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TOPIC HIGHLIGHT

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Vasoactive intestinal peptide signaling axis in human leukemia

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Abstract

The vasoactive intestinal peptide (VIP) signaling axis constitutes a master "communication coordinator" between cells of the nervous and immune systems. To date, VIP and its two main receptors expressed in T lymphocytes, vasoactive intestinal peptide receptor (VPAC)1 and VPAC2, mediate critical cellular functions regulating adaptive immunity, including arresting CD4 T cells in G_1 of the cell cycle, protection from apoptosis and a potent chemotactic recruiter of T cells to the mucosa associated lymphoid compartment of the gastrointestinal tissues. Since the discovery of VIP in 1970, followed by the cloning of VPAC1 and VPAC2 in the early 1990s, this signaling axis has been associated with common human cancers, including leukemia. This review highlights the present day knowledge of the VIP ligand and its receptor expression profile in T cell leukemia and cell lines. Also, there will be a discussion describing how the anti-leukemic DNA binding transcription factor, Ikaros, regulates VIP receptor expression in primary human CD4 T lymphocytes and T cell lymphoblastic cell lines (e.g. Hut-78). Lastly, future goals will be mentioned that are expected to uncover the role of how the VIP signaling axis contributes to human leukemogenesis, and to establish whether the VIP receptor signature expressed by leukemic blasts can provide therapeutic and/or diagnostic information.

Key words: Neuropeptides; Ikaros; Cancer; Hut-78; Epigenetics

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THE VASOACTIVE INTESTINAL PEPTIDE NEUROIMMUNE-NETWORK

Vasoactive intestinal peptide (VIP) is a 3.3 kDa protein originally discovered in swine intestines by Said *et al*[1]. Upon characterization, this small peptide consisting of 28 amino acids, had vasoactive properties when added to arteries, which prompted its name, VIP. Within 10 years after its discovery in 1970, VIP detection was measured in a number of human and rodent blood cells, including

mast cells[2], neutrophils[3], eosinophils[4], thymocytes[5] and T and B lymphocytes[6]. Additional discoveries revealed that biological fluids derived from immune-privileged organs (e.g. eye and spine) were rich in VIP[7], and importantly inhibited the proliferation of immune cells in mixed lymphocyte cultures[8]. Intriguingly, in addition to immune-privileged compartments that actively repeal immune cells, the immunosuppressive VIP peptide was also detected in secondary immune organs that actively recruit high numbers of immune cells. Two immune compartment examples are the mucosa associated lymphoid tissue (MALT) of the pulmonary and gastrointestinal tissues[9]. The immunoreactive (IR) VIP nerves detected within these compartments co-stained with markers for noradrenergic, non-cholinergic nerves that innervated these organs, thus identifying an additional neuronal source for the immunosuppressive VIP peptide, in addition to certain immune cells, including developing thymocytes, activated T cells and mast calls[5,10,11].

These studies detecting IR VIP$^+$ nerves within the eye and MALT represented the first major discovery that firmly established an anatomical basis for a neuroendocrine-immune network. Additional observations confirmed IR-VIP$^+$ nerves innervating additional immune organs, including the thymus, spleen, bone marrow, skin and Peyer's Patches within the gastrointestinal mucosa associated tissue. A second major contribution was the discovery that both immune and non-immune cells, in proximity to VIP$^+$ nerve endings, expressed receptors for the VIP neuropeptide[12]. A third important observation was that VIP possessed chemotactic properties for resting T lymphocytes and actively recruited them to Peyer's Patches located in the gut[13,14]. Lastly, VIP suppressed T lymphocyte activation by blocking interleukin (IL)-2, IL-4 and interferon (IFN)-γ production, inhibited apoptosis thereby enhancing T_{h2} memory cells and promoted the inducible FoxP3$^+$ regulatory T cell (iTreg) lineage (Figure 1). This collective body of research is the fundamental core for the field called neuroimmunomodulation, of which VIP has been firmly established as a master mediator in this regulatory axis (for review see[15-17]).

This review will focus on the VIP signaling axis and its relevance to human T cell leukemia. We will begin with a review of the VIP signaling axis in healthy T lymphocytes followed by the current understanding of VIP ligand and receptor expression profiles in T cell leukemia patients and cell lines. Penultimately, there will be a discussion on the contemporary dogma of the transcriptional regulation of VPAC1 by the anti-leukemic chromatin remodeling factor, Ikaros. Lastly, concluding comments will place into perspective a current working model that we expect will yield important insight into the potential role of the VIP signaling system in the diagnostic, treatment and clinical outcome of T cell acute lymphoblastic leukemia (ALL).

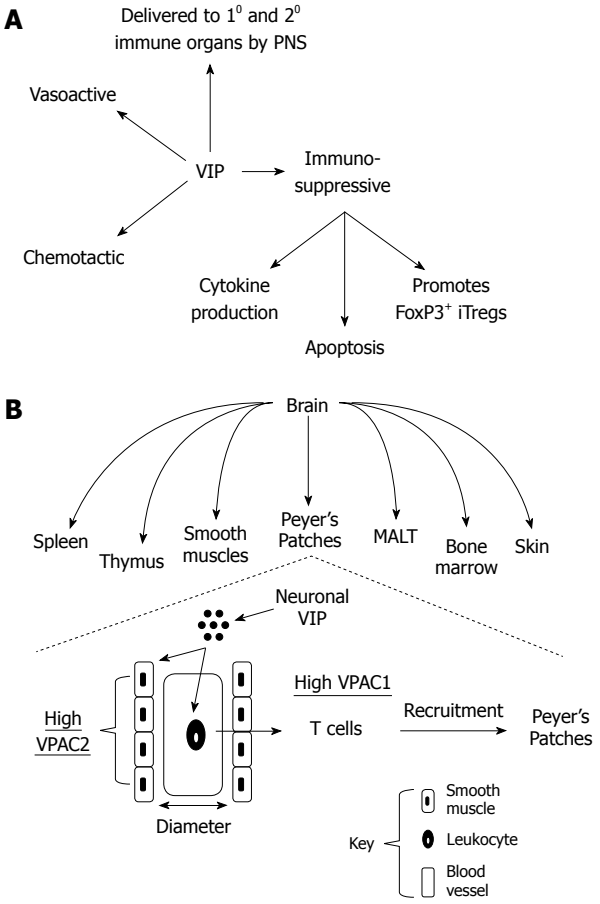

Figure 1 Neuroimmunomodulation by vasoactive intestinal peptide. A: Vasoactive intestinal peptide (VIP) is delivered to primary (1^0) and secondary (2^0) immune organs by the peripheral nervous system (PNS), which affects the metabolism of cells in close proximity through its vasoactive properties (vascular smooth muscle cells) and its chemotactic activities on resting T lymphocytes. During TCR signaling, VIP is immunosuppressive directly on T lymphocytes by: inhibiting proinflammatory cytokine secretion/production, inhibiting apoptosis and promoting FoxP3$^+$ inducible T regulatory cells; B: Delivery of VIP ligand to immune cells in indicated anatomical compartments (immune and non-immune) that represents a division of labor for VIP/vasoactive intestinal peptide receptor (VPAC)2 signaling (vasoactive; smooth muscle cells) and VIP/VPAC1 signaling (chemotactic and immunosuppressive; directly on lymphocytes) in an effort to effectively target trafficking naïve T cells to appropriate immune compartments such as Peyer's Patches within the gut. MALT: Mucosa associated lymphoid tissue.

VIP SIGNALING AXIS

VIP

The ligand, VIP, is classified as a neuropeptide member of the secretin superfamily that performs crucial biological activities, including regulation of the immune system[18]. The secretin superfamily is made up of nine diverse small peptides that share similar, as well as, distinct biological activities. Cloning of the human VIP gene occurred in 1995 and is located on chromosome 6q25 (Entrez Gene ID 7432)[19]. Rat and mouse VIP genes had been previously cloned in 1991, and are positioned on syntenic regions of the rat chromosome 1p11 (Entrez Gene ID 117064) and on the mouse chromosome 10A1 (Entrez Gene ID 22353), respectively[20]. The structure of the human VIP gene consists of 7 exons interrupted by 6 introns and spans 9 kb. The VIP gene is translated into a 170 amino acid preproprotein and proteolytically tailored to generate at least two biologically active peptides, called VIP and peptide histidine methionine

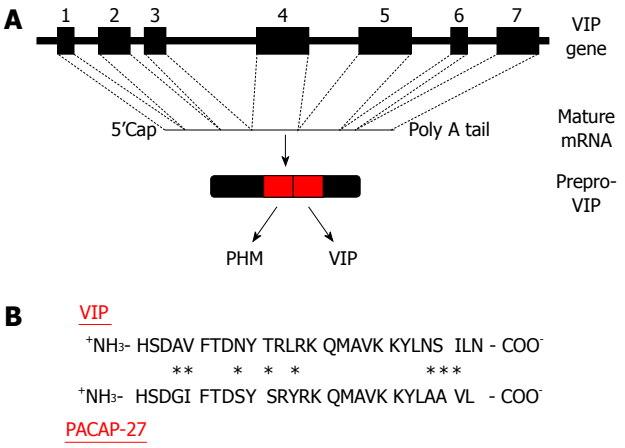

Figure 2 Molecular biology of vasoactive intestinal peptide. A: Vasoactive intestinal peptide (VIP) is transcribed from a gene consisting of 7 exons and translated into a 170 amino acid prepropeptide that produces at least two biologically active peptides as shown; B: The amino acid comparison between pituitary adenylate cyclase activating polypeptide (PACAP)27 and VIP with bold letters representing identical amino acids between peptides, and asterisks indicating amino acid differences.

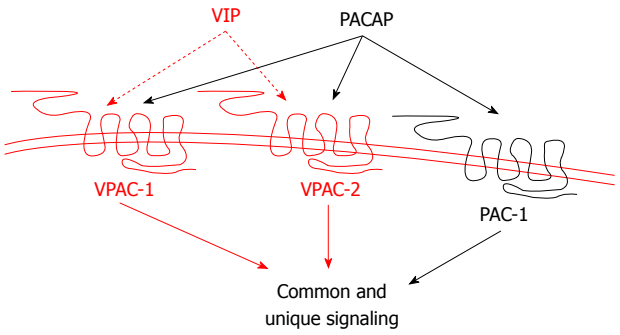

Figure 3 Binding selectivity of the vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptors. Pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1) selectively binds pituitary adenylate cyclase activating polypeptide (PACAP) with 1000-fold greater affinity than vasoactive intestinal peptide (VIP), whereas vasoactive intestinal peptide receptor (VPAC)1 and VPAC2 bind VIP and PACAP with equal affinity.

(PHM). Thus, the VIP gene appears to be organized into exon modules in which exon 5 exclusively encodes the VIP peptide, and exon 4 the PHM peptide (Figure 2). VIP shares nearly 70% amino acid sequence identity with another secretin family peptide called pituitary adenylate cyclase activating polypeptide (PACAP). The rodent (Adcyap1) (Entrez Gene ID mouse - 11516; rat - 24166), and human (ADCYAP1) (Entrez Gene ID 116) PACAP genes were cloned in the early 1990s[21-23], and have a similar gene structure and translational processing to VIP, generating at least three biologically active peptides called PACAP-38 (38 amino acids in length), PACAP-27 and PACAP related peptide. PACAP has remained nearly unchanged (96% identical) for over 700 million years of evolution and is considered the progenitor of the secretin superfamily of peptides[24]. PACAP-27 and VIP possess 68% amino acid sequence identity and VIP is thought to have evolved by exon duplication from PACAP concomitant with the evolution of the adaptive immune system as invertebrates evolved into vertebrates around 500 million years ago[24]. A co-evolution of VIP and its receptors with the establishment of the adaptive immune system may explain why VIP/PACAP modulates numerous immune functions such as proliferation[25], cytokine expression[26], inhibition of apoptosis[27] adhesion[14] and chemotaxis[13]. VIP is delivered by peripheral neurons to immune organs (and non-immune organs), in addition to being secreted by resting and activated leukocytes[28]. VIP is one of the most abundant peptides in immune organs such as the spleen, thymus and MALT[29]. This chemotactic and immunosuppressive neuropeptide ameliorates several autoimmune and inflammatory disease models in mice, including rheumatoid arthritis[30,31], atopic dermatitis[32], Crohn's disease[33], multiple sclerosis (MS)[34] host *vs* graft disease, and antagonists to VIP receptors inhibit the proliferation of many common, solid-tissue, human cancers, including 51 of 56 human lung cancer cell lines[35].

Recently, VIP and PACAP were discovered to increase the generation of inducible $CD4^+/CD25^+$ regulatory T cells (iTregs) that are positive for FoxP3 expression[36].

VIP receptors

The receptors that bind VIP and PACAP receive their name based on their affinity for these two biologically active peptides, respectively. For example, pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1) binds PACAP with a 1000-fold greater affinity than VIP, and is therefore categorized as the selective VIP/PACAP receptor[24]. In addition to PAC1, there are two non-selective receptors that bind VIP and PACAP with equal affinity, called VIP/pituitary adenylate cyclase activating polypeptide receptor (VPAC)1 and VPAC2 (Figure 3). All three receptors share a presumed similar 7-transmembrane structure based on hydropathy plots, with three external (EC1-3) and three internal loops (IC1-3), an extended N-terminal extracellular ectodomain and a relatively short intracellular C-terminal domain[37,38]. The VPAC1 (Vpr1) and VPAC2 (Vpr2) genes were cloned in rodents (Vpr1 - Entrez Gene ID mouse - 22354; rat - 24875; Vpr2 - Entrez Gene ID mouse 22355; rat 29555) and humans (VPR1 - Entrez Gene ID - 7433; VPR2 - 7434) in the early 1990s, and have very similar genetic structures with the human VPAC1 gene consisting of 13 exons and 12 introns[39-41]. Human VPAC1 has been mapped to chromosome 3q22, and to a syntenic region on chromosome 9 in mouse. Upon ligand binding, VPAC1 and VPAC2 couple with at least three G proteins, including $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$ that regulate signaling molecules as diverse as adenylate cyclase, PKA, PKC, PLC, PLD and EPAC, and elevate the intracellular secondary messengers, cAMP, IP_3, DAG and Ca^{2+}, that appear to be largely cell-context dependent[17,42,43]. There is also solid evidence for nuclear factor κB dependent and independent signaling effects by VIP[44].

VIP receptor expression profile and its transcriptome in T lymphocytes

In naïve, mouse and human CD4 and CD8 T lympho-

cytes, the constitutively expressed VPAC1 receptor is 300-500-fold higher than VPAC2[12,45-47] (unpublished data) at the mRNA and protein levels that appears to be inversely related to the expression level of IL-2. Our laboratory has recently identified the VIP/VPAC1 transcriptome in naïve and activated mouse splenic CD4 T cells. In naïve T cells, VIP/VPAC1 signaling appears to induce directed cell movement through EGFR signaling. In early activated T cells (5 h), the cAMP dependent CREM/ICER transcription factor is upregulated, which has been shown to "short-circuit" helix-loop-helix transcription factors that are critical for pro-inflammatory cytokine expression[48]. These microarray observations, we propose, can explain at a molecular level how the VIP signaling axis can act in an anti-inflammatory manner, as well as, induce the differentiation of activated T cells toward different effector phenotypes, including T regulatory cells and T_{h17} cells[36,49]. Since developing and mature T lymphocytes are heterogeneous cell populations, it will be important to generate the next generation of anti-VIP receptor antibodies capable of detecting protein within these hematopoietic subpopulations to confirm which receptor is evoking VIP-initiated signal transduction and altering metabolic cellular changes. To this end, our laboratory has generated by gene-gun technology a highly specific mouse anti-VPAC1 polyclonal antibody capable of detecting cell-surface VPAC1 protein on primary CD4 and CD8 T lymphocytes (manuscript submitted).

During *in vitro* T cell activation (e.g. anti-CD3/anti-CD28) T lymphocytes engage the cell cycle to begin a proliferative program that results in a precipitous drop of VPAC1 mRNA levels $\geqslant$ 80% as assessed by qPCR[46,47]. Likewise, *in vivo* activation of ovalbumin-specific CD8 T cells (OT-I) also showed undetectable levels ($\geqslant$ 99%) of VPAC1 mRNA and protein using an adoptively transferred T_{h1} pathogen mouse model[50]. The mechanism for VPAC1 downregulation in mouse CD4 T cells is through a Src/ZAP70/JNK signaling pathway based on a pharmacological inhibitor study conducted by our laboratory[51]. Importantly, Anderson *et al*[52] recently demonstrated that VIP/VPAC1 signaling potently inhibited G_1/S transition in human CD4 T cells. Thus, VPAC1 signaling appears to block the very signal (TCR activation) that causes its downregulation at the mRNA and protein levels, and might be the reason why many human and rodent T cell leukemia cell lines and human T cell blasts from patients with T cell leukemias have significantly reduced levels of VPAC1 mRNA (see below). After the expansion phase of antigen-specific CD8 T cells, we have collected data in mice that VPAC1 levels are restored in primary, but not secondary memory pools. These data may suggest an interesting possibility that the *in vivo* timing of VIP/VPAC1 signaling during T cell activation or in memory cells can have significant consequences regarding proliferative expansion or recruitment/retention in certain immune compartments based on the number of times exposed to antigen. In sharp contrast to VPAC1, VPAC2 has been termed the inducible VIP receptor, and was shown to become upregulated on T_{h2} cells, but not T_{h1} cells. A cause and effect for VPAC2 upregulation has been confirmed in its ability to protect T_{h2} cells from apoptosis and therefore is a survival factor and promoter of T_{h2} memory cells[53]. We have confirmed that mouse VPAC2 upregulation indeed does not take place in activated antigen-specific CD8 T cells during an *in vivo* T_{h1} pathogen, *Listeria monocytogenes,* infection[50]. This observation supports the idea that VPAC2 is induced against T_{h2}, but not T_{h1}, pathogens. Clinical relevance for VIP to skew towards a T_{h2} lymphocyte lineage is shown by amelioration of T_{h1}-driven autoimmune disorders, including MS[34]. In summary, the timing and location of T cell activation is paramount to whether the VIP/VPAC1 signaling axis modulates the metabolism of the T cell population, as well as, whether VIP/VPAC2 signaling in T_{h2} cells can promote T cell survival.

VPAC receptor expression profile in hematopoiesis

Human hematopoietic stem cells that are enriched for $CD34^+$ cells derived from either bone marrow or cord blood (CB) have been shown to predominately express VPAC1 verses VPAC2 as assessed by semi-quantitative PCR, subtractive hybridization and western analysis[54,55]. Also, the immature, non-dividing $CD34^+CD38^-$ hematopoietic precursors express 4 times greater VPAC1 expression compared to the more mature $CD4^+CD38^+$ population, which contain elevated numbers of colony-forming cells (CFCs). The signaling induced by VPAC1 due to added VIP ligand (10^{-9} mol/L) to these hematopoietic stem precursor cells showed a synergic effect on myeloid and mixed colony growth of $CD34^+$ CB cells with little to no detectable effect on BM cells in the presence, but not absence of three early cytokines, FLT3 ligand, stem cell factor (STF) and thrombopoietin (TPO)[55]. Another study confirmed high levels of VPAC1 mRNA in BM cells, but exogenously added VIP (10^{-13} to 10^{-7} mol/L) instead suppressed erythroid and myeloid colony growth, with a concomitant increase in transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α from an unidentified stromal cell type (possibly macrophages)[54]. These authors concluded that the suppressive activities by VIP/VPAC1 signaling was partly due to the increase in TGF-β and TNF-α as neutralizing antibodies to these cytokines suppressed the effect by VIP. These two studies did in fact validate functional VPAC1 expression in early hematopoietic populations. Their disagreement regarding a positive or negative influence on colony formation of $CD34^+$ cells might be due to deriving these cells from different hematopoietic groups; bone marrow *vs* cord blood. Regardless, it was suggested that microenvironments immediately surrounding nerve endings that supply VIP in bone marrow would best allow for this neuropeptide to alter hematopoietic cellular growth as VIP is readily degraded in serum (10^{-11} mol/L), and the early cytokine signaling is inhibited by serum. Further research is needed to better understand the coordination power of VIP/VPAC1 signaling in the context of different hematopoietic microenvironments.

VPAC receptor expression profile during thymocyte development

It is agreed that peripheral, mature T cells from rodents and humans express higher levels of VPAC1 compared to VPAC2, however, there is some disagreement between their expression profile in developing mouse thymocytes, as well as, a potential species difference between rodents and humans[5,6,12,56-58]. Several labs have measured rat, mouse and human VPAC receptor mRNA by qPCR and RNase protection assays in thymocytes, and all agree on the expression of functionally active VIP receptors in total thymocytes. The discrepancy comes from distinguishing which VIP receptor, VPAC1 or VPAC2 (PAC1 is not expressed), was predominately expressed. Total thymocytes from rat and mouse revealed constitutive VPAC1 levels with increases in VPAC2 only upon TCR activation[5,57]. In contrast, human thymocytes showed greater VPAC2 verses VPAC1 mRNA expression, and TCR activation decreased VPAC1 but not VPAC2 mRNA message[58]. The latter study measured VPAC receptors by qPCR, which may account for the greater sensitivity for VPAC2 in the absence of TCR signaling, but does not explain the higher VPAC2 levels upon T cell activation. Additional studies further fractionated thymocytes into specific groups based on CD4 and CD8 expression. With the exception of rat double negative cells ($CD4^-CD8^-$, DN), double positive ($CD4^+CD8^+$, DP), single positive ($CD4^+CD8^-$, SP4) and SP8 subsets showed readily detectable VPAC1 mRNA message by PCR followed by southern hybridization confirmation[5]. This consistently high VPAC1:VPAC2 ratio in developing rat T cells was opposite for human thymocytes, which showed approximately 4-6-fold more VPAC2 mRNA compared to VPAC1 in DP, SP4 and SP8 subsets as assessed by qPCR[58]. DN cells contained low but equivalent levels of both receptors. These results suggest a species difference during T cell development. Furthermore, definite VIP receptor ratio discrepancies are seen within mouse thymocytes. For example, two studies using Balb/c mouse thymocyte subsets disagreed on the VPAC1:VPAC2 ratios in DN and SP8 thymocyte subsets. In contrast, their data did agree with respect to DP and SP4 subsets that showed higher VPAC2 *vs* VPAC1 mRNA levels[57,59]. These discrepancies can most likely be attributed to PCR primers used and/or experimental conditions such as media culture conditions. Our laboratory has collected data suggesting further discrepancies of VIP receptor expression in mouse thymocytes. Using the C57Bl/6 mouse strain instead of Balb/c mice, we detected more VPAC1 than VPAC2 in total thymocytes and greater VPAC1 than VPAC2 in all four major thymocyte populations: DN, DP, SP4 and SP8, respectively by qPCR (Manuscript submitted). In addition, we further subdivided this population based on CD44, CD25 and CD117 expression called DN1-4 subsets[60] that revealed a fascinating VIP receptor reversal with high VPAC1 mRNA expression found in the earliest T cell progenitor ($CD44^+/CD25^-/CD117^+$, DN1) subset that became transiently silenced in DN2 ($CD44^+/CD25^+$) and DN3 ($CD44^-/CD25^+$) subsets with the concomitant induction of VPAC2 mRNA. DN4 cells showed the restoration back to high VPAC1 and low VPAC2 expression as observed for the later thymocyte populations. It is enticing to speculate that the VIP receptor ratio during T cell development may contribute to a T_{h1} skewing in C57BL/6 mice (high VPAC1:VPAC2) *vs* a T_{h2} skewing in Balb/c mice (low VPAC1:VPAC2). This idea is supported by the VPAC2 transgenic mouse model where forcing the expression of VPAC2 in a C57BL/6 T_{h1} skewed this mouse strain towards a T_{h2} phenotype[61] (see below). Functionally, two reports have shown evidence that VPAC2 mediates IL-2 suppression upon TCR activation in DP cells, and that VPAC2 signaling enhances DP →SP4 differentiation without altering apoptosis, viability, proliferation or cell numbers[57,59]. A third study revealed that VPAC1 signaling was contributing to the protection of spontaneous and glucocorticoid-induced apoptosis[62]. In summary, there appears to be functional VIP receptors expressed on developing thymocytes, but their expression ratio may be species specific, and VIP signaling influences IL-2 expression, differentiation and protection from apoptosis.

Genetically altered VPAC2 mice

While VPAC1 knockout and transgenic mice have not yet been reported, we and others have created VPAC2 knockout and transgenic C57BL/6 mouse strains. Opposite phenotypes were observed for the VPAC2 knockout and transgenic mouse models that provided further evidence for VIP/VPAC2 signaling playing an important role in immune responses[61,63]. Mice that developed in the absence of the immune-inducible VPAC2 receptor demonstrated enhanced delayed type hypersensitivity (DTH), which is mediated primarily by activated T cells and macrophages[63]. In contrast, there was a significant decrease in immediate type hypersensitivity (IH). These mice also demonstrated a polarization toward a T_{h1} response as evidenced by an increase in the T_{h1} cytokine, IFN-γ, and a decrease in T_{h2} cytokines, IL-4 and IL-5 as determined by *ex vivo* experiments of TCR stimulated $VPAC2^{-/-}$ CD4 T cells[63]. In VPAC2 transgenic mice under the control of the LCK promoter, VPAC2 protein was predominately expressed in the helper T cell compartment (25 fold higher in CD4 *vs* CD8 T cells). VPAC2 transgenic mice exhibited a shift in CD4 T cell polarization towards a T_{h2} phenotype as evidenced by (1) a depressed DTH response and an enhanced IH response; (2) an increased number of eosinophils and serum IgE and IgG_1 levels; and (3) higher T_{h2} cytokines, IL-4 and IL-5, and lower T_{h1} cytokine, IFN-γ production by activated CD4 T cells[61]. Thus, VPAC2 significantly modulates CD4 T cell responses. VPAC1 expression levels were consistent with wild type levels in both genetically mutated mouse models. In review, VPAC2 knockout and transgenic mice show a reciprocal differentiation influence towards a

T_{h2} polarization with respect to cytokine expression and delayed-type hypersensitivity through an unknown mechanism. In addition to T_{h2} differentiation, VIP/VPAC2 signaling protects T_{h2}, but not T_{h1}, cells from apoptosis and appears to contribute to this memory cell pool.

VIP$^{-/-}$ *mice and immunity*

The targeted removal of the VIP gene has been engineered[64-68]. A cadre of studies marshaled by James Waschek and other colleagues has focused on pulmonary disorders and asthma. These studies have uncovered an inflammatory component to the VIP$^{-/-}$ knockout mouse. Homozygous VIP$^{-/-}$ knockout mouse have enhanced lymphocyte and eosinophil infiltration into the lung. Moreover, microarray analyses have revealed that lung tissue in the absence of VIP show elevated inflammatory genes representing a chemokine (Ccr6), protease (Mcpt8) and two TNF superfamily members. These data suggest that VIP normally suppresses inflammation in tissues such as lung[68]. Evidence for similar inflammatory exacerbations in VIP$^{-/-}$ mice was observed in gastrointestinal disorders (Crohn's disease) as well[69].

VIP signaling axis and cellular proliferation

The effect on cellular proliferation and cell cycle entry by the VIP signaling axis is complex. In rat neurons, it is well-established that VIP induces proliferation[70,71], whereas it is a potent inhibitor of proliferation in human vascular smooth muscle and CD4 T cells[52,68]. These apparent nonsensical influences toward cellular mitotic control is contributed to by the differential expression of at least three different receptors capable of binding VIP (VPAC1, VPAC2 and PAC1), as well as a fourth called formyl peptide receptor-like 1 (FPRL-1)[43]. Once bound, the ability for these receptors to engage signal transduction cascades is cell-specific as they differentially couple multiple G proteins[17]. VPAC1 expressed on a lymphoblastic T cell line (H9) can transmit alternate internal signals by differentially coupling to $G_{\alpha s}$ or $G_{\alpha i}$ based on whether PHM or VIP binds. However, irrespective of the particular G protein pathway activated, both ligands increased proliferation as assessed by BrdU incorporation[42]. The fact that VIP and PHM evoked different pathways suggests that VPAC1 (and possible other family receptors) can distinguish subtle residue differences in natural ligands thereby tailoring the signaling cascade elicited. In addition to VIP and PHM, activated mast cells and rat basophilic leukemia cell lines secrete a truncated VIP_{10-28} that acts as a potent VPAC1 antagonist, with low VPAC2 binding[11]. Couple this complexity to at least one splice variant of VPAC1, two for VPAC2 and 11 for PAC1, and the ability for the VIP ligand released by neurons innervating an immune organ can have a multitude of functional consequences[72,73]. Unpublished data from our laboratory has identified up to four additional VPAC1 splice variants present in lymphoid and brain cells. The VIP field, therefore, is in its infancy with respect to understanding the biochemical and cellular effects of the VIP signaling axis.

VIP signaling axis and T cell leukemia

VIP signaling is evident in most common types of human cancer, including breast, prostate, lung, and colon[74,75]. These cancer etiologies have been shown to predominately express functional VPAC1, with only the rare human leiomyomas expressing functional VPAC2 receptor. PAC1 receptors are typically expressed in paragangliomas, pheochromocytomas and endometrial carcinomas. Antagonists that inhibit all three VIP receptors have been shown to be effective at suppressing the proliferation of these common cancers, as well as, CNS, melanoma, ovarian and renal tumors and leukemia[35,76]. These reports indicate that VIP and their related peptides enhance the survival and/or promote cellular proliferation in most cancers. In contrast, the VIP receptor(s) responsible for promoting cellular proliferation has not been strenuously studied T cell leukemia. In 1992, Sue O' Dorisio's group showed functional VIP binding sites that evoked increases in i[cAMP] levels in 22 out of a 32 patient cohort diagnosed with ALL of T or B cell origin[77]. The receptor identity, however, was unknown as the VIP receptors had yet to be cloned at the time of this study. Once the VIP receptors had been cloned later in the 1990's, the identity of the VIP receptor(s) expressed in human T cell leukemia blasts could be determined. This research has been conducted primarily utilizing a handful of human leukemic T cell lines, including Stanford University Pediatric (SUP) T1, Molt-4b, Jurkat, Hut-78 and H9 lines (Table 1). Based on these four parent cell lines (H9 is a derivative of Hut-78 cells) there appears to be either high levels of VPAC2 mRNA expression (Sup T1 and Molt 4b with an immature phenotype), or low levels of both VPAC1 and VPAC2 mRNA (Jurkat, Hut-78 and H9 cells with a mature phenotype)[42,78-81]. Our laboratory has verified that CD4^{+}/CD19^{-} cells recovered from biopsied lymph tissue from 2 human T cell leukemia patients also expressed high levels of functional VPAC2 receptor with exceeding low levels of VPAC1 mRNA as assessed by qPCR and cAMP ELISA (manuscript in preparation). In rodent leukemia T cell lines of various etiologies, all cell lines studied exclusively expressed VPAC2, some of which were validated to be functional[57,82-84]. A VPAC2 predominant expression profile in cancer is unusual as VPAC2 expressing tumors are rare[74], and that the expression profile of healthy peripheral lymphocytes express extremely high VPAC1 at both the mRNA and protein levels[45]. Malignant T cells from ALL patients occur due to a blockade in thymocyte development (thymic in origin), or from a blockade in HSC within the bone marrow (prethymic)[85]. These hyperproliferating, low VPAC1:VPAC2 ratio expressing leukemic blasts egress from the thymus and enter the vasculature and bone marrow, where they co-mingle with healthy HSC (CD34^{+}/CD38^{-}) and peripheral mature T

Table 1 Vasoactive intestinal peptide receptor expression in T cell lines

Name	Procedure	Receptor	Ref.
Human T cell lines			
Sup T1	RT-PCR and Northern	VPAC2	Xia *et al*[78] 1996
Molt-4b	q-PCR	VPAC2	Summers *et al*[129] 2003
Jurkat	RT-PCR and q-PCR[1]	Low levels of both	Finch *et al*[80] 1989
Hut-78	RT-PCR and q-PCR[1]	Low levels VPAC1 > VPAC2	Xia *et al*[81] 1996
H9	RT-PCR	VPAC1	Goursaud *et al*[42] 2005
Mouse T cell lines			
EL-4.IL-2	Northern/ RT-PCR	VPAC2	Waschek *et al*[82] 1995, Xin *et al*[57] 1997
MBI-1.15	Northern	VPAC2	Waschek *et al*[82] 1995
BW5147	Northern	VPAC2	Waschek *et al*[82] 1995
CTLL-2	Northern	ND	Waschek *et al*[82] 1995
CTLL-M	Northern	ND	Waschek *et al*[82] 1995
DBA/2	Northern	VPAC2	Waschek *et al*[82] 1995
YAC-1	Northern	ND	Waschek *et al*[82] 1995
F10	Northern	VPAC2	Waschek *et al*[82] 1995
BL/VL3	cAMP	VPAC2	Abello *et al*[83] 1989
NS8	cAMP	VPAC2	Robberecht *et al*[130] 1989
TL-2	cAMP	VPAC2	Robberecht *et al*[130] 1989
D10.TCR31	RT-PCR	VPAC2	Xin *et al*[57] 1997
D10.G4.1	RT-PCR	VPAC2	Xin *et al*[57] 1997
Rat T cell lines			
GK 1.5	RT-PCR	VPAC2	Xin *et al*[57] 1997
3.155	RT-PCR	VPAC2	Xin *et al*[57] 1997

[1]Quantitative polymerase chain reaction (qPCR) is unpublished data from our laboratory. RT-PCR: Reverse transcription-polymerase chain reaction; ND: Not detected. Receptor refers to which vasoactive intestinal peptide receptor is predominantly expressed.

cells that all express high VPAC1:VPAC2 ratios (Figure 4). One possible explanation for a low VPAC1:VPAC2 ratio in human T cell leukemia blasts compared to healthy peripheral T cells, might be due to normally low VPAC1:VPAC2 expression during T cell development as human thymocytes were found to express much higher levels of VPAC2 compared to VPAC1[58]. This altered expression profile in leukemia blasts for the VIP receptor signaling axis could contribute to a growth advantage as VPAC1 is a potent G_1/S transition arrestor by blocking the upregulation of several cyclins, while VPAC2 acts as a survival factor of mouse T_{h2} cells[36,52]. High levels of VIP ligand are also detected in human thymus. It is for these reasons the authors hypothesize that the increased expression of the VPAC2 receptor is a possible diagnostic marker for human (and rodent) T cell ALL. A similar supposition has previously been suggested by Waschek *et al*[82] where they rationalized that a potential molecular switch could take place during healthy T cell development, activation and/or homeostasis to explain the apparent VIP receptor reversal between healthy and leukemic T cells.

There is, however, some discrepancy with our proposal for high VPAC2 and low VPAC1 levels in ALL based on data showing human non-Hodgkin lymphoma patients exclusively expressing VPAC1 in 100% of the

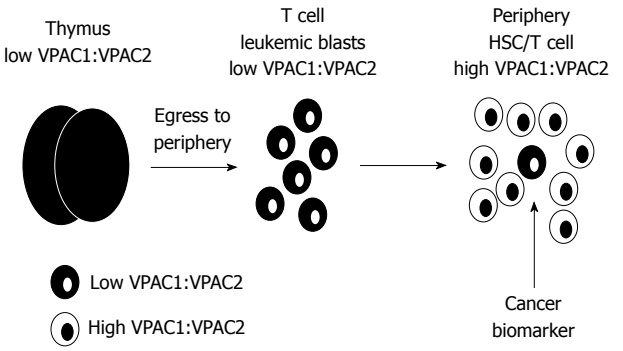

Figure 4 Working hypothetical model for differential vasoactive intestinal peptide receptor expression in T cell acute lymphoblastic leukemia blasts. The radical difference between low vasoactive intestinal peptide receptor (VPAC)1:VPAC2 ratio in developing thymocytes may act as a biomarker and prognostic indicator, readily distinguishable from peripheral hematopoietic stem cells (HSC) and mature T cells that express high VPAC1:VPAC2 ratios.

patient samples tested (6 out of 6)[75]. The authors did not distinguish between T *vs* B cell patients, however. Moreover, this study utilized ligand binding specificity for VIP receptor identification, which may contribute to discordant results compared to reverse transcription-polymerase chain reaction (RT-PCR) gene expression analysis, due to receptor internalization, dimerization and changes in ligand affinity[37]. Another study reported that Hut-78 T cells expressed 75 000 VPAC1 binding sites per cell based on RT-PCR, western analysis and ^{125}I-VIP binding measurements[81]. This high VPAC1 expression level is not consistent with our qPCR data showing < 1% that of healthy CD4 T cells (manuscript in preparation), which have been estimated to have only approximately 15 000 binding sites[14]. Also, H9 cells, a derivative of Hut-78s, were also estimated to have fewer binding sites of approximately 10 000 sites per cell[42]. Therefore, the levels of VPAC1 in Hut-78 T cells in all likelihood are lower than healthy primary T cells, and that the report suggesting they contain high levels of VPAC1 expression (75 000 sites/cell) is perhaps an overestimation.

IKAROS REGULATION OF VPAC1

IK and its role in T cell leukemia

IK is a kruppel-like, zinc-finger transcription factor that functions as a master regulator for the development and maintenance of the hemo-lymphoid compartment[86-88]. The IK gene generates at least 11 isoforms through alternative splicing[89]. All IK isoforms have a common C-terminus containing an activation domain and two zinc fingers that facilitate dimerization with other IK isoforms. All Ikaros protein products (at least 11) differ in their N-terminal domain consisting of 4 zinc-fingers, three of which are necessary to bind DNA[90]. Of the three genetically modified IK mouse models that have been generated[91-93], the more severe model resulted in the complete arrest of fetal and adult lymphocyte development. Importantly, heterozygous mice developed an aggressive lymphoblastic leukemia (100% penetrance) 3 to 6 mo

after birth[94]. In human leukemia patients, several reports have shown mutations in the IK gene[95-98]. More recently, it has been revealed that alternative splicing dysregulation alters the ratio between IK DNA binding to non-DNA binding isoforms[99]. Also, a 2009 study confirms that deletions/mutations in the IK gene is associated with a poor prognosis for B cell ALL patients[100], but interestingly IK mutations occurs a very small percentage of T cell ALL patients (≤ 4%)[101]. These mouse and human data have established IK as a master regulator for lymphopoiesis and an authentic tumor suppressor that sets the threshold for T cell activation, but indicates a species specific difference in Ikaros biology[102].

Mechanisms for transcriptional regulation by IK

Previous investigations in naïve mouse CD4 T cells have shown that IK recognizes at least five different chromatin remodeling and histone-modifying enzyme complexes. Regarding transcriptional permissive complexes, IK has been shown to bind the stimulatory chromatin remodeler, termed switch/sucrose nonfermentable[103]. Regarding transcriptional repressive complexes, IK has been shown to interact with the repressive nucleosome remodeling and deacetylase complex, c-terminal binding protein, c-terminal interacting protein, and mSin3a/b complexes[104-106]. Immunofluorescence staining shows IK protein present in a diffuse reticular nuclear pattern in naïve, non-cycling CD4 T cells. During T cell activation and entry into the cell cycle, IK is redistributed into a donut shaped nuclear pattern that co-localizes with pericentrimeric heterochromatin[86,107]. IK is differentially phosphorylated in a cell cycle dependent manner. The phosphorylation pattern of IK changes as T cells cycle from G_1 to G_2/M phase, and modulates DNA-binding affinity (36). IK is thought to act as an activator or repressor of gene expression based on its subnuclear distribution and binding partner(s) in naïve CD4 T cells[86,108]. There are also differences in basal Ikaros isoform expression levels between resting and activated mouse and human T cells. For example, mouse primary T cells express equivalent protein levels of IK-1 and IK-2 (IK-VI and IK-V based on Sinisa Dovat's nomenclature) irrespective of the activation status of T cells. In contrast, human primary T cells clearly show low levels of IK-1 and the largest known Ikaros isoform, Ikaros-H, that are upregulated upon TCR signaling[109]. Moreover, Sinisa Dovat's group has very nicely demonstrated how phosphorylation by casein kinase Ⅱ and dephosphorylation by PP1 serves to regulate IK's ability to bind DNA, regulate gene expression as well as dictate its subnuclear distribution[110,111].

Identification of Ikaros binding elements in the VIP receptor gene loci

VPAC1 and VPAC2 promoters possess a high frequency of putative Ikaros (IK) binding sites (5'-TGGGAT/A-3'). An inspection of a 5 kb nucleotide sequence of the human VPAC1 and VPAC2 promoters reveal 12 putative IK consensus sequences (5'-TGGGAA/T-3') spanning the transcriptional start site (Figure 5). In comparison, the rhodopsin gene that encodes a group I GPCR has only 1 putative IK binding site over a similar DNA length. Moreover, the entire gene loci of VPAC1 (55 kbp) and VPAC2 (48 kbp), including 10 kb immediately flanking these genes both upstream and downstream, possess 138 and 262 putative IK binding motifs, respectively. The random frequency of any 6 nucleotide DNA sequence being found on both strands of DNA in a 60 kb region is approximately 30 times. Using this random frequency as a comparison, there are 5-fold and 9-fold more IK consensus sequences present at these receptor gene loci. In addition, the IK binding elements are equally distributed throughout both VIP receptor loci with a nearly equal probability of finding an IK sequence on the template (60%) or non-template (40%) strand. Curiously, the 5 kb region mentioned above that spans the transcriptional start site for VPAC1 and VPAC2 has 12/12 (100%) and 8/12 (66%) IK binding motifs that are oriented on the non-template strand, respectively. We propose that this frequency of IK binding motifs preferentially on the non-template DNA strand is not a random event, but rather demarcates a powerful regulatory domain for Ikaros regulation of the VPAC1 and VPAC2 genes.

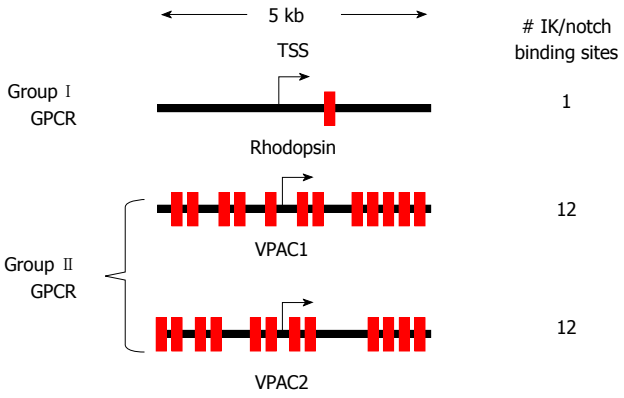

Figure 5 High Frequency IK binding sites at the vasoactive intestinal peptide receptor promoters. Schematic diagram of a 5 kb region for the vasoactive intestinal peptide receptor (VPAC)1 and VPAC2 promoters spanning the transcriptional start site (TSS). Red boxes are IK consensus sequences, with the number of IK binding sites per gene promoter indicated.

IK protein binds to the promoter regions of VPAC1

Evidence for IK protein binding to high-affinity IK-consensus elements in the promoter of VPAC1 is based primarily on electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation assays (ChIP). In 2002, we showed that nuclear protein from human Jurkat T cells, but not WI-38 fibroblasts, produced a retardation signal using a positive IK DNA probe (IKBS4[112]) or the most distal IK site within the VPAC1 promoter[113]. Jurkat protein was supershifted by anti-IK IgG but not a non-specific IgG confirming that IK protein was indeed part of the complex engaging the VPAC1 DNA probe. Moreover, recombinant GST-IK1 and IK2 bound to both probes and were competed away by unlabeled

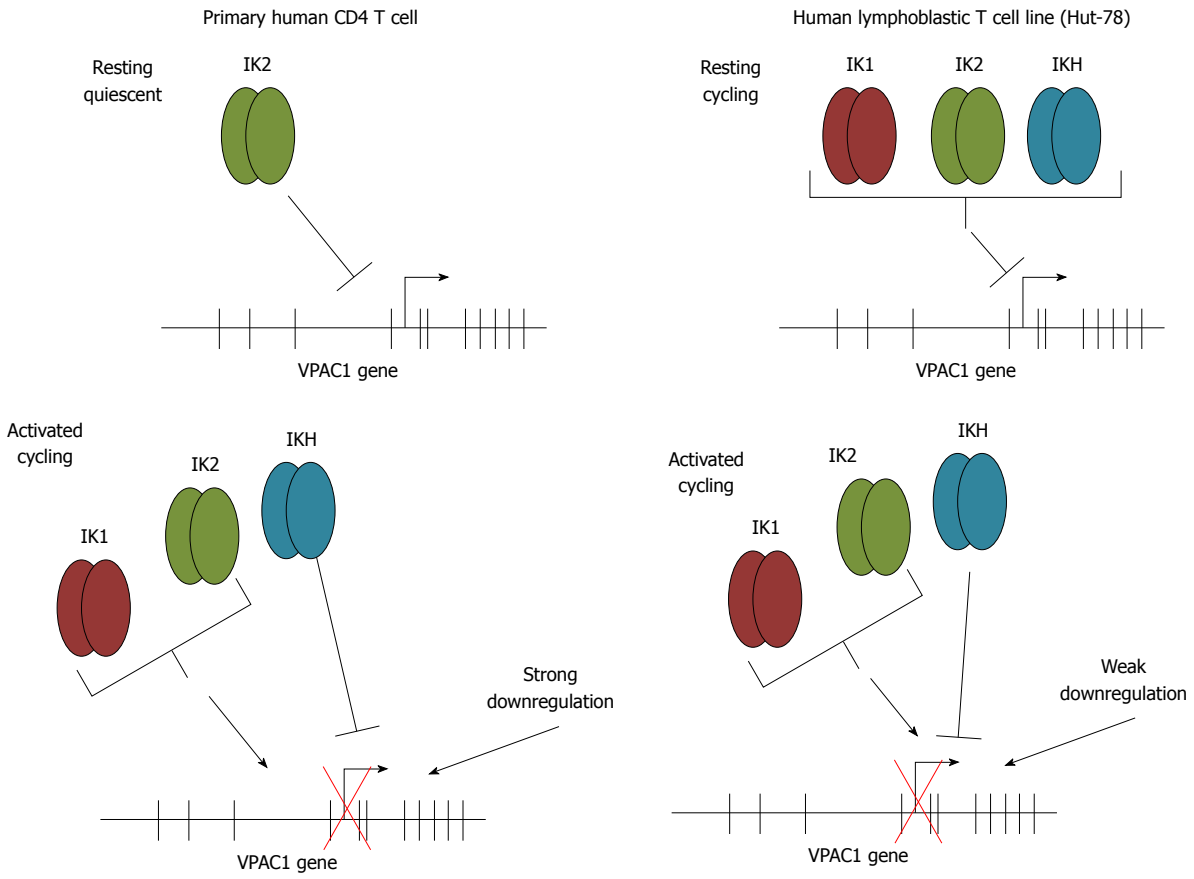

Figure 6 Ikaros engagement of the vasoactive intestinal peptide receptor 1 promoter in primary and T cell lines. Schematic representation of electrophoretic mobility shift assays and chromatin immunoprecipitation assays data comparing Ikaros engagement to the vasoactive intestinal peptide receptor (VPAC)1 promoter in primary CD4 T cells compared to the human lymphoblastic T cell line, Hut-78 cells. Top panels represent the expression profile for Ikaros in resting cells, and the bottom panels represent activated T cells.

probe, further validating that IK protein could positively recognize and bind to the VPAC1 promoter. Subsequent EMSA studies further validated these observations using nuclear protein from human primary T cells. Interestingly, only nuclear extracts from activated CD4 T cells, but not resting cells, showed a retardation signal with the VPAC1 DNA probe. Anti-IK antibody supershifted this signal whereas IgG did not. Collectively, these data reveal that human recombinant and endogenous IK protein from either activated CD4 T cells or from malignant T cell lines can bind to IK-consensus sequences within the VPAC1 promoter in a sequence dependent manner. This observation was confirmed by *in vivo* ChIP assays that allows for a "snap shot" to be taken within a cell to determine protein/DNA interaction by forming reversible cross links with formaldehyde. This study supported the EMSA data revealing VPAC1 DNA amplification of immunoprecipitated chromatin using the anti-IK-CTS pAB, but not anti-IK-H or IgG negative controls[109]. That IK-1 is upregulated during T cell activation supports the notion that IK-1 engages the VPAC1 promoter resulting in its repression. Possible mechanisms to explain the differential binding affinity for IK-2 verses IK-1 dimers might be differences in consensus sequence recognition, subnuclear distribution or post-translational modification pattern changes that renders it not conducive to engage the VPAC1 promoter position in euchromatin (Figure 6)[51].

Ikaros binding causes a functional change in VPAC1 expression

Using a negative IK cellular background of mouse NIH-3T3 cells, overexpression of DNA binding isoforms (IK-1 and IK-2), but not a DNA binding isoform that fails to enter the nucleus (IK-3), or the non-DNA binding isoform (IK-5), significantly downregulated VPAC1 expression by greater than 90% as assessed by qPCR[113]. These decreases in steady-state mRNA were paralleled at the protein level as well (50% decrease). Follow-up studies by our laboratory using a Hut-78 T lymphoblastic cell line background overexpressing the dominant negative IK-5 isoform introduced by nucleoporation resulted in a dramatic 15-fold induction of VPAC1 steady-state mRNA levels (manuscript in preparation, Figure 7). Surprisingly, the IK-2 DNA-binding isoform also increased VPAC1 levels by 2-fold, thus mimicking the positive upregulation that the DN IK-5 isoform displayed, albeit to a lower magnitude. These overexpression studies support a working model where decreasing the net DNA binding potential of the IK pool (IK-5), or altering the homo/heterodimer combination (IK-2, IK-H binds with

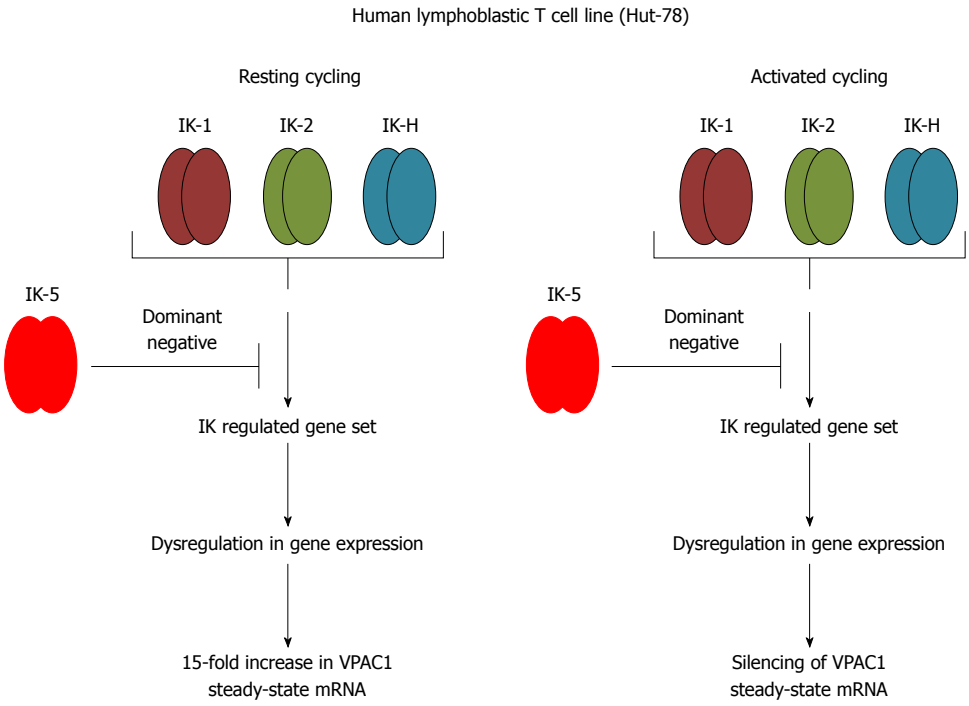

Figure 7 Working model for Ikaros mediated regulation of vasoactive intestinal peptide receptor 1 expression. IK-5 overexpression results in the upregulation of vasoactive intestinal peptide receptor (VPAC)1 steady-state levels in resting Hut-78 cells. The DNA binding activity of Ikaros therefore can alter the expression of growth modulating genes like VPAC1 in a direct (binding to the VPAC1 gene) or indirect mechanism (regulating a repressor/activator that binds the VPAC1 gene). We predict VPAC1 silencing will occur in activated IK-5 overexpressing cells.

greater affinity to IK-2 then IK-1[109]) results in greater IK-1 homodimers and causes elevated VPAC1 steady-state mRNA expression. We have unpublished data demonstrating IK enrichment to the VPAC1 promoter by ChIP assays in activated (PMA/ionomycin), but not resting, human Hut-78 T lymphoma cells could imply that dysregulating IK DNA binding by overexpressing IK isoforms result in increases in VPAC1 expression by an indirect manner. Future studies will investigate how IK dysregulation affects VPAC1 expression during T cell activation. We predict that VPAC1 expression will be silenced without functional IK DNA binding protein, which would support the idea that IK regulates transient gene expression changes and its plasticity upon resolution of T cell activation (memory cells).

FUTURE DIRECTIONS

Elevated VPAC2 expression in the diagnosis and therapeutic intervention of human T cell ALL

There still remains a critical gap in the fundamental knowledge base regarding VIP ligand and receptor expression levels in human T cell ALL. It is becoming readily apparent that functional VPAC2 expression is elevated in rodent and human T cell blasts with an immature phenotype. Unfortunately, there are only a few reports documenting VIP receptor expression in human leukemia, and none to our knowledge have conducted molecular measurements of mRNA and protein expression levels. It will be imperative to collect qPCR and flow cytometry data from a large human T cell ALL cohort to confirm high VPAC2 expression to support its use as a diagnostic tool and/or drug target for this particular leukemia etiology. Moreover, parallel studies showing a concomitant reduction of VPAC1 levels in T cell ALL patient samples would further imply that a low VPAC1:VPAC2 receptor ratio could be utilized as a leukemic indicator for routine diagnosis. Also, the specific VPAC1:VPAC2 receptor signature could be used to follow a cohort of human T cell ALL patients in an attempt to determine the extent to which this receptor ratio can predict patient outcome. Absence of such research will continue to put these human leukemia patients at risk.

Ikaros and Notch regulation of the VIP receptors

The Ikaros transcription factor binds a 6 nucleotide DNA sequence that is identical to the Notch trimer complex[114]. An antagonistic competition between Ikaros (differentiation) and Notch signaling (proliferation) to gain access to DNA binding sites in gene targets is thought to control the T cell developmental plan in the thymus. Gain of function in Notch signaling is observed in 60% of human T cell ALL, which may shift the delicate equilibrium of differentiation/proliferation toward cellular division. Future research to identify whether the Notch DNA binding trimer is actively displacing Ikaros protein from the VPAC receptor loci (and other gene targets) is an important question to answer.

A growth advantage for T cell leukemia blasts with a low VPAC1:VPAC2 ratio

Investigations focusing on how low VPAC1:VPAC2

expression levels alter VIP signaling and whether this chemical information is interpreted by leukemic blasts to initiate a survival/proliferative cellular program is paramount to uncovering future therapeutic drug targets downstream of VIP receptors. Additionally, antagonists and agonists to VIP receptors can be used in combination with other known chemotherapy drugs in an attempt to obtain greater apoptosis induction in leukemia blasts.

Functional significance of VPAC receptor expression in human B cell ALL

Lastly, VIP receptor expression data needs to be collected from B cell ALL patients as Ikaros mutations/deletions have been deleted in 30% of these patients[100]. A decrease in Ikaros protein would increase the relative Notch trimer complex binding to gene targets, including the VIP receptor loci, and again potentially causing a hyperproliferative phenotype. These expression changes in VIP receptors may allow for prognostic prediction and future drug targets downstream of VIP receptors. That VIP and its receptors are also expressed in myeloid and erythroid blood cell lineages, future research focused on these leukemic etiologies is expected to result in important insight in combating these types of human leukemias as well.

CONCLUSION

Numerous studies have demonstrated that a number of human cancers overexpress VIP, or pituitary adenylate cyclase-activating peptide (PACAP) receptors[74,75,80,115]. Interestingly, VIP and PACAP analogs have been shown to affect tumor growth in *in vitro* and *in vivo* animal tumor models, suggesting that these receptors could be used as novel therapeutic targets or for localization of tumors[116-119]. The effect of VIP varies with the type of tumor, by either directly promoting tumor growth[76,120-122], suppressing growth[123], or promoting its differentiation through VPAC1 receptor signaling[124,125]. More recently, VIP has been shown to modulate tumor cell migration[125]. However, the role of the VIP signaling pathway in human leukemia is unknown, and only a few *in vitro* studies have examined the role of this signaling pathway in the survival of leukemic blasts[126]. VIP has been shown to modulate EGFR/HER2[120], VEGF[127,128], FOS expression[48] in breast cancer cell lines. These findings further underscore the importance of this signaling pathway in human cancer and warrants further investigation.

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