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Vasoactive intestinal peptide receptor 1 is downregulated during expansion of antigen-specific CD8 T cells following primary and secondary *Listeria monocytogenes* infections

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ABSTRACT

As regulation of CD8 T cell homeostasis is incompletely understood, we investigated the expression profile of the vasoactive intestinal peptide (VIP) receptors, VPAC1 and VPAC2, on CD8 T cells throughout an *in vivo* immune response. Herein, we show that adoptively transferred CD8 T cells responding to a *Listeria monocytogenes* infection significantly downregulated, functionally active VPAC1 protein expression during primary and secondary expansion. VPAC1 mRNA expression was restored during contraction and regained naïve levels in primary, but remained low during secondary, memory generation. VIP co-administration with primary infection suppressed CD8 T cell expansion (≈50%). VPAC2 was not detected at any time points throughout primary and secondary infections. Collectively, our data demonstrate that functionally active VPAC1 is dynamically downregulated to render expanding CD8 T cells unresponsive to VIP.

1. Introduction

Vasoactive Intestinal Peptide (VIP) is delivered by the peripheral nervous system to primary and secondary immune organs (Bellinger et al., 1996; Said and Mutt, 1970). Innate and adaptive immune cells express two G-protein coupled receptors, vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor 1 (VPAC1) and VPAC2 (Delgado et al., 2004; Dickson and Finlayson, 2009; Smalley et al., 2009), allowing these cells to respond to the VIP ligand. Originally classified as a vasoactive peptide from the gut (Said, 1974), VIP has been re-categorized as an immunosuppressive neuropeptide (reviewed in Delgado et al., 2004). VIP is a neurotransmitter and mitogenic factor for neurons in the brain (Pincus et al., 1990), but is an anti-inflammatory mediator within the immune system (Bellinger et al., 1997; Ottaway and Greenberg, 1984; Xin et al., 1994). In addition to VIP^+ nerves, other sources of VIP are thymocytes and activated Th_2 CD4 T cells (Delgado et al., 1999a; Vassiliou et al., 2001).

Distinguishing whether VPAC1 or VPAC2 is responsible for VIP's effects is complicated due to the significant changes in their expression patterns depending on T cell activation status. For example, we have demonstrated that activated T cells downregulate VPAC1 mRNA expression through a Src kinase/ZAP70/JNK mediated mechanism (Vomhof-DeKrey and Dorsam, 2008; Vomhof-DeKrey et al., 2008). Regarding VPAC2, *in vitro* studies have shown that its mRNA expression is inducible upon T cell activation (Delgado et al., 1996; Voice et al., 2001). These observations were supported *in vivo* by Metwali et al., where they showed that schistosome infected mice had $VPAC2^+$ T cells present in granulomas, but splenic T cells were $VPAC2^-$ (Metwali et al., 2000). However, several other groups have failed to demonstrate VPAC2 upregulation by *in vitro* activation conditions (Metwali et al., 1996; Qian et al., 2001). Collectively, these discrepancies emphasize the need to study VIP receptor expression in a more physiologically relevant *in vivo* environment.

A great deal of research over the past decade has uncovered mechanisms by which VIP, binding to either VPAC1 or VPAC2, affect CD4 T cell functions, specifically Th_2 humoral immunity (Sharma et al., 2006; Voice et al., 2004, 2001). In contrast, very little data has been collected regarding the effects of VIP on cell-mediated immunity carried out by CD8 T cells. Therefore, in this study, we mapped VPAC1 and VPAC2 levels throughout a primary and secondary *Listeria monocytogenes* infection in the well established OT-I mouse model (Hogquist et al., 1994). To our knowledge, this is the first report of VIP receptor measurement in an *in vivo* CD8 T cell immune response. Functionally active VPAC1 receptor protein and mRNA became transiently silenced during CD8 expansion, and was restored during contraction. VPAC2 mRNA, surprisingly, was not detected during

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primary or secondary infections. Importantly, VIP co-administration during primary infection resulted in a significant suppression of CD8 T cell expansion (≈50%). Collectively, these data demonstrate that the immunosuppressive effects by VIP in resting antigen-specific CD8 T cells appear to be exclusively transmitted by the VPAC1 receptor.

2. Materials and methods

2.1. Mice

Wild type C57BL/6J, C57BL/6-Tg (TcraTcrb)1100Mjb/J (OT-I), and B6.PL-*Thy1*^{a}/CyJ (Thy1.1) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at NDSU. All animal studies were conducted with prior approval from the NDSU Institutional Animal Care and Use Committee, which sets best practice standards for the university under the guidance of the Office of Laboratory Animal Welfare.

2.2. Bacteria

act A$^-$ *L. monocytogenes*-OVA (LM-OVA) (Pope et al., 2001) was grown in TSB broth + 500 μg streptomycin, and then ~1×10^7 LM-OVA cells were injected retro-orbitally into recipient mice. *Listeria*-infected mice were housed under appropriate biosafety conditions. Mice between 6 and 28 weeks of age were used.

2.3. Flow cytometry

The following antibodies were utilized: CD44 (clone IM7, Biolegend), CD25 (clone PC61.5, eBioscience, San Diego, CA; clone 3C7, Biolegend), CD8a (clone 53-6.7, eBioscience), CD4 (clone RM4-4 or GK1.5, Biolegend), CD90.1 (clone OX-7, Biolegend), CD62L (clone MEL-14, Biolegend), CD127 (clone SB/199, Biolegend), TCR Vα2 (clone B20.1, Biolegend), CD69 (clone H1.2F3, Biolegend) and isotype controls IgG2aκ, IgG1κ, IgG2bκ and IgG (clones RTK2758, MOPC-21, eB149/10H5 and HTK888; Biolegend). At specific time points, splenocytes were stained with identifying (CD8 or CD4 or CD90.1) and activation marker (CD25, CD69, CD62L, or CD127) antibodies in order to numerate and determine the activation state of the Thy1.1$^+$/OT-I cells or polyclonally activated CD4 and CD8 T cells. For mVPAC1 antibody staining, total splenocytes (3×10^6) were resuspended in 100 μl PBS/0.5% BSA using 5 ml Falcon tubes and incubated with 1:100 melon gel (Bio-Rad, Hercules, CA) purified α-mVPAC1 polyclonal antibody (Aldevron, Fargo, ND) or rabbit serum for 30 min at 4 °C in the dark. Cells were washed twice with 1 ml PBS/0.5% BSA, centrifuged for 5 min at 500×g, supernatants aspirated off and incubated with secondary, PE-goat anti-rabbit IgG for 15 min at 4 °C in the dark. Cells were washed twice with 1 ml PBS/0.5% BSA, centrifuged for 5 min at 500×g, and resuspended in 300 μl PBS/0.5% BSA. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) or an Accuri C6 Cytometer (Ann Arbor, MI).

2.4. Polyclonal activation

C57Bl/6J mice were retro-orbitally injected with 10 or 50 μg anti-CD3 (Biolegend) or Armenian Hamster isotype (Biolegend, San Diego, CA) (Bemelmans et al., 1994). Twenty-four (24) hours later, mice were euthanized by rapid cervical dislocation and spleens harvested. Spleens were minced in RPMI with 10% defined FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin at room temperature. Dispersed splenocytes were passed through a 70 μm sieve (BD Biosciences, San Jose, California) and centrifuged at 500×g for 5 min. Splenocytes were stained with anti-CD4 or anti-CD8α magnetic beads and isolated by magnetic bead technology using an AutoMACs instrument (Miltenyi Biotec, Bergisch Gladbach, Germany) with the Possel (positive selection) program. Splenocytes were applied to the instrument twice as described by the manufacturer. Typical purity was >90% as determined by flow cytometry using anti-CD4 (clone RM4-4, Biolegend) or anti-CD8a (clone 53-6.7, eBioscience).

2.5. Adoptive transfers

Donor Thy1.1$^+$/OT-I mice were genotyped by flow cytometry using antibodies specific for CD90.1 and TCR Vα2. Donor mice were retro-orbitally bled and approximately 500 Thy1.1+/OT-I cells were adoptively transferred to naïve, C57BL/6J (Thy1.2) mice. One day post transfer, mice were immunized with 1×10^7 LM-OVA.

2.6. Purification of Thy1.1$^+$ adoptively transferred T cells

At different times post-infection (p.i.), mice were euthanized by rapid cervical dislocation and splenocytes were isolated as described above. Single cell suspensions were stained with anti-CD90.1 magnetic beads (Thy1.1) and isolated on an AutoMacs as described above. Typical purity was ≥90% by flow cytometry as described above with 2–3 applications over the magnetic column.

2.7. RNA isolation and qRT-PCR

Total RNA from enriched CD8$^+$, CD4$^+$ or CD90.1$^+$ T cells was isolated by sequential passes through a QIAshredder spin column followed by an RNeasy Micro column with on-column DNase I treatment as described by the manufacturer (Qiagen, Valencia, CA). After total RNA elution and a second DNase treatment (gDNA wipeout), cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit as described by the manufacturer (Qiagen). Real time reactions contained the following, 1× SYBRGreen Master Mix (Applied Biosystems, Inc., Carlsbad, California), 250 nM mVPAC1 (forward, 5′-GAT ATG GCC CTC TTC AAC AAC G-3′; reverse, 5′-GAA GTT GGC CAT GAC GCA AT-3′) or mHPRT (forward, 5′-CTG GTG AAA AGG ACC TCT CG-3′; reverse, 5′-TGA AGT ACT CAT TAT AGC AAG GGC A-3′) or 400 nM mVPAC2 (forward, 5′-CCA GAT GTT GGT GGC AAT GA-3′, reverse, 5′-GTA TGT GGA TGA GAT GCC AAT AGG-3′) primers and a 1/16 dilution of cDNA. The final volume of the reactions was 20 μl, and dissociation curves confirmed a single amplification species.

2.8. Intracellular cAMP competitive ELISA

Naïve, CD8$^+$ T cells and day 5, Thy1.1$^+$/OT-I cells p.i. were isolated as described above. Approximately, 5×10^6 cells/ml were seeded in a 96 well plate (100 μl), were pretreated with phosphodiesterase inhibitor (750 μM), 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO), and then treated with VIP (10^{-7} M) or water control for 15 min at 37 °C. Cells were lysed with 0.1 M HCl and the intracellular cAMP concentration (i[cAMP]) was measured by a competitive ELISA kit following the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). A cAMP standard curve was performed with all experiments showing linearity in a range of (0.5–500 pmol/ml). All unknown absorbance values were collected within this range using duplicate, replicates.

2.9. Statistics

All qPCR and i[cAMP] data are presented as means ± SEM and experiments are conducted at least 2–6 independent times unless otherwise mentioned in the figure legend. A two way *t*-test analysis was performed by the Origin® graphical software program to determine statistical significance ($p \le 0.05$), and is noted in the figure legends by asterisk symbols. Flow cytometry data is presented as representative for the indicated number of independent experiments.