot bind, nor is it activated by, the ligands for CD28 and CTLA-4. This can be explained by the lack of amino acid MYPPY motif in ICOS which is necessary for CD28 and CTLA-4 to bind their ligands CD80 (B7-1) and CD86 (B7-2). However, ICOSL has been described to bind weakly to both CD28 and CTLA-4 (Yao *et al.*, 2011).

Ligation of ICOS with ICOSL induces intracellular signalling through the phosphatidylinositol-3 kinase (PI3K) and AKT pathways (Hutloff *et al.*, 1999). PI3K-AKT signalling regulates numerous processes, including cell growth, differentiation, survival, proliferation, migration and cell metabolism (Slomovitz *et al.*, 2012). The cytoplasmic tail of ICOS carries a unique YMFM SH2 motif. Following ICOSL binding the p50α and p85α regulatory adaptor subunits are recruited to this YMFM SH2 motif. The preferred recruitment of p50α results in greater PI3K lipid kinase activity and phosphorylation of AKT (Fos *et al.*, 2008). In contrast, the p85α directly binds and translocates osteopontin (OPNi) to the nucleus resulting in preservation the Tfh lineage defining factor Bcl6. Another ICOS specific signalling pathway is the activation of the NFkB member TANK-binding kinase 1 (TBK1) that gets recruited by the ICOS proximal (IProx) motif. TBK1 was shown to directly control maturation, function and phenotype maintenance of germinal center Tfh cell and B cell responses (Pedros *et al.*, 2016, Wikenheiser *et al.*, 2016). The ICOS-ICOSL pathway also plays a central role in Th1 as well as Th2 responses (Greenwald *et al.*, 2002, Maazi *et al.*, 2015) during autoimmunity, bacterial infections (Marriott *et al.*, 2015), graft rejection (Sato *et al.*, 2013) and recently a growing body of literature is highlighting the role of ICOS signalling in tumor cell rejection (Hubbard *et al.*, 2005, Metzger *et al.*, 2016, Mo *et al.*, 2017, Odegard *et al.*, 2008, Zamarin *et al.*, 2017).

Two classes of therapeutic ICOS antibodies are currently being tested in clinical or pre-clinical approaches. These can be differentiated by their mode of action (see Figure 2). The first class of antibodies (Figure 2, A) induces Fc-mediated lysis of ICOS+- T cells (preferentially Treg cells). Simultaneous binding to ICOS and binding of the antibody's wild-type Fc part to Fc receptors (FcR) expressed on NK cells or macrophages, leads to antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). Lytic proteins, such as perforins or granzymes being released by NK cells or macrophages result in lysis of the ICOS+- T cells. The second class of ICOS antibodies (Figure 2, B) induces ICOS agonism upon crosslinking of ICOS+- T cells by binding to an ICOS antigen binding domain and a target antigen binding domain. The introduction of a genetically modified, inert Fc avoids Fc-mediated ADCC or ADCP (Delves *et al.*, 2012).

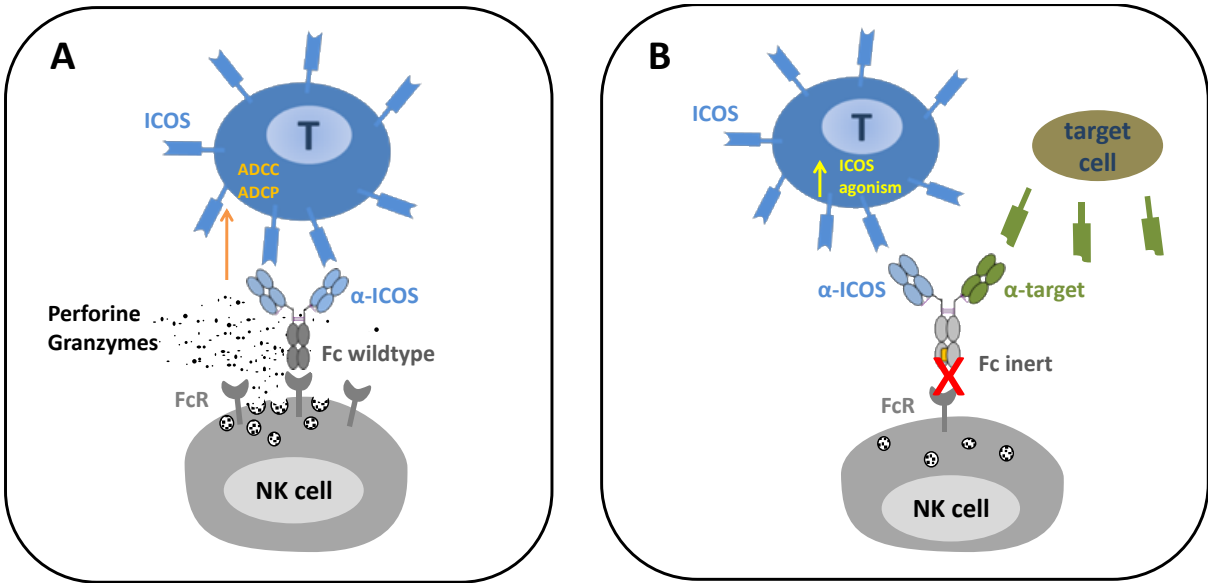

Figure 2: Schematic overview of two classes of therapeutic ICOS antibodies (A) ICOS antibodies mediating lysis of ICOS+- T cell via antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). Crosslinking of ICOS antibodies upon binding to ICOS+ on T cell and Fc receptor (FcR) on NK cell or macrophages results in lysis of ICOS+- T cell mediated by the release of lytic proteins (perforine and granzymes). (B) ICOS antibodies mediating ICOS agonism. Crosslinking of ICOS antibodies upon binding to ICOS+- T cell and target cell induced ICOS agonism of the ICOS+ T cell. No FcR-mediated effects due to inert Fc part. Figure has been generated based on information published by (Sainson *et al.*, 2018, Sazinsky, 2016).

3.4 T cell bispecific antibody therapy

T cell bispecific (TCB) molecules are a specific class of antibodies engineered to activate T cells upon simultaneous binding of a tumor surface antigen and the CD3ε subunit of the TCR complex. Simultaneous engagement of a tumor-associated antigen and CD3 results in MHC-independent recruitment of T cells to the tumor site; moreover, T cells become activated and ultimately induce tumor cell lysis by the secretion of cytotoxic granules and other cytokines (Dahlen *et al.*, 2018, Delves *et al.*, 2012, page 253, Martinez-Lostao *et al.*, 2015). Two main types of TCB can be differentiated: 1) Fc-free bispecific molecules as the CD19-CD3 targeting Blinatumomab (Sanford, 2015); 2) Fc-bearing IgG-like TCB, showing prolonged *in vivo* pharmacokinetics. One example of a Fc-carrying TCB is CEA-TCB, whose mode of action is affected via bivalent binding to human CEA and monovalent binding to CD3ε and is currently in clinical trials in late stage metastatic colorectal cancer (Bacac, Fauti, *et al.*, 2016, Bacac, 2016). Pre-clinical and first clinical studies using CEA-TCB showed a tumor-specific activation of T cells and as a consequence redirection of T cell cytotoxicity to

tumor cells. Furthermore, CEA-TCB resulted in the upregulation of the suppressive receptor PD-1 on T cells. Currently CEA-TCB is tested in combination with the checkpoint inhibitor anti-PD-L1 antibody atezolizumab to block suppressive signalling through PD-1/PD-L1 and thereby achieve maximal T cell mediated anti-tumor responses (Bacac, Fauti, *et al.*, 2016).

3.5 Combination therapies

The development of cancer immunotherapeutic agents relies on the continuous understanding the biology of cancer and immune cells. Successfully harnessing the immune system to treat cancer requires approaches that address the dynamics and flexibility of the immune system. Examples of constant immune adaption, as reported for example in the TCB-mediated upregulation of PD-1, made us understand that combination approaches are essential to achieve long-term or complete tumor remission. A growing body of combinations is currently being tested clinically or pre-clinically, including the concomitant administration of immune checkpoint inhibitors with surgery, radiation or chemotherapy (Mayes *et al.*, 2018, Pardoll, 2012). Very promising results could be observed when combining immune checkpoint inhibitors ipilimumab (anti-CTLA-4) with nivolumab (anti-PD-1), resulting in an objective response rate of 50-60 % in advanced-stage melanoma patients (Postow *et al.*, 2015).

In the course of anti-CTLA-4 clinical studies in bladder cancer and melanoma, ICOS is upregulated on effector T cells and serves as a biomarker for ipilimumab therapy efficacy in melanoma patients. Moreover, follow-up studies in mice revealed that agonizing the ICOS pathway by overexpression of ICOSL on tumor cells could further improve anti-CTLA-4 mediated anti-tumor response (Fan *et al.*, 2014, Fu *et al.*, 2011, Ng Tang *et al.*, 2013). Administration of ICOS signal alongside with anti-Ox40 therapy led to enhanced tumor rejection and survival (Metzger *et al.*, 2016). In summary, this supports the idea of ICOS being an attractive target for cancer immunotherapy approaches.

3.6 Objectives

Various pre-clinical studies show the relevance of agonizing T cell co-stimulatory receptors to improve current cancer immunotherapy approaches (Mahoney *et al.*, 2015, Mayes *et al.*, 2018). It has been found that combining CEA-TCB with the checkpoint inhibitor PD-L1 results in improved anti-tumor efficacy (Bacac, Fauti, *et al.*, 2016). Similarly, promising results have recently been reported on the combination of CEA-TCB with TNFR-superfamily member 4-1BBL (Sam, 2018). However, the combination with co-stimulatory IgG-like superfamily members has not been investigated yet.

The aims of this thesis are:

1. Gain a better understanding of:
    a. ICOS expression on healthy but also tumor-derived human T cells
    b. Kinetics of ICOS expression compared to other immunomodulatory receptors on human T cells
    c. ICOS expression at baseline and upon TCR or CD3 stimulation on effector and regulatory T cells
2. Generate and validate novel agonistic anti-ICOS antibodies
3. Test the hypothesis that agonizing ICOS signalling can further boost TCB-mediated anti-tumor therapy

Providing the first signal to T cells via TCB induces the expression of ICOS (Figure 3, step 1). Subsequent ICOS co-stimulation, using an agonistic ICOS antibody, is anticipated to enhance and prolong T cell immunity (Figure 3, step 2) resulting in an improved and long-lasting anti-tumor response (Figure 3, step 3).

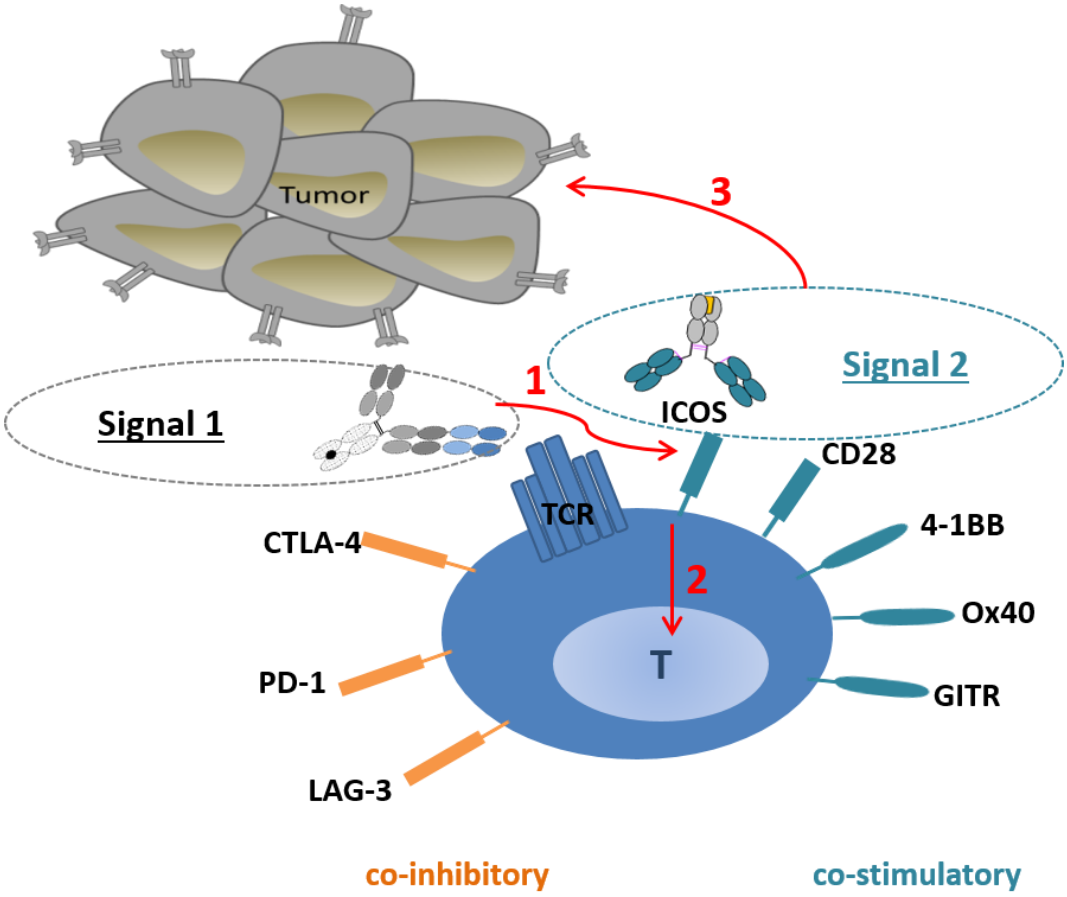

Figure 3: Hypothesis of ICOS signalling in the context of TCB-mediated anti-tumor therapy. TCB simultaneously binding a tumor surface antigen and the CD3ε subunit of the TCR complex provides the primary T cell signal (signal 1) and thereby induces the expression of ICOS (1). Co-stimulation with an agonistic ICOS antibody is anticipated to enhance T cell activation (2), finally expected to result in an improved and long-lasting anti-tumor response (3). Figure has been generated based on information published by (Corraliza-Gorjon *et al.*, 2017).

4 Material

4.1 Plastic ware

- 96-well flat-bottom or round-bottom cell culture plate, TPP, Switzerland
- Cell culture flask T25, T75, T150, TPP, Switzerland
- Cell strainer 70 μm, 40 μm,Fisher Scientific, Switzerland
- Eppendorf Safe-Lock Tube 1.5 ml, Eppendorf, Switzerland
- Eppendorf DNA LoBind Tube 1.5 ml, Eppendorf, Switzerland
- Falcon tubes 50 ml, 15 ml, TPP, Switzerland
- LS, MS, LD columns, Miltenyi Biotec GmbH, Switzerland
- NuPAGE® Novex® 3 – 8 % Tris-Acetate Protein Gels, Fisher Scientific, Switzerland
- NuPAGE® Novex® 4 – 12 % Bis-Tris Protein Gels, Fisher Scientific, Switzerland
- SepMate50 Tubes, StemCell Technologies, Germany
- Syringe 3 ml, 5 ml, BD, SWITZERLAND Bioscience

4.2 Kits and reagents

4.2.1 Kits

- Chromium Single Cell 3`Reagent Kit**,** 10x Genomics
- Cytotoxicity Detection Kit (LDH)**,** Roche, Switzerland
- FoxP3 intracellular staining kit**,** BioLegend, Switzerland
- LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit**,** Thermo Scientific, Germany
- NucleoSpin® Gel and PCR Clean-up**,** MACHEREY-NAGEL, Switzerland
- NucleoSpin® Plasmid**,** MACHEREY-NAGEL, Switzerland
- Taq PCR core kit**,** Qiagen, Switzerland
- Zombie UV™ Fixable Viability Kit**,** BioLegend

4.2.2 Reagents

- 1 x PBS (Phosphate-buffered saline), Gibco, UK
- 2 - beta Mercaptoetanol (MEthOH), Sigma-Aldrich AG, Switzerland

- Antibiotic-antimycotic 100 x (anti-anti)**,** Fisher Scientific, Switzerland
- BSA, Sigma-Aldrich AG, Switzerland
- CD CHO, Fisher Scientific, Switzerland
- Cell Dissociation Buffer, Fisher Scientific, Switzerland
- Cell Trace CFSE Dye, Fisher Scientific, Switzerland
- Collagenase D**,** Roche, Mannheim Germany
- D - (+) - Glucose, Sigma-Aldrich AG, Switzerland
- Dimethyl sulfoxide (DMSO), Sigma-Aldrich AG, Switzerland
- DNAse, Sigma-Aldrich AG, Switzerland
- Dulbecco's Modified Eagle Medium (DMEM), Fisher Scientific, Switzerland
- Ex-Cell 293 Serum-Free Medium for HEK 293 Cells, Sigma-Aldrich AG, Switzerland
- Fetal calf serum (FCS)**,** Gibco, UK
- G418, Geneticin®, Sigma-Aldrich AG, Switzerland
- GlutaMax^{TM}**,** Fisher Scientific, Switzerland
- Golgi Plug, BD, Switzerland
- Golgi Stop, BD, Switzerland
- Histopaque, Sigma-Aldrich AG, Switzerland
- Hyaluronidase, Sigma-Aldrich AG, Switzerland
- Hygromycin B, Sigma-Aldrich AG, Switzerland
- MACS Tissue Storage Solution, Miltenyi Biotec, GmbH, Switzerland
- Non-essential amino acids, Sigma-Aldrich AG, Switzerland
- Paraformaldehyde (10 % Formalin), Sigma-Aldrich AG, Switzerland
- Permeabilization buffer**,** BioLegend, Switzerland,
- Proleukine**,** FarmaMondo, USA
- Puromycin (c = 10 mg/ml)**,** InvivoGen, LabForce AG, Switzerland
- RPMI1640**,** Fisher Scientific, Switzerland
- Sodium bicarbonate NaHCO3**,** Sigma-Aldrich AG, Switzerland
- Sodium pyruvate**,** Sigma-Aldrich AG, Switzerland
- Triton X-100**,** BioRad AG, Switzerland
- Trypsin - EDTA, 0.05 %, Fisher Scientific, Switzerland
- Tween20**,** Roche, Switzerland
- Ultrapep Soy**,** Kerry Biosciences, Irland,

- Valproic acid**,** Sigma, Schweiz
- Zeocin**,** Fisher Scientific, Switzerland

4.2.3 Antibodies

- Anti-human CD197 (CCR7), G043H7, BioLegend, Switzerland
- Anti-human CD25, BC96, BioLegend, Switzerland
- Anti-human CD3, HIT3a, BioLegend, Switzerland
- Anti-human CD4, RPA-T4, BioLegend, Switzerland
- Anti-human CD45, HI30, BioLegend, Switzerland
- Anti-human CD45 (RO), UCHL-1, BioLegend, Switzerland
- Anti-human CD69, FN50, BioLegend, Switzerland
- Anti-human CD8, SK1, BioLegend, Switzerland
- Anti-human CTLA-4, L3D10, BioLegend, Switzerland
- Anti-human Fc $(Fab)^2$ PE conjugated, R&D Systems, Bio-Techne AG, Switzerland
- Anti-human FoxP3, 206D, BioLegend, Switzerland
- Anti-human ICOS, C398.4A, BioLegend, Switzerland
- Anti-human PD-1, EH12.2H7x, BioLegend, Switzerland
- LEAF anti-human CD28, CD28.2, BioLegend, Switzerland
- LEAF anti-human CD3, OKT3, BioLegend, Switzerland

4.1 Buffers and media

Table 1: Buffers and media

BUFFER	**SOURCE**
FACS Buffer	1 x PBS, 2 % FCS, 5 mM EDTA, 0.25 % sodium azide
Fixation Buffer	1 x PBS, 4 % PFA
MEDIA	**SOURCE**
Human T cell media	RPMI1640, 10 % FCS, 1 % GlutaMax^{TM}, 1 µM sodium pyruvate, 1 x NEAA

4.2 Cell lines

Table 2: Cell lines

CELL LINE	SOURCE	PROVIDER	MEDIA
3T3-hFAP	Mouse fibroblast, parental 3T3, transfected to stably overexpress human FAP	ATCC, CCL-92	DMEM, 10 % FCS, 1 % GlutaMax™, 1.5 µg/ml Puromycin
CHO-hICOS	Parental CHO-K1, Chinese hamster ovary cell line transfected to stably overexpress human ICOS	ATCC, CCL-61	DMEM, 10 % FCS, 1 % GlutaMax™, 6 µg/ml Puromycin
HEK293EBNA	Human embryonic kidney cell line	ATCC, CRL-1573	Ex-Cell293, 6 mM, 1 % GlutaMax™, 6 µg/ml G418
MKN45	Human gastric adenocarcinoma cell line	DSMZ ACC409	RPMI1640, 10 % FCS, 1 % GlutaMax™
MV3	Human melanoma metastatic cell line	CVCL_W280	DMEM, 10 % FCS, 1 % GlutaMax™

4.3 Tumor samples

Human tumor samples were received from University Hospital Zurich (Switzerland) based on ethical approval between USZ and Roche granted by the Kantonale Ethikkommission Zürich. The day of surgery or the day after tumor samples were shipped on ice. Tumor samples were stored in MACS Tissue Storage Solution until used for further processing.

4.4 Technical equipment

- Aekta Purifier, GE Healthcare, Switzerland, Protein purification
- Steri-Cycle i160, Thermo Scientific, Germany, CO^2 incubator
- Safe 2020, Thermo Scientific, Germany, Sterile safety hood
- Centrifuge 5810 R, Eppendorf, Switzerland, Centrifugation
- Centrifuge 5424 R, Eppendorf, Switzerland, Centrifugation
- Olympus CKX53, Olympus, Switzerland, Microscope
- Cedex HiRes, Roche, Switzerland, Cell counter
- Chromium Controller, 10x Genomics, Single cell emulsions

- Rocker 3D digital, IKA, Germany, Shaking table
- Quadro MACS, Miltenyi Biotec, GmbH, Switzerland, Magnetic separator
- Tecan Spark 10M, Tecan, Switzerland, Plate reader
- MixMate, Eppendorf, Germany, Samples mixing
- Platemaster, Gilson AG, Switzerland, Semi-automated pipetting of 96-well plates
- Dropsense BioPhotometer, Trinean, Switzerland, Protein/DNA concentration
- Mastercycler proS, Eppendorf, Switzerland, PCR
- Caliper LabChip GXII System, Perkin Elmer, USA, Capillary electrophoresis
- Biacore T200, GE Healthcare, Switzerland, SPR analysis
- FACS AriaIII, BD Bioscience, Switzerland, Flow cytometry, Cell Sorting
- FACS Fortessa LSR, BD Bioscience, Switzerland, Flow cytometry
- Vortex, VWR. Switzerland, Samples mixing

4.5 Software

- GraphPad Prism 6.07, Graphical and statistical analysis
- CloneManager 9, Planing of molecular cloning
- FACS Diva, FACS data analysis
- FlowJo V10, FACS data analysis
- Tibco Spotfire, Graphical and statistical analysis
- Biacore T200 Evaluation Software (version 3.1), SPR (analysis protein purification)
- LabChip GX, Capillary electrophoresis analysis

5 Methods

5.1 Generation of antibodies

5.1.1 Molecular cloning

The target gene sequence was cloned into a light chain or a heavy chain containing plasmids vectors (pSTAN1 and derivates; origin by Roche Innovation Center Zurich). Therefore, plasmids were enzymatically digested, followed by ligation with T4 ligase. Amplification of the plasmid was done by heat-shock transformation of XL-1 chemo competent bacteria with the ligated DNA and cultivated on an agarose plate containing the proper antibiotic for selection of positively transformed bacteria. Plasmid DNA was purified from bacteria inoculated dYT medium following the manufacturer`s instructions.

5.1.2 Transfection

Expression of human antibodies was achieved by transient co-transfection of HEK293 EBNA suspension cells with plasmid DNA using polyethylenimine (PEI, Polysciences Inc.). Therefore 2 x 10^7 cells resuspended in 20 ml CD CHO media were mixed with 10 µg plasmid DNA (1 µg/ml) and 27 µl PEI (1 mg/ml). After 3 h incubation at 37 °C, 5 % CO2, 135 rpm another 80 ml of ExCell media (6 mM L-glutamine, 5 g/l Ultrapep Soy, 1.2 mM valproic acid) was added and further incubated. 24 h post transfection, cells were supplied with feed and 3 g/l glucose. After six days incubation, antibodies secreted to the cell supernatant were harvested by centrifugation (210 x g, 30 min) and sterile filtered (0.22 µm filter „rapid“, TPP). Supernatant was stored at 4 °C for subsequent protein purification.

5.1.3 Protein purification

Secreted proteins were purified from cell culture supernatants by affinity chromatography using Protein A followed by size exclusion chromatography (SEC). For affinity chromatography, the sterile filtered supernatant was loaded on a Protein A MabSelectSure column (CV = 5 mL, GE Healthcare) equilibrated with 40 ml of 20 mM sodium phosphate, 20 mM sodium citrate, pH 7.5. Unbound protein was removed by washing with at least 10

column volumes of 20 mM sodium phosphate, 20 mM sodium citrate, pH 7.5. The bound protein was eluted using a linear pH-gradient of sodium chloride (from 20 mM to 100 mM) created across 15 column volumes of 20 mM sodium citrate, 100 mM sodium chloride, 100 mM Glycine, 0.01 % Tween20 pH 3.0. The column was then washed with 10 column volumes of 20 mM sodium citrate, 100 mM NaCl, 100 mM Glycine, 0.01 % Tween20, pH 3.0. The pH of collected fractions was adjusted by directly adding 2 M Tris, pH 8.0. The protein was concentrated and filtered using Amicon®Ultra-15 Ultracel 30 K columns prior to loading on a HiLoad Superdex 50/600 S200 column (GE Healthcare) equilibrated with 20 mM Histidine, 140 mM NaCl, 0.01 % Tween20, pH 6.0.

5.1.4 Quality control

The concentration of purified proteins was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence using the Dropsense biophotometer.

$$Protein\ concentration = \frac{(A280 - A320)}{Extinction\ coefficient}$$

Quality control of the antibodies was performed by the Process Biochemistry group of Roche Innovation Center Zurich. This comprised the purity, molecular weight and aggregate content of the antibodies using capillary electrophoresis (LabChipGXII (Caliper) and analytical size-exclusion column (TSKgel G3000 SW XL, Tosoh). Also the heat stability (heated up from 25 °C to 85 °C using a heating rate of 0.1 °C/min) of the antibodies was tested using an Optim2 device.

5.2 Cell culture techniques

5.2.1 Cultivation of tumor cell lines

All cell lines were cultured at 37 °C in a water-saturated atmosphere at 5 % CO2 (Steri-CycleTM i160). All manipulations were performed with sterile reagents under a laminar flow hood. Adherent cells were dissociated using 0.05 % Trypsin – EDTA or Cell Dissociation buffer (in case surface proteins were Trypsin sensitive; e.g. 3T3-hFAP) and split accordingly.

Cell number and viability was determined by trypan blue staining using the Cedex HiRes cell counter.

5.2.2 Human PBMC isolation

Human peripheral blood mononuclear cells (PBMC) were isolated either from fresh blood or buffy coat (from irreversibly anonymized donors obtained from Blutspendezentrum Zürich) by standard Histopaque density gradient centrifugation using SepMate tubes. In brief, human blood or buffy coat was mixed with equal volume of 1 x PBS and gently added to the SepMate tubes by pipetting down at the wall of the tube. After 10 min centrifugation at 1200 x g the supernatant was poured into a fresh tube, followed by several washing steps. Wash the cells by filling up the tube with 1 x PBS, centrifuge (400 x g, 10 min), aspirate the supernatant and resuspended the pellet. Repeat the washing step by resuspending the pellet with 1 x PBS and centrifuge (300 x g, 10 min), followed by a final washing step resuspending the pellet with 1 x PBS and centrifuge (350 x g, 10 min). In order to remove remaining red blood cells, a lysis step using ACK Lysis buffer was performed. Therefore, 5 ml of ACK Lysis Buffer were added. After 5 min incubation at 37 °C cells were washed with 1 x PBS and centrifuged (350 x g, 10 min).

5.2.3 Tumor tissue digest

Human tumors were minced into very small pieces using two sterile scalpels. Pieces were transferred into a fresh 50 ml Falcon tube, 10 ml of enzymatic digestion mix (RPMI1640, 2 % FCS, 10 U/ml DNaseI, 275 U/ml collagenase D, 471 U/ml Hyaluronidase) was added and incubated for 30 min at 37 °C on a rotator with medium speed. Thereafter the tumor pieces were filtered through a 70 µm mesh using the plunger of a 10 ml syringe and washed three times with cold 1 x PBS. Samples were centrifuged for 10 min at 250 x g and 4 °C, followed by resuspension of the pellet in the required buffer.

5.3 Flow cytometry

For this thesis, all data were acquired on a FACS Fortessa instrument, equipped with Diva Software. The FACS Fortessa comes with four lasers (violet (405 nm), blue (488 nm),

yellow/green (561 nm), red (633 nm)). The lasers with their adequate filter bands allow the simultaneous multi-parameter analysis and thereby the characterization of single cells and their antigens.
If not otherwise stated, all steps were carried out at 4 °C to prevent internalization of the antibodies. 96-well plates were centrifuged for 5 min at 350 x g, FACS tubes for 4 min at 400 x g. Plate-coating of antibodies was performed by diluting the antibody in 1 x PBS followed by either overnight incubation at 4 °C or 3 h incubation at 37 °C.

5.3.1.1 Surface staining

For the discrimination of live and dead cells, cells were labelled with the fixable Zombie UV viability dye. After washing with 1 x PBS, cells were incubated for 30 min at 4 °C with 50 µl of the antibody diluted at the appropriate concentration in FACS buffer, washed three times by adding 200 µl of FACS buffer, followed by centrifugation. If immediately acquired, cells were resuspended in 200 µl of FACS buffer. Otherwise, the cells were fixed by resuspending the pellet in 100 µl of 4 % PFA and incubating for 15 min at room temperature. After three times washing, cells were stored in FACS buffer until acquisition by flow cytometry (5.3).

5.3.1.2 Intracellular staining

For intracellular staining cells were resuspended in 1 x Perm/Fix buffer (FoxP3 intracellular staining kit, BioLegend) for 45 min at room temperature. After three times washing with 1 x Perm buffer, cells were incubated for 40 min at room temperature in 1 x Perm buffer containing the antibody for intracellular staining. Cells were washed three times using 1 x Perm buffer, followed by resuspension of the pellet in the required volume of FACS buffer for acquisition by flow cytometry (5.3).

5.4 Binding of antibody constructs to cells

Cells were harvested, counted, checked for viability and re-suspended at 10^6 cells per ml in FACS buffer. 100 µl of the cell suspension (containing 10^5 cells) were incubated in 96-well round-bottom plates for 30 min at 4 °C with increasing concentrations (7 pM – 120 nM) of the constructs. Thereafter, cells were washed twice with cold FACS buffer, re-incubated for a

further 30 min at 4 °C with a fluorochrome-conjugated, secondary antibody and washed again. If not acquired immediately, the cells were fixed by resuspending the pellet in 100 µl of 4 % PFA and incubating for 15 min at room temperature. After three times washing, cells were stored in FACS buffer until acquisition by flow cytometry (5.3).

5.5 T cell-mediated tumor cell lysis assay

ICOS signalling in the context of TCB-mediated T cell activation was assessed by T cell-mediated tumor cell lysis assay. Briefly, human PBMC were incubated with MKN45 tumor cell line expressing the tumor antigen CEA and a titration of CEA-TCB. Therefore, adherent tumor target cells were harvested with Trypsin - EDTA or cell dissociation buffer, resuspended at a density of 10^5 cells per ml and 100 µl seeded to the wells of a 96-well flat-bottom plate (containing 10^4 per ml per well). Cells were left overnight at 37 °C to adhere to the plate. On the day of the experiment, the assay plates were centrifuged and the medium was aspirated. Effector cells were isolated either from human fresh blood or buffy coat (see 5.2.2) and resuspended at 10^6 per ml. 100 µl of the cell suspension were added per well (containing 10^5 per ml per well), resulting in a final effector to target cell (E:T) ratio of 10:1. The indicated concentration of TCB was added to the corresponding wells to reach a final volume of 200 µl. Tumor cell lysis was assessed after 48 h of incubation by quantification of lactate dehydrogenase (LDH) released into the cell supernatant by apoptotic or necrotic cells (Cytotoxicity Detection Kit, Roche). Therefore, cells were centrifuged, 50 µl of cell medium were transferred to a fresh 96-well flat -bottom plate and mixed with 50 µl of catalyst - dye solution mix. Absorbance was measured immediately using an ELISA plate reader (TecanSpark 10 M). Maximal lysis of the target cells (= 100 %) was achieved by incubation of target cells with 1 % Triton X-100 for a minimum of 30 min before taking supernatant. Spontaneous lysis (= 0 %) refers to target cells co-incubated with effector cells in the absence of TCB.

Calculation of percentage of tumor cell lysis:

$$\%\ tumor\ cell\ lysis = \frac{absorbance - spontaneous\ lysis}{maximal\ lysis - spontaneous\ lysis}$$

5.6 T cell stimulation to study ICOS expression and signalling

For stimulation of human PBMC, anti-human CD3 antibody (clone OKT3) was coated to the flask or assay plate at a final concentration of 0.2 - 2 µg/ml by diluting the antibody in 1 x PBS followed by either overnight incubation at 4 °C or 3 h incubation at 37 °C. Thereafter the antibody solution was aspirated. Human PBMC, isolated as described in 5.2.2, were counted, resuspended at the appropriate concentration in human T cell media and added to the flask or assay plate, coated with anti-human CD3 antibody. Soluble anti-human CD28 antibody (clone CD28.2) was added at a final concentration of 2 µg/ml, followed by incubation of the cells for the indicated time points in a humidified incubator (37 °C, 5 % CO2).

Persistence of ICOS expression after stimulation was studied by performing a wash-out of anti-CD3 – anti-CD28 antibodies. Therefore, cells were transferred to a fresh assay plate, centrifuged and the pellet resuspended in 1 x PBS. After another centrifugation step, the pellet was resuspended in human T cell media followed by continued cultivation in a humidified incubator (37 °C, 5 % CO2). After incubation for the indicated time points, expression of activation markers was measured by surface staining with 25 µl of antibody mix containing anti-human CD45-, anti-human CD4-, anti-human CD8-, anti-human ICOS- and anti-human PD-1 antibody. Staining of CTLA-4 and FoxP3 was performed by subsequent intracellular staining.

5.7 Proliferation assay

Human PBMC were washed with 1 x PBS and resuspended at 10^6 per ml in 1 x PBS. Cell Trace CFSE dye (stock concentration 5 mM) was added to the cells at a final concentration of 1 µM, mixed immediately and incubated at 37 °C for 5 min. Labelling was stopped by adding half the volume of pre-warmed FCS, followed by 5 min incubation to remove remaining free dye. After three times washing using ice-cold PBS + 10 % FCS the cell pellet was resuspended in T cell media. ICOS signalling studies were performed by coating a suboptimal concentration of 0.5 µg/ml anti-CD3 antibody as well as the indicated concentration of ICOS antibody to the surface of an assay plate. CFSE+ cells were seeded at a final density of 5 x 10^4 cells per well to the wells of a 96-well plate containing the coated

antibodies and incubated for 5 days in a humidified incubator (37 °C, 5 % CO2). Thereafter, supernatant was removed; cells were washed and stained with 25 µl of antibody mix containing anti-human CD45-, anti-human CD4-, anti-human CD8 and anti-human ICOS antibody. Proliferation was measured by dilution of CFSE signal using flow cytometry.

5.8 Cell based co-culture assay

ICOS signalling in the context of TCB-mediated T cell activation was assessed in a cell co-culture assay. Therefore, adherent tumor target cells and target cells for crosslinking (e.g. 3T3-hFAP cells) were harvested with Trypsin - EDTA or cell dissociation buffer, irradiated with 5000 RAD and resuspended at 10^5 cells per ml. 100 µl of the tumor target cells and 100 µl target cells for crosslinking were seeded to the wells of a 96-well flat-bottom plate (containing 2 x 10^4 cells per well). Cells were incubated overnight at 37 °C to adhere to the plate. The day of the experiment, the assay plate was centrifuged and the medium was aspirated. 50 µl of targeted-ICOS antibodies were added to the adherent target cells at the indicated concentrations (range of 1.6 pM – 5000 pM) together with 50 µl of a fixed, suboptimal concentration of TCB. As controls, wells containing only the TCB molecule or only the targeted-ICOS antibodies were included. Effector cells, isolated either from human fresh blood or buffy coat (see 5.2.2), were resuspended at 10^6 per ml. 100 µl of the cell suspension (containing 10^5 cells per well) were added per well to obtain a final E:T ratio of 5:1. T cell activation was assessed after 48 h incubation in a humidified incubator (37 °C, 5 % CO2) by flow cytometric analysis, using antibodies recognizing the T cell activation markers CD69 (early activation marker) and CD25 (late activation marker). If differentiation of memory T cells was analysed, cells were incubated for a total of 72 h and surface staining of the T cell differentiation markers CD45RO and CCR7 was performed.

5.9 Cytokine analysis

For cytokine analysis from supernatant, cell based co-culture assay plates were incubated for 72 h, centrifuged and supernatant transferred to a fresh plate. If not measured immediately, supernatant was stored at - 20 °C for a maximum of 3 months. Detection of several cytokines from supernatant was performed using the bead based Mulitplex Kits (BioRad) following the manufacturer`s instructions. In short, capture antibody-coupled magnetic beads were first

incubated with 50 µl of cell culture supernatant, followed by detection using biotinylated detection antibodies. After addition of the reporter streptavidin-phycoerythrin conjugate (SA-PE), beads can be sorted and analysed by measuring fluorescence intensities.

5.10 Single cell RNA sequencing (scRNAseq)

Next generation sequencing was used to analyse genome wide expression of cell samples on a single cell level. Therefore, cells were sorted for CD45+ viable cells (using FACS Aria) and a total of 6 x 10^3 cells were further processed to have full library coverage. Following the 10x Genomics Chromium Single Cell 3`Reagent Kit protocol the partitioning of cells in nanolitre-scale Gel Bead-In-EMulsions (GEMs), RNA isolation, reverse transcription of DNA (cDNA), as well as amplification of cDNA was performed. For generation of the sequencing library PCR-amplified cDNA got enzymatically fragmented and random-primed following the protocol by 10x Genomics. Sequencing of the library was performed by ETH Zurich. Bioinformatic analysis was conducted with the help of the PS-BiOmics group at the Roche Innovation Center Basel using UMAP algorithm. In brief, the count matrix was analysed using Python. All data were corrected for total counts, data dimensionality reduced by principal component analysis. Gene set analysis was done using Enrichr server (http://amp.pharm.mssm.edu/Enrichr).

5.11 Statistical analysis

All data are presented as median +/- standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). The statistical significance of differences was determined using the indicated statistical test. P values < 0.05 were considered as significant and indicated with asterisks (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

6 Results

6.1 Assessment of ICOS and ICOSL expression on tumor and healthy donor peripheral immune cells

The expression of ICOS and ICOSL on various immune cell subsets was investigated by scRNAseq on tumor-infiltrating lymphocytes (TIL) from untreated melanoma metastases and autologous peripheral blood (as described 5.10). As shown in the tSNE plots in Figure 4, the expression of ICOS was exclusively found to be on CD3+- T cells from tumor, while very low to no ICOS expression could be identified on autologous blood T cells. ICOSL expression is absent on T cells but could be detected on myeloid cells, B cells and DC from tumor and autologous peripheral blood in equal frequencies.

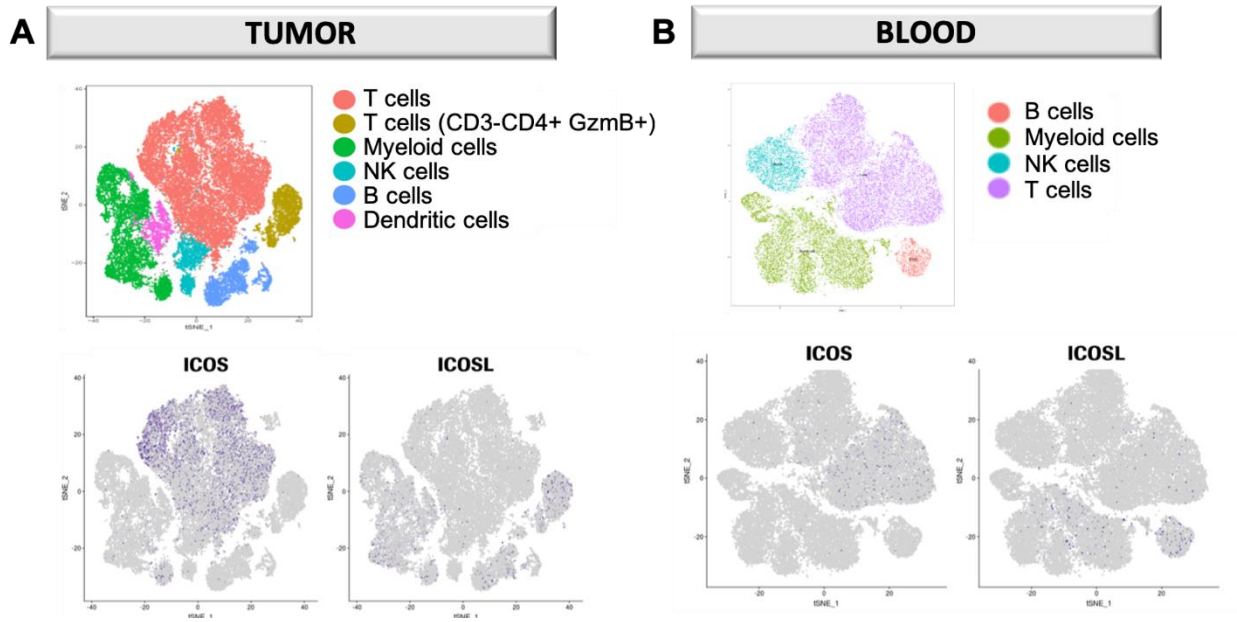

Figure 4: Analysis of ICOS and ICOSL expression on immune cells derived from melanoma metastases or autologous peripheral blood cells. scRNAseq was performed on viable, CD45+ sorted immune cells from melanoma patient tumor (A) or autologous blood samples (B). Two-dimensional tSNE plots showing the clustering of immune cell subsets (top) and the distribution of ICOS+ and ICOSL+ cells among immune cell clusters (bottom). A total of seven patients were analysed; shown is the overlay of all seven patient samples.

To study if the increased ICOS expression on melanoma metastases T cells is applicable to various tumor indications, ICOS expression was assessed on tumor-derived CD4+- and CD8+- T cells of melanoma, colorectal cancer, gastric cancer, pancreatic cancer patients as well as healthy donor PBMC by multi-color flow cytometry. Overall, a higher frequency of

ICOS+ cells was detected on human tumor-derived T cells compared to T cells from healthy donor PBMC (Figure 5). The increased frequency ICOS+ tumor-derived T cells was more pronounced on CD8+- T cells. Healthy donor CD4+- T cells showed a broader baseline expression of ICOS compared to CD8+- T cells, varying from 4 % ICOS+ to 75 % ICOS+ CD4+- T cells. Overall, a high donor variability could be observed across patients regarding both, frequency and expression levels of ICOS+- T cells.

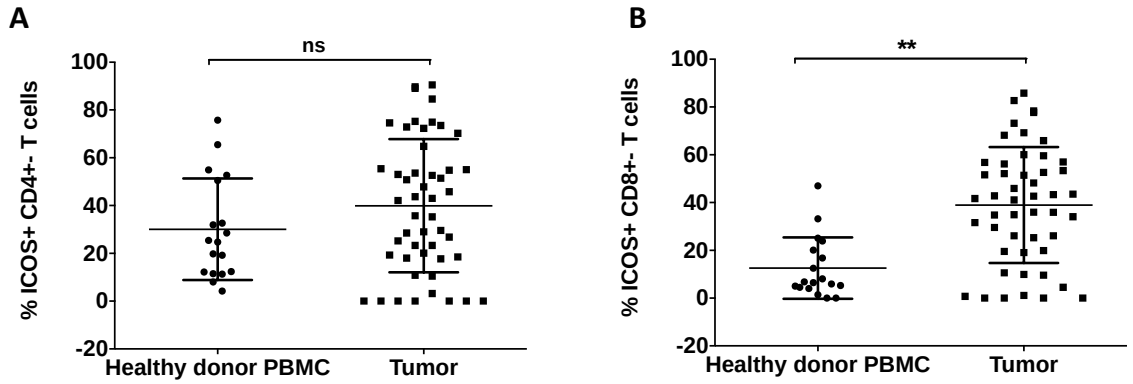

Figure 5: ICOS expression on healthy donor PBMC and human TIL. Human healthy donor PBMC as well as human tumor infiltrating lymphocytes (TIL) derived from various tumor indications were analysed by flow cytometry for the frequency of ICOS+ CD4+- T cells (A) and ICOS+ CD8+- T cells (B). A total of 18 healthy donors and 49 patients have been tested. Each dot indicates the technical triplicate of one donor or patient. For statistical analysis unpaired student T-test was applied (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

6.1.1 Co-expression of ICOS with other T cell immunomodulatory receptors on melanoma metastases TIL

To further characterize the phenotype of T cells from human melanoma metastases, ICOS expression was compared to the expression of the co-inhibitory receptors PD-1 and CTLA-4 on memory and naïve T cells. As shown in Figure 6, CTLA-4 and PD-1 are co-expressed with ICOS, both on CD4+- and CD8+- tumor-derived T cells with higher frequencies on memory T cells compared to naïve T cells. It was found that 50 – 60 % of human memory CD4+- TIL are positive for CTLA-4 and ICOS and ~ 30 % are positive for PD-1. Comparable frequencies of ~ 30 - 40 % CTLA-4+, ICOS+ and PD-1+ memory CD8+- TIL were observed. On naïve CD4+- and CD8+- T cells a frequency of ~ 20 - 30 % CTLA-4+, ICOS+ and PD-1+ cells was detected. Overall, the increase in frequency of CTLA-4- and

ICOS- positive memory T cells was higher on CD4+- compared to CD8+- melanoma metastases T cells.

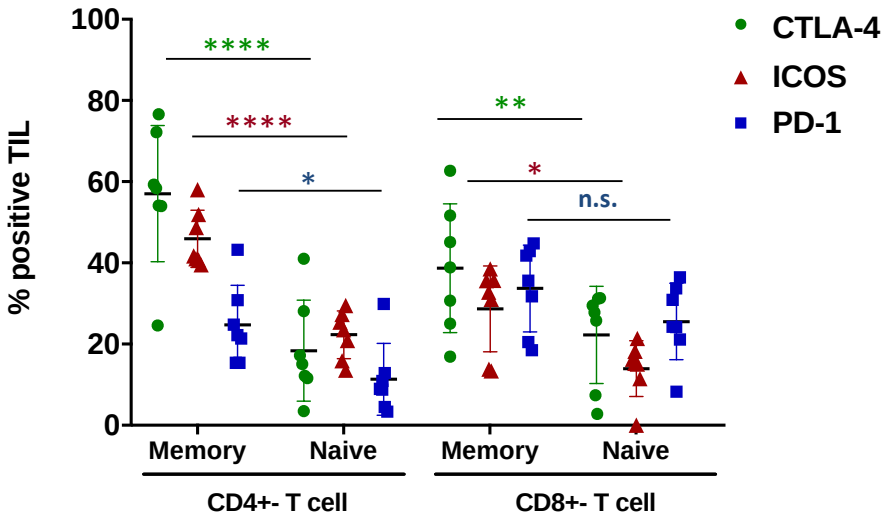

Figure 6: Expression of ICOS and co-inhibitory receptors on human melanoma metastases TIL. Expression of CTLA-4, ICOS and PD-1 on memory or naïve CD4+- and CD8+- melanoma metastases derived T cells assessed by scRNAseq and depicted as frequency positive TIL. A total of seven patients was tested. Each dot indicates the technical triplicate of one patient. Black horizontal line indicates the mean frequency among the total of seven tested patients +/-SD depicted by error bars. For statistical analysis two-way ANOVA including Bonferroni correction was applied (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$

In summary, these findings confirm the hypothesis that tumor infiltrating T cells express the co-stimulatory receptor ICOS, which could therefore serve as a target for cancer immunotherapy.

6.2 Kinetic of ICOS expression compared to other T cell immunomodulatory receptors

Besides understanding ICOS expression on healthy and tumor-derived T cell subsets, we assessed the kinetics of ICOS expression in comparison to the co-inhibitory receptors CTLA-4 and PD-1 (Figure 7). Upon *in vitro* T cell stimulation of human, healthy donor PBMC with anti-CD3 and anti-CD28 antibodies, ICOS is rapidly upregulated on CD4+- T cells with a frequency of almost 80 % ICOS+ after 24 h, while ~ 20 - 30 % of CD4+- T cells were positive for CTLA-4 and PD-1. After 48 h, the frequency of CTLA-4+, PD-1+ and

ICOS+ CD4+- T cells further increased to ~ 50 % CTLA-4+, ~ 75 % PD-1+ and ~ 90 % ICOS+ cells. Interestingly, on CD8+- T cells the kinetic of ICOS expression seemed to be slower as indicated by ~ 40 % ICOS+ cells after 24 h stimulation (compared to almost 80 % being ICOS+ CD4+- T cells after 24 h). Frequencies of CTLA-4+ and PD-1+ CD8+- T cells were similar to CD4+- T cells at that time point. A further increase of all receptors on CD8+- T cells was detected after 48 h, resulting in ~ 55 % CTLA-4+, ~ 50 % PD-1, and ~ 80 % ICOS+ CD8+- T cells. Overall, T cell stimulation with anti-CD3 and anti-CD28 antibody results in comparable expression of ICOS as well as CTLA-4 on CD4+- and CD8+- T cells, while PD-1 is higher expressed on CD4+- T cells.

In a second step, the persistence of expression in the absence of T cell stimulus was investigated. Therefore, a wash-out of anti-CD3 and anti-CD28 antibodies was performed, followed by further incubation of the pre-stimulated PBMC in culture media. Interestingly, the frequency of ICOS+ CD4+- and CD8+- T cells remained stable while a time-dependent decline of PD-1+ and CTLA-4+ T cells was detected.

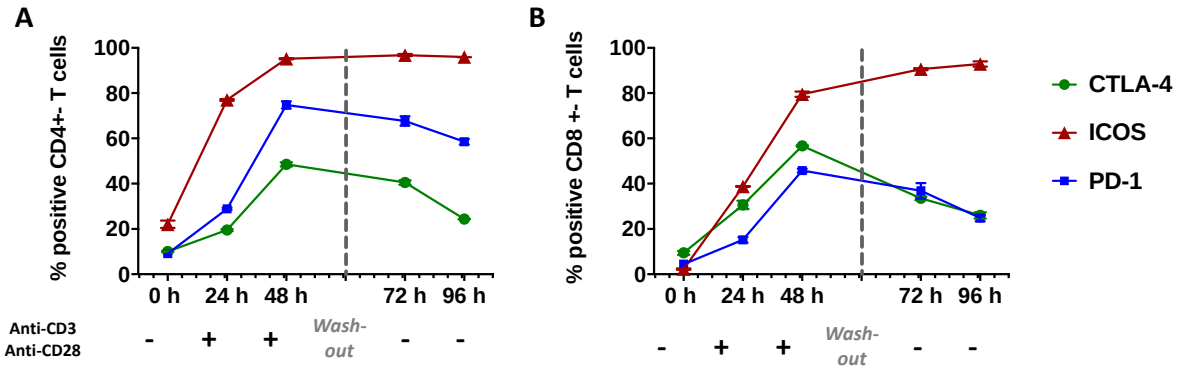

Figure 7: Kinetic of ICOS expression on human T cell subsets in comparison to the immunomodulatory receptors CTLA-4 and PD-1. Human PBMC were stimulated with plate-coated anti- CD3 (1 µg/ml) and soluble anti-CD28 (2 µg/ml) for a total of 48 h, followed by a wash-out and continued cultivation for another 48 h**.** Expression of the T cell molecules CTLA-4 (green), ICOS (red) and PD-1 (blue) was investigated by flow cytometry and is shown as percentage positive cells among CD4+- (A) and CD8+- T cells (B). A total of three independent experiments was performed in three replicates. Representative data of one experiment is shown as mean +/- SD.

6.3 ICOS expression upon various CD3-TCR stimulation on healthy donor T cells

Knowing that ICOS is expressed on the majority of human TIL and can be induced upon classical TCR engagement with anti-CD3 and anti-CD28 antibody, we wanted to characterize the role of ICOS co-stimulation in combination with TCB-mediated T cell activation. Therefore, we studied the expression of ICOS upon TCB treatment and compared it to classical TCR stimuli on healthy donor T cells.

6.3.1 TCB induces ICOS expression in a concentration dependent manner

Tumor cell lysis, detected by release of LDH, could be detected upon increasing concentrations of CEA-TCB. Moreover, the frequency of CD69- (early activation marker) and CD25- (late activation marker) positive T cells increased in a CEA-TCB concentration-dependent manner (Figure 8B). Likewise, ICOS was induced in a CEA-TCB concentration-dependent manner on human T cells. Upon saturating concentration of CEA-TCB comparable frequency of 80 – 90 % ICOS+ cell among CD4+- and CD8+- T cell could be detected.

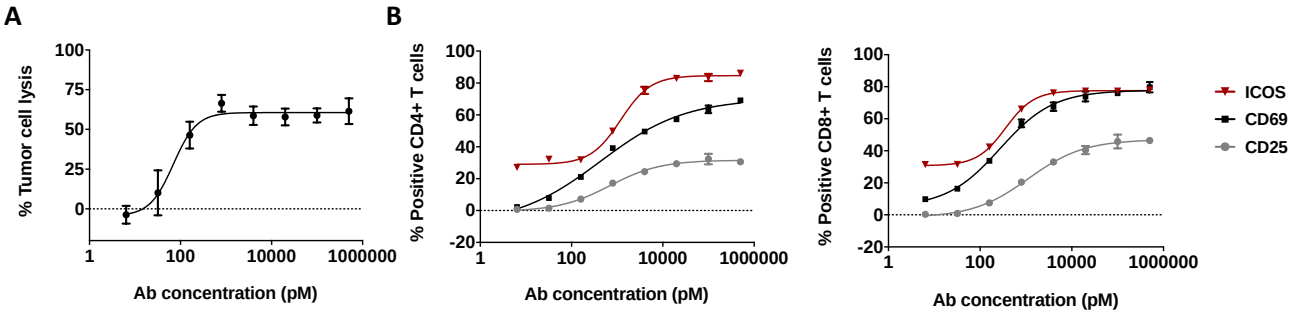

Figure 8: TCB induces tumor cell lysis and T cell activation in a concentration-dependent manner.
MKN45 tumor cells expressing CEA were incubated with human PBMC effector cells (E:T of 10:1) and a titration of CEA-targeting TCB for 48 h. **(A)** Tumor cell lysis detected by LDH release after 48 h incubation **(B)** T cell activation indicated by the percentage of CD25 (grey), CD69 (black) or ICOS (red) surface expression among CD4+- and CD8+- T cell subsets after 48 h incubation time. A total of three independent experiments was performed in three replicates. Representative data of one experiment is shown as mean +/- SD.

6.3.2 ICOS expression at baseline and upon CD3-TCR stimulation on effector and regulatory T cells

As ICOS is described to be expressed on Treg cells already at baseline (see 3.3), we wanted to better understand the role of ICOS expression on Treg cells compared to CD4+- and CD8+- effector T cells in the context of CD3-TCR complex stimulation. Moreover, we were interested, if the observed ICOS expression pattern among the different T cell subsets is a general finding or specific for the activation of T cells with the TCB. Human PBMC from healthy donors were therefore stimulated with a saturating concentration of anti-CD3 and anti-CD28 antibodies or with a saturating concentration of a medium and a high potency CEA-TCB.

As depicted in Figure 9 A, on freshly isolated human PBMC (grey squares) the expression of ICOS was highest on Treg cells with ~ 60 % ICOS+-Treg cells, followed by ~ 20 % ICOS+ CD4+- T cells and ~ 10 % ICOS+ CD8+- T cells. However, upon stimulation of T cells either with anti-CD3 - anti-CD28 antibodies or with CEA-TCB, ICOS is strongly upregulated on all T cell subsets with highest frequency on Treg cells (~ 90 - 100 %) followed by comparable frequencies of ICOS+ CD4+- and CD8+- T cells. Higher ICOS expression could be detected upon co-stimulation using the high potency CEA-TCB suggesting that stronger T cell activation translates into stronger ICOS up-regulation (Figure 9).

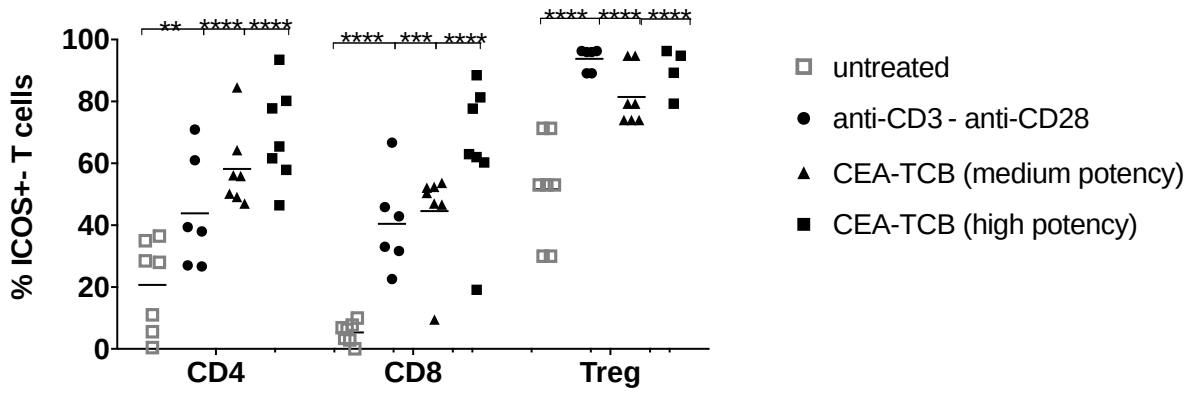

Figure 9: ICOS expression is increased on various stimulated T cell subsets. ICOS expression on human T cell subsets at baseline and upon activation for 48 h with anti-CD3 - anti-CD28 or CEA-TCB. Healthy donor PBMC either untreated, cultured *in vitro* with anti-CD3 (1 μg/ml) - anti-CD28 (2 μg/ml) or upon crosslinking of T cells and target cells using a saturating concentration of human medium potency and high potency CEA-TCB. Shown is the Frequency (%) of ICOS expression. A total of seven independent experiments on healthy donor samples was performed in three replicates. For statistical analysis two-way ANOVA using Bonferroni correction was applied (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

6.4 ICOS signalling depends on crosslinking and CD3 co-stimulation

ICOS signalling naturally occurs upon ligation of the ICOS receptors with its ligand ICOSL, causing the intracellular activation of the PI3K-Akt pathway, consequently resulting in enhanced T cell proliferation. To better understand the requirements for efficient ICOS downstream signalling, human T cells were stimulated with an agonistic ICOS IgG antibody in the presence or absence of a suboptimal anti-CD3 trigger and effects on the proliferation was analysed. The ICOS antibody was either administered in solution or bound to the plate (crosslinked). Figure 10 shows that simultaneous stimulation with anti-CD3 and anti-ICOS agonistic antibody led to increased T cell proliferation only if the ICOS antibody was crosslinked (blue bar). Addition of the ICOS antibody in solution does not induce further proliferation of T cells compared to CD3 alone (red bar). Moreover, the data shows that ICOS signalling depends on a concurrent TCR engagement. In the absence of anti-CD3 stimulation, the plate-coated ICOS antibody was not able to induce proliferation of T cells (grey bar).

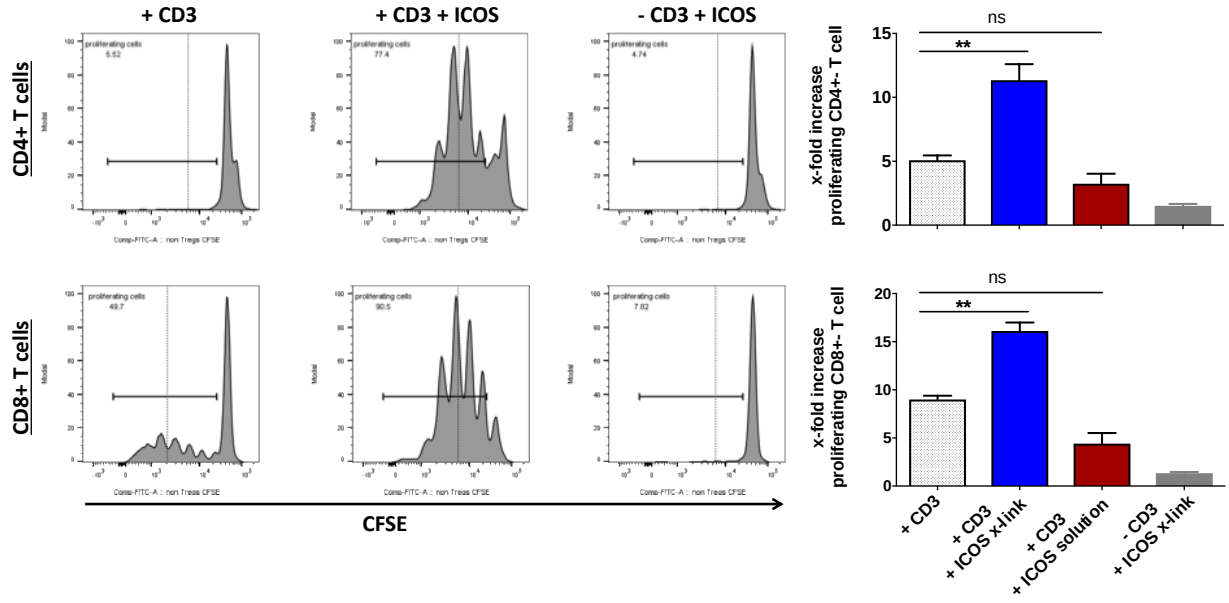

Figure 10: ICOS signalling requires concurrent CD3 signalling and crosslinking. T cell proliferation measured by CFSE dilution after 5 days stimulation with 0.5 µg/ml of anti-CD3 (clone OKT3) +/- anti-ICOS agonistic antibody either crosslinked (x-link) by coating the antibody to the bottom of the assay plate or added in solution. Shown are histograms of proliferation peaks and x-fold increase proliferation of human CD4+- and CD8+- T cells. T cell proliferation based on T cell counts were normalized to untreated cells (bar chart). A total of two independent experiments was performed in three replicates. Data is shown as mean +/- SD. For statistical analysis one-way ANOVA was applied using Dunnett`s multiple comparison (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

6.5 Generation of novel, agonistic ICOS antibodies

An agonistic ICOS antibody needs to fulfil two criteria to induce significant ICOS downstream signalling: (i) a concurrent TCR signal and (ii) the need crosslinking. Based on this, two targeted formats of anti-ICOS antibodies were cloned, produced and purified (as described in 5.1). A schematic structure of the antibodies is shown in Figure 11.
The described ICOS binding antibodies comprised an anti-human ICOS binding domain and a crosslinking domain that binds to a tumor-associated stroma antigen (hereafter referred to as stroma-target). As a control for crosslinking, ICOS binding antibodies comprising an untargeted control binding domain were generated.

The so called 1 + 1 format consisted of one fragment antigen binding (Fab) directed against ICOS and another Fab against the tumor-associated stroma target or the untargeted control domain (Figure 11, A, B), resulting in monovalent binding to ICOS and the corresponding

crosslinking domain. Bivalent ICOS binding was provided by the so called 2 + 1 formats. These antibodies contained two ICOS binding Fabs and a c-terminal Fc fused VH/VL against the stroma target or the untargeted control binding domain (Figure 11, C, D). All ICOS antibodies used in this work carried the P329GLALA mutation in the Fc part (Schlothauer *et al.*, 2016), which prevents binding to the Fc receptor (called silent Fc).

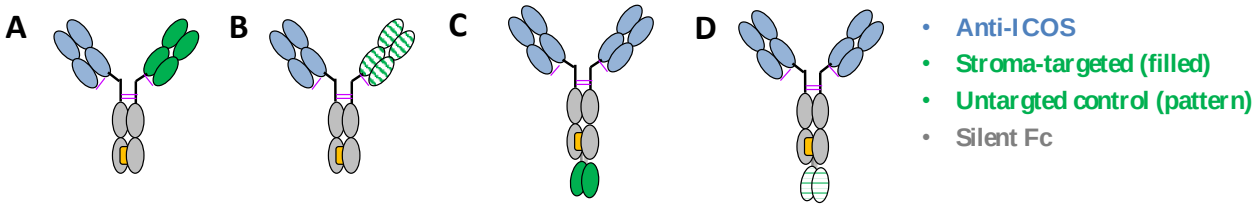

Figure 11: Schematic view of various targeted, agonistic anti-human ICOS antibodies. Targeted anti-ICOS antibodies, binding the human ICOS receptor and a targeting moiety, were cloned and produced either as monovalent 1 + 1 format or as bivalent 2 + 1 format. (A) stroma-targeted ICOS 1 + 1 (B) untargeted-control ICOS 1 + 1 (C) stroma-targeted ICOS 2 + 1 (D) untargeted-control ICOS 2 + 1. All shown antibodies carry a P329GLALA mutation in the Fc part (silent Fc).

All antibodies were produced by transiently transfecting HEK293EBNA cells and purified from cell culture supernatant by SEC and Protein A chromatography. The purity of the final product, including molecular weight, aggregate content as well as heat stability of the antibodies, was tested by the Process Biochemistry group of Roche Innovation Center Zurich.

6.5.1 Binding of targeted ICOS antibodies to cells

The binding of several targeted anti-ICOS constructs was tested on cell lines generated to overexpress either the human ICOS receptor or the stroma-target antigen (see 5.4). Human ICOS antibodies specifically bound to human ICOS (Figure 12, A) in a concentration-dependent manner. Moreover, ICOS binding was comparable for the monovalent and the bivalent ICOS antibodies. Specific binding to the stroma-target antigen was given for the stroma-targeted constructs, irrespective of the 1 + 1 or 2 + 1 format (Figure 12, B). As expected, none of the untargeted-control antibodies showed binding to target cells.

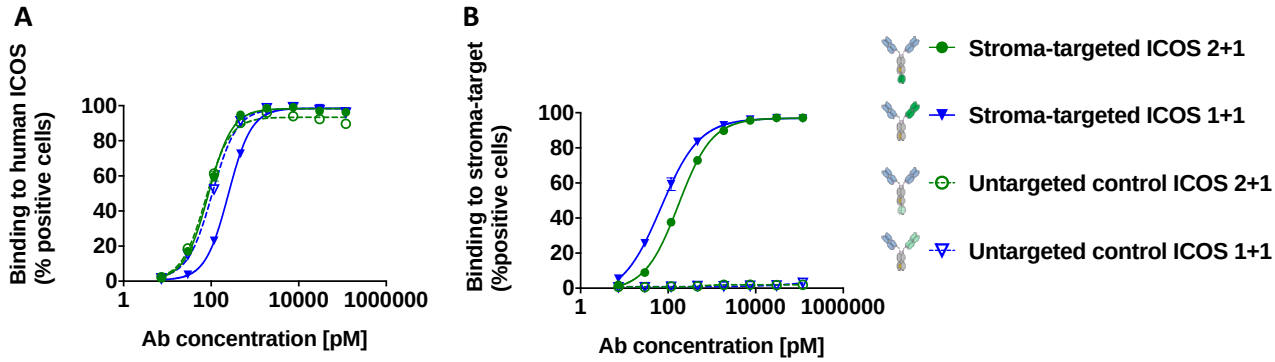

Figure 12: Percentage binding of human anti-ICOS antibodies to cells expressing ICOS or cells expressing the stroma target antigen. For binding analysis, (A) Chinese hamster ovary (CHO) cells expressing human ICOS and (B) 3T3 cells expressing the human stroma target antigen were incubated with a titration of targeted anti-human ICOS antibodies. Binding of antibodies was detected using a secondary, fluorophore-conjugated anti-human Fc antibody and is depicted as frequency positive cells (%). A total of two independent experiments was performed in three replicates and shown as mean +/- SD.

6.6 ICOS signalling enhanced TCB-mediated T cell activity

In a next step, we wanted to address how the human agonistic anti-ICOS antibodies can further boost CEA-TCB-mediated T cell activation in a cell based co-culture assay. Therefore, human PBMC were stimulated with a sub-optimal CEA-TCB concentration of 0.2 nM. Besides inducing ICOS expression (Figure 13), a sub-optimal CEA-TCB concentration ensured the sub-optimal activation of T cells that could be further amplified by ICOS co-stimulation.

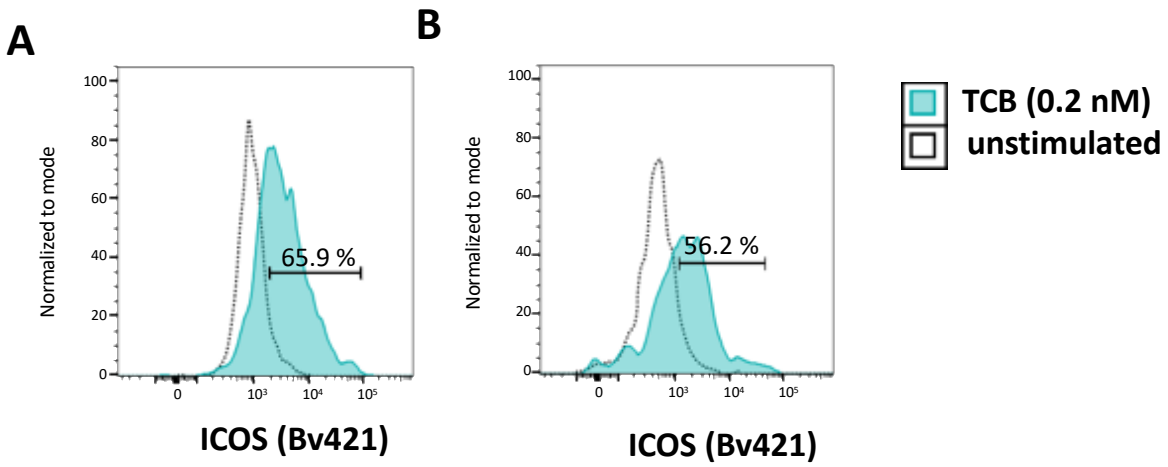

Figure 13: ICOS expression upon CEA-TCB-mediated T cell stimulation. Healthy donor PBMC were co-cultured with CEA-positive tumor cells, fibroblasts and stimulated with 0.2 nM CEA-TCB. (A) Histogram showing an overlay of ICOS surface expression peaks on CD4+- T cells (A) and CD8+- T cells (B) either unstimulated (unfilled peak) or after 48 h incubation with TCB (turquoise peak). A total of three independent experiments was performed in three replicates. Representative histogram of one donor is shown.

To determine the effect of ICOS co-stimulation, T cell activation after 48 h incubation of CEA-TCB stimulated cells in the presence or absence of a titration of stroma-targeted or untargeted control ICOS antibody was assessed by FACS analysis. Addition of stroma-targeted ICOS antibodies, either in the 2+1 or the 1+1 format, led to a concentration-dependent increase in expression of the T cell activation marker CD25 on both CD4+- and CD8+- T cells (Figure 14). The observed, concentration-dependent T cell activation was target-specific and was not seen with the untargeted-control ICOS antibodies.

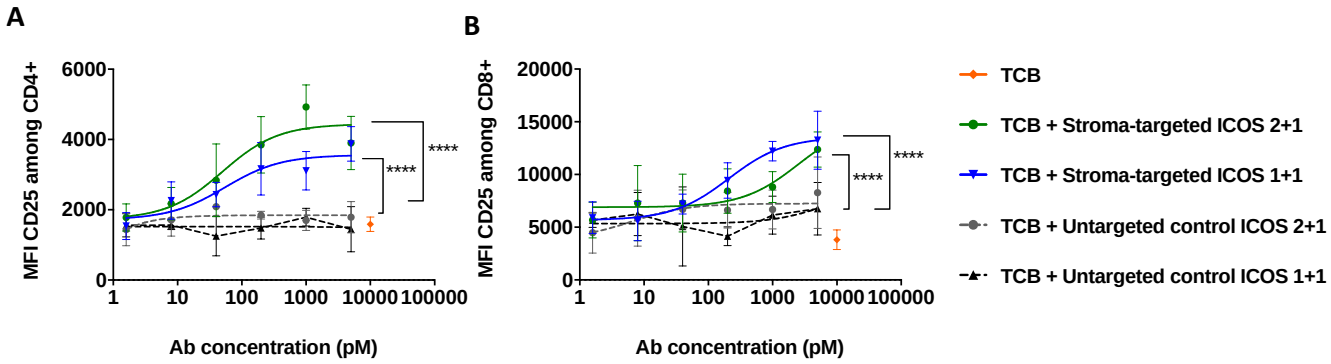

Figure 14: T cell activation upon ICOS co-stimulation in combination with CEA-TCB treatment. Healthy donor PBMC were co-cultured with tumor cells and fibroblasts, stimulated with 0.2 nM CEA-TCB in the presence or absence of a titration of stroma-targeted or untargeted control ICOS antibody. Surface expression of T cell activation marker CD25 was measured after 48 h incubation by FACS analysis. Median fluorescence intensity (MFI) of CD25 among CD4+- (A) and CD8+- T cells (B) is shown. A total of three independent experiments was performed in three replicates. Representative data of one experiment is shown as mean +/- SD. Significant differences were analysed by two-way ANOVA including Bonferroni correction (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

ICOS co-stimulation is also described to play an important role in T cell differentiation, including formation of memory T cell activation (Smith *et al.*, 2003). Consequently, after 72 h incubation we investigated the frequency of naïve (CD45RO-, CCR7+), central memory (Tcm, (CD45RO+, CCR7+)), effector memory (Tem, (CD45RO+, CCR7-)) and effector memory RA+ (Temra, (CD45RO-, CCR7-)) T cells. Upon co-stimulation with the stroma-targeted or the untargeted control ICOS antibodies in the 1 + 1 format an increase in the CD4+- and CD8+- Tcm cell population compared to CEA-TCB only treated cells could be detected. Moreover, ICOS co-stimulation with the monovalent ICOS antibodies resulted in decreased frequency of CD4+- Tem cell population, while a higher frequency of CD8+- Tem cells was found.

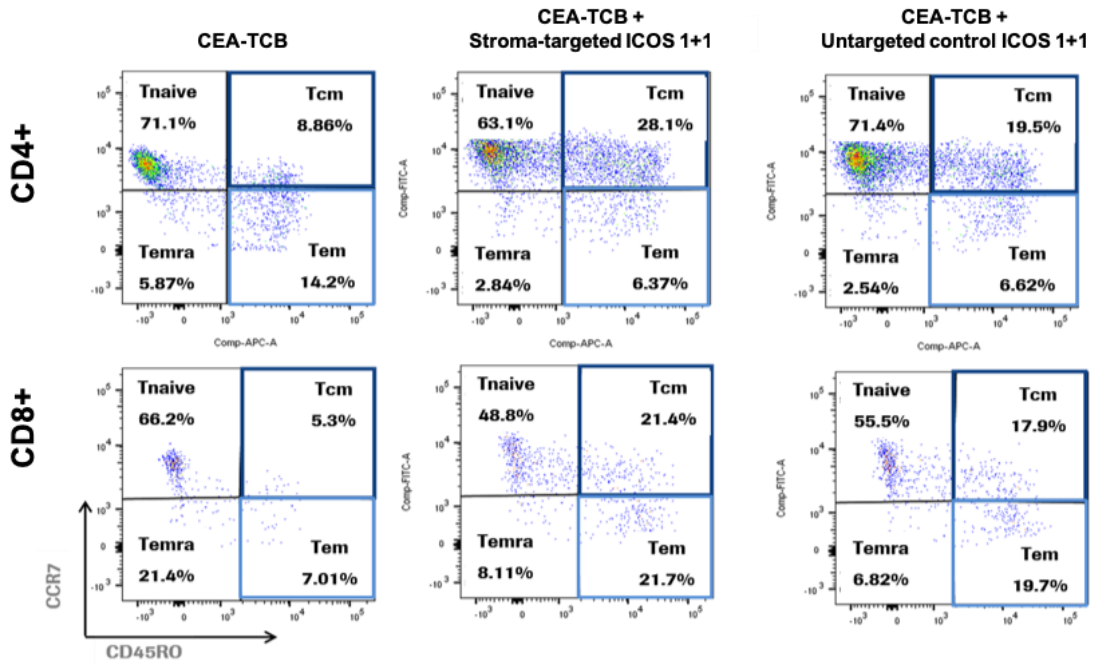

Figure 15: Memory T cell differentiation upon ICOS co-stimulation with CEA-TCB treatment Healthy donor PBMC were co-cultured with tumor cells and fibroblasts, stimulated with CEA-TCB (0.2 nM) and stroma-targeted or untargeted control ICOS 1+1 (1 nM) antibody. T cell differentiation after 72 h incubation is shown as FACS dot plot displaying the gating on naïve (CD45RO-, CCR7+)), central memory (Tcm (CD45RO+, CCR7+)), effector memory (Tem (CD45RO+, CCR7-)) and effector memory RA (Temra (CD45RO-, CCR7-)) T cells subsets. Frequency of memory T cell subsets upon treatment with CEA-TCB, CEA-TCB + stroma-targeted ICOS 1 + 1 antibody or CEA-TCB + untargeted-control ICOS 1 + 1 antibody is shown. A total of three independent experiments was performed. Representative data of one donor is shown.

The observed co-stimulatory effect of the ICOS antibodies in T cell differentiation was independent of the antibody format. A decrease in naïve T cell counts accompanied by an increase in CD4+- and CD8+-Tcm cell counts could be detected upon addition of the stroma-targeted ICOS antibody formats. Likewise, the untargeted-control ICOS antibodies resulted in increased Tcm cell count (Figure 16). Still, the number of cells undergoing differentiation towards Tcm cells was higher upon co-stimulation with the stroma-targeted ICOS antibodies.

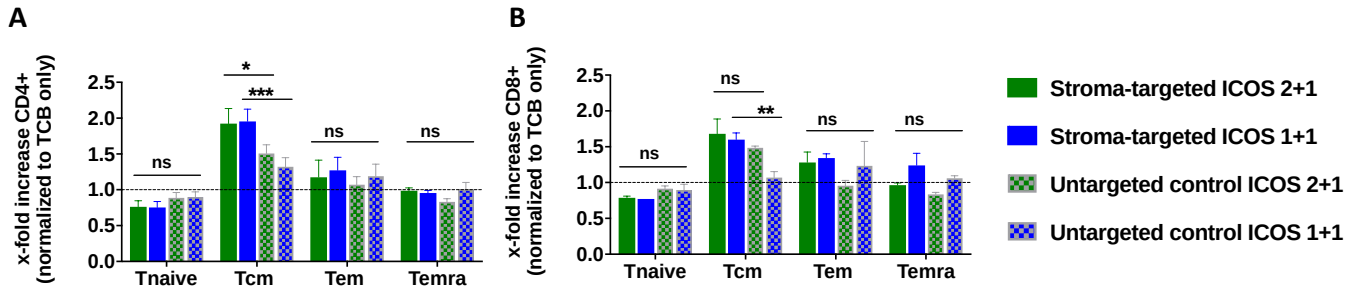

Figure 16: Memory T cell counts upon ICOS co-stimulation with- CEATCB treatment Healthy donor PBMC were co-cultured with tumor cells and fibroblasts, stimulated with CEA-TCB (0.2 nM) and stroma-targeted or untargeted control ICOS antibody. T cell differentiation after 72 h incubation is shown as a bar chart depicting the x-fold increase of naïve (Tn (CD45RO-, CCR7+)), central memory (Tcm (CD45RO+, CCR7+)), effector memory (Tem (CD45RO+, CCR7-)) and effector memory RA (Temra (CD45RO-, CCR7-)) T cells counts. Values are normalized to cell counts upon CEA-TCB only treated cells. Change in cell counts among the different T cell populations upon treatment with stroma-targeted or untargeted-control ICOS antibody formats is shown. A total of three independent experiments was performed in three replicates. Representative data of one experiment is shown as mean +/- SD. Significant differences were analysed by two-way ANOVA including Bonferroni correction (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$).

The synergy of the combination of CEA-TCB and ICOS agonism was also evidenced by increased cytokine secretion. Maximal cytokine values upon co-stimulation with the various targeted-ICOS antibodies after normalization to the level of cytokines induced by treatment with CEA-TCB only are shown in a heat map (Figure 17). Addition of the stroma-targeted ICOS antibodies resulted in enhanced cytokine secretion compared to untargeted-control ICOS antibodies. Interestingly, differences in the pattern of cytokine secretion could be observed between the stroma-targeted ICOS antibody formats. While the monovalent ICOS antibody format was significantly enhancing the secretion of IL-13, IL-17A, IL-5, and IL-6, the bivalent stroma-targeted ICOS antibody resulted in an increased secretion of GM-CSF, IFN-γ, IL-10, IL-2 and TNF-α. As seen in T cell differentiation the untargeted-control ICOS antibody in the 2 + 1 format showed weak baseline activation, indicated by a moderately increased secretion of the cytokines IFN-γ, IL-2, IL-4, and TNF-α compared to CEA-TCB only.

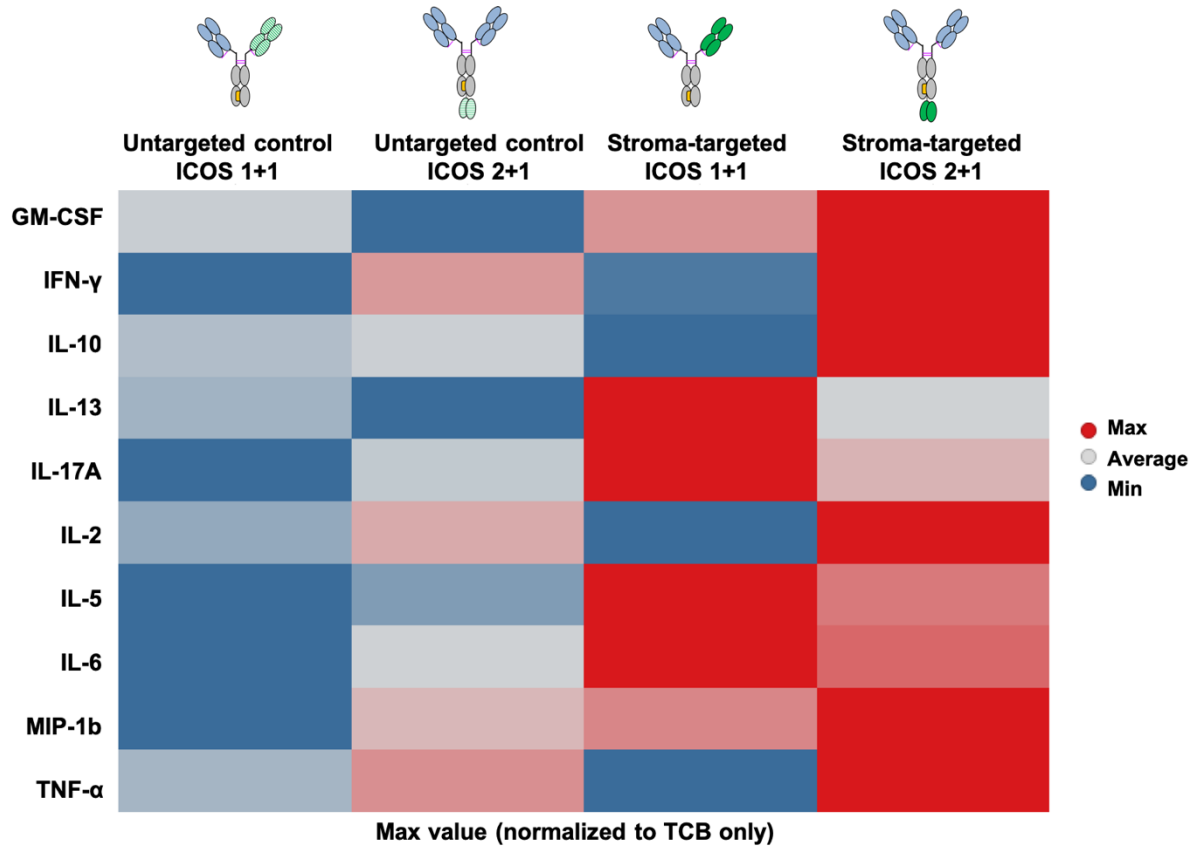

Figure 17: Cytokine secretion upon ICOS co-stimulation with CEA-TCB treatment Healthy donor PBMC were co-cultured with tumor cells and fibroblasts, stimulated with CEA-TCB (0.2 nM) and stroma-targeted or untargeted control ICOS antibody (1 nM). Secreted cytokines from cell culture supernatant after 72 h incubation were analysed by bead-based multi-parametric assay. A total of three independent experiments was performed in three replicates. Representative maximal cytokine values (max value) of one donor after normalization to cytokine values upon incubation with CEA-TCB only are displayed. The color code of the heatmap was generated using the GraphPad Prism algorithm and indicates the range of normalized cytokine values. For each of the depicted cytokines the range varies from maximal (max) cytokine values (red) to minimal (min) cytokine values (blue).

Based on these data, no clear format ranking of the various, targeted-ICOS antibodies could be done. Both formats of the stroma-targeted ICOS antibody equally increased TCB-mediated T cell activation and depended on crosslinking. For T cell differentiation no major difference in format could be detected. The only format difference was identified through the cytokine release pattern.

6.7 Single cell RNA sequencing analysis of regulated genes upon ICOS combination therapy

To gain a deeper understanding of genes and pathways altered in T cells as well as secondary effects on other immune cells upon ICOS co-stimulation, we performed a transcriptome analysis on single cell level of human PBMC co-cultured with tumor cells and treated *in vitro* with TCB alone or in combination with stroma-targeted ICOS antibody (described in 5.8 and 5.10).

As shown in previous experiments, the optimal time for detecting ICOS combination effects on T cells by FACS analysis was 40 – 48 h. Therefore, human PBMC either cultured *in vitro* with TCB alone, in combination with stroma-targeted ICOS antibody or without stimulus, were harvested after 42 h incubation.

Analysis of the scRNAseq data was done with the help of Petra Schwalie from the PS-BiOmics group at the Roche Innovation Center Basel. The clustering of all sequenced cells summarized in a two-dimensional UMAP plot is shown in Figure 18 A. Displaying the clustering by treatment groups (Figure 18 B) revealed a locational change from untreated cells (untreated) towards cells that were stimulated with TCB alone (TCB) and cell stimulated with TCB + stroma-targeted ICOS antibody (TCB + ICOS). Cells of the TCB or TCB + ICOS treatment groups were predominantly located in the memory and activation T cell clusters as well as the lysozyme+ myeloid cell cluster (M.LYZ). Differences in cell frequency among TCB monotherapy and the TCB + ICOS combination group are summarized in Figure 18 C, as the logarithm of ratio cell fraction. Upon combination with ICOS, a reduction in naïve T cells and NK cells as well as a higher frequency in the memory and activated CD4+- and CD8+- T cell clusters, classical CD14+ myeloid and LYZ+ myeloid cells could be detected. The strongest increase upon ICOS combination was observed in CD4+ memory T cells, myeloid LYZ+ TYROB+ and the myeloid progenitor DC cluster.

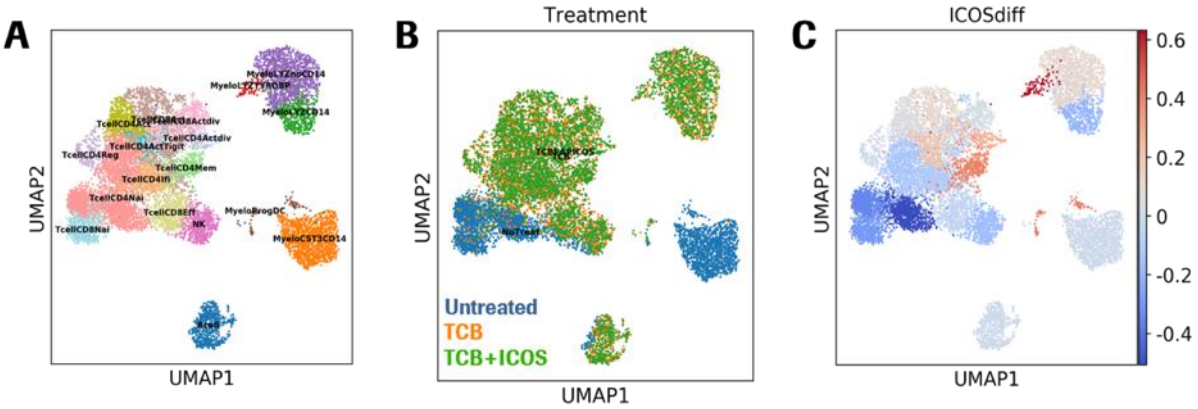

Figure 18: Single cell RNA sequencing of *in vitro* treated human PBMC (untreated, TCB treated (5 pM), TCB (5 pM) + ICOS (5 nM); incubated for a total of 42 h. 2-dimensional UMAP plot of CD45+ viable cells, analysed by scRNAseq. (A) Clustering of immune cell subsets based on the Louvain algorithm. (B) UMAP plot indicating cell distributing among the different treatment groups by color. (C) Fold change cell frequency upon TCB + ICOS vs. TCB indicated as log (ratio cell fraction). Experiment was performed as a single replicate on one donor. Analysis was performed with the help of PS-BiOmics group at the Roche Innovation Center Basel.

Gene signature changes upon ICOS co-stimulation were investigated by scRNAseq. For this purpose, gene signatures describing the functional phenotype of cells were analysed. Based on the work of Singer *et al.* (Singer *et al.*, 2017) this included signatures for cell activation (Act); cell dysfunction (Dys); both activation and dysfunction (Act/Dys); as well as naïve and memory cell phenotype. Shown in violin plots (Figure 19) is the distribution of cells among the treatment groups expressing genes that contribute to the signature. More cells with increased expression of genes contributing to the activation signature in CD4+- T cells, CD8+- T cells and B cells could be detected in the TCB + ICOS group. However, no significant change was discovered in the dysfunction cell signature upon ICOS co-stimulation. Signatures containing both, activation and dysfunction related genes were again higher in CD4+- T cells, CD8+- T cells and B cells.

Furthermore, we were curious if gene signature changes in the known main ICOS signalling pathways, TBK1 and PI3K, could be detected upon combination with the agonistic ICOS antibody (gene signatures were received from Reactome database). On CD4+- and CD8+- T cells an increase in cells contributing to the TBK1 gene signature upon ICOS co-stimulation could be detected. Surprisingly, no change in PI3K related gene signatures was found.

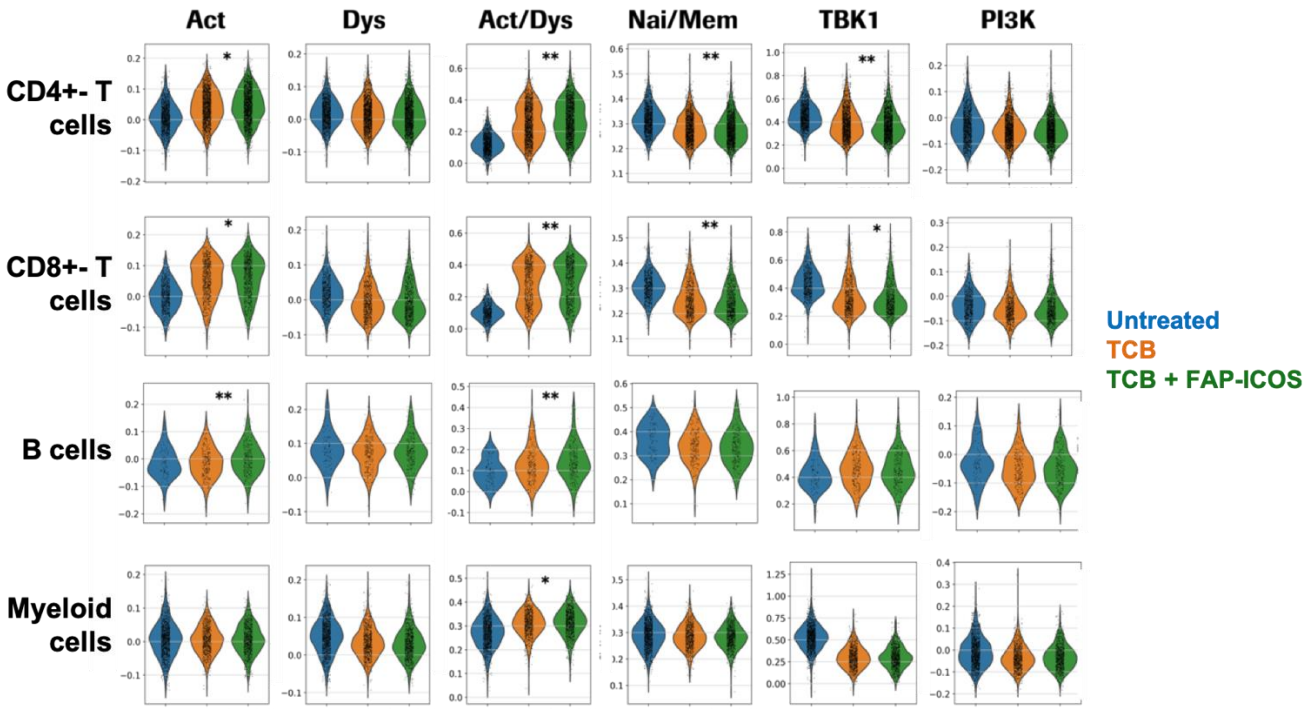

Figure 19: Gene signatures among treatment groups. Sequenced cells of the different treatment groups (indicated by color) were analysed with the help of PS-BiOmics group at the Roche Innovation Center Basel. Analysis of various signalling pathways was based on published gene signatures and are displayed as Violin Plots. Distribution of cell activation (Act), dysfunction (Dys), activation/dysfunction (Act/Dys), as well as naïve/memory cell signature (based on gene signatures described by Singer *et al.* (Singer *et al.*, 2017) on the indicated immune cell subsets. TBK1 and PI3K signatures are based on the Reactome database. Experiment was performed as a single replicate on one donor. Significant differences between the groups were analysed by two-sided, non-parametric Wilcoxon signed-rank test (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$).

In addition, we assessed the cytokine and chemokine pattern between the TCB monotherapy and the ICOS combination group. The distribution of cells expressing genes of cytokines and chemokines that were different between the treatment groups in analysed immune cell subsets is shown (Figure 20). In CD4+- T cells ICOS co-stimulation resulted in greater CCL2 and IFN-γ expression, while more cells with increased expression of CCL17 could be detected in the CD8+ cluster. Even though augmented activation signatures were discovered in B cells of the ICOS combination group, this could not be confirmed by higher cytokine or chemokine expression. In myeloid cells, more cells with higher CXCL10, CCL2, IFN-γ and IL-13 expression could be observed upon ICOS co-stimulation (as indicated by the asterisk).

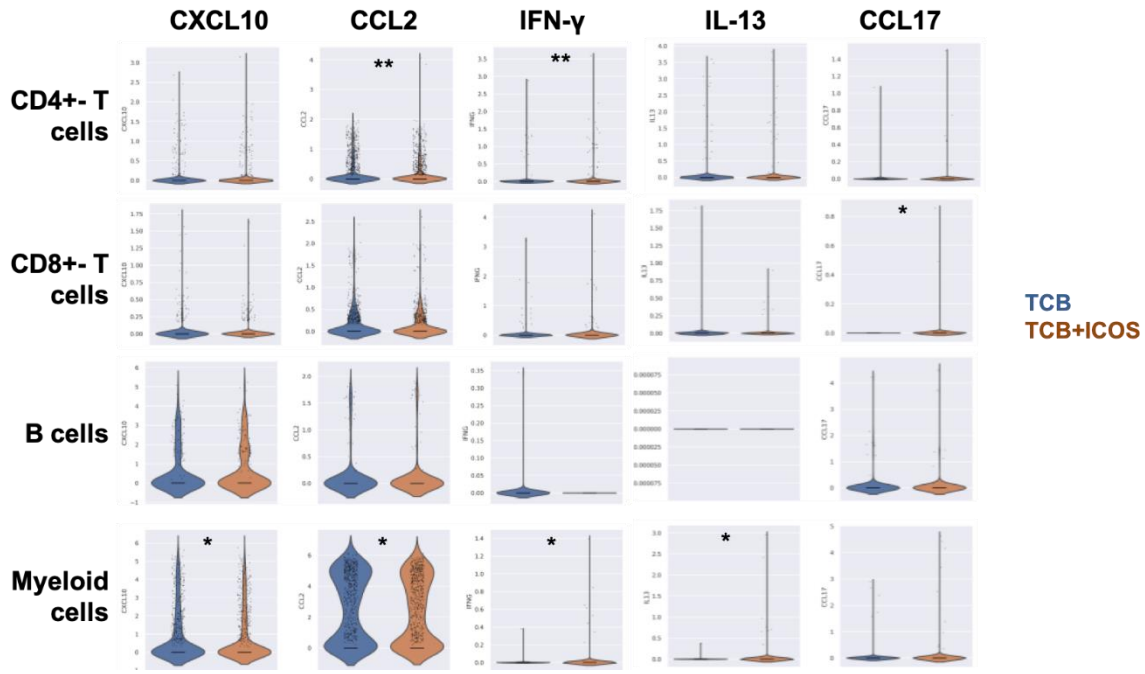

Figure 20: Cytokine signatures among treatment groups. Sequenced cells of the monotherapy (blue) and combination treatment group (orange) were analysed for altered cytokine expression of CXCL10, CCL2, IFN-γ, IL-13 and CCL17 with the help of PS-BiOmics group at the Roche Innovation Center Basel and are displayed as Violin Plots for the indicated immune cell subsets. Experiment was performed as a single replicate on one donor. Significant differences between the groups were analysed by two-sided, non-parametric Wilcoxon signed-rank test (ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$).

In summary, scRNAseq analysis revealed a very similar pattern between TCB treatment and ICOS co-stimulation. Changes upon ICOS signalling resulted in minor magnitude changes among gene signatures.

7 Discussion

The present work provides insights into the expression and induction of the T cell co-stimulatory receptor ICOS in human healthy and tumor infiltrating immune cells. It could be shown by systematic analysis that ICOS is expressed on tumor T cells and is inducible on T cells upon simultaneous binding of a TCB to tumor cells and healthy donor T cells. For the first time, we studied the effect of ICOS co-stimulation in combination with TCB using novel, targeted agonistic ICOS antibodies.

Affinity and format of bispecific antibodies can have a significant impact on the antibody`s therapeutic potency (Spiess *et al.*, 2015)**.** In contrast to co-stimulatory receptors belonging to the TNFR family, oligomerization of the antibody or ligand is not expected to be required for Ig-like superfamily members (Bodmer *et al.*, 2002, Mayes *et al.*, 2018). Since the natural ligand for ICOS is described to be expressed on the cell surface predominantly as a homodimer (Chattopadhyay *et al.*, 2006), we tested the bivalent and the monovalent ICOS binding antibody format to see if monovalent binding to ICOS and simultaneous binding to a target moiety (= crosslinking) could be equally (or even more) potent.

Functional assessment allowed no clear differentiation between the antibody formats. Both, the monovalent 1 + 1 and the bivalent 2 + 1 antibody format induced increased T cell activation but revealed different cytokine secretion patterns. Possible explanations are going to be discussed below. Nevertheless, slight unspecific activity was observed with the untargeted-control ICOS 2 + 1 antibody in late T cell responses, as T cell differentiation and cytokine secretion. Similar findings, stating a certain baseline activity of bivalent ICOS antibodies in the absence of crosslinking, could explain these observations (Takashi Tsuji, 2008).

7.1 ICOS expression and induction on healthy human T cells

ICOS, as a member of the IgG-like superfamily, is expressed on literally all activated T cell subsets. Consistent with literature, we could validate that ICOS baseline expression is highest on Treg cells, followed by conventional CD4+- and CD8+- T cells. Likewise, this study confirmed that ICOS expression is inducible upon TCR engagement and gets strongly

upregulated on CD4+- and CD8+- effector T cells as well as Treg subsets (Beier *et al.*, 2000, McAdam *et al.*, 2000). The fast kinetic of ICOS and the persistence of ICOS surface expression after wash out of anti-CD3 - anti-CD28 stimulus described in 6.2, indicates an important role of ICOS both during early and later stages of T cell co-stimulation.

The importance of ICOS co-stimulation in early stages of T cell activation is shown in a study of Tafuri *et al*. Proliferation and expression of activation markers of T cells of ICOS -/- mice were impaired at an early time point of 24 h stimulation, but could be recovered at 72 h probably by other molecules (Tafuri *et al.*, 2001). Also others reported defective proliferation, effector differentiation and cytokine immune functions of T cells stimulated in the absence of ICOS signalling (Coyle *et al.*, 2000, Dong *et al.*, 2001, Nurieva *et al.*, 2003).

A growing body of literature likewise suggests the importance of ICOS signalling in T cell memory differentiation. Burmeister *et al.* reported a reduced number of CD44+ CD62L+ memory T cells in mice carrying a genetic knock-out for either ICOS or ICOSL (Burmeister *et al.*, 2008). Human ICOS deficiency patients were reported to have a decreased CD4+ central and effector memory T cell compartment. Moreover, ICOS deficiency is often accompanied by impaired CD4+- and CD8+- effector T cell function (Grimbacher *et al.*, 2003, Takahashi *et al.*, 2009).

7.2 T cell subsets affected by ICOS co-stimulation

The expression level of ICOS is described to correlate with a distinct cytokine expression pattern: Lohning *et al.* reported that $ICOS^{high}$ cells mainly produce IL-10, $ICOS^{medium}$ cells produce IL-4, IL-5 and IL-13 and $ICOS^{low}$ cells produce IL-2, IL-6 and IFN-γ (Lohning *et al.*, 2003). We showed that upon TCB treatment, ICOS expression levels were comparable on CD4+- as well as CD8+- T cells and slightly higher on Treg cells. Still, *in vitro* combination of TCB and ICOS agonistic antibody revealed different cytokine patterns for the monovalent and the bivalent ICOS antibody. Co-stimulation by the stroma-targeted ICOS 2 + 1 antibody resulted in increased secretion of Th1 cytokines (IFN-γ, TNF-α, GM-CSF) as well as IL-2 and IL-10. In contrast, an enhanced secretion of Th2 cytokines (IL-5, IL-6, IL-13) and Th17 cytokine IL-17A was detected in combination with the monovalent stroma-targeted ICOS 1 + 1 antibody. We therefore conclude that the cytokine signature upon co-stimulation by our

agonistic ICOS antibodies cannot be correlated to the ICOS expression level. Instead, the various antibody formats, hence the degree of crosslinking, may result in altered intensity of subsequent ICOS downstream signalling. Moreover, the different antibody formats could cause altered cytokine kinetics. Since the cytokines have been analysed at one single point in time, this might also explain the differences.

Even though potentially interesting, the data must be interpreted with caution because cytokines from supernatant were analysed, which does not allow the identification of the specific T cell or immune cell subset (and its differentiation or activation status) that produced them. Further studies focused on intracellular cytokine T cell staining as well as a time-dependent assessment of their secretion are needed to better understand the underlying mechanisms upon format difference as well as identifying the T cell subsets being most responsive to ICOS co-stimulation.

7.3 Rationale of combining ICOS with TCB

TCB are able to tackle some immune escape mechanisms. Upon simultaneous binding of a tumor antigen and the CD3ε chain, TCB treatment represents an option of MHC-independent T cell activation, thereby overcoming lack of tumor- immunogenicity and T cell tolerance. Strong ICOS signalling is dependent on a concurrent first T cell signal. This implies the necessity of a tumor-inflamed setting. TCB-specific recruitement of T cells to the tumor site allows the transformation of poorly infiltrated or immune excluded tumors into inflamed tumors (Bacac, Klein, *et al.*, 2016).

In this study, it could be demonstrated that both, a classical anti-CD3 and anti-CD28 stimulation as well as TCB are able to induce ICOS expression. This finding is in line with recently published data by Bacac *et al.* showing that *in vitro* stimulation of human PBMC with anti-CD20-TCB likewise results in a time-dependent up-regulation of ICOS on human T cells (Bacac *et al.*, 2018). Therefore, up-regulation of ICOS is more a general consequence of TCB-mediated activation of T cells. Combining TCB treatment with tumor-targeted ICOS antibodies consequently could broaden the field of possible tumor indications applicable to ICOS co-stimulation. In addition to the up-regulation of ICOS, TCB treatment also induces the expression of the inhibitory immune checkpoint receptor PD-1, which in turn can dampen

the anti-tumor T cell response (Bacac, Klein, *et al.*, 2016). However, it was shown that TCB response can be enhanced by blocking the co-inhibitory receptor PD-1 expressed on activated T cells. Another promising approach of boosting T cell activity is the engagement of co-stimulatory receptors, for instance by combining TCB treatment with an agonistic ICOS antibody.
Providing additional co-stimulatory signal by an agonistic ICOS antibody to TCB treated cells can help in obtaining long-term protection by ICOS-mediated T cell memory formation (Burmeister *et al.*, 2008, Mahajan *et al.*, 2007, Marriott *et al.*, 2015, Moore *et al.*, 2011).
T cell activation without or with weak co-stimulation is reported to induce T cell anergy, a state of T cell unresponsiveness (Crespo *et al.*, 2013). ICOS co-stimulation could enhance TCB-mediated T cell response and potentially rescue TCB-treated T cells from anergy (Nurieva *et al.*, 2006).

7.4 Safety aspects of ICOS co-stimulation

7.4.1 Superagonism upon ICOS co-stimulation

Overstimulation of the immune system as occurred in 2006 during a Phase I clinical trial with the super-agonistic CD28 antibody TGN1412 cautions use of agonistic antibodies in human. TGN1412 was capable of activating CD28 irrespective of concurrent TCR activation, with the result of severe cytokine release syndrome and multiple organ dysfunctions (Hunig, 2016). Considering the structural and functional similarity of ICOS and CD28, the necessity of a concurrent CD3 signal and crosslinking of the antibody to ensure optimal ICOS signalling implies an important safety aspect. Reliance of ICOS signalling on concurrent TCR stimulation is in agreement with data previously published in a patent by Sazinsky *et al.* as well as reported by Wakamatsu *et al.* (Sazinsky, 2016, Wakamatsu *et al.*, 2013).

Crosslinking naturally occurs by receptor-ligand interactions delivered by T cell to APC engagement. Artificially mimicking this natural cell-cell contact for antibody based approaches can be achieved by introducing a crosslinking binding site (Mayes *et al.*, 2018). Besides providing full downstream signalling, a tumor-associated crosslinking target enables to have tumor-specific activity of the molecule. One example for a tumor-directed

immunotherapeutic agent is *ABBV-428*, a bispecific antibody targeting CD40 and a tumor-associated antigen (Dahlen *et al.*, 2018).

7.4.2 Risk of targeting ICOS+-Treg cells

As mentioned earlier, Treg cells are characterized by high baseline expression of ICOS expression. Stimulation of ICOS+-Treg cells with an agonistic antibody involves the risk of tumor-promoting, immunosuppressive T cell response.

Looking at the preclinical and clinical landscape, two key paradigms of ICOS agonistic antibodies can be described (shown in Figure 2): the first generation of agonistic antibodies usually exhibit a wild-type Fc, enabling the simultaneous binding to ICOS+- T cells and FcR positive cells, as NK cells or macrophages. Since the expression of ICOS on Treg at baseline is higher than the one on Teff cells, this is expected to result in a preferential depletion of $ICOS^{high}$ Treg cells. This is a mode of action that is described for JTX-2011, a dual activity ICOS agonistic antibody designed to stimulate T eff cells and deplete intratumoral Treg cells. The hypothesis of how the two mechanisms can coincide is based on the different ICOS expression density among Teff and Treg subsets as well as on receptor occupancy (Mayes *et al.*, 2018). The appropriate tumor indication with a favorable Teff to Treg ratio at baseline needs to be chosen accordingly. For instance cancer indication with high ICOS+-Treg infiltration (non-small cell lung cancer, head and neck squamous cell carcinoma) are well applicable to JTX-2011 ICOS+-Treg depletion mechanism (Sazinsky, 2016).
The second generation of agonistic ICOS antibodies usually comprises an ICOS- as well as a second targeting moiety that is used to crosslink the molecule. Here, the Fc part is usually modified to prevent any Fc-FcR interaction. One such example is the ICOS PD-L1 bispecific human IgG1 antibody that activates ICOS signalling through bridging together ICOS- and PD-L1-expressing cells. In a CT26 tumor model it was reported that the depletion of $ICOS^{high}$ Treg cells together with an improved Teff : Treg ratio (Sainson *et al.*, 2018).

TCB treatment results in an increased recruitement and proliferation of CD4+- and CD8+-effector T cells in the tumor microenvironment, but also increases the ratio of Teff to Treg in TIL upon therapy (Bacac, Fauti, *et al.*, 2016). According to this, we could confirm upon *in vitro* TCB stimulation a shift in the ratio of ICOS+-Treg to ICOS+-Teff in favour of CD4+-

and CD8+- effector T cells. Despite the higher ICOS expression level detected on human Treg, the combination with a TCB therefore allows the treatment with an agonistic ICOS antibody since starting numbers of Teff are higher than Treg.

7.5 ICOS co-stimulation in TCB-mediated anti-tumor efficacy

By scRNAseq transcriptome analysis we wanted to get more insight at a single cell level on the co-stimulatory effects of ICOS not only on T cells but also on other immune subsets that orchestrate anti-tumor immunity. Overall, gene signatures and cell frequency counts upon TCB monotherapy or in combination with ICOS agonistic antibody were very similar. Even though only a small percentage of cells responded to ICOS co-stimulation, transcriptome analysis could confirm results obtained from our previous *in vitro* experiments or being described for ICOS signalling. A slight decrease of naïve cells upon co-stimulation and an increase in activated and differentiated T cells could be observed based on cell frequency counts and signature distribution (Burmeister *et al.,* 2008, Okamoto *et al.*, 2004, Singer *et al.*, 2017).

Signalling pathway gene signature of ICOS co-stimulation and TCB seem to be similar. Both treatments similarly activated genes contributing to the main ICOS signalling pathway PI3K (Hutloff *et al.*, 1999). Surprisingly, on CD4+- and CD8+- T cells an increase in cells contributing to the TBK1 gene signature could be detected upon ICOS co-stimulation. Using a suboptimal TCB concentration for combination assays this finding is in contrast to literature, describing the stringent dependency on a concurrent strong CD3 stimulus for activation of the TBK1 pathway (Pedros *et al.*, 2016).

From a technical point of view, one has to bear in mind that even though ICOS-mediated co-stimulation can be detected after 42 h *in vitro* stimulation by increased T cell activation, this might not be the optimal timepoint for detection of gene signature changes. Signalling pathways, as PI3K, are early events of T cell activation implying a kinetic study to reveal the optimal timepoint to allow gene signature changes by scRNAseq.

Conclusively, one can assume that ICOS co-stimulation rather enhances the magnitude of the TCB signalling instead of completely activating an independent pathway. Taking into

consideration that the analysis was performed on one donor and at one time point only, this conclusion needs to be validated by more repetitions and with more exhaustive and time-dependent analysis to also take into account interindividual variability.

7.6 Combination of ICOS signalling and checkpoint blockade

T cell activation is a dynamic and flexible procedure of adaptive immune response driven by T cell co-stimulatory and co-inhibitory receptors. Positive and negative signals might be triggered on the same cell either simultaneously or consecutively. This contributes to the complexity of fully understanding T cell-mediated anti-tumor response.

Both, CTLA-4 and PD-1 mediate immune inhibition, but with different timing and anatomic location. CTLA-4 is known to regulate T cell proliferation early during the priming phase of immune response in lymphoid tissues, PD-1 preferentially functions during late effector phase on T cells within peripheral tissues. This diverse role is believed to be the reason for the success in combining checkpoint inhibitors against both pathways and thereby blocking early and late T cell inhibition (Mahoney *et al.*, 2015, Wei *et al.*, 2017). The co-expression of ICOS with PD-1 and CTLA-4 on activated healthy as well as on tumor-derived T cells detected in this study is in accordance with reports by other groups (Liu, 2016, Sazinsky, 2016) and provides the rationale for combining immune checkpoint inhibitors with ICOS co-stimulation.

Tang *et al*. detected elevated ICOS levels on blood T cells after anti-CTLA-4 therapy in melanoma and bladder cancer patients (Ng Tang *et al.*, 2013). This was followed by showing agonistic effects of ICOS activation in combination with anti-CTLA-4 therapy in a preclinical mouse model of melanoma and pancreatic cancer. Interestingly, combining ICOS co-stimulation with CTLA-4 therapy resulted in an increased Teff to Treg ratio (Fan *et al.*, 2014, Fu *et al.*, 2011). A similar finding was published by Zamarin *et al.* increasing CTLA-4 blockade by ICOS co-stimulation by using an oncolytic Newcastle disease virus expressing ICOSL. It is essential to point out that upon intra-tumoral therapy with ICOSL expressing virus, they could show that ICOS co-stimulation not only affects the tumor locally, but can also have systemic effects (Zamarin *et al.*, 2017). In the context of TCB combination this

strengthens our hypothesis of the generation of tumor-reactive, long-lived memory cells upon ICOS agonism.

A growing body of combinations using co-stimulatory antibodies together with PD-1 - PD-L1 blocking antibodies highlight the beneficial disease outcome potential (Hu-Lieskovan *et al.*, 2017). Two examples of ICOS antibodies currently being tested in combination with PD-1 blockade entered the clinics in 2016. The combination of agonistic ICOS IgG4 antibody GSK-3359609 with pembrolizumab is tested in different solid tumor indications (Liu, 2016). Moreover, advanced-stage solid tumors were tested with the combination of JTX-2011 ICOS agonistic IgG1 antibody with the PD-1 blocking antibody nivolumab (Mayes *et al.*, 2018). While GSK-3359609 acts as a true agonist, the JTX-2011 unifies Fc-mediated ADCC and agonistic properties. First data are expected in 2020 for both studies. Moreover, pre-clinical investigation of simultaneous binding to ICOS and PD-1 using a bispecific antibody resulted in an improved intra-tumoral Teff : Treg ratio (Sainson *et al.*, 2018).

Taking together the above given examples of successfully combining ICOS with checkpoint inhibition and given that ICOS is expressed along with PD-1 and CTLA-4 upon TCB stimulation, provides rationale for combining TCB treatment with the herein described agonistic ICOS antibodies and immune checkpoint inhibition.

7.7 Role of ICOS signalling in regard to tumor progression

Elevated ICOS expression levels on TIL compared to autologous as well as healthy donor PBMC were detected in this study, also stated by others (Sainson *et al.*, 2018, Sazinsky, 2016) . Several, contradicting reports on the correlation of ICOS expression and tumor outcome are published. A positive correlation of increased ICOS expression on primary tissue T cells and overall survival in untreated colorectal cancer patients was reported by Zhang et al (Zhang *et al.*, 2016). In contrary, a poor prognosis correlates with tumor infiltration of ICOS+- T cells in primary breast cancer patients, based on the proliferation of $ICOS^{high}$ Treg cells upon interaction with ICOSL+ plasmacytoid DCs (Faget *et al.*, 2013). Similarly, a study by Martin-Orozco et al. shows that ICOSL expression on melanoma cells is tumor promoting by directly supporting the expansion of ICOS+-Treg cells (Martin-Orozco

et al., 2010). It is thus envisionable that ICOS agonisation might induce detrimental effects to the cancer patient. This needs to be kept in mind while further evaluating this approach.

7.8 Outlook

In summary, this study showed that ICOS co-stimulation using novel agonistic, tumor-targeted antibodies has the potential to boost T cell activation. Current literature provides evidence that ICOS co-stimulation can fuel anti-tumor efficacy. As indicated before, the dynamics of the adaptive immune system manifests a time-dependent administration of co-stimulatory and co-inhibitory molecules. Further, in-depth investigation is needed to better understand the underlying mechanisms of ICOS co-stimulation in different tumor indications and among various patients, but also to identify possible additional combination partners, e.g. targeting the co-inhibitory signalling axis.

To address this, more repetitions with optimized assays conditions need to be done. Considering the minor changes upon ICOS co-stimulation detected by scRNAseq analysis, outliers could be the cells responding to ICOS therapy. In addition, more data at different time point could help to highlight the multiple levels of ICOS-mediated immune cell responses.

One question that remains is the impact of ICOS co-stimulation in TCB-mediated anti-tumor response. Does the higher T cell activation translate to an improved and long-lasting anti-tumor response? *In vitro* we were not able to detect enhanced T cell killing, most probably because ICOS signalling is rather affecting CD4+- Th cells instead of cytotoxic CD8+- T cells. An *in vivo* efficacy study would be a next step to determine the effect of ICOS co-stimulation on T cell memory formation to check our hypothesis that enhanced T cell immunity results in an improved and long-lasting anti-tumor response.

Given the reported expression of ICOS on distinct T cell subtypes, such as Th17, Tfh and Treg cells, but also on activated T conventional cells, one can easily imagine its complex role in T cell co-stimulation (Hutloff *et al.*, 1999, Paulos *et al.*, 2010, Strauss *et al.*, 2008, Weber *et al.*, 2015). Many more in-depth analyses of each of the subsets need to be done to unravel the net effect of using agonistic ICOS antibodies as cancer immunotherapy compounds.

Overall it could be shown that ICOS, despite its complex biological role, may serve as a promising candidate to further boost TCB-mediated T cell activation and thereby improve anti-tumor response.

8 Abbreviations

°C	Degree Celsius
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
APC	Antigen presenting cells
ATCC	American Type Culture Collection
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E:T	Effector to target
Fab	Fragment, antigen binding
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein-α
Fc	Fragment crystallizable
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
ICOS	Inducible T cell co-stimulator
ICOSL	Inducible T cell co-stimulator ligand
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-2	Interleukin-2
LDH	Lactate dehydrogenase
MEthOH	2-beta Mercaptoetanol
MFI	Median fluorescence intensity
MHC	Major Histocompatibility complex

NEAA	Non-essential amino acids
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ns	Not significant
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
RNA	Ribonucleic acid
Rpm	Rotations per min
RPMI	Roswell Park Memorial Institute
scRNAseq	single cell RNA sequencing
SEC	Size exclusion chromatography
TCB	T cell bispecific
TCR	T cell receptor
Tcyt	T cytotoxic cell
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	T helper cell
TIL	Tumor infiltrating lymphocyte
TNFR	Tumor necrosis factor receptor
Treg	T regulatory cell
U	Unit
VH/VL	Variable heavy / variably light
wt	wild-type

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11 Appendices

11.1 Poster presentation

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