Cytokine release in the context of T cell bispecific antibody therapies: biological mechanisms and mitigation strategies

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1. Abstract

T cell bispecific antibodies (TCBs) are a novel class of T cell engagers redirecting T cells towards tumor cells, facilitating the formation of a cytotoxic synapse and resulting in tumor cell lysis. On-target activity of TCBs may come with a risk of Cytokine Release Syndrome (CRS), characterized by elevated levels of pro-inflammatory cytokines in the serum, such as IL-6, IL-1β, TNF-α or IFN-γ and over activation of immune cells. Besides, the expression of the tumor-associated antigen on healthy cells may induce off-tumor activity of the TCB and contribute to inflammation. Despite the use of step-up dosing, glucocorticoids, or tocilizumab to manage or prevent these safety liabilities, they remain the major dose-limiting toxicities associated with the treatment of T cell engagers, highlighting the need to develop preventive mitigation treatments. To this aim, we investigated the biological mechanisms and the chronology of events involved in TCB-mediated cytokine release and explored mitigation strategies that might retain profound treatment efficacy while reducing cytokine release.

Using an *in vitro* co-culture of peripheral blood mononuclear cells (PBMCs) or total leukocytes (PBMCs + neutrophils) and target cells with the respective TCBs, or whole blood treated with a B cell depleting TCB, we confirmed the contribution of T cell and myeloid cells and revealed the role of neutrophils in the TCB-mediated cytokine release. In the same model, the use of anti-cytokine neutralizing antibodies provided insights into the chronology of events triggering the cascade of cytokines after TCB stimulation. Ultimately this work guided the evaluation of mitigation strategies directed against T-cell derived cytokine release by targeting kinases involved in signaling pathways downstream of the T cell receptor (TCR) after stimulation with TCBs. A novel small molecule-kinase inhibitors screen identified mTOR, JAK and Src inhibitors as candidates to switch-off T-cell derived cytokine release. We validated the effects of these kinase inhibitors and fine-tuned their effective doses in *in vitro* co-cultures of peripheral blood mononuclear cells (PBMCs) and tumor cells with the respective TCBs. *In vivo*, we used non-tumor or tumor-bearing-humanized NSG mice to assess the effect of mTOR, JAK and Src inhibitors on CD19-TCB-mediated cytokine release and anti-tumor efficacy.

Altogether, we confirmed the biological mechanisms of the TCB-mediated cytokine cascade and revealed the contribution of neutrophils. Our work on kinase inhibitors highlights their differential activities on the inhibition of cytokine release and/or T cell cytotoxicity and demonstrates the decoupling between both mechanisms. Our data open new horizons for the

prophylactic mitigation of CRS with the use of FDA approved mTOR and JAK inhibitors or the transient use of the Src inhibitor dasatinib. Finally, our results also indicate that dasatinib may serve as an “antidote” against adverse events related to the treatment with TCBs such as high grade CRS or unpredictable off-tumor activity of TCBs, a strategy which is now implemented the clinic.

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3. List of figures

4. List of abbreviations

BBB: Blood brain barrier

BCR: B-cell receptor

BiTE: Bi-specific T cell engager

CAB: Conditionally active biologic

CAR: Chimeric Antigen Receptor

CD: Cluster of differentiation

CEA: Carcinoembryonic antigen

CFSE: Carboxyfluorescein succinimidyl ester

CRS: Cytokine release syndrome

CTV: Cell trace violet

CXCL10: C-X-C motif chemokine ligand 10 (IP-10)

CXCL8: C-X-C motif chemokine ligand 8 (IL-8)

CXCR2: C-X-C motif chemokine receptor 2

DNA: Deoxyribonucleic acid

EMA: European medicines agency

FDA: Food and drug administration

FolR: Folate receptor

GrzB: Granzyme B

HLA: Human leukocyte antigen

ICANS: Immune effector cell associated syndrome

IFN-γ: Interferon-gamma

IL-1β: Interleukin-1β

IL-2: Interleukin-2

IL-6: Interleukin-6

IL-6R: Interleukin-6 receptor

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-32: Interleukin 32

JAK: Janus kinase

KI: Kinase inhibitor

MCP-1: Monocyte chemoattractant protein 1 (CCL2)

MIP-1α: Macrophage inflammatory protein-1α (CCL3)

MIP-1β: Macrophage inflammatory protein-1β (CCL4)

MHC: Major histocompatibility complex

mTOR: Mammalian target of rapamycin

NIR: Near infra red

NLR: Nuclight red

PBMCs: Peripheral blood mononuclear cells

PDX: Patient derived xenograft

PFN: Perforin

RBC: Red blood cells

RNA: Ribonucleic acid

scFV: Single chain fragment variable

TAA: Tumor-associated antigen

TCB: T cell bispecific antibody

TDB: T cell dependent bispecific antibody

TCR: T cell receptor

TDCC: T cell dependent cellular cytotoxicity

Tyrp1: Tyrosinase related protein 1

Tregs: T regulatory cells

TNF-α: Tumor necrosis factor-alpha

TKI: Tyrosine kinase inhibitor

UMAP: Uniform manifold approximation and projection

WT1: Wilms tumor 1

5. Introduction

5.1. T cell engaging therapies

In the field of cancer immunotherapy, redirecting T cell cytotoxicity towards tumor cells is a promising strategy to eradicate tumor cells. To this purpose, two main approaches are currently developed; one involving T cell genetic modification with chimeric antigen receptor (CAR) and the other one using T cell engagers linking the CD3ε chain of the T cell receptor to the targeted tumor antigen (1-6). They both act independently of the major histocompatibility complex on tumor cells, and of the specificity of the T cell receptor for tumor antigen, as depicted on Figure 1. CAR T cells and BiTE® directed against CD19 antigen have been approved, marketed and have shown remarkable efficacy to cure hematological tumors, opening an avenue for other types of T cell engaging therapies (7, 8). Currently, CAR T cells and T cell engagers against a wide spectrum of solid tumors and hematological tumors are under development and hold a great promise (9, 10).

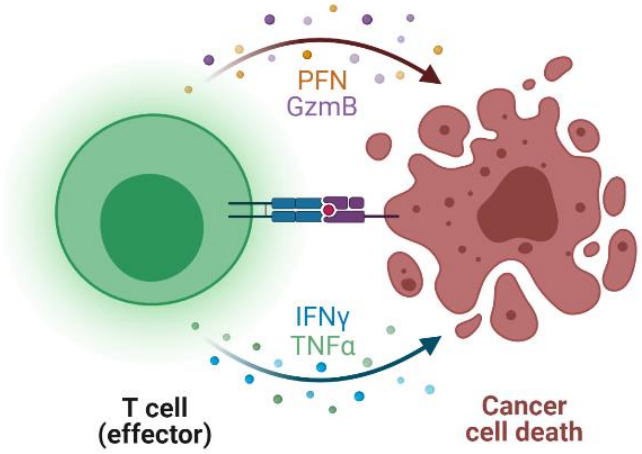

Figure 1. Antigen specific effector T cell recognize and kill cancer cells via the formation of a cytotoxic synapse and the release of perforin (PFN) and Granzyme B (GzmB)

5.1.1. CAR-T cells

CAR T cells are patient-derived T cells engineered with a chimeric antigen receptor targeted against tumor-associated antigens, schematized in figure 2. They can recognize tumor cells and form a cytotoxic synapse resulting in CAR T cell activation, proliferation, cytokine release and tumor cell killing. Tisagenlecleucel and axicabtagene are two CAR T cell products approved by

the European Medicine Agency (EMA) and US Food and Drug Administration (FDA) directed against the B-cell surface protein CD19 to treat patients with B cell malignancies. They differ by their intra-cellular co-stimulatory domain (4-1BB vs CD28) (11). So far, the success of CAR T cells has been restricted to B cell disorders. The development of CAR T cells directed against solid tumors is more difficult due to the heterogeneous expression of tumor-associated antigens, the challenging trafficking into tumor tissues and the potential immunosuppressive solid tumor microenvironment (12). This may be overcome by combination with immune checkpoint inhibitors.

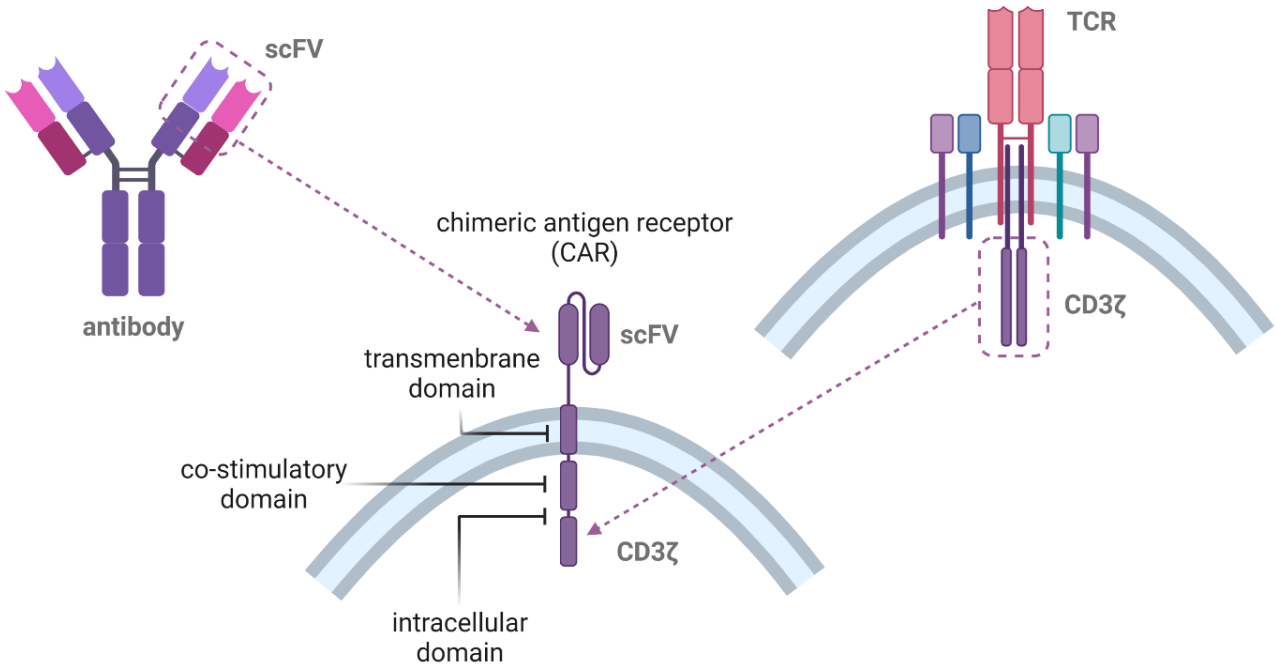

Figure 2. CAR-T cell construct, scFv: single-chain variable fragment, TCR: T cell receptor.

Having similar modes-of-action, CAR T cells and T cell engagers are associated with similar safety liabilities such as the induction of Cytokine Release Syndrome (CRS), or on-/off- target, off-tumor activity for CAR-T cells directed towards tumor antigens that may also be expressed at lower density on healthy tissues (13). The biological mechanisms triggering these safety liabilities as well as the mitigation strategies share commonalities between CAR T cell and T cell engagers. Since CAR T cells products are more advanced in the clinic, some findings may be applicable to T cell engagers. Nevertheless, the onset, intensity and duration of these toxicities differ between these two therapeutic modalities, with distinct PK/PD properties. Therefore, the

CAR T cell findings cannot be directly translated to T cell engagers, without at least verification and optimization for this different therapeutic modality

5.1.2. T cell engagers

T cell engagers represent an “off-the-shelf” approach to redirect T cells to tumor cells with reduced manufacturing costs compared to CAR T cells (9). They are bispecific antibodies targeting the CD3ε chain of the T-cell receptor and, simultaneously, the targeted tumor antigen, recruiting T cells to tumor cells and forming a cytotoxic synapse (10). The release of perforin and granzyme B results in tumor cell killing and is associated with T-cell activation, T-cell proliferation and cytokine release. Treatment with T cell engagers is therefore associated with a risk of cytokine storm, intrinsic to their mode-of-action (14, 15). Blinatumomab is a CD19/CD3 BiTE® antibody approved by the European Medicine Agency (EMA) and US Food and Drug Administration (FDA) directed against the B-cell surface protein CD19 to treat patients with B cell malignancies (16, 17). In the past years, T cell engagers have been developed in more than 50 different formats targeting solid and hematological tumor antigens. Three examples of common T cell engager formats are represented in Figure 3. In this work, we will focus on T cell engagers, and more specifically on T cell bispecific antibodies (TCBs).

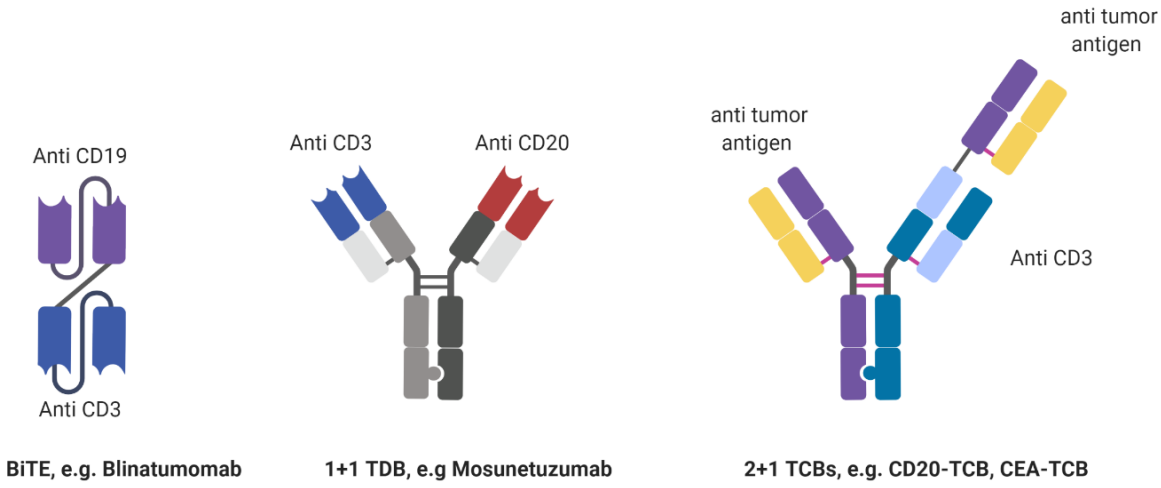

Figure 3. Examples of different T cell engager formats such as Bispecific T cell engagers (BiTE), 1+1 T cell Dependent Bispecific antibody (TDB) and 2+1 T cell bispecific antibodies (TCBs)

5.1.3. 2+1 format T cell bispecific antibodies (TCBs)

The T cell bispecific antibody platform consists of 2+1 format IgG molecules, targeting on both arms the tumor antigen and on one arm, the CD3ε chain of the T-cell receptor, as depicted in Figure 4 (3). These antibodies are engineered with the CrossMab technology that enables a correct antibody light-chain association with their respective heavy chain (18). They also carry a PGLALA mutation on the Fc portion that prevents FcγR signaling infusion reactions. The presence of an Fc portion increases their half-life in comparison to BiTE® molecules (19-21).

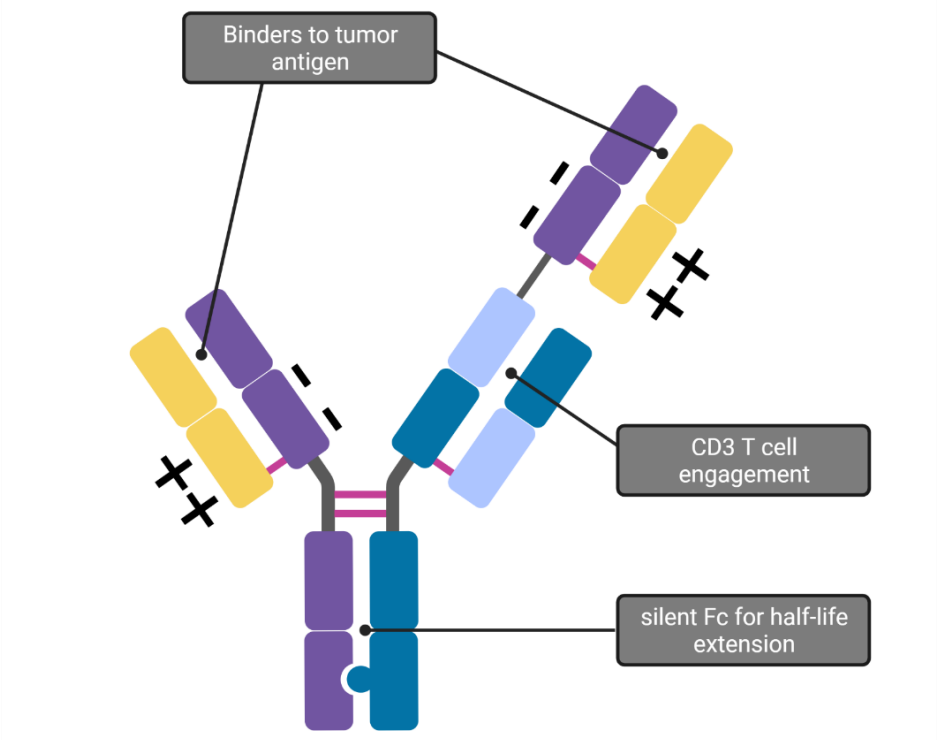

Figure 4. 2+1 T cell bispecific antibody format and specificity

Bacac *et al*. have already described CEA-TCB directed against CEA-positive solid tumor and CD20-TCB, directed against hematological tumors (1-3). By redirecting T cells to tumor cells, TCBs activate T cells and trigger the formation of a stable cytotoxic synapse in which perforin and granzyme B are responsible for the killing of cancer cells as shown in Figure 5. This biological mechanism goes along with the release of cytokines and chemokines, T cell activation and proliferation.

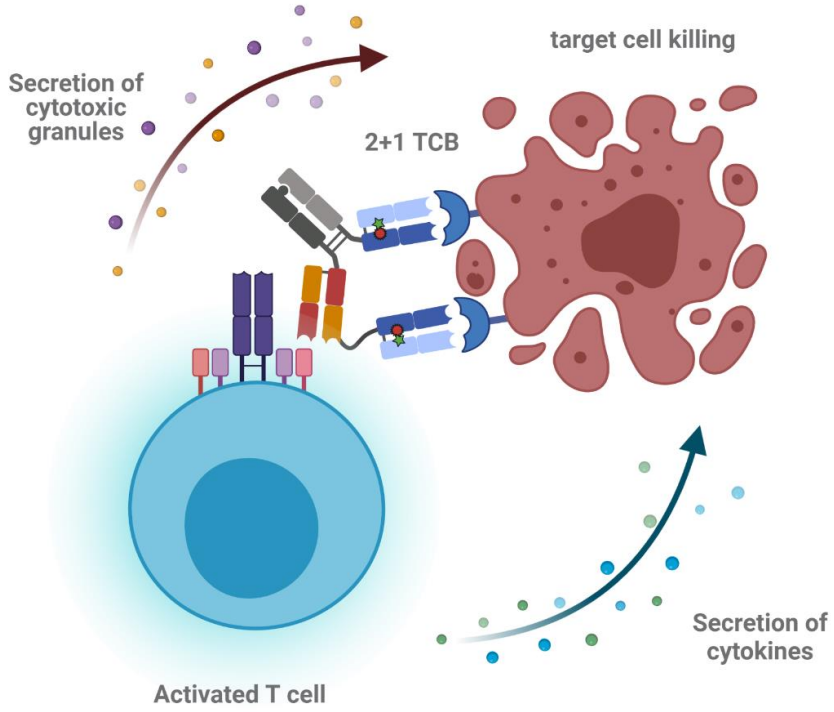

Figure 5. Summary of the TCB mode-of-action inducing tumor cell killing

Tyrp1-TCB and FolR-TCB are two other compounds targeted against Tyrp1- or folate receptor 1-expressing solid tumors respectively (22). HLA-A2 WT1-TCB is a TCR-like TCB recognizing WT-1 peptides presented by tumor cells on MHC class I (23). CD20-TCB and CD19-TCB are B cell depleting TCBs, directed against CD20 and CD19 antigens, respectively. CD20-TCB (glofitamab) in combination with obinutuzumab pre-treatment is tested in a pivotal phase 3 clinical trial for non-Hodgkin's lymphoma patients. Here, the dual targeting of CD20 by obinutuzumab followed by glofitamab results in safer and efficient depletion of B cells. Table 1 summarizes the indications investigated in the clinic for the different TCBs used in this work.

Table 1. TCBs and respective indications

TCBs	Indications
CD20-TCB (Glofitamab)	Phase I: Follicular Lymphoma Phase II: Non-Hodgkin's Lymphoma (NHL), Mantle Cell Lymphoma (MCL) Phase III : Diffuse Large B Cell Lymphoma (DLBCL)
CD19-TCB (tool compound)	Hematological malignancies
HLA-A2 WT1-TCB	Phase I: Acute Myeloid Leukemia (AML)
Tyrp1-TCB	Phase I: Metastatic melanoma (in combination with Tecentriq)
CEA-TCB (Cibisatamab)	Phase I: Colorectal cancer (CRC) (in combination with Tecentriq)
FolR-TCB (tool compound)	Folate receptor 1 expressed in ovarian, breast and lung cancer

5.2. Immune effector-cell related adverse events in the context of T cell engaging therapies: focus on T cell engagers

5.2.1. Off-/on-target off-tumor TCB activity

TCBs directed towards solid tumor antigens, such as FolR-TCB, Tyrp1-TCB and CEA-TCB, are associated with a risk of on-target off-tumor activity, unlike CD19- or CD20 TCB that deplete non-essential B lineage cells. Indeed the expression of tumor-associated antigens is often not restricted to tumor tissues and may be marginally expressed on healthy cells, such as epithelial tissues. In this case, the treatment with TCB may potentially trigger local cell death and inflammation, resulting in tissue damage compromising the patient's safety (Figure 6). As an example, treatment with CEA-TCB can potentially induce inflammation in the gastro-intestinal tract due to the expression of the CEA antigen on healthy enterocytes (24). One way to remediate on-target off-tumor TCB activity consists in fine-tuning binder affinities towards tumor antigen and CD3. Another way relies on the identification of target antigens whose expression is restricted to tumor tissues (4).

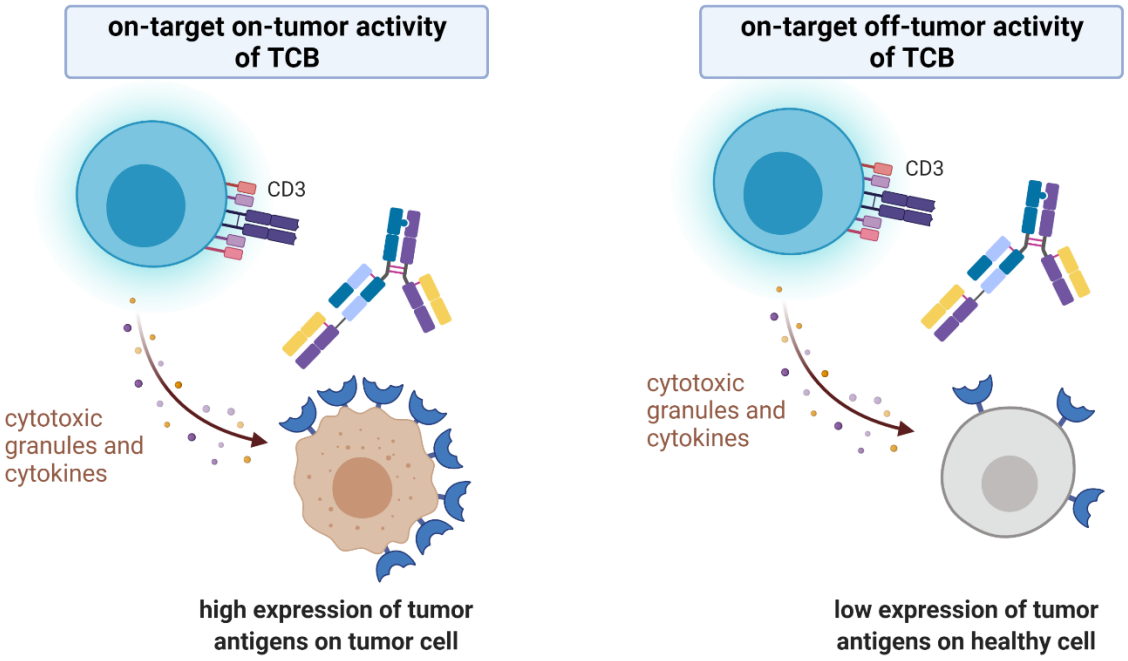

Figure 6. Illustration of on-target off-tumor activity

TCR-like TCBs such as HLA-A2 WT1-TCB may be associated with another problematic toxicity to manage in the clinic, represented by off-tumor off-target toxicity as observed with TCRs in the context of adoptive T cell therapy. A clinically relevant example of the risks associated with TCR-based therapies in the context of adoptive T cell therapy was identified when an unexpected cross-reactivity of an enhanced affinity TCR targeting an HLA-A*01-restricted epitope from MAGE-A3 resulted in severe cardiovascular toxicity through recognition of an unrelated HLA-A*01-associated peptide, A1-Titin (25). Similarly, a MAGE-A3 peptide-specific TCR for adoptive T cell therapy demonstrated an undetected cross-reactivity with MAGE-A12 responsible for severe neurotoxicity (26). A rapid blockade of T cell activation/proliferation at the onset of the off-target activity would have been essential to control such life-threatening toxicities. Recombinant TCR-based T cell engagers or TCR-like TCBs targeting intracellular proteins presented by MHC class I have the potential inherent risk of recognizing related undesired peptides in the context of MHC presentation (Figure 7).

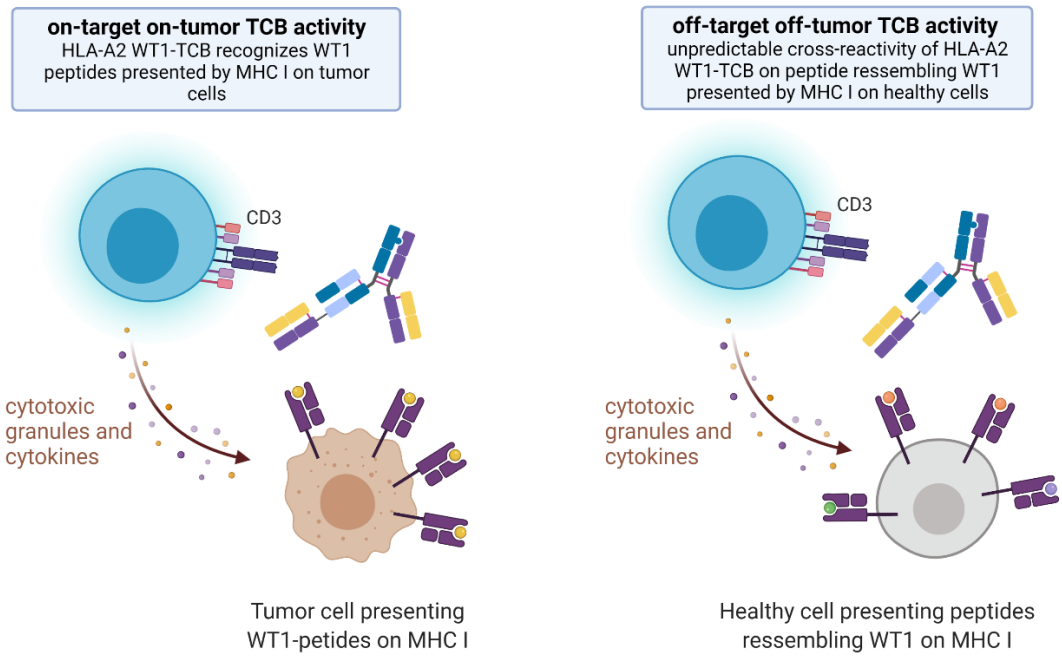

Figure 7. Illustration of off-target off-tumor TCB activity associated with HLA-A2 WT1-TCB as an example of TCR-like TCB

5.2.2. Immune effector Cell-Associated Neurotoxicity Syndrome (ICANS)

The ICANS has been reported as an adverse event after treatment with CD19 CAR-T cells and blinatumomab (27, 28). Mechanistic studies have shown that ICANS was associated with endothelial cell activation, leading to blood brain barrier disruption after treatment with CD19-CAR T cells (29). For blinatumomab, the incidence of ICANS seems to be less frequent than for CD19-CAR T cells and correlates with endothelial cell activation and T cell extravasation in the brain microvascular endothelium (11, 30). So far, ICANS was not reported with T cell engagers directed against solid tumors, nor with CD20-TCB, and will therefore not be investigated in the scope of this project focusing on the platform of 2+1 TCB molecules.

5.2.3. Cytokine Release Syndrome (CRS)

Treatment with T cell engagers also comes with a risk of Cytokine Release Syndrome (CRS) (31-34). The hallmark of CRS is a cytokine storm associated with an over activation of the immune system that can cause fever, hypotension and respiratory deficiency and, in the worst case, multi-organ failure (31, 35, 36). On-target activity of T cell engagers triggers T cell activation associated with the release of cytokines like IFN-γ, IL-2 and TNF-α that can further activate other immune cells, like myeloid cells, contributing to IL-6, TNF-α and IL-1β release and amplifying

the cytokine cascade (37-42) (Leclercq *et al.*, submitted). The rapid lysis of tumor cells after treatment with T cell engagers is also reported to contribute to the cytokine storm as well as the over-activation of surrounding immune cells (34). Nevertheless, this is a simplified mechanistic representation of the physiopathology of CRS, which is presumably much more complex, involving other immune and non-immune cell types (e.g. endothelial cells) and cytokines.

The severity, risk and incidence of CRS are likely to differ between hematological and solid tumor indications and depend on the type and potency of the CAR-T cell or T cell engager constructs. T cell engaging therapies against hematological tumors may be associated with a higher risk of CRS since on-target activity on B cells triggers an immediate release of cytokines in the blood. The occurrence and severity of CRS is also expected to correlate with the tumor load (43, 44). In addition, the onset of CRS varies between CAR-T cells and TCBs. For CAR-T cells, the peak of cytokine release seems to occur a few days after infusion and is associated with CAR-T cell proliferation and activation (11, 34). For TCBs, the peak of cytokine release can occur within hours after the first infusion, and is reduced upon the successive treatment cycles (3, 4, 39). Clinicians agreed on the ASTCT (American Society for Transplantation and Cellular Therapy) consensus to diagnose CRS severity at the patient bedside and recommend treatment interventions for syndrome management in the context of T cell engaging therapies (35, 45).

5.3. Clinical mitigation strategies

5.3.1. Glucocorticoids

Glucocorticoids like dexamethasone or methylprednisolone are used in the clinic for the mitigation of CRS. They are administered at high doses either as premedication or as mitigating agents. By strongly suppressing inflammatory reactions, glucocorticoids are the standard of care to manage CRS symptoms induced by T cell engaging therapies (13, 27, 31, 35, 46). In the clinic, the transient use of glucocorticoids was reported not to interfere with the response rate after treatment with CD19-BiTE® (14) or CD19 CAR T cells (47). In addition to preventing CRS, glucocorticoids can cross the brain blood barrier (BBB) and could mitigate neurotoxicity risks associated with T cell engaging therapies against hematological targets (45). However, it is still not yet clear whether continuous and systemic use of glucocorticoids might have a long-term impact on treatment efficacy since they are known to suppress T cell function (48, 49). Besides,

some patients experiencing CRS do not respond to glucocorticoids, suggesting that alternative approaches are required for the mitigation of CRS (4).

5.3.2. IL-6/ IL-6R blockade

IL-6 has been described as a key cytokine in the physiopathology of CRS and IL-6 levels were reported to correlate with the severity of CRS (34, 38, 39, 42, 50). Tocilizumab is the drug of choice to inhibit the IL-6-pathway by blocking the IL-6 receptor and is approved for the mitigation of low and high grade CRS for CAR-T cells (BLA 125276/S-114) (35). Siltuximab is another monoclonal antibody that binds IL-6 with a higher affinity than tocilizumab for IL-6 receptor. It could represent an attractive, yet investigational, approach to manage CRS. One advantage could be that siltuximab may prevent IL-6 crossing the brain blood barrier (BBB) as opposed to tocilizumab, targeting the receptor. Siltuximab could well prevent CAR-T cell induced neurotoxicity in addition to CRS management. Nonetheless, this hypothesis remains to be validated (45).

5.3.3. Step-up or fractionated dosing

Step-up or fractionated dosing of T cell engagers is now widely used to decrease the incidence of CRS, in particular the risk of grade 2 or higher CRS events that require patient hospitalization (35). Escalating the dose of T cell engager (step-up dosing) or dividing the dose into several lower doses (fractionated dosing) allows the administration of higher doses than the maximum tolerated flat dose by avoiding large peak release of cytokines. Dose fractionation is better tolerated than flat-dosing, as it reduces the first infusion cytokine release.

A clinical example of step-up dosing is the administration of 1000 mg obinutuzumab 7 days before the first cycle of escalating doses of glofitamab. In this clinical trial, glofitamab was given intravenously every 3 weeks for up to 12 cycles, with step-up doses starting at 2.5 mg, then 10 mg, and then 16 mg or 30 mg that were above the maximum tolerated flat dose (51, 52). The combination of obinutuzumab pre-treatment with step-up dosing of glofitamab resulted in a lower incidence of CRS grade 2 or higher. In this specific case, the pre-treatment with obinutuzumab also contributed to make the administration of glofitamab better tolerated in the step-up dosing phase, due to the de-bulking of peripheral B cells as well as the dual targeting of CD20 on tumor cells by both antibodies. Therefore combining step-up dosing with other prophylactic treatments

may even more efficiently prevent incidence of CRS while allowing administration of higher doses of T cell engagers.

5.4. Summary of the PhD goals

5.4.1. Investigation of the biological mechanisms triggering the TCB-mediated cytokine release cascade

The first goal of this project was to investigate the biological mechanisms involved in the cytokine release mediated by 2+1 TCBs and to identify the key cellular and molecular players. Therefore, we used an *in vitro* co-culture of peripheral blood mononuclear cells (PBMCs) or total leukocytes (PBMCs + neutrophils) with tumor cells and respective TCB. We assessed the contribution of T cells and monocytes by intra-cellular cytokine immunostaining. We confirmed the sequence of events triggering the cytokine cascade using neutralizing anti-cytokine antibodies targeted against T-cell derived cytokines. We then verified if neutrophils could play a role in TCB-mediated cytokine release by comparing PBMCs to total leukocytes from the same donor, used as effector cells in killing assays. The contribution of neutrophils was further demonstrated by single cell RNA sequencing of whole blood treated with a B cell depleting TCB. Altogether, our work sheds new lights on the role of neutrophils and confirms the contribution T cells and monocytes to the cytokine cascade driven by TCB-treatment. These results contribute to a better mechanistic understanding of CRS that may enable the development of alternative strategies to mitigate CRS along with the identification of biomarkers. These data could also contribute to the elaboration of predictive models of CRS and guide step-up dosing schedules.

5.4.2. Evaluation of mitigation strategies for on-/off-target off-tumor TCB activity using the Src/Lck inhibitor dasatinib

Since HLA-A2 WT1-TCB is associated with a risk of off-target off-tumor toxicity, with the unpredictable recognition of off-target peptides on MHC class I, we aimed to develop a “safety switch” for this specific program. Based on the recent screenings of tyrosine kinase inhibitor (TKI) libraries and findings from Mestermann *et al*. and Weber *et al*. showing that the Src inhibitor dasatinib can act as a pharmacological ON/OFF switch of CAR-T cell functionality, we evaluated the effect of dasatinib on TCB-induced T cell functionality, both, *in vitro* and *in vivo* (53-55). To determine the effective *in vitro* dose, we conducted a dose-response experiment on PBMCs stimulated with TCBs in the presence of tumor cells. We demonstrated that dasatinib

could reversibly switch-off the functionality of activated T cells previously stimulated with TCB at pharmacologically active doses, in an attempt to mimic the clinical situation (56). In non-tumor bearing humanized NSG mice, we showed that the combination of the short-lived small molecule dasatinib with a long-lived B-cell depleting TCB prevented the killing of B cells and cytokine release (56). Altogether, our data support that dasatinib may be used as an antidote for the mitigation of high grade CRS or adverse events related to on-target off-tumor toxicities for the platform of 2+1 TCB molecules.

5.4.3. Identification of JAK and mTOR kinase inhibitors to mitigate TCB-induced cytokine release while retaining anti-tumor efficacy

Despite the broad use of glucocorticoids, IL-6/IL-6R blockade, and step-up dosing, CRS remains one of the major dose-limiting toxicities associated to treatment with T-cell engagers. In addition, the management of CRS symptoms requires patient hospitalization that considerably increases treatment cost. Therefore, we aimed to evaluate prophylactic mitigation strategies that could prevent the incidence of CRS while retaining or improving efficacy of TCBs. A clinical example is the use of obinutuzumab pre-treatment in combination with glofitamab, resulting in a safer and efficacious profile (3). As the cascade of cytokines following treatment with TCBs is initiated by T-cells, we evaluated treatment interventions blocking T-cell derived cytokine release in order to prevent further activation of immune cells, while preserving T cell cytotoxicity. Li *et al.* had already shown that cytokine release could be uncoupled from T cell cytotoxicity (39). Therefore, we hypothesized that signaling pathways involved in cytokine release and T cell cytotoxicity downstream of TCR activation were uncoupled and aimed to identify kinase inhibitors that preferentially switch-off cytokine release while retaining T-cell cytotoxicity. To this purpose, we conducted a novel small molecule kinase inhibitor screen and identified FDA-approved mTOR and JAK inhibitors as lead candidates to prevent cytokine release and T cell proliferation following CD3 stimulation. We then validated the effects of these kinase inhibitors on TCB-mediated cytokine release, T cell activation and T cell cytotoxicity. We performed dose-response experiments on PBMCs stimulated with TCBs in the presence of tumor cells to determine the effective *in vitro* dose of mTOR and JAK inhibitors. We also compared their effects to those of dexamethasone and anti-TNF-α / IL-6R antibodies in the same system. *In vivo*, we first evaluated the effect of kinase inhibitors on cytokine release in humanized NSG mice upon the first treatment with a B cell depleting TCB. We then investigated whether transient prophylactic

treatment with kinase inhibitors, would affect the TCB anti-tumor efficacy using a lymphoma patient derived xenograft model in humanized NSG mice.

6. First manuscript

Title: Dissecting the mechanisms of cytokine release by T cell engagers highlights the contribution of neutrophils

Manuscript submitted to Oncoimmunology

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Keywords: Cancer immunotherapy, T cell engagers, T cell bispecific antibodies, Cytokine Release Syndrome, Neutrophil, PBMC

List of abbreviations

CAR: chimeric antigen receptor, CRS: cytokine release syndrome, NLR: NucLight Red, TAA: tumor-associated antigen, PBMCs: peripheral blood mononuclear cells, RBC: red blood cell, TCB: T cell bispecific antibody, TCR: T cell receptor, TDCC: T-cell dependent cellular cytotoxicity

Abstract

T cell engagers represent a novel promising class of cancer-immunotherapies redirecting T cells to tumor cells and have some promising outcomes in the clinic. These molecules can be associated with a mode-of-action related risk of Cytokine Release Syndrome (CRS) in patients. CRS is characterized by the rapid release of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-1β and immune cell activation eliciting clinical symptoms of fever, hypoxia and hypotension. In this work, we investigated the biological mechanisms triggering and amplifying cytokine release after treatment with T cell bispecific antibodies (TCBs) employing an *in vitro* co-culture assay of human PBMCs or total leukocytes (PBMCs + neutrophils) and corresponding target antigen-expressing cells with four different TCBs. We identified T cells as the triggers of the TCB-mediated cytokine cascade and monocytes and neutrophils as downstream amplifier cells. Further, we assessed the chronology of events by neutralization of T-cell derived cytokines. For the first time, we demonstrate the contribution of neutrophils to TCB-mediated cytokine release and confirm these findings by single cell RNA sequencing of human whole blood incubated with a B-cell depleting TCB. This work could contribute to the construction of mechanistic models of cytokine release and definition of more specific molecular and cellular biomarkers of CRS in the context of treatment with T-cell engagers. In addition, it provides insight for the elaboration of prophylactic mitigation strategies that can reduce the occurrence of CRS and increase the therapeutic index of TCBs.

Introduction

T Cell Bispecific antibodies (TCBs) or T cell engagers are bispecific antibodies capable of simultaneously binding a tumor-associated antigen (TAA) and the T cell receptor, which trigger T cell activation, proliferation, cytokine secretion and cytotoxicity towards tumor cells (1). We have previously described cibisatamab (CEA-TCB) and glofitamab (CD20-TCB) which harbor a 2+1 format with one binder for the CD3ε chain of the T cell receptor and two binders for the CEA or CD20 antigen (2-4). Their Fc region has been engineered with a P329G LALA mutation, preventing FcγR signaling without affecting functional binding to FcRn maintaining an IgG-like half-life (5-7). TCBs represent an accessible "off the shelf" alternative to Chimeric Antigen Receptor (CAR) T cells to eliminate tumors (8-13).

One of the major on-target safety liabilities associated with the use of TCBs is cytokine release, which can be excessive and initiate a Cytokine Release Syndrome (CRS) early after treatment (10, 14, 15). CRS is characterized by the clinical symptoms such as fever, hypotension and respiratory insufficiency, which are associated with a release of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α (10, 11). Clinicians have agreed on an ASTCT (American Society for Transplantation and Cellular Therapy) consensus to diagnose CRS severity at the patient bedside and recommend treatment for syndrome management (16, 17). Current approaches to manage CRS rely on glucocorticoids but also inhibition of IL-6R with tocilizumab, and blocking IL-6 with siltuximab are applied (16-22). If the symptoms do not resolve, patients receive supportive care to stabilize blood pressure and oxygen saturation (e.g. administration of vasopressors or oxygen). Dose-escalating regimens are also used to prevent the risk of high grade CRS for T cell engagers entering the clinic (23). Nevertheless, CRS still remains the dose-limiting toxicity associated with T-cell engaging therapies. This highlights the importance of better understanding the biological mechanisms and biomarkers involved in CRS in order to develop prophylactic treatments or build models that guide step-up dosing schedules (24). Therefore, we investigated the key cellular and molecular "triggers" and "amplifiers" involved in the cascade of TCB-mediated cytokine release.

To dissect in further detail the sequence of events in this cytokine release cascade, we used an *in vitro* T-cell dependent cellular cytotoxicity model ("TDCC"). In this system, peripheral blood mononuclear cells (PBMCs), monocyte-depleted PBMCs or total blood leukocytes (PBMCs and neutrophils) as effector cells were co-cultured with target cells expressing tumor-associated

antigens (TAAs) in the presence of a TCB. We used four different 2+1 format TCBs directed to solid tumor surface antigens (CEA, FolR1, Tyrp1) or an hematological tumor antigen, CD20.To clearly assess the role of neutrophils in cytokine release, we used a transcriptomic analysis by single cell RNA sequencing of human whole blood treated with CD20-TCB.

In line with the findings of Li. *et al*. and Godbersen-Palmer *et al*., we identified T cells as the trigger of the cytokine release induced by T cell engagers and monocytes as amplifier cells producing TNFα, IL-6 and IL-1β (25, 26). Furthermore, our study highlights for the first time the contribution of neutrophils to TCB-mediated cytokine release. Finally, our results also provide additional evidence that TCB-mediated cytokine release can be counteracted by treatment intervention targeted against T cell-derived cytokines, to mitigate CRS.

Material and methods

Antibodies

T cell bispecific antibodies (2+1 Format) are IgG1-based with bivalent binding entities to a target antigen and monovalent binding to the CD3ε chain of the T cell receptor. They have a silent Fc region engineered with a P329G LALA mutation, which prevents binding to the FcγR. DP47-TCB used as a control has the same IgG1-based format but bears two non-binding active binders in place of the target antigen binders. DP47-TCB, Tyrp1-TCB, CEA-TCB, FolR-TCB and CD20-TCB were produced internally. The commercial compounds adalimumab and tocilizumab were used to block TNF-α and IL-6, respectively. The anti-IFN-γ blocking antibody was purchased from BioXcell (BE0245).

Cell lines

CHO cells were engineered to stably express the Tyrp1, FolR or CEA antigen on the cell surface under Puromycin selection. CHO cells were cultured as adherent cells in T flasks (TTP), harvested with Trypsin (Gibco) and passaged twice per week at a density of 20 000 cells/cm^2 in DMEM/F12 medium (Gibco) containing 10% FBS (Gibco) and supplemented with 6 µg/mL Puromycin.

MKN45- and NucLight Red (NLR)-labelled MKN45 cells are human gastric cancer cell lines used as target cells in TDCC assays with CEA-TCB. MKN45 cells are adherent cells, harvested

with Trypsin (Gibco) and passaged twice per week at a density of 60 000 cell/cm^2 in RPMI Glutamax (Gibco) containing 10% FBS (Gibco).

PBMCs isolation

Human fresh blood was collected from anonymous healthy volunteers through the Roche internal employee donation program. Buffy coats were collected from anonymous healthy volunteers through the Zürich blood donation center, in accordance with the declaration of Helsinki. PBMCs were isolated from fresh whole blood or buffy coat by density gradient centrifugation. 20-25 mL of fresh blood or diluted buffy coat (1:2, PBS) were layered on 15 mL of Ficoll (Stemcell) in 50 mL tubes. Tubes were centrifuged at 2000 rpm for 30 min at RT without braking. The PBMC layer was removed, washed in PBS and centrifuged 3 times for 5 min at 1700 rpm, 1400 rpm and 1100 rpm (RT) to remove the remaining Ficoll. The PBMCs were washed again and centrifuged at 800 rpm (RT) for 10 min to remove remaining platelets. The PBMCs were counted using a Beckmann Coulter cell counter and diluted in medium to the targeted cell concentration.

Monocyte depletion

Monocyte-depleted PBMCs were isolated from fresh whole blood using RosetteSep^{TM} human monocyte depletion cocktail (Stemcell). Fresh blood was first incubated with an antibody cocktail (50 µL/mL of blood sample, 20 min, RT) which targets monocytic surface markers and crosslinks them together with red blood cells (RBC). Monocytes sediment together with RBCs at the bottom of the tube after density gradient centrifugation. The monocyte-depleted PBMC fraction was collected and the cells were washed 4 times as described above to remove any remaining Ficoll and platelets.

Isolation of total leukocytes

Total leukocytes were isolated from fresh whole blood by magnetic removal of RBCs using the EasySep^{TM} RBC depletion kit (Stemcell). Fresh whole blood was diluted 1:2 in PBS with 6 mM EDTA. 10 mL diluted fresh whole blood was incubated together with beads targeting RBCs (50 µL/mL of undiluted blood volume) in a 14 mL polystyrene tube (BD) using a magnetic tube holder (EasyEights^{TM}, Stemcell) for 5 min at RT. The cell suspension was carefully pipetted out and poured into a new 14 mL polystyrene tube. RBC magnetic isolation was then repeated three times (5 min, RT). The clear yellowish cell suspension was then collected and placed for 5 min in

the magnetic tube holder to remove remaining magnetic beads, washed with PBS twice by centrifugation at 800 rpm, for 10 min (RT) to remove the platelets. The total leukocytes were counted using a Beckmann Coulter cell counter and diluted in medium to reach the same lymphocyte concentration as in the PBMC preparation.

In vitro T cell dependent cellular cytotoxicity assays

One day before the assay, the target cells (MKN45 or transfected CHO cells) were plated in flat-bottomed 96-well plates (TPP) at a concentration of 25000 cells/well in 100 µL of culture medium. On day 0, it was replaced with 100 µL of fresh medium and 50 µL of a stock solution containing 6.0 x 10^6 lymphocytes/mL was placed in each well (300 000 lymphocytes/well, E:T =10:1). For each antibody, a series of 8 dilutions (1:10) was prepared and 50 µL were transferred to each well. At the 24 hrs and/or 48 hrs endpoints, the plates were centrifuged and supernatants were collected to measure LDH release (75 µL, RT) and cytokines (75 µL, stored at -80°C). The effector cells were washed twice in 100 µL PBS (1500 rpm, 5 min, RT) and stained for flow cytometry analysis.

In vitro TDCC assays in IncuCyte

For TDCC assays with 5000 adherent NLR-labelled MKN45 cells/well, the assay medium was replaced with fresh medium (100 µL/well) and 50 000 effector cells/well (50 µL) were transferred to obtain a final E:T ratio of approximatively 10:1. The kinase inhibitors (10 µL) followed by the antibody solutions (50 µL) were then added to initiate killing. The assay plates were covered with lids, and placed in the incubator or IncuCyte at 37°C, 5% CO_2 (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time).

Cytokine analysis

Cytokines were analyzed in the TDCC assay supernatants (stored at -80°C) using the Luminex technology with a human8Plex Assay kit (Biorad) and additional IL-1β and MCP-1 beads. Pre-diluted supernatants were incubated with beads for 1 hr and then centrifuged at 800 rpm. The plate was washed with wash buffer and detection antibodies were added for 1 hr before centrifugation at 800 rpm. The plate was washed again and streptavidin was added for 1 hr before centrifugation at 800 rpm. After another washing, samples were re-suspended in assay buffer

before being measured by fluorescence reading using a Luminex plate reader from Biorad. Data were analyzed using the Biorad Bio-Plex Software.

Intracellular cytokine staining

After 4-6 hrs of TCB stimulation, 20 µL of medium containing 1/150 (final dilution: 1/1500) Brefeldin A (Golgiplug, BD) and 1/100 (final dilution: 1/1000) Monensin A (Golgistop, BD) were added in each well to block cytokine secretion for 12-14 hrs. The PBMCs were collected, and washed twice in PBS by centrifugation at 1500 rpm for 5min (RT). 1/50 Fc-blocker solution (True Monocyte Staining, Biolegend) was added and incubated for 20min at RT in the dark to prevent nonspecific staining. The PBMCs were washed in PBS and centrifuged for 5 min at 1500 rpm (RT) and stained for 30 min at 4°C in the dark for the following surface markers: CD19 (pacific blue, Biolegend), CD4 (FITC, Biolegend), CD8 (BV605, Biolegend), CD14 (BV711, Biolegend), CD16 (BUV735, BD) and live dead (aqua zombie, Biolegend) in PBS. The PBMCs were fixed with BD Cytofix/Cytoperm (BD) (80 µL/well, 30 min at 4C). The PBMCs were washed with PBS, centrifuged and washed again with Perm/Wash buffer (BD) and incubated in Perm/Wash buffer (30 min, 4°C, no light). Perm/Wash buffer containing antibodies to IL-6 (PE, Biolegend), IL-1β (Alexa 647, Biolegend), TNF-α (APC-Cy7, Biolegend) and IFN-γ (BUV737, BD) was added (50 µL/well) and incubated for 30 min at 4°C in the dark. The PBMCs were washed with Perm/Wash buffer and with FACS buffer and re-suspended in 100 µL FACS buffer. Data acquisition was performed using a BD Fortessa flow cytometer and the DIVA software.

Target cell killing – LDH release

CytoTox-Glo™ (Promega) cytotoxicity assay was used to measure LDH release as a reporter of target cell killing. 75 µL supernatants were collected in a 96-well white plate and 25 µL of CytoTox-Glo reagents were added in each well. The plate was agitated for 15 minutes, 600 rpm, RT and luminescence was measured using a Perkin Elmer plate reader. Relative Luminescence Units (RLU) were then reported against the TCB concentration.

Flow cytometry analysis

After stimulation, PBMCs, monocyte-depleted PBMCs or total leukocytes were washed twice in PBS before centrifugation at 1500 rpm for 5 min (RT) and stained for the following surface markers: CD45 (Alexa fluor 700, Biolegend) CD4 (FITC, Biolegend), CD8 (BV605, Biolegend),

CD25 (BUV395, BD), CD69 (APC-Cy7, Biolegend) and live dead (aqua zombie, Biolegend) for 30 min at 4 °C in FACS buffer. The staining of TNFR1 and TNFR2 on T cells and monocytes was conducted using the following antibodies: CD4 (FITC, Biolegend), CD8 (BV605, Biolegend), CD14 (BV711, Biolegend), CD16 (BUV735, BD), CD120a (APC, Biolegend), CD120b (PE, Biolegend). Antibodies to CD16 (BUV395, BD), CD11b (Pe-Cy7, Biolegend) and CD62L (APC-Cy7, Biolegend) were used to analyze the phenotype of neutrophils. After staining, the cells were washed twice in FACS buffer and re-suspended in 100 µL/well FACS buffer for analysis. Acquisition was performed using a BD Fortessa coupled to an HTS platform.

Data analysis

Flow cytometry data were analyzed using Flowjo V10. Cytokine data were analyzed using the Bio-Plex software from Biorad. GraphPad Prism 8 was used to generate the graphs and for statistical analysis. EC_{50} values were determined using nonlinear regression curves, variable slope fit (four parameters) and least-square fit. For dose-titration curves, areas under the curves were calculated and used for statistical comparison. Data are shown as means with SD or SEM or as individual curves. The statistical tests used are indicated in the figure legends for each experiment.

Single cell RNA sequencing of whole blood

Whole blood from 4 donors was treated with 0.2 µg/mL CD20-TCB, or incubated in the absence of CD20-TCB. At baseline (before addition of TCB) and assay endpoints (2, 4, 6, and 20 hrs), blood was collected for total leukocyte isolation using EasySep^{TM} red blood cell depletion reagent (Stemcell). Briefly, cells were counted and processed for single cell RNA sequencing using the BD Rhapsody platform. To load several samples on a single BD Rhapsody cartridge, sample cells were labelled with sample tags (BD Human Single-Cell Multiplexing Kit) following the manufacturer's protocol prior to pooling. Briefly, $1x10^6$ cells from each sample were re-suspended in 180 µL FBS Stain Buffer (BD, PharMingen) and sample tags were added to the respective samples and incubated for 20 min at RT. After incubation, 2 successive washes were performed by addition of 2 mL stain buffer and centrifugation for 5 min at 300 g. Cells were then re-suspended in 620 µL cold BD Sample Buffer, stained with 3.1 µL of both 2 mM Calcein AM (Thermo Fisher Scientific) and 0.3 mM Draq7 (BD Biosciences) and finally counted on the BD Rhapsody scanner. Samples were then diluted and/or pooled equally in 650 µL cold BD Sample

Buffer. The BD Rhapsody cartridges were then loaded with up to 40 000 – 50 000 cells. Single cells were isolated using Single-Cell Capture and cDNA Synthesis with the BD Rhapsody Express Single-Cell Analysis System according to the manufacturer's recommendations (BD Biosciences). cDNA libraries were prepared using the Whole Transcriptome Analysis Amplification Kit following the BD Rhapsody System mRNA Whole Transcriptome Analysis (WTA) and Sample Tag Library Preparation Protocol (BD Biosciences).
Indexed WTA and samples tags libraries were quantified and quality controlled on the Qubit Fluorometer using the Qubit dsDNA HS Assay, and on the Agilent 2100 Bioanalyzer system using the Agilent High Sensitivity DNA Kit. Sequencing was performed on a Novaseq 6000 (Illumina) in paired-end mode (64-8-58) with Novaseq6000 S2 v1 or Novaseq6000 SP v1.5 reagents kits (100 cycles).

Single cell RNA sequencing data analysis

Sequencing data was processed using the BD Rhapsody Analysis pipeline (v 1.0 https://www.bd.com/documents/guides/user-guides/GMX_BD-Rhapsody-genomics-informatics_UG_EN.pdf) on the Seven Bridges Genomics platform. Briefly, read pairs with low sequencing quality are first removed and the cell label and UMI identified for further quality check and filtering. Valid reads are then mapped to the human reference genome (GRCh38-PhiX-gencodev29) using the aligner Bowtie2 v2.2.9, and reads with the same cell label, same UMI sequence and same gene are collapsed into a single raw molecule while undergoing further error correction and quality checks. Cell labels are filtered with a multi-step algorithm to distinguish those associated with putative cells from those associated with noise. After determining the putative cells, each cell is assigned to the sample of origin through the sample tag (only for cartridges with multiplex loading). Finally, the single-cell gene expression matrices are generated and a metrics summary is provided.
After pre-processing with BD's pipeline, the count matrices and metadata of each sample were aggregated into a single adata object and loaded into the besca v2.3 pipeline for the single cell RNA sequencing analysis (27). First, we filtered low quality cells with less than 200 genes, less than 500 counts or more than 30% of mitochondrial reads. This permissive filtering was used in order to preserve the neutrophils. We further excluded potential multiplets (cells with more than 5,000 genes or 20,000 counts), and genes expressed in less than 30 cells. Normalization, log-transformed UMI counts per 10,000 reads [log(CP10K+1)], was applied before downstream

analysis. After normalization, technical variance was removed by regressing out the effects of total UMI counts and percentage of mitochondrial reads, and gene expression was scaled. The 2,507 most variable genes (having a minimum mean expression of 0.0125, a maximum mean expression of 3 and a minimum dispersion of 0.5) were used for principal component analysis (PCA). Finally, the first 50 PCs were used as input for calculating the 10 nearest neighbours and the neighbourhood graph was then embedded into the two-dimensional space using the UMAP algorithm)(28) . Cell clustering was performed using the Leiden algorithm (https://www.nature.com/articles/s41598-019-41695-z) at a resolution of 2 (29).
Celltype annotation was performed using the Sig-annot semi-automated besca module, which is a signature-based hierarchical cell annotation method (27).To identify neutrophils, this signature was used: ELANE, MPO, PRTN3, CTSG, AZU1 and FCGR3B. FCGR3B was the most expressed gene and highly specific, together with negative expression of other lineage markers such as CD3D or CD79A. Finally, neutrophils were selected in order to generate further visualizations, such as the expression level of selected cytokines across conditions, by using a custom script with mainly besca and scanpy functions.

Results

T cell activation and target cell killing are associated with cytokine release upon first TCB stimulation

In an *in vitro* TDCC assay using PBMCs as effector cells, treatment with CEA-TCB resulted in a rapid dose-dependent T cell activation, as indicated by CD69 and CD25 upregulation on CD4+ and CD8+ T cells after 24 hrs, concomitant with the killing of MKN45 tumor cells [Fig 1A, B]. The negative control DP47-TCB bearing two non-binding active binders in place of the CEA binder did not produce these effects, showing that these were dependent on target engagement. Subsequently, IFN-γ, TNF-α, IL-6, IL-1β and IL-8 were detected in the supernatants of the CEA-TCB TDCC assays [Fig 1C].

As these cytokines are being found elevated in the serum of patients treated with TCBs and developing CRS, the TDCC assay system appears as a relevant assay system for mechanistic studies of TCB-mediated cytokine release (10). EC50 values for T cell activation measured by expression of CD25 on CD4+ and CD8+ T cells, for target cell killing and for cytokine release were calculated for CEA-TCB as well as for two other TCBs, Tyrp1- and FolR-TCB [Fig 1D].

FolR-TCB, which is the most potent of these TCBs, had the lowest EC50 for these three parameters followed by Tyrp1-TCB and finally CEA-TCB [Fig 1D]. For all three TCBs, the EC50 values indicate that target cell killing is a more sensitive parameter than T cell activation and cytokine release, suggesting that there are dose windows at which TCBs induce killing while not triggering cytokine release [Fig 1D]. This observation is also used for a MABEL starting dose selection (30).

To model a repeated TCB treatment, PBMCs harvested 4 days after a first stimulation were re-stimulated with CEA-TCB in the presence of fresh NLR-labelled MKN45 tumor cells. The real-time tumor cell killing was followed by Incucyte. After the second stimulation, the kinetics of tumor cell killing remained the same as after the first stimulation [Fig 1E]. However, TNF-α, IFN-γ, IL-2, and IL-6 levels detected 4 days after the second stimulation were significantly lower than 4 days after the first one, reflecting clinical experience with step-up fractionated dosing (31) [Fig 1F]. This data shows that CEA-TCB-induced cytokine release is decoupled from T cell cytotoxicity upon re-stimulation, as previously reported for other CD3 bispecific antibodies (25).

The *in vitro* TDCC assay system allows modelling TCB-mediated cytokine release early after T cell stimulation, upon initiation of target cell killing and is an appropriate tool to recapitulate the sequence of events and to identify key cellular and molecular players involved in TCB-mediated cytokine release.

Figure 1.

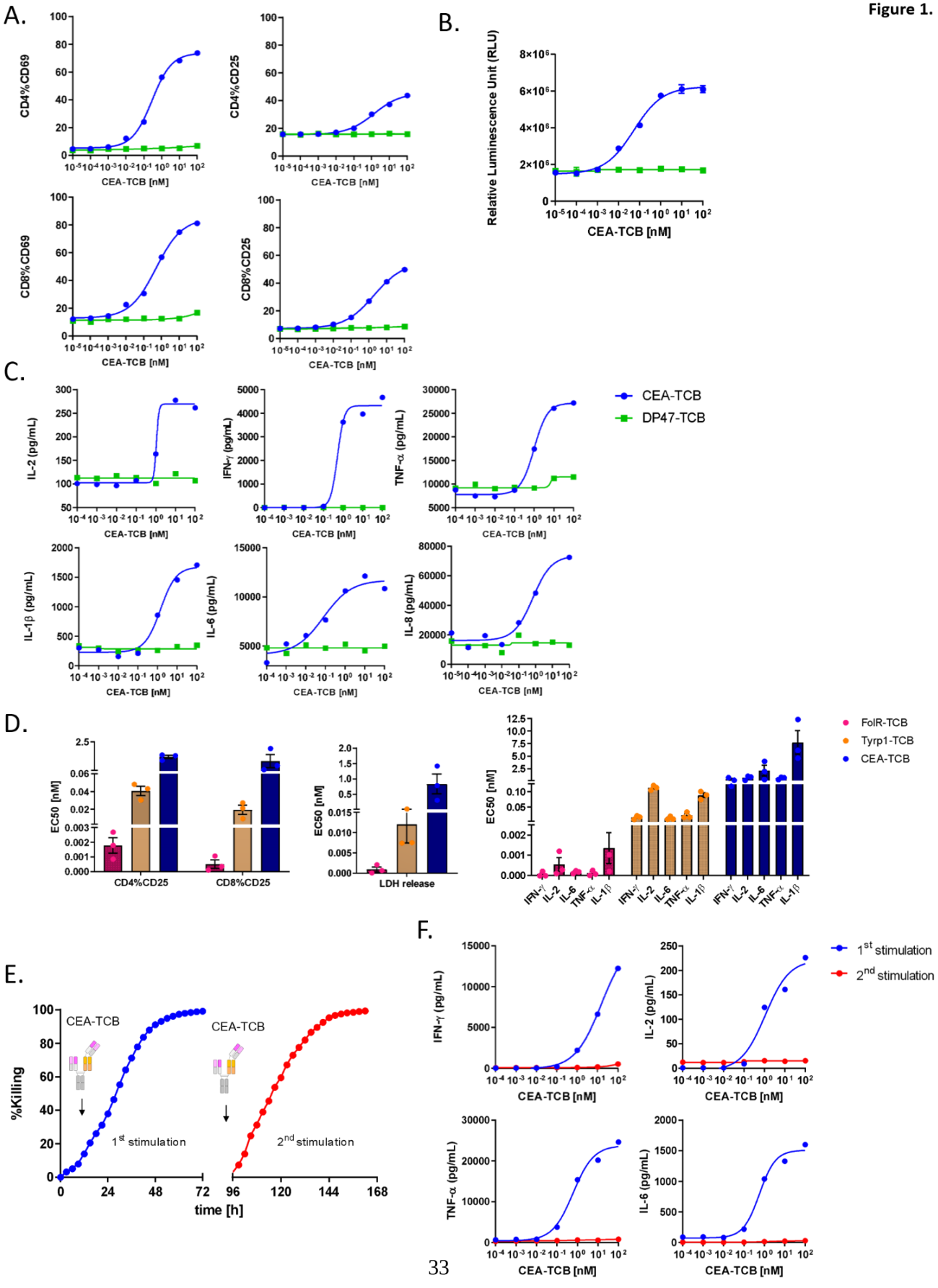

A.
CD4%CD69
CD4%CD25
CD8%CD69
CD8%CD25
CEA-TCB [nM]
B.
Relative Luminescence Unit (RLU)
CEA-TCB [nM]
C.
IL-2 (pg/mL)
IFN-γ (pg/mL)
TNF-α (pg/mL)
IL-1β (pg/mL)
IL-6 (pg/mL)
IL-8 (pg/mL)
CEA-TCB
DP47-TCB
D.
EC50 [nM]
CD4%CD25
CD8%CD25
LDH release
FolR-TCB
Tyrp1-TCB
CEA-TCB
IFN-γ
IL-2
IL-6
TNF-α
IL-1β
E.
%Killing
CEA-TCB
1st stimulation
2nd stimulation
time [h]
F.
IFN-γ (pg/mL)
IL-2 (pg/mL)
TNF-α (pg/mL)
IL-6 (pg/mL)
CEA-TCB [nM]
1st stimulation
2nd stimulation

Figure 1. Cytokine release is associated with TCB-induced T cell activation and cytotoxicity upon first stimulation.

A. PBMCs were stimulated with CEA-TCB in a TDCC assay using MKN45 target cells (E:T = 10:1) for 48 hrs. CD69 and CD25 expression on CD4+ and CD8+ T cells were measured by flow cytometry, cells from technical replicates were pooled. **B.** killing of MKN45 target cells was measured by LDH release. Mean of 2 technical replicates +/- SEM. **C.** Cytokines were analyzed in the supernatants by Luminex. **B-C.** data are shown for 1 donor representative of 3. **D.** EC_{50} values for CD25 expression on CD4+ and CD8+ T cells, LDH release and cytokine release in TDCC assays with FolR-TCB (CHOK1SV target cells), Tyrp1-TCB (CHOK1SV-Tyrp1 target cells), CEA-TCB (CHOK1SV-CEACAM5 target cells), mean of n=3 donors +/- SD (24 hrs). **E.** Real time killing of NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB during first and second stimulation (Incucyte). **F.** Comparison of cytokine release, 96 hrs after first and second stimulation with 10 nM CEA-TCB. Supernatants from technical replicates were pooled and analyzed by Luminex. **E-F**. 1 donor representative of 3.

T cells contribute to TNF-α and IFN-γ but not to IL-6 release

Since T cells are directly targeted by TCBs through CD3 engagement and TNF-α, IFN-γ and IL-6 are detected after TCB stimulation, we used intracellular cytokine staining and flow cytometry to determine whether these cytokines were produced by CD4+ and CD8+ T cells. When PBMCs were treated with 100 nM CEA-TCB, 24.9% of CD4+ cells and 18.9% of CD8+ cells were TNF-α positive after 20 hrs of incubation. Only 0.39% and 0.68% of CD4+ and CD8+ T cells, respectively, were TNF-α positive with the DP47-TCB negative control [Fig 2A]. With CEA-, FolR- and Tyrp1-TCB, TNF-α production by CD4+ and CD8+ T cells was dose-dependent [Fig 2B, Supp. Fig 1A, B].

Interferon-γ production by T cells was also detectable after stimulation with CEA-TCB [Fig 2C]. Among CD4+ and CD8+ T cells, 3.82% and 8.81%, respectively, were IFN-γ-positive 20 hrs after stimulation with 100 nM CEA-TCB. With DP47-TCB, respectively only 0.47% and 0.51% scored positive. The production of IFN-γ by CD8+ and CD4+ T cells was also TCB dose-dependent for all three TCBs tested. [Fig 2D, supp. Fig 1C, D].

The elevated levels of IL-6 in the supernatants of TDCC assays after TCB treatment suggested that T cells might contribute to its release. However, as found using intracellular immunostaining and flow cytometry, CD4+ and CD8+ T cells failed to produce IL-6 after stimulation with 100 nM CEA-TCB [supp. Fig 1E].

Altogether, these data demonstrate that on-target TCB activity triggers the dose-dependent release of TNF-α and IFN-γ but not of IL-6 by activated CD4+ and CD8+ T-cells.

Figure 2.

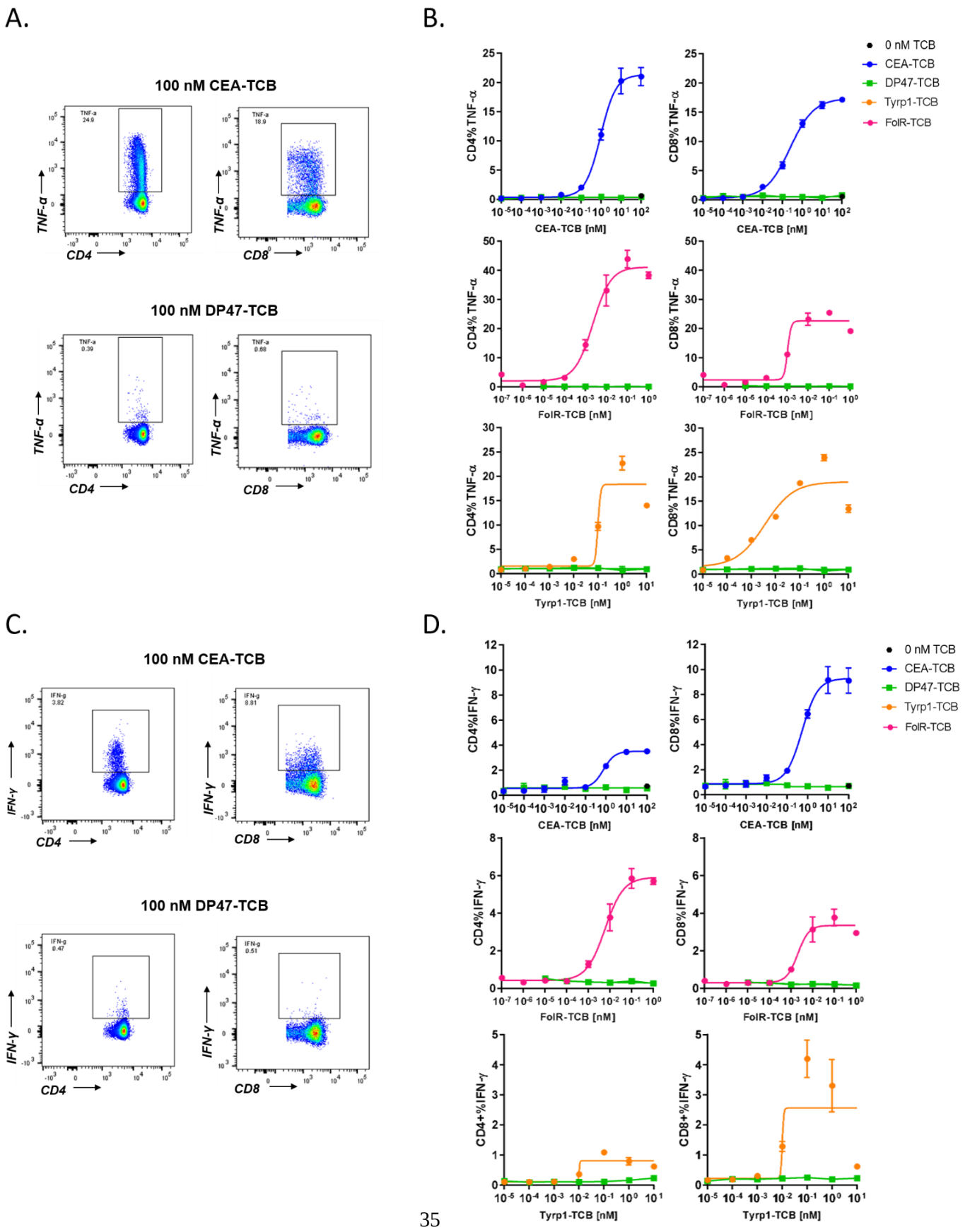

A.
100 nM CEA-TCB
TNF-a
24.9
TNF-α
CD4
TNF-a
18.9
TNF-α
CD8
100 nM DP47-TCB
TNF-a
0.39
TNF-α
CD4
TNF-a
0.68
TNF-α
CD8
B.
0 nM TCB
CEA-TCB
DP47-TCB
Tyrp1-TCB
FolR-TCB
CD4% TNF-α
CEA-TCB [nM]
CD8% TNF-α
CEA-TCB [nM]
CD4% TNF-α
FolR-TCB [nM]
CD8% TNF-α
FolR-TCB [nM]
CD4% TNF-α
Tyrp1-TCB [nM]
CD8% TNF-α
Tyrp1-TCB [nM]
C.
100 nM CEA-TCB
IFN-g
3.82
IFN-γ
CD4
IFN-g
8.81
IFN-γ
CD8
100 nM DP47-TCB
IFN-g
0.47
IFN-γ
CD4
IFN-g
0.51
IFN-γ
CD8
D.
0 nM TCB
CEA-TCB
DP47-TCB
Tyrp1-TCB
FolR-TCB
CD4%IFN-γ
CEA-TCB [nM]
CD8%IFN-γ
CEA-TCB [nM]
CD4%IFN-γ
FolR-TCB [nM]
CD8%IFN-γ
FolR-TCB [nM]
CD4+%IFN-γ
Tyrp1-TCB [nM]
CD8+%IFN-γ
Tyrp1-TCB [nM]

Figure 2. CD4+ and CD8+ T cells produce TNF-α and IFN-γ but not IL-6 upon TCB treatment.

PBMCs were stimulated with CEA-TCB, Tyrp1-TCB, FolR-TCB or DP47-TCB (negative control) in a TDCC assay using MKN45, CHOK1SV-Tyrp1 or CHOK1SV-FolR target cells respectively (E:T = 10:1). Golgistop and Golgiplug were added to block cytokine secretion and intra-cellular staining was performed 20 hrs after stimulation with TCB. Representative flow cytometry plots of **A.** TNF-α and **C.** IFN-γ positive cells were gated among CD4+ and CD8+ T cells after stimulation with 100 nM CEA- or DP47-TCB. Intracellular staining of **B.** TNF-α and **D.** IFN-γ by CD4+ and CD8+ T cells after stimulation with CEA-TCB, Tyrp1-TCB and FolR-TCB, means of 2 technical replicates +/- SEM for 1 donor representative of 3.

Monocytes contribute to TNF-α production together with T cells and are the main mediators of IL-6, IL-8 and IL-1β release.

In the same assay, we set the gating on CD14+CD16- classical monocytes to evaluate their contribution to cytokine production. When PBMCs were treated for 20 hrs with 100 nM CEA-TCB, 6.01% of CD14+CD16- monocytes were double positive for IL-6 and TNF-α, 3.94% for IL-1β and TNF-α and 26.5% for IL-1β and IL-6 [Fig 3A]. In comparison, these percentages were considerably lower (2.43%, 1.49% and 10.9%, respectively) when the cells were treated with 100 nM of the negative control DP47-TCB [Fig 3A]. This indicates that CD14+CD16- classical monocytes can simultaneously produce at least two of the three pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in response to TCB stimulation. For CEA-, Tyrp1- and FolR-TCB, this concomitant production of IL-1β, IL-6 and/or TNF-α by monocytes was dependent on the dose of TCB [Fig 3B, supp. Fig 2A and B]. For CEA-TCB, a higher background production of IL-1β, IL-6 and TNF-α by monocytes was observed in control conditions (in the absence of TCB or with DP47-TCB). We found that the interaction of monocytes with the MKN45 cell line triggered a baseline production of IL-1β, IL-6 and to a lower extent of TNF-α [Fig 3B, F].

To verify to which extent monocytes contribute to the release of IL-6, IL-1β and TNF-α, we set-up a TDCC assay comparing PBMCs with monocyte-depleted PBMCs from the same donor (Effector cells :Target cells=10:1) [Fig 3C]. Following stimulation with CEA-TCB, T cell activation profiles and tumor cell killing did not significantly differ neither in the presence or absence of monocytes in effector cells [Fig 3D, E]. In contrast, no IL-1β and drastically reduced IL-6 levels were released when the PBMCs were depleted of monocytes [Fig 3F, supp. Fig 3A]. This result indicates that monocytes are the main producers of IL-1β and IL-6.

TNF-α release was still detected when monocyte-depleted PBMCs were used as effector cells stimulated with CEA-TCB, but its levels remained lower than when PBMCs were used [Fig 3F]. This shows that monocytes contributed to TNF-α release, together with CD4+ and CD8+ T cells

[Fig 2A, B] as previously found by Li *et al.* and Godbersen-Palmer *et al*. (25, 26). In contrast, the levels of IFN-γ and IL-2 were not significantly impacted by the absence of monocytes in effector cells [Supp. Fig 3B].

Altogether, these results highlight that monocytes get indirectly activated after on-target activation of T cells and subsequently release IL-1β and IL-6, in addition to contributing to TNF-α production together with CD4+ and CD8+ T cells.

Figure 3.

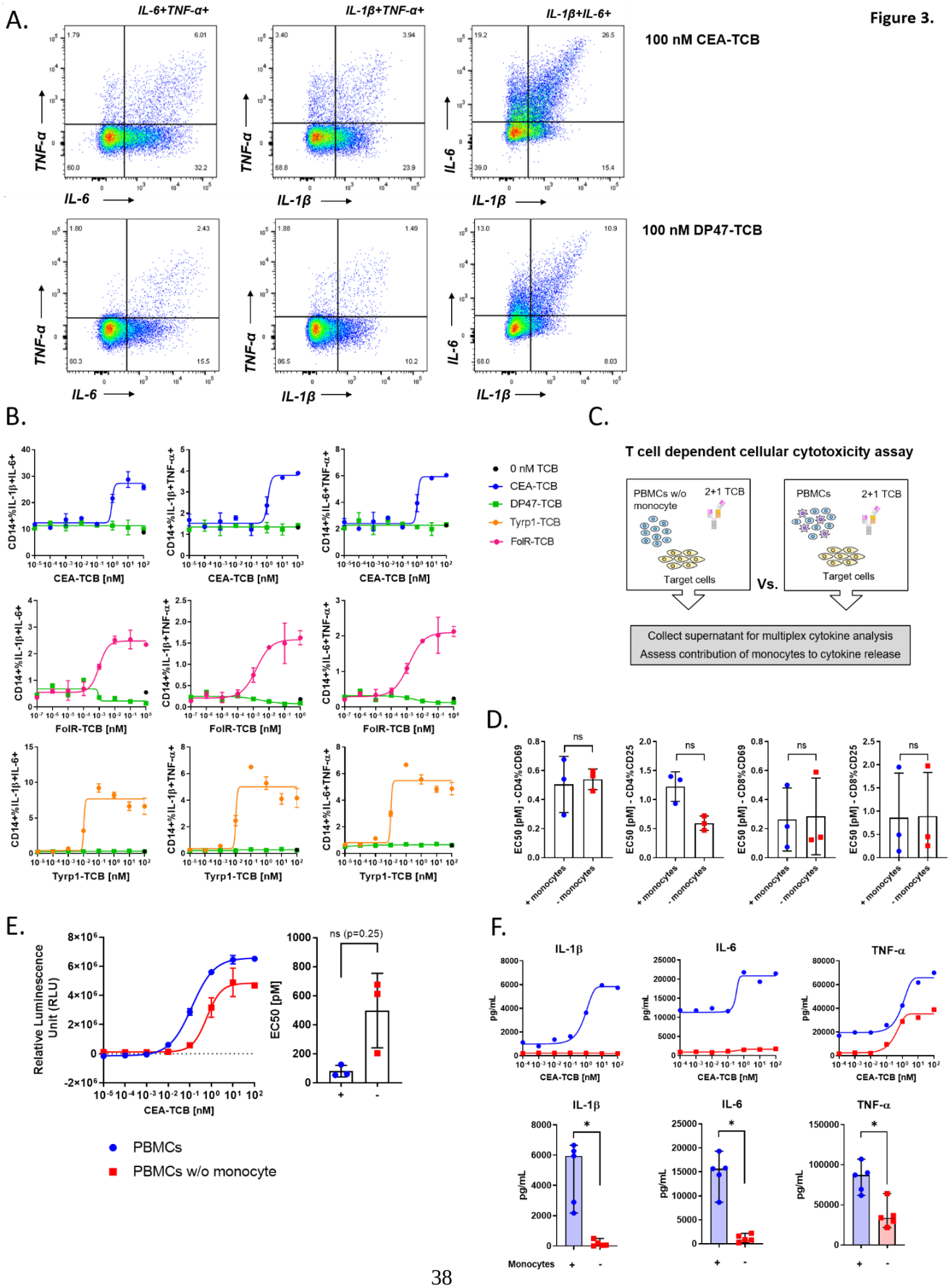

A.
IL-6+TNF-α+
IL-1β+TNF-α+
IL-1β+IL-6+
100 nM CEA-TCB
100 nM DP47-TCB
TNF-α
IL-6
IL-1β
B.
CD14+%IL-1β+IL-6+
CD14+%IL-1β+TNF-α+
CD14+%IL-6+TNF-α+
CEA-TCB [nM]
FolR-TCB [nM]
Tyrp1-TCB [nM]
0 nM TCB
CEA-TCB
DP47-TCB
Tyrp1-TCB
FolR-TCB
C.
T cell dependent cellular cytotoxicity assay
PBMCs w/o monocyte
2+1 TCB
Target cells
Vs.
PBMCs
Collect supernatant for multiplex cytokine analysis
Assess contribution of monocytes to cytokine release
D.
EC50 [pM] - CD4%CD69
EC50 [pM] - CD4%CD25
EC50 [pM] - CD8%CD69
EC50 [pM] - CD8%CD25
ns
+ monocytes
- monocytes
E.
Relative Luminescence Unit (RLU)
CEA-TCB [nM]
EC50 [pM]
ns (p=0.25)
PBMCs
PBMCs w/o monocyte
F.
IL-1β
IL-6
TNF-α
pg/mL
CEA-TCB [nM]
Monocytes

Figure 3. Monocytes produce IL-1β, IL-6 and contribute to TNF-α release upon TCB treatment.

PBMCs were stimulated with CEA-TCB, Tyrp1-TCB, FolR-TCB or DP47-TCB (negative control) in a TDCC assay using MKN45, CHOK1SV-Tyrp1 or CHOK1SV-FolR target cells respectively (E:T = 10:1). Golgistop and Golgiplug were added to block cytokine secretion and intracellular staining was performed after 20 hrs. **A**. Representative flow cytometry plots of TNF-α+IL-6+, TNF-α+IL-1β+, IL-1β+IL-6+, populations among CD14+ monocytes after treatment with 100 nM CEA- or DP47-TCB. **B.** Intracellular staining of IL-1β, TNF-α and IL-6 by CD14+ monocytes after treatment with CEA-TCB, FolR-TCB, Tyrp1-TCB and DP47-TCB. The black dots represent the cytokine levels in absence of TCB (CL: cell line + PBMCs only). Mean of 2 technical replicates +/- SEM, 1 donor representative of 3. **C.** PBMCs or monocyte-depleted PBMCs from the same donor were stimulated with CEA-TCB or DP47-TCB (negative control) in a TDCC assay using MKN45 tumor cells (E:T = 10:1). **D.** The expression of CD69 and CD25 expression on CD4+ and CD8+ T cells upon treatment with CEA-TCB was measured by flow cytometry after 24 hrs. EC_{50} values, mean of n = 3 donors +/- SD with ns = non-significant by Wilcoxon t test. **E**. The killing of MKN45 tumor cells was measured by LDH release. The dose-response plots show data for 1 donor representative of 3 and the bar plots show the EC_{50} values for n=3 donors, mean +/-SD with ns = non-significant by Wilcoxon t test. **F.** Cytokine levels were measured in the supernatants by Luminex (24 hrs). The dose-response plots show data for 1 donor representative of 5 and the bar plots show the means of n=5 donors +/-SD at 10 nM CEA-TCB. *$p < 0.05$ by Wilcoxon t test.

The release of IL-6 and IL-1β is dependent on TNF-α but not IFN-γ release

To verify if the production of monocyte-derived cytokines was dependent on TNF-α and IFN-γ produced by T cells after stimulation with TCB, we blocked each of the cytokines using 5 μg/mL anti-IFN-γ or adalimumab (anti-TNF-α) in TDCC assays with CEA-, Tyrp1- and FolR-TCB. The blockade of TNF-α but not of IFN-γ resulted in a decrease of IFN-γ, IL-2, IL-6 and IL-1β indicating that these T-cell or monocyte-derived cytokines are dependent on TNF-α release [Fig 4A]. The blockade of IFN-γ or TNF-α did not impair TCB-mediated T cell activation and tumor cell killing, as shown by the expression of CD25 and CD69 on CD4+ and CD8+ T cells as well as the LDH release measured in the supernatants of these TDCC assays, respectively [Fig 4B, C]. This indicates that these cytokines do not influence TCB-mediated killing. Interestingly, the expression of the TNF-α receptor CD120b (TNFR2) but not CD120a (TNFR1) was upregulated on CD4+ and CD8+ T cells and downregulated on CD14+ monocytes upon treatment with CEA-TCB [Fig 4D]. These results show that monocytes are activated by T cell-derived TNF-α, amplifying the release of TNF-α and mediating the production IL-6 and IL-1β. To note, the release of IFN-γ, TNF-α, IL-6 and IL-1β was not affected by IL-6R blockade, as indicated by the cytokine levels measured in a TDCC assay using Tyrp1-TCB in combination with Actemra (anti-IL-6R) [supp. Fig 4].

The use of neutralizing anti-cytokine antibodies confirmed the chronology of the events triggering TCB-mediated cytokine release as well as the dependence of IFN-γ, IL-2, IL-6 and IL-1β on the early release of TNF-α by T cells.

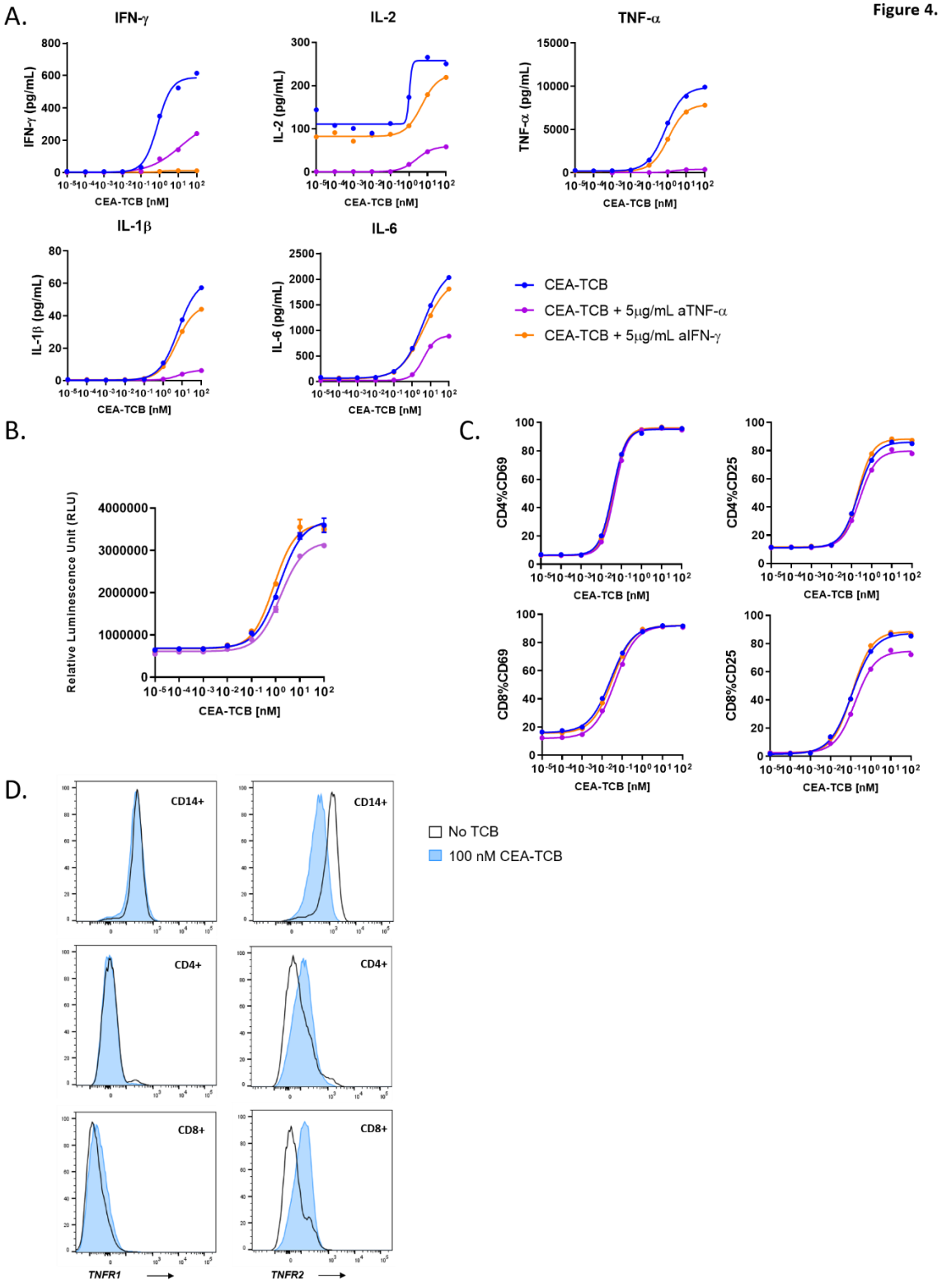


Figure 4.

Figure 4. The release of IL-6 and IL-1β is dependent on TNF-α but not IFN-γ release

PBMCs were stimulated with CEA-TCB in a TDCC assay using CHOK1SV-CEACAM5 target cells with CEA-TCB in the presence or absence of 5 μg/mL anti-TNF-α (adalimumab) or anti-IFN-γ. **A.** After 24 hrs, the release of IFN-γ, IL-2, TNF-α, IL-1β and IL-6 was measured in the culture supernatants by Luminex. **B**. The killing of CHOK1SV-CEACAM5 target cells was measured by LDH release (t=24 hrs). **C**. The expression of CD69 and CD25 on $CD4^+$ and $CD8^+$ T cells 24 hrs after treatment with CEA-TCB was measured by flow cytometry. **D**. Flow cytometry histogram plots showing the modulation of TNFR1 (CD120a) and TNFR2 (CD120b) (TNF-α receptors) on $CD14^+$ monocytes, $CD4^+$ and $CD8^+$ T cells after 24 hrs in TDCC assays with 100 nM CEA-TCB or in the absence TCB. **A-C,** data for 1 donor representative of 3.

Neutrophils are activated following TCB treatment and contribute to the release of IL-1β

Since neutrophils are the most abundant leukocyte population in blood, we aimed to assess their potential contribution to TCB-mediated cytokine release. For this purpose, we first conducted *in vitro* TDCC assays using total leukocytes (PBMCs and neutrophils) as effector cells co-cultured with CHOK1-Tyrp1 or CHOK1-FolR target cells and Tyrp1-TCB or FolR-TCB, respectively (E:T=10 lymphocytes : 1 target cell) [Fig 5A]. The expression of surface markers CD11b, CD62L and CD16 was measured by flow cytometry on neutrophils gated based on their FSC/SSC profile to assess the effect of TCB treatment on their phenotype. Both Tyrp1-TCB and FolR-TCB induced an upregulation of CD11b, which was associated with a downregulation of CD62L and CD16. There was no change in phenotype observed with the negative control DP47-TCB or in the absence of TCB [Fig 5B, supp. Fig 6A]. CD11b upregulation as well as CD62L and CD16 downregulation were also observed on the surface of neutrophils in whole blood treated with CD20-TCB [supp. Fig 7A]. This change in phenotype shows that neutrophils are activated following TCB stimulation.

In TDCC assays comparing PBMCs and total leukocytes as effector cells in the same experimental conditions, T cell activation and target cell killing remained unchanged [Fig 5C, D and supp. Fig 6B, C]. However, based on results from 7 blood donors, IL-1β but not IL-6 or TNF-α levels were significantly higher when neutrophils were present in the effector cells, suggesting that they contribute to IL-1β release together with monocytes [Fig 5E, supp. Fig 6D, 7B].

Figure 5.

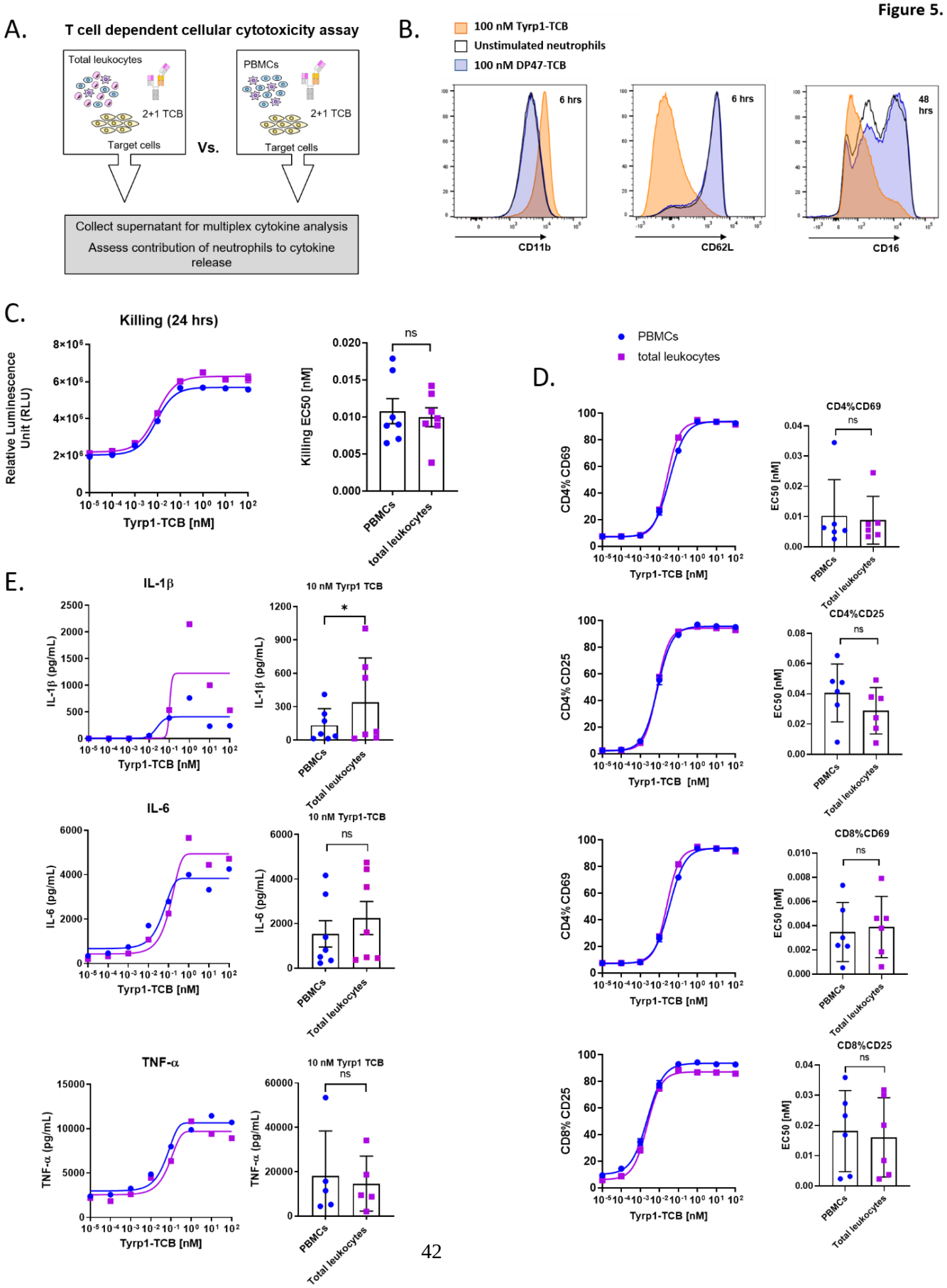

A.
T cell dependent cellular cytotoxicity assay
Total leukocytes
2+1 TCB
Target cells
Vs.
PBMCs
2+1 TCB
Target cells
Collect supernatant for multiplex cytokine analysis
Assess contribution of neutrophils to cytokine release
B.
100 nM Tyrp1-TCB
Unstimulated neutrophils
100 nM DP47-TCB
6 hrs
6 hrs
48 hrs
CD11b
CD62L
CD16
C.
Killing (24 hrs)
Relative Luminescence Unit (RLU)
Tyrp1-TCB [nM]
Killing EC50 [nM]
ns
PBMCs
total leukocytes
D.
PBMCs
total leukocytes
CD4% CD69
CD4%CD69
CD4% CD25
CD4%CD25
CD8% CD69
CD8%CD69
CD8% CD25
CD8%CD25
EC50 [nM]
Tyrp1-TCB [nM]
ns
PBMCs
Total leukocytes
E.
IL-1β
IL-1β (pg/mL)
10 nM Tyrp1 TCB
*
IL-6
IL-6 (pg/mL)
10 nM Tyrp1-TCB
ns
TNF-α
TNF-α (pg/mL)
10 nM Tyrp1 TCB
ns
Tyrp1-TCB [nM]
PBMCs
Total leukocytes

Figure 5. Neutrophils are activated following TCB treatment and contribute to IL-1β release.

A. PBMCs and total leukocytes (PBMCs + neutrophils) isolated from whole blood from one donor were stimulated with Tyrp1-TCB in a TDCC assay using CHOK1SV-Tyrp1 target cells (E:T=10:1). **B.** Flow cytometry histogram plots representing the expression of CD11b (6 hrs), CD62L (6 hrs) and CD16 (48 hrs) on neutrophils before or after incubation with Tyrp1- or DP47-TCB. **C.** The killing of CHOK1SV-Tyrp1 target cells was measured by LDH release in the presence or absence of neutrophils (24 hrs). **D**. The expression of CD69 and CD25 on $CD4^+$ and $CD8^+$ T cells upon treatment with CEA-TCB was measured by flow cytometry in the presence or absence of neutrophils (24 hrs). The dose-response plots show data for 1 donor representative of 6 or 7 and the bar plots show the mean of EC_{50} values +/- SD for n = 6 or 7 donors indicated by the symbols.* $p < 0.05$ by Wilcoxon t test. **E**. The levels of IL-1β, IL-6 and TNF-α were measured by Luminex at assay endpoint in the presence and absence of neutrophils (24 hrs). The dose-response plots show data for 1 donor representative of 7 and the bar plots show the mean of n = 7 donors treated with 10 nM Tyrp1-TCB. * $p < 0.05$ by Wilcoxon t test.

ScRNAseq of whole blood treated with CD20-TCB reveals the contribution of neutrophils to TCB-mediated cytokine release

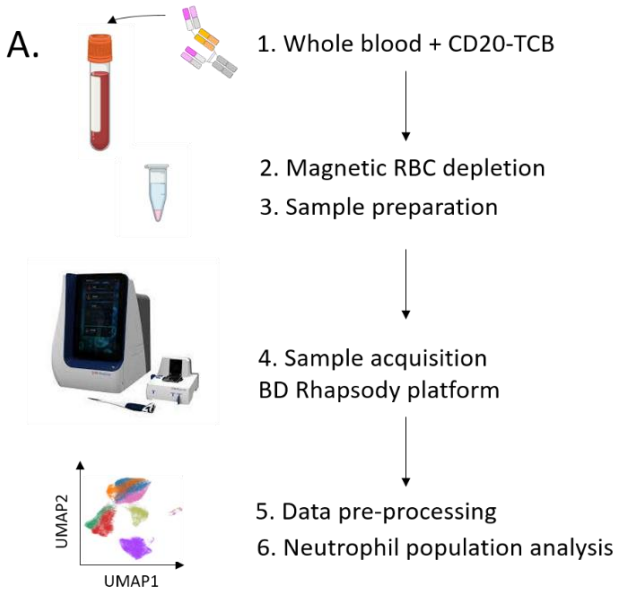
To confirm these findings, we conducted single cell RNA sequencing of whole blood using the BD Rhapsody platform at baseline and 2 hrs, 4 hrs, 6 hrs and 20 hrs after treatment with 0.2 μg/mL CD20-TCB [Fig 6A]. Neutrophil populations were identified based on the ELANE, MPO, PRTN3 CTSG, AZU1 and FCGR3B gene signature together with negative expression of lineage markers such as CD3D or CD79A. Of note, FCGR3B was the main gene expressed among those identified in the neutrophil gene signature and had high specificity. Additionally, the proportions of neutrophils were matching those measured by flow cytometry in the corresponding samples [supp. Fig 8.]. As shown in the UMAP plot, they clustered differently after 4 hrs, 6 hrs or 20 hrs stimulation with CD20-TCB, indicating a change in their phenotype [Fig 6B]. As a control, neutrophil populations from untreated samples incubated for 2 hrs, 6 hrs and 20 hrs overlapped with baseline samples, confirming that the observed change in phenotype was due to treatment with CD20-TCB [Fig 6B].

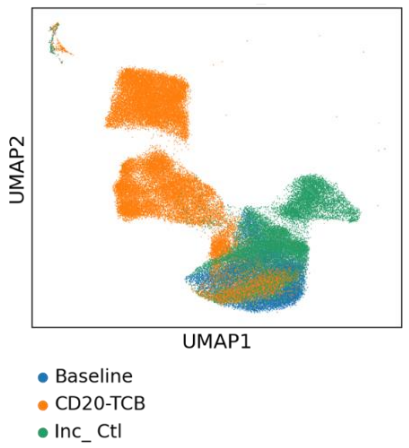
In line with our above findings, the IL-1β (IL1B) but not the IL-6 (IL6) gene was expressed in neutrophil populations after stimulation with CD20-TCB, as indicated by the UMAP plots [Fig 6C]. Only sparse expression of the TNF-α (TNF) gene was observed. Interestingly, the gene encoding the IL-8 receptor(CXCR2) was expressed at baseline and rapidly downregulated upon CD20-TCB treatment [Fig 6D]. The IL-8 gene (CXCL8) was induced early after TCB treatment, suggesting that neutrophil-derived IL-8 might act in an autocrine fashion. Along those lines, the TNF receptor genes (TNFRSF1A and TNFRSF1B) and IFN receptor genes (IFNGR1 and IFNGR2) were also found expressed on neutrophils at baseline and after treatment, suggesting that TNF-α and IFN-γ may signal in neutrophils. Additionally, we verified whether transcripts for

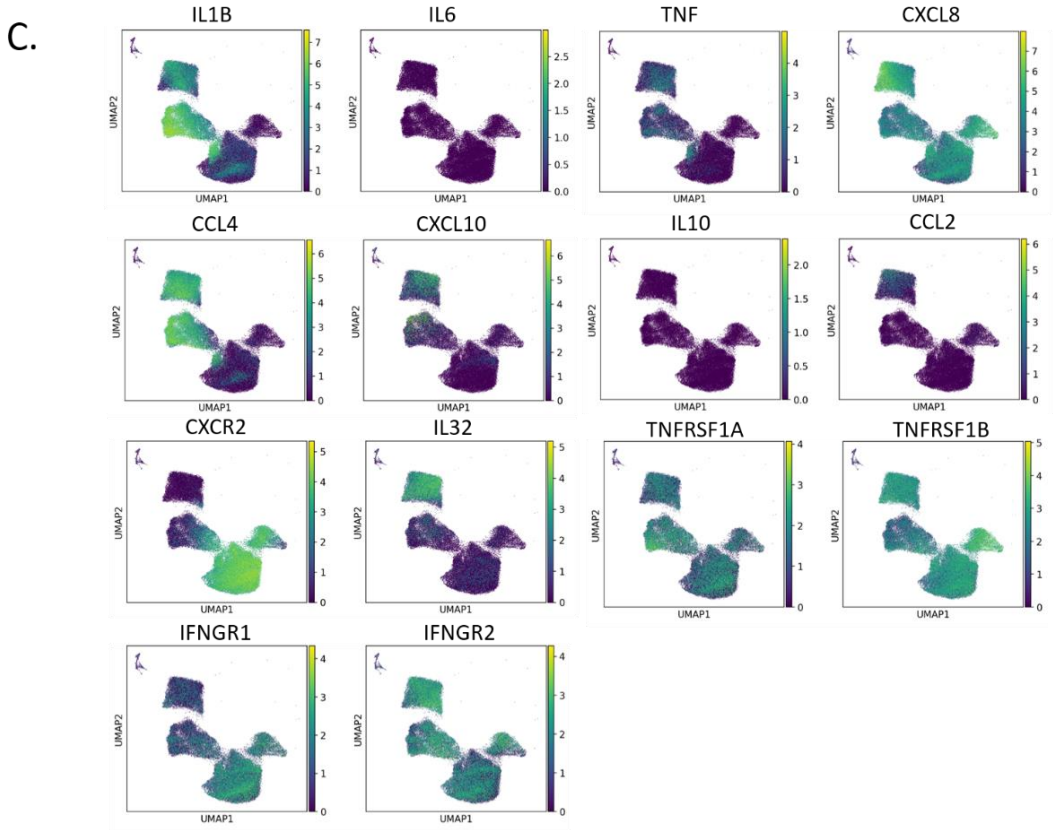
IL-10 (IL10), MCP-1 (CCL2), MIP-1β (CCL4), IP-10 (CXCL10), described as molecular players in the biology of TCB-mediated CRS, were induced in neutrophils. Among them, IL-8 (CXCL8),) and MIP-1β (CCL4) were upregulated upon treatment with CD20-TCB,implying that neutrophils may broadly contribute to TCB-mediated cytokine release [Fig 6C-D]. The IL-32 (IL32R) gene was also identified among the top 40 genes induced in neutrophils after CD20-TCB stimulation. In the kinetics of events, IL-1β (IL1B) and MCP-1 (CCL2) transcripts were upregulated already 2 hrs after treatment with CD20-TCB and peaked at 4 hrs, while IL-32 (IL32), IL-8 (CXCL8) and MIP-1β (CCL4) were mainly upregulated at later timepoints [Fig 6D, supp. Fig 9A-B]. These data demonstrate the rapid onset of cytokine release by neutrophils.

Overall, these results confirm the early contribution of neutrophils to IL-1β release at the transcriptional level and show that neutrophils broadly contribute to the cytokine release cascade following TCB treatment, notably through the release of CCL2, MIP-1β, IL-8 and IL-32.

Figure 6.


A.
1. Whole blood + CD20-TCB
2. Magnetic RBC depletion
3. Sample preparation
4. Sample acquisition
BD Rhapsody platform
5. Data pre-processing
6. Neutrophil population analysis
UMAP2
UMAP1


B.
UMAP2
UMAP1
Baseline
CD20-TCB
Inc_ Ctl


C.
IL1B
IL6
TNF
CXCL8
CCL4
CXCL10
IL10
CCL2
CXCR2
IL32
TNFRSF1A
TNFRSF1B
IFNGR1
IFNGR2
UMAP1
UMAP2

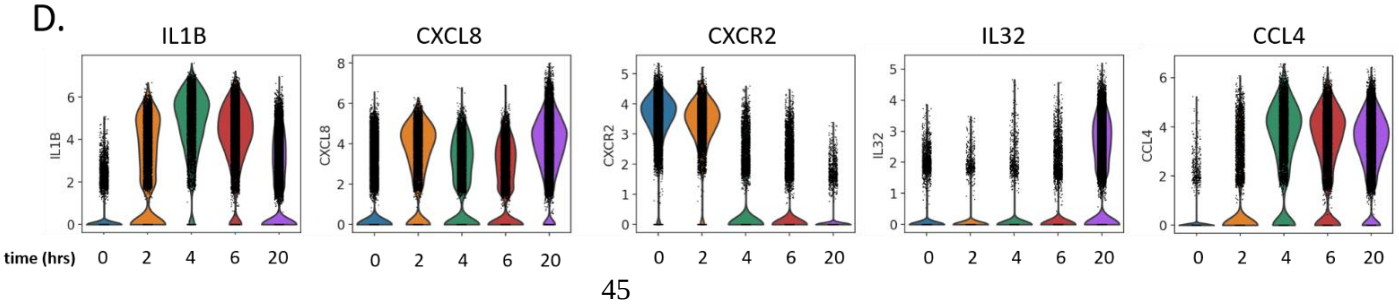

D.
IL1B
CXCL8
CXCR2
IL32
CCL4
time (hrs)
0
2
4
6
20

Figure 6. IL-1β and other cytokine and cytokine receptor genes are differentially upregulated in neutrophils from whole blood treated with CD20-TCB.

A. Single cell RNA sequencing of whole blood treated with 0.2 µg/mL CD20-TCB (baseline: n = 4 donors; 2 hrs: n= 2 donors; 4 hrs: n = 2 donors; 6 hrs: n = 2 donors; 24 hrs: n = 4 donors) was performed using the BD Rhapsody platform. **B.** UMAP plot of neutrophils colored by treatment, baseline and incubation control (inc_ctl). **C.** UMAP plots showing IL-1β (IL-1B), IL-6 (IL-6), TNF-α (TNF), IL-8 (CXCL8), MIP-1β (CCL4), IP-10 (CXCL10), IL-10 (IL10), MCP-1 (CCL2), IL-8R (CXCR2), IL-32 (IL32), TNFR1a (TNFRSF1A), TNFR1b (TNFRSF1B), IFNR1 (IFNGR1) and IFNR2 (IFNGR2) gene expression within the neutrophils clusters. **D.** Violin plots showing the expression distribution of IL-1β (IL-1B), IL-8 (CXCL8), IL-8R (CXCR2), IL-32 (IL32), MIP-1β (CCL4) genes within neutrophils at each treatment time point. Each dot is a cell. N=4 donors (baseline, and 24 hrs) and n=2 donors (2 hrs, 4 hrs and 6 hrs). Neutrophils were identified by scRNA sequencing of whole blood based on the ELANE, MPO, PRTN3 CTSG, AZU1 and FCGR3B gene signature.

Discussion

On-target activation of T cells is associated with a release of cytokines that can potentially result in a cytokine release syndrome, one of the major safety liabilities intrinsic to the mode-of-action of T cell engagers. The *in vitro* TDCC assay system allows to recapitulate TCB-induced cytokine release and to identify the contribution of the immune cell populations present among the effector cells. Early after activation with a TCB, CD4+ and CD8+ T cells release IFN-γ and TNF-α, but not IL-6. T cell-derived cytokines further activate T cells and other immune cells present in PBMCs. Among them, monocytes were identified as the main producers of IL-1β and IL-6 and found to contribute to TNF-α release together with CD4+ and CD8+ T-cells. This was clearly shown by intracellular cytokine immunostaining and the comparison of cytokine levels in supernatants from TDCC assays using either monocyte-depleted PBMCs or total PBMCs as effector cells. Our data with TCBs confirm the findings of Li J. *et al.* and Godbersen-Palmer C. *et al.*, who highlighted the contribution of myeloid cells to IL-6, IL-1β and TNF-α production *in vitro* and *in vivo* early after treatment with T cell engagers (25, 26).

Subsequently, we focused on the role of neutrophils in the cytokine release induced by T cell engagers, which had not yet been reported. Neutrophils show an activated phenotype in TDCC assays using total leukocytes as effector cells, or in whole blood treated with CD20-TCB. This suggested that they might well contribute to TCB-induced cytokine release. We first showed that neutrophils released IL-1β, by comparing IL-1β levels in culture supernatants from TDCC assays using either total leukocytes or PBMCs as effector cells. This result was confirmed by single cell RNA sequencing of whole blood treated with CD20-TCB. Additionally, genes of other cytokines such as IL-8 (CXCL8), IL-32 (IL32), MIP-1β (CCL4) were found rapidly upregulated in

neutrophils upon treatment with CD20-TCB, indicating a broader contribution of neutrophils to TCB-mediated cytokine release. Along with the upregulation of these cytokine genes, we also observed that the IL-8R genes were downregulated upon treatment with CD20-TCB, denoting that IL-8 might act there in an autocrine fashion. T-cell derived TNF-α and IFN-γ may as well stimulate neutrophils, as the genes coding for their receptors were expressed in neutrophils clusters identified in whole blood treated with CD20-TCB. Although they represent a large proportion of the white blood cells, the contribution of neutrophils to TCB-mediated cytokine release may be underestimated both in *in vitro* functional assays using PBMCs as effector cells and *in vivo* mouse models where the proportion of neutrophils does not translate to the human amounts.

Presently, CRS is managed in the clinic with the use of glucocorticoids and anti-IL-6 (tocilizumab) or anti-IL-6R (siluximab) antibodies (16). As demonstrated, IL-6 is released by myeloid cells downstream of the TCB-induced cytokine cascade, stimulated by TNF-□. Current therapeutic antibodies blocking the IL-6 pathway may mitigate IL-6-derived toxicities such as endothelial cell activation, which eventually can lead to vascular leakage, but may not affect the levels of other cytokines implicated in CRS (32). The use of anti-IL-1Ra (anakinra) antibody has been proposed for the mitigation of CAR-T cell-induced CRS and neurotoxicity (19, 33). With IL-1β produced by myeloid cells and neutrophils, downstream of TCB-induced T cell activation, the specific neutralization of IL-1Ra would likely mitigate IL-1β-driven toxicities but not strongly reduce the overall cytokine release.

It is confirmed that TCB-mediated cytokine release is first initiated by T cell-derived cytokines, as a result of on-target TCB activity. This highlights that prophylactic intervention at the T cell level could more broadly reduce the cytokine cascade. As shown by Li J. *et al.* and by our *in vitro* results, TNF-α blockade could reduce both IL-6 and IL-1β release by monocytes without impacting TCB-induced T cell cytotoxicity (26). Interestingly, etanercept and adalimumab were shown to counteract CRS in patients failing to respond to IL-6/ IL-6R blockade (34).

Our results indicate that IFN-γ is mainly released by T cells upon treatment with TCB. They also suggest that IFN-γ blockade may not efficiently reduce the release of other cytokines implicated in CRS. However, our model systems may not fully capture its potential effects since macrophages are lacking. As IFN- γ is known to activate macrophages, its blockade could reduce the release of macrophage-derived IL-6 and IL-1β s upon treatment with T cell engaging

therapies (33, 35). The neutralization of IFN-γ with emapalumab is indicated for the management of HLH/MAS symptoms that are closely related to CRS in patients treated with CAR-T cells (11, 36). Nevertheless, IFN-γ blockade was also shown to alter the recruitment of circulating T cells to the tumor site, suggesting that it might have a deleterious effect on anti-tumor efficacy, thus making it a less favorable prophylactic approach for the mitigation of CRS for T cell engagers targeting solid tumors (37). .

Step-up dosing of T cell engagers is also widely used to prevent the occurrence of CRS. As shown by others and in the present study, upon re-administration, peaks of inflammatory cytokines are attenuated while tumor cell killing remains effective (26, 38). However, despite the use of IL-6/IL-6R neutralizing antibodies, corticosteroids and step-up dosing, the risk of CRS still remains a major dose-limiting toxicity associated with the treatment of T cell engagers, highlighting that CRS mitigation by pre-treatment may open the way for clinical schedules that require less intense monitoring and get faster to the efficacious dose. Beside TNF-α blockade, promising T-cell targeted approaches for the mitigation of CRS include the use of kinase inhibitors targeting signaling pathways downstream of TCR stimulation (39, 40) (Leclercq *et al*., 2021, manuscript in review).

Our data on the mechanisms of TCB-induced cytokine release also provide insight to develop re-stimulation TDCC *in vitro* models that may help to guide escalation factor, and time intervals for step-up dosing schedules. Further investigations are required with respect to understanding biological mechanisms that counter balance cytokine release. Additionally, our work highlights the key cytokines upregulated intrinsically by the mode-of-action of TCBs, and may contribute to the selection of more specific clinical biomarker panels reflecting the cytokine release by monocytes and neutrophils, which are the major source of cytokines such as IL-1β, IP-10 or MIP-1β. Finally, exploring the phenotypes of neutrophils and monocytes and their propensities to produce high levels of pro-inflammatory cytokines could be beneficial for the identification of potential personalized biomarkers, that may predict whether a patient is at risk for CRS after treatment with T cell engaging therapies.

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Declaration of interest statement

All authors, except A. Odermatt, are employees of Roche or were employed by Roche at the time of this study and declare patent. All the authors, except A. Odermatt, G. Leclercq, N. Steinhoff, declare ownership of Roche stock.

Authors' contributions

- Concept & Experimental design G. Leclercq, H. Haegel, A. Schneider, C. Bossen, S. Danilin, N. Steinhoff, M. Bacac
- Acquisition of data: G. Leclercq, S. Danilin
- Data analysis and interpretation: G. Leclercq, L. Alberti-Servera, H. Haegel, A. Schneider, A. Giusti, M. Bacac, C. Klein
- Writing, review and/or revision of the manuscript: G. Leclercq, H. Haegel, A. Odermatt, L. Alberti-Servera, S. Danilin, C. Klein, M. Bacac
- Administrative, technical or material support: J. Challier, S. Danilin
- Study supervision: H. Haegel, A. Schneider, C. Klein, M. Bacac, P. Umana

Availability of data and material

All data relevant to the study are included in the article or uploaded as online supplementary information.

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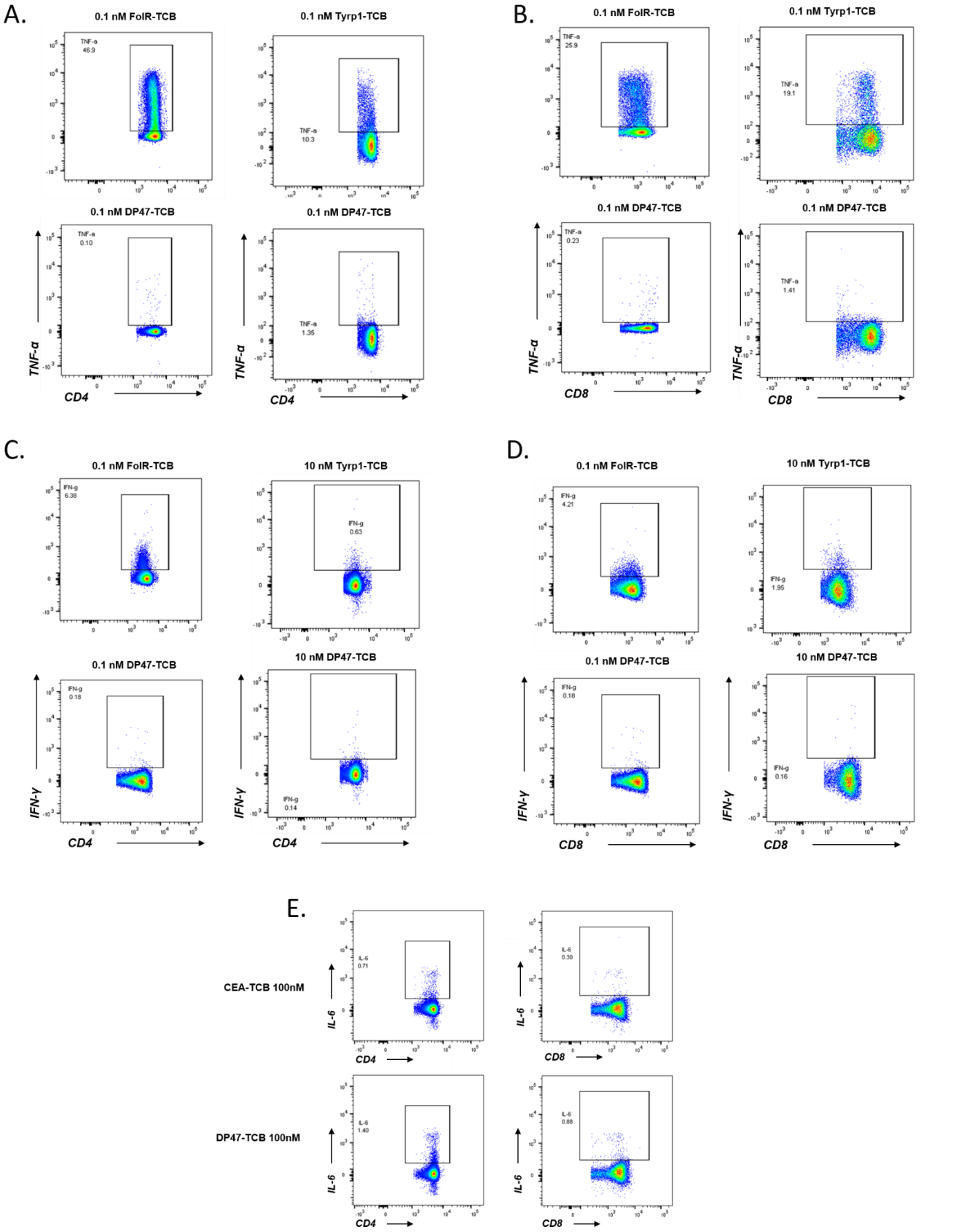
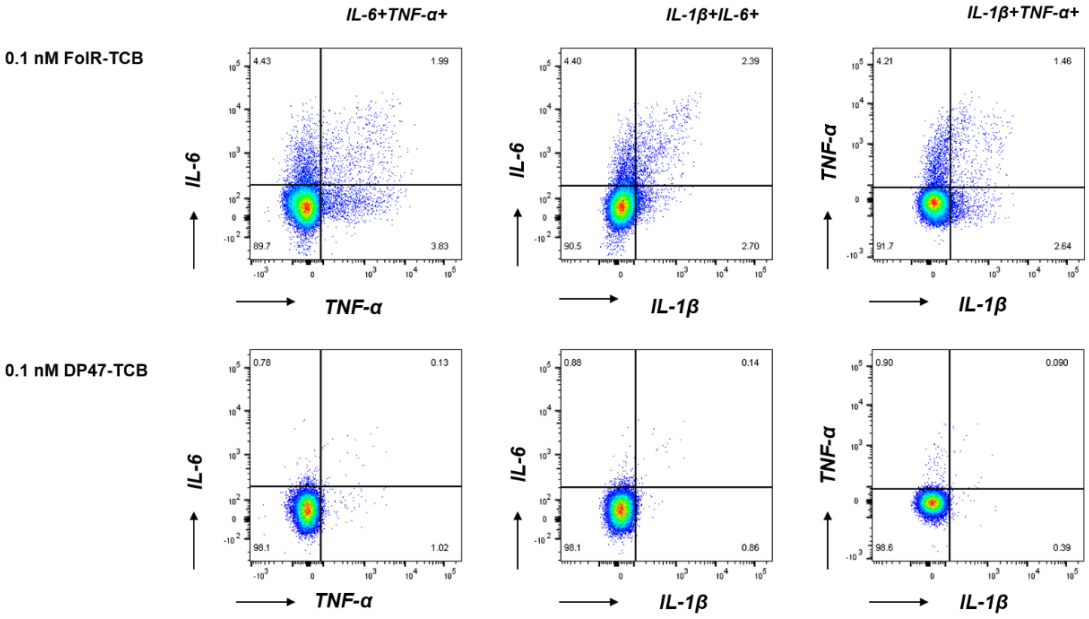
Supp. Fig 1.


A.
0.1 nM FolR-TCB
TNF-a 46.9
0.1 nM Tyrp1-TCB
TNF-a 10.3
0.1 nM DP47-TCB
TNF-a 0.10
0.1 nM DP47-TCB
TNF-a 1.35
TNF-α
CD4
B.
0.1 nM FolR-TCB
TNF-a 25.9
0.1 nM Tyrp1-TCB
TNF-a 19.1
0.1 nM DP47-TCB
TNF-a 0.23
0.1 nM DP47-TCB
TNF-a 1.41
TNF-α
CD8
C.
0.1 nM FolR-TCB
IFN-g 6.38
10 nM Tyrp1-TCB
IFN-g 0.63
0.1 nM DP47-TCB
IFN-g 0.18
10 nM DP47-TCB
IFN-g 0.14
IFN-γ
CD4
D.
0.1 nM FolR-TCB
IFN-g 4.21
10 nM Tyrp1-TCB
IFN-g 1.95
0.1 nM DP47-TCB
IFN-g 0.18
10 nM DP47-TCB
IFN-g 0.16
IFN-γ
CD8
E.
CEA-TCB 100nM
IL-6 0.71
IL-6 0.30
DP47-TCB 100nM
IL-6 1.40
IL-6 0.88
IL-6
CD4
CD8

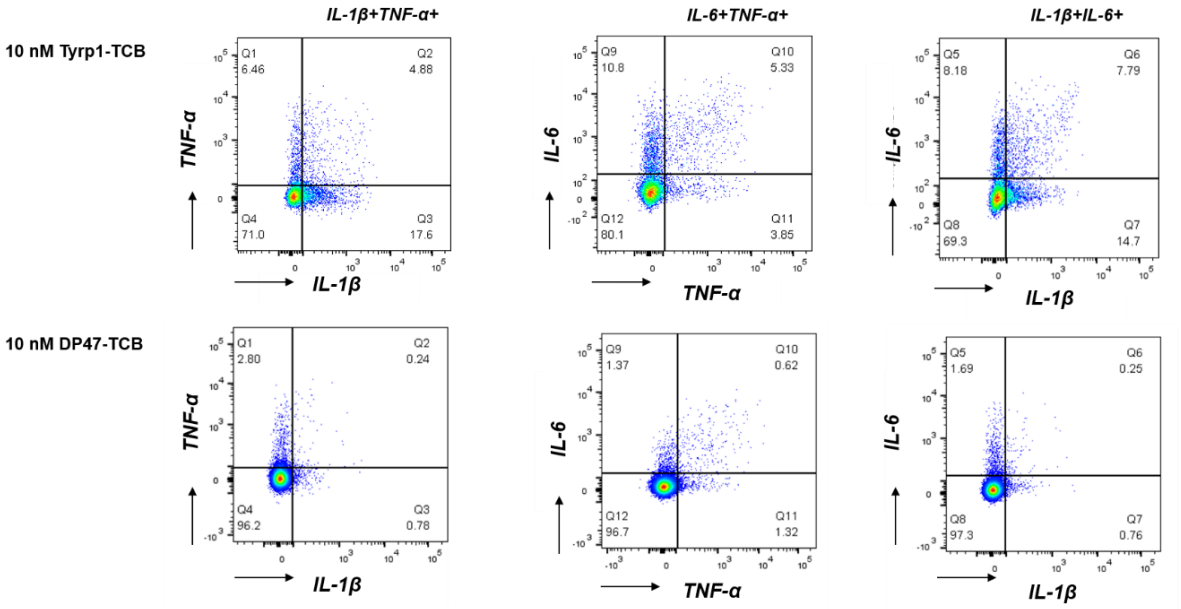
Supp. Figure 1. PBMCs were stimulated with Tyrp1-TCB, FolR-TCB or DP47-TCB (negative control) in a TDCC assay using CHOK1SV-Tyrp1 or CHOK1SV-FolR target cells respectively (E:T = 10:1). Golgistop and Golgiplug were added to block cytokine secretion and intra-cellular staining was performed at 20 hrs. **A, B.** Representative flow cytometry plots of TNF-α positive cells among CD4+ and CD8+ T cells after treatment with 0.1 nM FolR- and Tyrp1-TCB or 0.1 nM corresponding DP47-TCB. **C, D.** Representative flow cytometry plots of IFN-γ positive cells among CD4+ and CD8+ T cells after treatment with 0.1 nM FolR- and Tyrp1-TCB or 0.1 nM corresponding DP47-TCB. **E.** Representative flow cytometry plots of IL-6 positive cells among CD4+ and CD8+ T cells after treatment with 100 nM CEA- or DP47-TCB.

Supp. Fig 2.

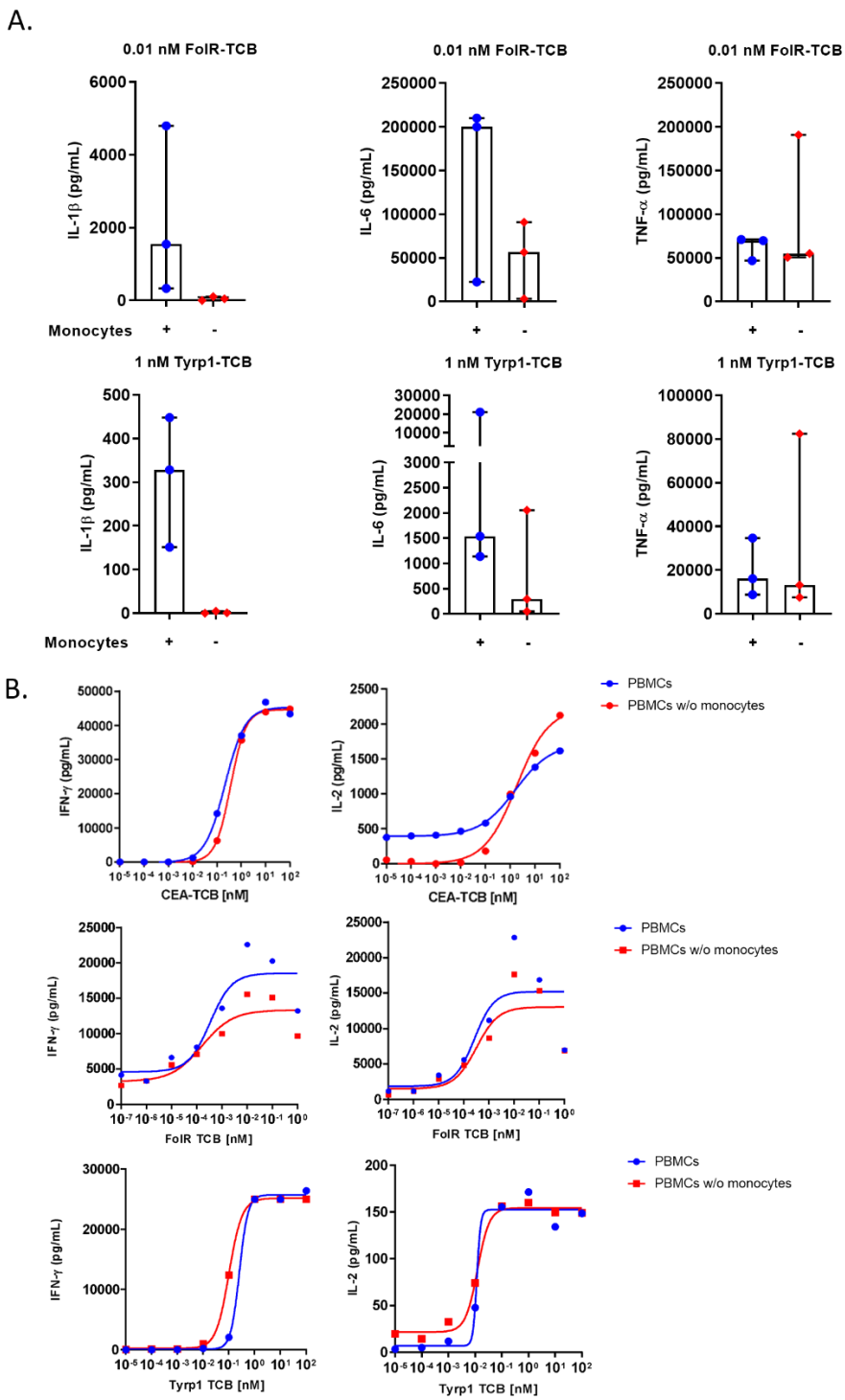
A.



B.

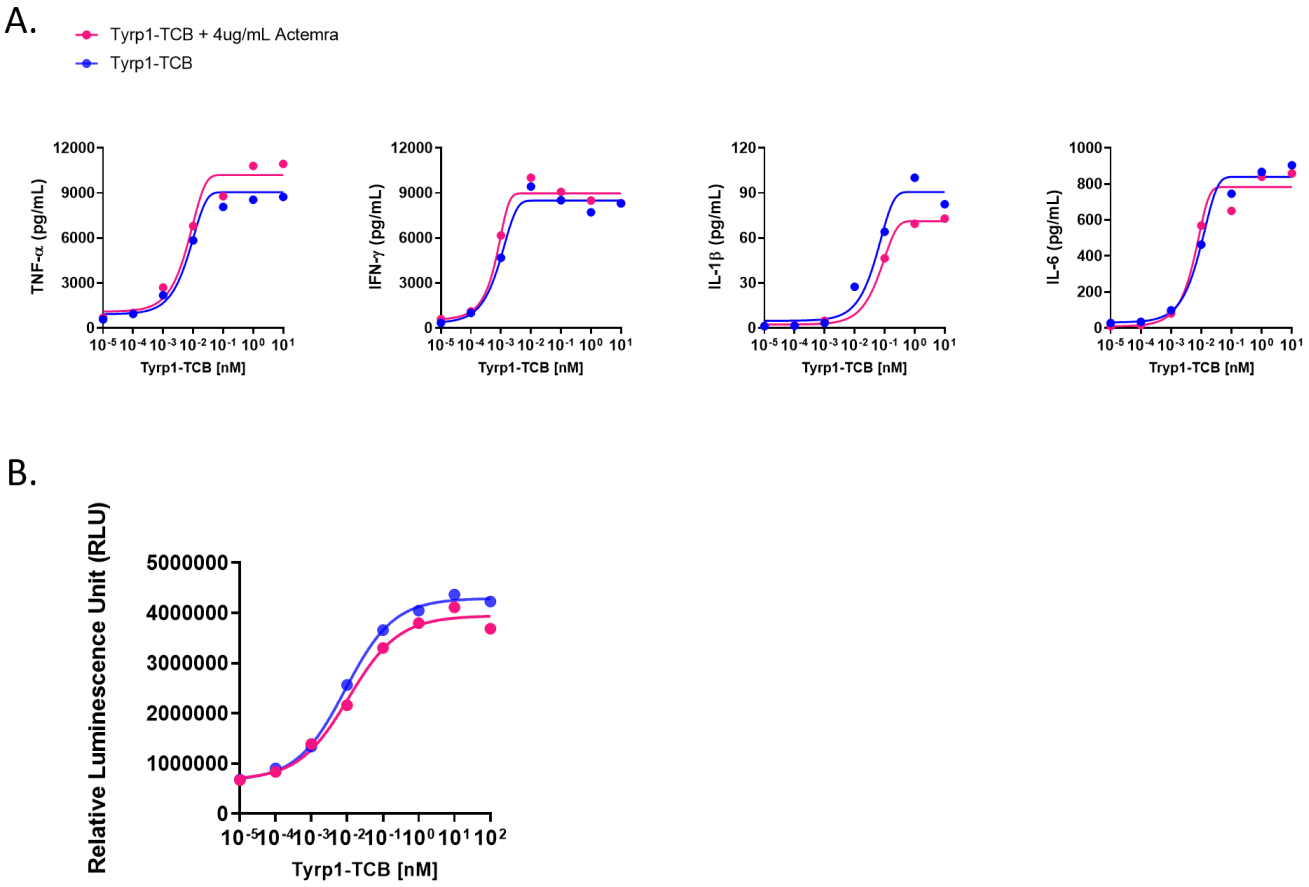


Supp. Figure 2. PBMCs were stimulated with Tyrp1-TCB, FolR-TCB or DP47-TCB (negative control) in a TDCC assay using CHOK1SV-Tyrp1 or CHOK1SV-FolR target cells respectively (E:T = 10:1). Golgistop and Golgiplug were added to block cytokine secretion and intra-cellular staining was performed at 20 hours. **A.** Representative flow cytometry plots of TNF-α+IL-6+, IL-1β+IL-6+, TNF-α+IL-1β+ populations among CD14+ monocytes after treatment with 0.1 nM FolR- or DP47-TCB. **B.** Representative flow cytometry plots of TNF-α+IL-1β+, TNF-α+IL-6+, IL-1β+IL-6+, populations among CD14+ monocytes after treatment with 0.1 nM Tyrp1- or DP47-TCB.



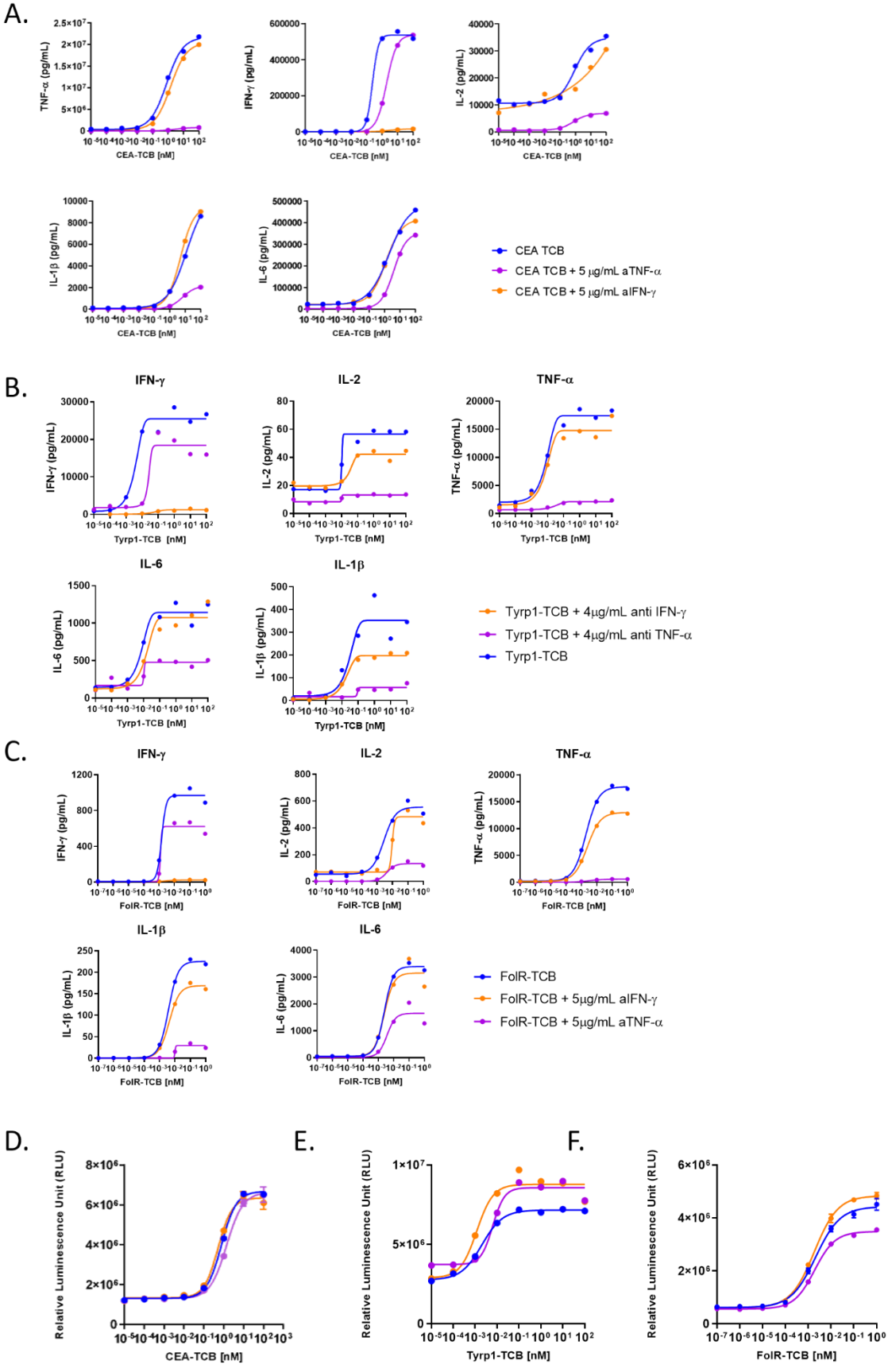
Supp. Figure 3. Cytokine production was compared between TDCC assays using either PBMCs or monocyte-depleted PBMCs from the same donor (E:T = 10:1). **A.** Levels of IL-1β, IL-6 and TNF-α measured by Luminex at assay endpoint (24 hrs) in TDCC assays using CHOK1SV-FolR or CHOK1SV-Tyrp1 target cells with 0.01 nM FolR-TCB or 1 nM Tyrp1-TCB, respectively. Mean of n=3 donors +/- SD. **B.** IFN-γ and IL-2 levels measured by Luminex at assay enpoint (24 hrs) in a TDCC assay using CHOK1SV-CEACAM5, CHOK1SV-FolR or CHOK1SV-Tyrp1 target cells with a dose range of CEA-TCB, FolR-TCB or Tyrp1-TCB. 1 donor representative of 3.

Supp. Fig 4.

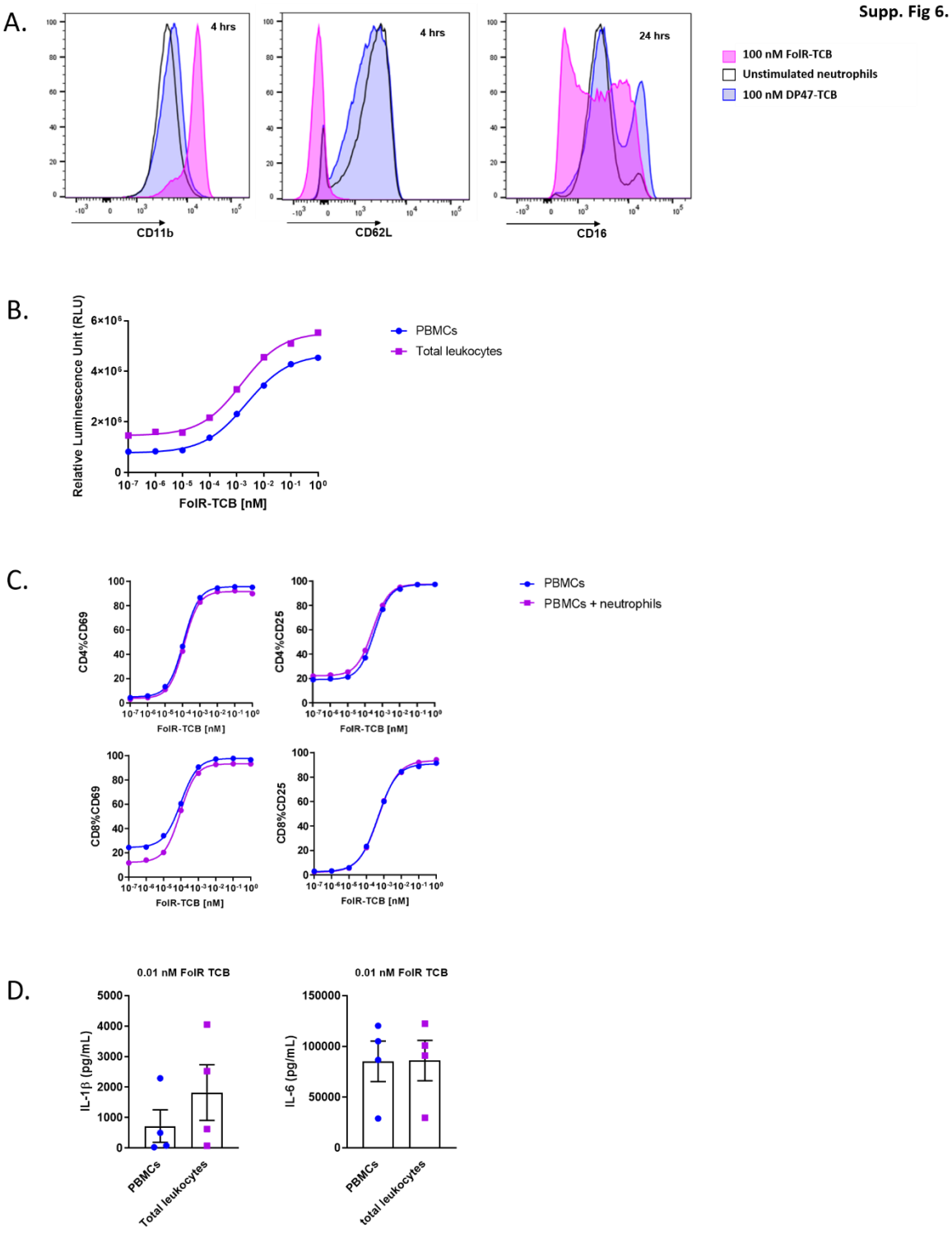


Supp. Figure 4. PBMCs were stimulated with Tyrp1-TCB and 4 µg/mL anti-IL-6R (actemra) in a TDCC assay using CHOK1SV-Tyrp1 target cells (E:T=10:1). **A.** Cytokines were detected by Luminex and **B.** LDH release was measured as a readout of target cell killing in the supernatant (24 hrs). 1 donor.

Supp. Fig 5.


A.
TNF-α (pg/mL)
IFN-γ (pg/mL)
IL-2 (pg/mL)
IL-1β (pg/mL)
IL-6 (pg/mL)
CEA-TCB [nM]
CEA TCB
CEA TCB + 5 μg/mL aTNF-α
CEA TCB + 5 μg/mL aIFN-γ
B.
IFN-γ
IL-2
TNF-α
IL-6
IL-1β
Tyrp1-TCB [nM]
Tyrp1-TCB + 4μg/mL anti IFN-γ
Tyrp1-TCB + 4μg/mL anti TNF-α
Tyrp1-TCB
C.
IFN-γ
IL-2
TNF-α
IL-1β
IL-6
FolR-TCB [nM]
FolR-TCB
FolR-TCB + 5μg/mL aIFN-γ
FolR-TCB + 5μg/mL aTNF-α
D.
Relative Luminescence Unit (RLU)
CEA-TCB [nM]
E.
Tyrp1-TCB [nM]
F.
FolR-TCB [nM]

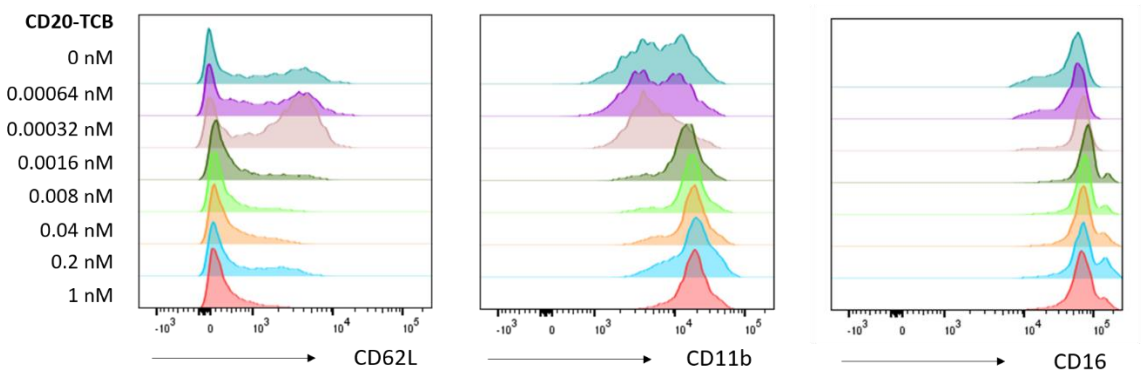
Supp. Figure 5. PBMCs were stimulated with CEA-, Tyrp1- and FolR- TCB and 5 µg/mL anti-IFN-γ or 5 µg/mL anti-TNF-α in a TDCC assay using CHOK1SV-CEACAM5, CHOK1SV-Tyrp1 and CHOK1SV-FolR target cells (E:T = 10:1). **A-C.** Cytokines were detected by Luminex and **D-F.** LDH release was measured as a readout of target cell killing in the supernatant (24 hrs for CEA-and FolR-TCB and 48 hrs for Tyrp1-TCB). 1 donor representative of 2. For each TCB, the experiment was conducted using two independent PBMC donors.



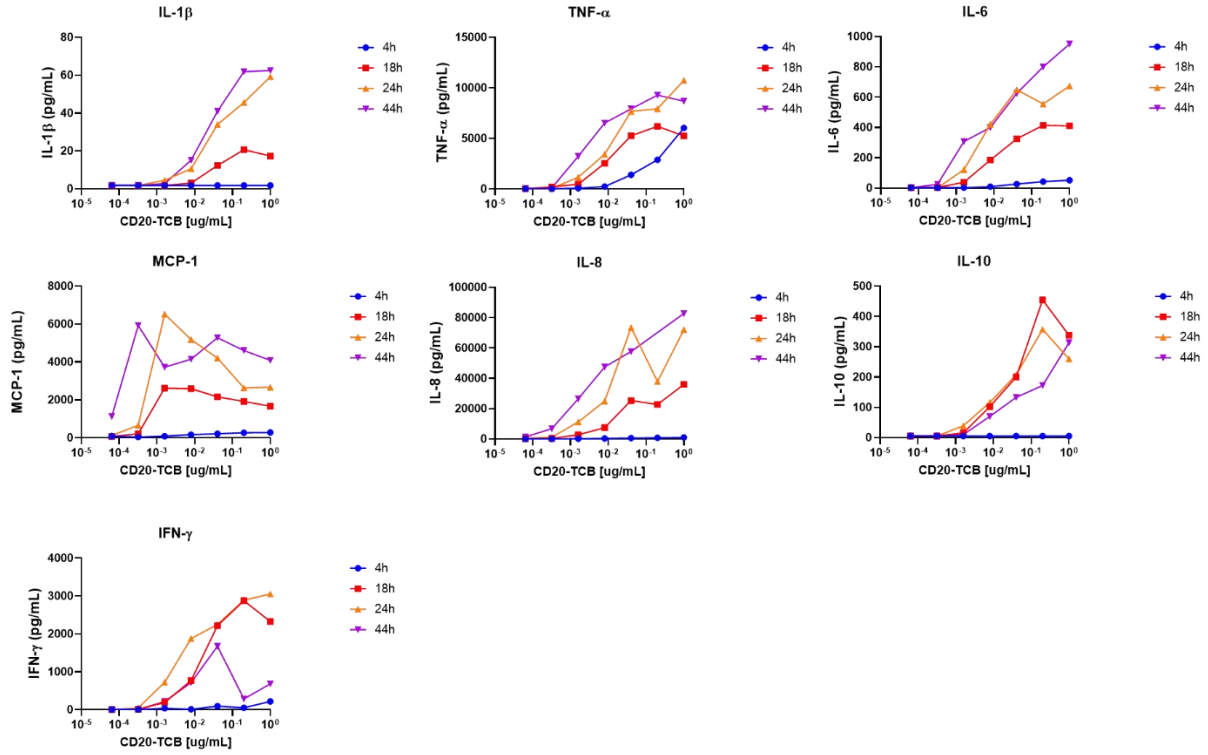
Supp. Figure 6. PBMCs and total leukocytes (PBMCs + neutrophils) isolated from whole blood from the same donor were stimulated with FolR-TCB in a TDCC assay using CHOK1SV-FolR target cells (E:T=10:1). **A.** Flow cytometry histogram plots representing the expression of CD11b (4 hrs), CD62L (4 hrs) and CD16 (24 hrs) on neutrophils following stimulation with 1 nM FolR- or DP47-TCB (negative control). **B.** The killing of CHOK1SV-FolR target cells was measured by LDH release in the presence (total leukocytes) and absence (PBMCs) of neutrophils (24 hrs). **C**. The expression of CD69 and CD25 expression on CD4+ and CD8+ T cells upon treatment with CEA-TCB was measured by flow cytometry in the presence and absence of neutrophils (24 hrs). **D.** The levels of IL-1β and IL-6 were measured by Luminex at assay endpoint in the presence and absence of neutrophils (24 hrs). Mean of n=4 donors treated with 0.01 nM FolR-TCB +/- SD.

Supp. Fig 7.

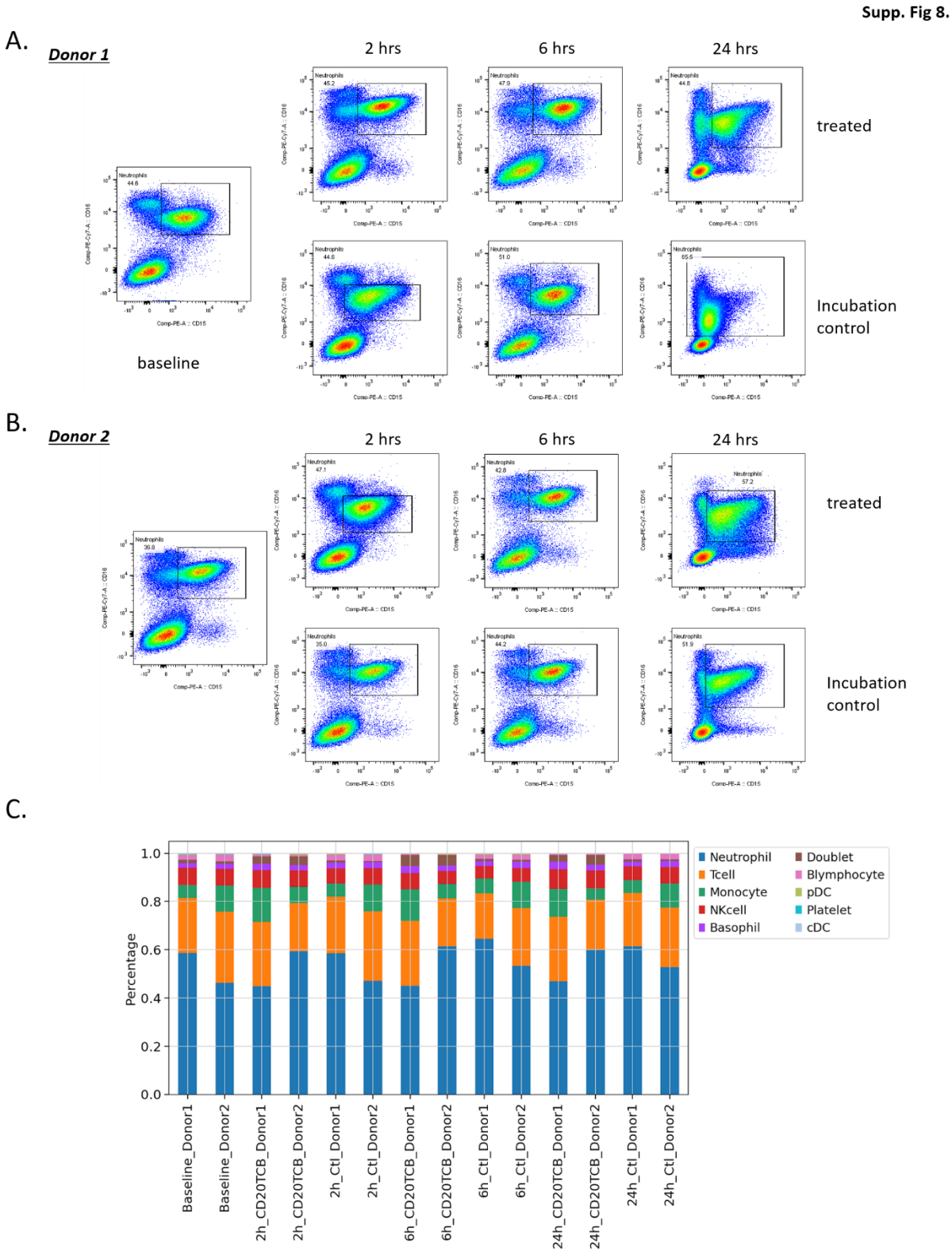
A.



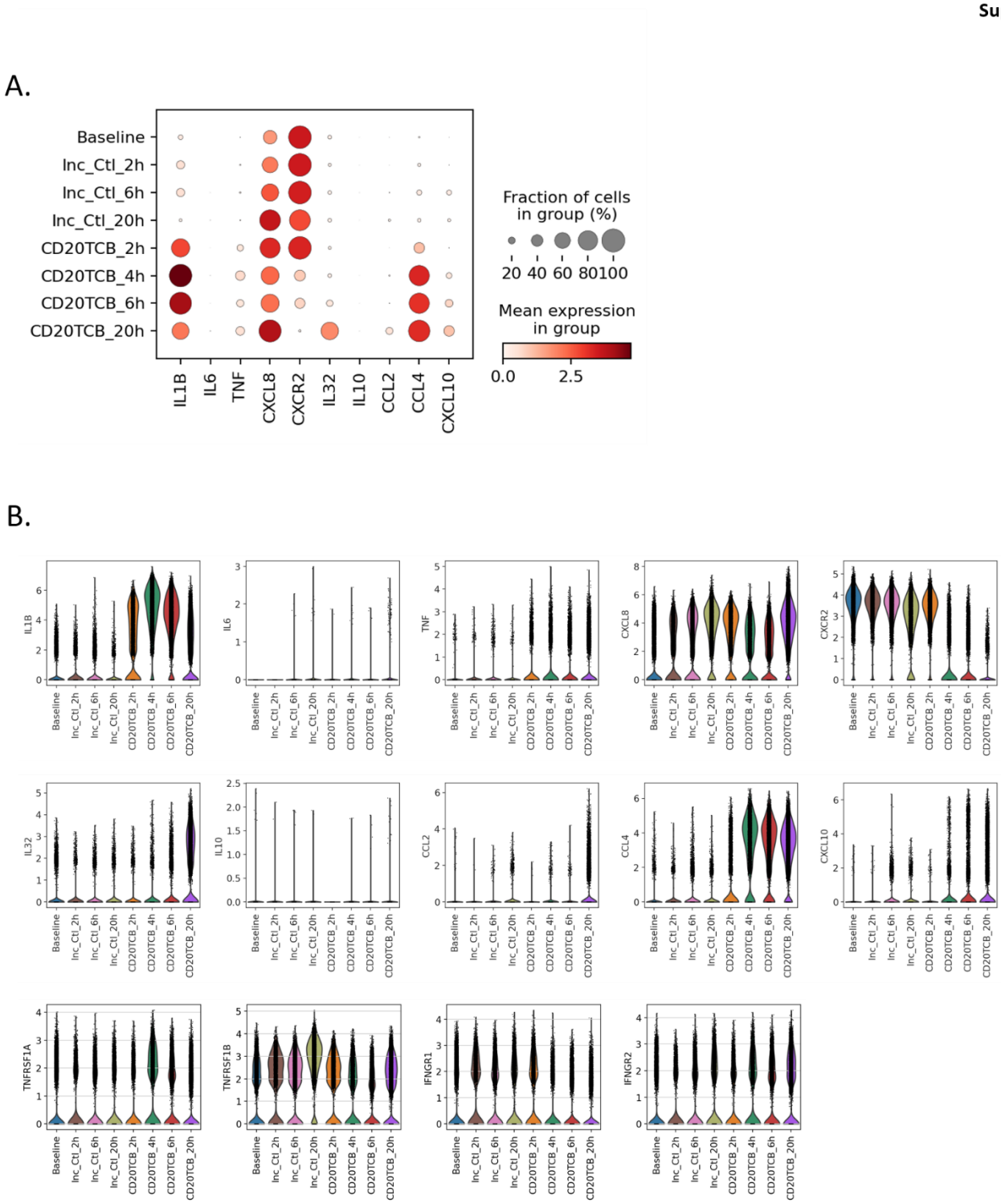
B.



Supp. Figure 7. Whole blood assay with CD20-TCB. **A.** Flow cytometry histogram plots representing the expression of CD62L (18 hrs), CD11b (18 hrs), and CD16 (18 hrs) on neutrophils following stimulation with escalating doses of CD20-TCB. **B.** Cytokine levels in the plasma at 4, 18, 24 and 44 hrs after CD20-TCB treatment measured by Luminex. 1 donor.



Supp. Figure 8. Neutrophils proportion in the blood measured by Flow cytometry matches neutrophils proportion in the single-cell RNA sequencing dataset. **A-B.** Proportion of CD16+CD15+ neutrophils in whole blood treated with 0.2 µg/mL CD20-TCB for 2 hrs, 4 hrs and 24 hrs and respective incubation controls measured by flow cytometry. **C.** Stacked barplot with cell type proportions in corresponding samples from donor 1 and donor 2 analyzed by single cell RNA sequencing. Neutrophils are annotated based on the ELANE, MPO, PRTN3 CTSG, AZU1 and FCGR3B gene signature.



Supp. Figure 9. A. Dotplot with c mean gene expression and fraction of positive cells per cytokine in neutrophils from whole blood after treatment with 0.2 μg/mL CD20-TCB for 2 hrs, 4 hrs, 6 hrs and 24 hrs and corresponding incubation controls. Mean of n= 4 donors (baseline, and 24 hrs) and n=2 donors (2 hrs, 4 hrs and 6 hrs). **B.** Violin plots showing the expression distribution of IL-1β (IL-1B), IL-6 (IL6), TNF-α(TNF), IL-8 (CXCL8), IL-8R (CXCR2), IL-32 (IL32), IL-10 (IL10), MCP-1 (CCL2), MIP-1β (CCL4), IP-10 (CXCL10), TNFR1a (TNFRSF1A), TNFR1b (TNFRSF1B), IFNR1 (IFNGR1) andIFNR2 (IFNGR2), genes within neutrophils at each treatment time point. N=4 donors (baseline, and 24 hrs) and N=2 donors (2 hrs, 4 hrs and 6 hrs). **A-B.** Neutrophils were identified by scRNA sequencing of whole blood based on the ELANE, MPO, PRTN3 CTSG, AZU1 and FCGR3B gene signature.

7. Second manuscript

Title: Src/Lck inhibitor dasatinib reversibly switches off cytokine release and T cell cytotoxicity following stimulation with T cell bispecific antibodies

Original research article

Journal for ImmunoTherapy of Cancer (JITC)

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Abstract

Background:T cell engagers are bispecific antibodies recognizing, with one moiety, the CD3ε chain of the T cell receptor and, with the other moiety, specific tumor surface antigens. Crosslinking of CD3 upon simultaneous binding to tumor antigens triggers T cell activation, proliferation and cytokine release, leading to tumor cell killing. Treatment with T cell engagers can be associated with safety liabilities due to on-target on-tumor, on-target off-tumor cytotoxic activity and cytokine release syndrome (CRS). Tyrosine kinases such as SRC, LCK or ZAP70 are involved in downstream signaling pathways after engagement of the T cell receptor and blocking these kinases might serve to abrogate T cell activation when required (online supplemental material 1). Dasatinib was previously identified as a potent kinase inhibitor that switches off CAR T cell functionality.

Methods: Using an *in vitro* model of target cell killing by human peripheral blood mononuclear cells, we assessed the effects of dasatinib combined with 2+1 T cell bispecific antibodies (TCBs) including CEA-TCB, CD19-TCB or HLA-A2 WT1-TCB on T cell activation, proliferation and target cell killing measured by flow cytometry and cytokine release measured by Luminex. To determine the effective dose of dasatinib, the Incucyte system was used to monitor the kinetics of TCB-mediated target cell killing in the presence of escalating concentrations of dasatinib. Last, the effects of dasatinib were evaluated *in vivo* in humanized NSG mice co-treated with CD19-TCB. The count of CD20+ blood B cells was used as a readout of efficacy of TCB-mediated killing and cytokine levels were measured in the serum.

Results: Dasatinib concentrations above 50 nM prevented cytokine release and switched off-target cell killing, which were subsequently restored on removal of dasatinib. In addition, dasatinib prevented CD19-TCB-mediated B cell depletion in humanized NSG mice. These data confirm that dasatinib can act as a rapid and reversible on/off switch for activated T cells at pharmacologically relevant doses as they are applied in patients according to the label.

Conclusion: Taken together, we provide evidence for the use of dasatinib as a pharmacological on/off switch to mitigate off-tumor toxicities or CRS by T cell bispecific antibodies.

Title: The Src/lck inhibitor dasatinib reversibly switches off cytokine release and T cell cytotoxicity following stimulation with T cell bispecific antibodies

Authors: G. Leclercq, H. Haegel, A. Schneider, A. Giusti, E. Marrer-Berger, C. Boetsch, A. Walz, V. Pulko, J. Sam, J. Challier, C. Ferlini, A. Odermatt, P. Umaña, M. Bacac, C. Klein

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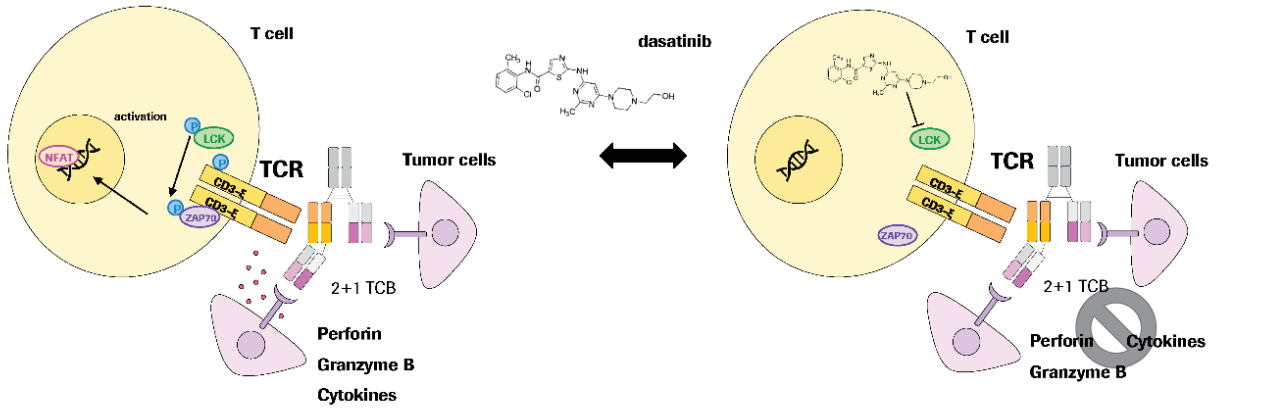

In brief: Dasatinib reversibly switches off T cell activation and proliferation, T cell cytotoxicity and cytokine release upon stimulation with T cell bispecific antibodies.

Online supplemental material 1 - graphical abstract.

Data availability statement

The authors declare that all relevant data to the study are included in the article or uploaded as online supplemental information.

Background

T cell bispecific antibodies (TCBs) or T cell engagers are bispecific antibodies that, with one binding moiety, recognize a tumor antigen expressed on tumor cells and, with the other binding moiety, the T cell receptor resulting in T cell activation and subsequent tumor cell killing.[1–5] We have described potent 2+1 TCBs, for example, cibisatamab (CEA-TCB)[6 7] or glofitamab (CD20-TCB),[8] based on a 2+1 format with one binder to the CD3ε chain of the T cell receptor and two binders to the specific tumor antigens. Their Fc region enables a longer half-life and is engineered with P329G LALA mutations to prevent FcγR signaling.[9 10] Crosslinking of the CD3ε chain with tumor antigens by simultaneous TCB binding triggers T cell activation, proliferation and cytokine secretion.[6 7] In contrast to chimeric antigen receptor (CAR) T cells, TCBs represent

an “off the shelf” therapy to eradicate tumors.[1 11 12] While lineage-specific antigens like CD19, CD20 or BCMA can be targeted with CAR T cells or TCBs as the respective cell types expressing these antigens are non-essential, the targeting of solid tumor antigens in epithelial tumors is more challenging due to their broader expression in normal tissues resulting in potential undesired on-target off-tumor toxicity.[13]

One of the most common mode-of-action related toxicities reported with T cell engagers is cytokine release syndrome (CRS).[14] This complex clinical syndrome is featured by fever and in the most severe cases by hypotension and/or hypoxia.[15] CRS is linked to a strong release of pro-inflammatory cytokines by T cells producing TNF-α, IFN-γ and GM-CSF16 17 and by myeloid cells producing TNF-α, IL-1β and IL-6.[18–2]

Several problems of toxicity grading of CRS were addressed as summarized in a recent publication of a consensus grading scale,[22] mainly driven by treatment interventions, with severe cases easily classified if managed with pressors and/or high-flow oxygen devices. Management of severe CRS also requires appropriate supportive care, high-dose glucocorticoids and benefit from anti-IL-6R/IL-6 treatment such as tocilizumab or silixumab.[16 23 24]

Another problematic toxicity to manage in the clinic is represented by off-tumor off-target toxicity as observed with TCRs in the context of adoptive T cell therapy. A clinically relevant example of the risks associated with TCR-based therapies in the context of adoptive T cell therapy was identified when an unexpected cross-reactivity of an enhanced affinity TCR targeting an HLA-A*01-restricted epitope from MAGE-A3 resulted in severe cardiovascular toxicity through recognition of an unrelated HLA-A*01-associated peptide, A1-Titin.[25] Similarly, a MAGE-A3 peptide-specific TCR for adoptive T cell therapy demonstrated an undetected cross-reactivity with MAGE-A12 responsible for severe neurotoxicity.[26] A rapid blockade of T cell activation/proliferation at onset of the off-target toxicity would have been essential to stop such life-threatening toxicities. Recombinant TCR-based T cell engagers or TCR-like TCBs targeting intracellular proteins presented by MHC class I have the potential inherent risk of recognizing related undesired peptides in the context of MHC presentation. Furthermore, on-target off-tumor toxicity may also occur when the tumor-associated antigens (TAAs) are expressed on healthy cells like in epithelial tissues, which may potentially trigger cell death and inflammation, resulting in irreversible tissue damage and compromising the patient’s safety.

Tyrosine kinases such as Lck, Fyn (Src family of protein kinases) or ZAP70 are involved in downstream T cell activation signaling pathways after engagement of the CD3ε chain of the T cell receptor.[27] Blocking these kinases might counteract T cell activation. Recently, screening of tyrosine kinase inhibitor (TKI) libraries was performed in an effort to identify TKI candidates inhibiting CAR T cell proliferation and activation.[28] Mestermann *et al*[29] and Weber *et al*[30] identified dasatinib as a potent candidate that switches off CAR T cell functionality by inhibiting Src/Lck phosphorylation and NFAT signaling.[31] Using an *in vitro* model of target cell killing by human peripheral blood mononuclear cells, we assessed the reversible effects of dasatinib combined with CEA-TCB, as an example of a solid-tumor targeting TCB,[7] CD19-TCB, as an example of blood cancer targeting TCB, or HLA-A2 WT1-TCB, as an example of TCR-like TCB on T cell activation and proliferation, target cell killing and cytokine release. "Killing assays" testing the dose-response effects of dasatinib were conducted to define the threshold at which TCB-induced T cell activation was fully inhibited. In humanized NSG mice, the combination of dasatinib with CD19-TCB successfully prevented TCB-mediated B cell depletion. These counteracting effects can be obtained at dasatinib concentrations corresponding to clinically relevant doses. Our data show that dasatinib can act as a reversible on/off switch for TCB-mediated T cell cytotoxicity and cytokine release. Dasatinib could be used either to block TCB-induced T cell activation in case of tissue toxicities or to reduce cytokine release if CRS symptoms are not manageable with standard interventions, as an alternative to TNF-α or IL-6R blockade.[19 32]

Methods

Reagents and antibodies

CEA-TCB (cibisatamab), HLA-A2 WT1-TCB (RG6007), CD19-TCB and DP47-TCB were produced internally in the 2+1 TCB format previously described (Bacac *et al.*, CCR for CEA and CD20-TCBs). Dasatinib (S1021) was purchased from Selleckchem.

Cell culture

The SKM-1 cell line (DSMZ# ACC547) is a human acute myeloid leukemia cell line. The cells were cultured in RPMI (11875101; Gibco) containing 20% FBS (26140079; Gibco) and split every 3 to 4 days (to 0.6 million cells/mL). To ensure sufficient MHC I levels, cells were used in

an assay 1–2 days after passaging. For the Incucyte experiment, SKM-1 labeled with NucLightRed (NLR) were used.

The NLR-labeled A375 cell line is an adherent melanoma cell line which is HLA-A2 and WT1 positive. It was transduced with a vector coding for histone-staining red fluorescent protein. The cells were cultured in DMEMF12 (11320033; Gibco) containing 10% FBS (26140079; Gibco) supplemented with 6 µg/mL puromycin and split every 3 to 4 days (to 20,000 cells/cm2). Cells were plated 1 day prior to the assay and pulsed with RMF peptides 2 hours before starting the assay.

The NLR-labeled MKN45 cell line is an adherent human gastric cancer cell line, which expresses high levels of the CEA antigen. It was transduced with a vector coding for histone-staining red fluorescent protein. The cells were cultured in RPMI Glutamax (61870036; Gibco) containing 10% FCS and split every 3 to 4 days (50,000 cells/cm2). The cells were plated 1 day prior to the assay.

The SU-DHL-8 cell line is a human large cell lymphoma cell line derived from peritoneal effusion in a 59-year-old male Caucasian patient (ATCC catalog number CRL-2961). The cells were cultured in RPMI (11875101; Gibco) containing 10% FBS (26140079; Gibco) and split every 3 to 4 days (to 0.8 million cells/mL).

Cell line authentication was performed at Microsynth.

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats donated by healthy donors (blood donation center in Zürich, in accordance with the Declaration of Helsinki) by Ficoll density gradient. Briefly, blood from buffy coat was diluted 1:1 with PBS and about 25 mL was layered on 15 mL of Ficoll (17-5442; GE-Healthcare) and centrifuged for 30 min at 2000 rpm without break. Lymphocytes were collected with a 10 mL pipette in a 50 mL tube, rinsed with PBS, and successively centrifuged at 1700 rpm (5 min), 1500 rpm (5 min), 1100 rpm (10 min) and 900 rpm (10 min) to remove remaining platelets.

Preparation of effector cells

PBMCs were counted and then adjusted to either 0.4×106 cells/mL, 1.0×106 cells/mL, 2.0×106 cells/mL or 6.0×106/mL in assay medium. Then 50 µL of the cell suspension was

transferred to the wells of the assay plates, corresponding to 20,000, 50,000, 100,000 or 300,000 cells/well.

Preparation of antibodies and dasatinib solution

HLA-A2 WT1-TCB, CEA-TCB, DP47-TCB and CD19-TCB were diluted in assay medium. A series of eight dilutions (1:10) was prepared by transferring and mixing 100 µL of 400 nM TCB solution to the subsequent wells containing 900 µL of assay medium. A 20× dasatinib solution was prepared in PBS from a 10 mM DMSO stock solution and transferred into the wells (10 µL/well).

For *in vivo* administration, dasatinib was formulated in 10% DMSO, 30% PEG300, 5% Tween 80% and 55% H2O in a stock solution of 10 mg/mL

Preparation of adherent tumor target cells

One day before the assay, adherent NLR-labeled A375 or NLR-labeled MKN45 target tumor cells were detached from the plate using 0.05% trypsin (25300096; Gibco). Cells were washed with PBS and the counts of viable cells (>90%) were determined by Trypan Blue staining using an EVE cell counter. Cells were re-suspended in pre-warmed assay medium (37°C) to obtain a cell density of 50,000 cells/mL. Then 100 µL of the cell suspension was transferred into a 96-flat-bottom well plate, corresponding to 5000 target cells per well.

Preparation of non-adherent tumor target cells

On the day of the assay, SKM-1 or SU-DHL-8 tumor cells were washed with PBS and the counts of viable cells were determined by Trypan Blue staining using an EVE cell counter (>90%). If required, the cells were labeled with Cell Trace CTV (C34557; Thermo Fisher) or CFSE (C34554; Thermo Fisher). Cells were re-suspended in pre-warmed assay medium (37°C) to obtain a cell density of 200,000 cells/mL. Then 100 µL of the cell suspension was transferred into a 96 U-bottom well plate, corresponding to 20,000 target cells per well.

For the Incucyte experiments, NLR-labeled SKM-1 cells were attached to a 96-well plate using rectronectin from Takara. Flat-bottom 96-well plates were coated with 50 µL of a rectronectin stock solution of 10 µg/mL (45 min, RT). The plates were washed with PBS, and the cells were plated (45 min, 37°C) before the assay.

Labeling of tumor cells

SKM-1 tumor cells were washed once with sterile PBS and labeled with Cell Trace CTV (C34557; Thermo Fisher) or CFSE dye (C34554; Thermo Fisher) (5 µM, 20 min at RT), washed with RPMI+20% FBS and counted.

Preparation of the killing assay

In assays with 20,000 SKM-1 tumor cells/well, 50 µL of the effector cell suspension (20,000, 100,000 or 200,000 cells/well) were added, followed by 50 µL of the antibody solutions. The final E:T ratio was 1:1, 5:1 or 10:1 and the total volume per well was 200 µL. The assay plates were covered with lids, and placed in the incubator, 37°C, 5% CO2.

Preparation of the killing assay using the Incucyte

The assay medium of tumor cells was replaced with fresh medium (100 µL/well). Then 50 µL of the effector cell suspension (50,000 cells/well) followed by 50 µL of the antibody dilutions were added to the assay plates containing 5000 adherent target cells/well. The E:T was approximately 10:1 and the total volume per well was 200 µL. The assay plates were incubated in the Incucyte for measurements of total red area/well every 3 hours, at 37°C, 5% CO2.

Flow cytometry readout: T cell activation and B cell count

At the assay endpoint, PBMCs were washed twice in PBS (1500 rpm, 5 min, RT) and stained with the following markers: CD4 (APC-Cy7, 317418; Biolegend), CD8 (BV605, 344742; Biolegend), CD25 (BUV395, 564034; BD), CD69 (PE, 310306; Biolegend) and Live Dead Near Infra Red (NIR) (L10119; Thermo Fisher) for 30 min at 4°C in FACS buffer. For blood staining, 25 µL of blood was lysed twice using BD Pharm Lyse buffer (555899; BD) (200 µL, 10 min, RT) and stained with the following markers: CD45 (Alexa 700, 304119; Biolegend), CD20 (APC, 302309; Biolegend) and Live Dead NIR (L10119; Thermo Fisher) for 30 min at 4°C in FACS buffer. Cells were then washed twice in FACS buffer (1500 rpm, 5 min, RT) and re-suspended in 100 µL/well FACS buffer for analysis. Sample acquisition was performed using an HTS plate reader connected to a BD Fortessa Flow cytometer.

Flow cytometry readout: intracellular cytokine staining

To block cytokine secretion, 20 µL of culture medium containing 1/150 (final concentration: 1/1500) Brefeldin A (Golgiplug, 555029; BD) and 1/100 (final concentration: 1/1000) Monensin A (Golgistop, 554724; BD) and CD107a (Alexa 647, 562622; BD) was transferred to each well for 24 hours. At the assay endpoint, PBMCs were collected, washed twice in PBS (5 min, 1500 rpm, RT) and stained with a mix of antibodies to the following surface markers: CD4 (BUV395, 564724; BD), CD8 (BV605, 344742; Biolegend) and live dead NIR (L10119; Thermo Fisher) in PBS (30 min, 4°C, no light) in PBS. PBMCs were centrifuged (5 min, 1500 rpm, RT) and fixed with Cytofix/Cytoperm buffer (554722; BD) (80 µL/well, 30 min, 4°C). PBMCs were centrifuged and then washed with Perm/Wash buffer (554723; BD) (5 min, 1500 rpm, RT). PBMCs were incubated in Perm/Wash buffer (30 min, 4°C, no light) and then stained with a mix of antibodies to cytokines: TNF-α (APC-Cy7, 502944; Biolegend) and IFN-γ (BV795, 612845; BD) in Perm/Wash buffer (50 µL/well, 30 min, 4°C, in the dark). PBMCs were centrifuged and washed with FACS buffer (5 min, 1500 rpm, RT). Last, they were re-suspended in 100 µL FACS buffer and sample acquisition was performed using and HTS plate reader connected to a BD Fortessa Flow cytometer.

Cytokine measurement

Cytokines were analyzed in the culture supernatants from the killing assays (stored at −80°C) by Luminex using a human8Plex Assay kit (M50000007A; Bio-Rad) and additional reagents for IL-1β (171B5001M; Bio-Rad) and MCP-1 (171B5021M) measurement. Pre-diluted supernatants were incubated with beads in a 96-well filter plate (1 hour, 800 rpm, RT, in the dark). The plate was washed twice using a vacuum manifold and the detection antibody solution was added (1 hour, 800 rpm, RT, no light). The plate was vacuumed and washed twice and the streptavidin solution was added (30 min, 800 rpm, RT, in the dark). The plate was vacuumed and washed twice and the samples were re-suspended in assay buffer. Sample acquisition was conducted using the Luminex equipment from Bio-Rad.

***In vivo* experiment**

Humanized NSG mice were ordered from the Jackson Laboratory. The Cantonal Veterinary Office, Zurich, Switzerland, approved the protocol (ZH225-17) in accordance with the Swiss Animal Protection Law. One day before treatment, humanized NSG mice were randomized based

on their T cell counts into three groups of four mice. One group was treated with dasatinib (50 mg/kg, orally) 1 hour before injection of CD19-TCB (0.5 mg/kg, intravenously) on day 0 and again 5 hours and 8 hours after injection. On days 1 and 2, dasatinib was given twice per day with intervals of 10–11 hours. Blood was collected by tail-vein bleedings or by terminal retro-orbital bleeding at 72 hours.

Data analysis

Flow cytometry data were analyzed using FlowJo V.10. Cytokine data were analyzed using the Bio-Plex software. GraphPad Prism V.8 was used to generate the graphs and for statistical analysis. For dose–titration curves, AUC were calculated and used for statistical comparison. Data are shown as means with SD or SEM or as individual curves. The statistical tests used are indicated in the figure legends for each experiment.

Results

Dasatinib is a potent inhibitor of TCB-mediated target cell killing at pharmacologically relevant doses

To assess the inhibitory effect of dasatinib on TCB-mediated target-cell killing, PBMCs were co-cultured with NLR-labeled SKM-1 cells and HLA-A2 WT1-TCB, in medium supplemented with escalating concentrations of dasatinib. The Incucyte system was used to capture the loss of red fluorescent protein signal over time as a readout of target-cell killing. A concentration of 100 nM (48.8 ng/mL) and 50 nM (24.4 ng/mL) dasatinib resulted in 92.2% and 95.5% inhibition of target-cell killing induced by 10 nM HLA-A2 WT1-TCB (figure 1A and table 1), while not directly affecting NLR-labeled SKM-1 growth (online supplemental figure 2A). A concentration of 25 nM (12.2 ng/mL) and 12.5 nM (6.1 ng/mL) dasatinib resulted in 87.1% and 80.2% inhibition of target-cell killing for 10 nM HLA-A2 WT1-TCB (table 1). The lower concentration of 6.25 nM dasatinib combined with 10 nM HLA-A2 WT1-TCB only partially inhibited killing (figure 1A and table 1). Similarly, 100 nM and 50 nM dasatinib significantly prevented HLA-A2 WT1-TCB-induced SKM-1 killing as well as T cell proliferation and activation in a killing assay using CSFE-labeled SKM-1 tumor cells co-cultured with PBMCs and HLA-A2 WT1-TCB (figure 1B–E and online supplemental figure 4A,B). Dasatinib did not affect T cell nor SKM-1 cell viability, nor the target expression on SKM-1 cells (online supplemental figures 2A,D,E and 3). Moreover, treatment with a concentration of dasatinib above 25 nM totally prevented the

release of IFN-γ, IL-2 and to a lower extent TNF-α (figure 1F–H and online supplemental figure 8A–D). The lower concentration of 12.5 nM and 6.25 nM dasatinib decreased but did not fully suppress cytokine release (figure 1F–H). Overall, these data show that dasatinib can fully prevent T cell mediated target-cell lysis triggered by PBMCs stimulated with HLA-A2 WT1-TCB at *in vitro* concentrations of 50 nM and above. The inhibitory effect of dasatinib on HLA-A2 WT1-TCB-induced T cell cytotoxicity and cytokine release was confirmed using another target cell line (A375) for HLA-A2 WT1-TCB. (online supplemental figures 1 and 2B).

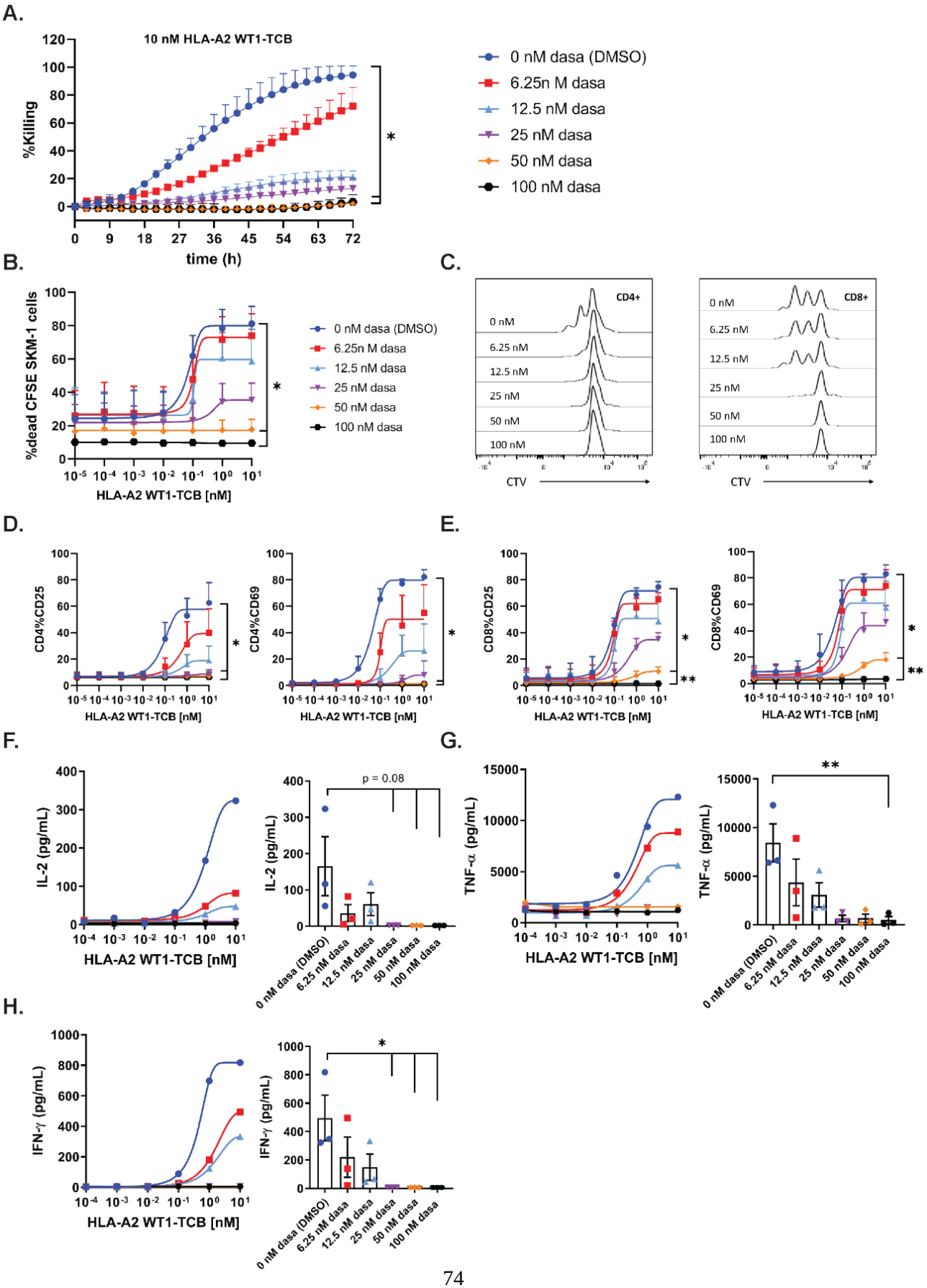
A.
10 nM HLA-A2 WT1-TCB
%Killing
time (h)
0 nM dasa (DMSO)
6.25n M dasa
12.5 nM dasa
25 nM dasa
50 nM dasa
100 nM dasa
B.
%dead CFSE SKM-1 cells
HLA-A2 WT1-TCB [nM]
C.
CD4+
CD8+
CTV
D.
CD4%CD25
CD4%CD69
E.
CD8%CD25
CD8%CD69
F.
IL-2 (pg/mL)
p = 0.08
G.
TNF-α (pg/mL)
H.
IFN-γ (pg/mL)

Figure 1. Dasatinib is a potent inhibitor of TCB-mediated target cell killing at the label pharmacological dose. (**A**) Real-time killing of NLR-labeled SKM-1 tumor cells by 10 nM HLA-A2 WT1-TCB in the presence of escalating concentrations of dasatinib. NLR-labeled SKM-1 tumor cells were co-cultured with peripheral blood mononuclear cells (PBMCs) and HLA-A2 WT1-TCB in media supplemented with dasatinib, E:T=2.5:1. Killing was followed by Incucyte (1 scan every 3 hours, zoom ×10, phase and red 400 ms acquisition time), mean of n=2 donors±SD with *p≤0.05, **p≤0.01 by one-way ANOVA (Friedman test). Effects of escalating concentrations of dasatinib on HLA-A2 WT1-TCB-induced killing (**B**), T cell proliferation (**C**) and T cell activation (**D, E**) in a killing assay where CFSE-labeled SKM-1 tumor cells were co-cultured with CTV-labeled PBMCs and HLA-A2 WT1-TCB, E:T=5:1. (**B**) The killing of CFSE-labeled SKM-1 cells was measured by flow cytometry at t=24 hours using a Live/Dead stain allowing exclusion of dead cells. (**C**) To assess the effect of dasatinib on T cell proliferation, the dilution of the CTV dye in CD4+ and CD8+ T cells was measured by flow cytometry at t=72 hours, histogram plots for 1 donor representative of 3. (**D, E**) The expression of CD25 and CD69 on CD4+ and CD8+ T cells was measured by flow cytometry (t=24 hours), mean of n=3 donors+SD with *p≤0.05, **p≤0.01 1 by one-way ANOVA (Friedman test). (**F, G, H**) The levels of IFN-γ, TNF-α and IL-2 were measured by Luminex in the supernatants after 24 hours. The dasatinib dose–response curves depict the data from 1 donor representative of 3. The graphs show the individual values for n=3 donors treated with 10 nM HLA-A2 WT1-TCB in the presence of escalating concentrations of dasatinib, mean of n=3 donors±SEM with *p≤0.05, **p≤0.01 by one-way ANOVA (Friedman test).

Table 1. The percentage of inhibition of tumor cell killing for each concentration of dasatinib was calculated over that in the absence of dasatinib for a fixed HLA-A2 WT1-TCB concentration of 10 nM in the assay of Figure 1A, mean of n= 2 donors.

dasatinib [ng/mL]	dasatinib [nM]	Inhibition [%]
48.80	100.00	94.20
24.40	50.00	95.90
12.20	25.00	87.90
6.10	12.50	80.10
3.05	6.25	33.00
0.00	0.00	0.00

Dasatinib rapidly switches off TCB-induced T cell functionality

To evaluate if dasatinib could act as a rapid and potent inhibitor of activated T cells, we first stimulated PBMCs cultured with SKM-1 tumor cells and HLA-A2 WT1-TCB for 24 hours before adding 100 nM dasatinib to the co-culture (figure 2A). The expression of CD69 and CD25 on CD8+ and CD4+ T cells at 24 hours showed a partially activated phenotype for T cells stimulated with HLA-A2 WT1-TCB in the absence of dasatinib (figure 2B,C). Along with activation of T cells, IFN-γ, TNF-α and IL-2 were also found in the culture supernatants after 24 hours of stimulation (figure 2D–F). Following a further 24 hours of incubation, this time in the presence of 100 nM dasatinib, the expression of the early activation marker CD69 and the late activation

marker CD25 on CD4+ and CD8+ T cells at 48 hours were lower than those measured at 48 hours in absence of dasatinib (figure 2B,C). Thus, treatment with 100 nM dasatinib rapidly inhibited further induction of activation markers in pre-activated T cells.We also measured the cytokine levels in the killing assay supernatants at 48 hours, to assess the impact of dasatinib on T cell-mediated cytokine release. No differences were observed for IFN-γ, TNF-α and IL-2 levels measured at 24 hours and 48 hours, as opposed to the dasatinib-untreated control where cytokine levels had largely increased at 48 hours (figure 2D–F). This indicated that the addition of 100 nM dasatinib at 24 hours had rapidly prevented the release of cytokines by activated T cells. We also assessed T cell proliferation 120 hours after addition of 100 nM dasatinib in the killing assay, measuring the CTV dye dilution peaks by flow cytometry. The treatment with 100 nM dasatinib decreased the proliferation of CD4+ and CD8+ T cells induced by 10 nM HLA-A2 WT1-TCB, with a stronger effect on CD4+ T cells (online supplemental figure 4C). In addition, CD4+ and CD8+ T cell counts were significantly higher in untreated cultures than in those treated with 100 nM dasatinib (figure 2G). Dasatinib appeared to impact more strongly the CD4+ than the CD8+ T cell proliferation (figure 2G and online supplemental figure 4C). These results showed that 100 nM dasatinib added to the killing assay inhibited TCB-induced T cell proliferation with an apparent stronger impact on CD4+ than on CD8+ T cells. As demonstrated in these experiments, dasatinib treatment rapidly resulted in the blockade of T cell activation, cytokine release and proliferation indicating a loss of T cell functionality.

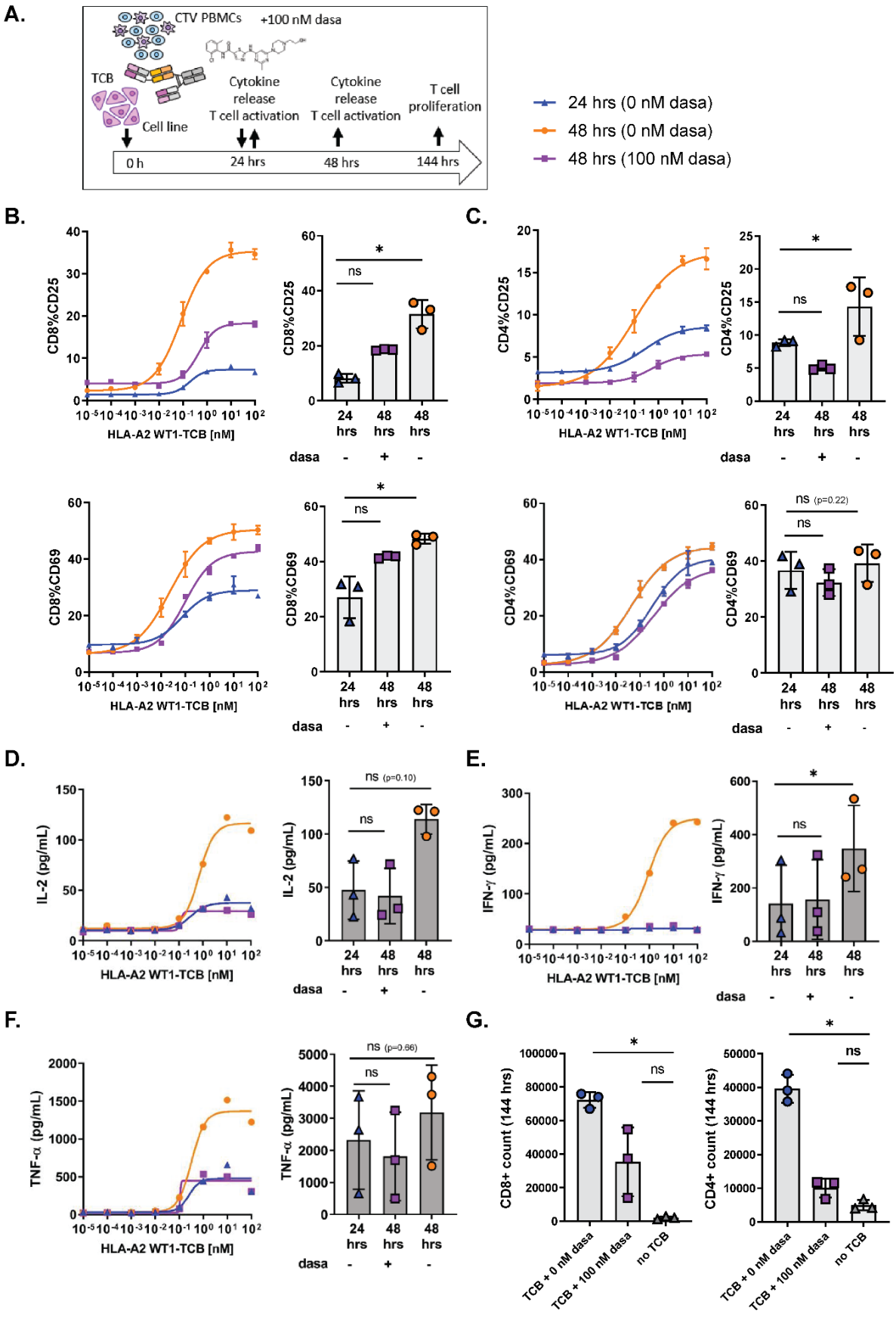
A.
CTV PBMCs
+100 nM dasa
TCB
Cell line
Cytokine release
T cell activation
Cytokine release
T cell activation
T cell proliferation
0 h
24 hrs
48 hrs
144 hrs
24 hrs (0 nM dasa)
48 hrs (0 nM dasa)
48 hrs (100 nM dasa)
B.
C.
CD8%CD25
CD4%CD25
CD8%CD69
CD4%CD69
HLA-A2 WT1-TCB [nM]
dasa
ns
ns (p=0.22)
D.
E.
IL-2 (pg/mL)
IFN-γ (pg/mL)
ns (p=0.10)
F.
G.
TNF-α (pg/mL)
ns (p=0.86)
CD8+ count (144 hrs)
CD4+ count (144 hrs)
TCB + 0 nM dasa
TCB + 100 nM dasa
no TCB

Figure 2. Dasatinib is a rapid and potent inhibitor of TCB-induced T cell functionality. (**A**) *In vitro* killing assay set-up and timelines. CTV-labeled peripheral blood mononuclear cells (PBMCs) were co-cultured with SKM-1 tumor cells (E:T=1:1) and HLA-A2 WT1-TCB. Dasatinib was added after 24 hours of activation. (**B, C**) The expression of CD25 and CD69 on CD8+ and CD4+ T cells was measured by flow cytometry after 24 hours and 48 hours of activation in the presence and absence of dasatinib. (**D, E, F**) The supernatants were collected at 24 hours and 48 hours, and the levels of IL-2, IFN-γ and TNF-α were measured by Luminex. (**G**) At 144 hours, the absolute T cell counts were measured by flow cytometry to assess the effect of dasatinib on CD4+ and CD8+ T cell proliferation. The dose–response curves depict the data from 1 donor representative of 3, mean of technical replicates±SD (**B, C**). The graphs show the means±SD of individual values from 3 donors treated with 10 nM HLA-A2 WT1-TCB with *p≤0.05, **p≤0.01 by one-way ANOVA (Friedman test). ns, not significant.

Dasatinib prevents TCB-induced cytotoxicity of activated T cells

To assess whether dasatinib could efficiently prevent TCB-mediated target cell killing by activated T cells, we set up an *in vitro* killing assay with two stimulation steps, in an attempt to mimic an ON/OFF switch of T cell cytotoxicity. PBMCs were first activated with HLA-A2 WT1-TCB for 20 hours in the presence of SKM-1 target cells labeled with CFSE in the absence of 100 nM dasatinib (ON). The PBMCs were then washed and re-stimulated with the TCB on SKM-1 cells labeled with CTV in the presence of 100 nM dasatinib (OFF switch). The use of CFSE-labeled and CTV-labeled SKM-1 tumors allowed to differentiate the tumor cells used in the first or second stimulation by flow cytometry (figure 3A). The first treatment with HLA-A2-WT1-TCB induced an upregulation of the early and late T cell activation markers CD69 and CD25 on CD8+ and CD4+ T cells (online supplemental figure 5), as well as the killing of CFSE-labeled SKM-1 target cells (figure 3B,C), while a non-targeted TCB, DP47-TCB, did not show any activity (online supplemental figure 6). T cells were therefore activated by HLA-A2 WT1-TCB and functional before the addition of dasatinib in the system. Following the second stimulation in the presence of 100 nM dasatinib, 87.30% of CTV-labeled SKM-1 cells were alive while only 2.04% were still alive following re-stimulation in the absence of dasatinib (figure 3B). Thus, addition of dasatinib on the second stimulation prevented HLA-A2 WT1-TCB-mediated killing (figure 3B,C). In addition, the release of IFN-γ, IL-2 and GM-CSF (known to be produced by T cells) and of TNF-α, IL-6 and IL-8 (produced by T cells and monocytes) was fully inhibited on re-stimulation in the presence of 100 nM dasatinib (figure 3D,E).18 19 This result emphasizes that dasatinib can switch off activated T cells, rapidly blocking TCB-mediated cytokine release as well as T cell cytotoxicity. To investigate how dasatinib could prevent T cell cytotoxicity, we measured the expression of CD107a by intracellular staining as a readout for T cell degranulation (online supplemental figure 7A) after stimulation with 10 nM HLA-A2 WT1-TCB in the presence or absence of 100 nM dasatinib. The addition of dasatinib prevented T cell

degranulation, as indicated by the inhibition of CD107a (online supplemental figure 7B,C). This result shows that dasatinib can prevent T cell degranulation and potentially the release of perforin and granzyme B that mediate the killing of target cells.

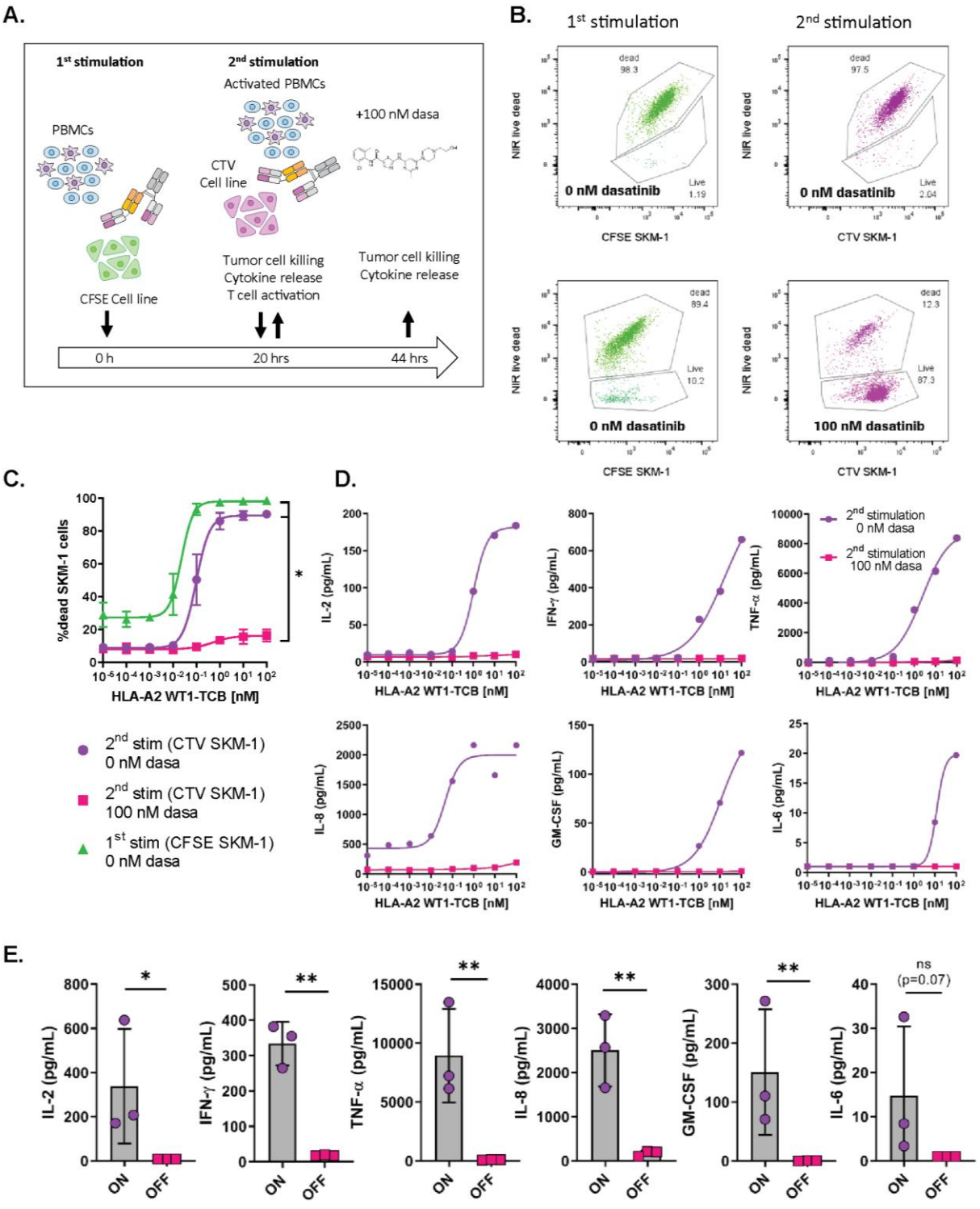

Figure 3. Dasatinib is a potent inhibitor of TCB-induced T cell cytotoxicity. (**A**) *In vitro* killing assay set-up and timelines. Peripheral blood mononuclear cells (PBMCs) were co-cultured with CFSE-labeled SKM-1 tumor cells (E:T=5:1) and HLA-A2 WT1-TCB. After 20 hours, the cells were washed and re-stimulated with HLA-A2 WT1-TCB on fresh CTV-labeled SKM-1 cells (E:T=5:1) in the presence or absence of 100 nM dasatinib for 24 hours. (**B**) The killing of SKM-1 cells was measured by flow cytometry at 20 hours (after 1st stimulation) and 44 hours (after 2nd stimulation) by exclusion of dead CFSE-labeled and CTV-labeled SKM-1 cells using a Live/Dead NIR dye. The flow cytometry dot plots are from 1 donor representative of 3 treated with 10 nM HLA-A2 WT1-TCB. (**C**) CTV-labeled and CFSE-labeled SKM-1 tumor cell killing before and after restimulation with HLA-A2 WT1-TCB in the presence or absence of dasatinib, mean of n=3 donors±SD with *p≤0.05, **p≤0.01 by one-way ANOVA (Friedman test). (**D, E**) IFN-γ, IL-2, TNF-α, IL-8, GM-CSF and IL-6 levels were measured by Luminex in the culture supernatants. The dose–response curves depict the data from 1 donor representative of 3 and the graphs show the mean±SD of the individual values from 3 donors treated with 10 nM HLA-A2 WT1-TCB with *p≤0.05, **p≤0.01 by one-way ANOVA (Friedman test).

Dasatinib reversibly stops TCB-induced tumor cell killing

We then verified whether the effect of dasatinib was reversible on its removal. To this aim, we set up a killing assay with repeated stimulations in the presence or absence of dasatinib and followed the killing kinetics using the Incucyte system (figure 4A). After each stimulation, effector cells were washed and re-stimulated on fresh NLR-labeled MKN45 target cells with 1 nM CEA-TCB in the presence or absence of dasatinib, in order to mimic an OFF/ON/OFF or ON/OFF/ON switch. A dose titration of dasatinib was conducted in this system, showing that 100 nM and 50 nM significantly prevented the killing of NLR-labeled MKN45 cells by 1 nM CEA-TCB (figure 4B and online supplemental figure 2C). In addition, dasatinib blocked CEA-TCB-mediated cytokine release (online supplemental figure 9). Adding 100 nM dasatinib in the co-culture during the first stimulation resulted in the inhibition of target cell killing, which was then reversed after dasatinib removal for the second stimulation (OFF/ON) (figure 4C). IFN-γ, IL-2 and TNF-α were not detected in the supernatants after the first stimulation in the presence of 100 nM dasatinib, indicating a full inhibition of T cell derived cytokine release (figure 4E). Removal of dasatinib after 3 days and re-stimulation with 1 nM CEA-TCB resulted in the release of IFN-γ, IL-2 and TNF-α, indicating that T cell functionality was restored on dasatinib removal (figure 4E). Subsequent addition of dasatinib at day 6 again inhibited T cell cytotoxicity until day 9 (figure 4C), showing an overall OFF/ON/OFF switch effect during the time course of this experiment. When dasatinib (100 nM) was added to the culture medium only on the second stimulation with CEA-TCB to prevent T cell cytotoxicity and then removed for the third stimulation, target cell killing was finally restored (figure 4D and online supplemental file 1), mimicking an ON/OFF/ON switch. Addition of 100 nM dasatinib for the second TCB stimulation prevented the release of IFN-γ, IL-2 and TNF-α, as expected (figure 4F). Cytokine release has

been shown to occur on initial exposure to T cell bispecific antibodies, but not or much less on subsequent dosing. Interestingly, low doses of dasatinib (<25 nM) added for the first TCB stimulation in the OFF/ON assay described in figure 4A partially inhibited target cell killing but seemed to equilibrate cytokine release between the first and second stimulation (online supplemental figure 10A–C).

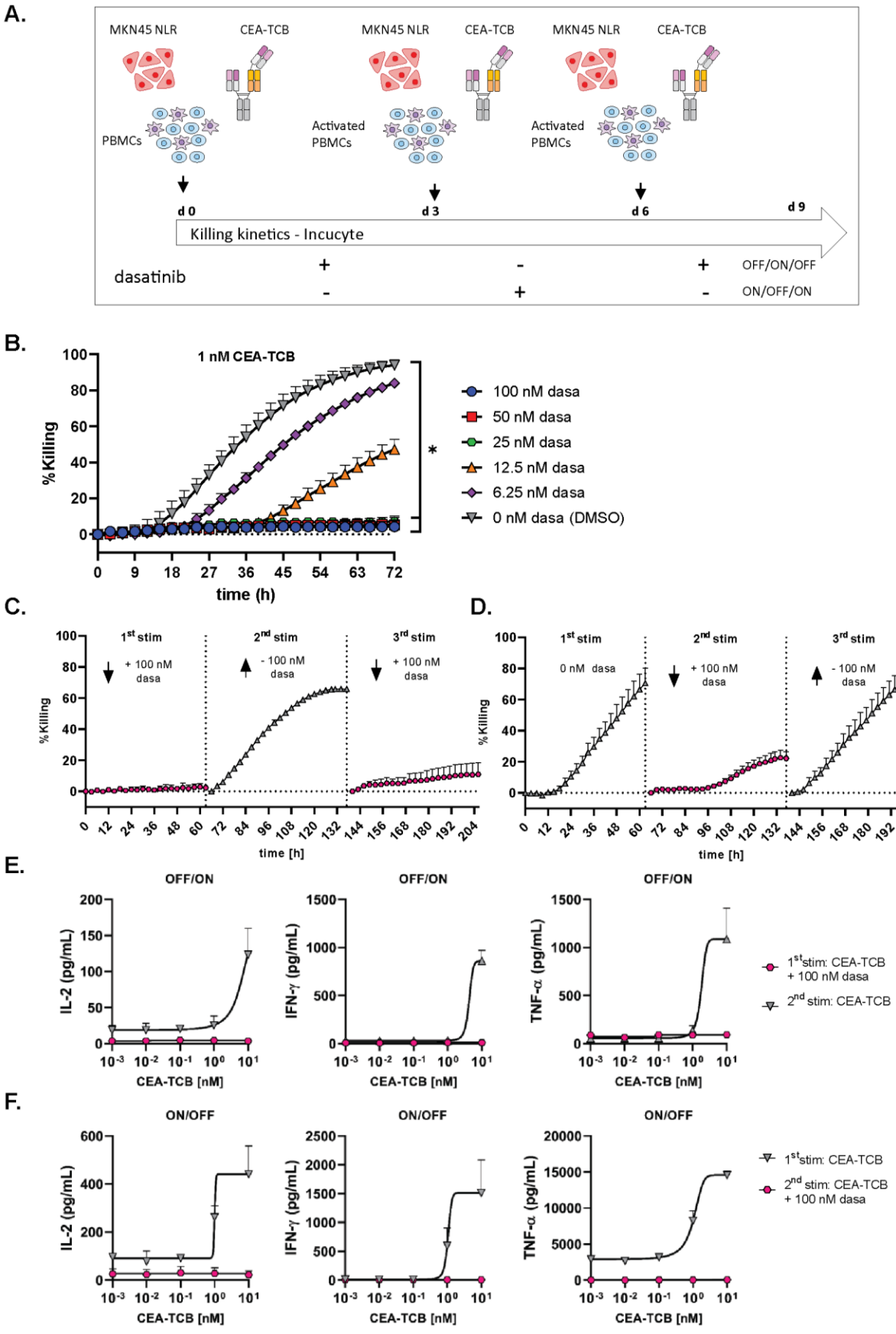
A.
MKN45 NLR
CEA-TCB
PBMCs
Activated PBMCs
d 0
d 3
d 6
d 9
Killing kinetics - Incucyte
dasatinib
+
-
OFF/ON/OFF
ON/OFF/ON
B.
1 nM CEA-TCB
% Killing
time (h)
100 nM dasa
50 nM dasa
25 nM dasa
12.5 nM dasa
6.25 nM dasa
0 nM dasa (DMSO)
C.
1st stim
2nd stim
3rd stim
+ 100 nM dasa
- 100 nM dasa
time [h]
D.
0 nM dasa
E.
OFF/ON
IL-2 (pg/mL)
IFN-γ (pg/mL)
TNF-α (pg/mL)
CEA-TCB [nM]
1st stim: CEA-TCB + 100 nM dasa
2nd stim: CEA-TCB
F.
ON/OFF
1st stim: CEA-TCB
2nd stim: CEA-TCB + 100 nM dasa

Figure 4. Dasatinib reversibly switches off TCB-induced T cell functionality. (**A**) Restimulation assay set-up. Peripheral blood mononuclear cells (PBMCs) were co-cultured with NLR-labeled MKN45 (E:T=10:1) target cells and CEA-TCB for 3 consecutive stimulations in the presence or absence of 100 nM dasatinib, mimicking an ON/OFF/ON switch (**C**) or OFF/ON/OFF switch (**D**), mean of n=3 donors+SD. (**B**) Effects of escalating concentrations of dasatinib on NLR-labeled MKN45 killing by 1 nM CEA-TCB on the first stimulation, mean of n=3 donors+SEM with *p≤0.05 by one-way ANOVA (Friedman test). Real-time killing was followed by Incucyte (1 scan every 3 hours, zoom ×10, phase and red 400 ms acquisition time). (**E, F**) The levels of IFN-γ, IL-2 and TNF-α were measured by Luminex in the culture supernatants after each stimulation of the ON/OFF and OFF/ON switch assays for a dose response of CEA-TCB, mean of n=2 donors+SEM.

Dasatinib prevents CD19-TCB-induced B cell depletion and cytokine release in humanized NSG mice

In line with the previous observations with CEA-TCB and HLA-A2 WT1-TCB, 100 nM dasatinib prevented CD19-TCB-dependent killing of SU-DHL-8 tumor cells and T cell activation as well as the release of TNF-α, IFN-γ and IL-2 *in vitro* (figure 5A,B and online supplemental figure 11A,B). To verify whether dasatinib could prevent CD19-TCB-induced B cell depletion and cytokine release *in vivo*, humanized NSG mice were either treated with vehicle or with 0.5 mg/kg CD19-TCB as a monotherapy or combined with 50 mg/kg dasatinib (figure 5C). To best translate the clinical pharmacodynamics and pharmacokinetics profile of dasatinib and to verify if the resulting exposure would be sufficient to prevent CD19-TCB-induced T cell cytotoxicity and cytokine release, dasatinib was given orally three times on day 0 and twice on days 1 and 2. As shown by the CD20+ B cell count measured in the blood at 48 hours, dasatinib prevented the killing of CD20+ B cells by CD19-TCB (figure 5D). At 72 hours, partial killing of B cells was nevertheless observed (figure 5D). The half-life of dasatinib being around 6 hours, the exposure of dasatinib was probably not sufficient any longer to continuously inhibit T cell cytotoxicity at the later timepoint. This is another indication that the inhibitory effect of dasatinib is rapidly reversible *in vivo,* as observed by Mestermann *et al*[29] and Weber *et al*[30] for CAR T cells.[33] Dasatinib blocked the release of IL-2, TNF-α, IFN-γ and IL-6 measured 1 hour 30 min and 6 hours after CD19-TCB treatment, indicating that dasatinib could also rapidly switch off CD19-TCB-dependent cytokine release (figure 5E,F). In line with the *in vitro* findings, the rapid onset of the activity of dasatinib allowed to prevent B cell depletion and cytokine release induced by the first infusion of CD19-TCB in humanized NSG mice. Collectively, these data demonstrate the favorable pharmacodynamic profile of dasatinib, efficiently preventing CD19-TCB-induced T cell cytotoxicity and cytokine release for at least 48 hours when administered twice per day at the dose of 50 mg/kg.

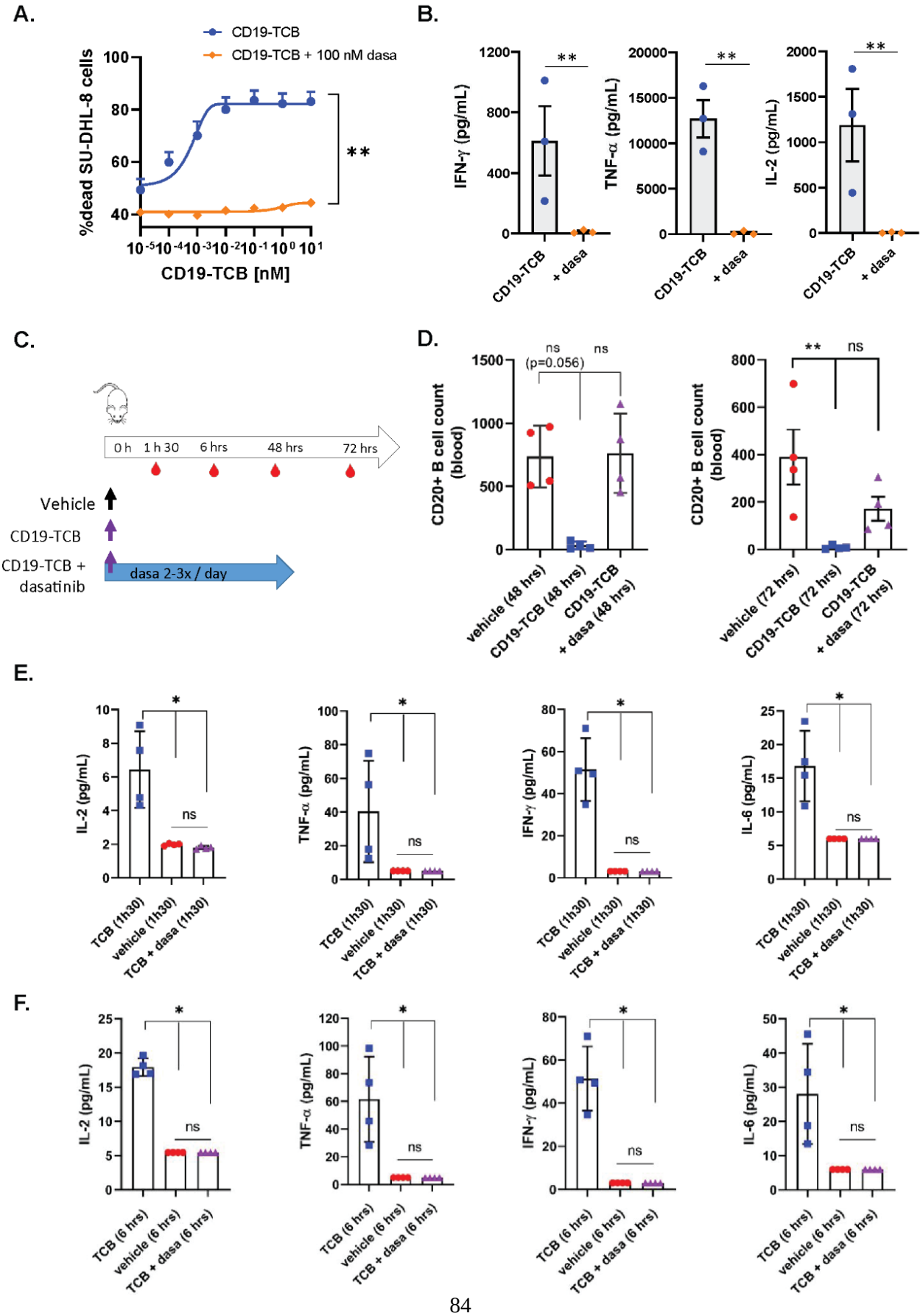
A.
CD19-TCB
CD19-TCB + 100 nM dasa
%dead SU-DHL-8 cells
CD19-TCB [nM]
**
B.
IFN-γ (pg/mL)
TNF-α (pg/mL)
IL-2 (pg/mL)
CD19-TCB
+ dasa
C.
0 h
1 h 30
6 hrs
48 hrs
72 hrs
Vehicle
CD19-TCB
CD19-TCB + dasatinib
dasa 2-3x / day
D.
CD20+ B cell count (blood)
ns
(p=0.056)
vehicle (48 hrs)
CD19-TCB (48 hrs)
CD19-TCB + dasa (48 hrs)
vehicle (72 hrs)
CD19-TCB (72 hrs)
CD19-TCB + dasa (72 hrs)
E.
IL-2 (pg/mL)
TNF-α (pg/mL)
IFN-γ (pg/mL)
IL-6 (pg/mL)
TCB (1h30)
vehicle (1h30)
TCB + dasa (1h30)
F.
TCB (6 hrs)
vehicle (6 hrs)
TCB + dasa (6 hrs)

Figure 5. Dasatinib prevents CD19-TCB-induced B cell depletion and cytokine release *in vivo*. Effect of 100 nM dasatinib on CD19-TCB-dependent SU-DHL-8 killing and cytokine release. Peripheral blood mononuclear cells were co-cultured with CTV-labeled SU-DHL-8 tumor cells (E:T=10:1) and escalating concentrations of CD19-TCB in the presence or absence of 100 nM dasatinib. (**A**) The killing of CTV-labeled SU-DHL-8 cells was measured by flow cytometry (t=24 hours) using a Live/Dead stain allowing exclusion of dead cells. (**B**) The levels of IFN-γ, TNF-α and IL-2 were measured in the culture supernatants by Luminex (24 hours, 10 nM CD19-TCB). (**A, B**) Mean of n=3 donors+SD with *p≤0.05, **p≤0.01, paired t-test. (**C**) Humanized NSG mice were co-treated with 0.5 mg/kg CD19-TCB (intravenously) and 50 mg/kg dasatinib (orally). (**D**) CD20+ B cell counts were measured by flow cytometry in blood collected at 48 hours and 72 hours. (**E, F**) The levels of IFN-γ, TNF-α, IL-2 and IL-6 were measured by Luminex in serum collected 1 hour 30 min and 6 hours post-treatment with CD19-TCB. (**D–F**) Mean of n=4 mice per group±SD with *p≤0.05, **p≤0.01 by one-way ANOVA (Kruskal-Wallis test). ns, not significant.

Discussion / Conclusion

In recent studies, Mestermann *et al*[29] and Weber *et al*[30] showed that the kinase inhibitor dasatinib allowed pharmacological control over activated CD19 CAR T cells. They demonstrated that dasatinib could reversibly switch off activated CAR T cells, enabling the mitigation of CRS and CAR T cell–associated neurotoxicity (CRES).[24] The authors suggested that the use of dasatinib would represent a more efficient approach to mitigate these life-threatening toxicities than the current approaches consisting of treatment with glucocorticoids or tocilizumab blocking IL-6R, which do not always result in recovery of symptoms in patients treated with CAR T cells.[34] Our work demonstrates that dasatinib reversibly suppresses T cell bispecific antibody-dependent cytotoxicity and cytokine release *in vitro* and *in vivo*, in line with recent *in vitro* findings of Leonard *et al*[35] with blinatumomab. Dasatinib therefore represents an attractive way to improve the safety profile and tolerability of T cell engagers.

We show that the rapid blockade of T cell activities with dasatinib could be a way to revoke inflammation and tissue damage in cases of severe TCB-mediated off-tumor toxicity. *In vitro*, 50 nM dasatinib was found to prevent the killing of TAA-expressing cells by CEA-TCB-stimulated or HLA-A2 WT1-TCB-stimulated T cells. To verify whether these *in vitro* concentrations would translate into pharmacologically active doses, we compared the *in vitro* dose with the C_{min}, C_{max} and steady-state concentrations measured in patients exposed to labeled pharmacological doses of dasatinib. Wang *et al* reported that the PK parameters derived from 146 patients treated with 100 mg dasatinib QD were a Cmin value of 2.61 ng/mL and a C_{max} value of 54.6 ng/mL.[36] Hence, the *in vitro* doses of 50 nM (24.4 ng/mL) and 100 nM (48.8 ng/mL) appear translatable to the dasatinib dosing regimen of 100 mg QD.[36] In humanized NSG mice, the

PK/PD profile of dasatinib seemed favorable as it prevented CD19-TCB-induced B cell depletion and cytokine release.

Addition of dasatinib 20 hours or 4 days after T cell stimulation with HLA-A2 WT1-TCB or CEA-TCB in killing assays was shown to rapidly stop further T cell activation and proliferation, as well as the release of cytokines including IFN-γ, IL-2 and TNF-α. Moreover, the presence of dasatinib on restimulation of activated T cells on fresh SKM-1 cells totally averted HLA-A2 WT1-TCB-mediated target cell killing and T cell degranulation. Altogether, these data reveal that dasatinib can quickly switch off TCB-mediated T cell cytotoxicity at pharmacological label doses in cases where undesired side effects occur, such as tissue toxicity.

Another application for dasatinib could be the mitigation of severe T cell engager–induced CRS.[37] The cytokine cascade is first initiated by T cells, which release cytokines that trigger activation of other immune cells, thus amplifying the cascade.[19] Myeloid cells were shown to be key mediators of IL-6 and IL-1β release and to be activated via TNF-α. Li *et al*[19] also showed that blocking TNF-α decreased cytokine release following treatment with a HER2 T cell–dependent bispecific antibody.[32] *In vitro,* TNF-α blockade partially inhibited the release of IL-6 and IL-1β but not of the T cell–derived cytokines IL-2 or IFN-γ, and did not block T cell cytotoxic activity (19 and *manuscript in preparation*). In our study, dasatinib rapidly and fully switched off the release of IL-2, IFN-γ, IL-6, TNF-α and IL-1β, in addition to blocking T cell cytotoxicity. Importantly, dasatinib was also able to rapidly stop both the cytokine release and cytotoxicity of pre-activated T cells, which might be important for the mitigation of high-grade CRS as well as tissue toxicities.

Tumor-associated antigen distribution in healthy and malignant tissues as well as the susceptibility of developing CRS is very heterogeneous among individuals and personalized prediction is not yet optimal. Consequently, dasatinib represents an attractive potential antidote against TCB-induced life-threatening safety liabilities like off-tumor related toxicities or high-grade CRS. Due to its short half-life as a small molecule, it can be tightly regulated in contrast to the administration of TNF-α or IL-6R blocking antibodies. Dasatinib (Sprycel) is indicated for the first-line treatment of Philadelphia chromosome-positive chronic myelogenous leukemia and acute lymphoblastic leukemia.[36] The dasatinib regimen of 100 mg once a day is associated with favorable PK parameters and a fast absorption rate of 30 min to 4 hours, suggesting that it could rapidly induce a pharmacological on/off switch after administration.[36] Furthermore, dasatinib's

toxicity profile is well characterized and treatment-associated adverse events, including cytopenias and pleural effusions, are manageable by dose modification or interruption.[38] Overall, this may support a straightforward implementation of dasatinib for the management of extreme cases of life-threatening CRS and on-target tissue toxicities.

Other Src kinase inhibitors were reported to block CAR T cell functionality like nintedanib, imatinib, ponatinib or saracatinib as well as the BTK inhibitor ibrutinib. However, none of them appeared as potent as dasatinib.[28 35 39 40] Our work highlights that a short-term intervention with dasatinib could be used as a rapid safety switch to mitigate high-grade CRS and/or adverse events related to on-target off-tumor activity of T cell engagers. We showed that dasatinib blocked TCB-mediated target cell killing on first infusion in a mouse model, but whether intermittent dosing of dasatinib would negatively affect T cell engagers' efficacy in a tumor model, as can be anticipated from the data presented, remains to be investigated. Indeed, the combination of blinatumomab and dasatinib to treat patients with relapsed or MRD-positive Philadelphia chromosome-positive leukemia was found to be safe and associated with efficacious responses.[41 42] Recently, Weber *et al*[33] showed that treatment with dasatinib could improve CAR T cell functionality, by inducing rapid and transient ON/OFF switches preventing exhaustion. While, for TCBs, our results suggest that dasatinib treatment would have a reversible but detrimental effect on efficacy, it is reasonable to assume that, like for CAR T cells, pulsed treatment with dasatinib may be able to reduce T cell engager–induced T cell exhaustion as well. To specifically reduce the risk of CRS and improve its mitigation, further mechanistic studies are required to identify kinase inhibitors that might differentially regulate T cell activation and cytokine release induced by CD3 bispecific antibodies.

Ethic statement

- **Patient consent for publication:** Not required.
- **Ethics approval:** The Institutional Animal Care and Use Committee of RICZ and the Cantonal Veterinary Office of Zurich approved the study protocol (license ZH225-17) in accordance with the Swiss Animal Protection Law. All the experiments were performed according to committed guidelines (GV-Solas; Felasa; TierschG) and under the AALAC accreditation.

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Contributors

Concept and experimental design: GL, HH, VP, CF, JS, CK. Acquisition of data: GL. Data analysis and interpretation: GL, HH, AS, AMG, VP, CF, CK, CB, A-CW, EM-B. Writing, review and/or revision of the manuscript: GL, HH, EM-B, CF, AO, CK, MB. Administrative, technical or material support: JC. Study supervision: CK, HH, AS, MB, PU.

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Competing interests

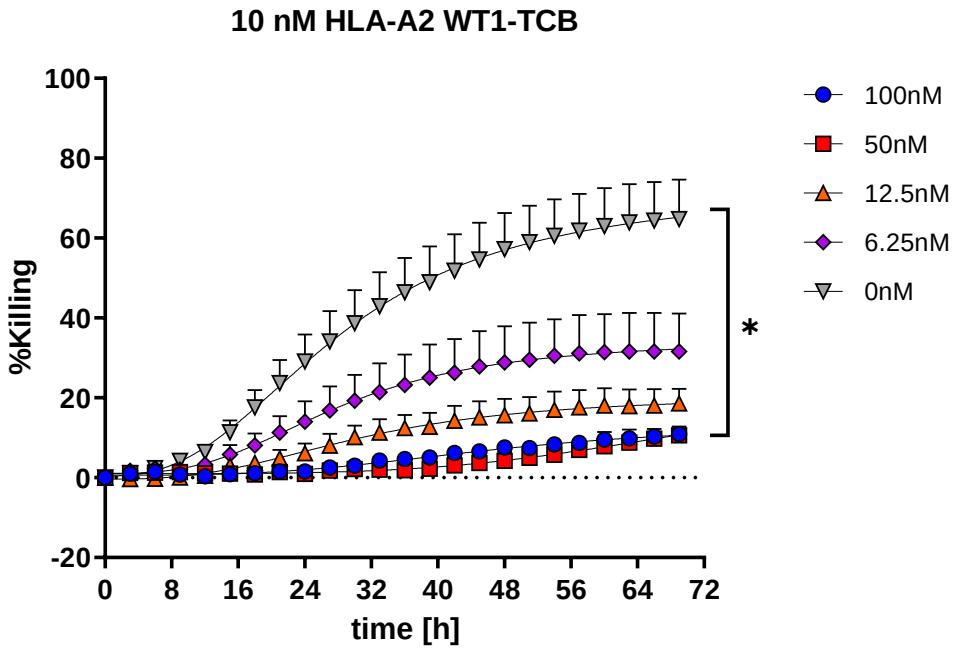
GL, HH and CK declare patent application related to the work described. All authors, except AO, are employees of Roche or were employed by Roche at the time of this study. All the authors, except AO, GL and HH, declare ownership of Roche stock.

Provenance and peer review

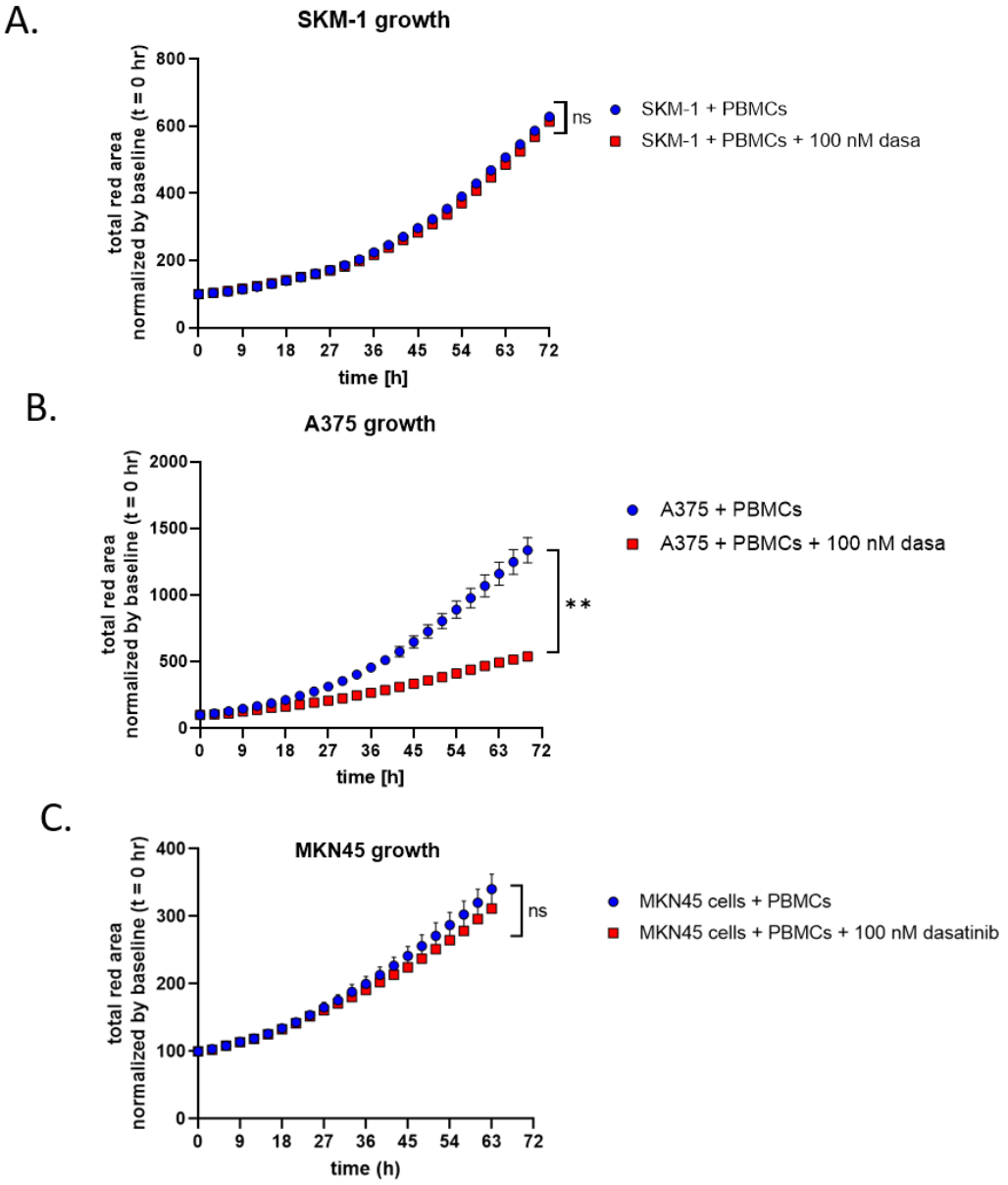
Not commissioned; externally peer reviewed.

Supplemental material

Supplemental materials



Supp. Figure 1. Real time killing of NLR-labelled A375 by 10 nM HLA-A2 WT1-TCB in the presence of escalating concentrations of dasatinib. NLR-labelled A375 target cells were co-cultured with PBMCs and HLA-A2 WT1-TCB in medium supplemented with dasatinib, at E:T=10:1. Killing was followed by Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentages of killing were measured by normalizing the total red areas with values at t = 0 hr and the control wells containing target cells, PBMCs and dasatinib for each time points. Mean of n=3 donors +/- SEM with * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test).


A.
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total red area
normalized by baseline (t = 0 hr)
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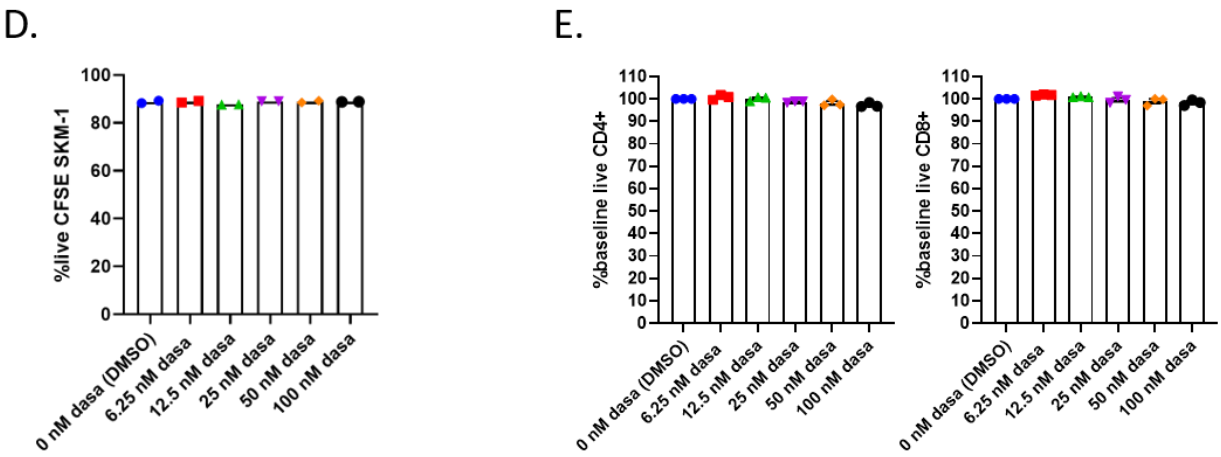
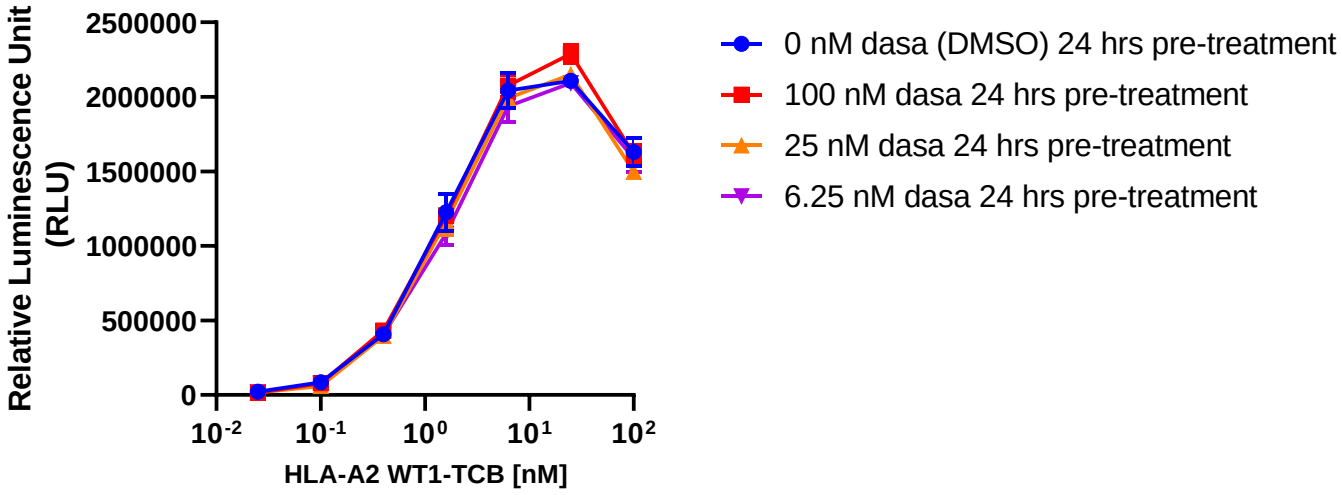
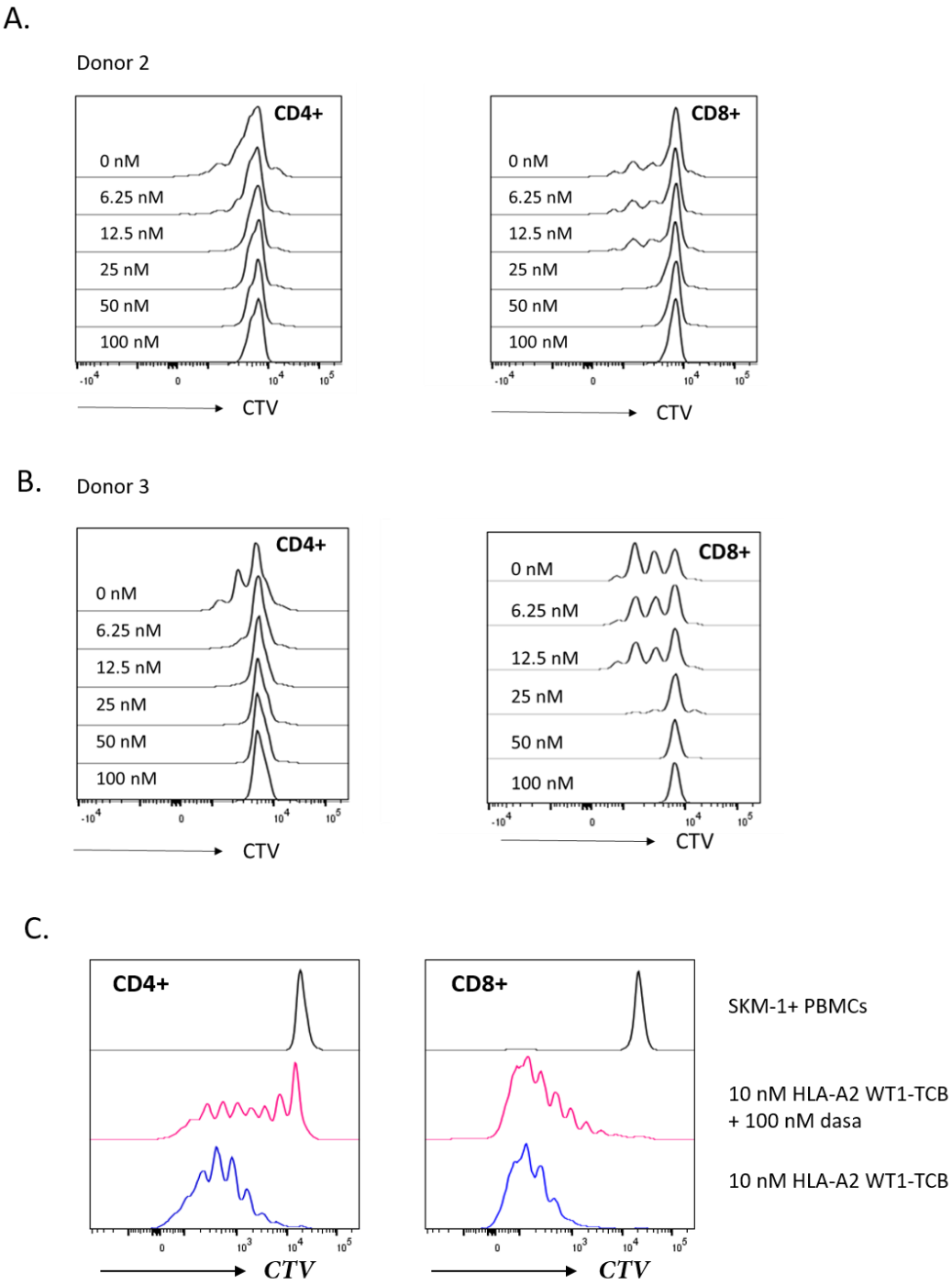
ns
SKM-1 + PBMCs
SKM-1 + PBMCs + 100 nM dasa
B.
A375 growth
total red area
normalized by baseline (t = 0 hr)
time [h]
**
A375 + PBMCs
A375 + PBMCs + 100 nM dasa
C.
MKN45 growth
total red area
normalized by baseline (t = 0 hr)
time (h)
ns
MKN45 cells + PBMCs
MKN45 cells + PBMCs + 100 nM dasatinib


D.
%live CFSE SKM-1
0 nM dasa (DMSO)
6.25 nM dasa
12.5 nM dasa
25 nM dasa
50 nM dasa
100 nM dasa
E.
%baseline live CD4+
%baseline live CD8+
0 nM dasa (DMSO)
6.25 nM dasa
12.5 nM dasa
25 nM dasa
50 nM dasa
100 nM dasa

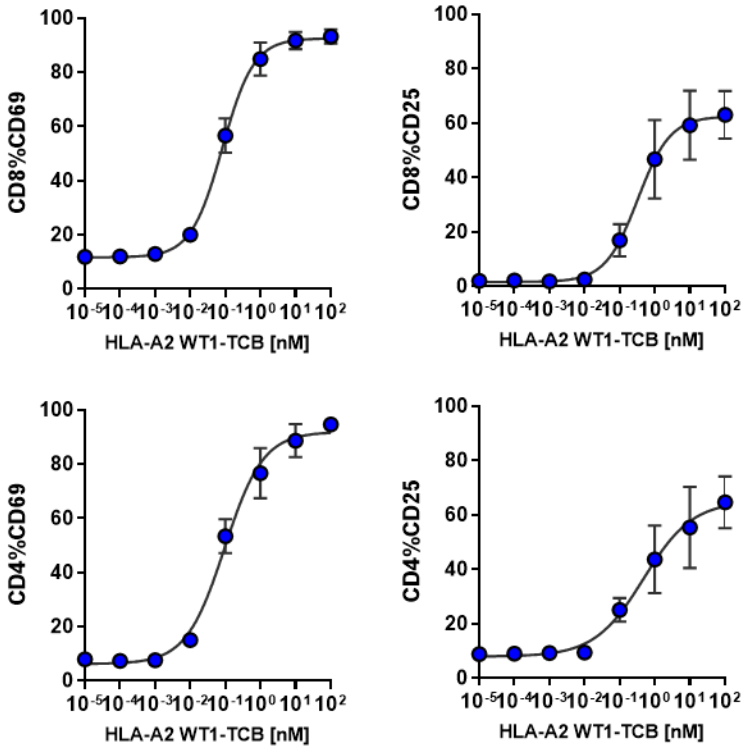
Supp. Figure 2. Dasatinib does not affect T cell viability and SKM-1 and MKN45 tumor cell growth. (**A**) Effect of 100 nM dasatinib on NLR-labelled SKM-1 tumor cells growth. (**B**) Effect of 100 nM dasatinib on NLR-labelled A375 tumor cell growth. (**C**) Effect of 100 nM dasatinib on NLR-labelled MKN45 tumor cell growth. Tumor cell growth was followed by Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time) and the total red area was normalized by the baseline at t=0 hr. Mean of n=2 donor +/- SD * $p \leq 0.05$, ** $p \leq 0.01$ by paired t test. (**D**) Effect of escalating concentrations of dasatinib on the viability of CFSE-labelled SKM-1 cells. At 24 hrs, cell viability was measured by flow cytometry using a Live/Dead stain. Mean of n=2 donors. (**E**) Effect of escalating concentrations of dasatinib on CD4+ and CD8+ T cell viability. At 24 hrs, the viability of CD4+ and CD8+ T cells was measured by flow cytometry using a Live/Dead stain and normalized by that in the absence of dasatinib (DMSO control), mean of n=3 donors.



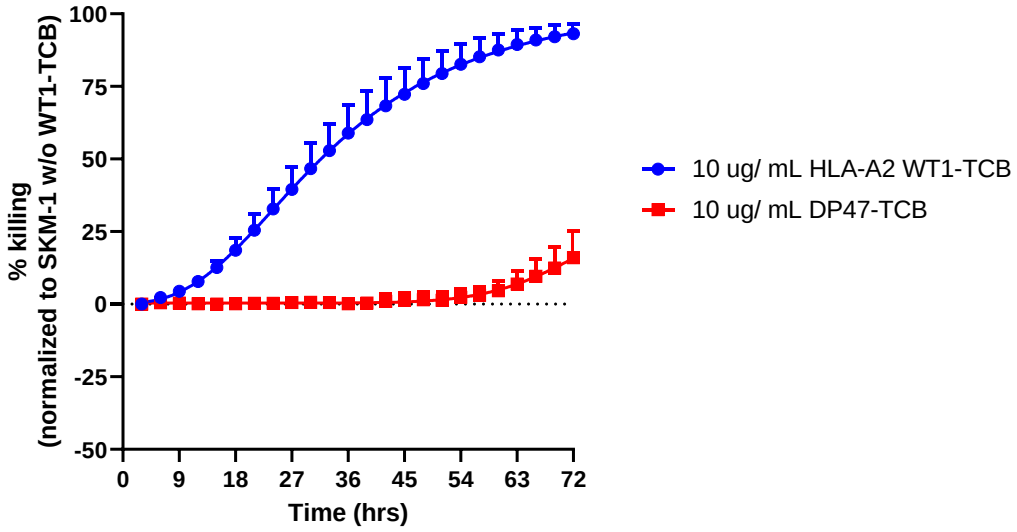
Supp. Figure 3. Effect of dasatinib on WT1 target expression by SKM-1 tumor cells. SKM-1 cells were incubated in medium supplemented with 6.25, 25 or 100 nM dasatinib for 24 hrs. At 24 hrs, a Jurkat NFAT reporter cell assay (E:T= 3:1) was conducted in the presence of escalating concentrations of HLA-A2 WT-TCB and the Luminescence was acquired with a Perkin Elmer plate reader. Induction of the luciferase reporter gene was similar irrespective of SKM-1 cell pre-treatment with dasatinib, showing that WT1 expression was not modified.



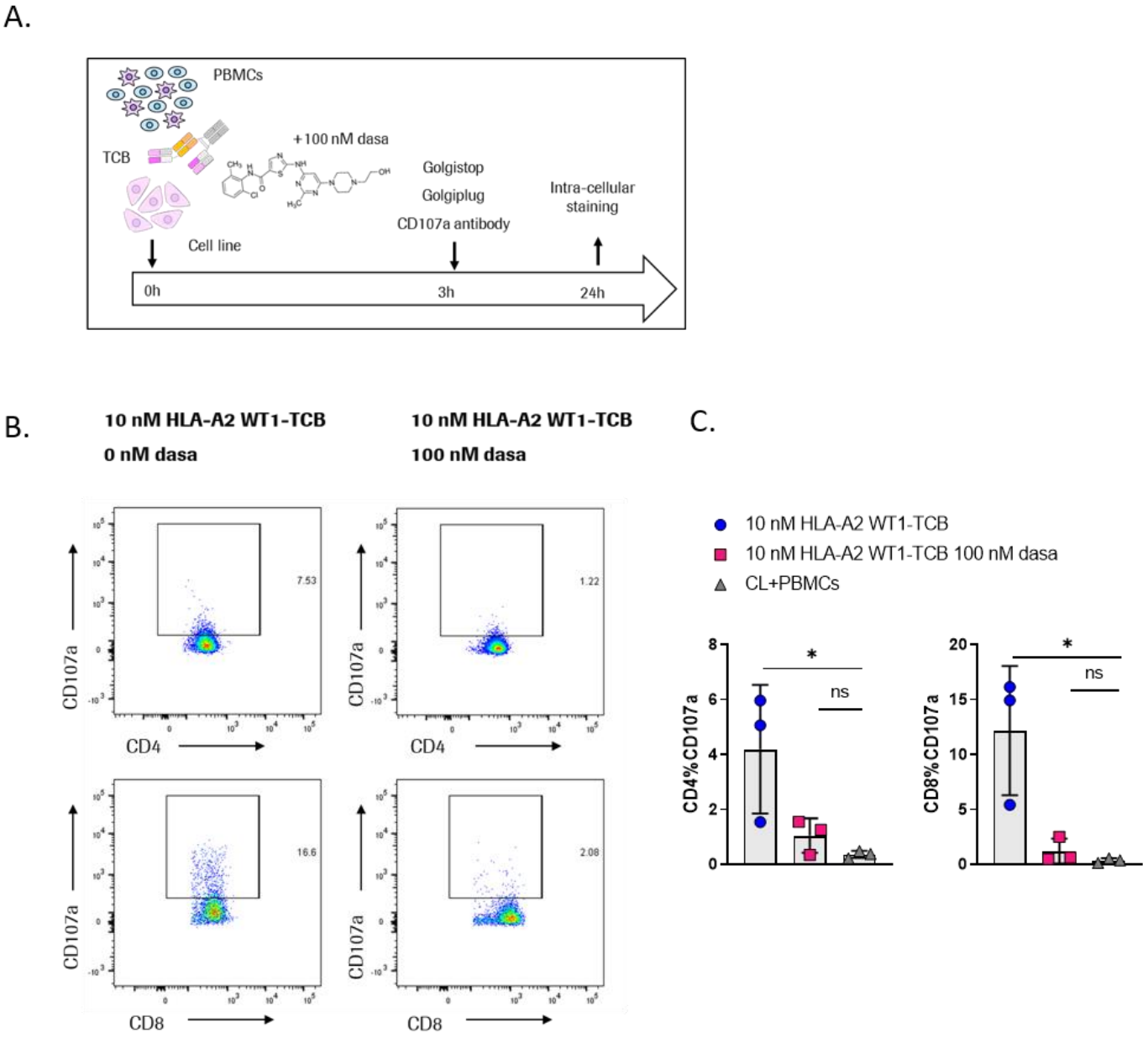
Supp. Figure 4. (**A, B**) Effect of escalating concentrations of dasatinib on CD4+ and CD8+ T cells proliferation. CFSE-labelled SKM-1 tumor cells were co-cultured with CTV-labelled PBMCs and HLA-A2 WT1-TCB, at E:T=5:1. To assess the effect of dasatinib on T cell proliferation, the dilution of the CTV dye in CD4+ and CD8+ T cells was measured by flow cytometry at t=72 hrs. Histogram plots are shown for 2 donors additional to the one shown on Figure 1. (**C**) Dasatinib rapidly stops CD4+ and CD8+ T cell proliferation. CTV-labelled PBMCs were co-cultured with SKM-1 tumor cells (E:T=1:1) and HLA-A2 WT1-TCB. Dasatinib was added after 24 hrs of activation. To assess the effect of dasatinib on T cell proliferation, the dilution of the CTV dye in CD4+ and CD8+ T cells was measured by flow cytometry at t= 144 hrs. Histogram plots are shown for 1 donor representative of 3.



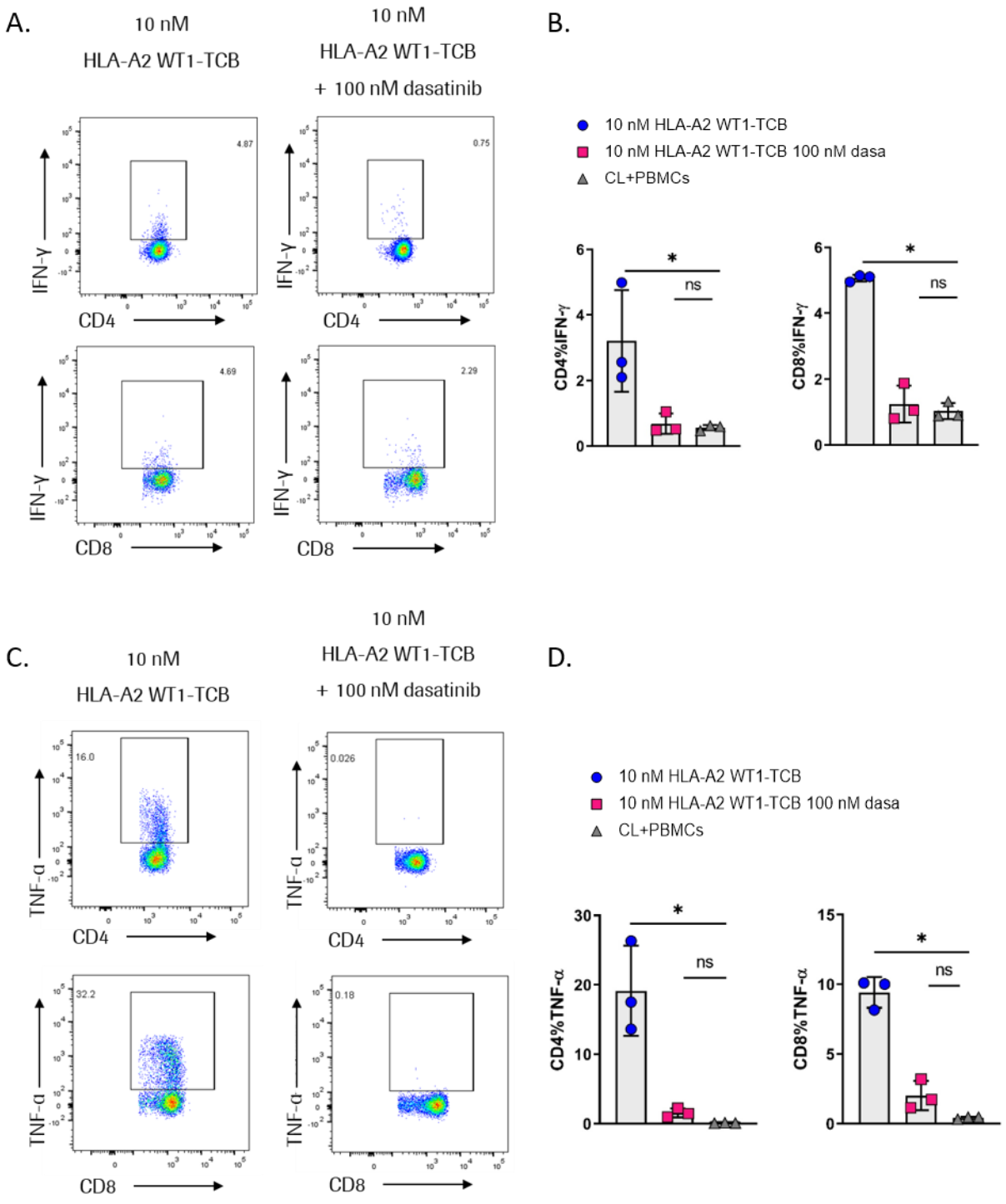
Supp. Figure 5. HLA-A2 WT1-TCB-induced T cell activation upon first stimulation (24 hrs) in the assay described in figure 3 (E:T = 5:1). (A, B) The expression of CD25 and CD69 was measured on CD4+ and CD8+ T cells by flow cytometry. Mean of n = 3 donors +/- SD.



Supp. Figure 6. Effect of a non-targeting TCB (DP47-TCB) vs. HLA-A2 WT1-TCB on killing of SKM-1 tumor cells. NLR-labelled SKM-1 tumor cells were co-cultured with PBMCs and HLA-A2 WT1-TCB or DP47-TCB, E:T=2.5:1. Killing was followed by Incucyte (1 scan every 3 hrs, zoom 10x, phase and red 400 ms acquisition time). The percentage of inhibition of tumor cell killing was calculated over that in the absence of TCB for a fixed HLA-A2 WT1-TCB or DP47-TCB concentration of 10 µg/mL. Mean of n= 3 donors + SEM.

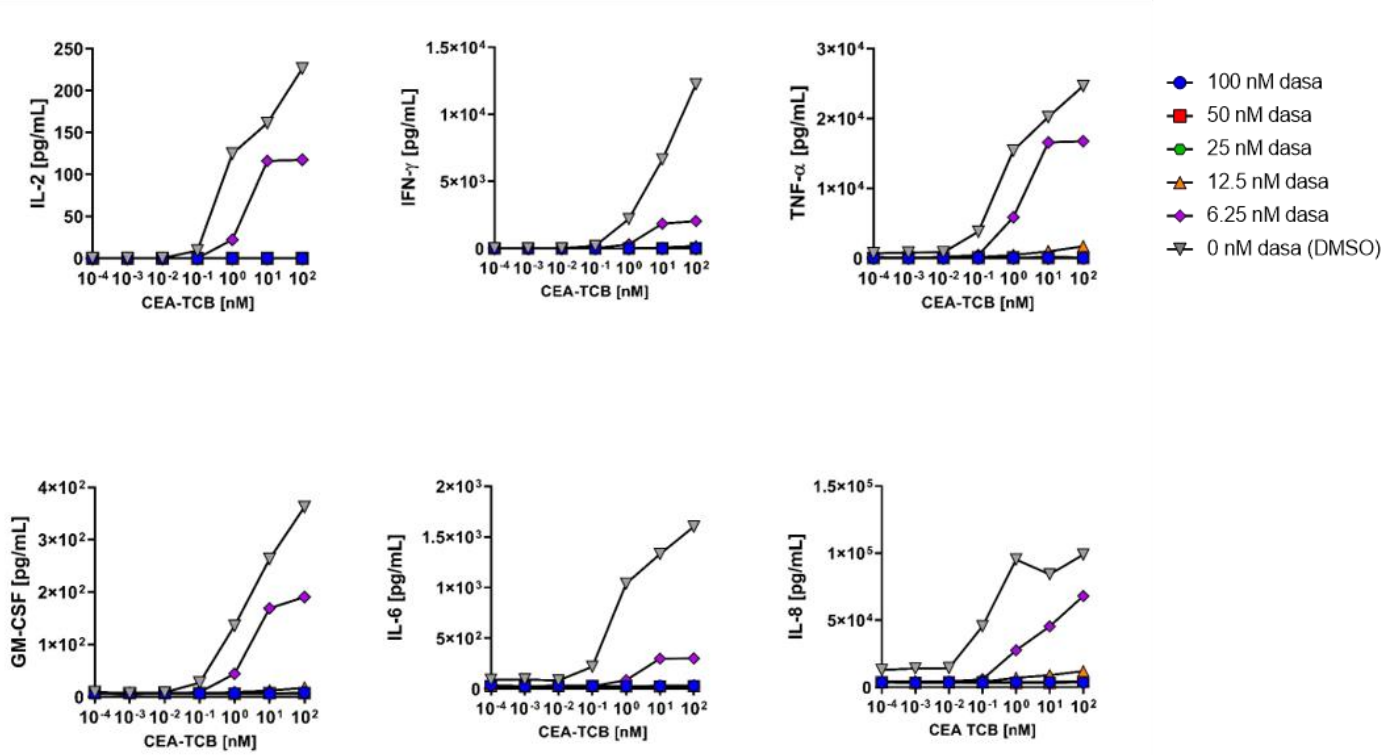


Supp. figure 7. Dasatinib prevents HLA-A2 WT1-TCB induced T cell degranulation. (**A**) PBMCs were co-cultured with SKM-1 target cells (E:T=5:1) and HLA-A2 WT1-TCB in the presence or absence of 100 nM dasatinib. Golgistop, Golgiplug and anti-CD107a antibody were added after 3 hrs of activation with the TCB. (**B**) Representative FACS plots of CD107a+ populations among CD4+ and CD8+ T cells for 1 donor representative of 3, 10 nM HLA-A2 WT1-TCB. (C)Percentages of CD107a+ cells among CD4+ and CD8+ T cells treated with 10 nM HLA-A2 WT1-TCB in the presence and absence of 100 nM dasatinib. Mean of n = 3 donors +/- SD with with * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test). ns = not significant

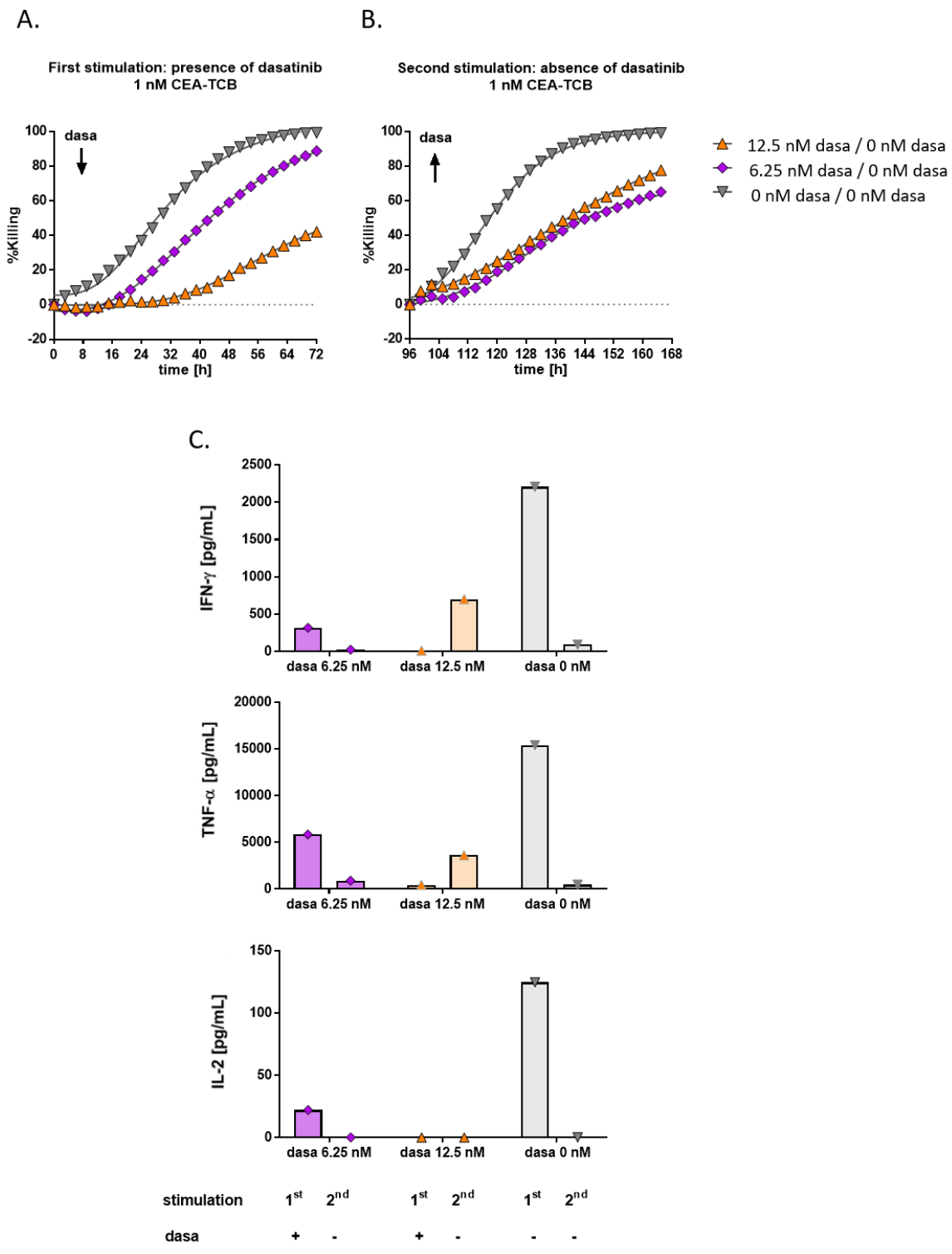


Supp. Figure 8. Dasatinib prevents the release of IFN-γ and TNF-α by CD4+ and CD8+ T cells after stimulation with HLA-A2 WT1-TCB. PBMCs were co-cultured with SKM-1 target cells (E:T=5:1) and HLA-A2 WT1-TCB in the presence or absence of 100 nM dasatinib. Golgistop and Golgiplug were added 3 hours after activation with the TCB. (**A, C**) Representative FACS plots of IFN-γ and TNF-α populations among CD4+ and CD8+ T cells for 10 nM HLA-A2 WT1-TCB. (**B, D**) Percentages of IFN-γ- and TNF-α-positive cells among CD4+ and CD8+ T cells for 10 nM HLA-A2 WT1-TCB in the presence and absence of 100 nM dasatinib. Mean of n = 3 donors +/- SD with * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test). ns=not significant

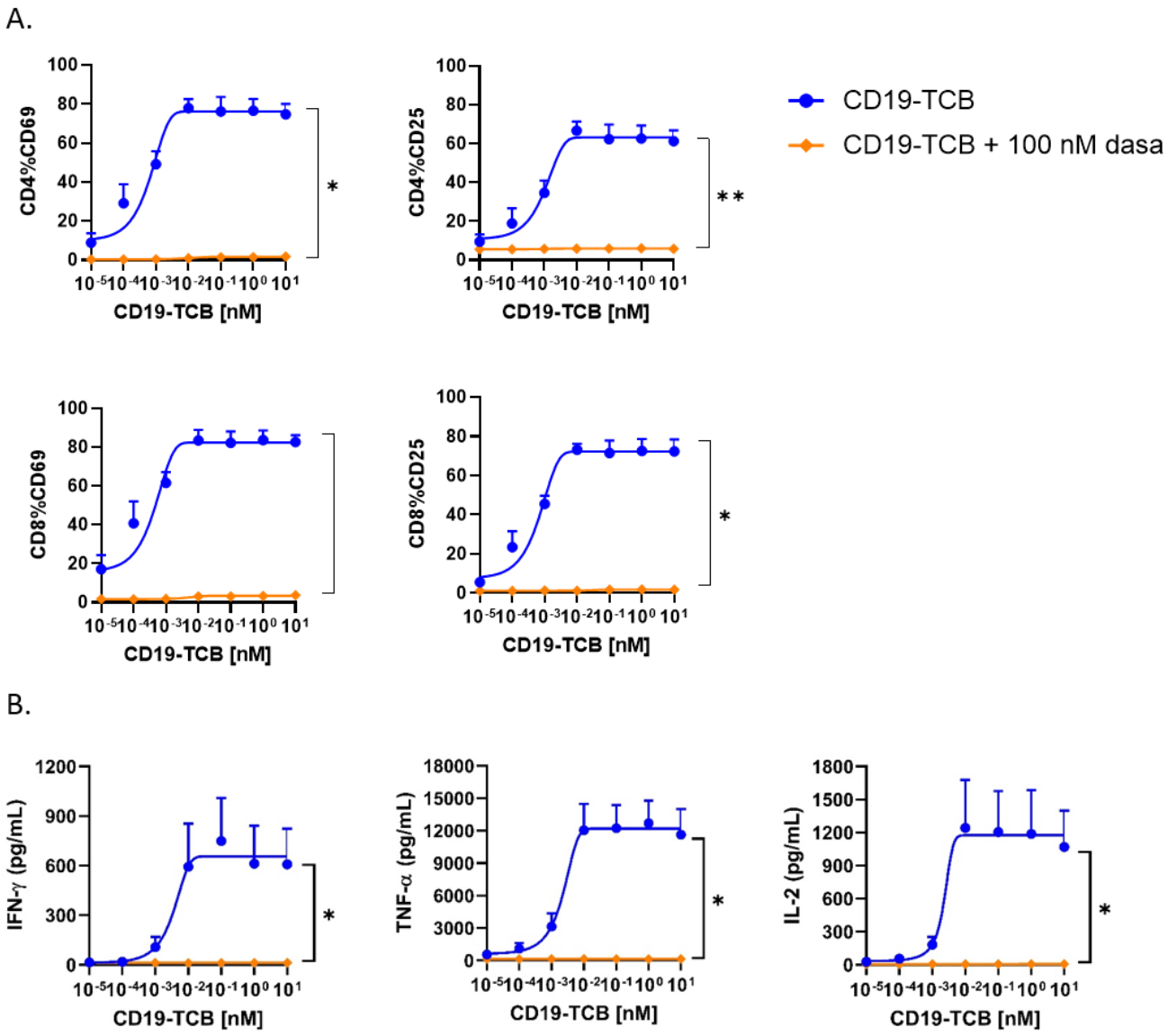
A.



Supp. Figure 9. (**A**) Effect of escalating concentrations of dasatinib on CEA-TCB-mediated cytokine release upon the first stimulation, in the assay described in figure 4 The levels of IFN-γ, TNF-α, IL-2, GM-CSF, IL-6 and IL-8 were measured in the supernatants of the assay at t=72 hrs by Luminex (1 donor representative of 2).



Supp. Figure 10. Low concentrations of dasatinib equilibrate TCB-induced cytokine release. (**A, B**) PBMCs were co-cultured with NLR-labelled MKN45 (E:T=10:1) target cells and 1 nM CEA-TCB for 2 consecutive stimulations in the presence or absence of 6.25 nM or 12.5 nM dasatinib upon the first stimulation. Real time killing was followed by Incucyte (1 scan every 3 hrs, zoom 10x, phase and red 400 ms acquisition time). Means of technical replicates +/-SD. (**C**) The levels of IFN-γ TNF-α and IL-2 were measured in the culture supernatant by Luminex after both stimulations (1 donor).



Supp. Figure 11. Effect of 100 nM dasatinib on CD19-TCB dependent T cell activation and cytokine release induced by CD19-TCB. PBMCs were co-cultured with CTV-labelled SU-DHL-8 tumor cells (E:T=10:1) and escalating concentrations of CD19-TCB in the presence or absence of 100 nM dasatinib. (**A-B**) The expression of CD25 and CD69 on CD4+ and CD8+ T cells was measured by flow cytometry and the levels of IFN-γ, TNF-α and IL-2 were measured by Luminex in the supernatants at t=24 hrs. Means of n = 3 donors +/- SD with *p ≤ 0.05, ** p ≤ 0.01 by 1 way ANOVA (Friedman test).

Supp. Documentation 1. Analysis reports for SKM-1 and MKN45 cell line typing from Microsynth.(cf online version)

Supp. Video 1. The reversible effect of dasatinib on CEA-TCB-mediated killing of NLR-labelled MKN45 cells. Real time killing of NLR-labelled MKN45 cells by 1 nM CEA-TCB upon first stimulation in the absence of dasatinib (ON), second stimulation in the presence of dasatinib (OFF) and third stimulation in the presence of dasatinib (OFF), as shown on Fig 4. Incucyte video (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time) for 1 donor representative of 3. (cf online version)

8. Third manuscript

Title: JAK and mTOR inhibitors prevent cytokine release while retaining T cell bispecific antibody in vivo efficacy

Original research article
Journal for ImmunoTherapy of Cancer (JITC)
Submitted: August, 31st 2021
Manuscript under review

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Keywords: Cancer immunotherapy, T cell engagers, T cell bispecific antibodies, kinase inhibitors, Cytokine Release Syndrome, toxicity, drug development, T cell receptor signaling

Declaration

- **Ethics statements**

The *in vivo* work on mice was conducted in accordance with the Swiss Animal Protection Law and under the AAALAC accreditation.

- **Availability of data and material**

All data relevant to the study are included in the article or uploaded as online supplementary information.

- **Competing interests**

G. Leclercq, H. Haegel, C. Klein, A. Toso, T. Zimmermann declare patent applications related to the work described. All authors, except A. Odermatt, are employees of Roche or were employed by Roche at the time of this study. All the authors, except A. Odermatt, G. Leclercq and N. Steinhoff declare ownership of Roche stock. All funding for the studies were provided by Roche. The authors do not declare a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

- **Funding**

All funding for the studies were provided by Roche. The authors do not declare a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

- **Authorship contributions**

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Acquisition of data: G. Leclercq, T. Zimmermann
Data analysis and interpretation: G. Leclercq, T. Zimmermann, A. Toso, L. Green, H. Haegel, A. Schneider, A. Giusti, V. Pulko, J. Sam, N. Steinhoff, M. Bacac, M. Stern, C. Klein
Writing, review and/or revision of the manuscript: G. Leclercq, H. Haegel, A. Toso, C. Klein, M. Bacac, A. Schneider, A. Odermatt, M. Stern
Administrative, technical or material support: J. Challier, T. Zimmermann, A. Freimoser-Grundschober, L. Larivière
Study supervision: C. Klein, H. Haegel, A. Schneider, A. Giusti, M. Bacac, P. Umaña

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List of abbreviations

TCB: T cell bispecific antibody, CAR: chimeric antigen receptor, CRS: cytokine release syndrome, CTV: cell trace violet, NIR: near infra-red, NLR: nuc light red, TAA: tumor associated antigen, TCR: T cell receptor, PBMCs: peripheral blood mononuclear cells, PDX:

Abstract

Background: T cell engaging therapies, like CAR-T cells and T cell bispecific antibodies (TCBs), efficiently redirect T cells towards tumor cells, facilitating the formation of a cytotoxic synapse and resulting in subsequent tumor cell killing, a process that is accompanied by the release of cytokines. Despite their promising efficacy in the clinic, treatment with TCBs is associated with a risk of Cytokine Release Syndrome (CRS). The aim of this study was to identify small molecules able to mitigate cytokine release while retaining T cell-mediated tumor killing.

Methods: By screening a library of 52 FDA-approved kinase inhibitors for their impact on T cell proliferation and cytokine release after CD3 stimulation, we identified mTOR, JAK and Src kinases inhibitors as potential candidates to modulate TCB-mediated cytokine release at pharmacologically active doses. Using an *in vitro* model of target cell killing by human peripheral blood mononuclear cells, we assessed the effects of mTOR, JAK and Src kinase inhibitors combined with 2+1 T cell bispecific antibodies (TCBs) including CEA-TCB and CD19-TCB on T cell activation, proliferation and target cell killing measured by flow cytometry and cytokine release measured by Luminex. Last, the combination of mTOR, JAK and Src kinase inhibitors together with CD19-TCB was evaluated *in vivo* in stem cell humanized NSG mice.

Results: The effect of Src inhibitors differed from those of mTOR and JAK inhibitors with the suppression of CD19-TCB-induced tumor cell lysis *in vitro*, whereas mTOR and JAK inhibitors

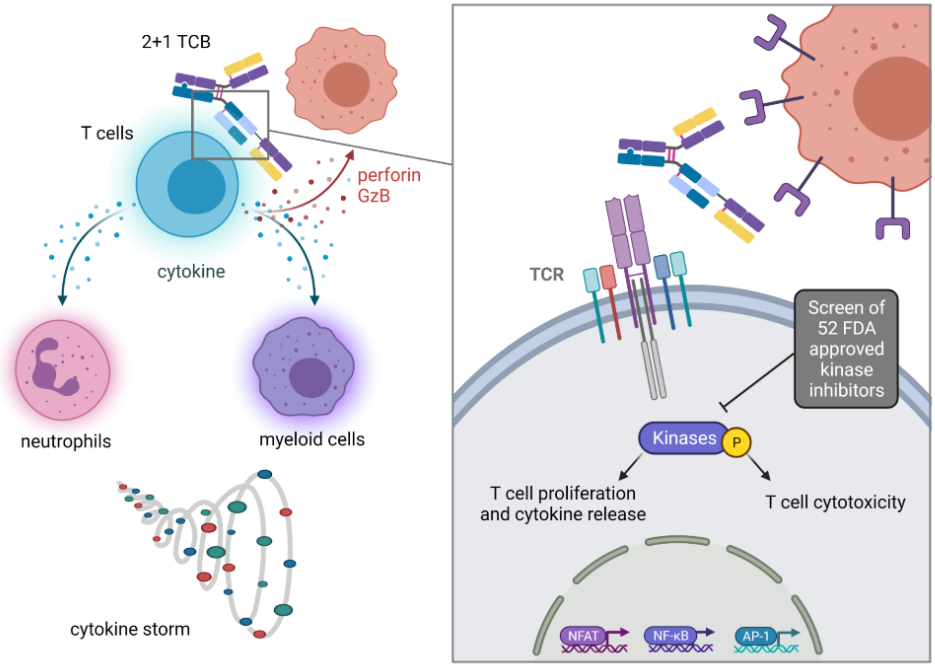
primarily affected TCB-mediated cytokine release. Importantly, we confirmed *in vivo* that transient exposure to Src, JAK and mTOR inhibitors strongly reduced CD19-TCB-induced cytokine release while retaining T cell-mediated anti-tumor efficacy in humanized mice.

Conclusions: Taken together, these data support further evaluation of the use of Src, JAK and mTOR inhibitors as prophylactic treatment to prevent occurrence of CRS.

Graphical abstract

JAK and mTOR inhibitors prevent cytokine release while retaining T cell bispecific antibody in vivo efficacy

Gabrielle Leclercq, Hélène Haegel, Alberto Toso, Tina Zimmermann, Luke Green, Nathalie Steinhoff, Johannes Sam, Vesna Pulko, Anneliese Schneider, Anna-Maria Giusti, John Challier, Anne Freimoser-Grundschober, Laurent Larivière, Alex Odermatt, Martin Stern, Pablo Umaña, Marina Bacac, Christian Klein



Key points

• Targeting JAK, mTOR and Src pathways reveals the uncoupling of TCB-induced T cell cytotoxicity and cytokine release.

• mTOR and JAK inhibitors prevent TCB-mediated cytokine release upon first infusion while preserving TCB-efficacy.

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Background

T cell engaging bispecific antibodies have raised major interest for the treatment of hematological and solid tumors (1-3). We have developed T cell bispecific antibodies (TCBs), e.g. cibisatamab (CEA-TCB) (4, 5) or glofitamab (CD20-TCB) (6), harboring a 2+1 format with one binder to the CD3ε chain and two binders to specific tumor antigens. Crosslinking of CD3 with tumor antigens triggers T cell activation and proliferation, cytokine release and tumor cell killing (7-11). In contrast to chimeric antigen receptor (CAR) T cells, TCBs represent an easily accessible "off the shelf" alternative to eradicate tumors (7, 12, 13). Their Fc region is engineered with P329G LALA mutations preventing FcγR signaling pathways and enabling a longer half-life than Fv-based formats such as BiTE® (Bi-specific T-cell engagers) antibodies (14, 15). Treatment with TCBs can be associated with adverse events related to their mode-of-action such as the Cytokine Release Syndrome (CRS), which is due to on-target activity (16, 17). This complex clinical syndrome featured by fever and hypotension and/or hypoxia is hardly predictable (18). The main driver of CRS is a strong release of pro-inflammatory cytokines such as TNF-α and IFN-γ (produced by T cells) (19) and TNF-α, IL-1β and IL-6 (produced by myeloid cells) (20-23). The symptoms of CRS can vary from mild to severe, and are classified in different grades (24). CRS can be managed with glucocorticoids and/or anti-IL-6/R treatment such as tocilizumab or siltuximab (19, 25, 26). If symptoms are not resolved, patients receive supportive care to stabilize blood pressure and oxygen saturation (e.g. administration of vasopressors or oxygen). Despite the broad use of prophylactic glucocorticoids and step-up-dosing approaches to decrease CRS incidence and severity in the clinic, this complication remains the major dose-limiting toxicity associated with T cell engaging therapies. There is a continued need to develop alternative treatments with the goal to improve CRS management and to reduce patient hospitalization, ideally through prophylactic treatments to prevent or reduce the occurrence and severity of CRS. To identify potential candidates that inhibit both T cell proliferation and cytokine release while retaining T cell-mediated tumor killing, we screened 52 FDA-approved tyrosine kinase inhibitors (TKI) on CD3-stimulated T cells, mimicking TCB stimulation (27). We selected JAK, mTOR, Src and MEK inhibitors as potent candidates to exert control over T cell proliferation and cytokine release. Using an *in vitro* model of target cell killing by human peripheral blood mononuclear cells, we tested the effect of mTOR, JAK and Src inhibitors on CD19-TCB-induced cytokine release, T cell cytotoxicity and activation. In line with previous reports for CAR-T cells,

dasatinib was found to fully switch-off CD19-TCB-induced T cell functionality (28-31). In contrast, mTOR inhibitors (temsirolimus, sirolimus and everolimus) and JAK inhibitors (ruxolitinib, baricitinib, and tofacitinib) were found to prevent CD19-TCB-induced cytokine release while not blocking TCB-mediated tumor cell killing at pharmacologically relevant doses. *In vivo*, mTOR and JAK inhibitors prevented cytokine release in humanized NSG mice treated with CD19-TCB, while not impairing B cell depletion. In lymphoma PDX-bearing humanized NSG mice, transient treatment with kinase inhibitors inhibited cytokine release but did not impair CD19-TCB anti-tumor efficacy. The use of mTOR or JAK inhibitors therefore represents a promising strategy to prevent CRS upon TCB treatment.

Material and methods

Kinase inhibitors and antibodies

CEA-TCB (cibisatamab) and CD19-TCB were produced internally in the 2+1 TCB format previously described (Bacac et al, CCR 2016 for CEA-TCB). Dasatinib (S1021), ponatinib (S1490), bosutinib (S1014), sirolimus (S1039), temsirolimus (S1044), everolimus (S1120), ruxolitinib (S1378), baricitinib (S2851), fedratinib (S2736), tofacitinib (S2789), trametinib (S2673), dexamethasone (S1322) were purchased from Selleckchem. Methylprednisolone was purchased from Pfizer. The compounds from the library used in the screen were produced internally or purchased from Selleckchem, LC laboratories, Ambeed or Apollo Scientific.

Cell culture

The SU-DHL-8 cell line is a human large cell lymphoma cell line (ATCC catalog number CRL-2961). The NALM-6 cell line is an acute lymphoblastic leukemia cell line (ATCC CRL-3273). SU-DHL-8 and NALM-6 cells were cultured in RPMI Glutamax (61870036, Gibco) containing 10 % FBS (26140079, Gibco) and split every three to four days (to 0.8 million cells/mL) or on the day prior the assay. SU-DHL-8 and NALM-6 cell lines provided from ATCC are routinely authenticated by short tandem repeat profiling prior to delivery. The diffuse large B cell lymphoma PDX was obtained from a patient who relapsed after R-CHOP treatment, and purchased from the Charles University in Prague. For *in vitro* use, the cells were thawed on the day of the assay and cultured in RPMI Glutamax (61870036, Gibco) containing 10% FBS (26140079, Gibco). For *in vivo* use, the cells were thawed, counted and suspended in a 50:50 mix of RPMI (1530586, Gibco) and Matrigel (354234, Corning).

PBMCs and pan-T cells isolation

PBMCs were isolated from buffy coats donated by healthy volunteers (blood donation center in Zürich, in accordance with the Declaration of Helsinki) by Ficoll density gradient. T cells were isolated from fresh human PBMCs by negative magnetic isolation using the pan T cell isolation kit from Miltenyi Biotec (130-096-535). Before the assay, T cells and fresh or thawed PBMCs were counted and adjusted to 4.0 x106/mL in assay medium. 50 µL of the cell suspension were transferred to the wells of the assay plates, corresponding to 200 000 cells / well. For the proliferation assay, T cells were previously stained with the Cell Trace Violet (CTV) dye (Thermo Fisher, C34557) (5 µM, 20 min at RT).

***In vitro* killing assays**

NALM-6, SU-DHL-8 or lymphoma PDX were labelled with the CTV dye (Thermo Fisher, C34557) (5 µM, 20 min at RT). 20 000 NALM-6, SU-DHL-8 or lymphoma PDX cells were transferred into each well (100 µL) followed by 200 000 effector cells/well (50 µL) to obtain a final E:T ratio of 10:1, respectively. The kinase inhibitors (10 µL) followed by the antibody solutions (50 µL) were then added. The assay plates were covered with lids, and placed in the incubator at 37°C, 5% CO2.

Flow cytometry

At assay endpoint, PBMCs were stained with the antibodies to CD4 (FITC, 317408, Biolegend), CD8 (BV605, 344742, Biolegend), CD25 (BUV395, 564034, BD), CD69 (PE, 310306, Biolegend) and Live Dead NIR (Thermo Fisher, L34975) in FACS buffer (30 min, 4°C). Cells were then washed twice in FACS buffer and re-suspended in 100 µL/well FACS buffer for analysis. Killing of CTV-labelled tumor cells was measured by gating of dead NIR-positive cells among CTV positive tumor cells. Proliferation of CTV-labelled T cells was assessed by dilution of the CTV dye.

To measure B cell count in the blood, 25 µL of blood was lysed twice using BD Pharm Lyse buffer (555 899, BD) (200 µL, 10 min, RT). To measure B cell count in the spleen, spleens were processed, lysed with BD Pharm Lyse buffer (555 899, BD) (2 mL, 10 min, RT) and cells were counted and suspended uniformly. Cells were then stained with the following the antibodies to CD45 (Alexa 700, 304119, Biolegend), CD20 (APC, 302309, Biolegend) and Live Dead NIR

(L10119, Thermo Fisher) in FACS buffer (30 min, 4°C). Cells were then washed twice in FACS buffer and re-suspended in 100 µL/well FACS buffer for analysis. The acquisition of samples was performed using high throughput screening plate reader connected to a BD Fortessa Flow cytometer and the DIVA Software.

Cytokine analysis

Cytokines were analyzed in the culture supernatant mouse serum samples (stored at - 80°C) by Luminex using a human 8 plex assay kit (Biorad) with additional IL-1β and MCP-1 beads, or human 23/40 plex assay kit (Biorad). Pre-diluted supernatants/ serum were incubated with beads in a 96-well filter plate (1 hr, 800 rpm, RT, no light). The plate was washed twice using a vacuum manifold and the detection antibodies solution was added (1 hr, 800 rpm, RT, no light). The plate was vacuumed and washed twice and the streptavidin solution was added (30 min, 800 rpm, RT, no light). The plate was vacuumed and washed twice and the samples were re-suspended in assay buffer. The sample acquisition was conducted using the Luminex equipment from Biorad.

***In vivo* studies in mice**

Humanized NSG mice were ordered from the Jackson Laboratory. The Institutional Animal Care and Use Committee of RICZ and the Cantonal Veterinary Office of Zurich approved the study protocol (license ZH225-17) in accordance with the Swiss Animal Protection Law. All the experiments were performed according to committed guidelines (GV-Solas; Felasa; TierschG) and under the AALAC accreditation. One day before treatment, humanized NSG mice were randomized based on their T cell counts into groups of 3-4 mice in the non-tumor bearing mouse study, or based on their tumor volumes into groups of 7-8 mice in the tumor-bearing mouse study. Blood was collected by tail-vein bleeding or by terminal retro-orbital bleeding. Tumor volumes were calculated from caliper measurements conducted 2-3 times per week. CD19-TCB (0.5 mg/kg), or vehicle were administered intravenously (i.v.) and kinase inhibitors as well as dexamethasone and methylprednisolone were given orally (p.o.). In both studies, mice treated with kinase inhibitors, methylprednisolone or dexamethasone 1 hour before and 6-7 hours after the first treatment with CD19-TCB on day 0. On day 1 and 2, the Src inhibitors and JAK inhibitors were given twice per day and the mTOR inhibitors, methylprednisolone or dexamethasone were given once per day. In the PDX-bearing mouse study, humanized NSG mice received a total of 4 cycles of CD19-TCB (1 treatment/week). 1 hr prior the second to last TCB

treatment, mice were also treated with the lowest dose of the compounds. The doses of the different compound tested were the following: mTOR inhibitors: 5 mg/kg, JAK inhibitors: 30 mg/kg, Src inhibitor: 20 or 50 mg/kg, dexamethasone: 2 times 1 mg/kg (day 0), 0.5 mg/kg (day 1), 0.25 mg/kg, (day 2 and other) methylprednisolone: 2 times 10 mg/kg (day 0), 5 mg/kg (day 1), 2.5 mg/kg (day 2 and other).

Data analysis

Flow cytometry data were analyzed using FlowJo V10. Cytokine data were analyzed using the Bio-Plex software. The graphs and statistical analysis were generated with GraphPad Prism 8 or Tibco Spotfire. For dose-titration curves, AUC were calculated and used for statistical comparison and EC50 values were calculated by non-linear regression using the variable slope methods with 4 parameters. Data are shown as means with SD or SEM or as individual curves. The statistical tests used are indicated in the figure legends for each experiment.

Additional methods are described in supplementary. The graphical abstract and Fig 4A were made with Biorender.

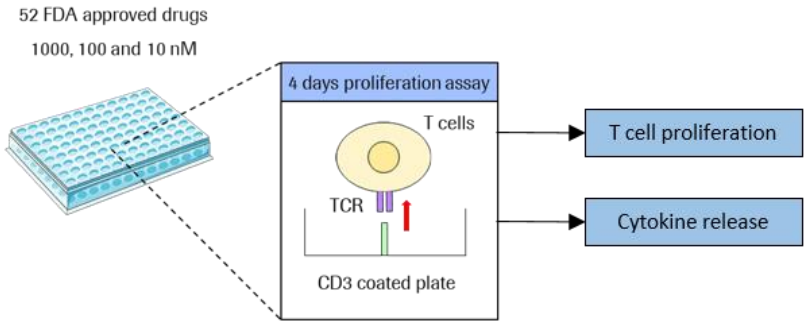
Results

A small molecule screening of 52 FDA-approved kinase inhibitors identifies mTOR, JAK, MEK and Src kinase inhibitors as potential candidates to pause TCB-induced T cell proliferation.

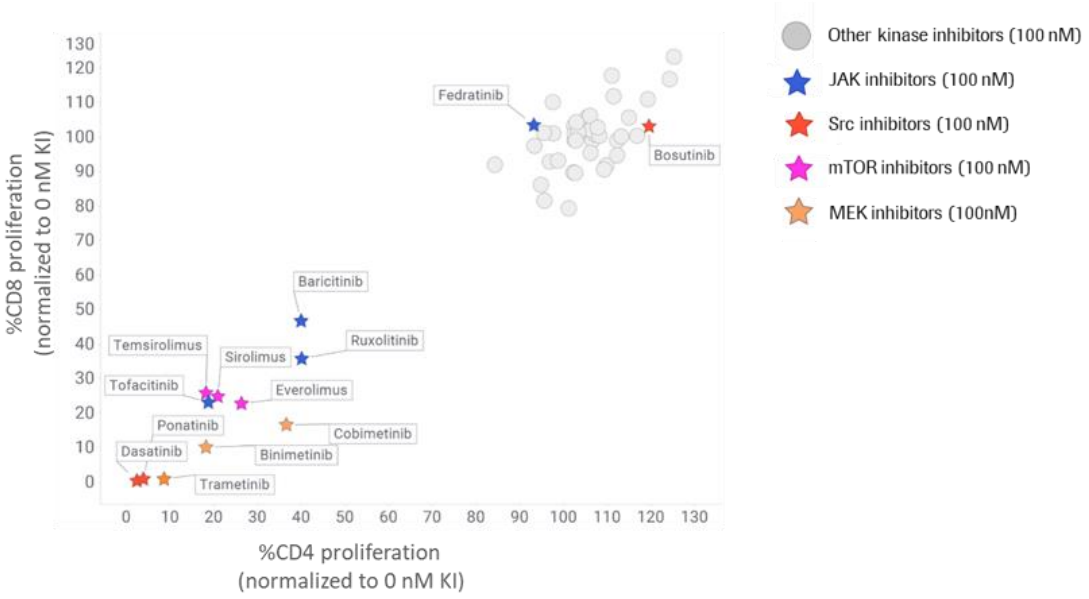
To identify families of kinase inhibitors that could potentially prevent T cell proliferation and cytokine release following CD3 stimulation, we screened a library of 52 FDA-approved kinase inhibitors at three different concentrations: 1000, 100 and 10 nM (Fig 1A). CTV-labelled T cells were stimulated on CD3-coated plates in the presence and absence of the kinase inhibitors (Fig 1A). At 96 hrs, the dilution of the CTV dye was measured by flow cytometry as a readout for CD4+ and CD8+ T cell proliferation. As a result, mTOR, JAK, Src and MEK inhibitors strongly reduced CD4+ and CD8+ T cell proliferation when used at a concentration of 100 nM (Fig 1B, C and S1A). In line with findings on T cell proliferation, the Src, MEK and mTOR inhibitors dose-dependently reduced T-cell derived cytokine as well as Granzyme B secretion, with the exception of the JAK inhibitors (Fig 1C, S1B). The screening of 52 FDA-approved kinase inhibitors enabled to select JAK inhibitors (ruxolitinib, baricitinib and tofacitinib), mTOR inhibitors (sirolimus, everolimus and temsirolimus), MEK inhibitors (trametinib, cobimetinib and

binimetinib) and Src inhibitors (dasatinib and ponatinib) as potential drugs to fine tune TCB-induced T cell proliferation and cytokine release (Fig 1B). We also considered the less potent Src inhibitor bosutinib and JAK inhibitor fedratinib, which inhibited T cell proliferation only at a higher concentration of 1000 nM (Fig 1C, S1A). In the next experiments, we applied the dose of 100 nM, defined from dose-responses experiments of Src, mTOR and JAK inhibitors conducted in a killing assay using CEA-TCB (Fig S2- S6). Since the effects of MEK1/2 inhibitor trametinib on CEA-TCB-mediated cytokine release correlated with an inhibition of tumor cell killing, we did not further investigate this class of kinase inhibitors for the mitigation of CD19-TCB related adverse events (Fig S2). This work focuses on mTOR and JAK inhibitors in comparison to the effects of the Src inhibitor dasatinib, previously described for TCBs and CAR-T cells (30-33).

A.


52 FDA approved drugs
1000, 100 and 10 nM
4 days proliferation assay
T cells
TCR
CD3 coated plate
T cell proliferation
Cytokine release

B.


%CD8 proliferation
(normalized to 0 nM KI)
%CD4 proliferation
(normalized to 0 nM KI)
Fedratinib
Bosutinib
Baricitinib
Ruxolitinib
Temsirolimus
Sirolimus
Tofacitinib
Everolimus
Ponatinib
Cobimetinib
Dasatinib
Binimetinib
Trametinib
Other kinase inhibitors (100 nM)
JAK inhibitors (100 nM)
Src inhibitors (100 nM)
mTOR inhibitors (100 nM)
MEK inhibitors (100nM)

C.

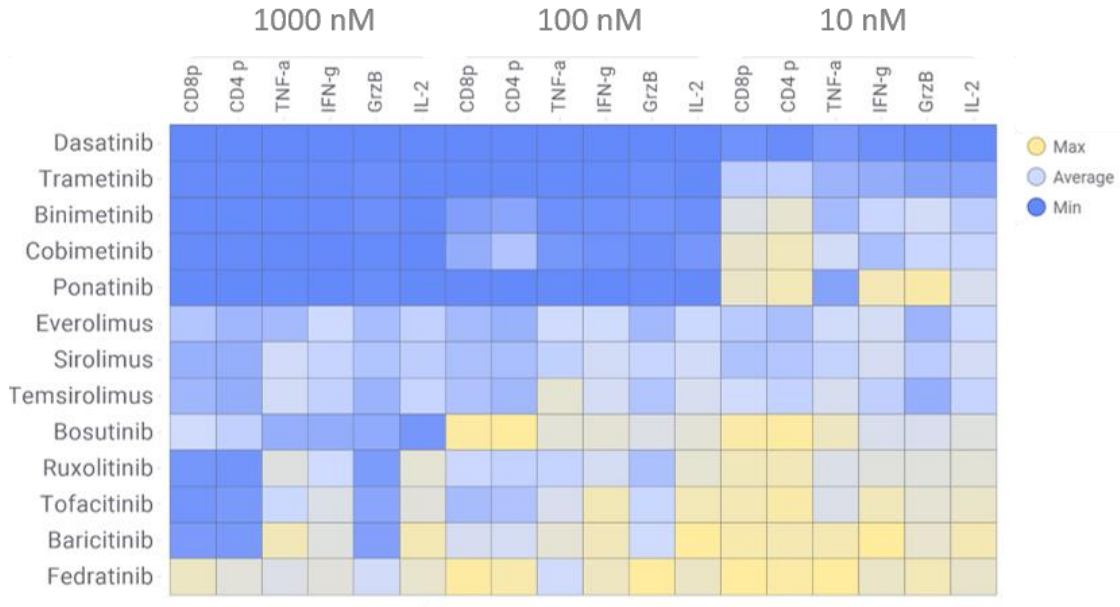
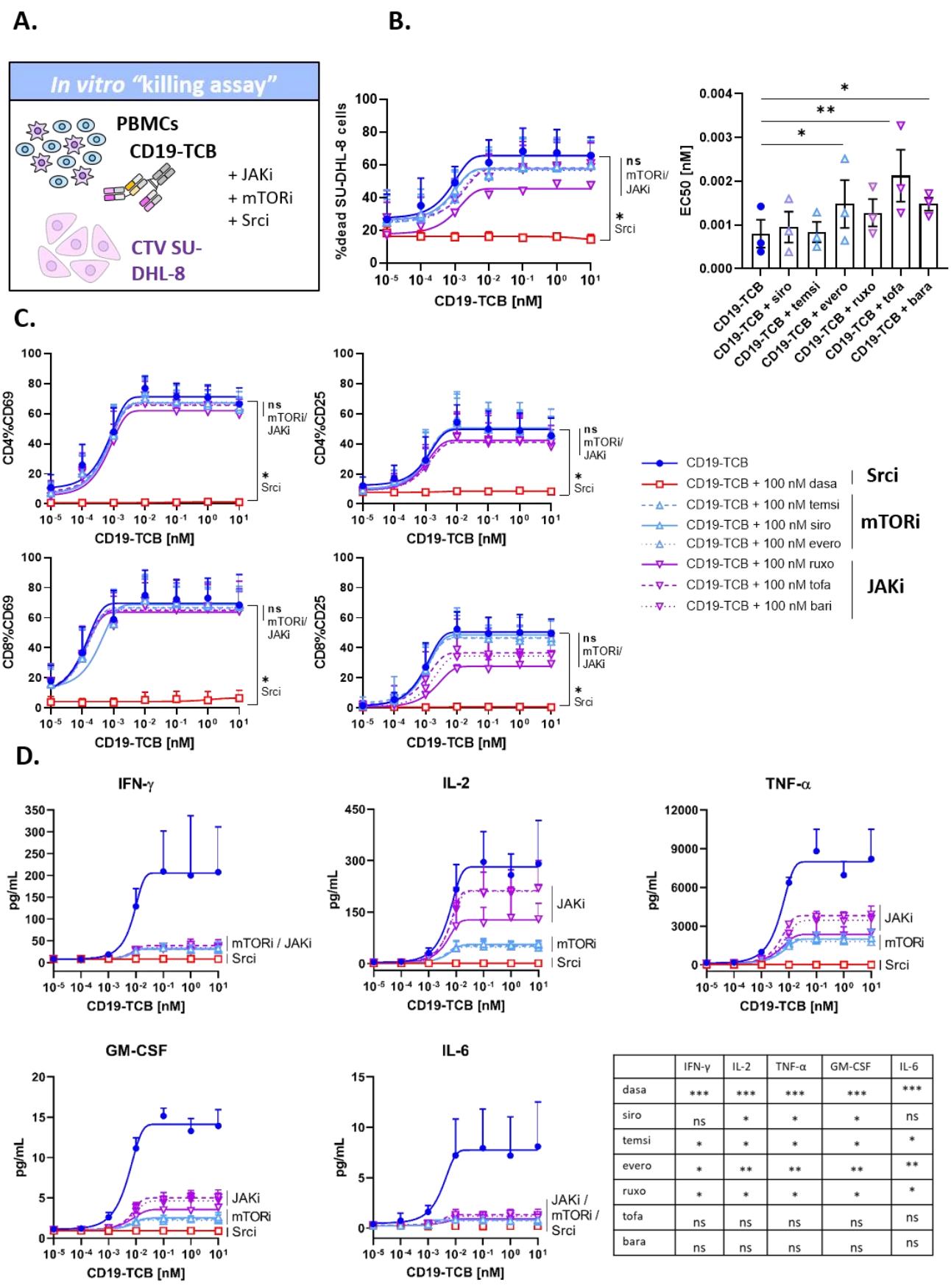

1000 nM
100 nM
10 nM
CD8p
CD4 p
TNF-a
IFN-g
GrzB
IL-2
Dasatinib
Trametinib
Binimetinib
Cobimetinib
Ponatinib
Everolimus
Sirolimus
Temsirolimus
Bosutinib
Ruxolitinib
Tofacitinib
Baricitinib
Fedratinib
Max
Average
Min

Figure 1. High throughput screening of 52 FDA approved kinase inhibitor to identify candidates reducing TCB-induced T cell proliferation and cytokine release.

(**A**) CTV-labelled pan T cells were stimulated on CD3 coated plate in the absence and presence 10, 100 and 1000 nM of each kinase inhibitor to mimic the TCB stimulation. (**B**) The dilution peaks of the CTV dye were measured by flow cytometry at 96 hrs to evaluate the effect of the different kinase inhibitors on CD4+ and CD8+ T cell proliferation. The proliferation of CD4+ and CD8+ cells were normalized to proliferation of untreated T cells. mTOR, JAK, Src and MEK inhibitors were identified as hits of the screen. (**C**) The effects of escalating concentrations of the selected mTOR, JAK, Src and MEK inhibitor candidates on TNF-α, IL-2, granzyme-B (GrzB), IFN-γ and CD4+ (CD4 p) and CD8+ (CD8 p) proliferation are depicted in a heat map. The levels of TNF-α, IL-2, granzyme-B (GrzB) and IFN-γ were measured in the supernatants by CBA (24 hrs) and normalized to untreated T cells. Median of technical triplicates, 1 donor.

The differential activities of Src, mTOR and JAK inhibitors reveal the uncoupling of CD19-TCB-induced cytokine release and cytotoxicity.

We compared the effects of mTOR and JAK inhibitors to Src inhibitors on CD19-TCB-induced T cell activation, cytotoxicity and cytokine release in a killing assay co-culturing CTV-labelled SU-DHL-8 tumor cells with PBMCs and CD19-TCB in the presence and absence of 100 nM of either JAK (ruxolitinib, tofacitinib and baricitinib), mTOR (sirolimus, temsirolimus and everolimus) or Src (dasatinib) inhibitors (Fig 2A). As a result, neither the JAK nor the mTOR inhibitors prevented killing of SU-DHL-8 tumor cells, nor CD4+ and CD8+ T cell activation as opposed to the Src inhibitor dasatinib, as indicated by the dose-response curves of dead CTV-labelled SU-DHL-8 cells and the expression of CD25 and CD69 on CD4+ and CD8+ T cells (Fig 2B, C and S7). Consistently with the switch-off of T cell functionality, the Src inhibitor dasatinib blocked CD19-TCB induced cytokine release (Fig 2B-D). Interestingly, the mTOR inhibitors reduced IFN-γ, IL-6, GM-CSF, IL-2 and TNF-α and the JAK inhibitors reduced IFN-γ, IL-6, GM-CSF and to a lower extent IL-2 and TNF-α (Fig 2D). When supplemented on pre-activated PBMCs, sirolimus and ruxolitinib also rapidly suppressed cytokine release while not preventing further killing of CTV-labelled NALM-6 tumor cells, mimicking a clinical intervention for the mitigation of CRS (Fig S8). In summary, the use of Src, mTOR and JAK inhibitors support the uncoupling of CD19-TCB-induced cytokine release and T cell cytotoxicity, opening new avenues for the mitigation of CD19-TCB-induced cytokine release.



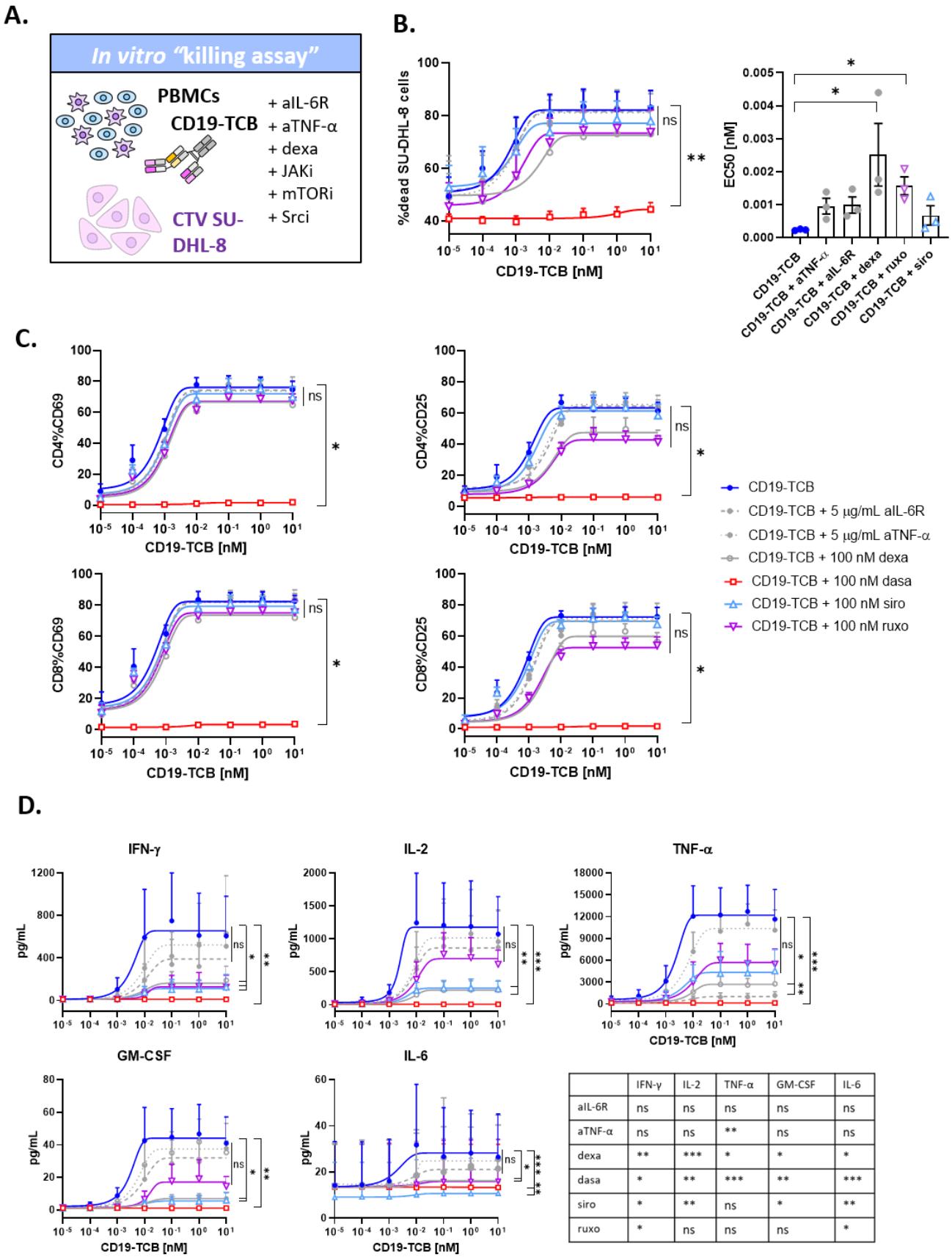
	IFN-γ	IL-2	TNF-α	GM-CSF	IL-6
dasa	***	***	***	***	***
siro	ns	*	*	*	ns
temsi	*	*	*	*	*
evero	*	**	**	**	**
ruxo	*	*	*	*	*
tofa	ns	ns	ns	ns	ns
bara	ns	ns	ns	ns	ns

Figure 2. mTOR and JAK but not Src inhibitors prevent CD19-TCB-induced cytokine release while not affecting tumor cell killing and T cell activation and rapidly abolish CD19-TCB induced cytokine release.

(**A**) PBMCs were stimulated on CTV-labelled SU-DHL-8 tumors cell by CD19-TCB in the presence of Src inhibitor (dasatinib), mTOR inhibitors (sirolimus, temsirolimus, everolimus) and JAK inhibitors (ruxolitinib, tofacitinib, baricitinib). (**B**) Effect of JAK, mTOR and Src inhibitors on CD19-TCB-induced killing of SU-DHL-8 tumor cells. At 24 hrs, technical replicates were pooled and stained with Live/Dead NIR, allowing measurement of dead SU-DHL-8 cells by flow cytometry. The EC50 values of each individual killing curve for n = 3 donors are summarized in the bar plot. (C) The levels of CD25 and CD69 on CD4+ and CD8+ T cells induced by CD19-TCB were measured by flow cytometry (24 hrs). (**B-C**) Mean of n=3 donors + SD with * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test). (**D**) The culture supernatants were pooled and the levels of TNF-α, IFN-γ, IL-2, IL-6 and GM-CSF were measured by Luminex (24 hrs), mean of n=3 donors +/-SD. The statistical difference to CD19-TCB treatment are summarized in the table with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by 1 way ANOVA (Friedman test).

mTOR and JAK inhibitors suppress CD19-TCB induced cytokine release, comparably to dexamethasone and more robustly than IL-6R and TNF-a blockade with similar effect on T-cell cytotoxicity and T cell activation.

The effects of mTOR, JAK and Src inhibitors on CD19-TCB-induced cytokine release, T cell activation and tumor cell killing were compared to those of IL-6R blockade and dexamethasone, commonly used in the clinic for the mitigation of CRS, and to TNF-α blockade, currently being explored for the mitigation of CRS (21, 34). PBMCs were co-cultured with SU-DHL-8 tumor cells and CD19-TCB in the presence and absence of 100 nM of the different kinase inhibitors, 100 nM dexamethasone or 5 μg/mL anti-IL-6R or anti-TNF-α (Fig 3A). As expected, 100 nM dasatinib completely inhibited SU-DHL-8 tumor cells killing, T cell activation and cytokine release, as opposed to anti-IL-6R, anti-TNF-α, dexamethasone and the mTOR and JAK inhibitors which only modulated cytokine release while not strongly affecting tumor cell killing and T cell activation (Fig 3B-D). mTOR inhibitors (sirolimus, temsirolimus and everolimus) suppressed CD19-TCB-induced IFN-γ, TNF-α, IL-2 and GM-CSF release to a broader extent than TNF-α, IL-6R blockade and ruxolitinib. Indeed, the JAK inhibitor ruxolitinib reduced IFN-γ and TNF-α but had a lower effect on IL-2 and GM-CSF release. (Fig 3D). Collectively, these data demonstrate that the JAK and mTOR inhibitors more broadly reduce CD19-TCB-induced cytokine secretion than TNF-α or IL-6R blockade while retaining CD19-TCB-mediated tumor cell killing and T cell activation. Furthermore, the mTOR inhibitors appear as the preferred candidates to prevent cytokine release while not affecting EC50 values of CD19-TCB-induced SU-DHL-8 tumor cell killing, as observed with ruxolitinib and dexamethasone.



	IFN-γ	IL-2	TNF-α	GM-CSF	IL-6
aIL-6R	ns	ns	ns	ns	ns
aTNF-α	ns	ns	**	ns	ns
dexa	**	***	*	*	*
dasa	*	**	***	**	***
siro	*	**	ns	*	**
ruxo	*	ns	ns	ns	*

Figure 3. The effects of mTOR and JAK inhibitors on CD19-TCB induced cytokine release, T cell activation and tumor cell killing are comparable to dexamethasone and more potent than IL-6R and TNF-a blockade in reducing cytokine release.

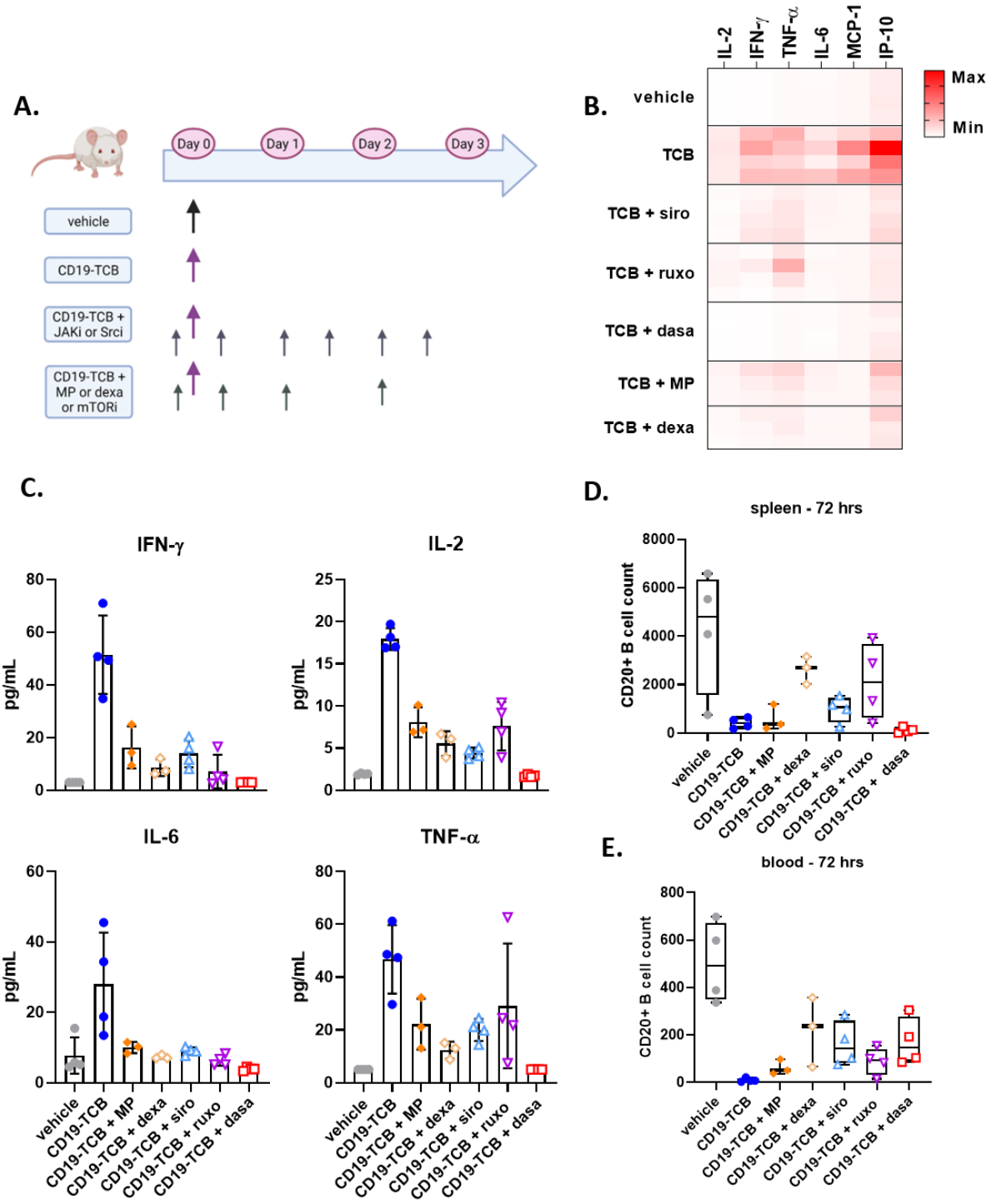
(**A**) PBMCs were stimulated on CTV labelled SU-DHL-8 tumors cell by CD19-TCB in the presence of 100 nM dasatinib (Src inhibitor), 100 nM sirolimus (mTOR inhibitor), 100 nM ruxolitinib (JAK inhibitor), 100 nM dexamethasone or 5 μg/mL of anti-IL-6R or anti-TNF-α. (**B**) At assay endpoint (24 hrs), the killing of CTV-labelled SU-DHL-8 cells was measured by gating on dead NIR-positive cells. The EC50 values of each individual killing curve for n = 3 donors are summarized in the bar plot. (**C**) In addition, the expression of CD25 and CD69 on CD4+ and CD8+ T cells was measured by flow cytometry. (**D**) Finally, the culture supernatants from technical replicates were pooled and the levels of TNF-α, IFN-γ, IL-2 and GM-CSF were measured by Luminex (24 hrs). (**B-D**) Mean of n = 3 donors +/-SEM with * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ by 1 way ANOVA (Friedman test). For cytokine data, the statistical differences to CD19-TCB treatment are summarized in the table.

mTOR and JAK inhibitors suppress CD19-TCB-induced cytokine release in humanized NSG mice while minimally interfering with B cell depletion

To verify if the Src, mTOR and JAK inhibitors would suppress CD19-TCB induced cytokine release *in vivo,* humanized NSG mice were either treated with vehicle or 0.5 mg/kg CD19-TCB as a monotherapy or combined with sirolimus, ruxolitinib or dasatinib (Fig 4A). In parallel, the effect of dexamethasone and methylprednisolone were also evaluated in this model. The different kinase inhibitors and glucocorticoids were given orally to reproduce the clinical route of administration. As a result, the mTOR inhibitor sirolimus reduced the levels of IFN-γ, IL-2, IL-6 and TNF-α upon infusion with CD19-TCB, comparably to dexamethasone, methylprednisolone and less efficiently than dasatinib (Fig 4B, C). In line with *in vitro* observations, the JAK inhibitor ruxolitinib preferentially reduced IFN-γ and IL-6 with a milder effect on TNF-α and IL-2 release (Fig 4B, C). Additionally, the other JAK inhibitor baricitinib behaved like ruxolitinib and the other mTOR inhibitors temsirolimus and everolimus like sirolimus in reducing CD19-TCB-induced cytokine release (Fig S9). Importantly, the CD20+ B cell counts measured in the spleen and peripheral blood (72 hrs) at study termination showed that sirolimus and ruxolitinib induced a moderate, yet not significant, inhibitory effect on CD19-TCB-mediated B cell depletion (Fig 4D, E). Dasatinib did not fully block CD19-TCB activity as indicated by CD20+ B cell count measured 72 hrs after CD19-TCB administration, consistent with our recent findings showing that dasatinib switches-off CD19-TCB activity up to 48 hrs upon treatment with CD19-TCB (Fig 4D, E) (32, 33).

Overall, this experiment demonstrates that the pharmacodynamic profile of mTOR and JAK inhibitors *in vivo* is favorable to control CD19-TCB-mediated cytokine release, comparably to

dexamethasone or methylprednisolone and less intensively than dasatinib. In line with previous *in vitro* findings, the mTOR inhibitors more broadly reduced cytokine release than the JAK inhibitors. When applied continuously during 3 days, the different kinase inhibitors only minimally reduced CD19-TCB-mediated cytotoxicity, suggesting that transient treatment for the mitigation of CRS should not impair anti-tumor efficacy.



	IFN-γ	TNF-a	IL-2	IL-6	CD20 count (blood)	CD20 count (spleen)
vehicle	**	***	***	*	**	*
CD19-TCB + MP	ns (p>1)	ns (p=0.25)	ns (p=0.36)	ns (p=0.29)	ns (p=0,85)	ns (p>1)
CD19-TCB + dexa	ns (p=0.41)	*	ns (p=0.07)	ns (p=0.07)	ns (p=0,07)	ns (p=0.21)
CD19-TCB + siro	ns (p>1)	ns (p=0.17)	*	ns (p=0.21)	ns (p=0,45)	ns (p=0.27)
CD19-TCB + ruxo	*	ns (p=0.33)	ns (p=0.20)	**	ns (p=0,13)	ns (p>1)
CD19 - TCB + dasa	**	***	****	****	ns (p=0,30)	ns (p=0.16)

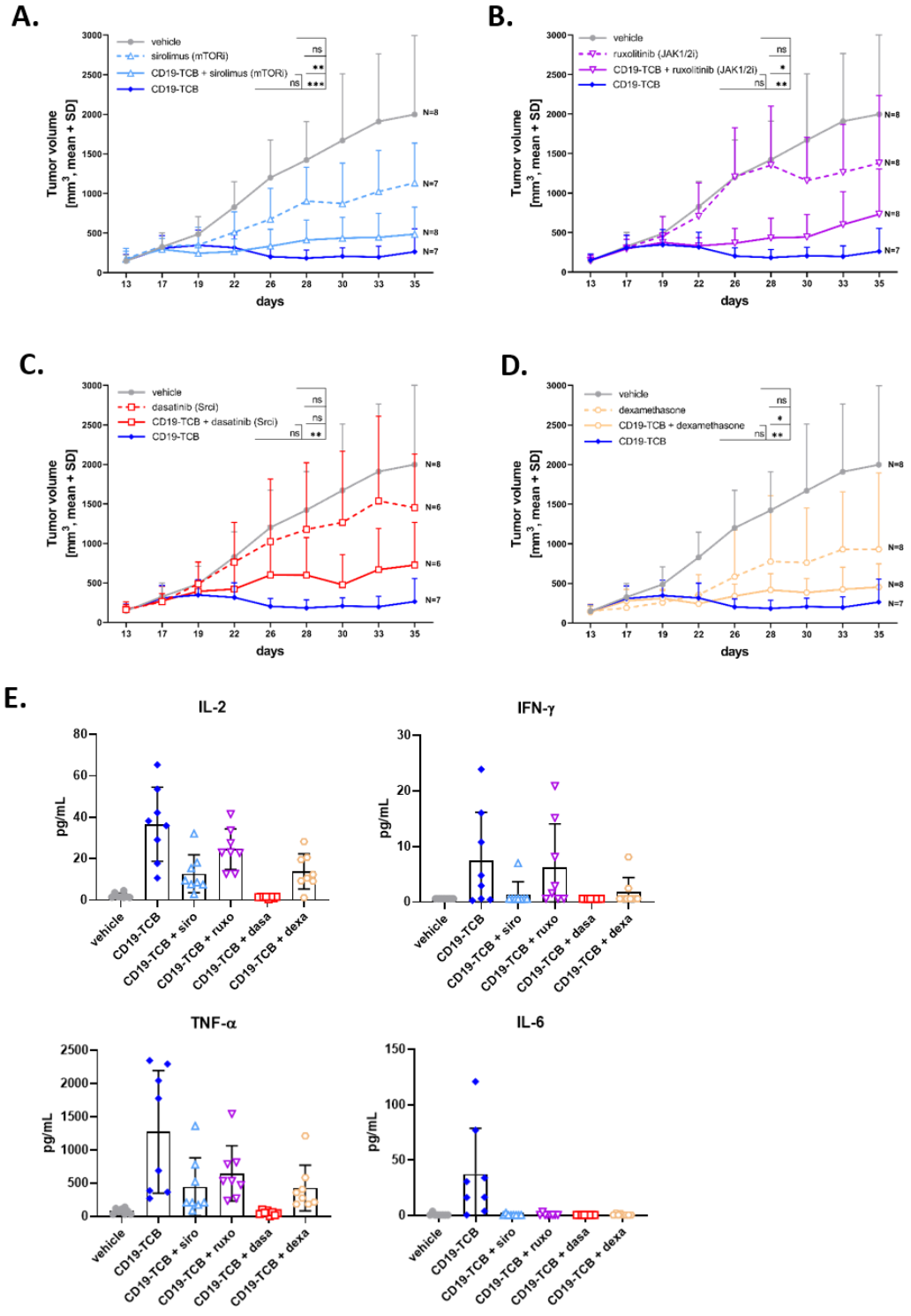
Figure 4. mTOR and JAK inhibitors mitigate CD19-TCB-induced cytokine release in huNSG mice while not preventing CD19-TCB-induced B cell depletion.

(**A**) *In vivo* experiment timelines and dosing schedule. Humanized NSG mice were co-treated with 0.5 mg/kg CD19-TCB (i.v.) alone or in combination with 50 mg/kg dasatinib (p.o.), 30 mg/kg ruxolitinib (p.o), 5 mg/kg sirolimus (p.o.), 2 times 1 mg/kg, 0.5 mg/kg and 0.25 mg/kg dexamethasone (p.o), or 2 times 10 mg/kg, 5 mg/kg, 2.5 mg/kg methylprednisolone (p.o.). Figure made with Biorender. (**B, C**) Serum was collected from blood 6 hrs post infusion with CD19-TCB and cytokine levels were measured by Luminex. The cytokine levels for each mice were either compared across different treatment groups in a heat map or shown as mean of individual values (bar plots). (**D, E**). The counts of CD20+ B cells were measured in the blood and spleen collected at termination (72 hrs) to assess the effect of kinase inhibitors on CD19-TCB-dependent B cell depletion. (**C-E**) The statistical differences to CD19-TCB monotherapy treatment are summarized in the table. Mean of n = 4 or 3 mice +/- SD with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ by 1 way ANOVA (Kruskal Wallis test).

mTOR and JAK inhibitors do not suppress CD19-TCB anti-tumor activity in lymphoma PDX bearing mice, similarly to dexamethasone.

We then evaluated the impact of Src, mTOR and JAK inhibition in comparison to dexamethasone on CD19-TCB anti-tumor activity in humanized NSG mice engrafted with a lymphoma patient derived xenograft (PDX). *In vitro*, these lymphoma cells were efficiently depleted by CD19-TCB in a killing assay (Fig S10). Therefore, mice were treated with either vehicle, CD19-TCB as a monotherapy or sirolimus, ruxolitinib, dasatinib, dexamethasone alone or in combination with CD19-TCB (Fig S11A). The different kinase inhibitors and dexamethasone were given one hour prior to the first treatment with CD19-TCB and then once or twice per day for three days to prevent cytokine release, predominantly occurring upon the first infusion. Moreover, they were also administered one hour before each subsequent treatment to prevent eventual residual cytokine secretion (Fig S11A). Both, dexamethasone and sirolimus, given as a single agent, induced a reduction in tumor growth, yet not significant (Fig 5A, D).When combined with CD19-TCB, the resulting anti-tumor efficacy was slightly but not significantly reduced, however sirolimus and dexamethasone suppressed IL-2, IFN-γ, TNF-α and IL-6 release upon the first infusion (Fig 5A, D, E). Additionally, co-treatment with ruxolitinib minimally interfered with CD19-TCB anti-tumor activity and decreased IL-6 and to a lower extent IFN-γ, TNF-α and IL-2 release (Fig 5B, E). Lastly, dasatinib strongly reduced T-cell derived cytokines (IFN-γ, TNF-α and IL-2) upon the first infusion while moderately affecting CD19-TCB anti-tumor efficacy. Yet, this effect was not significant (Fig 5C, E). This indicates that the transient use of dasatinib did not continuously block CD19-TCB-induced T cell cytotoxicity, in agreement with the reversible inhibitory properties of dasatinib (32, 33). Additionally, cytokine levels were much lower 6 hrs upon the second infusion of CD19-TCB, suggesting that treatment with kinase inhibitors upon

the first infusion should be sufficient to prevent cytokine release (Fig S12). Finally, the transient use of ruxolitinib and sirolimus, similarly to dexamethasone, decreased T-cell mediated cytokine release upon the first infusion of CD19-TCB, resulting in a better safety profile as indicated by the overall survival curves and changes in body weight (Fig S11B, C).



	IFN-y	TNF-a	IL-2	IL-6
vehicle	ns (p=0.06)	****	****	***
CD19-TCB + siro	ns (p=0.09)	*	*	***
CD19-TCB + ruxo	ns (p=0.64)	ns (p=0.43)	ns (p=0.45)	***
CD19 - TCB + dasa	ns (p=0.06)	****	****	****
CD19-TCB + dexa	ns (p=0.30)	*	*	**

Figure 5. mTOR and JAK inhibitors do not inhibit CD19-TCB anti-tumor efficacy *in vivo*.

Humanized NSG mice were engrafted with a lymphoma PDX (5 million cells, s.c.). When tumors reached 200 mm3, mice were randomized in groups of 8 or 7 based on their tumor size. They were treated with vehicle (i.v.), 5 mg/kg sirolimus (p.o.), 30 mg/kg ruxolitinib (p.o.), 20 mg/kg dasatinib (p.o.), 2 times 1 mg/kg, 0.5 mg/kg, 4 times 0.25 mg/kg dexamethasone (p.o.) alone or in combination with 0.5 mg/kg CD19-TCB (i.v.), 0.5 mg/kg CD19-TCB (i.v.) as a monotherapy. (**A-D**) Tumor growth curves were plotted from tumor volumes measured using a Caliper, mean of n= 6-8 mice + SD with * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ by 1 way ANOVA (Kruskal Wallis test). (**E**) The levels of IL-2, IFN-γ, IL-6 and TNF-α in the serum of the mice collected 6 hrs post first infusion with CD19-TCB. The statistical differences to CD19-TCB treatment are summarized in the table. Mean of n= 6-8 mice +SD with * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ by 1 way ANOVA (Kruskal Wallis test).

Discussion

By screening a library of 52 FDA-approved kinase inhibitors for their impact on T cell proliferation and cytokine release after CD3 stimulation, we identified mTOR, JAK and Src kinases inhibitors as potential candidates to modulate TCB-mediated cytokine release at pharmacologically active doses, as indicated in Table 1. The effect of Src inhibitors differed from those of JAK and mTOR inhibitors with the suppression of CD19-TCB-induced tumor cell lysis *in vitro* (Fig S13). In contrast, JAK and mTOR inhibitors, comparably to dexamethasone, more broadly reduced TCB-mediated cytokine release than anti-TNF-α or anti-IL-6R antibodies, but did not have a major impact on T cell killing (Fig S13). Of note, the mTOR inhibitors did not affect EC50 values of killing curves upon CD19-TCB treatment, suggesting that they might be the preferred candidates over dexamethasone and JAK inhibitors for the prophylactic mitigation of CRS after treatment with TCBs. Importantly, JAK, mTOR and Src inhibitors also prevented cytokine release from PBMCs previously activated by a TCB. When used as intervention treatment for CRS, it is reasonable to think that kinase inhibitors would not only prevent release of T-cell derived cytokines but also – as a downstream consequence of the former - myeloid cell-derived cytokines known to be the key mediators of CRS (20, 21, 35-37). *In vivo*, the combination of PK/PD properties of small molecules kinase inhibitors and CD19-TCB favorably reduced cytokine release upon the first infusion in non-tumor bearing humanized NSG mice, with comparable effects like glucocorticoids. In lymphoma PDX-bearing humanized NSG mice, transient treatment with Src, mTOR and JAK inhibitors upon the first infusion inhibited cytokine release while minimally impairing CD19-TCB anti-tumor efficacy, comparably to dexamethasone. (38-40). Glucocorticoids and anti-IL-6R antibodies are the standard of care for the management of CRS, with only a small percentage of patients being refractory and requiring

alternative treatments. Nevertheless, CRS remains the main dose limiting toxicity for T-cell engaging therapies, and the requirement for prompt intervention to manage higher grade CRS prevents these therapies from achieving broader uptake. Therefore combination strategies to reduce the occurrence of CRS and increase or sustain treatment efficacy, as described by Bacac *et al*. with the use of obinutuzumab pre-treatment and CD20-TCB, remain to be investigated for other indications (6). Altogether, our data emphasizes that prophylactic treatment with kinase inhibitors would not impair the long-term anti-tumor efficacy of TCBs.

Since the complex biology of CRS involves many more cytokines than the described TNF-α, IL-6, IFN-γ and IL-1β, JAK inhibitors blocking inflammatory pathways, such as the JAK/STAT pathway, may have superior efficacy than selective antagonistic antibodies specifically blocking IL-6/IL-6R, TNF-α, GM-CSF or IL-1β (21, 23, 34, 36, 41, 42). In line with pre-clinical findings from Kenderian *et al*. showing that ruxolitinib prevented CD123-CAR-T cell induced cytokine release *in vivo* and with findings from Huarte *et al*. demonstrating that ruxolitinib prevented the cytokine storm in models of hyper inflammation, our data confirm that JAK inhibitors prevented TCB induced-cytokine release, however, less efficiently than mTOR or Src inhibitors (42, 43). Indeed, they retained the release IL-2, GM-CSF and only partially suppressed the release of TNF-α, suggesting that a combination with an anti-TNF-α antibody may be advantageous for the management of TCB-induced cytokine release. This approach was used to manage grade 3 CRS in two patients treated with CAR-T cell against CD7+ T lymphocytes (44). In line with this, Uy *et al*. reported that prophylactic treatment with ruxolitinib decreased cytokine secretion, but did not lead to discernable improvement in clinical severity of CRS in patients receiving flotetuzumab, emphasizing that ruxolitinib alone may not be sufficient to suppress CRS (45). Itacitinib, a more selective JAK1 inhibitor, is being explored for the prevention of CD19-CAR-T cell induced CRS in patients with hematological malignancies (35, 46).

Dasatinib appears less attractive than JAK, mTOR inhibitors or dexamethasone for the mitigation of TCB-mediated cytokine release since it fully suppresses T cell cytotoxicity *in vitro*. The intervention with dasatinib was initially developed as a potential safety trigger switching off T-cell cytotoxicity in case of severe CRS or on-target off-tumor activity mediated by CAR-T cells or T cell engagers where a rapid T cell off-switch would be not be achieved with glucocorticoids (29-32) (Leclercq *et al*., JITC, 2021). Uninterrupted administrations of dasatinib before and after treatment with CD19-TCB were shown to switch-off cytokine release and B-cell depletion for up

to 48 hrs. Nevertheless, short treatment interventions with dasatinib upon first and subsequent CD19-TCB administrations in mice engrafted with a lymphoma PDX minimally interfered with anti-tumor efficacy, while fully switching-off cytokine release more profoundly than JAK, mTOR inhibitors or glucocorticoids. Thus, dasatinib may be used transiently as prophylaxis medication to prevent CRS upon first infusion with TCB. Additionally, Foà *et al.* showed that the use of dasatinib as induction therapy in patients with philadelphia chromosome positive leukemia followed by a consolidation therapy with concomitant treatment of blinatumomab and dasatinib was safe and efficacious (47). Along those lines, recent findings from Weber *et al*. demonstrated that reversible ON/OFF switches with dasatinib prevents CAR-T cell exhaustion and improves their long-term functionality (48).

For the first time, we described the use of mTOR inhibitors sirolimus, temsirolimus and everolimus to block TCB-induced cytokine release. Based on the *in vitro* comparison, mTOR inhibitors appear to be the most potent inhibitors to strongly reduce cytokine release while not interfering with T cell killing. *In vivo*, the effect of sirolimus favorably prevented CD19-TCB-mediated cytokine release while retaining efficacy with comparable effect to JAK, Src inhibitors and dexamethasone. Altogether, this suggests that mTOR inhibitors may be the preferred candidates for prophylaxis of CRS. Since mTOR inhibitors are used as anti-tumor agents in various solid cancers, a combination with a TCB to prevent CRS while maintaining efficacy may be of particular interest in such indications (49, 50). In line with this, for a melanoma patient who underwent kidney allograft rejection while receiving pembrolizumab, Esfahani *et al*. showed that sirolimus did not interfere with pembrolizumab-mediated anti-tumor activity while preventing organ rejection (51). This further supports the mTOR pathways as a target to mitigate inflammation-driven adverse events related to treatment with immunotherapies while retaining their efficacy. Taken together, these preclinical data support the further investigation of mTOR, Src and JAK inhibitors for the prophylaxis of T cell induced cytokine release after treatment with TCBs.

Table 1. *In vitro* effective doses of ruxolitinib, sirolimus, everolimus, temsirolimus and dasatinib as well as their respective clinical dose(s) and corresponding exposure(s).

Kinase inhibitor	Target	*In vitro* effective dose [nM]	*In vitro* effective dose [ng/mL]	Clinical dose	Clinical exposure	references
Ruxolitinib	JAK1/2	50 - 100	15.3 - 30.6	5 mg / day (oral)	C_{max} = 195 (35.4) ng/mL[a] $t_{1/2}$ = 2.6 (40) h[a]	NDA 2021092 (52)
				10 mg / day (oral)	C_{max} = 368 (29.9) ng/mL[a] $t_{1/2}$ = 3.4 (41) h[a]	
				25 mg / day (oral)	C_{max} = 934 (55.9) ng/mL[a] $t_{1/2}$ = 3.0 (22) h[a]	
Sirolimus	mTOR	10 - 100	9.1 - 91.4	2 mg / day (oral)	C_{max} = 12.2 +/- 6.2 ng/mL t_{max} = 3.01+/- 2.4 h	NDA 21-083/S-034 NDA 21-110/S-045
				5 mg / day (oral)	C_{max} = 37.4 +/- 21 ng/mL t_{max} = 1.84 +/- 1.3 h	
Everolimus	mTOR	10 - 100	9.6 - 95.8	5 mg / day (oral)	C_{max} =28.7 ng/ mL[a] $t_{1/2}$ = 31.2 (4.79) h[b]	NDA 21-560 (49)
Temsirolimus	mTOR	10 - 100	10.3 – 103.0	25 mg/week (i.v.)	C_{max} = 585 (83) ng/mL[b] $t_{1/2}$ = 17.3 (5.9) h[b]	NDA 22-088 (53)
Dasatinib	Src/Lck	50 - 100	24.4 - 48.8	100 mg / day (oral)	C_{max} = 54.6 ng/mL[a] (56) t_{max} = 1.00, (0.5, 4.00) h[c]	NDA 21-986 NDA 22-072 (54)

a = geometric mean (percent coefficient of variation [%]), b = mean (standard deviation), c = median (min, max)

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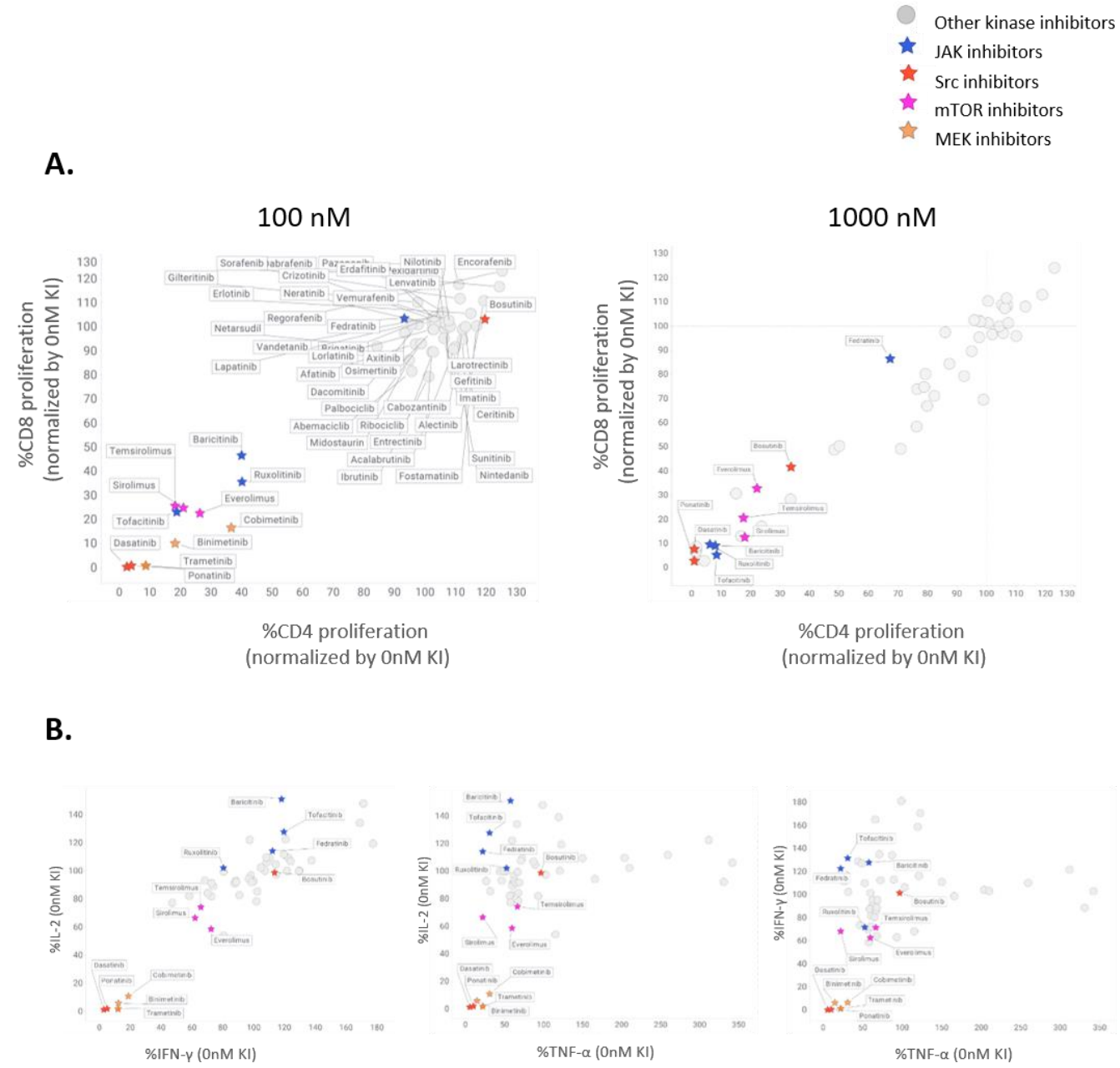
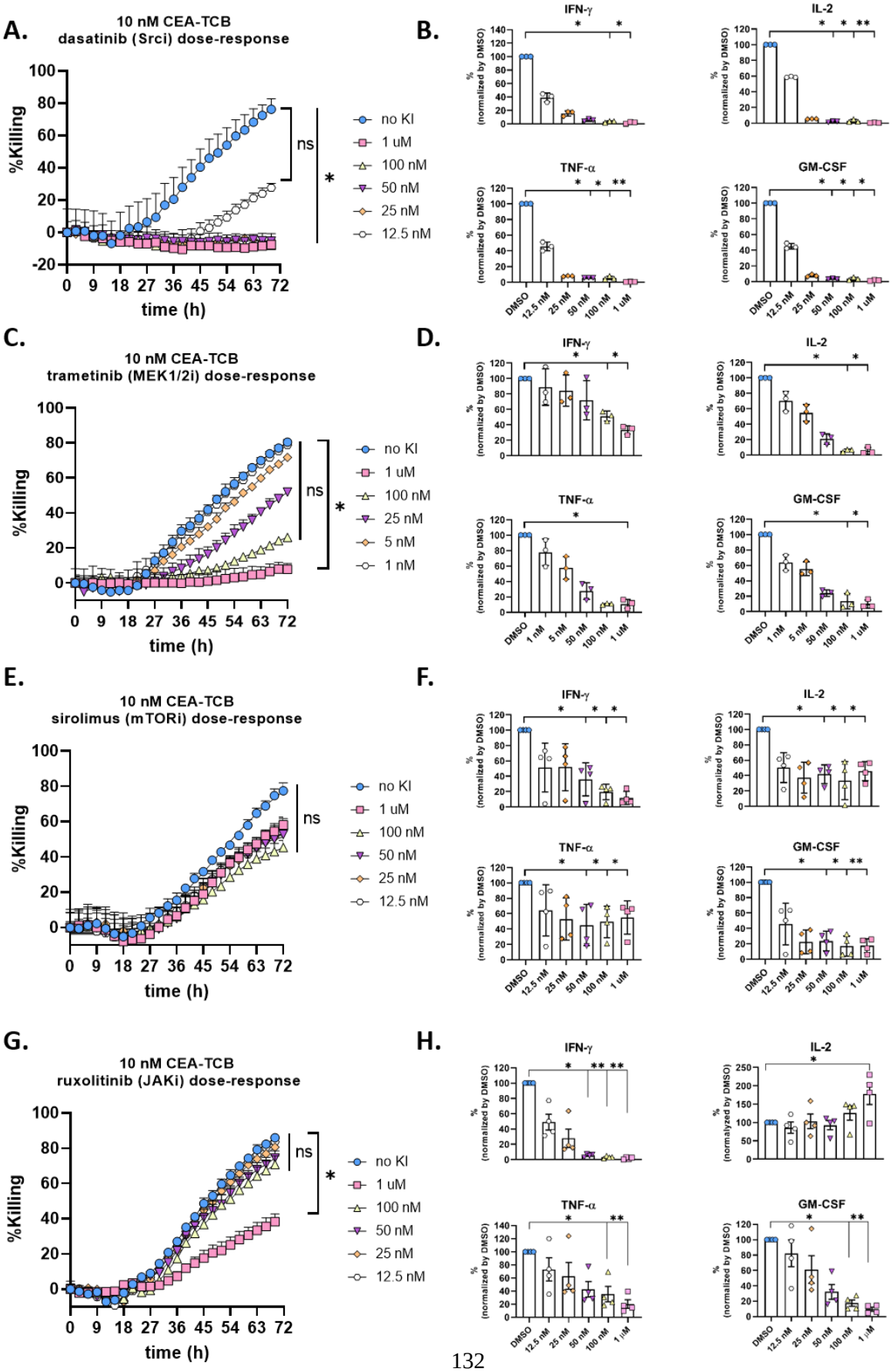
Supplementary data



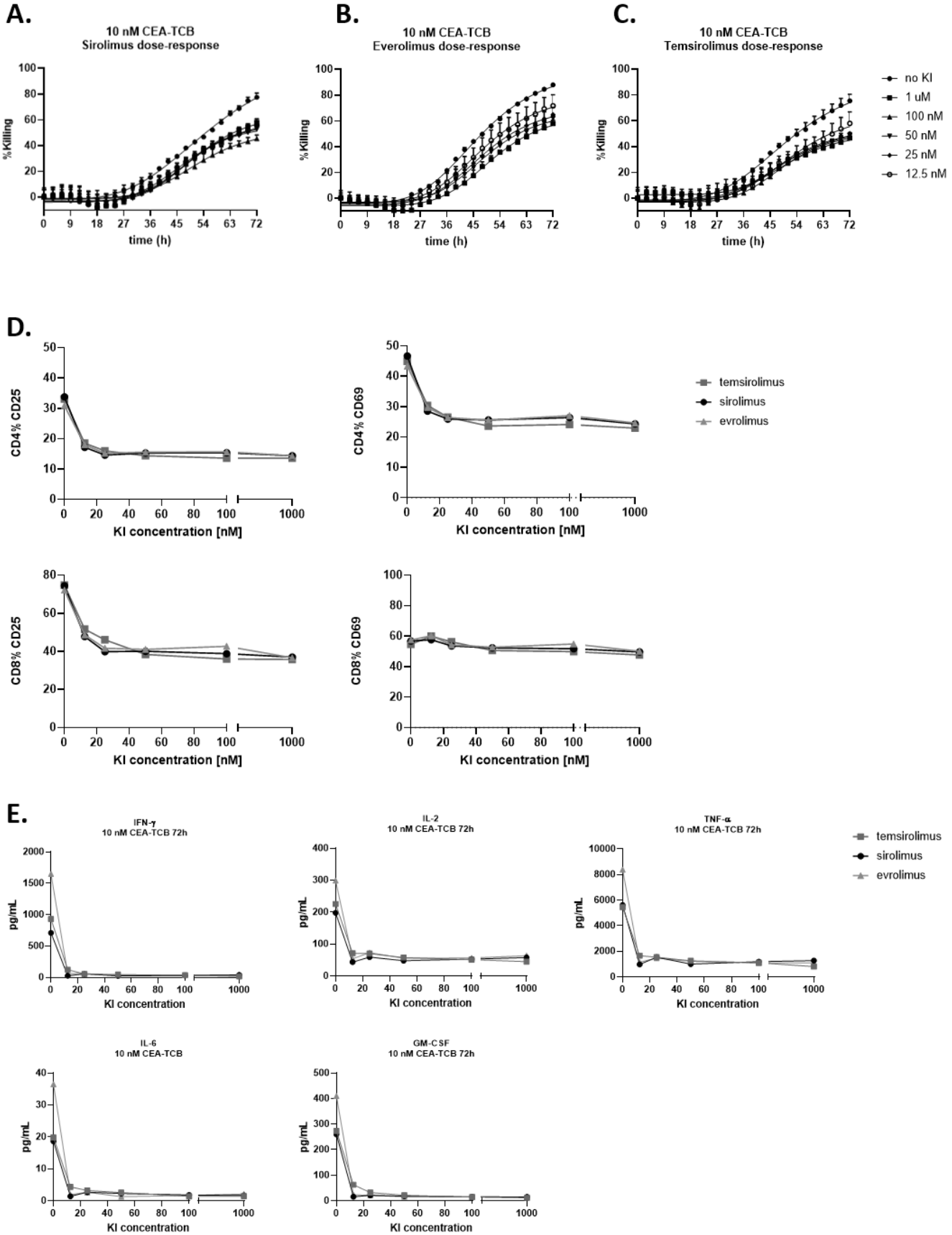
Supp. Figure 1. Selection of the hits of the screen of 52 FDA approved kinase inhibitors based on their effect on T cell activation and cytokine release.

CTV-labelled pan T cells were stimulated on CD3 coated plate, mimicking the stimulation with TCB, in the absence and presence of 100 nM or 1000 nM kinase inhibitors. The dilution of the CTV dye in $CD4^+$ and $CD8^+$ was measured by flow cytometry as a readout of T cell activation. (**A**) Effect of the 52 kinase inhibitors on $CD4^+$ and $CD8^+$ T cell proliferation. (**B**) Effect of the 52 kinase inhibitors (100 nM) on T cell-derived IL-2, TNF-α and IFN-γ release. Median of technical triplicates, 1 donor. The levels of TNF-α, IL-2, IFN-γ were measured in the supernatants by CBA normalized to untreated T cells (24 hrs).


A.
10 nM CEA-TCB
dasatinib (Srci) dose-response
%Killing
time (h)
ns
*
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
B.
IFN-γ
IL-2
TNF-α
GM-CSF
% (normalized by DMSO)
DMSO
12.5 nM
25 nM
50 nM
100 nM
1 uM
C.
10 nM CEA-TCB
trametinib (MEK1/2i) dose-response
%Killing
time (h)
ns
*
no KI
1 uM
100 nM
25 nM
5 nM
1 nM
D.
IFN-γ
IL-2
TNF-α
GM-CSF
% (normalized by DMSO)
DMSO
1 nM
5 nM
50 nM
100 nM
1 uM
E.
10 nM CEA-TCB
sirolimus (mTORi) dose-response
%Killing
time (h)
ns
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
F.
IFN-γ
IL-2
TNF-α
GM-CSF
% (normalized by DMSO)
DMSO
12.5 nM
25 nM
50 nM
100 nM
1 uM
G.
10 nM CEA-TCB
ruxolitinib (JAKi) dose-response
%Killing
time (h)
ns
*
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
H.
IFN-γ
IL-2
TNF-α
GM-CSF
% (normalized by DMSO)
DMSO
12.5 nM
25 nM
50 nM
100 nM
1 uM

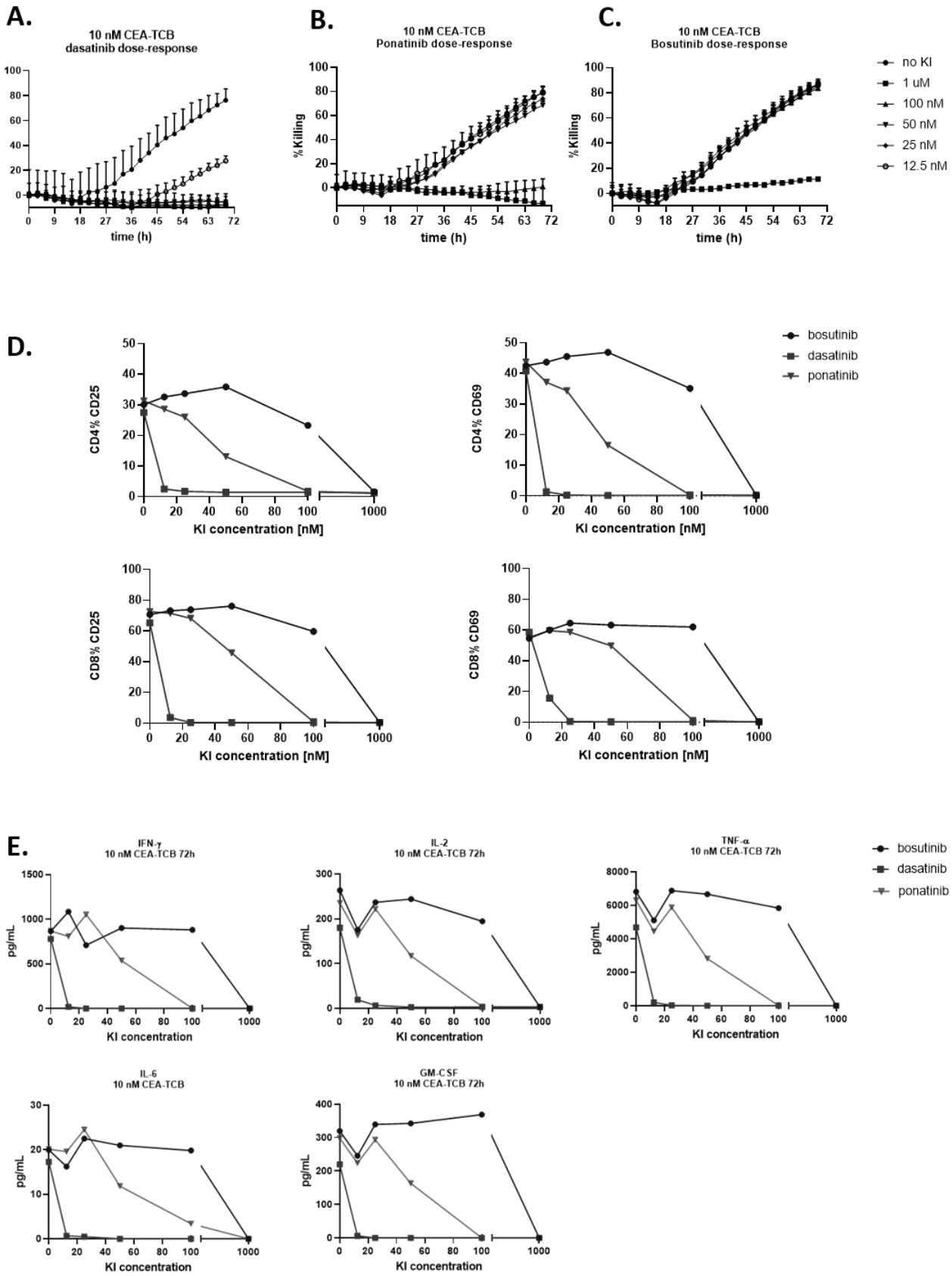
Supp. Figure 2. Validation of the top kinase inhibitor candidates on CEA-TCB-induced tumor cell killing and cytokine release.

PBMCs were stimulated on NucLightRed (NLR)-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of the selected kinase inhibitors. The real-time killing of NLR-labelled MKN45 cells was measured by IncuCyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time) to assess the impact of the different kinase inhibitor families on CEA-TCB-mediated killing. The percentage of killing was measured by normalizing the total red area / well to values at t = 0 hr and control wells containing the cell line, PBMCs and the KI for each time points. Effect of escalating doses of (**A**) Src (dasatinib), (**C**) MEK (trametinib), (**E**) mTOR (sirolimus) and (**G**) JAK (ruxolitinib) inhibitors on real-time killing of NLR-labelled MKN45 cells by 10 nM CEA-TCB, mean of technical replicates +/-SEM for one donor representative of 3 or 4 with * $p \leq 0.05$ by 1 way ANOVA (Friedman test). (**B, D, F, H**) The levels of IFN-γ, TNF-α and IL-2 were measured in the supernatants (72 hrs) by Luminex, to evaluate the impact of escalating concentrations of the different kinase inhibitors on CEA-TCB-induced cytokine release, mean of n= 3 or 4 donors +/-SD with with * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test).


A.
10 nM CEA-TCB
Sirolimus dose-response
B.
10 nM CEA-TCB
Everolimus dose-response
C.
10 nM CEA-TCB
Temsirolimus dose-response
% Killing
time (h)
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
D.
CD4% CD25
CD4% CD69
CD8% CD25
CD8% CD69
KI concentration [nM]
temsirolimus
sirolimus
evrolimus
E.
IFN-γ
10 nM CEA-TCB 72h
IL-2
10 nM CEA-TCB 72h
TNF-α
10 nM CEA-TCB 72h
IL-6
10 nM CEA-TCB
GM-CSF
10 nM CEA-TCB 72h
pg/mL
KI concentration
temsirolimus
sirolimus
evrolimus

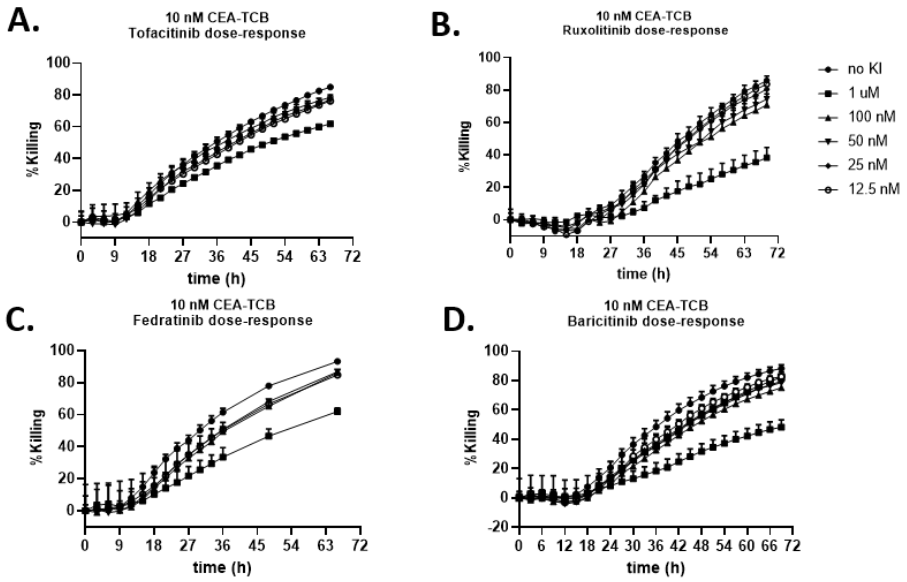
Supp. Figure 3. Dose-response of sirolimus, everolimus and temsirolimus in a killing assay with CEA-TCB.

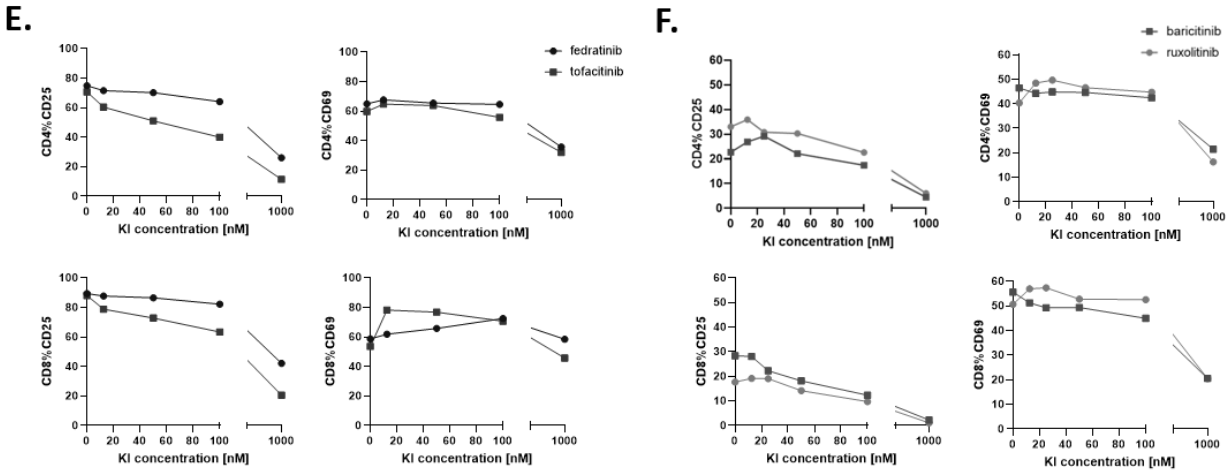
PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of mTOR inhibitors. The real-time killing of NLR-labelled MKN45 tumor cells by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentage of killing was measured by normalizing the total red area / well to values at t = 0 hr and control wells containing the cell line, PBMCs and the TCB for each time points. Effect of escalating doses of sirolimus (**A**), everolimus (**B**) and temsirolimus (**C**) on real-time killing of NLR-labelled MKN45 cells by 10 nM CEA-TCB, mean of technical replicates + SEM for 1 donor representative of 2. (**E**) The expression of CD25 and CD69 on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry (72 hrs) as a readout of T cell activation for 1 donor representative of 2. (**D**) The levels of IFN-γ, TNF-α, IL-2, IL-6 and GM-CSF were measured in the supernatants (72 hrs) by Luminex for 1 donor representative of 2.

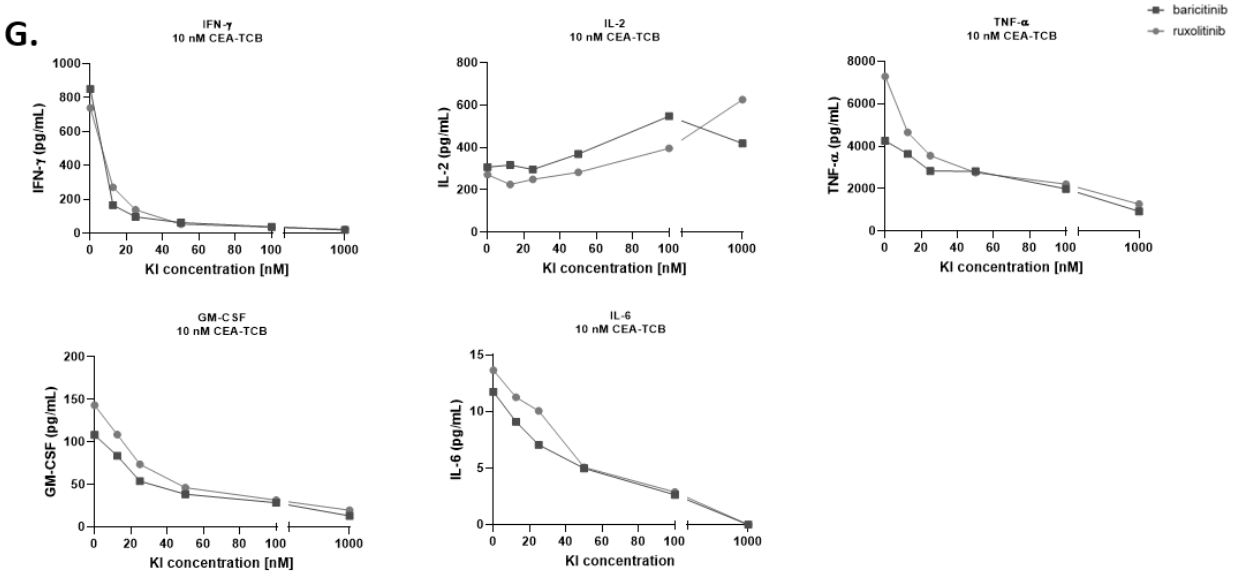

A.
10 nM CEA-TCB
dasatinib dose-response
% Killing
time (h)
B.
10 nM CEA-TCB
Ponatinib dose-response
% Killing
time (h)
C.
10 nM CEA-TCB
Bosutinib dose-response
% Killing
time (h)
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
D.
CD4% CD25
CD4% CD69
CD8% CD25
CD8% CD69
KI concentration [nM]
bosutinib
dasatinib
ponatinib
E.
IFN-γ
10 nM CEA-TCB 72h
IL-2
10 nM CEA-TCB 72h
TNF-α
10 nM CEA-TCB 72h
IL-6
10 nM CEA-TCB
GM-CSF
10 nM CEA-TCB 72h
pg/mL
KI concentration
bosutinib
dasatinib
ponatinib

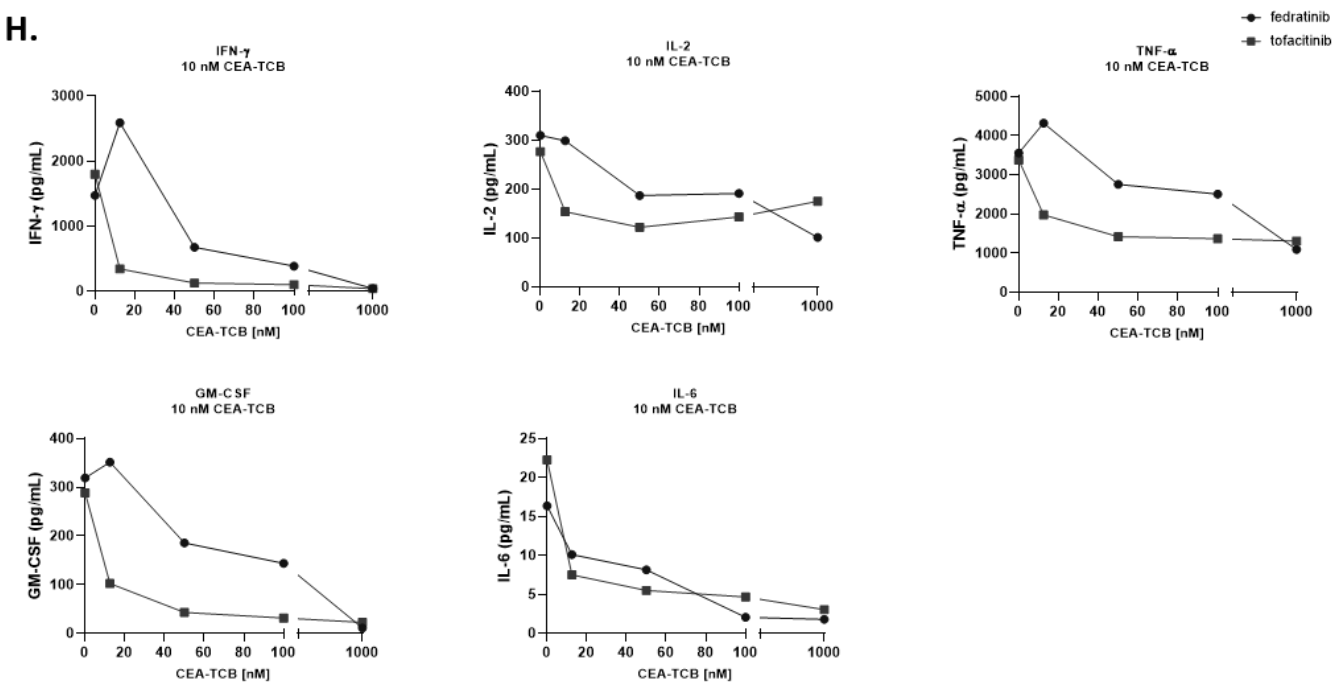
Supp. Figure 4. Dose-response of dasatinib, bosutinib and ponatinib in a killing assay with CEA-TCB.

PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of Src kinase inhibitors. The real-time killing of NLR-labelled MKN45 by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentage of killing was measured by normalizing the total red area / well to values at t = 0 hr and control wells containing the cell line, PBMCs and the TCB for each time points. Effect of escalating doses of dasatinib (**A**), ponatinib (**B**) and bosutinib (**C**) on real-time killing of NLR-labelled MKN45 cells by 10 nM CEA-TCB, mean of technical replicates + SEM for 1 donor representative of 2. (**D**) The expression of CD25 and CD69 on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry (72 hrs) as a readout of T cell activation for 1donor representative of 2. (**D**) The levels of IFN-γ, TNF-α, IL-2. IL-6 and GM-CSF were measured in the supernatants (72 hrs) by Luminex for 1donor representative of 2.


A.
10 nM CEA-TCB
Tofacitinib dose-response
% Killing
time (h)
B.
10 nM CEA-TCB
Ruxolitinib dose-response
% Killing
time (h)
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
C.
10 nM CEA-TCB
Fedratinib dose-response
% Killing
time (h)
D.
10 nM CEA-TCB
Baricitinib dose-response
% Killing
time (h)

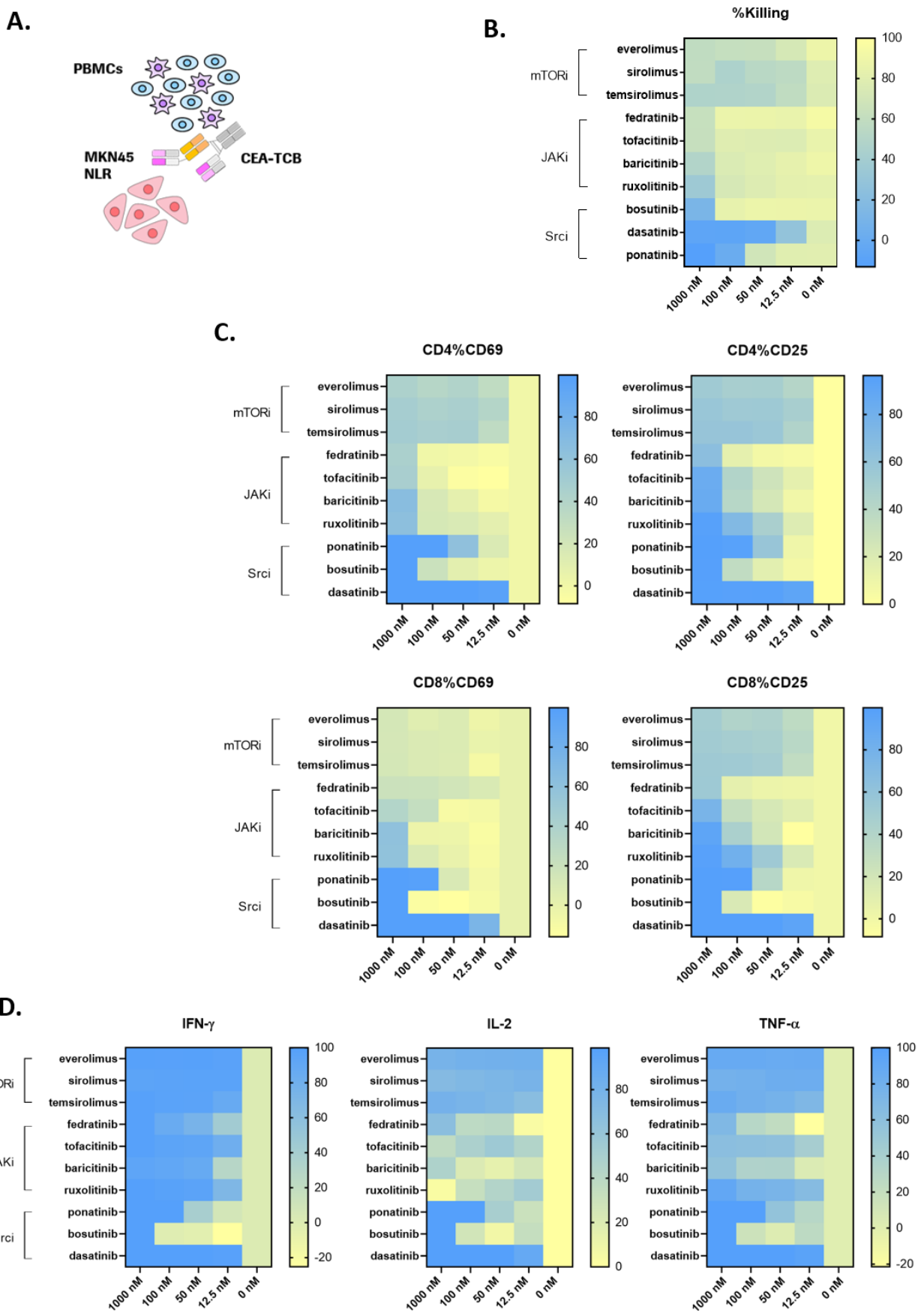

E.
CD4%CD25
CD4%CD69
CD8%CD25
CD8%CD69
KI concentration [nM]
fedratinib
tofacitinib
F.
CD4%CD25
CD4%CD69
CD8%CD25
CD8%CD69
KI concentration [nM]
baricitinib
ruxolitinib


G.
IFN-γ
10 nM CEA-TCB
IFN-γ (pg/mL)
IL-2
10 nM CEA-TCB
IL-2 (pg/mL)
TNF-α
10 nM CEA-TCB
TNF-α (pg/mL)
GM-CSF
10 nM CEA-TCB
GM-CSF (pg/mL)
IL-6
10 nM CEA-TCB
IL-6 (pg/mL)
KI concentration [nM]
KI concentration
baricitinib
ruxolitinib



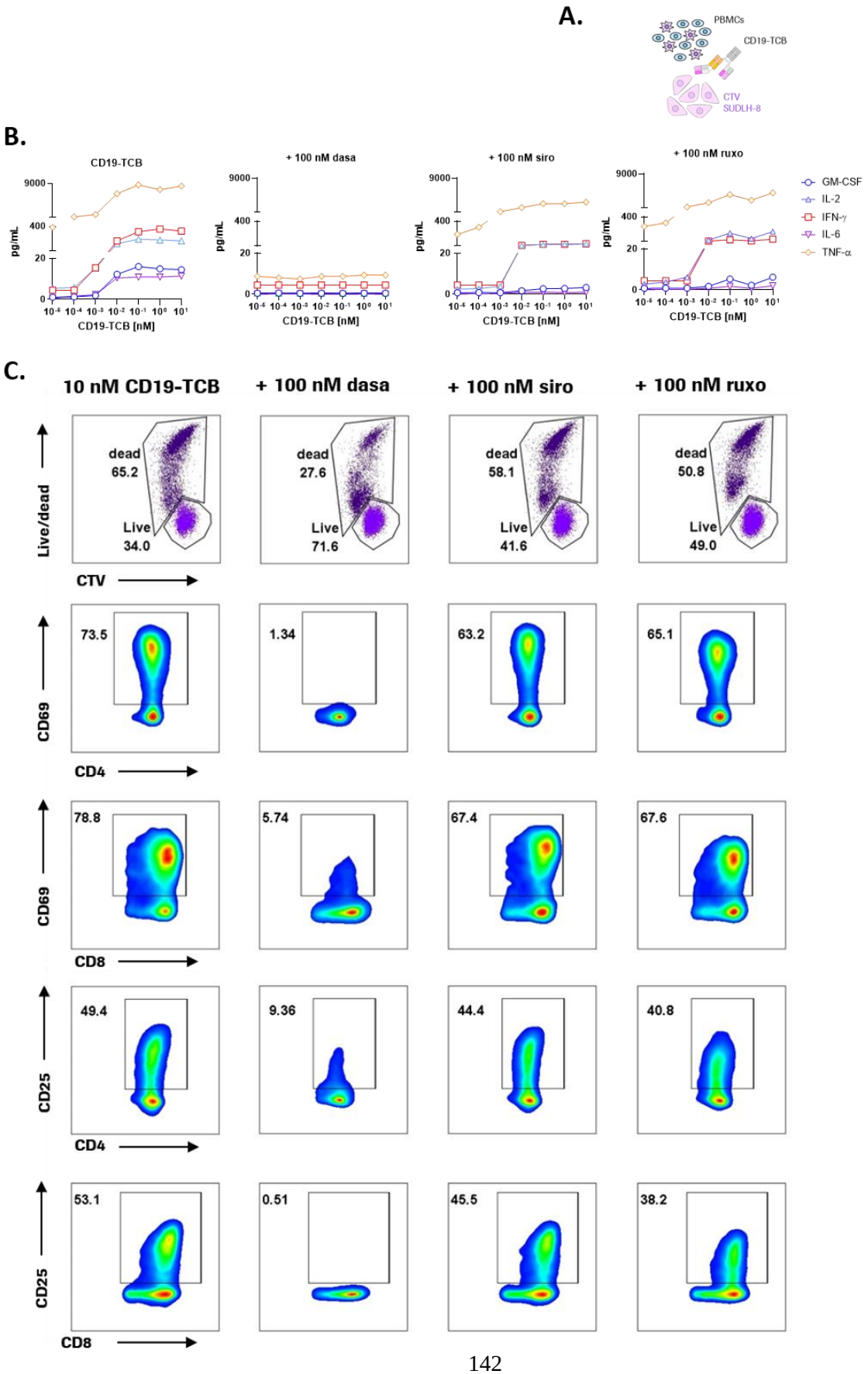
Supp Figure 5. Dose-response of ruxolitinib, tofacitinib, fedratinib and barictinib in a killing assay with CEA-TCB.

PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of JAK kinase inhibitors. The real-time killing of NLR-labelled MKN45 by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentage of killing was measured by normalizing the total red area / well to values at t = 0 hr and control wells containing the cell line, PBMCs and the TCB for each time points. Effect of escalating doses of tofacitinib (**A**), ruxolitinib (**B**), fedratinib (**C**) and baricitinib (**D**) on real-time killing of NLR-labelled MKN45 cells by 10 nM CEA-TCB, mean of technical replicates + SEM for 1 donor representative of 2. (**E, F**) The expression of CD25 and CD69 on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry (72 hrs) as a readout of T cell activation for 1 donor representative of 2.(**G, H**) The levels of IFN-γ, TNF-α, IL-2. IL-6 and GM-CSF were measured in the supernatants (72 hrs) by Luminex for 1 donor representative of 2.


A.
PBMCs
MKN45
NLR
CEA-TCB
B.
%Killing
mTORi
JAKi
Srci
everolimus
sirolimus
temsirolimus
fedratinib
tofacitinib
baricitinib
ruxolitinib
bosutinib
dasatinib
ponatinib
1000 nM
100 nM
50 nM
12.5 nM
0 nM
C.
CD4%CD69
CD4%CD25
CD8%CD69
CD8%CD25
D.
IFN-γ
IL-2
TNF-α

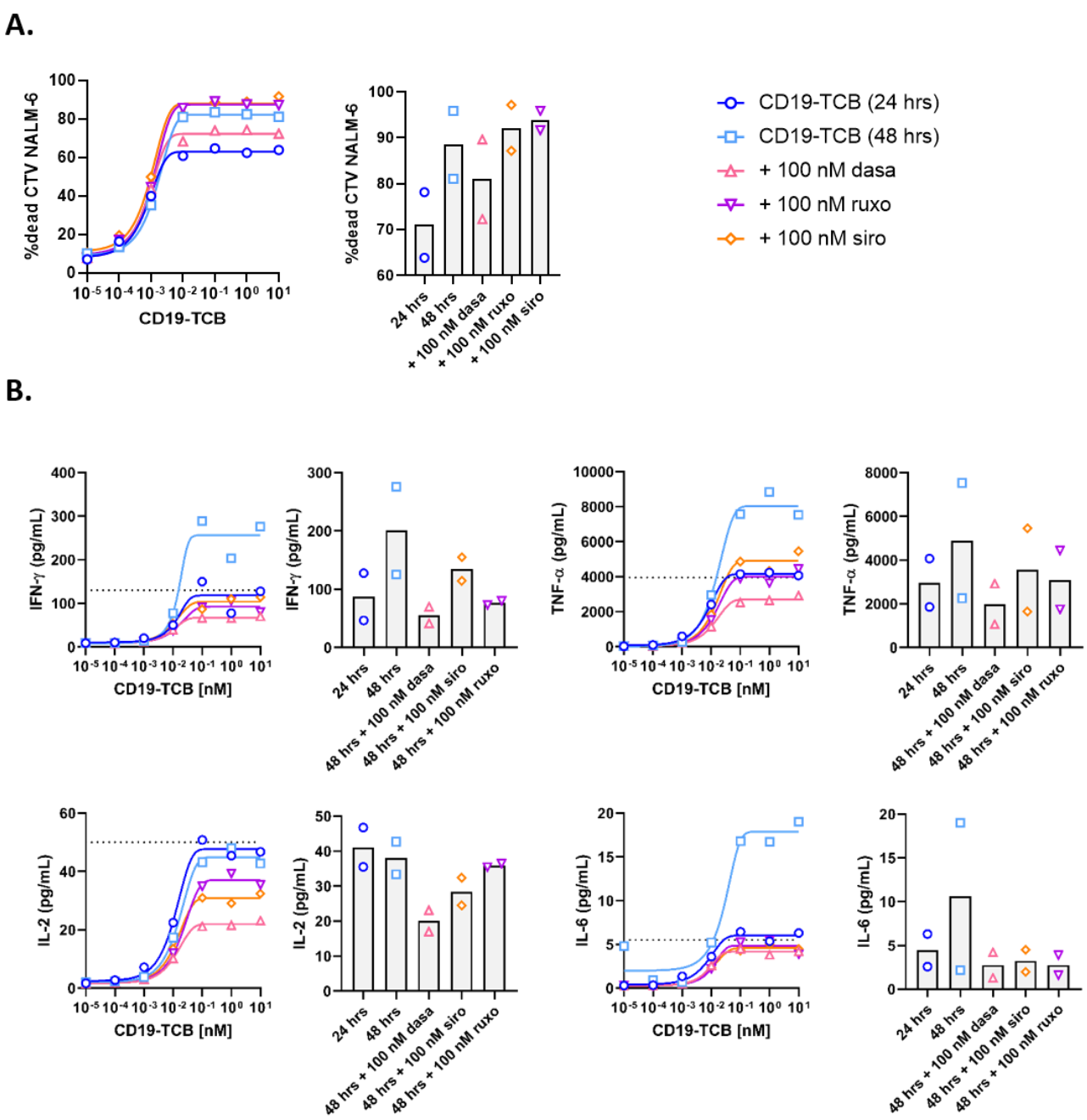
Supp. Figure 6. Effect of mTOR, JAK and Src inhibitors on CEA-TCB-mediated target cell killing and T cell activation vs. cytokine release.

(**A**) PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of kinase inhibitors. The real-time killing of NLR-labelled MKN45 by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentage of killing was measured by normalizing the total red area / well to values at t = 0 hr and control wells containing the cell line, PBMCs and the TCB for each time points for each time points. (**B-C**) Heat maps summarizing the effect of escalating concentrations of Src, MEK, mTOR and JAK inhibitors on CEA-TCB-induced tumor cell killing, T cell activation, and cytokine release at 72 hrs. Technical replicates were pooled and the expression of CD69 and CD25 on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry at 72 hrs. The culture supernatants from technical replicates were pooled and the levels of TNF-α, IFN-γ, IL-2, IL-6 and GM-CSF were measured by Luminex (72 hrs). 1 donor representative of 2.


A.
PBMCs
CD19-TCB
CTV
SUDLH-8
B.
CD19-TCB
+ 100 nM dasa
+ 100 nM siro
+ 100 nM ruxo
pg/mL
CD19-TCB [nM]
GM-CSF
IL-2
IFN-γ
IL-6
TNF-α
C.
10 nM CD19-TCB
+ 100 nM dasa
+ 100 nM siro
+ 100 nM ruxo
Live/dead
dead
65.2
Live
34.0
dead
27.6
Live
71.6
dead
58.1
Live
41.6
dead
50.8
Live
49.0
CTV
CD69
CD4
73.5
1.34
63.2
65.1
CD69
CD8
78.8
5.74
67.4
67.6
CD25
CD4
49.4
9.36
44.4
40.8
CD25
CD8
53.1
0.51
45.5
38.2

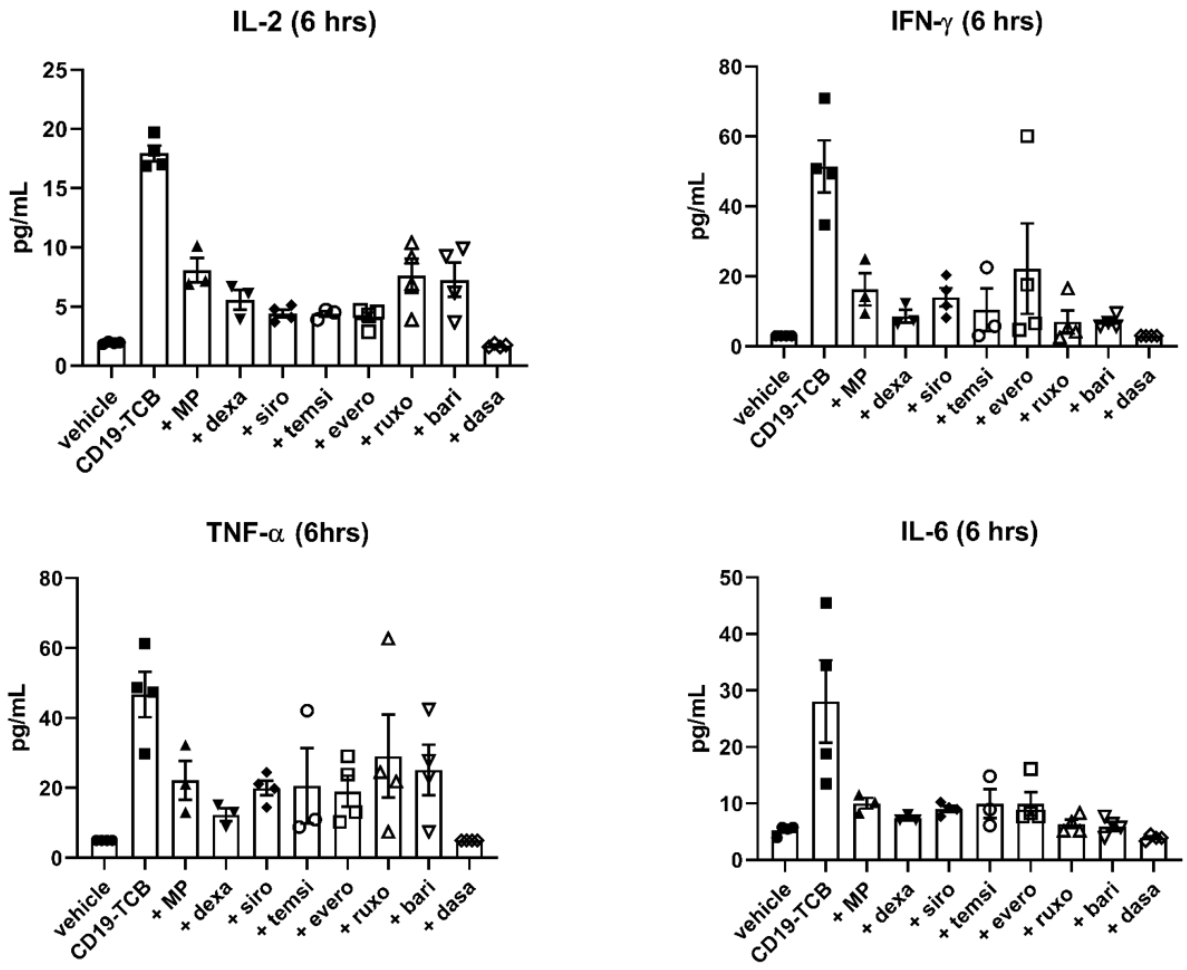
Supp. Figure 7. Effect of mTOR, JAK and Src inhibitors on CD19-TCB-induced T cell cytotoxicity, activation and cytokine release.

(**A**) PBMCs were stimulated on CTV-labelled SU-DHL-8 tumors cell by CD19-TCB in the presence of 100 nM dasatinib, 100 nM sirolimus and 100 nM ruxolitinib. (**B**) The levels of IFN-γ, TNF-α, IL-2. IL-6 and GM-CSF were measured in the supernatants (72 hrs) by Luminex, 1 donor representative of 3. The killing of SU-DHL-8 tumor cells as well as T cell activation was measured by flow cytometry at 24 hrs. (**C**) Representative flow cytometry plots of dead and live CTV-labelled SU-DHL-8 cells, CD25 and CD69 positive populations among $CD4^+$ and $CD8^+$ T cells upon treatment with 10 nM CD19-TCB in the presence and absence of 100 nM dasatinib (dasa), sirolimus (siro) and ruxolitinib (ruxo), 1 donor representative of 3.



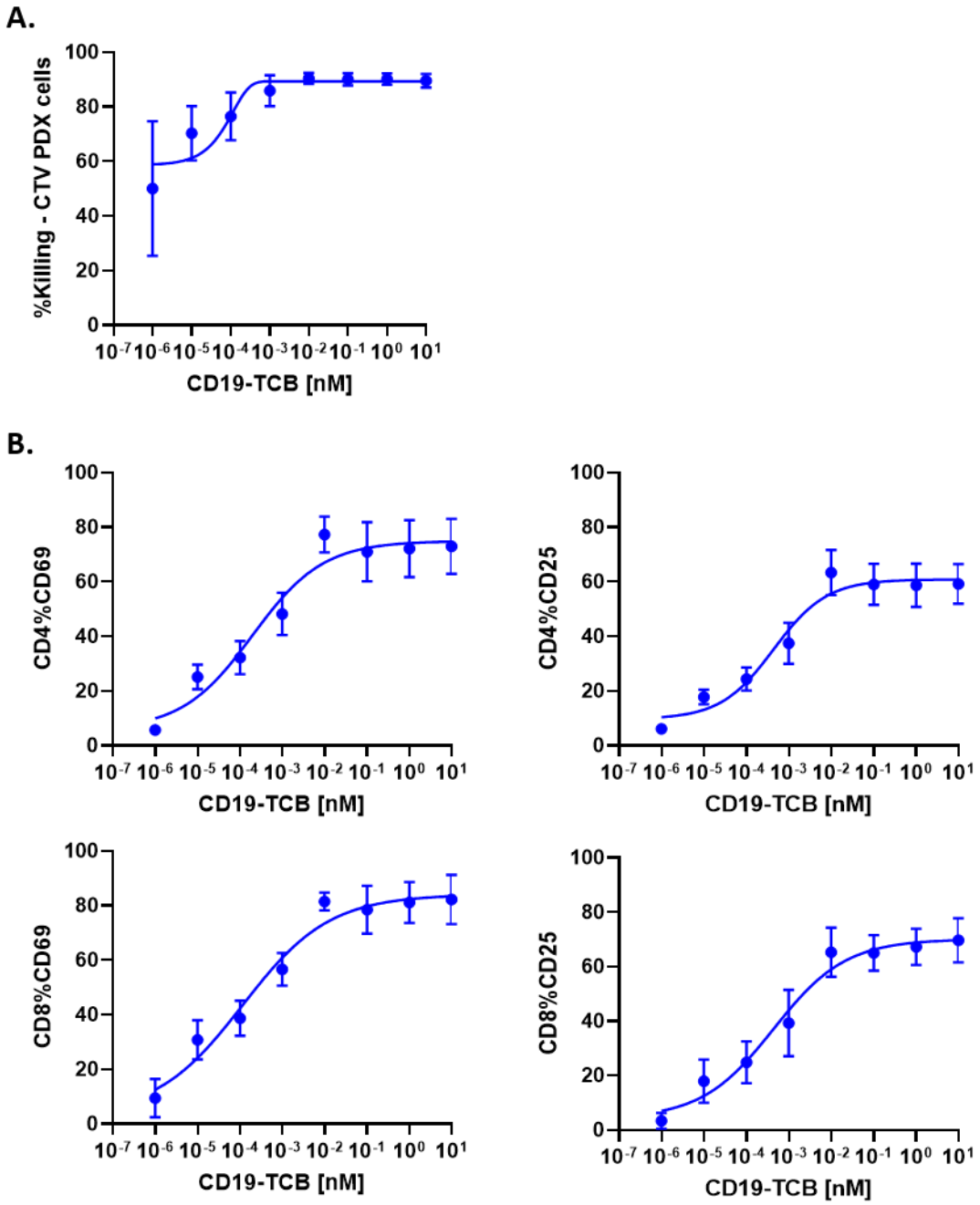
Supp. Figure 8. Effect of mTOR, JAK and Src inhibitors on activated PBMCs.

PBMCs were stimulated on CTV labelled NALM-6 tumors cell with CD19-TCB for 24 hrs. At 24 hrs, 100 nM sirolimus (siro), 100 nM ruxolitinib (ruxo) and 100 nM dasatinib (dasa) were added in the assay. (**A**) The killing of CTV labelled NALM-6 cells was measured by flow cytometry by exclusion of NIR positive cells at 24 hrs and 48 hrs. (**B**) The levels of TNF-α, IFN-γ, IL-2, and IL-6 were measured by Luminex before (24 hrs) and after addition (48 hrs) of the different kinase inhibitors in the pooled supernatants. (**A, B**) The dose-response plots depict data from 1 donor representative of 2 and the bar plot data from n=2 donors treated with 10 nM CD19-TCB.



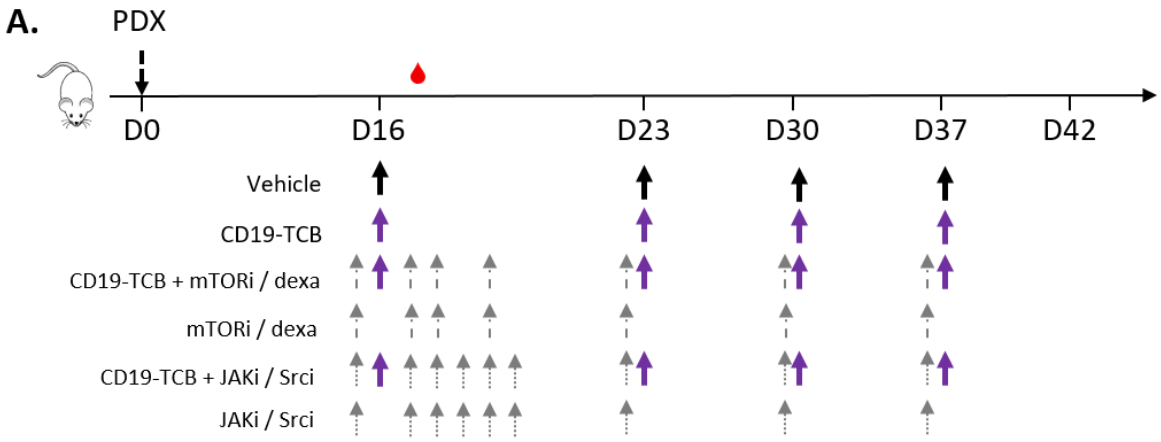
Supp Figure 9. Effect of mTOR (temsirolimus, sirolimus, everolimus), JAK (ruxolitinib and baricitinib) and Src (dasatinib) inhibitors on CD19-TCB induced cytokine release in non-tumor bearing huNSG mice.

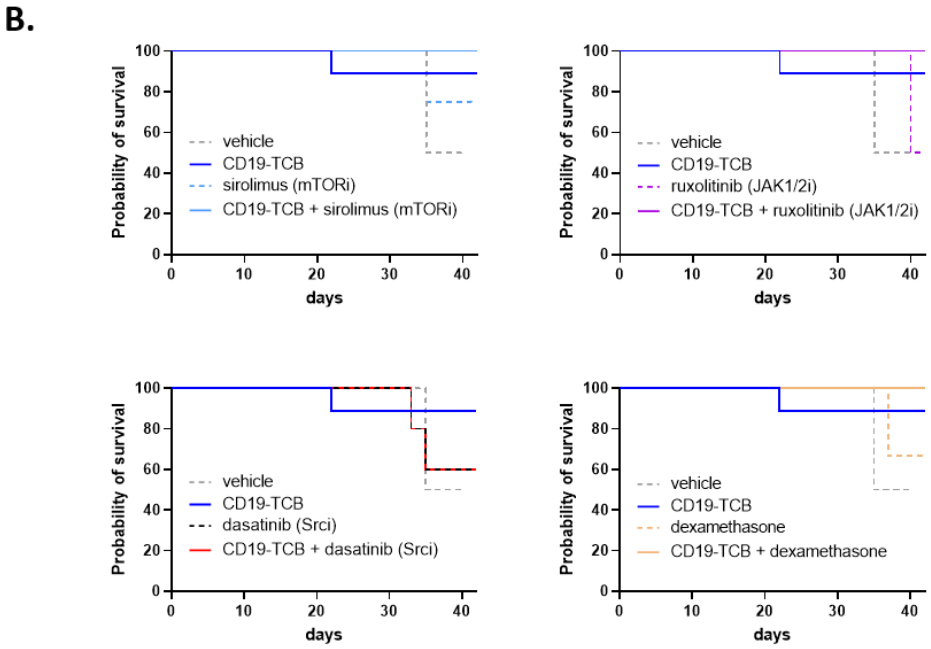
Humanized NSG mice were either pre-treated with 0.5 mg/kg CD19-TCB alone or in combination 50 mg/kg dasatinib (dasa) (p.o.), 30 mg/kg ruxolitinib (ruxo) or baricitinib (bari) (p.o), 5 mg/kg sirolimus (siro), temsirolimus (temsi) or everolimus (evero) (p.o.), 2 times 1 mg/kg, 0.5 mg/kg and 0.25 mg/kg dexamethasone (dexa) (p.o), or 2 times 10 mg/kg , 5 mg/kg, 2.5 mg/kg methylprednisolone (MP) (p.o.). Serum was collected from blood samples 6 hrs post infusion with TCB and the levels of TNF-α, IFN-γ, GM-CSF and IL-6 were measured by Luminex for n= 3-4 mice. Mean of n = 3-4 mice +/- SEM.

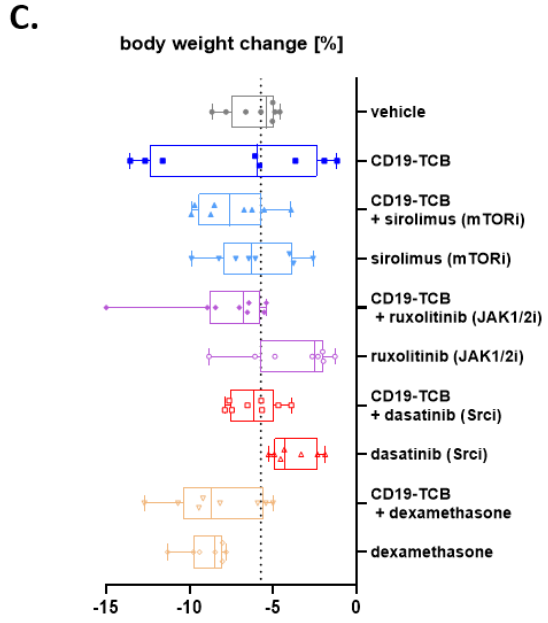


Supp. Figure 10. CD19-TCB kills lymphoma PDX cells *in vitro*.

Lymphoma PDX cells were thawed on the day of the assay, labelled with the CTV dye and cultured with PBMCs (E:T=10:1) in the presence of CD19-TCB for 24 hrs. (**A**) Killing of CTV labelled PDX cells was measured by flow cytometry, mean of n=3 PBMCs donors +/-SD. (**B**) The expression of CD69 and CD25 on CD4+ and CD8+ T cells was measured by flow cytometry as a readout for T cell activation, mean of n=3 PBMCs donors +/- SD.

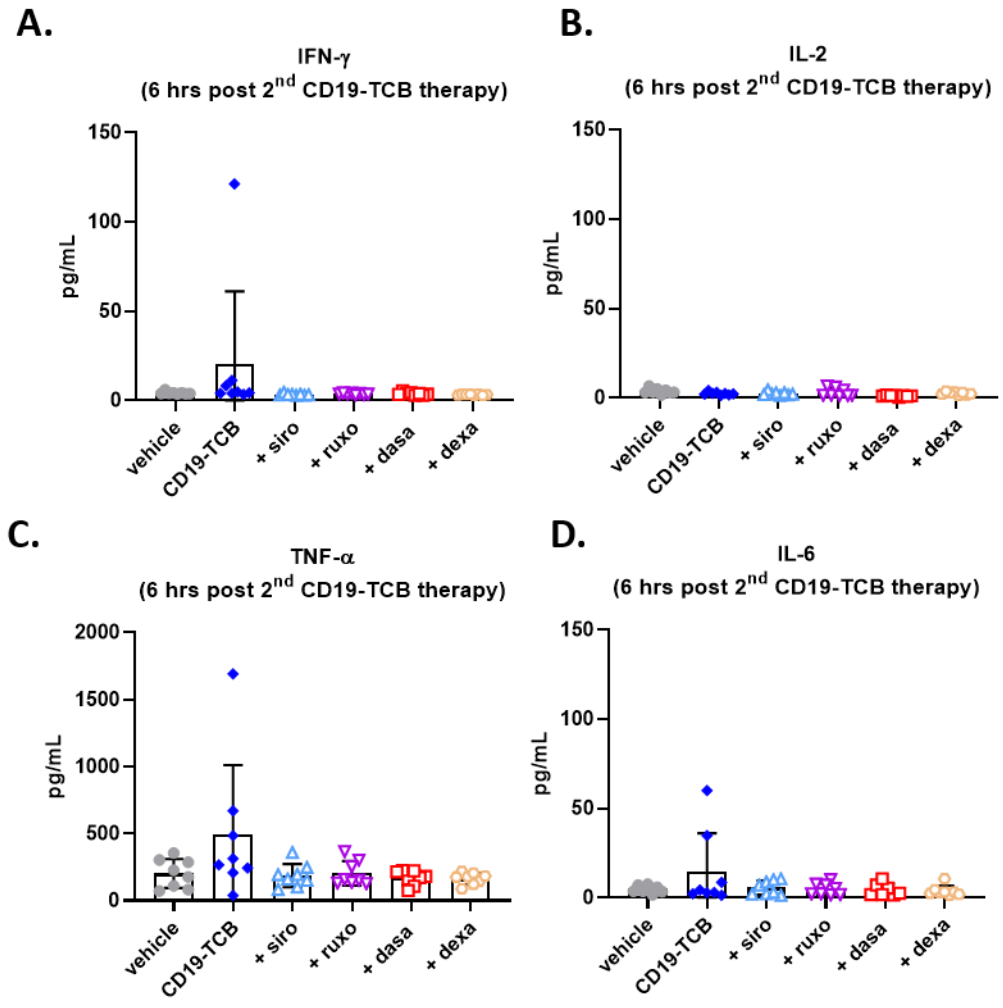

A.
PDX
D0
D16
D23
D30
D37
D42
Vehicle
CD19-TCB
CD19-TCB + mTORi / dexa
mTORi / dexa
CD19-TCB + JAKi / Srci
JAKi / Srci


B.
Probability of survival
days
vehicle
CD19-TCB
sirolimus (mTORi)
CD19-TCB + sirolimus (mTORi)
ruxolitinib (JAK1/2i)
CD19-TCB + ruxolitinib (JAK1/2i)
dasatinib (Srci)
CD19-TCB + dasatinib (Srci)
dexamethasone
CD19-TCB + dexamethasone


C.
body weight change [%]
vehicle
CD19-TCB
CD19-TCB + sirolimus (mTORi)
sirolimus (mTORi)
CD19-TCB + ruxolitinib (JAK1/2i)
ruxolitinib (JAK1/2i)
CD19-TCB + dasatinib (Srci)
dasatinib (Srci)
CD19-TCB + dexamethasone
dexamethasone

Supp. Figure 11. JAK and mTOR inhibitors co-treatment with CD19-TCB lead to 100% survival.

(**A**) *In vivo* experiment timelines and dosing schedule. Humanized NSG mice were engrafted with a lymphoma PDX (5 million cells, s.c.). When tumors reached 200 mm^3, mice were randomized in groups of 8 or 7 based on their tumor size. They were treated with vehicle (i.v.), 5 mg/kg sirolimus (p.o.), 30 mg/kg ruxolitinib (p.o.), 20 mg/kg dasatinib (p.o.), 2 times 1 mg/kg, 0.5 mg/kg, 4 times 0.25 mg/kg dexamethasone (p.o.) alone or in combination with 0.5 mg/kg CD19-TCB (i.v.), 0.5 mg/kg CD19-TCB (i.v.) as a monotherapy. (**B**) The survival curves of the different groups of mice treated with vehicle, the different kinase inhibitors, dexamethasone or CD19-TCB alone or co-treated with JAK (ruxolitinib), mTOR (sirolimus), Src (dasatinib) or dexamethasone and CD19-TCB are depicted. (**C**) Body weight loss for each individual animal. The change in body weight [%] is measured as a percentage of the body weight before first treatment with TCB for each mouse. Box and whiskers showing minimum to maximum values of n=6-8 mice per group.



Supp. Figure 12. Cytokine levels upon second treatment with CD19-TCB in lymphoma PDX bearing mice.

(**A-D**) Levels of IFN-γ, IL-2, TNF-α and IL-6 were measured in serum from blood collected 6 hrs post 2nd treatment with CD19-TCB using a multiplex cytokine kit. Mean of n=7-8 mice +/-SD.

Mitigating agent	T cell cytotoxicity	T cell activation	Cytokine release
aTNF-α			
aIL-6R			
Dexamethasone			
Methylprednisolone			
mTOR inhibitor			
JAK inhibitor			
Src inhibitor			

Supp. Figure 13. Comparative summary the *in vitro* effects of kinase inhibitors, dexamethasone, anti-TNF-α and anti-IL-6R on T cell cytotoxicity, T cell activation and cytokine release. Red shows an inhibitory effect, blue shows no effect.

Supplementary material and methods

Cell culture

The NLR-labelled MKN45 cell line is an adherent human gastric cancer cell line (DMSZ, ACC409). It was transduced with a vector coding for histone-staining red fluorescent protein. The cells were cultured in RPMI Glutamax (61870036, Gibco) containing 10% FBS (26140079, Gibco) and split every three to four days (50 000 cells / cm^2). The cells were plated 1 day prior to the assay. NLR-labelled MKN45 cells were authenticated at Mycrosynth.

In vitro killing assay in IncuCyte

For killing assay with 5000 adherent NLR-labelled MKN45 cells / well, the assay medium was replaced with fresh medium (100 µL / well) and 50 000 effector cells / well (50 µL) were transferred to obtain a final E:T ratio of approximatively 10:1. The kinase inhibitors (10 µL) followed by the antibody solutions (50 µL) were then added to initiate killing. The assay plates were covered with lids, and placed in the incubator or IncuCyte at 37°C, 5% CO2 (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time).

Preparation of antibody solutions

CEA-TCB and CD19-TCB were prepared in assay medium. A series of 8 dilutions (1:10) was prepared by transferring and mixing 100 µL of 400 nM or 40 nM TCB solution to the subsequent wells containing 900 µL of assay medium. For *in vivo* use, CD19-TCB was formulated in histidine buffer in a 0.1 mg/mL stock solution.

Preparation of kinase inhibitor solutions

Twenty-fold concentrated solutions of the different kinase inhibitors were prepared in PBS from a 10 mM DMSO stock solution. 10 µL of the 20x solution were transferred to each well (e.g. 2000 nM solution for 100 nM final concentration, 0 nM corresponding to the DMSO control). For *in vivo* use, the different kinase inhibitors were formulated in a mix of 40% PEG400, 10 % DMSO, 5% Tween and 45 % sterile water.

Preparation of adherent tumor cells

One day before the assay adherent NLR-labelled MKN45 target tumor cells were de attached from the plate using 0.05% trypsin. Cells were washed with PBS and the count of viable cells was determined by Trypan Blue stain exclusion using EVE cell counter (>90%). Cells were suspended in pre-warmed assay medium (37°C) to obtain a cell density of 50 000 cells / mL. 100 µL of the cell suspension were transferred into a 96-flat-bottom well plate, corresponding to 5000 target cells per well.

Preparation of non-adherent tumor cells

On the day of the assay, NALM-6, SU-DHL-8 or freshly thawed lymphoma PDX cells were washed with PBS and the count of viable cells was determined by Trypan Blue stain exclusion using EVE cell counter (viability >90%). The cells were labelled with Cell Trace Violet (CTV) dye (Thermo Fisher, C34557) (5 µM, 20 min at RT), washed and suspended in pre-warmed assay medium (37°C) to obtain a cell density of 200 000 cells / mL. 100 µL of the cell suspension were transferred into a 96 U-bottom well plate, corresponding to 20 000 target cells per well.

PBMCs isolation from blood

Blood from buffy coat was diluted 1:1 with PBS and about 25 mL were layered on 15 mL of Ficoll (17-5442, GE-Healthcare) and centrifuged for 30 min at 2000 rpm without break. Lymphocytes were collected with a 10 mL pipette in a 50 mL tube, rinsed with PBS, and successively centrifuged at 1700 rpm (5 min), 1500 rpm (5 min), 1100 rpm (10 min) and 900 rpm (10 min) to remove remaining Ficoll and platelets. PBMCs from different donors were frozen in FBS containing 10% DMSO at -80°C.

9. Discussion

9.1. Mechanisms of TCB-mediated cytokine release and mitigation strategies using neutralizing anti-cytokine antibodies

In the first part of this PhD work, we attempted to gain a better understanding of the mechanisms of the cytokine release cascade induced by TCBs. We confirmed the role of T cells as the main trigger of the cytokine cascade mediated by 2+1 TCBs and of myeloid cells as amplifier cells, in line with findings from other groups (39, 42). For the first time, we described the contribution of neutrophils to TCB-mediated cytokine release, notably, with the release IL-1β, one of the key cytokines involved in the physiopathology of CRS(41). We confirmed the contribution of neutrophils to cytokine and chemokine release by single cell RNA sequencing of whole blood treated with CD20-TCB at different timepoints. This experiment also allowed to reveal the expression of IL-8 receptors, TNF-α receptors and IFN-γ receptors on neutrophils, suggesting that IL-8, TNF-α and IFN-γ might play a role in neutrophil activation and recruitment. Importantly, this work may also contribute to the identification of cellular and molecular biomarkers of CRS (Leclercq *et al.*, submitted).

After treatment with TCBs, IL-6 and IL-1β are released by myeloid cells downstream of T cell derived TNF-α. The blockade of IL-6/IL-6R or IL-1Ra therefore intervenes downstream of the cytokine cascade by mitigating IL-6 or IL1-β toxicities. Norelli *et al.* and Giavridis *et al.* developed mouse models mimicking CAR-T cell-induced CRS and ICANS and showed that IL-1Ra and IL-6R blockade prevented CRS but that only IL-1Ra blockade protected the mice from neurological adverse events in addition (40, 41). Anakinra (anti-IL1Ra antibody) then arises as an efficient drug for the mitigation of both CRS and ICANS (49, 57).

The blockade of TNF-α acting upstream of the cytokine cascade was shown to reduce monocyte-derived IL-6 and IL-1β release, in agreement with previous work showing that TNF-α is responsible for monocyte activation (39, 42). In line with our findings, the prophylactic administration of an anti-TNF-α antibody before treatment with HER2-TDB (T cell dependent bispecific antibody), both, in *in vitro* co-cultures of PBMCs and HER2-expressing cells and in immunocompetent MMTV-HER2 transgenic mice resulted in a significant decrease in HER2-TDB-mediated IL-6 and IL-1β release but not in IFN-γ release (39). This data confirms the role of TNF-α in cytokine production by monocytes, and, in addition, suggest that acting upstream of the cytokine cascade may be an efficient strategy to mitigate CRS. Whether the prophylactic anti-

TNF-α treatment with etanercept or adalimumab may affect T cell bispecific antibody long-term efficacy remains to be evaluated.

It has been shown that the blockade of T cell-derived GM-CSF prevented myeloid and T cell-induced cytokine cascade in preclinical models with CAR-T cells. *In vitro*, the blockade of GM-CSF reduced CD19 CAR T cell-mediated cytokine release. *In vivo*, GM-CSF CRISPR-knockout CD19 CAR T cells or CAR T cells combined with anti-GM-CSF (lenzilumab) reduced cytokine release and neurotoxicity in mice engrafted with patient-derived xenografts, while retaining the anti-tumor efficacy (58, 59).

IFN-γ is released by T cells upstream of the cytokine cascade and its blockade did not reduce monocyte-derived IL-6 and IL-1β *in vitro*, suggesting that TNF-α blockade may be a more efficient mitigation strategy for TCB-mediated cytokine release. IFN-γ has been shown to be responsible for macrophage activation and its blockade with emapalumab is approved for the mitigation of the macrophage activation syndrome (MAS), which is closely related to CRS upon treatment with CD19 CAR T cells (38, 60). However, IFN-γ blockade was also found to decrease the accumulation of CD8+ T cells within tumors upon treatment with an anti-HER2-TDB, by preventing the release of essential chemokines involved in T cell recruitment. These findings support that prophylactic IFN-γ blockade for the prevention of cytokine release by macrophages may affect anti-tumor efficacy when combined with T cell engagers, especially those directed against solid tumors where T cell recruitment and infiltration is a critical step (61).

Altogether, these data allowed us to propose the mechanistic model of cytokine release depicted in Figure 8.

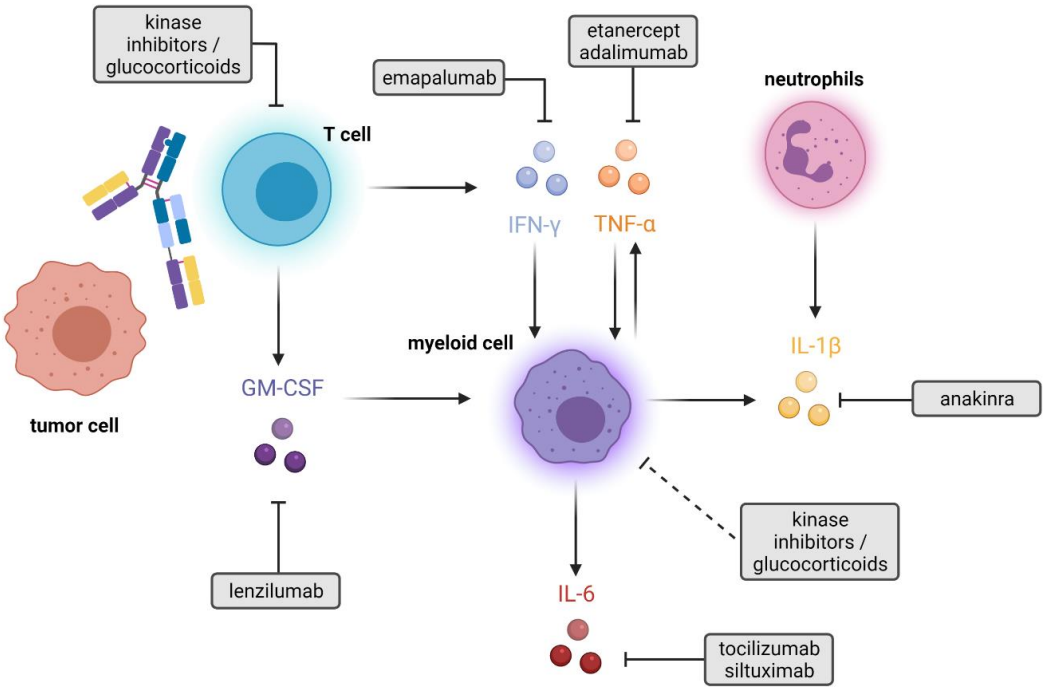


Figure 8. Mechanistic model of cytokine release induced by T cell engaging therapies and current strategies used to mitigate CRS

Based on the observation that cytokine release is mainly observed upon the first treatment cycle and attenuated upon subsequent cycles, step-up or fractionated dosing of T-cell engagers is often used to avoid large peak release of cytokines and reduce the incidence of CRS. *In vitro* experiments also show that cytokine release occurs mainly after the first TCB treatment and is considerably reduced upon restimulation (39) (Leclercq *et al.*, submitted). Lowering CD3 stimulation upon first treatment reduces this initial cytokine release without hampering T cell cytotoxicity upon restimulation. This allows to split the dose of T cell engager between several dosings at short intervals (fractionated dosing) or to increase the initial dose (step up dosing) without inducing excessive cytokine release, thereby widening the therapeutic window. The mechanism of cytokine release inhibition with the preservation of the cytotoxic activity upon restimulation with T cell engagers is not yet fully understood and requires further investigation. Our following work with kinase inhibitors has clearly shown that cytokine release could be uncoupled from T cell cytotoxicity. Other biological mechanisms explaining why T cell engagers are better tolerated by step-up dosing than flat dosing regimen remain to be investigated and

could be of interest as a follow-up of this work, helping to guide the definition of an optimal dosing schedule.

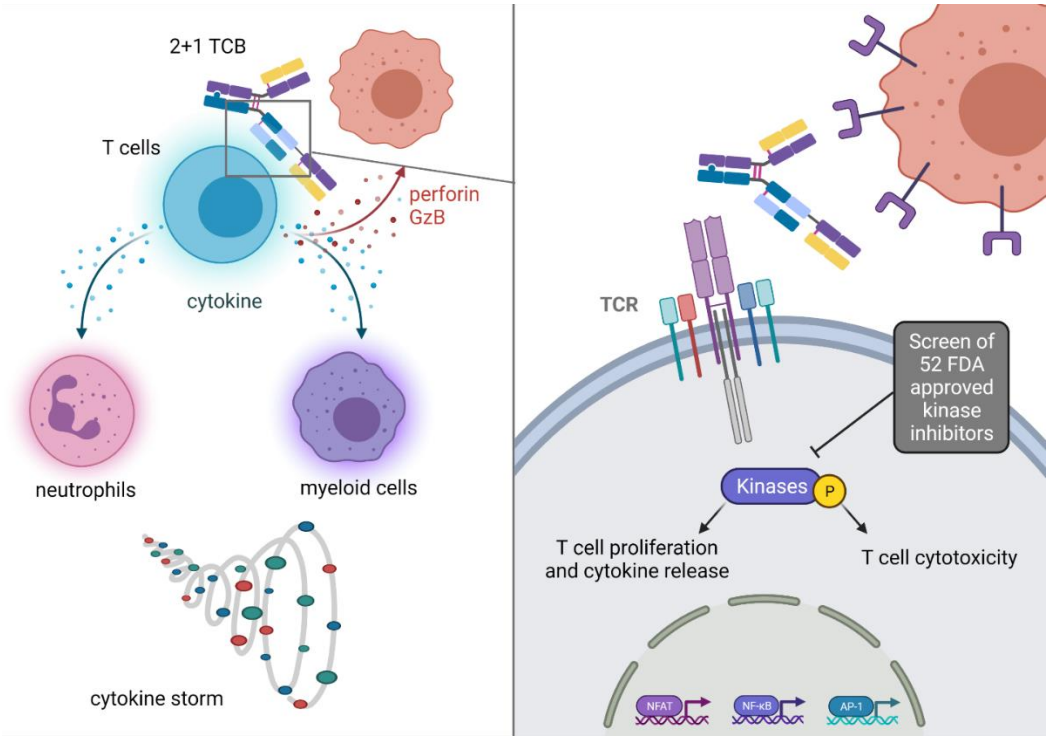


Figure 9. Schematic representation of TCB-induced signaling pathways downstream of T cell receptor (TCR) activation

The next steps of the project focused on identifying strategies that could be used to mitigate CRS by acting upstream of the cytokine cascade, at the level of T cells, to prevent further release of cytokines by myeloid cells and neutrophils, as illustrated in Figure 9.

9.2. The Src/Lck inhibitor dasatinib reversibly switches-off T cell functionality after treatment with TCBs

Based on the the work from Mestermann *et al*. and Weber *et al.* demonstrating that dasatinib could reversibly switch-off CAR-T cell functionality and cytokine release by preventing the phosphorylation of Src and Lck kinases downstream of CAR activation, we assessed the effect of dasatinib combined with 2+1 TCBs (53-55). Our data show that dasatinib reversibly switches-off TCB-mediated T cell cytotoxicity, T cell activation and cytokine release after stimulation with CEA-, CD19- or HLA-A2 WT1-TCB (Figure 10) (56, 62). The use of dasatinib was developed as a safety switch for HLA-A2 WT1-TCB, a TCR-like TCB, associated with the potential unpredictable recognition of WT1 similar peptides presented by MHC class I on healthy cells.

Additionally, it could be used to stop TCB activity in cases of high grade CRS that might occur in clinical trials.

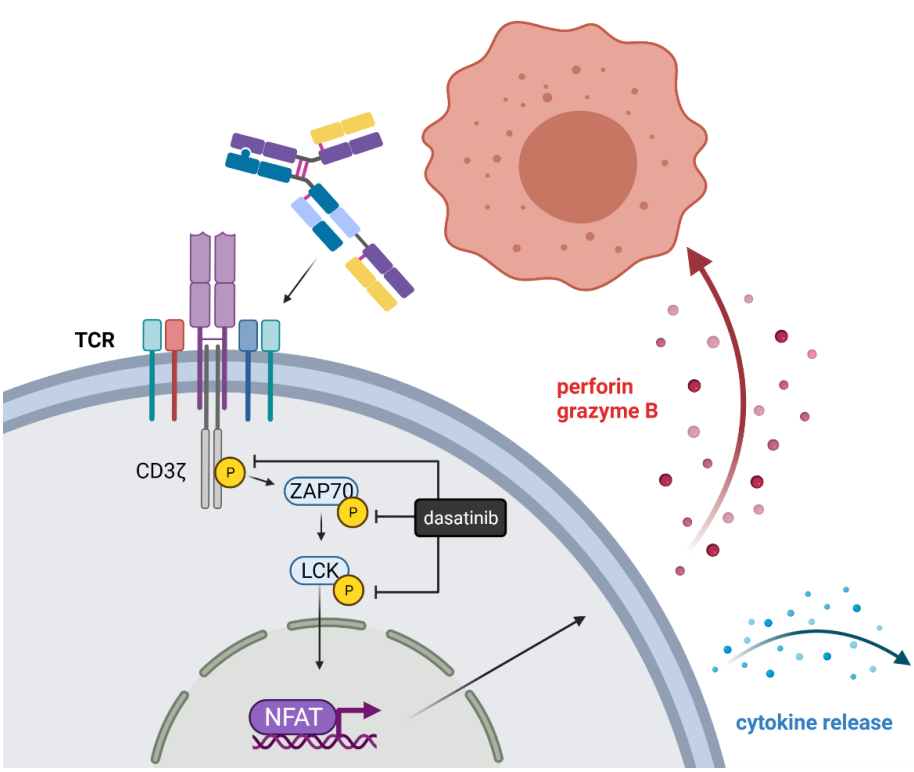


Figure 10. Mode-of-action of dasatinib

In the clinic, Foà *et al.* used dasatinib as induction therapy for 85 days in patients with Philadelphia chromosome positive leukemia, followed by a consolidation therapy with concomitant treatment with blinatumomab and dasatinib (63, 64). This approach was successful with an overall survival of 95% and a disease free survival of 88% at a median follow-up of 18 months and, importantly, associated with few toxic events. Another cohort of patients with relapsed Philadelphia chromosome positive leukemia was treated with a combination of blinatumomab and tyrosine kinase inhibitors targeted against BCR-ABL (dasatinib, ponatinib, imatinib). None of the patients from the dasatinib group developed CRS in comparison to the groups receiving other tyrosine kinase inhibitors (ponatinib, imatinib) for which grade 1 or 2 CRS events were reported. This observation suggested that dasatinib was the most efficient inhibitor to reduce incidence of CRS (15, 65). In both clinical trials, the combination of blinatumomab and dasatinib was associated with a safe and efficacious response. In the study described by Foà *et al.*, the de-bulking of tumor cells using dasatinib in the induction phase may have contributed to reduce the risk of CRS in the consolidation phase combining blinatumomab

with dasatinib. Although the *in vitro* continuous exposure of dasatinib was shown to suppress blinatumomab-mediated T cell activity, it is likely that the *in vivo* PK/PD properties of dasatinib inducing rapid ON/OFF switches during the consolidation phase may explain the safety profile of blinatumomab treatment (66). One could also hypothetize that the transient ON/OFF switches with dasatinib may have even prevented T cell exhaustion thereby enhancing T cell functionality, as recently described by Weber *et al.* in the field of CAR T cells (67). The trial reported by Foà *et al.* benefited from both the direct anti-tumor effect of dasatinib in the induction phase and its effect on cytokine release during the consolidation phase, making the treatment with blinatumomab better tolerated. In the field of T cell-engaging therapies directed against solid tumors, T-cell infiltration in the tumor is a major challenge. Since T-cell derived chemokines are known to favor T cell recruitment in the tumor, dasatinib may not be optimal in this context. Futhermore, dasatinib was shown to fully switch-off TCB-mediated T-cell cytotoxicity, in addition to cytokine release (56). Therefore, it might not be the optimal kinase inhibitor for the preventive mitigation of TCB-mediated cytokine release.

9.3. mTOR and JAK inhibitors reveal that signaling pathways involved in T cell-cytotoxicity are uncoupled from T-cell proliferation and cytokine release downstream of TCR stimulation by TCB

As a follow-up on the work on dasatinib, we aimed to assess other potential prophylactic strategies to avoid the occurrence of CRS. Based on the fact that T cell cytotoxicity and cytokine production induced by T cell engagers appeared to be independent mechanisms, we aimed to identify other kinase inhibitors that may specifically inhibit cytokine production without affecting T cell cytotoxicity (39). Following the screening of 52 FDA approved kinase inhibitors, we identified Src, mTOR and JAK inhibitors as candidates that reduced T cell proliferation and cytokine release after CD3 stimulation (Figure 11). In line with previous findings, the Src inhibitor dasatinib was found to be the most potent candidate to switch off T cell proliferation and cytokine release after TCB treatment. Importantly, the mTOR and JAK inhibitors were shown to preferentially block cytokine release while retaining T cell cytotoxicity after stimulation with TCBs. The comparison of Src inhibitors to mTOR and JAK inhibitors opens new avenues for the mitigation of CRS.

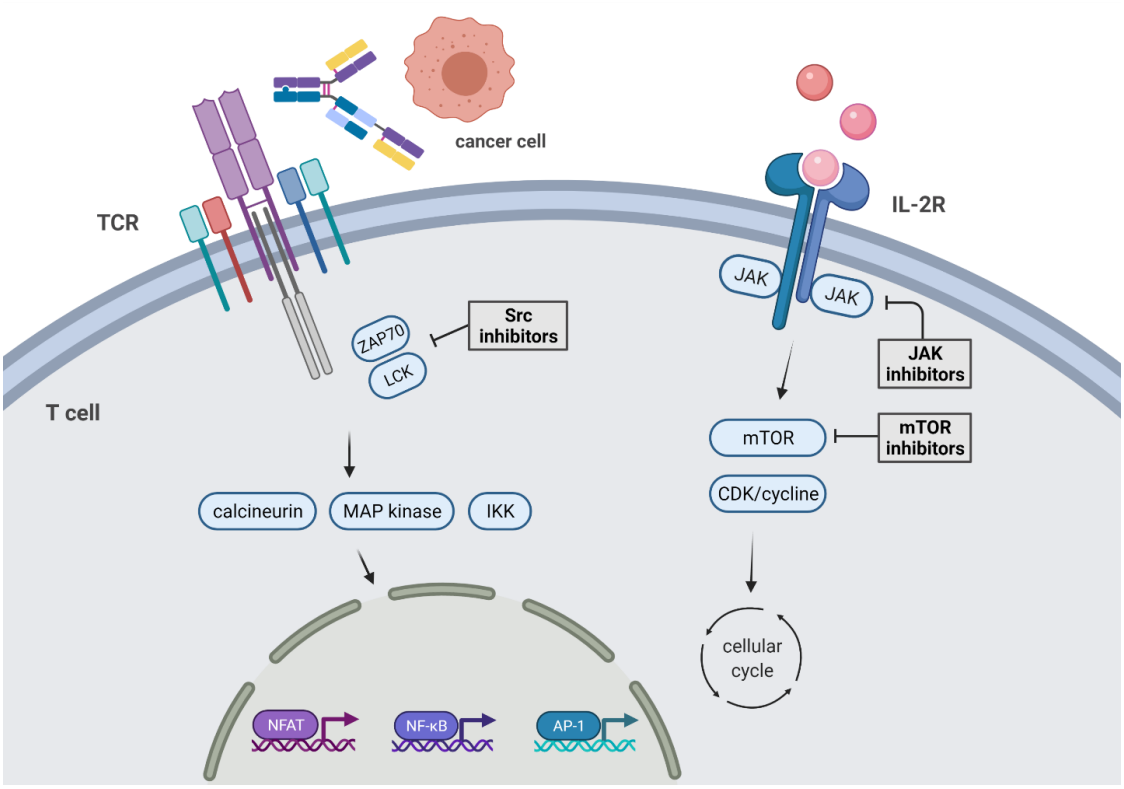


Figure 11. Representation of the targets of the different families of kinase inhibitors after stimulation with TCB

Our work on JAK inhibitors confirms pre-clinical findings from Kenderian *et al*. demonstrating that ruxolitinib prevented CRS symptoms and cytokine release after CD123 CAR T cell therapy in an acute myeloid leukemia xenograft mouse model. Mice treated with CD123 CAR T cells in combination with ruxolitinib exhibited less severe weight loss and attenuated cytokine release which did not impact treatment efficacy and was associated with long term survival (68). Itacitinib, a JAK1 inhibitor, was also shown to prevent IL-6, IFN-γ, IL-2 and IL-8 release in *in vitro* co-culture of CD19+ lymphoma cells with CD19 CAR T cells and did not impair anti-tumor activity in immunodeficient NSG mice inoculated with CD19+ expressing Nalm6 lymphoma cells (69). Itacitinib is currently evaluated for the prevention of CRS after treatment with CD19 CAR T cell directed against hematological malignancies (70). Since many cytokines involved in the cytokine release signal through the JAK/STAT pathways, there is a compelling rationale in using JAK inhibitors for the mitigation of CRS induced by T cell engaging therapies (71, 72). Whether the selective JAK1 inhibitor itacitinib is more efficient than pan JAK inhibitors such as ruxolitinib remains to be evaluated.

In the clinic, Uy *et al*. reported that prophylactic treatment with ruxolitinib decreased cytokine secretion, but did not lead to a discernable improvement in the severity of CRS in patients receiving flotetuzumab, emphasizing that ruxolitinib alone may not be sufficient to suppress CRS symptoms (73). Besides, ruxolitinib was used for the management of CRS symptoms in a patient refractory to glucocorticoids after CD22/CD19 CAR T cells treatment (74). In this study, ruxolitinib was reported to reduce the body temperature, cytokine levels (IL-6, IL-8, IL-10 and TNF-α), ferritin and CRP levels while retaining the anti-leukemic effect of CAR-T cells. This patient received dexamethasone prior to ruxolitinib, which may have enhanced the effects of ruxolitinib. In another study, ruxolitinib combined to etanercept (anti-TNF-α) was used for the management of grade 3 CRS in two patients after treatment with CD7-targeted universal CAR T cells (75).

Overall, JAK inhibitors efficiently reduce cytokine release while not affecting the efficacy of T-cell engaging therapies. However, ruxolitinib alone appears unsufficient to mitigate CRS clinical signs. The benefits of combining JAK inhibitors with other mitigating agents such as low dose glucocorticoids or anti-TNF-α antibodies remain to be evaluated for a better mitigation of clinical signs.

For the first time, we have identified mTOR inhibitors as a potential mitigation strategy preventing T-cell mediated cytokine release while not interfering with *in vivo* and *in vitro* anti-tumor efficacy after treatment with T-cell bispecific antibodies (Leclercq *et al*. submitted). Based on the *in vitro* comparison with JAK and Src inhibitors as well as glucocorticoids, mTOR inhibitors (sirolimus, temsirolimus, everolimus) appear as the most attractive candidates that retain tumor cell killing while strongly reducing cytokine release. In the lymphoma PDX-bearing humanized NSG mouse model used in our study, sirolimus favorably prevented CD19-TCB-mediated cytokine release while minimally affecting efficacy, comparably to dexamethasone. Since mTOR inhibitors stand-alone are used as anti-tumor agents in various solid cancers, their combination with TCBs to prevent CRS while maintaining efficacy may be of particular interest in such indications (76). We showed that mTOR inibitors are comparable to glucocorticoids in reducing TCB-induced cytokine release but have slightly different effects on T cell cytotoxicity. *In vitro,* mTOR inhibitors do not seem to interfere with TCB killing efficacy unlike dexamethasone. *In vivo,* both agents had a direct anti-tumor effect in lymphoma PDX-bearing huNSG mice, which made it difficult to assess how much they might interfere with TCB-

mediated T-cell cytotoxicity. Based on our results, mTOR inhibitors have a milder inhibitory effect on T-cell cytotoxicity than glucocorticoids, however this remains to be further investigated *in vivo.* For instance, one could look at T cell infiltration early after first treatment with TCB in the presence and absence of glucocorticoids or mTOR inhibitors. So far, mTOR inhibitors will most likely not replace glucocorticoids for the mitigation of CRS in the clinic. Nevertheless, refractory patients to glucocorticoids may benefit from them. Additionally, it could be of interest to evaluate the combination of low-dose glucocorticoids with sirolimus for the mitigation of TCB-induced cytokine release.

The *in vitro* comparison of mTOR and JAK inhibitors to Src inhibitors confirmed that signaling pathways involved in TCB-mediated cytokine release and T cell proliferation are uncoupled from T cell cytotoxicity after TCR stimulation with TCB. The use of mTOR inhibitors appears promising in TCB programs against solid cancer indications where they may have a direct anti-tumor effect in addition to mitigating CRS. In contrast, JAK inhibitors may need to be combined with other treatments to efficiently prevent CRS symptoms. This question remains to be investigated.

9.4. Future directions: towards engineering of safer T-cell engagers

Our work reveals the uncoupling of T cell cytotoxicity and cytokine release after TCR stimulation. One of the critical features of T-cell engagers is the affinity of the CD3 binder (77-79). *In vitro,* lower CD3 binder affinity is associated with reduce cytokine release and target cell killing. Nevertheless, this effect is not observed *in vivo* where low CD3 binder affinity retains anti-tumor efficacy while reducing cytokine release, enhancing the safety profile of T cell engagers. This appears to be linked to more favorable *in vivo* bio-distribution, leading to increased concentration of the T cell engager in the tumor and resulting in better anti-tumor efficacy (80). Additionally Dang *et al* described that lower CD3 binder affinity does not trigger the activation and proliferation of Tregs, preventing their infiltration in tumor tissues (81). For HER2-TDB, high affinity to the tumor-associated antigen was a critical parameter for *in vitro* and *in vivo* efficacy. Nevertheless, it also correlated with a more severe toxicity profile, including higher cytokine release and damages to normal tissues expressing HER2 antigens. Altogether, fine-tuning the affinities to both, the tumor target and the CD3, appears to be critical in maximizing the therapeutic window of novel T cell engagers.

Along those lines, new formats of TCBs are currently being engineered and evaluated in an attempt to increase their tolerability by avoiding off-tumor activity. One approach relies on protease activated TCBs where masking of the anti-CD3 Fab fragment with an anti-idiotypic mask was proven to enhance selectivity and safety of TCBs, as the mask needs to be cleaved by tumor-specific proteases to activate the TCB (82). Another approach relies on pH-dependent TCBs engineered with the Conditionally Active Biologic (CAB) technology, where the CD3 binder is only active under acidic intra-tumoral pH and remains inactive in healthy tissues (83-85).

10. Conclusion

To sum-up, our work provides a better mechanistic understanding of TCB-mediated cytokine release and highlights the contribution of neutrophils as a novel cellular biomarker of CRS. It supports that TCB-mediated cytokine release is initiated by T-cell derived cytokines, in particular TNF-α, which further amplifies the cascade. Therefore, the use of mitigation strategies blocking T-cell derived cytokine release may be attractive to prevent CRS after treatment with TCBs. The comparison of mTOR and JAK inhibitors to Src kinase inhibitors confirms the uncoupling of T cell cytokine release and cytotoxicity, opening new avenues for the mitigation of TCB safety liabilities. The Src inhibitor dasatinib could be used as an antidote to treat high grade CRS or adverse events related to on-/off-target off-tumor activity, as described in the specific case of TCR-like TCBs (e.g. HLA-A2 WT1-TCB). Along those lines, the prophylactic use of the mTOR or JAK inhibitors would rather stand as a strategy to reduce cytokine release upon first infusion with TCB while retaining T-cell cytotoxicity.

11. Curriculum vitae

Resume - Gabrielle Leclercq

Junior scientist in immunology and engineer in chemical engineering, looking for opportunities in the field of pharmaceutical sciences.

Personal information

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Work experience

2018 – Now **PhD student, Roche Glycart**, Schlieren, Switzerland

Roche Innovation Center Zürich (RICZ) & University of Basel.
Supervisors: Dr. H. Haegel, Pr. A. Odermatt

- Investigating cellular and molecular mechanisms involved in T cell bispecific antibody-induced cytokine release
- Mitigation strategies to prevent Cytokine Release Syndrome (CRS) in the context of T cell bispecific antibodies
- *In vitro* skills: cell culture, immune cell isolation, multiplex cytokine analysis, multi-color flow cytometry, IncuCyte, ScRNA sequencing.
- *In vivo* skills (mouse): LTK1 certificate, monitoring, i.v./i.p./s.c/p.o. injections

2016-2018 **Associate scientist** - **Merck**, Aubonne, Switzerland

Departement of Bioprocess Sciences (BPS)

- USP (Upstream process) development to produce drug substance for clinical supply
- Process scale-up and transfer of technology (mammalian cell amplification, production in 2L, 200L, 2000L single use bioreactors)
- GLP and GMP documentation

2015 **Intern (6 months)** - **Merck**, Corsier-sur-Vevey, Switzerland

Departement of Bioprocess Sciences (BPS)

- High throughput screening to identify substances enhancing the glycosylation profile of monoclonal antibodies.

2013 **Intern** - **Roche Diagnostics**, Penzberg, Germany

Department of rare reagents and surface treatment

- Iron oxide magnetic particles synthesis using high pressure batch reactors

Education

2014-2016	Master of Science in Chemical Engineering and Biotechnology at Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
2016	Master thesis at Ecole polytechnique de Montréal, Montréal, Canada
2013-2014	Erasmus exchange program at the Technische Universität München (TUM), Munich, Germany
2011-2014	Bachelor of Science in Chemistry and Chemical Engineering at Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
2011	Scientific Baccalauréat with honors, Lycée Charles Nodier, Dole, France

Languages: English (fluent), german (conversational), french (native speaker)

Softwares: gSuite, MS Office, Abode, FlowJo, GraphPad Prism, IncuCyte

Trainings

- Master course in immunotherapy, Université Paris Descartes, 2020
- High dimensional parameter flow cytometry course from BD
- LTK1 for *in vivo* experimental work
- European Network of Immunology Institutes (ENII) summer school, May 2019

Conferences

Cytokine Release Syndrome Summit, internal Roche meeting, 2019 and 2020

- Contribution to the presentation of pre-clinical data

Society of Cancer Immunotherapy (SITC), November 2020

- Short oral presentation and poster: The Src/Lck inhibitor dasatinib reversibly switches off cytokine release and T-cell cytotoxicity following stimulation with T-cell bispecific antibodies

pRED Sciences Days, Internal Roche meeting, March 2021

- E-poster - young scientist session: Uncoupling of T-cell bispecific antibody-induced cytokine release and cytotoxicity revealed by differential activities of mTOR, JAK1/2 and Src inhibitors

Annual meeting of the association for Cancer Immunotherapy, CIMT, May 2021

- Short E-talk: Uncoupling of T-cell bispecific antibody-induced cytokine release and cytotoxicity revealed by differential activities of mTOR, JAK1/2 and Src inhibitors

Immunotherapy of Cancer Conference 8 (ITOC8), October 2021

- E-poster - young researcher session: The use of FDA approved JAK, mTOR and Src inhibitors to regulate T cell-bispecific antibody-induced cytokine release while retaining T cell cytotoxicity

BioSafe Meeting, November 2021(oral presentation)

Manuscripts

G. Leclercq, H. Haegel, A. Schneider, A. Giusti, E. Marrer-Berger, C. Boetsch, A. Walz, V. Pulko, J. Challier, J. Sam, C. Ferlini, A. Odermatt, M. Bacac, P. Umaña, C. Klein. **Src/Lck inhibitor dasatinib reversibly switches off cytokine release and T-cell cytotoxicity following stimulation with T-cell bispecific antibodies**, 2021 JITC.

G. Leclercq, L. Alberti-Servera, S. Danilin, J. Challier, N. Steinhoff, C. Bossen, A. Odermatt, V. Nicolini, P. Umaña, C. Klein, M. Bacac, A. Giusti, A. Schneider, H. Haegel. **Dissecting the mechanism of cytokine release induced by T-cell engagers highlights the contribution of neutrophils,** 2021 (*manuscript submitted to Oncoimmunology*)

G. Leclercq, H. Haegel, A. Toso, T. Zimmermann, Luke Green, N. Steinhoff, J. Sam, V. Pulko, A. Schneider, A. Giusti, J. Challier, A. Freimoser-Grunschober, L. Larivière, A. Odermatt, M. Stern, P. Umaña, M. Bacac, C. Klein. **The use of FDA approved JAK, mTOR and Src inhibitor to regulate T cell-bispecific antibody-induced cytokine release while not preventing T cell cytotoxicity**, 2021 (*manuscript accepted at JITC*)

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