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Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan

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Genital human papillomavirus (HPV) infection is the most common sexually transmitted infection, and virtually all cases of cervical cancer are attributable to infection by a subset of HPVs (reviewed in ref. 1). Despite the high incidence of HPV infection and the recent development of a prophylactic vaccine that confers protection against some HPV types[2], many features of HPV infection are poorly understood. It remains worthwhile to consider other interventions against genital HPVs, particularly those that target infections not prevented by the current vaccine. However, productive papillomavirus infection is species- and tissue-restricted, and traditional models use animal papillomaviruses that infect the skin or oral mucosa[3]. Here we report the development of a mouse model of cervicovaginal infection with HPV16 that recapitulates the establishment phase of papillomavirus infection. Transduction of a reporter gene by an HPV16 pseudovirus was characterized by histology and quantified by whole-organ, multispectral imaging. Disruption of the integrity of the stratified or columnar genital epithelium was required for infection, which occurred after deposition of the virus on the basement membrane underlying basal keratinocytes. A widely used vaginal spermicide, nonoxynol-9 (N-9), greatly increased susceptibility to infection. In contrast, carrageenan, a polysaccharide present in some vaginal lubricants, prevented infection even in the presence of N-9, suggesting that carrageenan might serve as an effective topical HPV microbicide.

The development of an assay for HPV genital tract infection was made possible by our recent discovery of efficient methods for the *in vitro* production of high-titer papillomavirus pseudoviruses[4,5]. Like authentic papillomavirus, the pseudovirus capsid is composed of the two viral structural proteins, L1 and L2. However, the pseudoviruses carry an encapsidated plasmid that encodes a reporter gene rather than the papillomavirus genome. Although some uncertainty remains, pseudoviruses are thought to faithfully recapitulate the initial phases of papillomavirus infection, which are not species restricted[4–10].

In models that use authentic papillomaviruses, experimental infection is carried out in conjunction with deliberate abrasion of the epithelium[11], and preliminary analysis indicated that the intact mouse genital epithelium was resistant to infection by the HPV16 pseudovirus. However, we found that gentle mechanical abrasion of the genital epithelium with a Cytobrush cell collector permitted detectable levels of pseudovirus infection. Systemic progesterone treatment potentiated infection (**Supplementary Fig. 1** online), with 4 d of pretreatment being optimal (data not shown). An initial screen of various reporter proteins (**Supplementary Table 1** online) revealed that DsRed-Express red fluorescent protein (RFP) gave the clearest results histologically (**Supplementary Fig. 2** online), whereas a tandem dimer of RFP, tdTomato[12], proved optimal for multispectral imaging. Consistent with *in vitro* infection[4], substantial RFP fluorescence was first detected 48 h after inoculation and did not peak until 72 h after inoculation (**Supplementary Fig. 3** online). These pilot experiments led to the development of a general protocol that included progesterone treatment for 4 d, abrasion of the genital tract immediately before exposure to the pseudovirus, and tissue analysis for reporter gene expression 3 d after inoculation[13]. Under these conditions, infection was restricted to keratinocytes, as confirmed by costaining for keratin 6 (ref. 14; **Supplementary Fig. 4** online). The data presented here are confined to HPV16 pseudoviruses. However, HPV31, HPV45 and bovine papillomavirus type 1 pseudoviruses behaved similarly in the model.

To generate a more quantitative assay for cervicovaginal infection, we developed a method for measuring the total reporter gene expression in whole tissue samples using a multispectral fluorescence imaging device. For these analyses, the entire vagina and cervix were assessed for reporter gene expression, which generated data on the distribution and intensity of infection and the mean intensity per pixel, thus allowing quantitative comparison between specimens (**Fig. 1**).

As with *in vitro* infection, genital tract infection by the HPV16 pseudovirus requires incorporation of the minor structural protein, L2, into the capsid along with the major structural protein, L1 (ref. 15; **Supplementary Fig. 5** online). As with *in vivo* infection by authentic virus, systemic immunization with HPV16 L1 virus-like particles (VLPs)[16] protected the mice against subsequent challenge with HPV16 pseudovirus[2,17,18] (**Supplementary Fig. 6** online). These results

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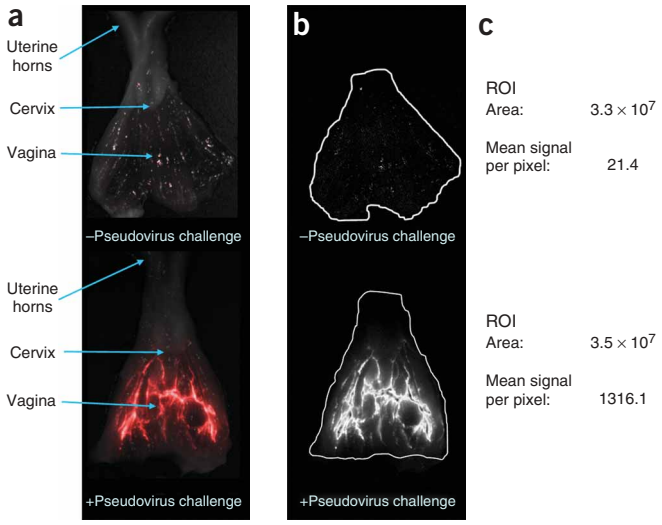


Figure 1 Quantitative analysis of murine reproductive tract infection. Conceptrol-treated mice were mock infected (top) or challenged with HPV-16-tdTomato pseudovirus (bottom). After 3 d, the entire reproductive tract was dissected out and the ventral wall of the vagina and cervix incised sagitally. (**a**) Composite Maestro image (mucosal epithelium facing up) with unmixing algorithm applied. Red signal represents location of infection compared to background autofluorescence. (**b**) Unmixed tdTomato signal converted to grayscale. Outline of tissue denotes ROI. (**c**) ImageJ analysis. Mean signal per pixel within the ROI was computed.

imply that *in vivo* transmission of the reporter gene occurs by capsid protein–mediated transduction, rather than by DNA transfection.

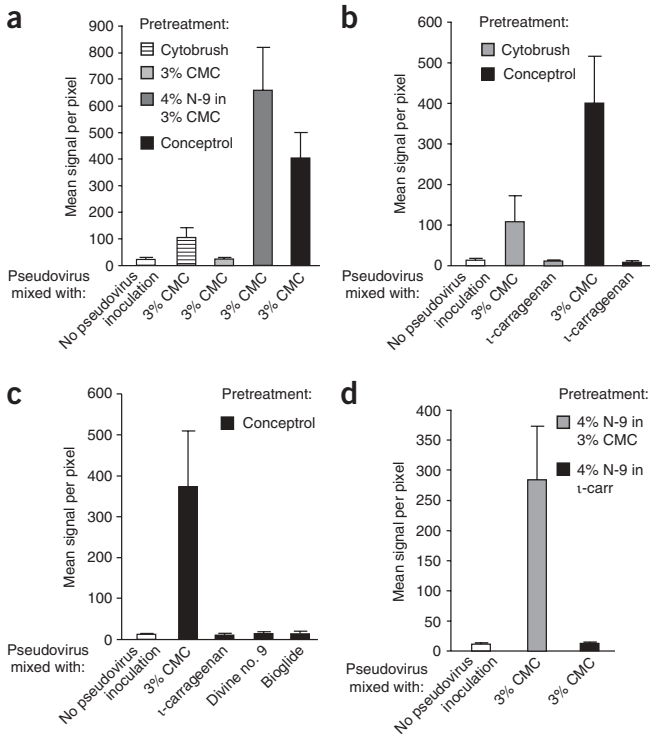
We also determined whether chemical disruption of the genital epithelium could promote infection. N-9 is a nonionic, membrane-active surfactant that is widely used as a spermicide and is known to disrupt the normal architecture of animal and human genital epithelium[19,20]. A formulation of 3% carboxymethylcellulose (CMC) designed to mimic the viscosity of a typical vaginal lubricant gel was made with or without 4% N-9. The gels were instilled in the vagina 6 h before the mice were inoculated intravaginally with pseudovirus. The mice pretreated with CMC alone were not detectably infected, whereas those pretreated with either Cytobrush or with CMC and N-9 were highly susceptible to infection ($P = 0.05$, 0.003, respectively) (**Fig. 2a**). Indeed, reporter signal intensity in the latter group was an average of fivefold stronger than infection-related signal induced by Cytobrush treatment ($P = 0.008$). Conceptrol, an over-the-counter, CMC-based spermicide that contains 4% N-9, also sensitized the genital tract to pseudovirus infection to a greater degree than did Cytobrush treatment ($P = 0.02$).

Our laboratory recently reported that a wide range of genital HPV types can be potently inhibited *in vitro* by carrageenan, an inexpensive polysaccharide whose gelling properties have led to its incorporation into some over-the-counter vaginal lubricants[9]. To test whether carrageenan can block infectivity *in vivo*, we challenged mice with HPV16 pseudovirus premixed 1:1 with either 1% ι-carrageenan or a 3% CMC preparation to control for the viscosity of the carrageenan preparation. Carrageenan prevented infection in the genital mucosa rendered susceptible to infection by either mechanical disruption (Cytobrush) or chemical disruption (N-9) (**Fig. 2b**). Two commercial carrageenan-containing lubricants (Divine No. 9 and BIOglide) that showed strong inhibitory activity in an *in vitro* pseudovirus assay[9]

Figure 2 Effects of mechanical disruption, N-9 and carrageenan on HPV16 pseudovirus infection of the mouse cervicovaginal mucosa. Multispectral imaging results (representative of two or three separate experiments), expressed as mean signal per pixel, for mice (six per group) are indicated on the *y* axis and gels used to prepare the pseudovirus inoculum are indicated on the *x* axis. Method of pretreatment is indicated by the key. Error bars, s.e.m. (**a**) Comparison of the potentiation of infection by mechanical and chemical disruption. (**b**) Protection provided by carrageenan when mixed with the inoculum. (**c**) Protection provided by over-the-counter lubricants when mixed with the inoculum. (**d**) Protection provided by carrageenan when mixed with N-9 during pretreatment.

similarly prevented detectable infection *in vivo* (**Fig. 2c**). To more closely mimic the conditions under which carrageenan might be used in common practice as a topical microbicide to prevent genital HPV transmission, N-9 in carrageenan or N-9 in control CMC gel was applied intravaginally 6 h before pseudovirus challenge. As expected, the CMC-based gel containing N-9 rendered the mucosa susceptible to significant HPV pseudovirus infection ($P = 0.03$), while the carrageenan-based gel prevented detectable infection (**Fig. 2d**). When each of the carrageenan conditions were compared to the negative controls, P values were >0.1.

To understand the mechanism underlying the increased susceptibility to pseudovirus infection induced by physical or chemical disruption, we tracked by confocal microscopy the location of virus particles in intact and disrupted stratified squamous or simple columnar epithelium of the cervicovaginal mucosa over the course of 3 d. After inoculation into the undisrupted vagina (**Fig. 3a**), viral capsids were bound to material in the vaginal vault but were not detected on or below the apical surface of the intact stratified squamous epithelium. Consistent with multispectral imaging results, cells did not become RFP positive at 72 h under these conditions. In Cytobrush-treated mice (**Fig. 3b**), broad areas of squamous epithelial ulceration were observed at early time points. These areas showed robust binding of viral capsids to exposed basement membrane, as confirmed by a coincident staining pattern of laminin-5 (ref. 21), a structural



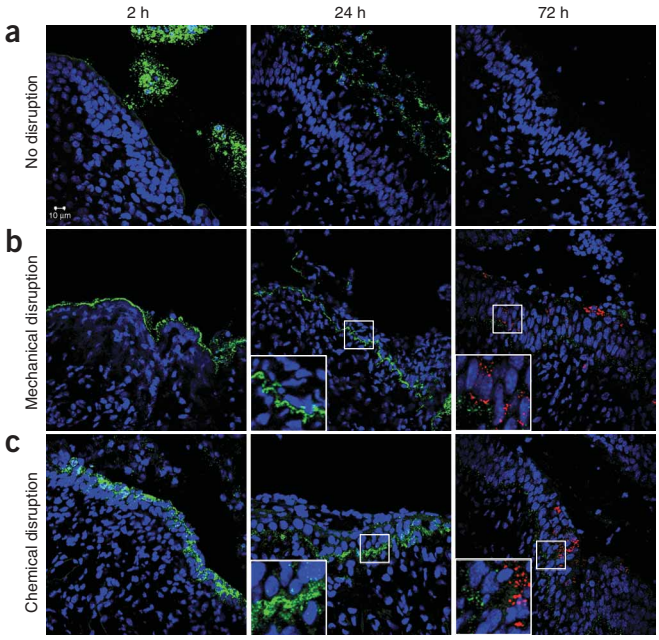


Figure 3 HPV16-RFP pseudovirus trafficking and infection in the vaginal stratified squamous epithelium as visualized in genital tissue sections by indirect immunofluorescence microscopy. Time after pseudovirus inoculation into the vagina is indicated at the top. Green signal indicates the physical presence of pseudovirus capsids and red signal indicates cells infected by pseudovirus. (**a**) Intravaginal inert vehicle (CMC) 6 h before inoculation. (**b**) Cytobrush disruption in conjunction with inoculation. (**c**) Intravaginal Conceptrol 6 h before inoculation.

component of the basement membrane (**Supplementary Fig. 7** online). In contrast, there was no detectable basement membrane binding in areas where the Cytobrush failed to disturb the epithelium. At early time points in N-9 treated mice (**Fig. 3c**), capsids were widely distributed along the basement membrane and on overlying keratinocytes that remained *in situ*, suggesting the capsids had traversed an intact, but compromised, epithelium. At 24 h after inoculation in both the Cytobrush- and N-9–treated mice, keratinocytes had begun to migrate back over denuded areas of basement membrane where capsid binding was still evident. As expected, RFP-positive cells were detected at 72 h in both the Cytobrush- and N-9–treated mice.

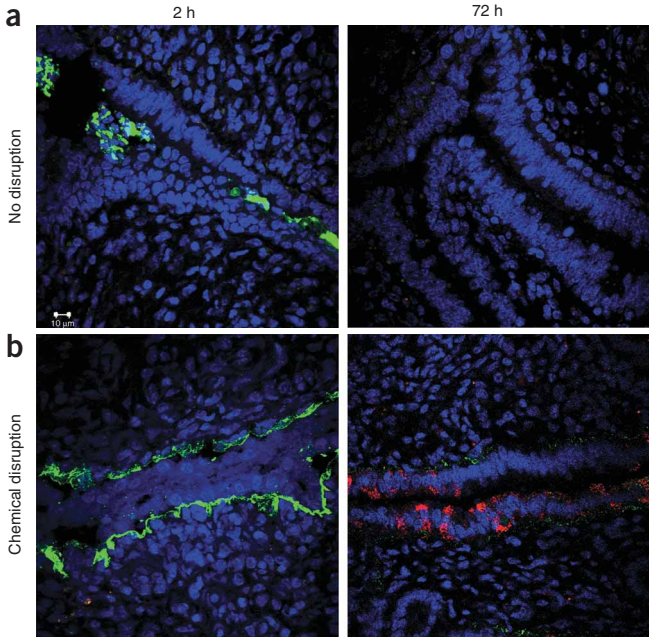
Similarly, after deposition of the inoculum directly into the undisrupted cervical canal, the capsids did not penetrate, or even bind to, the apical surface of the intact simple columnar epithelium adjacent to the transformation zone (**Fig. 4a**). By contrast, an endocervical pretreatment with N-9 led to extensive disruption and disorganization of the epithelium and widespread binding of capsids to the basement membrane (**Fig. 4b**). In the undisturbed endocervical mucosa at 72 h, virtually none of the pseudovirus inoculum remained detectable. In the N-9 treated group at 72 h, the epithelium had regenerated and reorganized so that it was largely indistinguishable from the undisrupted group. However, substantial capsid binding to the basement membrane remained visible, and there was intense and widespread infection of the columnar epithelium. The mice in this experiment were not pretreated with progesterone; pilot experiments demonstrated that pretreatment with either estrogen or progesterone did not influence the result (data not shown). The data in **Figures 3** and **4** were generated using indirect immunofluorescence and direct fluorescence imaging, respectively. Both experiments were repeated using the alternate method, and the same results were noted (data not shown).

Figure 4 Alexa Fluor 488–conjugated HPV16-RFP pseudovirus trafficking and infection in the columnar epithelium of the cervical transformation zone as visualized in genital tissue sections by direct fluorescence microscopy. Time after pseudovirus inoculation into the endocervical canal is indicated at the top. Green signal indicates the physical presence of pseudovirus capsids and red signal indicates cells infected by pseudovirus. (**a**) Endocervical inert vehicle (CMC) 6 h before inoculation. (**b**) Endocervical N-9 6 h before inoculation.

The remarkable inability of the HPV capsids to bind to or infect intact epithelia, even if only one layer thick, may have evolved to facilitate preferential infection of basal cells of a stratified squamous epithelium, the only tissue that supports productive infection. Initial binding to the basement membrane and then adsorption to the basal surface of the epithelial cells when they re-establish contact with the basement membrane during repair of the damaged epithelium might further promote preferential infection of basal keratinocytes. We observed marked sensitivity to N-9–mediated disruption and infection in the columnar epithelium adjacent to the transformation zone, where most HPV-induced cervical cancers arise. It would thus be desirable to extend these studies to primates, whose reproductive tract anatomy more closely resembles that of humans.

Overall, these results raised the possibility that use of over-the-counter N-9-containing vaginal contraceptives is a risk factor for genital HPV infection in women. N-9 has some inhibitory activity *in vitro* against enveloped viruses such as HIV and herpes simplex virus, but clinical studies have found that N-9 is either not protective or increases the risk of HIV infection[22]. Papillomaviruses are non-enveloped, and N-9 has been reported not to inhibit papillomavirus infection *in vitro*[23]. Thus, the ability of N-9 to disrupt the epithelium is not balanced by inhibitory activity against the papillomavirus capsid.

Carrageenan, whose regular use in the vaginal tract is well tolerated, is currently being studied as a candidate topical microbicide against HIV infection[24,25]. Our *in vitro* data indicate that it is active against papillomaviruses at concentrations that are about 1,000 times lower than those required to inhibit HIV[9], suggesting that a controlled clinical trial of carrageenan as an HPV topical microbicide is warranted. If a trial confirmed broad spectrum activity against genital



HPV types, carrageenan might become a useful adjunct to the current prophylactic HPV vaccines, which target a narrower spectrum of genital HPVs[2]. Because spermicidal activity of N-9 is retained in carrageenan-based gels[26], carrageenan might also be considered as a protective additive in over-the-counter vaginal contraceptives.

METHODS

Pseudovirus production. HPV16 pseudoviruses were produced according to the standard production protocol published on our laboratory website (http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm). A nucleotide map of the HPV16 packaging plasmid, p16sheLL, can be found at http://home.ccr.cancer.gov/lco/packaging.htm. Nucleotide maps of the reporter plasmids used for DsRed-Express (Clontech) red fluorescent protein (RFP) (plasmid p8RwB) and tdTomato (plasmid ptwB) are available at http://home.ccr.cancer.gov/lco/target.htm. Additional information about the performance of different reporter plasmids in this challenge model can be found in **Supplementary Table 1**. Pseudovirus titer, which represents a quantum measure of the number of capsids capable of infectious entry, was determined by reporter gene expression in 293TT cells as detailed in Note 10 of the standard production protocol, and titer is referred to in terms of infectious units per milliliter (IU/ml).

Coupling of Alexa Fluor 488 dye to HPV16-RFP pseudovirions was performed according the manufacturer's instructions for protein labeling (A10235, Molecular Probes). The dye-coupled capsids were purified by gel filtration over a column of 2% 50- to 150-μm agarose beads (Agarose Bead Technologies). Retitering of the dye-conjugated pseudovirion preparation confirmed that its infectivity remained comparable to that of nonlabeled pseudovirus.

Reagents. CMC and ι-carrageenan (Sigma-Aldrich) preparations were produced by dissolving the powdered form in deionized water. The N-9 (US Pharmacopeia) preparations were made by forcefully mixing the N-9 stock (40% in distilled H_2O) 1:10 with the CMC and ι-carrageenan preparations using a positive-displacement pipette. Conceptrol (McNeil), Divine No. 9 (Divine Corporation) and BIOglide (Joydivision International AG) were not modified before being used in the challenge model as described.

Mouse model and HPV pseudovirus challenge. Six- to eight-week-old female BALB/cAnNCr mice were obtained from the National Institutes of Health and housed and handled in accordance with their guidelines. Experimental protocols were approved by the National Cancer Institute's Animal Care and Use Committee. Unless otherwise noted, all mice received 3 mg of Depo-Provera (Pfizer) diluted in 100 μl of sterile PBS in a subcutaneous injection 4 d before pseudovirus challenge.

For vaginal challenge, mice designated for N-9 pretreatment received 50 μl of the N-9 containing compound intravaginally 6 h before intravaginal inoculation with pseudovirus. The material was delivered with an M50 positive-displacement pipette (Gilson), and standard dissecting forceps were used to occlude the vaginal introitus to achieve maximal retention of the material. Mice designated for mechanical disruption underwent a procedure in conjunction with pseudovirus inoculation in which a Cytobrush cell collector (Cooper-Surgical) was inserted in the vagina and twirled clockwise and counterclockwise 10 times. The pseudovirus inoculum was a 20-μl dose composed of 5 μl of purified pseudovirus with a titer of $\sim 5 \times 10^9$ IU/ml mixed with 15 μl of a 3% CMC preparation, with the exceptions of experiments shown in **Figure 2b,c**, in which 5 μl of inoculum was mixed with 5 μl of the indicated preparation, and in **Figure 3**, in which 15 μl of inoculum was mixed with 5 μl of 4% CMC. In the N-9–pretreated mice, this dose was delivered as a one-time, atraumatic, intravaginal inoculation using an M20 positive-displacement pipette. In the Cytobrush-treated mice, the inoculum was delivered in two doses, 10 μl before and 10 μl after Cytobrush treatment, using an M20 positive-displacement pipette. Unless otherwise indicated, the reproductive tract was harvested on day 3 post-challenge after the mice were euthanized by CO_2 inhalation. For endocervical challenge, the endocervical canal was pretreated by direct instillation of 15 μl of 1% CMC or 15 μl of 1% CMC with 4% N-9. Six hours after pretreatment, 7 μl ($\sim 1.4 \times 10^7$ IU) of pseudovirus mixed with 7 μl of 1% CMC was also deposited directly into the endocervical canal. See ref. 13 for a detailed protocol describing how to accomplish vaginal and endocervical challenge.

Histological analysis. The reproductive tract tissue samples were embedded and frozen in OCT freezing media (Electron Microscopy Sciences). A cryotome was used to cut 5–7-μm-thick sections, which were collected on slides and fixed in a 2% paraformaldehyde/PBS solution for ~20 min before being transferred to a solution of PBS with 200 μM glycine and 0.1% NaN_3. The slides were then washed in PBS and mounted with a DAPI-containing mounting medium (Prolong Gold with DAPI, Molecular Probes) to visualize nuclei in blue. Microscopy was performed on a Zeiss LSM 510 confocal system interfaced with a Zeiss Axiovert 100M microscope. The images were collated in Adobe Photoshop, and color levels were adjusted uniformly across experiments before conversion to TIFF format.

Histological analysis of HPV pseudovirus capsid trafficking in tissues was accomplished by either of two methods. For indirect immunofluorescence imaging, tissue sections from HPV16-RFP pseudovirus–challenged mice were fixed in 2% paraformaldehyde/PBS, then incubated for 45 min at 37 °C with a rabbit polyclonal serum against HPV16L1/L2 VLPs at 1:1,000 in PBS containing 0.1% Brij58, followed by 30 min at 37 °C with an Alexa Fluor 488–conjugated donkey anti-rabbit secondary antibody (Molecular Probes) at 1:1,000. For direct fluorescence imaging, tissue sections from Alexa Fluor 488–conjugated HPV16-RFP pseudovirus–treated mice were simply fixed and mounted as described in the preceding paragraph.

Multispectral fluorescence imaging and statistical analysis. The reproductive tract of each mouse, from the external genitalia to the lower half of the uterine horns, was excised and stored in PBS on ice for <6 h before imaging. A Maestro (CRi, Woburn, MA) imaging device with a green excitation filter and a 580-nm long-pass emission filter was used to obtain images from 550 nm to 900 nm in 10-nm wavelength increments. Using the spectral signature of RFP in infected tissues as signal and the background autofluorescence in uninfected tissues as noise, a spectral unmixing algorithm was applied to the composite images to determine the intensity and location of infection. The open-source software Image J (http://rsb.info.nih.gov/ij/) was used to calculate the mean signal per pixel in a region of interest (ROI) in the grayscale representation of unmixed signal. The mean of the numbers thus generated represents the result of each particular experimental condition. In some cases, to determine whether the difference between these means was statistically significant, an unpaired Student's *t*-test was performed and the results reported in the text as a *P* value.

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

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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