# CORRELATES OF HUMORAL MUCOSAL IMMUNITY IN PERIPHERAL BLOOD

# B LYMPHOCYTES IN HUMAN PERIPHERAL BLOOD ARE INDICATORS OF RECENT MUCOSAL IMMUNE RESPONSES AND REPRESENT A LINK BETWEEN SYSTEMIC AND MUCOSAL HUMORAL IMMUNITY

By

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TITLE: B lymphocytes in human peripheral blood are indicators of recent mucosal immune responses and represent a link between systemic and mucosal humoral immunity.

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**Abstract**

Several labs have previously demonstrated that humoral immune responses at one mucosal tissue can disseminate to other mucosal sites, giving rise to the theory of the common mucosal immune system (CMIS). Evidence that demonstrates a similar link between systemic immune responses and mucosal protection is lacking despite indications that both mucosal and systemic memory B cells share common circulatory pathways. The focus of this study is to determine the contribution of blood-borne B cells to mucosal immunity in humans, and how B cells trafficking through blood can be induced to traffic to mucosal tissues.

To address these aims, I have performed several analyses of active and inactive peripheral blood memory B cell (MBC) populations in normal, healthy humans. Analysis of recently activated blood B cells confirmed the revealed that the majority of pre-plasma cells (PPC) in blood of healthy, normal humans secrete IgA, and that the majority of IgA-positive PPC secrete primarily polymeric IgA. A large fraction of blood PPC also express CCR10, and this population contains the highest fraction of IgA-expressing and pIgA-secreting PPC in blood. In contrast, most $CCR10^-$ PPC secrete IgG, although a small fraction secretes and stains positive for IgA, and analysis of $\alpha_4\beta_7$expression by blood MBC revealed that CCR10 is more inclusive of IgA-switched and pIgA-secreting PPC in blood. The data presented in this study demonstrate that $IgA^+/CCR10^+$ PPC represent the mucosal subset of PPC in the blood of healthy humans, and can be investigated as representative of recent or ongoing mucosal immune responses.

*In vitro* polyclonal stimulation of blood MBC through CD40 was able to induce CCR10 expression by blood MBC treated with IL-21, IL-2, IL-10 with additions of other germinal center (GC) cytokines such as IL-5 and TGF-β enhancing CCR10 induction. Surprisingly, CCR10 expression by IgG MBC stimulated under these conditions was similar to that of IgA MBC, demonstrating that CCR10 expression is not limited to IgA-switched B cell clones. The results of this study demonstrate that CCR10 expression is inducible by many GC factors, and importantly, is not limited to IgA-switched B cells.

Systemic immunization of healthy volunteers with tetanus toxoid/diphtheria vaccine induced a robust systemic IgG response, but also resulted in a post-immunization mobilization of IgA PPC in blood. These PPC were not specific for tetanus or diphtheria, and in several volunteers showed specificity for mucosal pathogens such as poliovirus (PV) and herpes simplex virus (HSV) glycoproteins. In addition, stimulation of anti-HSV IgG memory was also observed. Thus we have demonstrated that a systemic immune response is capable of inducing antibody relevant to mucosal immunity, possibly exposing a mechanism through which systemic and mucosal humoral immune responses are linked.

The studies presented here demonstrate the presence of mucosal B cell memory in blood and thus provide new insight into ways to assess and manipulate mucosal immunity.

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| | |
|---|---|
| APC | Antigen-presenting cell |
| BALT | Bronchus-associated lymphoid tissue |
| CD | Crohn's disease |
| CMIS | Common mucosal immune system |
| DC | Dendritic cell |
| dIgA | DimericIgA |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISPOT | Enzyme-linked immunosorbent spot assay |
| FAE | Follicle-associated epithelium |
| FDC | Follicular dendritic cell |
| GALT | Gut-associated lymphoid tissue |
| GC | Germinal center |
| Hib | *Haemophilus influenza* b |
| HIV | Human immunodeficiency virus |
| HPV | Human papillomavirus |
| IBD | Inflammatory bowel disease |
| Ig | Immunoglobulin |
| IgA-PC | IgA-secreting plasma cell |
| IgM-PC | IgM-secreting plasma cell |
| J-chain | Joining chain |
| MALT | Mucosa-associated lymphoid tissue |
| MBC | Memory B cell |

| | |
|---|---|
| MEC/CCL28 | Mucosally expressed chemokine |
| MLN | Mesenteric lymph node |
| NALT | Nasopharyngeal-associated lymphoid tissue |
| PP | Peyer's patches |
| PPC | Pre-plasma cell |
| PC | Plasma cell |
| pIgA | Polymeric IgA |
| pIgM | Polymeric IgM |
| pIgR | Polymeric immunoglobulin receptor |
| PP | Peyer's patches |
| SC | Secretory component |
| SCID | Severe combined immunodeficiency |
| SIgA | Secretory IgA |
| sIg | Surface Immunoglobulin |
| TECK/CCL25 | Thymus-expressed chemokine/ |
| $T_{FH}$ | Follicular helper-T cells |
| UC | Ulcerative colitis |
| VCAM | Vascular-cell adhesion molecule |
| VIP | Vasoactive intestinal peptide |

# CHAPTER 1

## General Introduction

## 1.1 Overview of the mucosal immune system

In humans, the respiratory, digestive, and urogenital surfaces represent a highly vulnerable port of pathogen entry resulting from exposure to the external environment. Unlike the skin, the mucosal surfaces must be able to balance absorption of molecules essential for normal host function while at the same time excluding pathogens. Animal studies have revealed that immediately following birth these surfaces are exposed to commensal flora and dietary antigens to which immunological tolerance is maintained in order to prevent injury to the host, while allowing for the development of normal systemic and mucosal immune responses against pathogens (Berg *et. al*, 1975, Carter *et. al*, 1971, Schlessinger *et. al*, Šterzl *et. al*, 1970, Tlaskalova-Hogenova *et. al*, 1980). The combination of high antigenic burden and the need for non-inflammatory immune exclusion is reflected through the divergence of the human immune system into two arms; i) the systemic immune system and; ii) the common mucosal immune system (CMIS).

The CMIS consists of several major subdivisions, each associated with a specific mucosal surface, defined as "common" due to the ability of immune effectors activated at one site to disseminate to others (McDermott *et. al*, 1979, McDermott *et. al*, 1980, Rudzik *et. al*, 1975). These major sub-compartments are bronchus-associated lymphoid tissue (BALT), nasopharyngeal-associated lymphoid tissue (NALT), and gut-associated lymphoid tissue (GALT). Evidence suggests that activation in these sub-compartments

provides effector cells with a unique homing phenotype which permits their re-entry into the relevant effector site following activation (Quiding-Jarbrink *et. al*, 1995, Quiding-Jarbrink *et. al*, 1995, Quiding-Jarbrink *et. al*, 1997). In addition to these gross divisions, isolated inductive immune sites known as Peyer's patches (PP) can also be identified in multiple mucosal tissues. Collectively, the lymphoid tissues dedicated to mucosal defense are known as the mucosa-associated lymphoid tissues (MALT).

The branches of MALT give rise to immune effector cells with functions and capabilities that cooperate with tissue-specific characteristics including secretion of tissue-specific chemokines and expression of mucosal addressins and the polymeric immunoglobulin receptor (pIgR) (Macpherson *et. al*, 2008, Mestecky, 2005).

### 1.2 Secretory IgA and local mucosal immunity

IgA is the predominant immunoglobulin (Ig) isotype at mucosal tissues. IgA is a non-complement fixing immunoglobulin isotype which is secreted in a dimeric form (dIgA) by IgA plasma cells (IgA-PC) at mucosal surfaces (Macpherson *et. al*, 2008). dIgA is composed of two IgA1 or IgA2 monomers joined by a short polypeptide chain called the joining chain (J-chain), which also links IgM monomers together to form polymeric IgM (pIgM).

dIgA is secreted by sub-laminar PC and is transported into the luminal cavities via receptor-mediated transcytosis through epithelial cells following interaction with the pIgR (Kaetzel, 2005), a receptor for polymeric immunoglobulin expressed at the basolateral surface of mucosal epithelial cells. Following transcytosis, the extracellular

domain of the pIgR is cleaved off and remains associated with dIgA as a molecule known as secretory component (SC) (Kaetzel, 2005). This dIgA-SC complex is known as secretory IgA (SIga) and the presence of SC confers several biological properties to SIga including resistance to proteolytic degradation compared to monomeric IgA and IgG both *in vitro* (Lindh, 1975, Mestecky *et. al*, 1991, Renegar *et. al*, 1998) and *in vivo* (Chintalacharuvu *et. al*, 1997, Crottet *et. al*, 1998, Ma *et. al*, 1998), and may provide additional loci for non-specific pathogen neutralization through the presence of additional acetylated and galctosylated side-chains (Adlerberth *et. al*, 1996, Royle *et. al*, 2003, Wold *et. al*, 1990). Secretory IgA contributes to non-inflammatory immune exclusion through a variety of innate and adaptive functions including exclusion of microbes, viruses and toxins, inhibition of indigenous microbial populations, enhancement of mucosal immune development in infants, limiting epithelial penetration by HIV, and intracellular neutralization of viruses and LPS (Bomsel *et. al*, 1998, Brandtzaeg, 2002, Brandtzaeg, 2007, Fujioka *et. al*, 1998, Hocini *et. al*, 1997, Macpherson *et. al*, 2005, Mazanec *et. al*, 1995, Shen *et. al*, 2010). Polymeric IgA lacking SC has been shown to facilitate clearance of those antigens that are able to penetrate the innate and adaptive features of mucosal immunity and epithelial organization (Mazanec *et. al*, 1993, Robinson *et. al*, 2001).

Polymeric IgM associated with SC has also been documented in mucosal secretions, although in lower proportions than IgA (Brandtzaeg, 1981, Brandtzaeg, 1985). In newborns and IgA-deficient patients, IgM is of greater importance than in healthy adults (Brandtzaeg *et. al*, 1999, Brandtzaeg *et. al*, 1991, Plebani *et. al*, 1983).

IgG can also be found in mucosal secretions in approximately the same concentrations as IgM (Mestecky, 2005). The ratio of IgA to IgG is highly variable from tissue to tissue, and difficulties in methodology of collection and testing make accurate, reproducible quantification difficult (Mestecky, 2005). This is especially true when assaying female genital tract secretions which fluctuate with the progression of the menstrual cycle (Griffin *et. al*, 1993). It is becoming apparent that many reproductive tract secretions, including uterine cervical fluid, urine, vaginal washes, and seminal plasma frequently contain higher proportions of IgG than IgA (Kutteh *et. al*, 1996, Mestecky, 2005, Svanborg Eden *et. al*, 1985). Unlike IgA, transport of IgG into mucosal secretions is not pIgR-mediated, but rather results from nonspecific transport via paracellular transport, fluid-phase endocytosis, or through Fc-receptor mediated transport (Mestecky, 2005, Spiekermann *et. al*, 2002).

IgE and IgD are the least-prevalent immunoglobulins in human mucosal secretions, although presence of IgE in respiratory and gastrointestinal secretions is associated with allergic responses at these sites (Brown *et. al*, 1975, Jonard *et. al*, 1984, Mygind *et. al*, 1975). There is little evidence of specific transport of IgE into secretions, and thus detection of IgE in secretions may be a result of increased epithelial permeability due to allergy, rather than a cause. Low concentrations of IgD can also be found in milk and saliva, but there is no evidence of active transport of this isotype at mucosal surfaces (Leslie *et. al*, 1977).

## 1.3 IgA immune responses in mucosal tissues

### *1.3.1 GALT*

Murine GALT PP have been the most extensively characterized immune inductive tissues (Iijima *et. al*, 2001, Mowat, 2003). Thus the majority of this discussion will focus on murine GALT immunology. PP have been identified as crucial for normal induction of SIga responses (Mestecky, 2005).

The surface of PP is covered by specialized epithelial cells, known as follicle-associated epithelium (FAE). Specialized epithelial cells called M cells act as a portal for antigen uptake, and lie over a "pocket" within the FAE that is filled with T and B cells, and APC (Mestecky, 2005, Owen *et. al*, 1974). M cells are specialized for luminal antigen uptake and transport, but not processing, and thus play a major role in delivering intact luminal antigen to APC underlying the mucosal epithelium (Mestecky, 2005, Owen *et. al*, 1988). This system permits sampling of intact luminal antigen but can also be a target for pathogen entry, as is the case with *Salmonella* strains and several reoviruses which initiate infection through M cells (Jepson *et. al*, 2001). Following antigen transport by M cells PP APC process and present antigen to resident lymphocytes. Lymphoid ($CD8\alpha^+$) DC are believed to initiate Th1-type cellular responses, while myeloid ($CD11b^+$) DC seem to induce Th2 cells that are responsible for IgA immune responses (Banchereau *et. al*, 2000). In support of these observations, evidence indicates that exposure to innocuous food antigens induces myeloid DC to secrete IL-10 and TGF-β (Iwasaki *et. al*, 1999, Iwasaki *et. al*, 2000) and thus may induce IgA switch in activated naïve B cells

(Dullaers *et. al*, 2009). Distinct follicles analogous to B cell follicles observable in lymph nodes and spleen can be observed in PP, and are believed to be the source of mucosal IgA-PC (Mestecky, 2005). Adjacent to these B cell zones are T cell-enriched zones believed to induce and support both cellular and IgA responses (Farstad *et. al*, 1997, Farstad *et. al*, 1997). Once activated in PP antigen-specific B cells emigrate to the circulation via lymphatic drainage, circulate through the bloodstream, and home to mucosal effector sites, usually mucosal lamina propria (McGhee *et. al*, 1989, Paul, 2008).

### *1.3.2 BALT*

In comparison to the gastrointestinal and urogenital surfaces the respiratory mucosa is home to fewer immunoglobulin producing cells but can rapidly respond to inhaled antigen encounter with increases in pIgR expression and influx of B cells into the lungs (Jaffar *et. al*, 2009). Evidence from human and animal studies demonstrate that exposure to respiratory pathogen such as rhinovirus, RSV, and influenza results in increases in IgA secretion in bronchial and nasal secretions (Igarashi *et. al*, 1993, Joo *et. al*, 2010, Walsh *et. al*, 1999).

PC found underlying respiratory mucosa and less mature B cells residing in the upper respiratory BALT predominantly express IgA or IgM (Otsuki *et. al*, 1989). Evidence has been presented to support the hypothesis that the respiratory epithelium plays a role in polarizing B cell isotype switch to IgA through secretion of IL-5, TGF-β, IL-6 and IL-10 (Salvi *et. al*, 1999). Transepithelial transport of locally-produced pIgA occurs via the same mechanism as in the digestive tract, and results in release of SIga into

the respiratory lumen (Jaffar *et. al*, 2009). In the human lung IgA PC are primarily found in close proximity to the airway epithelium (Bienenstock *et. al*, 1973). Thus, in contrast to the GALT, which maintains IgA-PC induction through epithelial actions as well as the presence of PP, in healthy humans the BALT may rely exclusively on epithelial cytokine release to maintain an IgA-polarizing immune environment. This is analogous to development of SIga-producing PC in human tonsils, which are elicited through interactions of lymphoid effectors and epithelial cells (Harabuchi *et. al*, 1996). B cells and BALT are located close to or within the lung epithelium rather than in isolated lymphoid follicles as is seen in the digestive tract, supporting the hypothesis that epithelial interactions may play a role in directing B cell differentiation (Bienenstock *et. al*, 1973, Schmedtje *et. al*, 1973, Sue-Chu *et. al*, 1998). Studies of T-cell receptor knockout mice that demonstrate T cells are not necessary for the induction of IgA responses (Franco *et. al*, 1997, Gardby *et. al*, 1998) further support the observations drawn from lack of BALT in normal human lung tissue, specifically that other parts of the respiratory tract are capable of sustaining IgA-based immunity. It has been suggested that airway DCs and epithelial cells might perform this role as both are capable of processing and presenting antigen as well as secreting factors inducing IgA switch (Salvi *et. al*, 1999).

The protective effects of IgA in the upper respiratory tissues are emphasized by observations of some IgA-deficient individuals, who suffer from an increased prevalence of respiratory infections and associated disorders (Aghamohammadi *et. al*, 2009).

However, IgA-deficient individuals may receive protection by a compensatory increase in IgM, the majority of which appears to be locally produced (Cardinale *et. al*, 1992).

### *1.3.3 Female Reproductive Tract*

Animal and human studies have shown that the IgG:IgA ratio (Blandau *et. al*, 1973), expression of pIgR in cervical tissue (Sullivan *et. al*, 1984), and IgA transudation (Sullivan *et. al*, 1983) fluctuate significantly during the different phases of the menstrual cycle. The majority of IgG present in reproductive secretions is believed to originate in the serum and reaches the reproductive tissues via transudation (Bouvet *et. al*, 1994, Mestecky, 2005), although local IgG PC have also been identified (Kutteh *et. al*, 1988). The majority of IgA in the reproductive tract is polymeric and bears SC, reflecting a local origin although there is a small fraction of IgA monomers present (Kutteh *et. al*, 1988). Under normal conditions the vagina, fallopian tubes, and the cervix are the primary sites for PC homing and Ig production (Kutteh *et. al*, 1988). Unlike the secretions in the digestive and respiratory systems where the dominant secretion is SIga, human genital tract secretions contain approximately equal concentrations of IgA (secretory and monomeric) and IgG (Blandau *et. al*, 1973, Kutteh *et. al*, 1996).

The distribution of IgA1 and IgA2 in the human female genital tract strongly resembles that of the lower intestinal tract but differs from that found in serum or other tissues. In humans the lymphoid tissues of the large intestine are slightly enriched for IgA2 PC precursors over IgA1 PC precursors, a distribution shared only by the reproductive tract (Mestecky *et. al*, 1986) consistent with the CMIS model. The rectal

lymphoid tissues have been exploited as immune inductive sites for immune responses in the female genital tract in animal studies (Kutteh *et. al*, 2001), although such attempts have produced highly variable results depending on the type of antigen delivered and the stage of the menstrual cycle (Kozlowski *et. al*, 2002, Kutteh *et. al*, 2001). Interestingly, several studies have demonstrated that intranasal immunization with cholera toxin B or cholera toxin B/*V. cholerae* induces anti-cholera toxin B-specific IgA and IgG in the human female genital tract (Johansson *et. al*, 1998, Kozlowski *et. al*, 2002). The ability of the intranasal immunization route to elicit both secretory IgA responses at the reproductive mucosa as well as serum IgG responses demonstrates the ability of non-local antigen challenge to contribute to host defense at different points in the CMIS. In particular, the contribution of serum IgG to reproductive tract secretions is the most apparent interaction between the systemic and mucosal humoral immune compartments, and further demonstrates the uniqueness of the genital tract as a "receiving" site for immune responses generated in other tissues (Russell *et. al*, 2002). It is becoming evident from recent studies of genital tract infections that mucosal exposure to HIV, HPV, and other sexually-transmitted infections generates only limited local IgA and IgG responses (Russell *et. al*, 2002), emphasizing the importance of understanding the efficacy of non-genital vaccination routes in enhancing female genital tract immunity.

### *1.3.4 Male Reproductive Tract*

The penile urethra is home to the majority of PC with an abundance of IgA and IgM PC found along the lamina propria of the urethra and surrounding the periurethral

glands, which contribute a mucin-like substance to semen (Griffin *et. al*, 1993). The urethral tissue is also enriched for pIgR expression relative to other male reproductive tissues, and the urethral epithelium stains positive for IgA, IgM, and IgG (Griffin *et. al*, 1993). Based on these observations it has been suggested that urethral mucin and antibody secretion combine to form an immunologic barrier along the length of the penile urethra similar to the sIg-rich mucus of the cervix (Tjokronegoro *et. al*, 1975). Nearly all epithelial cells in the prostate express pIgR (Cunningham *et. al*, 2008), and both IgA and IgM PC are normally found in the normal prostate, suggesting that it may have an active role in immunity in the male reproductive system. Recent evidence that neutralizing sIgA in the male reproductive tract was correlated with increased clearance of *C. muridarium* indicates that neutralizing antibodies transported by the prostate would increase protection against infection in the male reproductive tract (Cunningham *et. al*, 2008). With the exception of these two sites, expression of pIgR and PC is not observed in other tissues of the male reproductive tissues (Griffin *et. al*, 1993, Mestecky, 2005). Seminal plasma from healthy male donors has been shown to contain IgG, IgA, and IgM (Friberg, 1980, Uehling, 1971). However, with the identification of PC and pIgR in the penile urethra it is now unclear if these immunoglobulins originate in the prostate or the urethra.

## 1.4 B cell responses

### *1.4.1 B Cell Activation*

Newly formed naïve B cells enter circulation from the bone marrow expressing the pan-B cell surface markers CD19 and CD20 (Allen *et. al*, 2007, McHeyzer-Williams *et. al*, 2005) and low to intermediate expression of CD38 (van Lochem *et. al*, 2004). Once in circulation, these cells traffic into peripheral lymphoid organs through high endothelial venules. If they encounter cognate antigen in the context of antigen-presenting cells and activated antigen-specific T cells, they become activated and migrate into the B-cell zone of local germinal centers (GC) (Allen *et. al*, 2007, McHeyzer-Williams *et. al*, 2005).

During the GC reaction, naïve B cells undergo somatic hypermutation which increases Ig affinity for the activating antigen, class-switch recombination to IgA, IgG, or IgE isotype, and survival of the activated cell. At this point activated B cells begin expressing CD27, which several studies have identified as a memory marker for circulating B cells (Caraux *et. al*, 2010, Klein *et. al*, 1998, Tangye *et. al*, 1998, Xiao *et. al*, 2004). $CD27^+$ B cells possess mutated Ig genes, switched isotypes, and preferential capacity *in vitro* differentiation into plasma cells when compared with $CD27^-$ blood B cells (Tangye *et. al*, 1998). During GC reactions B cells also increase expression of CD38, a surface marker indicative of recent activation (Malavasi *et. al*, 2008).

Some GC emigrant B cells retain expression of IgM in the absence of CD27. The identity and function of these cells still remains undetermined, but observations that they

are less dependent on GC interactions suggest that they may be comprised of T-cell independent B cells (Weller *et. al*, 2001).

Observations of GC responses have identified three populations of B cells that exit lymphoid organs into circulation. $CD19^{+}/CD27^{+}/CD38^{-/int}$resting memory B cells do not secrete antibody and form the memory pool for subsequent encounters of the relevant antigen. Two antibody-secreting populations can be identified, the plasmablast (PB) and the plasma cell (PC). Plasma cells frequently lack pan-B markers such as CD19, and express high levels of CD27, CD38, and CD138 (Perez-Andres *et. al*, 2004). Plasmablasts also express high amounts of CD27 and CD38, but in contrast with PC express low levels of CD19, and lack CD138. While resting memory B cells are predominantly surface $Ig^{+}$, most PB/PC express little to no surface immunoglobulin (Caraux *et. al*, 2010). Following antigen-specific responses, these three populations migrate into blood. PB/PC are functionally similar and difficult to distinguish, and along with resting memory B cells frequently express a variety of adhesion and chemokine receptors directing their subsequent tissue-specific or BM migration (Caraux *et. al*, 2010, Kunkel *et. al*, 2002). Typical phenotypes observed along the B cell differentiation pathway and their corresponding tissue compartments are presented in Figure 1.1.

### *1.4.2 IgA switch*

Antigen uptake by M cells and activation of local T cells triggers local germinal center (GC) formation in ILF (Mestecky, 2005). Alternatively, follicular dendritic cells (FDC) located in M cell pockets can migrate to MLN following antigen uptake from M

cells and trigger GC formation in the node, after which activated antigen-specific B cells home back to mucosal tissues through the blood (Mestecky, 2005). During these GC responses, IgA switch by naïve B cells can be induced by TGF-β (Cazac *et. al*, 2000, Coffman *et. al*, 1989, Islam *et. al*, 1991) and IL-6 (Fujihashi *et. al*, 1991, McGhee *et. al*, 1991), and can be enhanced by IL-10 (Defrance *et. al*, 1992, Fayette *et. al*, 1997). Other cytokines including IL-5 and IL-21 have also been implicated in IgA-switching and terminal differentiation of activated B cells (Dullaers *et. al*, 2009, Schoenbeck *et. al*, 1989, Sonoda *et. al*, 1992, Sonoda *et. al*, 1989). The importance of MALT-derived cells in inducing IgA switch is emphasized by *in vitro* studies of PP-derived DC which preferentially induced IgA switch by CD40-activated naïve B cells (Fayette *et. al*, 1997). Additionally, other secreted proteins such as vasoactive intestinal peptide (VIP) have been shown to induce *in vitro* activated B cells to switch to IgA1 and IgA2 (Kimata *et. al*, 1995). It has been suggested that DC may also influence the generation of IgA2 versus IgA1 responses, although the signals involved in such control are currently unknown (Fayette *et. al*, 1997).

### *1.4.2 Adhesion molecules directing B cell migration*

Following activation in GALT, activated B cells exist through draining microlymphatic vessels. Human memory B cells draining from GALT are characterized by strong expression of $\alpha_4\beta_7$ (Farstad *et. al*, 1997, Farstad *et. al*, 1997) as compared to similarly located naïve B cells which express lower levels of this heterodimer, and analysis of MLN populations provides similar results (Farstad *et. al*, 1997). Following

transit through the thoracic duct and re-entry into blood, these cells are believed to be predisposed to returning to the intestinal lamina propria through interaction with MAdCAM-1 (Butcher *et. al*, 1996). Consistent with this model, enteric stimulation in humans is followed by appearance of $\alpha_4\beta_7$-expressing antigen-specific B cells in blood, while parenteral immunization mainly induced L-selectin over $\alpha_4\beta_7$ (Kantele *et. al*, 1997, Quiding-Jarbrink *et. al*, 1997). Expression of $\alpha_4\beta_7$ remains high on gut lamina-propria PC (Kunkel *et. al*, 2003), suggesting that it may contribute to local retention of effector cells.

Activation in human NALT induces co-expression of L-selectin and $\alpha_4\beta_7$ on antigen-specific B cells and leads to increased antibody production in adenoids and nasal mucosa (Quiding-Jarbrink *et. al*, 1995), and animal models have demonstrated that human tonsillar B cells transferred to severe combined immunodeficiency (SCID) mice migrated to the lung but not the gut (Nadal *et. al*, 1991). These studies support the view that airway and gut homing of activated B cells utilizes different adhesion molecule profiles, reflecting compartmentalization of different mucosal effector sites. Surprisingly, NALT activation of B cells leads to significant accumulation of antigen-specific effectors in the urogenital tract, reflected by high levels of specific antibody in reproductive secretions of intranasally immunized mice and monkeys (Brandtzaeg, 1997, Johansson *et. al*, 2001), and human studies have demonstrated that intranasal vaccination results in a significant antigen-specific response at urogenital sites (Johansson *et. al*, 1998). The heterodimer $\alpha_4\beta_1$ has been proposed to mediate migration to non-intestinal mucosal tissues through interaction with bronchial/nasal-expressed vascular-cell adhesion molecule (VCAM)-1 (Bentley *et. al*, 1993), although no direct evidence supporting this

hypothesis exists. The role of $\alpha_E\beta_7$, an integrin expressed on intraepithelial T cells in the gut (Cepek *et. al*, 1994) is also unclear but evidence suggests it is not directly involved in lymphocyte migration to the intestine (Strauch *et. al*, 1994)). Animal studies suggest it may be involved in recruitment or retention of antigen-specific T cells following intravaginal virus encounter (Suvas *et. al*, 2007, Tang *et. al*, 2010). Work in our lab has demonstrated that αE-positive MBC and pre-plasma cells (PPC) are absent from circulation in normal, healthy humans (Fernandes *et. al*, 2010).

### *1.4.3 Chemokines directing B cell homing to mucosal tissues*

The thymus-expressed chemokine (TECK/CCL25) appears to mediate a central role in recruiting/retaining both T and B cells in the human small intestinal lamina propria. TECK is produced exclusively by small intestinal crypt epithelium (Bowman *et. al*, 2002, Kunkel *et. al*, 2000) and its' ligand, CCR9, is expressed by virtually all activated B cell/PC in the intestinal lamina propria (Kunkel *et. al*, 2002, Kunkel *et. al*, 2003). The structurally-related mucosally-expressed chemokine (MEC/CCL28) is expressed by all mucosal tissues (Lazarus *et. al*, 2003) and has been proposed to be a unifying mucosal chemokine for the CMIS. IgA plasmablasts and PC from each mucosal tissue express CCR10 (Lazarus *et. al*, 2003), and thus it is theorized to direct lymphocyte homing to multiple tissues with tissue specificity conferred by co-expression of tissue-specific addressins (e.g. $\alpha_4\beta_7$) or other chemokine receptor/chemokine signaling pathways (e.g. CCR9/TECK).

### 1.5 Parenteral immunization and mucosal immunity

Development of vaccine formulations for mucosal delivery has become an important goal for research, particularly for mucosally-encountered pathogens such as HIV, influenza, and HPV. Mucosal vaccination has several benefits, including painless administration, rapid potential for mass immunization in the case of highly dangerous outbreaks, and reduced cost of production, storage, and delivery, but currently the weight of evidence suggests that parenteral immunizations can also contribute to antibody-mediated mucosal immunity.

Parenteral immunization has been shown to be capable of inducing IgA responses as well as IgG responses measurable by an increase in pIgA in serum soon after immunization (Tarkowski *et. al*, 1990), some of which may reach the mucosa from the vasculature (Renegar *et. al*, 1991)). Human studies of parenteral immunization have been shown to lead to an increase in antigen-specific serum IgG correlated with an increase in specific IgG in the reproductive tract (Ashley *et. al*, 1998, Bergquist *et. al*, 1997, Kozlowski *et. al*, 2002, Rudin *et. al*, 1998). The majority of reproductive tract IgG is believed to be of seru origin, accumulating through transudation, FcRγ-mediated transport, and possibly through non-specific transport (Bouvet *et. al*, 1994, Mestecky, 2005). Parenteral immununization has also been shown to stimulate IgA-secreting cells in circulation correlated with increases in salivary IgA (Engstrom *et. al*, 2002), and recall responses to poliovirus as measured by the appearance of $\alpha_4\beta_7$ expressing IgA-MBC (Butcher *et. al*, 1996). Conjugate *H. influenzae* type b (Hib) vaccines administered

parenterally induce both IgG in serum and SIga in saliva (Pichichero *et. al*, 1983). In the case of the Hib vaccine, the mechanism by which both IgG and IgA responses are generated is currently unknown. These demonstrations of protection by systemically-delivered vaccines for mucosal pathogens exist for emphasize the availability of the systemic route to improve mucosal protection.

In contrast to these findings, a recent analysis of the polyclonal, non-specific antibody response to tetanus immunization suggested that parenteral stimulation does not affect the IgA response as measured in blood (Mei *et. al*, 2009). However, studies of the ability of a parenteral immunization to elicit nonspecific antibody production have provided support for the hypothesis that mucosal immunity might be stimulated or refreshed in a non-specific fashion (Bernasconi *et. al*, 2002). The presence of rotavirus-specific MBC lacking specific mucosal markers in blood has been confirmed in a study of RV-infected children, demonstrating that circulatory pathways are shared by systemic and mucosal memory B cells (Feng *et. al*, 2006, Jaimes *et. al*, 2004), providing a potential mechanism by which a parenteral immune response could impact mucosal antibody production.

### 1.6 Rationale

Due to sIgA's central role in mediating non-inflammatory immune exclusion at mucosal surfaces, it is essential to understand the activation requirements, trafficking patterns, and means by which these cells can be manipulated through immunization to provide immune protection at mucosal tissues. There remains uncertainty regarding the

identity and trafficking properties of sIgA-PC precursors, particularly with respect to a functional definition. Sampling mucosal secretions yields variable results depending on the methods used to collect them. Therefore, we argue that being able to positively identify and isolate B cell subsets with mucosal phenotype in blood would greatly simplify assessment of mucosal vaccine efficacy or antibody responses linked to autoimmune disorders at mucosal tissues. Analysis of the phenotype, specificity, and function of circulating B cell subsets is necessary before these subsets can be assayed as correlates of mucosal immunity.

Despite the recent suggestion that parenteral stimulation does not impact mucosal immunity (Mei, Yoshida, 2009), these conclusions were based primarily on phenotypic markers and thus may have underestimated contributions to mucosal antibody production. In particular, the authors found that the frequency of IgG and IgA-switched plasmablasts had not been altered by tetanus toxoid immunization, as assessed by intracellular flow cytometry. This approach neglects the specificity of the antibody-secreting cells in blood, which may be altered as a response to immunization without any overall change in the frequency of isotypes being produced. Studies rely on isotype to define a cell as mucosal would thus be unable to identify changes in functional indicators such as production of pIgA or secretion of Ig specific for mucosal pathogens. The work of Bernasconi (2003) and Odendahl (2005) both demonstrate that bystander activation in the context of a systemic tetanus boost does in fact occur, underlying the challenge of assessing bystander activation without assessing antigen-specific responses.

Similarly, assessing the ability of parenteral immunization to elicit mucosal

antibody responses might provide a mechanism by which parenteral stimulation can affect mucosal IgA production. The tetanus immunization model has been shown to be an effective means of eliciting multiple antibody specificities, and the widespread prevalence of HSV (Fatahzadeh and Schwartz, 2007) and PV vaccine use offer indicators of mucosal function in addition to a phenotype consistent with mucosal relevance.

The loss of CCR9 and CCR10 expression on virus-specific memory B cells in circulation in the absence of infection (Jaimes, Rojas, 2004) raises the question of whether CCR10 is inducible based on exposure to environmental cues such as cytokines. Although multiple mechanisms for IgA induction have been elucidated, there is relatively little data available on the conditions required for induction of mucosal homing molecules such as CCR10 on B cells. Recently, intestinal DCs have been shown to induce CCR9 expression on T cells through production of retinoic acid (Iwata, Hirakiyama*et al.*, 2004), and TGF-β and IL-21 were shown to induce CCR10 expression and IgA switch on *in vitro* activated naïve B cells (Dullaers, Li, 2009). The prevalence of these cytokines at mucosal inductive sites, as well as other cytokines such as IL-5 and IL-6, and their associated roles in inducing J-chain expression and IgA switch begs the question of whether they are involved in inducing a mucosal homing phenotype.

### 1.7 Central Hypotheses

Based on these observations, we hypothesize that precursors of mucosal plasma cells traffic through blood and can be isolated and functionally analyzed to describe immunological responses recently initiated at mucosal surfaces. We also hypothesize that due to shared circulatory routes, these precursors can be activated

nonspecifically by systemic immune responses. Finally, we hypothesize that a functional, mucosal homing phenotype will be expressed by activation of resting B memory cells in vitro, modeling the non-specific activation of mucosal memory B cells during parenteral immunization.

Thus, the studies described here attempt to use phenotypic and functional analysis to identify active mucosal B cells in blood of normal, healthy adult individuals. Furthermore, it will describe the ability of parenteral immunization to elicit a mucosal immune response measureable in cellular constituents of blood. Finally, conditions under which MBC can be induced to express CCR10 will be examined, in order to ascertain whether non- specific stimulation can induce a mucosal homing phenotype on blood MBC.

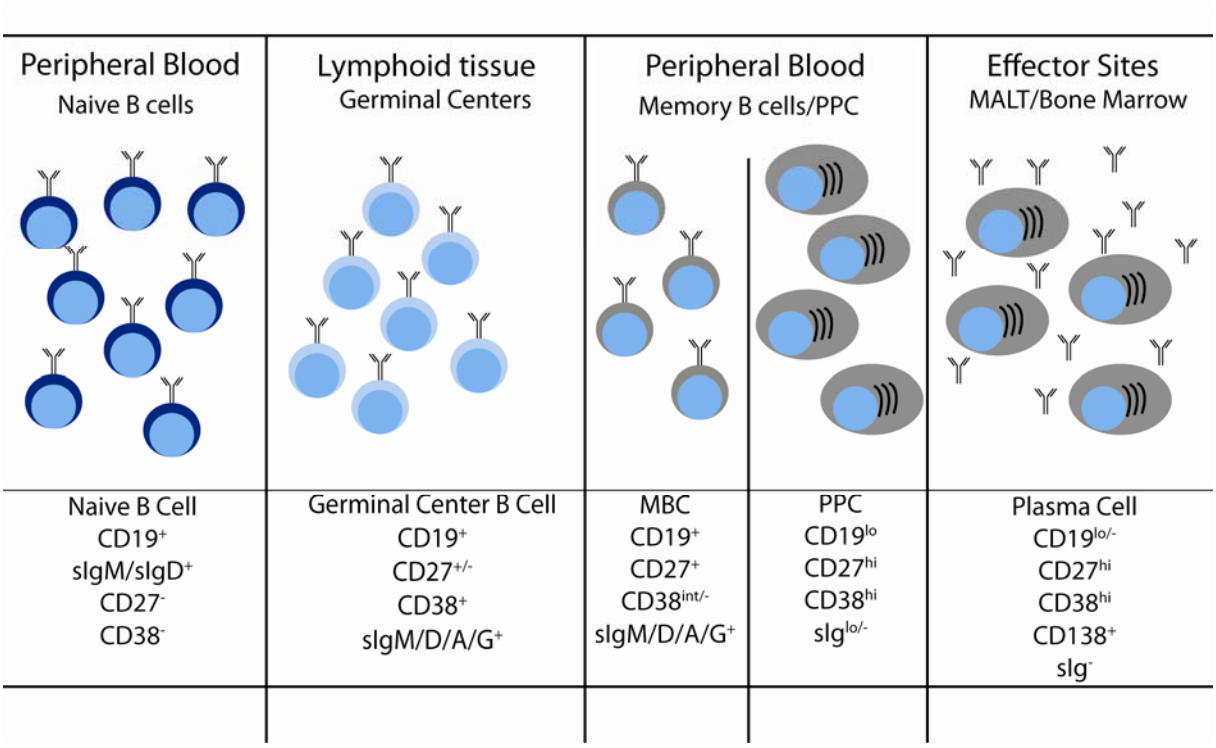


**Figure 1.1 Human B-cell Phenotypes.** Phenotypes of human B cell subsets present in peripheral blood, germinal centers, and effector sites.

# CHAPTER 2

## Stimulation of Anti-Polio and Anti-HSV IgA Pre-Plasma Cell Response in Blood following Parenteral Immunization with Tetanus-Diphtheria Vaccine

I have carried out all of the work outlined in this manuscript, with the exception of administration of the vaccine. Vaccines were administered by Marie Kancer, RN, in the clinic of Dr. Susan Wasserman.

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Stimulation of anti-polio and anti-HSV IgA Pre-plasma cell response in blood following parenteral immunization with tetanus-diphtheria vaccine. J.R. Fernandes, S. Wasserman, D.P. Snider. Feb;28(6):1493-8. 

## 2.1 Introduction

In order to deal with the high antigenic burden of mucosal surfaces, plasma cells residing in the mucosal lamina propria secrete polymeric IgA (pIgA) at high rates (up to 4 gm/day), and this IgA is capable of mediating non-inflammatory immune exclusion at mucosal surfaces (Cerutti *et. al*, 2008). Although the importance of the contribution of these plasma cells to maintaining mucosal homeostasis is recognized, there remains considerable uncertainty regarding the migration routes of these cells and their precursors, and how immunity to mucosal pathogens is maintained in the absence of antigenic stimulation.

Recently, Bernasconi and others have suggested that antibody-mediated immunity can be sustained through persistent bystander activation of memory B cells (MBC) (Bernasconi *et. al*, 2002, Odendahl *et. al*, 2005). In such a scenario, polyclonal activators such as microbial products can activate B cells through TLR4 and TLR9, and T helper cells activated in response to specific antigens can support proliferation and differentiation of other MBC via co-stimulatory molecules such as CD40 or CD27 and by providing cytokine support (Krieg, 2002, Lanzavecchia, 1983). Such polyclonal activation of B cells may help explain findings that parenteral immunization with tetanus/diphtheria is followed by the appearance of MBC specific not only for tetanus, but also for other commonly encountered pathogens such as measles and rubella (Bernasconi *et. al*, 2002). The authors postulated that ongoing polyclonal activation of B cells in a nonspecific fashion could sustain humoral immunity over a lifetime. These and other studies (Odendahl *et. al*, 2005) clearly demonstrated that systemic immunization

with typical antigens induces IgG producing blood cells against the vaccine and other antigens, but those IgG responses are against non-mucosal pathogens. We sought to extend these observations to mucosal IgA producing B cells, and antigens relevant to mucosal protection.

The presence of mucosally-relevant B cells in blood has been observed in humans. An examination of the kinetics of circulating B cell populations in children over the acute and convalescent phases of rotavirus (RV) infections (Jaimes *et. al*, 2004) revealed that during the acute phase of infection RV-specific MBC and plasmablasts expressed both CCR9 and CCR10, presumably directing their migration to the gut, where mature RV-specific plasma cells bearing both markers were observed. Subsequently, when the infection passed into convalescence, RV-specific B cells in blood were of an MBC phenotype and lacked CCR9 and CCR10 expression. These findings underscore a very important and previously unrecognized observation that mucosally-relevant MBC, in the absence of infection, can circulate through systemic lymphoid tissues, due to loss of functional markers for mucosal homing.

We have recently demonstrated that, in normal, healthy humans, there is a subset of activated B cells or plasma blasts in blood that secrete polymeric IgA (Fernandes *et. al*, 2010). We refer to these as pre-plasma cells because of their expression of proliferative markers, relative absence of plasma cell phenotype, but with intracellular IgA and the ability to secrete IgA. This subset can be identified in blood by expression of CCR10, a chemokine receptor whose ligand, Mucosal-epithelium Associated Chemokine

(MEC) has been shown to be expressed in a variety of mucosal tissues including the salivary and mammary glands, and small and large intestine (Pan *et. al*, 2000, Wurbel *et. al*, 2000). Although the identification of a mucosal plasma cell precursor population in blood is a significant step forward in understanding how recently activated B cell blasts traffic to mucosal tissues, the presence of such a subset in blood from normal individuals is one clear indication that while the mucosal and systemic humoral immune compartments are not completely distinct from one another, although generally they have been considered functionally distinct entities.

Although a recently published study concluded that mucosal immunity is not impacted by systemic antigen encounter (Mei *et. al*, 2009), this study did not assess any functional indicators of mucosal relevance. This is of significant concern, as the expression of markers such as the $\alpha_4\beta_7$ heterodimer was shown to be indicative of activation at mucosal inductive sites, not necessarily of relevance to defense at mucosal tissue (Kantele *et. al*, 1997, Kantele *et. al*, 1999, Quiding-Jarbrink *et. al*, 1997). Thus, it is essential to include analyses of function that reflect applicability to mucosal defense, such as specificity for mucosal pathogens, production of polymeric IgA, or both.

The work presented here provides the first investigation of mucosally-relevant function amongst circulating PPC following systemic immunization. In contrast to previous findings, here we report that a parenteral tetanus/diphtheria immunization is followed by mobilization of PPC that secrete IgA, some of which are specific for the mucosal pathogens PV and HSV. Therefore, we conclude that systemic antigen encounter

does have the ability to influence mucosal antibody production. This is an important observation when considering new vaccine strategies to elicit or boost mucosal antibody production, or when considering the underlying aspects of certain autoantibody-mediated pathologies.

## 2.2 Materials and Methods

### *2.2.1 Study Subjects and Vaccination*

Study subjects were normal, healthy male adult donors (18-55 years of age) who had not received a tetanus/diphtheria booster in the prior 5 years. All subjects received an intramuscular immunization with tetanus/diphtheria vaccine: Td-ADSORBED (Sanofi-Aventis, Bridgewater, NJ). This study was approved by the McMaster Research Ethics board and documented informed consent was obtained from all subjects prior to beginning the study. A total of 20 subjects were enrolled and all completed the protocol.

### *2.2.2 Specimen Preparation and B cell Isolation*

Blood samples were collected 7 days before, 7 days after, and 28 days after vaccination. At each timepoint, 100 mL of blood was collected from each subject. All samples were collected in_Vacutainer tubes (BD Biosciences, Oakville, Canada), with ACD (acid-citrate dextrose) as anticoagulant, and processed on the same day. B cells were isolated from fresh blood samples using the Human B Cell Negative Selection RosetteSep cocktail (StemCell Technologies, Vancouver, Canada). Whole blood was incubated with the RosetteSep cocktail at room temperature for 20 minutes, layered over Ficolle-Paque Plus (VWR International, Canada) , and centrifuged for 20 minutes at 1800 x *g*. The resulting PBMC were resuspended and washed twice in 2% FCS/PBS before staining for flow cytometric analysis or FACS. The isolated cell suspensions were always > 85% B cells as confirmed by CD19 staining in flow cytometry, with > 95%

viability as by trypan blue staining. Preliminary experiments indicated no loss of PPC phenotypic cells during this isolation procedure (data not shown).

### *2.2.3 Immunophenotyping*

The rosette-isolated, blood B cell preparations were surface stained using various mouse anti-human monoclonal antibodies, in order to phenotype blood B cell subsets. The following antibodies were used: anti-CD19-PE-Cy7, anti-CD27-PE, and anti-CD38-APC (BD Biosciences, Oakville, Canada). Data was collected on a Becton Dickinson LSR II cytometer using FACSDiva software (BD Biosciences) and analysed using FlowJo software v8.2 (Treestar Inc.).

### *2.2.4 FACS*

Pre-plasma cell (PPC) populations were purified from total B cell preparations by staining against CD19, CD27, and CD38 using antibodies described above, and then isolation by FACS. Sorting was carried out on a high speed digital FACSVantage sorter (BD Biosciences, Oakville, Canada) to yield PPC ($CD19^{lo}/CD27^{hi}/CD38^{hi}$) populations with >95% purity. The resulting purified populations had > 90% viability as assessed by trypan blue exclusion, and contained <5% $CD3^{+}$ T cells.

### *2.2.5 ELISPOT*

Duplicate microwells on Acrowell 96-well filter plates (VWR International, Canada) were incubated at 37°C with various antigens diluted in BBS, pH 8.2. Antigens were tetanus toxoid (Wako Biochemical, Richmond, USA), diphtheria toxoid (Sigma-

Aldrich, Oakville, Canada), formalin-inactivated poliovirus (a kind gift from P. Austin, Sanofi-Aventis), or recombinant HSV-1 and HSV-2 glycoprotein D (Raybiotech Inc., USA) for antigen specific ELISPOT tests. For IgA or IgG ELISPOT wells were coated with polyclonal anti-IgG (Caltag Labs, Burlingame, USA) or anti-IgA (Southern Biotech, Birmingham, USA) that were pre-adsorbed to remove cross-reactivity against other Ig isotypes. All ELISPOT wells were and blocked with 1% BSA in TBS following initial coating with antigens or anti-Ig. Control wells received BSA block only. Plates were washed with 0.05% Tween/PBS, and FACS-sorted PPC were cultured at 37°C at a density of 1000 cells/well, in 200 uL of IMDM containing 10% FCS, 2mM L-glutamine, 50 μM 2-ME, 100 U/mL penicillin and 100 μg/mL streptomycin for 36 hours. 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 to IgG or IgA (Caltag labs, and the ELISPOT Blue Colour Module, R&D Systems, Minneapolis, USA). The total number of IgA or IgG spots were counted manually using a dissecting microscope or in cases of high spot counts, an ImmunoSpot automated spot counter and Immunospot version 4.0 software (Cellular Technologies, Limited) was used. Counts were expressed as spot number per 1000 PPC. Manual and ImmunoSpot counting were found to agree closely in preliminary test samples.

### *2.2.6 Statistical Analysis and Software*

For all analyses listed, data is presented as mean ± SEM. Student's *t* test was used to analyze the difference between groups at a 95% confidence interval. All statistical analyses were performed using GraphPad Prism 4.0 Software (GraphPad Software, Inc.).

## 2.3 Results

### *2.3.1 Memory B cell and Pre-plasma Cell Frequencies in Peripheral Blood Following Systemic Tetanus Boost*

In order to assess the effects of parenteral tetanus/diphtheria boost on circulating B cell subsets, blood B cells were stained and analyzed by flow cytometry to determine frequencies for naïve, memory B cells, and pre-plasma cells (PPC) 7 days before, 7 days after, and 28 days after immunization. We have previously performed analysis of circulating B cell subsets (Fernandes *et. al*, 2010) using CD19, CD27 and CD38 to distinguish between naïve ($CD19^{+}/CD27^{-}$), MBC ($CD19^{+}/CD27^{int}/CD38^{-/lo}$) and PPC ($CD19^{int}/CD27^{hi}/CD38^{hi}$). It should be noted that the CD19+CD27- subset includes both mature naïve B cells and a small fraction of transitional cells (Wirths *et. al*, 2005). For simplicity we refer to these cells as naïve cells in the remainder of this paper. Others have used similar staining protocols and have variously referred to PPC as plasmablasts, antibody secreting cells, or at times plasma cells (Gonzalez-Garcia *et. al*, 2008, Jaimes *et. al*, 2004, Mei *et. al*, 2009, Wirths *et. al*, 2005). Prior to immunization, the majority of B cells in blood exhibit the naïve phenotype, while approximately 1/5 (21.3 ± 4.2%, n = 20) express CD27, a marker for both MBC and PPC (Table 2.1). The $CD27^{+}$ subset prior to immunization was comprised of 87.4 ± 1.2% MBC and 4.3 ± 0.6% PPC. Thus, in normal human subjects prior to immunization only a small fraction of blood B cells (1.2%) are PPC. PPC are mostly plasmablasts, that are either moving out of tissues where plasma cell differentiation is occurring, or are activated MBC, stimulated in lymphoid tissues and

appear in blood as plasmablasts (Tarlinton *et. al*, 2008). On day +7 following tetanus/diphtheria boost, there was a significant ($p < 0.001$, $n = 20$) increase (almost 3 fold more than at day -7) in the frequency of PPC among the $CD27^+$ subset concomitant with a decrease in MBC frequency (Table 2.1). By day 28, this burst of PPC was seen to subside, and frequencies of MBC and PPC in blood returned to pre-immunization levels (Table 2.1, Figure 2.1). Although the fraction of $CD27^+$ cells and the absolute number of $CD19^+$ cells in blood did not change 7 days following immunization (Table 2.1), the absolute numbers of MBC and PPC were significantly different between day -7 and day +7 (MBC = 1.48 vs 1.38 x $10^6$/100 mL blood, $p < 0.05$, $n = 20$; PPC = 0.07 vs 0.21 x $10^6$/100 mL blood, $p < 0.001$, $n = 20$). Thus, the changes in MBC and PPC frequencies observed post immunization reflects an internal remodeling of the $CD27^+$ B cell subsets in blood, and a significant increase in PPC numbers in blood. The kinetics of this PPC burst we observed were consistent with those reported by others in similar immunization studies for antibody secreting cells in PBMC (Bernasconi *et. al*, 2002, Mei *et. al*, 2009, Odendahl *et. al*, 2005), and demonstrates that systemic immunization by a common vaccine exerts a measurable influence on the frequencies of inactive (MBC) and active (PPC) components of B cell memory in blood.

### *2.3.2 Immunoglobulin Secretion by Pre-plasma cells and Memory B cells in Peripheral Blood Following Systemic Tetanus Boost*

Having observed that a parenteral boost led to measurable changes in PPC frequencies in blood, we sought to examine the nature of the PPC burst and whether such

a response led to a shift in secretion of different antibody isotypes. FACS-sorted PPC were placed into isotype-specific ELISPOT to assay IgA and IgG secretion. In contrast to previous finding by others who examined IgG producing cells by cytoplasmic stain (Mei *et. al*, 2009), we observed that on day 7 post-immunization, the frequency of IgG-secreting PPC was not significantly different from day 7 pre-immunization (Figure 2.2), despite the documented ability of the tetanus/diphtheria booster to generate high levels of tetanus-specific IgG producing cells (Bernasconi *et. al*, 2002, Mei *et. al*, 2009, Odendahl *et. al*, 2005). More strikingly, we observed a significant ($p < 0.001$, $n = 20$) increase in IgA-secreting PPC on day 7 post-immunization (Figure 2.2). A strong correlate was observed between the frequency of PPC and the frequency of IgA PPC on day 7 post-immunization (Figure 2.3). These data demonstrate that systemic tetanus/diphtheria immunization provided a stimulus that not only resulted in increased absolute numbers of PPC in blood, but also increased the frequency of IgA-secreting PPC in blood.

### *2.3.3 Antigen-specific B Cell Responses following Systemic Tetanus Boost*

Although isotype-specific ELISPOT analysis of PPC demonstrated a burst of IgA-PPC on day 7 following immunization, a more functional indicator was necessary to conclude that the IgA-PPC burst observed was relevant to mucosal tissues. We characterized the antigen specificities of PPC in blood in order to determine whether the PPC burst was solely specific for the immunizing antigens. At all three timepoints, we investigated PPC for secretion of tetanus- and diphtheria-specific IgG and IgA as a measure of the antigen-specific immune response. We did not observe tetanus- or

diphtheria-specific IgG or IgA secretion prior to immunization in any subjects (Figure 2.2). Consistent with previous studies (Bernasconi *et. al*, 2002, Mei *et. al*, 2009, Odendahl *et. al*, 2005), a highly significant ($p < 0.0001$, $n = 20$) anti-tetanus IgG response was observed on day 7 following immunization (Figure 2.2). This response constituted the majority of IgG-secreting PPC on day 7, and no tetanus-specific IgA was observed (Figure 2.2), a result consistent with previous observation using ELISPOT to identify IgG secreting cells from PBMC (Bernasconi *et. al*, 2002, Odendahl *et. al*, 2005). While we did observe a small increase in PPC in the diphtheria-specific IgA ELISPOT on day 7, the modest average number observed was typically close to background counts and did not account for the large increase in counts for the day 7 IgA-PPC burst (Figure 2.2). By day 28 following immunization, active, antigen-specific responses were no longer measurable, indicating that the active phase of the response to the immunization had subsided, including the IgA PPC response.

### *2.3.4 Antibody to PV and HSV antigens produced by PPC Following Systemic Tetanus Boost*

Having observed that the IgA-PPC burst on day 7 was not explained by response to either of the immunizing antigens, we investigated whether the IgA-secreting cells produced antibody relevant to mucosal antigens. Secretion of IgG and IgA against two mucosally-relevant pathogens, HSV and PV, was examined by using ELISPOT. No HSV- or PV-specific antibody secreting cells were observed pre-immunization in any subjects (Figure 2.4). On day 7 following immunization we observed a significant ($p < 0.005$, $n = 7$) number of PV-specific IgA secreting PPC in 7 subjects out of 10 tested

(Figure 2.4). Additionally, we also detected a significant ($p < 0.01$, $n = 6$) number of HSV-specific IgA secreting PPC in 6 subjects out of 10 tested (Figure 2.4). Although no PV-specific IgG secreting PPC were detected in any subjects, we did observe HSV-specific IgG secreting PPC ($p < 0.01$, $n = 3$) in 3 of the 6 subjects also positive for HSV-IgA (Figure 2.4). Similar to the tetanus- and diphtheria-specific responses, HSV- and PV-specific PPC were no longer detectable on day 28 post-immunization (Figure 2.4), indicating that the kinetics of the mucosal PPC response was similar to that of the response against the immunizing antigens, and the total IgA PPC response (Figure 2.2). These results provide clear evidence that systemic immunization is capable of inducing mucosal PPC response in blood, and provide the first clear, functional evidence that mucosal IgA antibody production can be modulated by a systemic response to a non-mucosal antigen.

Table 2.1: Effect of Systemic Tetanus/Diphtheria Immunization on $CD27^{\pm}$ B Cell Subsets in Blood

| **Total Blood B Cells** | **d-7** | **d7** | **d28** |
|---|---|---|---|
| % Naïve | 77.2 ± 6.1 | 77.0 ± 5.6 | 76.9 ± 4.5 |
| % $CD27^{+}$ | 21.3 ± 4.2 | 21.0 ± 4.9 | 22.2 ± 3.8 |
| Absolute number CD19+B cells /100 mL blood x $10^6$ | 8.0 ± 0.2 | 8.0 ± 0.2 | 7.9 ± 0.2 |
| **$CD27^{+}$Blood B Cells** | **d-7** | **d7** | **d28** |
| % MBC | 87.4 ± 1.2 | 81.0 ± 1.6* | 91.6 ± 3.2 |
| % PPC | 4.3 ± 0.6 | 11.9 ± 1.7* | 3.2 ± 0.4 |

*= Comparison of MBC or PPC d7 frequency with d-7 and d28 frequencies, difference is significant at $p < 0.001$ for $n = 20$

Values are mean ± SEM

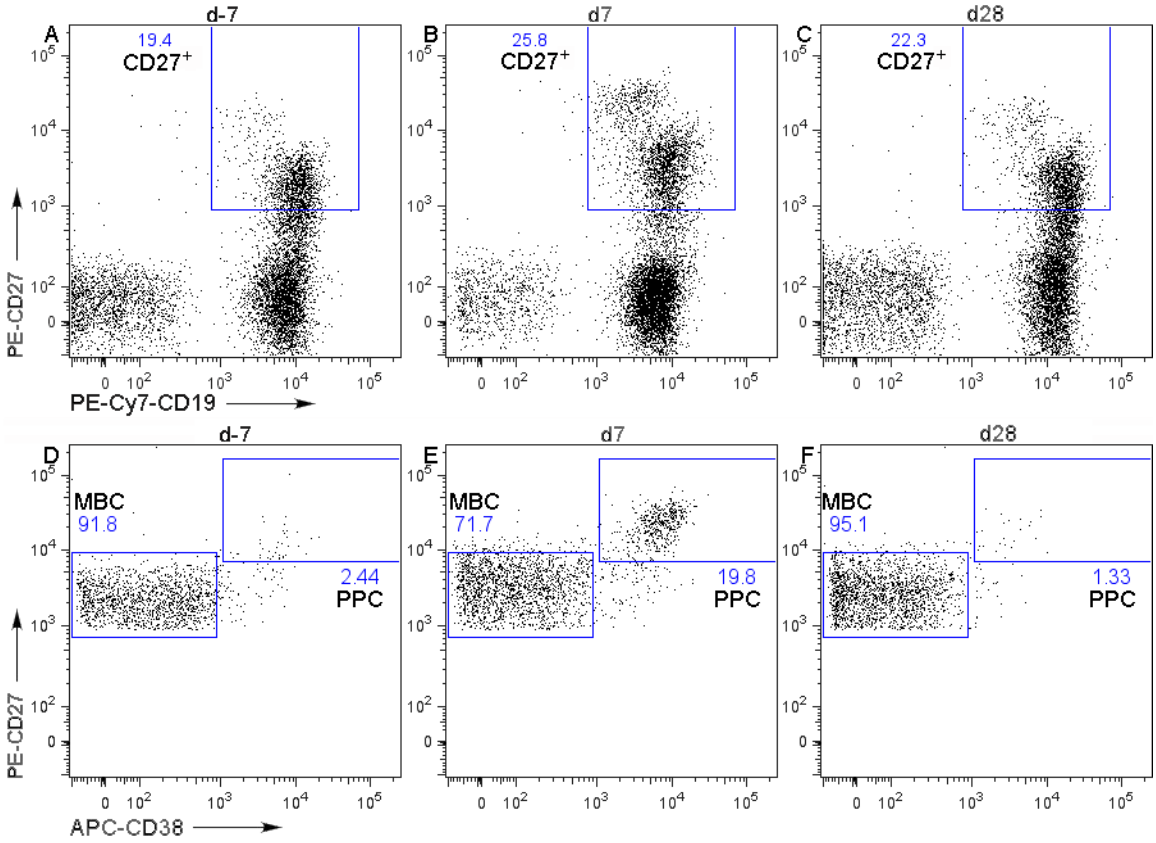


**Figure 2.1 Frequencies of Blood B Lymphocyte subsets Following Systemic Immunization with Tetanus/Diphtheria.** B cells from peripheral blood were isolated by rosette methods and stained with monoclonal antibodies against CD19, CD27 and CD38. Samples were taken 7 days before immunization (A,D), and then 7 (B,E) and 28 days (C,F) after immunization. Frequencies are shown for total $CD19^{+}/CD27^{+}$ B lymphocytes (A,B,C), and those of the MBC and PPC subsets (D,E,F). The latter were distinguished based on expression of CD27 and CD38, with MBC $CD27^{int}/CD38^{-/lo}$ (D,E,F, left regions) and PPC $CD27^{hi}/CD38^{hi}$ (D,E,F, right regions). Data shown is from one subject, representative of 20.

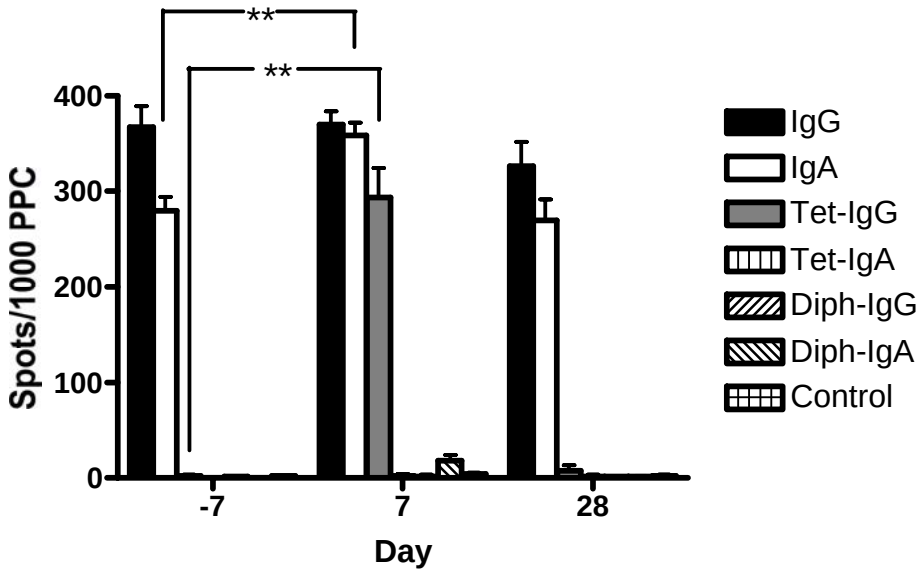


**Figure 2.2 IgG, IgA and Tetanus-Specific Antibody Secretion by Blood PPC Following Systemic Immunization with Tetanus/Diphtheria Vaccine.** The frequency of PPC secreting IgG or IgA, and those secreting Tetanus- or Diphtheria-specific IgG or IgG were enumerated by ELISPOT. Timecourse (x axis) indicates day relative to immunization (day 0). Mean background spots in control wells were < 10/1000 PPC for all ELISPOT tests. N = 20 for tetanus-specific analyses, n = 14 for diphtheria-specific analyses. Data bars indicate mean and error bars are SEM. ** = $p < 0.0001$ for comparison of day -7 and day 7 for both total IgA and tetanus-IgG ELISPOT counts. Same significance for both comparison betwee day 7 and day 28.

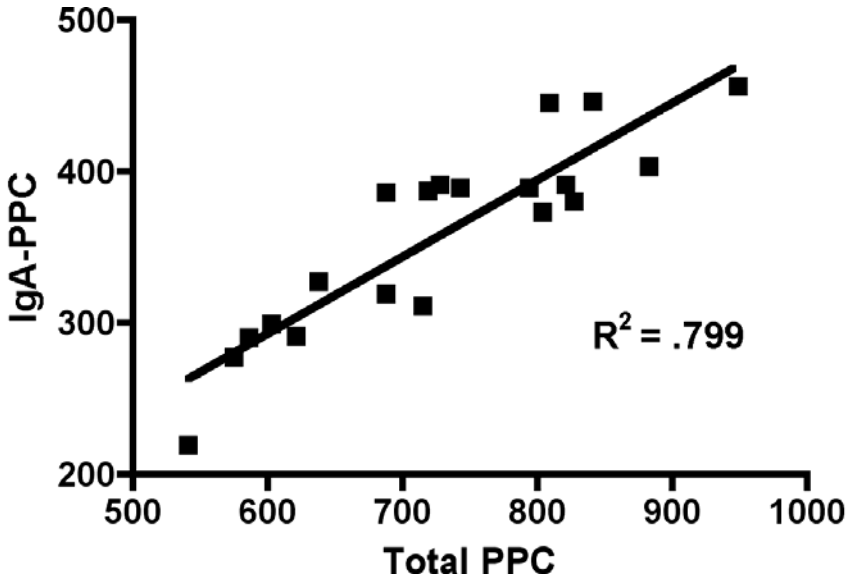


**Figure 2.3 Correlate of Total PPC with IgA-PPC at day 7 post immunization.** The number of PPC in blood are compared to IgA spot counts per 1000 plated PPC, within individual samples taken from subjects immunized 7 days previously. A significant positive correlate was found indicating association between increased total PPC and IgA producing PPC.

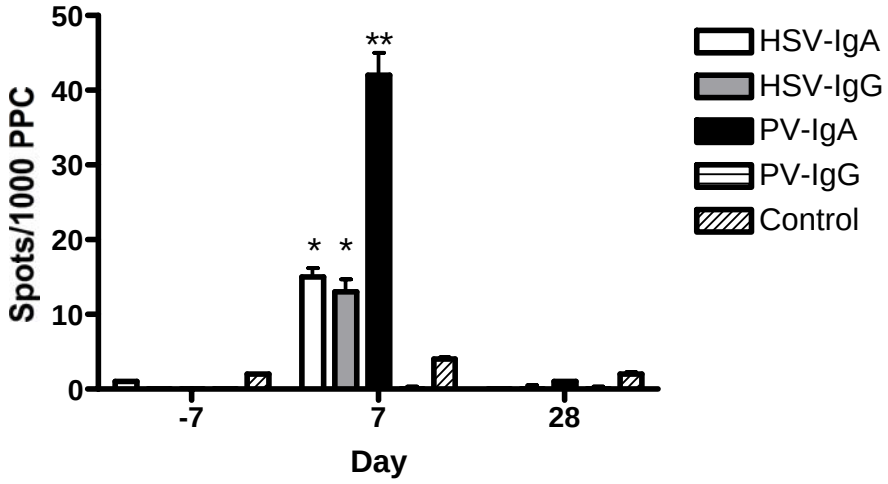


**Figure 2.4 Blood PPC Secreting IgA and IgG Antibody to PV and HSV Following Systemic Immunization with Tetanus/Diphtheria.** PPC were FACS-sorted from purified B cells. Timecourse (x axis) indicates day relative to immunization (day 0). HSV- and PV-specific IgA or IgG secreting PPC were enumerated by ELISPOT. Mean background spots in control wells were < 5 spots/1000 PPC. Data for HSV-IgA, HSV-IgG, and Polio-IgA are from 6, 3, and 7 individuals respectively, in which spot counts above control were observed. Data from non-responders were not included. Error bars are SEM. * = $p < 0.01$ for comparison of day -7 and day 7 or day 7 and day 28, for both HSV-IgA and HSV-IgG. ** = $p < 0.005$ for comparison of day -7 and day 7 or day 7 and day 28 for PV-IgA.

## 2.4 Discussion

Systemic booster immunization with tetanus/diphtheria results in a response by activated IgG B cells in blood, with antigen specificity unrelated to the immunizing antigen (Bernasconi *et. al*, 2002, Odendahl *et. al*, 2005). However, no examination of IgA antibody in a bystander response has been reported. Mucosally-relevant MBC have been shown to circulate in blood long after acute immune response to mucosal pathogens (Jaimes *et. al*, 2004). In addition, IgG-producing cells in blood have been demonstrated as indicators of recently initiated immune response to vaccination or infection (Jaimes *et. al*, 2004, Odendahl *et. al*, 2005). Since mucosal IgA producing MBC are in circulation and could enter peripheral lymph nodes from blood, we sought to determine whether mucosal IgA responses do occur as a result of booster vaccination.

Here, we report that 7 days after parenteral immunization with tetanus/diphtheria there is a significant increase in the number of blood PPC ($CD19^{mod}$, $CD27^{hi}$, $CD38^{hi}$) and a corresponding reduction in the number of non-activated MBC. This increase in blood PPC includes a large fraction of PPC secreting tetanus-specific IgG, and a significant increase in IgA-secreting PPC. The levels of IgA secreting PPC post immunization correlates with increased PPC numbers in blood, supporting the idea that increased IgA PPC is a function of mechanisms that drive the overall increase in blood PPC. Most important, we also observed significant numbers of PPC that were specific for polio virus and Herpes simplex virus that secreted IgA, among a large fraction of subjects that were vaccinated.

Our results are largely consistent with those of other groups investigating circulating B cell subsets and antibody secreting cells following parenteral immunization (Bernasconi *et. al*, 2002, Mei *et. al*, 2009, Odendahl *et. al*, 2005). We focused our attention on the PPC within the burst of cells in blood post vaccination. The increased ratio of PPC to MBC may reflect an altered ratio of MBC/PPC exiting activated lymphoid tissues, and a homeostatic mechanism that maintains constant B cell numbers in blood. Our data also indicate that PPC frequencies increased more than two fold by 7 days post vaccination, but that IgG producing PPC frequencies did not change from pre-vaccination levels. Since the IgG PPC compartment did not increase in frequency, the remaining increase in the PPC burst must be non-IgG PPC. Our data indicate that a substantial portion of the PPC increase comes from IgA producing PPC, but IgM producing PPC cannot be ruled out. It is also possible, that a large fraction of the total PPC burst at day 7 post immunization, are less differentiated IgG PPC, that do not yet secrete any IgG, thus explaining the high frequency of PPC phenotype in blood, but no indication of increased IgG secreting PPC. Further, the frequency of tetanus-specific IgG secreting PPC were nearly equivalent to the frequency of all IgG secreting PPC on day 7, indicating that almost all IgG producing PPC at day 7 were against the vaccine tetanus antigen, as others have concluded for antibody secreting cells examined in PBMC preparations (Bernasconi *et. al*, 2002, Odendahl *et. al*, 2005). Our analyses provide a novel perspective on the effect of systemic vaccination on the $CD27^+$ subsets of B cells in blood. More work is required to examine further details on the differentiation states of the PPC subsets that enter blood following systemic immunization.

Our observation of increased numbers of IgA secreting PPC after immunization appears to contrast observations in a recent study by Mei et al (Mei *et. al*, 2009), where intracellular staining was used to identify IgA producing cells. In other work, we have demonstrated that a large fraction of PPC with surface IgA expression secretes polymeric IgA, and that a majority of these cells express CCR10 (Fernandes *et. al*, 2010), a well-described mucosal homing chemokine receptor that others have identified on PPC that express surface IgA (Kunkel *et. al*, 2003, Mei *et. al*, 2009). Further, the IgA produced by isolated PPC is predominantly polymeric, so it was predictable that increased IgA secreting PPC would contain cells that provide IgA antibody to mucosally relevant antigens.

Our observation of the PPC post vaccine burst, containing cells that secrete IgA antibody against PV and HSV is the first direct evidence that mucosal IgA production can be induced by parenteral immunization. We reasoned that the high prevalence of HSV seropositivity (estimated at between 45 and 98% worldwide (Fatahzadeh *et. al*, 2007) and the previous widespread use of the oral PV vaccine, would likely result in IgA or IgG PPC responses from many of our randomly selected subjects. Our results show clearly that the day 7 response to tetanus vaccination included many PPC that secrete PV-specific IgA, HSV-specific IgA, and HSV-specific IgG, in many of our subjects. Given that there were no detectable HSV- or PV-specific PPC in any of these subjects prior to immunization, we have clear evidence that the recall response to systemic tetanus/diphtheria vaccination causes the increase in mucosal IgA or IgG producing cells in blood.

Several issues require comment in evaluating the findings of this study. Some subjects did not demonstrate any PV- or HSV-specific antibody production by PPC on day 7, but all subjects showed increases in total PPC and IgA PPC increases. Without knowing which childhood vaccines subjects received, or knowing specifically if or when subjects were exposed to different pathogens, it is difficult to assign significance to a lack of response in an antigen-specific assay such as our PV- and HSV-specific ELISPOTS. This does not indicate that a mucosal IgA response was not initiated in these subjects, but only that PV- and HSV-specific MBC were not activated as part of that response. Analyses of the type of vaccines received and previous infection history should be considered in future analyses in order to determine why some individuals may show respond with antibodies against certain mucosal pathogens but not others.

One significant question to resolve is the origin of the IgA mucosal PPC that move into blood during the response to systemic immunization. Two competing concepts are possible to explain their presence. The first, is the bystander activation of mucosal MBC within peripheral lymphoid tissue, resulting in random generation of IgA or IgG mucosal PPC that exit to blood, as proposed by Bernasconi and Lanzavecchia,who argue for a strong correlate between MBC proliferation and plasmablasts in blood (Bernasconi *et. al*, 2002, Tarlinton *et. al*, 2008). We would favour this based on our characterization of mucosal IgA PPC that have a clear characteristic of activated, recently divided, polymeric IgA secreting, plasma blasts, with few characteristic markers of plasma cells (Fernandes *et. al*, 2010). The second is the model in which the non-tetanus-specific antibody-secreting cell populations in blood consists of

plasma cells (perhaps long lived) that are displaced from BM niches in response to an influx of newly activated, antigen-specific IgG blasts (Gonzalez-Garcia *et. al*, 2006, Gonzalez-Garcia *et. al*, 2008, Mei *et. al*, 2009, Odendahl *et. al*, 2005). Although it has been shown that there is a fixed capacity of BM for plasma cells, it is not clear that mucosal IgA producing cells are likely to be displaced from the BM. In fact, mucosal tissues are a far richer source of IgA plasma cells and so would represent a more likely source than bone marrow (Kunkel *et. al*, 2003). A study comparing antigen-specific and non-specific antibody-secreting cells demonstrated that proposed emigrant BM cells contained a significant population of HLA-DR$^{hi}$/Ki67$^{+}$/CD138$^{-}$ cells (Gonzalez-Garcia *et. al*, 2008), a phenotype consistent with plasmablast that some argue are early less differentiated forms of plasma cells (Wirths *et. al*, 2005). Thus we refer to the responding plasmablasts to post vaccination as pre-plasma cells. It is therefore evident that more study is required to define where the post-immunization blood PPC go and where the mucosal PPC originate.

It has been previously shown that a parenteral vaccine is capable of activating pre-existing memory B cells bearing mucosal homing markers (Kantele *et. al*, 2005, Kantele *et. al*, 1997), however, these studies were performed in individuals were immunized mucosally and then received secondary parenteral exposure to the same antigens. Thus, the response was antigen specific and not as our observation here with B cell antibody response to non-cognate antigen. Those studies do provide clear evidence that parenteral immunization can involve mucosally relevant responses from memory cells however, indicating again that mucosal MBC can migrate through systemic

lymphoid tissues. Here, we present the first clear evidence that a mucosal antibody producing B cells increase in blood in response to parenteral vaccination with completely unrelated antigens. If as we suspect the mucosal PPC come from mucosal MBC then this would have important implications for designing mucosal vaccines or boosting mucosal immunity, and may provide an explanation for idiopathic exacerbations in autoantibody-mediated mucosal immune pathologies such as celiac disease.

# CHAPTER 3

## Polymeric IgA-secreting and Mucosal Homing Pre-plasma Cells in Normal Human Peripheral Blood

I have carried out all of the work outlined in this manuscript.

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Polymeric IgA-secreting and mucosal homing pre-plasma cells in normal human peripheral blood. J.R. Fernandes and D.P. Snider. Jun;22(6):527-40. © Oxford University Press. (2010)

## 3.1 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, allergens, 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 and the use of the pIgr to transport polymeric IgA through mucosal epithelial cells to the external surfaces (Goldblum, 1990, Kaetzel, 2005). This polymeric IgA is produced by plasma cells that differentiate from activated pre-plasma cell (PPC) blasts within the tissue directly beneath the epithelial layer (Kaetzel, 2005). The known large quantity of polymeric mucosal IgA produced in normal healthy subjects contrasts the fact that serum IgA is predominantly composed of monomeric IgA in similar subjects (Woof *et. al*, 2006). This raises an interesting question, whether the blood of normal healthy subjects contains PPC that are in transit to mucosal tissues, with both appropriate cell surface homing structures and the ability to produce polymeric IgA. 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 plasma cells in blood (Feng *et. al*, 2006, Hieshima *et. al*, 2004, Hieshima *et. al*, 2003, Jaimes *et. al*, 2004, Johansen *et. al*, 2005, Mei *et. al*, 2009, Wilson *et. al*, 2004), but no

study has correlated surface markers with the functional identification of polymeric IgA secretion. Polyclonal stimulation of PBL has previously revealed the presence of B cells capable of secreting polymeric IgA (Kutteh *et. al*, 1980, Moldoveanu *et. al*, 1984). These and other studies looked at polyclonal activators of both memory and naïve B cells, such as PWM that activates in the presence of T cell, and is not necessarily related to the active polymeric IgA 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 (Feng *et. al*, 2006, Jaimes *et. al*, 2004, Mei *et. al*, 2009). However, no studies have specifically identified the PPC subset of blood and examined polymeric IgA 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 polymeric IgA 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 plasma cell precursors in blood that are destined to seed mucosal tissues with polymeric IgA-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: naïve B cells (and a small population of transitional B cells) do not express CD27, whereas and antigen-experienced B cell lineages express CD27. Naïve B lymphocytes circulate through secondary lymphoid organs, where interaction with their cognate ligands, T lymphocytes and other cell types facilitates their activation, immunoglobulin class switching and somatic

hypermutation in the germinal center (GC) reaction (Agematsu *et. al*, 2000, Tangye *et. al*, 1998). B cells activated through GC reactions express CD27 and can be further subdivided into two functionally distinct subsets: memory B cells (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 immunoglobulin as they begin to differentiate into plasma cells (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 (Jego *et. al*, 1999, Medina *et. al*, 2002). 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 (Wirths *et. al*, 2005). 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 (Jaimes *et. al*, 2004). 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 $\alpha_4\beta_7$ 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 (Bargatze *et. al*, 1995, Berlin *et. al*, 1995, Streeter *et. al*, 1988). Known as a central mediator of T lymphocyte migration to the gut, $\alpha_4\beta_7$ expression has also been detected on *in situ* derived PC from the gut (Farstad *et. al*, 1995). Several studies have demonstrated expression of $\alpha_4\beta_7$ on antibody-secreting,

blood cells (ASC) following mucosal immunization (Quiding-Jarbrink *et. al*, 1997), and that expression of this marker is dependent on the route of antigen encounter (Kantele *et. al*, 1997, Kantele *et. al*, 1999). Although it is tempting to conclude that the $\alpha_4\beta_7$ heterodimer is a marker of mucosal B cells in transit through blood to mucosal tissues, there is evidence to suggest that $\alpha_4\beta_7$ expression is a better indicator of B lymphocytes that have been activated at a mucosal immune induction site (Quiding-Jarbrink *et. al*, 1997). Thus, expression of $\alpha_4\beta_7$ on human blood MBC or PPC is not necessarily a clear indicator that those cells will produce polymeric IgA or necessarily migrate into mucosal tissues.

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 (Butcher *et. al*, 1996). 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 (Campbell *et. al*, 2000). CCR9, the only known receptor for the thymus- and gut-restricted chemokine TECK, is expressed by circulating memory T cells bearing the $\alpha_4\beta_7$ integrin, and intestinal T cells (Kunkel *et. al*, 2000, Wurbel *et. al*, 2000). CCR10 responds to MEC, a chemokine expressed by epithelial cells in a variety of mucosal tissues including the salivary and mammary glands, small and large intestine (Pan *et. al*,

2000, Wang *et. al*, 2000). $CCR9^+$ and $CCR10^+$ PCs have been identified in mucosal tissues in both humans and animals (Kunkel *et. al*, 2003), 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 polymeric IgA, typically as dimers. Polymeric IgA 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 express surface IgA (sIgA) and intracellular IgA (icIgA), and exclusively secretes polymeric IgA. This provides the first function-phenotypic identification of the circulating, activated, B cell subset that represents the active normal mucosal IgA response in humans.

## 3.2 Materials and Methods

### *3.2.1 Specimen Preparation and B cell Isolation*

Blood samples from normal, healthy adults 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 ACD (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 minutes, layered over Ficoll-Paque Plus (VWR International, Canada), and centrifuged for 20 minutes at 1800 x *g*. The resulting B cells were resuspended 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.

### *3.2.2 Immunophenotyping*

The blood B cell preparations were surface stained using various mouse anti-human monoclonal antibodies. 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-$\alpha_4$-PE, anti-$\beta_7$-APC, anti-$\alpha_E$-PE, anti-CCR9-PE, anti-CCR10-PE, anti-CCR10-APC, anti-HLA-DR-APC-Cy7. All antibodies were purchased from BD Biosciences (Oakville, Canada) except anti-CD27-Alexa700 (EMD Biosciences, San Diego, USA), anti-IgA-FITC (DakoCytomation, Fort Collins, USA), and anti-CCR antibodies (PE and APC conjugates, R&D Systems, Minneapolis, USA). Specific phenotyping procedures used 4, 5, or 6 colour analysis to determine various B cell subsets. Data was collected on a Becton Dickinson LSR II cytometer using FACSDiva software (BD Biosciences, Oakville, Canada) and analysed using FlowJo software (Treestar Inc.).

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

### *3.2.3 FACS*

Memory B cell (MBC) and pre-plasma cell (PPC) populations, or PPC with specific surface markers (sIgA or CCR10) were purified from total B cell preparations by

staining against CD19, CD27, CD38, IgA or CCR10, using antibodies described above, and then isolation by FACS. Sorting was carried out on a high speed digital FACSVantage sorter (BD Biosciences, Oakville, Canada), with simultaneous two stream sorting of MBC and PPC. Typical yields for MBC ($CD19^{+}/CD27^{int}/CD38^{-/+}$) and PPC ($CD19^{lo}/CD27^{hi}/CD38^{hi}$) were >95% pure. In some experiments, PPC were identified and sorted by a $CD19^{lo}/CD27^{hi}$ phenotype that is clearly equivalent to the $CD19^{lo}/CD27^{hi}/CD38^{hi}$ phenotype (Figure 3.1).

### *3.2.4 Cell Culture*

FACS-sorted MBC were suspended in RPMI containing 10% FCS, 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin prior to activation cultures. As previously described(Hirano *et. al*, 2003), MBC were cultured in triplicate for 10 days at a density of $2 \times 10^4$ cells/well in a final volume of 200 μL in the presence mitomycin-C-treated L/CDw32 cells (ATCC), polyclonal B cell activators anti-CD40 mAb (mouse IgG1, clone G28.5) and anti-CD27 mAb (BD Biosciences, Oakville, Canada), and recombinant human IL-2 and IL-10 (R&D Systems, Minneapolis, USA). 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 (see below).

FACS-sorted PPC were cultured in IMDM containing 10% FCS, 2mM L-glutamine, 50 μM 2-ME, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were carried out in 364-well microculture plates (BD Biosciences, Oakville, Canada) at a

density of 5000 cells/well in a final volume of 50 μL for 36 hours and supernatants were collected analysed for IgA or IgG isotype by ELISA, in some cases by size exclusion chromatography with ELISA.

### *3.2.5 Size Exclusion Chromatography of Culture Supernatants*

MBC and PPC culture supernatants were concentrated 5x using BD Vivaspin (100 MW cutoff) centrifugal concentrators, and then fractionated through a 5ml 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, 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 dimmer and monomer. These standards were run prior to each supernatant analysis.

### *3.2.6 ELISA*

96-well Maxisorp plates (VWR International, Mississauga, Canada) were coated with polyclonal goat anti-human IgA (Southern Biotech, Birmingham, USA) or polyclonal goat anti-human IgG (Caltag Labs, Burlingame, USA), blocked with 1% BSA in TBS, and incubated with column-fractionated culture supernatants or an isotype standard (Jackson ImmunoResearch, West Grove, USA). Reactions were developed using biotinylated polyclonal goat anti-human secondary antibodies against IgA (Southern Biotech, Birmingham, USA) or IgG (Caltag Labs, Burlingame, USA) and streptavidin-alkaline phosphatase (Sigma-Aldrich, Oakville, Canada), followed by

incubation with p-nitrophenyl phosphate (Sigma-Aldrich, Oakville, Canada) at 37° C for 30 minutes. Absorbance at 405 nm was measured using a SAFIRE plate reader and XFluor software.

### *3.2.7 ELISPOT*

Acrowell 96-well filter plates (VWR International, Mississauga, Canada) were incubated at 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/well in 200 uL of IMDM containing 10% FCS, 2mM L-glutamine, 50 μM 2-ME, 100 U/mL penicillin and 100 μg/mL streptomycin for 36 hours. 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, Minneapolis, USA). 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.

### *3.2.8 Statistical Analysis*

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

## 3.3 Results

### *3.3.1 Pre-plasma cell and Memory B cell 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 immunoglobulin secreting MBC from human spleen (Avery *et. al*, 2005, Ellyard *et. al*, 2004). We refer to these cells as pre-plasma cells (PPC). These analyses exclude the transitional B cell subset in blood that has high CD38 expression, because that subset does not express CD27 (Carsetti *et. al*, 2004). Figure 3.1 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 ($CD27^+CD38^{lo}$) and PPC ($CD27^{hi}CD38^{hi}$) that are present at frequencies of 89.7 ± 1.1% and 6.1 ± 1.2% (n = 8) of the $CD27^+$ pool, respectively (Figure 3.1, panel C). Thus, PPC represent approximately 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 (Figure 3.1, panel G). This allowed us to identify PPC as $CD27^{hi}$ in subsequent phenotypic analysis and sorting experiments. Analysis of forward and side-scatter properties of B cell subsets indicated that PPC exhibited larger size and cytoplasmic complexity compared to resting MBC (Figure 3.1, panels D & E), as previously described for $CD38^{hi}$ cells from human spleen (Ellyard *et. al*, 2004). This is consistent with the activated or "blast" state of our identified PPC in blood.

We have defined PPC as $CD19^{+}CD27^{hi}(CD38^{hi})$, but this phenotype potentially does not exclude more differentiated cells that could be equally qualified as plasma blasts or plasma cells in blood. For instance, Mei et al recently defined blood plasma cells as intracellular $Ig^{high}$, surface $CD62^{-}$, HLA-$DR^{low}$ in phenotype, while grouping blood plasma blasts/plasma cells in the phenotype $CD19^{+}CD27^{hi}$ (Mei *et. al*, 2009). We therefore investigated our identified PPC for expression of CD138 and HLA-DR to determine what fraction may have a phenotype closer to plasma cells. The CD138 marker is considered specific for fully differentiated plasma cells in tissue, but recent data indicate that typically less than 2% of the $CD27^{+}$ fraction of blood from normal lactating women expresses the CD138 marker, and that would mean less than 0.1% of PBMC (Tuaillon *et. al*, 2009). 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 plasma cells are almost undetectable. Differential expression of HLA-DR was found when comparing $CD27^{hi}$ (PPC) and $CD27^{mod}$ (MBC) fractions of blood (Figure 3.2). The PPC had a reproducible fraction of cells with low-moderate expression of HLA-DR compared to 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 to typical HLA-DR expression on MBC varied between 1:10 and 1:4, comparable to results found by Mei et al (Mei *et. al*, 2009), where they concluded the intracellular $Ig^{+}$, HLA-$DR^{low}$ cells were plasma cells and those with normal levels of

HLA-DR were plasma blasts. We next double stained MBC and PPC with Ki67, a marker of proliferating or recently proliferating cells (Wirths *et. al*, 2005), and HLA-DR (Figure 3.2). These data clearly show that there is high expression of Ki67 in the majority of PPC, whereas MBC have low expression of Ki67. A noticeable correlate between Ki67 and HLA-DR stain was found in 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$^{+}$CD27$^{hi}$) 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 plasma cells.

### *3.3.2 Phenotypic analysis of surface IgA and intracellular IgA, mucosal relevant, adhesion molecules and chemokine receptors on PPC and MBC in normal blood*

Blood B cell populations were stained and analyzed by flow cytometry to determine the expression of surface markers relevant to mucosal function. The first markers investigated were the heterodimeric adhesion molecules $\alpha_4\beta_7$ and $\alpha_E\beta_7$ that are thought to have roles in mucosal migration of T cells (Berlin *et. al*, 1993, Hamann *et. al*, 1994, Smyth *et. al*, 2007, Zhou *et. al*, 2008). All B cell subsets expressed $\alpha_4$ integrin chain as expected, but the relative expression of the dimeric $\alpha_4\beta_7$ varied between subsets (Figure 3.3). Both naïve and resting MBC had equivalent levels of expression of $\alpha_4\beta_7$ dimer (Figure 3.3A,B), however a substantial fraction of MBC did not have $\beta_7$ expression. In contrast, PPC had a distinct expression pattern (Figure 3.3C), with

approximately one half (49.3 ± 9.4%, n=5) expressing high levels of $\alpha_4\beta_7$ dimer, and the remaining cells lacking $\beta_7$ chain expression but with high levels of $\alpha_4$ chain expression, presumably in combination with other β chains. We did not observe any expression of $\alpha_E$ on naïve, MBC or PPC cells (n=5, data not shown). Percentages of $\alpha_4\beta_7$ dimer among these subsets is summarized on Table 3.1. Thus, approximately one half of PPC had a distinct pattern and high level of $\alpha_4\beta_7$ dimer expression, and this would be consistent with a high potential for mucosal homing preference for those PPC expressing high levels of $\alpha_4\beta_7$.

The next markers analyzed were the mucosal chemokine receptors CCR9 and CCR10. The ligands for these receptors are the gut-restricted chemokine CCL25 (TECK) and the pan-mucosal chemokine CCL28 (MEC), respectively (Kunkel *et. al*, 2002, Kunkel *et. al*, 2003). Thus, these receptors are potentially important markers of B cells with mucosal homing potential. Very few if any naïve B cells expressed either CCR9 or CCR10, but a small population of $CCR9^+CCR10^-$ MBC was consistently identified, and very few PPC were found to express CCR9 (Table 3.1). A large fraction of PPC (33.7 ± 3.7%, n = 10) expressed CCR10 (Figure 3.4, Table 3.1), but very few MBC (2.9 ± 0.5 %, n = 5) expressed CCR10, and no CCR9/CCR10 co-expression was observed by either MBC or PPC. The expression of CCR10 by a large fraction of PPC indicates that many circulating, antibody secreting B cells are capable of responding to chemokines expressed by a majority of mucosal tissues.

Expression of surface IgA (sIgA) and surface IgG (sIgG) was examined among B cell subsets to further determine mucosal relevance and function (Table 3.1). Both MBC and PPC subsets had significant proportions of surface $IgA^+$ cells with more than one half PPC sIgA positive, consistent with published data identifying large $sIgA^+$ cells (Kunkel *et. al*, 2003) or more recently intracellular IgA expression by $CD19^+CD27^{hi}$ cells (Mei *et. al*, 2009). The fraction of naïve 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 3.1).

### *3.3.3 Polymeric IgA secretion by PPC and sIgA+ PPC in normal blood.*

No previous work has determined the frequency of blood PPC secreting IgA, nor associated IgA secretion with blood PPC using the recently defined surface phenotype $CD19^+CD27^{hi}$. Some previous studies have examined proportions of IgA, IgG and IgM secreted by whole mononuclear cell preparations from blood, and after immunization or viral infection (Feng *et. al*, 2006, Jaimes *et. al*, 2004, Mestecky *et. al*, 2004, Moldoveanu *et. al*, 1995). Other work has identified polymeric IgA production by PBMC and cells from various mucosal tissues, or IgA production by sIgA+ cells, but not by PPC (Kutteh *et. al*, 1980, Layward *et. al*, 1992, Moldoveanu *et. al*, 1984, Tarkowski *et. al*, 1991, Wilson *et. al*, 2004). Thus we prepared B cells from the blood of normal subjects, and used FACS to simultaneously isolate >98% pure PPC and compared them to resting > 98% pure MBC ($CD19^+CD27^{mod}$) for functional secretion of polymeric IgA. ELISPOT analysis demonstrated that on average (n=18), 66% of FACS sorted PPC secreted either

IgG or IgA, but as expected no sorted resting MBC secreted IgG or IgA above control background (Table 3.2). IgG producing PPC were detected at a slightly higher frequency than IgA producing PPC, by an average ratio of 1.4:1. These data on functional secretion of IgA versus IgG by $CD19^{+}CD27^{hi}$ PPC, are consistent with previous studies of PBMC (Mestecky *et. al*, 2004, Moldoveanu *et. al*, 1995). 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 (Moldoveanu *et. al*, 1995).

Secretion of polymeric IgA 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 polymeric (≥ 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 polymeric IgA, with little or no monomeric IgA evident (Figure 3.5A). In vitro stimulated MBC also produced mostly polymeric IgA, but had a more prominent fraction of monomeric IgA compared to PPC (Figure 3.5A). Thus, FACS purified PPC secrete polymeric IgA, not monomeric IgA. Since unstimulated MBC do not secrete IgA or IgG, it follows that among $CD27^{+}$ B cells in normal blood, the $CD27^{hi}CD38^{hi}$ PPC subset contains all the functional mucosal IgA producing cells.

We observed an average frequency of 54% of PPC that were $sIgA^{+}$, compared to 34% $CCR10^{+}$, and 49% $\alpha_4\beta_7^{+}$ (Table 3.1). Since the $sIgA^{+}$ 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 sIgA expression (Kunkel *et. al*, 2003) and IgA expression on the cell surface cannot predict polymeric versus monomeric IgA production. We therefore sorted PPC ($CD19^{+}CD27^{hi}$) cells that expressed sIgA 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 $sIgA^{+}$ cells. In addition, we took supernatants from cultured $sIgA^{+}$ and $sIgA^{-}$ 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 $sIgA^{+}$ sorted fraction of PPC, while none were present among the $sIgA^{-}$ sorted fraction (Table 3.2). The inverse observation was found for IgG secreting cells that were only detected in the $sIgA^{-}$ sort fraction of PPC. Thus, sIgA expression directly identifies IgA producing PPC in normal human blood, as assessed by ELISPOT. The vast majority of this IgA secreted by $sIgA^{+}$ PPC was dimeric or larger, with only a very small fraction of monomeric IgA (Figure 3.5B). Since no IgA production was found in the $sIgA^{-}$ fraction of PPC, it was obvious that $sIgA^{+}$ PPC are the dominant source of polymeric IgA.

Our ELISPOT data contrast the flow cytometric, intracellular IgA staining of whole PBMC preparations that was recently published by Mei et al (Mei *et. al*, 2009) and the much earlier microscopic intracellular IgA staining data for PBMC by Kutteh et al

(Kutteh *et. al*, 1982), where intracellular IgA cells largely outnumbered intracellular IgG cells.Our analysis of intracellular IgA expression by PPC demonstrates that the $sIgA^+$ subset of PPC contains all of the $icIgA^+$ cells (Figure 3.6). These results are consistent with our ELISPOT results (Table 3.2) which demonstrate that all IgA-secreting cells are contained within the $sIgA^+$ PPC fraction. The presence of $sIgA^+$ PPC that do not secrete IgA is consistent with previous suggestions that there may be different functional populations within the IgA PPC compartment (Conley *et. al*, 1982).

### *3.3.4 Mucosal homing integrin and mucosal chemokine receptor expression by $sIgA^+$ PPC.*

Surface IgA, integrin $\alpha_4\beta_7^+$, and chemokine receptors CCR10 are potential markers of mucosal relevance. Given our observation of exclusive polymeric IgA production by $sIgA^+$ PPC, and the relatively high frequency of the two homing markers on PPC (Table 3.1), we reasoned that defined combinations of sIgA with either of the two homing markers should provide the best surface phenotypic identification of mucosal PPC in normal blood. We therefore performed multi-colour flow cytometric analysis, to look at co-expression of CCR10 and $\alpha_4\beta_7^+$ with sIgA on normal blood PPC. The results indicated that the greatest fraction of $sIgA^+$ PPC (~ 72%) expressed CCR10, and this was two-fold higher than the fraction (~ 36%) that expressed $\alpha_4\beta_7$ (Table 3.3). These data confirm previous observation of CCR10 expression by $sIgA^+$ large lymphoblasts (Kunkel *et. al*, 2003). Within the $sIgA^+$ PPC, those that expressed CCR10 and no $\alpha_4\beta_7$ were 6 times more frequent than those that expressed $\alpha_4\beta_7$ but no CCR10. In addition,

approximately 80% of $sIgA^+\alpha_4\beta_7^+$ PPC co-express CCR10. These data indicate clearly that within normal blood PPC, the $CCR10^+$ PPC are more clearly associated with sIgA than $\alpha_4\beta_7$. However, a substantial fraction of $sIgA^+$ (~20%) did not express either CCR10 or $\alpha_4\beta_7$, indicating that as much as 1/5 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 sIgA or by way of comparison sIgG (Table 3.4). On average, the largest fraction (67%) of $CCR10^+$ PPC expressed sIgA and the majority of $CCR10^+$ PPC were $icIgA^+$ (Figure 3.7), while less than 33% of $CCR10^+$ PPC had sIgG (Table 3.4). However a substantial fraction (35%) of $CCR10^-$ PPC were also $sIgA^+$, an observation similar to that stated above for $sIgA^+$ PPC (Table 3.3). In addition, a large fraction of $CCR10^+$ PPC did not express sIgA or sIgG. 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 3.5). 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 $sIgA^+$ cells and IgA secreting cells in the $CCR10^-$ subset compared to the $CCR10^+$ subset, we examined the production of polymeric IgA in the two subsets of cells purified by FACS and cultured in vitro. The vast majority of IgA produced by $CCR10^+$ PPC was polymeric (Figure 3.5C). A similar profile was true for $CCR10^-$ PPC, but cells in these cultures produced much less IgA (Figure 3.5C), presumably because there were fewer sIgA producing cells present (Table

3.5). Thus, the $CCR10^+$ PPC subset include most of the polymeric IgA secreting cells and nearly all express sIgA, and although there is a smaller clearly defined fraction of $CCR10^-$ cells that secrete polymeric IgA, the larger $CCR10^+$ PPC subset represents a majority of functional mucosal PPC in normal blood.

Table 3.1: Surface Ig, $\alpha_4\beta_7$ and CCR Expression on Circulating B Cell Subsets

| | % $IgG^+$ | % $IgA^+$ | % $\alpha_4\beta_7^+$ | % $CCR9^+$ | % $CCR10^+$ |
|---|---|---|---|---|---|
| Naïve | 1.7 ± 0.4 | 1.6 ± 0.6 | 93.6 ± 1.2 | 2.7 ± 0.8 | 2.4 ± 1.1 |
| MBC | 23.8 ± 2.2 | 25.6 ± 2.1 | 54.1 ± 2.4 | 4.97 ± 2.3 | 2.9 ± 0.5 |
| PPC | 8.6 ± 1.8 | 54.0 ± 4.3 | 49.3 ± 9.4 | 1.81 ± 0.4 | 33.7 ± 3.7 |

Identified Subsets: Naïve: $CD19^+CD27^-$; MBC: $CD19^+CD27^{int}$; PPC: $CD19^+CD27^{hi}$

Data are mean ± SEM. Background was 1% for all markers.

For sIgG, sIgA, and CCR10, n = 10.

For $\alpha_4\beta_7$; and CCR9, n = 5.

Table 3.2: IgA and IgG Secretion by FACS sorted PPC or $sIgA^{\pm}$ PPC

| PPC | | MBC | | $IgA^{+}$ PPC | | $IgA^{-}$ PPC | |
|---|---|---|---|---|---|---|---|
| IgA | IgG | IgA | IgG | IgA | IgG | IgA | IgG |
| 277±17* | 371 ± 24* | 1 ± 1 | 1 ± 1 | 693±36^ | 1 ± 0 | 1 ± 0 | 550 ± 25^ |

Data are Mean spots per 1000 plated PPC ± SEM

^ $p < 0.001$ for n = 6 comparisons of IgA or IgG secretion by $sIgA^{+}$ versus $sIgA^{-}$ PPC

* $p < 0.001$ for n = 18 comparisons of IgA or IgG secretion by PPC versus MBC

Table 3.3: Expression of CCR10 and $\alpha_4\beta_7$ on Surface $IgA^{\pm}$ PPC

| $CCR10^+/\alpha_4\beta_7^-$ (%) | $\alpha_4\beta_7^+/CCR10^-$ (%) | $CCR10^+/\alpha_4\beta_7^+$ (%) | $CCR10^-/\alpha_4\beta_7^-$ (%) |
|---|---|---|---|
| 44.0 ± 5.6 | 7.3 ± 2.3 | 28.6 ± 7.8 | 20.1 ± 5.6 |

Data are Mean ± SEM

$p < 0.002$, for n = 4 comparisons of $CCR10^+/\alpha_4\beta_7^-$ vs. $\alpha_4\beta_7^+/CCR10^-$

$p < 0.05$, for n=4 comparisons of $CCR10^+$ vs. $\alpha_4\beta_7^+$

Table 3.4: Surface Ig Expression on Circulating $CCR10^{+/-}$ PPC

| $CCR10^{+}$ | | | $CCR10^{-}$ | | |
|---|---|---|---|---|---|
| % $sIgA^{+}$ | % $sIgG^{+}$ | % $sIgA^{-}/sIgG^{-}$ | % $sIgA^{+}$ | % $sIgG^{+}$ | % $sIgA^{-}/IgG^{-}$ |
| 66.8± 7.5 | 2.8 ±0.8 | 29.5 ± 6.7 | 34.7 ± 5.9 | 19.4 ± 4.5 | 44.7 ± 5.4 |

Data are mean ± SEM for n = 6.

Table 3.5: IgA and IgG Secretion by $CCR10^{\pm}$ and $CCR10^{-}$ PPC

| $CCR10^{+}$ PPC | | $CCR10^{-}$ PPC | |
|---|---|---|---|
| IgA | IgG | IgA | IgG |
| $779 \pm 20^{+}$ | $0 \pm 0$ | $281 \pm 39$ | $530 \pm 58^{+}$ |

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

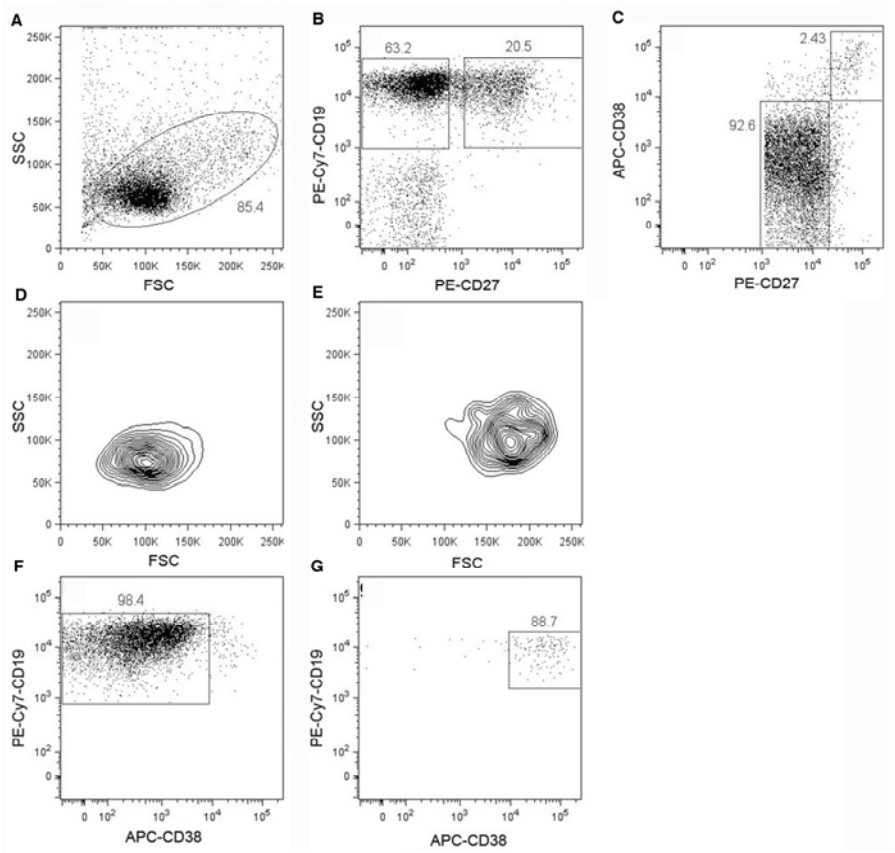


**Figure 3.1 Typical identification of MBC and PPC in B lymphocyte preparations from normal subjects.** Purified B cells were stained with monoclonal antibodies against CD19, CD27 and CD38. MBC and PPC were analyzed by gating with forward- and side-scatter region as shown (A), then gating on CD27+ cells (B) to examine expression of CD27 vs CD38 (C). Among $CD27^{+}$ B cells, MBC and PPC can be distinguished as $CD38^{lo/-}$ and $CD38^{hi}$, respectively. Light scatter of MBC (D) was demonstrated to be lower than that of PPC (E), an observation consistent with the larger blast form of antibody-secreting cells. Panels (F) and (G) show CD38 expression by MBC and PPC as identified by relative expression of CD27. MBC (F) were identified and gated as $CD19^{+}/CD27^{int}$ and are $CD38^{lo}$, while PPC (G) were identified and gated as $CD19^{+}/CD27^{hi}$, and are $CD38^{hi}$. Data are representative of 8 samples.

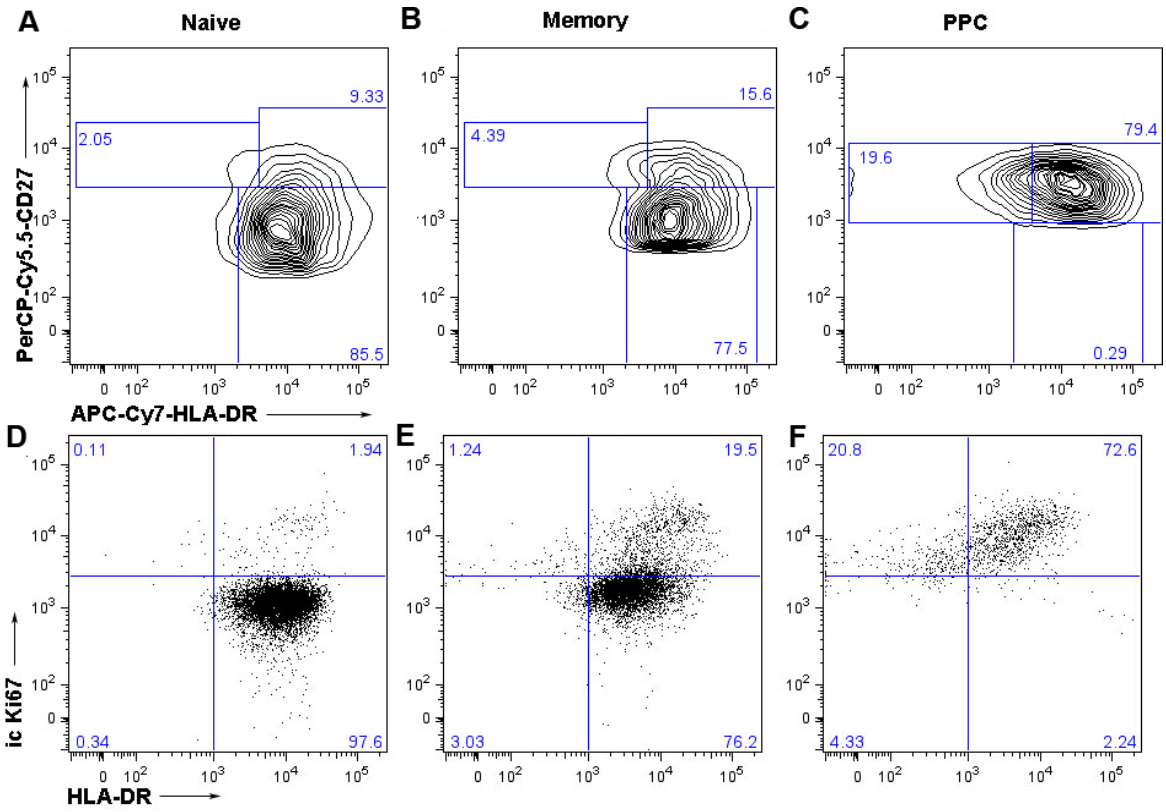


**Figure 3.2 HLA-DR$^{low/int}$ subset of PPC are Present in B cell and PPC Isolates**. Rosette isolated B cells were surface stained with HLA-DR antibody and intracellular stained with Ki67antibody to identify further activated and differentiated Naïve B cells, MBC and PPC in blood. Upper left region in C indicates low/intermediate HLA-DR expression and high Ki67 expression by a subset of PPC compared to normal HLA-DR expression on the majority of PPC (upper right). The lower right region in A,B shows HLA-DR and Ki67 expression on CD27$^{-/int}$, normal resting naïve and MBC. The HLA-DR$^{hi}$ Ki67$^{lo}$ population is missing in CD27$^{hi}$ PPC (C).

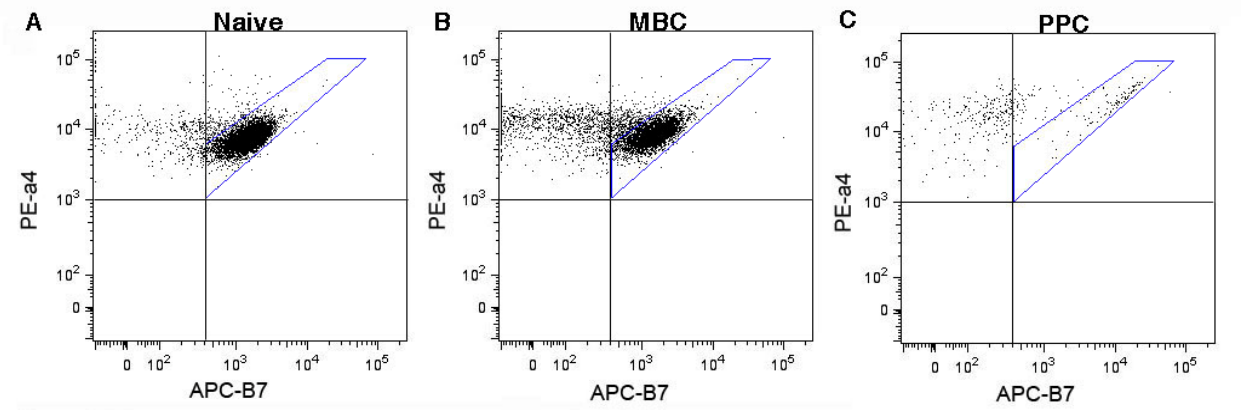


**Figure 3.3 Expression of $\alpha_4\beta_7$ integrin and CCR10 by naïve B cells, MBC, and PPC.** Vertical and horizontal lines indicate negative cutoff for background PE- $\alpha_4$ and APC$\beta_7$ - fluorescence. The region displayed represents all cells positive for $\beta_7$ integrin chain. A majority of naïve (A) and MBC (B) express low or moderate levels of $\alpha_4\beta_7$ with a small fraction $\alpha_4^+$ $\beta_7^-$ (upper left quadrant). Both MBC (B) and PPC (C) have clear populations of $\alpha_4^+$ $\beta_7^-$ with increased levels of $\alpha_4$ chain compared to naïve cells (A). A large fraction of PPC (C) expressed elevated high levels of $\alpha_4\beta_7$ compared to MBC or naïve cells. Figures are from one sample representative of five samples with similar results.

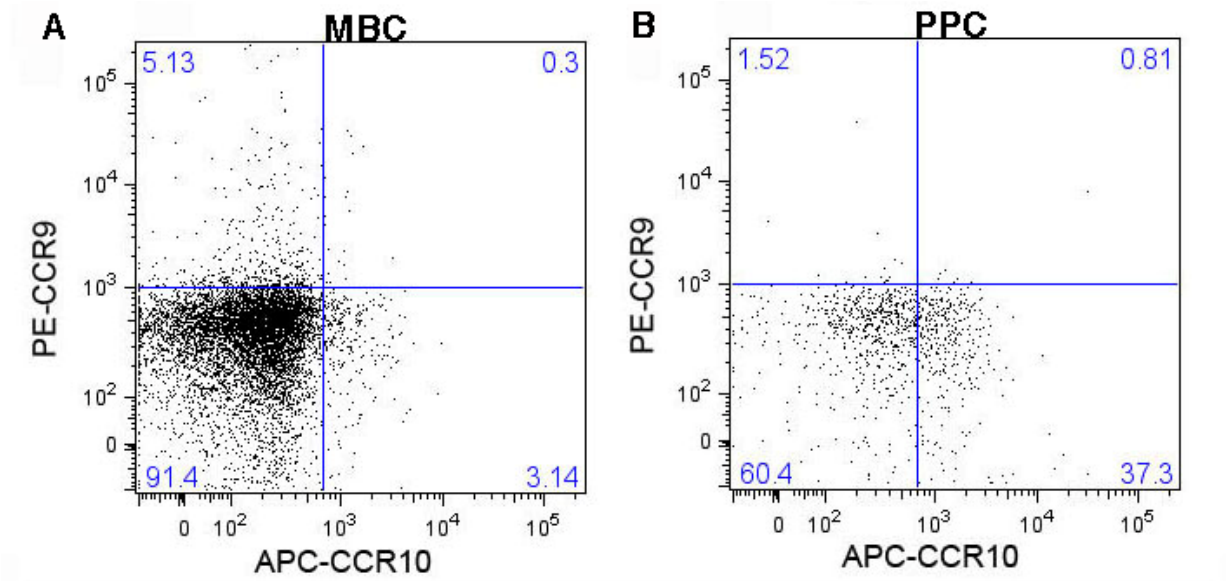


**Figure 3.4 A Large Fraction of PPC, but not MBC, express the mucosal chemokine receptor CCR10.** Small frequencies of CCR9- and CCR10-expressing MBC were observed in peripheral blood (Panel A). Similar analysis of PPC revealed that a large fraction of CCR10-expressing cells could be found in circulation (Panel B). Results are representative of 4 samples. Negative fluorescence cutoffs were established using fluorescence minus-one (FMO) control samples.

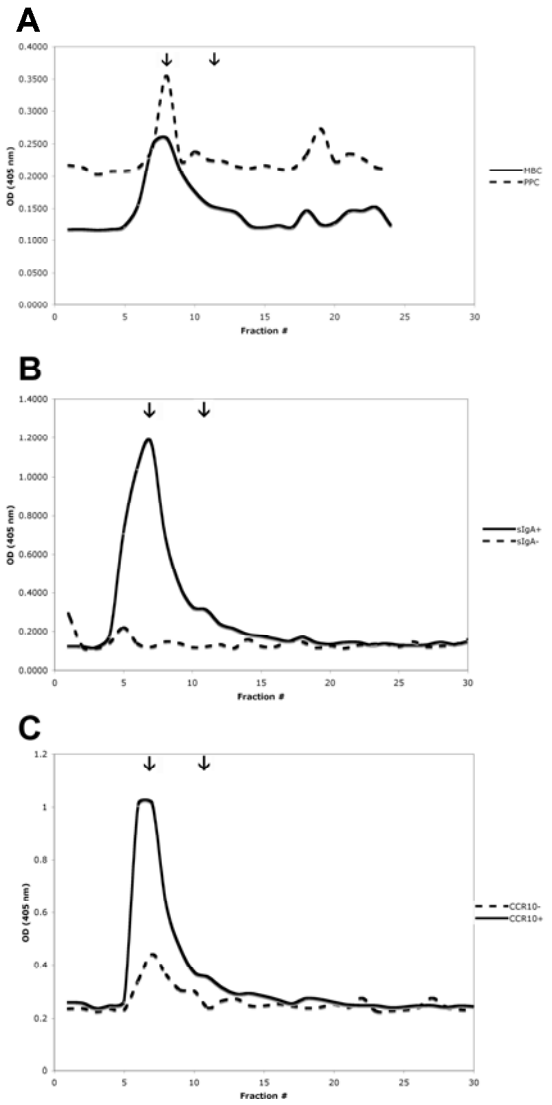


**Figure 3.5 sIgA$^+$ PPC and CCR10$^+$ PPC both secrete predominantly polymeric IgA.** Culture supernatants were collected from FACS-sorted PPC or sorted and activated MBC (A); sIgA$^+$ and sIgA$^-$ PPC (B); or CCR10$^+$ PPC and CCR10$^-$ PPC (C). Resulting supernatants were fractionated on a G-200 column, and assayed for the presence of IgA by ELISA. The left arrowhead indicates the peak elution fraction for dimeric IgA standard, and the right arrowhead indicates the peak elution fraction for a monomeric IgA standard. Results are single example, representative of 3-5 cell preparations for each comparison.

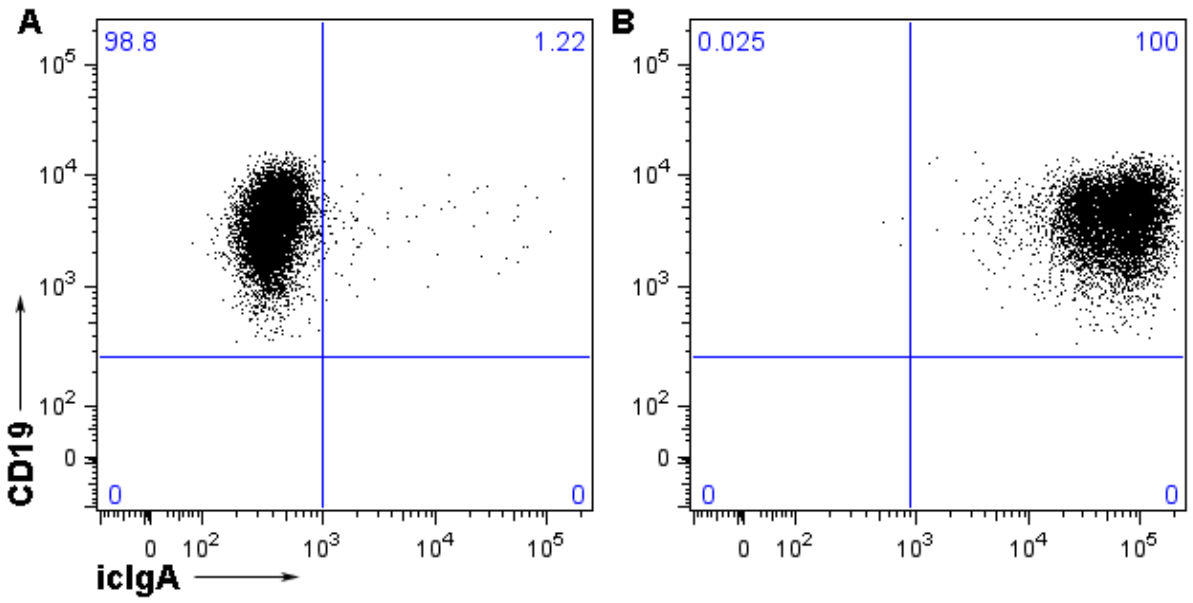


**Figure 3.6 sIgA$^+$ PPC contain all icIgA$^+$ PPC in blood.** sIgA$^+$ PPC were isolated by FACS, fixed, permeabilized, and stained for intracellular IgA expression. Analysis revelaed that all sIgA$^+$ cells were icIgA$^{hi}$ (Panel B), and no icIgA$^+$ PPC were observed in the sIgA$^-$ PPC fraction (Panel A). Results are representative of 3 samples. Negative fluorescence cutoffs were established using fluorescence minus-one (FMO) control samples.

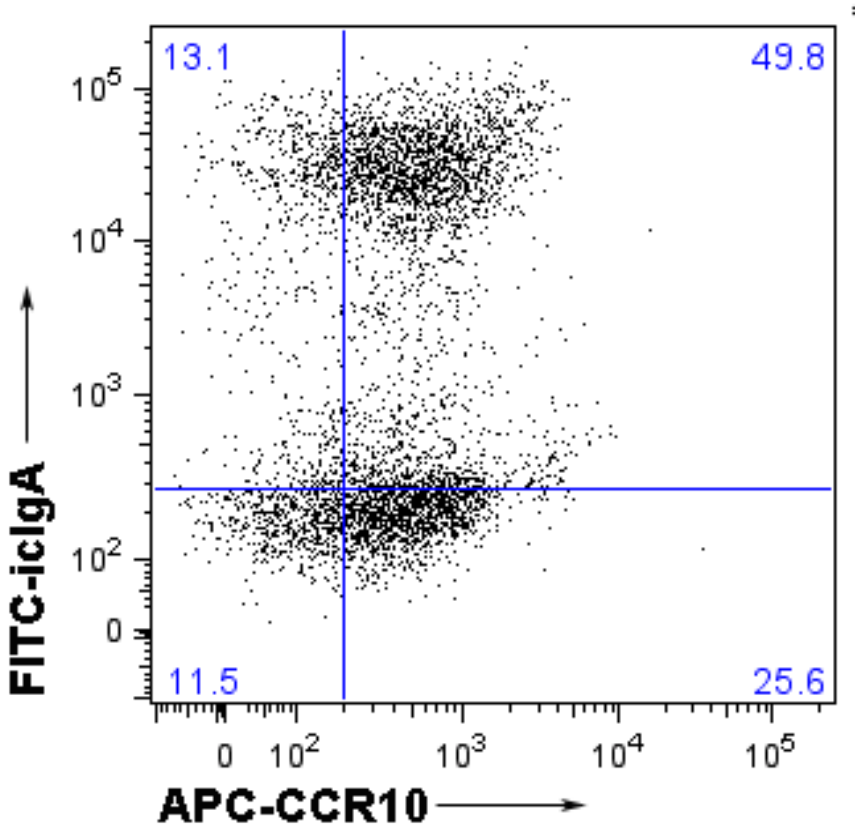


**Figure 3.7 CCR10$^+$ PPC are predominantly icIgA$^+$.** Rosette isolated B cells were surface stained for B cell markers and CCR10, then fixed, permeabilized, and stained for intracellular IgA. The upper right quadrant shows PPC positive for surface CCR10 and intracellular IgA. Results are representative of 4 samples. Negative fluorescence cutoffs were established using fluorescence minus-one (FMO) control samples.

## 3.4 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 and others have demonstrated that altered numbers of circulating MBC and PPC are indicators of recent or ongoing immune responses following vaccination, and we have confirmed this in separate work (Bernasconi *et. al*, 2002, Fernandes *et. al*, 2010, Odendahl *et. al*, 2005). Others have shown that antigen-specific PPC are mobilized in peripheral blood in the case of both acute and chronic infection (Jaimes *et. al*, 2004). 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 $CD27^{hi}$ PPC and $CD27^{int}$ MBC in circulation. As others (Bernasconi *et. al*, 2002, Odendahl *et. al*, 2000) 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.

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 pIgR, which confers enhanced stability and antimicrobial properties to secretory IgA (Layward *et. al*, 1992). It is secretory IgA that contributes to host defense in multiple innate and adaptive roles including, immune exclusion of microbes, viruses, and pathogenic toxins (Brandtzaeg, 2007), inhibition of indigenous microbial overgrowth (Macpherson *et. al*, 2005), enhancement of mucosal immune development in neonates (Brandtzaeg, 2002), limiting epithelial penetration by HIV (Hocini *et. al*, 1997), and providing intracellular neutralization of virus and LPS (Bomsel *et. al*, 1998, Fujioka *et. al*, 1998, Mazanec *et. al*, 1995). It was recently demonstrated that even polymeric IgA, lacking bound SC, can facilitate removal of antigens from the lamina propria (Mazanec *et. al*, 1993, Robinson *et. al*, 2001).

Despite this breadth of evidence confirming a central role of polymeric IgA in mucosal defense, investigations of blood B cells for subsets that secrete polymeric IgA has been performed in only limited study (Kutteh *et. al*, 1980, Kutteh *et. al*, 1982, Woof *et. al*, 2006). In fact, almost all of the evidence currently available regarding IgA B cells in blood depends solely on surface IgA or intracellular IgA expression to correlate phenotype with mucosal function, or was derived from the study of subjects during or post infection, where 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 (Delacroix *et. al*, 1983) and the earlier data (Kutteh *et. al*, 1982) had demonstrated both polymeric 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 polymeric IgA, 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: $CD27^{hi}sIgA^{+}CCR10^{+}$ that secrete polymeric IgA. Here, we show that all polymeric IgA secreting PPC are contained within the $sIgA^{+}$ PPC, and a majority of these cells are $CCR10^{+}$. Our data also indicates that $sIgA^{+}$ PPC produce little if any monomeric IgA, so the cells responsible for production of IgA monomers likely do not circulate freely in normal healthy subjects, and are likely sequestered in the bone marrow (Kutteh *et. al*, 1982).

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 means that these mucosal PPC can be used to examine the ongoing IgA response that occurs in healthy subjects. Thus, blood $sIgA^{+}$ or $IgA^{+}CCR10^{+}$ 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 (Mora *et. al*, 2005) 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 contrasts results from the recently published work of Mei et al. (Mei *et. al*, 2009) in regard to the relative frequency of IgA vs IgG producing cells in blood from normal healthy subjects. Their data is 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) indicates that the whole PPC ($CD19^{+}CD27^{hi}$) fraction contains slightly more IgG secreting cells than IgA. The difference between results cannot be directly tested, since it is not possible to perform intracellular IgA stain and test for IgA secretion by the same cell. We did look at intracellular IgA staining of identified PPC, and observed that the frequency of intracellular IgA cells was the same as the frequency of $sIgA^{+}$ cells. When we did icIgA stain of $sIgA^{-}$ cells we did not observe any significant fraction of $icIgA^{+}$ cells (Figure 3.6). Thus, we have similar observations to Mei, in regard to $icIgA^{+}$ PPC at high frequency in blood, but our ELISPOT data demonstrates that there is a significant fraction of $sIgA^{+}$ PPC that do not secrete antibody, consistent with the data that suggest that IgA2 PPC do not actively secrete IgA (Conley *et. al*, 1982). Previous work indicated that surface expression of IgA, particularly IgA2, is not necessarily predictive of secretion (Conley *et. al*, 1984). Thus, in normal human blood, $icIgA^{+}$ 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. Indeed, a previous study has demonstrated that cytoplasmic-$IgA2^{+}$ cells do not secrete when stimulated with PWM *in vitro* (Conley

*et. al*, 1982) indicating that these cells may not be secretors at all, even under conventional stimulation that includes T cell support of antibody production.

Mei et al. provide data indicating both plasma cells and plasmablast phenotypes may be present within the $CD19^{+}CD27^{hi}$ blood cells from normal subjects, based on differential HLA-DR expression, and lack of CD62L and integrin $\alpha_4\beta_7$. Our data indicates that a small fraction of the $CD19^{+}CD27^{hi}$ (PPC) did have reduced expression of HLA-DR, similar to Mei, but we found no expression of CD138, the canonical marker of plasma cells. 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 $CD19^{+}CD27^{hi}$ phenotype are all plasmablasts, and are not plasma cells, but there is a fraction of PPC (10-20%) of more differentiated, HLA-$DR^{low}$ plasmablasts. Since our cell isolation approach by B rosette and FACS did not alter the ratio or recovery of these cell subsets (Figure 3.2), we are confident 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 express CCR9

(Bowman *et. al*, 2002, Lazarus *et. al*, 2003). This was confirmed later in humans, where a distinct fraction (>30%) of IgA PC from small intestinal tissue were found to express CCR9 (Kunkel *et. al*, 2003). However, CCR9 expression was rarely seen on IgA PCs in other segments of the gut (Kunkel *et. al*, 2003), and was not expressed by IgA PCs in the lungs (Kunkel *et. al*, 2003, Lazarus *et. al*, 2003). 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(Kunkel *et. al*, 2000, Papadakis *et. al*, 2000). CCR10 is now understood to have a broader distribution of expression on B cells within various mucosal tissues, compared to 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 (Hieshima *et. al*, 2003, Pan *et. al*, 2000, Wang *et. al*, 2000), 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 are $CCR10^+$ (Kunkel *et. al*, 2003).

Our data on PPC co-expression of sIgA and CCR10 on blood PPC is clearly in agreement with the similar data of both Kunkel and Mei(Kunkel *et. al*, 2003, Mei *et. al*, 2009), although neither of these investigators commented on the small but significant fraction of $sIgA^+CCR10^-$ cells in their identified plasmablast/plasma cells. We clearly show that this smaller subset of PPC secretes polymeric IgA. Some of the $IgA^+CCR10^-$ cells we identified expressed $\alpha_4\beta_7$ (Table 3.3), but ~20% of $sIgA^+$ PPC expressed neither CCR10 nor $\alpha_4\beta_7$. Thus, our data indicates that in blood from normal healthy subjects

CCR10 identifies the largest fraction of polymeric IgA producing mucosal PPC. Our data also reveals the presence of a $CCR10^{+}/icIgA^{-}$ population (Figure 3.7), which may also contribute to the less than 100% IgA secretion rate of purified $CCR10^{+}$ PPC (Table 3.5). The smaller subset of $IgA^{+}CCR10^{-}$ PPC may also represent a distinct mucosal population, but there is also evidence of polymeric IgA-producing cells in non-mucosal tissues (Tarkowski *et. al*, 1991), raising the possibility that CCR10 might distinguish between mucosal and non-mucosal pIgA producing PPC in blood.

We did not detect any CCR9 expression by blood PPC from healthy subjects. This directly contrasts the results of Kunkel (Kunkel *et. al*, 2003) who found up to 25% of large $CD19^{+}sIgA^{+}$ PBMC expressed CCR9. However, the subjects used for that study were not well described, and others detected CCR9 on circulating B cells after viral infection (Jaimes *et. al*, 2004). 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 plasma cells, as others have reported up to 50% of small-intestinal PCs co-express both CCR10 and CCR9 (Kunkel *et. al*, 2003). 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 $\alpha_4\beta_7$ 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 (Quiding-Jarbrink *et. al*, 1997). We observed that less than 10% of IgA+ PPC expressed high levels of $\alpha_4\beta_7$ in the absence of CCR10, while 44% of IgA+ PPC expressed CCR10 in the absence of $\alpha_4\beta_7$ (Table 3.3). Thus, high levels of $\alpha_4\beta_7$ on its own is a poor marker of mucosal PPC in normal human blood. In addition, we observed that the majority of naïve B cells and MBC in blood express low to intermediate levels of $\alpha_4\beta_7$ integrin (Figure 3.2). These observations are consistent with the hypothesis that activation of memory B cells in mucosal associated lymphoid tissues (MLN or Peyer's patches) would cause upregulation of this integrin, but that high levels of $\alpha_4\beta_7$ are not always (or continuously) expressed by mucosal PPC

Here, we report that nearly 40% of $sIgA^+$ PPC co-express CCR10 with high $\alpha_4\beta_7$ (Table 3.3). 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 (Campbell *et. al*, 1998, Lloyd *et. al*, 1996). As a marker co-expressed with CCR10, $\alpha_4\beta_7$ would permit pIgA secreting PPC to enter the human intestinal mucosa where MAdCAM-1 is known to be expressed (Briskin *et. al*, 1997). Further analysis of other 60% of $sIgA^+CCR10^+$ PPC for other adhesion molecules such as VCAM-1 (salivary gland, trachea, bronchioles) (Bentley *et. al*, 1993) may reveal similar populations with different presumptive homing patterns.

Our results also indicate the presence of small (3-5% of MBC), but consistently observed subpopulations of $CCR9^{+}CCR10^{-}$ and $CCR9^{-}CCR10^{+}$ MBC, consistent with previous observations (Kunkel *et. al*, 2003). We FACS purified the total MBC ($CD19^{+}CD27^{int}$) population and activated them in cultures that provided appropriate stimulation for proliferation and differentiation to secrete IgG or IgA (Hirano *et. al*, 2003). Following *in vitro* activation of the MBC we did observe production of polymeric IgA that predominated over a smaller but distinct monomeric IgA production (Figure 5A). Nearly 25% of MBC express sIgA (Table 3.1), so there is not a strong correlate between $sIgA^{+}$ MBC and the expression of CCR10 or CCR9. While it is possible that the small fractions of $CCR10^{+}$ or $CCR9^{+}$ MBC were responsible for the production of polymeric IgA, 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 (Layward *et. al*, 1992). We obtained blood from normal healthy individuals, so our 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 memory B cells will require specific isolation of $sIgA^{+}$ MBC that express CCR9 or CCR10, comparing them to those that do not have these markers.

In summary, we have demonstrated a high frequency population of $IgA^+$ PPC, the majority of which express CCR10 and that can functionally contribute to mucosal defense through secretion of polymeric IgA. The PPC we observed appear to be plasmablasts, with a small fraction indicating further differentiation toward plasma cells. Thus the PPC fit a 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 (Brandtzaeg, 2002, Jaimes *et. al*, 2004). 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 polymeric IgA, including perhaps the identification of a IgA-secreting population in peripheral blood that is responsible for glomerular deposition of polymeric IgA in IgA nephropathy (Layward *et. al*, 1992).

# CHAPTER 4

# *In vitro* activation with IL-21 induces CCR10 expression on IgA and IgG memory B cells

## 4.1 Introduction

Several studies have demonstrated *in vitro* that the nature of the activating cells and cytokine milieu can imprint mucosal homing receptors on B cells (Mora, Iwata *et al.*, 2006, Shirakawa, Nagakubo *et al.*, 2008). B cell responses are heterogeneous with respect to effector populations produced; some activated cells proliferate and form the future memory B cell (MBC) population, some are activated to generate short-lived plasma cells (PC), and some develop into long-lived PC that migrate to either bone marrow or mucosal tissues (Liu and Banchereau, 1997).

The exact conditions under which MBC can be activated to yield a mucosal-homing PC precursor are not yet completely understood but several studies have demonstrated that mucosal MBC circulate in blood but do not bear homing markers expressed during infection (Jaimes, Rojas *et al.*, 2004, Fernandes and Snider, 2010). T cell-dependent mucosal antibody responses are generated through germinal center (GC) reactions, primarily in the mucosa-associated lymphoid tissue (MALT) (Hornquist, Ekman *et al.*, 1995, Lycke, Eriksen *et al.*, 1987, Dullaers, Li *et al.*, 2009). The GC reaction is multifactorial, with a variety of cells including antigen-presenting cells (APC), T cells, and B cells interacting in close physical proximity to define the nature of the immune response. $CD4^+$ T cells, follicular helper T cells ($T_{fh}$) cells in particular, have been implicated as important controllers of GC responses and are known to secrete many cytokines with associated functions on B cells such as IL-2, IL-10, IL-21, and TGF-β

(Hornquist, Ekman, 1995, Vogelzang, McGuire *et al.*, 2008, Breitfeld, Ohl *et al.*, 2000, King, Tangye *et al.*, 2008).

Recent evidence from others and our lab indicates that MBC specific for mucosal pathogens may be activated following a systemic immune response, as measured through the appearance of recently activated PPC secreting both IgG and IgA against mucosal pathogens, and the presence of antigen-specific MBC in blood following resolution of an acute enteric infection (Jaimes, Rojas, 2004, Gonzalez, Jaimes *et al.*, 2003, Feng, Jaimes *et al.*, 2006, , Fernandes, Wasserman *et al.*, 2010) . The appearance of these cells in blood demonstrates that the mucosal and systemic immune compartments may not be entirely separate. Taking into consideration the large amount of evidence demonstrating that mucosal homing phenotypes can be induced at inductive sites physically removed from the effector sites, these recent observations of bystander activation may provide a means by which MBC relevant for mucosal immunity can be induced to traffic to mucosal tissues from a non-mucosal inductive site.

Many groups have demonstrated the ability of the activating microenvironment to "imprint" cells with a mucosal homing phenotype. Vitamin A has been demonstrated to induce expression of CCR9 and $\alpha_4\beta_7$ on B cells, permitting their migration/retention to/in the gut (Mora, Iwata, 2006). A similar study demonstrated that vitamin D3 is capable of causing B cells to express CCR10, a receptor for mucosally-expressed chemokine (MEC), which is produced at all mucosal tissues (Shirakawa, Nagakubo, 2008). While these studies demonstrate the effect of local tissue factors on imprinting B cell homing

patterns, they do not address the abilities of common cytokines produced by T cells in peripheral lymphoid tissues during an immune response.

Although germinal center reactions form in response to a specific antigen, the resulting cytokine microenvironment could polarize mucosal homing potential of cells activated either specifically or nonspecifically as a result of the initial response. A greater understanding of how the microenvironment affects mucosal homing molecule expression may provide a mechanism through which mucosal MBC could be activated non-specifically in the periphery and conferred with a mucosal homing phenotype.

TGF-β is secreted in large amounts in MALT and by mucosal epithelium and is known to be a potent IgA switch factor for naïve B cells (Cazac and Roes, 2000, Coffman, Lebman *et al.*, 1989). IL-5 and IL-6 are recognized as terminal differentiation factors for PCs in humans (Strober, 1990, Fujihashi, McGhee *et al.*, 1991). Recent evidence has demonstrated that, in combination with IL-21, TGF-β can induce expression of CCR10 on naïve blood B cells activated in an *in vitro* culture system (Dullaers, Li, 2009). We sought to extend Dullaers' findings to determine whether cytokines commonly produced by T cells in GC can provide a mechanism through which an MBC could be induced to express a mucosal homing phenotype in the absence of antigen-specific activation. Thus, in order to mimic circumstances of polyclonal activation of circulating memory B cells, we investigated the ability of IL-21 to act with TGF-β or IL-5 in our *in vitro* culture system to induce CCR10 expression on circulating MBC. A model of this proposed phenomenon can be seen in Figure 4.1. We also examined whether IgA and IgG

MBC respond differently to such stimulation to examine whether CCR10 expression is imprinted at primary activation, or if it can be gained by any MBC during recall immune responses.

## 4.2 Materials and Methods

### *4.2.1 Specimen Preparation and B cell Isolation*

Blood samples from normal, healthy adults 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 ACD (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 minutes, layered over Ficoll-Paque Plus (VWR International, Canada), and centrifuged for 20 minutes at 1800 x *g*. The resulting B cells were resuspended 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.

### *4.2.2 Immunophenotyping*

The blood B cell preparations were surface stained using various mouse anti-human monoclonal antibodies. The following antibodies were used: anti-CD19-PE-Cy7, anti-CD27-PE, anti-IgG-APC, anti-IgG-FITC, anti-IgA-FITC, anti-CD38-APC, anti-CD138-PerCP-Cy5.5, and anti-CCR10-APC. All antibodies were purchased from BD

Biosciences (Oakville, Canada) except anti-IgA-FITC (DakoCytomation, Fort Collins, USA), and anti-CCR10-APC (R&D Systems, Minneapolis, USA). Specific phenotyping procedures used 4, or 5 colour analysis to determine various B cell subsets. Data was collected on a Becton Dickinson LSR II cytometer using FACSDiva software (BD Biosciences, Oakville, Canada) and analysed using FlowJo software (Treestar Inc.).

*4.2.3 FACS*

Memory B cell populations were purified from total B cell preparations by staining against CD19, CD27, CD38, IgA or IgG, using antibodies described above, and then isolation by FACS. Sorting was carried out on a high speed digital FACSVantage sorter (BD Biosciences, Oakville, Canada), with simultaneous two stream sorting of IgA and IgG MBC subsets. Typical yields were >95% pure.

*4.2.4 Cell Culture*

FACS-sorted Total, $sIgA^+$ or $sIgG^+$ MBC were suspended in RPMI containing 10% FCS, 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin prior to activation cultures. As previously described(32), MBC were cultured in triplicate for 6 days at a density of $2 \times 10^4$ cells/well in a final volume of 200 μL in the presence of mitomycin-C-treated L/CDw32 cells (ATCC), polyclonal B cell activators anti-CD40 mAb (mouse IgG1, clone G28.5) and recombinant human IL-2 and IL-10 . Some cultures were additionally stimulated with recombinant IL-21 alone or in combination with recombinant IL-5 or recombinant TGF-β. All cytokines were obtained from R&D

Systems, Minneapolis, USA. Post-culture viable cells were collected and analysed flow cytometry.

### 4.2.5 *Statistical Analysis*

For all analyses listed, data is presented as mean ± SEM. One-way ANOVA with Tukey's post-hot was used to analyze the difference between groups. All analyses were performed using GraphPad Prism 5.0 Software (GraphPad Software, Inc.)

## 4.3 Results

### *4.3.1 In vitro stimulation of PB MBC induces multiple phenotypes*

Phenotypic analysis of MBC was performed at 6 days post-culture to determine the ability of our *in vitro* system to generate a blood PPC-like phenotype from FACS-sorted blood MBC. At day 6, following stimulation of blood MBC with anti-CD40 in the presence of IL-2 and IL-10 (base conditions) two major populations of PPC were evident (Figure 4.2, panel A). The majority of cells were $CD19^{+}/CD27^{hi}$ ($CD27^{hi}$) (Table 4.1), and these cells were also $CD38^{hi}$ (data not shown), consistent with the blood PPC phenotype observed in our lab. A second smaller population of cells was observed that were $CD19^{+}/CD27^{int}/CD38^{int}$ ($CD27^{int}$) (Figure 4.2, panel A), consistent with the phenotype of blood MBC. Analysis of light-scatter by these two populations revealed that the $CD27^{hi}$ cells were larger, consistent with a blast phenotype, while the $CD27^{int}$ population exhibited scattering properties consistent with the resting MBC population. We have previously demonstrated in our lab that these PPC-like, CD27int cells do secrete antibody, as measured by ELISPOT and ELISA (Fernandes and Snider, 2010).

Thus, these findings confirm that stimulation with our base culture conditions generate antibody-secreting cells with a phenotype similar to that of blood PPC and non-secreting cells phenotypically resembling blood MBC.

### *4.3.2 Addition of IL-21 induces a third population of cells following in vitro stimulation of blood MBC*

IL-21 has been identified as a potent stimulator of B cell proliferation and differentiation in several *in vitro* systems (Bryant, Ma *et al.*, 2007, Kuchen, Robbins *et al.*, 2007, Ettinger, Sims *et al.*, 2007) and has been identified as a major secreted product of $T_{fh}$ cells (Bryant, Ma, 2007). Thus, we examined the effect of IL-21 on the output of our culture system.

Addition of IL-21 resulted in several changes to post-culture population frequencies. A significant ($p<0.05$, $n=5$) increase in the frequency of $CD27^{hi}$ cells and decrease ($p<0.05$, $n=5$) in the $CD27^{int}$ population was observed relative to base conditions (Table 4.1). We also observed the appearance of a third population which was $CD19^{lo}/CD27^{int}$ ($CD19^{lo}$) (Figure 4.2, panel B) that exhibited light-scatter properties similar to that of the $CD27^{hi}$ population (data not shown).

### *4.3.3 Stimulation with IL-21 alone or with IL-5 or TGF-β induces different phenotypes from blood MBC*

IL-21 is abundantly expressed by $T_{fh}$ in lymphoid follicles and has been shown to synergize with TGF-β to induce CCR10 expression on *in vitro*-stimulated naïve B cells (Dullaers, Li, 2009). IL-5 has been identified as a terminal differentiation factor for human plasma cells (Strober, 1990) and is also produced by helper T cells in GC reactions (Johansson-Lindbom, Ingvarsson *et al.*, 2003). Thus, we examined the effect of

combining IL-21 with IL-5 or TGF-β to identify their effects on MBC differentiation *in vitro*.

Addition of IL-5 had a significant effect on the frequencies of $CD19^{lo}$ and $CD27^{hi}$, but not $CD27^{int}$ subsets on day 6, relative to cells stimulated with IL-21. The $CD19^{lo}$ subset was significantly increased relative to base conditions or addition of IL-21 alone (Table 4.1, $p<0.05$, $n=5$), and the $CD27^{hi}$ subset was significantly decreased relative to cultures where IL-21 was added alone. (Table 4.1, $p<0.05$, $n=5$). Relative to base culture conditions, IL-5 did not significantly affect the frequency of the $CD27^{hi}$ subset, but the $CD19^{lo}$ and $CD27^{int}$ subsets were significantly increased and decreased, respectively (Table 4.1).

Addition of TGF-β also had a significant effect on post-culture frequencies (Table 4.1). Stimulation with IL-21 alone or IL-21/IL-5 generated fewer cells that were $CD27^{int}$ when compared to TGF-β stimulated cells. In addition, $CD27^{hi}$ cell generation was significantly reduced relative to all other culture conditions. Finally, addition of TGF-β yielded the greatest increase in frequency of the $CD19^{lo}$ subset (Table 4.1). Thus, IL-21, IL-5 and TGF-β exhibited marked differences in their abilities to generate activated B cell subsets from FACS-sorted MBC that are analogous to those observed in peripheral blood.

Population frequencies resulting from different culture conditions are summarized in Table 4.1 and Figure 4.3. These results demonstrate that polyclonal activation in our culture system gives rise to populations phenotypically similar to MBC and PPC in

blood. Our results also indicate that a third population of $CD19^{lo}$ cells is generated that displays phenotypic characteristics common to both blood MBC and PPC.

*4.3.4 IgA and IgG MBC respond similarly to IL-21, IL-5, and TGF-β*

Of the three populations generated by our culture system, two have *in vivo* analogs (Avery, Ellyard, 2005). Since factors present during primary activation are responsible for processes such as Ig switch and differentiation commitment we examined the effects of these culture conditions on post-culture sIg expression by FACS-sorted IgA or IgG MBC to determine if they would respond differently under these conditions.

Addition of IL-21 alone or in combination with IL-5 had no effect on sIg expression by $CD27^{int}$ cells following stimulation (Figure 4.4, panels B/C/F/G). Addition of TGF-β to cultures resulted in significantly ($p<0.05$, $n=5$) higher expression of sIg than in any other culture conditions (Figure 4.4, panels D/H,).

sIg expression by the $CD27^{hi}$/PPC fraction was not altered by the addition of IL-21 or the combination of IL-21 and IL-5 (Figure 4.5, panels B/C/F/G). Nearly all of the $CD27^{hi}$ subset generated with the addition of TGF-β retained sIg expression (Figure 4.5, panels D/H). Examination of the $CD19^{lo}$ subset revealed that both IL-21/IL-5- and IL-21/TGF-β-treated cultures had significantly increased sIg expression relative to cultures treated with IL-21 alone, (Figure 4.6, panels B/C/E/F, $p<0.05$, $n=5$).

Our findings indicate that although stimulation under different conditions generates a heterogeneous population, IgA and IgG MBC respond similarly under these conditions.

*4.3.5 Induction of CCR10 on IgA and IgG MBC following in vitro stimulation*

The recent investigation in which naïve B cells were induced to express CCR10 demonstrated synergy between IL-21 and TGF-β (Dullaers, Li, 2009). We examined whether CCR10 expression can be induced on blood IgA or IgG MBC under similar activation conditions. We also investigated synergy between IL-21 and IL-5, a cytokine implicated in terminal plasma cell differentiation.

Both IgA and IgG $CD19^{lo}$ cells showed the greatest increase in CCR10 expression, with >90% positive for CCR10 expression across all culture conditions (Figures 4.7). $CD27^{hi}$ cells also expressed CCR10 at high frequencies (Figures 4.5 & 4.7). When examining post-culture $CD27^{int}$ cells, cultures stimulated with IL-21/TGF-β resulted in the highest frequency of CCR10 expression (Figures 4.7, $p<0.05$, $n=5$). Among both IgA and IgG MBC, the base culture conditions were the only instance in which CCR10 expression was not observed (Figure 4.7).

These data demonstrate that that blood IgA and IgG MBC express CCR10 following *in vitro* activation in the presence of common follicular cytokines. They also demonstrate that IL-21 and TGF-β act in concert to induce CCR10 on the majority of *in vitro* activated IgA and IgG MBC. This effect was observed on all cell types generated by our culture system.

Table 4.1: Phenotypes induced by *in vitro* stimulation of MBC

| | **%CD19$^{lo}$** | **%CD27$^{int}$** | **%CD27$^{hi}$** |
|---|---|---|---|
| Base conditions | 8.0 ± 1.2 | 31.1 ± 3.3 | 51.2 ± 3.2 |
| + IL-21 | 14.9 ± 1.8 | 10.1 ± 1.7 | 66.5 ± 2.9 |
| + IL-21 & IL-5 | 25.7 ± 1.9 | 13.3 ± 1.5 | 49.8 ± 3.5 |
| + IL-21 & TGF-β | 45.8 ± 2.4 | 37.2 ± 2.77 | 6.3 ± 1.6 |

Data are mean frequency ± SEM, n = 8

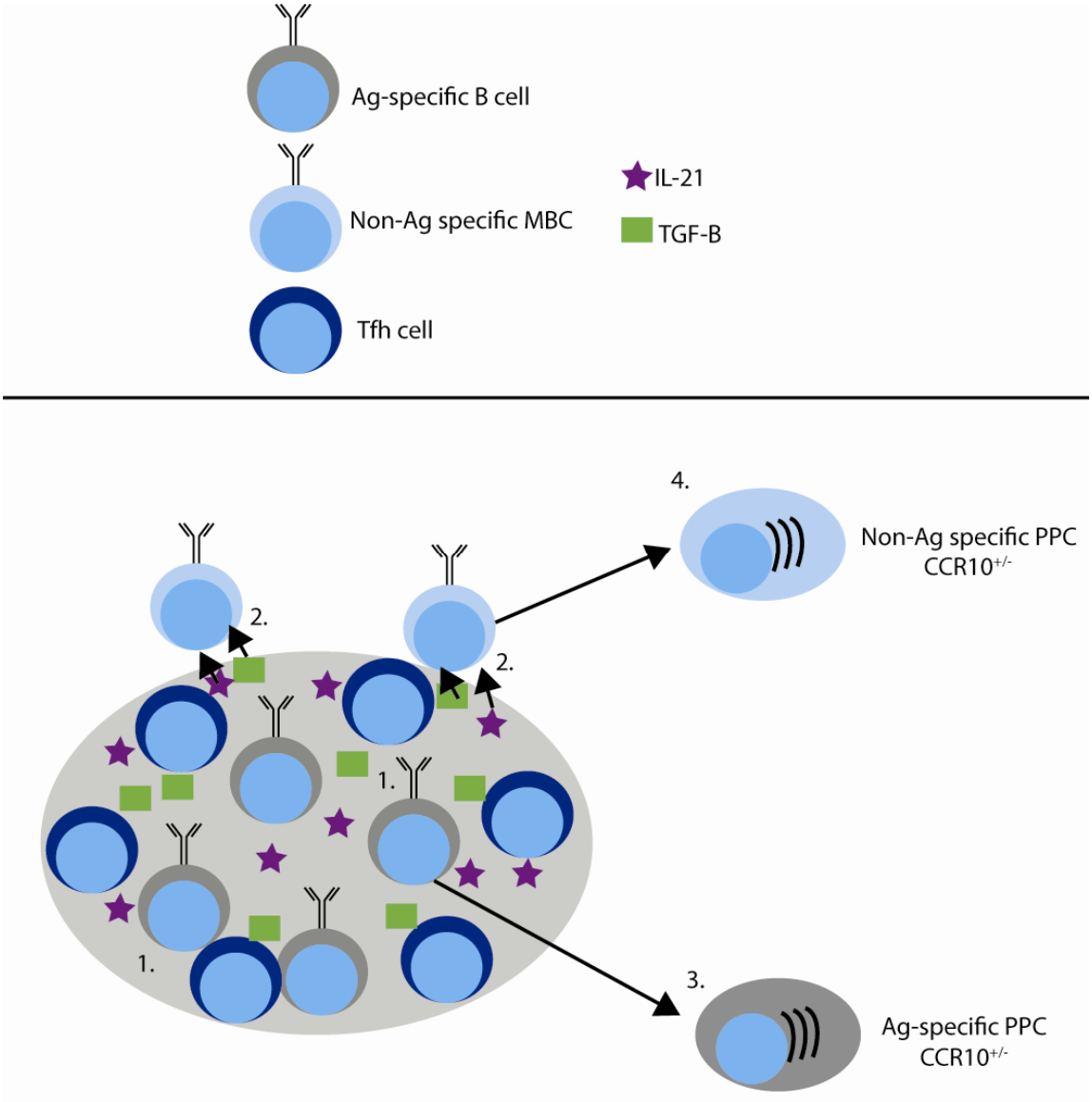


**Figure 4.1 Proposed model for non-specific induction of CCR10 on circulating MBC**. During a typical immune response, antigen-specific B cells are activated by antigen-presenting cells and $T_{fh}$ cells in germinal centers (1). Non-antigen specific MBC located in the same inductive tissues may be nonspecifically activated by the GC microenvironment (2). The GC reaction thus may give rise to two populations of activated B cells; antigen-specific PPC activated in the GC(3), and a non-antigen specific PPC activated nonspecifically (4), some of which may express CCR10.

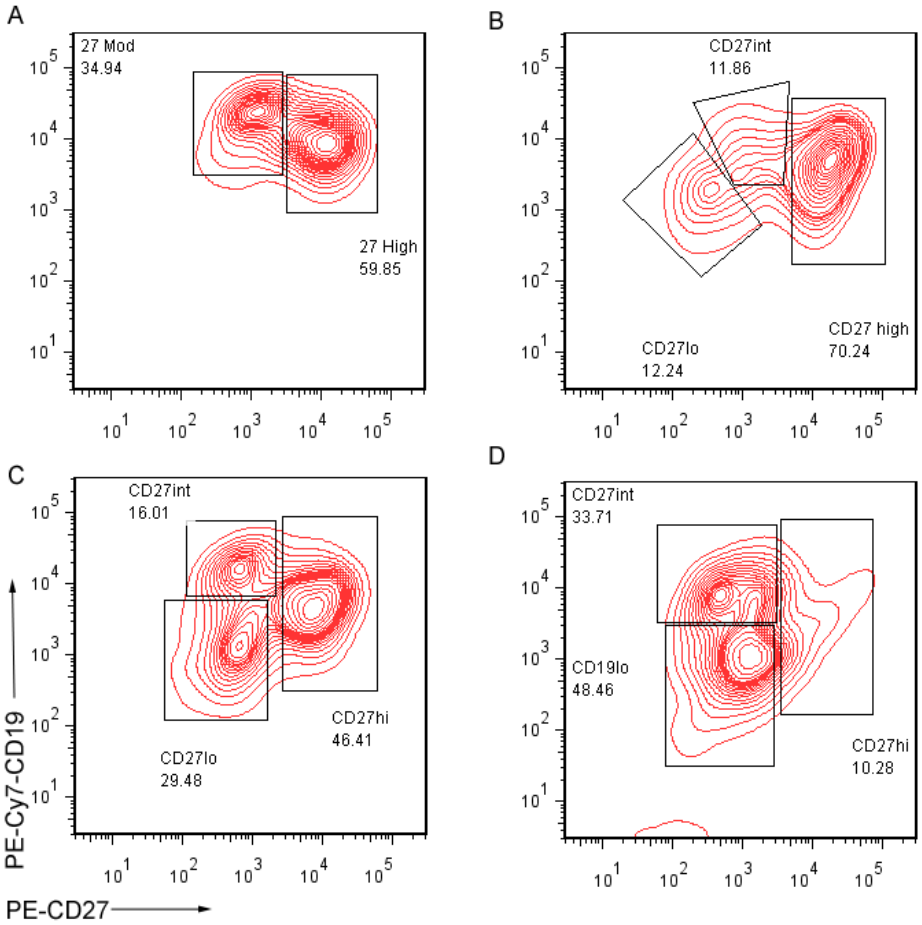


**Figure 4.2 Typical phenotypes generated from *in vitro* MBC activation**. FACS-purified MBC were cultured in the presence of L/CDw32 cells with anti-CD40, IL-2 and IL-10 alone (A), or with the additions of IL-21 (B), IL-21 and IL-5 (C), or IL-21 and TGF-β (D) and stained for B cell markers on day 6 post-culture. Analysis revealed the presence of 2 major populations under base conditions. Addition of IL-21, with or without other cytokines, resulted in the appearance of a third subset (B-D). Results are representative of 6 samples.

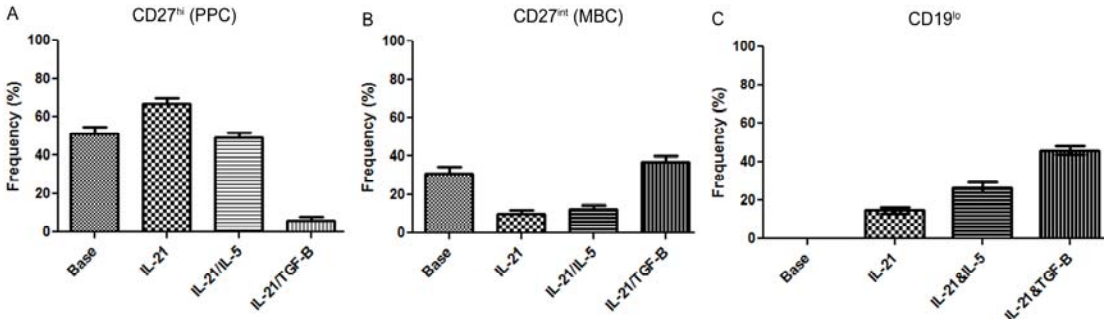


**Figure 4.3 *In vitro* activation of MBC with follicular cytokines results in multiple post-culture phenotypes.** FACS-sorted MBC were activated through CD40 ligation with IL-2 and IL-10 alone (Base) or in combination with IL-21, IL-21 and IL-5, or IL-21 and TGF-β for 6 days. Flow cytometric analysis was performed to compare the effects of these cytokines on MBC differentiation into $CD27^{hi}$ (PPC, panel A), $CD27^{int}$ (MBC, panel B), and $CD19^{lo}$ (panel C) phenotypes. Bars represent mean ± SEM, n = 5.

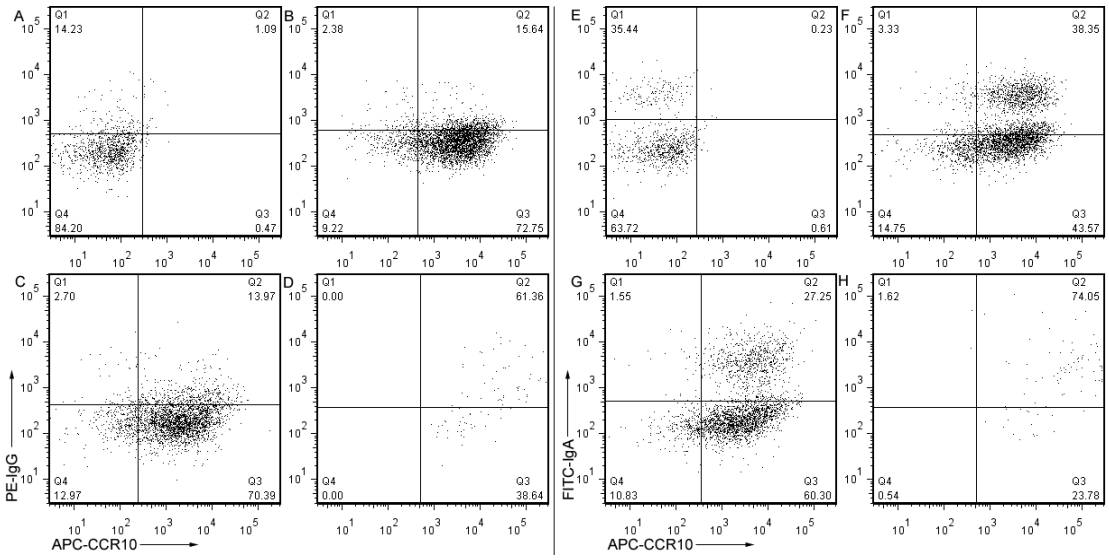


**Figure 4.4 – IL-21 induces CCR10 expression on *in vitro*-generated IgG and IgA MBC.** CD27$^{int}$ cells were generated by activation of FACS-sorted IgG (left panels) and IgA (right panels) MBC with anti-CD40, IL-2 and IL-10 (A/E) alone or in combination with IL-21 (B/F), IL-21 and IL-5 (C/G), or IL-21 and TGF-β (D/H) for 6 days. sIg and CCR10 expression was determined by flow cytometry. Negative cutoffs were determined using fluorescence-minus-one controls. Images shown are representative of 5 independent experiments.

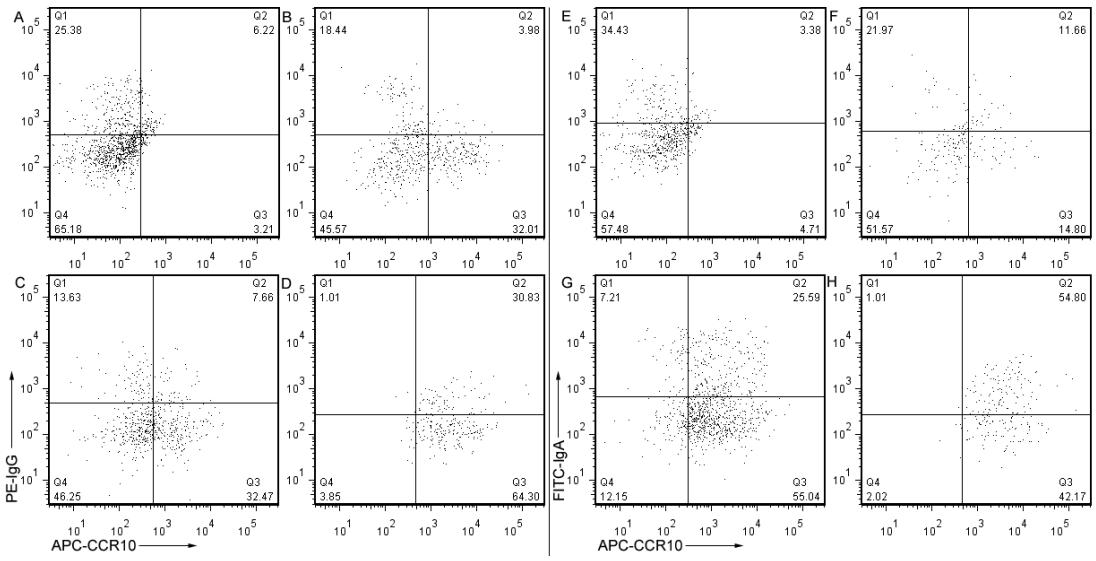


**Figure 4.5 – IL-21 induces CCR10 expression on *in vitro*-generated IgG and IgA PPC.** CD27$^{hi}$ cells were generated by activation of FACS-sorted IgG (left panels) and IgA (right panels) MBC with anti-CD40, IL-2 and IL-10 (A/E) alone or in combination with IL-21 (B/F), IL-21 and IL-5 (C/G), or IL-21 and TGF-β (D/H) for 6 days. sIg and CCR10 expression was determined by flow cytometry. Negative cutoffs were determined using fluorescence-minus-one controls. Images shown are representative of 5 independent experiments.

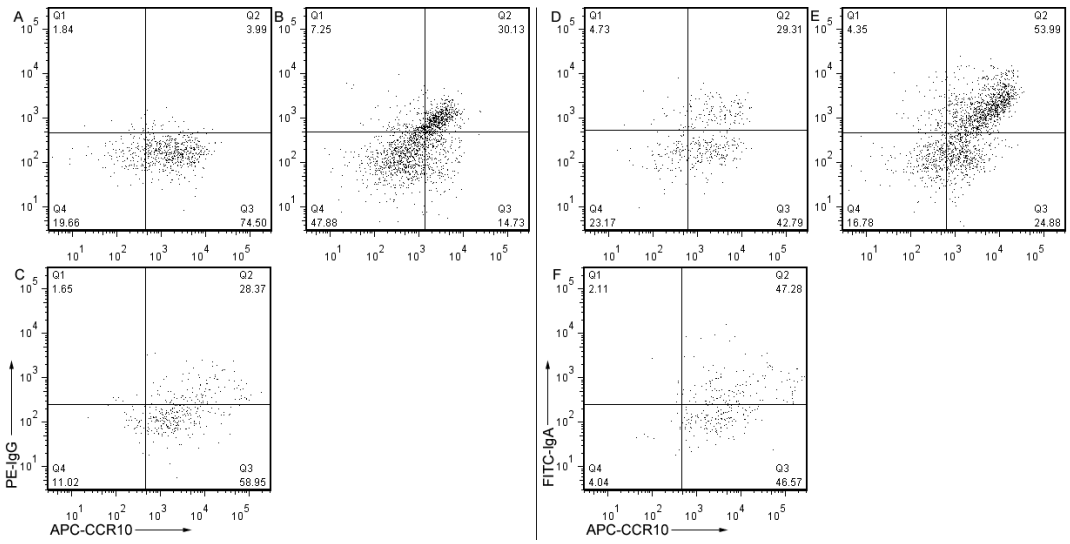


**Figure 4.6 – IL-21 induces CCR10 expression on *in vitro*-generated IgG and IgA CD19$^{lo}$ cells.** CD19$^{lo}$/CD27$^{int}$ cells were generated by activation of FACS-sorted IgG (left panels) and IgA (right panels) MBC with anti-CD40, IL-2, and IL-10 in combination with IL-21 (A/D), IL-21 and IL-5 (B/E), or IL-21 and TGF-β (C/F) for 6 days. sIg and CCR10 expression was determined by flow cytometry. Negative cutoffs were determined using fluorescence-minus-one controls. Images shown are representative of 5 independent experiments.

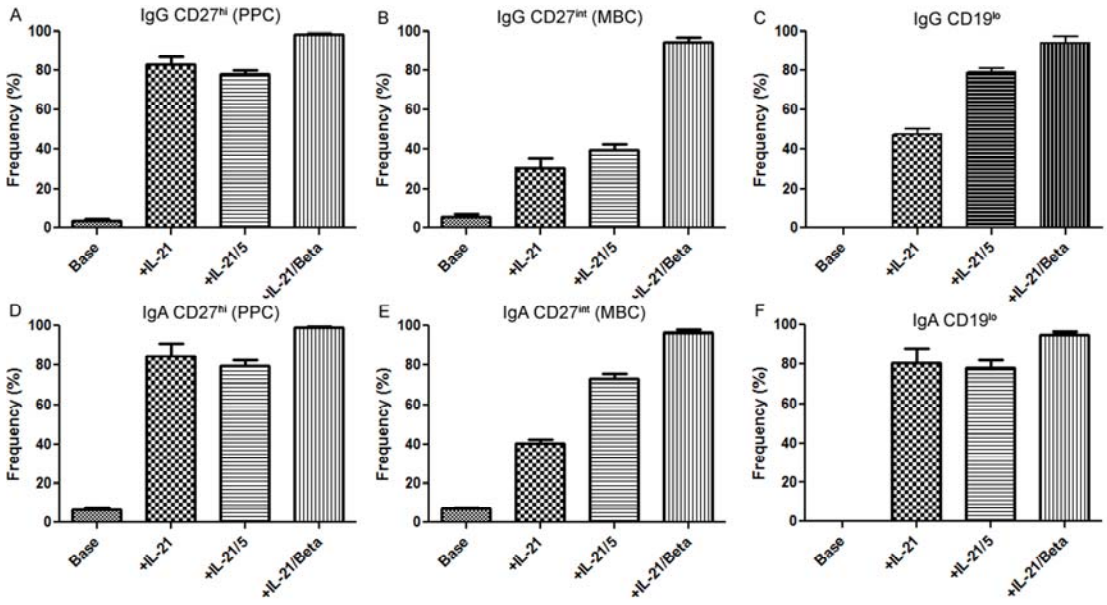


**Figure 4.7 – IL-21 is an inducer of CCR10 expression on *in vitro* activated IgA and IgG MBC.** FACS-sorted IgG (top row) and IgA (bottom row) MBC were activated with anti-CD40 and with IL-2 and IL-10 (base) alone or in combination with IL-21, IL-21 and IL-5, or IL-21 and TGF-β for 6 days as indicated. CCR10 expression was determined on indicated subsets by flow cytometry. Bars represent mean ± SEM, n = 5.

## 4.4 Discussion

Our results demonstrate that a variety of cytokines have the capacity to upregulate CCR10 on IgA and IgG MBC *in vitro*. We also report that addition of IL-21, alone or in combination with IL-5 or TGF-β, causes MBC to differentiate into populations with distinct phenotypic profiles. Surprisingly, we have identified conditions under which a majority of IgG-switched B cells can be induced to express CCR10, a phenotype not observed in B cells analysed e*x vivo*.

Addition of IL-21 to our base culture protocol increased the frequency of $CD27^{hi}$ cells generated in culture, and we suggest based on their phenotype and antibody secretion that these cells are representative of blood PPCs. IL-21 addition also resulted in the appearance of a subset of cells not induced by base conditions or observable in blood. This subset expresses low levels of CD19 and intermediate levels of CD27 and may represent a step in differentiation towards a plasma cell phenotype, although evidence of a $CD19^{lo}$ PC has not yet been reported. This interpretation is supported by the observation that adding both IL-5, a terminal B cell differentiation factor, significantly increased the frequency of the $CD19^{lo}$ subset. In the absence of more definitive indicators such as CD138 expression or HLA-DR downregulation it is not possible to confirm this hypothesis. Although we were not able to observe CD138 expression on post-culture cells, others have demonstrated that it is possible to generate CD138-expressing PC from a similar culture method (Borte, Pan-Hammarstrom *et al.*, 2009).

The majority of cells with the $CD19^{lo}$ phenotype expressed surface immunoglobulins in contrast with the mature PC phenotype. It is possible that sIg reduction is a late-stage event in the *in vitro* differentiation of PC and this might explain why more cells differentiated into the $CD19^{lo}$ phenotype when stimulated with IL-5, a terminal differentiation factor, but continued to express Ig on the cell surface. In fact, addition of IL-21 alone or in combination with IL-5 to cultures resulted in significantly higher surface sIgA than sIgG in all three populations generated (Figures 4.4 & 4.5). Post-culture FACS combined with other markers of PC phenotype such as Blimp-1 expression might provide an alternative to CD138 expression as a marker of terminal differentiation and permit further identification and characterization of these cells.

The functional capability and *in vivo* significance of the *in vitro* generated $CCR10^{+}$ cells is currently unknown. It is important to recognize that we did not assess whether $CCR10^{+}$ expressing cells generated by our culture system are capable of responding to the CCR10 ligand MEC.

Based on our own observations of blood MBC and PPC, and on previous B-cell analyses already published, we did not expect to see equivalent CCR10 expression on IgG and IgA MBC. This expression of CCR10 on IgG MBC is of particular interest, as this phenotype has not been previously described. While $IgA^{+}CCR10^{+}$ PPC/PC are commonly observed both in blood and at mucosal sites, the observation that CCR10 was so readily induced on IgG MBC under similar conditions suggests that the ability to express CCR10 is not unique to IgA-switched B cells. In fact, $IgM^{+}CCR10^{+}$ PC are

observed in the gut in humans, and the presence of IgG PC in the female reproductive tract, another site of MEC production (Brandtzaeg and Johansen, 2005, Fagarasan and Honjo, 2003, Kunkel, Kim *et al.*, 2003), suggests a possible *in vivo* correlate for this phenomenon. Although most cervical IgG is believed to have originated in the serum, CCR10 expression by IgG cells suggest it may be possible that small numbers of tissue-resident IgG-secreting PC are present. Thus, our results might indicate a mechanism by which IgG PCs gain access to the mucosal tissues.

The detection of CCR10-expressing IgG B cells may have relevance in the study of normal health as well as antibody-mediated autoimmune diseases. A recent study suggests that autoreactive IgG produced by gut-resident PC may have a directly pathogenic role in ulcerative colitis (Ebert, Geng *et al.*, 2009, Ebert, Geng *et al.*, 2006), and others have suggested that IgG may be an overlooked mediator of pathogenesis in inflammatory bowel disease in general (Brandtzaeg, Carlsen *et al.*, 2006). While these studies did not examine CCR10 expression, the presence of IgG PC in the gut demonstrates their ability to traffic to the mucosa, and this finding may provide insight into how they gain entry to mucosal sites. Without further study, however, it is impossible to reconcile these observations with the lack of *in vivo* and *ex vivo* evidence of $IgG^+$ $CCR10^+$ PPC.

The high frequency of CCR10 expression on these subsets in the presence different culture conditions may explain detection of circulating B cell populations where CCR10 expression is seen on the majority of PPC in blood (Fernandes and Snider, 2010).

As we tested cytokines produced in a variety of GC reactions, we propose that there are multiple mechanisms by which GC reactions can yield mucosal-homing B cell blasts. The variety of phenotypes emerging from different culture conditions may represent a mechanism by which a single MBC response can yield PPC or PC with multiple homing capabilities. As we and others have demonstrated the phenomenon of bystander activation of memory B cells, the results described here provide a possible explanation of how a peripheral immune response might lead to dissemination of antibody-secreting cells to distal mucosal sites. Additionally, the high frequency of $T_{fh}$-produced IL-21 may explain how mucosal inductive sites in one part of the the CMIS can seed distal effector sites (i.e. NALT activation leading to PPC/PC seeding of the female reproductive tract).

As this work is incomplete, there are several concerns and areas of interest that might be focused on in the future. As described above, the data presented above are phenotypic only. It would be of great interest to determine whether the different subsets show functions (i.e. antibody secretion, functional chemotaxis towards CCL28/MEC) compared to their *in vivo* correlates. While it may be difficult to attain mucosal PC from tissues, $CCR10^+$ PPC can be readily isolated from blood and used as an *ex vivo* correlate to compare functions.

The use of a co-culture system designed to mimic the cross-linking of CD40 in T-B cell interactions complicates these observations as the fibroblast cell line used may secrete factors such as TSLP, TGF-β, or vitamin D3 that might also influence these results, however the presence of a cell presenting activating antibody permits cross-

linking of CD40 on the surface of BC analogous to that in a GC reaction. There is currently no evidence regarding production of these molecules by the L/CDw32 cell line. As IL-21 was capable of inducing CCR10 expression without the addition of TGF-B or IL-5 in our system, future studies in which co-cultures are used should determine whether these cells secrete such factors constitutively or in response to IL-21 in order to determine whether they play a role in CCR10 induction. As we are attempting to loosely mimic a GC reaction, it would be interesting to observe whether or not expression of CXCR5, a chemokine receptor that directs homing and migration towards GC reactions, is different among the generated subsets as was previously reported for naïve B cells (Dullaers, Li, 2009). Similar experiments with activated GC $T_{fh}$ cells or their conditioned media might be more appropriate to investigate this phenomenon.

There are many other candidate cytokines produced by T cells participating in GC reactions that have direct effects on B cell differentiation, such as IL-4 and IL-6. Although we did not test IL-5 addition without IL-21, it might also be informative to observe its effect in the absence of IL-21. However, due to the large amount of IL-21 generated in GC reactions, and previous observations that IL-21 was required for TGF-β-mediated expression of CCR10 on naïve cells (Dullaers, Li, 2009), we were interested in the effects of IL-21 in combination with other cytokines. In addition, determining dose-dependency of each candidate cytokine might shed light on which factors are more/less efficient at inducing CCR10 expression.

In one respect, these results are at odds with our labs' previous investigations of blood PPC, where we demonstrated that in the steady state all $CCR10^+$ PPC in blood secrete IgA, not IgG, and were never $sIgG^+$. However, those analyses were performed in normal, healthy humans, thus it is not surprising as baseline induction of PPC trafficking into blood would presumably be taking place at mucosal tissues that would themselves be polarized to generate $CCR10^+IgA^+$ PPC.

We have identified conditions under which IgA and IgG MBC express CCR10. Our data reveals that IL-21 and TGF-β induce CCR10 expression on nearly all *in vitro* activated MBC more frequently than IL-21 alone, or combination with IL-5. Expression of CCR10 by IgG MBC is a novel finding and deserves further investigation particularly in the context of IgG PPC/PC trafficking to mucosal sites in both health and disease. Although these are preliminary observations, we suggest that GC reactions might be capable of inducing $CCR10^+$ PPC, whether they occur in MALT or peripheral lymphoid tissues.

# CHAPTER 5

## General Discussion

## *5.1 Preamble*

The work presented here describes correlates of mucosal immunity present in human blood. In particular, this work pertains to and extends the investigations of mucosal and circulating B cell subsets described by Butcher, Tangye, and Dullaers with respect to CCR10-expressing antibody secreting cells and memory B cells, and may have additional relevance to a number of mucosal vaccination strategies. We provide evidence that CCR10 marks the majority of pIgA-secreting PPC in blood and is a more appropriate marker of mucosal function on blood PPC than surface IgA or $\alpha_4\beta_7$ expression. Contrary to a recent investigation we report that a systemic immune response is associated with an increase in IgA-PPC in blood, some of which are relevant to mucosal rather than systemic protection. Our results also demonstrate that the phenotype of active mucosal PPC in blood can be induced *in vitro* by a variety of combinations of cytokines and that expression of CCR10 is highly inducible on IgA and, surprisingly, IgG MBC. The contents of this chapter will primarily discuss the relevance of these results on previous reports on correlates of mucosal immunity in blood, assessment of mucosal immune precursors and vaccinations, correlates of phenotypes generated in our *in vitro* assay and their possible relevance in immunity and autoimmunity, and linkage of the systemic and mucosal immune compartments.

### *5.2 Correlates of mucosal immunity in blood*

Our analysis of circulating PPC is assigns mucosal relevance to a subset of blood B cells in healthy humans based on functions specific to mucosal defense. Using a combination of flow cytometry, ELISPOT, size exclusion chromatography, and ELISA we have identified a population of pIgA-secreting cells found in normal human blood that express CCR10 and shown that $sIgA^+$ PPC negative for CCR10 expression predominantly produce mIgA (Chapter 2). Assessment of immunoglobulins in mucosal secretions yields highly variable results depending on the sampling method employed (Mestecky, 2005), thus a blood B cell subset that is easy to isolate and is amenable to *in vitro* study provides a reproducible means of identifying active immune responses at mucosal sites. Our confirmation that this subset predominantly secretes pIgA is further evidence that when assessing blood B cells the $CCR10^+$ PPC population in blood is the most relevant correlate of recent mucosal immune activity.

Intracellular staining prevents assessment of function provides insufficient evidence about mucosal relevance, as functional Ig secretion or secretion of polymeric antibody cannot be confirmed. Although other groups have used high intracellular immunoglobulin expression as a marker of PPC/PC differentiation (Mei *et. al*, 2009), earlier studies demonstrating that IgA2-switched PPC might not secrete *in vitro* indicate that identifying an intracellular-$Ig^{high}$ or surface $Ig^+$ PPC does not discern between actual secreting and non-secreting cells (Conley *et. al*, 1984, Conley *et. al*, 1982). A marker (CCR10) which identifies all polymer-secreting cells in blood provides a means of

separating this population from other PPC and permits downstream functional analysis. This approach generated data that suggest that this population can be used to investigate recent immune responses at mucosal surfaces through the use of antigen- or isotype-specfic ELISPOT assays. In humans most IgA-PC in mucosal tissues is of the IgA1 isotype except in the large intestine and female reproductive tract, leading to the hypothesis that the rectal lymphoid tissues may function as inductive sites for the female reproductive mucosa. Analysis of the CCR10 population could thus be refined to examine differences in isotype expression (i.e. IgA1 vs. IgA2) which might provide insight into the inductive sites contributing to an immune response, or to identify the frequency of secretors vs. non-secretors in responding B cells relative to induction site or secretion of pIgA vs. mIgA.

Jaimes *et al*. demonstrated loss of CCR10 on antigen-specific B cell populations following resolution of an acute rotavirus infection, despite the persistence of antigen-specific IgA MBC in blood (Jaimes *et. al*, 2004). The loss of CCR10 expression concurrent with resolution of the acute infection demonstrates the utility of this marker in providing a "snapshot" of active mucosal immune responses through blood lymphocytes, thus the persistent fraction of $CCR10^{+}$ PPC we have identified in blood is consistent with the high antigenic burden of mucosal surfaces. Furthermore, these observations suggest that this subset could be probed to identify specific responses, given the appropriate antigens for use in *in vitro* analysis.

### *5.3 Vaccine studies*

Efforts to create effective mucosal vaccines have been hindered by difficulties in inducing long-lasting immunity at mucosal surfaces. The appearance of antigen-specific, $CCR10^+$ PPC and MBC in blood following acute infection and lasting through convalescence demonstrates that mucosal infection can generate a local immune response that can be measured in blood (Jaimes *et. al*, 2004).

The work by Dullaers *et al*. demonstrates that naïve B cells can switch to IgA and express CCR10 following co-culture with follicular helper-T cells ($T_{FH}$) in an IL-21 and TGF-β dependent mechanism, and they argue that mucosal vaccination strategies should aim to generate immune responses that are similarly capable of inducing mucosal B cells *in vivo* (Dullaers *et. al*, 2009). Our data demonstrate that CCR10 can be induced on both IgA and IgG MBC activated *in vitro* and thus memory responses might be similarly influenced by vaccination or boosting strategies. As described above, CCR10 expression on an IgG-switched B cell is a novel observation and could have implications for both vaccine research and autoimmunity (see section 5.4).

The $CCR10^+$ PPC subset can also serve as a target for immunophenotyping to identify different combinations of homing and adhesion molecules. Several groups have shown that route of antigen exposure contributes significantly to the migratory patterns of responding cells (Quiding-Jarbrink *et. al*, 1995, Quiding-Jarbrink *et. al*, 1995, Quiding-Jarbrink *et. al*, 1997), thus this approach would permit comparison of multiple

immunization routes by using a comparatively less invasive sampling procedure. Rather than assaying all B cells in which mucosal precursors homing to mucosal tissues would be a relative minority, the CCR10 subset would be enriched for these cells and thus would facilitate analysis of other mucosal homing molecules. If combined with a measure of specificity such as a flurochrome-conjugated antigen, this would enable simultaneous cytometric screening of PPC specific for pathogens or vaccine antigens as well as homing molecules. Mucosal homing pairs have been proposed for some tissues, such as $CCR10^{+}/\alpha_4\beta_7^{+}$ targeting cells to the gut and $\alpha_4\beta_1$ directing migration into respiratory tissues (Bentley *et. al*, 1993, Farstad *et. al*, 1997, Farstad *et. al*, 1997, Kantele *et. al*, 1997, Kunkel *et. al*, 2003). Since local immunity is considered the primary goal for many mucosal vaccination strategies a greater understanding of the relationship between application site and the generation of mucosal effectors is essential to developing effective vaccines.

### *5.4 Autoimmunity*

Evidence has emerged to suggest that autoimmune pathologies at mucosal surfaces such as inflammatory bowel disease (IBD) may have extensive local humoral involvement.

In such situations $IgA^{-}/CCR10^{+}$ PPC might also bear investigation, such as the inflammatory phenotype associated with the IBD pathologies Crohn's disease (CD) and ulcerative colitis (UC). There is currently no data available demonstrating CCR10 expression on IgG-PPC in human blood, although this would not necessarily be expected

in a normal, healthy individual. Autoreactive IgG from sera and mucosa of Crohn's disease patients have been shown to mediate killing of colonocytes *in vitro* via a complement-dependant pathway, colonic biopsies from both UC and CD patients stain positive for autoreactive IgG CD patients stain positive for complement proteins (Das *et. al*, 2008, Ebert *et. al*, 2009). We have shown *in vitro* that there are multiple mechanisms capable of inducing CCR10 on IgG PPC. Thus, the involvement of IgG might be mediated by inappropriate expression of CCR10 on IgG cells either as a precursor or a downstream effect of the pathogenic changes in IBD. The inflammatory nature of these diseases, combined with documented complement deposition and autoreactive IgG antibodies suggests that IgG PPC are involved in IBD pathology and may provide therapeutic targets for treatment.

Identification of a population of circulating cells that can inform on such autoimmune responses would provide a means of identifying the root cause, given candidate antigens. Our evidence demonstrating that systemic immune responses are capable of affecting mucosal responses suggest that the converse may also occur. Careful examination of PPC in blood following recurrence of an autoimmune disorder might help clarify whether there is a concurrent immune response associated with autoimmune pathology.

## *5.5 Future Directions*

As described above, the literature primarily reports on phenotypes, particularly in recent years. Our results demonstrate that the most reliable indicator of mucosal origin and relevance on circulating B cells is the expression of CCR10. Further investigations of the bystander effect in blood or the efficacy of various vaccinations will benefit from the presence of an easily obtained population of cells that is amenable to phenotypic and functional analysis. Further analysis of CCR10-expressing PPC in blood might help further understand how cellular trafficking to various mucosal surfaces is accomplished. Finally, our preliminary results demonstrating CCR10 expression on IgG PPC are compelling as they demonstrate how a PPC expressing what is classically considered a non-mucosal immunoglobulin isotype might gain access to mucosal tissues, a finding that may have implications in vaccine design and studying autoimmune diseases.

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