Supplementary data

Materials & Methods

B16 *in vivo* tumor model

C57BL/6 mice were injected with $2x10^5$ B16-F10 in the right flank and peritumorally injected with 30µg anti-CD40 (FGK4.5) on d7, d10 and d13. Antibody solution was administered in 100µl. Tumor growth and survival was monitored throughout the experiment using a caliper and tumor size was calculated by the ellipsoid volume formula:

=4/3*π*a(radius of length)*b(radius of width)*c(radius of depth). Mice were sacrificed if the tumor exceeded $1cm^3$ or if ulcers developed.

In vitro specificity test for ^{125}I-CD40 antibody

ELISA plates with breakable wells were coated with 0.04, 0.4 or 4µg of recombinant murine CD40 (Fc Chimera R&D Systems) O/N in 4°C. The plate was washed with PBS 0.05% Tween before unspecific binding was blocked with a milk powder-based blocking solution (5%). Again, the wells were washed before 13nM radioactive ^{125}I-CD40 antibody was added. To saturate binding sites in the control wells, unlabeled anti-CD40 was used in approximately 5 times excess (66nM). Samples were incubated for 90 min in 4°C, washed before each separate well was measured for radioactivity content. All samples were run in triplicates.

Definition of target cells for *in vivo* CD40 expression

The investigated cell types in the spleen were defined as B-cells (B220+, CD11b-, CD11c-, F4/80-), conventional DCs (cDCs; $CD11c^{hi}$, CD11b+/-, B220-), monocyte-derived MΦ ($CD11c^{low/int}$, CD11b+, B220-, F4/80+), red pulp MΦ ($CD11c^{low/int}$, CD11b+, B220- , F4/80++) and marginal zone MΦ ($CD11c^{low/int}$, CD11b+, B220- , F4/80-).

Supplementary figure legends

Figure 1. Local low-dose anti-CD40 therapy generates distant anti-tumor effects and inhibits B16 tumor growth. (A) Mice carrying B16 tumors were subjected to local low-dose anti-CD40 therapy as described in materials and method. Data are shown as mean±SD of 9 mice/group (d16 Student´s t-test, ***p<0.001). (B) C57BL/6 mice were implanted with MB49 cells on d0 and on d1 of individual mice and subjected to 5 or 15µg of anti-CD40 antibody according to the illustration in 2E. Lines represents individual mice´s primary (black solid line) and secondary (red dotted line) tumor growth in Fig. 2F (n=9).

Figure 2. Retained CD40 specificity after radiolabeling and modulation of CD40 expression as well as immune cell distribution after repeated injections of FGK4.5 *in vivo*. (A) Verification of radioactively labeled ^{125}I-CD40 antibody by ELISA was performed as described in supplementary materials and method. Samples were run in triplicates. Data are shown as mean±SD (Student´s t-test). CPM; counts per minute. (B) Blood collected during the biodistribution study was fractionated by centrifugation and radioactivity was measured on individual samples. Data are presented as % injected dose/gram tissue, mean±SD of 4 mice/group. (C) *In vivo* CD40 expression on target cells was investigated in spleen after one, two or three doses of anti-CD40 antibodies. Four hours after treatment organs were digested with Liberase and stained for surface markers. Upper panel shows CD40 expression based on geometric mean and lower panel show the frequency of total cells. Statistical significance is calculated between rat IgG2a and anti-CD40 (mean±SD of 3 mice/group, Student´s t-test, *p<0.05, **p<0.01, ***p<0.001**).**

Figure 3. *In vivo* biodistribution of target organs 48h and 72h post injection of ^{125}I-CD40-specific and ^{131}I-rat IgG2a control antibody. Radioactive uptake of target organs (A) 48h and (B) 72h post injection of animals in Fig. 3. Data are presented as % injected dose/gram tissue (%ID/g), mean±SD of 4 mice/group. Statistical differences between anti-CD40 and rat IgG2a and between the two administration routes are depicted in Supplementary Table S3 (48h) and S4 (72h).