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Trpm4 differentially regulates Th1 and Th2 function by altering calcium signaling and NFAT localization

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Abstract

T helper cell subsets have unique calcium (Ca^{2+}) signals when activated with identical stimuli. The regulation of these Ca^{2+} signals and their correlation to the biological function of each T cell subset remains unclear. Trpm4 is a Ca^{2+}-activated cation channel that we found is expressed at higher levels in Th2 cells compared to Th1 cells. Inhibition of Trpm4 expression increased Ca^{2+} influx and oscillatory levels in Th2 cells and decreased influx and oscillations in Th1 cells. This inhibition of Trpm4 expression also significantly altered T cell cytokine production and motility. Our experiments revealed that decreasing Trpm4 levels divergently regulates nuclear localization of NFATc1. Consistent with this, gene profiling did not show Trpm4 dependent transcriptional regulation and T-bet and GATA-3 levels remain identical. Thus, Trpm4 is expressed at different levels on T helper cells and plays a distinctive role in T cell function by differentially regulating Ca^{2+} signaling and NFATc1 localization.

INTRODUCTION

T cell interactions with antigen initiate a cascade of signaling events that lead to an increase in intracellular calcium (Ca^{2+}) concentrations. TCR-stimulated influx of Ca^{2+} is required for T cell activation and downstream effector functions such as gene expression, cell motility, and cytokine production (1,2). The amplitude, duration, and kinetics of the Ca^{2+} signal encode information that plays an important role in altering the efficiency and specificity of gene expression, T cell motility, and subsequent T cell function (3–5).

Activation of polarized Th1 or Th2 cells results in the production of a discrete subset of cytokines that helps orchestrate the involvement of numerous immune cells. Th1 cells help clear intracellular pathogens by secreting IFN-γ and promoting macrophage activation. Th2 cells aid in the clearance of parasites by secreting IL-4, IL-5, and IL-13 and promoting Ab-mediated immunity by activating mast and B cells (6,7). Besides distinct cytokine production, T helper cell subsets exhibit unique intracellular Ca^{2+} patterns after stimulation with identical antigen. Th2 cells have lower sustained Ca^{2+} levels and fewer oscillations

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ACCESSION NUMBERS

Gene-expression data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE21996.

compared to Th1 cells (8–11). The biological importance of these unique intracellular Ca^{2+} signals in helper T cells remains unclear.

Ca^{2+} influx oscillations in T cells are proposed to be due to a cyclical interaction of Ca^{2+} release from intracellular stores and the CRAC channel and Ca^{2+}-activated K^+ current (K^{ca}) channels in the plasma membrane (2,12). Analysis of the differences in T helper cell Ca^{2+} signaling has shown Th1 cells have increased functional K_{ca} channels and Th2 cells have an increased rate of cytosolic Ca^{2+} clearance (11). More recent work using Jurkat T cells provided evidence that Ca^{2+} influx and oscillations are also regulated by Trpm4, a sodium (Na^+) channel that mediates cell membrane depolarization (13).

In electrically non-excited cells, such as T cells and mast cells, Trpm4 depolarizes the cell membrane by transporting Na^+ into the cell and this inhibits Ca^{2+} ions from entering the cell by decreasing the driving force of CRAC-mediated Ca^{2+} influx (13–15). Trpm4 has also been shown to play important roles in IgE-dependent mast cells activation and anaphylactic responses, IL-2 production in Jurkat T cells, dendritic cell migration, and the initiation of hemorrhages caused by spinal cord injury (13,15–17). Our examination by expression microarray, RT-PCR, and Western blot revealed that Trpm4 is expressed at higher levels in Th2 cells compared to Th1 cells and lead us to hypothesize that Trpm4 plays a role in the different Ca^{2+} influx patterns and cytokine differences between T helper cell subsets.

To test the hypothesis that Trpm4 regulates the unique intracellular Ca^{2+} signaling in helper T cells, we inhibited Trpm4 expression levels using either Trpm4 siRNA or a Trpm4 dominant negative (DN) mutant. The Trpm4 mutant is missing the first 177 amino acids at the N terminus and has been shown to successfully inhibit Trpm4 in Jurkat cells in a dominant negative manner (13). Inhibition of Trpm4 expression in Th2 cells resulted in an increase in Ca^2 influx and oscillations compared to controls whereas the opposite was seen with Th1 cells. Th2 cells with Trpm4 expression inhibited were less motile when compared to controls and the converse was true in Th1 cells. ELISPOT analysis revealed significant changes in IL-2, IL-4, and IFN-γ production in Th1 and Th2 cells when Trpm4 expression was inhibited. Examination of expression microarrays did not reveal Trpm4 dependent transcriptional regulation. We did not see differences in the transcription factors T-bet or GATA-3, but Trpm4 significantly affected NFATc1 nuclear localization in Th1 and Th2 cells. Together, these findings show Trpm4 affects Th1 and Th2 cellular motility and cytokine production through differential regulation of Ca^{2+} signaling and NFATc1 localization.

MATERIALS AND METHODS

T Cell Isolation and Maintenance

T cells were isolated and maintained as previously published (10). In brief, primary 2.102 T cells were isolated from the spleens of RAG1-deficient 2.102 TCR-transgenic mice (18) and T cells were enriched using CD4+ beads and magnetic cell sorting (Miltenyi Biotec). CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and spleen cells, containing the endogenous Hbb^d epitope that stimulates 2.102 cells, were isolated and irradiated (2000 RAD) for use as antigen presenting cells. All mice used in this study were between 5 to 10 weeks of age. 2.102 T cells (1×10^6) and irradiated CBA/J spleen cells (5×10^6), were added to a 12-well cell culture plate with the addition of the T cell specific cytokines and antibodies described below. Primary T cells were cultivated in media containing Iscove's modified Dulbecco's medium supplemented with 2 mM GlutaMAX (Invitrogen Life Technologies), 5×10^{-5} M 2-ME, and 50 μg/ml gentamicin.

T Cell Polarization

T cells were polarized to Th1 or Th2 cells *in vitro* by the addition of (a) 10 ng/ml IL-12 (R&D Systems) and 10 μg/ml anti–IL-4 mAb (clone 11B11) for Th1 cell polarization, (b) 10 ng/ml IL-4 (R&D Systems) and 10 μg/ml anti–IL-12 mAb (Clone TOSH) for Th2 cell polarization. IL-2 (100 U) was added to the media 48 hours after initial stimulation and T cell cultures were split 1:2. Cells were restimulated after 7 days in culture and the same cytokines and antibodies described above were added again. The polarized T cells were used on day 14.

Constructs

The dominant negative form of the Trpm4 gene (missing the first 177 amino terminal amino acids) was amplified from the full-length gene in the Trpm4-pBlue vector and digested and ligated into the pMI-DsR vector (Trpm4-IRES-DsRed) using the *EcoRI* and *MfeI* sites.

Transfection and Cell Sorting

T cells were retrovirally transfected as previously described (19). In brief, the Platinum-E packaging cell line (a gift from Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) was transfected with 30 μg of retroviral construct DNA with Lipofectamine 2000 (Invitrogen), and viral supernatant was collected 48 h after transfection. For T cell activation, $3–5 \times 10^5$ T cells were stimulated with 6.5×10^6 irradiated B6.K splenocytes loaded with 10 μM $Hb_{64–76}$. At 20 and 24 h after activation, retroviral supernatant was added to the T cell cultures and spun for 45 min at 1800 rpm at 25°C in the presence of Lipofectamine 2000 (Invitrogen) and 125 U/ml IL-2. At 5 days after activation, T cells were sorted for equivalent levels of DsRed using the cells were sorted on a MoFlow cell sorter (Cytomation, Fort Collins, CO).

siRNA Experiments

siRNA specific for Trpm4 or Thy1 were purchased from Dharmacon and prepared according to the manufacturers instructions. Thy1 was chosen as a control because it is a cell surface protein not involved with calcium influx and its levels can be easily evaluated with using flow cytometry. T cells were polarized as described above and the siRNA was performed using the Amaxa Mouse T cell Nucleofector Kit. Briefly, six million T cells were resuspended in 100μl of the Nucleofector Solution and placed in a cuvette with 4 ug of Trpm4 or Thy1 siRNA and electroporated with the CD4+ Amaxa nucleofector mouse CD4 T cell program. Electroporated cells were incubated in Nucleofector Medium for 4 hours and then washed and grown in Iscove's modified Dulbecco's medium. Electroporated cells were used in experiments 48 hours after the siRNA transfection.

Ca^{2+} Imaging

Ca^{2+} imaging was performed as published previously (10). In brief, immediately before imaging polarized T cells were incubated with 1μM Fura-2 AM (Molecular Probes) for 30 minutes at 37°C in Ringers imaging solution (150mM NaCl, 10mM glucose, 5mM HEPES, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$), washed, and then incubated in Ringers solution for another 30 minutes at 37°C. After washing, 100,000 Fura-2 loaded T cells were pipetted onto 100,000 adherent antigen presenting cells (Hi7-E^k) that had been loaded with 1μM $Hb_{64–76}$ overnight. The $Hb_{64–76}$ peptide was synthesized using standard Fmoc chemistry, purified by reverse phase HPLC, and confirmed using MALDI mass spectrometry as has been previously described (20). The location of the antigen presenting cells and T cells were monitored by visualizing the cells with transmitted light as well as DsRed every 3 seconds. All imaging was done in 8 chamber coverglass slides (Lab-Tek, Nalge Nunc).

Ca^{2+} imaging was performed at 37°C using a temperature controlled environmental chamber on a Zeiss axiovert 200M microscope equipped with a xenon arc lamp. Fura-2 loaded cells were excited using 340 and 380 excitation filters (71000a set, Chroma Technology Corp) and a polychroic mirror (73100bs, Chroma Technology Corp). Fluorescence was passed through a 510±40 wide band emission filter (Chroma Technology Corp) and captured by a Cascade 512B camera (Roper Scientific). Ratio measurements (340/380) were recorded at 3-second intervals over 30 minutes using a 20× Fluar objective (Zeiss; N.A. 0.75).

Western Blots

T cells lysates were boiled for 5 minutes at 100°C and resolved on a 10% SDS-PAGE gel (~5×10^6 cells/sample) and transferred onto a nitrocellulose membrane (Bio-Rad). The blots were blocked with 1:1 PBS:Blocking buffer (Licor Biosciences) for 1 hour and probed (1:1000 in PBS-T) with primary antibodies (Rabbit polyclonal anti-Trpm4; abcam #ab63080-100 or mouse monoclonal anti-GAPDH; Ambion #AM4300) overnight at 4°C. The membranes were probed (1:10,000 in PBS-T+SDS) with secondary antibodies (Goat anti-rabbit 680 or Goat anti-mouse 800; Licor Biosciences) for 1 hour at room temperature. Bands were visualized and quantified with the Odyssey infrared imaging system (Licor Biosciences).

Cytokine Measurements

T cell cytokine expression was assessed by ELISPOT. T cells were stimulated for 24 hrs with irradiated splenocytes or Hi7-E^k cells. ELISPOT measurement of cytokine levels was performed by 24 hour T cell stimulation with at least 3 replicates in 96-well Multiscreen IP plates (Millipore) that had been coated overnight with anti-IL4 (clone 11B11; eBioscience), anti-IFN-γ (clone R4-6A2 eBioscience), or anti-IL2 (clone JES6-5H4; BioXCell), and labeled with biotinylated anti-IL4 (BVD6-24G2 eBioscience), anti-IFN-γ (XMG1.2 eBioscience), or anti-IL2 (JES6-5H4 Biolegend) and streptavadin-conjugated HRP (Southern Biotech). Plates were developed with BCIP/NBT (Sigma) and scanned on a CTL Immunospot reader. Data was analyzed using CTL Immunospot 4.0 (Cellular Technology Ltd.).

Gene Expression Analysis

For microarray analysis RNA was isolated using RNeasy kits (Qiagen) and gene expression analysis was performed using mouse expression 430 2.0 arrays (Affymetrix). Data were normalized and expression values were modeled using DNA-Chip Analyzer (dChip)(21). For quantitative real-time PCR analysis, total RNA and cDNA were prepared from T cells with the RNeasy Mini Kit (Qiagen) and Superscript II reverse transcriptase (Invitrogen) according to manufactures directions. The SYBR Green PCR master mix and an ABI7000 machine (Applied Biosystems) were used according to the manufacturer's instructions. PCR conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 two-step cycles consisting of 15 s at 95 °C and 1 min at 60 °C. The values of each sample were normalized to β-actin.

NFATc1 nuclear localization

T cells were washed and incubated with the 1 μM SYTO13 (Molecular Probes) for 20 min at room temperature to stain the nucleus. A total of 2×10^5 T cells/sample was stimulated by Hi7 cells (1 μM Hb (64–76)) for 20 min at 37°C in 8-chamber coverglass slides (Lab-Tek, Nalge Nunc International). Cells were fixed with 100 μl of BD Cytofix (BD Biosciences) for 20 min at room temperature and washed twice with PBS. Cells were permeabilized for 10 minutes at room temperature in 2% triton and then cells were stained (1:100) for 1 h at room temperature in 100 μl of permeabilization buffer (.5% saponin and 1% BSA) with the NF-

ATc1 primary Ab (7A6; Santa Cruz Biotechnology). Cells were then washed, and stained (1:200) in 100 μl of permeabilization buffer with the secondary Ab (goat anti-mouse Alexa Fluor 546; Molecular Probes). After two washes, the cells were resuspended in imaging buffer (1% human serum albumin, 1 mM $CaCl_2$, 2 mM $MgCl_2$) and viewed using an Olympus Fluoview 1000 confocal microcope at 37°C with a 60x PlanApo N oil objective (Olympus: N.A 1.42). Quantification of nuclear localization was performed using the Olympus Fluoview software (colocalization function).

Data Analysis

Fluorescent images were analyzed using MetaMorph (Molecular Devices) and the 340/380 ratio is displayed on a pseudocolor scale, with calculations done on randomly selected cells. Standard deviations were calculated by measuring the sy.x value, the standard deviation of the vertical distances of the data points from the regression line (GraphPad Prism; GraphPad Software Inc). The linear regression line was fit using the 10 to 25 minute time point data. Measuring aspects of T cellular motility were performed by tracking the center of Fura-2 fluorescence with Pic Viewer Software (John Dempster, University of Strathclyde) (22). Only cells with tracks longer then ten minutes were used in the analysis. All significant values were determined using the unpaired two-tailed t test or a Mann-Whitney rank sum test (Graphpad Software Inc).

RESULTS

Trpm4 Expression Levels are Higher in Th2 cells Relative to Th1

T helper subsets have been shown to be functionally unique and exhibit distinct intracellular Ca^{2+} profiles (8–11). Both Th1 and Th2 cells have similar initial peak Ca^{2+} influx but Th1 cell sustained Ca^{2+} levels remain higher than Th2 cells on average and Th1 cells have more oscillations (Fig. 1A, 1B).

To examine Ca^{2+} related gene expression, microarray analysis of Th1 and Th2 cell samples was performed, focusing on genes encoding proteins involved in Ca^{2+} signaling. We found some K^+, Na^+, and Ca^{2+} ion channel genes that were differentially expressed (Fig. 1C). We were intrigued to see that the depolarizing Na^+ channel Trpm4 was expressed at higher levels in Th2 cells compared to Th1 cells. Differences in helper T cell Ca^{2+} influx and oscillation have been shown to be due in part to differences in K_{ca} levels (11) and more recently Ca^{2+} influx has been proposed to be due to the interactions between Trpm4 and the repolarizing potassium channels (11,13). The difference in Trpm4 expression in Th1 and Th2 cells was confirmed by RT-PCR and Western blot (Fig. 1D–F).

We hypothesized that the higher expression of Trpm4 in Th2 cells played a role in causing the lower sustained Ca^{2+} levels and fewer oscillations seen in Th2 cells. To test this we inhibited Trpm4 expression using siRNA or a DN Trpm4 mutant in both Th1 and Th2 cells. The dominant negative form of Trpm4 is missing the first 177 amino acids at the N terminus (13). Trpm4 requires multimerization to form functional channels and this DN Trpm4 mutant has been shown to successfully inhibit Trpm4 in Jurkats (13). We expressed the DN mutant using the pMI-DsRed retroviral vector, allowing us to sort a cell population expressing the vector and confirm inhibition of Trpm4 while imaging Ca^{2+} influx. These two methods yielded similar results, providing additional strength to the described findings and the specificity of the Trpm4 siRNA.

Inhibition of Trpm4 expression in Th2 Cells Increased Ca^{2+} Levels and Oscillations

Trpm4 was inhibited using siRNA in 2.102 T cells that had been polarized for two weeks. Thy1 was used as a siRNA control because it is a cell surface protein not involved with

calcium influx and its levels can be evaluated with using flow cytometry. Successful inhibition of Trpm4 expression was confirmed by Western blot (Fig. 1E, 1F). A similar decrease in the surface Thy1 levels was also observed by flow cytometry (data not shown). Western blot analysis showed a two fold higher level of Trpm4 in Th2 cells relative to Th1 cells which is similar to that which was seen with genechip and RT-PCR. Single cell intracellular Ca^{2+} analysis was performed by adding Fura2 labeled 2.102 Th2 cells to a monolayer of Hi7-E^k DCEK/ICAM antigen presenting cells that had been pulsed with 1 μM $Hb_{64–76}$ peptide overnight (Fig. 2). Ca^{2+} influx was increased in the Trpm4 siRNA cells compared to control Th2 cells (Fig. 2A, 2B; Videos S1 and S2).

The Ca^{2+} profiles of the Th2 control cells (Thy1 siRNA) have low levels of sustained Ca^{2+} with few oscillations similar to cells that have not undergone any siRNA procedure (Fig. 1B, 2C). The Trpm4 siRNA resulted in an increase in Th2 cell peak and mean Ca^{2+} levels and an increase in oscillations (Fig. 2D). Thus, inhibition of Trpm4 expression resulted in Th2 cells with a Ca^{2+} profile that is more ‘Th1-like’. The average curve shows the increase in the sustained Ca^{2+} levels is consistently higher in the Trpm4 siRNA group over time (Fig. 2E). The peak and mean Ca^{2+} levels and oscillations were all significantly higher than controls when Trpm4 was inhibited (Fig. 2F–2H).

To confirm these findings and allow us to examine only cells with Trpm4 expression inhibited, polarized T cells were transfected with either the DN Trpm4 or vector only. Transfected T cells (DsRed positive) were sorted for equivalent levels of DsRed (Fig. 3A) and single cell Ca^{2+} influx was measured. Ca^{2+} profiles of Th2 cells transfected with the vector have low levels of sustained Ca^{2+} and few oscillations (Fig. 3B). In contrast, the Th2 cells transfected with DN Trpm4 have higher levels of sustained Ca^{2+} and oscillations (Fig. 3C). The average Ca^{2+} profile (Fig. 3D) shows the increased mean Ca^{2+} over time for both the DN Trpm4 and vector only groups. Statistical analysis of the initial peak values were not significantly different between groups (Fig. 3E) but analysis of the sustained mean Ca^{2+} levels were significantly higher in the DN Trpm4 cells (Fig. 3F). We also found significantly higher levels of oscillations in the DN Trpm4 cells (Fig. 3G), although there is more variability in oscillation values with some cells having dramatically higher levels of oscillation than others even within the same treatment group. The findings with the DN Trpm4, higher calcium mean and oscillation levels, were similar to that seen with inhibition of Trpm4 expression using siRNA (Fig. 2). Thus, inhibition of Trpm4 expression in Th2 cells results in a ‘Th1-like’ pattern of Ca^{2+} influx using two separate methods.

Inhibition of Trpm4 expression in Th1 Cells Decreases Ca^{2+} Levels and Oscillations

Because inhibiting Trpm4 expression in Th2 cells resulted in a dramatic change to a ‘Th1-like’ calcium profile, we determined to examine the effects of inhibition of Trpm4 expression in Th1 cells. The level of Trpm4 expression in Th1 cells is ~3 fold lower than that of Th2 cells (Fig. 1D). We examined the role of Trpm4 in Th1 polarized 2.102 cells by inhibiting Trpm4 expression with siRNA. In contrast to what was seen in Th2 cells, inhibition of Trpm4 expression in Th1 cells resulted in a decrease in Ca^{2+} influx (Fig. 4). Ca^{2+} influx was lower in the Trpm4 siRNA cells compared to control cells (Fig. 4A, 4B; Videos S3 and S4). The Th1 control cells (Thy1 siRNA) have frequent oscillations and higher sustained levels of Ca^{2+} (Fig. 4C) and the Trpm4 siRNA results in decreased mean Ca^{2+} levels (Fig. 4D, 4E). The peak and mean Ca^{2+} levels and oscillations are all significantly lower when Trpm4 expression is inhibited compared to the control (Fig. 4F–4H).

Polarized Th1 cells were transfected with DN Trpm4 or the vector to confirm the Trpm4 siRNA findings and to examine only cells expressing the DN Trpm4. Transfected T cells (DsRed positive) were sorted and single cell Ca^{2+} influx was measured. The Ca^{2+} profile of

the Th1 cells transfected with vector have higher mean levels of Ca^{2+} and frequent oscillations (Fig. 5A). Transfection with DN Trpm4 results in a decrease in Ca^{2+} influx and fewer oscillations (Fig. 5B). The Th1 average curve shows consistently lower levels of sustained Ca^{2+} levels when Trpm4 is inhibited (Fig. 5C). The peak and mean Ca^{2+} levels are both significantly lower with the transfection of DN Trpm4 (Fig. 5D, 5E). Statistical analysis of oscillations also shows a significantly lower value with the transfection of DN Trpm4 (Fig. 5F). Inhibition of Trpm4 expression with the DN construct showed a similar phenotype as seen with the Trpm4 siRNA (Fig. 4). Thus, inhibition of Trpm4 expression in Th1 cells results in a significantly lower level of Ca^{2+} influx using two separate methods.

Inhibition of Trpm4 expression alters T helper cell velocity and motility

The divergent Ca^{2+} patterns in Th1 and Th2 cells seen when Trpm4 expression was inhibited were intriguing and to determine the biological effects we examined T cell motility. T cell motility has been reported to correlate with Ca^{2+} levels (3). Higher levels of Ca^{2+} prolong the interactions cells have with antigen *in vitro* and *in vivo* and result in altered gene expression and T cell function (5). Th2 cells have been shown to be significantly more motile compared to Th1 cells (10). Our analysis showed that there is a decrease in Th2 cell track length when Trpm4 expression is inhibited by siRNA (Fig. 6A, 6B). Statistical analysis revealed that Th2 cells that had Trpm4 expression inhibited have significant differences in velocity and motility (Fig. 6E, 6F) but no detectable differences in the meandering index (Fig. 6G).

When examining Th1 cell motility the opposite was seen, inhibition of Trpm4 expression in Th1 cells caused an increase in cell track length compared to the control group (Fig. 6C, 6D). Further analysis showed that inhibition of Trpm4 expression in Th1 cells caused significant differences in velocity and motility values (Fig. 6E, 6F) but no difference in the meandering index (Fig. 6G). Thus, the changes in Ca^{2+} influx caused by inhibition of Trpm4 expression were large enough to effect motility. Higher levels of Ca^{2+} influx in Th2 cells with inhibition of Trpm4 expression correlated with decreased motility. Lower levels of Ca^{2+} influx in Th1 cells with inhibition of Trpm4 expression correlated with increased motility.

Inhibition of Trpm4 expression alters Th1 and Th2 cell cytokine production

To examine the relationship between Trpm4 and T helper cell function we analyzed cytokine production. IL-2 secretion and the Th2 and Th1 cytokines IL-4 and IFN-γ were measured by ELISPOT (Fig. 7). Inhibition of Trpm4 expression in Th2 cells by siRNA or DN Trpm4 resulted in a significant increase in IL-2 secreting cells (Fig. 7A) and a significant decrease in IL-4 producing cells (Fig. 7B). There was minimal IFN-γ production by Th2 cells and no change in this with Trpm4 inhibition (Fig 7C).

The number of IL-2 secreting Th1 cells decreased significantly when Trpm4 expression was inhibited by either siRNA or DN Trpm4 (Fig. 7D). IFN-γ secreting cells were significantly lower for the Trpm4 siRNA group but not when the cells were transfected with DN Trpm4 (Fig. 7E). There was minimal IL-4 production by Th1 cells and no change in this with Trpm4 inhibition (Fig 7F). Thus, for both Th2 and Th1 cells, the Ca^{2+} influx differences caused by inhibition of Trpm4 expression led to significant differences in IL-2, IFN-γ, and IL-4 producing cells.

Transcription regulation when Trpm4 expression inhibited

To determine if inhibiting Trpm4 expression with siRNA resulted in specific transcriptional regulation of a gene or set of genes we performed genechip analysis. Comparison of the Th1 siRNA genechips (Thy1 vs Trpm4 siRNA) at a two-fold cutoff level only revealed one over-

expressed gene besides the expected difference in Thy1 (Table 1A). That gene, Peroxisome proliferator activated receptor binding protein, binds DNA and can mediate transcription but has no direct connection to Ca^{2+} regulation. Comparison of the Th2 siRNA genechips at a two-fold cutoff level did not result in any over-expressed genes besides the expected difference in Thy1 (Table 1A).

We also examined the effect of the dominant negative Trpm4 on gene expression at both 4-hr and 12-hour stimulation time points. At the two-fold cutoff level for Th1 cells there were 7 over or under-expressed genes besides Trpm4 (Table 1B). None of the genes had a direct connection with Ca^{2+} regulation. For Th2 cells there are 12 over or under-expressed genes when using a two-fold cutoff. There is a change in the Th2 cytokine IL-5 as well as IL-21, but no other genes that have a direct connection to Ca^{2+} regulation. At the two-fold cutoff level there was not any overlap between the Th1 or Th2 genes in the siRNA or either of the two DN Trpm4 time points, suggesting that these candidate genes may not be strongly correlated to Trpm4 expression. Thus, we did not see any strong candidate genes that were regulated by decreasing Trpm4 expression, suggesting that Trpm4 is not directly affecting Ca^{2+} signaling via transcription regulation.

T-bet and GATA-3 protein levels are the same when Trpm4 is inhibited

Because the transcription factors T-bet and GATA-3 have numerous effects upon T helper cell function and development, we determined to examine their protein expression levels when Trpm4 expression was inhibited. T helper cell polarization changes in T-bet and GATA3 levels result in changes in chromatin structure at the IFN-γ and IL-4 genes (23). Comparison of GATA-3 levels in Th2 cells were the same when comparing Thy1 and Trpm4 siRNA samples (Figs. 8A). Th1 T-bet levels also showed no difference between Thy1 and Trpm4 siRNA samples (Figs. 8D). Thus, the T helper cell transcription factors GATA-3 and T-bet showed no detectible differences when Trpm4 expression levels are inhibited and suggest that Trpm4 inhibition is not causing a lack of polarization to T helper subtypes.

NFAT localization differentially regulated by Trpm4

Nuclear factor of activated cells (NFAT) is a family of transcription factors with a calcineurin and DNA binding domain. NFAT has a strong correlation with IL-2 production and was initially identified as an inducible factor that could bind the IL-2 promoter (24). NFAT proteins are known to be regulated by Ca^{2+} signaling and can bind to both the IFN-γ and IL4 promoters in early stages of naïve T cell activation. T helper cell differentiation results in T-bet or GATA3 silencing of the Th1 or Th2 locus and then NFAT predominantly binds to either the IFN-γ and IL-4 promoters in Th1 and Th2 cells (23,25). To determine if the differences in cytokine production levels were due to changes in NFAT, we examined Th1 and Th2 NFATc1 nuclear localization.

In Th2 cells, comparison of the Thy1 siRNA group to Trpm4 siRNA group revealed a significant increase in NFATc1 localization (Fig. 8B, 8C). Conversely, in Th1 cells we saw a significant decrease in NFATc1 localization in the Trpm4 siRNA group (Fig. 8E, 8F). Thus, inhibition of Trpm4 expression results in significantly altered Th1 and Th2 NFATc1 levels that correlate with the measured IL-2 differences. We also see decreased IL-4 production in Th2 cells and decreased IFN-γ production in Th1 cells with inhibition of Trpm4 expression.

DISCUSSION

Activation of Th1 and Th2 cells with identical peptide and antigen presenting cells results in dramatically different Ca^{2+} signaling and cytokine production (8–11). The amplitude and intensity of the T cell Ca^{2+} signal are critical in setting the threshold for transcription of different genes and functional outcomes (26). In this study, we show that inhibition of Trpm4 expression differentially changes the Ca^{2+} profile and NFATc1 nuclear localization in Th1 and Th2 cells and this results in T helper cell functional changes in motility and cytokine production (IL-2, IL-4, and IFN-γ).

Two reported explanations for the differences normally seen in helper T cell Ca^{2+} influx patterns is increased levels of functional K_{ca} channels in Th1 cells and increased Ca^{2+} clearance in Th2 cells (11). The more recent identification and characterization of Trpm4 has provided insights into its role in Ca^{2+} influx and oscillations. Trpm4 has been proposed to act in concert with the CRAC channel and repolarizing K_{ca} channels to control membrane potential and Ca^{2+} oscillations in lymphocytes (13). This Trpm4 work was generated from patch clamp experiments done on Jurkat T cells using PHA stimulation. T helper subsets have unique Ca^{2+}signals with differences in levels of influx as well as oscillations. Th2 cells have lower sustained levels of Ca^{2+} influx and fewer oscillations compared to Th1 cells (8–11). Using microarray, RT-PCR, and Western blot we discovered that Trpm4 was expressed higher in Th2 cells compared to Th1 cells, stimulating our interest in the role Trpm4 plays in Ca^{2+} influx in polarized T helper cells stimulated by antigen presenting cells.

In single cell Ca^{2+} experiments we investigated the role of Trpm4 in Th2 and Th1 cells by suppressing Trpm4 levels either with siRNA or a DN Trpm4 mutant. Inhibition of Trpm4 expression in Th2 cells causes an increase in Ca^{2+} levels and oscillations, resulting in Th2 cells with a Ca^{2+} profile more similar to Th1 cells. Inhibition of Trpm4 by siRNA in Th2 cells resulted in levels of Trpm4 protein similar to control Th1 cells when measured by Western blot. Even in Th1 cells, where Trpm4 expression levels are low, we found Trpm4 plays an important role in Ca^{2+} influx regulation and T cell function. Inhibition of Trpm4 expression in Th1 cells caused a decrease in Th1 cell Ca^{2+} levels and oscillations, resulting in Th1 cells with a Ca^{2+} profile more similar to Th2 cells. Thus, we found that Trpm4 plays a divergent role in Ca^{2+} influx in Th2 and Th1 cells. We are uncertain why a decrease in Trpm4 levels in Th1 cells causes a calcium profile more similar to Th2 cells, but lowering the Trpm4 levels below a threshold level may result in increased levels of Ca^{2+} clearance and decreased oscillations as seen in Th2 cells. We also over expressed Trpm4 in Th1 cells and did not see any changes in the calcium profile (data not shown), suggesting that Trpm4 regulation in Th1 and Th2 is not strictly due to expression levels. The contrasting influence of Trpm4 in Th1 and Th2 cells may be due in part to the Trpm4 expression differences between T helper subsets, increased functional K_{ca}, or Ca^{2+} clearance differences resulting in different dependence upon Trpm4 for depolarization. It has also been proposed that Th2 signaling is unique in that it may employ a voltage gated Ca^{2+} channel which would increase the Ca^{2+} differences between subsets, but the evidence for a Th2 voltage gated Ca^{2+} channel remains controversial (1,27,28).

Inhibition of Trpm4 expression in Th1 and Th2 cells resulted in Ca^{2+} differences that were large enough to significantly alter T cell motility. Previous T cell studies have shown that increases in intracellular Ca^{2+} concentrations result in the delivery of a ‘stop’ signal and motility is reduced (3,29,30). In dendritic cells Trpm4 has been shown to prevent high levels of Ca^{2+} and is essential for proper dendritic cell migration (16). Consistent with these studies, we found inhibition of Trpm4 expression resulted in an increase in Th2 Ca^{2+} levels and decreased motility and velocity. In Th1 cells, even though there are low levels of Trpm4 expression to begin with, decreased Trpm4 expression caused a reduction in Ca^{2+} levels and

an increase in cell motility and velocity. Thus, regulation of Ca^{2+} influx by Trpm4 is dramatic enough to change critical immune functions such as DC migration (16) and T cell motility.

An important Ca^{2+} dependent function that Trpm4 regulates is cytokine production. IL-2 and IL-4 have been shown to be dependent upon Ca^{2+} sensitive transcription factors, with the amplitude and duration of the Ca^{2+} signal being critical (31). The IL-4 promoter has two NFAT binding sites and cytokine production is dynamically dependent upon changes in Ca^{2+} levels and shifts in the ratio of NFAT isoforms (32–35). IFN-γ has also been shown to have NFAT-binding sites in its promoter region although the extent of NFAT regulation of IFN-γ is less clear (25,36–38). Of interest here, we have found that the inhibition of Trpm4 expression results in altered Ca^{2+} influx patterns and nuclear localization of NFATc1 in T helper cells and significant alterations in the levels of IL-2 and T helper cell signature cytokine production. Our microarray analysis did not show any dramatic differences in transcription factor expression between groups, but insights into Trpm4 regulation of cytokines may best be determined by analysis using cytokine promoter gene reporters. Our ELISPOT analysis showed an increase in IL-2 producing Th2 cells and a decrease in IL-2 producing Th1 cells. These IL-2 changes correlate with the Ca^{2+} changes and NFATc1 localization measured. T lymphocyte activation and NFAT dependent IL-2 production is well studied and has been shown to be closely tied to the shape and intensity of the Ca^{2+} signal (4,39). Changes in IL-2 levels influence T cell proliferation, memory cell formation, and regulation of inflammatory immune response (40). We also found a decrease in Th2 production of its signature cytokine IL-4 and no changes in IFN-γ production. In Th1 cells we saw no changes in IL-4 production and a decrease in the production of its signature cytokine IFN-γ that correlates with the decreased levels of NFATc1 measured.

In summary, here we report for the first time a divergent role for Trpm4 regulation of Th2 and Th1 function by altering Ca^{2+} signaling and NFATc1 nuclear localization. We found that Trpm4 expression levels were higher in Th2 cells compared to Th1 cells. Two independent methods, siRNA and a dominant negative construct, were used to inhibit Trpm4 expression. We found that inhibition of Trpm4 expression resulted in increases in Ca^{2+} levels and NFATc1 nuclear localization in Th2 cells as well as decreased cell motility, increased IL-2 production, and decreased IL-4 production. Inhibition of Trpm4 expression in Th1 cells caused a decrease in Ca^{2+} levels and NFATc1 nuclear localization, resulting in an increase in cell motility, and a decrease in IL-2 and IFN-γ production. Thus, the differential regulation of T helper cell Ca^{2+} influx and NFATc1 localization by Trpm4 provides a novel means in which T helper cell Ca^{2+} signaling, NFATc1 nuclear localization, and subsequent biological functions are controlled.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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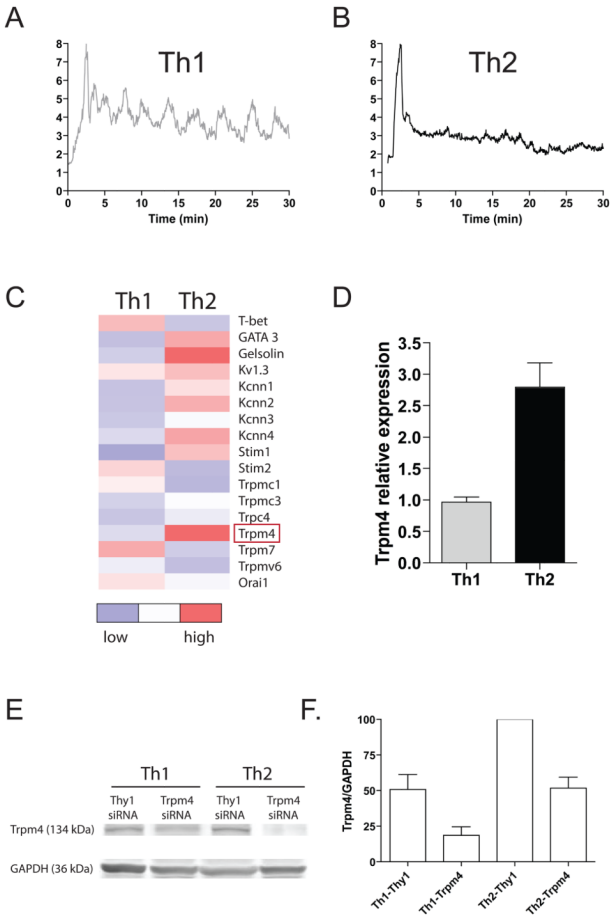

Figure 1. Gene Expression Levels of Trpm4 are Higher in Th2 Cells Relative to Th1 Cells

(A) Representative Th1 Ca^{2+} profile showing that after the initial peak Th1 cells maintain high sustained levels of Ca^{2+} and have numerous oscillations. (B) Representative Th2 Ca^{2+} profile showing that after the initial peak Th2 cells have low levels of sustained Ca^{2+} and few oscillations. (C) Expression microarrays showing Ca^{2+} related genes; fold differences between helper T cell subsets are illustrated from low to high by color (blue to red). The data represents three individual gene arrays for each Th1 and Th2 sample. (D) RT-PCR confirmation of relative Trpm4 expression levels (Trpm4/β-actin) in Th1 and Th2 cells. Data shows the mean ± S.E.M. from three independent experiments. (E) Representative Western blot of Trpm4 siRNA from Th1 and Th2 cell samples. Both the control siRNA (Thy1) and Trpm4 siRNA groups are shown for comparison. (F) Normalized intensity values (Trpm4/GAPDH) from three independent Western blots for Th1 and Th2 cells. Values are displayed as the mean ± S.E.M. and the control siRNA (Thy1) and Trpm4 siRNA groups are shown.

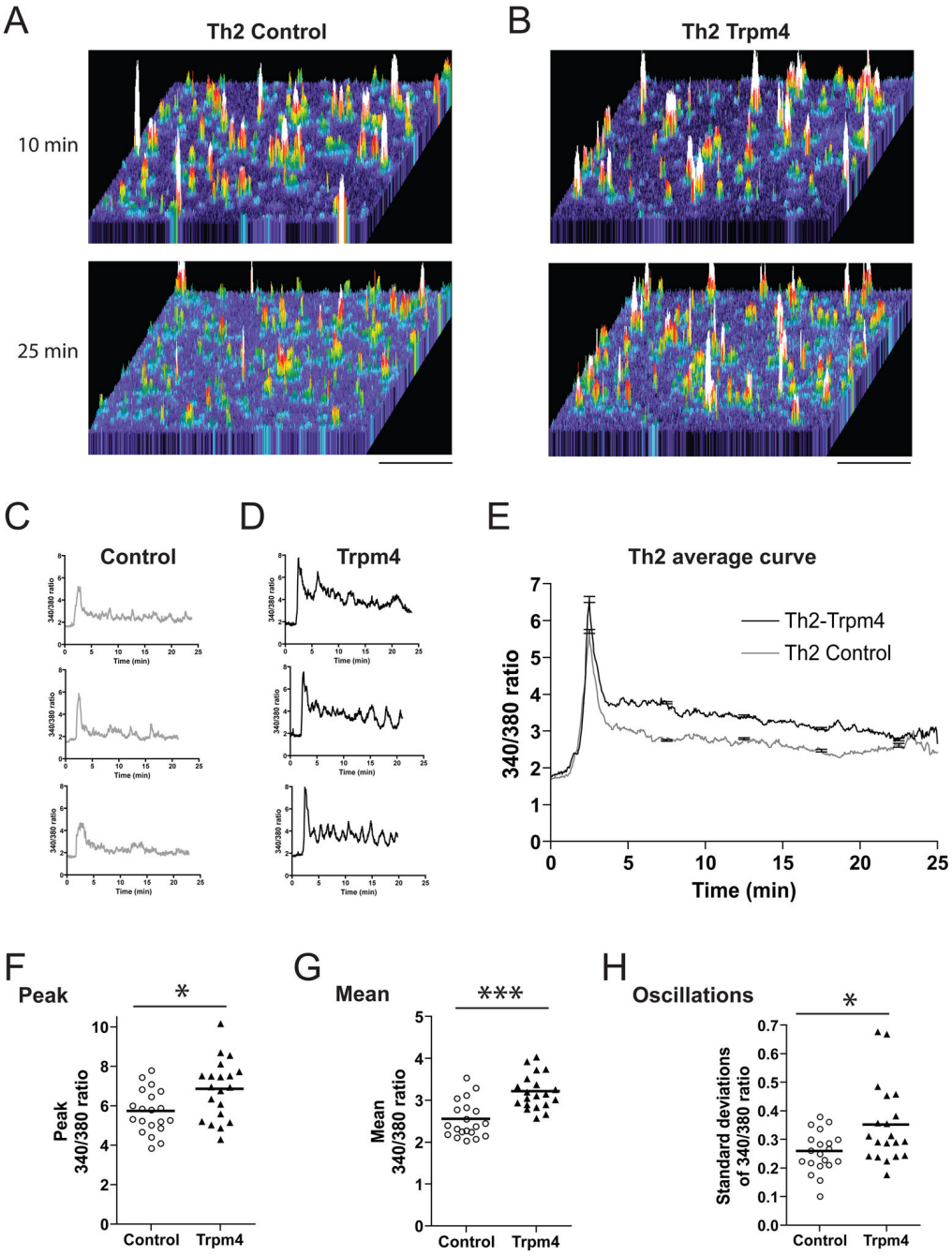

Figure 2. Trpm4 siRNA of Th2 Cells Results in Increased Peak, Mean, and Oscillatory Ca^{2+} Levels

(A) Th2 control (Thy1 siRNA) intensity profile of single cell Ca^{2+} analysis at 10 minute and 25 minute time points. Scale bar is 100um. (B) Th2 intensity profile (Trpm4 siRNA) of single cell Ca^{2+} analysis at 10 minute and 25 minute time points. Scale bar is 100um. (C) Representative Ca^{2+} profiles of control Th2 cells (Thy1 siRNA). (D) Representative Ca^{2+} profiles of Th2 cells with inhibition of Trpm4 expression by siRNA. (E) Curves showing the average Ca^{2} levels (340/380 ratio) at each time point over a 25 minute time span (n=20). Values are displayed as the mean ± S.E.M. Error bars are shown every 5 minutes and omitted elsewhere for clarity. Data (n=20) are from three independent experiments. (F)

Comparison of control Th2 siRNA (Thy1 siRNA) and Trpm4 Th2 siRNA peak Ca^{2+} levels ($P<.05$). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (G) Comparison of control Th2 (Thy1 siRNA) and Trpm4 Th2 siRNA mean Ca^{2+} levels ($P<.0001$). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (H) Comparison of control Th2 (Thy1 siRNA) and Trpm4 Th2 siRNA oscillation (standard deviation) Ca^{2+} levels ($P<.05$). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (see also Figs. S1–S3 and Videos S1 and S2).

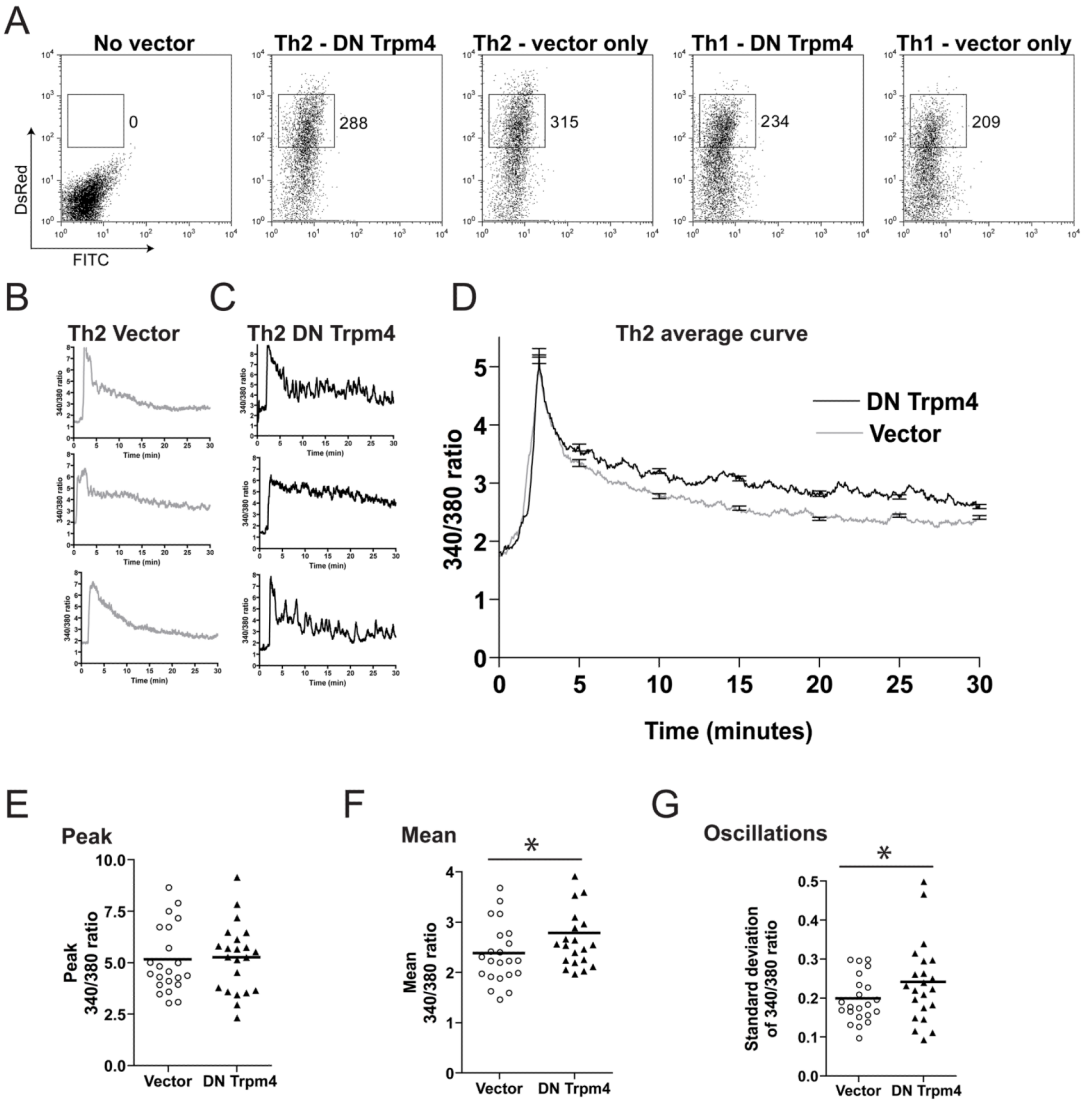

Figure 3. Th2 Cells Transfected with DN Trpm4 Have Increased Mean Ca2+ Levels and oscillations

(A) Histograms showing the levels of DsRed in DN Trpm4 transfected cells. Equivalent levels (MFU) of DsRed cells were taken for each group. (B) Representative Ca2+ profiles of Th2 cells transfected with the vector only. (C) Representative Ca2+ profiles of Th2 cells transfected with DN Trpm4. (D) Curves showing the average Ca2+ levels (340/380 ratio) at each time point over a 30 minute time span (n=20). Values are displayed as the mean ± S.E.M. Error bars are shown every 5 minutes and omitted elsewhere for clarity. Data are from three independent experiments. (E) Comparison of Th2 vector and DN Trpm4 peak Ca2+ levels (NS). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (F) Comparison of Th2 vector and DN Trpm4 mean Ca2+ levels (P<.05). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (G) Comparison of Th2 vector and DN Trpm4 oscillation (standard deviation) Ca2+ levels (P<.05). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments.

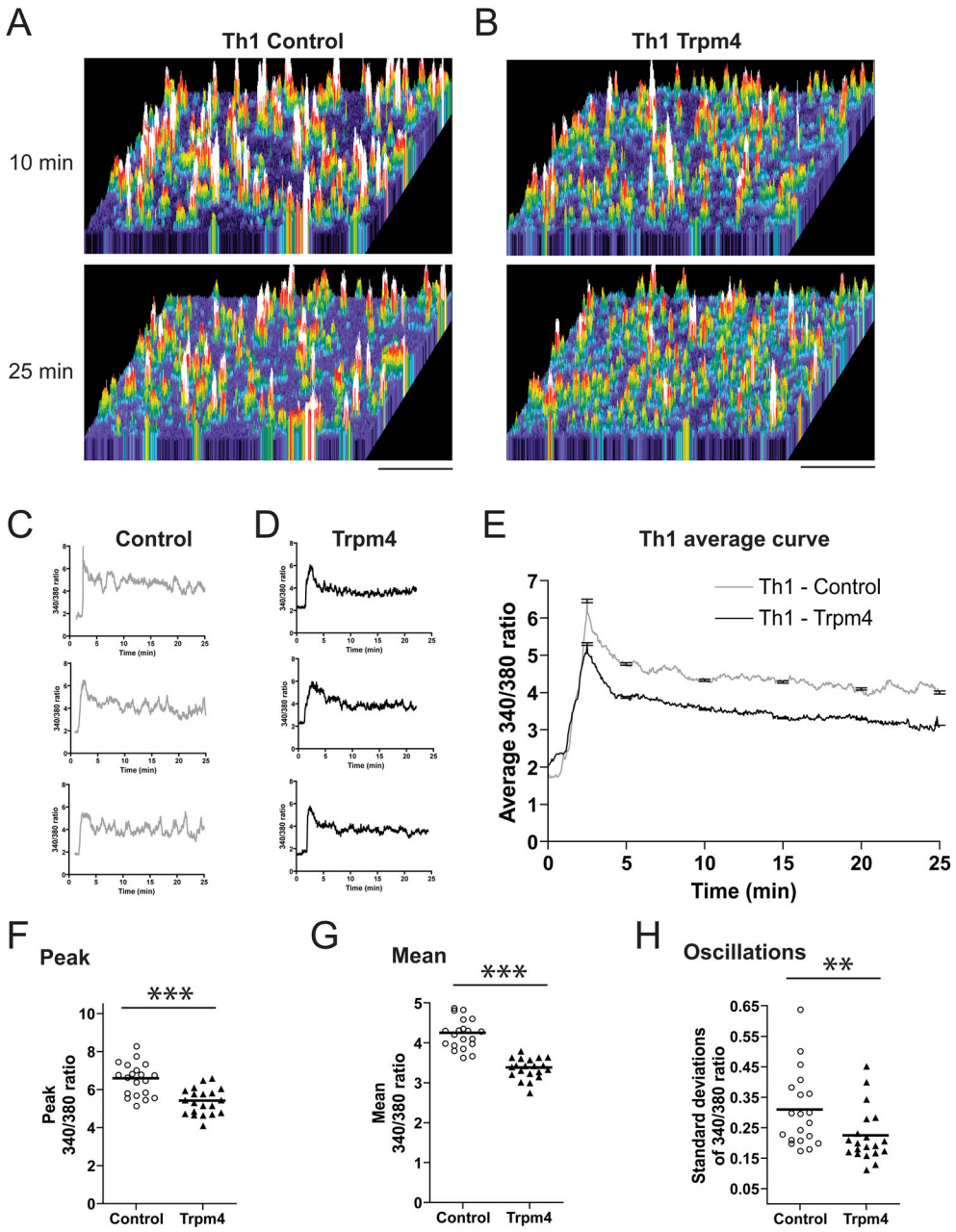

Figure 4. Trpm4 siRNA of Th1 Cells Results in Decreased Peak, Mean, and Oscillatory Ca^{2+} Levels

(A) Th1 control (Thy1 siRNA) intensity profile of single cell Ca^{2+}analysis at 10 minute and 25 minute time points. Scale bar is 100um. (B) Th1 Trpm4 intensity profile of single cell Ca^{2+}analysis at 10 minute and 25 minute time points. Scale bar is 100um. (C) Representative Ca^{2+} profiles of control Th1 cells (Thy1 siRNA). (D) Representative Ca^{2+} profiles of Th1 cells with siRNA for Trpm4. (E) Curves showing the average Ca^{2+} levels (340/380 ratio) at each time point over a 25 minute time span (n=20). Values are displayed as the mean ± S.E.M. Error bars are shown every 5 minutes and omitted elsewhere for clarity. Data (n=20) are from three independent experiments. (F) Comparison of control Th1 (Thy1 siRNA) and Trpm4 Th1 siRNA peak Ca^{2+} levels (P<.0001). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent

experiments. (G) Comparison of control Th1 (Thy1 siRNA) and Trpm4 Th1 siRNA mean Ca^{2+} levels (P<.0001). Values are displayed as scatter plots with a line marking the mean. Data are from three independent experiments. (H) Comparison of control Th1 (Thy1 siRNA) and Trpm4 Th1 siRNA oscillation Ca^{2+} levels (P<.001). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (see also Figs. S4 and S5 and Videos S3 and S4).

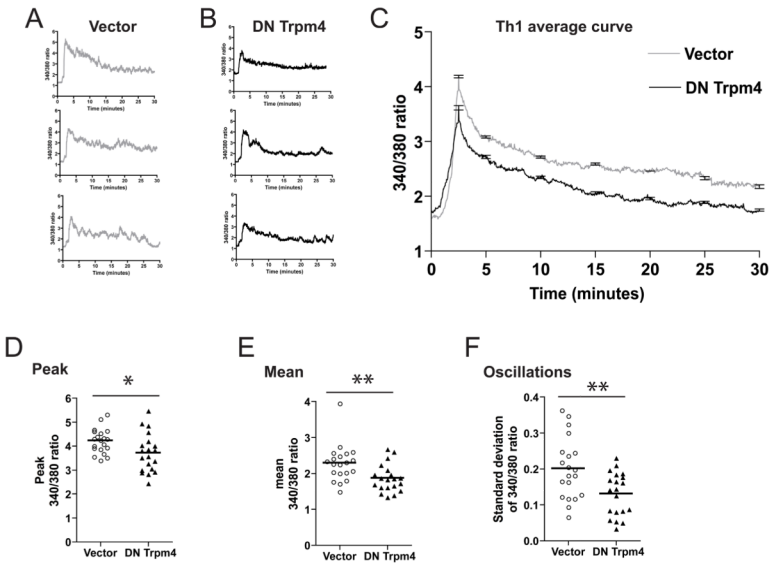

Figure 5. Th1 Cells Transfected with DN Trpm4 Have Decreased Peak and Mean Ca2+ Levels and oscillations

(A) Representative Ca2+ profiles of Th1 cells transfected with the vector only. (B) Representative Ca2+ profiles of Th1 cells transfected with DN Trpm4. (C) Curves showing the average Ca2+ levels (340/380 ratio) at each time point over a 30 minute time span (n=20). Values are displayed as the mean ± S.E.M. Error bars are shown every 5 minutes and omitted elsewhere for clarity. Data are from three independent experiments. (D) Comparison of Th1 vector and DN Trpm4 peak Ca2+ levels (P<.05). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (E) Comparison of Th1 vector and DN Trpm4 mean Ca2+ levels (P<.01). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments. (F) Comparison of Th1 vector and DN Trpm4 oscillation Ca2+ levels (P<.01). Values are displayed as scatter plots with a line marking the mean. Data (n=20) are from three independent experiments.

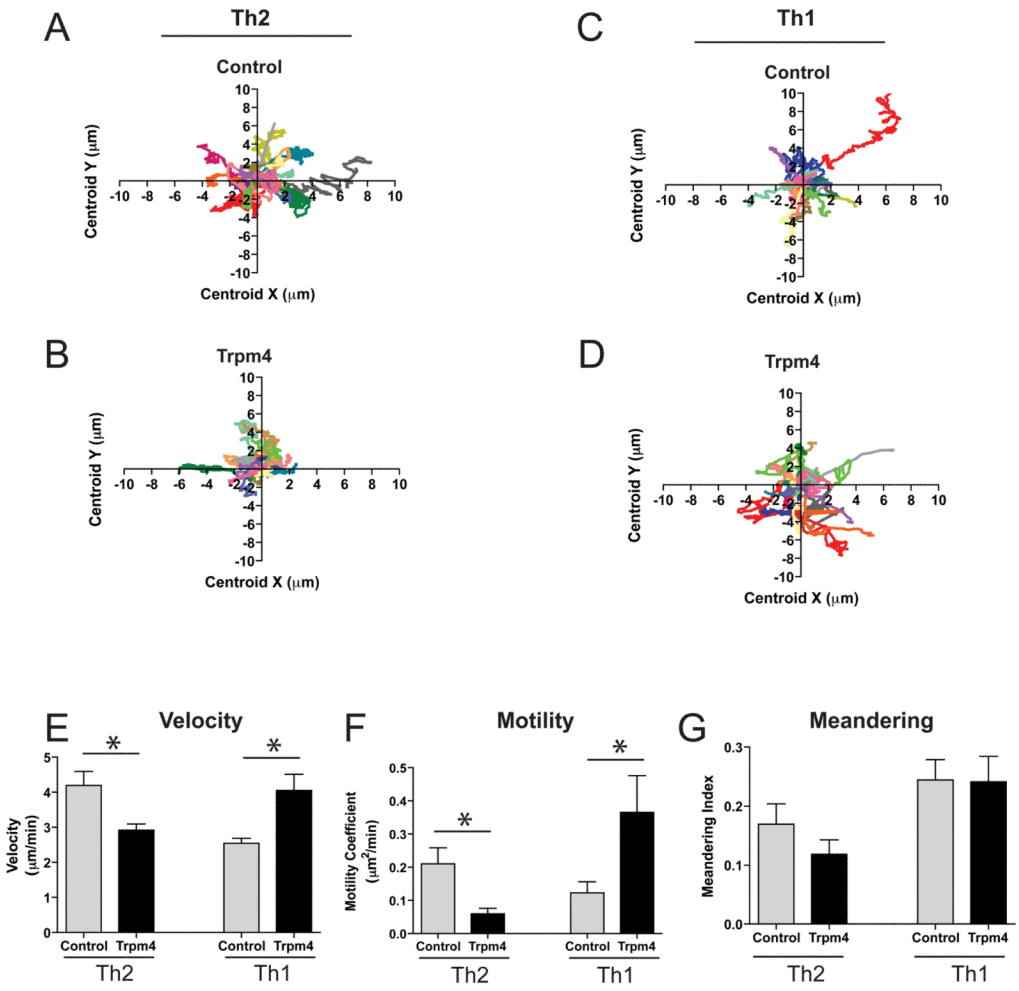

Figure 6. Inhibition of Trpm4 expression in Th2 Cells Results in Decreased Velocity and Motility and the converse in Th1 cells

Flower plots showing (A) Th2 control (Thy1 siRNA) and (B) Th2 Trpm4 siRNA cell displacement tracks. Flower plots showing (C) control Th1 (Thy1 siRNA) and (D) Th1 cells with Trpm4 siRNA displacement tracks. (E) Histogram showing the mean velocities (μm/min) from three separate experiments (n=20; P<05). Values are displayed as the mean ± S.E.M. (F) Histogram showing the motility coefficient (μm^2/min) from three separate experiments (n=20; P<.05). Values are displayed as the mean ± S.E.M. (G) Histogram showing the meandering index from three separate experiments (n=20; NS). Values are displayed as the mean ± S.E.M.

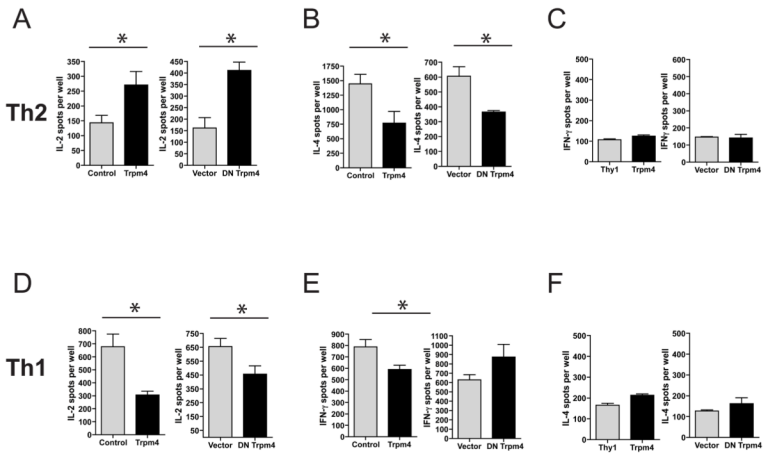

Figure 7. Inhibition of Trpm4 expression alters Th1 and Th2 IL-2, IL-4, and IFN-γ cytokine production

(A) ELISPOT analysis of IL-2 producing cells in Th2 cell culture that had inhibition of Trpm4 expression by the Trpm4 siRNA ($P<.05$) or DN vector ($P<.05$). Values are displayed as the mean ± S.E.M. and are from three independent experiments. (B) ELISPOT analysis of IL-4 producing cells in Th2 cell culture that had inhibition of Trpm4 expression by the Trpm4 siRNA ($P<.05$) or (D) DN vector ($P<.05$). Values are displayed as the mean ± S.E.M. and are from three independent experiments. (C) ELISPOT analysis of IFN-γ producing cells in Th2 cell culture that had inhibition of Trpm4 expression by Trpm4 siRNA (NS) or the DN Trpm4 vector (NS). Values are displayed as the mean ± S.E.M. and from three independent experiments. (D) ELISPOT analysis of IL-2 producing cells in Th1 cell culture that had inhibition of Trpm4 expression by Trpm4 siRNA ($P<.05$) or the DN vector ($P<.05$). Values are displayed as the mean ± S.E.M. and are from three independent experiments. (E) ELISPOT analysis of IFN-γ producing cells in Th1 cell culture that had inhibition of Trpm4 expression by Trpm4 siRNA ($P<.05$) or the DN Trpm4 vector (NS). Values are displayed as the mean ± S.E.M. and from three independent experiments. (F) ELISPOT analysis of IL-4 producing cells in Th1 cell culture that had inhibition of Trpm4 expression by Trpm4 siRNA (NS) or the DN Trpm4 vector (NS). Values are displayed as the mean ± S.E.M. and from three independent experiments.

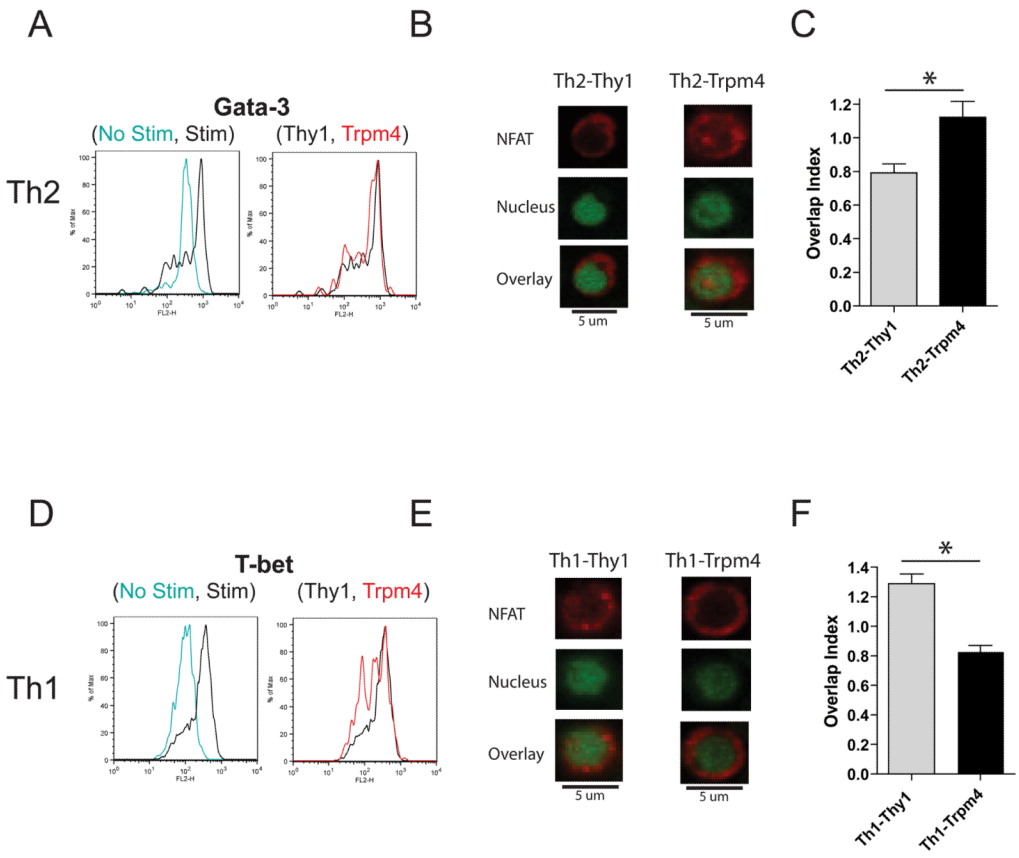

Figure 8. Transcription factor analysis shows NFATc1 localization differences caused by inhibition of Trpm4 expression

(A) Analysis of GATA-3 levels in Th2 cells when Thy1 or Trpm4 are inhibited by siRNA. Figure is representative of three independent experiments. (B) Representative confocal images of NFATc1 (red) and Syto13 nuclear stain (green) in Th2 cells with Thy1 or Trpm4 inhibited by siRNA. Overlays of the NFATc1 and Syto13 are shown at the bottom. (C) Histogram of overlap index in Th2 cells with Thy1 or Trpm4 inhibited by siRNA. Results are significantly different and data (mean ± S.E.M.) is from three independent experiments (n=175). (D) Analysis of T-bet levels in Th1 cells when Thy1 or Trpm4 are inhibited by siRNA. Figure is representative of three independent experiments. (E) Representative confocal images of NFATc1 (red) and Syto13 nuclear stain (green) in Th1 cells with Thy1 or Trpm4 inhibited by siRNA. Overlays of the NFATc1 and Syto13 are shown at the bottom. (F) Histogram of overlap index in Th1 cells with Thy1 or Trpm4 inhibited by siRNA. Results are significantly different and data (mean ± S.E.M.) is from three independent experiments (n=115). (see also Fig. S6)

Table 1
Genes over and under-expressed when Trpm4 inhibited

(A) Genes over and under-expressed when comparing siRNA inhibition of Thy1 or Trpm4 expression. The genes from the Th1 comparison (Th1-Thy1 vs Th1-Trpm4) are listed first followed by the Th2 comparison (Th2-Thy1 vs Th2-Trpm4). Only genes higher than the 2-fold cutoff are shown. Results presented are from one gene chip for each group (two gene chips total). (B) Genes over and under-expressed when comparing inhibition of Trpm4 expression using a DN vector. The genes from the 12- hour Th1 comparison (Th1-Vector vs Th1-DN) are listed first followed by the Th1 4-hour comparison. The genes from the 12-hour Th2 comparison (Th2-Vector vs Th2-DN) are listed next followed by the Th2 4-hour comparison. Only genes higher than the 2-fold cutoff are shown. Results presented are from one gene chip for each group (four gene chips total).

Probe set	Gene	Accession	Fold change
A. siRNA gene chips			
Th1-Thy1 vs Th1-Trpm4			
1421906_at	peroxisome proliferator activated receptor binding protein	AF332074	3.63
1423135_at	thymus cell antigen 1, theta	AV028402	2.65
Th2-Thy1 vs Th2-Trpm4			
1423135_at	thymus cell antigen 1, theta	AV028402	4.66
B. Dominant negative gene chips			
Th1-Vector vs Th1-DN (12 hour)			
1435549_at	transient receptor potential cation channel, subfamily M, member 4	BI685685	6.85
1418739_at	serum/glucocorticoid regulated kinase 2	NM_013731	−8.85
Th1-Vector vs Th1-DN (4 hour)			
1425137_a_at	histocompatibility 2, D region locus 1	BC011215	−2.81
1444411_at	Adult male corpora quadrigemina cDNA, RIKEN full-length enriched library	B230312F19	−2.81
1448164_at	kelch domain containing 3	NM_027910	−4.43
1428505_at	RIKEN cDNA 2310015N07 gene	AK009370	−4.76
1437264_at	cDNA sequence BC051142	AV278321	−6.17
1456496_at	death-associated kinase 3	AW050029	−10.52
Th2-Vector vs Th2-DN (12 hour)			
1416774_at	wee 1 homolog (S. pombe)	NM_009516	−2.34
1451768_a_at	solute carrier family 20, member 2	AF196476	−2.79
1454920_at	ubiquitin-like, containing PHD and RING finger domains 2	BQ266387	−4.99
Th2-Vector vs Th2-DN (4 hour)			
1428859_at	polyamine oxidase	AK010469	7.01
1459363_at	spinocerebellar ataxia 2 homolog (human)	AV318787	2.79
1433639_at	RIKEN cDNA 5730593F17 gene	AW548096	2.41
1439415_x_at	ribosomal protein S21	AV151252	2.33

1452798_s_at	RIKEN cDNA 2310009E07 gene	AK009250	2.22
1450550_at	interleukin 5	NM_010558	−2.97
1427351_s_at	immunoglobulin heavy chain 6 (heavy chain of IgM)	BB226392	−2.23
1450334_at	interleukin 21	NM_021782	−2.12
1421227_at	granzyme E	NM_010373	−6.44