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Rhesus macaque rectal and duodenal tissues exhibit B-cell sub-populations distinct from peripheral blood that continuously secrete antigen-specific IgA in short-term explant cultures

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Abstract

It is becoming increasingly obvious that evaluation of a vaccine aimed at preventing HIV infection should include assessment of induced immunity at mucosal sites of viral entry. Among the most salient immune responses are viral-specific antibodies. A recent report on IgA-secreting plasma cells in human duodenal explants prompted us to examine similar duodenal and rectal biopsies of rhesus macaques, a key animal model for pre-clinical HIV/SIV vaccine studies, and characterize the local resident B-cells. Here we report that non-human primate rectal explants possess similar levels of B-cells as duodenal explants. We characterize the antibody isotype expression on mucosal memory B-cells and show for the first time that the B-cell memory subsets of the duodenum and rectum are distinct from those of PBMC, not only by essentially lacking CD27⁺ cells, as previously reported for uninfected macaques (Titanji et al., 2010), but also in being mostly IgD[−]. SIV- and SHIV-infected macaques had fewer total IgA-secreting cells in rectal tissue compared to naïve macaques. As expected, the fractions of B-cells with surface expression of IgA were dominant in the rectal and duodenal explants whereas in PBMC IgG surface expression was dominant among IgD[−] B-cells. Mucosal antibody secreting cells were found to be predominantly plasma cells/plasma blasts based on their lack of response to stimulation. Importantly, short-term culture of rectal explants of SIV- and SHIV-positive animals led to secretion of Env-specific IgA into the culture supernatant which could be easily measured by ELISA. Collection of such culture supernatant over several days allows for accumulation of mucosal antibody in amounts that should enable antibody purification, characterization, and use in functional assays. Rectal explants can be readily obtained and unequivocally identify the mucosal tissue as the source of antibody. Overall they facilitate evaluation of mucosal vaccines.

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Keywords

SIV/SHIV infected rhesus macaques; mucosal explant cultures; memory B-cells; IgA; AIDS vaccine

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Introduction

Current efforts in HIV vaccine development include eliciting protective immune responses at mucosal sites [1], the main route of acquisition [2]. We are developing a prophylactic HIV vaccine based on replication-competent Adenovirus (Ad)-recombinant priming and envelope protein boosting [3]. Advantages of this strategy include replication of the vector at multiple mucosal sites and its targeting and persistence in macrophages and professional antigen presenting cells in rectal tissue [4]. The approach has induced localized mucosal SIV-specific IgA antibody in a rhesus macaque model that correlated with reduced viremia following single high-dose SIV_{mac251} and SHIV_{89.6P} rectal challenges [5, 6] and delayed acquisition following repeated low-dose rectal challenges with SIV_{mac251} [7].

HIV vaccine candidates are commonly evaluated in non-human primates, which effectively model HIV infection of people [8] and allow sampling of multiple tissues. In this model humoral mucosal immunity is routinely evaluated by measuring antigen-specific antibody in mucosal secretions obtained using swabs [5–7], Weck-cel sponges [9, 10], or lavages [11, 12]. An advantage of these methods is that samples can be collected throughout the course of a study with little trauma to the mucosal surface [5–7, 13]. However frequent blood contamination and the possibility that the Ig being measured arose through exudation makes determination of the Ig origin difficult. Additionally, the amount of Ig obtained by these methods is small, limiting extensive characterizations and downstream functional assays.

Recently it was reported that human duodenal explants harbor long-lived IgA secreting plasma cells [14]. Culture of this tissue may yield large amounts of secreted antibody. A limitation of duodenal explants however, is that they can only be obtained at necropsy or by endoscopy [15], outside the reach of many labs. We hypothesized that rectal explants should be similar to duodenal explants with regard to IgA-secreting B-cells. Together with easier accessibility, rectal explants should provide similar advantages as duodenal explants. We herein investigated this hypothesis. B-cells at both sites were characterized by flow cytometry for memory phenotype and surface Ig expression. Antibody secreting cells (ASC) were enumerated by ELISPOT. Secretion of predominantly IgA into the supernatant medium of explant cultures was confirmed, and antibody levels obtained over time were determined. We show that rectal explant cultures provide a ready source of mucosal IgA in quantities that should be sufficient for antibody isolation, characterization, and use in functional assays.

Material and Methods

Animals

Indian rhesus macaques were maintained at Advanced BioScience Laboratories, Inc. (ABL), according to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International and the Guide for the Care and Use of Laboratory Animals of the NIH. Animal protocols were approved by the ABL Animal Care and Use Committee prior to implementation. Rectal and/or duodenal pinch biopsies were randomly obtained as subsets from 4 groups of macaques. SIV_{mac251}-infected macaques ($n = 18$) had been used for *in vivo* titration experiments and were in the early chronic viremia phase. Their viral loads ranged from 1.4×10^4 to 3.3×10^8 (geometric mean of 4.4×10^6 SIV RNA

copies/ml plasma). SHIV_{SF162P4}-infected macaques ($n = 15$) had been vaccinated by an Ad-HIV_{env} prime/Env protein boosting regimen prior to SHIV infection. The samples were obtained at necropsy, late in the course of infection when the macaques exhibited undetectable or low viremia, ranging from <50 to 1.2×10^5 SHIV RNA copies/ml plasma (geometric mean of 4.2×10^2 , calculated by setting < 50 copies = to 50). Vaccinated macaques ($n = 4$) had received Ad-SIV_{env} followed by boosting with SIV gp120 protein. Samples were obtained at necropsy, 2 weeks later. Naïve macaques ($n = 6$) comprised the fourth group.

Sample collection

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by centrifugation over ficoll-paque Plus (GE Healthcare, Sweden) [5, 16]. Rectal pinch biopsies, 10/macaque, were obtained from macaques restrained using Domitor or Ketamine/xylazine with Antesedan or Yohimbine as a partial reversal agent and Isoflurane inhalant anesthesia. The animal was maintained in a prone position, perineum elevated. A speculum with light source was inserted into the rectum. A 3 mm Radial Jaw disposable biopsy instrument was advanced into the rectum approximately 3–5 cm. Biopsies were obtained circumferentially and placed in RPMI1640 medium. Duodenal pinch biopsies (10/macaque) were obtained following euthanasia using an intravenously administered overdose of Beuthanasia. A section of duodenum was isolated and opened sagittally. An Olympus 2 mm biopsy instrument was used to obtain pinches from the dissected section of duodenum. Rectal secretions were obtained using cotton-tipped swabs and placed in 1 ml of PBS containing 0.1% bovine serum albumin, 0.01% thimerosal, and 750 Kallikrein inhibitor units of aprotinin. The swabs were stored at -70°C prior to assay.

Flow cytometric evaluation of B-cell subsets

Rectal and duodenal pinch biopsies (4/macaque) were rinsed with pre-warmed RPMI1640 (Invitrogen) containing $2\times$ Antibiotic-Antimycotic solution, 2-mM L-Glutamine (Invitrogen) and 2 mg/ml Collagenase (Sigma-Aldrich). Prior to incubation (25 min at 37°C) the pinches were minced using a scalpel and a 19G needle, transferred in 10 ml of the same media to a 50 ml tube and pulse vortexed every 5 min. The digested tissue was passed 5 times through a blunt end cannula. The liberated cells and tissue debris were passed through a $70\text{-}\mu\text{m}$ cell strainer, and washed in R10 (RPMI1640 containing $2\times$ Antibiotic-Antimycotic solution, L-glutamine and 10% FBS).

Isolated single cells from pinches and PBMC were washed with PBS and surface stained with the following antibodies: CD2 (Qdot605, S5.5), CD14 (Qdot605, Tuk14), and Aqua viability from Invitrogen; CD19 (PE-Cy5, J1-119) from Beckman Coulter (Miami, FL); CD20 (EF650NC, 2H7) and CD27 (PerCP-eF710, O323) from eBioscience (San Diego, CA); CD21 (PE-Cy7, B-ly4), IgM (APC, G20-127), and IgG (APC-Cy7, G18-145) from BD Biosciences (San Jose, CA); and IgD (Texas-Red, polyclonal) and IgA (FITC, polyclonal) from Southern Biotech (Birmingham, AL). After a final wash, cells were resuspended in PBS containing 2% Formaldehyde (Tousimis, Rockville, MD). Within 2 hours of staining, cells were diluted in sheath fluid, passed through a $35\text{-}\mu\text{m}$ cell strainer, and a minimum of 10,000 live cells in the lymphocytic gate were acquired on a 4-laser LSRII SORP (BD Biosciences). Analysis was performed with FlowJo (v9.5.2, Tree-Star Inc.). Gates were set according to combinations of FMO, isotype and internal controls.

Explant culture

Mucosal pinches were washed and cultured in sodium bicarbonate buffered R10 (5ml 7.5% Na_2HCO_3 (Invitrogen)/500mL R10) at 37°C and 5% CO_2 in 48-well plates. Four pinches per well were cultured in the presence (Stim) or absence (Un-Stim) of 50 ng/ml hIL-21

(Peprotech), 0.1 µg/ml CpG ODN2006 (MWG Operon) and 0.5 µg/ml sCD40L (Peprotech). Media was replaced with 0.6 ml fresh R10 at the end of the day. Supernatants were collected the following day (day 1) and thereafter as indicated in the figures. Explants were kept in culture, replacing complete medium on each collection day. Culture supernatants were centrifuged and preserved at -20°C for further analysis.

Quantification of ASC in mucosal biopsies

Following three days of culture with or without stimulation, single cells were isolated from rectal and duodenal pinches by incubating in R10 containing 1 mg/ml collagenase (37°C for 30 min) under constant rotation. The digested tissues were passed through a cannula eight times and filtered through a 40-µm cell strainer (BD Biosciences). The filtered suspensions were centrifuged and the cell pellet was resuspended in 40% Percol (Sigma-Aldrich). After centrifugation the Percol was removed, and the cell pellet was washed and counted. Total IgG and IgA and SIV Env-specific IgA ASC were quantified by ELISPOT [17]. Spots were counted using an automated ELISPOT reader (Axioplan 2 imaging; Zeiss, Germany). Total and specific IgG and IgA ASC from triplicate wells were recorded. Results are expressed as % Env-specific ASC: (specific ASC/total ASC) x100.

Quantification of secreted antibody in explant media and swabs

Rectal swab samples were thawed and passed through a 5-µm PVDF microcentrifugal filter unit (Millipore). The flow-through was collected and stored at -20°C until analysis. Total and SIV Env-specific IgA in culture media and the extracted swabs were quantified by ELISA as previously described [16]. Concentrations of total IgA (in µg/ml) were determined using a standard curve made with purified Rhesus IgA (NIH Non-Human Primate Reagent Resource). Concentrations of SIV Env-specific IgA (in ng/ml) were determined using a pool of Rhesus macaque serum obtained from SIV_{mac251}-infected macaques that had been depleted of IgG and titrated specifically for SIV Env-specific IgA [16, 18, 19]. Standard curves for SIV Env-specific IgA ranged from 50 to 1.56 ng/ml and in preliminary tests using rectal secretions from 18 naïve macaques all sample concentrations were below 1 ng/ml. Final SIV Env-specific concentrations in explant media and rectal secretions were calculated by normalizing Env-specific values to total IgA and reported as % Env-specific IgA (ng Env-specific IgA/µg total IgA).

Statistical analysis

All graphs and statistics were obtained using Prism v5.04 or v6.0 (GraphPad). Differences between individual measures were assessed using a paired test. Differences between groups were assessed using ANOVA. P-values < 0.05 were deemed significant.

Results

Total B-cell populations of duodenal and rectal explants are similar

B-cells of paired biopsies from SHIV_{SF162P4}-infected macaques with low viral loads were evaluated by flow cytometry. The total B-cell population of each tissue was identified (Fig. 1A–C). Total B-cell percentages in duodenal samples showed greater variation than those of rectal explants (Fig. 1D). Nonetheless the means were not significantly different.

Memory B-cell distributions of duodenal and rectal samples are distinct from PBMC

B-cell memory subsets in PBMC of macaques were previously delineated using CD21 and CD27 [20]. We subsequently confirmed the same memory B-cell subpopulations in blood, bone marrow, and lymph nodes of chronically SIV-infected rhesus macaques [21]. The four PBMC subpopulations: naïve (CD21⁺CD27⁻), and resting (CD21⁺CD27⁺), activated

(CD21⁺CD27⁺) and tissue-like (CD21⁺CD27⁻) memory, are shown (Fig. 2A). Although the different subsets displayed varying levels of IgD⁺ cells, the CD21⁺CD27⁻ naïve B-cell subset contained a significantly higher fraction (Fig. 2B) as expected. Naïve cells, by definition, have not undergone class-switching [22, 23]. In contrast, B-cells from rectal and duodenal samples of the same animals were distinctly different and contained almost no resting (CD21⁺CD27⁺) or activated (CD21⁺CD27⁻) memory subpopulations (Fig. 2D), consistent with a previous report for uninfected macaques [20]. Here the fraction of IgD⁺ surface expressing cells found on “naïve” (CD21⁺CD27⁻) B-cells from rectal and duodenal biopsies were similar to each other and about four-fold lower than that of PBMCs (Fig. 2B,D) suggesting that they had undergone class-switching and were not really naïve. Representative dot plots of CD21 gated IgD⁺ B cells of PBMC and a duodenal sample from the same macaque are shown in Figures 2C and F, respectively. Together with the nearly total lack of resting and activated memory B-cells, the low levels of IgD⁺ cells in the “naïve” and “tissue-like” subpopulations suggest that B-cell memory subsets as defined by CD21 and CD27 are inappropriate for characterizing B-cells of duodenal and rectal samples.

IgA predominates in duodenal and rectal biopsies

We further investigated the proportions of IgM, IgA and IgG expressing IgD⁺ CD21/CD27 defined B-cells. Among IgD⁺ B-cell subsets of PBMCs, IgM-expressing cells were lower than IgA-expressing cells which were significantly lower than the IgG-expressing cells across all subsets (Fig. 3A). Similar to PBMCs, duodenal and rectal biopsies contained low proportions of IgD⁺ IgM surface-expressing cells. However, in contrast to PBMCs, duodenal and rectal biopsies contained significantly higher proportions of IgD⁺ IgA surface-expressing cells relative to IgG (Fig. 3B–C). Thus in both duodenal and rectal biopsies IgA-expressing cells predominate independent of CD21/CD27 classifications. Overall the proportions of different Igs were similar in duodenal and rectal explants.

Rectal biopsies contain Env-specific IgA ASC

Expression of intracellular immunoglobulin is a hallmark of both Ig-secreting plasma blasts and plasma cells [24]. Further, immunoglobulins of plasma cells are located only intracellularly [25], while pre-plasma cell blasts that differentiate into plasma cells can exhibit both surface and intracellular Ig expression depending on their maturation state [26]. To avoid this complexity of both intracellular and surface staining, we used ELISpot analysis to confirm the predominance of IgA ASC in mucosal samples, measuring total IgG- and IgA-secreting cells in rectal explants of naïve macaques. The explants were cultured for 3 days in the presence or absence of a B-cell stimulation cocktail known to induce maturation [17]. Mean levels of total IgG-secreting cells were 10 fold lower than those secreting IgA ($p < 0.01$; repeated measures ANOVA), with no difference seen between stimulated and un-stimulated cells (Fig. 4A). We did not evaluate IgG-secreting cells in subsequent assays.

We examined total IgA ASC in mucosal biopsies of SHIV- (Fig. 4B–C) and SIV- (Fig. 4E–F) infected macaques. No significant differences were observed in total IgA ASC between rectal and duodenal explants from either SHIV- or SIV-infected macaques. Total IgA ASC were slightly decreased in stimulated rectal explants of SHIV-infected macaques ($p = 0.031$; Wilcoxon signed rank test; Fig. 4B), leading to the speculation that the rectal explants harbored more plasma cells than plasma blasts. This was not seen in the ELISPOT data for the other rectal and duodenal tissues investigated (Fig. 4D, E, F), and needs to be confirmed by study of additional macaques. We next assessed whether these mucosal explants contained Env-specific IgA-secreting cells. Rectal explants from both SHIV- and SIV-infected macaques contained levels of Env-specific IgA ASC similar to those of duodenal explants (Fig. 4D, G). No increase was seen following B-cell stimulation.

Env-specific IgA levels in rectal and duodenal explant cultures are not increased by B-cell stimulation

We compared IgA levels in the culture medium of rectal and duodenal explants of SIV-positive animals cultured with and without B-cell stimulation. Following one day of culture, comparable levels of SIV Env-specific IgA were observed in both rectal and duodenal explants (Fig. 5A). Sequential collection of culture medium to day 5 showed continual secretion of Env-specific IgA by both rectal and duodenal explants, although antibody levels gradually declined (Fig. 5B–C). This progressive decrease in IgA levels can be attributed to a decrease in viable cells, evidenced by direct viability counts as well as by staining for caspase 3 activity by flow cytometry (data not shown). Improved viability and maintenance of IgA expression by single-cell suspension cultures of human duodenal explants was previously reported using irradiated PBMC feeder cells [14], although this was not attempted here. Overall, no differences were observed between un-stimulated and stimulated cultures.

Env-specific IgA in rectal explant culture medium compared to that in rectal swabs

Env-specific IgA in the medium of rectal explants from vaccinated and SIV-infected macaques was compared to that extracted from rectal swabs from the same animal. In each group of macaques levels of Env-specific IgA in rectal explant supernatants were higher than those obtained from rectal swabs although not significantly so (Fig. 6A,B). Combining the vaccinated and SIV-infected groups yielded a marginally non-significant difference of Env-specific IgA in culture supernatants compared to swabs (Fig. 6C; $p = 0.052$). Nevertheless, collection of IgA secreted by 14 explants over days 1–5 gave a 3-fold higher total than that obtained for rectal swabs collected on a single occasion ($3.53 \text{ ng}/\mu\text{g}$ vs $1.03 \text{ ng}/\mu\text{g}$; $p < 0.001$), indicating a potential advantage to accumulating IgA in culture supernatants over time for further characterization.

Discussion

HIV and SIV infect the gastrointestinal tract mucosa, leading to massive depletion of CD4^+ T-cells [27–30]. Consequently, gut T-cell populations of both humans and non-human primates have been extensively studied. Fewer studies have examined B-cells at different intestinal sites, especially in rhesus macaques, a key animal model for pathogenesis and pre-clinical vaccine studies. Our interest in developing a mucosally effective HIV vaccine prompted us to examine new strategies to quantify vaccine-induced mucosal immunity. We investigated the suitability of rectal biopsies for assessment of mucosal antibody responses. No such comparison of B-cells in duodenal and rectal tissues has been reported, although a few studies have examined B-cells at one or the other site. In one study, lowered proportions of total B-cells were reported in the jejunum compared to the duodenum, ileum and colon [30]. Cholera toxin (CT)-specific IgA ASC were reportedly induced in the rectum and vagina of nonhuman primates following CT vaccination [31]. Others concluded that the rectal lamina propria of infected macaques exhibited decreased IgA-positive cells in comparison to naïve macaques [32, 33]. The absence of comparisons of paired duodenal and rectal samples from the same macaque led us to investigate whether rectal biopsies might contain long-lived IgA^+ ASC as reported for human duodenal samples [14].

Flow cytometric analyses of paired duodenal and rectal biopsies showed no differences in total (Fig. 1D), or IgD^+ B-cell percentages (Fig. 2B). A distinct phenotypic difference of memory B-cells between the mucosal tissues and PBMC was seen (Fig. 2A–B). Duodenal and rectal memory B-cells essentially lacked CD27 and most were IgD^- , independent of infection status (data not shown). We previously reported that unstimulated macaque bone marrow tissue-like memory B-cells ($\text{CD21}^-\text{CD27}^-$) secrete immunoglobulin, confirming a

plasma cell phenotype [20]. Here, the high proportion of switched IgD⁺ cells in the CD21⁺CD27⁺ and CD21⁺CD27⁻ subpopulations of rectal and duodenal tissue, suggests that the four memory B cell subpopulations of PBMC cannot be applied to mucosal tissue.

The gut mucosa is replete with IgA ASC [14, 34, 35]. This was confirmed here by surface staining of IgD⁺ B-cells (Fig. 3) and by ELISPOT (Fig. 4). Although comparing IgA ASC levels between naïve and SIV-infected macaques was not a goal of our study, we observed fewer total IgA ASC in the rectal mucosa of SHIV- and SIV-infected macaques compared to naïve macaques (Fig. 4A, B, E). Env-specific IgA ASC (Fig. 4D, G) were readily detected, whereas others have reported a lack of IgA induction in duodenal biopsies following SIV infection [36]. This difference may have resulted from fewer pinches used or different culture conditions in the previous study [36]. We observed a rapid acidification of the culture medium even after one day in culture which required sodium bicarbonate buffering. Our ELISPOT and ELISA techniques may also have provided greater sensitivity compared to the western blot used previously. Importantly, we used SIV-infected macaques early in chronic infection before significant B-cell dysfunction had occurred, perhaps facilitating the ready detection of SIV Env-specific IgA.

We previously showed our B-cell stimulation cocktail converts memory B-cells to plasma blasts/plasma cells [17]. No increase in IgA ASC or antibody production following stimulation was detected in rectal or duodenal explants (Fig. 4–5), indicating both sites already harbor IgA-secreting plasma cells as reported for human duodenal mucosa [14]. Inguinal lymph nodes, not studied here, have also been shown to exhibit increased frequencies of memory B cells associated with increased SIV-specific antibodies in SIV-infected macaques [37]. APRIL and IL-6, necessary for long-term plasma cell survival in human duodenal mucosa [14], did not increase IgA levels in the culture media of macaque explants (data not shown), suggesting that explant cells already provided these factors. Single cell suspensions of human duodenal explants have exhibited sustained expression of IgA in the presence of irradiated PBMC feeder cells, indicating that PBMC can also provide essential factors for maintenance of IgA ASC [14]. We did not attempt such experiments and therefore do not know if such would have altered the non-significant progressive decrease in levels of IgA observed in Figure 5.

Measurement of IgA in cultured explant supernatants may provide greater sensitivity compared to rectal swabs, however, the difference in viral-specific IgA levels as illustrated in Figure 6C only neared statistical significance ($p=0.052$). Assessment of greater numbers of paired samples will be needed to confirm this difference. It is clear, however, that greater quantities of IgA can be accumulated from explant cultures by collecting the supernatant over time (Fig. 5 B,C), compared to what can be acquired from a single swab. Such acquisition of additional sample would facilitate characterization of mucosal antibody.

Intrarectal administration of a neutralizing monoclonal dimeric IgA1 specific for the HIV V3 loop previously protected 5/6 macaques from SHIV acquisition following rectal challenge [38]. The protection was correlated with virion capture activity and inhibition of transcytosis. It will be of interest to determine if vaccine elicited mucosal antibody mediates similar protective activity. But mucosal antibodies will require purification to confirm functionality [39]. Factors such as secretory leukocyte protease inhibitor (SLPI) and human lactoferrin in mucosal secretions have been shown to inhibit HIV [40,41], thus resembling virus neutralization [42]. The short-term culture system used here should facilitate purification and characterization of mucosal IgA and elucidation of its role in protective efficacy.

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References

1. Demberg T, Robert-Guroff M. Mucosal immunity and protection against HIV/SIV infection: strategies and challenges for vaccine design. *Int Rev Immunol*. 2009; 28:20–48. [PubMed: 19241252]
2. Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu Rev Med*. 2011; 62:127–139. [PubMed: 21054171]
3. Patterson LJ, Robert-Guroff M. Replicating adenovirus vector prime/protein boost strategies for HIV vaccine development. *Expert Opin Biol Ther*. 2008; 8:1347–1363. [PubMed: 18694354]
4. Patterson LJ, Kuate S, Daltabuit-Test M, Li Q, Xiao P, McKinnon K, et al. Replicating adenovirus-simian immunodeficiency virus (SIV) vectors efficiently prime SIV-specific systemic and mucosal immune responses by targeting myeloid dendritic cells and persisting in rectal macrophages, regardless of immunization route. *Clin Vaccine Immunol*. 2012; 19:629–637. [PubMed: 22441384]
5. Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, et al. Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. *J Virol*. 2009; 83:791–801. [PubMed: 18971271]
6. Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, et al. Multiple vaccine-elicited nonneutralizing anti-envelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. *J Virol*. 2010; 84:7161–7173. [PubMed: 20444898]
7. Xiao P, Patterson LJ, Kuate S, Brocca-Cofano E, Thomas MA, Venzon D, et al. Replicating adenovirus-simian immunodeficiency virus (SIV) recombinant priming and envelope protein boosting elicits localized, mucosal IgA immunity in rhesus macaques correlated with delayed acquisition following a repeated low-dose rectal SIV(mac251) challenge. *J Virol*. 2012; 86:4644–4657. [PubMed: 22345466]
8. Lifson JD, Haigwood NL. Lessons in nonhuman primate models for AIDS vaccine research: from minefields to milestones. *Cold Spring Harb Perspect Med*. 2012; 2:a007310. [PubMed: 22675663]
9. Kozlowski PA, Lynch RM, Patterson RR, Cu-Uvin S, Flanigan TP, Neutra MR. Modified wick method using Weck-Cel sponges for collection of human rectal secretions and analysis of mucosal HIV antibody. *J Acquir Immune Defic Syndr*. 2000; 24:297–309. [PubMed: 11015145]
10. Lai L, Kwa SF, Kozlowski PA, Montefiori DC, Nolen TL, Hudgens MG, et al. SIVmac239 MVA vaccine with and without a DNA prime, similar prevention of infection by a repeated dose SIVsmE660 challenge despite different immune responses. *Vaccine*. 2012; 30:1737–1745. [PubMed: 22178526]
11. Crotty S, Lohman BL, Lu FX, Tang S, Miller CJ, Andino R. Mucosal immunization of cynomolgus macaques with two serotypes of live poliovirus vectors expressing simian immunodeficiency virus antigens: stimulation of humoral, mucosal, and cellular immunity. *J Virol*. 1999; 73:9485–9495. [PubMed: 10516057]
12. Wright PF, Kozlowski PA, Rybczyk GK, Goepfert P, Staats HF, VanCott TC, et al. Detection of mucosal antibodies in HIV type 1-infected individuals. *AIDS Res Hum Retroviruses*. 2002; 18:1291–1300. [PubMed: 12487817]
13. Manrique M, Kozlowski PA, Cobo-Molinos A, Wang SW, Wilson RL, Montefiori DC, et al. Immunogenicity of a vaccine regimen composed of simian immunodeficiency virus DNA, rMVA, and viral particles administered to female rhesus macaques via four different mucosal routes. *J Virol*. 2013; 87:4738–4750. [PubMed: 23408627]
14. Mesin L, Di Niro R, Thompson KM, Lundin KE, Sollid LM. Long-lived plasma cells from human small intestine biopsies secrete immunoglobulins for many weeks in vitro. *J Immunol*. 2011; 187:2867–2874. [PubMed: 21841131]

15. Serra S, Jani PA. An approach to duodenal biopsies. *J Clin Pathol.* 2006; 59:1133–1150. [PubMed: 16679353]
16. Patterson LJ, Daltabuit-Test M, Xiao P, Zhao J, Hu W, Wille-Reece U, et al. Rapid SIV Env-specific mucosal and serum antibody induction augments cellular immunity in protecting immunized, elite-controller macaques against high dose heterologous SIV challenge. *Virology.* 2011; 411:87–102. [PubMed: 21237474]
17. Brocca-Cofano E, McKinnon K, Demberg T, Venzon D, Hidajat R, Xiao P, et al. Vaccine-elicited SIV and HIV envelope-specific IgA and IgG memory B cells in rhesus macaque peripheral blood correlate with functional antibody responses and reduced viremia. *Vaccine.* 2011; 29:3310–3319. [PubMed: 21382487]
18. Bertley FM, Kozlowski PA, Wang SW, Chappelle J, Patel J, Sonuyi O, et al. Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *J Immunol.* 2004; 172:3745–3757. [PubMed: 15004179]
19. Manrique M, Kozlowski PA, Wang SW, Wilson RL, Micewicz E, Montefiori DC, et al. Nasal DNA-MVA SIV vaccination provides more significant protection from progression to AIDS than a similar intramuscular vaccination. *Mucosal Immunol.* 2009; 2:536–550. [PubMed: 19741603]
20. Titanji K, Velu V, Chennareddi L, Vijay-Kumar M, Gewirtz AT, Freeman GJ, et al. Acute depletion of activated memory B cells involves the PD-1 pathway in rapidly progressing SIV-infected macaques. *J Clin Invest.* 2010; 120:3878–3890. [PubMed: 20972331]
21. Demberg T, Brocca-Cofano E, Xiao P, Venzon D, Vargas-Inchaustegui D, Lee EM, et al. Dynamics of memory B-cell populations in blood, lymph nodes, and bone marrow during antiretroviral therapy and envelope boosting in simian immunodeficiency virus SIVmac251-infected rhesus macaques. *J Virol.* 2012; 86:12591–12604. [PubMed: 22973034]
22. Meffre E, Milili M, Blanco-Betancourt C, Antunes H, Nussenzweig MC, Schiff C. Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development. *J Clin Invest.* 2001; 108:879–886. [PubMed: 11560957]
23. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med.* 1998; 188:1679–1689. [PubMed: 9802980]
24. Odendahl M, Mei H, Hoyer BF, Jacobi AM, Hansen A, Muehlinghaus G, et al. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood.* 2005; 105:1614–1621. [PubMed: 15507523]
25. Dorn I, Schlenke P, Mascher B, Stange EF, Seyfarth M. Lamina propria plasma cells in inflammatory bowel disease: intracellular detection of immunoglobulins using flow cytometry. *Immunobiol.* 2002; 206:546–557.
26. Fernandes JR, Snider DP. Polymeric IgA-secreting and mucosal homing pre-plasma cells in normal human peripheral blood. *Intern Immunol.* 2010; 22:527–540.
27. Veazey RS, DeMaria M, Chalifoux LV, Shvets DE, Pauley DR, Knight HL, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science.* 1998; 280:427–431. [PubMed: 9545219]
28. Mattapallil JJ, Smit-McBride Z, McChesney M, Dandekar S. Intestinal intraepithelial lymphocytes are primed for gamma interferon and MIP-1beta expression and display antiviral cytotoxic activity despite severe CD4(+) T-cell depletion in primary simian immunodeficiency virus infection. *J Virol.* 1998; 72:6421–6429. [PubMed: 9658083]
29. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med.* 2004; 200:749–759. [PubMed: 15365096]
30. Schultheiss T, Stolte-Leeb N, Sopper S, Stahl-Hennig C. Flow cytometric characterization of the lymphocyte composition in a variety of mucosal tissues in healthy rhesus macaques. *J Med Primatol.* 2011; 40:41–51. [PubMed: 20698929]

31. Eriksson K, Quiding-Jarbrink M, Osek J, Moller A, Bjork S, Holmgren J, et al. Specific-antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination. *Infect Immun*. 1998; 66:5889–5896. [PubMed: 9826370]
32. Vajdy M, Veazey RS, Knight HK, Lackner AA, Neutra MR. Differential effects of simian immunodeficiency virus infection on immune inductive and effector sites in the rectal mucosa of rhesus macaques. *Am J Pathol*. 2000; 157:485–495. [PubMed: 10934152]
33. Jackson S, Moldoveanu Z, Mestecky J, Pitts AM, Eldridge JH, McGhee JR, et al. Decreased IgA-producing cells in the gut of SIV-infected rhesus monkeys. *Adv Exp Med Biol*. 1995; 371B:1035–1038. [PubMed: 7502747]
34. Mora JR, von Andrian UH. Differentiation and homing of IgA-secreting cells. *Mucosal Immunol*. 2008; 1:96–109. [PubMed: 19079167]
35. Macpherson AJ, Hunziker L, McCoy K, Lamarre A. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes Infect*. 2001; 3:1021–1035. [PubMed: 11580989]
36. Schafer F, Kewenig S, Stolte N, Stahl-Hennig C, Stallmach A, Kaup FJ, et al. Lack of simian immunodeficiency virus (SIV) specific IgA response in the intestine of SIV infected rhesus macaques. *Gut*. 2002; 50:608–614. [PubMed: 11950804]
37. Hong JJ, Amancha PK, Rogers K, Ansari AA, Villinger F. Spatial alterations between CD4+ T follicular helper, B, and CD8+ T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *J Immunol*. 2012; 188:3247–3256. [PubMed: 22387550]
38. Watkins JD, Sholukh AM, Mukhtar MM, Siddappa NB, Lakhashe SK, Kim M, et al. Anti-HIV IgA isotypes: differential virion capture and inhibition of transcytosis are linked to prevention of mucosal R5 SHIV transmission. *AIDS*. 2013; 27:F13–F20. [PubMed: 23775002]
39. Keren DF. Models to follow secretory IgA response to mucosal infections. *Pathol Immunopathol Res*. 1987; 6:128–136. [PubMed: 3321019]
40. Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, et al. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. *Oral Dis*. 1997; 3(Suppl 1):S64–69. [PubMed: 9456660]
41. Harmsen MC, Swart PJ, de Bethune MP, Pauwels R, De Clercq E, The TH, et al. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. *J Infect Dis*. 1995; 172:380–388. [PubMed: 7622881]
42. Wei Q, Moldoveanu Z, Huang WQ, Alexander RC, Goepfert PA, Mestecky J. Comparative Evaluation of HIV-1 Neutralization in External Secretions and Sera of HIV-1-Infected Women. *Open AIDS J*. 2012; 6:293–302. [PubMed: 23346267]

Highlights

Rhesus macaque mucosal memory B-cells, unlike those of PBMC, lack CD27⁺ cells.

Duodenal/rectal explants of SIV/SHIV-infected macaques contain IgA secreting cells.

Mucosal explant culture supernatants yield significant levels of viral-specific IgA.

Figure 1. Gating strategy for mucosal B-cells and distribution of total B-cells at mucosal site (A–C) Representative B-cell gating of a SHIV-challenged duodenal biopsy. B-cells were first gated on single viable lymphocytes as shown. CD2⁺ and CD14⁺ cells were gated out and B-cells were identified by CD19 and CD20 staining. (D) Total B-cell distribution in matched duodenal and rectal samples from SHIV-challenged (n = 11) macaques show no significant difference between the two anatomical sites.

Figure 2. Memory B-cell distribution in PBMC and at mucosal sites

(A) Representative dot plot of B-cells in PBMC ($n = 8$, SHIV-challenged) showing the established memory populations: naïve, resting, activated, and tissue-like memory cells, based on CD21 and CD27 gating. (B) In PBMC the CD21⁺/CD27⁻ naïve subset contains significantly more IgD⁺ B-cells than the other populations ($p < 0.0001$ for each). (C) Representative dot plot of CD21 gated IgD⁺ B cells in PBMC. (D) Representative dot plot of mucosal memory B-cell distribution in a duodenal biopsy. (E) IgD⁺ B-cell proportions are lower at the mucosal site (duodenum and rectum) compared to B-cell populations within PBMCs. The fractions of IgD⁺ (CD21⁺CD27⁻) naïve B-cells were higher than the fractions of IgD⁺ (CD21⁻CD27⁻) tissue-like memory B-cells ($p < 0.01$ in both duodenal and rectal samples). No significant difference in B-cell IgD⁺ expression was observed between matched samples of duodenum and rectum ($n=11$, SHIV-challenged). (F) Representative dot plot of CD21 gated IgD⁺ B cells in a duodenal biopsy. Error bar = SEM.

Figure 3. Distribution of IgM, IgA and IgG isotype expression among memory B-cell populations in PBMC and mucosal tissue

(A) Isotype expression on IgD⁺ B-cells in the four memory B-cell populations of PBMC (n = 8, SHIV-challenged). Proportion of IgG expressing cells is significantly higher than IgA in all memory populations (p < 0.0025). (B) Isotype expression among the two major memory populations in duodenum. The proportion of IgA expressing cells is significantly higher than that of IgG (p < 0.0001) with no difference between the memory populations. (C) Isotype expression among memory B-cells in rectum. The proportion of IgA expressing cells is significantly higher than that of IgG (p < 0.0001). In addition proportions of IgA expressing cells are higher in the CD21⁻CD27⁻ (tissue like) population compared to the CD21⁺CD27⁻ (naïve) population (p < 0.05). The converse is observed in the proportion of IgG expressing cells (p < 0.05). In B–C percentages were arcsine transformed prior to analysis by repeated measures ANOVA. Overall proportions were similar between Duodenum and Rectum (n=11, SHIV-challenged). Error bar = SEM.

Figure 4. Rectal biopsies contain Env-specific IgA secreting cells

(A–G) Rectal and/or duodenal explants were either polyclonally stimulated for 3 days (Stim) or not (Un-Stim) and thereafter ASC were enumerated by ELISPOT. (A) Average total IgG and IgA producing cells per 10^6 rectal cells from 5 naïve rhesus macaques are shown. IgG producing cells are on average 10 fold less than IgA producing cells ($p < 0.01$ by repeated measures ANOVA). (B–D) Total and HIV Env-specific IgA ASC were enumerated in SHIV-infected rhesus macaques ($n = 7$ for rectal biopsies and $n = 3$ for duodenal biopsies). Total IgA ASC in rectal samples of SHIV-infected macaques decreased following B cell stimulation ($p = 0.031$). (E–G) Total and SIV Env-specific IgA ASC were enumerated in SIV-infected rhesus macaques ($n = 6$). No differences were seen following B cell stimulation. Error bar = SEM.

Figure 5. Levels of Env-specific IgA measured over time are unchanged by B-cell stimulation
Rectal and/or duodenal explants from SIV-infected rhesus macaques were either polyclonally stimulated (Stim) or not (Un-Stim). Culture media were collected on the indicated days and levels of IgA determined by ELISA. **(A)** Mean level of Env-specific IgA in rectal explants cultured for one day is similar to that of duodenal explants ($n=5$, $p>0.05$). **(B, C)** The levels of Env-specific IgA in rectal ($n = 5$) **(B)** and duodenal ($n = 5$) **(C)** explant cultures over time are similar regardless of B-cell stimulation. Geometric means were calculated after substituting all zero values with 0.01; results were similar over a range of substituted values and are approximately equal to the medians. Error bar = geometric SEM.

Figure 6. comparison of Env-specific IgA levels in rectal explant culture supernatants and rectal swabs

(A) Env-specific IgA levels of paired day one rectal explant cultures (R Pinch) and rectal swabs (R swabs) from vaccinated macaques (n=4) and (B) from SIV-infected macaques (n=18). (C) Env-specific IgA levels of explant supernatants and rectal swabs compared in the combined vaccinated and SIV-infected animals (n=22). Env-specific IgA in rectal explant cultures (R Pinch) was marginally higher than that in paired rectal swabs (R Swab) although not significantly so ($p=0.052$). Zero values were replaced with 0.05 for graphing to allow visualization of values otherwise hidden.