

Chemical control of protein stability and function in living mice

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Conditional control of protein function *in vivo* offers great potential for deconvoluting the roles of individual proteins in complicated systems. We recently developed a method in which a small protein domain, termed a destabilizing domain, confers instability to fusion protein partners in cultured cells. Instability is reversed when a cell-permeable small molecule binds this domain. Here we describe the use of this system to regulate protein function in living mammals. We show regulation of secreted proteins and their biological activity with conditional secretion of an immunomodulatory cytokine, resulting in tumor burden reduction in mouse models. Additionally, we use this approach to control the function of a specific protein after systemic delivery of the gene that encodes it to a tumor, suggesting uses for enhancing the specificity and efficacy of targeted gene-based therapies. This method represents a new strategy to regulate protein function in living organisms with a high level of control.

Effective methods to regulate gene expression or protein function within mammalian hosts are essential for understanding basic biological mechanisms, as well as for directing gene-based therapies. Generating knockouts of a specific gene and subsequent monitoring of that perturbation *in vivo* is a powerful technique for understanding ways in which proteins contribute to complex biological processes^{1–4}. However, the gene of interest may be essential for development, or cellular or molecular compensation may occur during embryonic development, precluding or confounding the study of the protein's function in an adult organism^{5,6}. Strategies to overcome this limitation have introduced elements of spatial and temporal control over gene expression⁷. The Cre-*loxP* system^{8,9} and the tetracycline-responsive transcriptional switch¹⁰ have been used with great success; however, with these methods, either the perturbations are irreversible, or their inherent reversibility is dependent upon stability of the protein product, the degradation of which can take days. Small-molecule activators or inhibitors of protein function target the protein product directly^{11–15}; however, the discovery process for these is arduous, and many small molecules lack either specificity¹⁶ or optimal properties for use *in vivo*^{17,18}.

We recently described a general method to achieve post-translational regulation of protein function in cultured cells¹⁹. We engineered small, inherently unstable domains that confer instability to any fused partner proteins. When expressed in cells, these fusions are rapidly and constitutively degraded in a proteasome-dependent fashion. Addition of a stabilizing ligand that binds specifically to this destabilizing domain protects the fusion protein from degradation, allowing the protein of interest to perform its normal cellular function. Specifically, a point mutant (L106P) of the 107-amino acid protein FK506- and rapamycin-binding protein (FKBP) confers instability to fusion partners, and this instability is reversed by a synthetic ligand named Shield-1. The combination of genetic manipulation with small-molecule regulation endows this system with the specificity of gene targeting and the speed, tunability and reversibility inherent to small-molecule control. Here we describe the use of this system in living mice.

RESULTS

Tunable regulation of protein stability *in vivo*

To assess the potential of this destabilizing domain technology in mice, we attempted to conditionally regulate luciferase stability, using bioluminescence as an indicator of intracellular luciferase abundance. The FKBP L106P destabilizing domain was cloned at the amino terminus of a thermostable luciferase (L106P-tsLuc) and stably integrated into the HCT116 colon cancer line. Cells expressing the fusion protein were tested for conditional regulation of luciferase activity and then xenografted into immunodeficient mice. Shield-1 was delivered intraperitoneally (i.p.) at a dose of 10 mg kg⁻¹, and luciferase activity was measured in the mice by *in vivo* bioluminescence imaging. Maximum expression was observed 12 h after treatment with Shield-1, and bioluminescent signals returned to background within 48 h (Fig. 1a). These data suggest that Shield-1 is delivered systemically and maintained at sufficient levels within target cells to stabilize the fusion protein for a considerable period of time before being cleared. Quantification of this data shows an approximate tenfold increase in signal upon treatment with Shield-1 (Fig. 1b), in agreement with the dynamic range observed for luciferase activity in this cell line in culture (Supplementary Fig. 1 online). Although this

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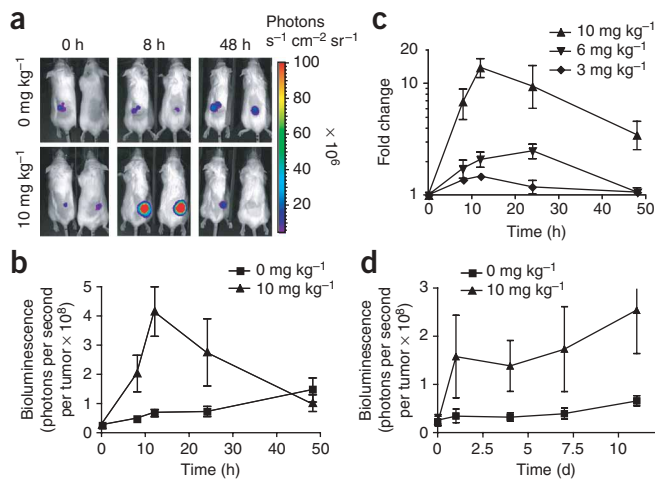


Figure 1 Conditional regulation of protein stability *in vivo*. (a) Bioluminescence imaging of SCID mice bearing HCT116 L106P-tsLuc xenografts (50–100 mm³) either untreated (top) or treated i.p. with Shield-1 (10 mg kg⁻¹, bottom). (b) Quantification of tumor signals from a. (c) Quantification of bioluminescence in mice similar to those in a were treated i.p. with Shield-1 at 3 mg kg⁻¹, 6 mg kg⁻¹ or 10 mg kg⁻¹. (d) Quantification of bioluminescence in mice bearing HCT116-tsLuc xenografts either untreated or treated with Shield-1 (10 mg kg⁻¹) every 48 h. Data in b–d are presented as the average bioluminescence detected within regions of interest drawn around the tumors ± s.e.m. (n = 4–10).

dynamic range is smaller than that observed for HCT116 cells expressing the L106P domain fused to the tomato fluorescent protein (which showed an approximately 50-fold increase in fluorescence with little detectable background signal; **Supplementary Fig. 2** online), it is sufficient for studying the dynamics and kinetics of protein stabilization *in vivo*.

Small molecules are attractive as modulators of protein function, owing, in part, to their ability to regulate protein activity in a dose-dependent manner¹⁹ (**Supplementary Figs. 1 and 2**), thus we tested for dose-dependent protein stabilization in the more complex environment of a living biological system. Mice bearing HCT116 L106P-tsLuc xenografts were treated with various doses of Shield-1 (3–10 mg kg⁻¹). As expected, bioluminescence increased with increasing doses of Shield-1 (**Fig. 1c**). The ability to tunably regulate protein abundance in living systems should prove to be a powerful perturbation technique, especially when the function of a target protein is dependent upon its intracellular concentration^{20,21}.

In cases where a gene is essential for development, Shield-1 abundance must be maintained until a time at which protein function may be abrogated. Therefore, we wanted to ascertain whether repeated Shield-1 delivery would allow for constant protein expression *in vivo*. Mice treated with Shield-1 (10 mg kg⁻¹) once every 48 h maintained increased bioluminescence over the course of several weeks (**Fig. 1d**), suggesting that an infrequent and low-dose treatment regimen with Shield-1 is sufficient to maintain protein expression levels *in vivo*. Notably, we observed no adverse effects on the mice (for example, changes in feeding behavior, body weight or overall activity),

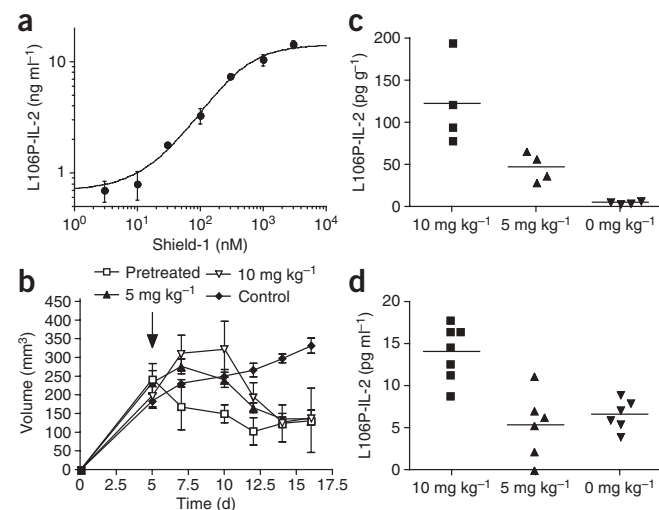
consistent with microarray analysis of mRNA levels in cells treated with Shield-1 that showed no appreciable cellular response to treatment²², as well as with clinical studies of a structurally similar FKBP ligand in humans²³.

Conditional control of a secreted protein reduces tumor burden

We next applied this method of conditionally stabilizing a biologically relevant protein *in vivo* to link protein abundance with a physiological response. Interleukin-2 (IL-2) is an instrumental cytokine in inducing the differentiation and proliferation of a variety of lymphocyte populations²⁴. Recombinant IL-2 is used in a variety of cancer therapies; furthermore, IL-2 fusion proteins impart tumor-protective immunity to a number of therapeutic applications²⁵. Regulation of IL-2 would represent the first use of this destabilizing domain technology for secreted proteins, which control physiological processes ranging from metabolism to growth and differentiation. Our previous results showing regulation of transmembrane glycoprotein abundance at the cell surface in culture¹⁹ and our observation of conditional secretion of *Gaussia* luciferase (**Supplementary Fig. 3** online) suggested that destabilizing domains might successfully regulate proteins that are trafficked through the secretory pathway.

The sequence encoding the L106P destabilizing domain was inserted between the sequence encoding the IL-2 signal peptide and the sequence encoding the secreted protein (L106P-IL-2) and stably introduced into HCT116 cells. After exposure to various concentrations of Shield-1, there was a dose-dependent increase in the L106P-IL-2 fusion protein in the cell culture medium (approximately 25-fold; **Fig. 2a**). L106P-IL-2 was further determined to be functionally active in a natural killer cell proliferation assay (**Supplementary Fig. 4** online). HCT116 L106P-IL-2 cells were subcutaneously

Figure 2 Conditional regulation of a secreted immunomodulatory protein leads to a reduction of tumor burden *in vivo*. (a) IL-2 concentration in culture medium of HCT116 L106P-IL-2 cells treated with various concentrations of Shield-1. Data are represented as means ± s.e.m. (n = 3). (b) Tumor volumes in CD1 *nu/nu* mice bearing subcutaneous HCT116 L106P-IL-2 tumors. Mice were either untreated or treated i.p. with Shield-1 at 5 mg kg⁻¹ or 10 mg kg⁻¹ every 48 h beginning 5 d after transplantation (arrow). Alternatively, mice received HCT116 L106P-IL-2 cells that had been pre-treated with 1 μM Shield-1 for 24 h and that were then treated with Shield-1 (10 mg kg⁻¹) every 48 h beginning on day 0 (squares). Data are represented as means ± s.e.m. (n = 5). (c) Concentration of IL-2 per gram tumor tissue, as determined by ELISA (n = 4), in tumors from mice treated with Shield-1 after transplantation (48 h after the start of Shield-1 treatment). (d) Concentration of IL-2 in the serum of mice treated with Shield-1 after transplantation and bled 48 h after the start of Shield-1 treatment, as determined by ELISA.



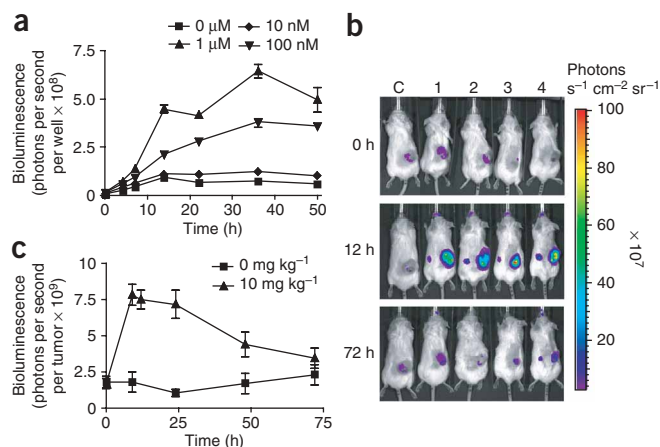


Figure 3 Systemic targeted delivery of a conditionally stabilized protein. (a) Bioluminescence of HCT116 cells infected with vvDD L106P-tsLuc and then mock treated or treated with Shield-1 at 1 μM , 100 nM or 10 nM concentrations. Data are represented as means \pm s.e.m. ($n = 3$). (b) Bioluminescence imaging of SCID mice bearing subcutaneous HCT116 xenografts (50–100 mm^3) that had received a single tail vein injection of vvDD L106P-tsLuc (1×10^8 plaque-forming units per mouse) and that 72 h later had been either untreated (C, control) or treated with Shield-1 (10 mg kg^{-1} , 1–4). (c) Quantification of the bioluminescent signal produced from regions of interest drawn around tumors in b. Data represent means \pm s.e.m. ($n = 4$).

L106P-IL-2 tumors, indicating that Shield-1 does not induce IL-2 production from other cells (data not shown).

Systemic delivery of a conditionally regulated protein

We previously described a viral gene delivery system for the systemic treatment of cancer based on a replication-selective (oncolytic) strain of vaccinia virus (hereafter vvDD), with deletions of viral thymidine kinase and growth factor genes, restricting viral replication to cancer cells²⁷. Intravenous delivery of vvDD results in initial infection of both tumor cells and cells in other tissues followed by rapid replication and spread of the viral vector (and any transgene in the viral genome) within the tumor and viral clearance from other tissues. Because vvDD can express transgenes in tumor cells after systemic delivery, it is an attractive model system to examine whether our technology might also be used to control protein function in an experimental model of a therapeutic application.

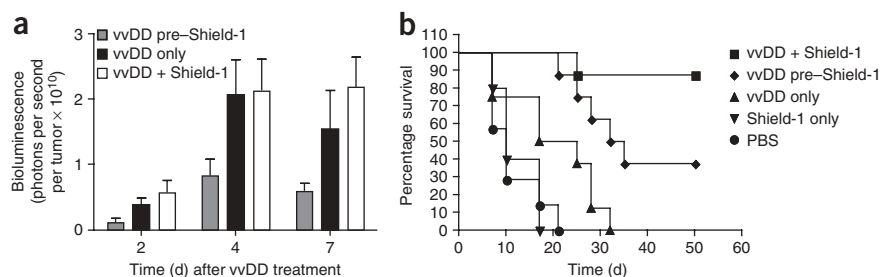
We established that infection of cultured cells with vvDD did not affect the kinetics or dynamic range of L106P-regulated protein stability (Supplementary Fig. 5 online), and Shield-1 did not affect the replication of the virus (data not shown). We next constructed strains of vvDD expressing either an L106P-tsLuc or an L106P-tomato fluorescent fusion protein and verified Shield-1 dependent activity in cultured cells (Fig. 3a and Supplementary Fig. 6 online). Whereas the dynamic range observed for L106P-tsLuc bioluminescence was modest, conditional regulation of virally delivered L106P-tomato resulted in a greater than 100-fold increase in fluorescence upon treatment with Shield-1, with no background signal observed (Supplementary Fig. 6). We then assayed for Shield-1-dependent bioluminescence of virally delivered L106P-tsLuc in a mouse tumor model (Fig. 3b,c). By allowing 72 h for viral infection to establish within the tumor and be cleared from most other tissues before Shield-1 treatment, we were able to selectively stabilize the protein of interest specifically in the tumor as the target tissue.

To further test the ability of this approach to regulate therapeutic proteins, we applied this technology to the cytokine tumor necrosis factor- α (TNF- α). Despite the proven cytotoxic effects of TNF- α against primary tumors, its systemic toxicity has limited the clinical use of TNF- α to local applications (for example, isolated limb or organ perfusion)²⁸. In addition, TNF- α shows antiviral effects to which the vaccinia strain Western Reserve (the backbone for vvDD) is susceptible²⁹. A gene encoding a L106P-TNF- α fusion protein was inserted into a strain of vvDD constitutively expressing luciferase, and dose-dependent control of the secreted L106P-TNF- α fusion protein from cultured cells was verified (Supplementary Fig. 7 online). Mice bearing large (150–250 mm^3) subcutaneous HCT116 tumors were then treated with a single intravenous injection of the L106P-TNF- α virus or a PBS control. Shield-1 (10 mg kg^{-1}) was delivered i.p. to the mice every 48 h, starting either 1 d before vvDD treatment (resulting in constitutive viral expression and stabilization of TNF- α), 3 d after treatment

xenografted into mice able to produce functional B and natural killer cells, and the ability of these cells to establish tumors and proliferate under Shield-1 treatment was monitored. Tumors did not develop in mice that received HCT116 L106P-IL-2 cells pretreated with Shield-1 and that were then subjected to Shield-1 treatment (10 mg kg^{-1}) every 48 h, causing constitutive L106P-IL-2 secretion (Fig. 2b). To determine whether regulated secretion of L106P-IL-2 from an established tumor might lead to a reduction in tumor burden, we established tumors in mice for 5 d. Mice were then treated with Shield-1 i.p. at either 5 mg kg^{-1} or 10 mg kg^{-1} every 48 h, and tumor volume was monitored over time. Tumor regression was observed in both treated groups, and by day 14 after implantation (that is, day 9 after start of Shield-1 dosing), the tumor burden in these two groups was reduced to levels identical to that of mice pretreated with Shield-1 (Fig. 2b). As expected, tumors in the untreated control group continued to increase in size over the course of the experiment (Fig. 2b). At day 16, all Shield-1-treated groups showed significantly reduced tumor burden relative to controls ($P = 0.0019$ for 10 mg kg^{-1} , $P = 0.0002$ for 5 mg kg^{-1} and $P = 0.0046$ for pretreated groups).

This destabilizing domain technology allows dose-dependent regulation of protein abundance *in vivo*; however, we did not observe any appreciable difference in the reduction of tumor burden between mice treated with 5 mg kg^{-1} or 10 mg kg^{-1} Shield-1. Analysis of L106P-IL-2 abundance within the tumors of treated and untreated mice confirmed dose-dependent production of L106P-IL-2 at the tumor site (Fig. 2c). Tumors from mice treated with Shield-1 at 10 mg kg^{-1} contained significantly higher amounts of IL-2 than did tumors from mice treated at 5 mg kg^{-1} ($P = 0.032$), which, in turn, produced more IL-2 than did tumors from untreated mice ($P = 0.0028$). Serum levels of IL-2 were also measured. Shield-1 treatment at 10 mg kg^{-1} ($n = 7$) produced significantly higher amounts of serum IL-2 relative to control mice ($n = 6$, $P = 0.0004$), whereas treatment at 5 mg kg^{-1} ($n = 6$) did not produce any increase in serum IL-2 abundance (Fig. 2d). These observations suggest that treatment with Shield-1 results in tunable, dose-dependent secretion of a cytokine *in vivo*, and that different biological effects are achieved by varying the dose of Shield-1. A dose of 5 mg kg^{-1} Shield-1 was capable of producing the same robust antitumor response as 10 mg kg^{-1} Shield-1 without the potential for toxicity associated with systemic distribution in conventional high-dose recombinant IL-2 therapies²⁶. In addition, mice bearing tumors derived from HCT116 cells alone and treated with 10 mg kg^{-1} Shield-1 ($n = 5$) showed the same serum IL-2 abundance as did untreated mice bearing HCT116

Figure 4 Antitumor benefit of conditional regulation of a targeted gene therapy vector. CD1 *nu/nu* mice bearing subcutaneous HCT116 tumors (150–250 mm³) were treated via a single tail vein injection with either PBS or the vaccinia strain vvDD expressing both luciferase as well as the L106P–TNF- α fusion protein (1×10^8 plaque-forming units per mouse). Treated mice also received Shield-1 (10 mg kg⁻¹) every 48 h by three different protocols: either 24 h before vvDD therapy (vvDD pre–Shield-1); 72 h after vvDD therapy (vvDD + Shield-1) or not at all (vvDD only) ($n = 8$ mice per group). Tumor-bearing mice that did not receive vvDD therapy but were treated with Shield-1 (10 mg kg⁻¹) served as a negative control (Shield-1 only, $n = 5$). (a) Viral load within the tumor, as assayed by measuring constitutive viral gene expression (bioluminescence) for each group at the indicated time points. (b) Kaplan–Meier survival curves of mice undergoing the indicated treatments. Mice were killed once tumor volume reached 1.44 cm³. All surviving mice at 50 d had no detectable tumor.



(resulting in stabilization of TNF- α only after vvDD gene expression is localized to the tumor) or not at all.

When Shield-1 dosing began before virus delivery, resulting in constitutive secretion of TNF- α , we observed only modest amounts of virus within the tumor (Fig. 4a), as measured by constitutive viral luciferase expression. It is likely that secretion of TNF- α from infected cells rapidly targets these cells for destruction, limiting the ability of the virus to establish within the tumor. Mice treated with Shield-1 before vvDD therapy had significantly enhanced survival relative to mice treated with vvDD alone (Fig. 4b, $P = 0.017$). Shield-1 treatment starting 72 h after vvDD resulted in significantly greater levels of viral gene expression in the tumor than when Shield-1 treatment was started before vvDD therapy (Fig. 4a; $P = 0.035$ at 2 d, $P = 0.035$ at 4 d and $P = 0.002$ at 7 d). When Shield-1 administration was delayed until 3 d after virus treatment, initial delivery and persistence of viral infection measured by luciferase expression within the tumor were equivalent to those in mice treated with virus alone (Fig. 4a). Addition of Shield-1 72 h after vvDD (vvDD + Shield-1) produced a further significant increase in survival relative to mice treated with Shield-1 before vvDD ($P = 0.031$). In addition, the antitumor effects observed in this experimental group were significantly greater than those seen in any other treatment regimen ($P < 0.05$). Seven of the eight mice showed complete and durable responses (Fig. 4b), with no observable adverse physiological effects. Shield-1 delivered alone had no effect on tumor burden (Fig. 4b). The ability to regulate the stability of physiologically relevant or therapeutic proteins in this experimental setting illustrates the potential of this method to enhance a variety of biological therapies where reversible control of the timing and level of protein activity may be advantageous.

DISCUSSION

We have demonstrated that the FKBP-derived L106P destabilizing domain, in concert with Shield-1, can confer conditional stability to a protein of interest with excellent kinetics and dose control in complicated living organisms. We demonstrate regulation of secreted proteins in a dose-dependent manner with conditional secretion of the physiologically relevant protein IL-2, resulting in biological effects (tumor regression) *in vivo*. By regulating the secretion of TNF- α , a protein of therapeutic value, we were able to show the benefits of controlling protein stability in a gene delivery vehicle, resulting in greatly enhanced antitumor effects once TNF- α was stabilized in the target tissue.

Central to any method of protein regulation involving the use of small molecules is the specificity of the perturbation. Our recent microarray analysis of cells treated with various concentrations of Shield-1 supports the notion that the small molecule causes little, if

any, off-target cellular perturbation²². Here we show that treatment with Shield-1 alone does not improve the therapeutic outcome in tumor-bearing mice, and Shield-1-treated mice maintain normal body weight, activity, and feeding behaviors. These observations suggest that any physiological responses observed are a direct result of the stabilization of a specific protein in a defined experimental context.

To date, we have successfully applied this approach to the regulated stability of more than 30 proteins, either in cultured cells¹⁹ or, now, *in vivo*. We have used this technology to confer conditional stability to proteins of various subcellular localizations¹⁹, and here we report the conditional regulation of secreted proteins. The broad applicability and simplicity of this approach suggest that this system may allow rapid and precise control of protein stability and function in a number of biological settings. The ability to regulate protein function *in vivo* with the precision and control conferred by this technology will probably prove useful for validating potential therapeutic targets, as well as advance our understanding of basic biological processes and pathways.

METHODS

Cell culture and transductions. We cultured cell lines (American Type Culture Collection) in DMEM with 10% FBS (Invitrogen), 2 mM glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (all from Gibco). We cloned the genes encoding thermostable luciferase³⁰ or human IL-2 into pBMN L106P iBlasticidin and used the construct to generate amphotropic retrovirus¹⁹. We incubated HCT116 cells with retrovirus and polybrene (6 μ g ml⁻¹) for 4 h at 37 °C and then selected for infected cells with blasticidin (5 μ g ml⁻¹) (Invitrogen). We treated cells grown in 96-well plates (2×10^4 cells per well) with various doses of Shield-1 as indicated in Figures 2–4 and either measured bioluminescence with an IVIS 50 (Xenogen Product from Caliper Life Sciences) after luciferin addition (300 μ g ml⁻¹) or collected media for ELISA (see below).

Shield-1 formulation and delivery. We formulated Shield-1 in 50% *N,N*-dimethylacetamide and 50% of a 9:1 PEG-400:Tween-80 mixture. We administered Shield-1 (40 μ l) i.p. at 3 mg kg⁻¹, 6 mg kg⁻¹ or 10 mg kg⁻¹.

Mouse models. We gave severe combined immunodeficient (SCID) or CD1 *nu/nu* mice (Charles River Laboratories) subcutaneous dorsal injections of $\sim 1 \times 10^7$ cells and allowed tumors to establish as indicated in the text and Figures 1–4. We gave mice an i.p. injection of luciferin (225 mg kg⁻¹), anesthetized them (2% isoflurane) and placed them on the warmed stage (37 °C) of an IVIS 100 or IVIS 200 (Xenogen Product from Caliper Life Sciences) for imaging. We measured the light produced as photons s⁻¹ for designated regions of interest. We determined tumor volumes by caliper measurement, and we killed the mice for survival assays when tumors reached 1.44 cm³. We collected serum samples by retro-orbital bleedings, collected tumors post-mortem and homogenized them for ELISA assays of cytokines

IL-2 and TNF- α (BD Biosciences). All experiments were run with approval from the Stanford University Administrative Panel on Laboratory Animal Care.

Vaccinia virus strains. We transfected CV1 cells with pSC-65 p7.5 L106P-tsLuc or pSC-65 p7.5 L106P-TNF- α pSE/Luc and simultaneously infected them with viral growth factor-deleted Western Reserve vaccinia (VSC20) (both pSC-65 and VSC20 were kindly provided by B. Moss). We integrated gene cassettes into the gene coding for viral thymidine kinase by homologous recombination and selected for them by resistance to bromodeoxyuridine on 143B TK⁻ cells. We purified single viral plaques in 143B TK⁻ cells.

vvDD assays in cultured cells. We incubated HCT116 cells in a 96-well plate (2×10^4 cells per well) with vvDD carrying an L106P fusion (multiplicity of infection >1) for 1 h at 37 °C. We removed the virus and treated the cells with Shield-1. For luminescence assays, we incubated cells with luciferin ($300 \mu\text{g ml}^{-1}$) and imaged them with an IVIS 50. We detected TNF- α in cell culture medium by ELISA.

Statistical analyses. We used two-tailed, unpaired Student's *t*-tests, except for comparison of survival curves, when we used the Gehan-Breslow-Wilcoxon test. We considered $P < 0.05$ significant.

Requests for materials. Shield-1 requests should be directed to T.J.W.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

L.A.B. and M.A.S. designed and performed research and analyzed data. S.H.T. designed, coordinated and performed research and analyzed data. C.H.C. and T.J.W. helped design and interpret experiments and analyze data. All authors wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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