Polymeric IgA-secreting and mucosal homing pre-plasma cells in normal human peripheral blood

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Abstract

Clear identification of recently activated mucosal B cells in human blood would greatly facilitate study of mucosal vaccines, immune response to infection and the ongoing mucosal IgA response. We examined blood lymphocytes from normal, healthy individuals to identify IgA-secreting pre-plasma cells’ (PPC) functional and phenotypic relevance to mucosal antibody production, in the absence of infection, disease or recent vaccination. PPC are the most recently activated B lymphocytes in blood and are considered in transit between lymphoid tissue and effector tissues, where they terminally differentiate into plasma cells. We observed that all IgA-secreting PPC expressed surface IgA (sIgA) and intracellular IgA (icIgA) and secreted primarily polymeric IgA (pIgA), as determined by flow cytometry, ELISPOT and size exclusion chromatography. A large sub-population of PPC in blood expresses the mucosal chemokine receptor CCR10 and contains the largest fraction of sIgA and icIgA PPC that secrete pIgA. The majority of CCR10° PPC expresses high levels of Ki67, indicative of recently activated blasts. In contrast, most CCR10− PPC secrete IgG, but a small population secretes pIgA and stains for icIgA. The mucosal integrin α4β7 was detected on a subset of PPC, but this subset did not account for all CCR10+ PPC or all PPC with slgA expression. Our data clearly demonstrate that PPC defined by surface expression of CD19, CD27hi, IgA and CCR10 secrete only pIgA and are the dominant mucosal PPC subset in human blood. These mucosal PPC can now be investigated routinely as indicators of recent human mucosal IgA responses.

Keywords: B lymphocyte, IgA, mucosal antibody, plasmablast

Introduction

The human immune system has evolved complex mechanisms that protect the various mucosal epithelial surfaces that are in contact with the external environment and are constantly exposed to pathogens and antigens from endogenous flora. The mucosal humoral immune response protects those surfaces and enables management of the high antigenic load through production of polymeric IgA (pIgA) and the use of the pIgAR to transport pIgA through mucosal epithelial cells to the external surfaces (1, 2). This pIgA is produced by plasma cells (PC) that differentiate from activated pre-plasma cell (PPC) blasts within the tissue directly beneath the epithelial layer (1). The known large quantity of mucosal pIgA produced in normal healthy subjects contrasts the fact that serum IgA is predominantly composed of monomeric IgA (3). This raises an interesting question concerning the identification of PPC in normal healthy people that are in transit to mucosal tissues, with both appropriate cell surface homing structures and the ability to produce pIgA. Those cells would represent the ongoing mucosal IgA response in healthy people that have normal immune status. Their study could potentially provide important information on the specificity and functions of normal mucosal IgA.

Recent work has assigned some mucosal homing markers to plasmablasts or PC in blood (4–10), but no study has correlated surface markers with the functional identification of pIgA secretion. Polyclonal stimulation of PBL has previously revealed the presence of B cells capable of secreting pIgA (11, 12). These and other studies looked at polyclonal activators of both memory B cells (MBC) and naive B cells, such as PWM that activates in the presence of T cells, and are not necessarily related to the active pIgA production by the PPC population in blood. Most recent studies rely on post-infection or post-vaccination analysis of human blood to identify IgA antibody relevant to mucosal response and the cells that produce that IgA (4, 7, 9). However, no studies have specifically identified the PPC subset of blood and examined pIgA secretion by these cells as a functional marker.
of mucosal PPC in blood. We propose that both phenotypic markers relevant to mucosal homing and identification of plgA production are required to identify mucosal PPC in blood. Development of effective mucosal vaccines or investigation of a patients' mucosal immune status would benefit greatly from the clear identification of the activated PC precursors in blood that are destined to seed mucosal tissues with plgA-secreting cells.

In humans, circulating B lymphocytes can be broadly divided into two large subsets based on the expression of the B cell marker CD27: naive B cells (and a small population of transitional B cells) do not express CD27, whereas antigen-experienced B cell lineages express CD27. Naive B lymphocytes circulate through secondary lymphoid organs, where interaction with their cognate ligands, T lymphocytes and other cell types facilitates their activation, Ig class switching and somatic hypermutation in the germinal centre (GC) reaction (13, 14). B cells activated through GC reactions express CD27 and can be further subdivided into two functionally distinct subsets: MBC with moderate CD27 expression that do not actively produce antibody but provide rapid immune responses on re-challenge and PPC with high CD27 expression that secrete Ig as they begin to differentiate into PC. Both MBC and PPC exit lymph nodes to the circulation following their activation and differentiation. Additionally, activated and differentiating B cells gain expression of CD38, with high expression seen on PPC (15, 16). Plasmablasts are likely the major subset in the PPC of blood since a large fraction of CD38* blood B cells express markers indicating recent proliferation (17). Others have demonstrated that blood MBC and PPC subsets defined by differential expression of CD27 and CD38 are both representative of recent active humoral immune responses (7). Our data presented here confirm these phenotypes with positive identification of the mucosal PPC subset in normal human blood.

One of the best characterized molecules directing blood lymphocyte migration at mucosal sites is the integrin α4β7 heterodimer whose ligand is the mucosal addressin cell adhesion molecule (MAdCAM-1) that is constitutively expressed on venules in the intestinal lamina propria and Peyer's patches (18–20). Plasmablasts are likely the major subset in the PPC of blood since a large fraction of CD38* blood B cells express markers indicating recent proliferation (17). Others have demonstrated that blood MBC and PPC subsets defined by differential expression of CD27 and CD38 are both representative of recent active humoral immune responses (7). Our data presented here confirm these phenotypes with positive identification of the mucosal PPC subset in normal human blood.

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Working in concert with adhesion molecules, chemokine receptors play an important role in mediating migration of lymphocytes to specific tissue sites, because ligation by tissue-specific chemokines increases the avidity and affinity of integrins for their ligands expressed on endothelium in specific tissues (25). Recent studies indicate that certain chemokines [thymus-expressed chemokine (TECK/CCL25) and mucosae-associated epithelial chemokine (MEC/CCL28)] direct lymphocytes to traffic from blood into the mucosal epithelial tissues of the gut, respiratory and urogenital tracts through ligation of the chemokine receptors CCR9 and CCR10 (26). CCR9, the only known receptor for the thymus- and gut-restricted chemokine TECK, is expressed by circulating memory T cells bearing the α4β7 integrin and intestinal T cells (27, 28). CCR10 responds to MEC, a chemokine expressed by epithelial cells in a variety of mucosal tissues including the salivary and mammary glands, and small and large intestines (29, 30). CCR9* and CCR10* PC have been identified in mucosal tissues in both humans and animals (31), and as a result, these two chemokine receptors have gained interest as indicators of mucosal relevance on circulating B lymphocyte subsets. However, there is only scattered evidence identifying CCR9 or CCR10 expression on human B lymphocyte subsets and little correlation of those markers with other mucosal markers or functions.

Numerous studies (mostly in non-human mammals) infer a mucosal tissue-specific 'type' for lymphocytes based on surface phenotype. However, proper identification of mucosal cell subsets requires coincident identification of mucosal relevant function with phenotypic markers. The most important function would be production of plgA, typically as dimers. plgA is the predominantly produced mucosal Ig isotype and its unique structure is required for the mucosal secretory function, making it an ideal functional indicator of mucosal relevance. Here, we describe a large, recently proliferative subset of CCR10-expressing PPC in the blood of healthy human volunteers, which comprises the majority of PPC that expresses surface IgA (sIgA) and intracellular IgA (icIgA) and exclusively secretes plgA. This provides the first function-phenotypic identification of the circulating, activated, B cell subset that represents the active normal mucosal IgA response in humans.

Methods

Specimen preparation and B cell isolation

Blood samples from normal, healthy volunteers were obtained with permission from the McMaster University Medical Centre Histocompatibility Laboratory. In some cases, blood samples (20–100 ml) were collected from normal, healthy adult volunteers. All samples were collected in acid citrate dextrose Vacutainer tubes (BD Biosciences, Oakville, Canada) and processed on the same day as collected. This study was approved by the McMaster Research Ethics Board and informed consent was obtained from all subjects.

B cells were isolated from fresh blood samples using the Human B Cell Negative Selection RosetteSep cocktail (Stem Cell Technologies, Vancouver, Canada). Whole blood was incubated with the RosetteSep cocktail at room temperature for 20 min, layered over Ficoll-Paque Plus (VWR International, Mississauga, Canada) and centrifuged for 20 min at 1800 × g. The resulting B cells were re-suspended and washed twice in 2% FCS/PBS before staining for flow cytometric analysis or FACS. The isolated cell suspension was always >85% CD19+ B cells, as determined by flow cytometry.
Functional identification of mucosal PPC in human blood

Immunophenotyping

The blood B cell preparations were surface stained using various mouse anti-human mAbs. The following antibodies were used: anti-CD19–PE–Cy7, anti-CD27–PE, anti-CD27–Alexa700, anti-CD27–PerCP–Cy5.5, anti-IgG–FITC, anti-IgA–FITC, anti-CD38–APC, anti-CD27–PE, anti-β7–APC, anti-CCR9–PE, anti-CCR10–PE, anti-CCR10–APC and anti-CD27hi/CD38hi. All antibodies were purchased from BD Biosciences except anti-CD27–Alexa700 (EMD Biosciences, San Diego, CA, USA), anti-IgA–FITC (DakoCytomation, Fort Collins, CO, USA) and anti-CCR antibodies (PE and APC conjugates, R&D Systems, Minneapolis, MN, USA). Specific phenotyping procedures used four, five- or six-colour analysis to determine various B cell subsets. Data were collected on a Becton Dickinson (BD) LSR II cytometer using FACS Diva software (BD Biosciences) and analysed using FlowJo software (Treestar Inc.).

Specific intracellular staining with anti-Ki67–PE (BD Biosciences) and anti-IgA–FITC (DakoCytomation) in combination with anti-CCR10–APC (R&D Systems) was performed using the BD Intracellular Staining kit. Briefly, B cell preparations were surface stained as described above and fixed with 1% paraformaldehyde. Cells were then permeabilized using BD Perm/Wash solution and then incubated with appropriate antibodies in BD Perm/Wash solution on ice for 30 min. The cells were pelleted and re-suspended in 1% BSA/PBS and data were collected on a BD LSR II cytometer using BD FacsDIVA software within 12 h of intracellular staining.

Fluorescence-activated cell sorter

MBC and PPC populations or PPC with specific surface markers (slgA or CCR10) were purified from total B cell preparations by staining against CD19, CD27, CD38, IgA or CCR10, using antibodies described above, and then isolated by FACS. Sorting was carried out on a high-speed digital FACS Vantage sorter (BD Biosciences), with simultaneous two-stream sorting of MBC and PPC or PPC subsets. Typical yields for MBC (CD19+/CD27+/CD38+1) and PPC (CD19+/CD27+/CD38+) were >95% pure. In some experiments, PPC were identified and sorted by a CD19+/CD27+/phenotype that is clearly equivalent to the CD19+/CD27+/CD38+ phenotype (Fig. 1).

Cell culture

FACS-sorted MBC were suspended in RPMI containing 10% FCS, 2 mM L-glutamine, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin prior to activation cultures. As previously described (32), MBC were cultured in triplicate for 10 days at a density of 2 × 10⁴ cells per well in a final volume of 200 μl in the presence of mitomycin-C-treated L/CDw32 cells (American Type Culture Collection), polyclonal B cell activators anti-CD40 mAb (mouse IgG1, clone G28.5) and anti-CD27 mAb (BD Biosciences), and recombinant human IL-2 and IL-10 (R&D Systems). Post-culture viable cells were collected and analysed by ELISPOT (see below), and supernatants were collected and analysed for IgG or IgA isotype by ELISA, in some cases by size exclusion chromatography with ELISA.

FACS-sorted PPC were cultured in IMDM containing 10% FCS, 2 mM L-glutamine, 50 μg ml−1 penicillin and 100 μg ml−1 streptomycin. Cultures were carried out in 364-well microculture plates (BD Biosciences) at a density of 5000 cells per well in a final volume of 50 μl for 36 h and supernatants were collected and analysed for IgA or IgG isotype by ELISA, in some cases by size exclusion chromatography with ELISA.

Size exclusion chromatography of culture supernatants

MBC and PPC culture supernatants were concentrated 5× using BD Visiprep (100 MW cutoff) centrifugal concentrators and then fractionated through a 5 ml Sephadex G-200 column and collected in 200 μl fractions. The presence of IgA or IgG in each fraction was determined by isotype-specific ELISA. A monomeric human IgG standard (Jackson ImmunoResearch, West Grove, PA, USA) was used to identify the peak fraction for an IgG monomer and a dimeric and monomeric human IgA (gift from S. Ketzel) was used to identify the peak elution fraction for IgA dimer and monomer. These standards were run prior to each supernatant analysis.

Enzyme-linked immunosorbent assay

Maxisorp 96-well plates (VWR International) were coated with polyclonal goat anti-human IgA (Southern Biotech, Birmingham, AL, USA) or polyclonal goat anti-human IgG (Caltag Laboratories, Burlingame, CA, USA), blocked with 1% BSA in Tris-buffered saline (TBS) and incubated with column-fractionated culture supernatants or an isotype standard (Jackson ImmunoResearch). Reactions were developed using biotinylated polyclonal goat anti-human secondary antibodies against IgA (Southern Biotech) or IgG (Caltag Laboratories) and streptavidin–alkaline phosphatase (Sigma-Aldrich, Oakville, Canada) followed by incubation with p-nitrophenyl phosphate (Sigma-Aldrich) at 37°C for 30 min. Absorbance at 405 nm was measured using a SAFIRE plate reader and XFluor software.

ELISPOT

Acrowell 96-well filter plates (VWR International) were incubated for 37°C with polyclonal anti-IgG or anti-IgA and blocked with 1% BSA in TBS. Plates were washed with 0.05% Tween–PBS, and FACS-sorted PPC were cultured at 37°C at a density of 1000 cells per well in 200 μl of IMDM containing 10% FCS, 2 mM L-glutamine, 50 μg ml−1 penicillin and 100 μg ml−1 streptomycin for 36 h. Cells were then removed and the plates were washed with 0.05% Tween–PBS. Antibody secretion was revealed using polyclonal biotinylated goat anti-human secondary antibodies and the ELISPOT Blue Colour Module (R&D Systems). The total number of IgA or IgG spots were counted manually using a dissecting microscope or an ImmunoSpot automated spot counter and ImmunoSpot version 4.0 software (Cellular Technologies, Limited) and expressed as a number per 1000 PPC.

Statistical analysis

For all analyses listed, data are presented as mean ± SEM. Student’s t-test was used to analyse the difference between
groups. All analyses were performed using GraphPad Prism 4.0 Software (GraphPad Software, Inc.)

Results

PPC and MBC identification and frequencies in blood

We first determined that staining of blood CD19+ B cells identified subsets expressing high levels of CD27 and CD38 that corresponded to the previously identified, activated and Ig-secreting MBC from human spleen (33, 34). We refer to these cells as PPC. These analyses exclude the transitional B cell subset in blood that has high CD38 expression, because that subset does not express CD27 (35). Figure 1 illustrates typical staining profiles of blood CD19+ B cells for CD27 and CD38 expression, distinguishing resting MBC from PPC. CD27 is expressed by 23.1 ± 3.9% (n = 8) of circulating CD19+ B cells. This population contains both resting MBC (CD27+CD38lo) and PPC (CD27hiCD38hi) that are present at frequencies of 89.7 ± 6.1% and 6.1 ± 1.2% (n = 8) of the CD27+ pool, respectively (Fig. 1, panel C). Thus, PPC represent ~1.4% of the total B cells in normal human blood. We were able to consistently identify nearly all CD27 bright cells as CD38 bright (Fig. 1, panel G). This allowed us to identify PPC as CD27hi in subsequent phenotypic analyses and sorting experiments. Analysis of forward and side scatter properties of B cell subsets indicated that PPC exhibited larger size and cytoplasmic complexity compared with resting MBC (Fig. 1, panels D and E), as previously described.
for CD38hi cells from human spleen (34). This is consistent with the activated or ‘blast’ state of our identified PPC in blood.

We have defined PPC as CD19+CD27hi(CD38hi), but this phenotype potentially does not exclude more differentiated cells that could be equally qualified as plasmablasts or PC in blood. For instance, Mei et al. (9) recently defined blood PC as intracellular Ig^high, surface CD62^/C0 and HLA-DR^low in phenotype while grouping blood plasmablasts/PC in the phenotype CD19+CD27hi. We therefore investigated PPC for expression of CD138 and HLA-DR to determine what fraction may have a phenotype closer to PC. The CD138 marker is considered specific for fully differentiated PC in tissue, but recent data indicate that typically <2% of the CD27+ fraction of blood from normal lactating women expresses the CD138 marker and that would mean <0.1% of PBMC (36). We examined CD138 expression by CD27+ cells and found no positive staining above background (data not shown) and conclude that in blood from normal healthy subjects fully differentiated PC are almost undetectable. Differential expression of HLA-DR was found when comparing CD27hi (PPC) and CD27int (MBC) fractions of blood (Fig. 2A–C). The PPC had a reproducible fraction of cells with low–moderate expression of HLA-DR compared with MBC. Importantly, this fraction of cells was maintained throughout the B cell isolation procedure and cell sorting, indicating that more differentiated/activated forms of PPC were not lost during any isolation procedure we used. The ratio of PPC with low–moderate HLA-DR compared with typical HLA-DR expression on MBC varied between 1:10 and 1:4, comparable to results found by Mei et al. (9), where they concluded the intracellular Ig^+, HLA-DR^low cells were PC and those with normal levels of HLA-DR were plasmablasts.

We double stained naïve, MBC and PPC with Ki67, a marker of proliferating or recently proliferating cells (17) and HLA-DR (Fig. 2D–F). These data clearly show that there is high expression of Ki67 in the majority of PPC, whereas only a small fraction of MBC expresses Ki67. A clear correlate between Ki67 and HLA-DR expression was found for PPC, indicating that most HLA-DR^hi cells were also brightest for Ki67 and moderate stained HLA-DR cells also had moderate Ki67. Thus, our data argue that normal blood PPC (CD19+CD27hi) have all recently proliferated, so are most likely plasmablasts, with a majority in a relatively undifferentiated state, and a small fraction are more differentiated plasmablasts but not PC.

Phenotypic analysis of surface Ig, mucosal relevant adhesion molecules and chemokine receptors on PPC and MBC in normal blood

Blood B cell populations were stained and analysed by flow cytometry to determine the expression of surface markers relevant to mucosal function. The first markers investigated were the heterodimeric adhesion molecules α4β7 and αEβ7 that are thought to have roles in mucosal migration of T cells (37–40). All B cell subsets expressed α4 integrin chain as expected, but the relative expression of the dimeric α4β7 varied between subsets (Fig. 3). All naïve and resting MBC had equivalent levels of expression of α4β7 dimer.

![Fig. 2. Expression of HLA-DR and Ki67 by blood PPC. CD27 and HLA-DR co-staining of isolated PBMC (A), rosette-isolated B cells (B) and FACS-isolated PPC (C). Data are gated on CD27+ cells. Upper left region indicates cells with CD27hi expression and reduced HLA-DR expression that are enriched by PPC isolation (C). Isolated B cells were surface stained with HLA-DR antibody and intracellular stained with Ki67 antibody and further stained with CD19 and CD27 antibody to identify (D) CD27+ (naive), (E) CD27int (MBC) and (F) CD27hi PPC in blood. Upper left region in (F) indicates cells with reduced HLA-DR and Ki67 expression but are still positive for Ki67. Data are from a representative sample of several cell preparations.](https://academic.oup.com/intimm/article-abstract/22/6/527/703312)
Expression of sIgA, surface IgG (sIgG) and surface IgM (sIgM) were examined among B cell subsets to further determine mucosal relevance and function (Table 1). Both MBC and PPC subsets had significant proportions of sIgA+ cells with more than one-half PPC sIgA positive, consistent with published data identifying large sIgA+ cells (43) or more recently iclGA expression by CD19+CD27hi cells (9). Both MBC and PPC had sIgG-positive subsets, as expected. sIgM-positive PPC and MBC were also detected. For PPC, the frequency of sIgM cells was higher than that of sIgG but only less than one-half of the frequency of sIgA. The fraction of naive cells with sIgG or sIgA did not substantively exceed background, consistent with the expected lack of expressions of IgG and IgA among cells with no Ig class switch (Table 1).

**Fig. 3.** Expression of α4β7 integrin and CCR10 by naive B cells, MBC and PPC. Vertical and horizontal lines indicate negative cutoff for background PEα4 and APCβ7 fluorescence. The region displayed represents cells positive for β7 integrin chain. A majority of naive cells (A) and MBC (B) expresses low or moderate levels of α4β7, with a small fraction α4β7- (upper left quadrant). Both MBC (B) and PPC (C) have clear populations of α4β7+ with increased levels of α4 chain compared with naive cells (A). A large fraction of PPC (C) expressed elevated high levels of α4β7 compared with MBC or naive cells. Figures are from one sample representative of five samples with similar results.

**Table 1.** Surface Ig, α4β7 and CCR expression on circulating B cell subsets

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<tr>
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<th>% IgG+</th>
<th>% IgA+</th>
<th>% IgM+</th>
<th>% α4β7+</th>
<th>% CCR9+</th>
<th>% CCR10+</th>
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<tr>
<td>Naive</td>
<td>1.7 ± 0.4</td>
<td>1.6 ± 0.6</td>
<td>88.6 ± 6.3</td>
<td>93.6 ± 1.2</td>
<td>2.7 ± 0.8</td>
<td>2.4 ± 1.1</td>
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<tr>
<td>MBC</td>
<td>23.8 ± 2.2</td>
<td>25.6 ± 2.1</td>
<td>50.6 ± 2.8</td>
<td>54.1 ± 2.4</td>
<td>4.97 ± 2.3</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>PPC</td>
<td>8.6 ± 1.8</td>
<td>54.0 ± 4.3</td>
<td>24.2 ± 3.6</td>
<td>49.3 ± 9.4</td>
<td>1.81 ± 0.4</td>
<td>33.7 ± 3.7</td>
</tr>
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Identified subsets: naive, CD19+CD27hi; MBC, CD19+CD27int; PPC, CD19+CD27hi. Data are mean ± SEM. Background was 1% for all markers. For sIgG, sIgA and CCR10, n = 10. For α4β7 and CCR9, n = 5. For sIgM, n = 4.
are consistent with previous studies of PBMC (44, 45). By approximate calculation, the number of IgA- plus IgG- secreting PPC in our study is only slightly higher than the counts of IgG plus IgA ASC in PBMC isolates from pre-immunized patients (45).

Secretion of pIgA is a functional marker of mucosal IgA production. We therefore examined culture supernatants from FACS-isolated PPC and 6-day culture supernatants from in vitro-activated MBC, for the presence of pIgA (>dimeric) and monomeric IgA, using size exclusion chromatography and ELISA. Our data comparing \( n = 4 \) cell isolates clearly indicated that nearly all IgA production by blood PPC was pIgA, with little or no monomeric IgA evident (Fig. 5A). In vitro-stimulated MBC also produced mostly pIgA but had a more prominent fraction of monomeric IgA compared with PPC (Fig. 5A). Thus, FACS-purified PPC secrete pIgA, not monomeric IgA. Unstimulated MBC do not secrete IgA or IgG, and the CD27hiCD38hi PPC subset contains all the functional mucosal IgA-producing cells.

We observed an average frequency of 54% of PPC that were slgA\(^+\), compared with 34% CCR10\(^+\) and 49% \( \alpha_4\beta_7\)\(^+\) (Table 1). Since the slgA\(^+\) fraction was highest among PPC subsets, it was possible that all IgA-secreting PPC would be included within this fraction. However, some IgA-secreting cells may have reduced slgA expression (43) and IgA expression on the cell surface cannot predict pIgA versus monomeric IgA production. We therefore sorted PPC (CD19\(^+\)CD27hi) cells that expressed slgA and those that did not and then counted IgA and IgG ELISPOTS for both sorted subsets to determine if all functional secretors of IgA were present among the slgA\(^+\) cells. In addition, we took supernatants from cultured slgA\(^+\) and slgA\(^-\) PPC and determined the distribution of polymeric and monomeric forms of IgA using size exclusion chromatography and ELISA.

The results of the ELISPOT analyses indicated that all IgA-secreting PPC were present in the slgA\(^+\)-sorted fraction of PPC, while none was present among the slgA\(^-\) sorted fraction (Table 2). The inverse observation was found for IgG-secreting cells that were only detected in the slgA\(^-\) sort fraction of PPC. Thus, slgA expression directly identifies IgA-secreting PPC in normal human blood, as assessed by ELISPOT. The vast majority of this IgA secreted by slgA\(^+\) PPC was dimeric or larger, with only a very small fraction of monomeric IgA (Fig. 5B). Since no IgA production was found in the slgA\(^-\) fraction of PPC, it was obvious that slgA\(^+\) PPC are the dominant source of pIgA.

Our ELISPOT data contrast the flow cytometric, icIgA staining of whole PBMC preparations that was recently published by Mei et al. (9) and the much earlier microscopic icIgA staining data for PBMC by Kutteh et al. (48), where icIgA\(^+\) cells largely outnumbered intracellular IgG cells. One possible explanation is that the slgA and IgA secretion do not correlate with icIgA staining, meaning perhaps that some IgA-producing cells do not secrete IgA or express slgA. Our analysis of icIgA expression by PPC demonstrates that the slgA\(^+\) subset of PPC contains all the icIgA\(^+\) cells and importantly that no slgA\(^-\) cells

### Table 2. IgA and IgG secretion by FACS-sorted PPC versus MBC or slgA\(^+\) versus slgA\(^-\) PPC

<table>
<thead>
<tr>
<th></th>
<th>IgA</th>
<th>IgG</th>
<th>MBC</th>
<th>IgA</th>
<th>IgG</th>
<th>IgA(^+) PPC</th>
<th>IgA (\sim) IgA(^-) PPC</th>
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<tbody>
<tr>
<td>IgA</td>
<td>277</td>
<td>371</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>693</td>
<td>550</td>
</tr>
<tr>
<td>IgG</td>
<td>17</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>36</td>
<td>25</td>
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Data are mean spots per 1000 plated PPC \(\pm\) SEM.

\(^*\)P < 0.001 for \( n = 18 \) comparisons of IgA or IgG secretion by slgA\(^+\) versus slgA\(^-\) PPC.

\(^*\)P < 0.001 for \( n = 6 \) comparisons of IgA or IgG secretion by PPC versus MBC.

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**Fig. 4.** A large fraction of PPC, but not MBC, express the mucosal chemokine receptor CCR10. Small frequencies of CCR9- and CCR10-expressing MBC were observed in peripheral blood (A). Similar analysis of PPC revealed that a large fraction of CCR10-expressing cells could be found in circulation (B). Results are representative of four samples. Negative fluorescence cutoffs were established using fluorescence minus-one (FMO) control samples.

**Table 2.** IgA and IgG secretion by FACS-sorted PPC versus MBC or slgA\(^+\) versus slgA\(^-\) PPC
Functional identification of mucosal PPC in human blood

Mucosal homing integrin and mucosal chemokine receptor expression by slgA+ PPC

slgA, integrin α4β7+ and chemokine receptors CCR10 are potential markers of mucosal relevance. Given our observation of exclusive plgA production by slgA+ PPC and the relatively high frequency of the two homing markers on PPC (Table 1), we reasoned that defined combinations of slgA with either of the two homing markers should provide the best surface phenotypic identification of mucosal PPC in normal blood. We therefore performed multicolour flow cytometric analysis to look at co-expression of CCR10 and α4β7+ with slgA on normal blood PPC. The results indicated that the greatest fraction of slgA+ PPC (~72%) expressed CCR10, and this was 2-fold higher than the fraction (~36%) that expressed α4β7 (Table 3). These data confirm previous observation of CCR10 expression by slgA+ large lymphoblasts (43). Within the slgA+ PPC, those that expressed CCR10 and no α4β7 were six times more frequent than those that expressed α4β7 but no CCR10. In addition, ~80% of slgA+α4β7+ PPC co-express CCR10. These data indicate clearly that within normal blood PPC, the CCR10+ PPC are more clearly associated with slgA than α4β7. However, a substantial fraction of slgA+ (~20%) did not express either CCR10 or α4β7, indicating that as much as one out of five functional mucosal PPC may not express either of the markers for mucosal homing.

We also performed a reciprocal examination of CCR10+ and CCR10− PPC looking for expression of slgA or by way of comparison of slgG (Table 4). On average, the largest fraction (67%) of CCR10+ PPC expressed slgA, while <33% of CCR10− PPC had slgG (Table 4). However, a substantial fraction (35%) of CCR10+ PPC were also slgA+, an observation similar to that stated above for slgA+ PPC (Table 3). In addition, a large fraction of CCR10− PPC did not express slgA or slgG. We therefore sorted CCR10+ PPC and CCR10− PPC and determined which of these two subsets secreted IgA or IgG. The results indicated clearly that CCR10+ PPC secrete IgA, but not IgG (Table 5). However, a substantial number of CCR10− PPC also secreted IgA. No IgG was secreted by CCR10+ cells, but rather all IgG-secreting cells appeared in the CCR10− fraction of PPC. Since there were a smaller but significant number of slgA+ cells and IgA-secreting cells in the CCR10+ subset compared with the CCR10− subset, we examined the production of plgA in the two subsets of cells purified by FACS and cultured in vitro. The vast majority of IgA produced by CCR10+ PPC was polymeric (Fig. 5C). A similar profile was true for CCR10− PPC, but cells in these cultures produced much less IgA (Fig. 5C), presumably because there were fewer slgA-producing cells present (Table 5). Finally, we examined iclgA stain along with CCR10 stain and observed that the majority of CCR10+ PPC was iclgA+ (Fig. 7), whereas one out of five iclgA+ cells did not express CCR10. Thus, the CCR10+ PPC subset includes most of the plgA-secreting cells and nearly all express slgA, and although there is a smaller clearly defined fraction of

express iclgA (Fig. 6). These results are consistent with our ELISPOT results (Table 2), which demonstrate that all IgA-secreting cells are contained within the slgA+ PPC fraction. However, those data also indicate that not all IgA+ PPC isolated for ELISPOT secreted IgA. By negative inference, there may be slgA+ PPC that do not secrete IgA and that would be consistent with previous suggestions that some subsets of iclgA+ cells are non-secretors (49).
CCR10+ cells that secrete pIgA, the larger CCR10+ subset represents a majority of functional mucosal PPC in normal blood.

Discussion

Analysis of circulating B cells that have previously encountered antigen can provide important information regarding ongoing or previous humoral immune responses in both the systemic and mucosal immune compartments. Isolated MBC from circulation can provide insight into previous pathogen exposures and vaccination if cultured and stimulated in vitro. However, circulating PPC can provide an important insight into active, recently initiated immune responses. Bernasconi et al. (50) have demonstrated that altered numbers of circulating MBC and PPC are indicators of recent or ongoing immune responses following vaccination (51) and we have confirmed this in separate work. Others have shown that antigen-specific PPC are mobilized in peripheral blood in the case of both acute and chronic infection (7). Our phenotypic analysis focused on the PPC compartment of blood from normal, healthy subjects with no infection or recent vaccination. We demonstrate that the PPC compartment is readily available for analysis of current ongoing antibody production in normal, healthy subjects. Our results are in agreement with other analyses of circulating B cell subsets and confirm the presence of CD27hi PPC and CD27int MBC in circulation. As others (50, 52) have speculated, we concluded from our data that PPC and MBC could be identified solely on the basis of the differential expression of CD27. We have now applied these phenotypic analyses to identify mucosal PPC in normal human blood.

Table 3. Expression of CCR10 and α4β7 on sIgA+ PPC

<table>
<thead>
<tr>
<th></th>
<th>CCR10+/α4β7− (%)</th>
<th>α4β7+/CCR10− (%)</th>
<th>CCR10+/α4β7+ (%)</th>
<th>CCR10−/α4β7− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIgA+</td>
<td>44.0 ± 5.6</td>
<td>7.3 ± 2.3</td>
<td>28.6 ± 7.8</td>
<td>20.1 ± 5.6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.
P < 0.002 for n = 4 comparisons of CCR10+/α4β7− versus α4β7+/CCR10−.
P < 0.05 for n = 4 comparisons of CCR10− versus α4β7+.

Table 4. Surface Ig expression on circulating CCR10+ and CCR10− PPC

<table>
<thead>
<tr>
<th></th>
<th>CCR10+ (%)</th>
<th>CCR10− (%)</th>
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<tbody>
<tr>
<td>sIgA+</td>
<td>66.8 ± 7.5</td>
<td>34.7 ± 5.9</td>
</tr>
<tr>
<td>sIgG+</td>
<td>2.8 ± 0.8</td>
<td>19.4 ± 4.5</td>
</tr>
<tr>
<td>sIgA+ / sIgG+</td>
<td>29.5 ± 6.7</td>
<td>44.7 ± 5.4</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for n = 6.

Table 5. IgA and IgG secretion by CCR10+ and CCR10− PPC

<table>
<thead>
<tr>
<th></th>
<th>CCR10+ PPC</th>
<th>CCR10− PPC</th>
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<tbody>
<tr>
<td>IgA</td>
<td>779 ± 20*</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IgG</td>
<td>281 ± 39</td>
<td>530 ± 58*</td>
</tr>
</tbody>
</table>

Data are mean spots per 1000 plated PPC ± SEM.
*P < 0.001 for n = 4 comparisons of CCR10+ PPC and CCR10− PPC for IgA or IgG.

CCR10− cells that secrete pIgA, the larger CCR10+ PPC subset represents a majority of functional mucosal PPC in normal blood.
The contribution of B cells to mucosal immunity is manifested primarily through secretory mechanisms found uniquely at mucosal epithelial surfaces. IgA dimers (or higher polymers) but not monomers are transcytosed from the basolateral to apical face of epithelial cells and are released into the lumen bearing a cleavage product of the plgR, which confers enhanced stability and anti-microbial properties to secretory IgA (47). It is secretory IgA that contributes to host defense in multiple innate and adaptive roles, including immune exclusion of microbes, viruses and pathogenic toxins (53); inhibition of indigenous microbial overgrowth (54); enhancement of mucosal immune development in neonates (65); limiting epithelial penetration by HIV (56) and providing intracellular neutralization of virus and LPS (57–59). It was recently demonstrated that even plgA, lacking bound secretory component, can facilitate removal of antigens from the lamina propria (60, 61).

Despite this breadth of evidence confirming a central role of plgA in mucosal defense, investigations of blood B cells for subsets that secrete plgA have been performed in only limited study (3, 11, 48). Previous studies have demonstrated that plgA is the dominant isotype produced in primary IgA responses, regardless of the route of immunization and nature of the antigen (62), but these studies did not identify the cellular origin of the plgA produced. However, recent studies of B cells in blood focused on slgA or iclG expression to correlate phenotype with mucosal function and were derived from the study of subjects during or after infection. Detection of IgA against the mucosal pathogen presumed, but do not demonstrate, that the IgA antibody is polymeric. Given that serum IgA is predominantly monomeric (63) and the earlier data (48) had demonstrated both plgA and monomeric IgA were produced by PBMC (not purified B cell subsets), it is necessary to clearly define only those subsets that make and secrete plgA when identifying and analyzing mucosal B cell subsets in blood. Our work here addresses this issue in regard to PPC subsets in normal healthy subjects and clearly demonstrates the existence of a mucosal PPC phenotype: CD27hiIgA+CCR10+ that secretes plgA. Here, we show that all plgA-secreting PPC are contained within the slgA+ PPC, all slgA+ PPC are also iclG+ and a majority of these cells is CCR10+. Our data also indicate that slgA+ PPC produce little if any monomeric IgA, so the cells responsible for production of IgA monomers likely do not circulate in large number, in normal healthy subjects, and are likely sequestered in the bone marrow (48).

We purposefully examined blood in normal healthy subjects free of infection, disease or recent immunization. Our observation and identification of the mucosal PPC in these subjects mean that these mucosal PPC can be used to examine the ongoing IgA response that occurs in healthy subjects. Thus, blood slgA+CCR10+ PPC may now be routinely studied for questions related to IgA response to normal mucosal flora and the role of mucosal IgA in immune homeostasis of human mucosal tissues. Just as important, current vaccine efforts have turned to monitoring mucosal IgA production in a variety of mucosal infections, including HIV. However, access and measures of mucosal samples of secreted IgA are not very reliable or convenient in humans (64) and therefore the blood surrogate of mucosal PPC secreting IgA could be used more reliably to test vaccine efficacy and monitor response to mucosal infection.

Our ELISPOT data for IgA secretion by PPC apparently contrast results from the recently published work of Mei et al. (9) in regard to the relative frequency of IgA- versus IgG-producing cells in blood from normal healthy subjects. Their data are based on intracellular staining of PBMC preparations and indicates that in normal subjects cytoplasmic IgA-producing cells are much more frequent than those with cytoplasmic IgG. In contrast, our functional data of IgA secretion (ELISPOT) indicate that the whole PPC (CD19+CD27hi) fraction contains slightly more IgG-secreting cells than IgA. The difference between results cannot be directly tested, since it is not possible to perform iclG stain and test for IgA secretion by the same cell. We did look at iclG-staining of identified PPC and observed that all the slgA+ cells were iclG+ but no slgA− cells were iclG+ cells (Fig. 6). Thus, we have similar observations to Mei, in regard to iclG+ PPC at high frequency in blood, but our ELISPOT data demonstrate that there is a significant fraction of slgA+ PPC that do not secrete antibody.

Previous in vitro work indicated that IgA2 PPC do not actively secrete IgA (49) and that surface expression of IgA, particularly IgA2, is not necessarily predictive of secretion (65). Thus, in normal human blood, iclG+ cells are not all secretors, and it may be that these PPC are IgA2 producers with an earlier differentiated state or with a specific functional phenotype. Thus, our data are consistent with both IgA1 and IgA2 PPC in circulation. Further work is required to demonstrate the distribution of IgA1 and IgA2 intracellular, surface and secreting cells among various subsets of blood PPC.

Mei et al. (9) provide data indicating both PC and plasmablast phenotypes may be present within the CD19+CD27hi blood cells from normal subjects based on differential HLA-DR expression and lack of CD62L and integrin α4β7. Our data indicate that a small fraction of the
CD19+CD27hi (PPC) did have reduced expression of HLA-DR, similar to Mei, but we found no expression of CD138, the canonical marker of PC. Our analysis of the Ki67 marker for recent proliferation clearly showed that all PPC are actively or recently divided cells, with small fraction having lower expression of Ki67 that correlated with reduced HLA-DR expression. Mei did not report on CD138 expression by normal subjects, although CD138-positive cells did appear in blood 7 days after tetanus toxoid immunization. We therefore conclude that the PPC we identify in the blood of normal subjects using the CD19+CD27hi phenotype are all plasmablasts and are not PC, but there is a fraction of PPC (10–20%) of more differentiated, notype are all plasmablasts and are not PC, but there is a fraction of PPC (10–20%) of more differentiated, HLA-DRlo plasmablasts. Since our cell isolation approach a fraction of PPC (10–20%) of more differentiated, notype are all plasmablasts and are not PC, but there is a fraction of PPC (10–20%) of more differentiated, HLA-DRlo plasmablasts. Since our cell isolation approach by Rosette and FACS did not alter the ratio or recovery of these cell subsets (Fig. 2), we are confident that our data are an accurate reflection of recently proliferating and functionally mucosal PPC in blood.

There is increasing evidence to support the notion that CCR9 and/or CCR10 can be used as markers of B cells that function predominantly in the mucosa. Murine studies have demonstrated that PC in Peyer’s patch and mesenteric lymph node (MLN) express CCR9 (66, 67). This was confirmed later in humans, where a distinct fraction (>30%) of IgA PC from small intestinal tissue was found to express CCR9 (43). However, CCR9 expression was rarely seen on IgA PC in other segments of the gut (43) and was not expressed by IgA PC in the lungs (43, 67). Consistent with this restricted receptor expression are observations that TECK (CCL25), the CCR9 ligand, is expressed at high levels by crypt epithelial cells and endothelial cells in the gut, but not in other mucosal tissues (27, 68). CCR10 is now understood to have a broader distribution of expression on B cells within various mucosal tissues, compared with CCR9. Production of the CCR10 ligand MEC (CCL28) has been demonstrated in the large and small intestine, trachea and bronchi, mammary glands and salivary glands (6, 29, 30), suggesting a common mucosal homing potential for CCR10+ cells. Indeed, analysis of human tissue samples from various mucosal sites has demonstrated that the majority of mucosal tissue IgA PC is CCR10+ (43).

Our data on PPC co-expression of slgA and CCR10 on blood PPC are clearly in agreement with the similar data of both Kunkel and Mei (9, 43), although neither of these investigators commented on the small but significant fraction of slgA+CCR10− cells in their identified plasmablast/PC. We clearly show that this smaller subset of PPC secretes plgA. Some of the IgA+CCR10− cells we identified expressed α4β7 (Table 3), but ~20% of slgA+ PPC expressed neither CCR10 nor α4β7. Thus, our data indicate that in blood from normal healthy subjects, CCR10 identifies the largest fraction of plgA-producing mucosal PPC. Our data also reveal the presence of a CCR10+/slgA+ population (Fig. 7), which may also explain how <100% CCR10+ PPC secrete IgA as measured by ELISPOT (Table 5). The smaller subset of slgA+CCR10− PPC may also represent a distinct mucosal population, but there is also evidence of plgA-producing cells in non-mucosal tissues (46), raising the possibility that CCR10 might distinguish between mucosal and non-mucosal plgA-producing PPC in blood.

We did not detect any CCR9 expression by blood PPC from healthy subjects. This directly contrasts the results of Kunkel (43) who found that up to 25% of large CD19+slgA+ PPC expressed CCR9. However, the subjects used for that study were not well described, and others detected CCR9 on circulating B cells after viral infection (7). Our observation on the lack of CCR9 expression by PPC in blood from normal healthy subjects is consistent with one current hypothesis that the main function of CCR9 is to ensure retention of gut-resident PC, as others have reported that up to 50% of small intestinal PC co-express both CCR10 and CCR9 (43). Lack of CCR9 on blood PPC in normal healthy subjects would be expected if it is expressed only by resident small intestinal PC in healthy humans.

While α4β7 has been demonstrated to be central to T cell migration into the gut, it is not clear for B cell subsets, particularly in human blood. There is considerable evidence to suggest that the location of antigen encounter, and not the tissue of antigenic relevance, is responsible for the expression of adhesion molecules on antigen-experienced B cells (22). We observed that <10% of IgA+ PPC expressed high levels of α4β7 in the absence of CCR10, while 44% of IgA+ PPC expressed CCR10 in the absence of α4β7 (Table 3). Thus, high levels of α4β7 on its own are a poor marker of mucosal PPC in normal human blood. In addition, we observed that the majority of naive B cells and MBC in blood expresses low to intermediate levels of α4β7 integrin (Fig. 2). These observations are consistent with the hypothesis that activation of MBC in mucosal-associated lymphoid tissues (MLN or Peyer’s patches) would cause up-regulation of this integrin but that high levels of α4β7 are not always (or continuously) expressed by mucosal PPC.

Here, we report that nearly 40% of slgA+ PPC co-express CCR10 with high α4β7 (Table 3). According to the multistep model of lymphocyte migration, interactions mediated by integrins initiate rolling of lymphocytes on endothelium, where endothelial-expressed chemokines can interact with their cognate receptors on the rolling lymphocyte, strengthening the integrin interactions and thus triggering adhesion and diapedesis (69, 70). As a marker co-expressed with CCR10, α4β7 would permit plgA-secreting PPC to enter the human intestinal mucosa where MadCAM-1 is known to be expressed (71). Further analysis of the other 60% of slgA+CCR10− PPC for other adhesion molecules such as vascular cell adhesion molecule-1 (salivary gland, trachea and bronchioles) (72) may reveal similar populations with different presumptive homing patterns.

Our results also indicate the presence of small (3–5%) of MBC but consistently observed sub-populations of CCR9+CCR10− and CCR9+CCR10+ MBC, consistent with previous observations (43). We FACS purified the total MBC (CD19+CD27+hi) population and activated them in cultures that provided appropriate stimulation for proliferation and differentiation to secrete IgG or IgA (32). Following in vitro activation of the MBC, we did observe production of plgA that predominated over a smaller but distinct monomeric IgA production (Fig. 5A). Nearly 25% of MBC express slgA (Table 1), so there is not a strong correlation between slgA+ MBC and the expression of CCR10 or CCR9. While it is possible that the small fractions of CCR10+ or CCR9+ MBC were...
Functional identification of mucosal PPC in human blood

responsible for the production of plgA, the evidence is not clear. For instance, it is also likely that some MBC down-regulate CCR10 and/or CCR9 expression as they transition into a resting phenotype. The best evidence that this occurs, as the examination of MBC in rotavirus (RV)-infected during the convalescent phase of infection demonstrated, where RV-specific MBC lack CCR9 and CCR10 expression, even though all RV-specific B cells expressed at least one of the markers during acute infection (47). We obtained blood from normal healthy individuals, so observed low expression of CCR9 and CCR10 detection on circulating MBC is consistent with their patterns of expression on this population in blood in the absence of active infection. Further analysis of mucosal MBC will require specific isolation of slgA+ MBC that express CCR9 or CCR10, comparing them to those that do not have these markers.

In summary, we have demonstrated in normal human blood a high-frequency population of surface and intracellular IgA+ PPC, the majority of which expresses CCR10 and that can functionally contribute to mucosal defense through secretion of plgA. The PPC we observed are likely recently proliferating plasmablasts, with a small fraction indicating further differentiation towards PC. Thus, these IgA-producing PPC fit the description of recently activated mucosal plasmablasts in blood. Our results were obtained in normal, healthy individuals, so the identified mucosal PPC should reflect the normal level of mucosal plasmablasts in blood and should be taken in contrast to previous observations made in virus-infected individuals (7, 55). Our functional definition of mucosal PPC will allow meaningful observations of ongoing mucosal IgA antibody responses in healthy subjects and comparison of homeostatic IgA responses to those after infection and vaccination. Many other applications may follow for diseases involving plgA, including perhaps the identification of an IgA-secreting population in peripheral blood that is responsible for glomerular deposition of plgA in IgA nephropathy (47).

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References


