Large intestine–targeted, nanoparticle-releasing oral vaccine to control genitoreal viral infection

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Both rectal and vaginal mucosal surfaces serve as transmission routes for pathogenic microorganisms. Vaccination through large intestinal mucosa, previously proven protective for both of these mucosal sites in animal studies, can be achieved successfully by direct intracolorectal (i.c.r.) administration, but this route is clinically impractical. Oral vaccine delivery seems preferable but runs the risk of the vaccine’s destruction in the upper gastrointestinal tract. Therefore, we designed a large intestine–targeted oral delivery with pH-dependent microparticles containing vaccine nanoparticles, which induced colorectal immunity in mice comparably to colorectal vaccination and protected against rectal and vaginal viral challenge. Conversely, vaccine targeted to the small intestine induced only small intestinal immunity and provided no rectal or vaginal protection, demonstrating functional compartmentalization within the gut mucosal immune system. Therefore, using this oral vaccine delivery system to target the large intestine, but not the small intestine, may represent a feasible new strategy for immune protection of rectal and vaginal mucosa.

Mucosal immunization has proven to be crucial for inducing mucosal protection1–5 and contributes to rapid and long-lasting mucosal protection, in contrast to systemic immunization6. It has been shown that antigen–specific functional CD8+ cytotoxic T cells in the mucosa are essential for protection of rhesus macaques from CD4+ T cell depletion by simian-human immunodeficiency virus3, whereas human studies indicate that a higher frequency of the antigen–specific mucosal CD8+ T cells correlates with a lower degree of herpes simplex viral infectivity as well as reduced severity of the disease7. In the mucosal tissues of HIV-infected long-term nonprogressors, there exist immunodominant CD8+ T cells, and their presence is strongly correlated with HIV-1 control8,9. A variety of approaches have been proposed and employed to induce protective mucosal immunity against viral transmission through either the rectal or vaginal route1–3,9–11. However, a potent but clinically practical genitoreal vaccination strategy remains unestablished for several reasons. The large intestinal mucosa is an optimal site to induce both rectal and vaginal immunity. I.c.r. vaccination directly at the large intestinal mucosa induces robust cellular and humoral immune responses in the regional lymph nodes4 more effectively than vaccination at a distant mucosa (for example, intranasal) or by a parenteral route1–5. However, for mass human vaccination, i.c.r. administration seems to be clinically too cumbersome and unpalatable. In addition, this procedure, although generally low risk, has the potential to be physically traumatic. The intranasal route, although practical and relatively easy, poses the risk of inoculum invasion into the central nervous system by olfactory nerve transport12. The oral route is the safest and most practical; however, except for a few live attenuated vaccines inducing systemic responses, simple oral delivery is ineffective at protecting either rectal or genital mucosa13. The failure is mostly attributed to pH-dependent or enzymatic destruction of the vaccine in the proximal gut and, probably, inadequate antigen uptake in the large intestine.

We aimed here to discover a way to selectively deliver a vaccine to the large intestinal mucosa through the oral route, which to our knowledge has not previously been accomplished. To mimic the ‘gold standard’ i.c.r. immunization while circumventing the limitations of oral delivery, we encapsulated a peptide or protein vaccine into biologically compatible poly(ε-lactic-co-glycolic acid) (PLGA) nanoparticles14,15 to be used for site-specific immunization. The depot effect of PLGA nanoparticles offers an additional feature in that its controlled release of entrapped vaccines over extended time periods provides a longer antigen exposure to the immune system. PLGA particle size, adjustable during manufacturing, was engineered in nanometers because size-dependent mucosal uptake is most effective within nanometer ranges and impeded when the size is over 1 μm (ref. 16). Selective combinations of Toll-like receptor (TLR)
ligands can induce synergistic activation of T cells\textsuperscript{17-19}. As vaccine adjuvants, we used macrophage-activating lipoprotein (MALP-2) for TLR2, polyinosine-polycytidylic acid (poly(I:C)) for TLR3 and CpG oligodeoxynucleotides (ODN) for TLR9, which have been shown to synergistically induce mucosal antiviral protection after i.c.r. immunization\textsuperscript{20}. To bypass the denaturing effects of digestive low pH and enzymatic destruction and to selectively deliver the particles to the lower gastrointestinal tract intact, we coated the PLGA nanoparticle surface with methacrylate-based polymer Eudragit FS30D\textsuperscript{21}, an anionic tripolymer comprising poly(methyl acrylate, methyl methacrylate, methacrylic acid) in a 7:3:1 ratio. The ratio of free carboxyl groups to m particles, that released a substantial amount of phages and secondarily in CD11c\textsuperscript{+}CD11b\textsuperscript{+} dendritic cells (DCs) in the lamina propria of the large intestine (Supplementary Fig. 4a). Uptake of PLGA nanoparticles by DCs (derived from bone marrow) was also confirmed \textit{in vitro} by flow cytometry (Supplementary Fig. 4a), fluorescence microscopy (Supplementary Fig. 4b) and transmission electron microscopy (Supplementary Fig. 4c).

We subsequently confirmed that antigen-specific T cell responses could be induced in the colon after a single i.c.r. delivery (Fig. 1b), but not after subcutaneous administration (Supplementary Fig. 5). Of PLGA nanoparticles encapsulating PCLUS3-18II1B (a CD4\textsuperscript{+} T cell helper epitope fused with an HIV Env CD8\textsuperscript{+} cytotoxic T lymphocyte epitope) and TLR ligands (MALP2 plus poly(I:C) plus CpG)\textsuperscript{22}. Therefore, PLGA nanoparticles can serve as an effective vaccine delivery system when they are deposited in the large intestinal lumen.

**RESULTS**

**Proof-of-principle study on the PLGA nanoparticle vaccine**

Uncoated PLGA nanoparticles were manufactured in the range of 300–500 nm (418±88 nm) (mean±s.d.) in diameter (Supplementary Fig. 2a), with 90% encapsulation efficacy (Supplementary Fig. 2b). We first conducted a proof-of-principle experiment in which fluorescein-conjugated BSA formulated in PLGA (PLGA/FITC-BSA) nanoparticles were delivered directly to the colon of mice by i.c.r. administration. At day 3, a proportion of the cells isolated from the large intestinal lamina propria were detected positive for fluorescence (Fig. 1a), indicating an uptake of the nanoparticles. Nanoparticle uptake was primarily found in CD11b\textsuperscript{+}B220\textsuperscript{+} macrophages and secondarily in CD11c\textsuperscript{+}CD11b\textsuperscript{+} dendritic cells (DCs) in the lamina propria of the large intestine (Supplementary Fig. 3a). Transmission electron microscopy showed PLGA nanoparticles in the cytoplasm (Supplementary Fig. 3b). Uptake of PLGA nanoparticles by DCs (derived from bone marrow) was also confirmed \textit{in vitro} by flow cytometry (Supplementary Fig. 4a), fluorescence microscopy (Supplementary Fig. 4b) and transmission electron microscopy (Supplementary Fig. 4c).

Oral delivery of nanoparticle-releasing microparticle vaccines The Eudragit FS30D polymer served to make 10- to 50-µm particles, too large to be phagocytosed, that released a substantial amount of their contents as quickly as 1 h at pH 7.4, in contrast to at pH 2.5, at which the particles were stable (Supplementary Fig. 6). After oral delivery of Eudragit FS30D containing PLGA/FITC-BSA nanoparticles, nanoparticle uptake occurred almost exclusively in the large intestine. Small intestine Large intestine

**Figure 1** i.c.r.-delivered nanoparticles enter the large intestinal mucosa, and orally delivered PLGA nanoparticle-releasing FS30D microparticles selectively target the large intestinal mucosa for uptake. (a) Colorectal mucosal uptake of PLGA nanoparticles after i.c.r. delivery of PLGA nanoparticles containing FITC-BSA (PLGA/FITC-BSA). Cells were isolated from the colorectum 3 d after administration and measured for fluorescence-positive cells. P < 0.01 between PLGA/FITC-BSA treated and PBS treated. Representative of three independent experiments, n = 12–15 per group. (b) Induction of antigen-specific colorectal mucosal T cells after i.c.r. delivery of PLGA nanoparticles encapsulating PCLUS3-18II1B and MALP2 plus poly(I:C) plus CpG vaccine (PLGA/PeptAg+TLRL). Three weeks after one immunization, colorectal cells were isolated and measured for P18-I10 specific CD8\textsuperscript{+} T cells by tetramer staining. P < 0.01 between PLGA/PeptAg+TLRL and PLGA alone. Representative of two independent experiments. n = 10 per group. (c) Gut mucosal uptake of PLGA particles after oral delivery of FS30D/PLGA or L100-55/PLGA. Cells were isolated from the small and large intestine at day 2 for measurement of fluorescence-positive cells. **P < 0.02 indicates the difference from small intestine. ***P < 0.001 the difference from the small intestine. n = 9–12 per group. (d) Representative examples of flow cytometry from experiments shown in c. i.r.c., intracolorectal; s.c., subcutaneous; p.o., per os (oral). Numbers in quadrants (a,b,d) indicate the percentage of cells in the marked gate. Error bars are s.e.m.
intestine (Fig. 1c,d). The cecum is the first part of the large intestine encountered by the particles, but in humans, where the cecum is relatively small, the starting point of nanoparticle release and selective uptake in the large intestine may differ somewhat. We further contrasted Eudragit FS30D with another Eudragit formulation, L100-55, that starts to dissolve at pH > 5.5, allowing early release. We found that oral administration of L100-55-coated nanoparticles resulted in primary uptake in the small intestine (Fig. 1c,d). Likewise, uncoated PLGA nanoparticles, to the extent that any successfully traversed the stomach, were primarily delivered to the small intestine and not the colon (Supplementary Fig. 7). Thus, the distribution of nanoparticles was altered by coating them with FS30D, such that a majority reached and were taken up by cells in the large intestine (Fig. 1c,d and Supplementary Fig. 7). These results validate the approach of coating PLGA nanoparticles with FS30D to protect them during transit through the upper gastrointestinal tract for improved delivery to the large intestine.

We next used a luciferase reporter system to confirm the large intestine–targeted delivery with FS30D. We detected luciferase expression specifically in large intestinal sections after oral administration of FS30D-coated PLGA nanoparticles containing a luciferase DNA plasmid (Supplementary Fig. 8).

After confirming that formulated vaccine components within the nanoparticle-releasing microparticles retained TLR agonist activity in in vitro settings or after oral administration (Fig. 2a), we examined intestinal immune responses in mice after two immunizations with the two different Eudragit coatings of the PLGA nanoparticle vaccine given orally with a 2-week interval. Three weeks after the second immunization with the oral FS30D-coated vaccine, we detected tetramer-positive CD8+ T cells in the large intestine, indicating successful induction of colorectal immunity (Fig. 2b,c). In contrast, the L100-55–coated vaccine induced a minimal number of colonic antigen-specific CD8+ T cells (Fig. 2b,c). In fact, the L100-55–coated vaccine induced a T cell response primarily in the small intestine, where the FS30D-coated vaccine was only marginally effective (Fig. 2d). Without Eudragit coating, PLGA nanoparticle vaccines did not elicit significant immune responses in the large intestine but did induce responses in the small intestine (Fig. 2e,f). These results

**Figure 2** Orally delivered, FS30D-coated PLGA nanoparticle vaccine induces antigen-specific T cells in the large intestine, whereas L100-55–coated vaccine induces T cells in the small intestine. (a) Activation of DCs after a 20-h incubation with supernatants from FS30D-coated PLGA containing PCLUS3-1BI1B plus MALP2, poly(I:C) and CpG (FS30D/PLGA/PeptAg+TLRL), antigen (FS30D/PLGA/PeptAg) or vaccine only (PeptAg+TLRL) dissolved in PBS at pH 7.4 for 16 h (top graphs). Intracellular interleukin-12 (IL-12) was measured by flow cytometry (*n* = 6 per group). The nanoparticle-releasing microparticles were also given orally, and DC activation *in vivo* was assessed *ex vivo* (bottom graphs), *n* = 5 per group. MHC, major histocompatibility complex. (b–d) Induction of T cell responses after oral delivery of FS30D/PLGA/PeptAg+TLRL or L100-55/PLGA/PeptAg+TLRL. Oral administration was conducted twice with a 2-week interval. Tetramer-positive cells in the colorectum (b and c) or upper part of the small intestine (d) were measured 3 weeks later. The i.c.r. group was immunized with vaccine only without nanoparticles but formulated in DOTAP. **P < 0.01, ***P < 0.001 indicate the significant difference between the group with asterisks and each of the groups without asterisks. There are no differences between the two groups with asterisks. A representative experiment on tetramer results in the colorectum is shown in b, and all such experiments are summarized with statistics (*n* = 8–12 per group) in c. In d, *P* < 0.001 for L100-55 versus other groups. *n* = 8–12 per group. (e,f) T cell responses induced after oral delivery of uncoated or FS30D-coated PLGA/PeptAg+TLRL. Tetramer-positive cells in the upper small intestine (e) and the colorectum (f) were measured. **P < 0.01 indicates a significant difference between groups (*n* = 7 per group). Numbers in quadrants (a,b,d–f) indicate the percentage of cells in the marked gate. Error bars are s.e.m.
**Figure 3** Orally delivered FS30D-coated PLGA nanoparticle peptide vaccine confers T cell–mediated resistance to virus infection at the rectal or vaginal mucosa. (a, b) Viral load in ovaries on a log scale after FS30D/PLGA/PeptAg+TLRL or L100-55/PLGA/PeptAg+TLRL was given orally to mice twice with a 2-week interval, followed by i.c.r. (a) or intravaginal (b) challenge with $2 \times 10^7$ or $1 \times 10^7$ PFU of vPE16, respectively, 3 weeks after the last immunization. Ovaries (where this virus primarily replicates) were removed at day 6 for viral titer assessment. **P < 0.01, ***P < 0.001 indicate the significant difference in viral titer between the group with asterisks and each of the groups without asterisks (*n = 12–15 per group). PFU, plaque-forming units. Error bars are s.e.m.

affirm that the enteric coating with FS30D is essential for oral delivery of PLGA nanoparticles to the large intestine.

**Induction of T cell immunity against viral infection**

To evaluate the protective efficacy of this vaccine, after prime and boost oral immunization we challenged mice rectally with a replication-competent vaccinia virus, vPE-16, which expresses the HIV Env protein containing the epitope P18-I10 used in the peptide vaccine. Mice immunized with the L100-55–coated vaccine were not protected from the virus challenge. However, the FS30D-coated vaccine reduced viral load almost equally as well as the peptide vaccine given i.c.r. (Fig. 3a). Therefore, oral delivery of FS30D-coated PLGA vaccine targeting the large intestine is effective for local colorectal vaccination against viral infection.

It has been previously shown that i.c.r. vaccination with adenovirus-based vaccines induces effective protection against virus challenge (vaccinia and herpes simplex virus-2) not only in the rectal but also in the vaginal mucosa. To determine whether vaginal mucosal protection can be induced by the orally administered FS30D-coated PLGA vaccine, we immunized mice orally with the vaccine (FS30D-coated PLGA containing PCLUS3-181HB plus TLRL ligands) twice with a 2-week interval, followed by intravaginal challenge with vPE-16. Compared to i.c.r. immunization, oral delivery of FS30D-coated vaccine induced almost equal clearance of virus after vaginal challenge (Fig. 3b), whereas, again, the L100-55 formulation that delivers the vaccine to the small intestine was not effective. Thus, delivery to the large intestine was more effective than to the small intestine in protecting against both genital and rectal challenge. This efficacy was largely T-cell mediated, as the virus does not incorporate the HIV-1 Env protein gp160 into virions and is not sensitive to gp160 neutralization.

**Induction of antibody immunity against viral infection**

The humoral response also has a key role in protection in the gut and in genital mucosal immunity. Therefore, we examined whether encapsulating whole viral proteins in the FS30D-coated PLGA vaccine can induce antibody-mediated protective immunity at both mucosal sites after challenging mice with the pathogenic vaccinia strain WR. Vaccinia A33 and L1 are immunogens of the extracellular enveloped virion and intracellular mature virion, respectively. Antibody responses induced by the combination of both types of viral antigens encoded by plasmid DNA or as recombinant proteins can protect animals from lethal challenge by WR. Of note, cytotoxic T lymphocytes specific for the vaccinia protein A33 or L1 have not been reported in BALB/c mice. TLR ligands have been shown to activate B cells directly and contribute to antigen-specific antibody response, including in the gut. Here we found that the triple combination of MALP-2, poly(I:C) and CpG ODN TLR ligands previously shown to synergistically activate naive T cells could also synergistically activate B cells (Supplementary Fig. 9a), as determined by CD69 upregulation. i.c.r. immunization with the combination of recombinant A33 and L1 mixed with the triple combination of MALP-2, poly(I:C) and CpG ODN TLR ligands in DOTAP transfection reagent induced antibody responses in the blood (Supplementary Fig. 9b).

The ability of PLGA nanoparticles containing the above vaccine components to induce antibody responses was assessed by immunization through either the i.c.r. or subcutaneous routes (Supplementary Fig. 10a), and the results further showed that CD4+ T cells were also activated by such a vaccine regimen (Supplementary Fig. 10b). We therefore constructed vaccine microparticles of FS30D-coated PLGA nanoparticles containing A33 protein plus L1 protein plus MALP-2,

![Image](https://example.com/image.png)

**Figure 4** Orally delivered FS30D-coated PLGA nanoparticle peptide vaccine confers antibody-mediated resistance to virus infection in the rectal or vaginal tract. FS30D-coated PLGA containing antigen proteins A33, and L1 and TLR ligands (ProtAg+TLRL) was administered orally with a 2-week interval. (a) Local IgA (top) and IgG (bottom) antibodies against both A33 and L1 (together) measured at 3 weeks after the last immunization. Both FS30D/PLGA/ProtAg+TLRL p.o. and ProtAg+TLRL i.c.r. groups have significantly higher antibody titers than the other groups (P < 0.02). **P < 0.01 and ***P < 0.001 indicate difference (for both sites) from each of the bars without asterisks. Results from two independent experiments (n = 10 per group). (b) Disease course of the mice assessed by weight loss after challenge with WR by the i.c.r. (4 x 10^7 PFU) or intravaginal (1 x 10^7 PFU) route 3 weeks after the last immunization. **P < 0.02, ***P < 0.001 indicate the differences between the FS30D/PLGA/ProtAg+TLRL and unimmunized groups in weight loss (n = 10–14 per group). $, 75% mortality; †, 50% mortality. Error bars are s.e.m.
poly(I:C) and CpG ODN TLR ligands and administered them orally. Vaccinia-specific IgA and IgG antibody responses were significantly induced in both the large intestine (tested in tissue homogenates) and vaginal tracts (tested in vaginal washes) (Fig. 4a). Further, orally immunized mice resisted the WR virus challenge by either the rectal or vaginal route (Fig. 4b), as determined by weight loss and by 0% mortality for immunized mice challenged by either route compared to 75% mortality for i.c.r. challenge without preimmunization and 50% mortality for intravaginal challenge without preimmunization). Therefore, the FS30D-coated PLGA vaccine system can also be used to induce antibody-mediated mucosal protection at both mucosal transmission sites.

DISCUSSION

As direct rectal or vaginal vaccine delivery may be impractical for mass human vaccination, despite the efficacy of the i.c.r. route, oral vaccination is a desirable alternative, potentially allowing for safe, simple and rapid delivery. We hypothesized that if the vaccine is able to survive the passage to and is not absorbed before reaching the large intestine, the vaccination should mimic the efficacy of i.c.r. vaccination. Accordingly, we designed a two-part PLGA nanoparticle–releasing FS30D microparticle system and showed that this new system can bypass the small intestine and deliver orally administered vaccines highly specifically into the large intestinal mucosa and induce almost equal protective immunity in not only the rectal but also the vaginal mucosa.

Uncoated PLGA particles given orally either failed to traverse the stomach or reached only the small intestine and did not elicit significant colonic responses, only small intestinal immune responses. These results underline the need for combinatorial use of Eudragit FS30D and PLGA to ensure specific targeting of an orally delivered vaccine to the large intestine. Our study also indicates that activities of MALP-2, poly(I:C), and CpG ODN TLR ligands were maintained after encapsulation within PLGA; thus, PLGA is an ideal delivery vehicle for vaccines containing TLR agonists.

Further, we designed a contrasting coated nanoparticle (L100–55–coated PLGA nanoparticle) vaccine that delivered antigen to and induced T cell responses in the small intestine selectively, providing a way to compare these small- and large-intestinal mucosal sites for the first time with the same vaccine. Thus, we have made a fundamental discovery of cellular immune compartmentalization within the gut mucosal immune system, which is reminiscent of the subcompartmentalized gut humoral immunity, highlighting the need for site-specific delivery of vaccines to the large intestinal mucosa for protection against infections transmitted by the rectal or vaginal route, and we have demonstrated a new way to accomplish this via the more practical oral route. Our findings thus strengthen the conception that the common mucosal immune system is somehow subcompartmentalized.

There are many potential applications of our delivery technology beyond vaccination against viral infection. This strategy is applicable for many forms of vaccines such as DNA, recombinant proteins, peptides and a wide variety of adjuvants, and it may be adapted in the development of vaccination strategies combating certain sexually transmitted infections caused by not only viruses but possibly also other types of pathogens. It also suggests a new approach to the development of a preventive or therapeutic vaccination against mucosal malignancies such as colorectal as well as cervical cancer. With this technology, in-depth study of the mucosal immunological subcompartmentalization between the large and small intestines can be easily conducted. For example, aside from vaccination, one could use this approach to localize the site of induction of oral tolerance. The technology may be incorporated with other technologies to devise therapies in which selective targeting within the gut mucosa is needed.

Our targeted nanoparticle-releasing microparticle system lends itself to practical large-scale clinical applications because of its great stability in a dry-powder formulation, easy shipment and storage without refrigeration and long shelf life, and economical large-batch good manufacturing process processing. These features would be highly desired for effective industrial manufacturing and clinical management. PLGA is a component in several US Food and Drug Administration–approved products. Although fine tuning of the formulation may be necessary to account for longer transit times in the gut of nonhuman primates and humans, as well as slightly higher pH values (around 7.3) in the distal small intestine of humans, this study is a promising proof of concept that this type of formulation may be practical and effective for oral vaccination to induce lower gastrointestinal immunity without the need for intrarectal delivery. Capsules either coated with or composed of Eudragit have been investigated in humans for site-targeted release in the distal gastrointestinal tract. Clinical approaches can be extended to consider packaging PLGA nanoparticles within these colon-specific capsules, which may represent a preferable carrier for targeted colon vaccination in humans.

In conclusion, we have demonstrated functional immune compartmentalization of the gut mucosa and developed a nanoparticle–releasing microparticle oral delivery system and demonstrated that it is an easy, noninvasive vaccination strategy effective against viral infection occurring through the rectal or vaginal mucosa. Such a vaccination strategy would represent a conceptually novel approach to the development of vaccines against mucosal infections and potentially against mucosal cancers and to the advanced study of immunobiological mechanisms involving mucosal compartmentalization.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Q.Z., R.I.M. and J.A.B. designed the experiments, interpreted the data and wrote the manuscript. Q.Z. executed many of the experiments, Z.W. conducted some of the experiments and J.T., R.C.W., J.K. and B.E. produced nanoparticle-releasing microparticle vaccines and were involved in micro- and nanoparticle characterization and in vitro release experiments. T.C. participated in experiment design and the initial experiments. G.Z. performed electron microscopy. D.M.K. provided CpG ODN and contributed to analysis and discussion. I.M.B., S.G. and Y.S. participated in planning and discussion. J.A.B. oversaw the overall execution of the projects.
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. Female BALB/c (6–8 weeks old) were purchased from the Frederick Cancer Research Center or Taconic and housed in pathogen-free conditions in the US National Cancer Institute Animal Facility. All animal experiments were approved by the Animal Care and Use Committee of the National Cancer Institute.

Reagents. PCLUS3-18IIIB (KQIINMWQEVKAMYAPPISGQIRRIQRGPG RAFTVIGK) and P18-I10 (RGPGRAFVTI) were synthesized by NeoMPS. TLR ligands including macropage-activating lipoprotein (MALP-2) and polyinosine-polycytidylic acid (poly(I:C)) were purchased from Invivogen. Equimolar mixtures of the phosphorothioate CpG ODNs 1555 and 1466 were used as previously described. For in vitro stimulation of DCs, we added MALP2, poly(I:C) and CpG ODN dosed at 0.1 µg ml⁻¹, 25 µg ml⁻¹ and 2 µg ml⁻¹, respectively, to culture.

PLGA and Eudragit particles. We manufactured PLGA (Lakeshore Biomaterials) nanoparticles with the NanoDRY technology (US Patent application 20050175707). Detailed manufacturing conditions and settings are described in Supplementary Methods. PLGA/FITC-BSA is composed of 1–2% of FITC-BSA (Sigma-Aldrich), and 10 µg of the nanoparticles was dosed for i.c.r. (or 20 µg p.o.) delivery to examine mucosal uptake. PLGA-encapsulated vaccine is composed of peptide antigen (PeptAg: 100 µg of PCLUS3-18IIIB per 10 mg of PLGA nanoparticle; ProtAg: A33+L1 10 µg each) and TLR ligands (TLRL: 0.5 µg of MALP-2, 100 µg of poly(I:C) and 10 µg of CpG ODN). Eudragit FS30D or L100-55 (Evonik) were used to coat PLGA, and 20 µg of microparticles containing 200 µg of PeptAg or 20 µg of A33+L1, 1 µg of MALP-2, 200 µg of poly(I:C) and 20 µg of CpG ODN vaccine were used for each oral delivery.

Reagent delivery and virus challenge. For oral delivery, we suspended micro/nanoparticles in 50 µl of PBS and administered them orally through an animal feeding needle. For i.c.r. delivery, reagents were suspended in 100 µl of PBS and delivered with a polished pipette tip through the anal canal. Peptide and TLR ligands as vaccines were mixed with 20 µg of DOTAP liposomal transfection reagent (Roche Diagnostics) before i.c.r. delivery. To challenge mice with vaccinia, we gave mice 2 × 10⁷ or 1 × 10⁷ PFU of the virus by either the i.c.r. or intravaginal route, respectively. After WR challenge, mice were weighed daily for 2 weeks and killed when their weight loss was over 25%.

Flow cytometry. Antibodies for flow cytometry were specific for the following proteins: CD11b (1:200, M1/70, BD Biosciences), CD11c (1:200, H3-6, BD Biosciences), B220 (1:200, RA3-6B2, BD Biosciences), CD4 (1:500, GK1.5, eBioscience), CD8α (1:500, 53-6.7, eBioscience), CD69 (1:200, H1.2F3, eBioscience), IL-12 p70/40 (1:200, C15.6, BD Biosciences). Soluble tetramer P18-I10 (H-2Dd) was kindly provided by the NIH Tetramer Core Facility and used to determine the frequency of antigen-specific CD8+ T cells isolated from the intestine. Sample data were acquired on a FACSCalibur and analyzed with FlowJo software (TreeStar). To measure TLR ligand activity in stimulating DCs, Eudragit/PLGA/Pept+TLRL microparticles were incubated with PBS (pH 7.4) for 16 h and centrifuged at 2,000 r.p.m. to remove aggregates. The supernatants were added to bone marrow–derived DC cultures. After a 20-h incubation, DCs were stained for intracellular IL-12 with IL-12p70/40 antibodies. For identification of FITC-expressing cells, isolated mucosal cells or in vitro cultured DCs were examined by flow cytometry.

Antibody ELISA. ELISA was performed as previously described. In brief, we serially diluted samples and incubated them in plates precoated with WR proteins A33 and L1 (BEI Resources). We then incubated the plates with horseradish peroxidase–conjugated detection antibodies to mouse IgA (1:5,000, 1040-05) or IgG (1:5,000, 1030-05) (both from Southern Biotechnology Associates) and developed color by incubating TMB (Biolegend) in darkness. The optical density was read at 450 nm. Antibody titers were derived from the inverse dilution at which the sample yielded an optical density twice that of the background of control specimens from unimmunized mice.

Statistical analysis. Comparisons among means of groups were determined by one-way analysis of variance post hoc with Bonferroni correction. Analyses were performed with SPSS (SPSS). P values < 0.05 were considered statistically significant.

Additional methods. Detailed methodology is described in the Supplementary Methods.