Supplemental Figure legends

Supplemental Figure 1**: p55TNFR levels and response to acute TNF injection in *Tnfrsf1α*$^{+/+}$, $^{+/-}$ and $^{-/-}$**

A-C: Serum creatinine, hexosaminidase and LDH levels of *Tnfrsf1α*$^{+/+}$ (n=8), $^{+/-}$ (n=7) and $^{-/-}$ (n=2) after i.p. injection with 50µg TNF. **D-F:** p55TNFR levels measured in kidney, spleen and lung of *Tnfrsf1α*$^{+/+}$ (n=6), *Tnfrsf1α*$^{+/-}$ (n=5) and *Tnfrsf1α*$^{-/-}$ (n=4) mice measured by ELISA in kidney (**D)** spleen **(E)** and lung **(F)**. *Tnfrsf1α*$^{+/-}$ mice express approximately half of the WT levels of p55TNFR. *Tnfrsf1α*$^{-/-}$ mice show no measurable expression of p55TNFR. (**G**) qPCR on RNA samples from liver of *Tnfrsf1α*$^{+/+}$ (n=9), *Tnfrsf1α*$^{+/-}$ (n=5) and *Tnfrsf1α*$^{-/-}$ (n=3) animals. Again intermediate levels of p55TNFR were measured in *Tnfrsf1α*$^{+/-}$ mice compared to *Tnfrsf1α*$^{+/+}$ and *Tnfrsf1α*$^{-/-}$ mice. (**H)** FACS measurement of membrane p55TNFR expression on BMDMs.

Supplemental Figure **2: Signal transduction and gene expression in *Tnfrsf1α*$^{+/+}$, $^{+/-}$ and $^{-/-}$**

A: Western blot showing IκB degradation in thioglycolate -elicited macrophages at different time points after TNF stimulation (10 ng/ml). Densities of the bands were normalized to the actin bands. After 15 min, IκB was almost completely degraded in the *Tnfrsf1α*$^{+/+}$ samples compared to the *Tnfrsf1α*$^{+/-}$.

B-D: Corresponding blots for Main Figures 2A, 2B and 2C. BMDMs were stimulated with TNF (10 ng/ml) and activation of NFκB (**B**), JNK2 (**C**) and ERK (**D**) was analyzed. **E:** Induction of IL6 is observed 8 and 24 h after TNF stimulation (10 ng/ml) in supernatants of BMDMs . At 24 h, IL6 levels in *Tnfrsf1α*$^{+/-}$ mice are significantly lower than in *Tnfrsf1α*$^{+/+}$ mice. **F:** Mice ($n \geq 3$) were injected with TNF (5 µg) and 5 minutes later, livers were excised, and IκB was measured by Western blotting.. **Left panel** shows Western blots and **right panel** shows the quantified results. *Tnfrsf1α*$^{+/+}$ (black), *Tnfrsf1α*$^{+/-}$ (blue) and *Tnfrsf1α*$^{-/-}$ (red). Levels in untreated mice were set as 1.0. **G-I:** Mice ($n \geq 3$) were injected *i.p.* with 30 µg mTNF, *i.e.* an LD_{100} in this acute model, and expression of NFκB-dependent genes in the liver was measured by qPCR 0 h, 1 h and 6 h after challenge. Results are shown for CXCL9 (**G**), cFlip (**H**) and Nox2 (**I**). Asterisks represent significant differences between *Tnfrsf1α*$^{+/-}$ and *Tnfrsf1α*$^{+/+}$ mice. Data of *Tnfrsf1α*$^{-/-}$ mice are not shown because they are all flat and show no responsiveness to TNF. **J-M:** Intermediate levels of

TNF-induced cytokines IL1α, MIP1b, MCP-1 and CCL5/Rantes in serum of *Tnfrsf1α*$^{+/-}$ mice. Mice (n ≥ 3 in all groups) were injected *i.p.* 30 μg of TNF and multiple cytokines were measured at different time points. All the cytokines were induced about half maximally in *Tnfrsf1α*$^{+/-}$ compared to the *Tnfrsf1α*$^{+/+}$ and *Tnfrsf1α*$^{-/-}$mice. Asterisks represent significant differences between *Tnfrsf1α*$^{+/-}$ and *Tnfrsf1α*$^{+/+}$ mice.

Supplemental Figure **3: TNF-induced inflammatory gene induction in intestinal tissue of *Tnfrsf1α*$^{+/+}$, $^{+/-}$ and $^{-/-}$**

A-D: qPCR analysis of induction of NF-kB driven genes, IL6, IL1β, A20 and IkBα in the intestinal epithelium of *Tnfrsf1α*$^{+/+}$ and *Tnfrsf1α*$^{+/-}$ mice (n=4 per timepoint).

Supplemental Figure **4: Generation of *Tnfrsf1α* conditional knockout mice (see also Materials and Methods)**

A: Genomic structure of the mouse *Tnfrsf1α* locus and description of targeting vector for the generation of the *Tnfrsf1α* conditional knockout locus before neoR gene deletion.. Exons 2-5 are floxed. Probe locations and expected restriction fragments are depicted. FRT sites (grey diamonds) and loxP sites (white triangles). **B**. Southern blot analysis of representative wild type (lane 1) and recombinant (lane 2) ES cells clones. *EcoRI* digested genomic DNA hybridized with probe A (left panel). Recombinant clones have a 7.1 kb band in addition to the 21 kb band of the wild type allele. *HindIII* digested genomic DNA hybridized with probe B (right panel). Recombinant clones have an 8.8 kb band in addition to the wild type 17 kb allele. **C**. Flow cytometric analysis for the detection of p55TNFR in BMDMs of *Tnfrsf1α*$^{+/+}$ (black line), *Tnfrsf1α*$^{flx/flx}$ (green line), *Tnfrsf1α*$^{-/-}$ (red line), *Tnfrsf1α*$^{flx/\Delta}$ (purple line) and *Tnfrsf1α*$^{\Delta/\Delta}$ (blue line). **D**. Flow cytometric analysis for the detection of p55TNFR in BMDMs of *Tnfrsf1α*$^{+/+}$ (black line), *Tnfrsf1α*$^{flx/flx}$ (green line), *Tnfrsf1α*$^{-/-}$ (red line), LysM-Cre *Tnfrsf1α*$^{flx/flx}$ (cyan and yellow), *Tnfrsf1α*$^{flx/\Delta}$ (purple) and *Tnfrsf1α*$^{\Delta/\Delta}$ (blue line). **E-F**. DNA PCR analysis for the detection of recombination (deletion) events in different tissues of Villin-Cre *Tnfrsf1α*$^{flx/flx}$ (**E**) and Alfp-Cre *Tnfrsf1α*$^{flx/flx}$ (**F**).

Supplemental Figure **5: p55TNFR expression in the intestinal epithelium is crucial for TNF-induced toxicity.**

A: Q-PCR for p55TNFR expression. Significantly more p55TNFR expression in ilea of reactivated mice (*Villin-Cre Tnfrsf1α* $^{flxneo/+}$) compared to non-reactivated mice, both in rest and after TNF challenge (left panel). Restored liver p55TNFR expression in *Alfp-Cre Tnfrsf1α* $^{flxneo/+}$ mice (right panel)**. B:** Relative expression of IL17 and IL6 and IL1β mRNA in intestinal tissue of *Villin-Cre Tnfrsf1α* $^{+/flxNeo}$ (black bars, n=4) and *Tnfrsf1α* $^{+/\mathrm{flxNeo}}$ mice (grey bars, n=6) 2 h after TNF challenge. **C.** *Tnfrsf1α* $^{\mathrm{flx/flx}}$, and *Villin-Cre* or *Alfp-Cre* or *LysM-Cre Tnfrsf1α* $^{\mathrm{flx/flx}}$ were iv injected with 7 µg TNF and lethality was monitored. **D**: Relative expression of IL17 and IL6 and IL1β mRNA in intestinal tissue of Villin-Cre *Tnfrsf1α* $^{\mathrm{flx/flx}}$ (black bars, n=7) and *Tnfrsf1α* $^{\mathrm{flx/flx}}$ mice (grey bars, n=7) 2 h after TNF challenge. **E:** Relative intestinal permeability of Villin-Cre *Tnfrsf1α* $^{\mathrm{flx/flx}}$ (black bars) compared to *Tnfrsf1α* $^{\mathrm{flx/flx}}$ (grey bars) when mice were injected with 7 µg TNF and orally gavaged with 4kD FITC-dextran (0 h; n=4 and 8 h; n=9). All data represent means ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test and log-rank test for survival).

Supplemental Figure **6: Lethal response to TNF + GalN and to TNF + ActD of *Tnfrsf1α*** $^{+/+}$ **and** $^{-/-}$ **mice**

A: Lethality response of mice to different doses of TNF in combination with 20 mg GalN. Mortality was scored for 72 h, after which no further deaths occurred. All *Tnfrsf1α* $^{-/-}$ mice survived, and there was no significant difference in mortality of *Tnfrsf1α* $^{+/-}$ and *Tnfrsf1α* $^{+/+}$ mice. **B-C:** Survival and body temperature of *Tnfrsf1α* $^{+/+}$ (black, n=9) and *Tnfrsf1α* $^{+/-}$ (blue, n=9) mice *i.p.* injected with 20 µg ActD plus 1 µg TNF. There was no significant difference in final survival, but significant difference in survival time between *Tnfrsf1α* $^{+/+}$ and *Tnfrsf1α* $^{+/-}$ mice.

Supplemental Figure **7: Toxicity profiles of daily TNF + IFNγ injections in mice.**

A: Safety studies with *Tnfrsf1α* $^{+/+}$ and *Tnfrsf1α* $^{+/-}$ mice were performed both in tumor-free and in B16BL6 melanoma tumor bearing mice as fdescribed in Supplamentary Materials and Methods section. (1) groups of tumor-free mice were treated systemically (*i.p.*) with different doses of TNF + 5000 IU IFNγ daily for 10 days. (2) Safety studies in tumor-bearing *Tnfrsf1α* $^{+/+}$ and

Tnfrsf1α$^{+/-}$ mice The groups of mice were injected daily with different doses of TNF + 5000 IU IFNγ near the tumors when they had a TSI of about 70 mm^2, i.e. on day 0. The daily injections were given for 10 days.

Safety studies with anti-mp55TNFR antibodies in tumor-bearing *Tnfrsf1α*$^{+/+}$ mice, Mice were *i.p* injectied with antibody (control or anti-mp55TNFR) at 10 mg/kg every day starting at day 0 and *s.c.* injections of different doses of TNF + 5000 IU IFNγ close to the tumor daily from day 0 to day 9, when the tumors had a TSI of about 70 mm^2.

Safety studies with anti-hp55 antibodies in tumor-bearing *Tnfrsf1α*$^{+/+}$ mice, Mice were *i.p* injected of m5R16 antibody at 10 mg/kg every day starting at day 0 and *s.c.* injections of different doses of TNF + 5000 IU IFNγ close to the tumor daily from day 0 to day 9, when the tumors had a TSI of about 70 mm^2.

In these safety studies, the numbers of deaths were recorded daily and the experiment was considered finished 10 days after the last injection. Tumours had fully regressed in all of the safety studies performed.

B. Left upper panel: (A) the TNF+IFNγ anticancer response of *Tnfrsf1α*$^{+/+}$ tumor cells in *Tnfrsf1α*$^{+/+}$. $^{+/-}$ and $^{-/-}$ mice is not determined by the *Tnfrsf1α*$^{+/+}$ genotype of the tumor cells, but by the genotype of the host mice (**left panel**, data not shown) and (B) tumor regression by treatment with TNF+IFNγ is independent of the p55TNFR status of the tumor cells, but by the p55TNFR status of the host mice (**right panel**): response of fibroblast tumors in Nu/Nu mice. *Tnfrsf1α*$^{+/+}$ (black, ▲, n=11), *Tnfrsf1α*$^{+/-}$ (blue, ●, n=12) and *Tnfrsf1α*$^{-/-}$ (red, ■, n=12+) fibroblast tumor cells were inoculated in Nu/Nu mice and 10 days later treatment was started by *paralesional s.c.* injection with 10 µg TNF + 5000 IU IFNγ per mouse daily for 10 days. The three tumor cell types regress similarly, showing that the expression level of p55TNFR on the tumor cells is irrelevant for TNF-induced tumor regression. A representative picture of a necrotic tumor shows the effective tumor necrosis. **Lower panel:** Summary of the tumor experiments. Genetic status of the *Tnfrsf1α* genes in mice and tumors are as follows: *Tnfrsf1α*$^{+/+}$ (black), *Tnfrsf1α*$^{+/-}$ (blue) and *Tnfrsf1α*$^{-/-}$ (red).

Supplemental Figure **8: Generation of human *Tnfrsf1α* knockin mice (*Tnfrsf1α* "humanized" mice) (see also Materials and Methods)**

A: Genomic structure of the wild type mouse *Tnfrsf1α* locus. E and Nc refer to *EcoRI* and *NcoI* restriction sites respectively. **B-C**: Homologous recombination of the targeting vector allows the generation of a "humanized" *Tnfrsf1α* locus that is shown before (**B**) and after (**C**) neo^R gene deletion. Exons are represented by boxes. Grey lines and boxes represent human sequences, black represent mouse sequences. **D**: Southern blot analysis of representative wild type (lane 1) and recombinant (lane 2) ES cells clones. Left panel: *EcoRI*-digested genomic DNAs hybridized to probe A. Recombinant clones have a 7.1 kb band in addition to the 21 kb band of the wild type allele. Right panel: NcoI-digested genomic DNAs hybridized to probe B. Recombinant clones have a 13 kb band in addition to the 8.7 kb band of the wild type allele. **E**. The h*Tnfrsf1α* recombinant locus produces h*Tnfrsf1α* mRNA. mRNA from liver, spleen and brain tissues of wt and h*Tnfrsf1α* KI mice were isolated and the specific mp55TNFR and hp55TNFR transcripts were detected by RT-PCR using exon-2-specific primers, designed for the mRNA produced by the genetically modified locus or the mp55TNFR wt locus. Primer sequences for hp55TNFR were: sense: GTGCTCCTGGCTCTGCTGAT and antisense: GCACTTGGTACAGCAAATCG.

Supplemental Materials and Methods

Listeria monocytogenes model

Mice were infected i.p. with 10^2, 10^4 or 10^5 CFU of virulent *Listeria monocytogenes* strain L028 (kindly provided by Dr. G. Milon, Pasteur Institute, France). Survival was monitored daily for 20 days, after which no further deaths occurred.

Acute TNF-bolus model

Mice were injected intraperitoneally (*i.p.*) with mTNF diluted in 500 µl low endotoxin PBS. For most acute injections 30 µg TNF was used, which is the LD_{100} for $Tnfrsf1\alpha^{+/+}$ mice. Body temperature was monitored for 72 h, after which body temperatures of surviving mice returned to normal and no further deaths occurred.

TNF + GalN model and TNF + ActD model

Mice were injected *i.p.* with 0.3 µg or 0.5 µg TNF plus 20 mg D-(+)-galactosamine D (GalN) (Sigma). Mortality and temperature were monitored for 24 h. In the TNF + ActD model, 1 µg TNF was injected *i.p.* in combination with 20 µg of Actinomycin D (ActD) (Sigma), and mortality and hypothermia were monitored for 32 h, by which time all mice had died. For a better characterization of the induction of apoptosis, we chose the TNF + GalN model, in which apoptosis is induced specifically in liver. Mice were injected with 1 µg TNF and 20 mg GalN and sacrificed when their temperature dropped to 30°C (7 h and 7.5 h in $Tnfrsf1\alpha^{+/+}$ and $Tnfrsf1\alpha^{+/-}$ mice, respectively). Blood and liver samples from the $Tnfrsf1\alpha^{-/-}$ mice (which were unresponsive to TNF + GalN) were taken 8 h after challenge.

Antibody treatment in the acute TNF model

The protective power of the hamster anti-mp55TNFR was tested by pretreating mice (-30 min) with a single injection of PBS, 10 mg/kg control hamster antibody or a dilution of anti-mp55TNFR antibody. Mice were challenged with 30 ug mTNF *i.p.* and survival was followed in function of time.

To test the efficacy of the m5R16 F(ab)' PEG anti-hp55TNFR antibody, h$Tnfrsf1\alpha$KI mice were pretreated (-30 min) either with PBS or a single *i.p.* injection of anti-hp55TNFR antibody (10 mg/kg or 4 mg/kg) or 10 mg/kg control antibody, then received *i.p.* injections with 50 µg mTNF per mouse and survival was followed in function of time. None of the antibodies had protective effects against TNF in $Tnfrsf1\alpha^{+/+}$ wild-type mice (data not shown).

Cumulative sickness score

Mice were monitored every hour and scored for the following sickness parameters: physical inactivity, ruffled fur and diarrhea. For each of these parameters a mouse scored either 0 (no physical inactivity, ruffled fur or diarrhea) or 1. At a given time point, a mouse can have a score between 0 (minimal score) and 3 (maximal score).

Body temperature measurement and blood collection

Rectal body temperature was measured with a digital thermometer (Comark electronics, Littlehampton, UK). Blood was taken with a glass capillary from the tail vein and allowed to clot for 30 min at 37°C. The clot was removed and serum was prepared by centrifugation at 20,000g for 10 min. Serum was stored at –20°C.

Intestinal permeability

FITC-labeled dextran (4 kDa, Sigma) was administered to mice by gavage at 150 mg/kg body weight. Five hours later, blood was obtained by heart puncture in EDTA-coated tubes (Sarstedt) and plasma was prepared. Leakage of FITC-labeled dextran into the circulation was determined by measurement of the fluorescence with λex/λem = 488/520 nm. Values were normalized to the lowest value.

Measurement of serum AST, ALT, Creatinine, Hexosaminidase, LDH

Serum levels of the liver-damage markers ALT and AST and creatinine were determined by a standard clinical automatic analyzer (Hitachi). Hexosaminidase (HEX) activity was measured in 10 µl plasma, as described by (Kuenzler et al. 2002). LDH was measured in 1 µl plasma using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the guidelines of the manufacturer.

NOx determination in serum samples

The NO_x concentration in the serum, which is the combined concentration of the stable NO metabolites nitrate and nitrite, was determined as described (Granger et al. 1991).

SRBC immunization and ELISA for SRBC-specific antibodies and immunohistochemistry

Mice at the age of 8-10 weeks were injected *i.p.* with 2.5×10^8 sheep red blood cells (SRBCs) in 0.9% saline. Serum samples were collected 15 days after immunization. Serum anti-SRBC IgG

levels were measured by ELISA by standard methods. Optical density values were converted to arbitrary units (A.U.) with reference to a pool of sera obtained after immunization of *Tnfrsf1α*$^{+/+}$ mice with SRBCs. Spleens from immunized mice were frozen in OCT compound (BDH) and 6-µm cryostat sections were placed on gelatinized slides. For detection of germinal centers, sections were fixed for 10 min in acetone containing 0.5% H_2O_2 to block endogenous peroxidase activity, blocked with phosphate buffered saline containing 5% FBS, and incubated with 25 µg/ml peanut agglutinin-horse radish peroxidase antibody (PNA-HRP, SIGMA)(*29*). Bound peroxidase activity was detected by staining with diaminobenzidine (DAB; Sigma). For detection of follicular dendritic cells, sections were fixed in acetone and blocked with acetone/H_2O_2, and 5 µg/ml rat anti-CD35 (CR1; clone 8C12, Pharmingen BD) was applied. Conjugation to alkaline phosphatase was performed using Vectastain ABC-AP kit (Vector Laboratories) according to the manufacturer's specifications, and alkaline phosphatase activity was visualized with NBT-BCIP. Stainings were visualized with an E-800 Eclipse microscope (Nikon).

Antitumor experiments

Three different types of antitumor experiments were performed.

(1) Subcutaneous B16BL6 melanoma. The murine B16BL6 melanoma cell line was a gift from M. Mareel (Ghent, Belgium) by courtesy of I. Fidler (Dallas, USA). Cultured B16BL6 cells were washed three times in PBS, counted, and their density adjusted with PBS to $6x10^6$ cells/ml. Mice were injected *s.c.* in the shaved right thigh with 100 µl of this solution. Treatment was started 12 days later by *s.c*. injection with 0.1 ml of a mixture of TNF and IFNγ close to the tumor. This treatment was repeated daily for 10 days. Tumor size index (TSI) was defined in mm^2 as the product of the longest diameter of the tumor and the diameter perpendicular to it. Mortality was usually monitored until 10 days after the last injection.

(2) Subcutaneous Lewis Lung Carcinoma. LLC cells were washed three times with PBS, counted, and their density adjusted with PBS to $6x10^7$ cells/ml; 100 µl of the cell suspension was injected *s.c.* in the shaved right thigh of mice. TNF/IFNγ treatment and follow-up was the same as for the B16BL6 melanoma model.

(3) Fibroblast tumors in Nu/Nu mice. Pieces of ears and tails of *Tnfrsf1α*$^{+/+}$, *Tnfrsf1α*$^{+/-}$ and *Tnfrsf1α*$^{-/-}$ mice were trypsinized and put into culture. The outgrowing cells were immortalized

by transfection with a plasmid expressing SV40 large T antigen. An additional transformation with the *ras* oncogene was performed to make these cells tumorigenic. The immortalized cell lines were grown in culture, trypsinized, and their density adjusted to $5x10^7$ cells/ml in PBS. Mice were injected *s.c.* with 100 µl of tumor cell suspension and afterwards treated with TNF/IFNγ as described in the B16BL6 melanoma section.

TNF dosage in antitumor experiments

Since the effective therapeutic dose of TNF/IFNγ is close to the MTD, and since we were interested in demonstrating a significant increase in safety, we used antitumor doses of TNF close to the MTD (always in combination with 5000 IU IFNγ). Such doses result in excellent TNF-induced tumor regression and enable comparison of the safety of the therapy in the different studied groups. In tumor-bearing $Tnfrsf1\alpha^{+/+}$ mice, daily injection of 15 µg TNF + 5000 IU IFNγ for 10 days led to complete tumor regression but caused considerable mortality. Based on our safety studies, we chose to use three types of doses. (1) In $Tnfrsf1\alpha^{+/-}$ mice and control $Tnfrsf1\alpha^{+/+}$ mice, taking into account the increase in MTD of 8-12x (this study), we used a very high dose of TNF, *i.e.* 50 µg daily for 10 days in the B16BL6 and LLC models. (2) In the experiments with different *Tnfrsf1α* tumor genotypes in Nu/Nu mice, which were performed to demonstrate that the expression of p55TNFR on the tumor cells *per se* is irrelevant for tumor regression, we used a sub-lethal dose of 10 µg TNF daily for 10 days. (3) In the antibody experiments, taking into account the great protection of the anti-mp55TNFR and the anti-hp55TNFR in the acute TNF model, we decided to perform the antitumor experiments with a high and very effective dose of TNF, i.e. 25 µg TNF + 5000 IU IFNγ for 10 days. (4) In the experiments with *Villin-Cre* $Tnfrsf1\alpha^{flx/flx}$ mice, we used we used a lethal dose of 12 µg TNF daily for 10 days. The differences in TNF-dosing caused by the use of a more potent batch of TNF than the one used in previous experiments (L929 cytotoxicity assay exhibited 2x activity compared to previous batch, data not shown), so we re-titrate TNF. Safety studies with 5000 IU IFNγ + 10 µg TNF injected paralesionally or ip appeared to be cause lethality (100%) in the test experiments (data not shown).

Safety studies with TNF/IFNγ in mice

Safety studies with *Tnfrsf1α*$^{+/+}$ and *Tnfrsf1α*$^{+/-}$ mice were performed both in tumor-free and in B16BL6 melanoma tumor bearing mice as follows. (1) Different groups of tumor-free mice were treated systemically (*i.p.*) with different doses of TNF + 5000 IU IFNγ daily for 10 days. (2) Safety studies in tumor-bearing *Tnfrsf1α*$^{+/+}$ and *Tnfrsf1α*$^{+/-}$ mice involved *s.c.* inoculation of 600,000 tumor cells in the shaved right thigh on day –10. Again, different groups of mice were injected daily with different doses of TNF + 5000 IU IFNγ near the tumors when they had a TSI of about 70 mm², i.e. on day 0. The daily injections were given for 10 days.

Safety studies with anti-mp55TNFR antibodies in tumor-bearing *Tnfrsf1α*$^{+/+}$ mice involved *s.c.* inoculation of 6x10^{5} tumor cells in the shaved right thigh on day –10, *i.p* injections of antibody (control or anti-mp55TNFR) at 10 mg/kg every day starting at day 0 and *s.c.* injections of different doses of TNF + 5000 IU IFNγ close to the tumor daily from day 0 to day 9, when the tumors had a TSI of about 70 mm².

Safety studies with anti-hp55TNFR antibodies in tumor-bearing *Tnfrsf1α*$^{+/+}$ mice involved *s.c.* inoculation of 6x10^{5} tumor cells in the shaved right thigh on day –10, *i.p* injections of m5R16 antibody at 10 mg/kg every day starting at day 0 and *s.c.* injections of different doses of TNF + 5000 IU IFNγ close to the tumor daily from day 0 to day 9, when the tumors had a TSI of about 70 mm².

In these safety studies, the numbers of deaths were recorded daily and the experiment was considered finished 10 days after the last injection. Tumors had fully regressed in all of the safety studies (supplemental figure 7A).

Antibody treatment in antitumor experiment

To study the effect of the anti-mp55TNFR antibodies in an antitumor setting, *Tnfrsf1α*$^{+/+}$ mice were inoculated with 6x10^{5} B16BL6 melanoma cells on day -10. TNF/IFNγ (or PBS) was injected daily from day 0 until day 9 and antibody at a dose of 10 mg/kg (or PBS) was daily administered *i.p.* from day 0 until day 9. Tumor regression was measured and lethality recorded until day 18. Tumor-bearing mice that had been treated with PBS had large tumors by day nine and were euthanized at that day.

To study the effect of the anti-hp55TNFR antibodies in an antitumor setting, h*Tnfrsf1α*KI mice were inoculated with $6x10^5$ B16BL6 melanoma cells on day -12. TNF/IFNγ (or PBS) was injected daily from day 0 until day 9 and antibody at a dose of 10 mg/kg (or PBS) was daily administered *i.p.* from day 0 until day 8. Tumor regression was measured and lethality recorded until day 18. Tumor-bearing mice that had been treated with PBS had large tumors by day nine and were euthanized that day.

Stimulation of fibroblasts with TNF and TNF/CHX

Primary fibroblasts were derived from $Tnfrsf1\alpha^{+/+}$, $^{+/-}$ and $^{-/-}$ mice. After 2-3 passages, 50.000 cells were seeded in 96-well plates and were stimulated with different concentrations of TNF. 24h later, supernatant was collected and IL-6 concentration was determined by 7TD1 bioassay. In parallel, 50.000 cells were seeded in 96-well plates and stimulated with different concentrations TNF in combination with 10µg/ml CHX. Cell survival was measured 24 h later by hexosaminidase assay.

Isolation of bone marrow derived macrophages (BMDM)

To generate macrophages, bone marrow was isolated from eight-week-old mice and cultured in DMEM medium supplemented with 10% FCS, penicillin, streptomycin, L-glutamine and 20 ng/ml M-CSF (Peprotech). Medium was refreshed every other day. After seven days in culture, macrophages were harvested using a non-enzymatic dissociation buffer.

Isolation of thioglycolate-elicited peritoneal macrophages (TEPMs)

TNFR1 $^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were injected with thioglycolate medium. After 3 days peritoneal lavage was performed and cells were counted and plated at a concentration of $2x10^6$ cells per well in a 6-well plate. Three hours later cells were washed with PBS and stimulated with 2000IU TNF for 5, 10, 15, 20 or 25 minutes.

Detection of caspase-activity in cell lysates

Liver lysates were prepared by lysing the cells in a buffer containing 1% NP-40, 200 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 µg/ml leupeptin, aprotinin (0.27 trypsin inhibitory U/ml), and 100 µM PMSF. Caspase-3 and -7 activity was determined by incubating lysates containing 100 µg total protein with 50 µM of the fluorogenic substrate FAM-DEVD-AMC (Immunochemistry Technologies, LLC) in 200 µl cell-free system buffer comprising 10 mM Hepes pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT. The release of fluorescent 7-amino-4-methylcoumarin was measured for 1 h at 2 min intervals by spectrofluorometry. Data are expressed as the increase in fluorescence as a function of time.

IL6 induction in BMDMs

BMDMs were plated at $5x10^5$ cells per well in a six-well plate and allowed to adhere overnight. Next, they were stimulated with TNF (10 ng/ml) and supernatant was collected after 0, 8 and 24 h. IL6 levels were determined as described above.

EMSA in BMDMs

BMDMs were plated at $1x10^7$ in a 10-cm^2 dish and allowed to adhere overnight. Next day the cells were harvested as described above, or stimulated with TNF (10 ng/ml) for 15, 30, 45 or 60 min and then harvested. Cell pellets were allowed to swell for 15 min in a hypotonic buffer consisting of 10 mM HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM Pefabloc, 0.5 mM DTT and 0.15 IU/ml Aprotinin. NP-40 was added to a final concentration of 0.65% and cells were vortexed for 10 seconds. Nuclei were pelleted by centrifugation and lysed in a buffer containing 20 mM HEPES pH 7.5, 1% NP40, 1 mM $MgCl_2$, 400 mM NaCl, 10 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM pefablock, 0.5 mM DTT and 0.15 IU/ml aprotinin. Ten micrograms of this nuclear extract was incubated for 30 min with a ^{32}P-labeled NFκB consensus oligo in binding buffer (50% glycerol, 10 mM Tris-HCl pH 7.6, 500 mM KCl, 10 mM EDTA, and 1 mM DTT) in a final volume of 20 µl for 30 min at room temperature. The nucleotide–protein complex was separated in a 5% polyacrylamide gel in 0.5×TBE buffer (100 mM Tris-HCl, 100 µM boric acid, and 2 mM EDTA) at 150 V on ice. The

gel was dried and radioactive bands were detected by autoradiography. Densities of the bands were analyzed with ImageJ software.

Western blot (WB)

Tissue samples for IκBα and caspase-8 WB were homogenized in ice-cold HEPES buffer (20 M Hepes pH 7.5, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2.6H_2O$, 0.5 mM EDTA, 0.1 mM EGTA, and 0.5 mM DTT) supplemented with 1 mM PMSF, 0.3 mM aprotinin, 1 mM leupeptin and a protease inhibitor cocktail (Complete, Roche). All homogenates were centrifuged for 30 min at 20,000g and 4°C, and supernatant was stored at –80°C. For IκBα, 100 µg (lung) or 150 µg (liver) of total protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For caspase-8, half of the 6-well lysate was seperated on a 12.5% SDS-Page. Blots were blocked for 2 h at room temperature in 5% non-fat dry milk and 0.1% Tween-20, followed by overnight incubation at 4°C with rabbit anti-IκBα antibodies (Santa Cruz) , mouse anti-caspase-8 (C15, Alexis) and monoclonal anti-actin diluted 1/1500 in 5% BSA and 0.1% Tween-20. Blots were washed and incubated with goat-anti-rabbit IRDye 800 and goat-anti-mouse IRDye 680 (1:10,000) (LI-COR, Westburg b.v.) and scanned at the appropriate wavelengths for two-color detection using the Odyssey Imager (LI-COR), followed by quantification. Densities of the bands were analyzed with ImageJ software.

MAPK-P western blots

BMDMs were plated at $5x10^5$ cells per well in a six-well plate and allowed to adhere overnight. They were then stimulated with TNF (10 ng/ml) and harvested after 0, 5, 10, 15, 30, 60 and 120 min. Harvesting was done by washing with PBS and detaching cells with a rubber policeman. Cells were lysed in 1x Laemli buffer. Western blot was done as described below. Antibodies were purchased from the Phospho-Mapk family kit (Cell Signaling Technology).

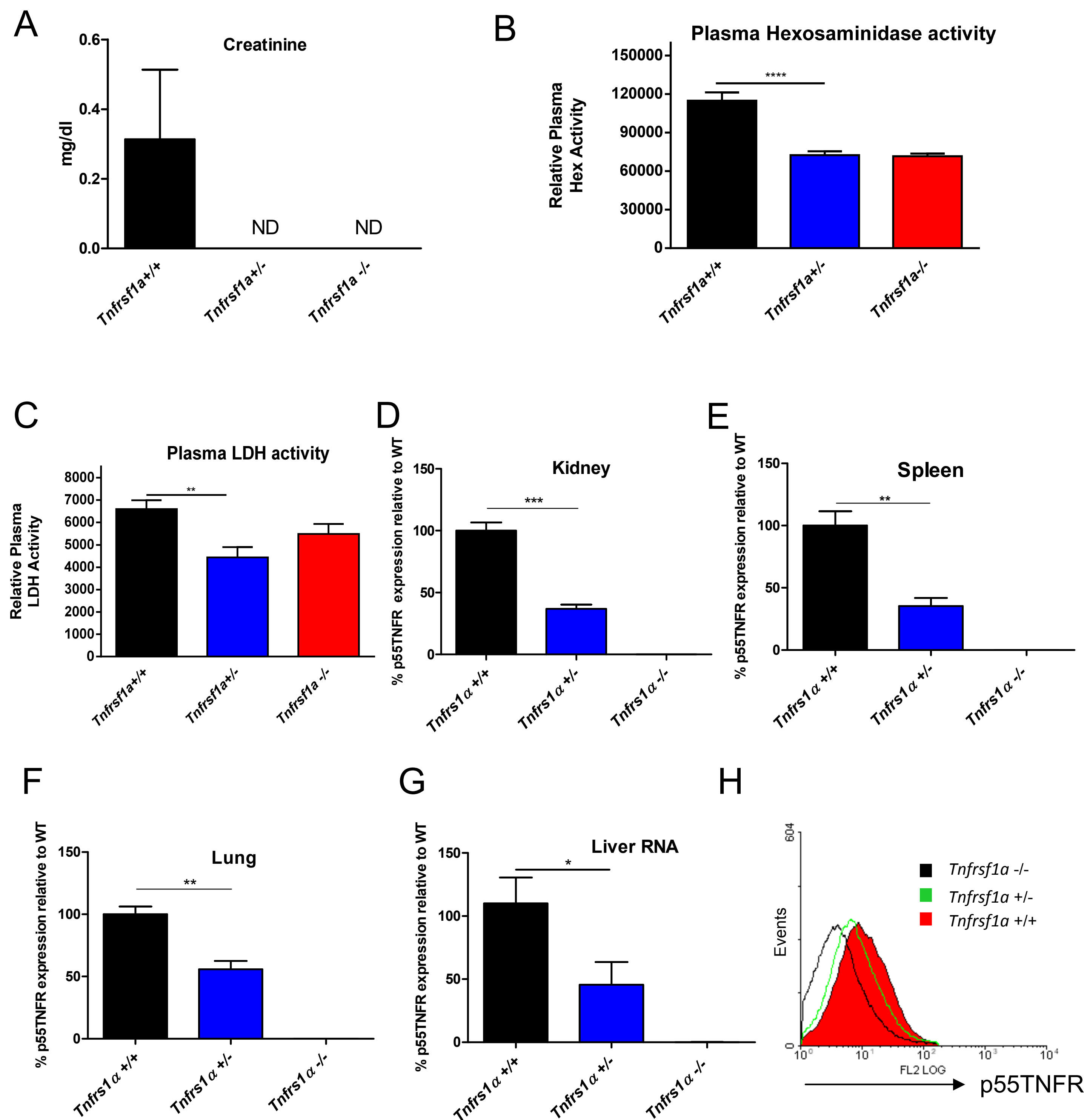
IκB degradation in BMDMs and TEPMs

BMDMs were plated at $5x10^5$ cells per well in a six-well plate and allowed to adhere overnight. Then they were stimulated with TNF (10 ng/ml), and harvested and lysed as described above after 0, 5, 10, 15, 20 and 25 min. IκB was detected by western blot, as previously described.

Isolation of Intestinal Epithelial Cells

The ileum was removed, flushed with HBSS/2% FBS, opened longitudinally, and cut into 0.5-cm pieces. The tissue was further washed and incubated in HBSS/2% FBS, 0.5 mM EDTA, and 1 mM DTT, at 37 °C in a shaking water bath for 45 min. The cell suspension released upon vigorous shaking was layered on a discontinuous 25%/40% Percoll gradient (Sigma) and centrifuged at 600 × g for 10 min. Intestinal epithelial cells (IEC) were collected from the interphase. The purity of the population was assessed by FACS analysis for the epithelial marker E-cadherin (FITC-conjugated mAb; BD) and was consistently ⊠90%.

Supplemental figure 1



Supplemental figure 2

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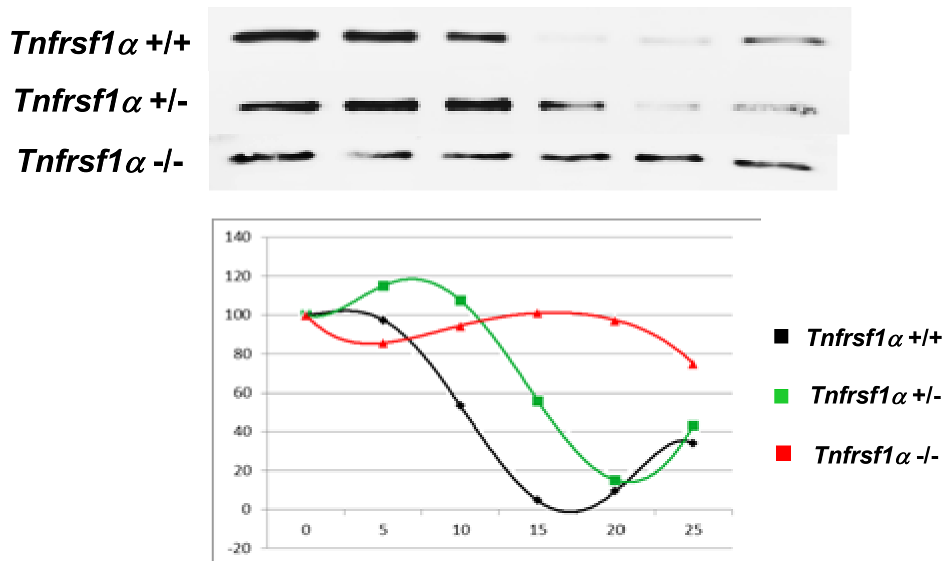

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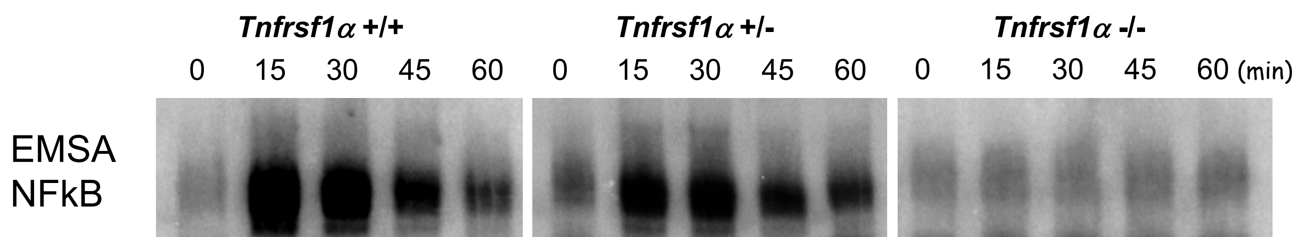

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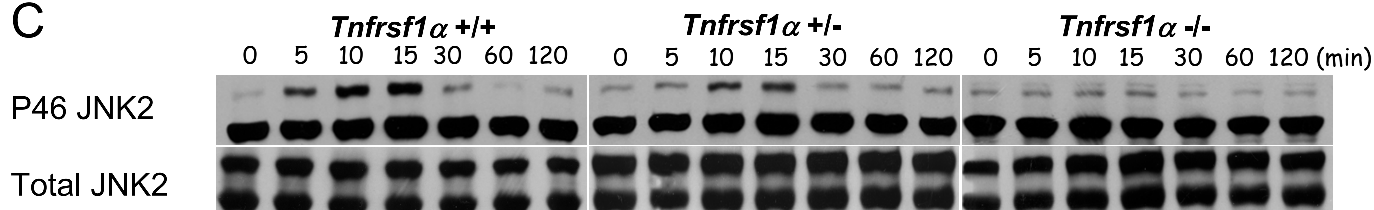

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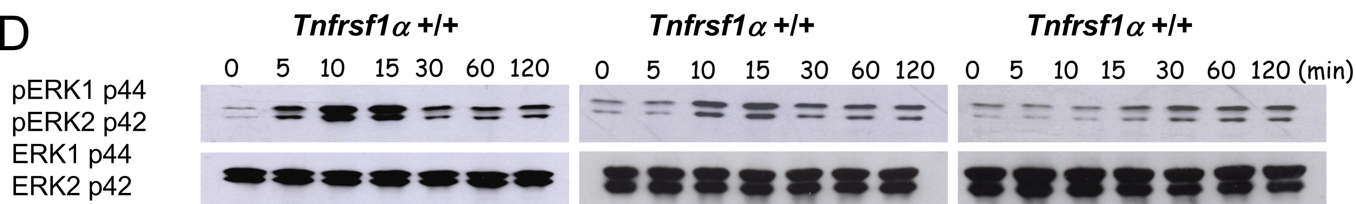

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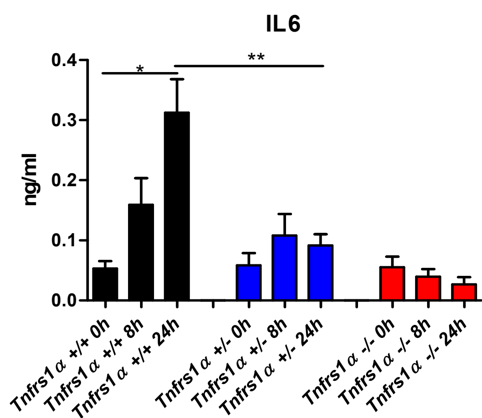

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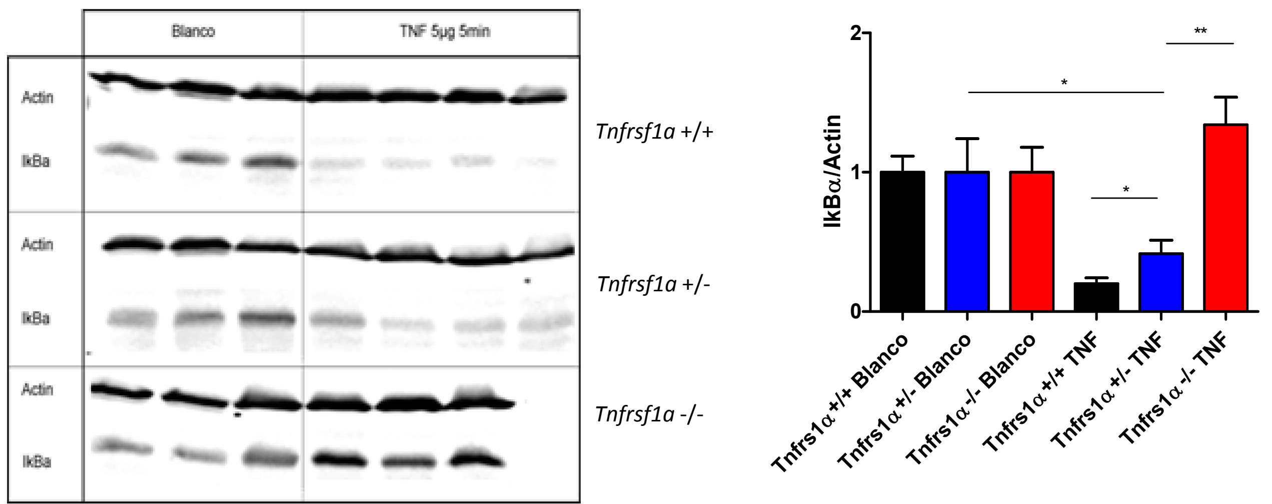

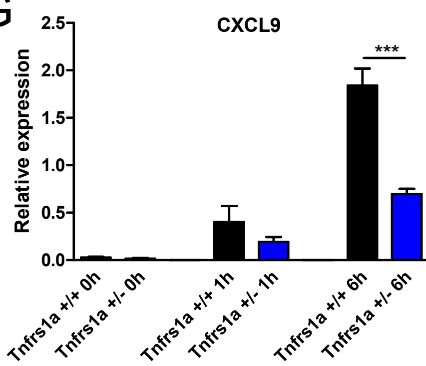

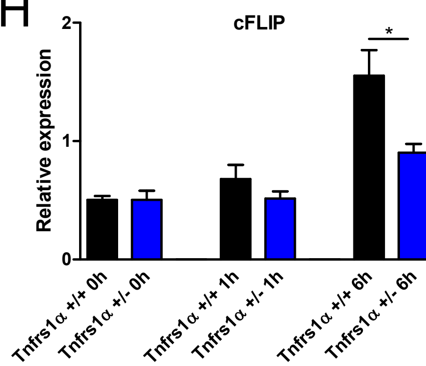

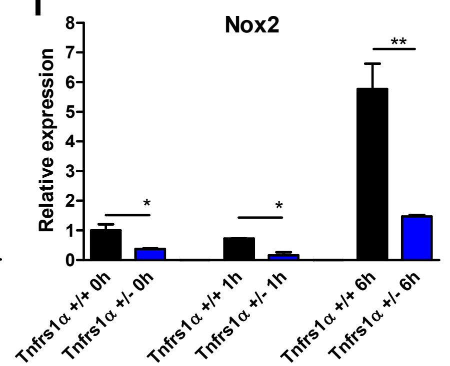

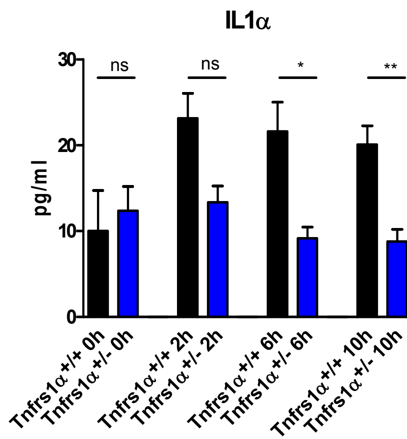

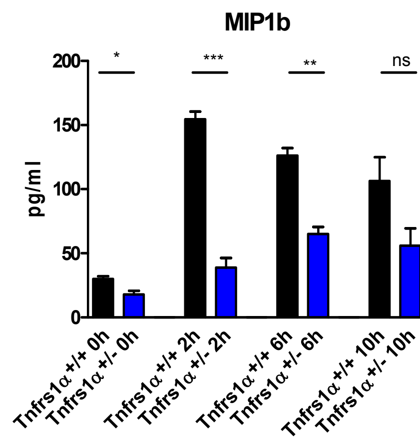

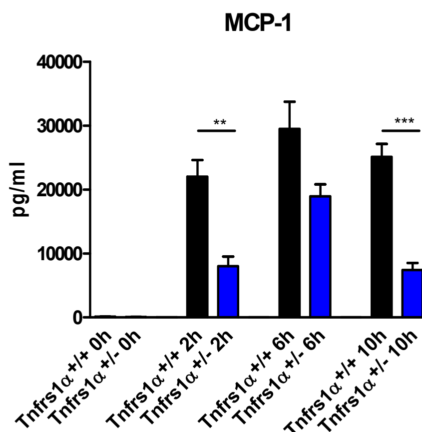

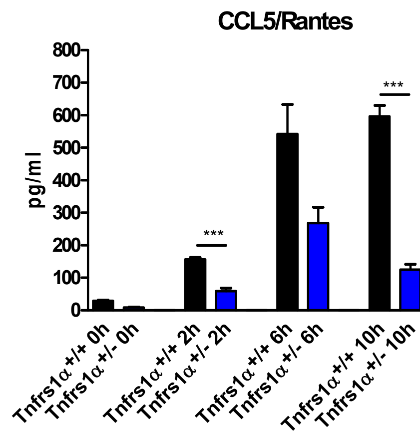

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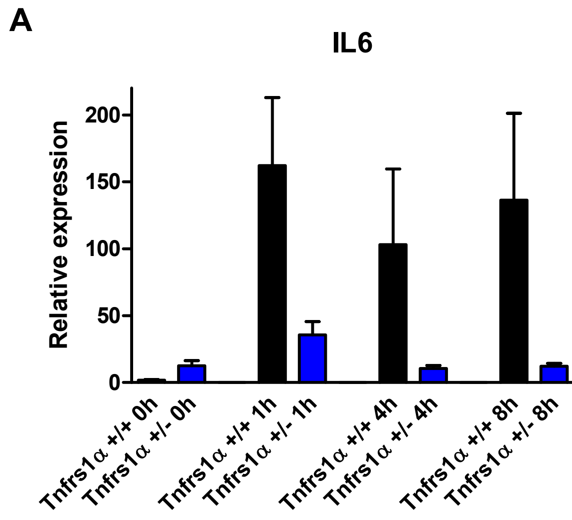

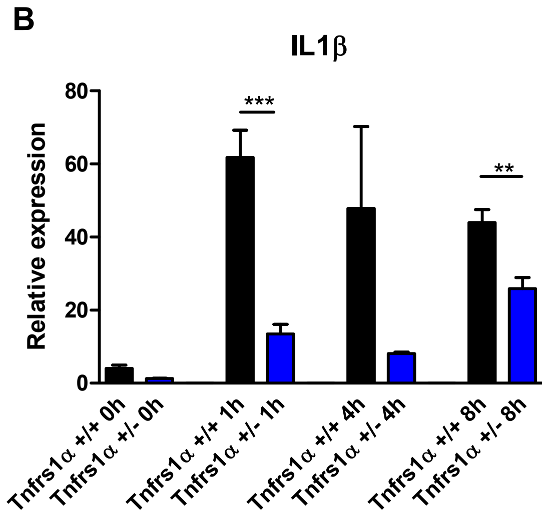

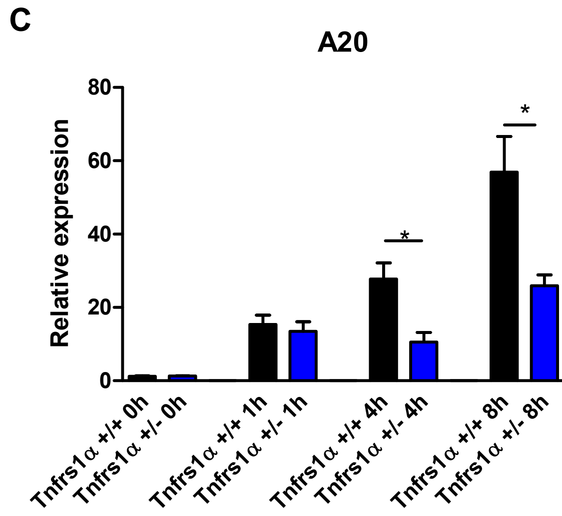

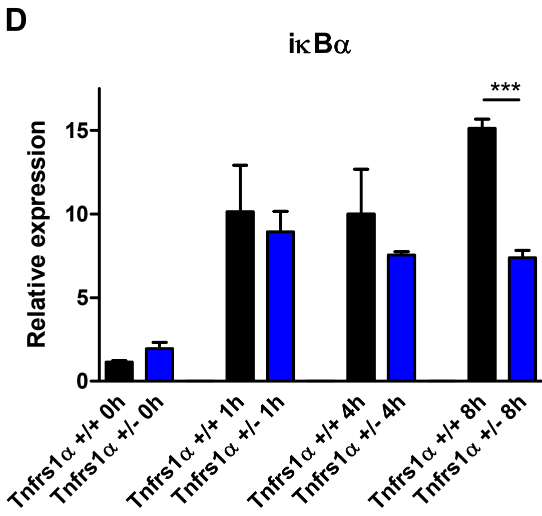

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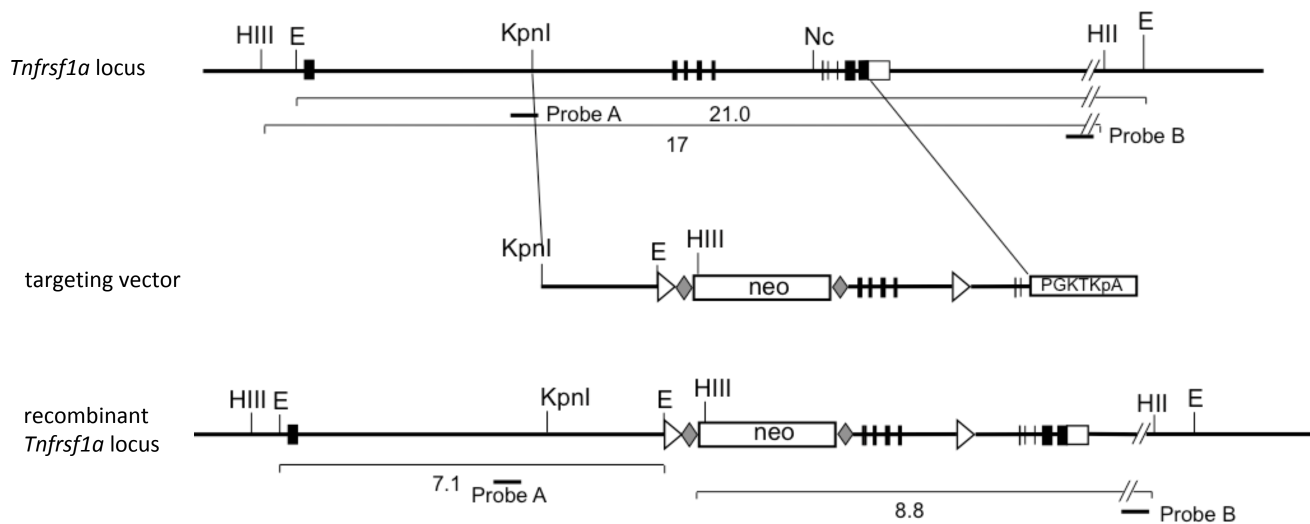

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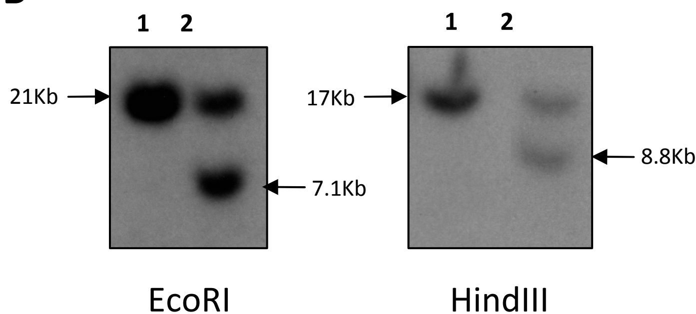

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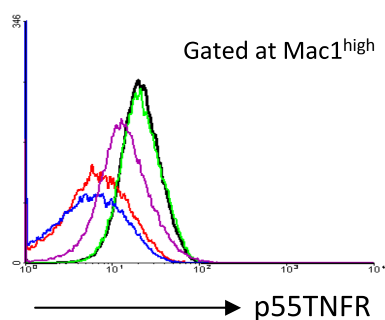

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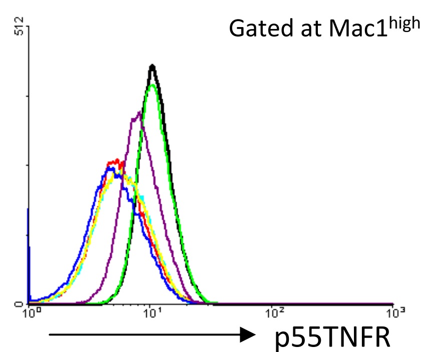

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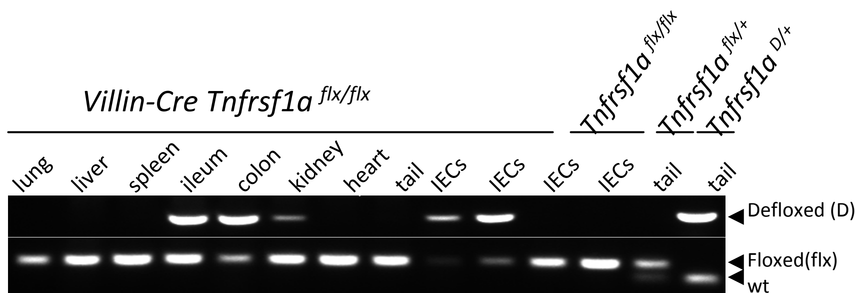

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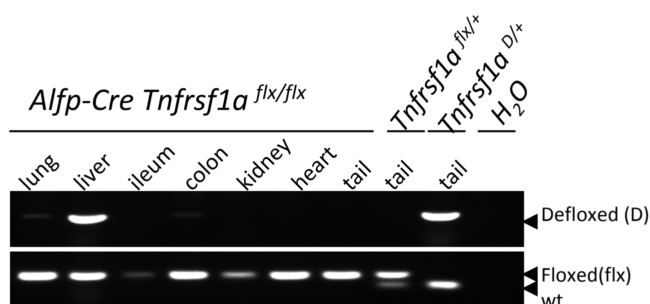

Supplemental figure 5

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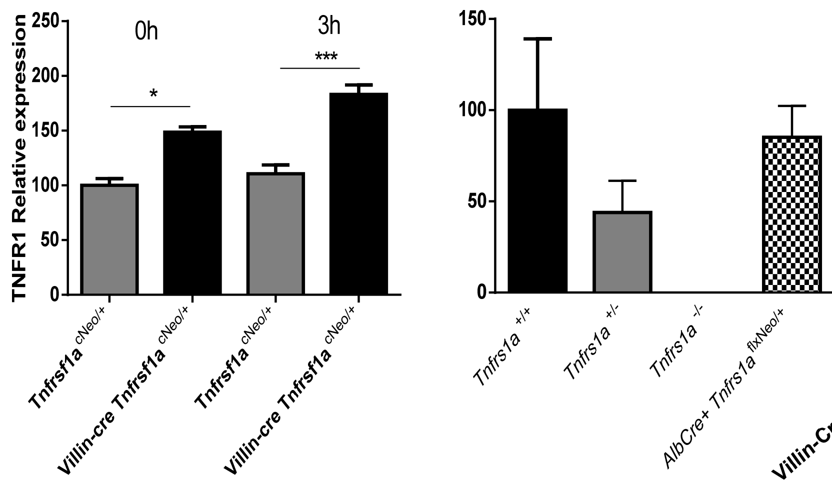

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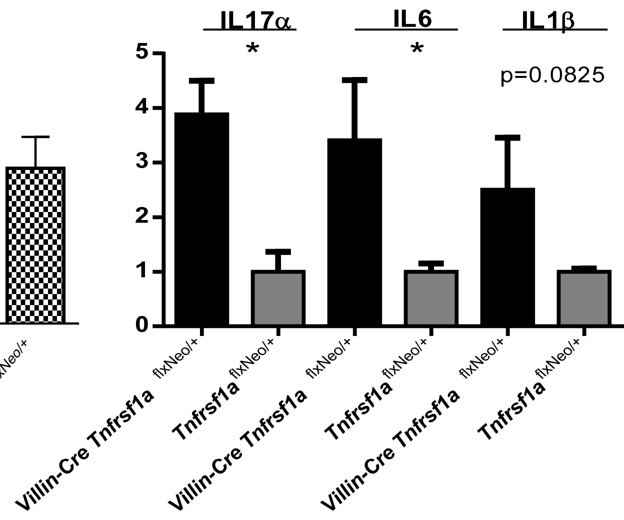

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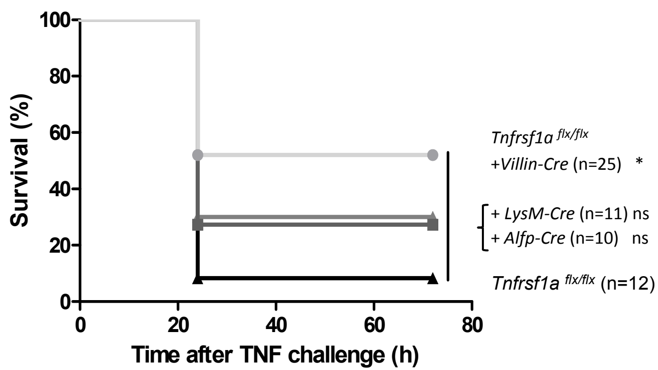

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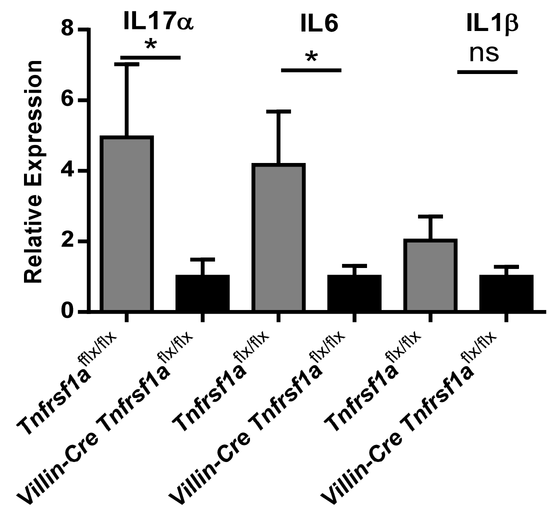

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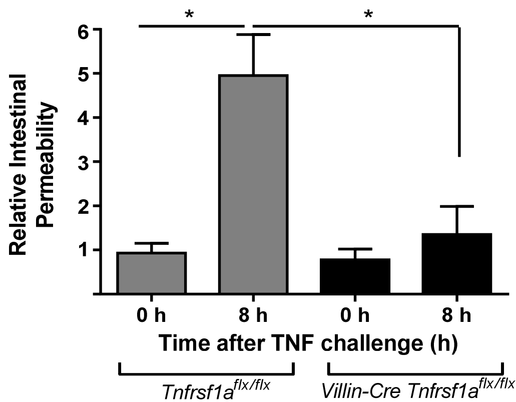
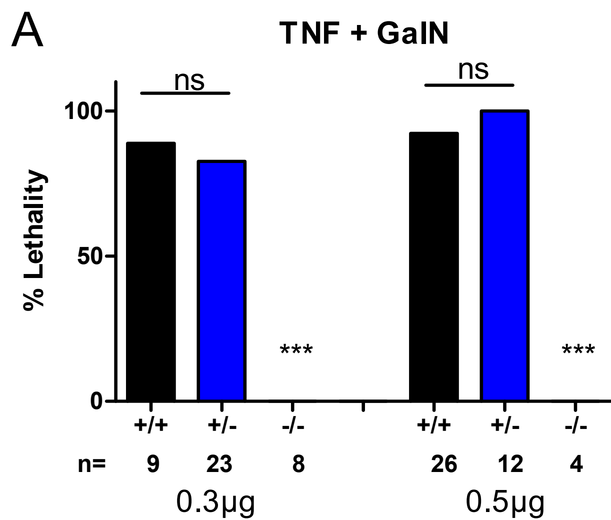
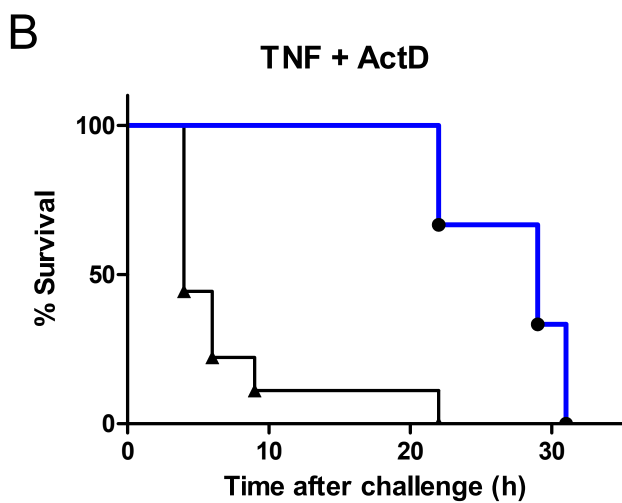
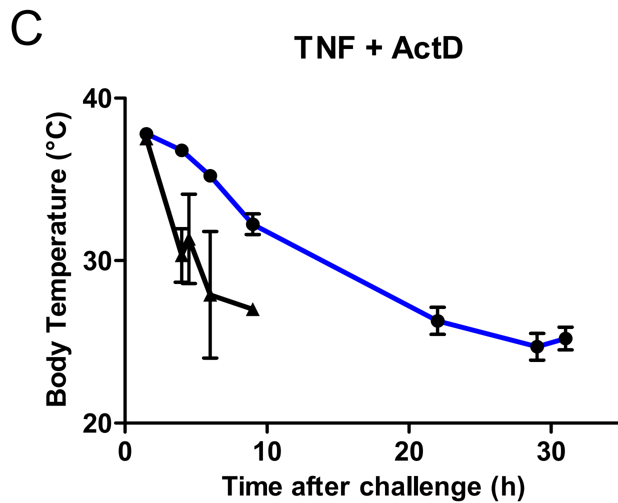


Supplemental figure 6







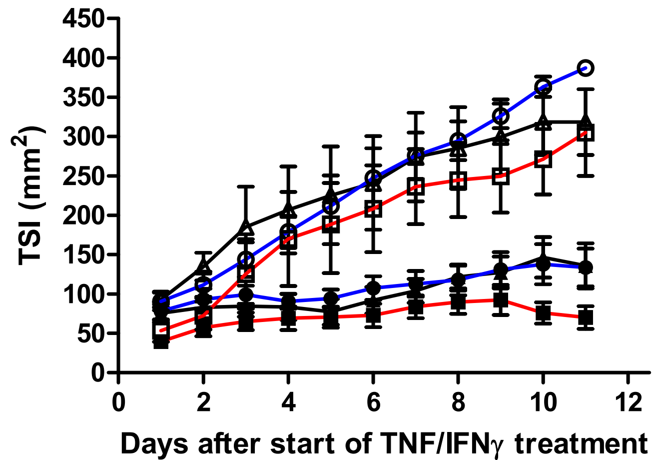
Supplemental figure 7

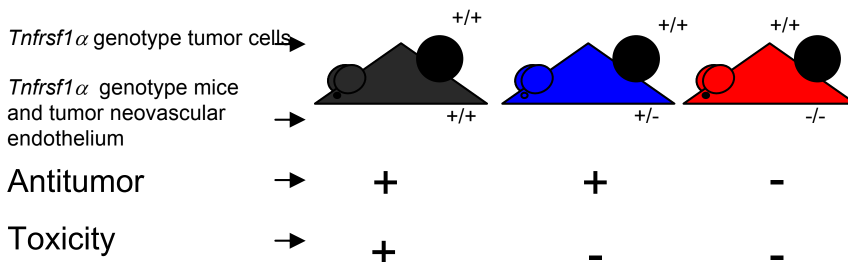
A

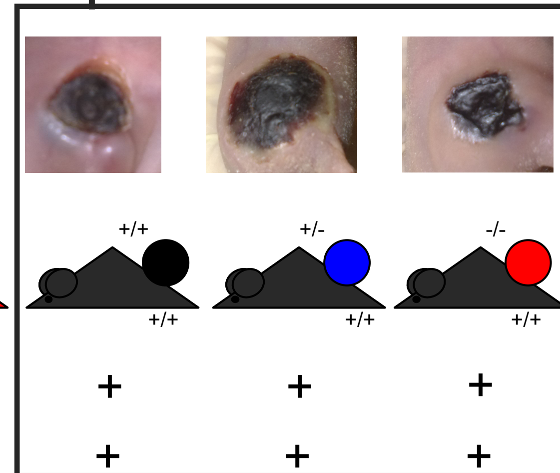
Dose TNF (µg) /mouse/day +5000 IU IFNg	WT mouse *Tnfrsf1a* expressing mice						Human *Tnfrsf1a* KI mice		
	Tumor-free healthy mice		Tumor-bearing mice						
			Genetic		anti-mouse P55TNFR AB		anti-human P55TNFR Fab		
	+/+	+/-	+/+	+/-	+/+ Ctrl AB	+/+ mP55 AB	PBS	Ctr Fab	hP55 Fab
10	0/6	-	4/21	0/5	-		2/6	-	-
12,5	2/6	-	8/11	-	-	0/5	-	-	-
15	3/6	-	13/16	-	-		4/6	-	-
17,5	6/6	-	12/12	-	-		-	-	-
20	5/5	-	12/12	-	-		6/6	-	-
22,5	-	-	5/5	-	-		-	-	-
25	-	-	10/10	0/5	5/5	0/6	4/4	12/12	0/12
50	-	0/10	-	0/5	-	3/6	-	-	0/6
75	-	-	-	1/4	-	6/6	-	-	6/6
100	-	0/6	-	7/12	-	-	-	-	-
125	-	-		8/8	-	-	-	-	6/6
150	-	0/5	-	8/12	-	-	-	-	-
200	-	4/5	-	4/5	-	-	-	-	-
LD50	**15**	**181,25**	**11,44**	**93,8**	**12,5**	**50**	**12,5**	**12,5**	**62,5**
µg/mouse/day	**12,1x**		**8,2x**		**4x**		**5x**		

B

Tumor regression in *Tnfrs1α* mutant tumors







Supplemental figure 8