# Anti-tumor effects of RTX-240: an engineered red blood cell expressing 4-1BB ligand and interleukin-15

## Supplementary material

**Supplementary table 1**: Antibodies used for flow cytometry

| **Anti-mouse antibodies** | **Clone** | **Source** |
|---|---|---|
| CD3 | 17A2 | BioLegend |
| CD8 | 53-6.7 | BD Biosciences / BioLegend |
| NK1.1 | PK136 | BioLegend |
| CD44 | IM7 | BioLegend |
| CD45.2 | 104 | BioLegend |
| CD45 | 30-F11 | BD Biosciences |
| Ki67 | B56 | BD Biosciences |
| KLRG1 | 2F1/KLRG1 | BioLegend |
| EOMES | Dan11mag | Thermo Fisher Scientific |
| F4/80 | EMR1 | BioLegend |
| CD27 | LG.3A10 | BioLegend |
| CD11b | M1/70 | BioLegend |

| **Anti-human antibodies** | **Clone** | **Source** |
|---|---|---|
| Anti-human CD3 | UCHT1 | BD Biosciences |
| Anti-human CD56 | 5.1H11 | BioLegend |

| | | |
|---|---|---|
| Anti-human CD45RO | UCHL1 | BioLegend |
| Anti-human CD62L | DREG-56 | BioLegend |
| Anti-human CD8 | RPA-T8 | BioLegend |
| Anti-human TRAIL | RIK-2 | BioLegend |
| Anti-human CD56 | 5.1H11 | BioLegend |
| Anti-human NKp44 | 44.189 | Thermo Fisher Scientific |
| Anti-human 4-1BB | 4B4-1 | BioLegend |
| Anti-human IFNγ | 4S.B3 | BioLegend |
| Anti-human GZMB | GB11 | BioLegend |
| Anti-mouse IL-15Rα | DNT15Ra | Thermo Fisher Scientific |
| Anti-human 4-1BBL | 5F4 | BioLegend |

IFNγ, interferon γ; NK, natural killer

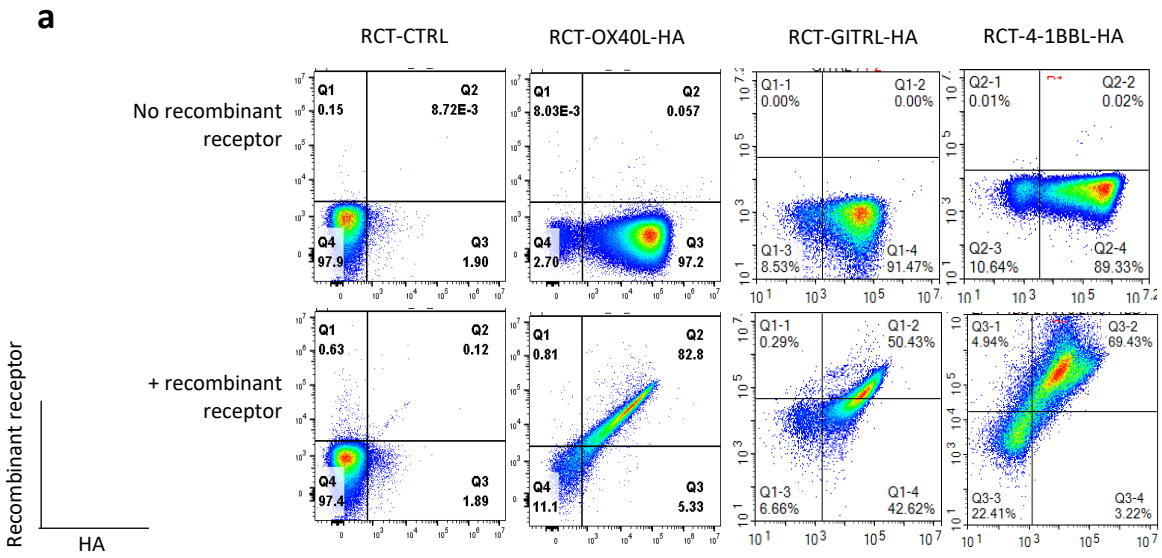
**Supplementary table 2:** Gating strategies for flow cytometry

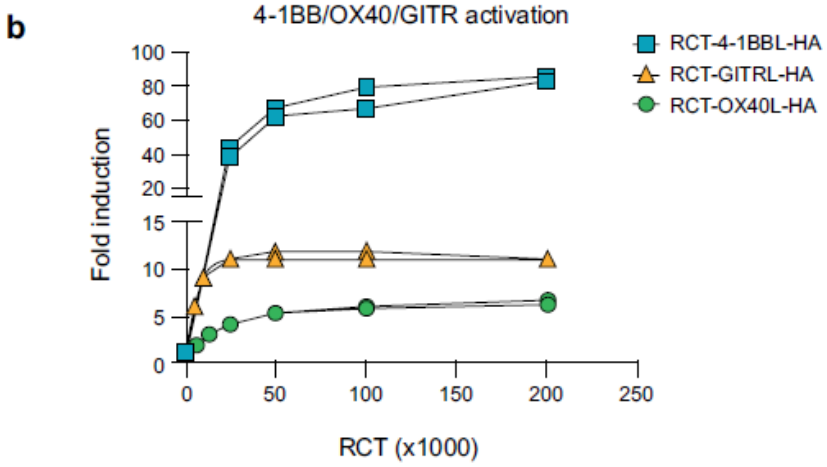
| **Cell type** | **Gating strategy (murine)** |
|---|---|
| All cells | |
| All immune cells | CD45.2+ of live cells (LD-negative population) |
| CD8 | CD8+ of live CD45.2+ |
| Proliferating CD8+ T cells | Ki67+ of CD8+ T cells |
| Activated CD8+ T cells | CD44+ or IFNγ of CD8+ T cells |
| Liver CD8+ T cells associated with liver injury | Eomes+/KLRG1+ of CD8+ T cells |
| Liver macrophages | F4/80+ of live CD45.2+ |
| NK | NK1.1+ of live CD45.2+ |
| Terminally differentiated NK | CD11b+/CD27-/KLRG1+ of NK1.1+ |

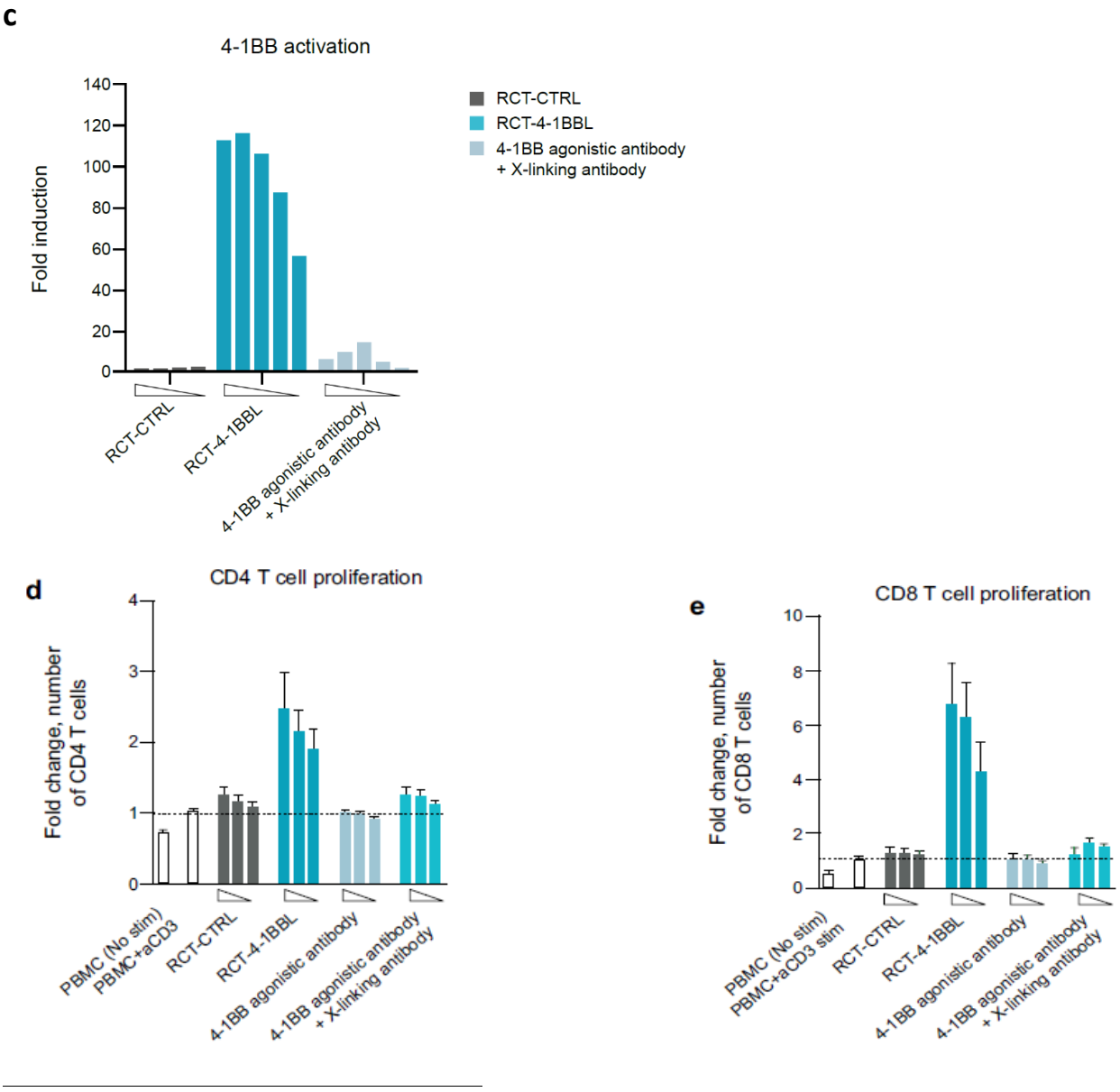
| **Cell type** | **Gating strategy (human)** |
|---|---|
| Leukocytes | FSC/SSC gating |
| Live cells | LD-negative population |
| CD8 | CD3+/CD8+ of live cells |
| Proliferating CD8+ T cells | CTFR diluted of CD3+/CD8+ |
| Activated CD8 | GZMB+ of CD3+/CD8+ |

| | |
|---|---|
| CD8 memory T cells | CD45RO+/CD8+/CD3+ |
| Proliferating memory CD8+ T cells | CTFR diluted of CD45RO+/CD8+/CD3+ |
| TEM | CD45RO+/CD62L- of CD8+ T cells |
| NK | CD56+/CD3- of live cells |
| Proliferating NK cells | CTFR diluted of CD56+/CD3- |
| Activated NK | TRAIL+ or NKp44+ or 4-1BB+ or GZMB+ or IFNγ of NK |

CTFR, CellTrace™ Far Red dye; IFNγ, interferon γ; NK, natural killer


a
RCT-CTRL
RCT-OX40L-HA
RCT-GITRL-HA
RCT-4-1BBL-HA
No recombinant receptor
+ recombinant receptor
Recombinant receptor
HA
Q1 0.15
Q2 8.72E-3
Q4 97.9
Q3 1.90
Q1 8.03E-3
Q2 0.057
Q4 2.70
Q3 97.2
Q1-1 0.00%
Q1-2 0.00%
Q1-3 8.53%
Q1-4 91.47%
Q2-1 0.01%
Q2-2 0.02%
Q2-3 10.64%
Q2-4 89.33%
Q1 0.63
Q2 0.12
Q4 97.4
Q3 1.89
Q1 0.81
Q2 82.8
Q4 11.1
Q3 5.33
Q1-1 0.29%
Q1-2 50.43%
Q1-3 6.66%
Q1-4 42.62%
Q3-1 4.94%
Q3-2 69.43%
Q3-3 22.41%
Q3-4 3.22%


b
4-1BB/OX40/GITR activation
RCT-4-1BBL-HA
RCT-GITRL-HA
RCT-OX40L-HA
Fold induction
RCT (x1000)
100
80
60
40
20
15
10
5
0
0
50
100
150
200
250

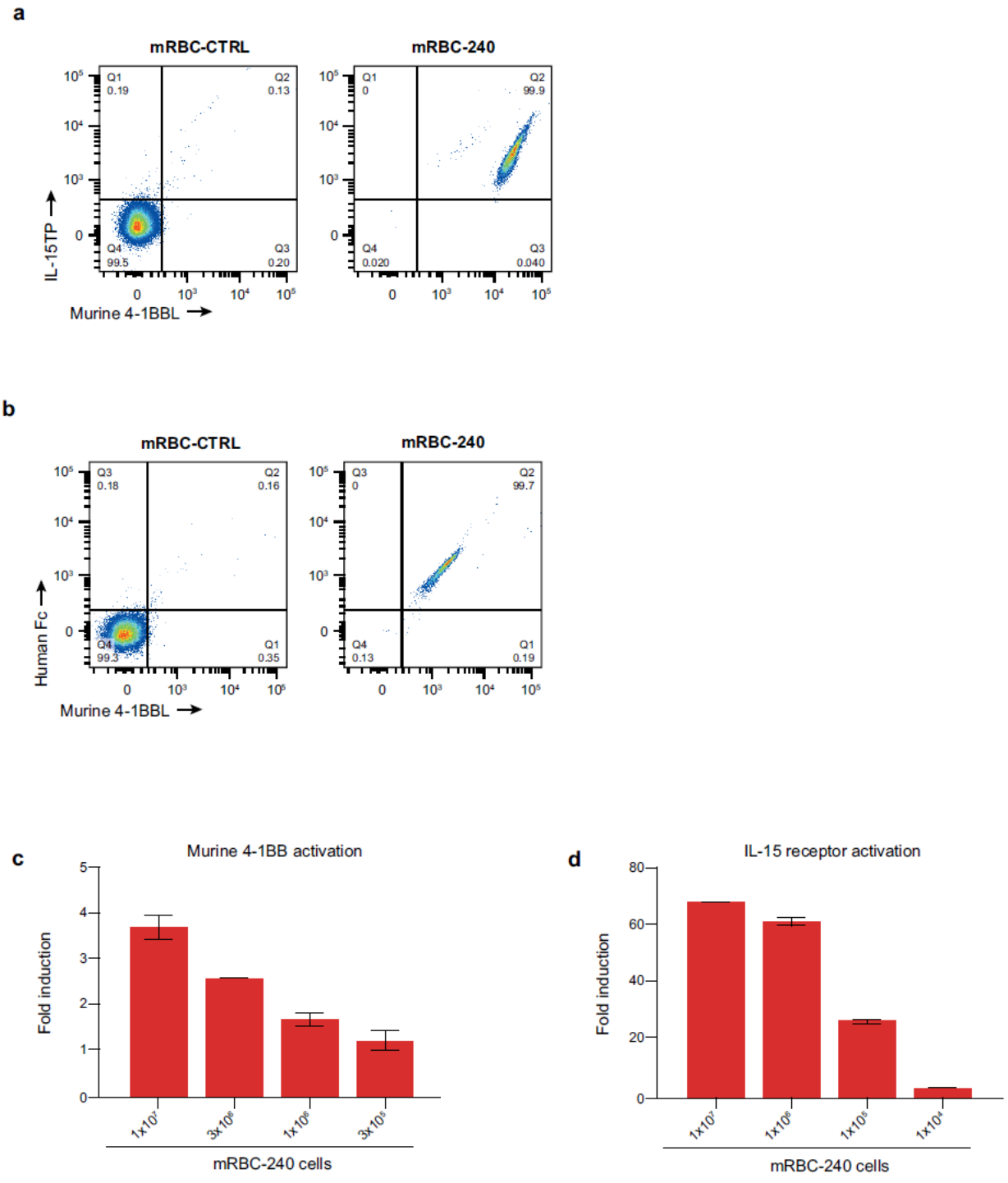


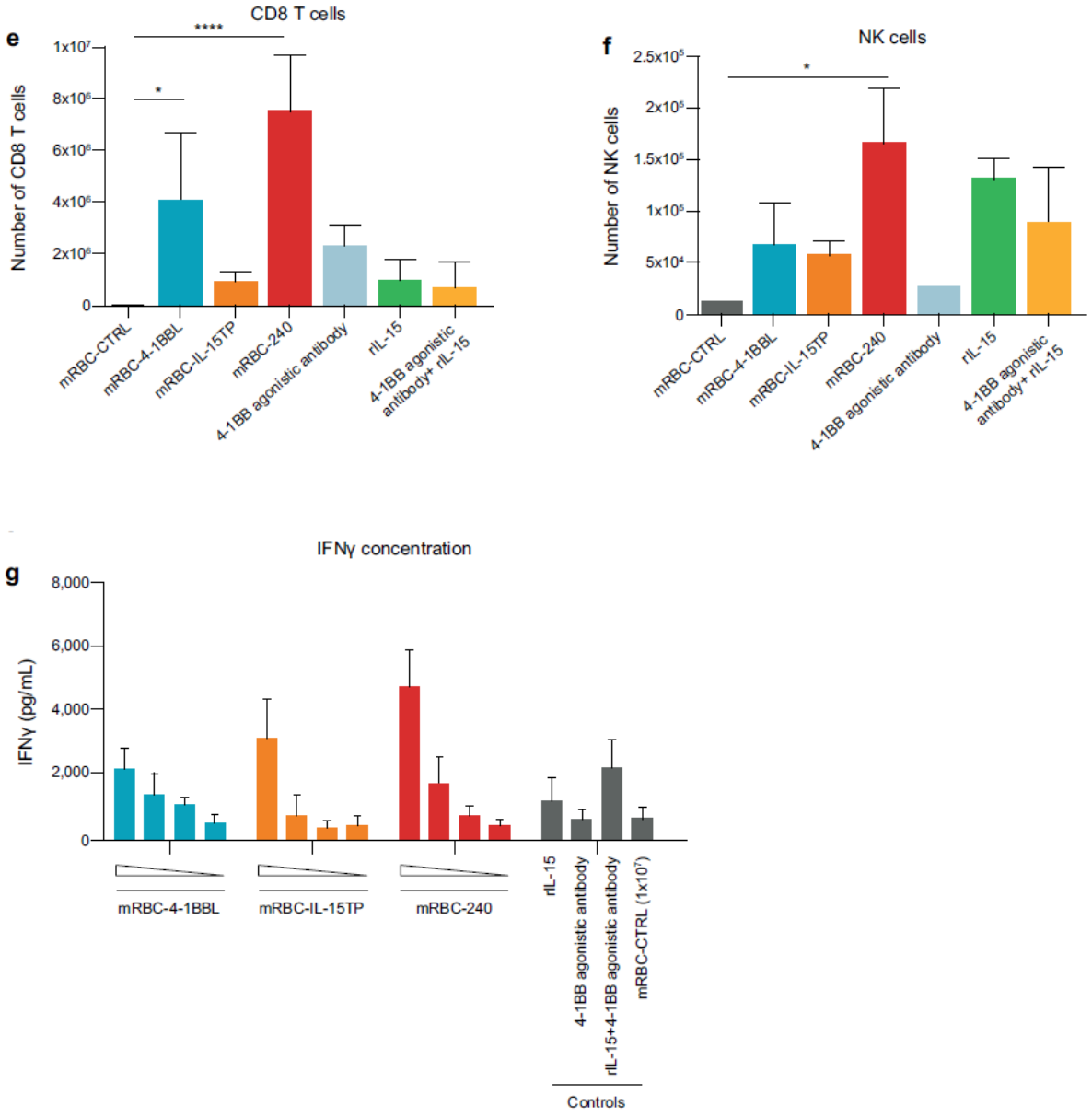

**Supplementary figure 1:** Engineered red blood cells expressing functional costimulatory ligands

**a** Representative flow cytometry plots for RCT-CTRL, RCT-OX40L-HA, RCT-GITRL-HA and RCT-4-1BBL-HA incubated in PBS with or without His-tagged recombinant receptors, stained with anti-HA antibody and anti-His antibody, and evaluated for expression of HA-tagged therapeutic proteins (top panel) and binding to recombinant His-tagged receptors (lower panel). **b** RCTs

expressing 4-1BBL, GITRL or OX40L were incubated with NFκB reporter Jurkat cells expressing 4-1BB, GITR or OX40, respectively, for 6 hours, and NFκB activation was measured. Bars indicate technical replicates. **c** NFκB activation in reporter Jurkat cells incubated for 6 hours with engineered RBCs (2-fold dilutions from 4 x $10^5$ cells) or 4-1BB agonistic antibody or isotype control antibody (10-fold dilutions from 100 nM) with crosslinker (10-fold dilutions from 250 nM). **d, e** 2 x $10^5$ PBMCs were cultured for 3 days with 0.5 μg/mL anti-CD3 and engineered RBCs (2-fold dilutions from 1 x $10^5$) or 4-1BB agonistic antibody (10-fold dilutions from 1 μM) with crosslinker (10-fold dilutions from 2.5 μM), then (**d**) CD4+ and (**e**) CD8+ T cell numbers were measured by flow cytometry, and fold change over PBMC+aCD3 was calculated

PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; RBC, red blood cell; RCT, red cell therapeutic


a
mRBC-CTRL
mRBC-240
Q1 0.19
Q2 0.13
Q4 99.5
Q3 0.20
Q1 0
Q2 99.9
Q4 0.020
Q3 0.040
IL-15TP
Murine 4-1BBL
b
mRBC-CTRL
mRBC-240
Q3 0.18
Q2 0.16
Q4 99.3
Q1 0.35
Q3 0
Q2 99.7
Q4 0.13
Q1 0.19
Human Fc
Murine 4-1BBL
c
Murine 4-1BB activation
Fold induction
mRBC-240 cells
d
IL-15 receptor activation
Fold induction
mRBC-240 cells

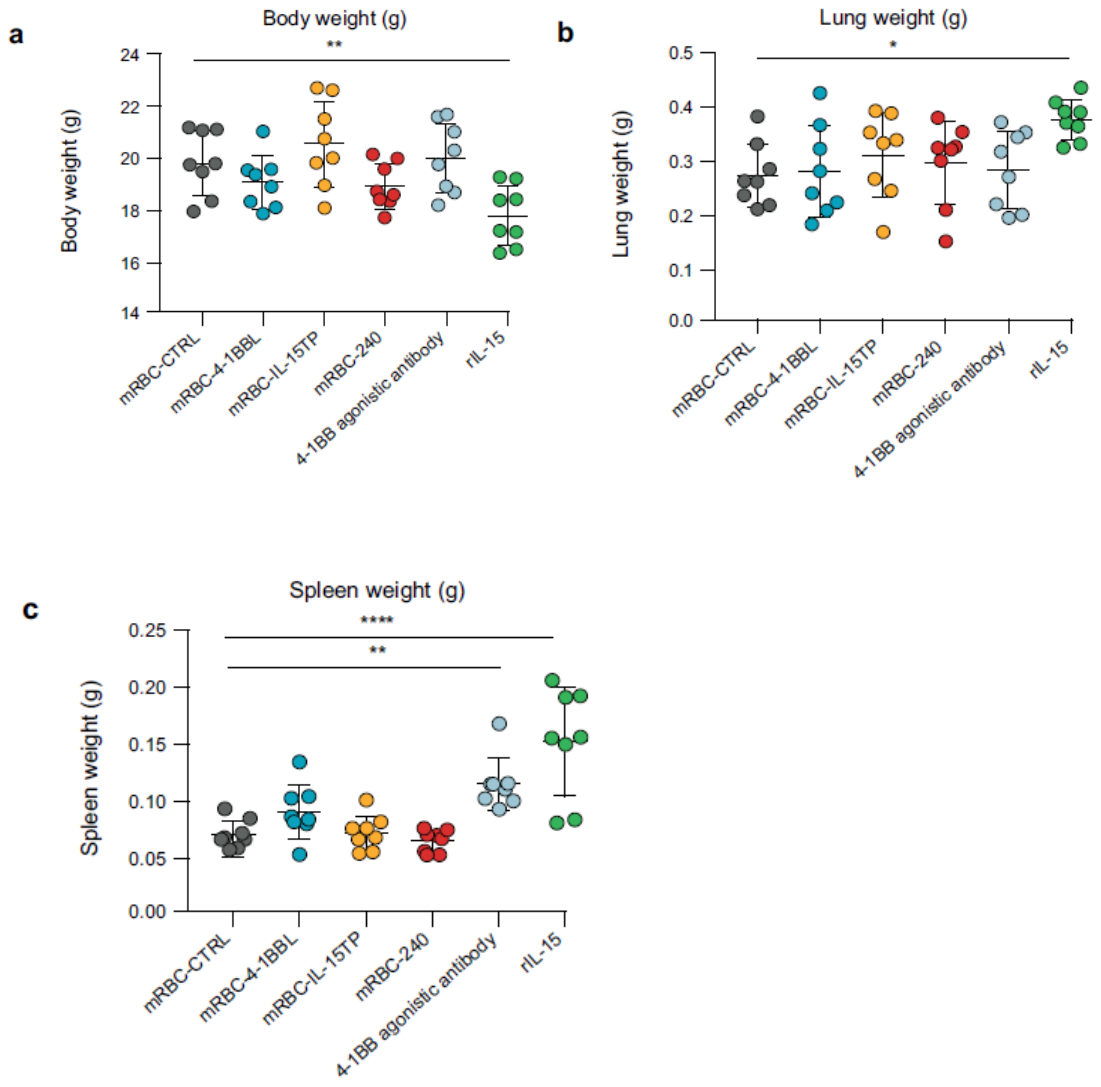



**Supplementary figure 2:** mRBC-240 engages 4-1BB and IL-15 receptor and activates CD8+ T cells and NK cells in vitro

**a** mRBC-CTRL or mRBC-240 were stained with anti-mouse 4-1BB Ligand and anti-mouse IgG2a antibodies (IL-15 detection) and expression of 4-1BBL and IL-15TP was determined by flow cytometry. **b** mRBC-CTRL or mRBC-240 were incubated with 1 µg of recombinant human Fc-tagged mouse 4-1BB receptor and binding was detected using anti-mouse IgG2a antibody to

detect mRBC-240 and anti-human Fc antibody to detect the mouse 4-1BB receptor. **c** 3T3 cells expressing 4-1BB receptor were incubated with mRBC-240 for 24 hours and a fold change of the luminescence signal over mRBC-CTRL is represented. **d** HEK-Blue IL-2 SEAP cells were incubated mRBC-240 for 20 hours and a fold change of the luminescence signal over mRBC-CTRL is represented. The number of (**e**) CD8+T cells or (**f**) NK cells following a 4-day stimulation of mouse splenocytes with mRBC-CTRL, mRBC-4-1BBL, mRBC-IL-15TP or mRBC-240 in the presence of 1 µg/mL of anti-CD3. 4-1BB agonistic antibody (10 µg/mL) or recombinant (r) IL-15 (100 ng/mL) are used as controls. Comparisons were analyzed by a one-way ANOVA and compared with mRBC-CTRL and showing as *, $p<0.05$, ****, $p<0.0001$. **g** The concentration of IFNγ (measured by ELISA) in the supernatant after a 24-hour co-culture of mouse splenocytes with either mRBC-4-1BBL, mRBC-IL-15TP,mRBC-240 or controls, in the presence of 2 µg/mL anti-CD3. 4-1BB agonistic antibody (10 µg/mL) and rIL-15 (100 ng/mL) were used as controls. Bars indicate (**c–d**) SD of 2–3 technical replicates and (**e–g**) SD of 3 biological replicates

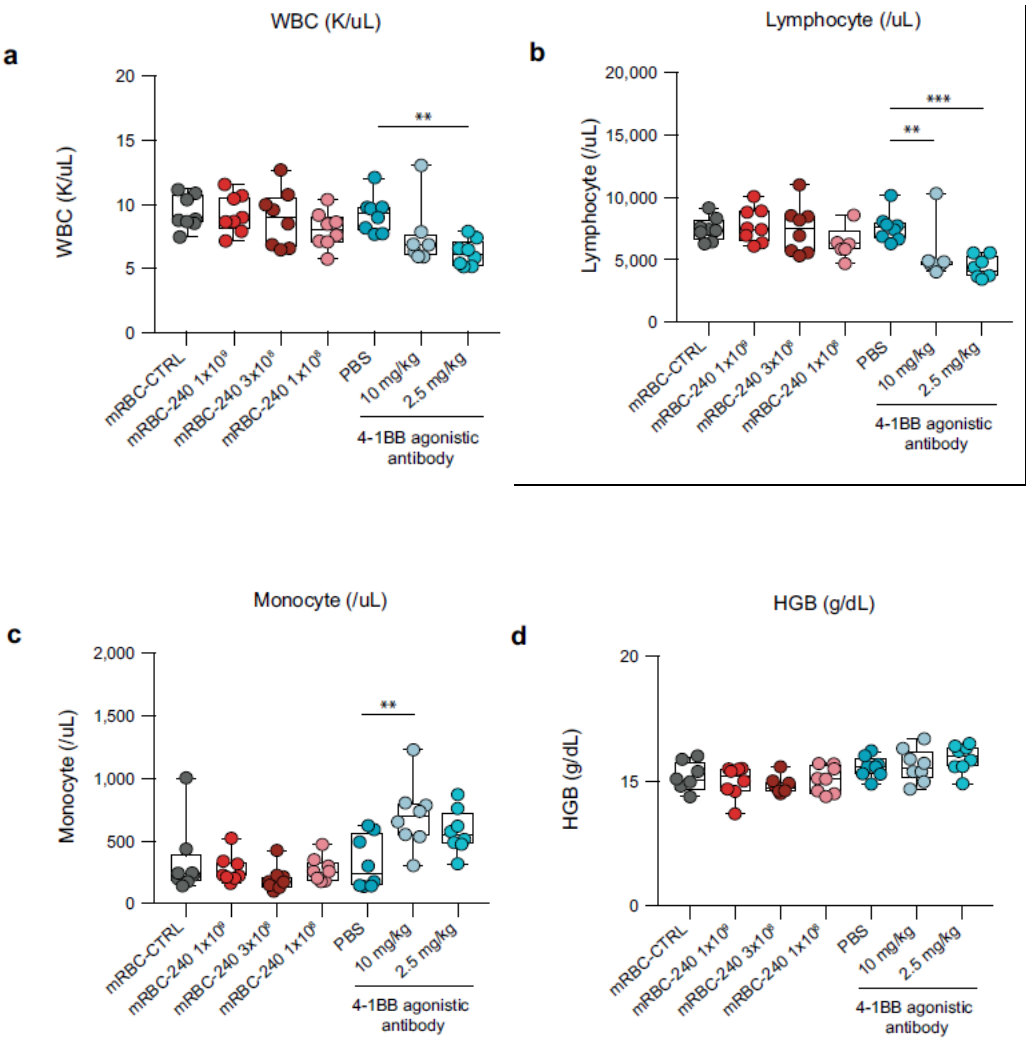
IFNγ, interferon-γ; IgG, immunoglobulin G; IL-15TP, trans-presented interleukin 15; NK, natural killer; RBC, red blood cell; SD, standard deviation




**Supplementary figure 3:** No changes in organ weights in mice following mRBC-240 treatment

C57BL/6 mice were inoculated intravenously with B16-F10 tumor cells and treated with 1 x $10^9$ of either mRBC-4–1BBL, mRBC–IL-15TP or mRBC-240 intravenously or with 2.5 mg/kg of 4-1BB agonistic antibody or 0.2 mg/kg of rIL-15 intraperitoneally on days 1, 5 and 8 post-inoculation. (**a**) Body weight, (**b**) lung weight and (**c**) spleen weight were determined on day 14. Data presented ± SD. All comparisons were analyzed by one-way ANOVA compared to mRBC-CTRL and showing as *, $p$<0.05, **, $p$<0.01, ****, $p$<0.0001

RBC, red blood cell; rIL-15, recombinant interleukin-15; SD, standard deviation




**Supplementary figure 4:** Complete blood count in mRBC-240-treated mice

The counts of (**a**) white blood cells (K/µL), (**b**) lymphocyte (per µL), (**c**) monocyte (per µL) and (**d**) hemoglobin (g/dL) were analyzed in the blood of C57BL/6 wild-type mice on day 18 following 4 doses of either mRBC-240 (1 x $10^9$, 3 x $10^8$ or 1 x $10^8$), mRBC-CTRL or 4-1BB agonistic antibody (10 mg/kg or 2.5 mg/kg) (*n*=8 mice/group). All comparisons were analyzed by one-way ANOVA and showing as **, $p<0.01$, ***, $p<0.001$

HGB, hemoglobin; PBS, phosphate-buffered saline; RBC, red blood cell; WBC, white blood cell