**Supplementary information**

**Systemic agonistic anti-CD40 treatment of tumor bearing mice modulates hepatic myeloid suppressive cells and causes immune-mediated liver damage**

José Medina-Echeverz1, Chi Ma1, Austin Duffy1, Tobias Eggert1, Nga Hawk2, David E. Kleiner3, Firouzeh Korangy1, Tim F. Greten1

1 Gastrointestinal Malignancy Section, Thoracic and Gastrointestinal Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

2 Experimental Transplantation and Immunology Branch, National Institutes of Health, Bethesda, MD, USA.

3 Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.



**Supplementary Figure S1. Systemic agonistic anti-CD40 induces liver toxicity.**

TF and EL4 TB C57BL/6 mice (n=3 mice/time point) received CD40 Ab. Mice were sacrificed at the indicated time points. Serum ALT and AST (A) levels were quantified. Data expressed as mean ± SEM, representative of two independent experiments. Representative hematoxilin and eosin staining of liver sections from BALB/c 4T1 TB mice 24 hours after IgG (B) or CD40 Ab (C). Images show a 20x magnification and yellow bar = 0.2mm. TB bone marrow chimeric mice (donor🡪recipient) received i.p. either CD40 Ab or IgG (n=4 mice/group) and were sacrificed 24 hours after antibody injection. Serum TNF- levels were quantified by ELISA (D). Data are expressed as mean ± SEM, representative of two independent experiments. \**P*<0.05, \*\* *P*<0.01, \*\*\* *P*<0.005 : Student’s t test.



**Supplementary Figure S2. Systemic administration of anti-CD40 agonist enhances liver inflammation in a model of hepatic metastases.**

3x105 EL4 cells were inoculated into the spleens of C57BL/6 mice. 3 weeks after injection mice received either IgG or anti-CD40 agonist (n=4 mice/group). Data were generated 24 hours after either IgG or anti-CD40 treatment. (A) Representative image of EL4-induced hepatic metastases. ALT (B) and AST (C) serum levels. Cumulative data of two independent experiments are expressed as mean ± SEM. *p* value is considered not statistically significant: Student’s t test.

**Supplementary Figure S3. Agonistic CD40 antibody induces CD11b+ Gr-1+ cell maturation in the liver.**

(A, B) TF and EL4 TB mice received i.p. either CD40 Ab or IgG. Absolute number of liver CD11b+Gr-1high G-MDSC (A) and CD11b+Gr-1low M-MDSC (B) 24 hours after injection are shown. Cumulative data expressed as mean ± SEM, representative of 2 independent experiments. (C-J) EL4 TB mice received i.p. either CD40 Ab or IgG. (C-G) Absolute number of liver-infiltrating CD3-CD19+ B cells (C), CD11c+ DC (D), CD11b+F4/80+ macrophages (E), CD3+ T cells (F) and CD3-NK1.1+ NK cells (G) 24 hours after antibody injection are shown. Cumulative data expressed as mean ± SEM, representative of 2 independent experiments. (H-J) 4T1 TB mice received i.p. either CD40 Ab or IgG. Absolute number of hepatic CD11b+Gr-1+ (H), CD11b+Gr-1+CD40+ (I) and CD11b+Gr-1+CD80+ (J). Data expressed as mean ± SEM, representative of two independent experiments. \**P*<0.05, \*\* *P*<0.01: Student’s t test.

****

**Supplementary Figure S4. Representative gating of CD40 and CD80 expression on tumor-induced hepatic CD11b+ Gr-1+ cells after agonist CD40 antibody injection.**

Representative dot plot of liver mononuclear cells from EL4 tumor-bearing mice which received i.p either control IgG (A) or agonistic anti-CD40 (B). Dot plots show the expression of CD40 and CD80 surface markers gated on CD11b+ Gr-1+ cells.



**Supplementary Figure S5. Differential contribution of G-MDSC and MDSC subsets in agonistic CD40 antibody-mediated liver damage.**

Kinetics of CD11b+Gr-1high G-MDSC (A) and CD11b+Gr-1low M-MDSC (B) absolute numbers 3 and 24 hours after injection of either control IgG or agonistic anti-CD40 (n=2 mice/group). Statistics reflect whether absolute numbers vary among time points. Cumulative data are presented as mean ± SEM, representative of three independent experiments. (C) Mean Fluorescence Intensity of DCFDA gated on hepatic G-MDSC and M-MDSC 3 hours after IgG or agonistic CD40 antibody injection (n=2 mice/group). Data expressed as mean ± SEM are representative of at least 2 independent experiments. n.s. non significant, \* *P*<0.05; \*\* *P*<0.01; \*\*\* *P*<0.005: two-way ANOVA.



**Supplementary Figure S6. Purity and phenotype of tumor-induced hepatic CD11b+enriched cells used for congenic transfer experiments.**

As described in *Materials and Methods*, liver CD11b cells from B16 GMCSF TB B6-CD45.1 mice were isolated using CD11b beads. Representative dot plot shows CD11b and Gr-1 expression on total liver mononuclear cells before and after (A) CD11b enrichment. (B) Monocytic Ly6G- Ly6Chigh and granulocytic Ly6Clow Ly6Ghigh MDSC cell subsets gated on liver CD11b+ enriched cells prior to transfer. 5x107 B16 GMCSF-induced WT liver CD45.1+CD11b+ cells (n=3 mice) were injected i.v. into TF CD45.2+ *Cd40-/-* mice (n=6-8 mice/group). Then either IgG or CD40 Ab were injected i.p. TF WT and *Cd40-/-* mice received CD40 Ab as a control (n= 3mice/group).



**Supplementary Figure S7. Human anti-CD40 antibody modulates arginase expression in human MDSC.**

2x105 sorted CD14+HLA-DRhigh or CD14+HLA-DRlow cells from healthy donors (n=4) were cultured in complete RPMI medium at the presence of either agonistic anti-human CD40 antibody (5 μg/ml) or megaCD40L (0.1 g/ml) for 24 hours. Results show the fold change induction in Arginase mRNA expression. Data expressed as mean ± SEM are cumulative of 2 independent experiments. \* *P* <0.05; \*\* *P* <0.01: two-way ANOVA.

**Supplementary Table 1**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Age** | **Diagnosis** | **Stage** |
| Patient 1 | 52 | HCC | IV |
| Patient 2 | 46 | HCC | IV |
| Patient 3 | 48 | Colon | IV |
| Patient 4 | 63 | Pancreas | IV |
| Patient 5 | 62 | HCC | IV |
| Patient 6 | 63 | Pancreas | IV |
| Patient 7 | 53 | Pancreas | IV |
| Patient 8 | 66 | HCC | IV |
| Patient 9 | 55 | HCC | III |
| Patient 10 | 41 | Colon | IV |
| Patient 11 | 63 | Rectum | III |
| Patient 12 | 62 | HCC | IV |
| Patient 13 | 41 | Colon | IV |