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Thyroid stimulating hormone β-subunit splice variant is expressed in all fractional subsets of bone marrow hematopoietic cells and peripheral blood leukocytes and is modulated during bacterial infection

Austin Weber[a], **Hitesh N. Pawar**[a], **John R. Klein**[a,*]

[a]Department of Diagnostic and Biomedical Sciences, School of Dentistry, University of Texas Health Science Center at Houston, Houston TX, USA

Abstract

Thyroid stimulating hormone (TSH), a hormone produced in the anterior pituitary, is used to regulate thyroid hormone secretion. It has been known for over three decades that TSH is made by the cells of the immune system; however, the functional role of immune system TSH is unclear. We previously demonstrated that an alternatively-spliced isoform of TSHβ, referred to as the TSHβ splice variant (TSHβv), is the primary form of TSHβ made by hematopoietic cells in mice and humans. Most studies have linked TSHβv expression to myeloid cells of the immune system; however, it has recently been demonstrated that plasma cells in patients with Hashimoto's thyroiditis may be a source of immune system TSHβv. Here, we demonstrate that TSHβv is expressed in bone marrow precursors of lymphoid cells, monocytes, and granulocytes, as well as in mesenteric lymph node (MLN) cells. Plasma cells generated by in vitro culture with bacterial lipopolysaccharide (LPS), and MLN cells from mice infected with *L. monocytogenes* expressed TSHβv. There was an increase in the intensity of intracellular TSHβv expression in MLN cells following exposure to LPS, and in the proportion of TSHβv$^+$ CD138$^+$ MLN cells following *L. monocytogenes* infection. The number of TSHβv$^+$ cells increased in MLN cells, particularly among CD138$^+$ cells, following bacterial infection. This was confirmed by an increase in gene expression of BLIMP-1, the transcription factor for CD138, following infection. Levels of circulating thyroxine dropped significantly in mice 24 hrs post-infection. These findings suggest that immune system TSHβv may contribute to the host immune response during bacterial infection.

Keywords

bacterial infection; bone marrow; leukocyte; pituitary hormone; thyroid

*Corresponding author: john.r.klein@uth.tmc.edu.

Author's Contributions

JRK, conceptual design; AW and HNP, executed experiments.

Declarations of Interest: None

1. Introduction

Thyroid stimulating hormone (TSH), a glycoprotein hormone produced by the anterior pituitary in the hypothalamus-pituitary-thyroid (HPT) axis, consists of an α-subunit and a β-subunit. Hormone specificity is determined by TSHβ, which in mice is coded for by exons 4 and 5 of the TSHβ gene (Gordon et al., 1988). Upon stimulation by thyrotropin releasing hormone from the hypothalamus, TSH is released into the circulation and binds to TSH receptors on thyroid follicular cells, causing the release of triiodothyronine (T3) and thyroxine (T4). T4 is converted into T3, the more biologically active form of thyroid hormone, at the cellular level.

It has been known for over three decades that TSH is produced by peripheral blood leukocytes (PBL) (Harbour et al., 1989; Kruger et al., 1989; Smith et al., 1983) and bone marrow (BM) hematopoietic cells (Vincent et al., 2009). Studies in our laboratory identified an alternatively-spliced variant of TSHβ (TSHβv), and demonstrated that this is the primary form of TSHβ made by BM cells and PBL in mice and humans (Schaefer and Klein, 2009; Vincent et al., 2009). TSHβv is unique in that in mice the polypeptide is coded for only by exon 5. Exon 4, which is used by the native form of TSHβ, is deleted by alternative splicing(Klein, 2019). Uniquely, the 3’ end of intron 4 is used to code for the TSHβv signal peptide (Baliram et al., 2013; Baliram et al., 2016; Vincent et al., 2009). A molecule similar to mouse TSHβv has been identified in human leukocytes, as well as across many different species, and has been linked to Hashimoto’s thyroiditis (HT) and thyroid tissue destruction (Liu et al., 2012; Liu et al., 2019; Liu et al., 2015; Schaefer and Klein, 2009).

What remains unclear is how TSHβv contributes functionally to the overall health and disease of the organism. Although it might be a factor in the initiation and/or perpetuation of autoimmune thyroiditis, as suggested above, it may also have a role in microregulating the overall metabolic response of the host by altering thyroid hormone output independently of TSH produced by the HPT axis. This could occur by competitive binding of TSHβv for TSH receptors in the thyroid, either under normal homeostatic conditions or during times of immune stress (Bagriacik et al., 2001; Baliram et al., 2017; Klein, 2006; Schaefer and Klein, 2011). There is now accumulating evidence for the latter in both humans and mice (Liu et al., 2019; Montufar-Solis and Klein, 2016).

Several studies have reported TSHβv to be primarily produced by cells of the macrophage/monocyte lineages in BM and PBL (Baliram et al., 2016; Montufar-Solis and Klein, 2016). In humans, TSHβv also has been reported to be produced by plasma cells in the thyroid of patients with HT (Liu et al., 2015). In an effort to reconcile those differences, we reexamined the expression of TSHβv in mouse BM cells and mesenteric lymph node (MLN) cells as a source of PBL, using a monoclonal antibody made in our laboratory to region of TSHβ coded for by exon 5. As shown here, we observed high-level expression of TSHβv in BM T cell and B cell precursors and MLN cells. Moreover, expression of TSHβv increased in T cells and B cells following three days of systemic infection with *L. monocytogenes*. These findings collectively suggest that TSHβv produced by peripheral lymphoid cells may be part of the host immune defense mechanism during bacterial infection.

2. Materials and methods

2.1 Mice, cell lines, and cell isolations

Adult female C57BL/6 mice, 24 weeks of age, purchased from Harlan (Indianapolis, IN), were used. Animals were used in accordance with The University of Texas Health Science Center at Houston Institutional Animal Welfare Committee permit No. HSC-AWC-18-0012. For BM cell recovery, mice were euthanized and BM cells were flushed from right and left femurs. MLN cells were used as a source of peripheral leukocytes. Cell lines were the mouse AM alveolar macrophage cells line, provided by Dr. Chinnaswamy Jagannath, Department of Pathology, The University of Texas Health Science Center at Houston. EL4 mouse T cell thymoma cells (TIB-39) were purchased from American Type Culture Collection, Manassas, VA. Peripheral leukocytes were isolated as described elsewhere (Montufar-Solis and Klein, 2016).

2.2 Bacteria and infection

Animals were infected by i.p. injection of 1.0×10^9 colony forming units (CFU) *L. monocytogenes*, a dose we have previously used in mice (Montufar-Solis and Klein, 2016). Studies by others have demonstrated that C57BL/6 mice are more resistant to infection than most other strains (Cheers et al., 1978), and that i.p. infection is usually done in the range of 10^6 - 10^9 CFU (Kernbauer et al., 2013; Lindback et al., 2011). To alleviate pain from bacterial infection, mice are treated with buprenorphine, 0.05 – 0.1 mg/kg body weight given i.p. 2-3 times per day as needed. In the present study, treatment was deemed not to be necessary during the three days of infection.

2.3 Antibodies, flow cytometry, ELISA assay, and real-time PCR

Immunoprofiling of cells was done with the following antibodies: PE-anti-CD3 (145-201), PE-anti-CD19 (1D3) (Tonbo Bioscience, San Diego, CA), PE-CD138 (281-2) (BD Pharmingen, San Diego, CA), anti-TSHβ (1B11), streptavidin-APC and isotype control antibodies (eBioscience, San Diego, CA). 1B11 anti-mouse TSHβ monoclonal antibody was made and biotinylated in our laboratory (Zhou et al., 2002). 1B11 is reactive to a 12 amino acid peptide (SDSIHEAVRTNY) coded for by exon 5 and located in the C-terminus end of the mouse TSHβ protein (Zhou et al., 2002). Using a Fasta peptide homology search program, only sequences of TSHβ in a number of other species (mouse, rat, human, dog, pig, bovine, chicken horse, llama, hamster, and opossum) were identified, this indicating that the likelihood of off-target relativities is extremely low.

One-color surface staining of BM and MLN cells was achieved by first reacting cells with anti-CD16/CD32 as a blocker of Fc-receptor reactivity followed by washing and reacting with a PE-labeled antibody. For intracellular staining, cells were stained for surface expression using a PE-labeled primary antibody (anti-CD3, anti-CD19, or anti-CD138). Cell membranes were permeabilized for 20 min at 4°C with cytofix/cytoperm (BD Bioscience, San Diego, CA), washed with permwash (BD Biosciences, San Jose, CA), reacted with biotinylated 1B11 at 4°C for 30 min, washed with permwash, and reacted at 4°C for 15 min with streptavidin-APC. Fluorescence was determined on a FACSCalibur flow cytometer with Cell-Quest software (BD Biosciences).

Circulating T4 levels were measured in a mouse thyroxine T4 ELISA kit (Mybioscience; San Diego, CA) using blood collected from the heart after euthanasia.

Real-time PCR was done as previously described (Montufar-Solis and Klein, 2016). Primer sets were:

CD138	Forward: 5’ CTCAGAGCCTTTTGGACAGG 3’
	Reverse: 5’ ATCCGGTACAGCATGAAAGC 3’
BLIMP-1	Forward: 5’ AAGTGTGCCCAGTGTCACAA 3’
	Reverse: 5’ CTGCATTGTCGCTGATGTCG 3’
CD19	Forward: 5’ GGGACCTGGACTGTGACCTA 3’
	Reverse: 5’ GGGCACATACAGGCTTTGTT 3’

3. Results

3.1 All major populations of bone marrow hematopoietic cells express TSHβv

BM hematopoietic cells consist of all of the progenitors of peripheral leukocytes. Developing monocytes, granulocytes, and lymphoid cells are present in the BM (Loken et al., 1987; Terstappen et al., 1992). Those populations can be distinguished by flow cytometry according to cell size, forward scatter (FCS), and granularity, side scatter (SSC). This is shown in Fig. 1 A, which includes a lymphoid precursor subset with relative small size and low granularity (Fig. 1A, gate 1), a monocyte precursor subset with larger size and mid-level granularity (Fig. 1A, gate 2), and a granulocyte precursor subset with medium cell size and high granularity (Fig. 1A, gate 3).

The majority of the cells in the lymphoid precursor population expressed TSHβv (Fig. 1B). Some but not all monocyte and granulocyte precursors expressed TSHβv (Fig. 1C–D). This was confirmed for lymphoid cell precursors stained for CD3 expression as a marker of T cell, which consisted of cells with high CD3 expression and cells with low CD3 expression (Fig. 1E). Despite the difference in CD3 expression, cells in both groups expressed equivalent levels of intracellular TSHβ expression as determined by mean fluorescence intensity (mfi) (Fig. 1F). Similarly, BM CD19 precursors consisted of cells with high and low CD19 expression (Fig. 1G). $CD19^{hi}$ cells expressed less intracellular TSHβv than $CD19^{lo}$ cells based on mfi (Fig. 1H).

3.2 Lymphocytes, monocytes, and granulocytes in MLNs express TSHβv

PBL in MLNs were stained for TSHβv expression. Similar to BM cells, MLN cells were defined according to three cell subsets based on cell size and granularity (Fig. 2A). The majority of cells in all three groups expressed TSHβv (Fig. 2B–D); the mfi of intracellular TSHβv expression is shown within the histograms. TSHβv is expressed in the majority of MLN $CD3^+$ T cells (Fig. 2E) and $CD19^+$ B cells (Fig. 2F), thus demonstrating that lymphoid cells and myeloid cells (monocytes and granulocytes) are sources of TSHβ. That finding was further confirmed for T cells using the murine EL4 T cell line, which expressed surface CD3 (Fig. 2G) and also expressed intracellular TSHβv (Fig. 2H). Both the AM macrophage cells

line (Fig. 2I) and the EL4 T cell line (Fig. 2J) expressed TRHR and TSHβv genes, but did not express the gene for full-length native TSHβ.

It was recently reported that plasma cells, a terminally-differentiated population of B cells, are a source of TSHβv (Liu et al., 2015). We stained MLN cells from normal mice for CD138, a marker of plasma cells, and for intracellular TSHβv expression. There were few plasma cells among lymphoid MLNs (Fig. 2K). However, proportionally more $CD138^+$ cells were present in the cell population located in gate 2 (Fig. 2L) and gate 3 (Fig. 2M). This is consistent with the fact that B cells become larger and more granular once they have differentiated into plasma cells.

3.3 LPS stimulation of MLN cells alters the expression of TSHβv

Based on the findings of others (Liu et al., 2015), we sought to determine whether there was an increase in the numbers of $CD138^+$ plasma cells and/or intracellular TSHβv expression in MLN cells after culture with *E. coli* LPS. As seen in Fig. 3, no differences were observed in the expression of CD138 between non-stimulated cells in the lymphocyte, monocyte, or granulocyte populations (Fig. 3B, D, and F) compared to LPS-stimulated cells (Fig. 3I, K, and M). However, the levels of intracellular TSHβv expression increased in all three cell populations following exposure to LPS as seen by an increase in mfi of LPS-stimulated cells (Fig. 3J, L, and N) compared to non-stimulated cells (Fig. 3C, E, and G). A partial exception to this was the bimodal expression of TSHβv in the granulocyte population of LPS-stimulated cells (Fig. 3N), which consisted of a subset of cells with a lower mfi than non-LPS-stimulated cells, and a subset with a higher mfi than non-LPS-stimulated cells. The reason for this is currently unclear; however, it is possible that the downward shift in intracellular TSHβv in LPS-stimulated granulocytes reflects the secretion of TSHβv following LPS stimulation.

3.4 Bacterial infection results in an increase in TSHβv expression in MLN cells

Mice were infected with 1.0×10^9 CFU *L. monocytogenes* by i.p. infection. Three days later, MLN cells were recovered and stained for surface expression of CD138 and intracellular TSHβv expression. No increase in TSHβv was observed in $CD138^+$ MLN lymphocytes in *L. monocytogenes*-infected mice (Fig. 4H) compared to non-infected mice (Fig. 4B). However, there was an increase in $CD138^+$, $TSH\beta v^+$ cells located in gate 2 (Fig. 4I) and gate 3 (Fig. 4J) of infected mice compared to non-infected mice (Fig. 4C and D). $CD138^+$ cells with those physical characteristics are likely to be plasma cells given that B cells increase in both size and granularity following differentiation into plasma cells.

To further examine the expression of TSHβv in lymphocytes in *L. monocytogenes*-infected and non-infected mice, we stained MLN cells for surface CD3 and CD19 expressions and intracellular TSHβv. There was an increase in the mfi of TSHβv in $CD19^+$ MLN cells from *L. monocytogenes*-infected mice (Fig. 4K) compared to non-infected mice (Fig. 4E), as well as for $CD3^+$ MLN cells from *L. monocytogenes*-infected mice (Fig. 4L) compared to non-infected mice (Fig. 4F). This also held true when all leukocyte populations were analyzed (mfi non-infected,981.4 ± 542, n = 5 vs. 2,654.5 ± 248, infected, n = 3; p = 0.024). Expression of BLIMP-1, a transcription factor for CD138, is increased in MLN cells 7 days

post-infection with *L. monocytogenes* (Fig. 4M). Circulating T4 levels dropped sharply in infected mice 24 hrs post-infection (Fig. 4N).

4. Discussion

Although it is well established that TSHβv is made by cells of the immune system in humans and mice (Schaefer and Klein, 2009; Vincent et al., 2009), the functional and physiological significance of that is only now becoming evident. Recent studies have demonstrated that changes in intrathyroidal TSHβv expression occur during bacterial infection, and that this may be due to TSHβv-secreting leukocyte homing to the thyroid (Montufar-Solis and Klein, 2016). TSHβv also has been shown to contribute to bone development and morphogenesis (Abe et al., 2003; Baliram et al., 2013; Baliram et al., 2016; Baliram et al., 2017). In HT, an autoimmune disease of the thyroid, circulating levels of TSHβv are altered, possibly by plasma cells that traffic to the thyroid (Liu et al., 2012; Liu et al., 2019; Liu et al., 2015). Yet, many unanswered questions still remain as to the role of TSHβv in health and disease.

Several studies, including those from our laboratory, reported that production of TSHβv is primarily the domain of monocytes and granulocytes, with little if any TSHβv made by lymphoid cells (Montufar-Solis and Klein, 2016). Other studies, however, have reported that TSHβv is made by lymphoid cells, in particular by plasma cells, the terminally-differentiated form of B cells (Liu et al., 2015). The purpose of the present study was two-fold. First, we examined the expression of TSHβv in BM cells and peripheral leukocytes, with particular interest in the expression in the lymphoid cell population. Second, we examined the expression of TSHβv in MLN cells in mice infected systemically with *L. monocytogenes.* The rationale for *L. monocytogenes* infection was that it generates a rapid and strong response that incorporates all branches of the immune system. As noted above, in an earlier study we found that expression of TSHβv was largely restricted to myeloid cells, the monocyte and granulocyte populations (Montufar-Solis and Klein, 2016). In that study, however, the majority of intracellular TSHβ staining was done with the M-16 antibody obtained from Santa Cruz Biotechnology, which we have subsequently found to be unreliable for TSHβ staining. In the present study, we used antibody 1B11, a monoclonal antibody made in our laboratory (Zhou et al., 2002). That antibody has strong reactivity to a portion of TSHβ that is present in the TSHβv polypeptide. To insure that the reactivity of 1B11 is for TSHβv, we used a commercial anti-TSHβ beta antibody made by Abeam (Thermo Scientific, clone ab231218). We observed similar staining patterns of anti-TSH beta antibody ab231218 and 1B11 on MLN cells (data not shown), this confirming that monoclonal antibody 1B11 specifically reacts with TSHβv.

The findings reported here indicate that all three primary subsets of leukocytes (lymphoid cells, monocytes, and granulocytes) in the BM and in MLNs, a peripheral immune compartment, are sources of TSHβv. In extending that, we made three additional observations. First, following *in vitro* stimulation of MLN cells with bacterial LPS, the intracellular fluorescence intensity of TSHβv increased in all populations, thus indicating that exposure to bacterial cell wall stimuli has a direct effect on the amount of TSHβv produced. Second, the increase in $CD138^{+}$ cells in granular (Fig. 4B, gate 3) MLN cells

from *L. monocytogenes*-infected mice is indicative of an increase in plasma cells in response to infection. Plasma cells have significantly more cytoplasmic granulation due to enhanced antibody secretion (Pracht et al., 2017). Third, the increase in intracellular TSHβv expression in both $CD19^+$ and $CD3^+$ MLN cells in infected mice, as demonstrated by higher mfi, points to involvement of those cells in response to bacteria.

Perhaps one of the most perplexing question pertaining to TSHβv is the observation that TSHβv is endogenously present in most of the cells of the immune system. This is evident by flow cytometry as reported here and by others (Baliram et al., 2013; Montufar-Solis and Klein, 2016), and has been documented in electron microscopic studies of BM macrophages (Baliram et al., 2013). Collectively, this suggests that peripheral leukocytes are primed and ready to release TSHβv on short notice. Questions remain as to why TSHβv would need to be secreted from intracellular stores rather than by cellular *de novo* manufacture as needed, what signal are used to trigger its release, in which sites it is released, and what the physiological outcome of TSHβ secretion is. We have proposed that immune system TSHβv is used to assist in regulating metabolism during times of immune stress, such as during viral or bacterial infection (Schaefer and Klein, 2011). This could occur by intrathyroidal release of TSHβv upon trafficking of select leukocyte populations to the thyroid, which may result in competition in binding between native TSHβ and TSHβv to the TSHR, and could account for the precipitous drop in circulating T4 24 hrs post-infection as shown here and as reported in mice challenged with alloantigen (Bagriacik et al., 2001).

5. Conclusions

We demonstrate that TSHβv is expressed in all major BM precursors leukocyte subsets, as well as in mature leukocytes in MLN. Additionally, there is an increase in CD138 expression, a marker of plasma cells, on $TSH\beta v^+$ cells in mice during an active *L. monocytogenes* infection. These findings collectively add to our understanding of the distribution of TSHβv-producing cells in mice in normal homeostatic conditions as well as during an active immune response to foreign antigen.

Acknowledgments

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Abbreviations:

BM	bone marrow
CFU	colony forming units
HT	Hashimoto’s thyroiditis
HPT	hypothalamus-pituitary-thyroid
LPS	lipopolysaccharide

MLN	mesenteric lymph nodes
MFI	mean fluorescence intensity
PBL	peripheral blood leukocyte
TSH	thyroid stimulating hormone
TSHβv	TSHβ splice variant
T3	triiodothyronine
T4	thyroxine

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Highlights

- The TSHβ splice variant (TSHβv) is expressed in lymphoid precursors (T cells and B cells), monocyte precursors, and granulocyte precursors in the bone marrow of normal mice.
- TSHβv is spontaneously expressed in lymphocytes, monocytes, and granulocytes, as well as in $CD138^{+}$ plasma cells, in mesenteric lymph node cells from normal mice.
- In vitro exposure of mesenteric lymph node cells to bacterial lipopolysaccharide resulted in an increase in the intracellular fluorescence intensity of TSHβv.
- Infection of mice with *Listeria monocytogenes* resulted in elevated expression of $TSH\beta v^{+}$ in $CD138^{+}$ plasma cells seven days post-infection.
- Levels of circulating thyroxine dropped significantly twenty-four hours post-*Listeria monocytogenes* infection.

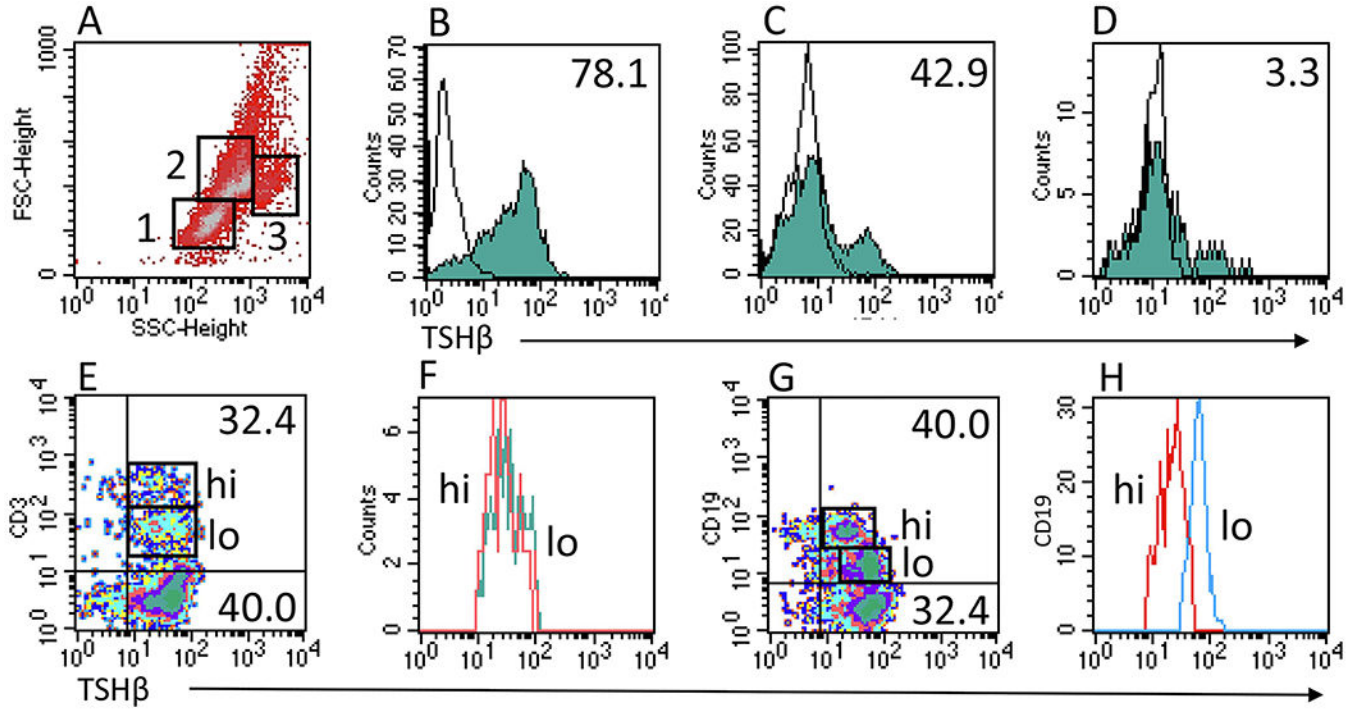

Fig. 1.

Expression of TSHβv in BM hematopoietic cells. (A) BM scattergram of cells defined by forward scatter (FSC) and side scatter (SSC) showing three distinct populations consisting of lymphoid precursors (gate 1), monocyte precursors (gate 2), and granulocyte precursors (gate 3). (B) Expression of TSHβv in BM lymphoid precursors in gate 1. (C) Expression of TSHβv in BM monocyte precursors in gate 2. (D) Expression of TSHβv in BM granulocyte precursors in gate 3. (E) Expression of TSHβv in two populations of $CD3^{+}$ T cell precursors defined by the intensity of CD3 fluorescence expression. (F) Comparison of TSHβv the mfi on $CD3^{hi}$ and $CD3^{lo}$ subsets. (G) Expression of TSHβv on two populations of $CD19^{+}$ BM precursors defined by the intensity of CD19 fluorescence expression. (H) Comparison of TSHβv expression on $CD19^{hi}$ and $CD19^{lo}$ subsets.

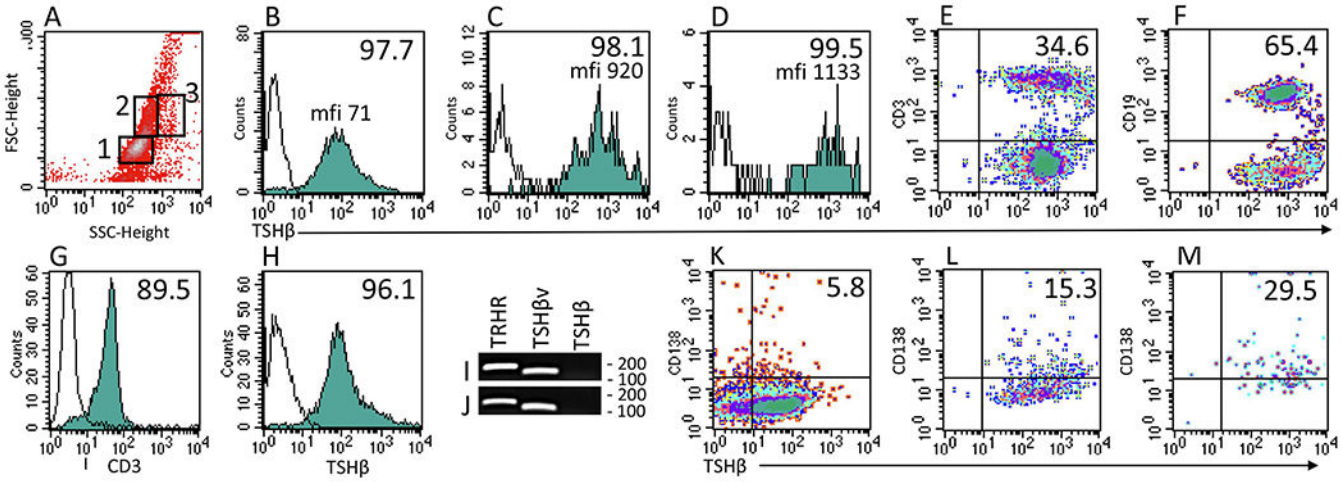

Fig. 2.

Expression of TSHβv in MLN cells from normal mice. (A) Scattergram of cells defined by forward scatter (FSC) and side scatter (SSC) showing three distinct populations consisting of lymphoid cells (gate 1), monocytes (gate 2), and granulocytes (gate 3). Intracellular expression of TSHβv in (B) lymphoid cells, (C) monocytes, (D) granulocytes, (E) $CD3^+$ T cells, and (F) $CD19^+$ B cells. Expression of (G) CD3 on EL4 T cells, and (H) intracellular TSHβv expression in EL4 T cells. PCR analysis of TRHR, TSHβv, and TSHβ gene expression in the mouse (I) AM macrophage cell line, and (J) EL4 T cells. CD138 expression on MLN (K) lymphoid cells, (L) monocytes, and (M) granulocytes, mfi, mean fluorescence intensity.

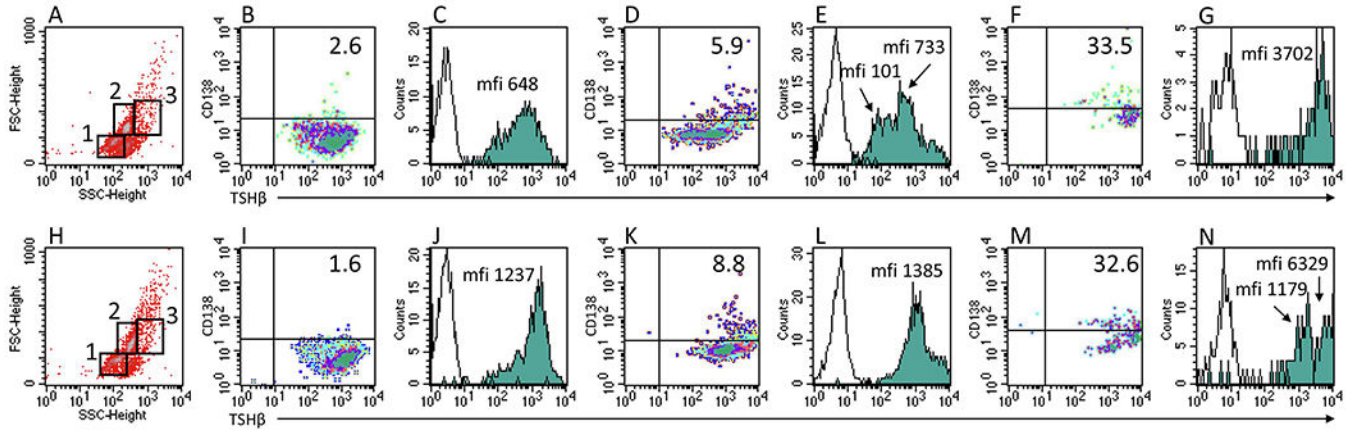

Fig. 3.

Expression of CD138 and TSHβv in MLN cells. MLN cells were cultured (A-G) without LPS, and with (H-N) 100 μg/ml LPS for 48 hrs. No differences were noted in the expression of $CD138^{+}$, $TSH\beta v^{+}$ (B vs. I) lymphocytes, (D vs. K) monocytes, or (F vs. M) granulocytes when stimulated with LPS. However, intracellular expression of TSHβv increased following exposure to LPS (J, L, N) compared to (C, E, G) non-stimulated cells, mfi, mean fluorescence intensity.

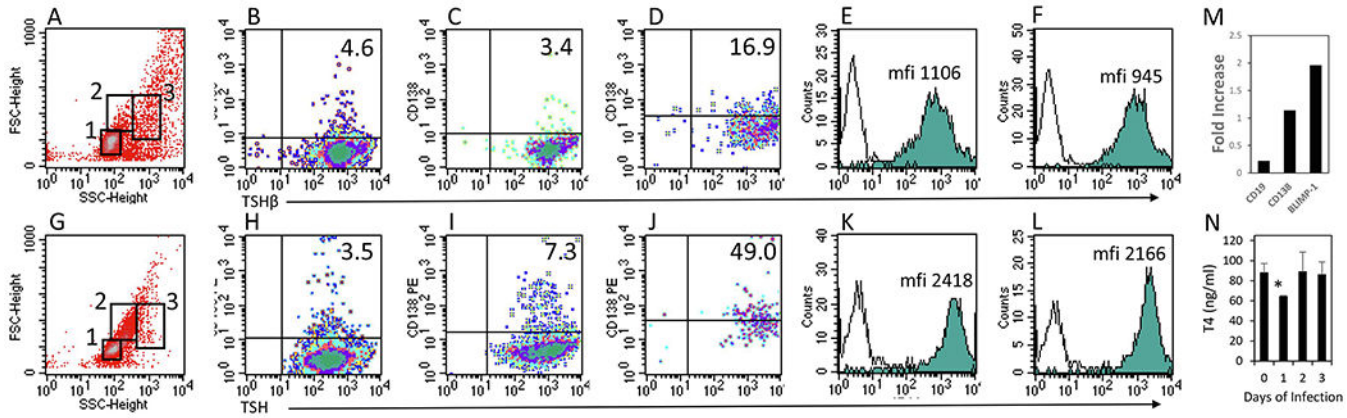

Fig. 4.

Effect of *L. monocytogenes* infection on TSHβv expression in MLN cell. MLN cells from (A-F) non-infected and (G-L) *L. monocytogenes*-infected mice. (B, C, D) Proportions of $CD138^+$, $TSH\beta v^+$ MLN cells in gated populations 1, 2, and 3 in panel A of non-infected mice, and (H, I, J) *L monocytogenes*-infected mice. Intracellular TSHβv expression in $CD19^+$ MLN cells from (E) non-infected mice compared to (K) *L. monocytogenes*-infected mice. Intracellular TSHβv expression in $CD3^+$ MLN cells from (F) non-infected mice compared to (L) *L. monocytogenes*-infected mice. (M) Fold increase in CD19, CD128, and BLIMP-1 gene expression in MLN cells from infected mice relative to non-infected mice. (N) Circulating levels of T4 were significantly lower in mice 24 hrs post-infection compared to non-infected mice and to mice 48 and 72 hrs post-infection. * $p = 0.02$. mfi, mean fluorescence intensity.