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Phenotypes and distribution of mucosal memory B-cell populations in the SIV/SHIV Rhesus macaque model

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Abstract

As vaccine-elicited antibodies have now been associated with HIV protective efficacy, a thorough understanding of mucosal and systemic B-cell development and maturation is needed. We phenotyped mucosal memory B-cells, investigated isotype expression and homing patterns, and defined plasmablasts and plasma cells at three mucosal sites (duodenum, jejunum and rectum) in rhesus macaques, the commonly used animal model for pre-clinical vaccine studies. Unlike humans, macaque mucosal memory B-cells lacked CD27 expression; only two sub-populations were present: naïve (CD21⁺CD27⁻) and tissue-like (CD21⁻CD27⁻) memory. Similar to humans, IgA was the dominant isotype expressed. The homing markers CXCR4, CCR6, CCR9 and $\alpha 4\beta 7$ were differentially expressed between naïve and tissue-like memory B-cells. Mucosal plasmablasts were identified as CD19⁺CD20⁺-HLA-DR⁺Ki-67⁺IRF4⁺CD138⁺ and mucosal plasma cells as CD19⁺CD20⁻HLA-DR⁺Ki-67⁻IRF4⁺CD138⁺. Both populations were CD39⁺-CD27⁻. Plasma cell phenotype was confirmed by spontaneous IgA secretion by ELISpot of positively-selected cells and J-chain expression by real-time PCR. Duodenal, jejunal and rectal samples were similar in B-cell memory phenotype, isotype expression, homing receptors and plasmablast/plasma cell distribution among the three tissues. Thus rectal biopsies adequately monitor B-cell dynamics in the gut mucosa, and provide a critical view of mucosal B-cell events associated with development of vaccine-elicited protective immune responses and SIV/SHIV pathogenesis and disease control.

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Author Contributions

TD designed and conducted experiments, analyzed data, and wrote the paper; VM conducted experiments; DV conducted statistical analyses; MRG directed research, analyzed results, and wrote the paper.

Disclosure of Conflicts of Interest

The authors have no conflicts of interest to report.

Keywords

SIV/SHIV rhesus macaque model; mucosal memory B cell phenotypes and distribution; homing markers; plasmablasts/plasma cells

1. Introduction

Human Immunodeficiency Virus (HIV) infection is taking a toll on families, healthcare systems, and economies worldwide, but cannot be cured with currently available antiretroviral therapy [1]. Thus development of a prophylactic vaccine is critical. The rhesus macaque currently provides the best available animal model for evaluating candidate vaccines pre-clinically [2,3,4], both systemically and mucosally [5]. The latter is particularly important since both HIV and SIV replicate in the gut prior to systemic spread [6–8]. The goal of vaccination is induction of immune memory, and systemic B-cell populations have been investigated in macaques [9,10]. However mucosal memory B-cells, plasmablasts (PB) and plasma cells (PC) have not been fully characterized in macaque species [9–14].

Here we obtained mucosal biopsies and necropsy samples from rhesus macaques previously vaccinated and/or challenged with SHIV_{SF162P4} or SIV_{mac251}, and uninfected animals and investigated B-cell memory sub-populations at different mucosal sites. We compared mucosal B-cells with those in peripheral blood by flow cytometry, characterizing B-cell memory sub-populations, isotype expression, homing profiles, and PB/PC. Our results provide an extensive framework for evaluating B-cell maturation during vaccination and/or infection, and for determining critical B-cell properties associated with protection from viral acquisition and control of disease progression.

2. Methods

2.1 Macaque samples

Rhesus macaque mucosal tissues and PBMC were obtained at necropsy from 12 SHIV_{SF162P4}-infected animals (duodenum, n=12; jejunum, n=8; rectum, n=12, and PBMC, n=8) from a vaccine study (Thomas et al. in preparation). Eight of the macaques had been mucosally vaccinated with a replicating Ad recombinant expressing rhFLSC (HIV_{BaL}gp120 linked to the first two domains of rhesus CD4), with or without Ad-recombinants expressing SIV_{gag} and SIV_{nef}, followed by boosting with the rhFLSC protein. Four of the macaques were naïve controls or received empty vector/adjuvant as controls. At the time of necropsy, six of the macaques had undetectable viral loads, while six had a geometric mean viral load of 6.9×10^3 SHIV RNA copies/ml plasma. These macaques were used in studies addressing total memory B-cells, Ig isotypes and homing marker expression.

Mucosal samples were obtained from 5 SIV_{mac251}-infected animals from a viral titration early in chronic infection (duodenum and rectum, n=5 each) and from 1 uninfected animal (duodenum and rectum, n=1 each). These animals were used to examine initial plasma cell staining with CD138 between duodenum and rectum.

Rectal biopsies were obtained 2 weeks post-challenge from 30 SIV_{mac251}-infected animals from an ongoing vaccine study (Tuero et al., in preparation), from 4 animals infected with SIV_{mac251} in a viral titration experiment, and from 5 uninfected animals. The vaccinated animals (n = 24) had received replicating Ad-recombinants expressing SIV_{smH4env/rev}, SIV_{239gag} and SIV_{nef} mucosally followed by boosting with either monomeric SIV_{mac251} gp120 (n = 12) or oligomeric gp140 (n = 12) prior to intrarectal challenge with SIV_{mac251}. Controls (n = 6) received empty vector and adjuvant only. These samples were used to further characterize total rectal plasma cells and plasmablasts. Comparison of data obtained from the infected and uninfected animals by the Mann-Whitney test did not reveal any statistical difference. Thus the data presented here are from the combined data set.

All animals were housed at Advanced BioScience Laboratories, Inc. (ABL; Rockville, MD) or the NIH Bethesda Animal Facility according to the rules and regulations set forth by the NIH Guide for the Care and Use of Laboratory Animals and the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International. Experimental protocols were reviewed and approved by the ABL and NIH NCI Animal Care and Use Committees prior to implementation of experimental protocols.

2.2 Tissue preparation

Mucosal tissues were rinsed with pre-warmed digestive medium (RPMI1640, anti-fungal-bacterial solution, 2-mM L-Glutamine (all Invitrogen) and 2 mg/ml Collagenase (Sigma-Aldrich, St. Louis)) and minced in 5 ml digestive medium using a scalpel and 19G needle. The minced material was transferred into a 50 ml tube (Greiner) and media was added to 10 ml. Following 20–25 min digestion at 37°C with pulse vortexing every 5 min, samples were transferred into 6-well plates and passed 5 times through a blunt end cannula attached to a syringe. Liberated cells and tissue debris were passed through a 70 µm cell strainer and washed with 30 ml of R10 (RPMI1640 containing anti-fungal-bacterial solution, L-glutamine and 10% FBS). Cells were resuspended in R10 and equally distributed among FACS tubes. PBMC were isolated using a Ficollpaque (GE healthcare) gradient.

2.3 Magnetic sorting of CD138⁺ cells for ELISpot and PCR

Tissues were digested as above; cells were passed through a 35 µm cell strainer and washed. Cells were resuspended in 100 µl PBS containing 1% BSA (PBS/BSA) and CD138-PE antibody was added. After 25 min incubation on ice, cells were washed in PBS/BSA and resuspended in 100 µl of PBS/BSA. 20 µl of anti-PE magnetic beads were added and cells were incubated for 15–20 min on ice. Cells were washed and resuspended in 1 ml PBS 0.5% BSA and magnetically separated using a Miltenyi Automacs (program Possld). Isolated cells were counted and samples from randomly selected animals were checked for purity by flow cytometry. IgG and IgA ELISpots were quantified on CD138⁺ positively-selected cells by plating in R10 overnight at 37°C at a density of 2000 cells/well in triplicate as previously published [15], except a different HRP substrate was used (KPL, Germantown, MD) and plates were blocked with 1% BSA/PBS.

Real time PCR was performed on aliquots of the same positively-selected cells. Total RNA was isolated using the NucleoSpin RNA XS kit (Macherey-Nagel, Clontech, Mountain

View, CA) according to the manufacturer's instructions. J-chain primers were designed using human and rhesus macaque reference sequences and primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers and amplicons were tested against the Rhesus genome using BLAT to ensure optimal primer binding and amplification (<http://genome.ucsc.edu/index.html>). PCR reactions were performed in 25 µl using SYBR GreenER (Invitrogen, Carlsbad, CA) with the primers (18s: forw 5'-GCCCGAAGCGTTTACTTTGA-3', rev 5'-TCCATTATTCCTAGCTGCGGTATC-3' and J-chain: forw 5'-CCGGATTAAGTTCCAGGATCA-3', rev 5'-ATGGTGAGGTGGGATCAGAA-3') and the following program: 50°C 2 min; 95°C 10 min; 40 cycles of 95°C 30s; 59°C 30s; 72°C 45s, followed by melting curve analysis on a Applied Biosystem ABI7500 PCR machine (Life Technologies). Expression level differences were assessed using the Ct method.

2.4 Flow Cytometry

Cells ($1-2 \times 10^6$ /tube) were stained with antibodies listed in Table 1. After a 25 min surface staining, cells were washed in PBS, fixed and permeabilized according to the manufacturer's instructions using Fix and Perm or a transcription buffer set for IRF-4 (BD Bioscience, San Jose, CA). After washing in Permwash solution, intracellular staining was conducted. Subsequently, cells were washed and resuspended in PBS containing 2% Formaldehyde (Tusimis, Rockville, MD) and acquired within 2 hours on a custom 4-laser LSR II (BD Bioscience). Samples were diluted in sheath fluid and passed through a 35 µm cell strainer. A minimum of 50000 live cells in the lymphocytic gate were acquired in DIVA. Analysis was performed in FlowJo, and data were exported into Excel and Graphpad Prism 6.

2.5 Statistical analysis

Differences in B-cell distribution were assessed using repeated measures ANOVA on arcsine-transformed data. Differences in the expression of isotypes were assessed using repeated measures ANOVA. For analysis of CXCR4, CCR6, CCR9 and $\alpha 4\beta 7$, data were arcsine-transformed and repeated measures ANOVA was used. For analysis of HLA-DR, Ki-67 and loss of CD21 expression the Wilcoxon signed rank test was used. For analysis of J-chain expression, repeated measures ANOVA on log-transformed data was used. For comparison of IRF-4⁺HLA-DR⁺ and IRF-4⁺HLA-DR⁻ populations, repeated measures ANOVA was used. P values were not corrected for multiple comparisons. Differences with a p value <0.05 were considered significant. Some values <0.05 indicate noteworthy differences but are less certain of reaching the usual level of statistical significance.

3. Results

3.1 Mucosal memory B-cells

Memory B-cell profiles of duodenum, jejunum and rectum, exclusively CD27⁻, differed strongly from those of PBMC and bone marrow [9,10]. Instead of four PBMC memory B-cell populations distinguished by CD21/CD27 (Fig.1A), we observed only two: CD21⁺CD27⁻ and CD21⁻CD27⁻, hereafter termed naïve and tissue-like, respectively (Fig.1) in accord with the earlier-defined PBMC memory sub-populations [9,10]. The comparison of duodenal and rectal memory populations was previously reported [16]. Here we added

analysis of jejunal tissue. Overall, the proportion of total B-cells was similar across the three mucosal sites (Fig.1C), however, the distribution of naïve vs tissue-like memory B-cells differed significantly between duodenum and rectum (Fig.1D,E). Naïve cells were elevated in duodenum, whereas tissue-like memory B-cells were increased in the rectum (Fig.1D,E). Jejunal cells displayed intermediate values.

3.2 Isotype expression of mucosal memory B-cells

In contrast to PBMC where IgD expression, indicative of unswitched B-cells, was highest in naïve cells (Fig.2A), in mucosal tissues IgD expression was low in the naïve population and even lower in tissue-like memory B-cells (Fig.2B,C). Here we again compared cells from three mucosal sites: duodenum, jejunum and rectum. Data for the duodenum and rectum were previously reported [16]. Frequencies of IgD-expressing cells were equivalent among the three tissues for both naïve and tissue-like B-cells (Fig.2B,C). The low IgD expression in CD21⁺CD27⁻ cells of gut mucosa suggests the term “naïve” might not be appropriate for this cell population. Compared to PBMC, where IgG expression was dominant among IgD⁻ B-cells in each memory sub-population, IgA⁺ cells dominated at mucosal sites (Fig.2A–C). In general, jejunal tissue exhibited modestly higher frequencies of Ig expressing cells than the other tissues. Among naïve cells (Fig.2B), IgM expression in jejunum was significantly higher compared to duodenum and rectum, with IgM expression in rectum significantly lower than in duodenum. IgA and IgG expression frequencies were significantly higher in jejunum compared to rectum. Differences among tissue-like memory B-cells were less pronounced (Fig.2C). IgM expression in jejunum was significantly higher compared to duodenum and rectum. Both duodenum and jejunum expressed significantly higher IgG frequencies compared to rectum.

Examination of Ig expression between memory sub-populations of each mucosal tissue revealed generally significantly higher frequencies in naïve compared to tissue-like B-cells. This was the case for IgD⁺ (Fig.2D), IgM⁺ (Fig.2E), and IgG⁺ cells (Fig.2G), although the latter expression frequencies were low. Surprisingly, frequencies of IgA⁺ cells were similar between naïve and tissue-like memory populations in duodenum and jejunum, with significantly higher expression seen in tissue-like memory B-cells in rectum (Fig.2F).

3.3 Homing of memory B-cells

In view of the differences in B cell distribution among the three mucosal tissues and between memory B cell sub-populations within the same tissue, we investigated the expression of several homing markers: CXCR4 (lymph node and bone marrow), CCR6 (Peyer's Patches (PP) and inflamed tissue), CCR9 (GALT) and $\alpha 4\beta 7$ (GALT) [17,18]. The three tissues displayed similar expression of CCR9 and $\alpha 4\beta 7$ on both naïve and tissue-like memory B-cells (Fig.3G,H,I,K). CXCR4 expression was similar across naïve cells of the mucosal tissues (Fig.3A) but significantly elevated in rectal tissue-like memory cells in rectum compared to duodenum (Fig.3B). CCR6 expression was elevated in naïve cells of jejunum compared to rectum (Fig.3D), but similar across tissue-like memory cells of the three tissues (Fig.3E). Within tissues, CXCR4 and CCR6 expression levels were significantly elevated on naïve compared to tissue-like memory B-cells (Fig.3C,F), whereas

the mucosal homing receptors CCR9 and $\alpha 4\beta 7$ were significantly elevated on tissue-like memory B-cells (Fig.3I,L).

For comparison we evaluated homing markers on eight matched PBMC samples. CXCR4 and CCR6 expression frequencies were highest on naïve and resting memory B-cells, while activated and tissue-like memory B-cells expressed CXCR4 and CCR6 at lower levels (Fig. 3M,N) in concert with the naïve and tissue-like memory B-cells of the mucosal tissues. CCR9 and $\alpha 4\beta 7$ were expressed at lower frequencies in all memory sub-populations in comparison to mucosal tissue-like memory B-cells (Fig.3O,P).

3.4 Identification and distribution of plasma cells and plasmablasts

We previously defined PB and PC populations in peripheral blood and bone marrow of rhesus macaques as CD38⁺CD138⁺ [9], however PB and long-lived terminally-differentiated PC were not differentiated. We subsequently used the proliferation marker Ki-67, to distinguish replicating PB from PC, and IgD, to identify class-switched cells (Thomas et al., manuscript in preparation). Further distinctions of PC and PB in mucosal tissues which harbor large numbers of antibody secreting cells (ASC) [19] were made possible using a combination of CD138, IgD, Ki-67, CD39, HLA-DR and the transcription factor, IRF-4.

We first identified PC in duodenum and rectum. B-cells were identified with CD19 and CD20, IgD⁺ B-cells were excluded, and the remaining cells were gated with CD20 vs CD138, confirming that CD138⁺ B-cells are CD20⁻ CD19⁺ (Fig.4A). There was no difference in frequency of CD20⁻CD138⁺ PC between duodenum and rectum (Fig.4B). PC are unable to present antigen. Using an antibody to HLA-DR, we verified that HLA-DR expression on CD20⁻ CD138⁺ PC was significantly lower than the high frequency expression on CD20⁺CD138⁻ B-cells within the same sample ($p < 0.0001$, Fig.4C). As terminally differentiated PC undergo little to no proliferation, the frequency of Ki-67 expression should be low. This was confirmed, with significantly lower expression of Ki-67 on CD20⁻CD138⁺ cells compared to CD20⁺CD138⁻ B-cells ($p < 0.0001$, Fig.4D). IRF-4 is a key transcription factor for PC differentiation, second only to the “master regulator” Blimp-1 [20–22]. It has been successfully used to identify PC by immunohistochemistry in non-human primates [22]. IRF-4 is highly expressed in PC co-expressing Blimp-1 and has been used to identify PC and PB at mucosal sites [21,23,24]. We confirmed high frequency expression of IRF-4 (>87%, data not shown) in CD19⁺CD20⁻ CD138⁺ cells. We previously showed that rhesus macaque bone marrow ASC are CD21⁻CD27^{dim/-} [9]. We confirmed this phenotype for mucosal CD20⁻CD138⁺ PC, significantly different compared to CD20⁺CD138⁻ B-cells ($p < 0.0001$, Fig.4E). Finally, we magnetically sorted CD20⁻CD138⁺ cells from rectal biopsies (Fig. 4F) and confirmed secretion of IgG (data not shown) and IgA (Fig.4G) by the unstimulated cells. Using PCR we also confirmed J-chain expression by the sorted cells. In contrast, negative control H9 T-cells failed to generate a PCR product. Setting the H9 cell value arbitrarily as 1, a >360-fold increase in geometric mean J-chain expression was observed ($p = 0.0001$). J-chain was selected due to the dominance of IgA expression at mucosal sites, as well as IgM (pentameric secretion) and IgG secreting cells [25].

PB were investigated in rectal tissue only. We initially evaluated the late activation molecule CD39 as it was described as PB-specific in human bone marrow but with no expression on PC [26]. We gated on IgD⁻ B-cells (Fig.4A) and then plotted CD39 vs Ki-67 to examine CD39⁺Ki-67⁺ and CD39⁻Ki-67⁺ populations (Fig.5A) for expression of HLA-DR vs IRF-4 (Fig.5B). PB were identified in the upper right quadrant containing IRF-4⁺ HLA-DR⁺ cells (Fig.5B). In both CD39⁺Ki-67⁺ and CD39⁻Ki-67⁺ populations we observed PB (IRF-4⁺HLA-DR⁺) and “transitional” PB or early stage PC (IRF-4⁺HLA-DR⁻). With loss of HLA-DR expression, independent of the expression of CD39, CD20 was further downregulated (Fig.5C, p=0.0011; E, p=0.0031). The loss of HLA-DR expression in this IRF-4⁺ population indicates that these cells can be considered as late stage PB in transition to terminally differentiated PC. PB express HLA-DR and are able to present antigen to other cells. In concert with the PC CD21⁻ CD27⁻ phenotype, the majority of IRF-4⁺ cells in both PB subsets were already CD21⁻CD27⁻ (Fig.5D,F). With the loss of CD20 expression and HLA-DR down-regulation, that phenotype increased and the difference compared to cells still expressing HLA-DR became significant (p=0.026, Fig.5D; p=0.0017, Fig.5F). While the majority of PC expressed the surface marker CD138, not all PB did (data not shown), indicating that PB can indeed vary in phenotype as CD39^{+/−} and CD138^{+/−} depending on their maturation status. Unfortunately PB could not be viably sorted due to the need for intracellular staining for their identification. Future experiments will be needed to confirm the PB phenotype identified here.

For comparison, we addressed PB/PC in peripheral blood where Ki-67⁺CD138⁺ cells are rare (unpublished). In view of the low-level of circulating CD138⁺ cells PC/PB were identified within the B-cell population by gating on IRF-4⁺ cells (Fig.5G), which are present at low frequency. As in the gut mucosa, the majority of PB/PC in blood are CD19^{dim/+}, CD20^{dim/+}, CD27^{dim/+} and they lack surface IgD expression (Fig.5G). To further distinguish PB/PC in PBMC, Ki-67 and HLA-DR can be used as in mucosal samples (data not shown).

4. Discussion

CD19 is a key marker for B-cell identification from early development to terminal differentiation [27]. Because CD19 antibody was reported to only weakly stain rhesus macaque B-cells [28], it has not been included in many recent studies. However as PB and PC down-regulate CD20 and are often described as CD19⁺ or CD19^{dim/+} [29], we included it in our B-cell gating strategy. Rhesus macaque memory B-cell sub-populations [9,10] differ from those of humans[30], largely defined as CD27⁺ [31]. However, in macaque PBMC a fairly large proportion of B-cells is double negative for CD21/CD27 [9,10]. The macaque mucosal memory B-cell profile presented here (Fig. 1) confirms a previous report of similarly double negative mucosal memory B-cells [10] essentially lacking CD27 in contrast to other reports [11,12]. We know that previous infection with SHIV_{SF162P4} does not affect CD27 expression, as data from 60 healthy rhesus macaques show the same staining pattern in the rectal mucosa (our unpublished data). However, we cannot rule out that tissue processing, including the use of collagenase, might lead to some loss of CD27 on the B-cell surface. Further, CD27 cleavage has been reported during T- and B-cell activation [32]. However, CD27⁻ memory B-cells have been reported in other species. Most rabbit mucosal B-cells are CD27⁻ but IgM⁺ [33]. Circulating CD27⁻ memory B-cells in humans

can represent up to one third of IgG1 and nearly half of IgG3 memory B-cells [34]. However, these CD27⁻ cells are generally found at low frequency in peripheral blood and tissue [35,36]. The origin of CD27⁻IgG⁺ memory B-cells in humans was reported to be the spleen, whereas CD27⁺IgG⁺ memory cells originate in germinal centers (GC); CD27⁻IgA⁺ originate in the gut [37]. Further investigation is needed to elucidate whether macaque CD27⁻ memory B-cells are associated with a specific activation history (T-cell dependent vs independent) or if they reflect a different point of origin. Additionally, future use of immunohistochemical staining of mucosal tissues could confirm the lack of CD27 on unprocessed memory B cells.

Before antigenic stimulation, naïve mature B-cells and activated GC B-cells commonly co-express IgD and IgM [38,39] as seen here in mucosal tissues. However, overall levels of IgD⁺ mucosal B-cells were low. “Naïve” CD21⁺IgD⁺ B-cell frequencies were significantly higher compared to tissue-like memory cells. But compared to naïve cells in PBMC, levels were almost 4-fold lower (Fig.2A,B). During class-switching to IgA and IgG, IgD expression is lost. In humans there are two types of IgM⁺ memory B-cells: one IgM⁺ only and one IgM⁺IgD⁺ [34]. The latter are CD27⁺ and the larger of the two populations [34]. As CD27⁺ memory B-cells were virtually absent in our mucosal samples, we treated all IgD⁺ B-cells as naïve, and focused on isotype expression of IgD⁻ cells, thus perhaps underreporting total IgM⁺ memory B-cells. Here, overall IgM expression on mucosal IgD⁻ cells was higher compared to B-cell populations in circulation (Fig.2A,B,C).

IgG-expressing mucosal B-cells were significantly elevated in naïve compared to tissue-like memory B-cells at all tissue sites (Fig.2B,C). These cells might subsequently undergo further maturation and class switching to IgA. In both mucosal memory B-cell populations the IgA isotype was dominant (Fig.2B,C) in agreement with previous reports on macaques [40] and humans [19]. Our short-term cultures of macaque mucosal explants have also confirmed by ELISA higher IgA expression compared to IgG [41,16]. However, an earlier study reported mainly IgM⁺ B-cells and few to no IgA⁺ B-cells in rectal lymphoid nodules of rhesus macaques [13]. We cannot address specific locations of the cells we investigated, but the gut mucosa may compartmentalize different B-cell subsets within particular niches. Overall our findings confirm the dominance of IgA in mucosal tissue [25,42], and extend this finding by addressing isotype expression on different memory B-cell subsets.

Nevertheless, we may have under-reported the frequency of IgA⁺ cells in healthy macaques, since the majority of animals studied were SHIV-or SIV-infected. Both HIV and SIV infection skew mucosal antibody responses away from IgA towards IgG and IgM [41,43].

The elevated frequencies of IgD, IgM, and IgG expressing cells in naïve versus tissue-like mucosal memory B-cells (Fig.2D,E,G), together with the equivalent IgA frequency in the two memory populations in duodenal and jejunal tissue and the elevated IgA frequency in rectal tissue-like memory B-cells (Fig.2F) led us to investigate homing receptors. We observed a higher frequency of CXCR4- and CCR6-expressing cells in naïve compared to tissue-like memory populations (Fig.3C,F). Both molecules play important roles in B-cell chemotaxis/homing and development. CXCR4 binds Stromal Derived Factor-1 (SDF-1/CXCL12), produced by stromal cells [44], and mediates bone marrow retention of precursor B-cells and PC [44,45]. It is abundantly expressed on naïve cells and promotes entry of B-

cells into lymph nodes and Peyer's patches (PP), as well as B-cell egress from PP into the lymph [46]. CCR6 which binds CCL20 (MIP-3 α) is a marker for mucosal homing to the gut and lung and to inflamed tissue [17,47,48] and is important for the organization of gut lymphoid tissues (PP, mesenteric lymph nodes and gut-associated lymphoid tissue) [47]. We also observed higher frequencies of CCR9⁺ and α 4 β 7⁺ memory B-cells in the tissue-like rather than naïve subpopulation of mucosal tissues (Fig.3I,L). Among mucosal homing markers, α 4 β 7 is dominant [18,19] and binds MadCAM-1. In rodents, homing to the gut is dependent on CCL25/TECK and CCL28, both known to enhance binding of α 4 β 7 to MadCAM-1 [25,49]. CCR9 (its ligand is CCL25) is often co-expressed on gut homing T-cells, and contributes to the localization of IgA⁺ PC to the small intestine [25,49]. The expression patterns of these chemokine receptors in mucosal B cell sub-populations suggest that the higher IgG and IgM expression in "naïve" cells might reflect homing of "fresh" B-cells, primed elsewhere but not yet class switched to a "residential" IgA phenotype. The naïve B-cells may have been primed at mucosal inductive sites (PP and mesenteric lymph node) before migrating into the lamina propria [50]. In studies of systemic versus oral immunization that induced antigen-specific IgA, the induction site rather than isotype commitment was shown to determine chemokine responsiveness and homing of B-cells [49]. Moreover, memory B-cells exhibit migratory plasticity, and can evolve from α 4 β 7^{low}CCR9^{low} to α 4 β 7^{high}CCR9⁺ at mucosal sites in the presence of retinoic acid or mesenteric lymph node dendritic cells [51]. Our data showing higher frequencies of α 4 β 7^{high}CCR9⁺ cells in the tissue-like memory population which has matured to the PB/PC stage (Fig.5) are in agreement with this concept. Unfortunately homing markers were not incorporated into our PB/PC panel; thus homing of macaque PC/PB warrants further investigation.

The overall higher frequencies of α 4 β 7 expression in both mucosal B-cell populations compared to the same subpopulations in PBMC (Fig.3L vs 3P) is in accord with this concept of B cell trafficking. Another study in naïve rhesus macaques showed that memory B-cell β 7 expression frequencies were similar in PBMC and jejunum, with no differences between jejunal sub-populations [11]. Here, using an α 4 β 7-specific antibody, we report differences between PBMC and mucosal tissues and between different B-cell populations within each mucosal tissue. The differences likely are due to the greater specificity of anti- α 4 β 7 for the homing integrin. β 7 can form a complex not only with α 4 (CD49d) but also with integrin alpha E (CD103) [52]. The complex formation with α 4 is controlled by β 1 expression [53].

In humans circulatory PB generally peak 6–10 days post-vaccination [46,54–57]. The main ASC populations have been reported as CD19⁺CD138^{+/–}CD38^{hi}Ki-67⁺ [55]. This illustrates the importance of the Ki-67⁺ phenotype and matches our observations that early PB can be CD138[–] and only later CD138⁺ (data not shown). Investigation of CD39, previously described as an exclusive PB marker in human bone marrow [26] and widely expressed on human peripheral blood B-cells [58–60], revealed that CD39 expression is not exclusively limited to mucosal PB, but seems to be a late stage B-cell activation marker. It is helpful but is not essential for PB identification in rhesus macaques. Further almost all IRF-4⁺ B-cells in PBMC were also CD39⁺ (data not shown) indicating that CD39 is not an ideal marker for distinguishing PB and PC.

Our findings regarding IRF-4 agree with previous results showing it is highly expressed on PC and can be used to identify PB/PC at mucosal sites and PBMC [21,23,24]. The PC defined here were HLA-DR⁻, did not proliferate (Ki-67⁻), and downregulated CD20, matching the profile of human PC [56,57]. The macaque mucosal PB remained HLA-DR⁺ and proliferated (Ki-67⁺), similar to human PB [56,57]. Overall, our results agree with the definition of human PB/PC. We define mucosal PB as CD19⁺CD20^{+/-}HLA-DR⁺Ki-67⁺IRF-4⁺CD138^{+/-} and mucosal PC as CD19⁺CD20⁻HLA-DR⁻Ki-67⁻IRF-4⁺CD138⁺ with both populations being CD39^{+/-}CD27⁻. The CD20 and HLA-DR down-regulation in late stage PB/PC is in agreement with previous publications [29,61]. In addition to phenotype and demonstration of ASC in the absence of stimulation (Fig.4G), we confirmed enrichment of ASC among CD138⁺ cells by expression of Jchain, necessary for the creation of polymeric IgA [25,42,62].

With regard to PBMC, the majority of IRF-4⁺ B-cells in humans are CD27^{+/bright}CD21^{dim/-} (Demberg and Mohanram, unpublished), whereas in monkey PBMC the IRF-4⁺ B-cells were CD27^{dim/-}CD21^{dim/-} (Fig.5G). The IRF-4^{hi} B-cells in PBMC are low in frequency compared to gut mucosa, in accord with the low frequency of PC/PB in circulation in humans. Overall the IRF-4⁺ B-cells in circulation expressed similar PC/PB phenotypes as their counterparts in tissue.

In summary we have shown that rhesus macaque memory B cell profiles are similar at three mucosal sites: duodenum, jejunum, and rectum. Thus easily obtained rectal biopsies should adequately reflect induction of memory B-cells in this animal model, precluding the need for more invasive procedures for monitoring gut B-cell maturation. Our characterizations of homing profiles and definitions of PB and PC will enrich the macaque model by facilitating investigations of SIV/SHIV pathogenesis and B cell dysfunction, induction of mucosal B-cell memory, and identification of B-cell responses associated with protection from viral acquisition and disease progression. A thorough understanding of both mucosal and systemic B-cell dynamics in the rhesus macaque is critical for making the greatest use of this commonly used animal model for pre-clinical HIV vaccine development.

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Highlights

Rhesus macaque memory B cells in duodenum, jejunum and rectum lack CD27 expression.

Naïve and tissue-like memory cells differentially express CXCR4, CCR6, CCR9 and $\alpha 4\beta 7$.

Mucosal plasmablasts are defined as $CD19^+CD20^{+/-}HLA-DR^+Ki-67^+IRF4^+CD138^{+/-}$.

Mucosal plasma cells are defined as $CD19^+CD20^-HLA-DR^-Ki-67^-IRF4^+CD138^+$.

Overall, rectal biopsies can adequately monitor B-cell dynamics in the gut mucosa.

Figure 1. Mucosal B-cell gating strategy and distribution of total and memory B-cells in PBMC and mucosal tissue of SHIV_{SF162P4}-infected macaques

(A) A representative rectal pinch sample is shown. B-cells were identified by gating on singlets, then lymphocytes, and exclusion of dead cells. Next CD2⁺ (NK and T-cells) and CD14⁺ (monocytes) cells were excluded and B-cells were identified as either CD19⁺CD20⁻ or CD19⁺CD20⁺. Memory B-cells were defined using CD21 and CD27 as previously published [9,10]. (B) Memory B-cell distribution in PBMC. (C) Total B-cell distribution between mucosal tissues. (D) Distribution of naïve (CD21⁺CD27⁻) memory B-cells among duodenum, jejunum, and rectum (p=0.005, Duodenum vs Rectum). (E) Distribution of tissue-like (CD21⁻CD27⁻) memory B-cells among the three tissues (p=0.0029, Duodenum vs Rectum). * indicates significant differences. Error bar = standard error of the mean (SEM).

Figure 2. Analysis of isotype expression on memory B-cells from different tissues of SHIV_{SF162P4}-infected macaques

(A) Isotype expression among memory B-cells in PBMC. (B,C) Isotype expression between naïve (B) and tissue-like (C) memory B-cells in duodenum (D), jejunum (J) and rectum (R). Significant differences were observed in B for expression of IgM (D vs J, $p < 0.0001$; J vs R, $p < 0.0001$; D vs R, $p = 0.046$), IgA (J vs R, $p = 0.039$), and IgG (J vs R, $p = 0.0081$). In C significant differences between tissues were seen for IgM (D vs J, $p = 0.011$; J vs R, $p = 0.04$), IgG (D vs R, $p = 0.0042$; J vs R, $p = 0.0015$). (D–F) Differential expression of IgD, IgM, IgA and IgG between memory B-cell populations within the same tissue. (D) The difference in

IgD expression between memory populations was significant for duodenum, jejunum and rectum ($p<0.0001$; $p=0.033$; $p=0.0003$ respectively). Similar differences were observed for IgM (E) in duodenum, jejunum and rectum ($p<0.0001$; $p<0.0001$; $p=0.0003$ respectively); and for IgG (G) duodenum, jejunum and rectum ($p=0.0025$; $p=0.0003$; $p<0.0001$ respectively). For IgA expression the only significant difference between memory populations was in rectum ($p<0.0001$). * indicates significant differences. Error bar = SEM.

Figure 3. Homing marker expression in memory B-cell populations of mucosal tissues from SHIV_{SF162P4}-infected macaques

Expression of CXCR4 on naïve (panel A) and tissue-like memory (panel B) B-cell populations was compared between tissues. No significant differences were observed among naïve cells of the three tissues. Significantly higher CXCR4 expression frequencies were seen in tissue-like memory B-cells of rectum vs duodenum ($p=0.0061$). Within each tissue CXCR4 was significantly more highly expressed on naïve B-cells compared to tissue-like memory cells ($p<0.0001$ for all; panel C). Expression of CCR6 on naïve (panel D) and tissue-like (panel E) memory B-cell populations was compared between tissues. The only

significant difference was observed between jejunum and rectum in the naïve B-cell population ($p = 0.0088$; panel D). Within each tissue CCR6 was significantly more highly expressed on naïve B-cells compared to tissue-like memory cells ($p < 0.0001$ for all; panel F). Expression of CCR9 on naïve (panel G) and tissue-like memory (panel H) B-cell populations was compared between tissues. No significant differences were observed between tissues. Within each tissue CCR9 was significantly more highly expressed on tissue-like memory B-cells compared to naïve cells ($p < 0.0001$ for all; panel I). Expression of $\alpha 4\beta 7$ on naïve (panel J) and tissue-like (panel K) memory B-cell populations was compared between tissues. No significant differences were observed between tissues. Within each tissue $\alpha 4\beta 7$ was significantly more highly expressed on tissue-like memory B-cells compared to naïve cells of duodenum and jejunum ($p < 0.0023$ for both) and rectum ($p < 0.0001$; panel L). (M) CXCR4, (N) CCR6, (O) CCR9 and (P) $\alpha 4\beta 7$ expression on B-cell memory populations of PBMC. * indicates significant differences. Error bar = SEM.

Figure 4. Identification and characterization of Plasma cells in mucosal tissue

(A) Plasma cells from a representative SIV_{mac251}-infected macaque were identified by gating on B-cells, eliminating IgD⁺ B-cells and gating on CD138. (B) Distribution of CD138⁺ cells in duodenum and rectum of SIV_{mac251}-infected macaques. No significant difference was observed between tissues. (C–E) Characterization of CD138⁺ cells in rectal samples of 34 SIV_{mac251}-infected and 4 uninfected macaques. No significant difference was observed between the macaque groups; thus data were combined. (C) Expression of HLA-DR was significantly downregulated on CD138⁺ cells ($p < 0.0001$). (D) Ki-67 was significantly downregulated on CD138⁺ cells ($p < 0.0001$). (E) The majority of CD138⁺ cells are CD21[−]CD27[−]. The difference between CD20⁺CD138[−] and CD20[−]CD138⁺ cells is significant ($p < 0.0001$). (F–G) CD138⁺ cells from uninfected rhesus macaques. (F) Pre- (left panel) and post-sort (right panel) purity of isolated CD138⁺ cells. (G) IgA ELISpot of sorted

CD138⁺ cells and H9 cells as negative control. * indicates significant differences. Error bar = SEM.

Figure 5. Identification and characterization of Plasmablasts in rectum and PBMC of SIV_{mac251}-infected rhesus macaques

(A) Plasmablasts were identified by two methods gating on (1) CD39⁺Ki-67⁺ and (2) CD39⁻Ki-67⁺. (B) both subsets (1, 2) were further gated on HLA-DR and IRF-4. Early plasmablasts were defined as HLA-DR⁺IRF-4⁺ (upper right quadrant) and transitional or late stage plasmablasts/early plasma cells (HLA-DR⁻IRF-4⁺), lower right quadrant. (C,D) Early and transitional plasmablasts from subset 1 were compared for downregulation of CD20 (C) and loss of CD21 expression (D). Late or transitional plasmablasts expressed significantly less CD20 ($p=0.0011$), and lost expression of CD21 ($p=0.026$). The same is

true for plasmablasts identified in subset 2 (E,F). Late or transitional plasmablast expressed significantly less CD20 (E, $p=0.0031$), and lost expression of CD21 (F, $p=0.0017$). (G) Identification and characteristics of Plasmablast/plasma cells in PBMC for comparison. These CD19⁺IRF-4⁺ cells were present at very low frequency in blood. They down-regulated CD20 expression, were CD27⁻IgD⁻, and like mucosal cells could be further distinguished by HLA-DR and Ki-67 expression (not shown). * indicates significant differences. Error bar = SEM.

Table 1

Antibodies used for Flow Cytometry

Antigen	Color	Clone	Host Species	Isotype	Supplier
CD2	Qdot605	S5.5	Mouse	IgG ₂ a	Invitrogen
CD3	BV605	SP34-2	Mouse	IgG ₁ λ	BD Bioscience
CD14	Qdot605/Qdot800	Tu14	Mouse	IgG ₂ a	Invitrogen
CD19	PeCy5	I3-119	Mouse	IgG ₁	Beckman Coulter
CD20	eF650NC	2H7	Mouse	IgG ₂ b,k	eBioscience
CD21	PeCy7	B-ly4	Mouse	IgG ₁ k	BD Bioscience
CD27	PerCP-eF710	O323	Mouse	IgG ₁ k	eBioscience
CD39	BV421	A1	Mouse	IgG ₁ k	Biologend
CD138	Pe/APC	DL-101	Mouse	IgG ₁ k	Biologend
CD184 (CXCR4)	BV421	12G5	Mouse	IgG ₂ a,k	Biologend
CD196 (CCR6)	Pe	11A9	Mouse	IgG ₁ k	BD Bioscience
CDw199 (CCR9)	FITC	112509	Mouse	IgG ₂ a	R&D Systems
α4β7	APC	A4B7	Rhesus recombinant	IgG ₁	NHP Reagent Resource
IgA	FITC	Polyclonal Fab	Goat	NA	Southern Biotech
IgD	Texas Red*	Polyclonal	Goat	NA	Southern Biotech
IgG	APC-Cy7	G18-145	Mouse	IgG ₁ k	BD Bioscience
IgM	APC	G20-127	Mouse	IgG ₁ k	BD Bioscience
Ki-67	Ax700	B56	Mouse	IgG ₁ k	BD Bioscience
IRF-4	eFluor660/FITC	3E4	Rat	IgG ₁ k	eBioscience
HLA-DR	Qdot800	Tu36	Mouse	IgG ₂ b	Invitrogen
Viability Dye	Aqua	NA	NA	NA	Invitrogen

* Green laser (532nm) or yellow-green laser (561nm) required