



Named Series: Neuropeptide Regulation of Immunity

TCR signaling and environment affect vasoactive intestinal peptide receptor-1 (VPAC-1) expression in primary mouse CD4 T cells

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ABSTRACT

Strict regulation of T cell function is imperative to control adaptive immunity, and dysregulation of T cell activation can contribute to infectious and autoimmune diseases. Vasoactive intestinal peptide receptor-1 (VPAC-1), an anti-inflammatory G-protein coupled receptor, has been reported to be downregulated during T cell activation. However, the regulatory mechanisms controlling the expression of VPAC-1 in T cells are not well understood. Therefore, mouse splenic CD4 T cells were treated in complete media \pm anti-CD3 for 24 h, total RNA isolated and VPAC-1 levels measured by qPCR. Surprisingly, we discovered that T cells incubated in complete media steadily upregulated VPAC-1 mRNA levels over time (24 h). Importantly, CD4 T cells isolated from blood also showed elevated VPAC-1 expression compared to splenic T cells. Collectively, these data support that the vascular environment positively influences VPAC-1 mRNA expression that is negatively regulated by TCR signaling. This research was supported by a national service award (1K01 DK064828) to G.D., the Center for Protease Research (2P20RR015566), and INBRE (P20 RR016741).

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1. Introduction

Vasoactive intestinal peptide (VIP) is a 28 amino acid (AA) molecule that performs crucial biological activities, including immunological protection (Nussdorfer and Malendowicz, 1998). VIP is part of the glucagon/PACAP/secretin (GPS) superfamily that consists of nine small peptides, which share similar but distinct biological roles (Dorsam et al., 2000). VIP binds two structurally similar receptors that share \approx 50% AA homology called vasoactive intestinal peptide receptor-1 (VPAC-1) and VPAC-2. VIP receptors belong to the GPS class IIA, seven transmembrane, G-protein coupled receptor (GPCR) superfamily and are encoded by separate genes. VIP and PACAP (Pituitary adenylyl cyclase-activating polypeptide) binding evokes three major signaling pathways: G_s /cAMP/PKA, G_q /PLC/ Ca^{2+} and PLD activation (O'Dorisio et al., 1981; Delporte et al., 1995; Xia et al., 1996; McCulloch et al., 2000, reviewed by Goetzl et al., 1995). Both peptide ligands modulate numerous T cell functions including, proliferation (Ottaway and Greenberg, 1984; Wang et al., 2000), trafficking (Ottaway, 1984), cytokine expression (Tang et al., 1996; Martinez et al., 1996; Voice et al., 2001), apoptosis (Delgado et al., 2002) and adhesion (Johnston et al., 1994; Xia et al., 1996; reviewed by Delgado et al., 2004).

An anatomical connection exists between the nervous and immune systems (Delgado et al., 2004). Autonomic noradrenergic and cholinergic nerves of the peripheral nervous system innervate the thymus, spleen, lymph nodes and the mucosa-associated lymphoid tissues (MALT) of the pulmonary and gastrointestinal systems (Felten et al., 1987). Immune cells that express VIP receptors in proximity to VIP positive nerve endings can respond to a “neuro-delivered” biologically active ligand (Delgado et al., 1996; Goetzl et al., 1998; Cozzi, 1999). VIP sources can also be non-neuronal as Th_2 , but not Th_1 , CD4 T cells synthesize and secrete VIP, acting in an autocrine or paracrine manner, to support the Th_2 immune response (Blum et al., 1992; Delgado and Ganea, 1999; Goetzl et al., 2001; Voice et al., 2001; Delgado and Ganea, 2001; Vassiliou et al., 2001).

The immunomodulatory actions of this “neuro-cytokine-like” ligand, VIP, on T cells has been demonstrated to act by suppressing the crucial T cell growth factor, IL-2, through a VPAC-1 signaling cAMP-dependent mechanism (Wang et al., 2000). Therefore, VIP/VPAC-1 signaling is thought to suppress bystander T cell activation (Lara-Marquez et al., 2001). Upon receiving appropriate signals for activation, CD4 T cells decrease VPAC-1 mRNA steady-state levels \geq 90% in mouse and human (Lara-Marquez et al., 2001; Voice et al., 2001) that facilitates optimal T cell activation. However, T cell regulatory mechanisms controlling the anti-inflammatory VPAC-1 GPCR are not well understood.

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In this study, evidence is presented demonstrating an unexpected upregulation of VPAC-1 steady-state mRNA levels in primary CD4 T cells exposed to RPMI complete media (Section 2), which is blocked by anti-CD3 treatment. The Src kinases, Fyn and Lck, appear to negatively regulate VPAC-1 expression as a selective Src kinase inhibitor, PP2 (Hanke et al., 1996), but not PP3 (Bain et al., 2003), resulted in a complete restoration in VPAC-1 levels. In addition, the environment of CD4 T cells can alter the expression levels of VPAC-1 as T cells isolated from blood showed elevated VPAC-1 mRNA levels compared to splenic T cells.

2. Materials and methods

2.1. Reagents

1 × PBS (without Ca²⁺ and Mg²⁺), pyrogen-free water, defined fetal bovine serum, 1 M HEPES, 40% Glucose, 1 M sodium pyruvate penicillin/streptomycin/ampotericin B were purchased from Hyclone. AIM-V, Opti-MEM, normal mouse serum, charcoal-stripped, and Nuclease-free water FBS were purchased from Invitrogen. RPMI 1640 media and DMEM was obtained from Cellgro. DNase I, QIAshredder, RNeasy kits were obtained from Qiagen. Magnetic columns, 30 μM sieves, anti-CD4-labeled magnetic beads were purchased from Miltenyi. Antibodies against mouse CD3, CD28, CD4-conjugated with PE/Cy5 and their respective isotype controls were obtained from Biologend. IL-2 ELISA kits and cAMP competitive ELISA kits were bought from Biosource. DNA oligo primers and fluorescent probes were from Integrated DNA Technologies. Taqman 2 × Universal master mix was obtained from Applied Biosystems Inc. DNase I kits and MELT Total RNA Isolation System kit came from Ambion and real time plates and caps from Fisher Scientific. M-MLV reverse transcriptase, deoxynucleotides and random primers were purchased from Promega. Protease Cocktail Inhibitor Set III, phorbol 12-myristate 13-acetate and all pharmacological inhibitors were bought from Calbiochem. SDS, Tris, glycine, sodium chloride, NP40, bromophenol blue, glycerol, pyronin Y and Tween 20 were purchased from VWR. Bisacrylamide, ammonium persulfate, TEMED, Silver Stain Plus, Kaleidoscope prestained standard, and DC protein assay were all obtained from Bio-Rad. Phytohemagglutinin (M form; PHA-m) and all other reagents used were obtained from Sigma.

2.2. Mice

Wild-type C57BL/6J mice were purchased from Charles River Hollister (Hollister, CA) or Jackson Laboratories (Bar Harbor, ME) and bred in a facility at North Dakota State University. Mice were housed in a ventilated Nalgene Armadio cabinet (VWR) as described by the manufacturer. Mice cages, water bottles and steel lids were purchased from Jackson Labs. Spleens and other organs were harvested by standard dissection techniques in a clean UV irradiated PCR quality hood. All mouse protocols were approved by our institutional IACUC board and met all federal guidelines.

2.3. T cell isolation, culture, activation and pharmacological inhibitor studies

Each independent experiment used 4–6 male or female mice between the ages of 6 and 32 weeks, and were euthanized by CO₂ narcotization followed by rapid cervical dislocation. Harvested spleens were minced in PBS at RT, and dispersed splenocytes were passed through a 30 μm sieve. Erythrocytes were lysed with 1 ml/spleen of lysis solution (0.155 M ammonium chloride, 0.01 M potassium carbonate and 0.1 mM EDTA) for 1 min, diluted to 50 ml with PBS and centrifuged at 500g for 5 min. To remove the adherent cells, splenocytes were placed in complete media (86% RPMI 1640, 10% dFBS, 10 mM HEPES, 0.4% Glucose, 1 mM sodium pyruvate and 1 × penicillin/streptomycin/ampotericin B) for 1 h at 37 °C 5% CO₂/95% air in a humidified incubator. Non-adherent splenocytes were passed through a 30 μm sieve, centrifuged as above and resuspended in 93 μl of PBS/0.5% BSA with 7 μl of anti-mouse CD4 magnetic beads/1 × 10⁷ cells and refrigerated (4–8 °C) for 20 min. CD4 T cells were purified by a Miltenyi Auto-MACS instrument using the positive selection option. Cells were counted with a hemocytometer using 0.2% Trypan blue that showed ≥ 90% cell survival. DMEM supplemented with 10% FBS, AIM-V, Opti-MEM were used to culture CD4 T cells for 24 h and used for total RNA isolation and qPCR analysis. Complete media supplemented with 10% charcoal-stripped FBS, or 10% heat-inactivated dFBS were also used to culture CD4 T cells for identical end-point analysis. Heat-inactivated dFBS was prepared by incubating 50 ml of dFBS in a 56 °C water bath for 30 min with intermittent mixing. Activation studies used 1 × 10⁶ or 4 × 10⁶ cells/ml/well in 24-well-tissue culture plates using complete media alone, ≤ 0.1% DMSO (vehicle control), 5 ng/ml PMA or 1 μg/ml plate-bound anti-CD3 ± 3.3 μM PP2 or PP3 unless otherwise noted. Cells seeded at 1 × 10⁶ cells/ml were in quadruplicate wells and pooled after incubation, while cells seeded at 4 × 10⁶ cells/ml in a single well gave similar VPAC-1 data. Plate-bound anti-CD3 was prepared by incubating 1 μg/ml/well or 4 μg/ml/well with complete media supplemented with either 10% dFBS or 10% normal mouse sera for ≥ 2 h at 37 °C. Cells were pretreated with PP2 or PP3 for 15–30 min prior to anti-CD3 treatment. Co-stimulation was performed by the addition of 2.5 μg/ml of soluble anti-CD28. Activation with 375 μg/ml of soluble PHA-m or var-

ious concentrations of PMA ± ionomycin were also attempted. After 24 h or indicated time intervals, cells were collected, centrifuged as above and used for total RNA isolation or antibody staining.

2.4. Fluorometric-based kinetic RT-PCR (qPCR)

Total RNA from primary, CD4 T cells were isolated by sequential passes through a QIAshredder spin column followed by a Mini Prep RNeasy column with on-column DNase I treatment as described by the manufacturer. Some experiments isolating total RNA from mouse tissue was conducted by MELT Total RNA Isolation System as described by the manufacturer. Following total RNA elution using nuclease-free water, a second DNase I treatment was performed using the DNA-Free kit or by a slightly modified procedure. Briefly, to each 50 μl total RNA eluant, 1 μl of DNase I, 6 μl of 10 × PCR buffer and 3 μl of nuclease-free water was added and incubated for 45 min at 37 °C. DNase I was inactivated by a 69 °C incubation for 20 min. RNA was precipitated by the addition of 2.5 volumes of 100% ethanol and a 1/10 dilution of a 3 M sodium acetate solution. Samples were incubated at –80 °C for ≥ 20 min, centrifuged at 18,000g at 4 °C for 15 min, washed with 1 ml 70% EtOH, air dried and reconstituted in 20 μl RNase-free water. Some samples were purified by a second RNeasy column instead of EtOH precipitation as described by the manufacturer with similar results. Purified total RNA (≤ 4 × 10⁶ cells) was used to generate cDNA using reverse transcriptase and random primers as described by the manufacturer. Real time reactions contained 10 μl of cDNA template with 15 μl of a 2 × master mix containing, 2 × ABI master mix, 500 nM primers (mVPAC-1, 1888–1212 bp, forward, 5'-AACTTAAAGGCCAGGTGAAAAT-3'; mVPAC-1, 1245–1268 bp, reverse, 5'-CTGCACCTCGCCATTG-3'; mHPRT, 636–655 bp, forward, 5'-CTGGTAAAAGGACCTCTCG-3'); mHPRT, 719–744, reverse, 5'-TGAAGTACTCATTATAGCAAGGGCA-3') and 400 nM of a 5'-labeled 6-carboxyfluorescein (FAM) and 3'-labeled quencher dye 6-carboxytetramethylrhodamine (TAMRA) labeled probe (mVPAC-1, 1218–1242 bp, 5'-FAMTTGTGGTGGCCATCCTCTACTGCTCC TAMRA-3'; mHPRT, 659–687 bp 5'-FAMTGTGGATACAGGCCAGACTTGTGGAT TAMRA-3'). Primer and probe sequences for VPAC-1 and HPRT were determined by PrimerExpress software. The VPAC-1 (Accession No. NM_011703) and HPRT (Accession No. NM_013556) qPCR amplicons were sequenced to confirm authenticity. Some reactions were conducted with total RNA in a one-step procedure with similar results. Reactions for both amplicons were conducted with nuclease-free water alone, and in the absence of reverse transcriptase to ensure ≥ 6 cycle thresholds compared to reactions in the presence of reverse transcriptase. This would verify ≤ 1.6% genomic DNA contamination in reactions as described by the manufacturer. The qPCR reaction was conducted using a 7500 ABI instrument with the following parameters: 2 min at 48 °C, 10 min at 94 °C to denature the reverse transcriptase and activate the Taq polymerase, followed by 40 cycles of 15 s at 94 °C and 60 s at 60 °C. Three serially diluted cDNA samples (1/4) were measured in duplicate for mVPAC-1 and mHPRT to ensure similar amplification efficiency on every plate, and relative VPAC-1 levels calculated by the ΔΔC_t method. Relative ΔΔC_t values were used (highest and lowest values excluded) to obtain average relative mVPAC-1 levels normalized to mHPRT. Data are represented as average ΔΔC_t ± SEM.

2.5. SDS–polyacrylamide gel electrophoresis

Normal fetal bovine and mouse sera, charcoal-stripped and heat-inactivated fetal bovine sera, and AIM-V medium protein concentrations were determined by a Bradford DC Protein Assay as described by the manufacturer. Ten micrograms of protein and 5% loading dye (0.375 M Tris–Cl, 30% glycerol, 10% SDS, 0.6% β-mercaptoethanol, 0.012% bromophenol blue, 0.012% pyronin Y, pH 8.8) were boiled at 115 °C for 10 min and separated on a 12% separating/5% stacking (separating: 1.5 M Tris base, 0.4% SDS, pH 8.7; stacking: 0.5 M Tris base, 0.4% SDS, pH 6.8) (Laemmli, 1970) SDS–PAGE gels using the Bio-Rad mini protean 3 apparatus and an SDS running buffer (1.5% Tris base, 7.2% glycine, 0.5% SDS). Proteins were separated for approximately 1.5 h at 100 v. Gels were silver stained as described by the manufacturer and documented by a Syngene digital camera.

2.6. Antibody staining

Enriched CD4 T cells were resuspended in a 200 μl PBS/0.5% BSA at 5 × 10⁶ cells/ml using 5 ml Falcon tubes. Cells were incubated with 5 μg/ml of anti-CD16/32 antibody on ice for 10 min to lower background fluorescent signal. A PE/Cy5-conjugated rat anti-mouse CD4 antibody (5 μg/ml) was added to cells and incubated for ≥ 30 min in the dark on ice. Cells were centrifuged for 10 min at 600g, supernatants aspirated and cells resuspended in 500 μl PBS/0.5% BSA. Propidium iodide was used at 2 μg/ml concentration to stain and exclude non-viable cells, which were routinely ≤ 5%. Flow cytometry was performed on a FACS Caliber (Becton–Dickenson). CD4 T cell purity was determined to be ≥ 95% pure versus no antibody control.

2.7. Measurement of T cell activation status

To quantify the extent of T cell activation, media supernatants were measured for mL-2 protein by ELISA (Biosource). Enriched mouse CD4 T cells were seeded at 10⁶ cells/ml/well in 24-well plates and treated as described above for 24 h.

Supernatants were stored at -20°C until assayed. Supernatant samples (50 μl) were assayed in duplicate in a mL-2 ELISA plate as described by the manufacturer. Optical density measurements were performed on an LD400 spectrophotometer at 405 nm (Beckman Coulter). Standard curves between 15.6 and 500 pg/ml were run with every ELISA experiment and routinely generated $r^2 \geq 0.95$.

2.8. Competitive cAMP ELISA

Purified CD4 T cells were seeded 1×10^6 cells/ml/well in 24-well plates and cultured with complete medium \pm plate-bound CD3. Cells were collected, counted on a hemocytometer and washed with PBS. Cells were centrifuged at 600g for 5 min, resuspended in HBSS/0.1% BSA and seeded into a 96-well plate at 12×10^6 cells/ml in a total volume of 100 μl . T cells are treated with 0.75 mM IBMX (3-Isobutyl-1-methylxanthine) for 30–60 min at 37°C 5% CO_2 /95% air in a humidified incubator. Exogenous VIP was added directly to wells for a final concentration of 1×10^{-6} M VIP concentration and incubated as above for 15 min. Cells were immediately lysed using 100 μl ice-cold $2\times$ lysis buffer (0.2 M HCl/2% Triton-100) and incubated with gentle rocking at RT for 10 min. Samples were frozen in the tissue culture plate until assayed. The detection of cAMP from samples was performed as described by the manufacturer. A standard curve between 0.078 and 20 pmol/ml was performed that generated an r^2 value of 0.99. This range was produced by acetylating the standards. All samples were acetylated also as described by the manufacturer. Optical density measurements were performed on an LD400 spectrophotometer at 450 nm (Beckman Coulter).

2.9. Statistical analysis

All data are presented as means \pm SEM and experiments were conducted at least three independent times unless otherwise noted in the figure legend. Statistical significance values ($p \leq 0.05$) are noted in the figure legends by asterisk symbols. A two way *t*-test analysis was performed by the Origin[®] graphical software program to determine statistical significance.

3. Results

3.1. Validation of CD4 T cell purity, phenotype and qPCR measurement of mVPAC-1 mRNA

To determine the purity and phenotype of isolated T cells, we conducted flow cytometry and IL-2 ELISA analyses. Murine, splenic, primary CD4 T cells were enriched by magnetic bead isolation (Miltenyi) to $\geq 95\%$ pure as assessed by flow cytometry (Fig. 1a). To demonstrate the extent of CD4 T cell activation in our *ex vivo* conditions, we measured IL-2 protein from cultured complete media by ELISA. We showed very little IL-2 secretion (<10 pg/ml, $n = 7$) from media control samples, which indicated a naïve phenotype (Muckenfuss et al., 2006). In contrast, 5 ng/ml PMA and 1 μg /ml ionomycin showed a robust increase in IL-2 secretion (1476 pg/ml, $n = 2$) confirming that these naïve CD4 T cells can be appropriately activated (Fig. 1b). Anti-CD3 \pm anti-CD28 treatment resulted in significantly elevated IL-2 levels (120 and 250 pg/ml, respectively; $n = 5$) and indicates that this *ex vivo* treatment successfully triggers TCR signaling and IL-2 secretion (Sun and Ganea, 1993; Schillace et al., 2005).

To confirm the accuracy and precision of our qPCR procedure, we quantitated VPAC-1 mRNA levels from eleven different mouse tissues. Relative steady-state mRNA levels of VPAC-1 normalized to the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT), are presented in Fig. 1c. The ranking in expression from lowest to highest for VPAC-1 is heart and kidney < stomach, testes < brain, lung, thymus < spleen < liver < small intestine, colon. These data are in agreement with another study using qPCR to measure mouse VPAC-1 mRNA tissue levels (Karacay et al., 2001). This agreement validates our qPCR procedure for the measurement of VPAC-1 mRNA levels.

3.2. VPAC-1 mRNA levels are increased by complete media incubation

A 24 h *ex vivo* incubation of purified CD4 T cells incubated with anti-CD3 resulted in what appeared to be a downregulation of VPAC-1 steady-state mRNA levels compared to complete media

as previously shown by others (Lara-Marquez et al., 2001; Voice et al., 2001). Unexpectedly, we noticed that VPAC-1 levels from splenic CD4 T cells used immediately after isolation were 80% lower than cells cultured in complete medium for 24 h (Fig. 2a; compare naïve vs. media). This increase in VPAC-1 expression occurred rapidly and linearly over the first 6 h and leveled off by 24 h, while anti-CD3 treatment showed a delay in inhibiting VPAC-1 levels during the initial 2 h, but was nearly abolished by 24 h (Fig. 2b). Moreover, CD4 T cells incubated for 6 and 24 h in complete medium prior to incubation with plate-bound anti-CD3 for 24 h also resulted in a similar inhibition of VPAC-1 (data not shown). To measure functionally active VPAC-1 protein levels, we utilized a competitive ELISA to measure intracellular cAMP produced from 1 μM of exogenously added VIP to CD4 T cells incubated in complete medium \pm anti-CD3. This study showed a 40% decrease in cAMP levels evoked from VIP treated anti-CD3 cells (Fig. 2c). These results support the qPCR data showing an 80% decrease in VPAC-1 mRNA levels. Also, due to the delay in protein translation and longer half-life for VPAC-1 protein, it is not surprising that a lag exists for VPAC-1 protein decrease. It is a major future goal to examine VPAC-1 protein at 48–96 h.

3.3. Culture medium supplemented with bovine or mouse serum proteins, but not serum-reduced Opti-MEM, upregulates VPAC-1 mRNA levels

Fig. 3a shows that complete medium (Section 2) supplemented with 10% normal mouse serum in place of defined fetal bovine serum (dFBS) also elevated VPAC-1 mRNA levels to the same extent. In addition, anti-CD3 treatment resulted in a similar 80% decrease in VPAC-1 mRNA after 24 h in mouse serum supplemented complete medium. This suggests that there are no heterologous factors or fetal factors influencing VPAC-1 mRNA expression. Heat-inactivated and charcoal-stripped dFBS were also tested to rule out complement proteins (heat-inactivated) and bioactive lipids and steroid hormones (charcoal-stripped).

The upregulation of VPAC-1 from complete media incubation led us to ask the question whether serum (e.g. growth factors) could upregulate VPAC-1 levels *in vivo*. Lara-Marquez et al. (2001), activated human CD4 T cells with anti-CD3/PMA isolated from human blood and showed a downregulation of VPAC-1 using an *ex vivo* procedure similar to that used in the present study. Perhaps these isolated T cells already had elevated levels of VPAC-1 due to their exposure to serum factors in the vasculature. To test this hypothesis, we isolated CD4 T cells from mouse blood and measured relative VPAC-1 levels. Fig. 3b shows that VPAC-1 mRNA is expressed at a higher level when isolated from the vasculature compared to the spleen (1.8-fold, $n = 3$). Our data reveal that VPAC-1 mRNA levels are modestly elevated from T cells isolated from blood compared to spleen, and supports an authentic and positive physiological influence on VPAC-1 expression by *in vivo* niches such as serum.

We were confident that the xenogenic culture system was not causing the complete medium-induced upregulation of VPAC-1, and therefore focused on whether serum proteins were responsible. To this end, we tested two commercially available culture media called “serum-free” AIM-V and “serum-reduced” Opti-MEM. Serum-free Aim-V medium showed a 3-fold upregulation (Fig. 3c) in VPAC-1 mRNA compared to the 6-fold increase observed with Media (see Fig. 2b, Media 24 h). This less robust increase in VPAC-1 levels by serum-free AIM-V medium suggested that non-serum factors were causing the effects on VPAC-1 expression. To clarify, the manufacturer states “serum-free” in its product title to indicate that serum need not be supplemented by the researcher. SDS-PAGE analysis clearly shows a similar protein pattern in AIM-V compared to bovine or mouse serum (10 μg /lane; Fig. 3d). The proteins added

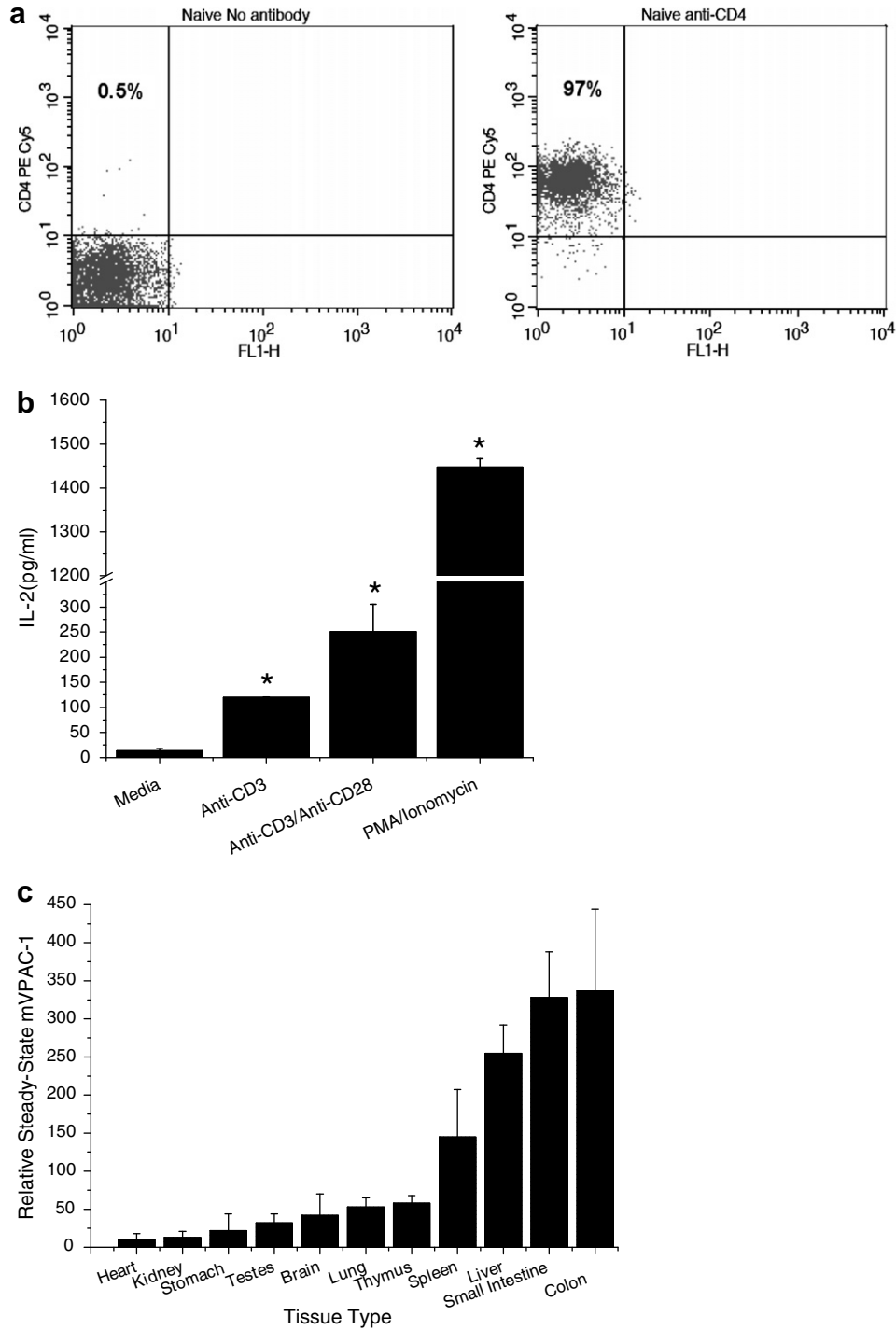


Fig. 1. Characterization of splenic, murine CD4 T cells and qPCR validation. Mouse splenocytes were used to isolate CD4 T cells by Automacs (Section 2). Purified CD4 T cells were stained with (a) (left panel) without or (right panel) with rat anti-CD4-PE as indicated. A representative scatter plot from three independent flow cytometry analyses is presented with the percentage of positive CD4 T cells indicated. (b) Purified CD4 T cells were seeded at 1 or 4×10^6 cells/ml treated with complete media, anti-CD3, anti-CD3/anti-CD28 or PMA/ionomycin for 24 h (Section 2). One asterisk symbol * indicates a statistical significance to media ($p \leq 0.05$). Supernatant IL-2 levels were measured by ELISA. Data are presented as means \pm standard deviation and is from 5 (media and anti-CD3) and 2 (anti-CD3/anti-CD28 and PMA/ionomycin) independent experiments. (c) Organs were harvested from C57Bl/6J mice and total RNA isolated (Section 2). Relative VPAC-1 levels normalized to HPRT are presented from 11 mouse tissues as indicated by qPCR. VPAC-1 tissue levels were calculated by the $\Delta\Delta C_t$ method with heart levels set arbitrarily to one.

by the manufacturer, however, are proprietary. In contrast, the reduced-serum Opti-MEM showed very little increase in VPAC-1 mRNA. As stated by the manufacturer, Opti-MEM contains significantly less protein (15 μ g/ml) compared to 10% bovine serum (50 mg/ml), 10% mouse serum (90 mg/ml) or AIM-V medium (34 mg/ml). SDS-PAGE analysis (Fig. 3d and data not shown) clearly

demonstrates this observation. Unfortunately, the Opti-MEM supplemented growth factors could not be ruled out for playing a role in VPAC-1 mRNA upregulation, as this recipe is also proprietary. In summary, only media containing significant protein levels showed an elevation in VPAC-1 mRNA expression further supporting a serum protein as the causative factor.

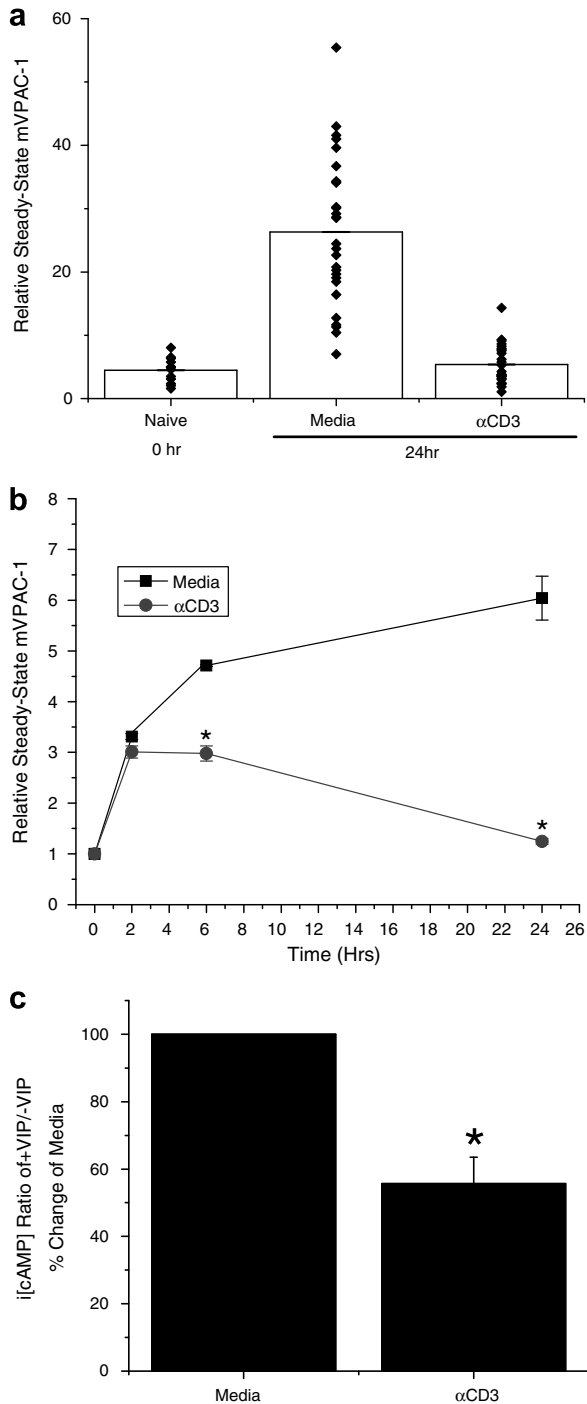


Fig. 2. Upregulation of VPAC-1 mRNA by complete media is inhibited by anti-CD3 treatment. Purified CD4 T cells were used to isolate total RNA and relative VPAC-1 levels were assessed by qPCR (Section 2). Data are presented as means \pm SEM unless otherwise noted. One asterisk symbol * indicates a statistical significance to complete media ($p \leq 0.05$). (a) VPAC-1 levels from 5×10^6 cells used immediately after T cell purification (naïve 0 h; $n = 12$) or seeded at 1 or 4×10^6 cells/ml \pm anti-CD3 treatment for 24 h (media 24 h; $n = 27$; anti-CD3 24 h $n = 26$). Data is presented as a bar graph for means and scatter data for individual experimental measurements. (b) VPAC-1 levels from cells seeded at 4×10^6 cells/ml \pm anti-CD3 treatment over the indicated time intervals. (c) Measurement of functionally active VPAC-1 receptor protein capable of eliciting an increase in cAMP levels. Purified CD4 T cells incubated \pm anti-CD4 for 24 h were incubated with 1×10^{-6} M VIP for 15 min (Section 2). Intracellular cAMP changes due to VIP addition from both populations were compared to their basal cAMP levels and graphed. Data are represented as means \pm SD from four independent experiments. The limit of detection was 0.078 pmol/ml.

3.4. RPMI 1640 medium, not serum, causes complete medium-induced upregulation of VPAC-1 levels

In an attempt to confirm that serum protein was the cause for complete medium-induced upregulation of VPAC-1, we tested whether diluting out dFBS would result in a predictable decline in VPAC-1 levels. Initially, we diluted serum by 12 orders of magnitude (10% to 10^{-11} % dFBS) supplemented with complete RPMI medium. Curiously, this showed only a downward trend in VPAC-1 levels that was not statistically significant (Fig. 4a). However, because this strategy showed significant cellular death with decreases in serum concentration (based on visual inspection of cell pellets), we also tried to spike serum into Opti-MEM that had shown superior viability compared to RPMI. Very surprisingly, this did not show an increase in VPAC-1 levels, and suggested an RPMI 1640 medium effect (Fig. 4b). To further test this hypothesis, we used DMEM/10% dFBS and showed little effect on VPAC-1 levels. We therefore conclude that serum has little effect on VPAC-1 mRNA levels, but rather substances in RPMI medium, and to a lesser extent AIM-V medium, are causing this effect. The identification of this RPMI or AIM-V substance (or substances) is presently unknown.

3.5. Src kinases are required for anti-CD3 mediated negative regulation of VPAC-1 mRNA levels

To demonstrate that the negative regulatory influence by anti-CD3 treatment on complete medium-induced upregulation of VPAC-1 expression is dependent on TCR signaling, a selective inhibitor against Src kinases was utilized to test whether a restoration in VPAC-1 levels was observed. The Src kinases, Fyn and Lck, are the most proximal upstream TCR signaling proteins that become activated upon anti-CD3 binding (Palacios and Weiss, 2004), and are inhibited by PP2 (Hanke et al., 1996), but not by PP3 (Bain et al., 2003). A 24 h *ex vivo* incubation of CD4 T cells with anti-CD3, showed a significant reduction of VPAC-1 levels compared to media control (mean = 81%; range = 60–95%; $n = 6$). In addition to the housekeeping gene, HPRT, 18S rRNA also generated similar relative VPAC-1 levels (data not shown) strongly suggesting a true inhibition in VPAC-1 levels due to anti-CD3 treatment. Increasing concentrations of PP2 (0.7–3.3 μ M), but not PP3 (3.3 μ M), completely restored VPAC-1 levels compared to anti-CD3 in a concentration-dependent manner (Fig. 5a). In addition, we have demonstrated that PP2, but not PP3, completely blocks IL-2 upregulation in the presence of TCR signaling (Fig. 5b). These functional IL-2 ELISA and qPCR analyses for VPAC-1 levels strongly support high specificity for PP2 and a low likelihood for off-target effects in our studies. Three other well established *ex vivo* T cell activation treatments showed similar low VPAC-1 levels, with PMA/ionomycin showing the greatest effect (Fig. 5c; mean = 99.3%, $n = 2$). The Src-kinase inhibitor, PP2 (3.3 μ M), was also able to effectively restore VPAC-1 levels in cells treated with anti-CD3/anti-CD28, albeit to a lesser extent and with greater imprecision, in cells treated with the non-specific, red kidney bean lectin, PHA-m (Fig. 5c). As expected, cells treated with PMA were not sensitive to PP2 as it circumvents the TCR receptor and Src kinases entirely (Castagna et al., 1982). Collectively, we conclude that anti-CD3 treatment of CD4 T cells negatively regulates RPMI-induced upregulation of VPAC-1 expression in a Src kinase-dependent mechanism.

4. Discussion

VPAC-1 levels were demonstrated to become elevated due to a 24 h incubation in RPMI complete medium, (Fig. 2a and b) which

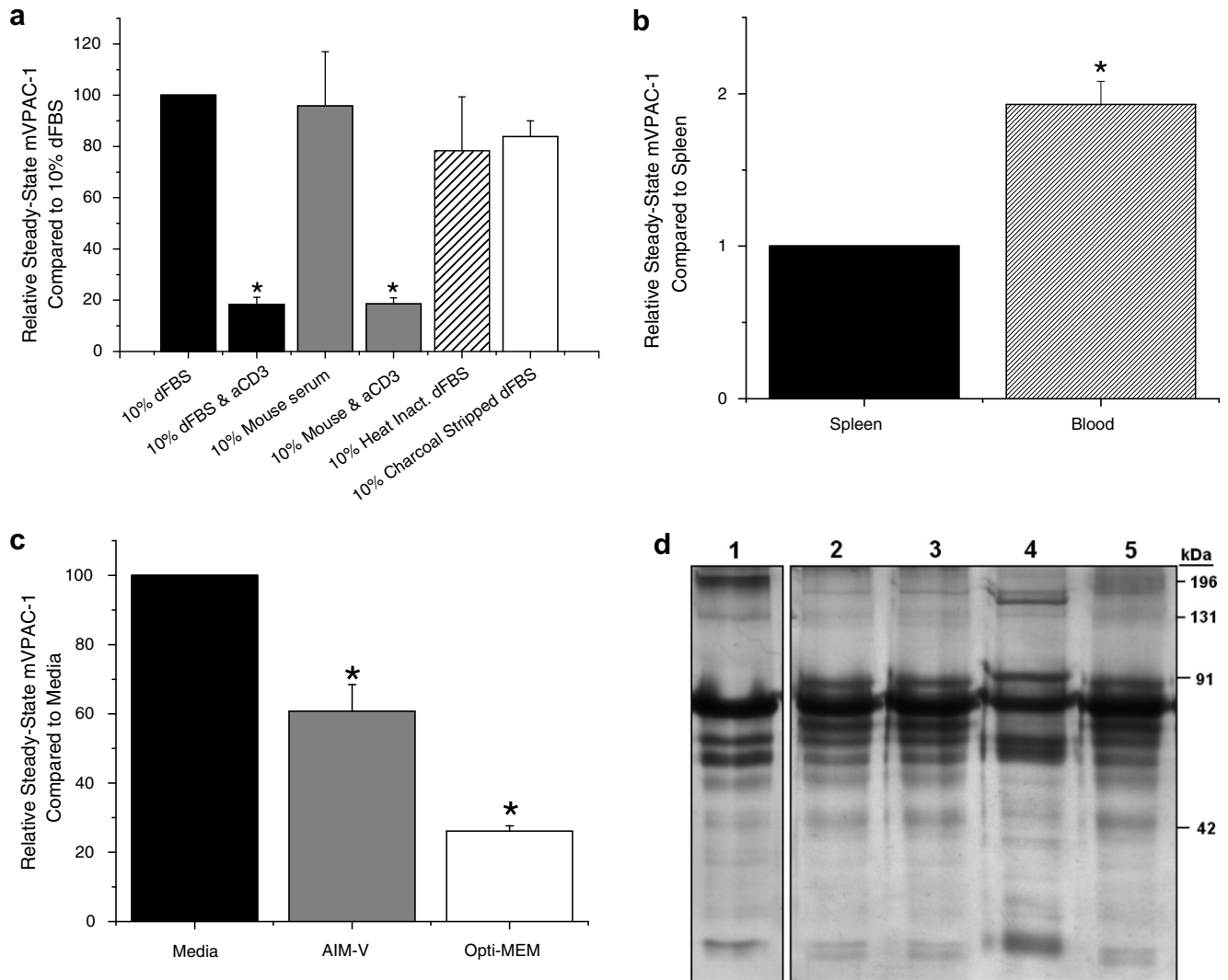


Fig. 3. Various treatments of bovine sera and mouse sera cause VPAC-1 upregulation. Purified CD4 T cells were used to isolate total RNA and relative VPAC-1 levels were assessed by qPCR (Section 2). An asterisk symbol * indicates a statistical significance to complete media ($p \leq 0.05$) for panels a and c. (a) VPAC-1 levels from cells seeded at 4×10^6 cells/ml in RPMI complete medium supplemented with the indicated sera for 24 h. Data are represented as means \pm SEM from two to three independent experiments. (b) Elevated VPAC-1 levels from CD4 T cells isolated from blood versus spleen. Immediately after isolation, purified CD4 T cells (5×10^6 cells) were used to isolate total RNA, and relative VPAC-1 levels were assessed by qPCR (Section 2). Data are presented as means \pm SEM from two independent experiments using 2–4 ml of pooled blood from 4–6 mice and a third independent experiment using 10 ml of pooled blood purchased from Bioreclamation, Inc. A $p \leq 0.05$ is considered statistically significant. (c) VPAC-1 levels from cells seeded at 4×10^6 cells/ml in the indicated media after 24 h incubation. Data are represented as means \pm SEM from two to three independent experiments. (d) SDS-PAGE analysis of various sera and serum-free AIM-V medium. Ten micrograms of protein were separated and visualized by silver staining. Protein gels were photographed by a Syngene digital camera. Lane 1, AIM-V; lane 2, dFBS; Lane 3 heat-inactivated dFBS; Lane 4, mouse sera; Lane 5, charcoal-stripped FBS.

suggested to us that (1) factors contained in RPMI or AIM-V may induce an upregulation in VPAC-1 levels, (2) CD4 T cell expression of VPAC-1 may significantly vary as a consequence of its environment (e.g. spleen vs. vasculature), and (3) TCR signaling is, more accurately described as, inhibiting this media-induced increase in VPAC-1 expression, rather than causing a bona fide downregulation of VPAC-1 mRNA levels. A study performed in 2001 that utilized blood derived human CD4 T cells showed a 91% downregulation in VPAC-1 steady-state levels after a 10 h *ex vivo* incubation with anti-CD3/PMA as compared to media (Lara-Marquez et al., 2001). Although the T cell treatments and incubation times were different, their data is in agreement with the present study as we also show lower VPAC-1 levels from various T cell treatments (e.g. PMA) after 24 h (Fig. 5c). Furthermore, this 2001 study also reported a substantial elevation in VPAC-1 levels from one of two different 10 h media (AIM-V) incubations (1621 ± 681 and 3229 ± 372 pg/100 rRNA) compared to cells used immediately

after isolation (1451 ± 493 pg/100 rRNA), suggesting a similar upregulation of basal VPAC-1 expression in AIM-V medium that we observed. Moreover, if VPAC-1 levels from their higher media control data (3229 copies/100 pg rRNA) were used to compare VPAC-1 levels from cells treated with anti-CD3, a 63% decrease in VPAC-1 would be the result. These differences would be closer to our observations of a mean of 81% with a range between 60–95% (24 h). In conclusion, the 2001 study by Lara-Marquez et al. and that of the present study, are, for the most part, in agreement. The present study further distinguishes that TCR signaling counteracts the positive regulatory influence on VPAC-1 expression from RPMI or AIM-V exposure, rather than downregulating VPAC-1 levels that began at a high initial expression level. In contrast, our data does not support a different study using naive, splenic CD4 T cells (Voice et al., 2001). This study illustrated a downregulation of VPAC-1 by qPCR that began at a high expression level at time zero and steadily declined by 95% over 18 h and remained low through

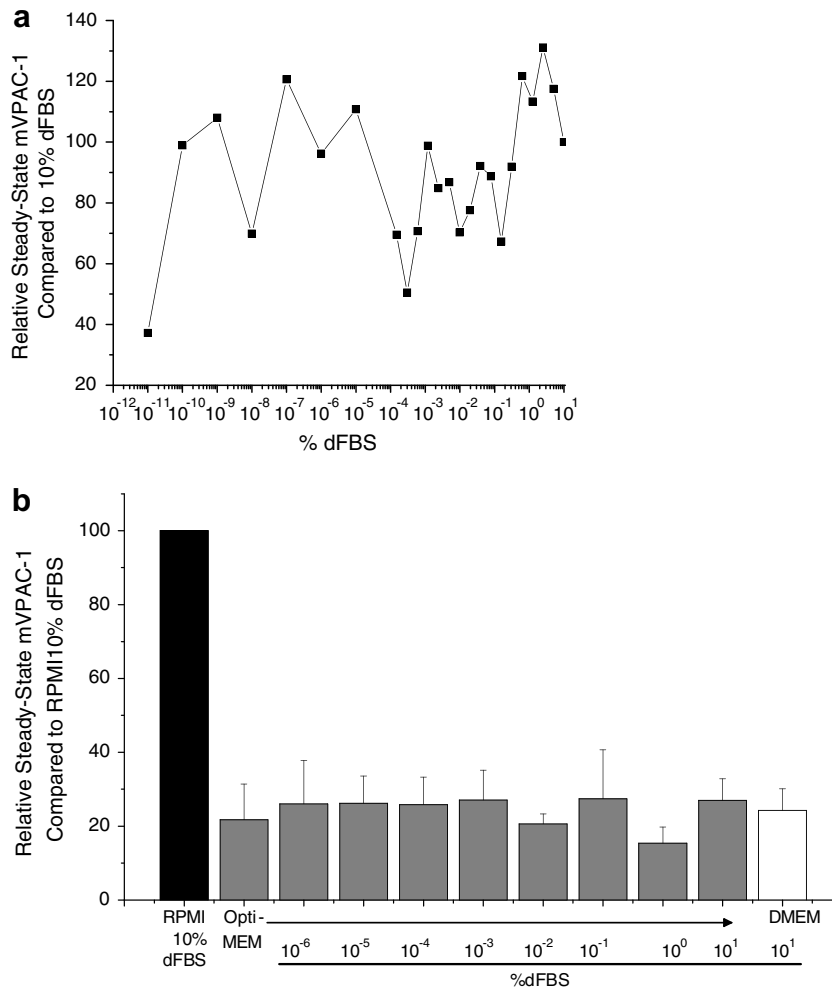


Fig. 4. Serum dilution or spiking does not affect VPAC-1 upregulation from complete media incubation. Purified CD4 T cells were used to isolate total RNA and relative VPAC-1 levels were assessed by qPCR (Section 2). (a) Four million CD4 T cells were treated for 24 h in complete media supplemented with the indicated serum concentration obtained by serial dilution. Data are represented from 1–3 independent experiments. There is no statistical significance with a calculated *p* value of 0.145. (b) Four million CD4 T cells were treated for 24 h in complete medium, DMEM/10% dFBS or Opti-MEM supplemented with the indicated percentage of dFBS generated by serial dilutions. Data are represented from 2–3 independent experiments.

96 h. However, this study utilized CD4 T cells isolated from a human VPAC-2 transgenic mouse model, which may explain such discordant data.

We show a nearly 2-fold elevation of VPAC-1 mRNA expression from CD4 T cells isolated from blood vs. spleen. VIP receptors have been shown by *in vivo* studies to be involved in cellular trafficking (Ottaway, 1984). CD4 T cells that had their VIP receptors downregulated on their plasma membranes by ligand treatment did not home to Peyer's patches as well as control. This apparent necessity for VPAC-1 receptors to assist CD4 T cells in trafficking to mesenteric lymphatic tissue and Peyer's patches was recovered within 24 h as VPAC-1 receptors resumed their normal plasma membrane expression level. We hypothesize that VPAC-1 levels may become elevated during T cell movement through the vasculature due to serum factors. This elevation may assist T cells to locate VIPergic nerves that innervate near high endothelial venules (HEV) and assist their movement from the vasculature into Peyer's Patches (Ottaway et al., 1987; Miura et al., 1997). This is further supported by studies where VIP causes increased adhesion of naïve, but not activated, CD4 T cells to the extracellular matrix protein, fibronectin (Johnston et al., 1994).

We also observed a 6-fold increase in VPAC-1 levels by RPMI complete medium. The class of molecule(s) responsible for this in-

crease by RPMI 1640 medium and AIM-V is presently unknown. It is entirely possible that a substance found in RPMI may also be present in whole blood, which explains our *in vivo* data showing nearly a two-fold increase in VPAC-1 levels. However, why fetal bovine serum would also not contain this substance is not understood. Perhaps this substance found in RPMI is lost or degraded upon fetal bovine serum isolation. A recent study showed that L-Arginine, an amino acid found in RPMI in high concentrations, has been shown to regulate the expression of CD3 zeta (Rodriguez et al., 2002). Whether amino acids or other RPMI specific factors are control VPAC-1 expression will require additional research. Moreover, it is not known whether this substance found in RPMI is present at similar concentrations in serum. Another possibility to explain the higher VPAC-1 expression level on CD4 T cells from blood vs. spleen might be due to a greater memory CD4 T cell population in blood than spleen. If true, it would indicate that memory CD4 T cells have elevated VPAC-1 levels. Experiments are presently ongoing to investigate these unanswered questions.

In summary, these data have demonstrated that CD4 T cells express VPAC-1 at differing levels dependent on the environment of the T cell. T cells incubated for 24 h in RPMI complete media or AIM-V show a substantial elevation in VPAC-1 mRNA levels as compared to naïve, splenic CD4 T cells measured immediately after

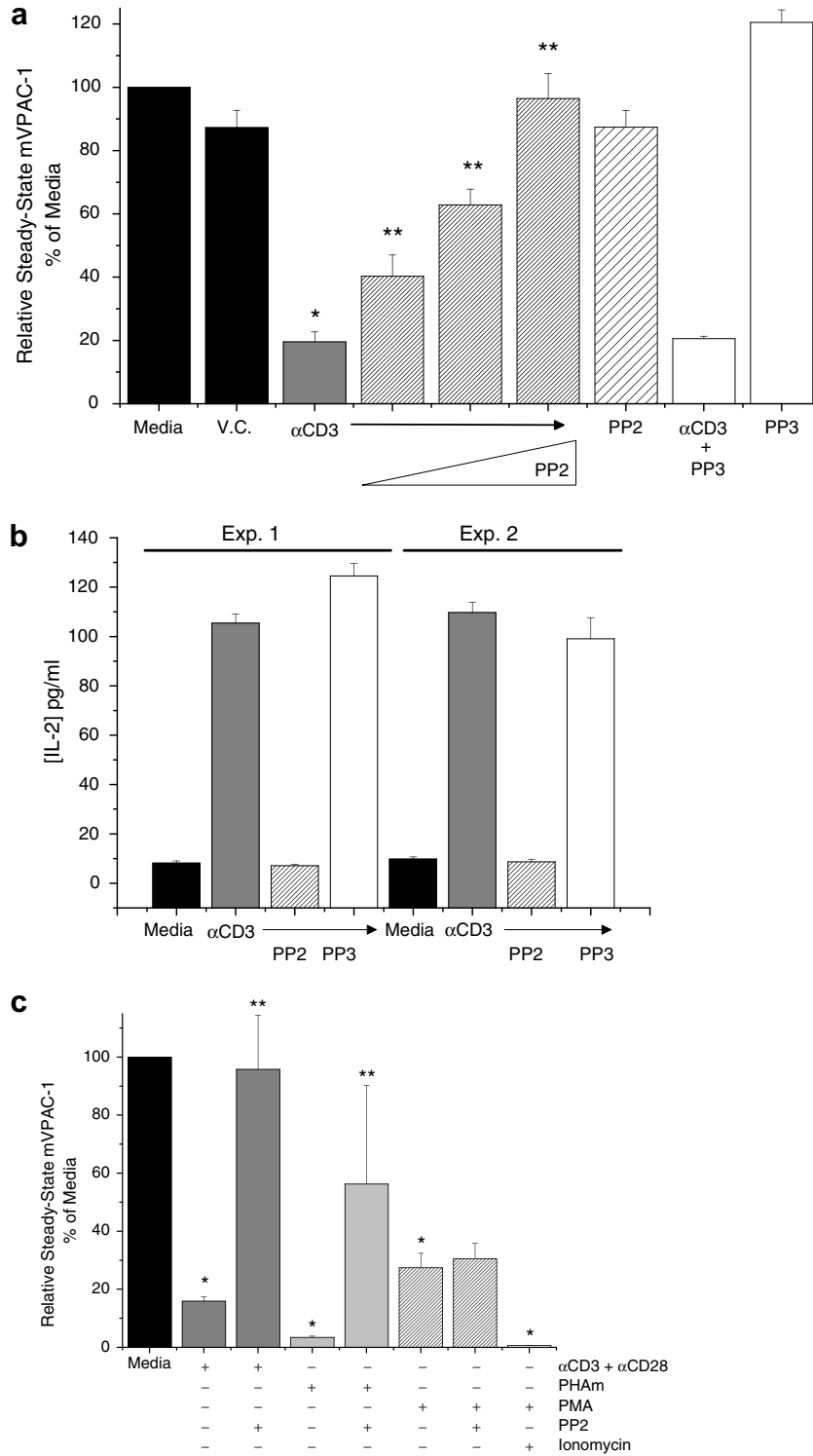


Fig. 5. TCR signaling is required for anti-CD3 negative regulation of VPAC-1 in complete medium. Purified CD4 T cells seeded at 1 or 4×10^6 cells/ml were used to isolate total RNA and relative VPAC-1 levels were assessed by qPCR (Section 2). Complete media levels were arbitrarily set to 100%. One and two asterisk symbols indicate a statistical significance to media alone (*) or anti-CD3 (**). Data are presented as means \pm SEM from two to five independent experiments unless otherwise indicated. (a) Cells were incubated for 24 h in complete media (media), $\leq 0.1\%$ DMSO (V.C.), $3.3 \mu\text{M}$ PP2 or PP3, or with anti-CD3 (αCD3) \pm increasing concentrations of PP2 (0.7 , 1.1 and $3.3 \mu\text{M}$, triangle) or $3.3 \mu\text{M}$ PP3 for 24 h. (b) Cells incubated in complete media $\pm 4 \mu\text{g}/\text{well}$ of plate-bound anti-CD3 and $3.3 \mu\text{M}$ of PP2 or PP3 (Section 2). Cultured media supernatants were collected and used for IL-2 measurements by ELISA. Data are represented from two separate experiments as means \pm SD. (c) Cells were incubated for 24 h with the indicated culture conditions (Section 2), data is from 3 experiments as means \pm SEM. One and two asterisk symbols indicate a statistical significance to media alone (*) or appropriate activating condition (**).

isolation or in the presence of TCR signaling. The upregulation of VPAC-1 in RPMI complete media is presently hypothesized to be a substance found in RPMI and AIM-V but not in commercially

available bovine or mouse serum, Opti-MEM or DMEM. TCR signaling appears to override the positive influence on VPAC-1 regulation through a Fyn and Lck Src kinase-dependent manner. Additional

research is necessary to fully understand the regulatory mechanisms of VPAC-1 in T cells. Such research will be essential to fill in a fundamental gap in knowledge regarding regulation of this anti-inflammatory GPCR.

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