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Identification of the Early VIP-Regulated Transcriptome and its Associated Interactome in Resting and Activated Murine CD4 T Cells

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Abstract

More than 40 years after the discovery of vasoactive intestinal peptide (VIP), its transcriptome in the immune system has still not been completely elucidated. In an attempt to understand the biological role of this neuropeptide in immunity, we chose CD4 T cells as a cellular system. Agilent Mouse Whole Genome microarrays were hybridized with fluorescently labeled total RNA isolated from resting CD4 T cells cultured $\pm 10^{-7}$ M VIP for five hours or PMA/ionomycin activated CD4 T cells cultured $\pm 10^{-7}$ M VIP for five hours. These VIP-regulated transcriptomes were analyzed by Significance Analysis of Microarrays (SAM) and Ingenuity Pathway Analysis (IPA) software to identify relevant signaling pathways modulated by VIP in the absence and presence of T cell activation. In resting CD4 T cells, VIP modulated 368 genes, ranging from 3.49 to -4.78 fold. In the PMA/ionomycin activated CD4 T cells, 326 gene expression levels were changed by VIP, ranging from 2.94 to -1.66 fold. IPA analysis revealed that VIP exposure alters cellular function through EGFR signaling in resting CD4 T cells, and modulates immediate early genes, *Fos* and *CREM/ICER*, in activated CD4 T cells. These gene expression changes are suggested to explain at a molecular level how VIP can regulate T cell homing to the gut and induce regulatory T cell generation.

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Keywords

Vasoactive Intestinal Peptide; Vasoactive Intestinal Peptide Receptor 1; Microarray; EGFR; Fos; T cell homing; regulatory T cells

1. Introduction

Vasoactive intestinal peptide (VIP) is a secreted, 28 amino acid protein that modulates numerous cell functions, such as migration and proliferation (Newman et al., 2005; Ottaway, 1987). Isolated in 1970 by Drs. Said and Mutt from porcine intestine (Said and Mutt, 1970), this peptide has a diverse tissue expression profile consistent with its pleiotropic activities. In the central nervous system, VIP acts as a neurotransmitter and potent neurotropic agent, and is also delivered to primary and secondary immune organs where it modulates immune function through a paracrine mechanism. VIP secretion by activated CD4 Th₂ cells is a secondary autocrine/paracrine source that can modulate immune function, and VIP has been suggested to function as a Th₂ cytokine with clinical potential in the treatment of certain autoimmune diseases (Ben-Horin and Chowers, 2008; Li et al., 2006). For example, VIP is protective against collagen induced arthritis (Delgado et al., 2001), inflammatory bowel disease (Abad et al., 2003), uveoretinitis (Keino et al., 2004) and experimental autoimmune encephalomyelitis (Gonzalez-Rey et al., 2006). The mechanism for this immunosuppressive effect by VIP is now thought to be due to the generation of antigen-specific regulatory T cells (Pozo et al., 2009). Unfortunately, more than 40 years after the identification of VIP, knowledge of its transcriptome in the CNS and the immune system has still not been well addressed. In the absence of such data, it will remain extremely difficult to fully understand the biological role of this neuropeptide.

A major impediment to obtaining a true VIP receptor-regulated transcriptome is the expression of several potential VIP-binding receptors in different combinations depending on the cell type. Thus, signal transduction elicited by these receptors would make it difficult to distinguish which receptor-mediated signaling pathway was responsible for gene expression changes. For example, vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor - 1 (VPAC1) and VPAC2 are expressed in the CNS and in the immune system, and bind VIP with a sub-nM K_d. These receptors belong to the secretin class IIB, G-protein coupled receptor (GPCR) superfamily, and elicit to varying extents, cAMP/PKA, PLD and PLC/Ca²⁺ activation (Delgado et al., 2004). A third receptor called pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1), also expressed in the CNS and the immune system, binds VIP with a μM K_d (Dautzenberg et al., 1999). Lastly, formyl peptide receptor-like 1 (FPRL1) exhibits weak affinity for VIP, and is widely expressed, including immune cells (El Zein et al., 2008). Therefore, in an attempt to continue the process of ascertaining the biological role of VIP in the immune system, as well as, satisfying the requirement for the high likelihood of a single VIP receptor (VPAC1) being predominately expressed, we chose CD4 T cells as a cellular system for determining a VIP-regulated transcriptome.

In support of identifying the VIP-regulated transcriptome using CD4 T cells, we offer the following rationale. First, we and others have demonstrated high, constitutive levels of VPAC1 with little, if any, detectable VPAC2 expression in both mouse and human resting CD4 T cells (Delgado et al., 1996; Lara-Marquez et al., 2001; Vomhof-DeKrey and Dorsam, 2008). Second, we have also shown that receptor reversal takes place during T cell activation with VPAC1 becoming downregulated and the inducible VPAC2 becoming upregulated, however, such changes peak between 12–96 hours post activation (Voice et al., 2004; Vomhof-DeKrey et al., 2008). Lastly, there has been no evidence that PAC-1 is expressed on T lymphocytes, with only weak expression of FPRL-1 (El Zein et al., 2008). Moreover, the FPRL-1 protein may

not be presented on the plasma membrane of leukocytes until after cellular activation. For these reasons, CD4 T cells were chosen to identify the VIP-regulated transcriptome.

In this study, murine CD4 T cells were isolated and used to identify global changes in gene expression in the absence (resting) and presence of PMA/ionomycin (activated) for five hours with and without 10^{-7} M VIP. Whole mouse genome arrays were utilized from six independent biological experiments with a 10% false discovery rate (FDR) as determined by the Significance Analysis of Microarrays (SAM). This analysis identified 103 and 58 unique genes differentially regulated ≥ 1.5 fold by VIP in resting versus activated CD4 T cells, of which 12 genes were commonly regulated. Ingenuity Pathway Analysis (IPA) identified a VIP interactome centered on the expression of epidermal growth factor receptor (*EGFR*) in resting CD4 T cells. In the presence of PMA/ionomycin, the VIP interactome supports a modest, but fundamental change in the repertoire of AP-1 and the inducible cAMP response element (*ICER*), which are transcriptionally competent nuclear master regulators. Collectively, these data encompass the mouse VIP-regulated transcriptome and interactome, and may provide important new knowledge regarding the molecular pathways controlled by VIP in T cell trafficking, while regulating Treg effector cell differentiation in activated cells.

2. Materials and Methods

2.1 Mice

Wild type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in ventilated cabinets (VWR) at North Dakota State University as previously described (Benton et al., 2009). Spleens from 6–32 week old males and females were harvested by standard dissection techniques in a sterile hood. All mouse protocols were approved by the NDSU IACUC board and met all federal guidelines.

2.2 Murine CD4 T cell Isolation

Mouse splenic CD4 T cells were purified as previously described (Vomhof-DeKrey and Dorsam, 2008). Briefly, each independent experiment (six biological replicates) used five to ten harvested spleens forced through a wire mesh in PBS at RT, and passed through a 70 μ m sieve. Erythrocytes were lysed, passed through a 70 μ m sieve a second time, and centrifuged as above. Cells were resuspended in 93 μ l of PBS/0.5% BSA with 7 μ l of anti-mouse CD4 magnetic beads/ 1×10^7 cells (Miltenyi Biotech; Auburn, CA) at 4–8°C for 20 minutes, and purified on a Miltenyi Auto-MACS instrument using the Possel program (Miltenyi Biotech; Auburn, CA). Some samples did not undergo erythrocyte lysis.

2.3 Antibody staining

To determine percent purity, 0.5×10^6 total splenocytes (pre-separation) and enriched CD4 T cells (post-separation) were resuspended in 100 μ l PBS/0.5% BSA, and incubated with 1:200 FITC conjugated rat anti-mouse CD4 antibody (clone RM4-4, Biolegend, San Diego, CA) for 30 min at 4°C in the dark as previously published (Benton et al., 2009). Cells were washed with 1 ml PBS/0.5% BSA, centrifuged for 5 min at $500 \times g$, and resuspended in 300 μ l PBS/0.5% BSA. Rabbit anti-mouse CD25 (IL-2 receptor alpha chain; PC61.5 clone) and anti-mouse CD62L (L-selectin; MEL-14 clone) antibodies purchased from Biolegend were used to stain isolated cells. Flow cytometry was performed on a Becton Dickinson FACS Calibur (Franklin Lakes, NJ) or an Accuri flow cytometer (Ann Arbor, MI). Note that the presence of anti-mouse CD4 magnetic beads did reduce the decade shift slightly for the FITC conjugated rat anti-mouse CD4 antibody (clone RM4-4), but still shows successful enrichment (Fig. 1A).

2.4 Quantitative RT- PCR

Total RNA was isolated using RNeasy mini columns (Qiagen, Valencia, CA) with on-column DNase I treatment performed as previously published (Vomhof-DeKrey and Dorsam, 2008). For cDNA synthesis, 0.5 microgram of total RNA was reverse transcribed using 250 ng of random hexamers and M-MLV reverse transcriptase according to the manufacturer's protocol (Promega; Madison, WI). Samples were incubated at 37°C for 60 minutes and used immediately or stored at -20°C until assayed. Five microliters of cDNA was used as template with 400 nM specific primers (IDT DNA technologies, Coralville, IA; Supplemental Table 1) and 7.5 µl of 2× Fast SYBR Green Mastermix. Samples were amplified in an ABI7500 instrument (Applied Biosystems, Foster City, CA), and relative changes were calculated by the $\Delta\Delta C_t$ method using β -actin (*Actb*) as a reference control gene.

2.5 cAMP quantitation

Intracellular cAMP concentration was measured by a competitive ELISA (Assay Designs; Ann Arbor, MI) as previously published (Vomhof-DeKrey et al., 2008). Briefly, stable CHO-K1 clones overexpressing human VPAC1 were seeded in 96 well tissue culture plates (Corning) in 100 µl at 5×10^6 cells/ml and incubated for 24 hours in culture media and G418 at 1 µg/ml. Cells were washed twice with 1× PBS and pretreated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in HBSS, 0.5% BSA (binding buffer) for 45 minutes at 37°C to limit cAMP catabolism. Cells were washed as above and incubated with binding buffer and increasing concentrations of exogenously added VIP ligand (10^{-14} to 10^{-6} M) for 15 minutes at 37°C. Cells were then washed with ice-cold PBS and lysed with 0.1 M HCl. The plate was incubated at RT with shaking for 30 minutes and immediately used for measurements in the cAMP competitive ELISA or stored at -20°C until assayed. Each ELISA measurement was accompanied with a cAMP standard curve (0.78 – 200 pmol/ml) and a positive (10^{-6} M forskolin) and negative control (water), and optical density (450 nm) was measured using a Multiskan Spectrum spectrophotometer (Thermo Scientific; Waltham, MA).

2.6 Murine CD4 T cell in vitro culture conditions

Cells were cultured at 2×10^6 cells/ml in RPMI supplemented with 10% defined fetal bovine serum, L-glutamine, 1× penicillin/streptomycin at 37°C in 5% CO₂. PMA (phorbol 12-myristate 13- acetate) and ionomycin were purchased from Sigma Aldrich (St. Louis, MO), and VIP was purchased from American Peptide Company (Sunnyvale, CA). Resting cells received water (vehicle control) or 10^{-7} M VIP for 5 hours, and DMSO vehicle control for PMA/ionomycin. For T cell activation conditions, cells were treated with 10 ng/ml PMA and 1 µM ionomycin in the presence or absence of 10^{-7} M VIP for 5 hours.

2.7 Global expression profiling

All microarray experiments and statistical analyses were performed by the Marshall University Genomics Core Facility (<http://musom.marshall.edu/genomics/>). To determine the effect of VIP on resting and PMA/ionomycin activated mouse CD4 T lymphocyte gene expression, we performed expression profiling using Agilent Mouse Whole Genome Arrays (Agilent, Santa Clara, CA) in a balanced block design. RNAs from individual VIP resting (CD4 T cells -/+ VIP) and activated (PMA/ionomycin -/+ VIP) were compared in a single array so that there were six biologically independent sets of array data for each comparison. Total RNA quality was determined on an Agilent 2100 Bioanalyzer. All samples had RNA integrity numbers (RIN) greater than 8. Total RNA (250 ng) was used for labeling using the QuickAmp labeling kit (Agilent) and cyanine (Cy)-3-CTP and Cy5-CTP (Perkin Elmer, Waltham, MA). Three of the six arrays contained control cRNA labeled with Cy5 and VIP-treated cRNA labeled with Cy3 (Perkin Elmer, Waltham, MA). The remaining three arrays were flip-labeled to minimize the effect of bias in dye incorporation. Following purification of labeled cRNAs, 825 ng of

Cy3- and Cy5-labeled cRNAs were combined and hybridized for 17 hours at 65°C in an Agilent hybridization oven. Microarrays were then washed and scanned using Agilent DNA Microarray Scanner.

2.8 Statistical Analysis of Microarray Data

Feature intensities were extracted in lowess-normalized form from the scanned image using Agilent Feature Extraction software. All extracted data were exported to Microsoft Excel (Microsoft Corporation, Redmond, WA) as tab delimited files. For each feature, a value was considered present if at least one channel had a signal well above background according to Feature Extraction. The log base 2 of the expression ratios of resting with VIP ligand (sample) to resting without VIP ligand (control) or the log base 2 of the expression ratios of activated with VIP ligand (sample) to activated without VIP ligand (control) were computed, and assembled into a single tab-delimited file for each comparison. Features for which less than 50% of the arrays produced a present value were removed from future analyses. Each file was then imported into the Multiple Experiment Viewer (MeV) v4.3 for statistical analysis (Saeed et al., 2006).

Calculated log ratios were compared for significant deviation from zero using one-class Significance Analysis of Microarrays (SAM) (Tusher et al., 2001). SAM was performed with the maximum number of unique permutations available, and delta values were chosen to give a median False Discovery Rate (FDR) of 10%. All other parameters were set to the MeV defaults. The resulting list of statistically significant probes was exported to tab-delimited format and opened using Microsoft Excel. For each feature, the mean log base 2 ratio across all arrays was computed and used to calculate the average fold change between samples and controls. For genes which were represented by multiple features on the array, the feature with the largest fold change was reported. The log base 2 ratios were used to calculate a 95% confidence interval for fold change. Features for which the confidence interval includes 1 were removed from the results. This initial expression profile ("no threshold" dataset) was subsequently filtered for a minimum fold change greater than 1.5 (threshold dataset). The "no threshold" data set was used in the IPA analysis of VIP-regulated interactome (Fig. 4–Fig 6). Microarray data may be accessed at the NCBI Gene Expression Omnibus (GEO) database (GSE18526).

2.9 Network analysis

Excel files containing gene names and fold change values from SAM analyses using a 10% FDR were uploaded onto the Ingenuity Pathway Analysis (IPA) website (version 7.5) (Ingenuity Systems, Inc, Mountain View, CA). Analysis settings included the reference set of the Ingenuity Knowledge Base (Genes only), which is a manually curated database of the scientific literature that documents the direct and indirect relationships between genes and gene products, such as protein-protein or protein-DNA interactions, activation/inhibition, localization, translocation or expression (Calvano et al., 2005). Summary of Analysis reports were generated for resting and activated datasets individually, and included the top five most significant Networks created by the proprietary IPA Network Generation Algorithm. Networks are constructed to contain up to 35 Focus Genes (VIP modulated gene targets) and are optimized for high degrees of interconnectivity among them. They present the interactions between "Focus Genes" and intermediate "non-Focus Genes" based on the premise that highly interconnected molecules may be more likely to characterize a biological function. IPA reports also contained the top five most significant Biological Functions (Diseases and Disorders, Molecular and Cellular Functions, Physiological System Development and Functions), Canonical Pathways and Toxicological Lists. Network Scores and p values were assigned by IPA software. The Core Analysis prepares a Summary of Analysis report including Networks of genes categorized by functional relationships. Networks are graphical displays that describe

the relationships between a subset of genes and their neighboring genes. For example, if two molecules have a relationship, such as a protein-protein interaction or one activates the expression of the other, they are connected by a line. If they are related to a third molecule and a fourth, then additional lines are drawn, and a network is formed. According to the degree of interconnectedness among the molecules observed, a higher or lower IPA network score is assigned. The top three Networks of each dataset were imported into the Path Designer to assign symbols to the molecules to designate their functions.

3. Results

3.1 Assessment of CD4 T cell purity, VIP receptor expression profile and phenotypic changes of activated cells

As this study aimed to identify VIP gene targets in primary CD4 T cells, isolation by magnetic bead technology was performed to obtain a high purity ($\geq 95\%$) of this cell population from total splenocytes as determined by flow cytometry (Fig. 1A). To evaluate the predominant VIP receptor expressed on CD4 T cells, the mRNA expression levels of four known VIP receptor genes, *Vpac1*, *Vpac2*, *Pac1* and *Fprl1* were measured by qRT-PCR. *Vpac1* was the predominately expressed VIP receptor, showing 208 fold higher levels than *Fprl1* and 3327 fold higher levels than *Vpac2* or *Pac1* (Fig. 1B). Figure 1C demonstrates that these cells were successfully activated by PMA/ionomycin treatment (Truneh et al., 1985) based on measured mRNA levels by qRT-PCR and microarray analysis that revealed an upregulation of IL-2 receptor alpha chain (*Il2ra*) transcript and a downregulation of L-selectin (*Sell*) transcript (Altman et al., 1992; Buhrer et al., 1990). To assess surface protein levels, flow cytometry was utilized, and as expected, after five hours of stimulation, the cells exhibited CD62L (*Sell*) shedding, but no changes in CD25 (*Il2ra*) protein expression could be detected at this time point. These data confirmed an early activation CD4 T cell phenotype in the cells assessed at 5 hours (Fig. 1D). Lastly, Figure 1E demonstrates that the synthetic VIP used in these studies was biologically active, inducing readily detectable intracellular cAMP ([cAMP]i) concentration in human VPAC1 overexpressing CHO-K1 clones (Fig. 1E). These data validated that we were able to obtain a highly purified, resting, splenic CD4 T cell population expressing high levels of VPAC1, which resulted in an early activated phenotype after PMA/ionomycin treatment (5 hours) that were assessed for gene expression changes using a functionally active VIP ligand.

3.2 Microarray analysis identifies a VIP transcriptome in resting and PMA/ionomycin activated mouse CD4 T cells

In order to identify the early genes modulated by VIP in CD4 T cells, we used DNA microarray expression profiling. Purified CD4 T cells were incubated *in vitro* in the absence and presence of PMA/ionomycin (resting and activated), in which each group was cultured without and with 10^{-7} M VIP for five hours. While this concentration is higher than that found physiologically, it is still well within the acceptable range generally used in the VIP field. Fluorescently-labeled cRNAs were prepared from total RNA and hybridized to Agilent Whole Mouse Genome Arrays containing 45,220 features which represent approximately 41,000 unique mouse genes and transcripts (Figure 2A). Microarray expression ratios were analyzed by the SAM program. Using a 10% FDR, 368 genes were identified as modulated by VIP in the resting cells as shown in the Venn diagram in Figure 2B. This dataset included 286 upregulated genes and 82 downregulated genes, ranging from 3.49 fold upregulated to 4.78 fold downregulated. Using the same FDR, 326 genes were identified to be modulated by VIP in the presence of PMA/ionomycin. These data identified 312 upregulated genes and 14 downregulated genes ranging from 2.94 fold upregulated to 1.66 fold downregulated. Using a 1.5 fold cutoff and deleting genes for which the confidence intervals included 1, two smaller datasets were generated and shown in Fig. 2B (right side). There were 12 transcripts in common between resting and

activated cells, which are listed in Figure 2C. Both resting and activated datasets with a 1.5 fold cutoff were uploaded into the Ingenuity Pathway Analysis search engine (<http://www.ingenuity.com/>). IPA grouped these transcripts into functional categories and defined their cellular locations. Graphical representation of functional group percentages and a list of fold changes with their cellular locations are shown in Figure 2D and Table 1. A complete list of SAM-filtered genes identified for resting and activated samples, including ESTs and accession numbers, are provided in Supplemental Tables 2 and 3, respectively. These data represent the early gene targets in the VIP transcriptome of murine CD4 T cells. We conclude that CD4 T cells interpret VIP, presumably induced by VPAC1 receptor signaling, by regulating two nearly unique transcriptomes (resting and activated) with $\geq 81\%$ uniquely modulated genes. It would therefore follow that cellular changes by VIP would be dictated primarily by the activation status of CD4 T cells.

3.3 Verification of VIP-regulated microarray gene expression changes by qRT-PCR

Microarray ratios obtained for several VIP-modulated transcripts were independently corroborated by qRT-PCR analysis using primary CD4 T cells. All 13 genes analyzed by qRT-PCR showed a change in expression by VIP treatment similar to fold changes obtained by microarray analyses (Figs. 3A and 3B). Collectively, we conclude that this VIP transcriptome represents an accurate and precise dataset of VIP regulated gene expression changes.

3.4 Network analyses of the VIP transcriptome in CD4 T cells

To interpret our data in the context of biological processes, pathways and networks, IPA Core Analyses were conducted using the 10% FDR SAM dataset without a threshold cutoff, thus increasing the probability of identifying biologically relevant signaling pathways modulated by VIP that might normally be ignored based on a purely statistical basis (i.e. using a 1.5 fold filter). The top three IPA generated gene networks for the resting and PMA/ionomycin activated datasets are shown in Figure 4 and Figure 5, with Table 2 listing the top five categories of functions and related diseases modulated by VIP. Consistent with the observation that VIP signaling promotes nearly unique transcriptomes dependent on PMA/ionomycin exposure, the IPA network analysis showed significantly different changes at the molecular and functional levels (60% and 40%, respectively), and completely different disease states (Table 2). The complete lists of genes in each functional category with p values assigned by the IPA software are provided in Supplemental Tables 4 and 5. Examples include lists of genes involved in cellular movement, homing and chemotaxis for the resting dataset (*APP*, *CHST2*, *EGFR*, *EPHB2*, *F2*, *FGFR1*, *IL5*, *PAK1*, *UPAR/PLAUR*, *PLG*, *PRKCA*, *PRNP*, *COX2/PTGS2*, *RNASE2*, *TNFSF11*, *VEGFA*), and genes involved in T cell proliferation the activated dataset (*CD14*, *CD28*, *CXCR4*, *DUSP10*, *F2RL1*, *FYB*, *GRAP2*, *IGF1*, *IL23A*, *KITLG*, *MYB*, *PDE4D*, *PTGS2/COX2*, *RGS2*, *SATB1*, *TCF7*, *TG*, *THY1*, *TNFRSF1A*, *TNFRSF1B*, *TSC22D3*, *TSLP*, *TXK*, *UBASH3A*). However, significant functions were also shared between the resting and activated datasets demonstrating that VIP signaling may control cell cycle checkpoints and apoptosis, as well as, regulate hematopoietic developmental programs through the small, but common gene set identified in Figure 2B and 2C. Taken together, VIP signaling affects similar and different CD4 T cell functions, which may translate to vastly different physiological states, dependent on the activation status of T cells.

We also investigated relationships between molecules without a bias towards functional categorization. To this end, we queried the same two resting and activated “no threshold” datasets by using IPA’s Build and Connect functions to reveal networks of only direct interactions (the indirect option was deselected). One large network, termed “VIP interactome,” for each dataset emerged, and these are shown in Figure 6. A major hub of connectivity for the resting sample network was *Egfr* with 11 edges (direct connections), while the activated sample network showed *Fos* as a hub of connectivity, as it appears to play quite

a significant role possessing 19 direct relationships. We conclude that VIP signaling may be altering cellular function through EGFR signaling in resting CD4 T cells, while altering immediate early genes (*Fos*, *FosB* and *CREM/ICER*) in activated CD4 T cells.

4. Discussion

To date, only small scale studies have been performed on the VIP transcriptome in Th₂ CD4 T cells, lung, colon and synovial fibroblasts (Abad et al., 2005; Arranz et al., 2008; Hamidi et al., 2008; Sharma et al., 2006). To our knowledge, the present study represents the largest microarray survey of a VIP transcriptome in any cell type, and thus presents a comprehensive set of early VIP gene targets in a T lymphocyte, most of which are new to the neuroimmunology field. However, several VIP targets identified in this study have also been previously published, and support the present study, though the direction of change observed was not always the same. For example, VIP was shown to inhibit *Cxcl10* (IP-10) expression in LPS-stimulated total splenocytes and purified CD11b macrophages (Jiang et al., 2002). In contrast, we show *Cxcl10* expression to be upregulated in PMA/ionomycin activated T cells. Another example is the transcription factor, *Egr3*, that has been shown to be downregulated by VIP in an anti-CD3 activated T cell hybridoma (Delgado and Ganea, 2001), while we did not show this gene being modulated by VIP in PMA/ionomycin activated CD4 T cells, but rather in resting cells. These types of discrepancies may be due to “real” differences like cell types, VIP receptor expression combinations and/or activating conditions, or “manipulative” differences through cell culture conditions. Nonetheless, the agreement between these VIP target changes in gene expression lends credence to both the current study and those previously reported in the literature.

It is now well-accepted that VIP-binding sites observed on mouse and human resting CD4 T cells are due to VPAC1 receptor expression (Johnson et al., 1996). Our data supports this observation as our quantitative analysis of enriched CD4 T cells clearly revealed an enormous skewing towards VPAC1 mRNA expression dwarfing that of other known VIP binding receptors: VPAC2, PAC1 and FPRL-1, respectively (Fig. 1B). Our experimental setup was purposefully short (5 hours) to limit potential changes in VIP receptor expression levels (Vomhof-DeKrey and Dorsam, 2008; Vomhof-DeKrey et al., 2008). For these reasons, we propose that the VIP transcriptome identified by this study is indicative of the VIP/VPAC1 receptor signaling axis in both resting and activated CD4 T cells. However, we cannot rule out the contribution of alternatively spliced variants of VPAC1 (Bokaei et al., 2006), and/or unknown VIP receptors expressed by resting CD4 T cells participating in the observed VIP-regulated transcriptome.

In the late 1980's, Ottaway et al. showed strong evidence that the presence of VIP binding sites was crucial for proper T cell homing to lymphatic compartments, most notably to mesenteric lymph nodes and Peyer's patches (PP) (Ottaway, 1984). VIP-induced chemotaxis was shown to be sensitive to tyrosine inhibition, pertussis and cholera toxins, but insensitive to PKC inhibition. Several groups subsequently showed that purified mouse and human T cells elicited a significantly repressed chemotactic response to VIP due to T cell receptor signaling (e.g. activation) (Johnston et al., 1994). As activated CD4 T cells show significant VPAC1 mRNA downregulation (Lara-Marquez et al., 2001; Vomhof-DeKrey et al., 2008), reduced VIP-induced chemotaxis of activated T cells supports a VPAC1 signaling dependent mechanism (Johnston et al., 1994). Importantly, there exists strong anatomical evidence for a VIP/VPAC1-induced chemotactic recruitment of naïve T cells to gut lymph and PP. VIPergic nerves have been identified in proximity with specialized endothelial cells leading to postcapillary venules of PP, thus providing a neuronally-supplied, paracrine signal for blood CD4 T cells to migrate into PP (Ottaway et al., 1987). These data imply that *in vivo* homing of resting, unactivated

CD4 T cells, in sharp contrast to activated CD4 T cells, positively respond to the VIP/VPAC1 signaling axis for proper trafficking to lymph compartments in the gut tract.

One major contribution of our IPA evaluated microarray study provides a possible molecular mechanism mediating VIP-induced homing of resting CD4 T cells. The VIP interactome in the absence of PMA/Ionomycin showed epidermal growth factor receptor (EGFR) as the most predominant hub of connectivity with 11 edges (Fig. 5). VIP addition induced mRNA expression of *Egfr* by 1.4 fold (range 1.3–1.6 fold; n=6) after 5 hours. This modest upregulation of *Egfr* mRNA coincided with the upregulation (1.3 fold; range 1.1–1.6; n=6) of *Adam15*, a disintegrin and metalloprotease 15 enzyme, a membrane-anchored glycoprotein that is responsible for proteolytic detachment of membrane-associated soluble EGFR ligands, and increased adhesion to integrins through its RGD domain (Charrier-Hisamuddin et al., 2008; Tanaka et al., 2004). The upregulation of *Egfr* and *Adam15* may represent a coordinated response to VIP resulting in the elevation of EGFR signaling. A future goal will be to determine whether different VIP exposure intervals, and different VIP concentrations will result in greater, fewer or different gene expression changes. Furthermore, there is evidence for a rapid and direct increase in EGFR signaling by VIP in human breast cancer cells, as VPAC1 signaling has been shown to transactivate EGFR (HER1) and HER2 proteins within minutes of VIP addition through a cAMP/PKA/Src dependent pathway (Valdehita et al., 2009). We conclude that rapid (min) and slow (hours) elevation of EGFR signaling may mediate CD4 T cell directed movement into PP induced by neuronally supplied VIP ligand.

Additional evidence supporting VIP-regulated trafficking of resting CD4 T cells is the identification of additional signaling molecules known to be involved in EGFR signaling that are also involved in regulating cell migration. First, growth factor receptor bound (Grb) 7, a cytoplasmic adaptor molecule containing a src homology (SH) 2 domain, has been shown to be recruited to tyrosine phosphorylated EGFR dimers to enhance cell migration (Holt and Daly, 2005; Margolis et al., 1992). In fact, *Grb7* has a conserved region encompassing a Ras-association (RA) and a pleckstrin homology (PH) domain that has high homology with the cell migration Mig10 protein in *C. elegans* (Manser et al., 1997). Second, p21 activated serine/threonine kinase 1 (*Pak1*), is part of the Rac/cdc42 GTPase signaling pathway, and essential for cell migration (Guo et al., 2007). The EGFR kinase inhibitor, ZD1839, blocked Pak1 activation and EGF-induced cytoskeletal rearrangements and tumor invasion in human head and neck cancer cells (Yang et al., 2004). Moreover, PAK1 also plays an essential role in neuronal guidance and cell migration in the CNS, where VIP acts as a neurotransmitter (Nikolic, 2008). Third, is the zinc finger transcriptional repressor, *Snail1*, whose protein expression is upregulated by EGFR activation mediated by several signaling molecules, including PAK1 (Hipp et al., 2008). PAK1 activity has been shown to alter the subcellular localization of Snail1 through phosphorylation (Yang et al., 2005). Snail1 is one of the most potent transcriptional repressors of E-Cadherin, a suppressor of tumor invasion, and the loss of this protein is a hallmark of epithelial-mesenchymal transition that assists in tumor metastasis (Birchmeier and Behrens, 1994). Fourth, the type I transmembrane amyloid precursor protein (APP), which causes neuronal plaques in Alzheimer's disease, plays a role in cell adhesion and migration in neurons and keratinocytes, and its structure would suggest a growth factor like molecule (Sabo et al., 2001; Sheng et al., 2009). Fifth, the urokinase-plasminogen system (uPA) has been demonstrated to act at the leading edge of polarized, migrating cells and is a tumorigenic marker (Kook et al., 1994; Nusrat and Chapman, 1991). Two uPA genes were differentially modulated by VIP. The urokinase-type plasminogen activator receptor (*uPAR/Plaur*) was upregulated, whereas plasminogen (*Plg*) was downregulated. *Plaur* can physically bind and therefore illicit enhanced EGFR signaling (Mazzei et al., 2006). Lastly, there were a number of other genes that encode for proteins related to cellular migration, adhesion and cytoskeletal reorganization. They were: *Scrt1* (Nakakura et al., 2001), *Wwc1* (Duning et al., 2008), *Unc5B* (Wang et al., 2009), *Palld*

(Goicoechea et al., 2009), *Lgals* (Gendronneau et al., 2008), *Tubb2a* (Valenzuela-Fernandez et al., 2008) and *Myo1b* (Tang and Ostap, 2001). *In toto*, we conclude that VIP induces a resting CD4 T cell program that promotes directed T cell migration (e.g. homing) through an EGFR centrally regulated signaling pathway. We further speculate that neuronally relevant genes (e.g. APP and PAK1) have been usurped by nature to regulate T cell biology by VIP/VPAC1 signaling, a well-established neuronal signaling axis. Future research will be essential to determine the regulatory controls and cellular context mediating this molecular mechanism *in vivo*.

VIP has been demonstrated to affect lipid metabolism by shifting the balance from adipogenesis towards lipolysis (Richter et al., 1989). Interestingly, this metabolic response may be consistent with a VIP-regulated migratory program in resting CD4 T cells. Support for this supposition is an elegant study that showed the need for eicosanoid production (PGD₂) as a necessary second signal for activated neutrophils to complete their transmigration from the vasculature to the interstitial stroma (Tull et al., 2009). It would therefore be enticing to speculate that the lipid metabolism effects by VIP, as determined by our IPA analysis (Fig 4 and Fig 5), is also needed for resting T cell transmigration during its normal trafficking route from the vasculature to lymph. Elevated PGD₂ production is dependent on the availability of its arachidonate (AA) precursor and enzymatic activity of prostaglandin synthetase I (COX1) and the inducible COX2 (*Ptsg2*) (Sandig et al., 2007). Our VIP interactome indicates an increase in *COX2/Ptsg2* message that might provide the necessary elevated enzyme activity needed for diverting AA release towards production of prostaglandins, a necessary signal for transmigration of activated neutrophils. A plausible explanation for how VIP might be shifting the balance towards lipid peroxidation and prostaglandin production is through vascular endothelial growth factor (*Vegfa*), previously shown to be upregulated by VIP and supported by this study, which binds to the VEGF receptor, and upregulates COX2 expression in certain cell types (Soltau and Dreys, 2009) (<http://www.genome.jp/kegg/pathway/hsa/hsa04370.html>). In contrast, VIP has been shown to downregulate COX2 expression in myeloid cells (macrophages), microglial cells and dendritic cells (Gonzalez-Rey and Delgado, 2008). Currently, these differences in VIP-mediated modulation of COX2 are unexplained, but may be due to cell-context dependent regulation of COX2 by the VIP signaling axis.

Treatment of CD4 T cells with PMA/ionomycin mimics TCR signal transduction that leads to an elevation of IL-2 and a proliferative, “activated” cellular program (Grier and Mastro, 1986). Significant alterations in the transcriptional profile occur during T cell activation, including increases in the transcriptional rate of certain *Jun* and *Fos* proteins that dimerize to form the immediate early transcription factor called AP-1 (Crabtree and Clipstone, 1994). These early changes in gene expression are necessary to induce the proliferative phenotype observed during TCR signaling. However, IL-2 expression and subsequent T cell proliferation can be blocked by an antagonistic signal transduction pathway. This opposing pathway elevates the intracellular second messenger, cAMP ([cAMP]_i), resulting in protein kinase A (PKA) activation, and leading to a “reshuffling” of the AP-1 composition due to subtle changes in *Jun* and *Fos* expression (Tamir et al., 1996). It would therefore follow that VIP treatment of CD4 T cells would lead to the activation of the cAMP/PKA pathway as observed for other cAMP inducing agonists. To support this supposition, our data shows increased *Fos* expression, coupled with a 30% increase in *FosB* expression. These data are supported by a study showing cell-permeable cAMP (dbcAMP) elevating *Fos* and *FosB* expression over that observed by activating conditions alone (Tamir et al., 1996). In contrast, these studies also showed differential changes in *Jun* (decrease) and *JunB* (increase) due to co-treatment with dbcAMP and TPA/ionomycin, but were measured at 1–3 hour versus our 5 hour time point. Such early changes in *Jun* and *Fos* expression by VIP have been reported by Wang *et al.* that demonstrated similar changes between 1–3 hours using primary CD4 T cells. However, this same group did not measure a change in *FosB* expression due to VIP treatment (Wang et al., 2000). Such

discrepancies in *Jun* and *Fos* expression due to cAMP inducing agents (VIP or dbcAMP) are most likely a result of temporal differences and/or the use of T cell lines versus primary CD4 T cells isolated from different mouse strains (C57Bl/6 or Balb/c). Nonetheless, modest changes of 30% in *Fos* and *FosB* by VIP during T cell activation can indeed cause drastic downstream phenotypic changes in proliferation and differentiation. The precedent for this suggestion is supported by subtle changes in *Sox2* gene expression (e.g. 50%), which can cause embryonic stem cells to proliferate and differentiate into lineage restricted phenotypes, whereas greater than 2 fold changes in *Sox2* induces apoptosis (Kopp et al., 2008). This phenomenon of small molar mass changes bringing about significant cellular responses is well known in the transcription field and has been coined squelching (Cahill et al., 1994).

In addition to *Fos* and *FosB*, VIP co-treatment with PMA/ionomycin changed the expression levels of an additional 13 transcription factors with most participating in direct interactions with *Fos* (Fig. 6). One of these genes is called cAMP responsive element modulator (*CREM/ICER*), is upregulated by VIP irrespective of the presence of T cell activation, and further supports VIP induction of gene expression changes due to the activation of the cAMP/PKA pathway. Interestingly, VIP upregulates two additional *Crem* isoforms during T cell activation. These *Crem* isoforms, measured only during T cell activation, are due to an alternative promoter induced by increases in [cAMP]_i that produces shorter *Crem* transcripts called inducible cAMP early repressor (*ICER*). *ICER* potentially blocks IL-2 expression and T cell proliferation by competing with basic helix loop helix transcription factors (AP-1 and CREB) at TRE responsive elements in the IL-2 promoter (and other mitogenic promoters), thus uncoupling crucial stimulatory transcriptional cassettes such as NFAT, NF-kB and CBP/p300 from physical contact with RNA Polymerase II (Masquillier and Sassone-Corsi, 1992). Ganea's group has also observed an elevation in *ICER* with VIP treatment using activated CD4 T cells, and thus supports our present finding (unpublished data from (Wang et al., 2000)).

Over the past decade, VIP has been clearly demonstrated to protect against several animal disease models that show pathological similarities to human inflammatory and autoimmune disease, including collagen induced arthritis (Delgado et al., 2001), inflammatory bowel disease (Abad et al., 2003), uveoretinitis (Keino et al., 2004) and experimental autoimmune encephalomyelitis (Gonzalez-Rey et al., 2006). This protective effect appears to be due to a shift from a Th₁ to a Th₂ immune response regulated by VIP, which has been substantiated by several laboratories through both *in vitro* and *in vivo* studies. One major cause for VIP-regulated protection is the tremendous induction of CD4⁺/CD25⁺/FoxP3⁺ regulatory T cells (Treg) from peripheral CD4⁺/CD25⁻/FoxP3⁻ T cells, which dampens both IL-2 production and Th₁ cell mediated immunity (Chen et al., 2008). In 2007, Bodor *et al.* showed that the transcriptional repressor ICER is pivotal in suppression of T cell proliferation and activation by silencing IL-2 expression (Bodor et al., 2007). The molecular mechanism of VIP immune protection and Treg differentiation may be due to its ability to alter early mitogenic transcriptional programs (*ICER*, *c-Fos* and *FosB*) capable of blocking cell-mediated immunity, while promoting a Th₂ polarization in certain settings. TGF-β induces Treg generation and our data shows TGF-β₃ being upregulated in resting, but not activated T cells (Shah and Qiao, 2008). This may present an intriguing possibility that the effect on T cells by VIP is dependent on previous temporal exposures. Thus, CD4 T cells in lamina propria, for example, that are bathed in high levels of VIP (Said and Mutt, 1970), may elevate their threshold for T cell activation, and exhibit tolerance to self antigens, through their ability to induce differentiation into ICER expressing Treg cells.

In this study, we have identified two, nearly unique, VIP-mediated transcriptomes in resting versus activated CD4 T cells. These data strongly support the conclusion that CD4 T cells respond differently to VIP based on their activation status. We propose that resting CD4 T cells mediate directed cell movement orchestrated by EGFR signaling. During T cell activation, an

immediate early transcriptional program appears to be initiated by VIP, a cAMP-inducing agent, involving a subtle change in AP-1 composition and ICER expression, among others, that is a driving force for immune protection against certain inflammatory disorders. These gene expression changes are also suggested to explain at a molecular level how VIP, presumably acting through VPAC1, can allow for naïve cell homing to the gut, while inducing an altered differentiation program during T cell activation of Treg effector cells. Additional studies will be crucial to validate these phenotypic cellular changes *in vivo*, which is a major future goal of our research effort.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

GPCR	G-protein coupled receptor
VIP	Vasoactive Intestinal Peptide
VPAC1	Vasoactive Intestinal Peptide Receptor 1
CNS	central nervous system
PP	Peyer's Patches
SAM	Significance Analysis of Microarrays
IPA	Ingenuity Pathway Analysis
Treg	regulatory T cell

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Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1E

Figure 1.

VIP receptor expression levels and phenotypic changes due to PMA/ionomycin treatment. Data is presented as means $\pm$ SEM from three independent experiments calculated by Origin software unless otherwise noted. **A.** Isolation of highly enriched murine CD4 T cells. Pre- and post anti-CD4 magnetic bead (proprietary clone, Miltenyi) samples were incubated with FITC conjugated anti-mouse CD4 antibody (RM4-4) and analyzed by a FACS Calibur (Materials and Methods). Histogram plots were generated by FloJo software and percent purity is indicated. This data is representative of six independent biological experiments. **B.** *Vpac1* is the predominant VIP-binding receptor expressed by CD4 T cells. MACS purified CD4 T cells were used to measure the relative expression levels of four known VIP receptors, *Vpac1*, *Vpac2*, *Pac1* and *Fpr11* by qRT-PCR. Data was normalized to *Actb*, and fold changes calculated by the $\Delta\Delta C_t$ method, with *Pac1* levels arbitrarily set to 1. The y-axis has a break from 30 to 3,000 as indicated by two slashes (/). **C.** Expected mRNA expression changes of *Il2ra* and *Sell* induced by PMA/ionomycin treatment. Purified CD4 T cells were cultured in the presence or absence of 10 ng/ml PMA and 1 μ M ionomycin for 5 hours. Real-time PCR analysis was performed as described in 1B. Microarray averages were calculated from normalized pixel intensity values calculated from six (microarray) or three (qRT-PCR) independent biological experiments. Black bars represent microarray values and open bars represent qRT-PCR values. **D.** Expected phenotypic surface expression levels of CD25 (*Il2ra*) and CD62L (*Sell*) before and after PMA/ionomycin treatment. Purified CD4 T cells were sequentially incubated with or without PMA/ionomycin for 5 hours followed by staining with anti-CD25 (left panel) or anti-CD62 (right panel), and analyzed by an Accuri flow cytometer (Ann Arbor, MI). The red

line represents cells incubated in media alone, and the black line represents cells incubated with PMA/ionomycin. These data show expected changes as there is complete shedding of CD62L with PMA/ionomycin. **E.** Use of biologically active VIP ligand. Chinese Hamster Ovary (CHO-K1) cells were stably transfected with human *Vpac1* cDNA. Cells were incubated with varying concentrations of VIP ranging from 10^{-14} to 10^{-6} M (Materials and Methods). After fifteen minutes, cells were lysed and [cAMP]i were measured by a competitive ELISA. Curve fitting was performed in Origin graphical software.

Fig. 2A

Fig. 2B

Fig. 2C

Gene	Fold-Change	
	Resting	Activated
ABCB1B	1.6	1.6
AGPAT9	2.3	1.8
ARHGEF10	1.5	1.5
FANCI	1.6	1.7
MPP7	1.6	1.8
PCSK1	-1.5	1.6
PPP2R3A	2.2	1.7
RUNDC3B	1.8	1.5
SGIP1	2.1	2.9
TRIM13	1.6	1.5
D330027G24RIK	2.3	2.0
9430027b09RIK	2.7	1.7

Figure 2. Identification of the early VIP transcriptome in resting and activated CD4 T cells. **A.** Five to ten C57BL/6J spleens were harvested for each independent experiment (n=6), and CD4 T cells were isolated by magnetic bead technology. Cells were cultured in the absence (resting) or presence of PMA/ionomycin (activated), and cultured with or without 10^{-7} M VIP for five hours (four groups). Total RNA was harvested from resting and activated samples, fluorescently labeled, and hybridized to Agilent Whole Genome Microarrays. These data were then analyzed by SAM and Ingenuity Pathway Analysis software (Materials and Methods). **B.** Venn diagrams of the number of transcripts uniquely or commonly (overlapping) modulated by VIP in resting and PMA/ionomycin activated CD4 T cells (left side) as identified by SAM analysis with a 10% FDR. The left diagram shows all VIP-modulated genes (no threshold applied), while the right panel shows the number of VIP modulated genes after a $\geq -/+ 1.5$ fold cutoff. **C.** Gene names that are commonly regulated by VIP (after a $\geq -/+ 1.5$ fold cutoff) with fold change values indicated. **D.** Pie charts showing the percentage changes of genes in fourteen functional categories identified by IPA for the resting and activated datasets.

Fig. 3A

Fig. 3B

Figure 3. qPCR corroboration of VIP modulated gene targets identified by microarray analysis. RT-PCR was utilized to measure transcript level changes in response to VIP treatment. Gene of interest values were normalized to *Actb* and data represents means $\pm$ SEM calculated by the $\Delta\Delta C_t$ method from six (microarray) or three (qPCR) independent experiments as indicated using **A.** resting, and **B.** activated samples. Black bars represent microarray values and open bars represent qRT-PCR values.

Figure 4.

IPA generated networks of VIP modulated genes and their corresponding networks in resting CD4 T cells. No user-defined cutoffs for expression values were used for network generation. Networks scores were generated by Ingenuity's Network Generation Algorithm, and were assigned the following scores, **A.** 36, **B.** 31 and **C.** 31, respectively. Open symbols are intermediate molecules, "non-focus genes," placed in the network by the Ingenuity software, but are not regulated by VIP. "Focus genes" are regulated by VIP and are denoted by red symbols for upregulated genes, and green symbols indicate downregulated genes. Symbols representing functional categories of the molecules are listed in the legend.

A. Cellular Movement
Cell-Mediated Immune Response
Hematological System Development /Function

B. Inflammatory Response
Lipid Metabolism
Small Molecule Biochemistry

C. Cell Cycle
Lipid Metabolism
Small Molecule Biochemistry

Figure 5.

IPA generated networks of VIP modulated genes and their corresponding networks in activated CD4 T cells. IPA analysis was conducted as described in Fig. 4. Network scores were **A.** 39, **B.** 35 and **C.** 32, respectively. The functional classifications denoted by the symbols are found in the legend for Figure 4.

Resting

Activated

Figure 6. Identification of the direct VIP interactome for resting and activated datasets. In IPA software, the identical datasets used in Figure 4 and 5 for resting or activated samples, were selected to Build a network using the Connect function in IPA for **A.** resting and **B.** activated datasets. The symbol legend is located in Figure 4.

Table 1

VIP-modulated genes in resting and PMA/ionomycin activated CD4 T cells grouped by function

<i>Symbol</i>	Entrez Gene Name	Resting Fold Change	Activated Fold Change
<i>Cytokines/Cytokine Receptors</i>			
<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10 (IP-10)		1.806
<i>Il5</i>	interleukin 5 (colony-stimulating factor, eosinophil)	1.697	
<i>Il5ra</i>	interleukin 5 receptor, alpha	1.523	
<i>Il7r</i>	interleukin 7 receptor	-1.53	
<i>Il17rc</i>	interleukin 17 receptor C		1.782
<i>Tnfsf11^a</i>	tumor necrosis factor (ligand) superfamily, member 11 (RANKL)	2.03	
<i>Tnfrsf19</i>	tumor necrosis factor receptor superfamily, member 19	-1.568	
<i>Tslp</i>	thymic stromal lymphopoietin		1.512
<i>Enzymes</i>			
<i>Agpat9^a</i>	1-acylglycerol-3-phosphate O-acyltransferase 9	2.346	1.825
<i>Chd3</i>	chromodomain helicase DNA binding protein 3	-1.53	
<i>Chst2</i>	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	1.502	
<i>Far2</i>	fatty acyl CoA reductase 2	1.638	
<i>Galc</i>	galactosylceramidase		1.588
<i>Gnat1</i>	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	2.161	
<i>Hsd3b4</i>	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4		1.71
<i>G-protein coupled receptors</i>			
<i>Ccr7</i>	chemokine (C-C motif) receptor 7		1.756
<i>Ccr8</i>	chemokine (C-C motif) receptor 8		1.825
<i>Lpar4</i>	lysophosphatidic acid receptor 4	1.598	
<i>Lrrc8d</i>	leucine rich repeat containing 8 family, member D		1.576
<i>Growth factors</i>			
<i>Pdgfb</i>	platelet-derived growth factor beta polypeptide		-1.584
<i>Vegfa^a</i>	vascular endothelial growth factor A	1.57	
<i>Ion channels</i>			
<i>Cnga1</i>	cyclic nucleotide gated channel alpha 1	-1.672	
<i>Gria3</i>	glutamate receptor, ionotropic, AMPA 3	-1.846	
<i>Scn3b</i>	sodium channel, voltage-gated, type III, beta	1.871	
<i>Kinases</i>			
<i>Ephb2</i>	EPH receptor B2	1.667	
<i>Mapk15</i>	mitogen-activated protein kinase 15	1.715	
<i>Mos</i>	v-mos Moloney murine sarcoma viral oncogene homolog	1.543	
<i>Pak1^a</i>	p21 protein (Cdc42/Rac)-activated kinase 1		1.624
<i>Prkca</i>	protein kinase C, alpha	1.878	
<i>Yes1</i>	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1		1.519
<i>Ligand-dependent nuclear receptors</i>			

<i>Symbol</i>	<i>Entrez Gene Name</i>	<i>Resting Fold Change</i>	<i>Activated Fold Change</i>
<i>Ar</i>	androgen receptor	1.649	
<i>Nr4a2</i>	nuclear receptor subfamily 4, group A, member 2	1.746	
Peptidases			
<i>Adamts15</i>	ADAM metallopeptidase with thrombospondin type 1 motif, 15	-4.011	
<i>Arhgef10</i>	Rho guanine nucleotide exchange factor (GEF) 10	1.502	1.51
<i>Mmp2</i>	matrix metallopeptidase 12 (macrophage elastase)		1.54
<i>Pcsk1</i>	proprotein convertase subtilisin/kexin type 1	-1.545	1.588
<i>Plg</i>	plasminogen	-2.426	
<i>Usp2</i>	ubiquitin specific peptidase 2		1.635
Phosphatases			
<i>Alpi</i>	alkaline phosphatase, intestinal	-1.505	
<i>Dusp10</i>	dual specificity phosphatase 10		1.617
<i>Minpp1</i>	multiple inositol polyphosphate histidine phosphatase, 1	1.79	
<i>Nudt4</i>	nudix (nucleoside diphosphate linked moiety X)-type motif 4	1.674	
<i>Ppp2r3a</i>	protein phosphatase 2 (formerly 2A), regulatory subunit B', alpha	2.242	1.716
<i>Ptpn4</i>	protein tyrosine phosphatase, non-receptor type 4 (megakaryocyte)		1.569
Transcription regulators			
<i>Crem</i>	cAMP responsive element modulator (ICER)		1.562
<i>Egr3</i>	early growth response 3	-1.874	
<i>Epas1^a</i>	endothelial PAS domain protein 1		1.808
<i>Fos</i>	v-fos FBJ murine osteosarcoma viral oncogene homolog		1.668
<i>Mitf</i>	microphthalmia-associated transcription factor		-1.593
<i>Myb^a</i>	v-myb myeloblastosis viral oncogene homolog (avian)	1.68	
<i>Tceal1</i>	transcription elongation factor A (SII)-like 1 (p21)	1.872	
<i>Tsc22d3</i>	TSC22 domain family, member 3		1.501
Transmembrane receptors			
<i>Cd14</i>	CD14 molecule		1.67
<i>Plaur^a</i>	plasminogen activator, urokinase receptor (uPAR)		1.636
Transporters			
<i>Abcb1b^a</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	1.617	1.602
<i>Abcb4</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 4	2.104	
<i>Azgp1</i>	alpha-2-glycoprotein 1, zinc-binding	1.625	
<i>Exoc4</i>	exocyst complex component 4	1.566	
<i>Slc27a2</i>	solute carrier family 27 (fatty acid transporter), member 2	1.634	1.541
<i>Syt13</i>	synaptotagmin XIII	3.492	
<i>Tmed3</i>	transmembrane emp24 protein transport domain containing 3		1.515
Others			
<i>Aim1</i>	absent in melanoma 1		1.554
<i>Arhgap15</i>	Rho GTPase activating protein 15	-1.78	
<i>Arhgap18</i>	Rho GTPase activating protein 18	1.727	
<i>Arhgap29^a</i>	Rho GTPase activating protein 29	-1.517	

<i>Symbol</i>	Entrez Gene Name	Resting Fold Change	Activated Fold Change
<i>Arrdc3</i>	arrestin domain containing 3		1.586
<i>Auts2</i>	autism susceptibility candidate 2	-1.53	
<i>Cilp2</i>	cartilage intermediate layer protein 2		-1.669
<i>Cldn5</i>	claudin 5	1.876	
<i>Coch</i>	coagulation factor C homolog, cochlin (Limulus polyphemus)		1.687
<i>Col11a2</i>	collagen, type XI, alpha 2	-1.535	
<i>Ctla2a</i>	cytotoxic T lymphocyte-associated protein 2 alpha	1.648	
<i>Cytip</i> ^a	cytohesin 1 interacting protein (<i>Pscdbp</i>)		2.035
<i>Dfna5</i>	deafness, autosomal dominant 5		1.652
<i>Diaph2</i>	diaphanous homolog 2 (Drosophila)	-1.558	
<i>Dnah17</i>	dynein, axonemal, heavy chain 17	1.857	
<i>Emb</i> ^a	embigin homolog (mouse)		1.688
<i>Eml1</i>	echinoderm microtubule associated protein like 1	1.775	
<i>Epb41</i>	erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)		1.56
<i>Fanci</i> ^a	Fanconi anemia, complementation group I	1.642	1.744
<i>Fndc5</i>	fibronectin type III domain containing 5	1.93	
<i>Frmpd1</i>	FERM and PDZ domain containing 1	1.529	
<i>Gckr</i>	glucokinase (hexokinase 4) regulator	1.54	
<i>Grap2</i>	GRB2-related adaptor protein 2		1.59
<i>Hist1h1c</i>	histone cluster 1, H1c		1.545
<i>Igsf3</i>	immunoglobulin superfamily, member 3		-1.512
<i>Impg2</i>	interphotoreceptor matrix proteoglycan 2	-1.787	
<i>Khdrbs3</i>	KH domain containing, RNA binding, signal transduction associated 3	1.751	
<i>Moblk2b</i> ^a	MOB1, Mps One Binder kinase activator-like 2B (yeast)	1.852	
<i>Mpp7</i>	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	1.59	1.822
<i>Myo1b</i> ^a	myosin IB	1.751	
<i>Nrip3</i>	nuclear receptor interacting protein 3	1.704	
<i>Parp8</i>	poly (ADP-ribose) polymerase family, member 8	2.142	
<i>Pdlim4</i>	PDZ and LIM domain 4	-1.608	
<i>Podxl2</i>	podocalyxin-like 2		1.726
<i>Prnp</i>	prion protein	1.571	
<i>Pvrl3</i>	poliovirus receptor-related 3	1.523	
<i>Ramp3</i>	receptor (G protein-coupled) activity modifying protein 3		1.755
<i>Rapgef6</i>	Rap guanine nucleotide exchange factor (GEF) 6	-1.783	
<i>Rgs2</i>	regulator of G-protein signaling 2, 24kDa		1.731
<i>Rundc3b</i>	RUN domain containing 3B	1.762	1.546
<i>Sgip1</i> ^a	SH3-domain GRB2-like (endophilin) interacting protein 1	2.08	2.941
<i>Sgtb</i>	small glutamine-rich tetratricopeptide repeat (TPR)-containing, beta		1.517
<i>Sh3bgrl2</i>	SH3 domain binding glutamic acid-rich protein like 2	1.842	

<i>Symbol</i>	Entrez Gene Name	Resting Fold Change	Activated Fold Change
<i>Sspn</i>	sarcospan (Kras oncogene-associated gene)	1.557	
<i>Tctex1d1</i>	Tctex1 domain containing 1	2.371	
<i>Tex11</i>	testis expressed 11	1.753	
<i>Trim13</i> ^a	tripartite motif-containing 13	1.604	1.519
<i>Vps37b</i>	vacuolar protein sorting 37 homolog B (S. cerevisiae)		1.562
<i>Ypel4</i>	yippee-like 4 (Drosophila)	1.608	
<i>Zbtb20</i> ^a	zinc finger and BTB domain containing 20	-1.664	
<i>Zcchc12</i>	zinc finger, CCHC domain containing 12	1.633	

^aTranscripts which were represented by more than one microarray feature

Table 2

Most significant biological functions as determined by IPA

Resting CD4 T cells -/+ VIP		PMA/ionomycin Activated CD4 T cells -/+ VIP	
<i>Molecular and Cellular Functions</i>			
<u>Name</u>	<u># of Molecules</u>	<u>Name</u>	<u># of Molecules</u>
Cell Death	68	Cellular Movement	60
Cellular Compromise	22	Cellular Growth and Proliferation	79
Cell Cycle	39	Cell Cycle	40
Carbohydrate Metabolism	14	Cellular Development	70
Lipid Metabolism	27	Cell Death	78
<i>Physiological System Development and Functions</i>			
<u>Name</u>	<u># of Molecules</u>	<u>Name</u>	<u># of Molecules</u>
Skeletal and Muscular System Development/ Function	31	Cell-Mediated Immune Response	71
Cardiovascular System Development/Function	28	Hematological System Development/Function	58
Tissue Morphology	38	Tissue Morphology	49
Hematological System Development/Function	31	Skeletal and Muscular System Development/ Function	28
Organismal Development	42	Hematopoiesis	42
<i>Diseases and Disorders</i>			
<u>Name</u>	<u># of Molecules</u>	<u>Name</u>	<u># of Molecules</u>
Neurological Disease	100	Immunological Disease	67
Developmental Disorder	32	Dermatological Diseases/Conditions	29
Endocrine System Disorder	64	Inflammatory Disease	69
Reproductive System Disease	37	Cancer	101
Genetic Disorder	161	Inflammatory Response	46