Supporting Information

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SI Materials and Methods

Mice. RIP1-Tag5 transgenic mice were used on a C3HeBFe background (kindly provided by D. Hanahan, Institut Suisse de Recherches Experimentales sur le Cancer, Lausanne, Switzerland). For adoptive transfer experiments, mice transgenic for a TCR that recognizes Tag presented by the MHC class I molecule H-2Kk (referred to as TagTCR8; kindly provided by T. Geiger, St. Jude Children's Research Hospital, Memphis, TN, and R. Flavell, Yale University, New Haven, CT) or the MHC class II molecule I-A (TagTCR1; kindly provided by I. Foerster, University of Düsseldorf, Düsseldorf, Germany) were used on a C3H background. All mice were kept under specific pathogen-free conditions at the University of Western Australia, and all experimental protocols were approved by the Animal Ethics Committee of the University of Western Australia.

Cell Lines. AG104A, mouse fibrosarcoma cells, were kindly provided by H. Schreiber (University of Chicago, Chicago, IL). Mouse brain endothelial cells (bEnd5) were kindly provided by B. Engelhardt (University of Bern, Bern, Switzerland). HUVEC were purchased from PromoCell.

Production of Recombinant Proteins. Mature murine TNFα and IFNγ with or without a C-terminal modified RGR peptide (CRGRRSTG, connected via a GGG linker) were cloned into Xho/BamH1 sides of the vector pET-44a (Novagen) to express soluble fusion proteins with N-terminal Nus•Tag/His•Tag. Briefly, after isopropyl-β-D-glactopyranoside (IPTG) induction overnight at 25 °C (TNFα) or for 4 h at 37 °C (IFNγ), cultures were centrifuged, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, and 1% Triton X-100 (vol/vol) at pH 8.0), incubated with 1 mg/mL lysozyme on ice for 30 min, sonicated, and purified by using Ni-NTA beads (Qiagen) following the manufacturer's instructions. Nus•Tag/His•Tag was cleaved with tobacco etch virus (TEV) protease overnight at 4 °C (TNFα) or for 4–6 h at 30 °C followed by room temperature overnight (IFNγ). Recombinant proteins from cleavage reactions were dialyzed overnight in PBS and purified twice by using Ni-NTA beads. Purity was assessed on Coomassie brilliant blue stained protein gels.

Treatment of Tumor-Bearing RIP1-Tag5 Mice. RIP1-Tag5 mice were treated in short- or long-term regimens. Short-term: Commencing at 27 wk of age, mice were treated for 2 wk with biweekly i.v. injections of recombinant proteins in 100 μL of volume at the indicated dose. At 29 wk of age, mice were killed and tumors were isolated for histology or RNA analysis. Long-term: 22- to 23-wk-old RIP1-Tag5 mice were treated with biweekly i.v. injections of proteins as described for short-term regimens and survival was monitored. For antibody depletion studies, anti-Lyt.2 (anti-$CD8^+$, rat anti-mouse 53–6.7), anti-L3T4 (anti $CD4^+$, rat anti-mouse GK1.5 hybridoma), or irrelevant rat IgG control antibodies (0.5 mg) were injected i.p. for three consecutive days before start of treatment, followed by weekly injections of antibodies (1 mg) for 8 wk. Depletion efficiency was monitored by FACS analysis. For vaccination studies, mice were primed with a single s.c. injection at the tail base of 50 μg of purified Tag protein mixed with 50 μg of CpG-ODN 1668 (Sigma). Thereafter, the same Tag/CpG mixture was injected i.p. every third week and survival was monitored. For adoptive transfer experiments, TagTCR8 splenocytes or TagTCR1 lymph node cells were activated in vitro for 3 d, with 10 U of rIL-2 per mL and 25 nM Tag peptide 560–568 (SEFLIEKRI for TagTCR8 cells) or 25 nM Tag peptide 362–384 (TNRFNDLLDRMDIMFGSTGSADI for TagTCR1 cells). Activated $CD4^+$ T cells (2.5×10^6) and 2.5×10^6 $CD8^+$ T cells were injected i.v. every 2 wk. For T-cell quantification, lymph node or tumor cell suspensions were stained with PE-conjugated anti-CD8 (Ly-2) and biotinylated anti-mouse Vβ8.1 and 8.2 (MR5.2, BD Pharmingen) followed by SA-Cy5 and analyzed in a two-color FACS setting.

Histology. For homing studies, recombinant TNFα and IFNγ fusion proteins were biotinylated by following the manufacturer's instructions (EZ-Link Sulfo-NHS-LC-Biotin; Pierce) and i.v. injected into tumor-bearing RIP1-Tag5 mice. After 10–30 min of circulation, mice were perfused with 10% neutral-buffered formalin, postfixed in 10% formalin (vol/vol) for 2 h, followed by incubation in 10% (wt/vol, 2 h) and 30% sucrose (wt/vol) overnight and embedded in OCT compound. Intratumoral cytokines were detected on 10-μm sections by using streptavidin (SA)-Alexa 488 (Invitrogen) or SA-Cyanin-3 (Cy3) (Jackson Immuno Research). For immunohistochemistry the antibodies used were the following: anti-CD8 (Ly-2; BD Pharmingen), anti-CD31 (Mec 13.3.; BD Pharmingen), anti-VCAM (clone 429; BD Pharmingen), ER-TR7 (Abcam), CD68 (FA-11; Abcam), CD11b (M1/70; BD Pharmingen), F4/80 (HP-198; ATCC), PDGFRβ (eBiosciences), and αSMA (Sigma). For secondary detection, Cy-3 or FITC-conjugated IgG F(ab′)2 fragment goat anti-rat (Jackson Immuno Research) was used. The αSMA staining was amplified by using the mouse on mouse (M.O.M.) kit (Vector). For lectin perfusion, mice were i.v. injected with 50 μg of FITC-labeled tomato lectin (*Lycopersicon esculentum*; Vector). After 10 min of circulation, mice were heart-perfused with 2% neutral-buffered formalin (wt/vol) and tumors were frozen in OCT. To evaluate vessel leakiness, 1 mg of 70-kDa Texas Red Dextran (Invitrogen) was injected i.v. and allowed to circulate for 10 min. Mice were heart-perfused with PBS followed by 2% neutral buffered formalin, and tumors were frozen in OCT. Apoptosis was assessed by using TUNEL staining (Roche). Images were recorded on a Nikon Ti-E microscope and quantified by using NIS software modules (version 3.0).

IFNγ in Vivo Detection. Briefly, 27-wk-old RIP1-Tag5 mice were i.v. injected with 75 μg of IFNγ fusion protein, and tumors were dissected after 2 h of circulation. Tumors were homogenized in lysis buffer (1× PBS, 0.1% Igepal, 1 tablet per 10 mL protease inhibitor; Roche), 100 μL per 10 mg of wet weight), sonicated, and cleared by centrifugation. Protein concentration, measured by BCA protein assay kit (Pierce), was adjusted to 20 μg of protein per μL. The cytometric mouse/rat bead-capture assay (Bender MedSystems) was used for quantitative detection of IFNγ in the tumor extract according to the manufacturer's instruction. Data were acquired by using a FACScan (BD Biosciences) and analyzed by using the manufacturer's software.

Isolation of Tumor Macrophages. Tumors were collected from untreated and TNFα treated mice and enzymatically digested with 0.2 mg/mL collagenase P (Roche), 0.8 mg/mL dispase (Invitrogen), and 0.1 mg/mL DNase I (Sigma) in PBS for 25 min at 37 °C and passed through a 70-μm membrane (BD Pharmingen). Cells were subsequently washed with FACS buffer [1% BSA (wt/vol, Sigma) in PBS], stained with Alexa Fluor 647-labeled CD68 antibodies (Biolegend) and sorted on a FACS Aria II (BD Biosciences).

Gene Expression analyses. RNA from CD68-positive macrophages was prepared by using the RNA Easy Mini Kit (Qiagen). For cDNA synthesis and qPCR the RT^2 system (Qiagen) was used according to the manufacturer's protocol. Angiogenic and inflammatory gene expression was assessed by using commercial arrays (SABiosciences) on the 7900HT cycler (Applied Biosystems). Data were analyzed by using software available online (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Array data were subsequently validated on two independent cDNA populations by qPCR using the Rotor Gene Real-Time PCR Detection system. Primer sequences were obtained from a public source (PrimerBank; ref. 1). All reactions were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT).

HUVEC Coculture Assay. FACS sorted macrophages from tumors were added to serum starved HUVECs with or without blocking antibodies for angiopoetin 2 (anti-human tie2, 10 μg/mL; R&D Systems) and mouse TNFα (MP6XT22, 1 μg/mL; R&D Systems) and incubated overnight at 37 °C, 5% CO_2. HUVECs were fixed and stained with goat anti-hVCAM (R&D Systems) followed by staining with biotinylated horse anti-goat IgG (Vector) and SA-Cy3 (Jackson Immuno Research). Multiple images were recorded on a Nikon Ti-E microscope, and VCAM-positive cells were quantified by using NIS software modules (version 3.0).

Flow Cytometry. For FACS analysis, cells were incubated with TNFα and IFNγ fusion proteins as indicated. MHC class I expression (H-2K^k) on AG104A cells was analyzed by using biotinylated H100-27.55 mAb; VCAM expression was detected by using biotinylated anti-VCAM antibodies (clone 429; BD Pharmingen). SA-Cy5 (eBioscience) was used as secondary antibody.

In Vivo CTL Assay. For the in vivo CTL assay, the F1 generation of RIP1-Tag5 and C57BL/6 mice (referred to as RIP1-Tag5/F1) were used. Briefly, 1×10^7 splenocytes per mL were loaded with H2-K^b-restricted Tag peptide IV (404-411, VVYDFLKL) or left without peptide. Targets were labeled with CSFE in a final concentration of 0.75 μM (high, with peptide IV) or 0.075 μM (low, without peptide IV). Cells (1×10^7) of each population was injected i.v. into recipient mice. CTL activity was assessed 18 h after the adoptive transfer by using FACS analysis.

T-Cell/Macrophage Coculture Assay. The macrophage-T-cell repression assay was adapted from ref. 2. TagTCR8 cells (4×10^4) were isolated from lymph nodes, labeled with CFSE (5 μM; Molecular Probes, Invitrogen) (3), and stimulated with 25 nM Tag peptide 560–568 and 10 U of rIL-2 per mL in the presence or absence of tumor-derived macrophages (ratio 1:1) in 96-well plates for 55 h. T cells were stained with PE-conjugated anti-CD8 (Ly-2) and biotinylated anti-mouse Vβ8.1 and 8.2 (MR5.2) followed by SA-Cy5 and analyzed in a three-color setting for cell proliferation.

Statistics. Cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test. Student's *t* test (two-tailed) was used unless indicated otherwise. A P value <0.05 was considered statistically significant. Error bars indicate SD unless stated otherwise.

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2. Denardo DG, et al. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov* 1:54–67.
3. Quah BJ, Warren HS, Parish CR (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* 2:2049–2056.

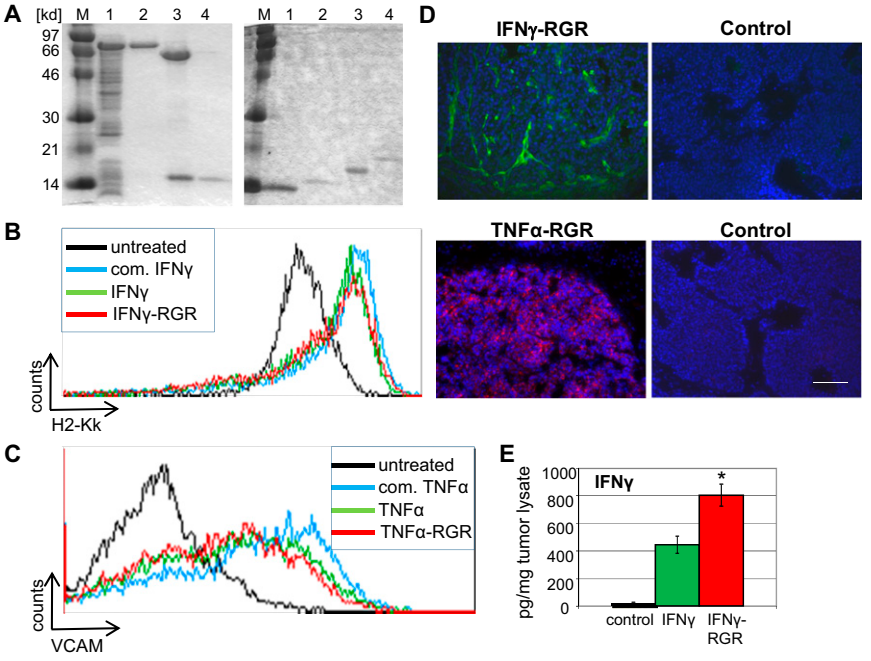

Fig. S1. Purification of recombinant IFNγ and TNFα fusion compounds, bioactivity, and tumor homing. (*A Left*) M, protein marker; 1, IFNγ–RGR fusion protein cloned into pET-44TEV, bacterial lysate; 2, His-tagged fusion compound purified by using a Ni-NTA column; 3, IFNγ–RGR released from Nus•Tag by TEV digestion; 4, IFNγ–RGR compound after repurification. (*A Right*) M, protein marker; purified proteins (1–4): 1, IFNγ; 2, IFNγ–RGR; 3, TNFα; 4, TNFα-RGR. (*B*) AG104A fibrosarcoma cells were incubated for 24 h with 200 U of commercial, recombinant murine IFNγ, bacterially expressed murine IFNγ, and IFNγ–RGR. MHC class I expression (H-2K^k) was measured by using flow cytometry. (*C*) Brain endothelial cells (bEnd5) were incubated for 2 h with 20 ng of commercial, recombinant murine TNFα or bacterially expressed murine TNFα and TNFα-RGR. Induction of VCAM was analyzed by FACS. (*D Upper*) RIP1-Tag5 mice were i.v. injected with 75 μg of biotinylated IFNγ–RGR. Mice were killed after 30 min, and tumor tissue was stained with FITC-streptavidin. (*Upper Left*) Homing of IFNγ–RGR to vascular structures. (*Upper Right*) Untreated control. (*Lower*) RIP1-Tag5 mice were i.v. injected with 10 μg of biotinylated TNFα-RGR, killed after 30 min, and tissue stained with Cy3-streptavidin. (*Left*) Intratumoral staining after TNFα-RGR injection. (*Right*) Untreated control. All tissue was counterstained with DAPI. (Original magnification: 10×.) (Scale bar: 200 μm.) (*E*) RIP1-Tag5 mice were i.v. injected with 75 μg of IFNγ or IFNγ–RGR or remained untreated (control). Tumors were isolated after 2 h, and IFNγ was measured in tumor lysates by using an IFNγ–specific bead-capture assay (*P = 0.004; IFNγ–RGR compared with IFNγ, n = 7). Homing/retention experiments could not be performed with TNFα/TNFα-RGR because of toxicity of TNFα at a dose ≥20 μg.

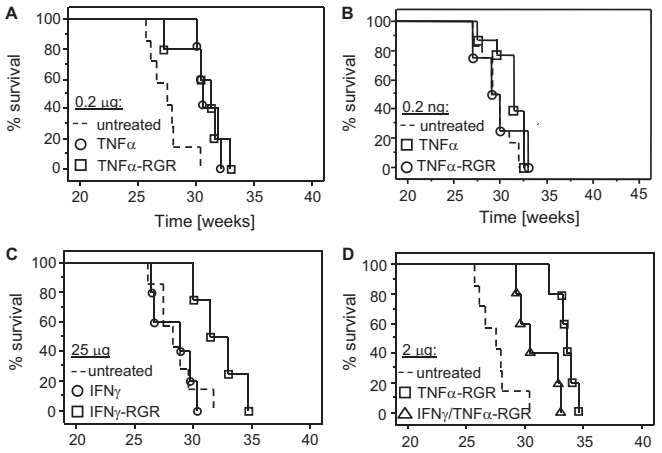

Fig. S2. Long-term treatment with IFNγ and TNFα and fusion compounds: Dose-dependent survival. Survival of 22-wk-old RIP1-Tag5 mice treated biweekly with 0.2 μg of TNFα or TNFα-RGR (P = 0.02 compared with untreated controls, n = 5–7) (*A*); 0.2 ng of TNFα or TNFα-RGR (not statistically significant to untreated controls, n = 8) (*B*); 25 μg of IFNγ or IFNγ–RGR (P = 0.025 compared with untreated, n = 5–7) (*C*), and a combination of 2 μg of IFNγ–RGR plus 2 μg of TNFα-RGR (P = 0.01 compared with TNFα-RGR monotherapy and untreated controls, n = 5–7) (*D*). Combination treatment of 2 μg of IFNγ plus 2 μg of TNFα was not well-tolerated, and mice were killed after three treatment cycles.

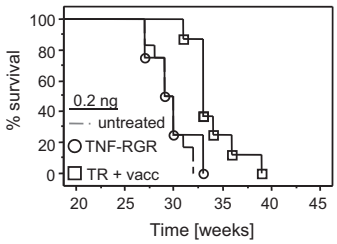

Fig. S3. Combination therapy with nanograms of TNFα-RGR. The 22-wk-old RIP1-Tag5 mice were left untreated or treated with 0.2 ng of TNFα-RGR alone or in combination with anti-Tag vaccine (P = 0.01 single versus combination treatment, n = 8–12).

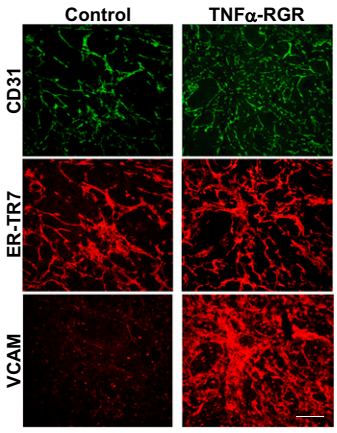

Fig. S4. Intratumoral TNFα activates tumor stroma. Tumor stroma in untreated and TNFα-RGR–treated RIP1-Tag5 mice is stained with CD31 (blood vessels; *Top*), ER-TR7 (reticular fibroblast marker, *Middle*), and VCAM-1 (activation, inflammatory marker; *Bottom*). (Original magnification: 10×.) (Scale bar: 200 μm.)

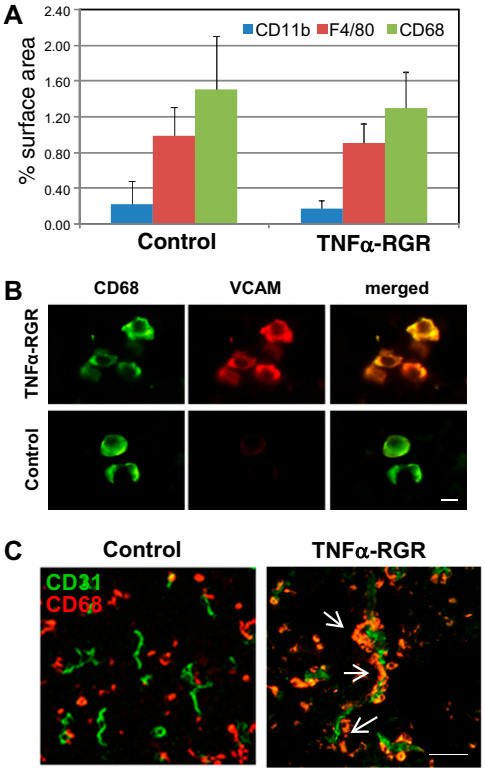

Fig. S5. Intratumoral macrophages change in phenotype but not frequency after treatment. (*A*) Quantification of $CD11b^+$, $F4/80^+$, and $CD68^+$ macrophages in untreated RIP1-Tag5 tumors (control) and after 2 wk of TNFα-RGR treatment (2 μg). (*B Upper*) CD68-positive macrophages strongly express VCAM after TNFα-RGR treatment. (*B Lower*) TNFα-treated control tumors. (Original magnification: 40×.) (Scale bar: 50 μm.) (*C*) $CD68^+$ tumor-resident macrophages in untreated and TNFα-RGR–treated tumors in relation to endothelial cells. Arrows point to macrophages that cluster around $CD31^+$ tumor vessels (Original magnification: 40×.) (Scale bar: 50 μm.)

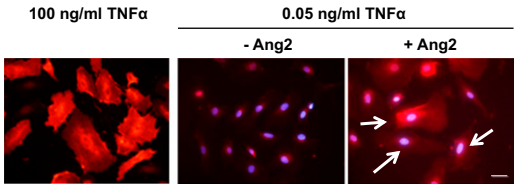

Fig. S6. Angiopoetin 2 sensitizes endothelial cells to low-dose TNFα-induced VCAM expression. HUVEC cells were treated overnight with a high dose of TNFα (100 ng/mL), which induces vessel inflammation measured by VCAM expression without recombinant angiopoetin 2 (Ang2). Low-dose TNFα (0.05 ng/mL) is not sufficient to induce VCAM expression in the absence of Ang2. A combination of low-dose TNFα (0.05 ng/mL) and Ang 2 (250 ng/mL) induces VCAM expression. Arrows point at VCAM-positive cells. (Original magnification: 40×.) (Scale bar: 50 μm.)

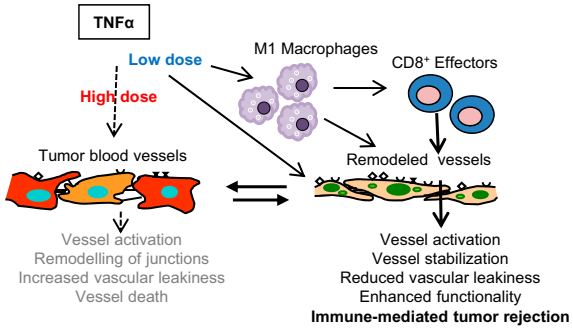

Fig. S7. Proposed model of TNFα action in the tumor environment. High-dose TNFα causes vessel activation, redistribution of cell junction and cytoskeletal proteins, and, ultimately, vessel death. Low-dose TNFα modulates both tumor vessels and tumor-resident macrophages. M1-like macrophages secrete immunostimulatory and angiogenic factors that alleviate T-cell suppression and may indirectly induce vessel remodeling/activation and, thus, increase vessel perfusion and extravasation of effector T cells. These effects favor immune-mediated tumor rejection, albeit transiently.

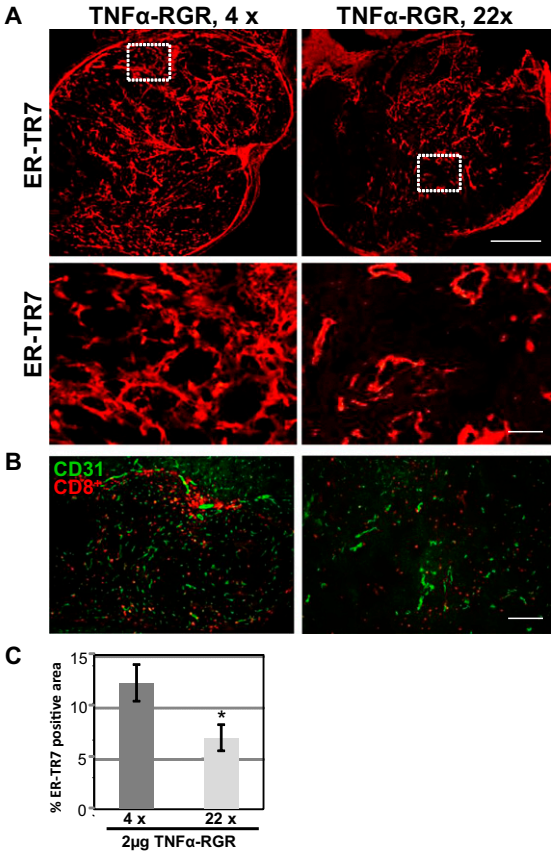

Fig. S8. Stromal destruction after long-term intratumoral TNFα-RGR treatment. (*A Upper Left*) Stroma (ER-TR7$^+$) in RIP1-Tag5 tumors after 4× i.v. injections of 2 μg of TNFα-RGR over a 2-wk treatment course. Corresponding inset (white dotted line) is depicted below. (*A Upper Right*) Stroma in RIP1-Tag5 tumors treated with 22× i.v. injections of 2 μg of TNFα-RGR over an 11-wk treatment course and higher magnification below. (Original magnification: 4x.) (Scale bar: 500 μm.). (*A Lower*) (Original magnification: 40×.) (Scale bar: 50 μm.) (*B*) Costaining for CD31 (blood vessels, green) and tumor-infiltrating CD8$^+$ T cells (red) after 4× and 22× i.v. injections of 2 μg of TNFα-RGR. (Original magnification: 10x.) (Scale bar: 200 μm.) (*C*) Quantification of stromal area positive for the fibroblast marker ER-TR7 in tumors after 4 or 22 injections of 2 μg of TNFα-RGR (n = 6, *P = 0.02).