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Influence of *Tmevpg1*, a long intergenic noncoding RNA, on the expression of *Ifng* by Th1 cells

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Abstract

The majority of the genome is noncoding and was believed to be nonfunctional. However, it is now appreciated that transcriptional control of protein coding genes resides within these noncoding regions. Thousands of genes encoding long intergenic noncoding RNAs (lincRNAs) have been recently identified throughout the genome, which positively or negatively regulate transcription of neighboring target genes. Both *TMEVPG1* and its mouse orthologue encode lincRNAs and are positioned near the interferon gamma gene (*IFNG*). Here we show that transcription of both mouse and human *TMEVPG1* genes is Th1 selective and dependent upon Stat4 and T-bet, transcription factors that drive the Th1 differentiation program. *Ifng* expression is partially restored in *Stat4*$^{-/-}$ *Tbx21*$^{-/-}$ cells through co-expression of T-bet and *Tmevpg1* and *Tmevpg1* expression contributes to but alone is not sufficient to drive Th1-dependent *Ifng* expression. Our results suggest that *TMEVPG1* belongs to the general class of lincRNAs that positively regulate gene transcription.

Introduction

The immune system is directly responsible for protection against invading pathogens including bacteria and viruses. Production of IFN-γ by CD4^{+} Th1 cells, CD8^{+} T cells, natural killer (NK) and NKT cells dictates the ability to clear intracellular infections and promote tumor immunity. *IFNG* expression is the result of successful coordination of histone modifications, trans-activating factors and cis regulatory elements including distal conserved noncoding sequences (1–3). A naïve CD4^{+} T cell initiates a Th1 polarizing program upon antigenic stimulation in the presence of the cytokine IL-12 leading to the activation of the JAK/STAT pathway components STAT 1 and STAT 4 (4). These molecules promote transcription of the master Th1 transcription factor, T-bet (5–6) contributing to maintenance of *IFNG* locus permissivity as well as repression of the *IL4* locus through covalent chromatin modifications (7). Understanding how the *IFNG* locus is regulated by noncoding elements of the genome, particularly in the context of Th1 cell differentiation, has been an area of extensive investigation.

Long intergenic noncoding RNAs (lincRNAs) are a new species of regulatory RNAs that exist throughout the genome but the complete understanding of their functions is

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incompletely understood (8). Initial findings indicate that thousands of lincRNAs exist in the vertebrate genome. In general, lincRNA genes are conserved across species, are positioned near their target protein coding genes and impact a variety of compartmentalized biological systems. For example, XIST is required for the process of X chromosome inactivation in females. With the assistance of six additional lincRNAs, XIST "paints" the inactive chromosome with repressive histone marks silencing transcription (9–11). HOTAIR and AIR regulate the HOXD and Igf2r loci, respectively, and are critical for development by modulating expression of these essential transcription factors. Recent evidence suggests that HOTAIR regulates expression of the HOXD locus by acting as a scaffold for histone modifying enzyme recruitment (12–15). LincRNA-p21 expression is enhanced by the potent tumor suppressor p53 and then subsequently represses transcription of genes downstream of p53 signaling (16). To date, few lincRNAs with enhancer function have been described (17–18). The function of lincRNAs in the immune system has not been actively investigated. A genome-wide screen identified a cluster of lincRNAs predicted to function in the immune system based upon the proximity to genes known to encode proteins with immune function (19). Additionally, independent noncoding RNA screens of activated $CD8^+$ T cells and SARS-coronavirus infected cells have been conducted (19); however, the functional biology of these lincRNAs has not been determined.

TMEVPG1, a lincRNA transcript was initially identified in the context of Theiler's virus infection whereby mice deficient in *TMEVPG1* expression were unable to control the intracranial viral infection (20). Here we describe *TMEVPG1* as a Th1 specific lincRNA that requires Stat4 and T-bet for active transcription and contributes to the transcription of the gene encoding IFN-γ.

Materials and Methods

Mice

BALB/c-J.*Stat4*$^{-/-}$*Tbx21*$^{-/-}$, DO11.10.*Stat4*$^{-/-}$, DO11.10.*Tbx21*$^{-/-}$ and wildtype mice were obtained from Christopher L. Williams in the Boothby laboratory. The BALB/c-J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were bred in the Vanderbilt University animal facilities. Research using mice complied with all relevant institutional and federal guidelines and policies.

Human and mouse lymphocyte culture conditions

Healthy human PBMC were isolated by Ficoll–Hypaque density centrifugation (GE Healthcare). $CD4^+$ T cells were sorted by positive selection and stimulated with immobilized anti-CD3 (OKT3; ATCC, Manassas, VA) and soluble anti-CD28 under Th1 (IL-12 10 ng/ml) or Th2 (IL-4 10 ng/ml) polarizing conditions for three days followed by two days of culture with IL-2. Murine BALB/c-J, BALB/c-J.*Stat4*$^{-/-}$*Tbx21*$^{-/-}$, DO11.10.*Stat4*$^{-/-}$, DO11.10.*Tbx21*$^{-/-}$ splenocytes (1×10^6 cells/ml) were stimulated with immobilized anti-CD3 (2C11; ATCC, Manassas, VA) or $OVA_{323–339}$ peptide antigen (10 μg/ml) under Th1 (10 ng/ml IL-12 and 10 ug/ml anti IL–4 11B11; ATCC), Th2 (10 ng/ml IL-4 and 10 μg/ml anti–IFN-γ, R4-642; ATCC), Th17 (10 ng/ml IL-6, 10 ng/ml IL-23, 1 ng/ml TGF_{β} and 10 ug/ml anti-IFN-γ) or Th22 (5 ng/ml IL-1β, 10 ng/ml IL-6 and 5 ng/ml TNF-α) polarizing conditions for three days. $CD4^+$ T cells were purified by positive selection (Miltenyi Biotec). Human or mouse cells were restimulated to generate effector cells by the addition of 50 ng/ml PMA and 1 μM ionomycin for 6 hrs or peptide antigen as described in the *Results* section.

RNA isolation and quantitative RT-PCR

Total RNA was isolated with Tri Reagent (Ambion, Inc.). cDNA was synthesized with the SSRIII kit (Invitrogen). Transcript levels were determined by SybrGreen quantitative RT-PCR (qPCR) using the following primer pairs: human *TMEVPG1* forward 5′aaacgctggaggagaagtca 3′ and reverse 5′ttctcctccagcgttttacg 3′ and mouse *Tmevpg1* forward 5′cctgaaaatcaccatgcaca 3′ and reverse 5′gttttcgggatgtcgtcaaa 3′. Human and murine message levels are expressed as the ratio to *GAPDH* (or *Gapdh*) transcript levels calculated directly from the C_t.

siRNA knockdown

Silencer® Select siRNA duplexes (Ambion, Inc.) were designed against the 5′ *Tmevpg1* sequence: siRNA 1 sense 5′ GAGAAGAGCCUGAGAGAAATT 3′ and antisense 3′ TTCUCUUCUCGGACUCUCUUU 5′; siRNA 2 sense 5′ GCAGACUAAACUAGAUAGUTT 3′ and antisense 3′ TTCGUCUGAUUUGAUCUAUCA 5′. *Ifng* siRNA (siRNA ID: 158238) and non-specific scramble siRNA (sense 5′ caacugggacacauguguutt 3′ and antisense 3′ ttguugacccuguguacacaa 5′) were used as controls. siRNA duplexes (30 pmoles) were nucleofected into cells according to the manufacturer's instructions (Lonza). After 4 hrs of recovery cells were cultured (5×10^5 cells/ml) under Th1 conditions for three days before restimulation. IFN-γ was measured in culture supernatants by ELISA (BD OptEIA).

Tmevpg1 sequencing and overexpression

Tmevpg1 cDNA clone AA162222 (Open Biosystems) was sequenced at the Vanderbilt Sequencing Facility. Full length *Tmevpg1* was cloned into the pcDNA3.1/myc-His A overexpression vector (Invitrogen). CMV-*Tmevpg1*, CMV-*Tbx21* or CMV-empty vectors were transfected (1 μg/10^6 cells) into polarized splenocytes using Amaxa Nucleofection (Lonzabio).

Statistical analysis

Statistical significance was determined by Student's T test.

Results

Selective expression of TMEVPG1 and its murine orthologue under Th1 polarizing conditions

Utilizing the UCSC genome browser configuration we identified a gene, AK124066, also named *TMEVPG1*, which is predicted to transcribe a spliced, noncoding mRNA transcript and is positioned approximately 170 kb from the *IFNG* coding region (Fig. 1*A*). The 33 kb long *TMEVPG1* gene is comprised of four exons and encodes an mRNA of 1791 bp in length. *TMEVPG1*, similar to *IFNG*, possesses multiple Th1 specific DNAse I hypersensitivity sites at its promoter as well as epigenetic histone marks, H3K9 acetylation and H3K4 mono- and tri-methylation, that are known to be associated with active transcription. Both *TMEVPG1* and its mouse ortholog are located on the opposing strand to *IFNG*. The transcriptional start site of mouse *Tmevpg1* is positioned 117 kb from the *Ifng* transcriptional start site and is spliced into a mature transcript 918 bp in length. The promoter region and first intron of *Tmevpg1* also exhibit considerable sequence conservation with human *TMEVPG1*. These data are consistent with the possibility that *TMEVPG1* encodes a lincRNA transcript selectively expressed in Th1 cells relative to Th2 cells.

To address this possibility, human $CD4^+$ T cells were stimulated *in vitro* under Th1 or Th2 polarizing conditions for three days, followed by two days of additional culture with IL-2 before restimulation with PMA and ionomycin. Transcript levels of *IFNG*, *TMEVPG1*, and *GAPDH* were determined by qPCR. Transcript levels of *IFNG* and *TMEVPG1* were substantially greater in Th1 cultures compared to Th2 cultures (Fig. 1*B* and 1*C*). We also determined transcript levels of *IFNG* and *TMEVPG1* in PBMCs from healthy human control subjects and compared transcript levels by linear regression. We found a positive correlation between *IFNG* and *TMEVPG1* transcript levels relative to *GAPDH* (Fig. 1*D*). Taken together these results demonstrate that, like *IFNG*, *TMEVPG1* transcript levels are selectively expressed in Th1 cultures relative to Th2 cultures. The linear regression analysis further indicates a strong association between *IFNG* and *TMEVPG1* transcript levels in PBMCs.

To assess whether murine *Tmevpg1* was also preferentially expressed in polarized Th1 cells, we measured *Tmevpg1* transcript levels in total splenocytes cultured under Th1 or Th2 polarizing conditions. RNA was isolated after three days in culture at the peak of *Ifng* expression during primary stimulation. *Tmevpg1* and *Ifng* message levels were measured by qPCR. Consistent with our results in human lymphocytes, *Tmevpg1* transcript levels were significantly greater in Th1 cells than in Th2 cells (Fig. 1*E*). *Tmevpg1* transcript levels were also analyzed in Th17 and Th22 polarized cells to confirm Th1 specificity (Fig 1*E*). We also restimulated Th1 effector cells with PMA and ionomycin and followed *Tmevpg1* transcript levels over time. IL-2 was added to these cultures to sustain viability. Restimulation resulted in a marked increase in *Tmevpg1* transcript levels that was sustained over several days (Fig. 1*F*). In contrast to what we observed in Th1 effector cultures, *Tmevpg1* transcript levels were undetectable in effector $CD8^+$ T cells polarized under Th1 culture conditions, which produced a significant amount of IFN-γ (1.7 fold *Gapdh* transcript levels measured by qPCR). We conclude from these experiments that Th1 selective *Tmevpg1* expression is conserved between murine and human lymphocytes but is not expressed by $CD8^+$ T cells under these culture conditions. Further, restimulation of Th1 cells results in greater *Tmevpg1* expression levels than observed in primary Th1 cultures implicating a role for *Tmevpg1* in effector Th1 cells.

Tmevpg1 is regulated by Th1 transcription factors

Th1 selective expression of IFN-γ in our model system is dependent upon the transcription factors Stat4 and T-bet. Because *Tmevpg1* also displays selective Th1 expression we determined if *Tmevpg1* expression was also dependent upon Stat4 and T-bet. To do so, we isolated splenocytes from DO11.10.*Stat4*$^{-/-}$ and DO11.10.*Tbx21* $^{-/-}$ (T-bet knockout) transgenic mice and stimulated the cells *in vitro* with $OVA_{323–339}$ peptide and IL-12. After three days, $CD4^+$ T cells were purified and restimulated with $OVA_{323–339}$ peptide for 48 hrs. As expected, *Ifng* transcript levels were substantially diminished in Th1 cells in the absence of Stat4 or T-bet compared to the DO11.10 wildtype control mice (Fig. 2*A*). *Tmevpg1* transcript levels were also substantially reduced in T cells deficient in either Stat4 or T-bet (Fig. 2*A*).

Influence of Tmevpg1 on Ifng transcription

LincRNAs have been clustered into two functionally distinct categories: repressors and enhancers of transcription of protein coding genes. We aimed to determine the function of *Tmevpg1* in Th1 cells via siRNA-mediated knockdown of *Tmevpg1*. Nucleofection of *Tmevpg1*-specific siRNA duplexes 1 and 2 resulted in a reduction of *Tmevpg1* (Fig. 2*B*) and *Ifng* (Fig. 2*C*) transcript levels relative to the scramble siRNA transfected polarized splenocytes. Knockdown of *Tmevpg1* by siRNA duplex 1 or siRNA duplex 2 resulted in a two-fold or four-fold reduction in IFN-γ protein, respectively, relative to transfection with a

non-specific scrambled siRNA (Fig. 2*D*). Knockdown with *Ifng* siRNA resulted in a similar decrease in IFN-γ protein concentrations. Nucleofection with both *Tmevpg1* siRNA duplexes 1 and 2 caused a comparable reduction in *Tmevpg1* transcript levels while siRNA knockdown of *Ifng* did not affect transcript levels of *Tmevpg1*. Our conclusion is that *Tmevpg1* plays a role in *Ifng* expression by Th1 cells.

Based upon the results from the siRNA knockdown experiments, we determined if overexpression of *Tmevpg1* was sufficient to induce *Ifng* transcription. Full length *Tmevpg1* was cloned into a CMV expression plasmid. Total BALB-c/J splenocytes were stimulated with anti-CD3 under non-polarizing conditions (Th0) or under Th1 or Th2 polarizing conditions for three days. $CD4^+$ T cells were isolated and CMV-*Tmevpg1* or CMV-empty vectors were then transfected (1 μg of plasmid/10^6 cells). After a period of rest, cells were restimulated with PMA and ionomycin. Nucleofection of CMV-*Tmevpg1* into primary Th1 cells resulted in an increased expression of *Tmevpg1* compared to the empty vector control (Fig. 3*A*). Ectopic expression of *Tmevpg1* in primary $CD4^+$ T cells resulted in no significant increase in IFN-γ protein in Th0, Th1, or Th2 cells relative to transfection with an empty vector control (Fig. 3*B*). We further examined whether *Tmevpg1* was sufficient to restore *Ifng* transcript expression in the absence of Stat4 and T-bet. CMV-*Tmevpg1*, CMV-*Tbx21* or CMV-empty vector plasmids were transfected into Th1 polarized BALB/c-J.*Stat4*$^{-/-}$*Tbx21* $^{-/-}$ splenocytes. After a period of rest, cultures were restimulated with PMA and ionomycin. *Tmevpg1*, *Tbx21* and *Ifng* transcript levels were determined by qPCR. We observed *Tmevpg1* expression to be restored by ectopic expression of T-bet alone in the BALBc/J.*Stat4*$^{-/-}$*Tbx21* $^{-/-}$ cells (Fig 4*A*). CMV-*Tmevpg1* and CMV-*Tbx21* co-transfection resulted in a substantial increase in *Ifng* transcript levels relative to transfection of CMV-*Tmevpg1* or CMV-*Tbx21* alone (Fig. 4*C*). We conclude from these experiments that overexpression of *Tmevpg1 in trans* alone is not sufficient to induce increased *Ifng* transcription in Th0, Th1, or Th2 cells or in *Stat4*$^{-/-}$*Tbx21* $^{-/-}$ cells except in the presence of T-bet.

Discussion

Presently, lincRNAs segregate into two functional categories that either repress or enhance the transcription of protein coding genes. To summarize our results, *Tmevpg1* and its human ortholog, are expressed selectively in Th1 cells relative to Th2, Th17 and Th22 cells and expression is dependent upon the Th1 specific transcription factors, Stat4 and T-bet. Our results also demonstrate that *Tmevpg1* influences *Ifng* transcription in response to the Th1 differentiation program. In contrast, ectopic expression of *Tmevpg1* does not increase *Ifng* transcript levels in Th0, Th1, or Th2 cells, however, *Tmevpg1* is able to partially restore *Ifng* expression when T-bet is also overexpressed. One possible interpretation is that *Tmevpg1* must be expressed from its endogenous locus, or in *cis*, to stimulate *Ifng* transcription. A second possible interpretation, which our data favor, is that *Tmevpg1* must act in concert with T-bet, or other critical trans-activating factors, to influence *Ifng* transcription. Other studies of enhancer lincRNAs are consistent with our results and these lincRNAs also fail to stimulate transcription of protein coding genes *in trans* or require additional transactivation factors to drive their transcription (17).

A general emerging model is that cell-type specific transcription factors bind to lincRNA promoters to drive their transcription. The lincRNAs bind to ubiquitous proteins required to establish the epigenetic code and by mechanisms that are incompletely understood direct these proteins to their target protein-coding genes (18, 20). This model does not rule out the possibility that these cell-type specific transcription factors also target protein-coding genes. Our results demonstrate that one lincRNA, *Tmevpg1*, contributes to *Ifng* expression as part

of the Th1 differentiation program. We predict that additional lincRNAs play critical roles in developmental programs required to establish the different functions of the immune system.

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Abbreviations used

lincRNA	long intergenic noncoding RNA

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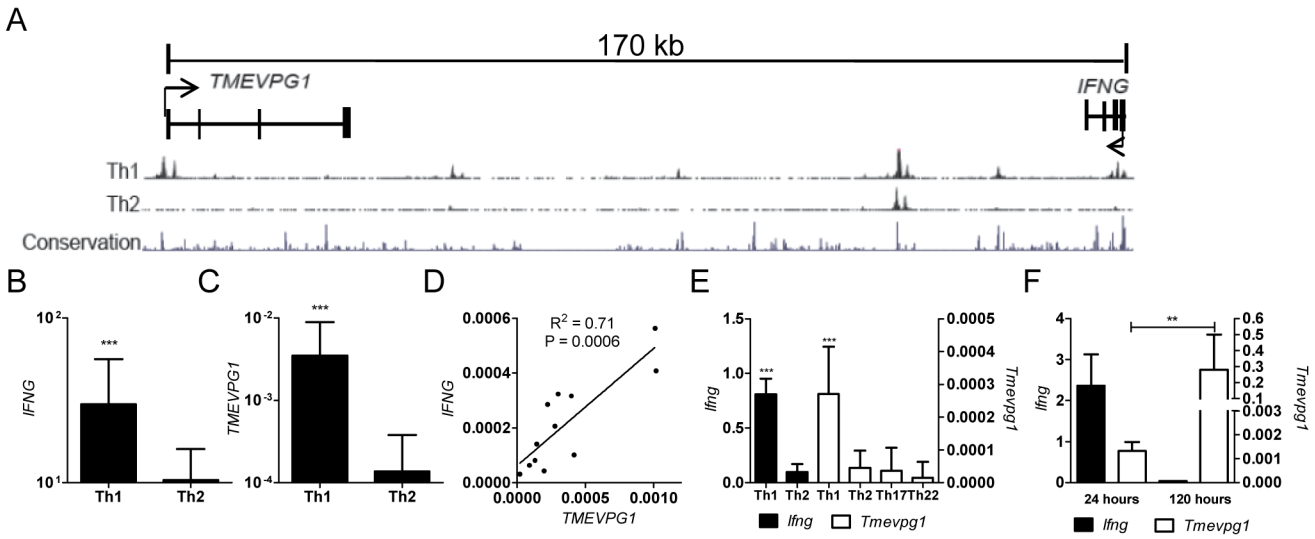

FIGURE 1.

TMEVPG1 is selectively expressed in Th1 cells and positively correlates with *IFNG* expression in human and mouse. *A*, Relative genomic positions of *TMEVPG1* and *IFNG* on human chromosome 12. Th1/Th2 DNAse I hypersensitivity sites and mammalian conservation peaks are also shown. Arrowheads indicate the orientation of transcription. *B* and *C*, Human $CD4^+$ T cells (n=12) were cultured under Th1 or Th2 polarizing conditions for three days. *B, IFNG* and *C, TMEVPG1* transcript levels relative to *GAPDH* were determined by SybrGreen qPCR. *D*, Relationship between *IFNG* and *TMEVPG1* transcript levels from whole blood samples (PAXgene collection tubes) were determined by linear regression analysis (n=12). *E*, *Ifng* and *Tmevpg1* transcript levels relative to *Gapdh* in Th1, Th2, Th17 or Th22 polarized cultures. *F*, *Tmevpg1* and *Ifng* transcript levels relative to *Gapdh* in $CD4^+$ effector Th1 cells after restimulation with PMA and ionomycin. Results are expressed as the mean ± SD of three independent experiments.**p ≤ 0.01 and ***p ≤ 0.001

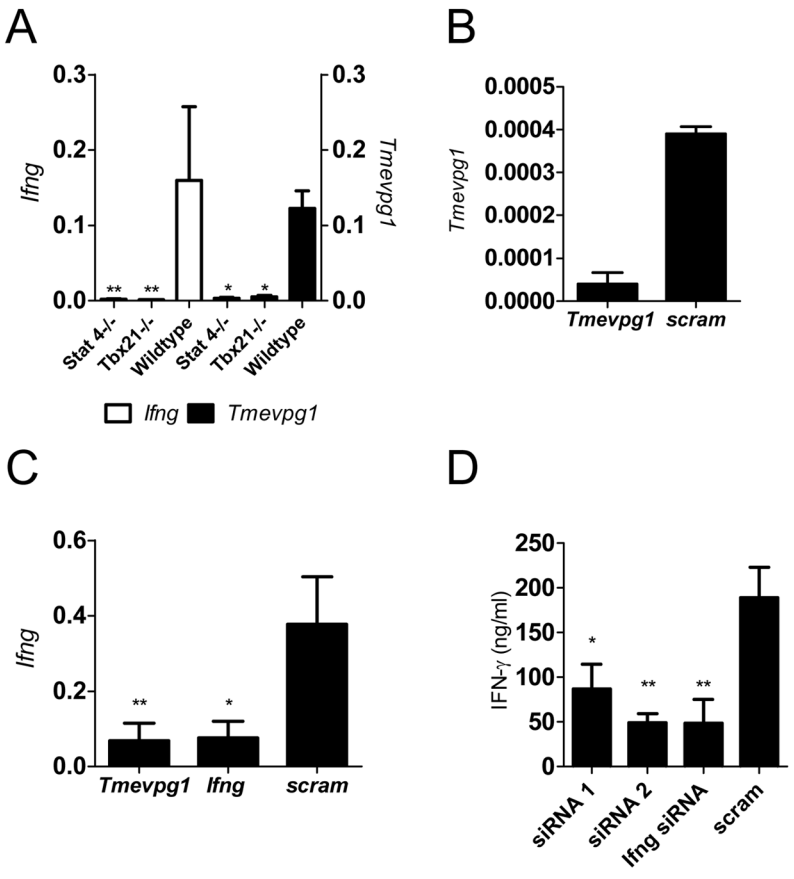

FIGURE 2.

Tmevpg1 induction is dependent upon Stat4 and T-bet transcription factors. *A*, *Ifng* and *Tmevpg1* expression in Th1 cells from DO11.10.*Stat4*$^{-/-}$, DO11.10.*Tbx21*$^{-/-}$ (T-bet knockout) and wildtype TCR transgenic mice relative to *Gapdh. B, C and D*, *Tmevpg1*-specific, *Ifng*-specific, or scrambled siRNA duplexes were introduced into Th1 polarized CD4^{+} T cells by nucleofection. B, *Tmevpg1*, C, *Ifng*, and D, IFN-γlevels (measured by ELISA) were determined after restimulation with PMA and ionomycin. Results are expressed as the mean ± SD of three independent experiments. *p ≤ 0.05 and **p ≤ 0.01

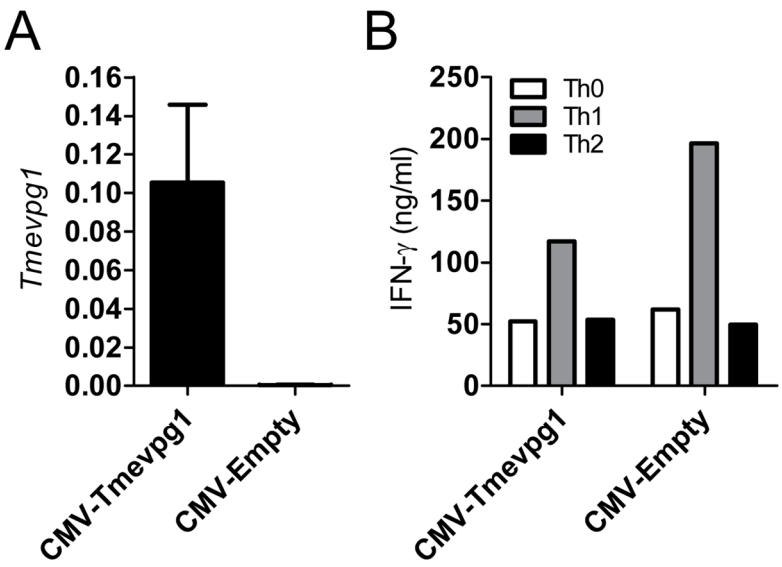

FIGURE 3.

Tmevpg1 is necessary but not sufficient for IFN-γ expression by Th1 cells. CMV-*Tmevpg1* or CMV-empty expression plasmids were introduced into polarized cultures by nucleofection and cultures were restimulated with PMA and ionomycin. *A, Tmevpg1* transcript levels are expressed relative to *Gapdh. B,* IFN-γ levels in Th0, Th1, and Th2 polarized cultures were determined by ELISA. Results represent the mean of at least three separate experiments

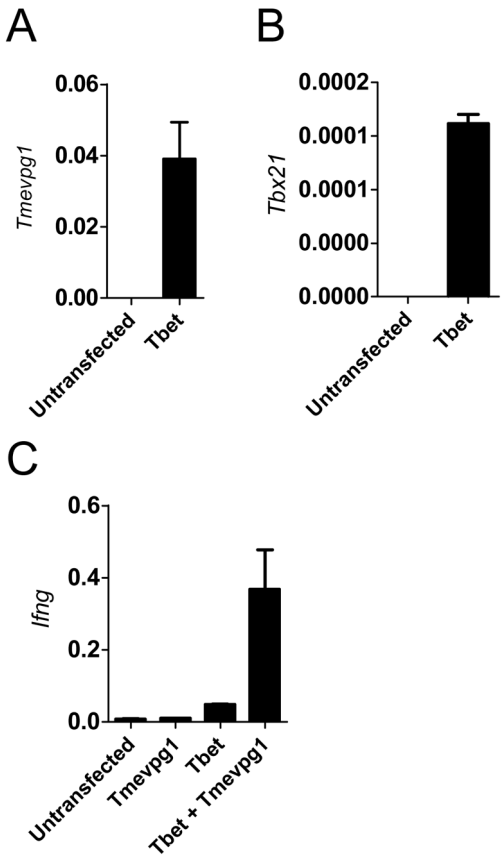

FIGURE 4.

Cooperative action of T-bet and *Tmevpg1* restores *Ifng* expression. CMV-*Tmevpg1*, CMV-*Tbx21 and/*or CMV-empty vector plasmids were transfected into Th1 polarized BALB-c/J.*Stat4*$^{-/-}$*Tbx21*$^{-/-}$ cells. Cells were restimulated with PMA and ionomycin. *A, Tmevpg1* B, *Tbx21* and *C*, *Ifng* transcript levels are expressed relative to *Gapdh*. Results represent the mean of at least three independent experiments.