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Characterization of Human IgA-Inducing Protein

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Characterization of Human IgA-Inducing Protein

by

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Dedication

This work is dedicated to my wife Janice, my daughter Jessica and my son Iain. You have inspired me to dare new heights, and given me the strength to attain them.

Characterization of Human IgA-Inducing Protein

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Over the last several years there has been a great deal of progress in characterizing the role of dendritic cells (DCs) in the activation and modulation of B cells. DC-secreted chemokines can induce B cell trafficking to the lymph nodes. DC-produced survival factors such as BAFF and APRIL have been shown to be essential for B cell maturation, but have also been implicated in class-switch recombination and B cell lymphoma survival. Recently added to this list of DC-derived factors effecting B cells is IgA-inducing protein (IGIP). Here we characterize production of IGIP by human DCs, and examine its capacity to induce IgA class switching and differentiation of naïve B cells *in vitro*. Monocyte derived DCs were cultured *in vitro* with TLR agonists (3,4,5, and 9), other factors including CD40L, GM-CSF, and IL-4, and the neuropeptide vasoactive intestinal peptide (VIP). Under *in vitro* stimulation with VIP and CD40L, IGIP mRNA expression was up-regulated as much as thirty five-fold above non-stimulated samples within 12-48 hours. Naïve B cells cultured with exogenous rhIGIP produced IgA in significantly greater quantities than non-stimulated controls, and I demonstrated that IGIP stimulation drives the production of μ-α switch circles from IgM^+/IgD^+ naïve human B cells, indicating its role as an IgA switch factor.

Additionally, the capacity of IGIP to elicit a mucosal IgA response was evaluated as part of a vaccine preparation, using a putative HIV-1 vaccine in a SCID-hu mouse model. SCID-hu mice were immunized with a dextran-based HIV-1 vaccine carrying gp120, with or without IGIP, and both serum and mucosal antibody responses were measured. While protection was sporadic, robust antibody responses were detected at both locations.

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Introduction

B CELLS

General Characteristics

B cells and their secreted antibodies comprise the humoral arm of the adaptive immune system, and protect extracellular spaces against infectious agents. B cells are mono-nuclear leukocytes of lymphoid lineage, and are marked by the cell surface expression of CD19, CD20, CD21, IgM, and IgD, amongst other molecules. B cells detect extra-cellular antigens via the B cell receptor (BCR), a complex of surface molecules including IgM. Signaling through the BCR, combined with co-stimulatory signals from an activated T helper cell, activate the B cell and lead to eventual differentiation into memory B cells or antibody secreting plasma cells.

B cell development

B cell derivation and development occur in the bone marrow in humans and mice, where stromal cells provide necessary stimuli such as stem cell factor (SCF) and Interleukin (IL)-7. Here, hematopoietic stem cells give rise to lymphoid progenitors, a portion of which will become committed to the B cell lineage of lymphocyte development (Jacobsen, Kravitz et al. 1996).

The first stage in B cell development is the pro-B cell, or progenitor cell committed to the B cell lineage. This has been further divided into early pro-B cells and late pro-B cells. The early pro-B cell stage is marked by the expression of surface proteins, such as CD19, which are characteristic of the B cell lineage. It is further marked by the beginning stages of immunoglobulin (Ig) gene rearrangement. The diversity (D) region of the Ig heavy chain gene is joined to the joining (J) region during this stage of development (Allman, Li et al. 1999). The variable (V) region is then joined to the D region during the late pro-B cell stage,

completing VDJ recombination. This step is critical in that successful VDJ recombination is necessary for the survival of the pro-B cell into the next stage of development(Hardy, Carmack et al. 1991).

Following the pro-B cell stage is the pre-B cell stage. This is again divided into sub-stages; the large pre-B cell and the small pre-B cell. A successful VDJ recombination leads to intracellular expression of the μ heavy chain, signaling an end to VDJ recombination and the beginning of several rounds of cell division. The results of this cell division are several small pre-B cells. It is in this stage that VJ recombination of the immunoglobulin light chain takes place, yielding a finished IgM upon completion. Expression of a completed IgM on the cell surface then signals the transition to an immature B cell (Hardy, Carmack et al. 1991).

Subsequent to surface IgM expression, immature B cells undergo negative selection for reaction to self-antigen. B cells reacting with high affinity to self-antigen are deleted via apoptosis to prevent the rise of a self-reactive population and the possible onset of autoimmune disease. Those immature B cells surviving negative selection are then released to the peripheral lymphoid organs, such as the spleen and lymph nodes and Peyer's patches, where they undergo further differentiation, culminating in the surface expression of IgD as well as IgM. Surface expression of IgD signifies the completion of B cell differentiation, and a mature B cell (Allman, Lindsley et al. 2001).

B cell activation

Activation of naïve B cells requires two distinct signals. The primary signal is antigen binding and cross-linking of the B cell receptor (BCR), which is composed of surface bound immunoglobulin (Ig) and the Igα and Igβ invariant chains (Borst, Jacobs et al. 1996). These chains bear immunoreceptor tyrosine-based activation motifs (ITAMs) which initiate a series of signaling cascades (Cornall, Cheng et al. 2000), such as the MAP kinase cascades, protein kinase C and calcineurin (Ballard 2001; Yokozeki, Adler et al. 2003). These

signaling cascades ultimately result in the activation and nuclear translocation of transcription factors such as NF-κB, NFAT and AP-1 (Ballard 2001; Yokozeki, Adler et al. 2003), which in turn induce transcription of proteins involved in cell proliferation and differentiation .

This signaling can be augmented by a number of co-receptors, the most important of which are the B cell co-receptor complex and CD45 (Pao and Cambier 1997; Hegedus, Chitu et al. 1999). The tyrosine phosphatase activity of CD45 is necessary to allow phosphorylation of the ITAMs in Igα/Igβ and the generation of the subsequent signaling cascade, making CD45 clustering with the BCR vital for proper B cell activation. The B cell co-receptor complex is composed of three surface bound proteins: the signaling protein CD19, the complement receptor protein CD21, and CD81 (Sato, Miller et al. 1997). Antigens encountered by B cells will frequently have complement components attached to their surfaces. CD21 binds the complement component C3d, resulting in a signaling cascade arising from the intracellular portion of CD19. A clustering of BCRs and B cell co-receptors yields an aggregate signal, originating from both Igα/Igβ and CD19, of greater strength than either receptor or co-receptor could generate singly.

The second signal required for B cell activation depends upon the antigen to which the cell is reacting. Thymus-dependent (Td) antigens require the interaction of the B cell with a helper $CD4^+$ T cell (Lane, Traunecker et al. 1992; Noelle, Roy et al. 1992). Antigen binding to the BCR, in addition to initiating an activation signal within the B cell, causes the antigen-immunoglobulin complex to be endocytosed. The resulting phagocytic vesicle fuses with a lysosome in the classic phagocytic pathway resulting in antigen degradation (Hiltbold and Roche 2002; Hsieh, deRoos et al. 2002; Pluger, Boes et al. 2002). The phagolysosome subsequently merges with an exocytic vesicle from the Golgi apparatus containing HLA-DR/CD74 (Gregers, Nordeng et al. 2003). Antigen becomes loaded onto HLA-DR after

CD74 degradation and CLIP removal by HLA-DM, and is subsequently presented on the cell surface (Pathak, Lich et al. 2001; Van Kaer 2001; Zarutskie, Busch et al. 2001). There, it can present antigen to a $CD4^+$ T cell. Interaction with a T cell specific for the presented antigen leads to T cell activation and CD40L expression on the T cell surface (Jaiswal and Croft 1997). The binding of CD40L on the activated T cell to CD40 on the B cell surface generates the necessary second signal for a Td antigen. This signal acts to drive the B cell into the cell cycle, promoting proliferation and enabling the cell to undergo class-switch recombination (CSR, see below). In addition to its effects on proliferation and CSR, CD40 signaling also triggers increased expression of co-stimulatory molecules of the B7 family, such as CD80 and CD86 (Lane, Traunecker et al. 1992; Noelle, Roy et al. 1992), which are important for T cell activation and survival. This reciprocal T cell-B cell co-stimulation leads to increased proliferation of both cell types specific for the antigen in question and the rapid expansion of the adaptive immune response.

Thymus-independent (Ti) antigens, however, do not require CD40 signaling and thus need no direct interaction between the B cell and a helper T cell. Ti antigens are further divided into two groups, dependent upon how the second signal is initiated. Ti-1 antigens invoke the second signal in the absence of CD40L by binding an activating receptor on the B cell surface directly (Anderson 1977). The receptors most frequently responsible for this signaling are the Toll-like receptor (TLR) family of pathogen pattern recognition receptors. B cells express TLR-4, recognizing lipopolysaccharide (LPS) on their cell surface, and TLRs 3 and 9, recognizing double-stranded RNA and CpG DNA, respectively, in endocytic vesicles (Leadbetter, Rifkin et al. 2002; Marshall-Clarke, Downes et al. 2007). Binding of the TLRs to their respective ligands initiates a signal cascade resulting in the nuclear translocation of large amounts of NF-κB and increased Bcl-2 expression, leading to cell survival, proliferation and CSR, and effectively replacing many of the intracellular events

induced by CD40 binding (Schjetne, Thompson et al. 2003; Ulevitch 2003). In very high concentrations, Ti-1 antigens can induce non-specific, polyclonal B cell responses(Mosier DE 1977). Cross-linking of multiple TLRs on a single B cell can induce proliferation and differentiation into antibody secreting cells in the absence of BCR binding.

Ti-2 antigens differ from Ti-1 antigens in that they only engage the B cell through the BCR, and not through any of the TLRs. Ti-2 antigens are generally large molecules or polymers with highly repetitive structures (Fuchs S 1974). These activate B cells by simultaneously cross-linking a large number of BCRs, thus providing sufficient signal to activate the cell in the absence of CD40 or TLR signaling. Antigen concentration for this activation is critical, as too little antigen will not generate sufficient stimulus to activate the cell, while to much BCR stimulation in the absence of CD40 signaling has been shown to lead to anergy. In the absence of other external factors, the antibodies produced against Ti-2 antigens are generally IgM. The presence of certain cytokines in the B cell microenvironment, however, can lead to CSR and the expression of IgG or IgA.

IMMUNOGLOBULINS

General characteristics

Immunoglobulins are proteins, secreted by B cells, which recognize a specific antigen. While the potential diversity of Ig specificities is very large, each B cell will secrete Ig with only a single specificity. Ig have two distinct basic functions, antigen binding and immune cell interaction, carried out by discrete parts of the molecule. Antigen binding, performed by the variable region of the Ig, coats and neutralizes virus particles, marks pathogens for destruction by phagocytes, and attracts proteins from the complement system to the pathogen surface to facilitate direct killing. Immune cell interaction is carried out by the Ig constant region, which binds to Ig receptors on phagocytic cells of the immune system.

This interaction facilitates the endocytosis and lysis of pathogens and the subsequent processing and presentation of antigens to cells of the adaptive immune system.

Immunoglobulin structure

Immunoglobulins are hetero-polymers consisting of two copies each of two different peptides (Harris, Larson et al. 1992) (Fig.1.1). The two heavy chains (approx. MW~50kDa) are joined at their respective hinge regions by disulfide bonds to form a homo-dimer. The light chains (approx. MW~25kDa) are subsequently attached by more disulfide bonds to each heavy chain, at the amino-terminal side of the hinge (Harris, Larson et al. 1992). The amino-terminal end of each heavy chain and light chain compose what is known as the variable region, encoded by the VDJ and VJ regions of the heavy and light chains, respectively. The portion of each chain downstream of the variable region is known as the constant region (Edelman 1991). In the light chain, this is encoded by one of two germline genes, κ and λ, in a roughly 2:1 ratio in humans, or nearly 20:1 in mice (Takeda, Sonoda et al. 1996). No functional difference between the two has been identified to date, but mechanism by which κ or λ expression is determined has been partially elucidated (Gorman, van der Stoep et al. 1996). B cells only express one light chain gene per cell, determined by a successful recombination during the pro-B cell stage of development, so both light chains in any given Ig will be identical (Arakawa, Shimizu et al. 1996).

Similar to the light chain, the heavy chain can be of several different types encoded by different germline genes. These genes have been assigned letters of the Greek alphabet, and are designated μ, δ, γ, ε and α. These genes encode the heavy chains for IgM, IgD, IgG, IgE and IgA, respectively. In contrast to the light chains, control over which heavy chain

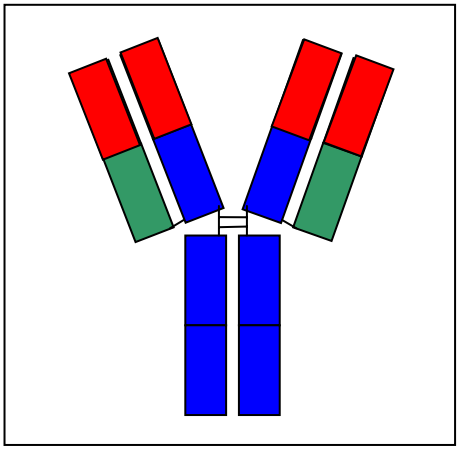


Figure 1.1: Immunoglobulin structure.

Block model of Ig structure showing the heavy chains in blue, light chains in green, and the variable regions in red. Lines connecting blocks represent the disulfide bonds that hold the four peptides together. This block model is representative of IgD, IgG and IgA. IgM and IgE monomers lack the hinge region have a extra Ig domain at the C-terminal end of the heavy chain.

gene is expressed at any given time is tightly controlled, and will be discussed below. The heavy chain determines the isotype of the Ig expressed by the B cell, and the role of that Ig in the humoral immune response, with disparate isotypes having significantly different interactions with other immune cells and innate immune proteins.

Both heavy and light chains are composed of repeated structural motifs known as Ig domains, which are characteristic of members of the Ig protein super-family (Brummendorf and Lemmon 2001). Ig domains are composed of seven to eight anti-parallel lengths of the protein arranged into a β-sheet. Two consecutive sheets are then joined face-to-face by a disulfide bond and bent into a roughly tubular structure known as a β-barrel (Ramsland PA 2002). Each variable region, on both the heavy and light chains, is composed of one Ig domain. The constant regions of light chains contain one additional Ig domain, whereas the heavy chain constant region contains three or four, designated C1-C4, with the hinge region falling between C1 and C2 if present.

Ig isotypes – IgM

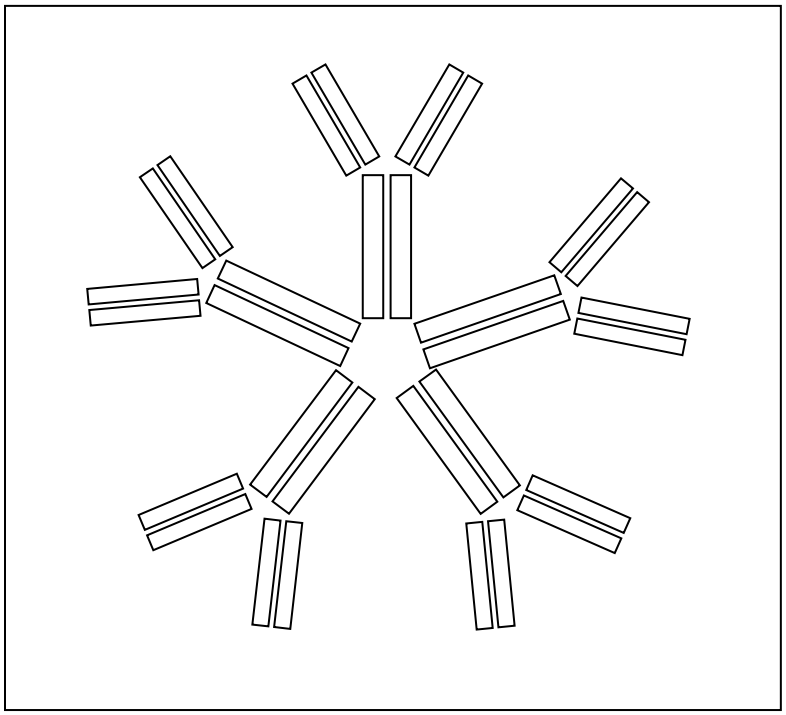
Also previously discussed, IgM is present on all pre-B and mature naïve B cells. Unlike IgD, however, IgM expression does not stop at activation, and IgM secreting plasma cells are not unusual. IgM is a common isotype expressed by B cells stimulated with Ti-2 antigens, as such B cells frequently do not receive the necessary input signals to undergo CSR. It represents about 10% of serum Ig. IgM is somewhat unusual in structure, as it and IgE lack the hinge region between the C1 and C2 Ig region, contains extra Ig domains in the C2 region, and has a fourth Ig constant region at its' carboxy-terminus.

Unique among the Ig isotypes, IgM is secreted as a pentamer (Fig. 1.2). In this configuration its heavy chains are joined at the C3 regions by the J chain, a joining protein co-expressed with polymeric Igs (Niles, Matsuuchi et al. 1995), as well as by inter-μ

disulfide bonds at the pentalrenate C-terminal residue (Beale and Feinstein 1969; Milstein, Richardson et al. 1975; Davis, Roux et al. 1989; Wiersma and Shulman 1995). The pentameric form makes serum IgM a very large molecule, about 900kDa, and as such it is not easily diffused across the endothelial barrier into the extra-vascular spaces. It will also not cross the blood - brain barrier, or the placenta.

In the serum, however, IgM excels in the roles of neutralization and complement fixation. Neutralization is the process of coating a pathogen or toxin in Ig, thus preventing it from attaching to its cell-surface receptor. Many bacterial pathogens are covered in a capsule that wards them against phagocytosis. Capsule components however tend to be long

Figure 1.2: Pentameric IgM.



carbohydrates or glycoproteins with highly repetitive structures, making then very good IgM-inducing Ti-2 antigens. IgM covering the surface of capsulated bacteria prevents them from attaching, overcoming the protection offered by their capsule proteins.

The complement system is a group of serum proteins that directly lyse pathogens without phagocytosis (Frank and Fries 1991). There are three complement pathways that can lead to lysis, the classical, the alternate and the lectin pathways. The classical pathway begins with Ig binding to the pathogen in question. Complement protein C1q then binds to the constant region of the bound Ig (Perkins and Nealis 1989). Different Igs attract (fix) C1q with greater or lesser affinities, making them better or inferior complement fixers. The pentameric nature of IgM allows it to fix up to six C1q molecules on a single Ig, greatly enhancing the process of building the complement complex and hastening pathogen lysis, and making it the most efficient complement fixer among the Ig isotypes.

Ig isotypes - IgD

As described under B cell maturation, cell surface co-expression of both IgD and IgM is the hallmark of a fully mature B cell (Abney, Cooper et al. 1978). Surface expression of IgD, combined with the lack of CD27 expression, is also the hallmark of a naïve, or non-activated B cell. Surface bound IgD can act as part of the BCR, signaling through Igα and Igβ, as does IgM (Blattner and Tucker 1984). IgD expression is generally halted upon B cell activation due to alternate splicing of the common IgM/IgD mRNA expressed in the naïve cell. Naïve B cells can be experimentally activated by cross-linking multiple surface IgD molecules, but it is unclear if this molecule plays a role in B cell activation *in vivo*. IgD is rarely secreted by activated B cells or plasma cells, and has no known role in the innate or adaptive immune response.

Ig isotypes – IgG

IgG is actually a complex of four closely related and highly similar Ig subclasses, designated IgG1 through IgG4, encoded by the heavy chain genes γ1 through γ4, respectively. The IgGs are by far the most common Ig in the serum, with concentration averaging around 13mg/ml, or about 80% of serum Ig. The IgGs are also the prevalent isotype in tissues and extra-vascular sites, owing to the efficiency with which they diffuse across the endothelial barrier. IgG is the only Ig isotype transported across the placenta via an IgG-specific receptor, protecting the developing fetus from infection long before the development of its' own functional immune system (Clark 1997).

The IgG subtypes can be grouped by their shared homology and role in the adaptive immune response. IgG1 and IgG3 are quite similar, while IgG2 is similar to IgG4. IgG1 and 3 are efficient in the neutralization and opsonization of pathogens, as well as complement fixation. IgG3, particularly, binds complement with such high affinity that it is nearly as efficient as IgM, despite being a monomer and only fixing one molecule of C1q at a time. IgG1 and IgG3 also have nearly equal affinity for the Fcγ receptors, Ig receptors specific for the γ heavy chain of IgG, and are the primary molecules involved in antibody-directed cellular cytotoxicity. Where these two differ is in the concentration of each one of them commonly found circulating in the blood. IgG1 is the most common isotype in the blood, accounting for roughly 75%, or 9mg/ml, of total IgG. IgG3 is fairly rare by comparison, at only 1mg/ml (Clark 1997).

While IgG2 and IgG4 are both as functional at neutralization as any other isotype, they are rather inefficient at most other Ig functions. These isotypes function poorly in pathogen opsonization due to a low affinity for Fcγ receptors. The sole exception to this is the FcγRIIa, which binds IgG2 with a very high affinity but is itself very uncommon, being present in only 50% of Caucasians and absent in all other groups. Additionally, these two

IgG subtypes are poorly transported across the placenta and do not fix complement well. While IgG2 is present in serum at moderate levels (~3mg/ml), IgG4 is rare (~0.5 mg/ml) (Clark 1997). One area in which IgG2 does excel, however, is in carbohydrate binding. Most Ig raised against bacterial lippopolysaccharide is IgG2.

Ig isotypes – IgE

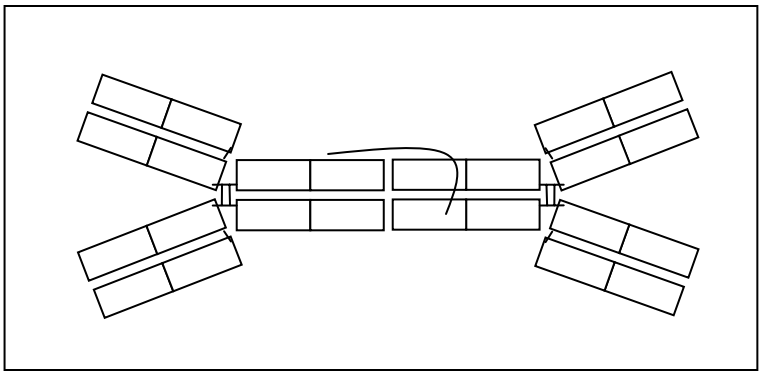
IgE is the least common of all the Ig isotypes, about 0.002% of serum Ig, with the narrowest range of function. IgE does a poor job of opsonization and neutralizing pathogens, owing primarily to its rarity in the serum at a scant $3x10^{-5}$mg/ml. IgE does not fix complement proteins, nor is it transported across the endothelium or epithelium. The primary role of IgE is in the detection of parasites, particularly in the skin and mucosal surfaces where the mast cell, basophiles and eosinophiles, to which most IgE binds, are located in greatest numbers (Gounni, Lamkhioued et al. 1994; Jankovic, Kullberg et al. 1997). Cross-linking of IgE bound to these cells with the antigen for which the IgE is specific signals and activates the mast cells through the FcεRI, causing the cell to de-granulate, or release its' stored granule proteins. Mast cell granules contain histamine and a number of other molecules that increase blood flow, cause tissue destruction, promote inflammation, cause smooth muscle contraction and kill parasites, all in an effort to rid the body of a parasite infestation.

Unfortunately, these same mechanisms can also recognize and react to harmless environmental molecules, such as the allergenic proteins of pollens and fungal spores. These inappropriate immune reactions are the basis of type I allergic reactions.

Ig isotypes – IgA

The final isotype, IgA, has a number of interesting characteristics. Like IgG, it has multiple subtypes, but only two, IgA1 and IgA2, as opposed to four for IgG. It can be

Figure 1.3: Dimeric IgA with J-chain.



expressed as a monomer, like IgG and IgE, or as a dimer joined at the C3 region by the J chain similar to IgM (Hendrickson, Conner et al. 1995) (Fig. 1.3). Monomeric IgA is present in the serum at modest levels (~2mg/ml, or 6% of serum Ig), but dimeric IgA is the prevalent Ig isoform in mucosal secretions. In fact, IgA is by far the most common isotype produced in the human body, at up to 1mg/kg/day (1-3 g/day for most people). Just as IgA is primarily localized to mucosal surfaces as opposed to the serum as with other isotypes, most dimeric-IgA secreting plasma cells are localized to the sub-epithelial regions of mucosal tissues, such as the lamina propria of the gut (van der Heijden, Stok et al. 1987), as opposed to the bone marrow where most other plasma cells, including monomeric-IgA secreting plasma cells, are sequestered (Macpherson, Hunziker et al. 2001). IgA1 is present at a higher concentration than IgA2 in both serum and most secretory Ig, but the ratio of IgA1:IgA2 is location dependent with IgA1:IgA2 being 2:1 in the serum and 3:2 in the upper gut. IgA2, however, is predominant in the lower gut, where a 2:3 ration of IgA1:IgA2 has been observed (Woof and Mestecky 2005). The primary difference between IgA subtypes is a fifteen amino acid deletion in the hinge region of IgA2. This alteration in amino acid sequence disrupts a bacterial peptidase recognition site, making IgA2 more resistant to cleavage than IgA1 (Senior, Dunlop et al. 2000). This is a significant advantage in the bacteria-rich environment of the gut and a likely selective basis for the increased presence of IgA2 in the intestinal lumen.

Dimeric IgA is actively transported across the epithelial barrier into the mucosal spaces. The Cα3 regions of the two IgA monomers binds to the polymeric-Ig receptor (pIgR) on the basal surface of an epithelial cell (Geneste, Iscaki et al. 1986; Geneste, Mangalo et al. 1986), and is transported through the cell in an endocytic vacuole in a process known as transcytosis. On the luminal side, the pIgR is cleaved by a thiol protease, freeing the dimeric IgA with a part of the pIgR still bound to the J chain. The released fragment of the pIgR is

then referred to as the secretory component, and the molecular complex of two IgA monomers, one J chain and secretory component is called secretory IgA (SIgA) (Mostov, Friedlander et al. 1984). Beyond its' role in transcytosis, secretory component has been shown to stabilize SIgA in the gut, making it resistant to denaturation in the acidic environment of the gastrointestinal tract. SIgA can even survive the low pH of the stomach, and is transferred from mothers to nursing infants in breast milk as a form of passive immunity to common gut pathogens.(Hansen 2002)

The role of IgA in the serum is largely redundant to that of IgG. It performs most of the same functions as IgG, with the exception of complement fixation, but generally with less efficiency. However, as IgA is the only Ig isotype present in the mucosa in appreciable quantities, its' role there takes on much greater significance. IgA, either monomers or SIgA, is not particularly good at fixing complement, but there is little complement in the mucosa to be fixed. Likewise, IgA is not an efficient opsonin, but phagocytes are encountered infrequently on luminal side of the epithelial barrier. The exception to this rule is alveolar macrophages patrolling the lung, which bear Fcα receptors to facilitate the phagocytosis of SIgA bearing pathogens (Hamre, Farstad et al. 2003). The primary role, then, of SIgA in the mucosa is that of non-inflamatory neutralization and agglutination (Fernandez, Pedron et al. 2003; Bollinger, Everett et al. 2006). SIgA binding to pathogens or toxins in the gut generally prevent them from crossing the epithelial barrier into the lamina propria, and invading the circulatory system and the rest of the body. SIgA bound particles are then flushed harmlessly out of gut by normal peristaltic processes and do no harm to the host. Additionally, SIgA plays an important role in maintenance the homeostatic balance of the normal gut microflora by trapping stray commensals in the mucus lining of the gut and preventing bacterial colonization of the gut epithelium.

CLASS SWITCH RECOMBINATION

General characteristics

As described in the previous section on B cell development, mature naïve B cells express both IgM and IgD on their surfaces. This is the result of genetic geography, which places the μ and δ heavy chain genes directly downstream of the VDJ gene segments. Upon activation however, many, or even most, B cells switch to expressing either IgG or IgA. The process by which the μ and δ genes, and potentially several others, are replaced with the heavy chain gene to be expressed is called class switch recombination, or CSR.

Structure of the Ig heavy chain genes

The Ig heavy chain genes (IgH) are arranged in a concatemer near the telomere on the long arm of chromosome 14 (Fig. 1.4). The Igμ and Igδ genes lie immediately downstream of the VDJ genes and are co-transcribed on the same mRNA in naïve B cells. Which one is expressed from any particular mRNA is a matter of alternate RNA splicing (Abney, Cooper et al. 1978; Blattner and Tucker 1984). B cells cease expression of IgD upon activation as a result of the alternate splicing that yields secreted, rather than membrane-bound, IgM. All the other IgH genes are expressed singly after being joined to the VDJ genes. Upstream of each IgH gene, with the exception of δ, are two regulatory elements that play an important role in CSR. These are the promoter of transcription for each heavy chain gene (I_H) and the switch region (S) (Jung, Rajewsky et al. 1993).

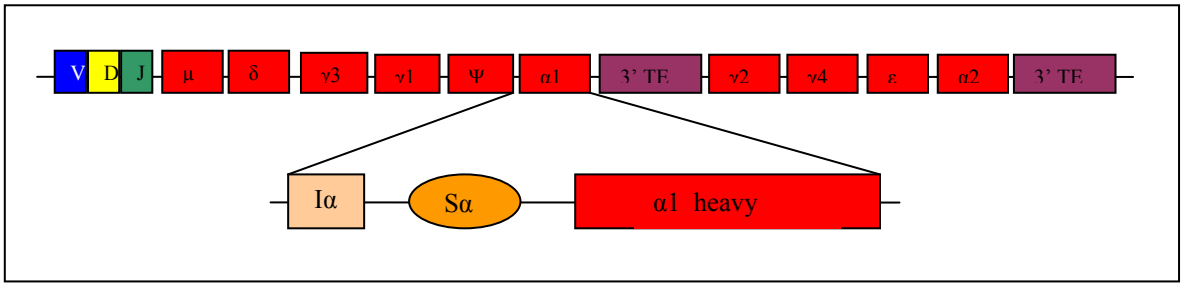


Figure 1.4: Schematic of the arrangement of the Ig heavy chain genes on the chromosome. The enlarged portion shows the arrangement of the promoter (Iα) and switch region (S) relative to the coding region. The 3' terminal elements (purple) contain terminators of transcription, which cause the RNA polymerase to dissociate from the coding strand DNA. Ψε denotes a nonfunctional ε pseudogene.

Critical events in CSR

The first critical event in CSR is full B cell activation by an appropriate second signal, such as CD40 ligation or TLR signaling, which causes the B cell to enter the cell cycle and begin division. This step is essential for a number of reasons. The chromatin remodeling necessary to gain access to the IgH genes downstream of δ can only take place when the chromatin is otherwise dissociating from the histones, which only occurs during DNA replication (Snapper, Marcu et al. 1997). Additionally, and equally important, CSR is completely dependent upon the DNA modification enzyme activation-induced cytidine deaminase (AID) (Revy, Muto et al. 2000). AID has been shown to be expressed exclusively in germinal center B cells, and is NF-κB dependent (Zhou, Saxon et al. 2003; Dedeoglu, Horwitz et al. 2004). This combination of requirements means that at least two to three rounds of cell division are necessary for class switching to occur.

The second critical event in CSR is the opening and transcription of the heavy chain gene that is to be expressed after CSR. AID acts by attacking the S regions ahead of the μ gene and the non-VDJ-associated IgH gene being actively transcribed. In order for the S region of the target IgH gene to be accessible to AID, it must be single stranded (Petersen-Mahrt, Harris et al. 2002; Ramiro, Stavropoulos et al. 2003; Sohail, Klapacz et al. 2003). This can only be accomplished by transcription in genomic DNA.

Direction of CSR

The identity of the IgH gene to target during CSR, and subsequently which Ig isotype the B cell will express, is determined by the switch factors present in micro-environment in which the B cell resides upon undergoing class switching. Switch factors are cytokines that bind B cell surface receptors, initiating signals that result in the nuclear localization and DNA binding of a multitude of transcription factors. Among other genes, these transcription

factors initiate the transcription of one or more IgH genes. These transcripts have no RNA coding for a variable region and cannot form a functional Ig, and are thus known as "sterile transcripts". As described above, the opening of the genomic DNA and transcription of the IgH gene is an essential step in CSR. The switch factor specific manner in which IgH gene transcription, if regulated, gives a great deal of control over which Ig isotype is expressed, and generally ensures that the resulting Ig is appropriate for the environment in which it will act.

Mechanism of recombination

Superficially, the mechanism of recombination of CSR shares some similarities with VDJ recombination. Both involve the looping out of a section of DNA, the creation of a double-strand break, and a break repair yielding a new connection and an excised loop of DNA (Fig. 1.5). Upon closer examination, however, there are several important differences in the two processes. Whereas VDJ recombination is dependent upon the recombinases RAG1 and RAG2, CSR is RAG independent (Lansford, Manis et al. 1998). Instead, CSR relies upon the previously described AID to induce double strand breaks (Revy, Muto et al. 2000). AID attacks the cytidine-rich S region between the IgH promoter and coding region. There, it removes the amino groups from the pyrimidine rings of cytidines, transforming the deoxy-ribocytidines into a deoxy-ribouridines (Petersen-Mahrt, Harris et al. 2002; Ramiro, Stavropoulos et al. 2003; Sohail, Klapacz et al. 2003). Normal DNA editing machinery acts to remove the uridine from the DNA, creating double stand breaks in the process. While this is taking place at the target gene, AID is simultaneously attacking the S region upstream of the μ coding region, creating a double strand break there, as well. The precise mechanism by which the correct ends are joined and these breaks are repaired has not yet been defined. However, the normal double strand break system using ku70 and ku80 has been implicated (Rolink, Melchers et al. 1996; Casellas, Nussenzweig et al. 1998; Manis, Gu et al. 1998).

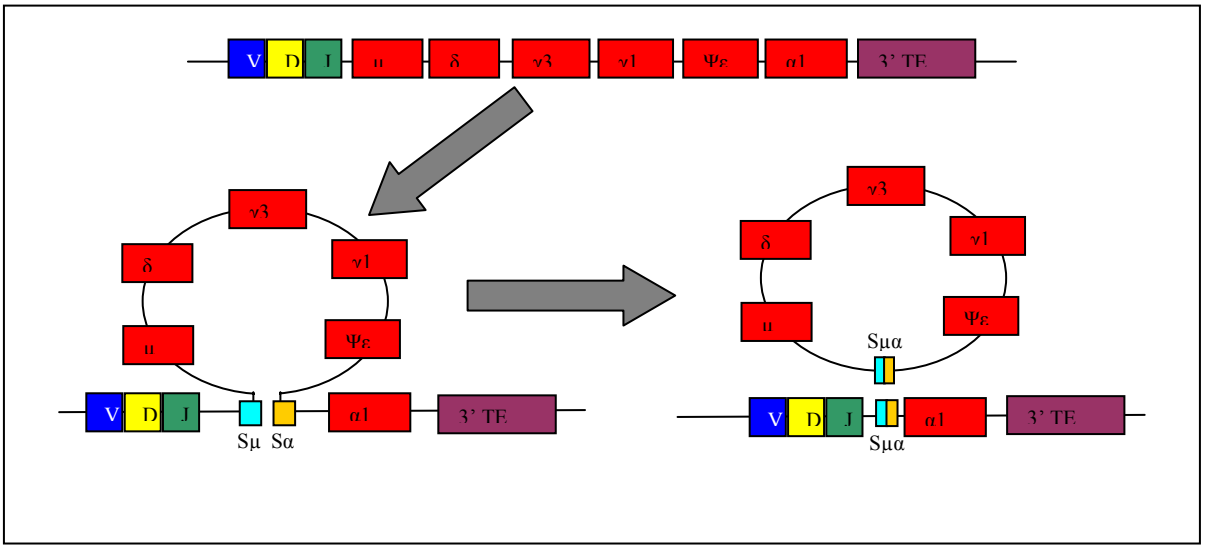


Figure 1.5: Mechanism of class switch recombination illustrating class switching to IgA1.

The free ends of the excised DNA are also joined together to prevent any erroneous recombination events, resulting in an excised loop of IgH DNA known as a switch-circle.

IgA switch factors

Numerous cytokines have been linked to the production of IgA at mucosal surfaces, including transforming growth factor (TGF)-β, vasoactive intestinal peptide (VIP), interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-15, interferon (IFN)-γ, B-cell activating factor of the TNF-family (BAFF) and a proliferation-inducing ligand (APRIL) (Snapper and Mond 1993; Ehrhardt and Strober 1995; Kramer, Sutherland et al. 1995; Yamamoto, Vancott et al. 1996; Lycke 1998; Boyaka, Lillard et al. 1999; Boyaka, Marinaro et al. 1999). Among these, only TGF-β and APRIL have been shown to promote IgA class switching independent of other cytokines. In addition, other factors not directly linked to IgA class switching, such as IL-2 and IL-10, can either increase the production of IgA in committed B cells or mitigate the negative effects of switch-directing cytokines (Arpin, Dechanet et al. 1995; Tangye, Avery et al. 2003). This is particularly important for factors such as TGF-β, which has been shown to be anti-proliferative (Letterio and Roberts 1998; Tangye, Avery et al. 2003), as progression through the cell cycle is required for class switching (Lundgren, Strom et al. 1995). While TGF-β has been definitively shown to induce class switching to IgA, the ubiquitous expression of this cytokine suggests that other factor(s) may be responsible for the compartmental nature of IgA secreting cells in the mucosa. Similarly, while APRIL has been shown to induce class switching to IgA in the Peyer's patches in response to the presence of bacterial components, it has not been shown to act in the mesenteric lymph nodes where a significant proportion of antigen specific IgA responses are generated.

The role of neuropeptides such as VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) in immune regulation may hold the key to our understanding of some aspects of host responses to infection. VIP has previously been shown to possess a number of potent immunomodulatory properties, including the capacity to inhibit cytokine and chemokine expression by macrophages (MΦ) and T cells, and to induce a Th2 phenotype in the immune response (Delgado, Abad et al. 2003; Ganea and Delgado 2003; Ganea, Rodriguez et al. 2003). Recent studies have shown that VIP and PACAP also have a variety of effects on DCs, depending on their maturation states. Exposing immature DCS to VIP or PACAP has been shown to generate production of Th2 cytokines, while stimulation with either neuropeptide strongly inhibits the ability of LPS-induced DCs to prime naïve T cells (Delgado, Reduta et al. 2004). Furthermore, DCs differentiated from peripheral blood monocytes in the presence of VIP were shown to be tolerogenic, inducing a regulatory phenotype in both $CD4^+$ and $CD8^+$ T cells (Dubois, Vanbervliet et al. 1997; Delgado, Gonzalez-Rey et al. 2005). Additionally, there is a well established link between VIP and IgA production (Boirivant, Fais et al. 1994; Kimata and Fujimoto 1994; Kimata and Fujimoto 1995), and VIP has been considered a switch factor for IgA (Fujieda, Waschek et al. 1996; Litinskiy, Nardelli et al. 2002). Subsequent work, however, has shown that B cells lack VIP receptors (Johnson, McCormack et al. 1996), suggesting that VIP modulation of B cells is an indirect effect. Here we demonstrate that DC-derived IGIP may be a vital link between VIP expression/signaling and mucosal IgA production.

Recent work in our laboratory has added another member to the list of IgA regulatory factors. Austin *et al.* described a novel protein, IgA-Inducing Protein (IGIP), isolated from a cDNA library produced from bovine Peyer's patch and mesenteric lymph node lymphocytes

activated under various conditions (Austin, Haas et al. 2003). Bovine IGIP, like BAFF and APRIL, was found to be produced primarily by DCs, in response to CD40L stimulation. Further, it was shown that recombinant bovine IGIP enhances IgA expression in IgM^+ peripheral blood B cells in an *in vitro* culture system.

DENDRITIC CELLS

General characteristics

Dendritic cells (DCs) are a small, but critical population of immune cells arising from precursors in the bone marrow. DCs serve as the primary antigen-presenting cell (APC) in the immune system, and the only cell type capable of activating naïve T cells (Banchereau and Steinman 1998). Two basic populations of DCs have been rcognized to date (Shortman and Liu 2002). Plasmacytoid DCs (pDCs) primarily respond to viral antigens by activating naïve T cells and expressing large quantities of type-1 interferons. Conventional DCs (cDCs) respond particularly well to extracellular bacterial antigen, but are also quite capable of presenting viral antigens if infected or if properly activated after phagocytosing extracellular virus. DCs generally express low constitutive levels of HLA-A,B,C and HLA-D, which are upregulated upon activation. Other dendritic cell type markers include DC-SIGN (dendritic cell-specific ICAM3-grabbing non-integrin, CD206), DEC-205 (CD205), and macrophage mannose receptor (Jiang, Swiggard et al. 1995; Sallusto, Cella et al. 1995; Valladeau, Ravel et al. 2000). Upon activation, DCs upregulate expression of T cell co-stimulatory molecules such as members of the B7 family, Ox40 and 4-1BB (Shuford, Klussman et al. 1997; Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998; Lane 2000).

Dendritic cell development

Dendritic cells were originally thought to be of a myeloid lineage, and there was good evidence to support this assertion. Mouse bone marrow myeloid precursors can be differentiated into DCs in the presence of granulocyte/monocyte-colony stimulating factor (GM-CSF) (Inaba, Inaba et al. 1993), and peripheral blood monocytes will differentiate into DCs in the presence of GM-CSF and IL-4 (Romani, Gruner et al. 1994; Sallusto and Lanzavecchia 1994). Additionally, adoptive transfer of myeloid precursors to mice with ablated immune systems was shown to reconstitute the DC populations (Traver, Akashi et al. 2000; Wu, D'Amico et al. 2001).

It has also been shown, however, that lymphoid progenitor cells can give rise to DCs. When lymphoid precursor cells are adoptively transferred from the thymus of normal mice to the thymus of ablated mice, the ablated thymus reconstitutes both the T cell and DC compartments (Ardavin, Wu et al. 1993; Wu, Vremec et al. 1995). Similar results were subsequently shown for lymphoid progenitors from normal donors injected into circulating peripheral blood of ablated recipients (Manz, Traver et al. 2001; Wu, D'Amico et al. 2001).

These seemingly contradictory findings have since been resolved. In fact, a subset both myeloid and lymphoid progenitors have the capacity to differentiate into any of the subsets of DCs. The requisite attribute, rather than lineage, is expression of Flt3. Any $Flt3^+$ progenitor of either origin may develop into any type of DC (D'Amico and Wu 2003; Chicha, Jarrossay et al. 2004; Shigematsu, Reizis et al. 2004). Most of the details of that development, however, remains unclear. It has been shown in a mouse model that a $CD45RA^-CD11c^{int}CD11b^+$ population represents cDC precursors, while a $CD45RA^+CD11c^{lo}CD11b^-$ population represents the pDC precursors (O'Keeffe, Hochrein et al. 2003), but the intermediate steps between this cell and the lymphoid or myeloid precursor have yet to be identified.

Dendritic cell activation

Dendritic cells have three activation, or maturation, states; immature, partially mature, and fully mature. Immature DCs are primarily resident in the peripheral tissues, where they patrol for pathogens or other foreign bodies. These DCs express low levels of HLA molecules, and no co-stimulatory molecules, but are very active in phagocytosis and pinocytosis. Phagocytosis is carried out through a number of cell surface structures. Molecules such as CD205 and CD206 bind and initiate uptake of carbohydrates and mannose moieties, respectively, which are common on the surfaces of bacterial and viral pathogens. Antigen can also be taken up by pinocytosis, after which it undergoes the same degradative processes in the endolysosome as actively phagocytosed pathogens.

Activation of immature DCs is accomplished via one of two pathways, TLR signaling or cytokine signaling. Tissue resident cDCs bear TLRs 1, 2, 4 and 6 on their surface and TLRs 3 and 9 in endocytic vesicles. Activation of any of these TLRs by the binding of their respective pathogen components induces a MyD-88 dependent DC maturation pathway (Muzio, Natoli et al. 1998; Kaisho and Akira 2001; Kaisho, Takeuchi et al. 2001). Activated DCs stop phagocytosis and pinocytosis, upregulate the HLA molecules, and begin to express co-stimulatory molecules like CD80 and CD86. They also begin to express chemokine receptors such as CCR7, which promote homing to the lymphoid organs (Hirao, Onai et al. 2000). At this point the DC is said to be partially activated. It has the capacity to activate naïve T cells, but not the ability to direct the subsequent immune response. Upon entry into the spleen or lymph node, the DC will begin secreting the chemokine CCL18 (also known as DC-CK), which attracts naïve T cells exclusively.

The same activation state can be induced by exogenous pro-inflammatory cytokines. TNF-α, for example, can drive cDCs to the partially activated state, and type-1 interferons can partially activate pDCs (Kalinski, Hilkens et al. 1999).

It is at this point the DC will interact with and activate the naïve T cell, the two being held together by cell adhesion molecules such as DC-SIGN on the DC and ICAM-3 on the T cell. Presented antigen borne on the DC HLA molecules is recognized by the TCR of specific T cells. The CD80 and CD86 on the DC surface bind CD28 on the T cell. Still other, tertiary co-stimulatory molecules on the DC surface, such as CD70 and CD134, bind the CD27 and CD252, respectively, on the T cell. This plethora of stimulatory signals activates the T cell, which then begins expression of the activation molecule CD154 (CD40L). CD154, subsequently, binds CD40 on the partially activated DC, inducing full activation (Caux, Massacrier et al. 1994; Schuurhuis, Laban et al. 2000). Upon full activation, the DC then expresses cytokines such as IL-10 or IL-12, which determine the nature of the T cell response.

Dendritic cell effects on B cells

While DC-B cell interactions have not yet been fully elucidated, it is clear that DCs can have a significant impact on B cell differentiation. Early studies showed that DCs, in the presence of known switch factors, could increase the proportion of IgA producing B cells eight- to ten-fold. The mechanism by which this enhancement took place has not yet been identified. Subsequent studies have found that a number of DC-derived soluble factors can act directly on B cells to enhance IgA production.

The B cell survival factors BAFF and APRIL, secreted by pDCs in response to type-1 interferons, were first shown to induce Ti CSR to IgA in the presence of TGF-β or IL-10 (Litinskiy, Nardelli et al. 2002). While these studies did not show that either BAFF or APRIL were IgA switch factors, it did implicate them in IgA production and gut homeostasis, and spur further investigation. APRIL has since been shown to be a legitimate IgA switch factor in Peyer's patches, being secreted by DCs in the gut in response to bacterial cell products (He, Xu et al. 2007).

Another observation linking DCs to B cells and IgA was that IgA levels, both in the gut and serum, were significantly decreased in mice lacking inducible nitric oxide synthase (INOS), but the adoptive transfer of INOS/TNF-α secreting DCs could restore IgA level to those of normal mice (Tezuka, Abe et al. 2007). Neither nitric oxide nor TNF-α are IgA switch factors, and no mechanism was revealed in those studies, but they did further the case for DC regulation of gut IgA homeostasis.

As described above, results from our recent studies have added IGIP to the list of DC-derived cytokines that influence B cell differentiation (Austin, Haas et al. 2003). While it shares its origin with other cytokines such as BAFF and APRIL, it differs in several important ways that will be detailed in the following sections.

HUMAN IMMUNODEFICIENCY VIRUS

General characteristics

Human immunodeficiency virus (HIV)-1 is a *Lentivirus*, in the family *Retroviridae* (Ratner, Haseltine et al. 1985). It is an enveloped virus approximately 100-120μm in size, with a conical shaped capsule and a genome-associated reverse-transcriptase (Gonda, Wong-Staal et al. 1985). The genome is composed of a single, positive-sense RNA 9.7 kilo-bases in size. Each capsid contains two genomic copies. The reverse-transcriptase of HIV-1 lacks fidelity and proof-reading capabilities, making it error prone. Replicated virus within a single host will not be identical, but rather a population of quasi-species, highly similar but distinct (Meyerhans, Cheynier et al. 1989). This property makes HIV-1 a rapidly evolving virus, greatly complicating the mounting of an effective immune response, or the design of an effective vaccine.

The primary receptor by which HIV-1 gains entry into the cell is CD4, which is born on a subset of T cells and macrophages. HIV-1 also uses a chemokine receptor as a

secondary receptor to facilitate cell entry. This can be either CCR5 or CXCR4, depending on wither the virus is T cell (T)-tropic or macrophage (M)-tropic (Collman, Hassan et al. 1989; Collman, Balliet et al. 1992).

HIV-1 has a typical retrovirus life-cycle (Freed 2001). After gaining entry into the cytoplasm, the reverse-transcriptase makes a double-stranded DNA copy of the single-stranded RNA genome, which takes on a circular conformation joining at the long terminal repeats. The capsid then degenerates, leaving the circular DNA genome to be transported to the nucleus. A virus-encoded integrase then integrates and linearizes the DNA genome into the host cell genome. The integrated linear DNA genome is then known as a pro-virus. Subsequently, whenever the cell becomes activated it will produce transcripts of the provirus, some of which will be translated into viral proteins and others of which will be incorporated into capsids as new viral genomes. Once capsids are complete and loaded with a genome, they are budded through the host cell membrane at a point where the viral envelope proteins are clustered, thus forming a new infectious viral particle.

Over the long term, HIV-1 infection leads to a significant loss of $CD4^+$ T cells (CDC 1981), through both the eventual lyses of infected cell and the induced apoptosis in bystander T cells. Given the critical role that $CD4^+$ helper T cells play in both the humoral and cell-mediated immune responses, by providing necessary cytokines to B cells and $CD8^+$ cytotoxic T cells, respectively, their loss has a crippling effect on the immune system and results in acquired immune deficiency syndrome, or AIDS.

While entire texts can be, and have been, written on the various aspects of HIV-1, a comprehensive review is beyond the scope of this work. Those aspects that require attention for our purposes here are the envelope protein, the animal models, and the difficulty of producing an effective vaccine.

HIV-1 vaccines

Since the time HIV-1 and HIV-2 were identified as the causative agents of AIDS, efforts have been ongoing to produce a protective HIV vaccine. To date, those efforts have been without success. This is not surprising when one considers the unique challenges that HIV presents to the vaccinologist. As previously mentioned, the error-prone reverse-transcriptase encoded on the HIV genome leads to a large degree of genetic variation. Phylogenies based on the *pol* gene sequence divide HIV into three groups, M, N and O, with M being the largest. Group M can be subdivided into nine separate clades (Walker and Korber 2001; Gaschen, Taylor et al. 2002). Further complicating vaccine design, the divergence in the envelope protein, which is the most likely target of a humoral immune response, is greater than that of the *pol* gene. Envelope proteins within a single clade have been shown to have up to 20% difference in amino acid sequence, and up to 35% difference has been measured between clades.

A related challenge to vaccine design is the immunogenicity of various HIV epitopes. The most immuno-dominant T cell epitopes in HIV tend to be in the more variable regions of expressed proteins. So, while an initial cell-mediated response may be vigorous and effective in reducing the number of infected cells, genetic drift and the rise of quasi-species during the course of in infection result in immune evasion, and the loss of control of viral replication (Phillips, Rowland-Jones et al. 1991; Allen, O'Connor et al. 2000; Barouch, Kunstman et al. 2002).

Compounding the difficulty of maintaining an effective T cell response to HIV is the presence of latent reservoirs of infection (Chun, Carruth et al. 1997; Chun, Engel et al. 1998). When an infected T cell is in a resting, or inactive state, transcription of viral genes is virtually non-existent. Viral proteins are thus not expressed and presented on the type-1

HLA molecules, effectively masking the presence of the virus from $CD8^+$ T cell immune surveillance.

The ideal HIV vaccine would be one that generated a sterilizing mucosal SIgA response, preventing the virus from crossing the epithelial barrier, thereby eliminating these sites of infection. Unfortunately, an effective way to elicit a mucosal response to vaccination has proven to be as elusive as an HIV vaccine.

HIV-1 envelope protein

The HIV-1 envelope protein (*env*) is expressed as a 160kDa poly-protein known as gp160 and glycosylated during translation on the rough endoplasmic reticulum (Willey, Bonifacino et al. 1988). After translation and glycosylation, it quickly forms oligomers of two to four units each, with three being the predominant number. It is then transported to the Golgi apparatus, where it is proteolytically cleaved by furin, or a furin-like protease, into gp120 and gp41 (Hallenberger, Bosch et al. 1992). These two proteins form a weak association, which is essential for their transport to the cell surface. Once there, gp41 will form the trans-membrane portion of the viral binding and fusion apparatus, while gp120 forms the receptor binding portion (McCune, Rabin et al. 1988).

As previously mentioned, gp120 is the most appealing target for an Ig based vaccine response. It is readily available on the viral surface and binds the cell surface receptor CD4, making it attractive for neutralization. Unfortunately, as was also previously mentioned, gp120 is not a reliable target, showing considerable structural variability even within one infected individual. This variability is not uniformly distributed throughout the molecule. The gp120 protein is divided into five variable regions (V1 - V5), separated by five fairly well conserved regions (C1 – C5) (Leonard, Spellman et al. 1990). The portions of gp120 that are important for CD4 binding have been mapped to the C3 and C4 domains, giving some hope that these regions of this important structure may be targeted for neutralization

(Thali, Moore et al. 1993). A recently resolved crystal structure of gp120 interacting with CD4 fragments, though, shows the binding site to be deep within a pocket and difficult to access (Kwong, Wyatt et al. 1998; Wyatt, Kwong et al. 1998). The successful deployment of a vaccine eliciting neutralizing antibodies to gp120 is dependent upon a novel approach antigen delivery and presentation.

Animal models of HIV-1 infection

One of the greatest barriers to the study of HIV-1 has been the lack of animal models. To date, HIV-1 has only been shown to establish infection in humans and chimpanzees (Novembre, Saucier et al. 1997). While the chimpanzee is an excellent model of human immunity, they present a number of challenges such as: their designation as an endangered species and the accompanying regulatory hurdles; their high cost to acquire, house and care for; their size, strength, and occasionally temperament; and the fact that HIV-1 infection does not generally induce any measurable disease (Arthur, Bess et al. 1989).

The only animal model of disease that has shown promise thus far for the study of HIV-1 is the humanized mouse. These are mice with a severe combined immune deficiency (SCID), generally lacking T cells and B cells. These mice can be reconstituted with a partially or fully functional human immune system, after which they are known as humanized mice, or SCID-hu mice.

One simple protocol by which partially functional SCID-hu mice may be produced is the intra-peritoneal injection of human peripheral blood mononuclear cells (PBMCs) into a SCID mouse (Mosier, Gulizia et al. 1991). This model is of limited utility for the study of HIV-1, as the $CD8^+$ T cell population tends not to migrate to the lymphoid organs, yielding a sub-optimal response.

A more productive method involves the implantation of human liver and thymus tissue into the peritoneal cavity of the SCID mouse (Namikawa, Kaneshima et al. 1988). If

properly vascularized, these implanted tissues will continue to grow and produce human immune cells, reconstituting a functional human adaptive immune system. A recent improvement to this system has been the incorporation of mice deficient in CD132, the cytokine receptor common gamma chain. These mice are crossed with SCID mice to produce SCID-CD132$^{-/-}$ mice, or NOG mice (Ito 2008). IL-7 is essential for the development of all common lymphocyte progenitors, and the IL-7 receptor signals through CD132. NOG mice are thus completely lacking lymphocytes (T cells, B cells, and NK cells), making them even more suitable for reconstitution.

This model does, however, have one significant limitation, the reconstituted human immune system fails to mount a primary response to HIV-1 infection (Ifversen, Martensson et al. 1995). It is thus limited to modeling an uncontrolled HIV-1 infection, as opposed to a primary infection in a normal healthy human. Despite that deficit, this model is useful for evaluating HIV-1 vaccines, given that the job of a vaccine is to elicit a primary response in the absence of infectious pathogen, and that recall responses in this model function normally.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

My hypothesis for the following work is that IGIP is a key component of both T cell-dependent and –independent B cell activation and class switching to IgA expression, and will promote an IgA response if included in a vaccine preparation. I have tested this hypothesis through the following specific aims.

Specific Aims

Specific Aim 1

I will determine whether IGIP is, in fact, an IgA switch factor that functions regardless of the presence of any other cytokine, or an expression factor that augments IgA expression in concert with other cytokines. I will also determine the extent, if any, to which IGIP synergizes with other cytokine such as TGF-β, IL-10, and IL-6 to augment IgA production.

Specific Aim 2

I will use purified populations of peripheral blood leukocytes to determine the cellular source(s) of human IGIP, and the conditions under which it is expressed. Purified T cells, B cells, monocytes and dendritic cells will be assayed for the production of IGIP transcripts and protein in response to various physiologic stimuli.

Specific Aim 3

I will evaluate the potential of using IGIP as a vaccine adjuvant. Recombinant IGIP will be added to an existing vaccine construct, and IgA responses and protection will be evaluated and compared to a vaccine lacking recombinant IGIP.

Materials and Methods

Reagents

The following reagents were used in the course of this work, and are listed with their working concentrations (where appropriate) and suppliers. $Pam3CSK_4$ (TLR2 ligand, 1μg/ml, Alexis Biochemicals), Poly I:C (TLR3 ligand, 10μg/ml, Sigma), LPS (TLR4 ligand, 100ng/ml, Sigma), Flagellin (TLR5 ligand, 1μg/ml, Alexis Biochemicals), CpG ODN 2006 (TLR9 ligand, 10μg/ml, IDT DNA) or CD40L(30ng/ml, Alexis Biochemicals), VIP (1μM, Calbiochem), rhTGF-β (10ng/ml, R&D Systems), TACI-Fc(soluble BAFF and APRIL neutralizing receptor, 20ng/ml, R&D Systems), rhIL-10 (25ng/ml, R&D Systems), anti-TGF-β mAb (30μg/ml, R&D Systems), TMZ-bl cell line, recombinant gp120, anti-HIV-1 gp120 antibody, HIV-1 (NIH AIDS Research and Reference Reagent Program, Germantown, MD), recombinant human HSP70 (Stressgen, Seattle, WA), Dextran (from *L. mesenteroides*, approx. WM 2,000,000, Sigma), EZ-Link LC-LC-Biotin, NeutrAvidin (Pierce, Rockford, IL), Phorbol-myristate acetate (PMA)(10ng/ml,Sigma) and Ionomycin(1μg/ml, Sigma). Treatment with PMA and Ionomycin is abbreviated PMA/I.

Isolation of naïve human B cells from peripheral blood

Total PBMCs were isolated from 100 ml whole blood from normal healthy donors via Accuprep (Accurate Chemical and Scientific Corp., Westbury, NY) gradient centrifugation, as previously described (Yagil-Kelmer, Kazmier et al. 2004). Peripheral blood mono-nuclear cells (PBMCs) were treated with biotinylated mouse monoclonal antibody (MAb) to human IgD (Southern Biotechnology Associates, Birmingham, AL), followed by anti-biotin Microbeads (Miltenyi Biotec, Auburn, CA). All reactions were carried out in sorting buffer (PBS pH 7.4, 0.5% BSA, 2mM EDTA) at 4^oC. The $sIgD^+$ B cells were then purified with an

automated magnetic cell sorter (AutoMACS, Miltenyi). The purity of sorted $sIgD^+$ cells, tested by flow cytometry and the absence of RT-PCR for IgG and IgA transcripts as previously described, exceeded 95% in all experiments (data not shown).

Isolation of peripheral blood mononuclear cell subsets

PBMCs were isolated by density gradient centrifugation. Populations of T cells, B cells, monocytes and NK cells were isolated with anti-human CD3, CD19, CD14 and CD56 Microbeads, respectively, by double-column sorting with an AutoMACS magnetic cell separator (Miltenyi). Isolated populations were shown to be ≥98% pure by FACS analysis (data not shown).

Preparation of monocyte-derived dendritic cells

PBMCs were labeled with anti-CD14 Microbeads (Miltenyi) and monocytes were separated by AutoMACS (Miltenyi). The $CD14^+$ monocytes were cultured with 10 ng/ml rhIL-4 (R&D Systems) and 1400 U/ml rhGM-CSF (Leukine, Immunex, Seattle, WA) for 6 days as previously described (37, 38). Non-adherent cells were removed and cultured for an additional 14 hours in complete RPMI 1640 (cRPMI), with or without 300 ng/ml of soluble rhuCD40L. Purity of isolated mDC populations was verified by flow cytometry with antibodies to DC-SIGN (R&D Systems) and HLA-DR (Southern Biotech).

Amplification of human IGIP

Pure mDC cultures were stimulated with 500 ng/ml rhCD40L (Axxora LLC, San Diego, CA), 20 ng/ml LPS or 10 ng/ml PMA and 1μg/ml Ionomycin for 14 hours. Total RNA was extracted with an RNeasy RNA extraction kit (Qiagen) and DNase-treated with a DNA Free DNase kit (Ambion, Austin, TX) as per manufacturer instructions. Human IGIP transcripts were amplified with a Titan One Tube RT-PCR kit (Roche, Indianapolis, IN) according to manufacturers instructions with forward primer 5'-AAT ATC ATT AAT TTG

CAC TGT-3' and reverse primer 5'-TTT TGC CTA CTT TAT TTC A-3'. Temperature cycling conditions were as follows: 50°C for 50min.; 95°C for 5 min.; 35 cycles of 95°C for 30 sec., 50°C for 1 min., 72°C for 2 min.; 72°C for 7 min.; 4°C hold. Fragments were visualized in a 1% w/v agarose gel containing 0.5μg/ml ethidium bromide.

RT-PCR assays

RT-PCR for hIGIP, BAFF, APRIL and IL-10 was performed at the UTMB Real-Time PCR Core Facility with primer and probe sets from BD Biosciences as follows:
hIGIP: Fwd-5'CCC ATC TCA GTG CTG GGA AA3' Rev-5'CTG ATG CAC AAC ACG TTT GCT3', Probe-5'CAC CAT GTG GAA ACC3', BAFF: Fwd-5'ACC GCG GGA CTG AAA ATC T3' Rev-5'GTT CTG ACT GGA GTT GCC TTC TC3', Probe-5'TGA ACC ACC AGC TCC3', APRIL: Fwd-5'CAC TCT GTC CTG CAC CTG GTT3' Rev-5'TCT GTC ACA TCG GAG TCA TCC T3', Probe-5'CAT TAA CGC CAC CTC C3', IL-10: Fwd-5'CCC CAA GCT GAG AAC CAA GAC3' Rev-5'TCC CCC AGG GAG TTC ACA3' Probe-5'CAG ACA TCA AGG CGC3'

Densitometry

Densitometry measurements were made with AlphaEaseFC Software (AlphaInnotech Corp., San Leandro, CA). Integrated Density Values (IDVs) were determined for each band in the gel, with the software correcting for background. Relative Expression (RE) was calculated for each band by dividing the IDV of IGIP by the IDV of G3PDH (housekeeping gene) in the same sample and PCR reaction ($RE_{IGIP} = IDV_{IGIP} / IDV_{G3PDH}$).

Expression of recombinant human IGIP

Human IGIP proved to be difficult to express *in vitro* due to issues with RNA stability. In order to stabilize the mRNA, it was necessary to link it to another mRNA and express the pair as a chimera joined by an inert linker. CD40L was chosen as the stabilizing

mRNA, since its encoded protein is required for IGIP-induced CSR and would already be added to all cultures. Human IGIP cDNA was ligated into the multiple cloning site (MCS) of the pCDNA5 expression vector (Invitrogen) containing an N'-terminal FLAG tag. Human CD40L was then ligated into the MCS at the 3' end of human IGIP, with a short inert linker in between. Sequence was verified and the plasmid was transfected into HEK293F cells with the Lipofectamine transfection reagent (Invitrogen) according to the manufacturers' instructions. Cells were cultured for three days in DMEM supplemented with 10% FBS, and supernatants were collected. Recombinant IGIP was purified with an affinity column specific for the FLAG peptide DYKDDDDK, constructed with anti-FLAG M2 agarose beads (Sigma) and dialyzed in sterile double distilled water overnight. The IGIP concentration was determined with the BCA protein assay (Pierce) according to the manufacturers' protocol. It should be noted here that the CD40L portion of the IGIP-CD40L chimeric protein lacked any activity, as it was found to be unable to support IgA CSR in the absence of additional rhCD40L.

ELISA

Sandwich capture ELISA for quantifying secreted Igs was performed as previously described (Engvall and Perlmann 1972). Briefly, Immulon II 96-well round bottom plates (Dynatech, Chantilly, VA) were coated overnight at 4^{o}C with 1μg/well polyclonal goat anti-human IgM(μ), IgG(γ) or IgA(α) (Southern Biotech). Plates were then washed three times with PBS (pH 7.4) containing 0.1% Tween-20 (PBST) and blocked with Superblock (Pierce Biotechnology Inc., Rockford, IL) for 2 hours at 37^{o}C and washed three times with PBST. Fifty μl of B cell culture supernatants or mouse sera, diluted 1:1 in PBST, were added to each well and incubated for 1 hour at 37^{o}C and washed three times. Purified human IgM, IgG, or secretory (S)IgA (Sigma-Aldrich, St. Louis, MO) from 1.0 μg/ml to 16ng/ml and assayed in parallel with the culture supernatants. Plates were then incubated with horseradish

peroxidase conjugated polyclonal goat anti-human IgM(μ), IgG(γ) or IgA(α) (Southern Biotech) at room temperature for 30 min. and wash three times. ABTS substrate (100μl, Sigma) was added to each well, and the absorbance was read at 405 nm with a Spectramax 340pc plate reader (Molecular Devices Corp., Sunnyvale, CA) after 15 min incubation. The concentrations of IgM, IgG and IgA were calculated based on the standard curves of purified human protein for each respective isotype (Sigma, St. Louis, MO) and analysis by linear regression. Correlation coefficients were >95% for each evaluation. Results are presented as the mean and standard error of the mean (SEM) for triplicate treatments, and are representative of three experiments with similar results.

Switch circle PCR

PCR for the joining region of the μ-α switch circle was performed as previously described (40). Briefly, phenol/chloroform extracted DNA was amplified with the following primers designed to span the Iα-Sμ joint of the switch circle: $I\alpha_{1/2}$ sense primer 5'-CAG CAG CCC TCT TGG CAG GCA GC-3', Sμ antisense primer 5'-TGA GTG CCC TCA CTA CTT GAG TCC CG-3'. A two-step PCR profile was used with the following temperature conditions: 94°C for 10min, 68°C for 10min, then 30 cycles of 94°C for 1min, 68°C for 2min, and 72°C for 3minutes. Products of this reaction were used as the template for a second reaction with the following primers, which are internal to those used in the previous reaction: $I\alpha_{1/2i}$ sense primer 5'-CTC AGC ACT GCG GGC CCT CCA-3' and $S\mu_i$ antisense 5'-CAG ACT GTC ATG GCT ATC AGG GGT GGC GGG G-3'. Temperature conditions were the same as the previous reaction. Products were visualized in a 1% agarose gel containing ethidium bromide under UV light. Gel was run with a mixture of 100bp marker and 1kb marker DNA ladders (New England Biolabs).

Preparation of AECM-dextran

Dextran purified form *Leuconostoc mesenteroides* with an average molecular weight of 2,000,000 Da was modified to aminoethylcarbamylmethyl (AECM)-dextran according to the following protocol. Dextran (4%w/v) was dissolved in 1M chloroacetic acid and 3M NaOH, and incubated at 25^{o}C for one hour. The reaction was stopped by adding 0.4% NaH_2PO_4 to 0.04% final concentration. pH was adjusted to 7 with 6M HCl. The resulting carboxymethylated dextran was dialyzed in 0.2M HCl for 5 days with daily buffer changes. After dialyzation, ethylenediamine hydrochloride was added at 0.2g/ml of dialyzate. 1-ethyl-3-(3-dimethlyaminopropyl) carbodiimide hydrochloride (EDC.HCl) was added to 1.5g/100ml slowly over four hours, while maintaining pH at 4.6 – 5. The resulting AECM-dextran was dialyzed for two days at 4^{o}C, with two buffer changes per day, then for three days at 4^{o}C in water. The AECM-dextran was then lyophilized.

Preparation of dextran-based gp120 vaccine

AECM-dextran, recombinant gp120, anti-FLAG antibody and recombinant human heat-shock protein (HSP)-70 were biotinylated with EZ-Link LC-LC-Biotin (Pierce) according to the manufacturers instructions, and dialyzed overnight at 4^{o}C in PBS. The biotinylated AECM-dextran was saturated with NeutrAvidin (1:1 w/w) and dialyzed again overnight at 4^{o}C in PBS. Biotinylated, avidinized AECM-dextran was then combined at a 1:1 ratio (original weight AECM-dextran/weight of additional components) with a 2:2:1 mixture (w/w) of recombinant gp120, rHSP-70 and anti-FLAG. This final product was dialyzed once more to remove any unbound gp120, HSP-70 or antibody. To half the preparation, recombinant IGIP bearing a FLAG epitope was added to the anti-FLAG antibody. Each half was then filter sterilized (0.22 microns) and prepared for injection at 1μg/ml.

Mouse vaccinations

SCID-hu mice were anesthetized with isoflourene in an aerosol chamber, pre-bled (ocular bleeds) to assess baseline IgM, IgG and IgA antibody levels, then injected with 100µl of AECM-dextran-based gp120 vaccine intraperitoneally (i.p.). Mice were bled again two weeks post-vaccination and serum levels of gp120 specific atibodies were assessed. Mice were then boosted with 100µl of dextran-based gp120 vaccine i.p. Serum levels of gp120-specific antibody were re-assessed at two weeks post-boost.

Challenge

Two weeks post-boost, mice were challenged with 200 $TCID_{50}$ HIV-1 (Chiang Mai) in sterile PBS by i.v. injection into the tail-vein.

HIV-neutralizing Ig assay

Serial dilutions of serum (1:10 out to 1:10000) were incubated with 200 $TCID_{50}$ of HIV-1 (Chiang Mai) in 50µl of cRPMI at 37^{o}C for two hours. Serum/virus samples were then placed over 10^5 TMZ-bl cells and incubated at 37^{o}C for four days. 100µl of freshly prepared Fast-Yellow Luciferase Substrate was then added to each well and incubated for two minutes. 100µl of supernatant from each well was then transferred to a Corning Costar black walled 96-well plate (Fisher) and luminescence was measured on a Fluostar Optima lumenometer (BMG Labtech, Durham, NC).

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Results

SPECIFIC AIMS 1 AND 2

The major source of IGIP in humans is the dendritic cell

In order to define the cellular sources of human IGIP transcripts, different cell populations were isolated from normal blood donors and stimulated with mitogens, calcium ionophore, or other key agonists, and RNA collected at optimal time points. mDCs were differentiated from peripheral blood monocytes as previously described (37, 38). T cells ($CD3^+$), B cells ($CD19^+$) and NK cells ($CD56^+$) were stimulated for 24 hrs with PMA and Ionomycin. Monocytes ($CD14^+$) were stimulated for 24 hrs with LPS. mDCs were stimulated for 24 hrs with VIP (Calbiochem). Additional samples of T cells and monocytes were also stimulated with VIP, as these cell types have previously been shown to be VIP reactive. RNA was collected with an RNeasy RNA extraction kit and IGIP transcripts quantified by RT-PCR. mDCs were shown to be the primary producers of IGIP among these cell types, with relatively low levels of transcription detected in purified B cells (Fig. 3.1a). IGIP production by cell populations and activation conditions are summarized in Table 1. In order to establish the kinetics of IGIP expression, mDCs were stimulated with VIP (1μM) and rhCD40L (30ng/ml) and harvested at 12, 18, 24, 48 and 72 hours. IGIP expression was first detected 12 hours after stimulation, peaking at 24 hours, and was no longer detectable by 72 hours (Fig.3.1b).

IGIP transcription is induced by vasoactive intestinal peptide

Monocyte derived DCs were stimulated with $Pam3CSK_4$, Poly I:C, LPS, CpG DNA, CD40L, SIgA, and VIP. Interestingly, TLR stimulation depressed IGIP expression below constitutive levels (Fig. 3.2a), as did stimulation with CD40L, SIgA, and PMA/I (Fig. 3.2b).

	T Cells	B Cells	NK Cells	Mono/Mac	DC
No Stim.	-	-	-	-	-
PMA/I	-	+	-	-	-
CD40L	-	-	-	-	+
VIP	-	-	-	+	++

Table 3.1. Summary of IGIP expression by cell type and stimulus.

B cells express low levels of IGIP mRNA under stimulation with Phorbol myristate acetate (1μg/ml) and calcium ionophore (100ng/ml) (PMA/I). DCs express low levels of IGIP mRNA under CD40L stimulation and high levels under VIP stimulation. Monocytes/macrophages express low levels of IGIP mRNA under VIP stimulation. Relative Expression (RE) was determined by densitometry. Data shown are from one experiment out of three with similar results, and are graded by the following scale: - = RE < 0.1; + = 0.1 < RE > 0.5; ++ = RE > 0.5.

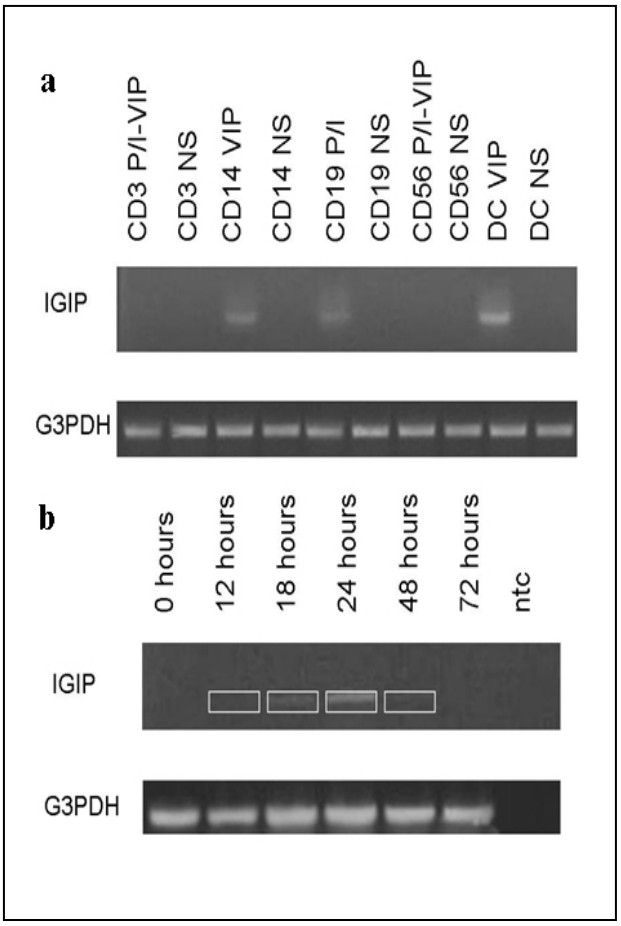

FIGURE 3.1: DCS ARE THE PRIMARY SOURCE OF IGIP.

a. Peripheral blood mononuclear cell populations were isolated by positive selection with an AutoMACS as described in the Materials and Methods section and stimulated for 24-48 hrs. RNA was collected and assayed for the presence of IGIP transcripts by RT-PCR. **b**. Peripheral blood monocytes were cultured in GM-CSF and IL-4 for 6 days. Non-adherent DCs were collected and stimulated with 1μM VIP. RNA was collected at the time points indicated and assayed for the presence of IGIP transcripts by RT-PCR. Data shown represents one experiment of three with similar results.

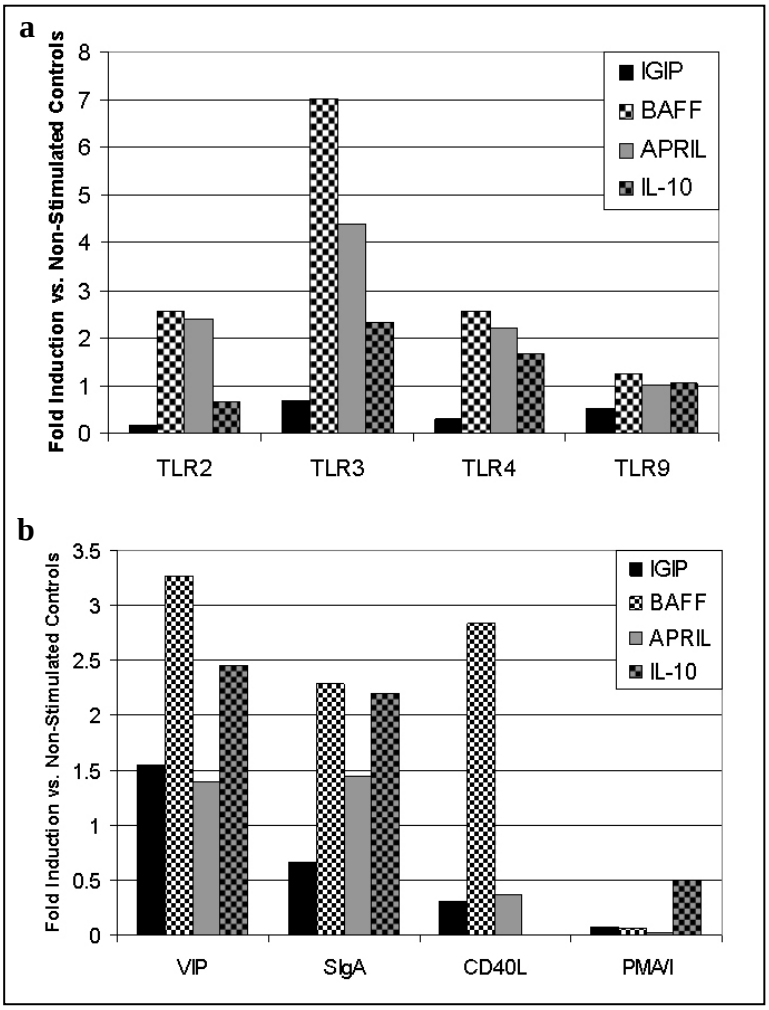

FIGURE 3.2: DCS EXPRESS IGIP MRNA IN RESPONSE TO VIP, BUT NOT TLR LIGAND, STIMULATION. Monocyte derived DCs were stimulated for 24 hrs. Cells were collected, RNA was extracted and cDNA produced. Real-Time PCR for IGIP, BAFF, APRIL and IL-10 was performed. **a.** Treatment of DCs with $Pam3CSK_4$ (1µg/ml), Poly I:C (10µg/ml), LPS (100ng/ml), or CpG DNA (10µg/ml) resulted in a decrease in IGIP transcripts, while BAFF and APRIL transcripts were both increased. **b.** DCs treated with VIP showed increased IGIP transcripts, while DCs treated with SIgA or CD40L showed increased BAFF and APRIL

transcripts. Results are presented as fold increase above non-stimulated controls, and represent one experiment of three with similar results.

Only VIP stimulation induced IGIP expression above basal levels. IGIP expression in response to VIP stimulation was found to increase in a dose-dependent manner up to 20μM, the highest concentration tested (Fig. 3.3). Interestingly, mDC expression of BAFF and IL-10 were shown to respond to VIP stimulation in a dose-dependent manner, with optimal expression achieved at 5μM. Induction of IGIP transcripts by VIP stimulation was detected by 18 hrs, peaked at 24 hrs, decreased substantially by 48 hrs, and were undetectable by 72 hrs, while very high concentrations (20μM) reduced expression below constitutive levels.

CD40L acts synergistically with VIP to induce IGIP expression

In order to investigate the role of mDC activation/maturation in VIP-induced IGIP expression, mDCs were treated with various TLR ligands, or CD40L trimer to crosslink CD40, in addition to VIP. Monocyte-derived DCs were cultured for 24 hours with $Pam3CSK_4$, Poly I:C, LPS, Flagellin, CpG ODN 2006 or CD40L in the presence of VIP. Stimulation with TLR ligands did not alter IGIP transcription when combined with VIP in culture. However, the combination of CD40L and VIP induced an approximately thirty-five fold increase of IGIP mRNA transcripts (Fig. 3.4).

Recombinant human IGIP induces expression of IgA in the presence of IL-10

In order to determine the effects of IGIP on IgA expression, naïve B cells were cultured with recombinant human IGIP and compared to cells cultured with TGF-β. Additionally, as IL-10 has a well-established role as an accessory factor in IgA expression but not CSR (40, 41), IL-10 was added to some cultures to assess its effect on IGIP-dependent IgA expression. Briefly, IgD^+ B cells were isolated from peripheral blood and cultured at 10^5/well in a 96 well plate in cRPMI with CD40L, IL-2, and TACI-Fc. Additionally, cells were stimulated

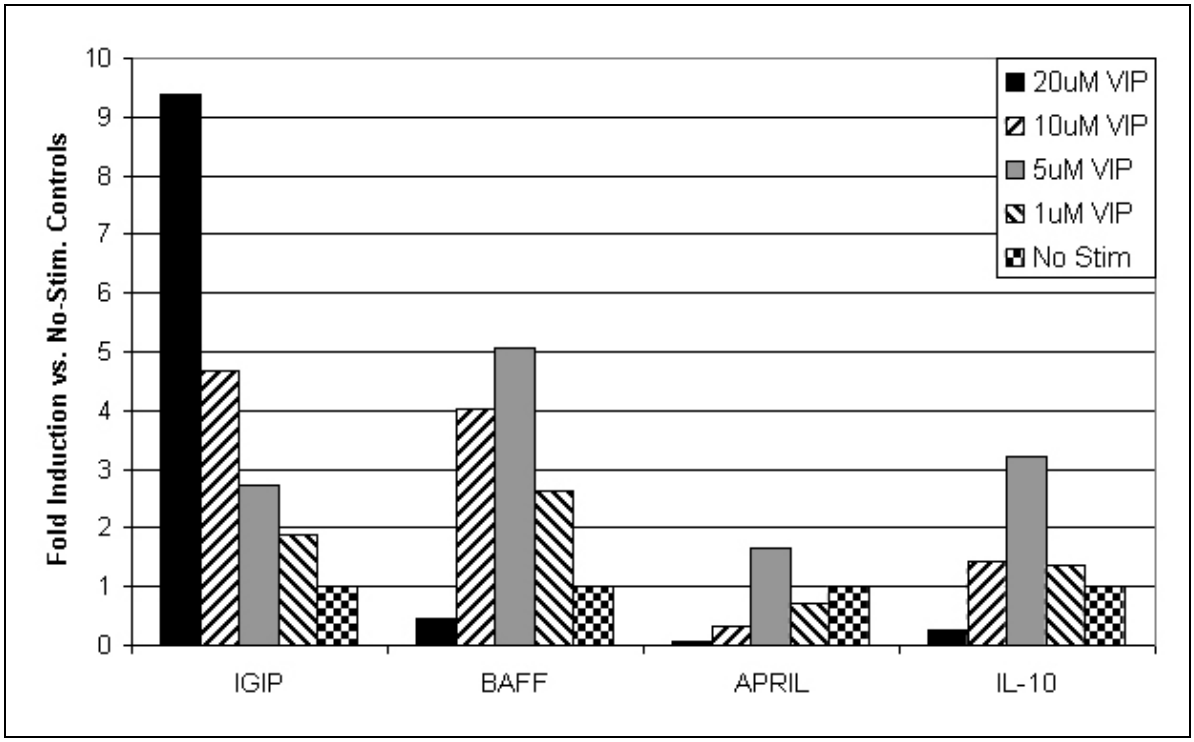

FIGURE 3.3: DCS EXPRESS IGIP IN RESPONSE TO VIP STIMULATION IN A DOSE-DEPENDENT MANNER. Monocyte derived DCs were stimulated with increasing concentrations of VIP, as indicated, for 48 hrs. RNA was extracted and cDNA amplified. Real-Time PCR was performed to determine expression levels of IGIP, BAFF, APRIL and IL-10. Results are presented as fold increase in expression above non-stimulated controls, and represent one experiment of three with similar results.

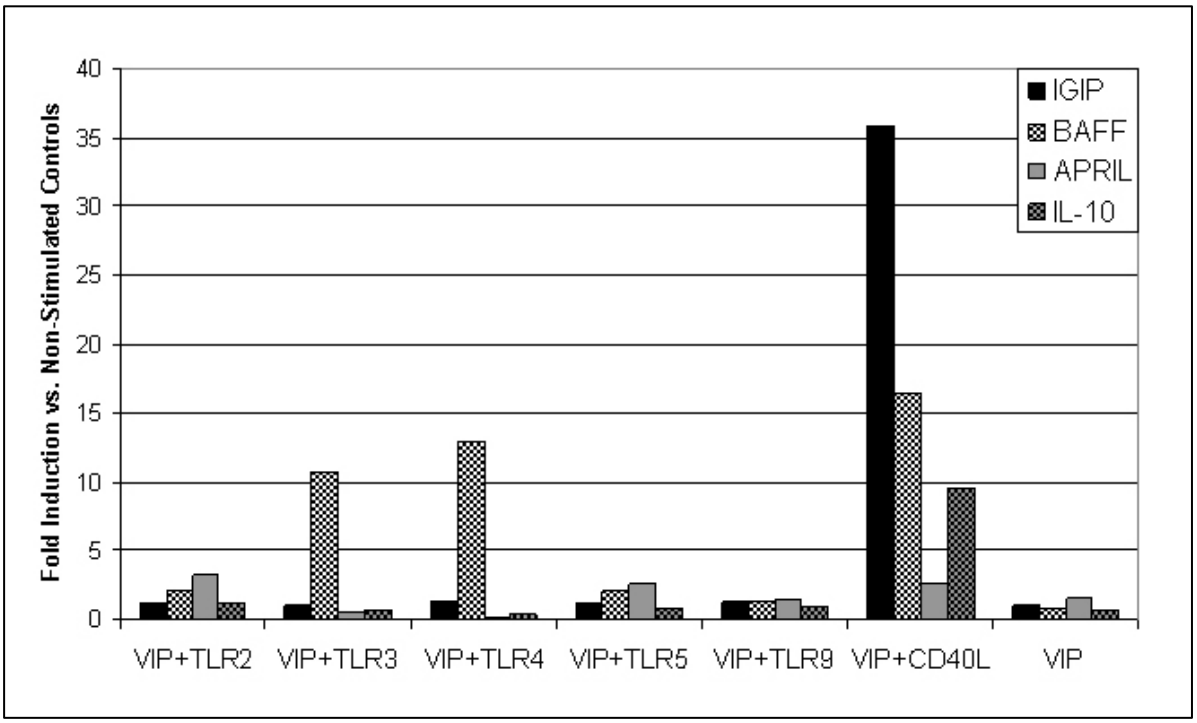

FIGURE 3.4: VIP ACTS SYNERGISTICALLY WITH CD40L TO UPREGULATE IGIP EXPRESSION.

Monocyte derived DCs were stimulated for 48hrs with 1μM VIP plus $Pam3CSK_4$ (1μg/ml), Poly I:C (10μg/ml), LPS (100ng/ml), Flagellin (1μg/ml), CpG DNA (10μg/ml) or rhCD40L(30ng/ml). Controls were stimulated with 1μM VIP alone, or left non-stimulated. RNA was extracted and cDNA amplified. Real-Time PCR was performed to determine expression levels of IGIP, BAFF, APRIL and IL-10. Results are expressed as fold induction over controls, and represent one experiment of three with similar results.

with rhIGIP (1 μg/ml), rhTGF-β, rhIL-10, rhIGIP plus IL-10, or rhTGF-β plus IL-10. An anti-TGF-β mAb was added to cultures not receiving exogenous TGF-β to eliminate any IgA CSR it may have initiated. Supernatants were collected at day 14, and IgA concentration was measured by sandwich-capture ELISA. In the presence of IL-10, rhIGIP plus CD40L stimulation yielded an IgA concentration of 307 ng/ml, as opposed to 3.35 ng/ml for CD40L alone (Fig. 3.5). In this regard, IGIP was found to have a similar capacity to induce IgA expression as TGF-β, which yielded 260 ng/ml in the same experiment. In the absence of IL-10, IGIP stimulation yielded an IgA concentration that was consistently, but not significantly ($p=0.052$), greater than control wells stimulated with CD40L alone. Culture supernatants were also assayed for IgM and IgG. IGIP was found to have no effect on either IgM or IgG expression under these stimulation conditions (data not shown).

IGIP is an IgA switch factor and not an accessory factor

In order to determine whether IGIP is a switch factor or an accessory factor that aids in IgA expression, we assayed whole genomic DNA of rhIGIP stimulated B cells for the presence of switch circles, the circular fragments of IgH genomic DNA that are excised during class-switch recombination (CSR). IgD^+ B cells were isolated from peripheral blood and cultured at 10^6/well in a 24 well plate in cRPMI with rhCD40L, anti-TGF-β neutralizing mAb and TACI-Fc, with or without rhIGIP (1μg/ml). As a positive control, additional IgD^+ B cells were cultured with rhCD40L and rhTGF-β. Cells were collected at day five and DNA was harvested by phenol/chloroform extraction. We assayed for the presence of switch circles with a nested PCR for the region of the switch circle comprised of the joint between the Iα and Sμ regions, with an expected product of ~1200bp. A PCR product of the appropriate

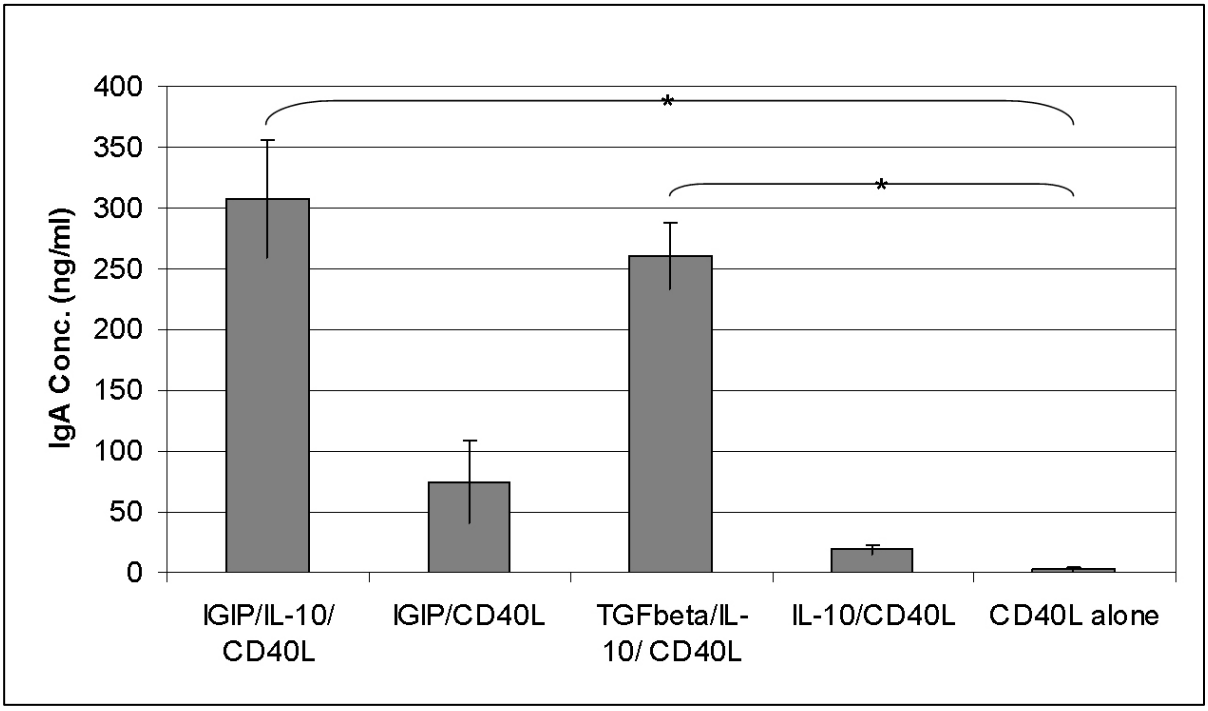

FIGURE 3.5: RECOMBINANT IGIP INDUCES IGA EXPRESSION IN THE PRESENCE OF IL-10.

IgD^+ B cells were stimulated with rhCD40L(30ng/ml), IL-2(50ng/ml) and TACI-Fc (20ng/ml). Additionally, cells were stimulated with rhIGIP(1μg/ml), rhTGF-β(1ng/ml), rhIL-10(50ng/ml), rhIGIP plus IL-10, or rhTGF-β plus IL-10. Supernatants were collected at day 14 and assayed for IgA concentration by ELISA. All samples were run in triplicate. Data is presented as the average IgA concentration in triplicate supernatants ± SEM. * = $p < 0.05$. Data presented is one representative experiment out of two with similar results.

size was detected in DNA extracted from cells that had been stimulated with rhIGIP and CD40L, as well as rhTGF-β and rhCD40L, showing the presence of μ-α switch circles and indicating μ-α CSR (Fig. 3.6). No product of the appropriate size was detected in DNA extracted from cells stimulated with CD40L alone.

SPECIFIC AIM 3

Dextran based delivery of gp120 is capable of eliciting an immune response

Despite previous success using a dextran construct to deliver antigen in a T-independent manner, there was some question at the outset of this work as to whether biotinylated gp120 would be effectively delivered by the dextran construct, or if the chemistry used to attach it would damage critical epitopes. To ascertain the feasibility of delivering of this particular antigen with this vaccine construct, we immunized mice with a gp120-containing construct to see if it would elicit an antigen-specific B cell response.

Fully reconstituted SCID-hu mice, approximately 20 weeks old, were given 100μl of dextran-based gp120 vaccine (1μg/ml) i.p. in sterile PBS. Blood samples were taken two weeks post-immunization, and the sera assayed for gp120-specific IgM, IgG and IgA. Mice were boosted with a second, identical dose and sera assayed again one week post-boost. While the initial response was lackluster, the levels of gp120-specific antibody in the sera one week after boost indicated a robust B cell response (Fig. 3.7).

Dextran-based gp120 vaccination is sporadically protective

Once it was determined that biotinylated gp120 was effectively delivered by our dextran-based vaccine construct, we wanted to assess its' capacity to elicit protective

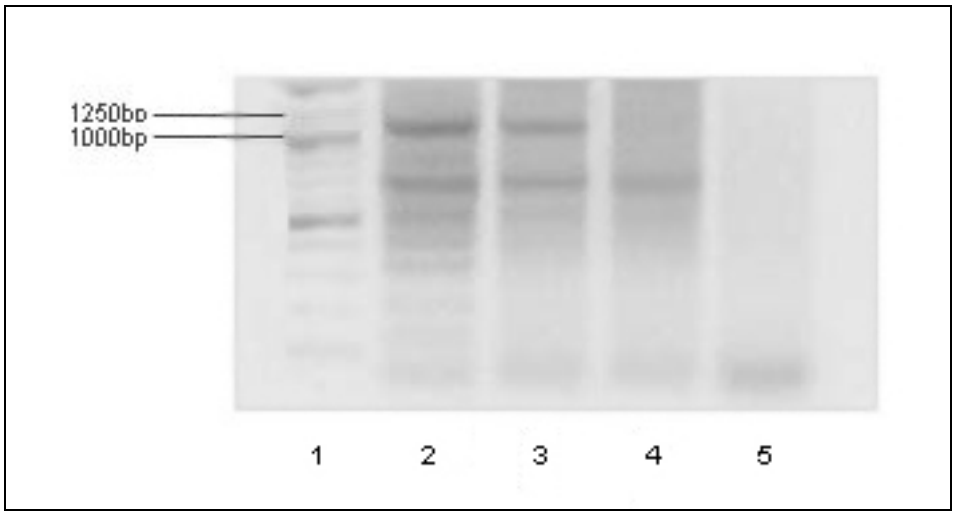

FIGURE 3.6: IGIP IS AN IGA SWITCH FACTOR.

IgD^+ B cells were cultured for 5 days with rhCD40L(30ng/ml), anti-TGF-β neutralizing mAb(30μg/ml) and TACI-Fc(20ng/ml), in the presence or absence of rhIGIP(1μg/ml). Positive control cells were incubated for 5 days with rhCD40L(30ng/ml) and rhTGF-β (1ng/ml). DNA was collected at day 5 and assayed for switch circles by nested PCR. The PCR product of 1182bp indicates the presence of μ-α switch circles (confirmed by sequencing) and IgA CSR. Data presented is one representative experiment out of three with similar results. Lane 1 = 100bp ladder, Lane 2 = IGIP+CCD40L, Lane 3 = TGF-b +CD40L, Lane 4 = CD40L alone, Lane 5 = No Template Control.

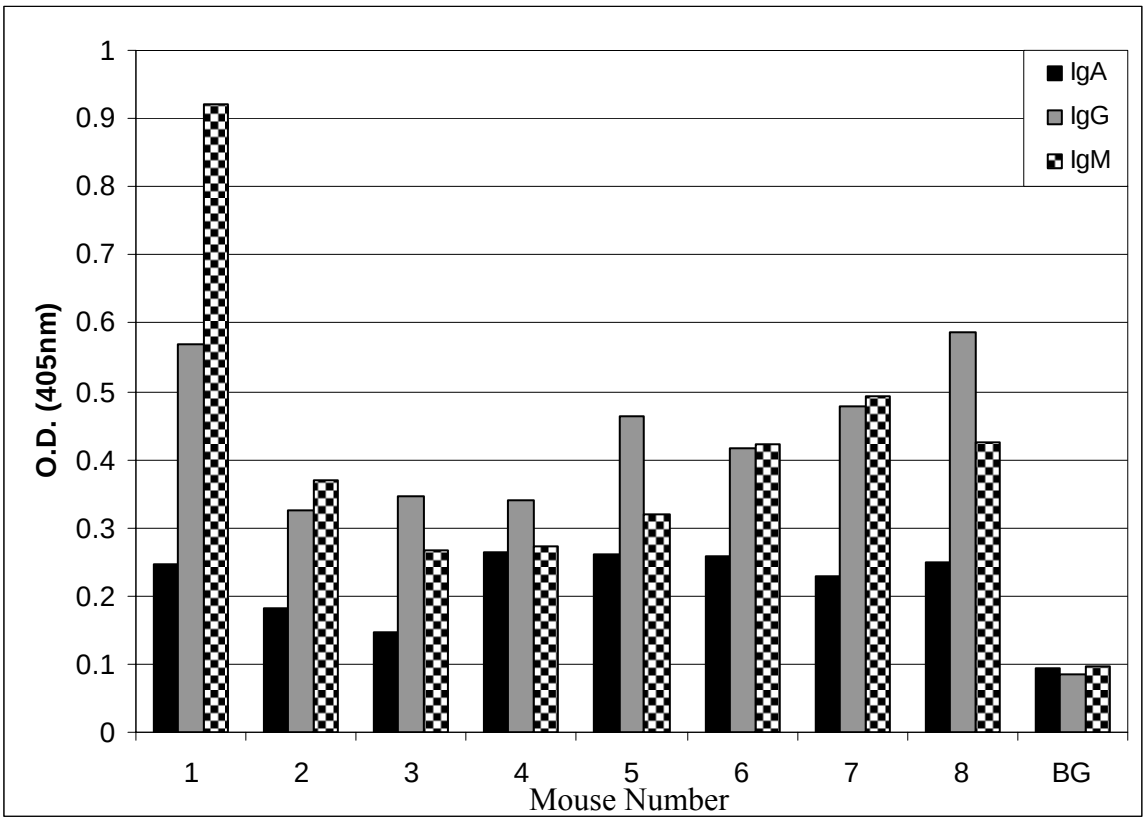

FIGURE 3.7: DEXTRAN-DELIVERED GP120 ELICITS A ROBUST SERUM ANTIBODY RESPOSNE. SCID-hu mice were immunized i.p. with 0.01μg of dextran-based gp120 vaccine. Blood samples were collected at 5 days post-immunization and sera were separated by centrifugation. 20μl of serum diluted 1:5 in PBS were assayed for gp120-specific IgA, IgG and IgM by sandwich-capture ELISA using gp120 as the target protein. Background (BG) was determined by running sera without the gp120 capture protein. Data are presented as O.D. at 405nm, and are from one experiment.

immunity to HIV-1. While gp120-based vaccines have previously had limited success in generating a sterilizing immunity, it was hoped that the intraperitoneal delivery of an antigen in a T-independent construct would produce a more poly-reactive antibody response.

To that end, previously vaccinated mice were challenged i.p. with 200 $TCID_{50}$ of HIV-1(Chiang Mai) two weeks post-boost. Blood was collected and separated fourteen days post-challenge. Sera were again assayed for gp120-specific IgM and IgG, as well as capacity to neutralize virus. Serum IgG and IgM specific for gp120 was still high (Fig. 3.8), but only one mouse (mouse #6) developed a neutralizing antibody to HIV-1 (Fig. 3.9), consistent with previous observations.

RNA was collected from PBMCs in the separated blood samples and assayed for HIV-1 p24 transcripts by RT-PCR. Products were visualized on agarose gel containing ethidium bromide and relative expression measured semi-quantitatively by densitometry, normalized to the housekeeping gene G3PDH. Consistent with the results from the neutralizing antibody assay, all mice except mouse #6 had high levels of expression of HIV-1 p24 (Fig. 3.10), indicating active HIV-1 replication.

Effects of IGIP on IgA induction by dextran-based gp120 vaccine

Our final objective was to assess the capacity of IGIP to enhance the mucosal SIgA response when included in a vaccine construct. Mice 1 though 4 were immunized with a vaccine construct containing IGIP, while mice 5 through 8 were immunized with vaccine containing no IGIP. Four weeks post –challenge, sera and oral swabs were collected from surviving mice and assayed for gp120-specific IgA by ELISA. Results from all mice showed both serum and mucosal IgA specific for gp120 (FIG. 3.11). Unfortunately, only one mouse from

the No-IGIP group remained at this point, making it impossible to draw any conclusions as to the effectiveness of IGIP in a vaccine preparation.

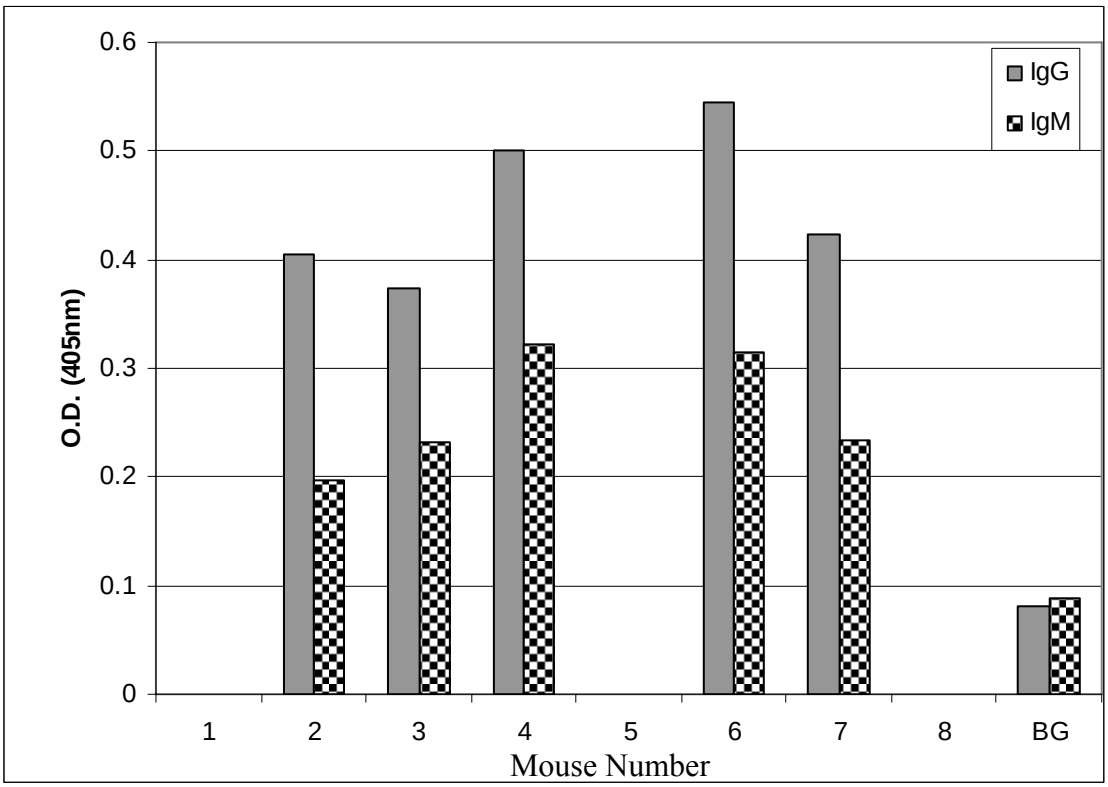

Figure 3.8: Serum anti-gp120 remains high after challenge. Vaccinated SCID-hu mice were challenged 14days post-boost with 200 TCID50 HIV-1 (Chiang Mai), delivered I.P. in PBS. Blood samples were collected and separated 14 days post-challenge. Sera were assayed for gp120-specific antibodies by ELISA as described above. Mice 1, 5, and 8 had expired in the intervening 14 days. Data are presented as O.D. at 405nm, with background as described above, and represent one experiment.

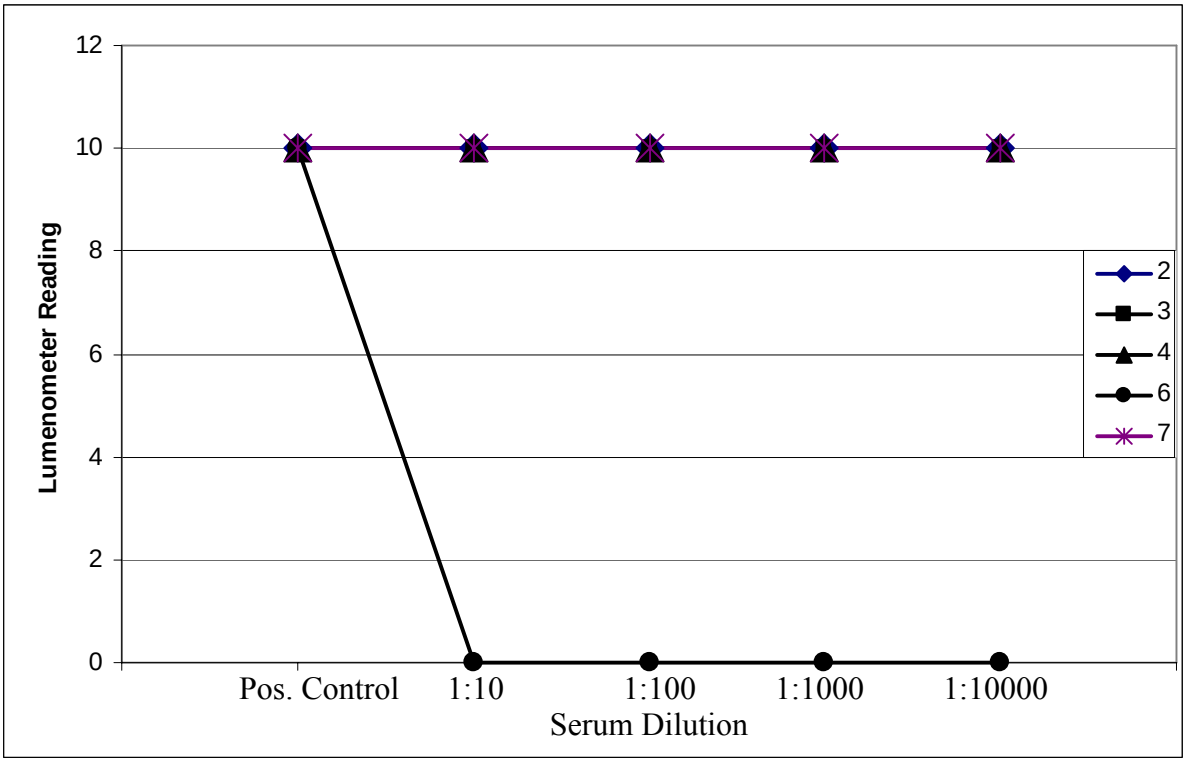

FIGURE 3.9: DEXTRAN-BASED GP120 VACCINATION HAS POTENTIAL TO ELICIT A PROTECTIVE RESPONSE. Sera from 14 days post-challenge were assayed for neutralizing antibody with a luciferase assay. Serum dilutions from 1:10 up to 1:10000 were incubated for one hour with 200 $TCID_{50}$ of HIV-1 Chiang Mai and then added to 10^5 TMZ-bl nef-reporter cells for 4 days. Cells were lysed and luciferase substrate added, then 100 µl transferred to a black-walled 96-well plate and luminescence was measured after 5 minutes. Data shown are from one experiment.

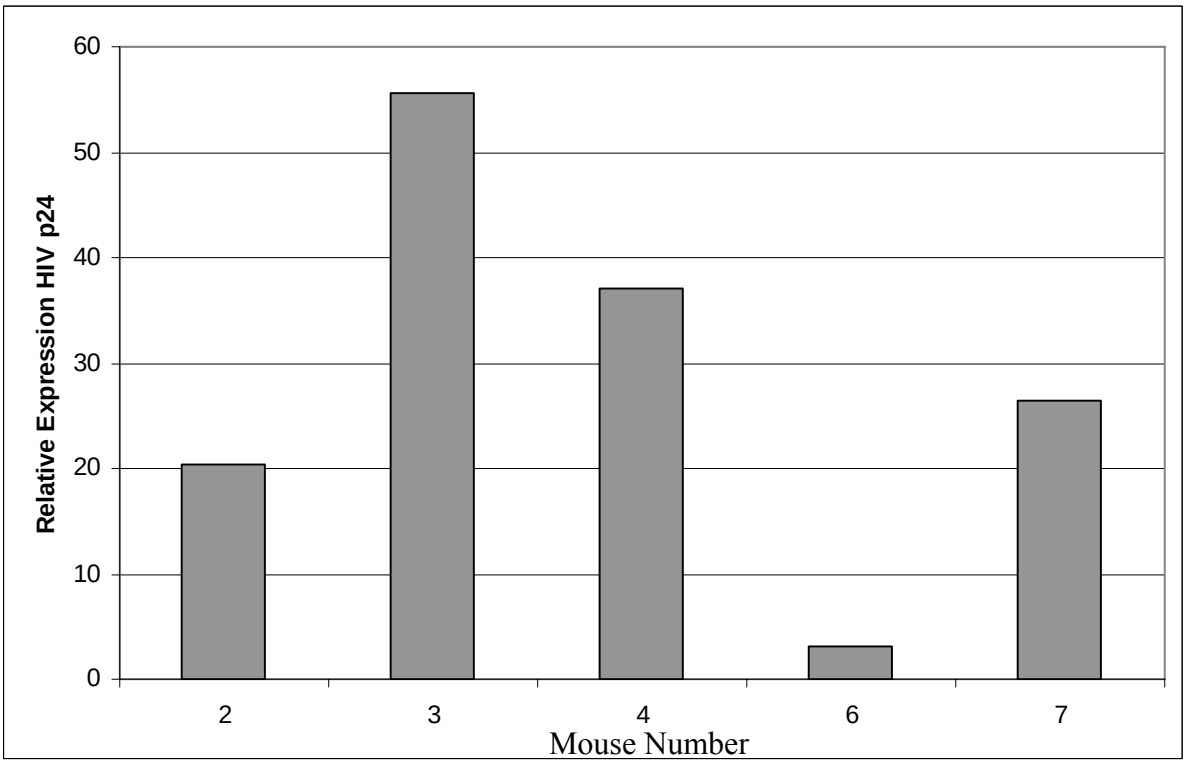

FIGURE 3.10: HIV-1 P24 TRANSCRIPTS IN PBMC OF SCID-HU MICE INDICATE ACTIVE HIV-1 REPLICATION. RNA was harvested from PBMC collected 14 days post-challenge and assayed for HIV-1 p24 by RT-PCR. Results were visualized on an agarose gel and expression was measured by densitometry. Results shown here represent Relative Expression of p24 normalized to expression of G3PDH, and are from one experiment.

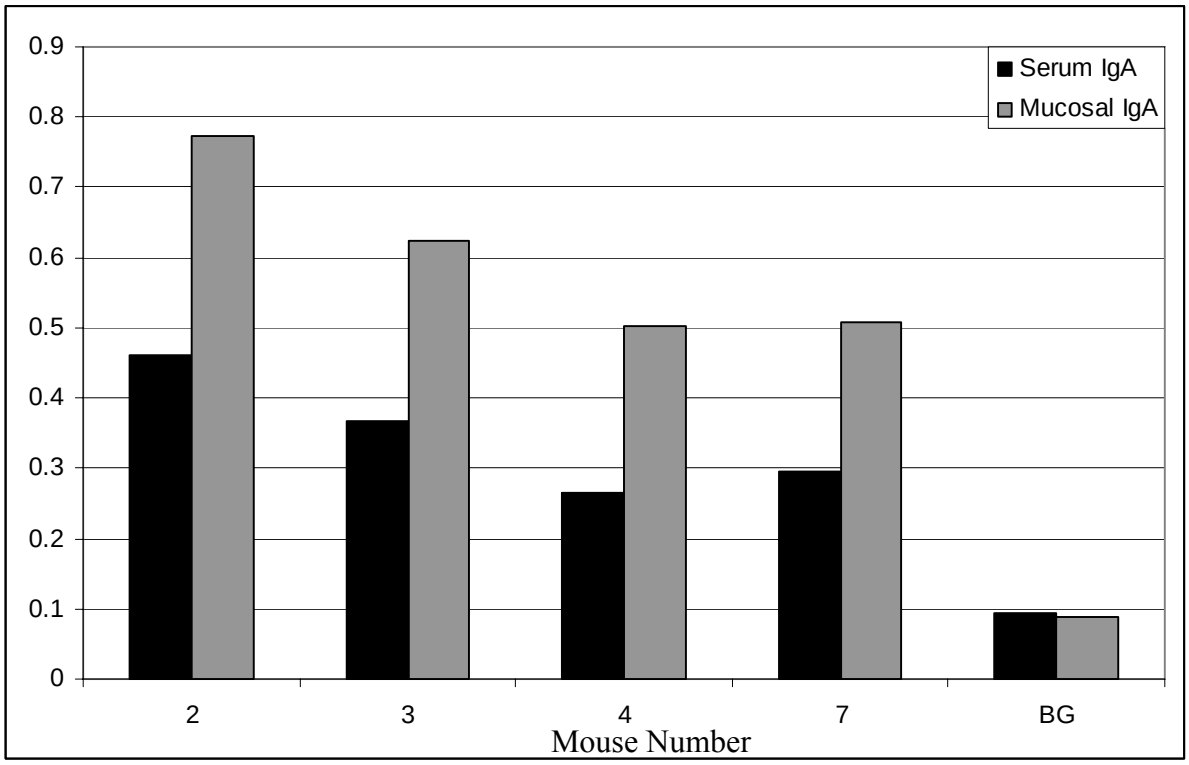

FIGURE 3.11: DEXTRAN-BASED GP120 VACCINATION ELICITS BOTH SERUM AND MUCOSAL IGA RESPONSES. Blood and oral swabs were collected 21 days post-challenge and assayed for gp120-specific IgA by sandwich-capture ELISA, as described above. Data are presented as O.D. at 405nm, and represent one experiment.

Discussion

Despite the localized IgA production at mucosal sites, the total amount of this isotype produced by the body on a daily basis and its' role in defense against mucosal infection, the regulation of IgA expression and secretion in humans and other species is not fully understood. To date, only TGF-β and APRIL have been definitively shown to induce CSR at the molecular level independent of other factors. Other DC-derived cytokines such as BAFF have been implicated in IgA CSR, providing the necessary NF-κB signaling in T-independent antigen-induced CSR, but these factors alone are insufficient to directly drive IgA class switching (21).

IGIP, a relatively small peptide characterized in the bovine system has recently been added to the list of factors that can positively regulate IgA expression (35). Previous studies indicated that bovine mDCs were the primary source of IGIP expression. In the present study, I have found that human IGIP is similar to that in the bovine system, both in expression and response. Monocyte-derived DCs, and to a lesser extent monocytes/macrophages, were identified as the primary producers of human IGIP. A relatively low level expression was also detected in activated B cells, suggesting a possible autocrine effect. IGIP expression was not detectable in other human lymphocyte populations. Regulation of IGIP mRNA was found to be somewhat more complex in humans than observed in the bovine. Bovine mDCs similarly derived from peripheral blood precursors were found to produce IGIP transcripts in response to stimulation with recombinant CD40L alone (35). In contrast, human mDCs required the presence of VIP to initiate IGIP expression, whereas CD40L was found to be insufficient as the sole stimulus. Furthermore, IGIP expression by human mDCs in response to VIP was dose-dependent

within a pharmacological level. While CD40L stimulation alone was found to be insufficient to induce IGIP expression in human mDCs, there was a synergistic effect of CD40L and VIP on IGIP production (Fig. 9), suggesting that high levels of IGIP expression may be dependent on T cell interaction. This stands in contrast to the regulation of other DC-derived IgA regulatory factors. BAFF and APRIL, for example, are fully expressed by DCs upon stimulation by a single factor, namely type-I interferons for plasmacytoid DCs (pDCs) and CD40L for mDCs.

In addition to the effects on IGIP expression by human mDCs, we found that BAFF expression is also induced by VIP stimulation in a dose dependent manner. The co-regulation of BAFF and IGIP may indicate a shared role for these two DC-derived cytokines in the regulation of B cell CSR. Interestingly, expression of APRIL by human mDCs does not appear to be influenced by VIP stimulation (Fig. 3.3).

Although it was not tested, the effects of PACAP are likely to be similar to VIP with regards to IGIP and BAFF expression. It has previously been shown that mouse mDCs express VPAC1 and VPAC2, two receptors shared by VIP and PACAP, and that stimulation with these two neuropeptides results in mDCs with the same phenotype (27).

As previously stated, there is a well established, but indirect, link between VIP and IgA production (29-33). Here we present strong evidence that IGIP may be the missing link in this mucosal IgA regulation scheme.

Among the other stimuli tested for their ability to induce IGIP expression was SIgA (Sigma), the rational being that signaling through CD89 (FcαR) may upregulate the expression of IgA regulatory proteins in response to the presence of SIgA. Interestingly, while the addition of SIgA did not significantly effect the expression of IGIP, it did upregulate BAFF expression by about two fold (Fig. 3.2).

In addition to characterizing IGIP expression, we also examined the effects of IGIP stimulation on B cells and IgA expression. Under the influence of exogenous IGIP stimulation, CD40L-activated IgD^+ B cells were shown to increase IgA production above CD40L-activated controls without IGIP (Fig. 3.5). The addition of IL-10 was shown to greatly enhance IGIP-dependent production of IgA, as has been shown for other cytokines involved in IgA expression (40, 41). IGIP was also shown to be a bona fide IgA switch factor. In the presence of IGIP and CD40L alone, IgD^+ B cells were shown to undergo μ-α CSR by the presence of Sα-Sμ switch circular extra-chromosomal DNA fragments (Fig. 3.6).

Our data support a role for VIP in the regulation of IgA expression via IGIP production from DCs. This clarifies the role of VIP in regulation of IgA responses. This pathway is unique in that IGIP expression does not appear to be regulated via recognition of pathogen associated molecular pattern receptors (PAMPS) on the DCs. Our observations in these studies may provide a link between VIPergic fibers and innervation of GALT with homeostatic production of IgA (independent of TLR stimulation) and potentially with natural baseline mucosal IgA production.

In the final set of studies from which this text was drawn, we attempted to evaluate the capacity of IGIP to induce a robust mucosal IgA response when included as part of a vaccine preparation. A vaccine platform known to elicit B cell responses in a T-independent manner was used to test this application, as well as to evaluate the feasibility of using biotinylated HIV-1 glycoprotein gp120 as a target antigen. Mice were immunized with this dextran-based vaccine via inrtraperitoneal injection in an attempt to increase the likelihood of a good mucosal response. Results we obtained in examining antigen-specific serum antibody were consistent with those of previous studies in which gp120 had been delivered via more conventional methods. Serum levels of both IgM and IgG were high, but most antibody was non-neutralizing. As has been previously shown, the immuno-dominant epitopes of gp120

are not protective. However, given the production of gp120-specific serum and mucosal antibodies, we were successful in showing that biotinylated gp120 is effectively delivered to APCs in the peritoneum, and capable of initiating an immune response. Additionally, we have shown that intraperitoneal delivery of dextran-based vaccines can elicit a robust IgA response in both the serum and mucosa. Unfortunately, we were unable to reach a conclusion about the capacity of IGIP to enhance an IgA response when included in a vaccine preparation due to the deaths of research animals.

There remain a number of research areas relating to IGIP that are unexplored. The receptor through which IGIP signals is a complete mystery, as is the downstream signal transduction cascade that leads to IgA CSR. Given the necessity of Smad signaling in the IgH α promoter region, it would seem that IGIP-R signaling would have to merge with TGF-βR1 signaling, but this remains to be explored. The *in vivo* expression characteristics of IGIP are unknown, in regards to its tissue localization and level of expression. It is presumed that IGIP expression would be restricted to mucosal sites at which VIP is expressed, but that too remains to be shown.

So where, then, does IGIP fit within the larger context of IgA regulation in the mucosa? It is likely that it plays a role in both the antigen-specific response to pathogens and in the generation of broadly reactive natural antibodies (Fig. 4.1). In a traditional antigen-specific response, IGIP fits neatly into the role presumed to be filled by TGF-β. It is entirely possible that it is cytokine expressed by the fully activated DC, rather than the T cell, that is driving CSR in the germinal centers in mucosa-associated lymph nodes. This is much more in line with the localization of IgA expression than the global nature of TGF-β expression, and fits better with the need for an activated B cell to undergo cell-cycle progression. The potential role for IGIP in the natural antibody response is less clear, but IGIP is expressed

under similar conditions as APRIL, and is likely present in the same cytokine microenvironments.

While much remains to be done, we feel this work adds significantly to the body of knowledge regarding the regulation of IgA and the maintenance of gut homeostasis. These are strangely under-represented in the broad context of biomedical research, especially given their importance to human health. It is our strong hope that this work will be carried on by those that follow, and that their efforts will be fruitful.

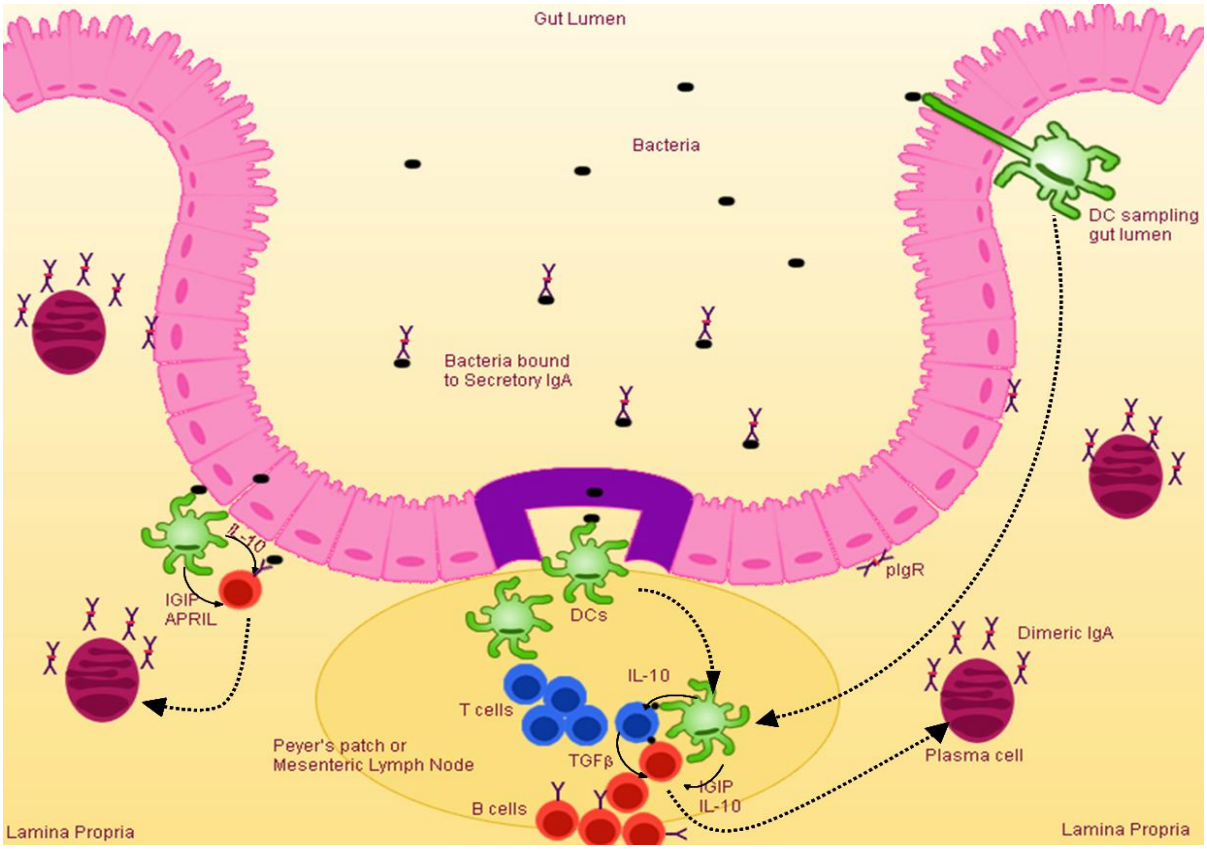

Figure 4.1: The role of IGIP in the regulation of IgA expression in the mucosa. IGIP likely plays a role in both the antigen-specific, T cell-dependent response to pathogens and in the T cell-independent generation of broadly reactive natural antibody. Large dashed arrows depict cell migration, and in the case of B cells, differentiation. Small solid arrows depict cytokine secretion.

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