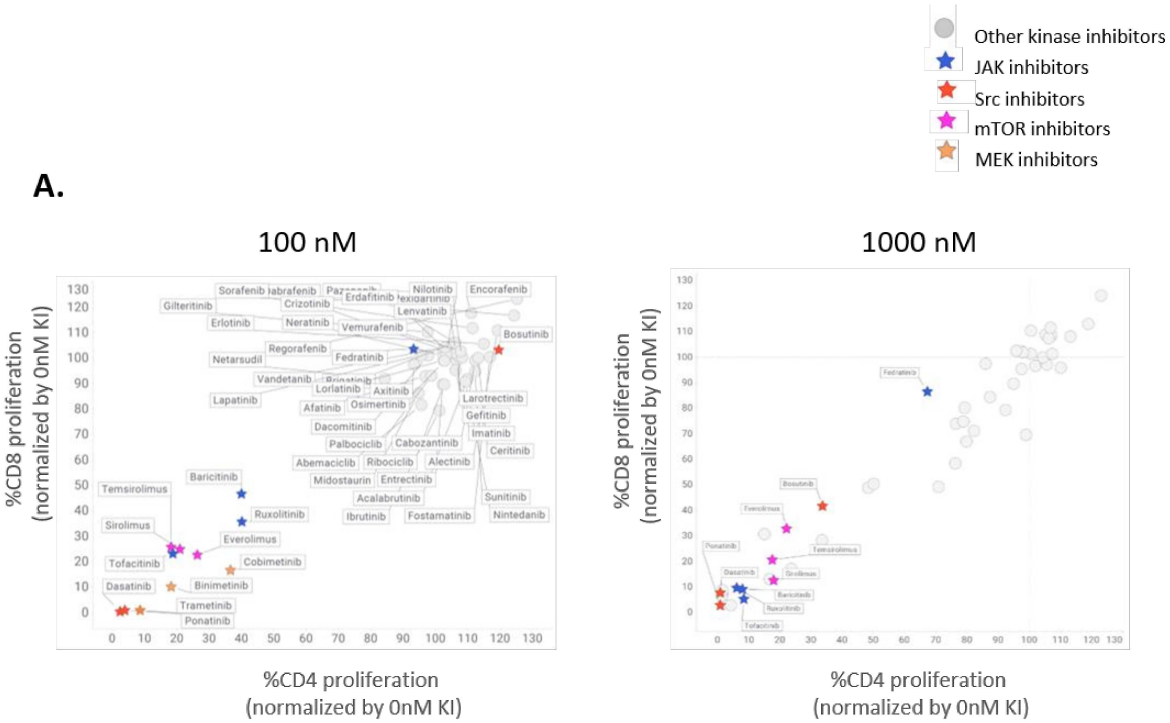
Supplementary material



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).

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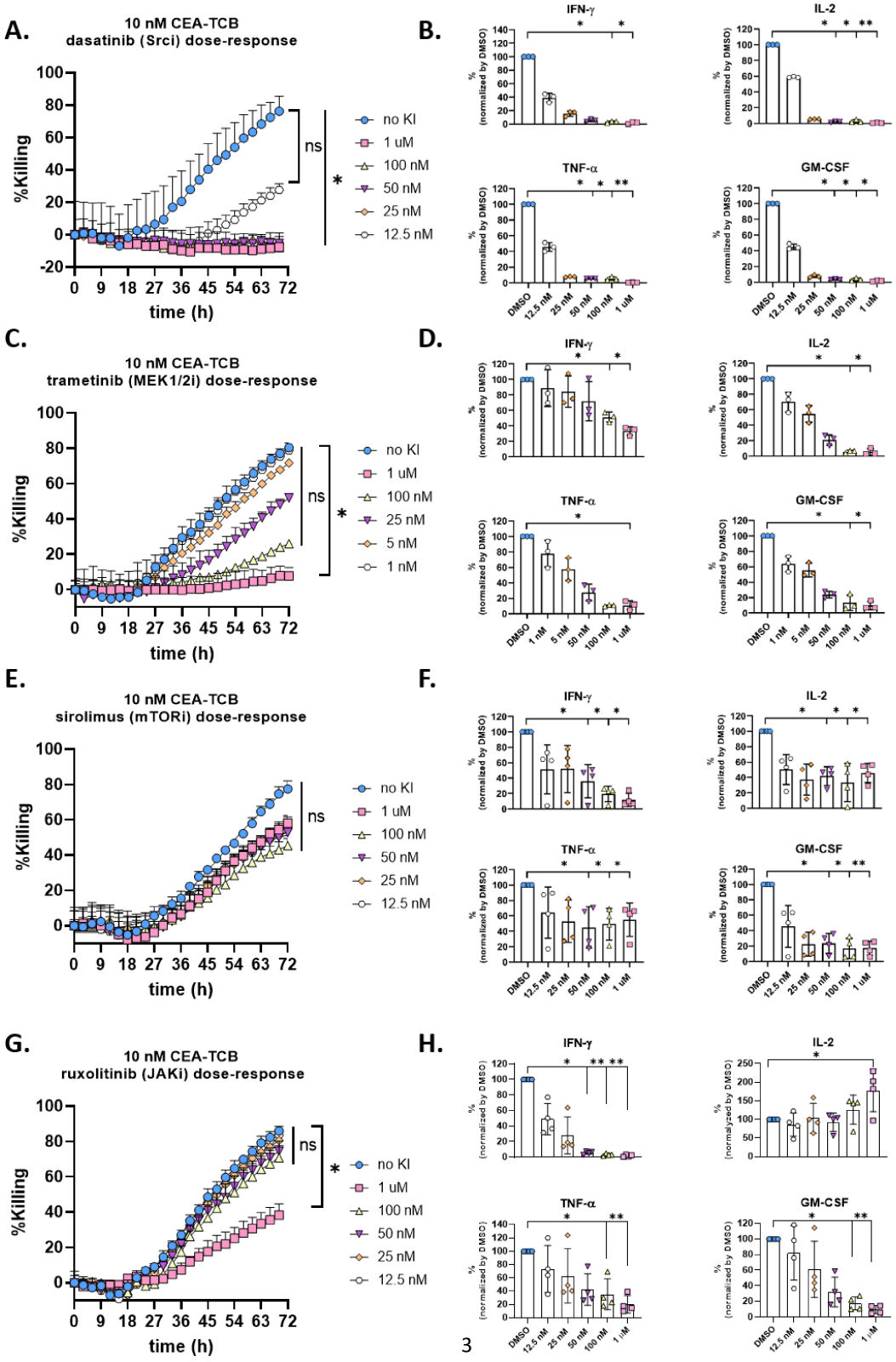
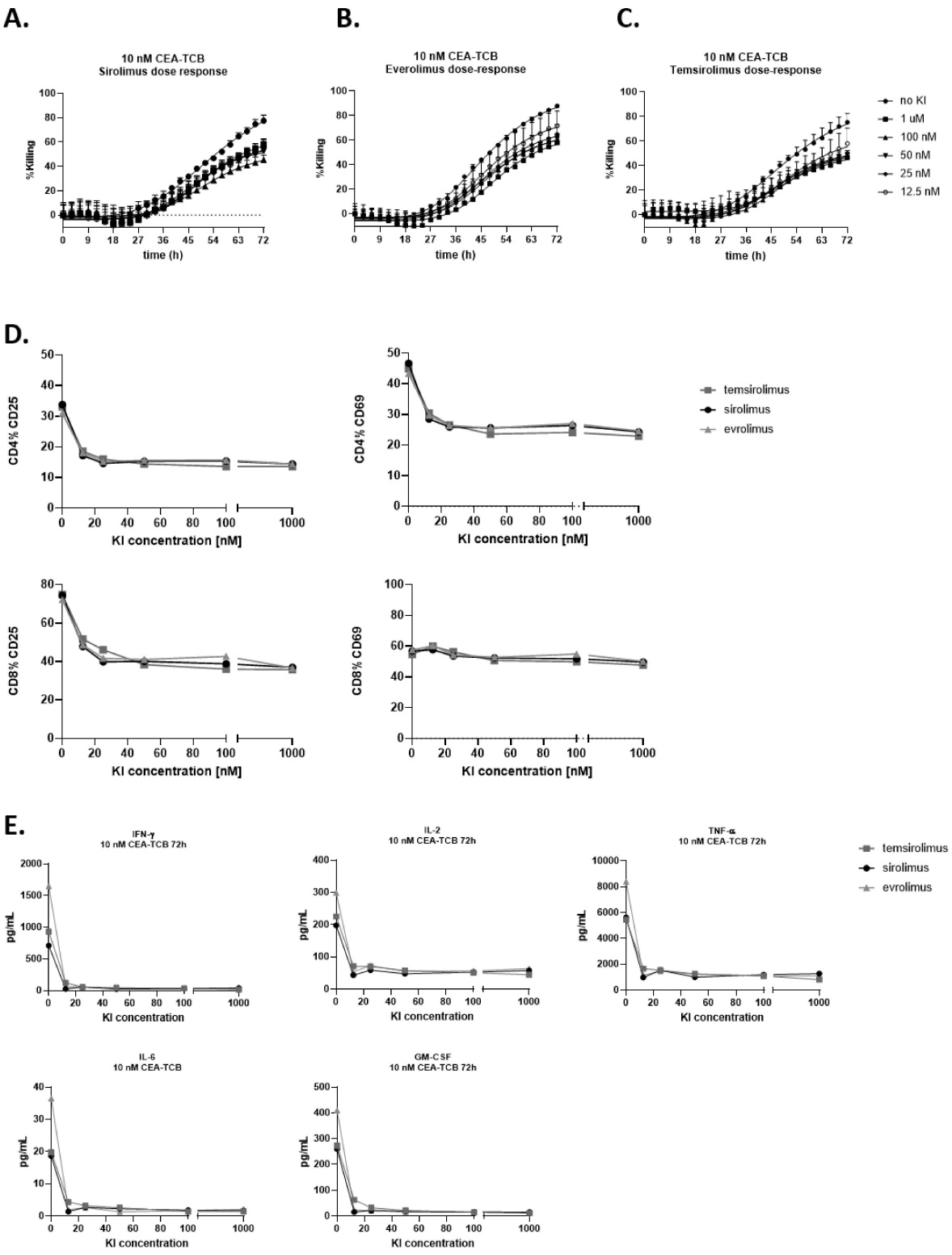

A.
10 nM CEA-TCB
dasatinib (Srci) dose-response
%Killing
time (h)
ns
*
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
B.
IFN-γ
IL-2
TNF-α
GM-CSF
% (normalized by DMSO)
DMSO
12.5 nM
25 nM
50 nM
100 nM
1 uM
C.
10 nM CEA-TCB
trametinib (MEK1/2i) dose-response
25 nM
5 nM
1 nM
D.
E.
10 nM CEA-TCB
sirolimus (mTORi)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

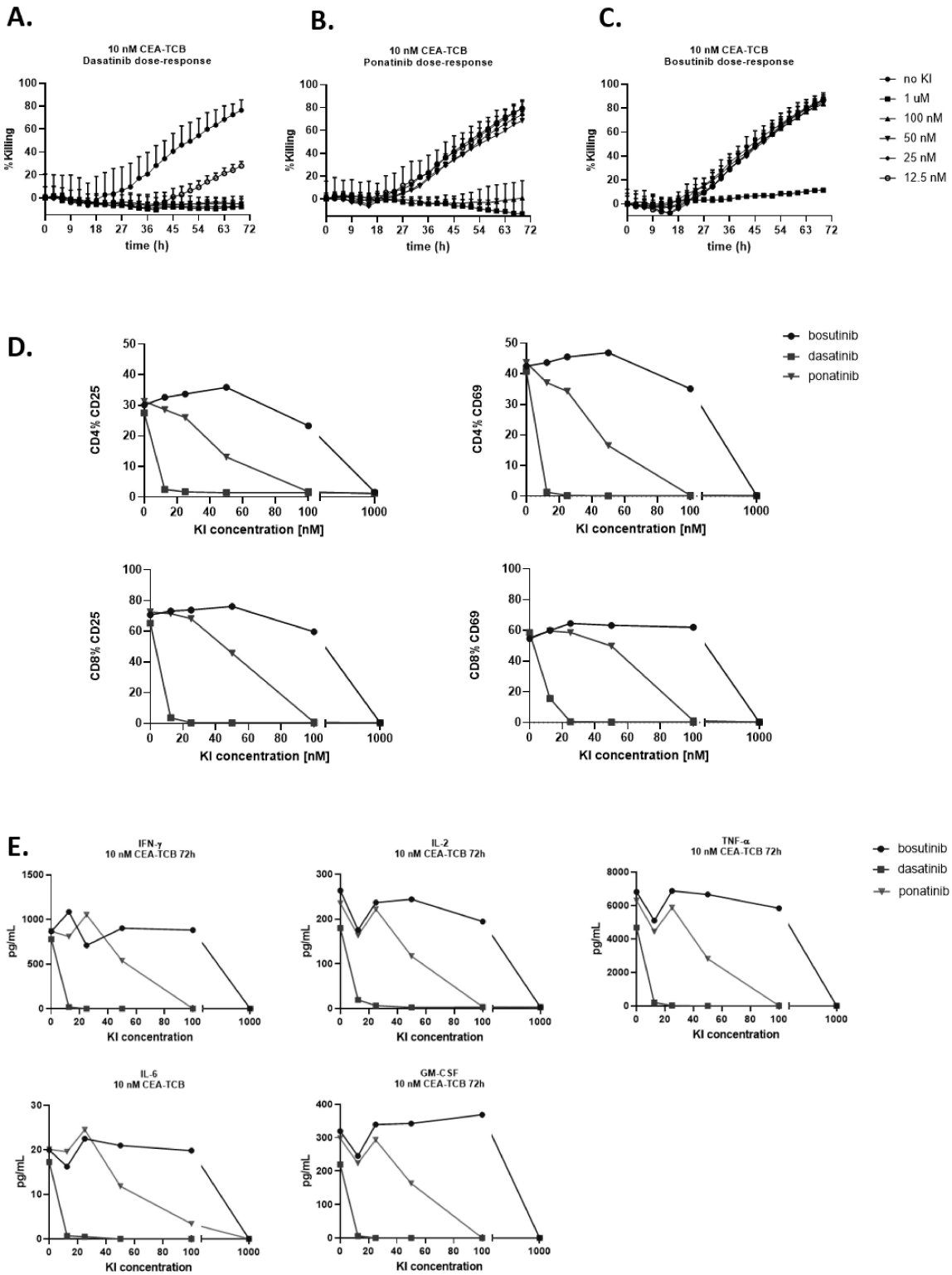
Supp. Figure 2. Validation of the top kinase inhibitor candidates on CEA-TCB-induced tumor cell killing and cytokine release.

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 +/-SD 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 * $p \leq 0.05$, ** $p \leq 0.01$ by 1 way ANOVA (Friedman test).


A.
10 nM CEA-TCB
Sirolimus dose response
%Killing
time (h)
B.
10 nM CEA-TCB
Everolimus dose-response
%Killing
time (h)
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

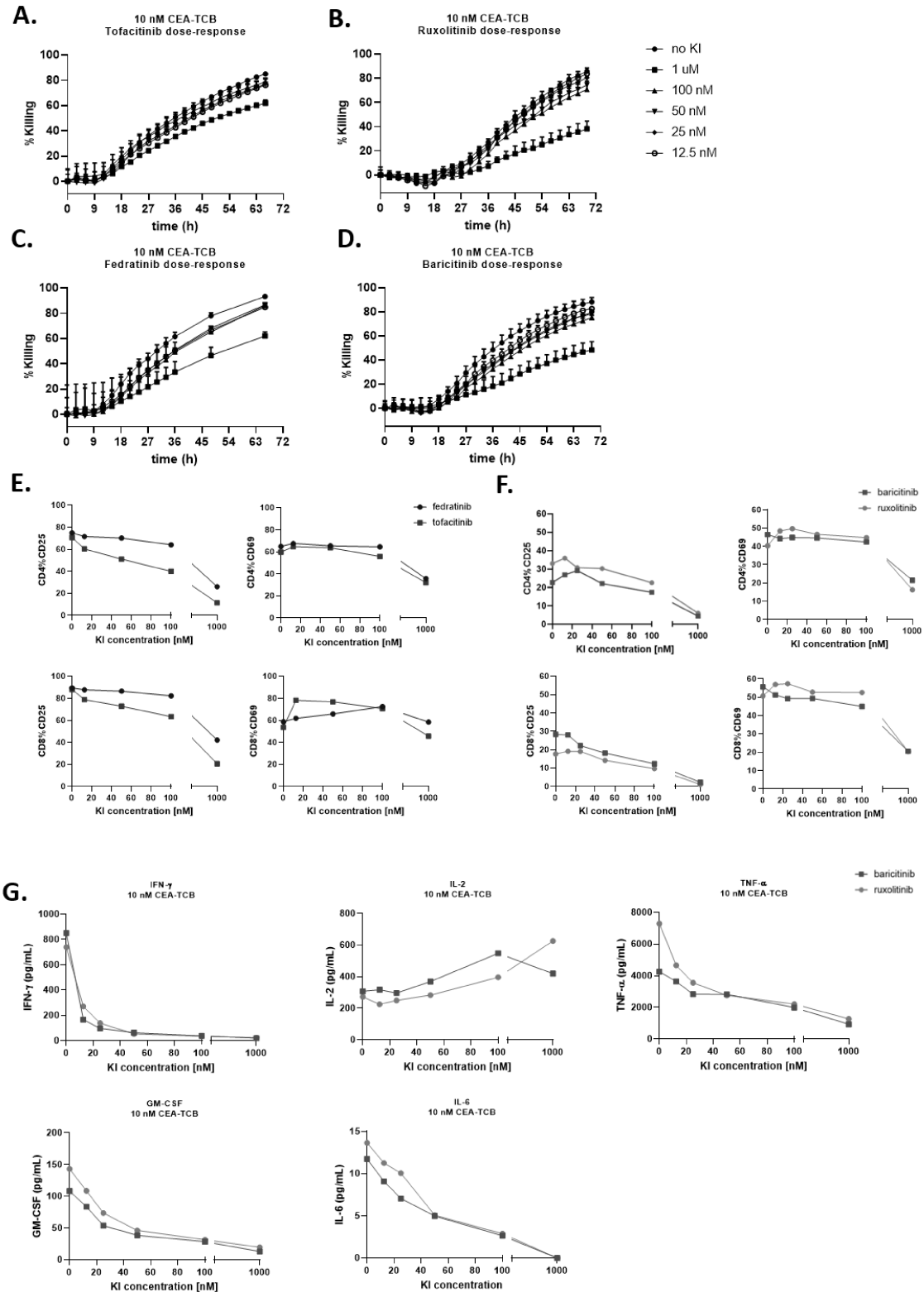
Supp. Figure 3. Dose-response of sirolimus, everolimus and temsirolimus in a killing assay with CEA-TCB.

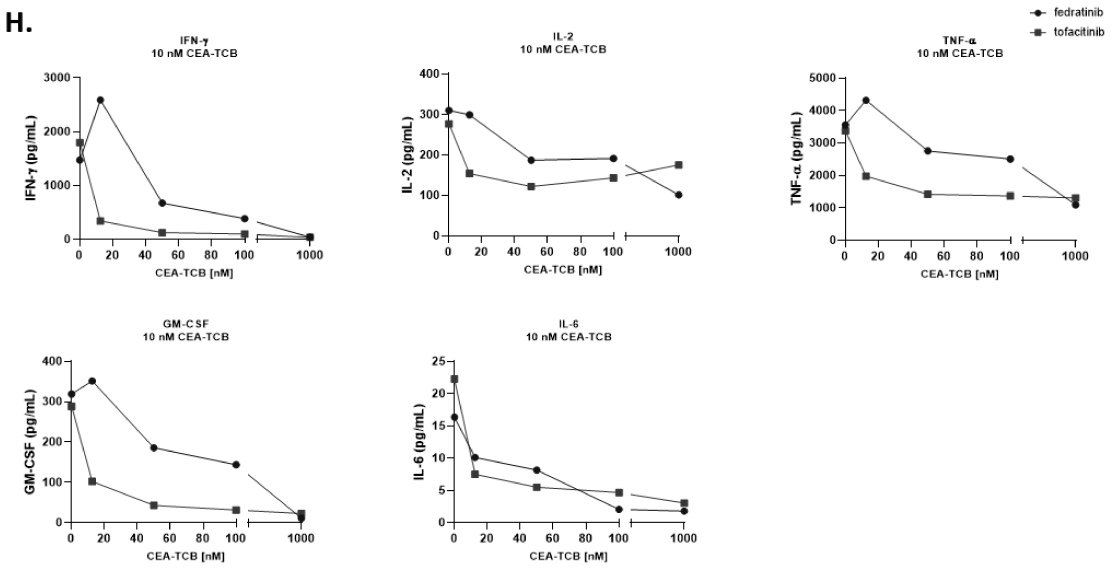
PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of mTOR inhibitors. The real-time killing of NLR-labelled MKN45 tumor cells by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The 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 + SD 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 1 donor representative of 2. (**E**) 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.

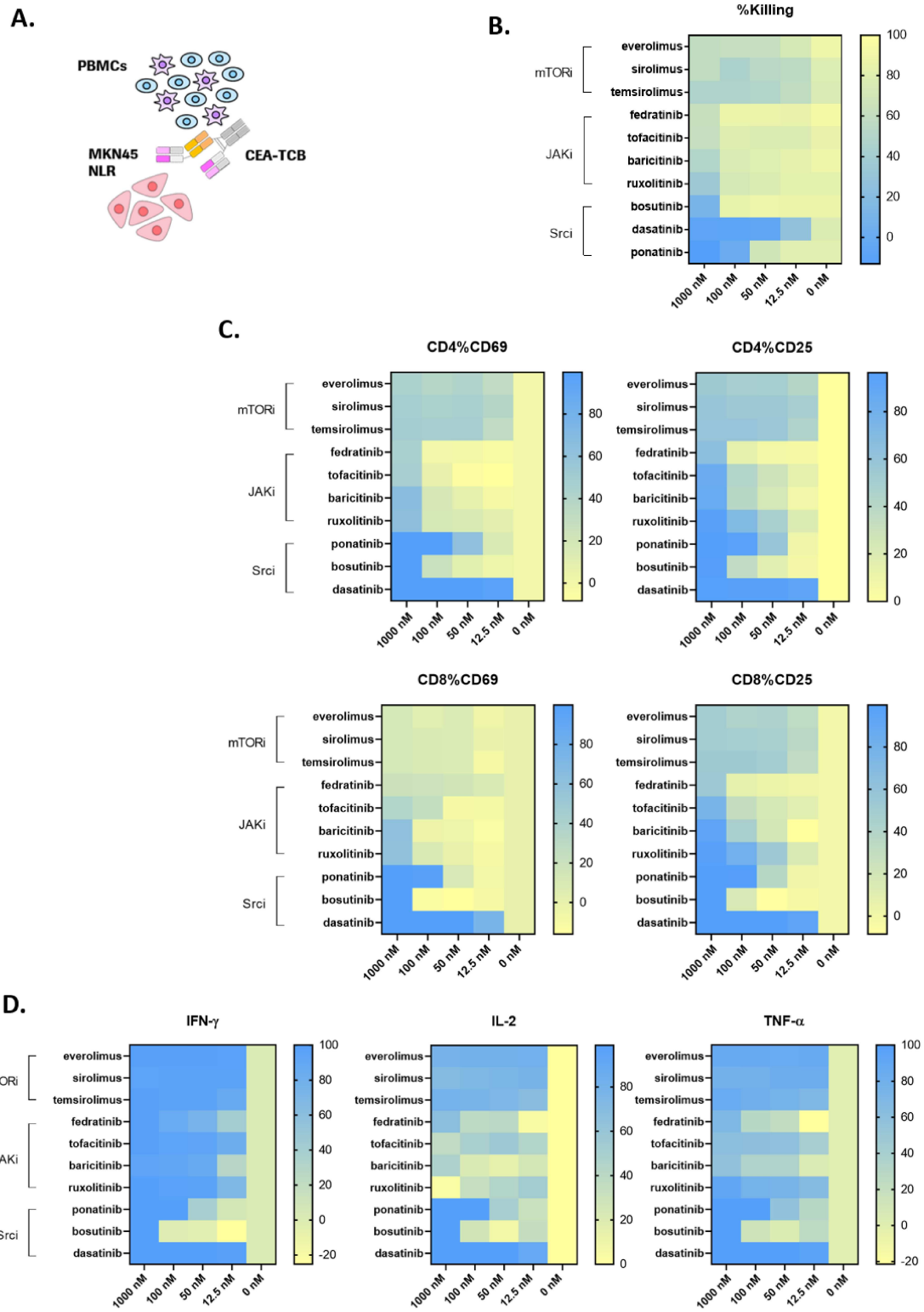
PBMCs were stimulated on NLR-labelled MKN45 tumor cells by 10 nM CEA-TCB in the presence of escalating doses of src kinase inhibitors. The real-time killing of NLR-labelled MKN45 by CEA-TCB was measured using an Incucyte (1 scan every 3 hrs, zoom 10x, phase and red, 400 ms acquisition time). The percentage 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 + SD 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. (**E**) 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
B.
10 nM CEA-TCB
Ruxolitinib dose-response
no KI
1 uM
100 nM
50 nM
25 nM
12.5 nM
% Killing
time (h)
C.
10 nM CEA-TCB
Fedratinib dose-response
D.
10 nM CEA-TCB
Baricitinib dose-response
E.
fedratinib
tofacitinib
CD4%CD25
CD4%CD69
CD8%CD25
CD8%CD69
KI concentration [nM]
F.
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



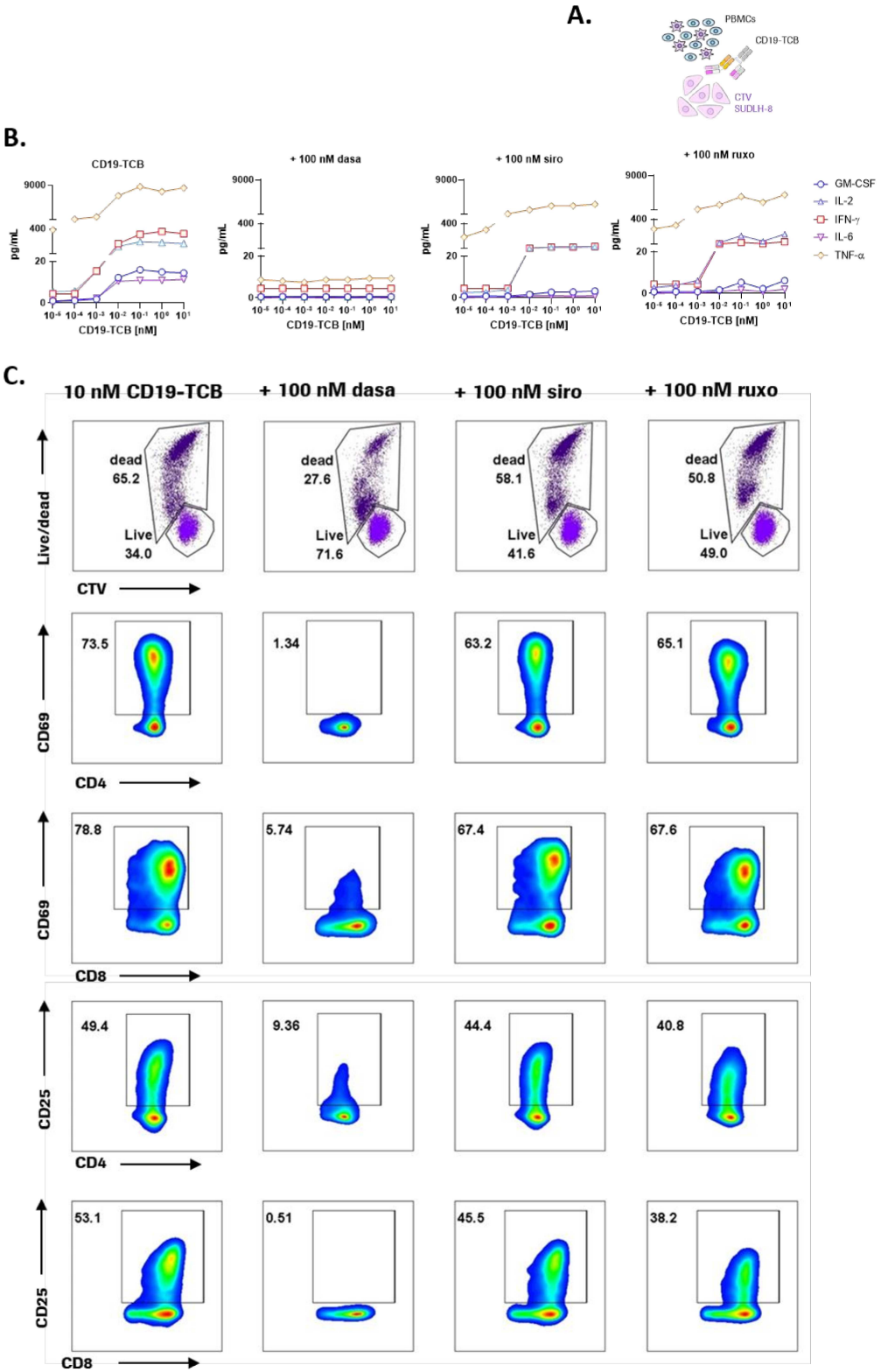
Supp Figure 5. Dose-response of ruxolitinib, tofacitinib, fedratinib and baricitinib in a killing assay with CEA-TCB.

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 + SD 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

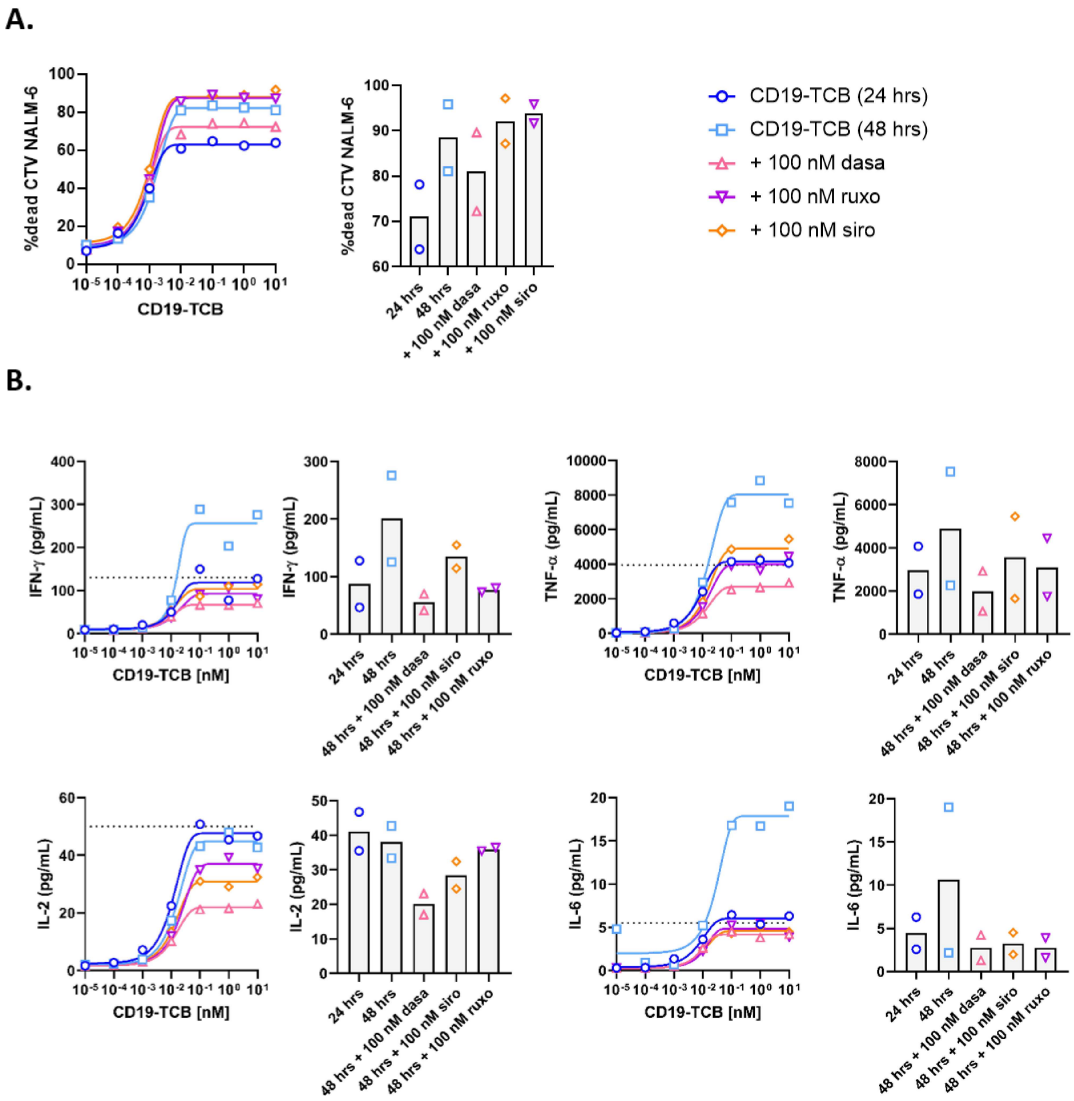
Supp. Figure 6. Effect of mTOR, JAK and Src inhibitors on CEA-TCB-mediated target cell killing and T cell activation vs. cytokine release.

(**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
Live
65.2
34.0
27.6
71.6
58.1
41.6
50.8
49.0
CTV
CD69
CD4
CD8
CD25
73.5
1.34
63.2
65.1
78.8
5.74
67.4
67.6
49.4
9.36
44.4
40.8
53.1
0.51
45.5
38.2

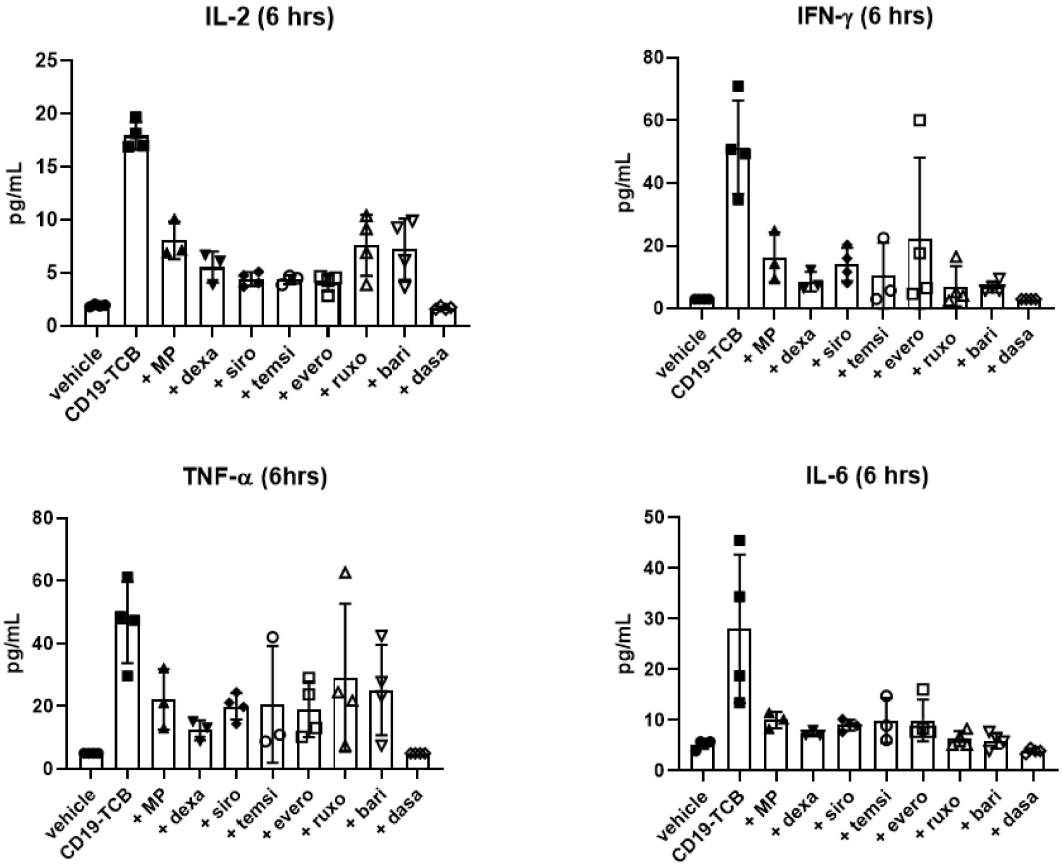
Supp. Figure 7. Effect of mTOR, JAK and Src inhibitors on CD19-TCB-induced T cell cytotoxicity, activation and cytokine release.

(**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.


A.
%dead CTV NALM-6
CD19-TCB
CD19-TCB (24 hrs)
CD19-TCB (48 hrs)
+ 100 nM dasa
+ 100 nM ruxo
+ 100 nM siro
24 hrs
48 hrs
B.
IFN-γ (pg/mL)
TNF-α (pg/mL)
IL-2 (pg/mL)
IL-6 (pg/mL)
CD19-TCB [nM]
48 hrs + 100 nM dasa
48 hrs + 100 nM siro
48 hrs + 100 nM ruxo

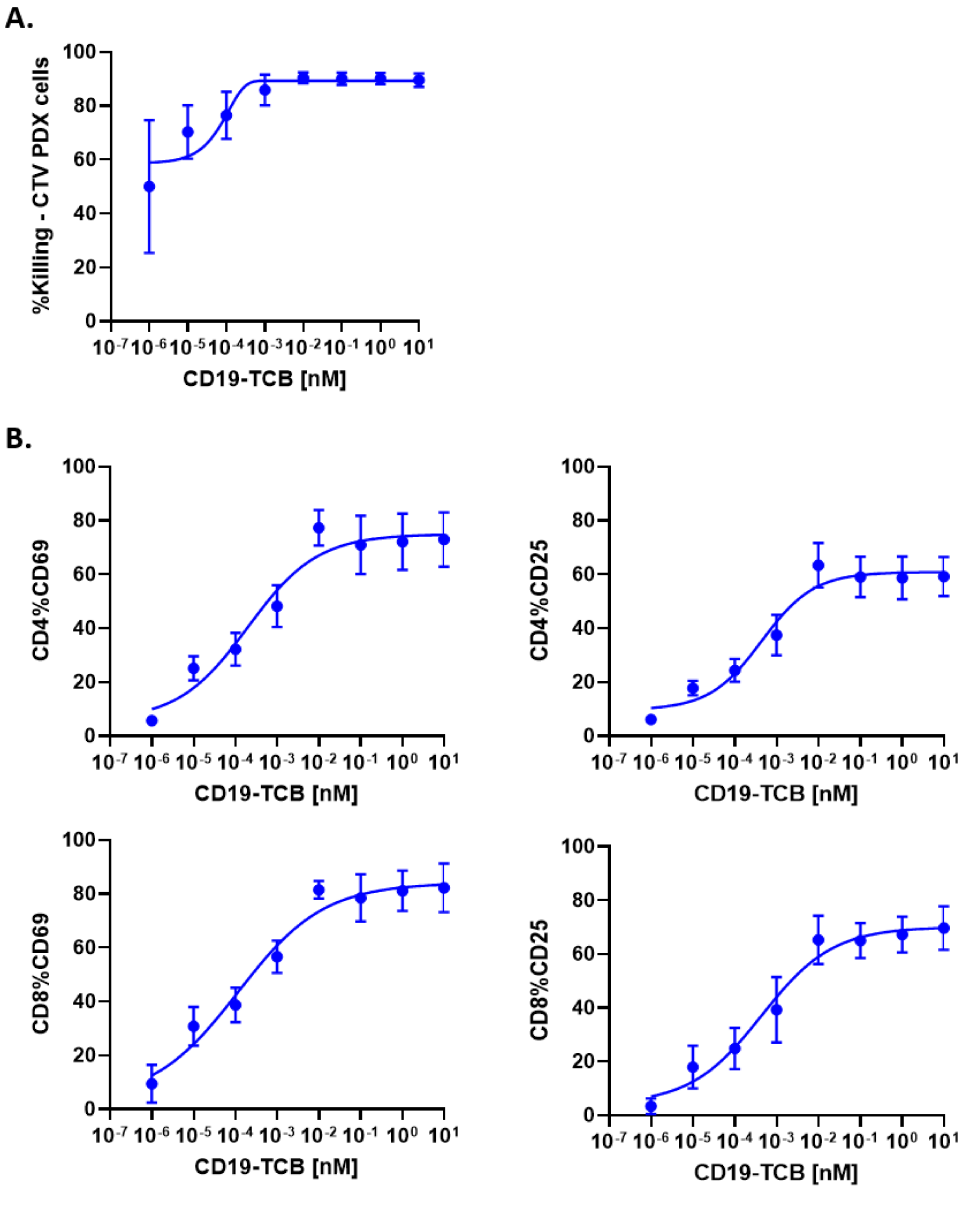
Supp. Figure 8. Effect of mTOR, JAK and Src inhibitors on activated PBMCs.

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.


IL-2 (6 hrs)
IFN-γ (6 hrs)
TNF-α (6hrs)
IL-6 (6 hrs)
pg/mL
vehicle
CD19-TCB
+ MP
+ dexa
+ siro
+ temsi
+ evero
+ ruxo
+ bari
+ dasa

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.

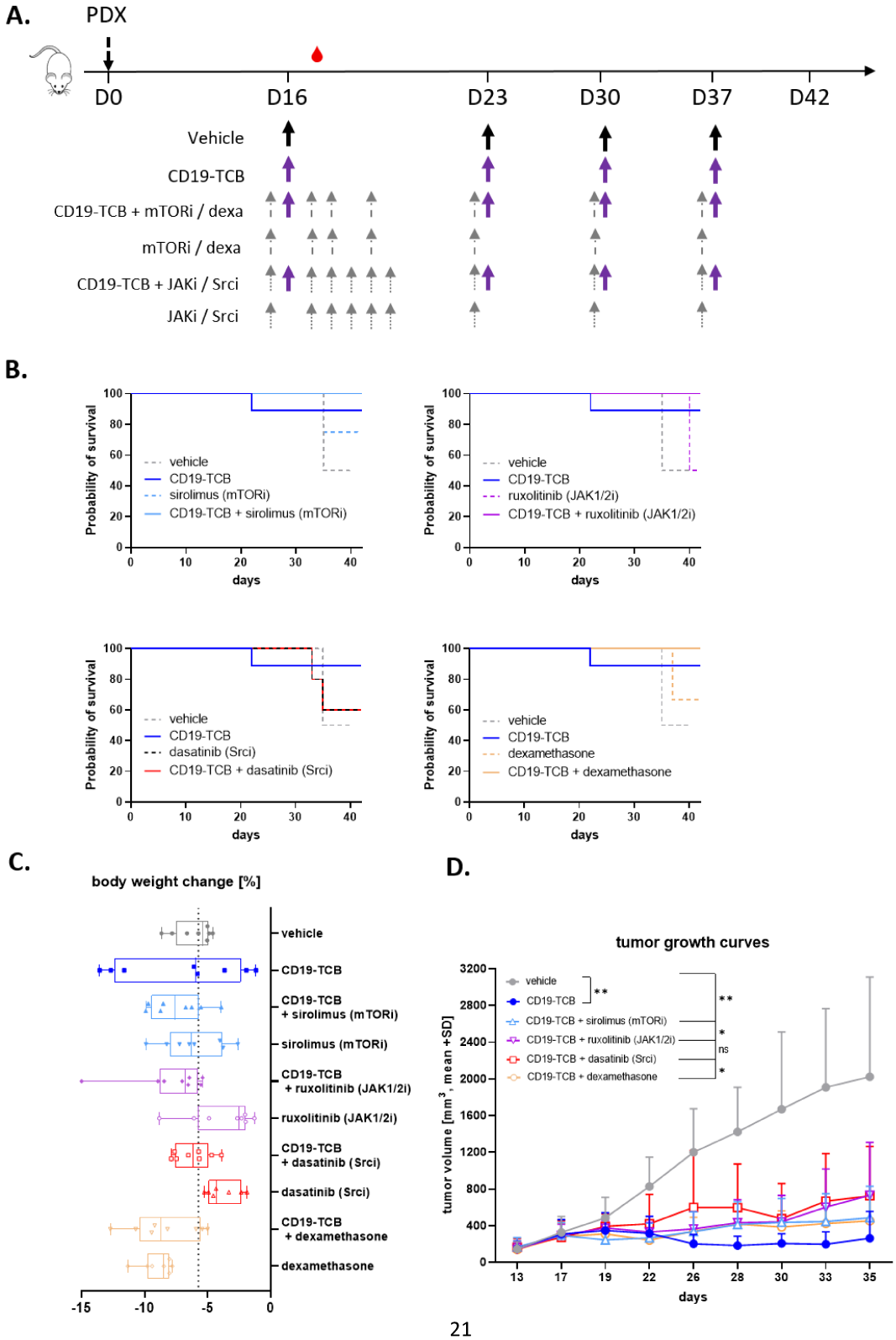
Humanized NSG mice were either pre-treated with 0.5 mg/kg CD19-TCB alone or in combination 50 mg/kg dasatinib (dasa) (p.o.), 30 mg/kg ruxolitinib (ruxo) or baricitinib (bari) (p.o), 5 mg/kg sirolimus (siro), temsirolimus (temsi) or everolimus (evero) (p.o.), 2 times 1 mg/kg, 0.5 mg/kg and 0.25 mg/kg 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 +/- SD.


A.
%Killing - CTV PDX cells
CD19-TCB [nM]
B.
CD4%CD69
CD4%CD25
CD8%CD69
CD8%CD25
CD19-TCB [nM]

Supp.

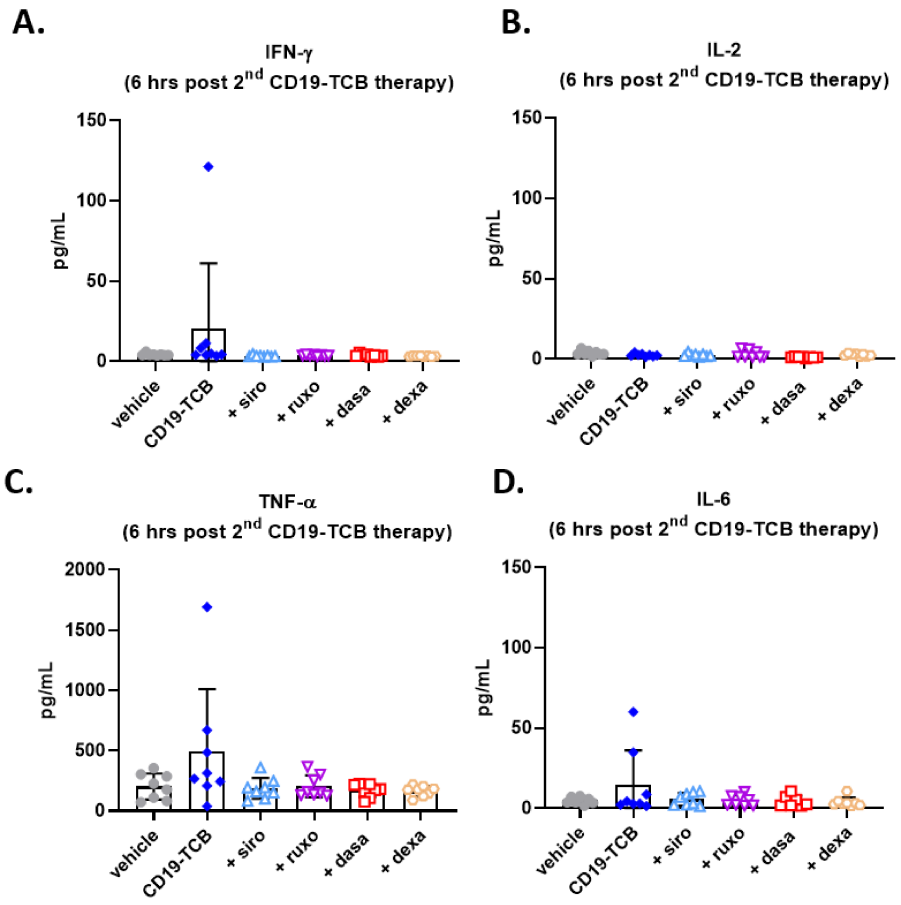
Figure 10. CD19-TCB kills lymphoma PDX cells in vitro.

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 [%]
D.
tumor growth curves
tumor volume [mm3, mean +SD]
CD19-TCB + sirolimus (mTORi)
CD19-TCB + ruxolitinib (JAK1/2i)
CD19-TCB + dasatinib (Srci)
CD19-TCB + dexamethasone
ns

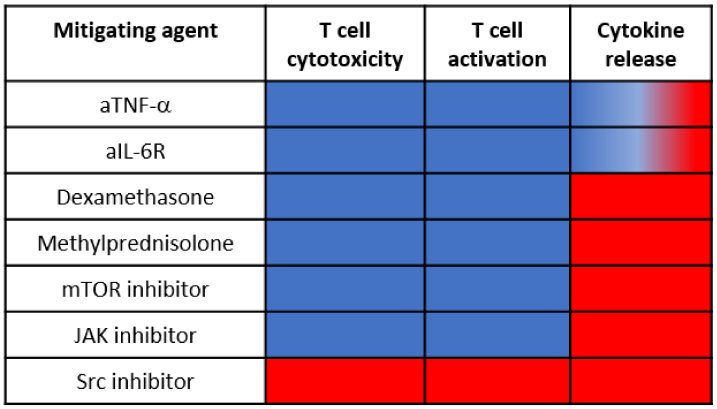
Supp. Figure 11. JAK and mTOR inhibitors co-treatment with CD19-TCB lead to 100% survival .

(**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 24 hrs after the first treatment with CD19-TCB. 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. (**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).



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.



Supp. Figure 13. Comparative summary of 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 brake. 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.