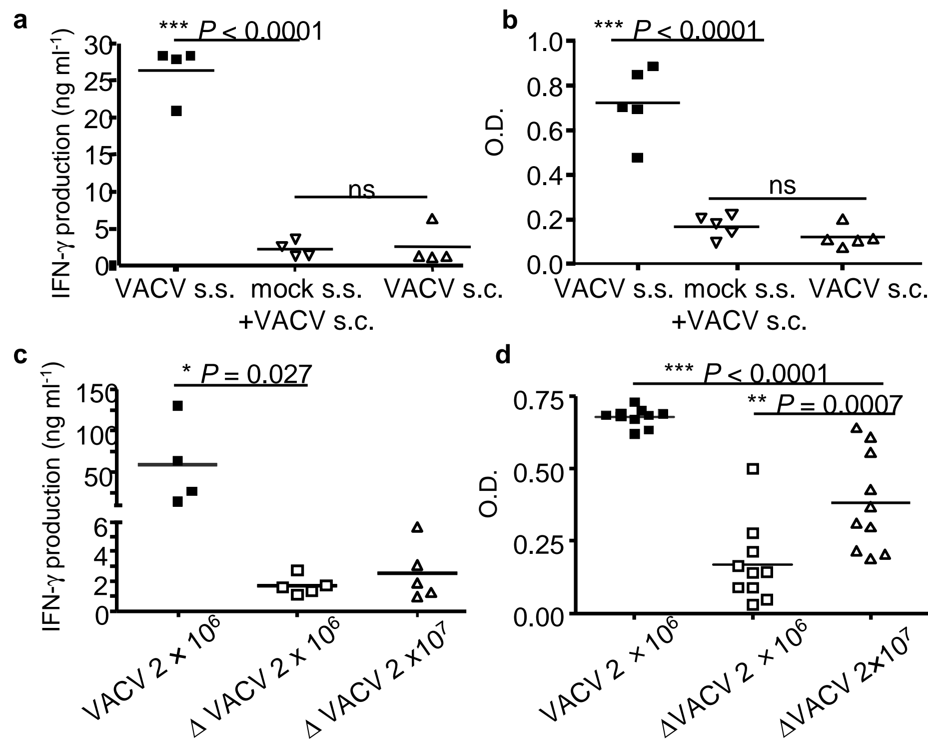
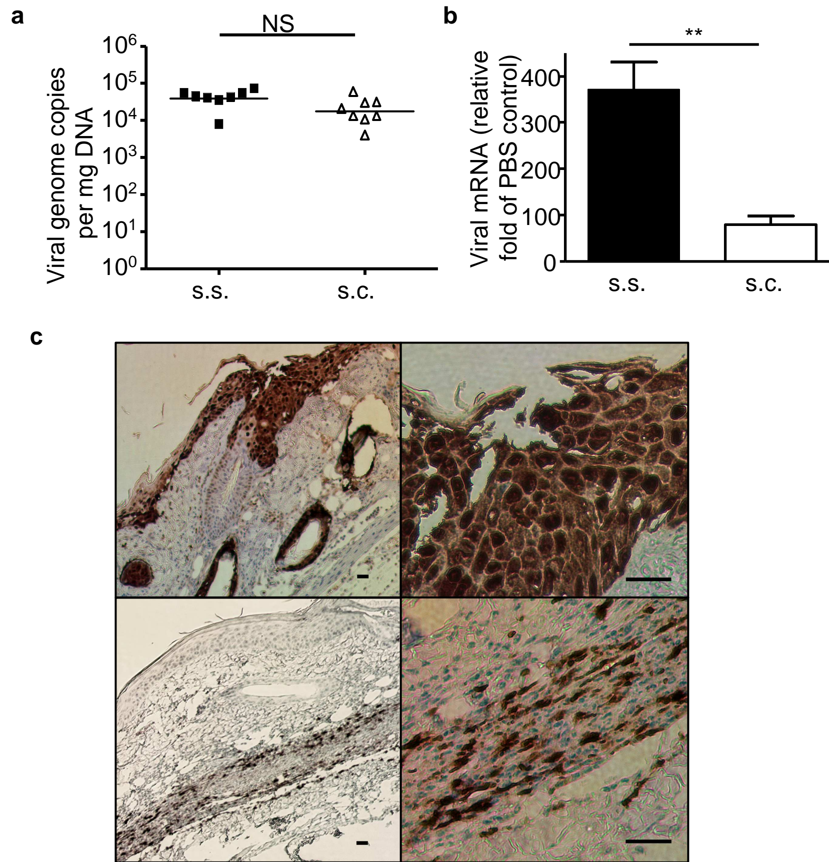
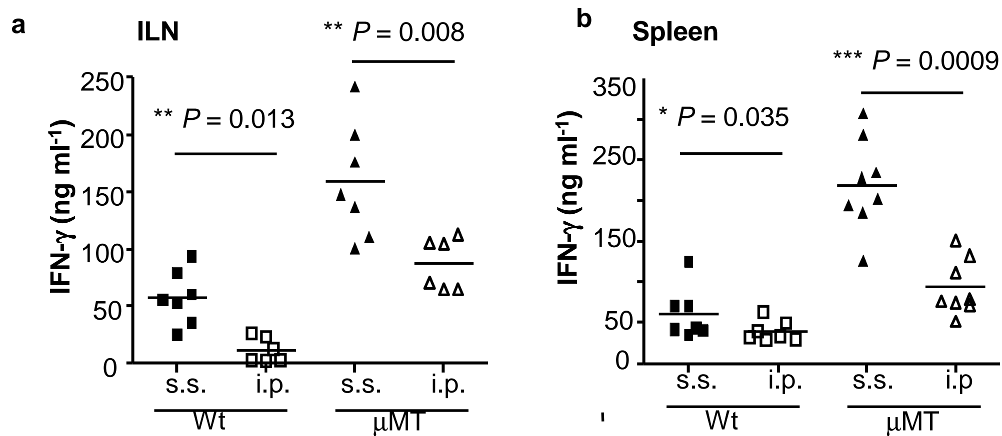
Physical disruption of skin during poxvirus immunization is critical for the generation of highly protective T cell-mediated immunity. Luzheng Liu, Qiong Zhong, Tian Tian, Krista Dubin, Shruti K. Athale, and Thomas S. Kupper



Supplementary Figure 1 Inoculation of live VACV in the context of superficially injured skin is critical to the induction of robust immune responses. (**a, b**) Mice were immunized with VACV via s.s., or s.c. injection with or without concurrent mechanical epidermal disruption (mock s.s.). Primary T cell response was assessed on d 7 p.i. by the production of IFN-γ from splenocytes using *in vitro* restimulation assay (**a**). Serum VACV-specific IgG was assessed 6 weeks p.i. (**b**). (**c, d**) Mice were immunized via s.s. with live or heat-inactivated (Δ) VACV at the indicated doses. Primary T cell response (**c**) and antibody response (**d**) were measured at 7 d and 6 weeks p.i., respectively. Data are representative results from 2–3 independent experiments.

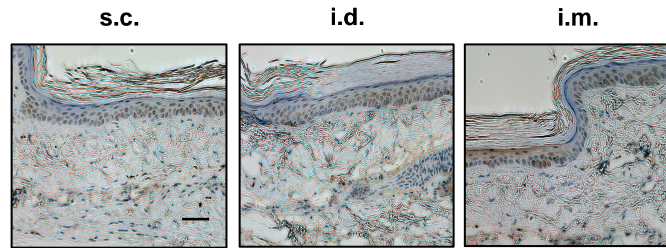


Supplementary Figure 2 VACV delivery via disrupted epidermis led to significantly higher level of viral gene expression compared to s.c. injection. C57BL/6 mice were immunized with 2×10^6 pfu VACV (EGFP-expressing) via s.s. or s.c. route. (a) The initial viral load measured at 30 min p.i.. Data represent the means ± SE of 8 individual mice in one experiment. (b) Early viral gene mRNA expression at the inoculation site measured by real-time RT-PCR at 8 h p.i.. Data represent the means ± SE of 6 individual mice in one experiment. (c) VACV-infected cells at the inoculation sited detected by IHC staining for EGFP at 3 d following immunization via s.s. route (upper panel), or s.c. route (lower panel). Scale bars represent 5 µm.

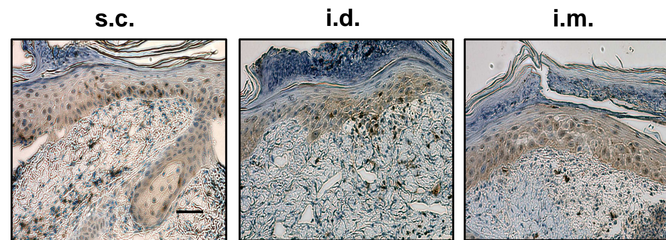


Supplementary Figure 3 VACV immunization via s.s. route generated significantly more robust recall memory response than i.p. immunization upon secondary cutaneous poxvirus challenge. C57BL/6 mice or μMT mice were immunized with 2×10^6 pfu VACV via s.s. route or i.p. injection, and challenged with secondary cutaneous VACV infection at 6 weeks p.i.. Recall T cell response in ILN (**a**) and spleen (**b**) were assessed by *in vitro* restimulation assay.

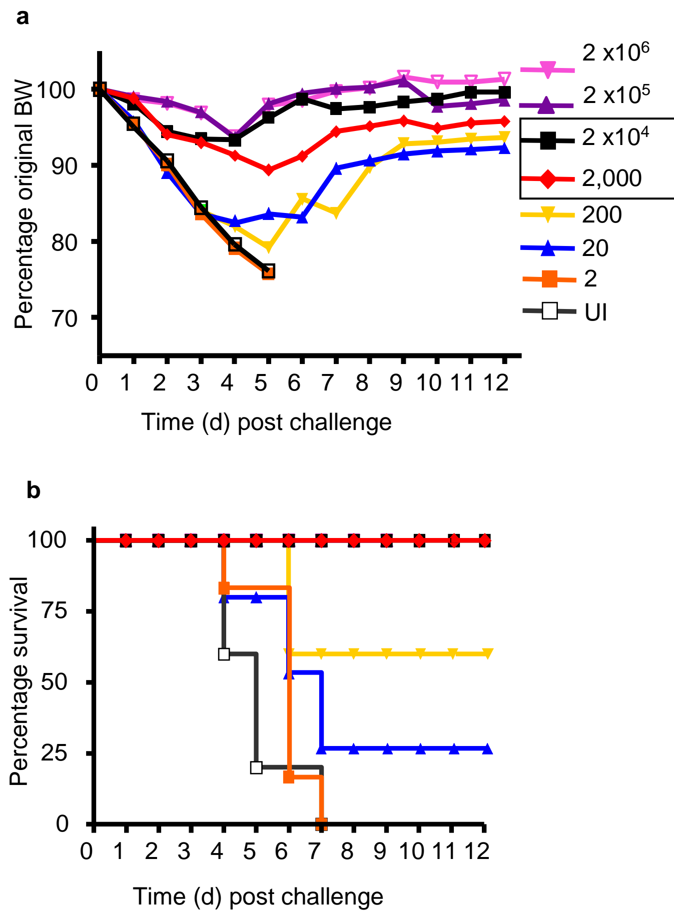
a Before challenge (14 weeks p.i.)



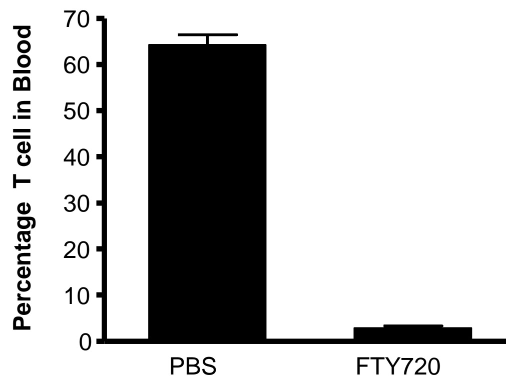
b 4 d after challenge



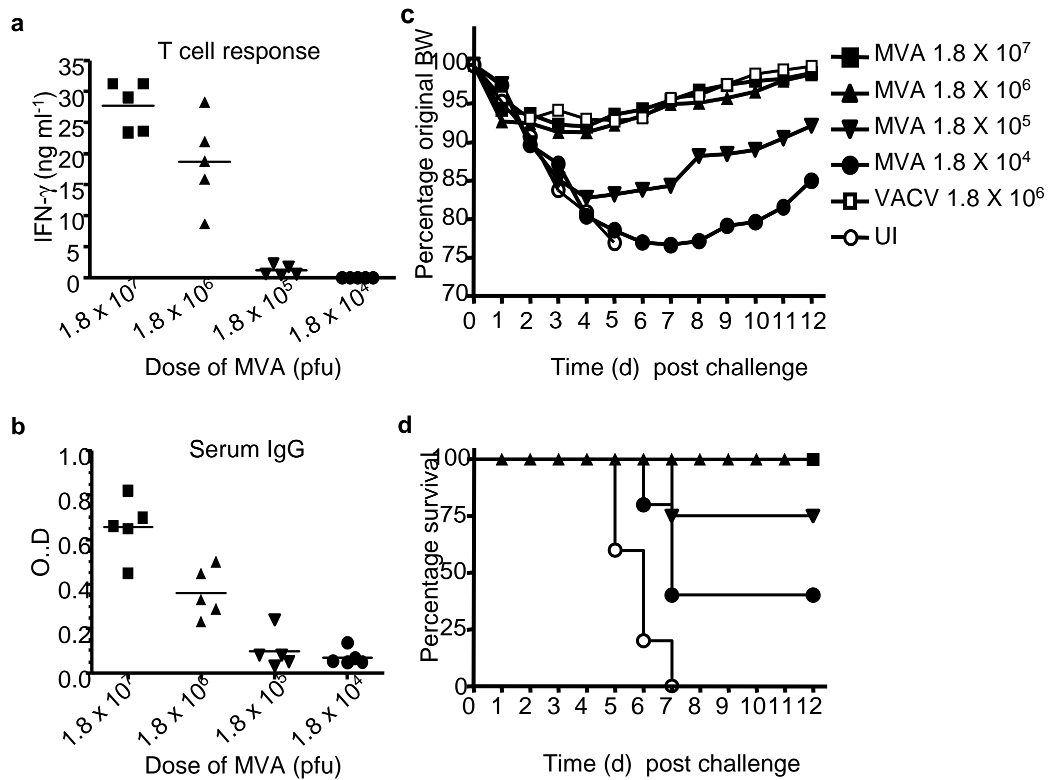
Supplementary Figure 4. VACV immunization via s.c., i.d., and i.m injection route failed to induce robust skin-infiltrating T_{EM}. C57BL/6 mice were immunized with 2 × 10^6 pfu VACV via the indicated injection routes and challenged cutaneously at 14 weeks post immunization. The presence of T_{EM} in skin before (**a**) and after (**b**) challenge was detected microscopically by IHC staining for $CD3^+$ cells. Photographs shown are the representative of 9 slides from 3 mice per group. Scale bar represents 5 μm.



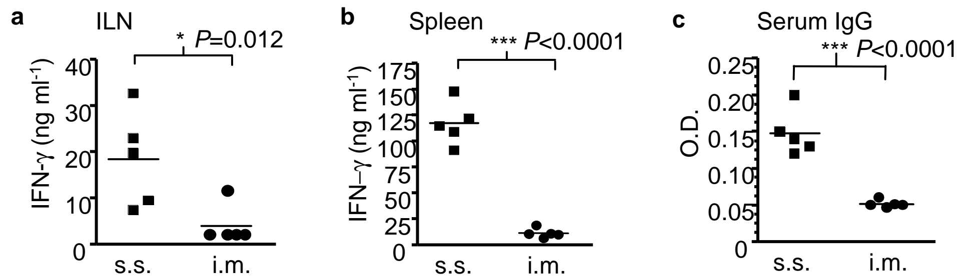
Supplementary Figure 5 Single-dose VACV immunization via s.s. route is highly efficacious to protect mice from lethal intranasal WR-VACV challenge. C57BL/6 mice were immunized with titrated doses of VACV via s.s. route and lethally challenged with WR-VACV intranasal infection at 6 weeks p.i.. Loss of BW (**a**) and survival (**b**) were monitored daily. *n*= 5 in each group. Data is representative of two independent experiments.



Supplementary Figure 6 FTY720 treatment led to blockage of lymphocyte egress from lymphoid tissues and development of lymphocytopenia. μMT mice were immunized with 2×10^6 pfu VACV via s.s. route and challenged intranasally with lethal dose WR-VACV at 16 weeks post immunization. Mice were treated daily with FTY720 or PBS (mock) starting from 2 d before the challenge. Percentage of T cells in peripheral blood was determined 2 h prior to the challenge to assess the blockage of T cell egress from lymphoid tissues. n = 10 in each group. Data is representative of 2 independent experiments.



Supplementary Figure 7 MVA immunization via s.s. route demonstrated dose-dependent immunogenicify and protection efficacy. C57BL/6 mice were immunized via s.s. route with the indicated does of MVA. (**a**) Primary VACV-specific T cell response was measured in ILN at day 7 p.i. by *in vitro* re-stimulation assay. (**b**) Serum vaccinia - specific IgG was measured at 6 weeks p.i. by ELISA. (**c,d**) Immunized mice were challenged with lethal dose of intranasal WR-VACV infection. BW (**c**) and survival (**d**) were monitored daily. VACV scarificed memory mice were included as controls. $n = 5$ in each group. Data is representative of two independent experiments.



Supplementary Figure 8 MVA immunization via s.s. route elicited stronger cellular and humoral immune response compared to the i.m. routes. C57BL/6 mice were immunized with 2×10^6 pfu MVA by the indicated routes. Primary T cell response was measured in spleen (**a**) and ILN (**b**) on day 7 p.i.. Serum VACV-specific IgG level was measured by ELISA 6weeks p.i. (**c**). $n = 5$ in each group. Data represent three independent experiments.

SUPPLEMENTARY METHODS:

Real-time RT-PCR to detect vaccinia mRNA expression. C57BL/6 mice were immunized with 2×10^6 pfu VACV at ~ 1 cm from the base of the tails via either s.s. or s.c.. Tail segments (1 cm long) were cut from the inoculation sites 8 h following immunization and immediately snap-frozen in liquid nitrogen. RNA extract and real-time RT-PCR were performed as previously described with minor modification[1]. The primers are specific for the ribonucleotide reductase Vvl4L of VACV. The sequences are: (forward) 5'-GAC ACT CTG GCA GCC GAA AT-3'; (reverse) 5'-CTG GCG GCT AGA ATG GCA TA-3'; Thermocycle conditions were 1 cycle of 95 ^oC for 2 min, followed by 45 cycles of amplification (95 ^oC for 15 s, 60 ^oC for 15 s, and 72 ^oC for 45 s), 1 cycle of 95 ^oC for 60 s, 1 cycle of 55 ^oC for 60 s, and 80 cycles of 55 ^oC for 10 s. The level of viral gene expression was normalized to that in the skin samples of mock (PBS)-immunized mice.

Detection of virus-encoded EGFP expression by IHC. Tail samples were harvested from the inoculation site 3 d after mice received 2×10^6 pfu VACV via s.s. or s.c. route. Tissues were preserved in 10% formalin, embedded in paraffin, sectioned (5µM) and stained with anti-EGFP at Brigham and Women's Hospital Histopathology Core as described previously with minor modification[1]: Briefly, slides were deparaffinized and pre-treated with 1mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) as per manufacturer's instructions followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with

Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity, followed by a 1:5 dilution of goat serum in 50 Mm Tris-Cl, pH 7.4, for 20 minutes to block non-specific binding sites. Primary murine anti-GFP antibody (Clone JL-8; Clontech, Palo Alto, CA) was applied at a 1:1000 dilution in DAKO diluent.. After washing in 50 mM Tris-Cl, 0.05% Tween 20, pH 7.4, anti-mouse horseradish peroxidase-conjugated antibody (Envision detection kit, DAKO) was applied for 30 min. Immunoperoxidase staining was developed using a diaminobenzidine (DAB)+ chromogen kit (DAKO) per the manufacturer and counterstained with hematoxylin.

Reference

1. Liu, L., Fuhlbrigge, R.C., Karibian, K., Tian, T. & Kupper, T.S. Dynamic programming of CD8+ T cell trafficking after live viral immunization. *Immunity* 25, 511-520 (2006).