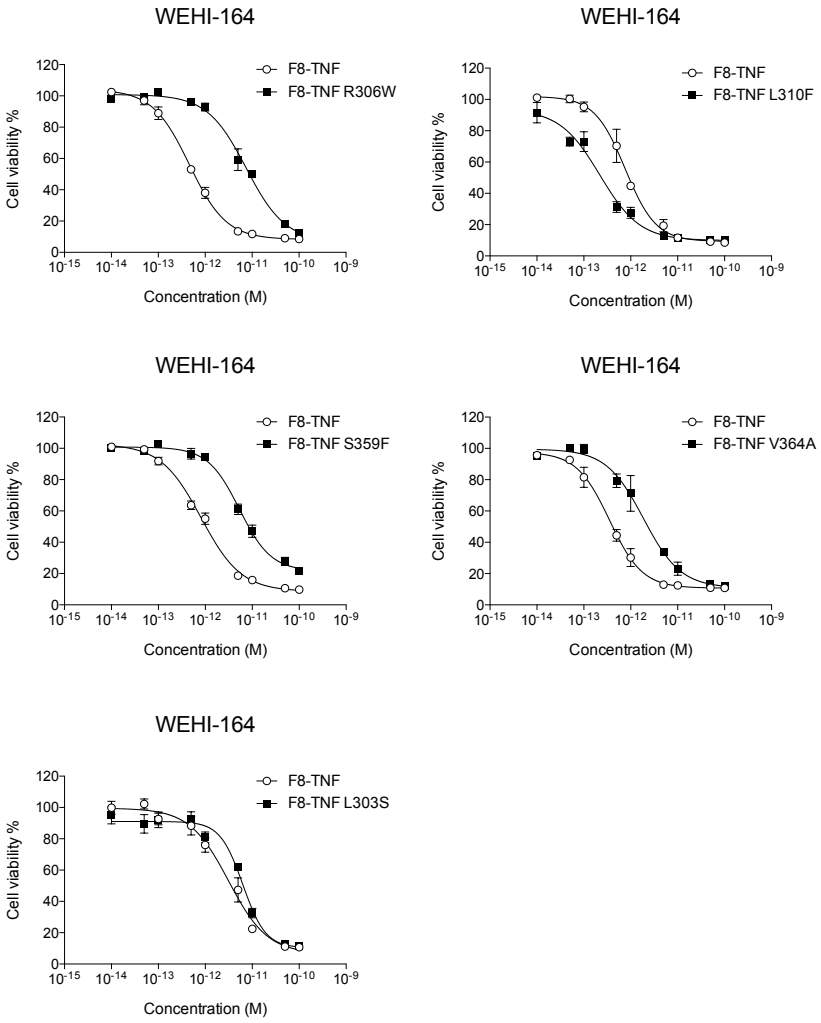
Supplementary Data

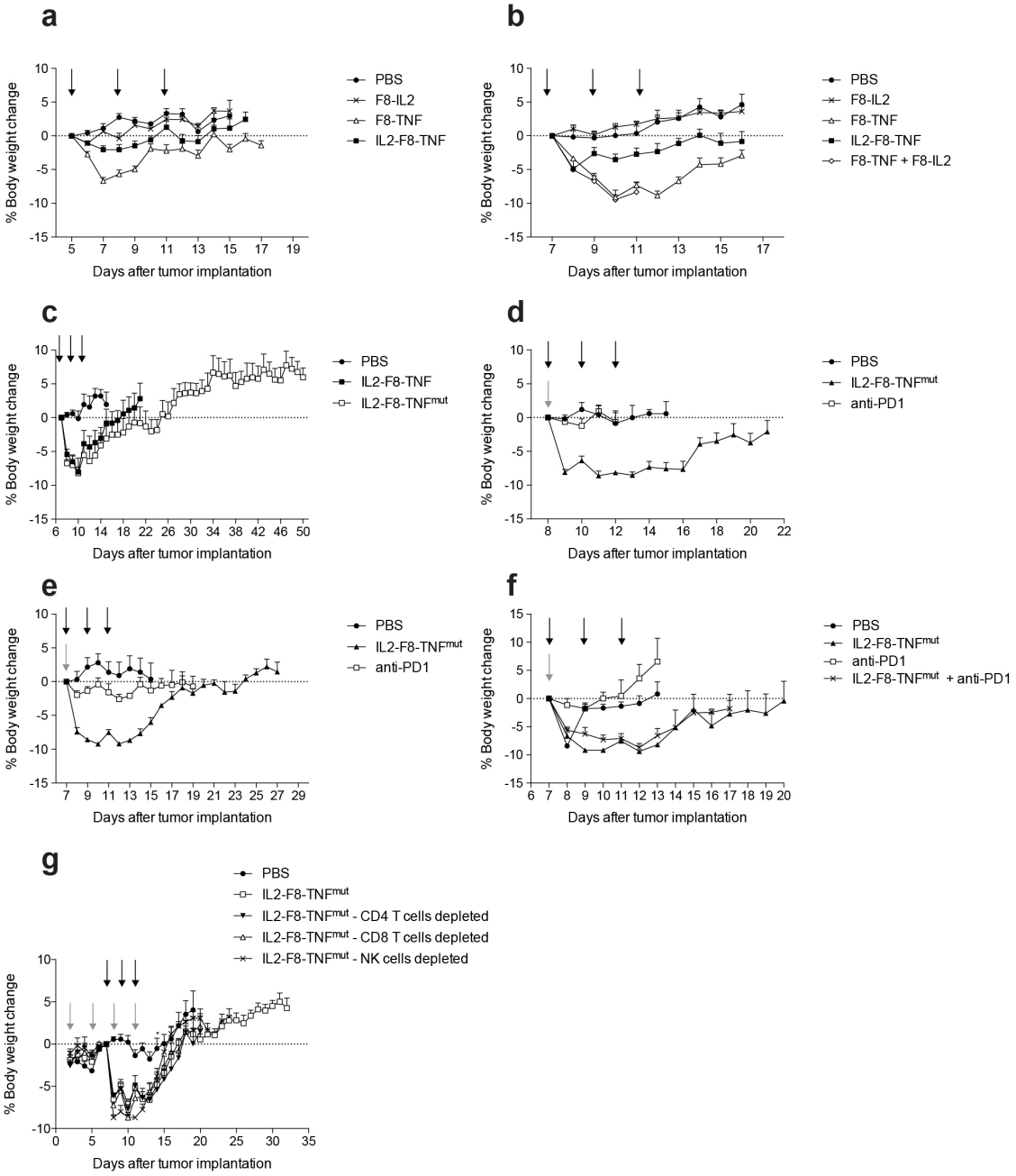
IL2 – 12aa linker – V_H – 14aa linker – V_L – 15aa linker – TNF - STOP

APTSSSTSSSTAEAQQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLPRMLTFKFY
LPKQATELKDLQCLEDELGPLRHVLDLTQSKSFQLEDAENFISNIRVTVVKLKGSDNTFE
CQFDDESATVVDFLRRWIAFCQSIISTSPQGDGSSGGSGGASEVQLLESGGGLVQPGGSLR
LSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTL
YLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGGGSGGGGSGGGGEIVLT
QSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS
GSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGLRSSS
QNSSDKPVAHVVANHQVEEQLEWLSQRANALLANGMDLKDNQLVVPADGLYLVYSQ
VLFKGQGCPDYVLLTHTVSRFAISYQEKVNLLSAVKSPCPKDTPEGAELKPWYEPIYLGG
VFQLEKGDQLSAEVNLPKYLDFAESGQVYFGVIAL*

Supplementary Figure S1: Amino acid sequence of IL2-F8-TNF. Starting from the N-terminus: murine IL2, the F8 antibody in scFv format and murine TNF.

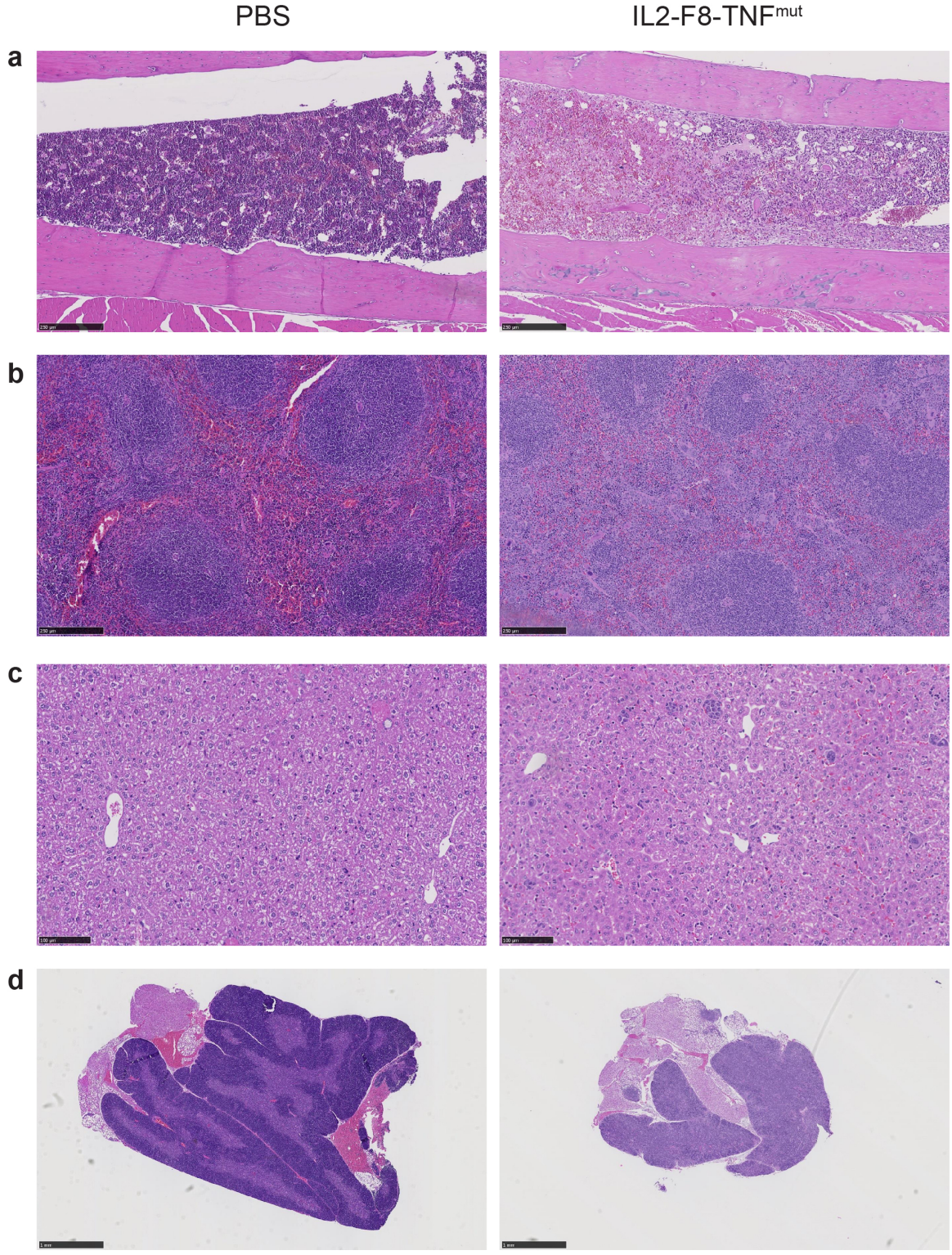


Supplementary Figure S2: murine TNF mutants screening. TNF bioactivity assay, based on the killing of WEHI-164 cells.

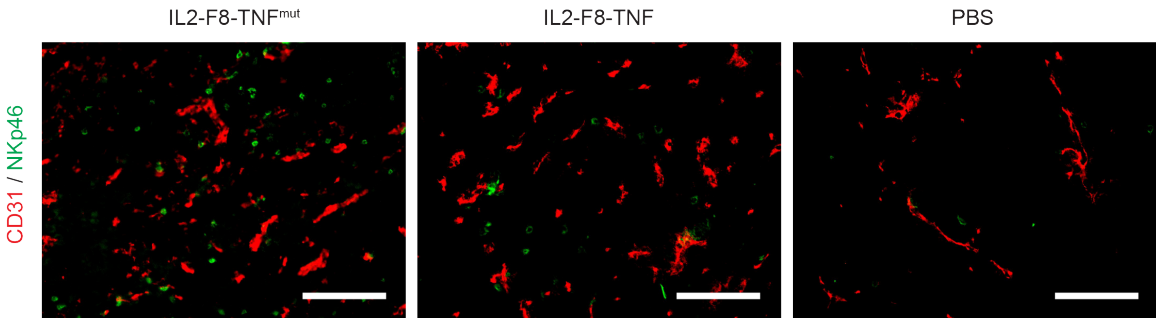


Supplementary Figure S3: Analysis of toxicity form therapy by observation of changes in the weight of mice. Change in weight of WEHI-164 bearing mice during immunocytokines treatment in the first therapy study (A), second therapy study (B). (C) Weight change during the

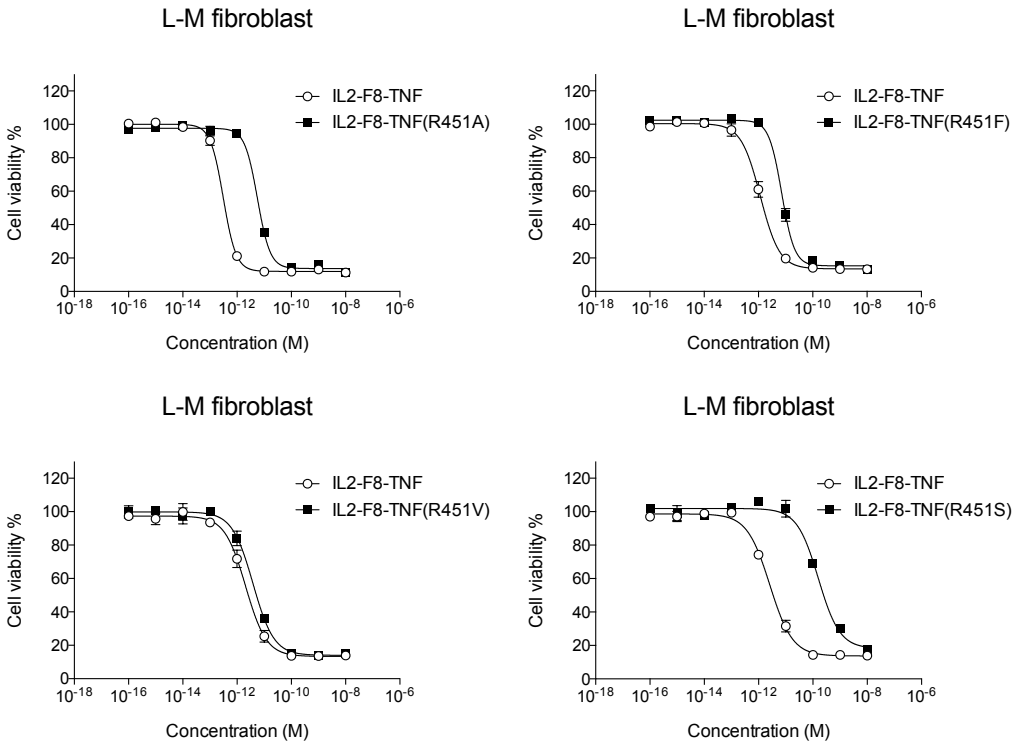
therapy of subcutaneous WEHI-164 lesions treated with either IL2-F8-TNF or IL2-F8-TNF^{mut}. (D) Weight change during the therapy of subcutaneous CT26 colon carcinomas. (E) Weight change during the therapy of subcutaneous C1498 acute myeloid leukemia chloroma lesions. (F) Weight change during the therapy of subcutaneous F9 teratocarcinomas. (G) Weight change during the depletion experiment of $CD4^+$ T cells, $CD8^+$ T cells and NK cells in WEHI-164 tumor bearing mice. Data represent mean % weight (± SEM), n = 4-6 mice per group.


PBS
IL2-F8-TNFmut
a
b
c
d

Supplementary Figure S4: Necroscopic analysis of organs after IL2-F8-TNF^{mut} (right panels) treatment compared to PBS (left panels). (A) H&E staining of bone marrow sections, scale bar = 250µm. (B) H&E staining of spleen sections, scale bar = 250µm. (C) H&E staining of liver sections, scale bar = 100µm. (D) H&E staining of thymus sections, scale bar = 1mm.



Supplementary Figure S5: Immunofluorescence analysis of tumor infiltrating NK cells. Staining on WEHI-164 tumor sections 24 hours after treatment with PBS, IL2-F8-TNF or IL2-F8-TNF^{mut}, with the monoclonal antibody anti-NKp46 (green, Alexa Fluor 488), anti-CD31 (red, Alexa Fluor 594), 20x magnification, scale bars = 100µm.



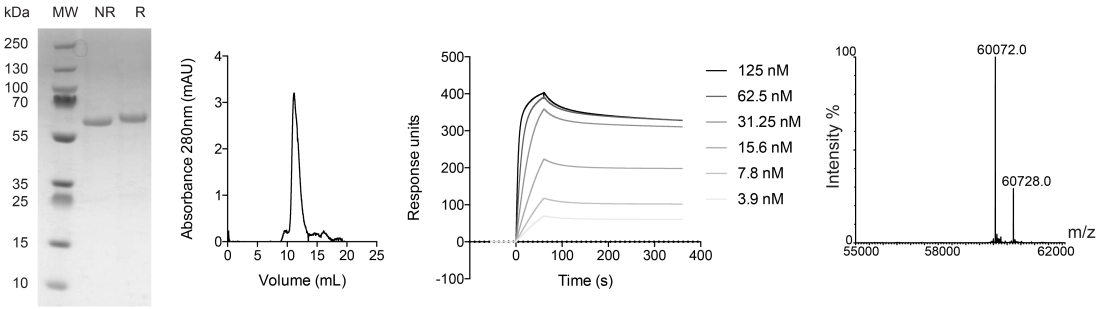
Supplementary Figure S6: human TNF mutants screening. TNF bioactivity assay, based on the killing of L-M cells.

a

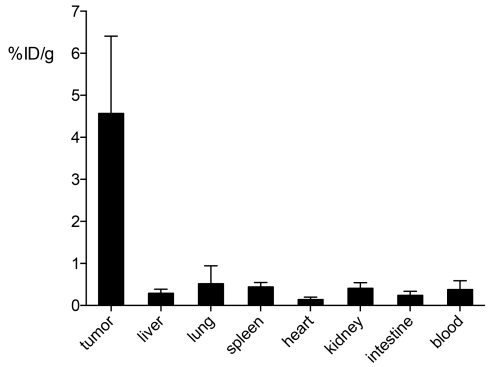
IL2 – 12aa linker – V_H – 14aa linker – V_L – 15aa linker – TNF - STOP

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKP
LEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT
GDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAIS
GSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVT
VSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRL
LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGS
SSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRAANALLANGVELRDNQLVVPSEGL
YLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLG
GVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL*

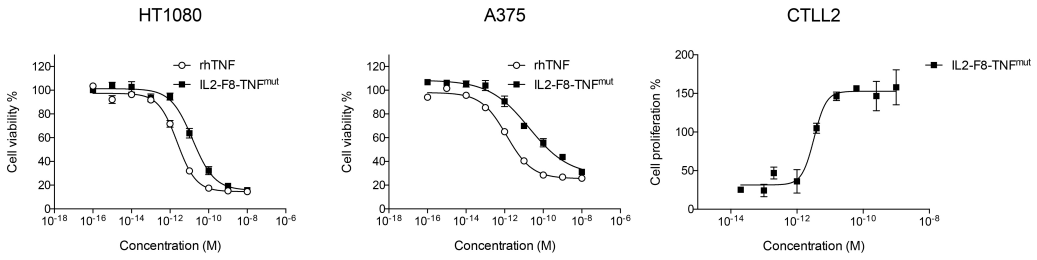
b



c



d



Supplementary Figure S7: Cloning expression and characterisation of fully-human IL2-F8-TNF^{mut}. (A) Amino acid sequence. Starting from the N-terminus: human IL2, the F8 antibody in scFv format and human TNF containing the R108A mutation. (B) Starting from left: SDS-Page analysis (MW: molecular weight, R: reducing conditions, NR: non-reducing conditions), size exclusion chromatography profile, BIAcore analysis on EDA-coated sensor chip and ESI-MS profile. (C) Quantitative biodistrubution analysis of radioiodinated IL2-F8-TNF^{mut} in immunocompetent mice bearing F9 teratocarcinoma tumors. Results are expressed as percentage of injected dose per gram of tissue (%ID/g ± SEM), (n = 3 mice per group). (D) TNF bioactivity assay, based on the killing of HT1080 and A375 cells and IL2 proliferation assay, based on the proliferation of CTLL2 cells.