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Human IgA inducing protein from dendritic cells induces IgA production by naïve IgD+ B cells[1]

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Abstract

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) and other factors, including CD40L, GM-CSF, and IL-4 as well as the neuropeptide vasoactive intestinal peptide (VIP). Under *in vitro* stimulation with VIP and CD40L, IGIP mRNA expression could be 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 greater quantities than non-stimulated controls. Finally, we demonstrate 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.

Keywords

Antibodies; B Cells; Human; Mucosa

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Introduction

Immunoglobulin (Ig) A is the primary Ig isotype present in secretions, where it acts as the first line of defense against pathogens at mucosal sites. IgA also plays an important role in maintaining gut homeostasis by ensuring the resident micro-flora does not invade the intestine(1). 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) (2–8). Among these, only TGF-β has 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 (9,10). This is particularly important for factors such as TGF-β, which has been shown to be anti-proliferative(11), as progression through the cell cycle is required for class switching (12). 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.

Dendritic cells (DCs) are potent regulators of the adaptive immune response, including the regulation of Ig, and particularly IgA, expression(13,14). Interstitial DCs (iDC) and monocyte-derived DCs (mDC) have the ability to regulate B cell differentiation through the expression of IL-10 and IL-12 following CD40 ligation (15–18), and have been linked to the regulation of antibody production both in the presence and the absence of T cells. Recent studies have also shown that human DCs up-regulate BAFF and APRIL expression upon exposure to a variety of stimuli including IFN-α, IFN-γ, CD40L or Toll-like receptor (TLR)-induced reactive oxygen species. (19–23).

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 (24–26). Recent studies have shown that VIP and PACAP also have a variety of effects on DCs dependent on their maturation states. Exposing immature DCS to VIP or PACAP has been shown to generate a Th2 response, while stimulation with either neuropeptide strongly inhibits the ability of LPS-induced DCs to prime naïve T cells (27). 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 (28). Additionally, there is a well established link between VIP and IgA production (29–31), and VIP has been considered a switch factor for IgA (32). Subsequent work, however, has shown B cells to be lacking in VIP receptors (33), 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.

Vitamin metabolites such as retinoic acid (RA), a vitamin A metabolite, have also been shown to play a role in mucosal IgA secretion. RA has been shown to induce the expression of mucosal homing chemokine receptors such as CCR9, as well as the mucosa-specific α4β7 integrin(34). While RA was not sufficient to induce IgA expression in naïve B cells in these experiments, it did synergize with as yet unidentified DC-derived factors to enhance 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 bovine Peyer's patch and mesenteric lymph node combined cDNA library with cells activated under various conditions (35). Bovine IGIP, like BAFF and APRIL, was found to be produced primarily by DCs, in response to CD40L stimulation. Further, we found that recombinant bovine IGIP enhances IgA expression in IgM^+ peripheral blood B cells in an *in vitro* culture system. Here, we demonstrate the effects of this peptide on human B lymphocytes and characterize the conditions under which IGIP is expressed and the conditions required for IGIP function.

Materials and Methods

Reagents

The following reagents were used for mDC or B cell stimulation experiments, and are listed with their working concentrations and suppliers. $Pam3CSK_4$ (TLR2 ligand, 1μg/ml, Alexxa Biochemicals), Poly I:C (TLR3 ligand, 10μg/ml, Sigma), LPS (TLR4 ligand, 100ng/ml, Sigma), Flagellin (TLR5 ligand, 1μg/ml, Alexxa Biochemicals), CpG ODN 2006 (TLR9 ligand, 10μg/ml, IDT DNA) or CD40L(30ng/ml, Alexxa 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) 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 (36). 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°C. $sIgD^+$ 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). $CD14^+$ 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'-AATATCATTAATTTGCACTGT-3' and reverse primer 5'-TTTTGCCTACTTTATTTCA-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'CCCATCTCAGTGCTGGGAAA3' Rev-5'CTGATGCACAACACGTTTGCT3', Probe-5'CACCATGTGGAAACC3', BAFF: Fwd-5'ACCGCGGGACTGAAAATCT3' Rev-5'GTTCTGACTGGAGTTGCCTTCTC3', Probe-5'TGAACCACCAGCTCC3', APRIL: Fwd-5'CACTCTGTCCTGCACCTGGTT3' Rev-5'TCTGTCACATCGGAGTCATCCT3', Probe-5'CATTAACGCCACCTCC3', IL-10: Fwd-5'CCCCAAGCTGAGAACCAAGAC3' Rev-5'TCCCCCAGGGAGTTCACA3' Probe-5'CAGACATCAAGGCGC3'

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 protein mRNA and express the pair as a chimera joined by an inert linker. CD40L was chosen as the stabilizing protein, as it is required for IGIP-induced CSR and would already be present in 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. 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 induce IgA CSR in the absence of additional rhCD40L.

ELISA

Sandwich capture ELISA was performed as previously described (39). Briefly, Immulon II 96-well round bottom plates (Dynatech, Chantilly, VA) were coated overnight at 4°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°C and washed three times with PBST. 50μl of B cell culture supernatants, diluted 1:1 in PBST, were added to each well and incubated for 1 hour at 37°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. Supernatant IgM, IgG and IgA concentrations were calculated based on the standard curve and analysis by linear regression with purified human protein for each respective isotype (Sigma, St. Louis, MO). 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 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'-CAGCAGCCCTCTTGGCAGGCAGC-3', Sμ antisense primer 5'-TGAGTGCCCTCACTACTTGAGTCCCG-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'-CTCAGCACTGCGGGCCCTCCA-3' and $S\mu_i$ antisense 5'-CAGACTGTCATGGCTATCAGGGGTGGCGGGG-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).

Results

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). 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, with relatively low levels of transcription detected in purified B cells (Fig. 1a). IGIP production by population and activation condition is 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.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. 2a), as did stimulation with CD40L, SIgA, and PMA/I (Fig. 2b). 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). 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. 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 any possible 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 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. 5). In this regard, IGIP was found to have a similar capacity to induce IgA expression as TGF-β plus IL-10, 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 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. 6). No product of the appropriate size was detected in DNA extracted from cells stimulated with CD40L alone.

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 has been definitively shown to induce CSR at the molecular level independent of other factors. Other DC-derived cytokines such as BAFF and APRIL 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 mDCs were the primary source of IGIP expression, much like the TNF family members BAFF and APRIL (35). In the present study we 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 to 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. 4), 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 cytokine, 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).

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. 2).

In addition to characterizing IGIP expression, we have 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. 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 Iα-Sμ switch circular extra-chromosomal DNA fragments (fig. 6).

In summary, our data support a role for VIP to regulate expression of IgA 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.

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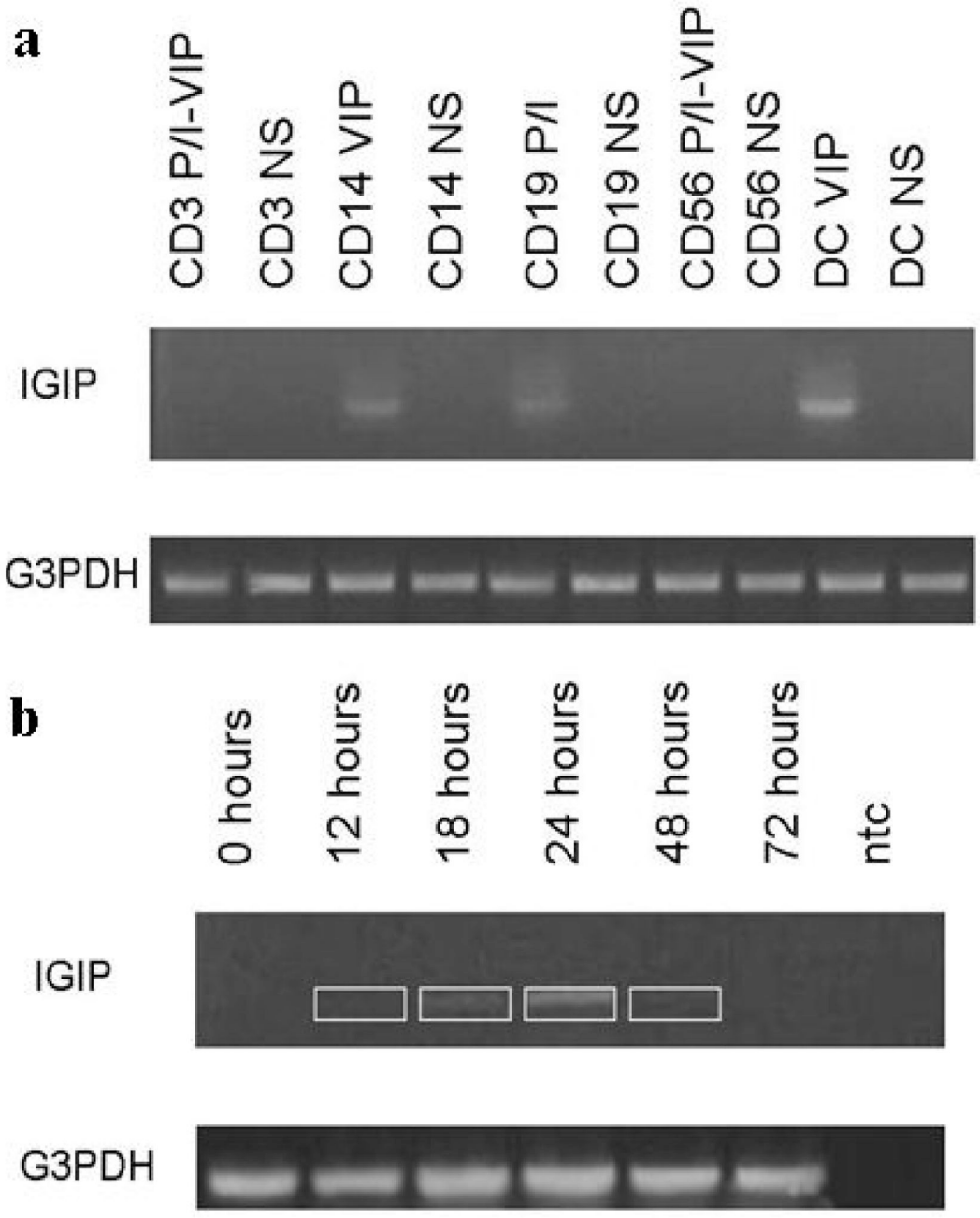

Figure 1. DCs are the primary source of IGIP

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 1 μM 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.

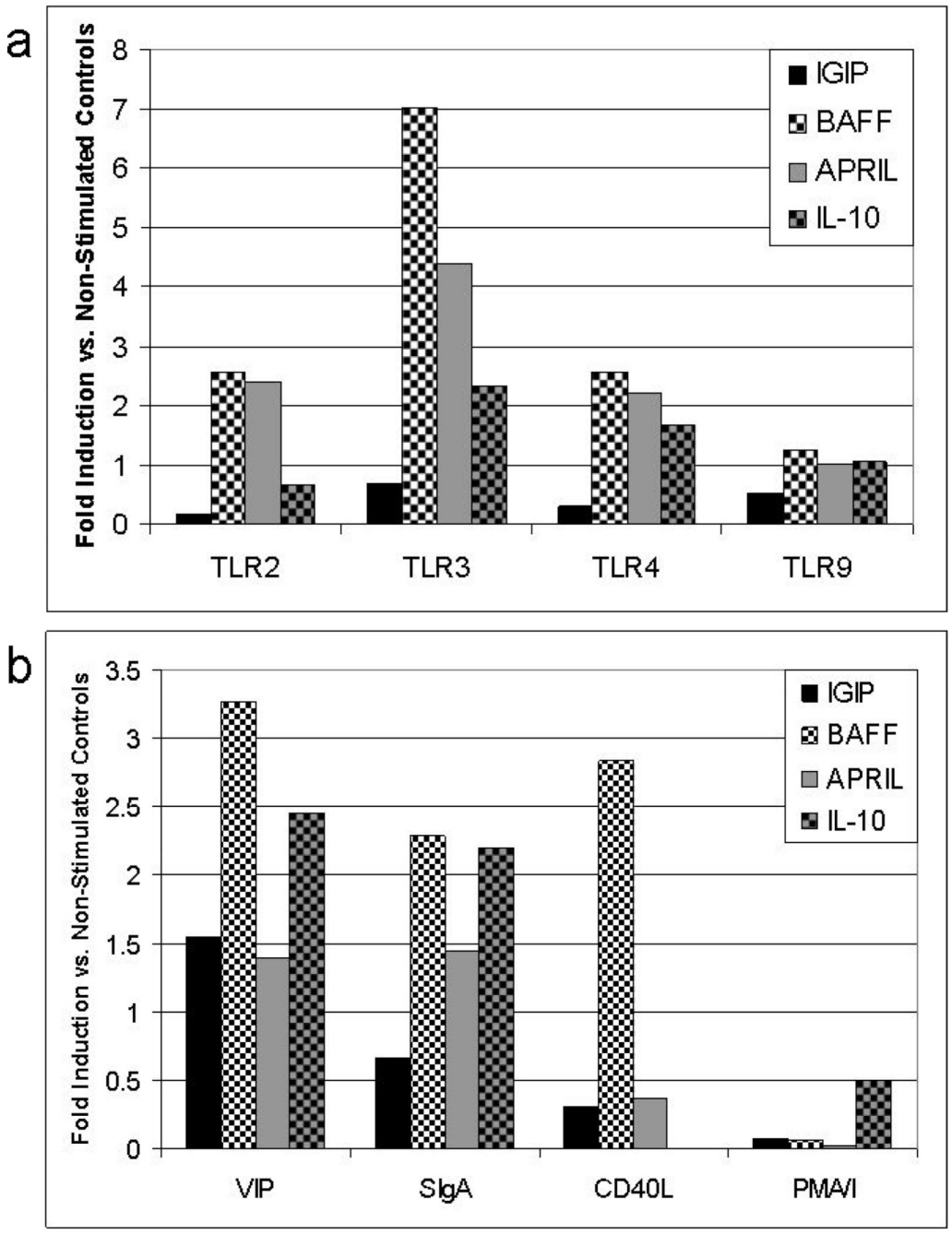

Figure 2. DCs express IGIP mRNA in response to VIP, but not TLR ligand, stimulation

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.

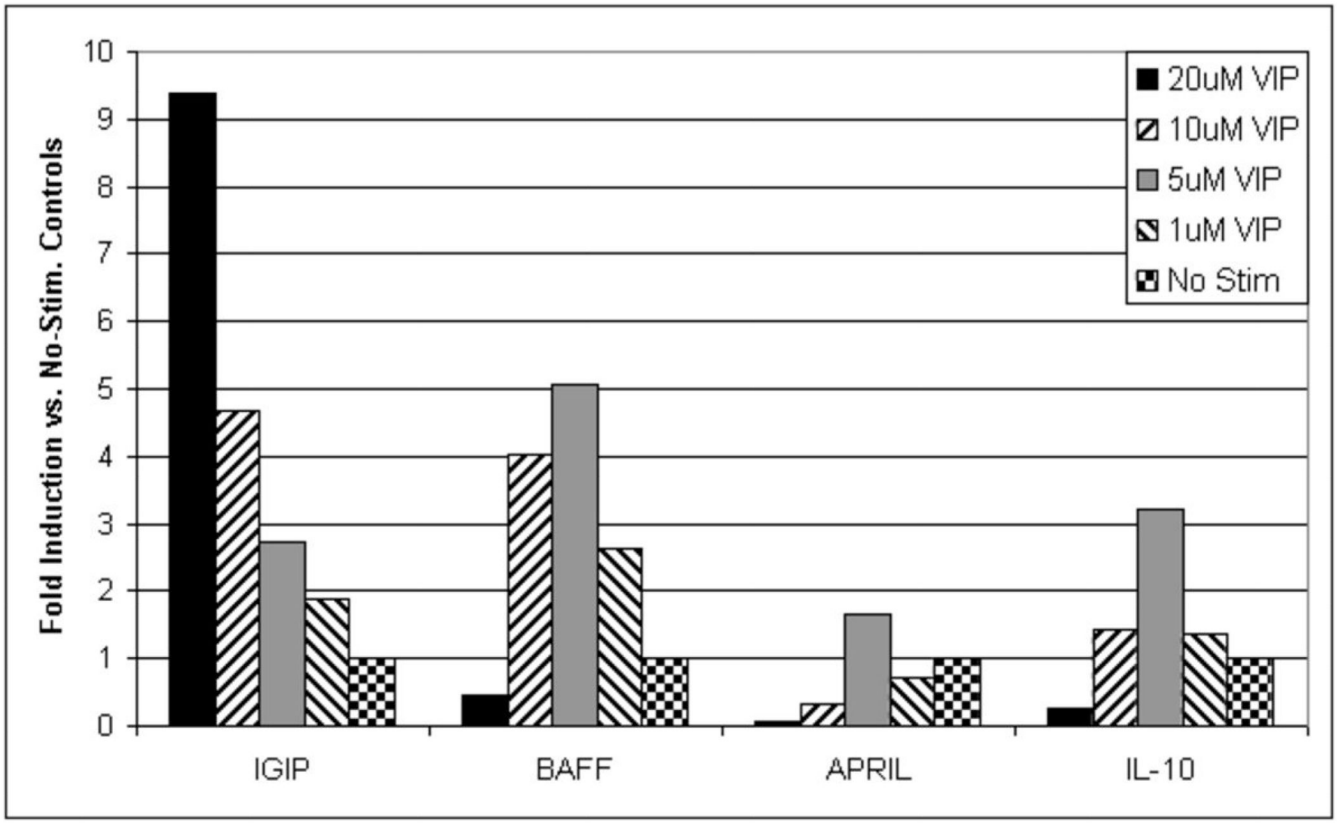

Figure 3. DCs express IGIP in response to VIP stimulation in a dose-dependent manner

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.

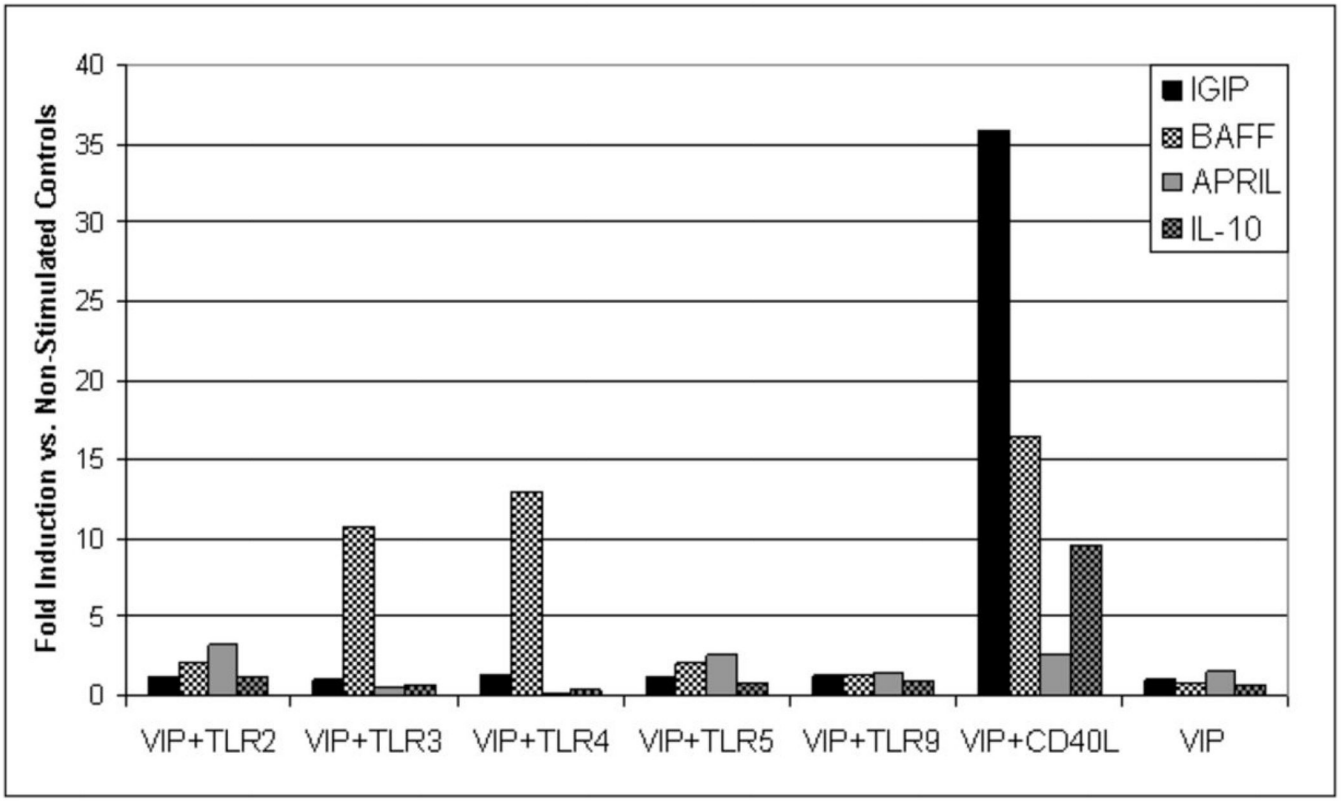

Figure 4. VIP acts synergistically with CD40L to upregulate IGIP expression

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.

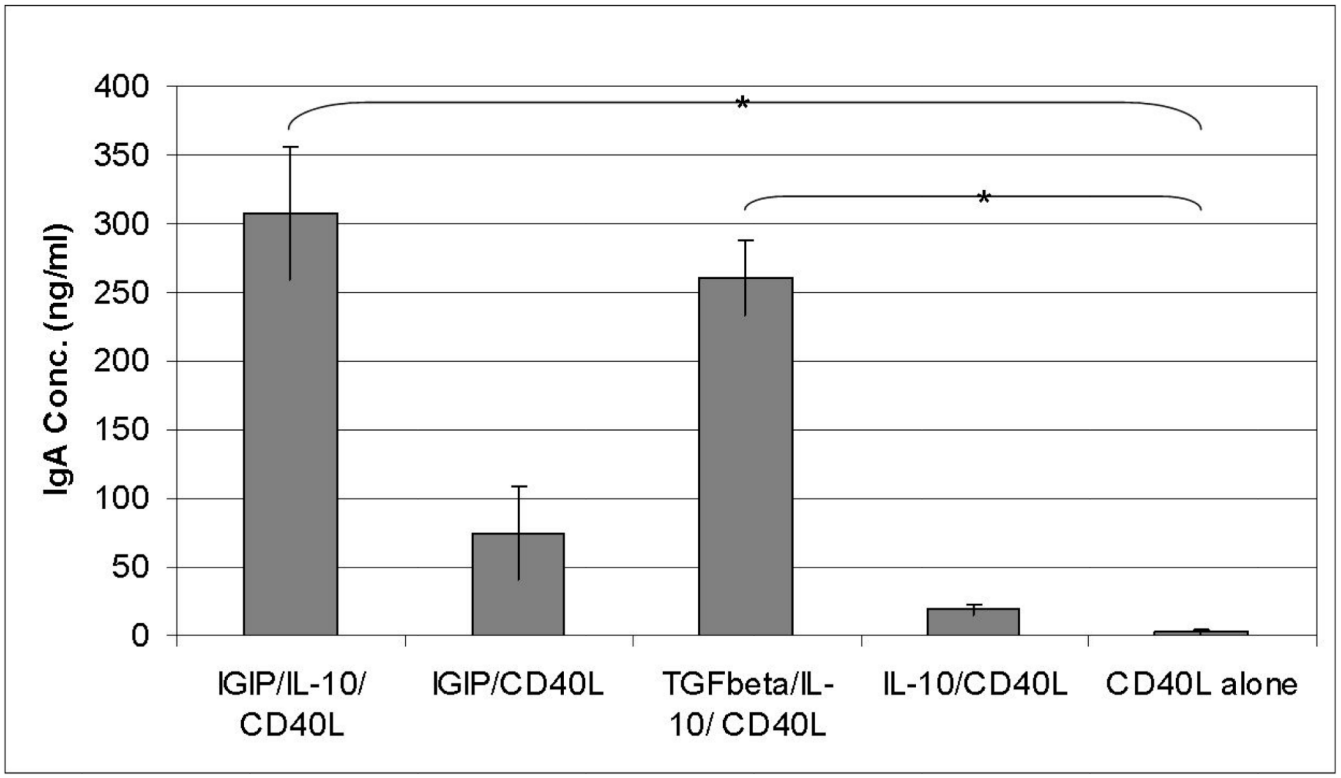

Figure 5. Recombinant IGIP induces IgA expression in the presence of IL-10

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.

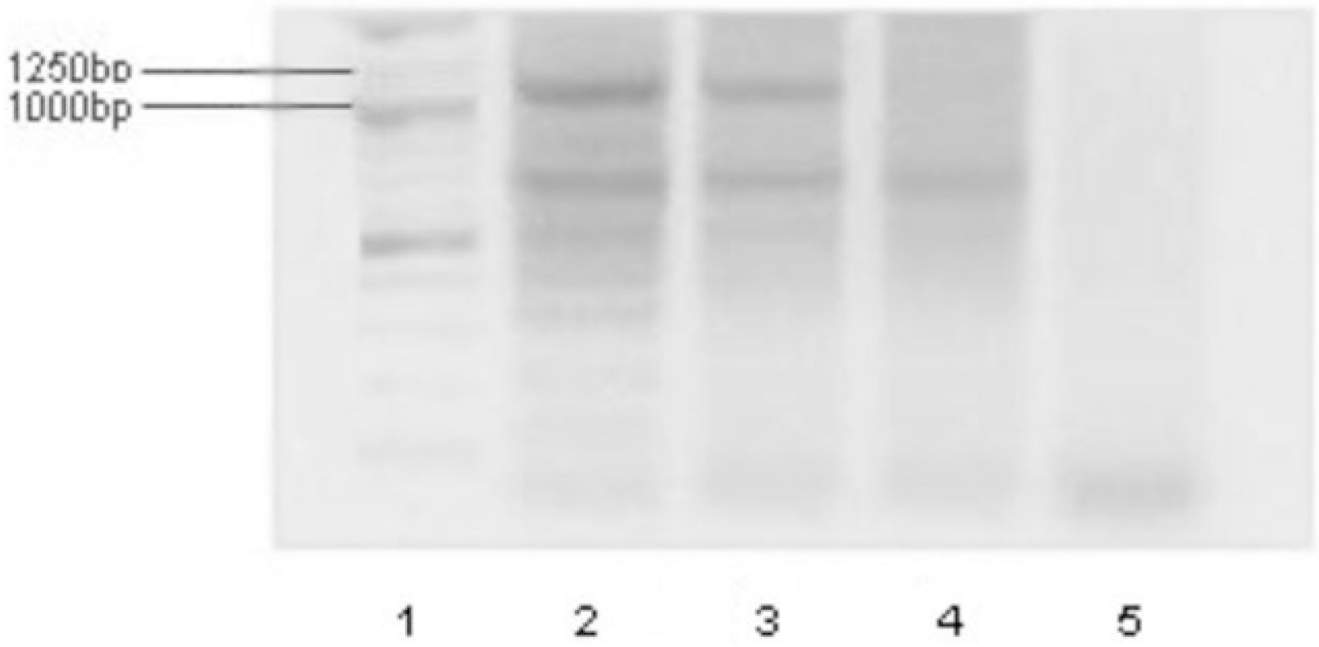

Figure 6. IGIP is an IgA switch factor

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.

Table 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.

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