Modulation of the anti-tumor immune response by complement

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Methods

Cell proliferation, apoptosis and angiogenesis. The morphology of tumors and spleens was assessed by light microscopy (Olympus BX 60) of hematoxylin and eosin–stained paraffin sections 5 mm in thickness. For analysis of the number of cells in the S phase of the cell cycle, paraffin sections from tumors and spleens were stained with monoclonal anti-BrdU (BMC 9318; Roche). The presence of apoptotic cells in tumor sections was assessed by staining for the activated caspase-3 site on cytokeratin 18. Both assays were done as described[1]. The microvascular density of engrafted tumors was evaluated by immunofluorescence staining for CD31 expression on endothelial cells in frozen sections. Bound biotinylated anti-CD31 (MEC 13.3; BD Biosciences) was visualized with a streptavidin-rhodamine complex (BD Biosciences). Fluorescence was evaluated by standard fluorescent microscopy (Olympus BX 60 microscope). The incorporation of BrdU into tumor cells and the microvascular density of tumors were quantified in five to ten microscopic fields (magnification, x400 for BrdU and x100 for microvascular density) with the use of ImageJ image analysis software (National Institutes of Health), and mean values were calculated. Apoptosis was assessed in a semiquantitative way; scores of 0–4 were assigned to sections depending on the size of the area occupied by apoptotic cells. All analyses were made by researchers 'blinded' to sample identity.

Quantitative real-time PCR analysis. Expression of C5aR mRNA was analyzed by quantitative real-time RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen), and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription of RNA into DNA according to the manufacturer's instructions. The SYBR Green PCRMaster Mix Kit (Applied Biosystems) was used for real-time PCR as described[2]. The absolute quantification method was used with the generation of standard curves for genes of interest and reference. Each amplification experiment was done in 96-well optical-grade PCR

plates covered with optical tape in the AbiPrism 7700 Sequence Detection System (Applied Biosystems). The following primers were used: forward, 5' – CGCTCCACCAAGACGCTCAA - 3'; reverse, 5' – GGGGCAGCCACGCTATCATC – 3'. The cDNA 'load' was normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the forward primer 5' - CCTGCACCACCAACTGCTTA - 3' and reverse primer 5' – CATGAGTCCTTCCACGATACCA - 3'. Data are presented as relative units normalized to those of 1 x 10^4 GAPDH mRNA molecules. Molecules were considered present if more than five copies of mRNA were detected for every 1 x 10^4 copies of GAPDH mRNA[3].

Preparation of dendritic cells and macrophages. Bone marrow cells were dispersed by vigorous pipetting and were cultured for 8 d in RPMI-1640 medium supplemented with penicillin (100 μg/ml), streptomycin (100 U/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 μM; Sigma) and 10% (vol/vol) heat-inactivated FBS in the presence of recombinant mouse granulocyte-macrophage colony-stimulating factor (20 ng/ml; 315-03; Peprotech). Cultures were replenished with granulocyte-macrophage colony-stimulating factor on days 3 and 6. In some experiments, maturation was induced by culture of the cells for 2 d in the presence of granulocyte-macrophage colony-stimulating factor (10 ng/ml), mouse tumor necrosis factor (20 ng/ml; 315-01A; Peprotech) and bacterial lipopolysaccharide (1 μg/ml; from Escherichia coli serotype 0111:B4; L2630; Sigma). Mouse peritoneal cells were obtained by washing of the peritoneal cavities of C57BL/6J mice with complete medium.

Immunoblot analysis. Whole-cell extracts were prepared from tumor tissue mechanically disrupted in lysis buffer (20 mM HEPES, pH 7.4, 0.2 mM EDTA, 420 mM NaCl, 1.5 mM $MgCl_2$ and 20% (vol/vol) glycerol) treated with protease and phosphatase inhibitors (1 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM β-glycerophosphate, and antipain dihydrochloride, aprotinin, bestatin and leupeptin (2 μg/ml each

of the final four reagents)). For each protein sample, 40 μg was separated by electrophoresis through a 12% polyacrylamide gel followed by transfer to a polyvinylidene difluoride membrane. Membranes were incubated overnight at 4 °C with mouse mAb to arginase I (8C9; Santa Cruz Biotechnology) or mouse mAb to β-actin (AC-15; Abcam). Primary antibody binding was detected with horseradish peroxidase–conjugated anti-mouse (cat. no. 170-6516; Bio-Rad Laboratories) and chemiluminescence (Amersham Pharmacia Biotech). Protein loading was normalized to the abundance of β-actin with Ponceau S–stained membranes used for verification. Protein expression was quantified by densitometry with ImageQuant software (Molecular Dynamics).

References

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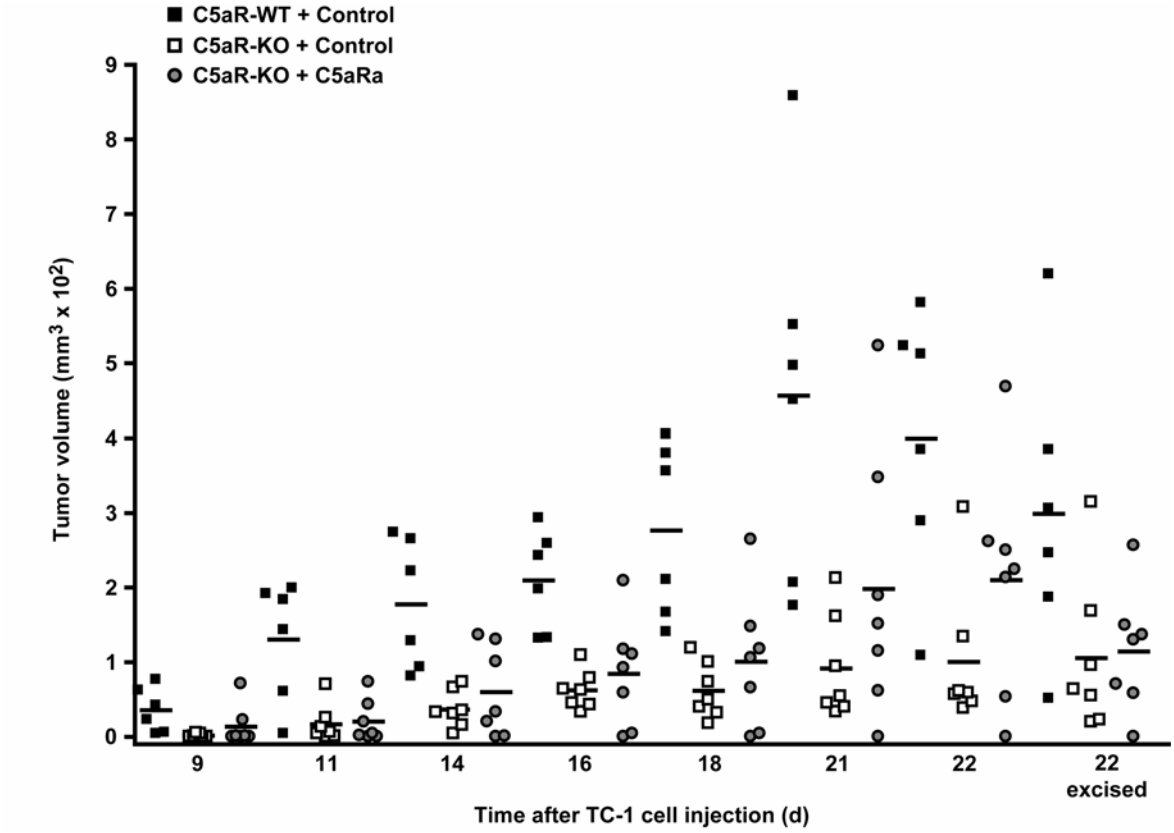
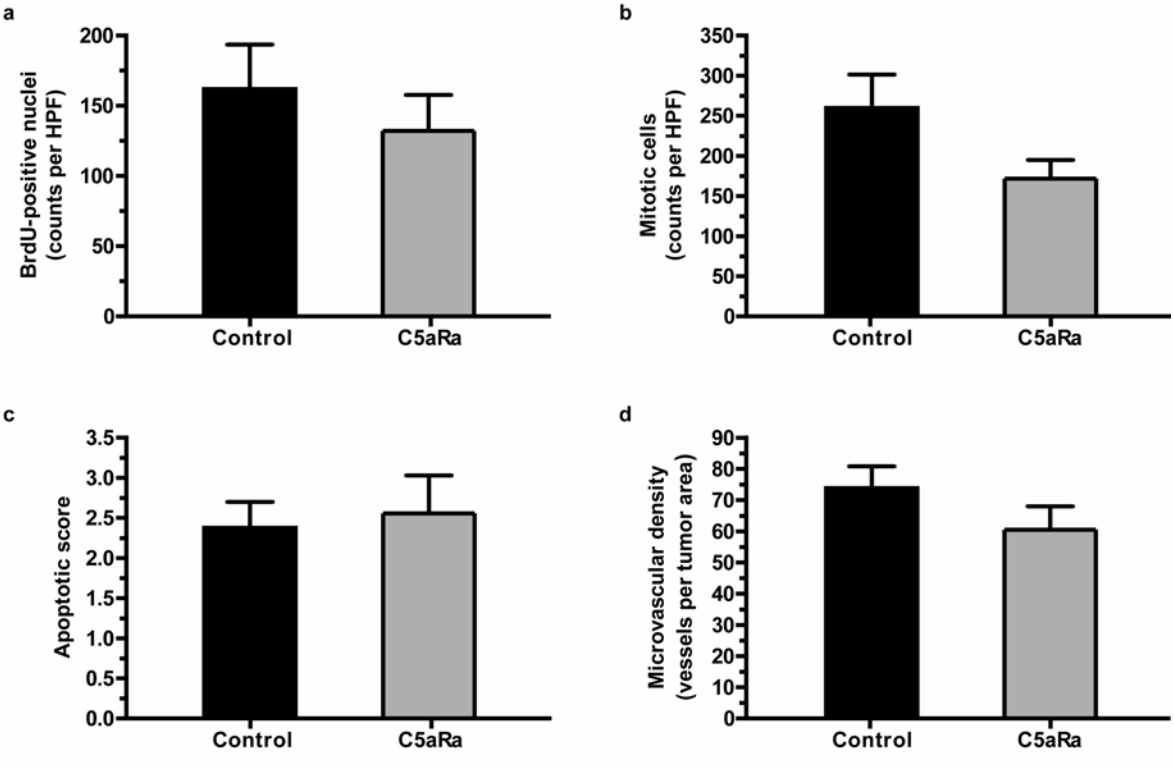
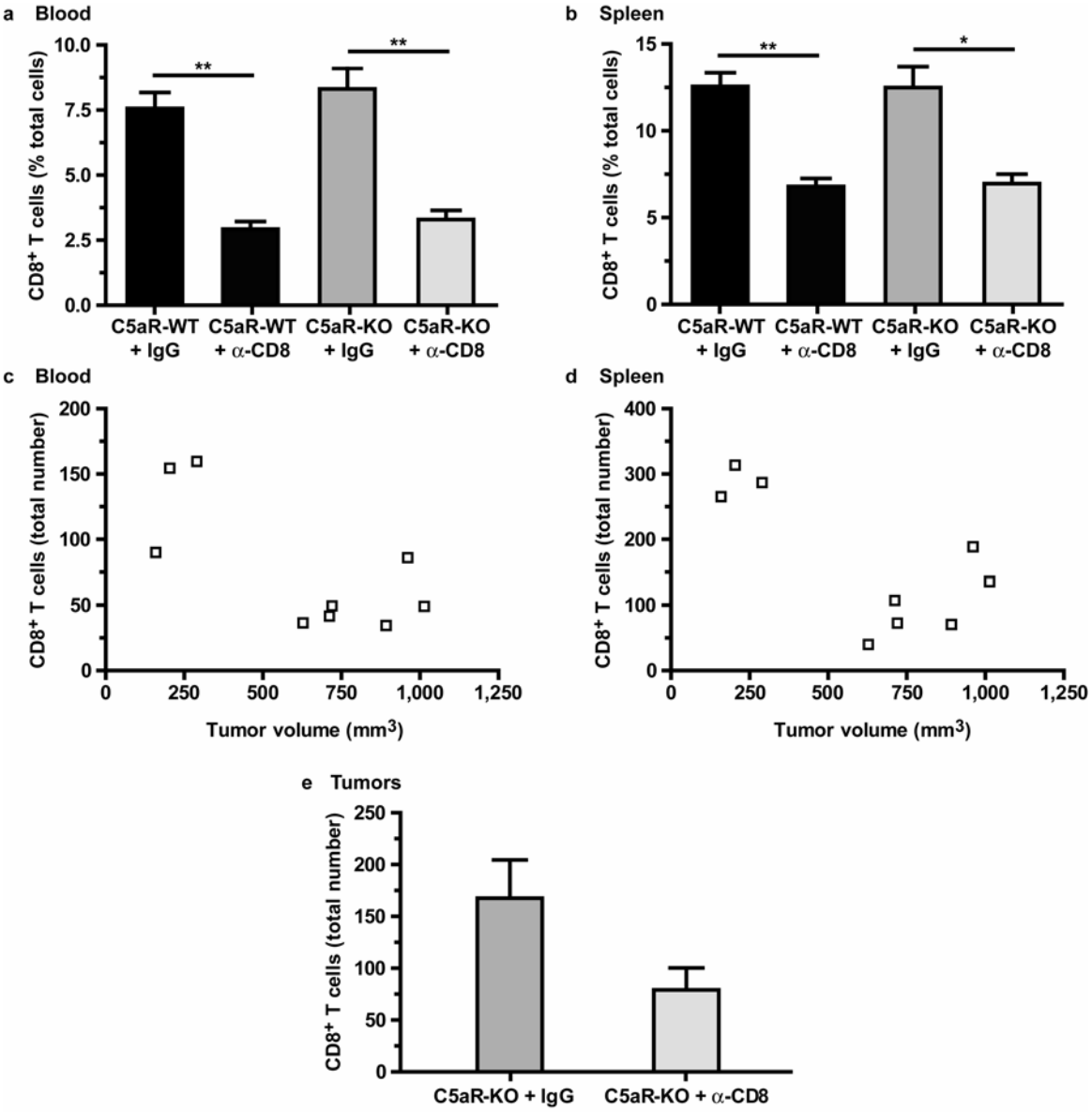
2. Conejo-Garcia, J.R. *et al.* Tumor-infiltrating dendritic cell precursors recruited by a β-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat. Med.* **10**, 950–958 (2004).
3. Benencia, F. *et al.* HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. *Mol. Ther.* **12**, 789–802 (2005).



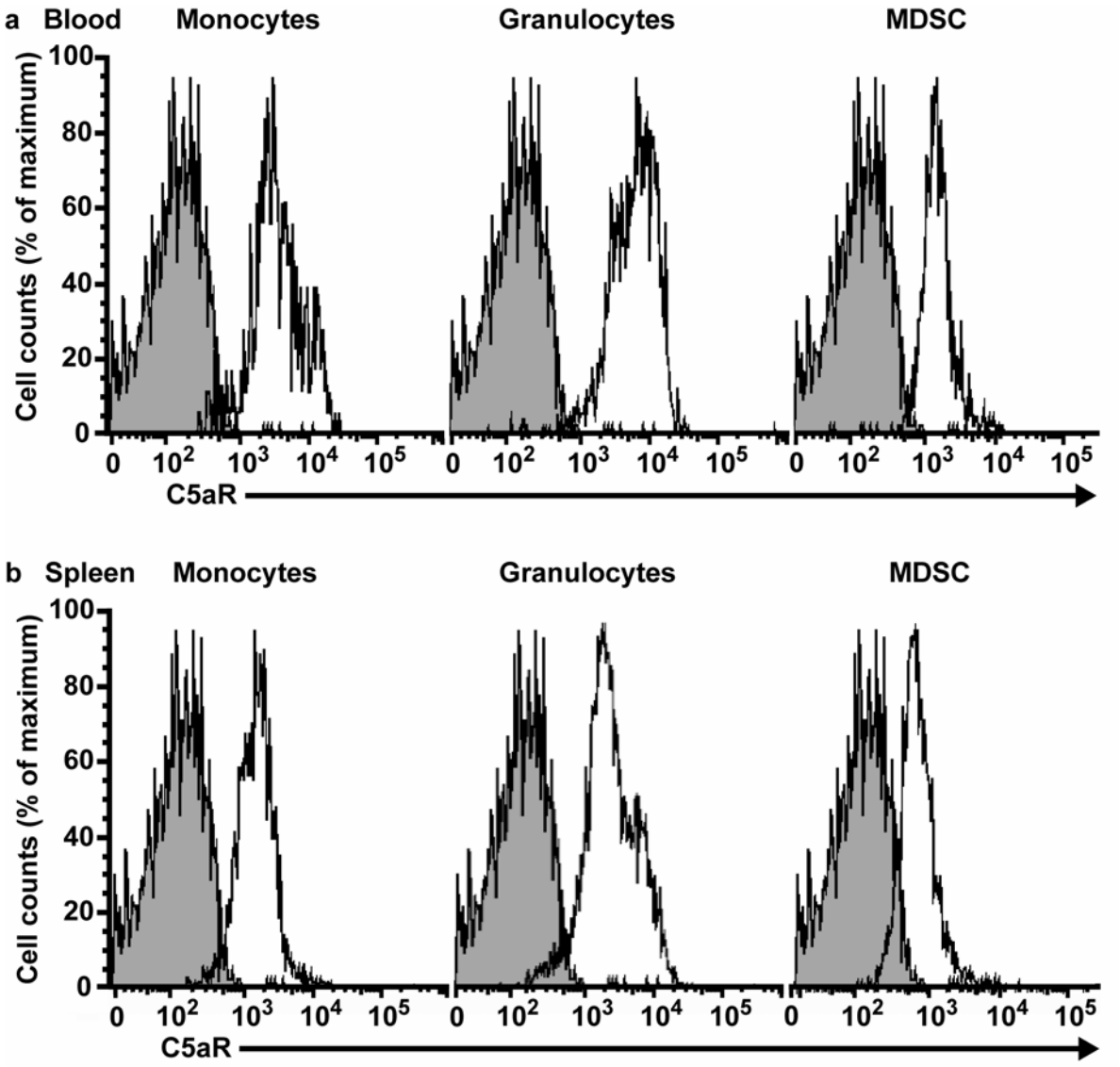
Supplementary Figure 1 C5aR antagonist does not exhibit cytotoxicity toward TC-1 cells. Tumor volumes of C5aR-deficient (C5aR-KO) mice and littermate wild-type control mice (C5aR-WT) treated with either control peptide (control) or C5aR antagonist (C5aRa). Far right (22 excised), volumes based on measurements obtained after mice were killed and the tumors were removed. Each symbol represents an individual mouse; small horizontal lines indicate the mean. $P < 0.0001$, C5aR-wild-type plus Control versus C5aR-deficient plus Control or C5aR-deficient plus C5aRa (two-way ANOVA). Data are representative of 1 experiment ($n \geq 6$ mice per cohort).



Supplementary Figure 2 Blockade of C5aR by antagonist does not significantly affect cell proliferation, apoptosis, or angiogenesis in tumors. Tumor sections from wild-type mice treated with C5aRa or PBS (Control) (1 experiment, $n \geq 9$ mice per cohort) were analyzed for (**a**) The number of BrdU-positive nuclei per high-power field (HPF; magnification, 400x; $P = 0.4770$), (**b**) The number of mitotic figures per HPF ($P = 0.0927$), (**c**) The amount of apoptosis ($P = 0.7580$), and (**d**) Microvascular density ($P = 0.2137$). *P* values were determined by *t*-test.



Supplementary Figure 3 Treatment of C5aR-deficient mice with CD8 antibody depletes $CD8^+$ T cells and accelerates tumor growth. (**a**,**b**) Flow cytometry of the percentage of $CD3^+CD8^+$ T cells in the blood (**a**) or spleen (**b**) of C5aR-deficient mice and C5aR-wild-type control mice bearing tumors and treated with either IgG or anti-CD8 (α-CD8). *, P = 0.0005; **, $P < 0.0001$ (*t*-test). (**c**) Tumor-infiltrating $CD3^+CD8^+$ T cells in the blood (**c**) or spleen (**d**) versus tumor volume for C5aR-deficient mice treated with α-CD8. $P = 0.0323$ and $r = -0.7094$ (Pearson correlation; **c**); $P = 0.0302$, $r = -0.7154$ (Pearson correlation; **d**). (**e**) Number of $CD8^+$ T cells, as determined by immunofluorescence, in tumors from C5aR-deficient mice treated with IgG or anti-CD8. $P = 0.0705$ (*t*-test). Data are based on studies shown in Fig. 4e and are therefore representative of 1 experiment (n ≥ 9 mice per cohort; **a**–**e**).



Supplementary Figure 4 C5a receptor is expressed on myeloid-derived cells. (**a**,**b**) Expression of C5aR (open histograms) versus isotype-matched control (shaded histograms) in monocytes, granulocytes, and myeloid-derived suppressor cells (MDSC) in the blood (**a**) or spleen (**b**) of a wild-type mouse without a tumor. Viable cells (**a**,**b**) were gated based on their scatter properties and CD45 expression, and MDSCs were identified as cells co-expressing CD11b and Gr-1. Data are representative of 1 experiment with 3 mice (**a**,**b**).