Supporting Information

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SI Materials and Methods

Cell Lines. All cancer cell lines were originally purchased from the American Type Culture Collection (1999–2002), except the PC3-PSMA line, which was a generous gift from Prof. Michel Sadelain of Memorial Sloan Kettering Cancer Center, New York. LNCaP cells were grown in RPMI medium 1640 supplemented with 1 mM sodium pyruvate and 10 mM Hepes pH 7.4. PC3-PSMA cells were grown in RPMI medium 1640 supplemented with 5 mg/L puromycin. VCaP cells were grown in DMEM. PC3 and DU145 cells were grown in MEM supplemented with 1% nonessential amino acids, 1% MEM vitamin mixture, 1 mM sodium pyruvate, and 10 mM Hepes pH 7.4. MCF7 cells were grown in RPMI medium 1640. All tissue culture media contained 10% FCS, penicillin (100 U/mL), and streptomycin (100 mg/L). PC3 and LNCaP cells were infected with lentivirus carrying Luc to generate PC3-Luc and LNCaP-Luc as previously described (18). Cell lines were cultured at 37 °C in 5% CO_2. Human PBMCs were isolated from blood buffy coats by Ficoll-Paque PLUS (GE Healthcare) density-gradient centrifugation and maintained as described (14). Cell lines were periodically tested to confirm the absence of mycoplasma. All cell culture reagents were obtained from Biological Industries Beit Haemek.

Chemicals. All solvents and reagents were used as supplied. The (1-[Bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxid hexafluorophosphate) (HATU), dimethylformamide (DMF; peptide synthesis grade), and acetonitrile (HPLC grade) were purchased from Bio-Lab Ltd.; diisopropylethylamine (DIPEA), piperidine, triisopropylsilane (TIS), D_2O, and trifluoroacetic acid (TFA) from Sigma-Aldrich; standard Fmoc-amino acids from Luxembourg Industries Ltd.; Fmoc-Cys(Trt)-Wang resin from GL Biochem; and orthopyridyl disulfide-PEG-NHS ester (molecular weight 2,000) from Creative PEGWorks. PMPA was from ENZO Life Science.

Synthesis of DUPA–Linker–DyLight 680.

DUPA ligand synthesis. The DUPA ligand was synthesized using the reported procedure (23).

Peptide synthesis. The peptide was synthesized following standard Fmoc solid-phase peptide synthesis (SPPS) procedures using commercially purchased Fmoc-Cys(Trt)-Wang resin as the solid support with loading of 0.47 mmol/g. The synthesis is performed typically on a 0.25-mmol scale. Initially, the Fmoc-Cys(Trt)-Wang resin was swollen in dichloromethane (DCM) for 2 h and in DMF for 20 min.

Fmoc removal. The Fmoc-peptidyl-resin was treated twice with 20% piperidine in DMF (10 mL) for 10 min and 15 min at room temperature. The Kaiser test (55) was performed to confirm the complete Fmoc removal. The solid support was then washed thoroughly with DMF (5 × 5 mL).

Coupling procedure. Fmoc-protected amino acid [5 equivalents (eq)], HATU (4.5 eq) and DIPEA (8 eq) were dissolved in DMF (6 mL). The reaction mixture was activated for 5 min at 0 °C before adding to the peptide resin with free amine. The resin was swirled for ∼45 min. The solid support was then washed thoroughly with DMF (4 × 5 mL). The completion of the coupling was monitored by Kaiser test.

Capping with acetic anhydride. After the first amino acid coupling, the resin was treated with acetic anhydride to block the unreacted free amine functional group. The resin was treated with a solution of acetic anhydride (10 eq) and DIPEA (8 eq) in DMF for 20 min and washed with DMF (5 × 5 mL) and DCM (3 × 5 mL).

Coupling of DUPA building block. The fully protected DUPA building block with free acid (5 eq), HATU (4.5 eq) and DIPEA (8 eq) were dissolved in DMF (6 mL). The combined reaction mixture was activated for 5 min at 0 °C. The preactivated DUPA building block was then added to the peptide resin bearing an N-terminal free amine. The reaction mixture was swirled for ∼60 min. The resin was washed thoroughly with DMF (5 × 5 mL). The completion of the DUPA building-block coupling was monitored by Kaiser test.

Peptide release from solid support. A freshly prepared solution (5 mL) of TFA/TIS/TDW (95: 2.5:2.5) was cooled to 0 °C and added to 200 mg resin-bound peptide. The mixture was swirled at room temperature for 4 h. The solution was then filtered and washed with 3 mL of TFA, which was concentrated under nitrogen atmosphere. The resulting peptide was precipitated by slow addition of ice-cold ether. The solution was centrifuged and the peptide washed twice with cold ether. A minimal volume of a 1:1 ACN/TDW mixture was used to dissolve the crude peptide, which was then lyophilized before purification (Fig. S2*A*). The formation of the product was confirmed by liquid chromatography (LC)-MS (Fig. S2*B*). DyLight 680 conjugation was carried out using the reported procedure (56).

Synthesis of DUPA–LinkerA. Parent DUPA peptide was synthesized using standard Fmoc SPPS procedures on Fmoc-Cys(Trt)-Wang resin as the solid support.

Swelling. The resin was swelled for at least 2 h in dichloromethane.

Fmoc removal. The resin was treated with a solution of 20% piperidine in DMF (2 × 20 min), and then washed with DMF (5 × 2 min).

Coupling of Fmoc-Asp(OtBu)-OH. The 3 eq of Fmoc-Asp(OtBu)-OH and 3 eq HATU were dissolved in 15 mL of DMF, and 8 eq of DIEA were added to the mixture. The solution was preactivated by mixing for 10 min at room temperature before it was added to the resin for 1 h. The coupling was repeated with a fresh mixture. The resin was washed with DMF (3 × 2 min) and DCM (2 × 2 min). A Keiser test was performed to ensure complete coupling.

Capping. The resin was treated with a solution of acetic anhydride (10 eq) and DIEA (8 eq) in DMF for 20 min and washed with DMF (3 × 2 min).

Fmoc removal. The resin was treated with a solution of 20% piperidine in DMF (2 × 20 min), and then washed with DMF (5 × 2 min).

Coupling of Fmoc-diaminopropionic acid. Three eq of Fmoc-diaminopropionic (DAP) acid, 3 eq HATU, and 8 eq *N*,*N*-diisopropylethylamine (DIEA) were dissolved in 15 mL of DMF. The solution was preactivated by mixing for 10 min at room temperature before it was added to the resin for 1 h. The resin was washed with DMF (3 × 2 min) and DCM (2 × 2 min), and a Keiser test was performed to ensure complete coupling.

Fmoc removal. The resin was treated with a solution of 20% piperidine in DMF (2 × 20 min), and then washed with DMF (5 × 2 min).

Peptide elongation. The same procedure was used for the coupling of Fmoc-Phe-OH, Fmoc-Phe-OH, Fmoc-8-aminooctanoic acid, and the OtBu-Glu(Fmoc)-OH.

Fmoc removal. The resin was treated with a solution of 20% piperidine in DMF (2 × 20 min), and then washed with DMF (5 × 2 min) and DCM (3 × 2 min).

Coupling of the DUPA. The 0.9 mL of triethylamine (6.6 mmol) was combined with 0.9 g (3 mmol) L-glutamic acid di-tert-butyl ester hydrochloride and dissolved in 15 mL DCM. This solution was added dropwise over 45 min to a solution of 5 mL DCM and triphosgene (0.35 g, 1.1 mmol) at 0 °C. After stirring for an additional 50 min. the mixture was added to the resin with another 0.9 mL of triethylamine. The resin was shaken for 3 h and washed with DMF (3 × 2 min).

Full cleavage. The resin was washed with DCM (3 × 2 min) and dried under vacuum. A solution of 2.5% TDW and 2.5% TIS in TFA at 0 °C was added. The reaction proceeded for 4 h at room temperature. The mixture was filtered and treated with a cooled solution of ether/hexane 1:1, and the peptides were precipitated by centrifugation. The crude peptides were dissolved in acetonitrile/TDW 1:1 and lyophilized. The crude product was purified by preparative RP-HPLC and coupled to DyLight 680 as described (56).

Synthesis of PPD. DUPA–linker was synthesized and the crude product was purified by preparative RP-HPLC (Fig. S3*A*). The formation of the product was confirmed by LC-MS (Fig. S3*B*). Then, 4.37 mg (1.2×10^{-4} mmol) of diconjugate 1:1 (24) were dissolved in 940 μL of 20 mM Hepes pH 7.4. Next, 1 mg (9.1×10^{-4} mmol, ∼5 eq) of DUPA-linker dissolved in 2 mL of 1:1 ACN (HPLC grade)/(20 mM Hepes pH 7.4) was added dropwise to the reaction. Then, to reach ∼10% total concentration of ACN in the reaction, an additional 4 mL of 20 mM Hepes pH 7.4 were introduced into the reaction mix. The reaction was completed as described by Joubran et al. (24).

Confocal Fluorescence Microscopy. The selectivity of DUPA ligand for PSMA-overexpressing cells was evaluated using DUPA labeled with DyLight 680 (DUPA–linker–DyLight 680). Uptake of DUPA–linker–DyLight 680 was tested in live PC3-PSMA, LNCaP, and MCF7 cells using confocal fluorescence microscopy (FluoView FV1000; Olympus). LNCaP and MCF7 cells were seeded (8,000 cells per well) in eight-well μ-Slides (80826; Ibidi) and grown for 72 h. Then, the medium was refreshed and DUPA–linker–DyLight 680 (70 nM) was added. Cells were followed by time-lapse microscopy for 5 h. Sulforhodamine (green) was added to the medium to ensure that micrographs were from cross-sections of the cells.

Cell Survival Assay. PC3-PSMA, LNCaP, VCaP, and MCF7 cells were seeded in 96-well plates at a density of 5,000 cells per well, in triplicate. PC3 was seeded at 1,000 cells per well, in triplicate. On the next day, the cells were treated with PPD/polyIC, PPD/polyI, or polyIC alone at the indicated concentrations. After the length of treatment indicated in the text, cell survival was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Immunoblot Analysis. LNCaP cells were seeded in six-well plates at a density of 10^6 cells per well. At 24 h later, cells were treated with PPD/polyIC as indicated and lysed with boiling Laemmli sample buffer. The lysed cells were subjected to Western blot analysis to examine the cleavage of caspase-3 and PARP using primary antibodies from Cell Signaling Technology: anti-caspase3 (catalog no. 96625), anti-cleaved caspase-3 (96615), and anti-PARP (95425). Expression levels were normalized to GAPDH expression using anti-GAPDH (catalog no. sc-25778; Santa Cruz).

Measurement of Secreted Cytokines by ELISA. PC3-PSMA and LNCaP cells were seeded in triplicate in 96-well plates (at a density of 2,000 cells per well for PC3-PSMA and 10,000 cells per well for LNCaP). At 24 h later, cells were treated with PPD/polyIC at the indicated concentrations. After 48 h or 72 h, medium was collected from each well and the concentrations of the secreted cytokines, IP-10 and RANTES, were measured using commercial ELISA kits (PeproTech).

Quantification of Cytokine Transcripts by qRT-PCR. Total RNA was extracted from PC3-PSMA and LNCaP cells as follows: PC3-PSMA (100,000 cells per well) and LNCaP cells (500,000 cells per well) were seeded in 12-well plates. On the next day, the cells were treated with PPD/polyIC as indicated. After 4 or 8 h, total RNA was extracted using the EZ-10 DNA Away RNA-MiniPrep Kit (Bio Basic).

Total RNA was extracted from stimulated PBMCs as follows: LNCaP cells were seeded at a density of 10^6 cells per well in polylysine-precoated six-well plates, where polylysine was used to allow strong adhesion of the cells to the plastic. On the next day, the cells were treated with PPD/polyIC as indicated. Forty-eight hours later, the conditioned medium was collected and transferred to new six-well plates. Freshly isolated PBMCs were added to the wells at a density of 10^7 cells per well and incubated for 24 h. Next, total RNA was extracted from the PBMCs using the EZ-10 DNA Away RNA-MiniPrep Kit (Bio Basic).

The RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was conducted using Fast SYBR Green (Applied Biosystems). The relative quantities of cytokine transcripts were normalized to GAPDH or HUPO transcripts and compared with untreated cells using the ΔΔ CT method. The primers used for the qRT-PCR are listed in Table S1.

Chemotaxis Assays. LNCaP cells were seeded at a density of 250,000 cells per well in polylysine-precoated 24-well plates. On the next day, the culture medium was replaced with fresh medium supplemented with only 0.15% FBS. The cells were then treated with PPD/polyIC at the indicated concentrations. Forty-eight hours later, the conditioned medium was collected from each well and analyzed for its ability to stimulate chemotaxis of PBMCs using Transwell plates [microporous polycarbonate membrane (0.5 μm) Corning; Costar] as follows: The conditioned medium was placed in the lower wells of the Transwell plate and freshly isolated PBMCs (in medium supplemented with only 0.15% FBS) were added at a density of 10^6 cells per well to the Transwell inserts placed on each well. After an incubation period of 3.5 h, the Transwell inserts were removed and the medium containing the migrated PBMCs was collected and subjected to FACS analysis, scatter-gating to quantify the lymphocyte subsets.

In Vitro Analysis of PPD/PolyIC Bystander Effects. PPD/polyIC bystander effects were analyzed in vitro using coculture systems, where treated PSMA-overexpressing cells were cocultured with PBMCs alone or with PBMCs and with cells not expressing PSMA (neighboring cancer cells). The bystander effects were assessed using cell lines that stably express luciferase (LNCaP-Luc, PC3-Luc, or MCF7- Luc). Survival of these cells, which correlates to the luciferase activity, was measured using the Luciferase Assay System (Promega).

LNCaP-Luc–PBMC coculture system. LNCaP-Luc cells were seeded (10,000 cells per well) in triplicate in 96-well plates precoated with polylysine. On the next day, the cells were treated with PPD/polyIC as indicated. Twenty-four hours later, freshly isolated PBMCs (10^5 cells per well) were added to the culture. Luciferase activity was analyzed 48 h later, as a measure of the survival of the LNCaP-Luc cells.

LNCaP–PBMC–PC3-Luc coculture system. LNCaP cells (6,000 cells per well) were seeded in triplicate in 96-well plates precoated with polylysine. On the following day, the cells were treated with PPD/polyIC as indicated. PC3-Luc cells (4,000 cells per well) were added to the culture 16 h later. Freshly isolated PBMCs (10^5 per well) were added to the culture 6 h after the PC3-Luc cells (i.e., 22 h after the initiation of PPD/polyIC treatment). Seventy-two hours later, the survival of the PC3-Luc cells (luciferase activity) was measured.

PC3-PSMA–PBMC–MCF7-Luc coculture system. PC3-PSMA cells (2,000 cells per well) were seeded in triplicate in 96-well plates. On the next day, the cells were treated with PPD/polyIC as indicated. Sixteen hours later, MCF7-Luc cells (4,000 cells per well) were added to the culture. Freshly isolated PBMCs were added to the culture (10^5 cells per well) 6 h later. Seventy-two hours later, the survival of the MCF7-Luc cells (luciferase activity) was measured.

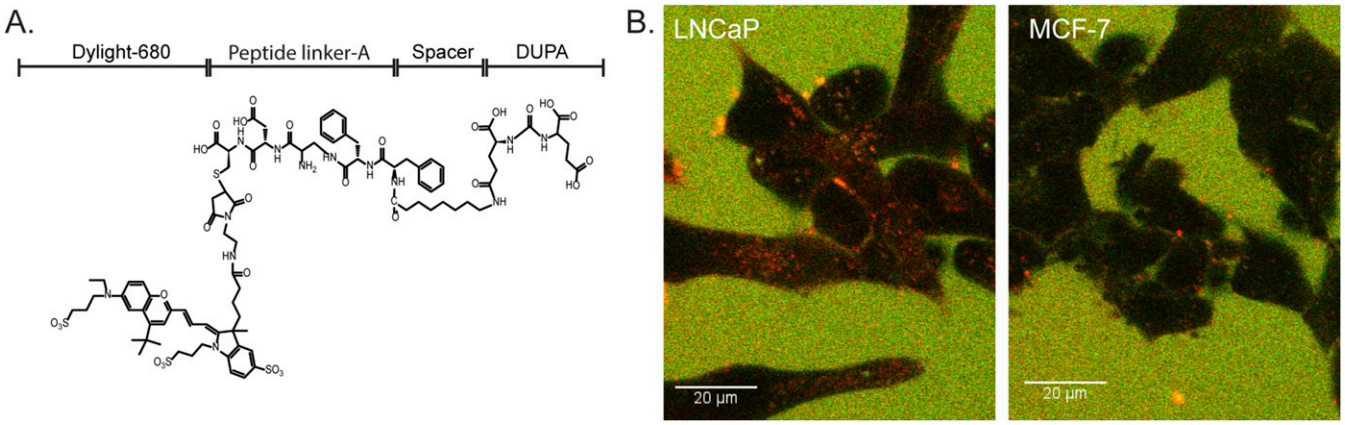

Fig. S1. DUPA–linkerA selectively enters PSMA-overexpressing cells. (*A*) Structure of DUPA–linkerA–DyLight 680 (23). (*B*) LNCaP or MCF7 cells were treated with 70 nM DUPA–linkerA–DyLight 680 for 5 h and visualized in a laser scanning confocal fluorescence microscope.

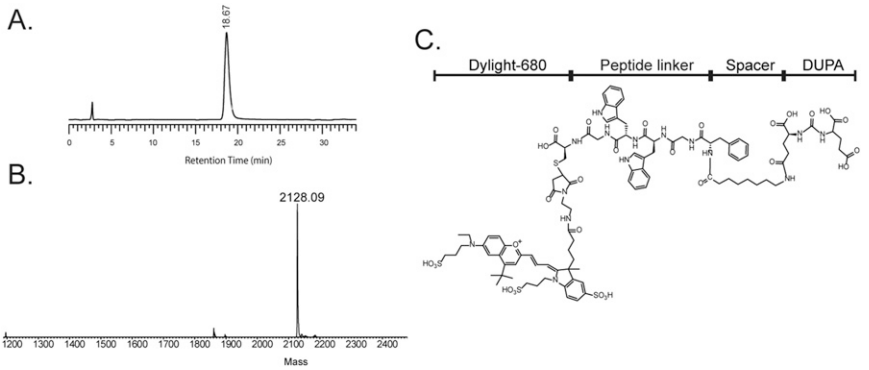

Fig. S2. Structure of DUPA–linker–DyLight 680. (*A*) RP-HPLC of DUPA–linker–DyLight 680. Analytical RP-HPLC analyses were performed on an Acquity UPLC H-Class with UV detection (220 nm) using an XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm) using a linear gradient of 5–60% acetonitrile over 30 min at 1 mL/min flow. (*B*) Deconvoluted mass spectrum of DUPA–linker–DyLight 680: observed 2128.09; calculated 2128.77. (*C*) Chemical structure of DUPA–linker–DyLight 680.

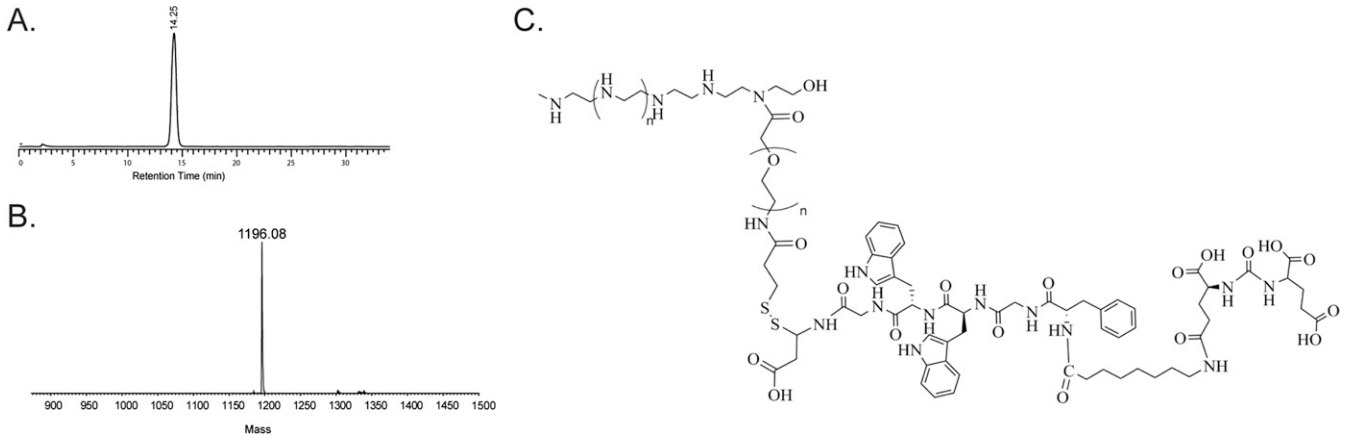

Fig. S3. Structure of PPD. (*A*) RP-HPLC of DUPA–linker. Analytical RP-HPLC analyses were performed on an Acquity UPLC H-Class with UV detection (220 nm) using an XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm) using a linear gradient of 5–60% acetonitrile over 30 min at 1 mL/min flow. (*B*) Deconvoluted mass spectrum of DUPA–linker: observed 1196.08; calculated 1197.48. (*C*) Chemical structure of PPD.

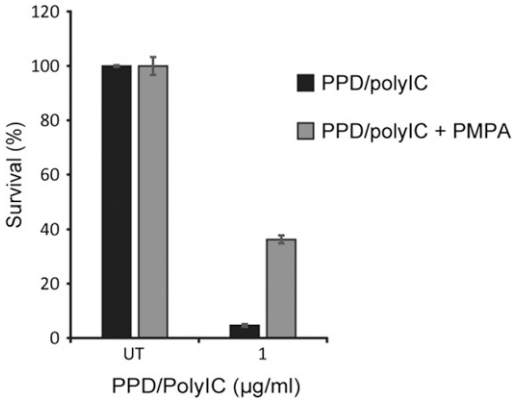

Fig. S4. Killing of LNCaP cells by PPD/polyIC is dependent on PSMA. LNCaP cells were incubated with PPD/polyIC in the absence or presence of a 100-fold molar excess of the PSMA inhibitor PMPA; cell survival was measured 72 h later using CellTiter Glo (Promega). The graph shows means plus SDs.

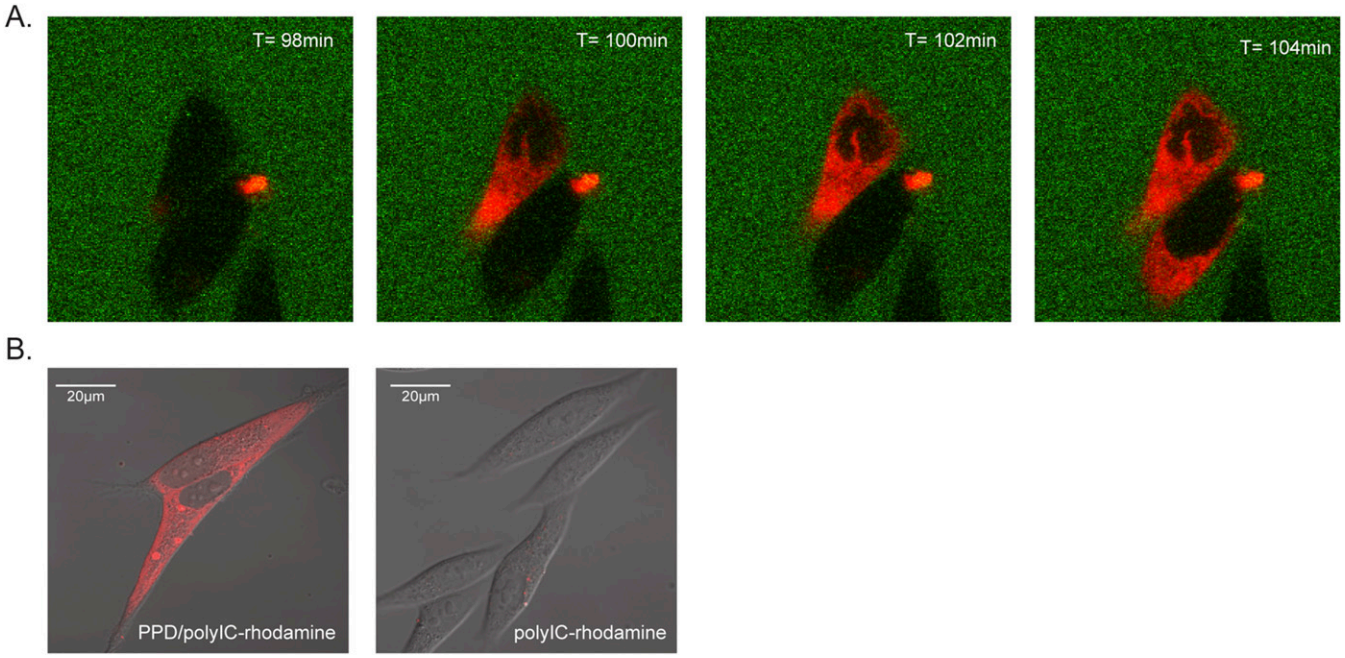

Fig. S5. Rapid entry of PPD/polyIC into LNCaP cells. (*A*) Time-lapse photography using rhodamine-labeled polyIC. LNCaP cells were treated with PPD/polyIC–rhodamine (1 μg/mL) at time 0. Entry of rhodamine into the cells was first noted after ~90 min, at which point photographs were taken every 2 min. The medium was stained with sulforhodamine (green) to ensure that photographs were of cross-sections of the cells. (*B*) Optical sectioning using rhodamine-labeled polyIC. LNCaP cells were treated as above, with PPD/polyIC–rhodamine or polyIC–rhodamine alone. Even after 5-h incubation, polyIC–rhodamine that was not complexed with PPD was barely detectable in the cells.

Table S1. Primers for qRT-PCR

Name	Sequence 5′ to 3′
IFN-β	F: ATGACCAACAAGTGTCTCCTCC
	R: GCTCATGGAAAGAGCTGTAGTG
GAPDH	F: GAGCCACATCGCTCAGAC
	R: CTTCTCATGGTTCACACCC
IFN-γ	F: GCTGTTACTGCCAGGACCCATA
	R: TCCGCTACATCTGAATGACCTG
IL-2	F: AGACCCAGGGACTTAATCAGCAA
	R: CAATGGTTGCTGTCTCATCAG
TNF-α	F: GTGCTTGTTCCTCAGCCTCTTC
	R: GGCCAGAGGGCTGATTAGAGAG
HUPO	F: GCTTCCTGGAGGGTGTCC
	R: GGACTCGTTTGTACCCGTTG