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CD4 T Cells Differentially Express Cellular Machinery for Serotonin Signaling, Synthesis, and Metabolism

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Abstract

CD4 T cells play a major role to orchestrate the immune response. Upon activation, CD4 T cells differentiate into effector T cell (Teff) or regulatory T cell (Treg) subsets that promote or suppress the immune response, respectively. Along with these unique immunological roles, CD4 T cell subsets have specific metabolic requirements and programs that can influence the immune response. We therefore examined the metabolite levels of Teff and Treg in detail. Surprisingly, the metabolite showing the largest difference between Teff and Treg was serotonin (5-HT), revealing a potentially distinct role for serotonin in CD4 T cell function. 5-HT is well known as a neurotransmitter and recently has been recognized to play a role in the immune response; however, little is known about the immune cell type-specific expression of the serotonergic machinery and receptors. We therefore examined the serotonergic-related machinery in Teff and Treg and found differential expression of the serotonin transporter SERT and 5-HT_{1a} and 5-HT₂ receptors. We also found that Treg express tryptophan hydroxylase, which converts tryptophan to serotonin, suggesting for the first time that Treg synthesize serotonin. Our results in this study expand the potential immunomodulatory role of serotonin in CD4 T cell biology and could ultimately aid the

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Authorship statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by VG, HW and DH. The first draft of the manuscript was written by VG and HW and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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development of novel immunomodulatory targets for treatment of autoimmune and neuropsychiatric disorders.

Keywords

Serotonin; 5-HT; lymphocytes; T cells; regulatory T cells; Treg

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter commonly known for its role in brain function and mood; however, brain-derived or central serotonin only accounts for 5% of the body's total serotonin (1–3). A role for serotonin in immunity is becoming increasingly recognized, as the use of serotonin modulating drugs such as serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors have been shown to be immunomodulatory and influence autoimmune and inflammatory processes (4–8). SSRIs bind to the serotonin transporter SERT (also called 5-HTT), an integral membrane protein that mediates the transport of serotonin and other ions into the cell (9, 10). SSRIs have even been described as a novel class of immunosuppressants, as they have been shown in animal models to suppress immune reactions involved in autoimmune disease and *in vitro* to suppress lymphocyte proliferation, cytokine secretion, and viability (7, 8, 11). Serotonin itself can be transported into the cell via SERT and stored in vesicles mediated by the vesicular monoamine transporter (VMAT), a process which has been largely characterized in neurons but is thought to occur in immune cells as well (12–14). Extracellularly, serotonin also binds to a large family of 5-HT receptors, the downstream effects of which are cell-type specific and not well understood for immune cells (6, 15, 16).

Serotonin is derived from the amino acid tryptophan. Tryptophan is involved in protein synthesis and the production of cellular metabolites, including kynurenine and 5-HT (17). The enzyme indolamine-2,3-dioxygenase (IDO) converts tryptophan to kynurenine in most tissues. The IDO/kynurenine pathway is an important metabolic pathway that generates nicotinamide adenine dinucleotide (NAD), an essential metabolic cofactor. In addition, the IDO pathway has been implicated in immunity, as IDO1 expression can be induced by inflammatory cytokines and has been found to play a role in immune tolerance and the maternal-fetal interface (18–20). Kynurenine and the other metabolites along the IDO pathway have also been shown to play an immunoregulatory role through binding to the aryl hydrocarbon receptor (Ahr) in various immune cell types (21, 22). Tryptophan conversion to 5-HT is mainly controlled by the enzyme tryptophan hydroxylase (TPH) (23). Because both tryptophan degradation pathways have been implicated to influence the immune response, a greater understanding of the role of tryptophan metabolism and the metabolites kynurenine and 5-HT in immunity is critical.

As CD4 T cells are the major orchestrators of the adaptive immune system, the regulation of the T cell response by 5-HT is of great interest. Naïve CD4 T cells are activated by cytokines in the environment and differentiate into T effector cells (Teff), including pro-inflammatory T helper 1 (Th1) and T helper 17 (Th17) cells which direct and coordinate the immune

response (24). Another subset of T cells, regulatory T cells (Treg), are responsible for suppressing and moderating the inflammatory response (25, 26). An appropriate balance between Teff and Treg is important, as imbalance can lead to either excessive inflammation or immune suppression. While previous studies have examined the presence of serotonergic machinery in T cells, these studies have been performed in T cell lines or total T cells (27–29). As Teff and Treg play distinct roles in the immune response, an understanding of the expression of serotonin-processing cellular machinery and levels of metabolites in different CD4 T cell populations (naïve, Teff, Treg) will provide a greater understanding of the role of serotonin in T cell biology. Here, we examine the expression of serotonergic machinery, including serotonin transport, receptors and intracellular storage in the CD4 T cell subsets.

2. Materials and Methods

2.1 Mice

Six to eight week old C57BL/6J mice from Jackson Laboratory were used for all experiments. All procedures were performed under Duke University Medical Center IACUC-approved protocols.

2.2 T cell isolation and differentiation

Naïve CD4+CD25– T cells were isolated *ex vivo* from the spleens of the mice described above by magnetic microbead negative selection with >98% purity (Miltenyi #130-104-454). The isolated CD4+CD25– T cells were then cultured on irradiated splenic feeder cells (300 Gy) at a ratio of 1:5 (200,000 T cells:1 million irradiated splenocytes in a 24-well plate) in RPMI supplemented with 10% FBS, sodium pyruvate, penicillin/streptomycin, HEPES and beta-mercaptoethanol and 2.5µg/mL of soluble anti-CD3 antibody (eBioscience). The naïve T cells were then differentiated *in vitro* by adding the following cytokines to each generate each subset: Th1, 10ng/mL IL-12 (R&D Systems), 10µg/mL anti-IL-4 (eBioscience, clone 11B11), 1µg/mL anti-IFNγ (eBioscience); Th2, 1000U/mL recombinant IL-4 (R&D Systems), 10µg/mL anti-IL-12 (eBioscience), 10µg/mL anti-IFNγ; Th17, 20ng/mL IL-6 (R&D Systems), 2.5ng/mL TGFβ (R&D Systems), 10µg/mL anti-IFNγ; Treg, 3ng/mL TGFβ. On day 3 post stimulation, cells were split 1:2 and expanded with IL-2 for an additional 2 days (30, 31). Cells were then harvested, washed 1x with PBS, pelleted and snap frozen.

2.3 Semi-quantitative real time PCR

RNA was extracted from cell pellets with the RNeasy RNA purification minikit (Qiagen). Reverse-transcription PCR was performed with iScript cDNA synthesis kit (Biorad). Real-time PCR was performed with iQSYBR Green detection chemistry (Biorad) using an iCycler (BioRad). Relative mRNA levels were normalized to actin RNA content. A CT value < 36 was reported as positive for RNA expression. Primer sequences are as follows:

Name	Sequence	Use
TPH1FW	GAA CAA AGA CCA TTC CTC CGA AAG AG	qPCR
TPH1 REV	GTG AGC TGA TCG GGC GAG TCC A	qPCR
HTR2A FW	CAG CCC TCC CTC CTC GTT TTG	qPCR
HTR2A REV	GCC GGAAGT TGT AGC AGA TGAAGT	qPCR
HTR1A FW	ACC CCA ACG AGT GCA CCA TCA G	qPCR
HTR1A REV	GCA GGC GGG GAC ATA GGA G	qPCR
HTR1B FW	CGA TGC GGT GGA GTA TTC TGC	qPCR
HTR1B REV	TAG CGG CCA TGA GTT TCT TCT TTT	qPCR
Slc6a4 FW	ACA ACA TCA CCT GGA CAC TCC ATT C	qPCR
Slc6a4 REV	CCG CAT ATG TGA TGA AAA GGA GGC T	qPCR
IDO1 FW	AAGGGCTTCTCCTCGTCTC	qPCR
IDO1 REV	AAAAACGTGTCTGGGTCCAC	qPCR
VMAT2 FW	TGTGAAGTCTGGTGTGTTAAGG	qPCR
VMAT2 REV	CATCACATCACAAGGCATCC	qPCR

2.4 Metabolomics

Nontargeted metabolomic analyses were performed as described previously (32). Briefly, CD4 T cells were isolated and differentiated as described above and then subjected to both GC/MS and LC/MS/MS platforms (by Metabolon Inc.) for determination of cellular metabolites. Data were then grouped by unsupervised clustering using MetaboAnalyst software. The samples were normalized using Bradford protein concentration and rescaled to set the median to 1.

2.5 Statistical analysis

Statistical analyses were primarily performed with Prism software (GraphPad) using 2-tailed Student's *t*-tests. Statistically significant results are indicated (* = $p < 0.05$). For the metabolomics data, pair-wise comparisons were performed with Welch's *t*-tests and/or Wilcoxon's rank sum tests using the program "R".

3. Results

3.1 Metabolomic profiling of CD4⁺ subsets reveals differences in tryptophan metabolic pathway

The balance of effector CD4 T cells (Teff; e.g. Th1 and Th17) and regulatory T cells (Treg) is critical in a variety of settings, particularly in the context of autoimmunity and inflammatory disease (25, 33–35). An understanding of the metabolic differences between Teff and Treg may lead to new strategies for treating inflammatory diseases by modulating the Teff:Treg balance through metabolic inhibition. While much of the previous work examining the T cell metabolic profiles has focused on glucose and lipid metabolism, there has been an increasing focus on differences in amino acid metabolism in the T cell subsets

(36–42). We therefore examined metabolite levels of Teff and Treg in detail, using unsupervised clustering of approximately 400 metabolites measured by high-resolution nontargeted Q exactive–mass spectrometry (QE-MS) metabolomics (32). Surprisingly, the metabolite showing the largest difference between Teff and Treg was serotonin (5-HT). While the levels of the serotonin precursor tryptophan were relatively similar between naïve T cells, Teff (Th1 and Th17) and Treg, 5-HT levels were markedly higher in Treg compared to naïve T cells and Teff (Figure 1). We also examined the other tryptophan metabolite kynurenine and found a trend toward higher levels in Teff, suggesting that Teff and Treg may utilize distinct pathways to metabolize tryptophan.

To assess whether Treg have the ability to synthesize serotonin from tryptophan, we performed qPCR analysis and found that Treg express high levels of the enzyme that converts tryptophan to 5-HT, tryptophan hydroxylase 1 (TPH1) (Figure 2). Teff also expressed TPH1 but to a much lesser extent than Treg (20–40 fold compared to 100 fold higher than naïve T cells). The expression of the other TPH isoform, TPH2, was not detected. Therefore, our data shows that Treg have the ability to synthesize 5-HT and contain high levels of intracellular 5-HT. Interestingly, while Teff did not contain significant levels of 5-HT, Th1 and Th17 cells showed higher levels of IDO, the enzyme that converts tryptophan to kynurenine. Th17, in particular, expressed significantly higher levels of IDO1.

3.2 Treg contains the machinery to synthesize, transport and store intracellular serotonin

Given that Treg contained high levels of 5-HT, we next examined if Teff and Treg have differences in other 5-HT-related machinery that might explain increased intracellular 5-HT levels in Treg. The serotonin transporter, SERT (*slc6a4*, also called 5-HTT) is expressed by neurons and cells of the GI tract but has more recently been reported in human blood lymphocytes (9, 43). We found that *Slc6a4* was expressed by naïve T cells, Teff and Treg with no significance differences between the subtypes (Figure 3A), suggesting that all of the examined CD4 T cell subtypes have a similar capacity to transport extracellular 5-HT into the cell. We next examined the expression of vesicular monoamine transporter 2 (VMAT2 or *slc18a2*), which is best known for its ability to transport monoamines, including serotonin, from the intracellular space into synaptic vesicles in the brain. Previous studies have suggested that human peripheral blood lymphocytes express VMAT2 but it has only been examined in the context of dopamine in bulk lymphocytes (13). Interestingly, Treg showed significantly higher expression of VMAT2 compared to naïve T cells and Teff (Figure 3A). Altogether, these data suggest that Treg have the capacity to generate 5-HT from tryptophan and store 5-HT in intracellular vesicles via VMAT2.

3.3 Teff and Treg have different expression patterns of 5-HT receptors

The finding that CD4+ T cells, particularly Treg, express 5-HT related machinery led us to hypothesize that serotonin effects on T cells via the 5-HT receptors may modulate T cell function. Indeed, previous studies have shown that lymphocyte cell lines and human T cells express several of the 5-HT receptors, including the 5-HT1, 5-HT2 and 5-HT7 receptors, although the downstream effects of these receptors in T cells are still the subject of investigation (44–47). Importantly, none of these studies explored differences between the CD4 T cell subsets. Therefore, we performed qPCR to examine if Teff and Treg have

differential expression patterns of the 5-HT receptors. While the expression of the 5-HT1a, 5-HT1b, 5-HT2 and 5-HT7 receptors were examined, only 5-HT1aR and 5-HT2R were expressed by the CD4 T cell subsets (Figure 3B). 5-HTR1a was expressed at a higher level in naïve T cells compared to Teff and Treg, while 5-HTR2 had significantly higher expression in Treg. Together, these data suggest that changes in serotonin receptor expression may play a role in Teff versus Treg differentiation and/or function.

4. Discussion

Understanding the mechanisms that control T cell function and differentiation is crucial to develop new strategies to modulate immune function and prevent autoimmune and inflammatory disease. One such emerging area of research is the role of serotonin in immunity (6, 48, 49). While earlier studies have explored the presence of serotonin-related machinery, as well as the expression of serotonin receptors, in T cells, these studies were primarily done in bulk lymphocytes, total T cells or T cell lines (6, 27, 28, 44, 46, 50). To our knowledge, no study had specifically examined the CD4 T cell subsets (Teff and Treg). Total lymphocytes or T cells were shown to express the 5-HT1a, 5-HT1b, 5-HT2, 5-HT3 and 5-HT7 receptors (27, 28, 44, 46, 47). Our results with the differentiated CD4 T cell subsets suggest that naïve CD4 T cells as well as Th1, Th17 and Treg express the mRNA for 5-HT1a and 5-HT2 receptors but not 5-HT1b, or 5-HT7. The effect of serotonin signaling on CD4 T cell fate and function will be important to explore in future studies. Previous work reported an increased expression of the 5-HT1a receptor in the peripheral blood lymphocytes of systemic lupus erythematosus patients, suggesting a possible role for serotonin signaling in autoimmunity (51). The role of 5-HT2 in T cells is less clear; one study showed that treatment of total T cells with a 5-HT2 receptor agonist increased the expression of inflammatory cytokines; however, another used a different agonist and found a reduction in splenic CD8 T cells (52, 53). Therefore, more research is needed to understand the role of 5-HT as a stimulator or a suppressor of T cell function, and in what subtypes and contexts.

Additionally, more research is needed to determine whether serotonin, tryptophan or the balance between the IDO and TPH metabolic pathways influences immune and inflammatory processes (19, 54). Tryptophan is an essential amino acid that can be degraded into either serotonin or kynurenine (17). The expression of IDO in dendritic cells has been shown to promote tolerance and inhibit T cell proliferation (18, 20). Several theories have been proposed, including that the metabolism of tryptophan removes the essential amino acid from the microenvironment, effectively inhibiting T cell proliferation (54, 55). Other data has linked the IDO pathway to the generation of Treg, as tryptophan starvation combined with kynurenine supplementation induced a regulatory phenotype in naïve T cells (56). There is also evidence that kynurenine interacts with the aryl hydrocarbon receptor to generate Treg and that serotonin upregulates Treg and inhibits Teff (57–59). In addition, our data suggests that Treg selectively express TPH-1. Previous work has shown that total CD3 T cells increase their expression of TPH-1 upon activation with concanavalin A as well as activated CD4 T cells stimulated with PMA and ionomycin (27–29). Whether the TPH-1 detected in these previous studies was a result of Treg expression will be important to explore in future studies. In addition, we found that the CD4 T cell subsets express VMAT2, while VMAT1 was previously shown to be expressed in total T cells (29). Therefore, much

more work is needed to understand the expression of the various serotonergic-related genes in the different T cell compartments, including CD8 T cells, memory T cells and follicular T helper cells, and confirm the expression of these proteins. Our data suggests that there may be a more direct role for serotonin in CD4 T cell biology, as Teff and Treg express distinct levels of the serotonin receptors and machinery. The high levels of serotonin and expression of TPH1 in Treg compared to Teff also points to a more direct role for serotonin or the removal of tryptophan in the CD4 T cell subsets.

It is becoming increasingly clear that the neurotransmitter serotonin plays a role in immune function. The presence and distinct expression of serotonin pathway components in Teff and Treg suggest a unique role for serotonin in these cell populations. Moving forward, it will be important to confirm these findings in human T cells and further examine the role of serotonin in specific T cell subsets and whether drugs that alter 5-HT signaling have differential effects on the balance of specific T cell subtypes in different disease settings.

5. Conclusions

These data suggest that tryptophan metabolism is differentially regulated in Teff and Treg and that serotonin may be selectively important for Treg. The results of this study expand the potential immunomodulatory role of serotonin in CD4 T cell biology and may ultimately support the development of new immunomodulatory targets for the treatment of autoimmune and neuropsychiatric disorders.

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Abbreviations:

Teff	effector T cell
Treg	regulatory T cell
5-HT	serotonin, 5-hydroxytryptamine
SSRIs	serotonin reuptake inhibitors
SERT	serotonin transporter
<i>slc6a4</i>	5-HTT
IDO	indolamine-2,3-dioxygenase
VMAT	vesicular monoamine transporter
NAD	nicotinamide adenine dinucleotide
Ahr	aryl hydrocarbon receptor
TPH	tryptophan hydroxylase

Th1	T helper 1 cell
Th17	T helper 17 cell

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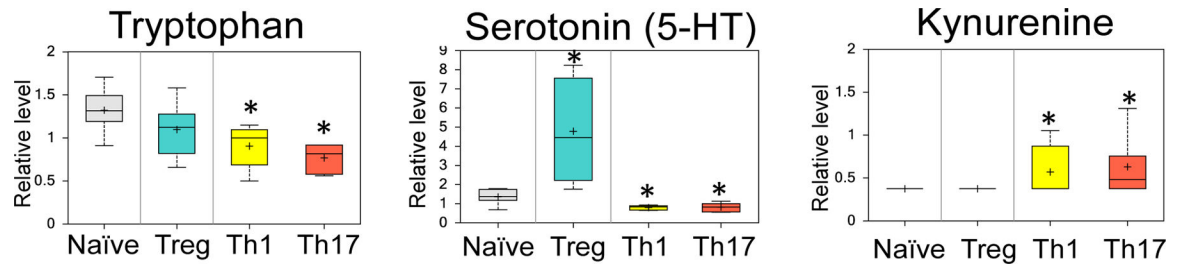


Figure 1. Metabolomic profiling of CD4⁺ T cell subsets reveals differences in tryptophan metabolic pathway.

Naïve CD4⁺CD25⁻ T cells were collected or polarized in vitro for 3 days, split 1:2, and cultured with IL-2 alone for an additional 2 days to generate Th1, Th17, or Treg. T cell subset lysates from 3 biological replicate samples were extracted and analyzed using high-resolution LC-QE-MS for determination of cellular metabolites. Relative levels of tryptophan, serotonin (5-HT) and kynurenine are shown. Samples are shown as box plots representing 6 biological replicates per sample. * = $p < .05$ compared with naïve.

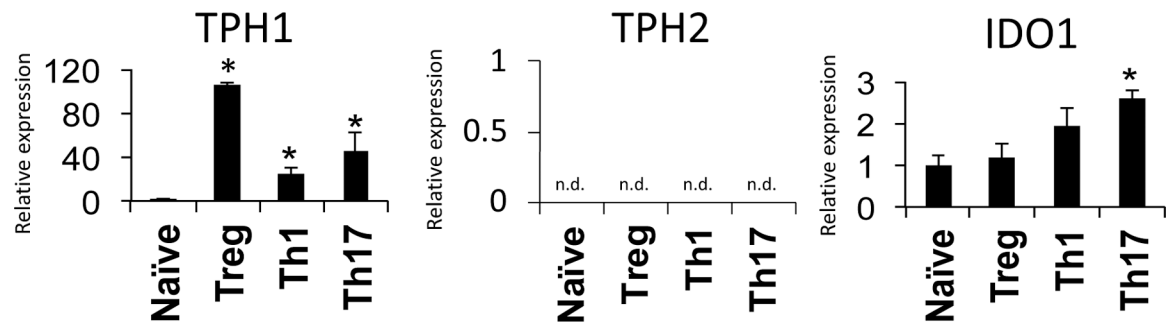


Figure 2. CD4⁺ T cell subsets have differential expression of enzymes in tryptophan metabolic pathway.

Naïve CD4⁺CD25⁻ T cells were collected or polarized in vitro for 3 days, split 1:2, and cultured with IL-2 alone for an additional 2 days to generate Th1, Th17, or Treg. T cell subset lysates from 3 biological replicate samples were extracted and subjected to qPCR to assess gene expression. The gene expression of tryptophan hydroxylase 1 (TPH1), tryptophan hydroxylase 2 (TPH2) and Indoleamine-pyrrole 2,3-dioxygenase (IDO1) are shown. Samples are normalized to naïve T cells. Data are shown as mean \pm SD and is representative of 3 independent experiments. n.d. = not detected. * = $p < 0.05$.

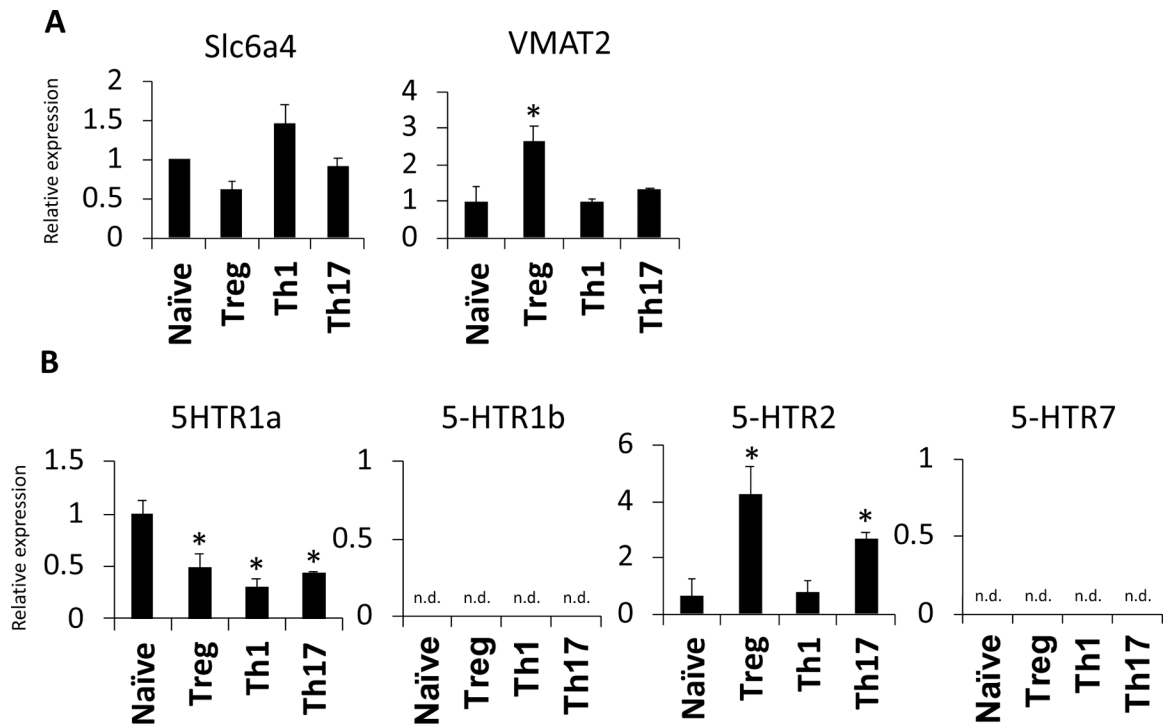


Figure 3. CD4 T cells differentially express serotonin-related genes.

Naive CD4⁺CD25⁻ T cells were collected or polarized in vitro for 3 days, split 1:2, and cultured with IL-2 alone for an additional 2 days to generate Th1, Th17, or Treg. T cell subset lysates from 3 biological replicate samples were subjected to qPCR to assess gene expression. (A) Relative expression levels of Slc6a4 VMAT2 are shown. (B) Relative expression levels of 5-HTR1a, 5-HTR1b, 5-HTR2 and 5-HTR7 are shown. Samples are normalized to naive T cells and data are shown as mean \pm SD and are representative of 3 independent experiments, n.d. = not detected. * = $p < .05$