HIV-1 vaccination by needle-free oral injection induces strong mucosal immunity and protects against SHIV challenge

Andrew T. Jones1,2, Xiaoying Shen3, Korey L. Walter4, Celia C. LaBranche5, Linda S. Wyatt6, Georgia D. Tomaras3, David C. Montefiori5, Bernard Moss6, Dan H. Barouch7, John D. Clements8, Pamela A. Kozlowski4, Raghavan Varadarajan9 & Rama Rao Amara1,2

The oral mucosa is an attractive site for mucosal vaccination, however the thick squamous epithelium limits antigen uptake. Here we utilize a modified needle-free injector to deliver immunizations to the sublingual and buccal (SL/B) tissue of rhesus macaques. Needle-free SL/B vaccination with modified vaccinia Ankara (MVA) and a recombinant trimeric gp120 protein generates strong vaccine-specific IgG responses in serum as well as vaginal, rectal and salivary secretions. Vaccine-induced IgG responses show a remarkable breadth against gp70-V1V2 sequences from multiple clades of HIV-1. In contrast, topical SL/B immunizations generate minimal IgG responses. Following six intrarectal pathogenic SHIV-SF162P3 challenges, needle-free but not topical immunization results in a significant delay of acquisition of infection. Delay of infection correlates with non-neutralizing antibody effector function, Env-specific CD4+ T-cell responses, and gp120 V2 loop specific antibodies. These results demonstrate needle-free MVA/gp120 oral vaccination as a practical and effective route to induce protective immunity against HIV-1.
Human immunodeficiency virus-1 (HIV-1) is most commonly transmitted across genital and rectal mucosal surfaces via sexual contact. Within the first days and weeks of infection, HIV-1 is localized to the mucosal tissue, replicating in resident target cells, before systemic dissemination and seroconversion. In addition, irrespective of the route of infection, HIV causes a rapid and profound depletion of CD4 T cells in the gut. Because of this, genital and gut mucosal infection, HIV causes a rapid and profound depletion of CD4+ T cells characterized in humans and non-human primates. Most sub-presenting cells, however, these populations have not been fully characterized in previous clinical trials.

Oral vaccines are attractive as they can induce strong immunity in the gut, are relatively non-invasive, and can be administered on a large scale. Oral vaccines generally are ingested and thus must survive the hostile acidic environment of the stomach to be sampled by the gut-associated lymphoid tissue (GALT) mainly in the distal regions of the small intestine. An alternative strategy of oral vaccination is to directly target the tissues within the oral mucosa for antigen delivery. Vaccination of the oral mucosa, primarily to the buccal (inner cheek) and sublingual (below the tongue) tissue, has been proposed to be a practical, safe, and non-invasive method of oral mucosal vaccination. The sublingual and buccal (SL/B) tissues contain numerous subsets of antigen-presenting cells, however these populations have not been fully characterized in humans and non-human primates. Most sub-lingual and buccal vaccinations are performed by the topical application of vaccines to oral tissues, allowing for natural absorption across the oral epithelium. The oral mucosa, unlike the simple columnar epithelium in the small intestine, consists of multilayered squamous epithelium, which can limit the natural uptake of vaccine antigens. Thus, oral vaccination approaches that enhance vaccine uptake may significantly increase vaccine responses.

To aid in antigen uptake, needle-free injectors can be used to deliver vaccines across the skin or oral epithelium into the underlying tissue, while retaining the non-invasive features of oral vaccination. Needle-free injectors have been investigated as a tool to deliver drugs and vaccinations, primarily through the skin, and are an attractive alternative to needle-based injections which carry disadvantages such as the need for trained healthcare workers to administer injects, the risks associated with needle-sticks and re-using needles, as well as the common fear of needles resulting in reduced patient compliance.

Here we evaluate the SL/B tissue as a route of needle-free oral HIV-1 vaccination in rhesus macaques. Vaccine components are delivered orally to the SL/B tissues via either needle-free injection (Needle-free SL/B) or topical application (Topical SL/B) and compared to the conventional intradermal/subcutaneous route (ID/SC) commonly used for needle-based immunizations. Vaccinations consist of two priming immunizations with modified vaccinia Ankara (MVA) engineered to express HIV-1 antigens (MVA-HIV) followed by boosting twice with a recombinant trimERIC gp120 immunogen (cycP-gp120), along with the Escherichia coli derived mucosal adjuvant double mutated heat-labile enterotoxin (dMLT), which has been shown to promote mucosal immune responses. MVA-HIV has been extensively characterized in non-human primate studies and is currently being tested in human clinical trials as an HIV-1 vaccine candidate, and cycP-gp120 has been previously shown to elicit tier-2 neutralizing antibodies in guinea pigs as well as promote highly-cross reactive V1V2-directed antibodies, a major correlate of protection in the RV144 trial, in rabbits and rhesus macaques.

To test the vaccine efficacy of MVA-HIV/cycP-gp120, animals are challenged intra-rectally 19 weeks after the last immunization with a heterologous, pathogenic, difficult to neutralize SHIV-SF162P3. Needle-free SL/B and ID/SC immunization both results in a significant delay in acquisition of infection compared to unvaccinated controls, with non-neutralizing antibody effector functionality and Env-specific CD4 T-cell responses being the predominant correlates of protection. These results demonstrate that oral needle-free delivery of MVA-HIV/cycP-gp120 vaccination induces a strong antibody response in mucosal and systemic compartments with protective potential against HIV-1. They also describe a novel method of needle-free vaccination that is practical and induces a strong antibody response in three major mucosal sites and serum.
Fig. 1 Dendritic cells in the sublingual and buccal (SL/B) tissue of rhesus macaques. Paraffin-embedded sublingual and buccal tissue sections were stained with a Hematoxylin and eosin and imaged by light microscopy (scale bar, 500 μm) or b anti-Langerin and analyzed by immunohistochemistry (scale bar, 200 μm). Arrows show Langerin+ cells stained in red. c Representative flow plots showing dendritic cell (DC) subsets in PBMC, sublingual tissue, buccal tissue, and the submandibular, submental, and inguinal lymph nodes. Top row; CD45+CD3−CD20−HLA-DR−CD14+CD16− cells were gated for plasmacytoid DC (CD123+BDCA-1+, orange) and conventional DC (CD123−BDCA-1+, blue) markers. Middle row; CD45+CD3−CD20−HLA-DR+ cells were gated for Dermal DCs (CD14+DC-SIGN+, green). Bottom row; frequencies of DC subsets in different tissues (*, p < 0.05, Wilcoxon matched-pairs test). Data representative of samplings from six independent animals, n = 5 (PBMC) n = 6 (buccal, sublingual), n = 3 (submandibular, submental, and inguinal lymph nodes). Samples from the same animal were paired together. d Detection of aldehyde dehydrogenase (ALDH) activity in buccal or submandibular DCs using the ALDEFLUOR™ fluorescent reagent. Control samples were used to set gating. Representative flow plots are shown on top measuring ALDH activity in conventional DCs in buccal tissue compared to submandibular lymph nodes. Bottom, frequencies of ALDEFLUOR+ conventional DCs (blue) and dermal DCs (green) in PBMCs (n = 2), buccal (n = 3) and sublingual tissue (n = 3), and submandibular (n = 3) and submental lymph nodes (n = 3) (*, p < 0.05; **, p < 0.01; Mann-Whitney test). PMBC, peripheral blood mononuclear cells; SL/B, sublingual and buccal tissue. Box and whiskers plot in c, d; box extends from 25th to 75th percentile, the line indicates median, whiskers indicate min and max values.

the submandibular and submental lymph nodes, however this activity was not found in DCs within the sublingual and buccal tissue themselves (Fig. 1d). However, it is possible that RALDH activity may be induced in tissue DC upon activation as we showed previously following Ad5 vaccination in mice.25  Taken together, these data demonstrate that the oral mucosa contains multiple antigen-presenting cell subsets with predominance of LCs, BDCA1+ DC and CD14+DC-SIGN+ DC, and the draining lymph nodes contain gut-homing induction potential, indicating the oral mucosa is a viable route for mucosal vaccination.

NHP study design. To test if needle-free delivery of vaccines to the SL/B mucosa enhances the humoral immunity, we used the Syrijet Mark-II Needless Injector (Keystone Industries), designed for use in dentistry to inject local anesthesia to the oral mucosa, to deliver MVA-HIV and protein vaccines to rhesus macaques.
The mucosal adjuvant dmLT (Fig. 2c)\(^\text{13,28}\). Doses were split in half trimeric clade B cycP-gp120 immunogen (strain JRFL) along with gag, pol, env (strain ADA) followed by two boosts with 100 (plaque-forming units) of MVA-HIV expressing HIV-1 clade B vaccines, currently being tested in human clinical trials as a thigh of ID/SC immunization.

between the sublingual and buccal tissue or the left and right thigh of ID/SC immunization. 19 weeks following the second cycP-gp120 immunization, animals were challenged intra-rectally with low dose pathogenic SHIV-SF162P3 weekly for up to six weeks. Cartoons depict MVA-HIV and virus-like particles, cycP-gp120, and dmLT.

To evaluate the SL/B route of oral vaccination, we immunized female rhesus macaques orally via either topical application to the sublingual and buccal (SL/B) tissue (Fig. 2a). First, we injected a rhesus macaque with 100 μl of sterile PBS and visualized the injection site before and after injection (Fig. 2b). The Syrijet injected effectively into both the sublingual and buccal tissue with minimal damage and bleeding, and no additional swelling or damage was reported in the following days post injection.

To evaluate the SL/B route of oral vaccination, we immunized female rhesus macaques orally via either topical application to the SL/B tissue (n = 4), needle-free injection to the SL/B tissue (n = 5), or intradermally (with MVA-HIV) and subcutaneously (with cycP-gp120 + dmLT) (n = 6). MVA-HIV and cycP-gp120 doses were split between the buccal and sublingual tissue (SL/B) or the left and right thigh (ID/SC). 19 weeks following the second cycP-gp120 immunization, animals were challenged intra-rectally with low dose pathogenic SHIV-SF162P3 weekly for up to six weeks. Cartoons depict MVA-HIV and virus-like particles, cycP-gp120, and dmLT. Antibodies were induced by two injections of cycP-gp120 (100 μg) with the mucosal adjuvant dmLT.

**Needle-free vaccination induces strong serum IgG responses.** Impressively, needle-free SL/B immunization generated a strong anti-ADA gp120 IgG response in animals before (wk 25) and two weeks post (wk 45) immunization with cycP-gp120 + dmLT. A pre-challenge serum IgG response was observed in MVA-HIV and cycP-gp120 in the ID/SC group was adjuvanted with 1 μg of dmLT per site, while subcutaneous cycP-gp120 immunization in the ID/SC group was adjuvanted with 1 μg of dmLT. Since we observed swelling of mouth following the first cycP-gp120 + dmLT boost, and to make the second boost comparable between needle-free SL/B and ID/SC immunization, we reduced the dose of dmLT for the second cycP-gp120 immunization to 1 μg per site\(^\text{30}\).

\(\text{Fig. 2 SL/B immunization with a needle-free injector induces strong systemic antibody responses.}\) a) Syrijet, the needle-free injector used to deliver immunizations to the sublingual and buccal tissue. Sterile water cartridges were modified to contain immunogens. b) Sublingual and buccal tissue of a rhesus macaque before and five minutes after 100 μl PBS injection via Syrijet. c) Study design. Rhesus macaques (n = 15) were immunized twice with MVA-HIV (1 × 10\(^8\) pfu) and boosted twice with recombinant trimeric gp120 (cycP-gp120) (100 μg) with the mucosal adjuvant dmLT. Animals were immunized via topical application to the sublingual and buccal (SL/B) tissue (n = 4), needle-free injection to the SL/B tissue (n = 5), or intradermally (with MVA-HIV) and subcutaneously (with cycP-gp120 + dmLT) (n = 6). MVA-HIV and cycP-gp120 were split between the buccal and sublingual tissue (SL/B) or the left and right thigh (ID/SC). 19 weeks following the second cycP-gp120 immunization, animals were challenged intra-rectally with low dose pathogenic SHIV-SF162P3 weekly for up to six weeks. Cartoons depict MVA-HIV and virus-like particles, cycP-gp120, and dmLT. d) Kinetics of anti-gp120 (ADA) serum IgG in vaccine groups (geometric mean ± SD) and for individual animals (line, geometric mean ± SD) at the peak time point (wk 25) and pre-challenge time point (wk 45) (*, p < 0.05; Mann–Whitney test). Dotted lines denote week of indicated immunization. e) Anti-dmLT serum IgG response in animals before (wk 25) and two weeks post (wk 45) immunization with cycP-gp120 + dmLT. f) Topical SL/B (n = 4); blue square, needle-free SL/B (n = 5); gray triangle, ID/SC (n = 6).
mean titer, 9.6 μg ml−1) that were boosted remarkably by 100-fold following the 1st protein boost (geo-mean titer, 1012 μg ml−1). These responses contracted by 10-fold over 8 weeks and were marginally boosted upon the 2nd protein boost (geo-mean titer, 276 μg ml−1). At the time of pre-challenge, 14 weeks after the final protein boost, anti-gp120 serum IgG titers had contracted about 10-fold (geo-mean titer, 258 μg ml−1). In contrast, topical SL/B immunization resulted in minimal or undetectable antibody responses following both MVA-HIV and cycP-gp120 immunizations. Remarkably, while gp120-specific IgG responses induced by the needle-free SL/B immunization were comparable with ID/SC group following MVA immunizations, they were 10-fold (geo-mean titer 1012 vs. 120 μg ml−1) and 3-fold higher (geo-mean titer 1012 vs. 300 μg ml−1) following the 1st and 2nd protein boosts, respectively. However, we are uncertain if this was because of the higher dose of the adjuvant used in the needle-free group during the 1st protein boost. Despite differences in the adjuvant doses, these data show that needle-free SL/B injection is an effective non-invasive method of generating high titers of vaccine-specific serum IgG. A dmLT is currently being investigated for its potential as a vaccine candidate against Enterotoxigenic Escherichia coli (ETEC) infection31, we also measured serum IgG responses against dmLT and found a strong anti-dmLT IgG response generated in needle-free SL/B immunized animals upon boosting with cycP-gp120 adjuvanted with dmLT (Fig. 2e). These results demonstrate that needle-free SL/B immunization is an effective route to non-invasively generate strong systemic vaccine-specific IgG antibody responses against multiple antigens.

**Needle-free vaccination induces strong mucosal IgG and IgA.**

As a major goal of mucosal vaccination is the generation of mucosal immune responses, we measured vaccine-specific IgG and IgA antibodies in the rectal, vaginal, and salivary secretions. Due to the variable amounts of immunoglobulin in secretions, antibody concentrations were normalized relative to the total IgG or IgA concentrations by calculating the specific activity (SA; ng gp120-specific IgG or IgA per total μg IgG or IgA)32. As with the systemic responses, needle-free SL/B immunization generated a strong gp120-specific IgG response in rectal, vaginal, and salivary secretions (Fig. 3a, b, c). Similar to serum IgG responses, the mucosal IgG responses also peaked after the 1st protein boost and the 2nd protein boost showed a small recall (<2-fold). At the peak (wk 25), the IgG SA was comparable between the three mucosal compartments (geo-mean of specific activity; rectal, 56; vaginal, 48; salivary, 51). Topical SL/B immunization generated minimal to undetectable mucosal antibodies, highlighting the importance of the needle-free injector to generate these responses. Additionally, while ID/SC immunization did result in mucosal IgG responses, these were significantly lower than needle-free SL/B responses at the peak time point (wk 25). Mucosal IgG responses after the first protein boost contracted and were modestly expanded by the subsequent boost before contraction to the pre-challenge time point. Interestingly, while the gp120-specific rectal and vaginal IgG antibodies were maintained until pre-challenge, IgG antibodies in the saliva declined and approached undetectable levels, suggesting the establishment and maintenance of vaccine-specific IgG antibody responses varied between mucosal compartments.

**Fig. 3 Needle-free SL/B immunization generates strong mucosal antibody responses.** a–c Anti-ADA gp120 IgG antibodies in rectal (a), vaginal (b), and salivary (c) secretions. d–f Anti-ADA gp120 IgA antibodies in rectal (d), vaginal (e), and salivary (f) secretions. Data represented as geometric mean ± SD specific activity for each group. Specific activity calculated as ng gp120-specific IgG or IgA antibody per μg total IgG or IgA isolated. Specific activity for individual animals is shown at the peak time point (wk 25); line indicates geometric mean. The shaded region in each graph indicates the specific activity cut-off value. g–h IgG specific activity against gp70-V1V2 (Clade B/Case A2) in rectal and vaginal secretions. a–h *p < 0.05; **p < 0.01; Mann–Whitney test. White circle, topical SL/B (n = 4); blue square, needle-free SL/B (n = 5); gray triangle, ID/SC (n = 6).
Needle-free SL/B immunization also generated vaccine-specific IgA antibodies in all three mucosal compartments, with strongest responses in vaginal secretions with all animals generating detectable vaginal IgA responses (geo-mean SA of 7.9) and weakest in saliva with only 2 out of 5 animals generating detectable IgA (Fig. 3d, e, f). The peak rectal and vaginal IgA response (wk 25) was significantly higher in needle-free SL/B compared to ID/SC immunized animals, which generated minimal mucosal IgA responses, and these responses were largely undetectable in topical SL/B immunized animals. Rectal IgA and salivary IgA levels contracted over time and were predominantly undetectable at the time of pre-challenge. However, vaginal IgA responses were still detectable at the time of pre-challenge, suggesting this route may generate durable vaginal antibody responses. In addition to gp120 specific antibodies, we also measured antibodies against the gp70-V1V2 scaffold antigen, which displays the variable loops 1–2 (V1V2) loops of gp120, as serum IgG antibodies directed against gp70-V1V2 were a major correlate of protection in the RV144 trial. Both needle-free SL/B and ID/SC immunization generated anti-gp70-V1V2 IgG in both rectal and vaginal secretions throughout the immunization regimen, however these responses were not long-lasting as they had contracted to undetectable levels by the pre-challenge time point (Fig. 3g, h). These results indicate that needle-free SL/B immunization is an easy and practical method of generating strong IgG responses in rectal, vaginal and oral mucosa, and strong IgA responses in vaginal mucosa.

Needle-free vaccination induces broad V1V2 and V2HS response. To address the global diversity of HIV-1, an effective vaccine should ideally recognize HIV-1 isolates from multiple strains and clades. To characterize the cross-reactivity of MVA/cycP-gp120 induced antibodies, we measured antibody binding to a global panel of gp120, gp140 and gp70-V1V2 scaffold proteins via binding antibody multiplex assay (BAMA). Upon boosting with cycP-gp120 we observed a strong cross-reactive antibody response against multiple clades of gp120 and gp140 antigens, reacted to by all immunized animals (Fig. 4a). Responses were significantly higher in 15/16 of the antigens tested for needle-free orally immunized animals compared to ID/SC immunized at this time point (wk 25), however at the pre-challenge time point both groups had similar levels of reactivity (Supplementary Fig. 2a). These results show the high broadly-reactive antibody response generated by MVA/cycP-gp120 regimen, a crucial component to an HIV-1 vaccine candidate.

As results from the RV144 trial suggest that antibodies directed towards the V1V2 loop of gp120 are associated with reduced risk of infection, the generation of these antibodies, especially broadly reactive V1V2-directed antibodies, is of great interest in HIV-1 vaccine development. To measure the cross-reactivity of V1V2-directed antibodies, sera IgG binding to a panel of 16 gp70-V1V2 scaffolds representing the global diversity of HIV-1 was quantified via BAMA. Two weeks post the first protein boost, needle-free SL/B immunized animals generated a substantial broadly cross-reactive gp70-V1V2 response against multiple clades of isolates, significantly higher than ID/SC immunization, demonstrating not only the high immunogenicity of the sublingual and buccal route, but also the broadening of the antibody responses upon needle-free oral delivery (Fig. 4b).

Similarly, the anti-Env response at the pre-challenge time point responses to gp70-V1V2 scaffolds had contracted in both groups, with no significant differences between the groups, suggesting that through contraction and further boosting in ID/SC immunized animals the V1V2 response leveled to a set point (Supplementary Fig. 2b).

To map the regions of gp120 targeted by the vaccine-induced antibody response, we measured binding via peptide microarray of sera to 15-mer peptides (overlapping by 12 amino acids) derived from 13 strains including consensus clade B Env (Supplementary Table 1). Both needle-free SL/B and ID/SC immunization resulted in a broad response against numerous regions of consensus clade B gp120, the strongest responses directed against the C1, C2, V3, and C5 regions of gp120 (Fig. 4c).

Binding responses against linear V2 hotspot epitope was developed against consensus B (Fig. 4c) as well as to other clade consensus and viral strains at lower magnitude (Supplementary Fig. 2c). Linear V2 binding was a subdominant response compared to V3 and C4 linear epitope binding, consistent with previous findings for HIV-1 Env vaccine-elicited antibody responses. Comparing needle-free SL/B to ID/SC peptide responses showed similar trends in both groups, with a high proportion of the IgG response directed towards the C1, V3, and C5 regions, however needle-free SL/B immunization resulted in a modestly larger proportions of response against the V2, C4, V5, and C3.1 regions (Fig. 4d).

We next measured antibody responses to the V2 Hotspot (V2- HS) peptide, a region of the V2 loop (spanning positions 166–178 of HIV-1 strain HXB2) in which antibody recognition correlated significantly with decreased risk of infection in the RV144 trial. Responses to the consensus B V2 peptides were detected in the peptide microarray analysis, primarily in the needle-free SL/B group, though these responses were modest compared to other regions. To determine responses to the V2-regions of the vaccine and challenge virus strains, we synthesized 13 amino-acid peptides corresponding to the V2 hotspot of cycP-gp120 (clade-B JRFL, E168K), MVA-HIV (clade-B ADA), and SHIV-SF162P3 (clade-B). Needle-free SL/B immunization generated a strong cross-reactive V2 hotspot response, recognizing not only the MVA-HIV and cycP-gp120 vaccine strains (ADA, JRFL E168K), but also the heterologous SHIV-SF162P3 (Fig. 4e), similar to previous studies examining antibody responses generated by cycP-gp120. ID/SC immunization resulted in minimal V2 responses compared to needle-free SL/B immunization, which is likely due to the overall antibody response being significantly lower in ID/SC immunized animals at this time point. The cross-reactivity of V2 antibody responses to heterologous strains detected in both linear peptide microarrays and in V2 hotspot ELISA demonstrates the remarkable ability of cycP-gp120 to generate V2-hotspot binding antibodies.

The generation of neutralizing antibodies to HIV-1 is a long-sought goal of HIV-1 vaccination. To test the presence of neutralizing antibodies generated by MVA-HIV/cycP-gp120 immunization, we measured the neutralizing activity of sera against a multi-clade panel of pseudoviruses that have high (SF162 LS, MWF965.26), moderate (Bal-L26), or low (ADA, JR-FL, TRO.11) neutralization sensitivity (tier-1A, 1B, 2, respectively). Needle-free SL/B immunization induced a significantly higher titer of neutralizing antibodies against the neutralization sensitive clade-B SF162 LS and the moderately resistant clade-B Bal-L26 isolate after the first and second protein boosts than ID/SC immunization (Fig. 4f). At the pre-challenge time point, however, both responses were not significantly different. Additionally, we detected no tier-2 (neutralization resistant) neutralizing antibody responses, which is in line with our previous results suggesting that mutations to cycP-gp120, or additional homologous immunizations, may be required to generate tier-2 neutralizing activity.

The results of the RV144 trial suggest that non-neutralizing antibodies may have a role in protection as vaccine-induced V1V2-directed antibodies were largely non-neutralizing. Furthermore, non-neutralizing antibodies have been shown to...
when measuring ADCVI activity, we found that sera from some
animals enhanced viral outgrowth rather than inhibiting replication,
whereas other sera inhibited up to 80% of viral outgrowth
compared to controls (effectors + targets + naive serum). ADCVI
activity was not related to the magnitude of the antibody response
at the pre-challenge time point (Supplementary Fig. 2d), indicat-
ing that antibody effector functionality is more dependent on
specificity than magnitude, and certain antibody responses may
be detrimental in combating HIV-1.

**Needle-free vaccination induces T cell responses in blood.** To measure the cellular immune responses after immunization, we
stimulated peripheral blood mononuclear cells (PBMCs) with
HIV-1 clade B consensus Gag and Env peptides and measured
cytokine production in T-cells (Live CD3+). For measuring
ADCVI activity, we found that sera from some

**Fig. 4 Serum IgG specificity, neutralizing activity, and effector function.** Binding of IgG antibodies at two weeks post the first cycP-gp120 immunization (wk 25) to a gp120 and gp140 antigens and b gp70-V1V2 scaffolds representing the global diversity of HIV-1 determined using Binding Antibody Multiplex assay (BAMA). Shaded areas indicate clade. c Binding of peak immune sera (wk 25) to 15mer peptides derived from clade B consensus gp120, measured by peptide microarray linear epitope mapping and reported as binding signal (Log2 fold difference post-immunization/baseline binding intensity). Magnitude of binding to each epitope is defined as the highest binding signal for a single peptide within the region of the epitope. d Representation of peptide array binding of each clade B consensus epitope as % of the total response. e Anti-V2 hotspot (HS) response (wk 25) against V2-HS peptides derived from strains JRFL (E168K), ADA, and SF162P3 measured by ELISA. Dotted lines connect data from same animal. Blue squares, needle-free SL/B; gray triangles, ID/SC. f Neutralizing antibodies over time against HIV-1 isolates MW965.26, SF162.LS, and Bal.L6, measured as ID50. g Antibody-dependent cell viral inhibition (ADCVI) measured at pre-challenge as % viral inhibition (mean ± S.D.). An average of two individual experiments is shown. Box and whiskers plots (a-c, f); box extends from 25th to 75th percentile, line indicates median, whiskers indicate min and max values (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; 2-Way ANOVA, multiple comparisons). ID50, serum dilution required to neutralize 50% infection
MVA-HIV resulted in IFN-γ production in CD4+ T-cells against both Gag and Env peptides, peaking one week after the second MVA-HIV immunization (wk 16), and responses were similar between needle-free SL/B and ID/SC groups (Fig. 5b). Similar to IFN-γ responses, Gag and Env specific TNF-α (Fig. 5c) and IL-2 (Fig. 5d) producing CD4+ T-cells were also observed, peaking again after the second MVA-HIV. Interestingly, unlike IFN-γ production, both TNF-α and IL-2 responses were expanded upon the first cycP-gp120 + dmLT boost. In contrast to vaccine-specific CD4 T-cell responses, we detected only low levels of vaccine-specific CD8 T-cell responses that peaked after the second MVA-HIV immunization and contracted to below detection over the course of the immunization (Fig. 5e). However, we did not detect vaccine-specific T-cell responses in the rectal or vaginal tissues after immunization, suggesting that stronger T-cell generating vaccine strategies, such as the utilization of DNA-primes, may be necessary to induce these responses. As a major concern with HIV-1 vaccines is the unwanted generation of an abundance of HIV target CD4+ T-cells in the mucosal tissue, we characterized the phenotypes of CD4 T-cells in rectal tissue at time of pre-challenge and found that needle-free SL/B, topical SL/B, and ID/SC immunized animals all had similar levels of activated (HLA-DR+CCR5+) CD4+ T-cells in the rectal tissue, indicating that immunization did not result in an accumulation of target cells in the rectal mucosa (Supplementary Fig. 3b,c). Taken together, these data show that needle-free SL/B immunization is capable of generating vaccine-specific functional T-cell responses in the blood, similar to responses generated by conventional ID/SC immunization.

MVA-HIV/cycP-gp120 vaccination protects from SHIV challenge. As a preliminary readout of the efficacy of MVA-HIV/cycP-gp120 vaccine, we challenged animals 19 weeks after the second protein boost with repeat low dose weekly intrarectal challenges of SHIV-SF162P3 for maximum of six exposures. The envelope in SHIV-SF162P3 virus is a tier-2 Env, heterologous to the vaccine strains, and we measured no detectable neutralization against this Env in vaccinated animals. Five unvaccinated female macaques were used as a control group. Following challenge, all unvaccinated animals were infected by the third challenge with 3 of the 5 becoming SHIV infected after the 1st exposure. Impressively, we detected a significant delay in acquisition of infection in both the needle-free SL/B immunized

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Fig. 5 Needle-free SL/B immunization generates vaccine-specific CD4 and CD8 T cells in the blood. PMBCs were stimulated with HIV-1 consensus B Gag and Env peptides and analyzed by flow cytometry for cytokine production. a Representative flow plots for IFN-γ and TNF-α cytokine expression on Live CD3+CD4+ cells in non-stimulated (NS), Gag, or Env stimulated PMBCs is shown. Kinetics of the total (Gag + Env) b IFN-γ, c TNF-α, and d IL-2 response in CD4+ T cells (mean ± S.D.), with the peak response (wk 16) highlighted for each animal (line denotes mean). e Kinetics of the total IFN-γ response in CD8+ cells (mean ± S.D.), with the peak response (wk 16) highlighted for each animal. White circle, topical SL/B (n = 4); blue square, needle-free SL/B (n = 5); gray triangle, ID/SC (n = 6).
(p = 0.019) and ID/SC (p = 0.0074) immunized animals, but not topical SL/B immunization (p = 0.20) (Log-rank (Mantel-Cox) test), with two ID/SC immunized animals remaining uninfected after the sixth challenge (Fig. 6a). Vaccine efficacy per exposure for each group was calculated to be 58% (Needle-Free SL/B) and 77% (ID/SC) (Fig. 6b). No differences in vaccine efficacy were observed between these two vaccine groups. Tracking viral loads following infection, we detected a trend for lower set-point viral loads in between these two vaccine groups. To identify immune response correlates of protection, we performed a principal component analysis (PCA) with the all immune responses measured in this study. PCA results showed that principal component 1 (PC1), containing ten variables, could separate animals with and without delayed acquisition (Fig. 6d), while a simulated PCA performed with the ten PC1 variables using randomized assay data showed no separation (Supplementary Fig. 4a). Loadings for PC1 included CD4+ T cell IFN-γ + TNF-α production two weeks after the second cycP-gp120 immunization (wk 33), and JRFL-E168K V2 hotspot reactivity in serum IgG (wk 33) with acquisition of infection (Kendall’s Tau Correlation Test). Needle-free SL/B (blue squares) and ID/SC (gray triangles) are combined for analysis (n = 11).

Fig. 6 Needle-Free SL/B and ID/SC immunization results in delayed acquisition of SHIV-SF162P3 infection. Animals were challenged weekly with an intrarectal low dose (1:100 dilution) of SHIV-SF162P3. 5 unvaccinated macaques were used as a control. a Acquisition of infection in topical SL/B, needle-free SL/B, or ID/SC immunized animals (Log-rank (Mantel-Cox) test). Dotted line, unvaccinated controls (n = 5); black line, topical SL/B (n = 4); blue line, needle-free SL/B (n = 5); gray line, ID/SC (n = 6). b Vaccine efficacy of ID/SC, needle-free SL/B immunization groups vs control animals (Log-rank (Mantel-Cox) test). c Kinetics of plasma viral loads in unvaccinated and vaccinated animals. d Top, Principal Comparison Analysis (PCA) plot showing PC1 and PC2 scores for delayed (>5 challenges to infect, n = 5) or non-delayed (<5 challenges to infect, n = 6) acquisition of infection in needle-free SL/B and ID/SC immunized animals. Bottom, loadings of immune response parameters in PC1. e-g Correlation analysis of ADCVI activity at pre-challenge, Env-specific CD4+ T cell TNF-α production two weeks after the second cycP-gp120 immunization (wk 33), and JRFL-E168K V2 hotspot reactivity in serum IgG (wk 33) with acquisition of infection. (Kendall’s Tau Correlation Test). Needle-free SL/B (blue squares) and ID/SC (gray triangles) are combined for analysis (n = 11)
strategically poor systemic immunity highlighting the unique ability vaccination induces strong humoral and cellular responses in the mucosa. Our results demonstrated that needle-free injection of the sublingual and buccal tissue is an easy, safe, and efficient method of vaccination for generating strong mucosal and systemic antibody responses. Needle-free SL/B immunization also resulted in induction of strong CD4 and CD8 T cell responses in blood that were comparable to systemic immunization. The strong immunogenicity of the SL/B tissue, which is often regarded as a site for immune tolerance induction, suggests the adjuvant activity of MVA-HIV and dmLT effectively overcomes the tolerogenic nature of the oral tissue to generate vaccine-specific T and B cell responses. The concept of needle-free delivery of vaccines is not new as recent studies examined needle-free injection through the skin as a method of vaccination in humans. However, to our knowledge this method of vaccination has not been explored for SL/B route in humans or non-human primates and thus our study represents the first demonstration that needle-free SL/B immunization as an attractive approach for mucosal vaccination with protective potential against HIV, warranting further studies to characterize this route for HIV/SIV and other mucosal pathogens.

An impressive finding of our study is that oral needle-free vaccination induces strong humoral and cellular responses in blood in addition to mucosal compartments. This was in contrast to previous oral topical vaccination studies, which showed relatively poor systemic immunity highlighting the unique ability of needle-free vaccination to generate systemic immunity. One question that remains to be addressed is how comparable are the systemic immune responses induced by the oral needle-free vaccination to systemic immunization. We are unable to determine this in this study due to the use of higher adjuvant dose during the first protective effect. Nevertheless, our data show that at least after MVA vaccinations they are comparable.

The mechanisms by which needle-free SL/B vaccination induces a strong mucosal and systemic immunity need further investigation. In addition, since we vaccinated via both buccal and sublingual routes simultaneously, we cannot conclude if buccal or sublingual vaccination alone is superior compared to each other. To gain insights into the role of different antigen-presenting cells in the mouth, we characterized different subsets of DC in the buccal and sublingual tissue. Our results showed the presence of multiple subsets of DCs, including Langerhans cells (LCs), CD14+ DC-SIGN+ dermal DCs, and BDCA-1+ (DC1c) conventional DCs (cDCs). These subsets have been shown to have multiple roles in the generation of tolerance or immunity. For example, LCs are generally considered to be tolerogenic and aid in the maintenance of the epithelial barrier, however there is increasing evidence that these cells have some functional plasticity and are capable of becoming immunogenic based on the specific context of inflammation. In contrast, cDCs are thought to be more involved with the induction of immunity and cross-presentation of antigens, and dermal DCs or similar CD14+ cells have been shown to be important in T-follicular helper cell formation and B-cell activation. Additionally, while these subsets were found in both buccal and sublingual tissue, we found that the buccal tissue had significantly higher proportion of both BDCA-1+ cDCs and dermal DCs than the sublingual tissue. The larger proportion of these subsets suggests the buccal tissue may be a preferred target of immunization, although most oral tissue immunization studies have targeted the sublingual tissue. Future studies should evaluate buccal vs sublingual only immunization to compare the differences in these tissues, as well as evaluate the effect different adjuvants have on the activation and function of the different dendritic cell subsets.
secretions, with most animals showing no detectable vaccine-specific rectal IgA. In contrast, all immunized animals had detectable anti-gp120 IgG in rectal secretions at the pre-challenge time point, and animals showing the greatest level of contraction in the rectal IgG response were significantly more likely to be infected (Supplementary Fig. 5d), highlighting the role of mucosal antibody responses in protection from mucosal pathogens. Thus, it is important to test the influence of different adjuvants on the magnitude and longevity of antibody responses induced by needle-free oral vaccination. The potential adjuvants include TLR7/8 agonists, TLR4 agonists and a combination of these. The development of improved vaccine regimens and adjuvants that improve the duration of the antibody response, especially in the mucosal secretions, may aid in long-term protection from infection. Additionally, unlike the rectal IgA responses, needle-free SL/B immunization resulted in a strong and long lasting HIV-1 specific vaginal IgA in protection from vaginal SHIV challenge5,27, indicating needle-free SL/B may be superior to ID/SC immunization in this context.

In conclusion, our study demonstrates the vaccine-mediated protection of MVA-HIV/cycP-gp120 immunization against a pathogenic, heterologous SHIV, as well as the viability and effectiveness of needle-free SL/B immunization as an alternative to conventional needle-based vaccination. Future studies will further evaluate the needle-free SL/B route of vaccination in comparison with conventional routes, including examining the effects of adjuvant dosage on vaccine responses as well as investigating this route in generating protective immune responses against other mucosal pathogens.

Methods

Ethics statement. All housing and experiments involving rhesus macaques were conducted at the Yerkes National Primate Research Center, and protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC) protocol YER-2003491. Experiments were carried out in accordance to USDA regulations and recommendations derived from the Guide for the Care and Use of Laboratory Animals. Rhesus macaques were housed in pairs in standard non-human primate cages and provided with both standard primate feed (Purina monkey chow) fresh fruit, and enrichment daily, as well free access to water. Immunizations, blood draws, and other sample collections were performed under anesthesia with ketamine (5 mg kg−1), and after blank bead subtraction.

Binding antibody detection assays. Rhesus macaque serum IgG binding to recombinant ADA gp120 (Immune Technology Corp) was measured by ELISA. Microtiter plates were coated with 0.5 μg ml−1 of ADA gp120 in PBS at 4°C overnight followed by 3-fold serial dilutions of rhesus macaque sera before detection. Inhibitory anti-rhesus IgG, which was raised against the TMB Microwell Peroxidase Substrate Kit (KPL). Additionally, serum IgG binding to dmlT was measured by ELISA using microtiter plates coated with 0.5 μg ml−1 dmlT in PBS and detected similarly to anti-gp120 IgG. Concentrations of gp120 or dmlT-specific IgG were quantified using a rhesus IgG standard based on microtiter plates coated with 1 μg ml−1 anti-rhesus IgG (Southern Biotech) and standardized using recombinant rhesus IgG (Southern Biotech) serially diluted 3-fold, starting with 100 ng ml−1. Peptides corresponding to the V2-hotspot region of HIV-1 strains JR-FL-E168K (N-RDKVQKEYALFYKLD-C), ADA (N-RDKVKKDYALFYRLD-C) were synthesized (Genemed peptide synthesis, San Antonio, TX). The mean values are presented with 95% confidence intervals.

Neutralization assays. Neutralizing antibody was measured in 96-well culture plates by using Tα1-regulated luciferase (Luc) reporter gene expression to quantify reductions in virus infection in TZM-bl cells. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. Assays were performed with HIV-1 Env-pseudo-typed viruses as described28,29. Test samples were diluted over a range of 1:20 to 1:4730 in cell culture medium and pre-incubated with virus (~150,000 relative light unit equivalents) for 1 h at 37°C prior to addition of cells. Following 48 h incubation, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the sample dilution at which relative lucinescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of RLU in cell control wells (ID50). Serum samples were heat-inactivated at 56 °C for 1 h prior to assay. Positive values were reported as being at least 3x baseline values standardized against the negative control virus, SVA-MLV.

Binding antibody multiplex assay (BAMA). BAMA assays were performed as follows29,30. Serial dilutions (starting at 1:80, five-fold dilutions) of rabbit or rhesus serum were reacted on beads coated to a panel of gp120 (strains S1802, BORI, BUX002, 254008, CNE20, TT31P.2792, B6240, A244), uncleaved trimeric gp140 (strains RPHA42159, AE011,com_v03, 1086.0, C. CH505TF, WITO4160, BF1266, 90046, SC42661), and gp70-V1V2 scaffold proteins (strains B.CaseA, 7000160141, CM244.c1, TV1.21, 1001428.2.42, CAP210.2.00.E8, C2101.c01, BUX0002000.03.02, BF266_431a, 96ZMG651.02, RPHA42597., C1086.B, 62357.14, 700001058, 1019084.B, TT31P.210.F2972) representing the global panel of HIV-1 Envs34. Detection of serum to beads was detected using a secondary biotin-conjugated anti-rhesus IgG and measured via fluorescent readout. The mixture of bead coated with 4 μg of peptide at 4°C overnight, and after blank bead subtraction.

Linear epitope mapping peptide microarray. Solid phase peptide microarray epitope mapping was performed as follows29,31. Briefly, array slides were prepared by JPT Peptide Technologies GmbH (Germany) by printing a library designed by Dr. B. Ederer, Los Alamos National Laboratory, onto Epoxy slide glasses (PolyAn GmbH, Germany). The library contains 15-mer peptides overlapping by 12, covering consensus Env (gp160) clade A, B, C, D, Group M, CRF1, and CRF2 and vaccine strains (gp120) LA244, 1T0233, MN, 10180, CTV1, and CZM651. Sera were diluted 1:50 and applied to the peptide array, followed by washing and detection using goat anti-human Fc-AP (Alkaline Phosphatase) at a dilution of 1:470 and detected at a wavelength of 655 nm with an InnoScan 710 AL scanner (Innopsys, France) using XDR mode. Scan images were analyzed using MagPix 8.0 software to obtain
binding intensity values for all peptides. Microarray data were then processing using R package pepStat to obtain binding signal for each peptide, which is defined as log (fluorescence of post-immunization signal/baseline signal). Binding magnitude to each identified epitope is defined as the highest binding signal by a single peptide within the epitope region.

Antibody-dependent cellular viral infection (ADCVI). Assays for ADCVI activity in rhesus macaque serum were performed using HIV SF162 infected CEM-NKr cells. Briefly, on day 1, CCR5+ CEM-NKr cells (a gift from Dr. Jim Hoxie, University of Pennsylvania) were infected with HIV SF162 at an MOI of 0.01. On day 2, cryopreserved PBMCs were thawed, washed, and added to wells of V-bottom plates in 100 µl of medium containing 1 × 10^4 cells. On day 3, 50 µl of 1:25 diluted sterile-filtered serum and 30 µl containing 1 × 10^5 cells were added to each well. A pool of monoclonal antibodies was used as a positive control. On day 7, the cells were washed twice to remove any gag-specific antibodies which may interfere with the assay readout. On day 10, the medium was harvested, treated with TritonX-100 detergent, and analyzed for viral content using a gag-p24 capture ELISA. The % inhibition of infection was determined by comparing the p24 concentration in wells that had been incubated with test serum to that in wells incubated with naive control macaque serum.

Antibody-dependent phagocytosis (ADP). Phagocytosis by serum antibodies was measured using the THP-1 monocytic cell line (ATCC) and fluorescent beads labeled with gp120 SHIV,2535 (Immune Technology). The THP-1 cells were maintained at a low density (0.5 × 10^6/ml) in RPMI 1640 medium containing HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.4 mg/ml tunicamycin, 1 mM sodium pyruvate and 10% FBS (all Gibco). For the ADP assay, 20 µl of 1 µM neutravidin-labeled Fluorospheres (Invitrogen) were washed in 1 ml of sterile 1% BSA in PBS in a sterile 1.5 ml microcentrifuge tube that had been preblocked with BSA overnight at 4°C. The beads were resuspended in 200 µl of 1% BSA containing 7 µg of biotinylated anti-HIV tag mouse monoclonal antibody (ThermoFisher) and mixed at 1100 rpm for 1 hr at room temperature. The beads were then washed, resuspended in 200 µl of 1% BSA containing 14 µg of HIVgp120 SHIV 2535 protein (Immune Technology), and mixed at 1100 rpm overnight at 4°C. The following day, beads were washed and resuspended in 5.5 ml medium. The beads were added in a 25 µl volume to wells of V-bottom plates containing 25 µl of diluted sterile-filtered test serum, pooled serum from naive control rhesus macaques or a positive control anti-HIV Env human monoclonal IgG antibody (3BNC117; AIDS Reagent Program). Serum samples were tested in triplicate at 5-fold dilutions starting with a 1:20 final dilution. After a 30 min incubation at 37°C and 5% CO_2, 2 × 10^4 THP-1 cells in 50 µl were added to each well. The plates were then incubated for 5 h. The assay was terminated by centrifuging the plates at 300 × g for 5 min, removing the medium, and resuspending the cells in 200 µl of 1% paraformaldehyde. After storage at 4°C overnight, the cells were analyzed for fluorescence by flow cytometry. To determine the phagocytic score, the percentage of cells containing fluorescent beads (head-positive) were counted (head-negative cells were a test multiple). The % binding signal by a single peptide within the epitope region was calculated by subtracting the value obtained for naive control serum at the same dilution. The results are presented for 1:20 serum dilutions, which produced peak results. The 3BNC117 antibody (NIH AIDS Reagent Program) produced a score of 6.5 at 1 µg/ml.

Flow cytometry and T-cell responses. PBMCs were stimulated with Gag (clade B consensus) and Env (clade C consensus) peptides (2 µg/ml) per well (3BNC117; AIDS Reagent Program) along with anti-CD28 (BD) and anti-CD49d (BD) co-stimulatory molecules (1 µg/ml final each) for two hours before the addition of GolgiStop (BD) and GolgIPlug (BD) and incubated for an additional four hours. PBMCs were then surface stained with antibodies against CD3 (BD, SP34-2, Cat# 562381, 1:400 dilution), CD4 (BD, SK3, Custom, 1:2000 dilution), CD8 (BD, RPA-T8, Custom, 1:600 dilution), and LIVE/DEAD Fixable stain (ThermoFisher), and analyzed by flow cytometry using a FACSCalibur (BD) and a Gallios (Beckman Coulter). All antibodies used at a dilution of 1:40.

Rhesus macaque tissue digestion and flow cytometry. Uninfected rhesus macaques scheduled for necropsy were euthanized and their buccal tissue, sublingual tissue, and submandibular, submental, and inguinal lymph nodes were collected. Paraffin-embedded tissues were sectioned and stained with Hematoxylin and eosin. Paraffin tissue was characterized via immunohistochemistry with an antibody to Langerin to identify the tissue parenchyma. Sublingual tissue, lingual tissue, and submandibular, submental, and inguinal lymph nodes were digested with 200 U/ml Collagenase IV (Worthington) and 0.03% DNAse-I (Life) for two hours before mechanical disruption with a syringe and needle (gauge 12), passage through a cell strainer, and washed with RPMI + 10% FBS. Alternatively, buccal and sublingual tissue were paraffin-embedded and analyzed via H&E stain or immunohistochemistry with an anti-Langerin monoclonal antibody (Adcam, Cat# ab192027). Single-cell suspensions were analyzed via flow cytometry with the following anti-human monoclonal antibodies: CD3 (BD, DCN46, Cat# 564127), CD4 (BD, Biologend, L61, Cat# 333151), CD123 (BD, 73G, Cat# 562391), CD45 (BD, D058-1283, Cat# 5638661), CD14 (BD, M52E, Cat# 5640555), CD65 (BD, 3G, Cat# 5636991), CD3 (BD, SP3-2, Cat# 561805), CD20 (BD, 2H7, Cat# 560651), and LIVE/DEAD Fixable stain (ThermoFisher). All antibodies used at a dilution of 1:40. Characteristics of retinoic acid induction in macaque cells was performed using the ALDEFLUOR kit (StemCell) per the manufacturer’s instructions. After incubation with the ALDEFLUOR reagent in test and control settings, cells were stained and analyzed via flow cytometry. ALDEFLUOR+ cell gating was based on the control samples. %ALDEFLUOR+ cells were calculated by subtracting the ALDEFLUOR− cell frequency in the control sample gate from the test sample gate.

Statistical analysis. Statistical analysis was performed using Graphpad Prism v7.0. Additional univariate correlation analysis between number of challenges and individual immune measurements was by Kendall’s Correlation test performed using R and subsequently confirmed using SAS. Principal component analysis (PCA) was performed in R using the prcomp package. Variables for PCA were pre-selected by ranking of separation between groups, which is defined as the between-group variance divided by within-group variance for each variable.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this Article.

Data availability

All data associated with this manuscript are available upon request.

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Author contributions
A.T.J., R.V. and R.R.A. designed the studies. A.T.J. led the animal studies, processed samples, performed assays, and interpreted data. X.S. and G.D.T. performed BAMA experiments, linear peptide array mapping, and PCA modeling. K.L.W. and P.A.K. performed mucosal antibody characterization, ADP, and ADCVI assays. C.C.L. and D.C. M. performed neutralizing antibody assays. L.S.W. and B.M. generated and provided the MVA-HIV. D.H.B. provided the SHIV-SF162P3, J.D.C. provided the dmlT adjuvant, R. V. provided plasmid encoding JRFL-cycP-gp120 and contributed to discussions. A.T.J. and R.R.A. led the studies and wrote the paper along with all co-authors.

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