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Trpm4 Differentially Regulates Th1 and Th2 Function by Altering Calcium Signaling and NFAT Localization

K. Scott Weber,* Kai Hildner,† Kenneth M. Murphy,*‡ and Paul M. Allen*

Th 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 with 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 in Th cells and plays a distinctive role in T cell function by differentially regulating Ca\(^{2+}\) signaling and NFATc1 localization. The Journal of Immunology, 2010, 185: 2836–2846.

To test the hypothesis that Trpm4 regulates the unique intracellular Ca\(^{2+}\) signaling in Th cells, we inhibited Trpm4 expression levels using either Trpm4 small interfering RNA (siRNA) or a Trpm4 dominant-negative (DN) mutant. The Trpm4 mutant is missing the first 177 aa at the N terminus and has been shown to successfully inhibit Trpm4 in Jurkat cells in a DN manner (13). Inhibition of Trpm4 expression in Th2 cells resulted in an increase in Ca\(^{2+}\) influx and oscillations compared with controls, whereas the opposite was seen with Th1 cells. Th2 cells with Trpm4 expression inhibited were less motile when compared with 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 factor 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, Auburn, CA). CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and spleen cells, containing the endogenous Hb6-76 epitope that stimulates 2.102 cells, were isolated and irradiated (2000 rad) for use as APCs. All mice used in this study were between 5 and 10 wk of age. The 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 Abs described below. Primary T cells were cultivated in media containing IMDM supplemented with 2 mM GlutaMAX (Invitrogen Life Technologies, Carlsbad, CA), 5 × 10^{-7} M 2-ME, 10% fetal cell serum, and 50 μg/ml gentamicin.

T cell polarization

T cells were polarized to Th1 or Th2 cells in vitro by the addition of the following: 1) 10 ng/ml IL-12 (R&D Systems, Minneapolis, MN) and 10 μg/ml anti-IL-4 mAb (clone 11B1) for Th1 cell polarization; 2) 10 ng/ml IL-4 (R&D Systems) and anti-IL-12 mAb (TOSH hybridoma supernatant (19)) for Th2 cell polarization. IL-2 (100 U) was added to the media 48 h after initial stimulation, and T cell cultures were split 1:2. Cells were restimulated after 7 d in culture, and the same cytokines and Abs described above were added again. The polarized T cells were used on day 14.

Constructs

The DN 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 (20). In brief, the Platinum-E packaging cell line (a gift of T. Kitamura, University of Tokyo, Japan) was transfected with 30 μg retroviral construct DNA with Lipofectamine 2000 (Invitrogen), and viral supernatant was collected 48 h after transfection. For T cell activation, 3–5 × 10^5 T cells were stimulated with 6.5 × 10^5 irradiated B6.K splenocytes loaded with 10 μM Hb64–76. 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 (100 U) was added to the media 48 h after initial stimulation, and T cell cultures were split 1:2. Cells were restimulated after 7 d in culture, and the same cytokines and Abs described above were added again. The polarized T cells were used on day 14.

Ca^{2+} imaging

Ca^{2+} imaging was performed as previously published (10). In brief, immediately before imaging, polarized T cells were incubated with 1 μM Fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in Ringers imaging solution (125 mM NaCl, 10 mM KCl, 5 mM HEPES, 5 mM MgCl₂, 1 mM MgCl₂, 2 mM CaCl₂), washed, and then incubated in Ringers solution for another 30 min at 37°C. After washing, 100,000 fura 2-loaded T cells were pipetted onto 100,000 adherent APCs (Hb7-E²) that had been loaded with 1 μM Hb64–76 overnight. The Hb64–76 peptide was synthesized using standard Fimoc chemistry, purified by reverse-phase HPLC, and confirmed using MALDI mass spectrometry, as has been previously described (21). The location of the APCs and T cells was monitored by visualizing the cells with transmitted light as well as DsRed every 3 s. All imaging was done in 8-chamber coverglass slides (Lab-Tek; Nalgene Nunc International, Rochester, NY).

Cytokine measurements

T cell cytokine expression was assessed by ELISPOT. T cells were stimulated for 24 h with irradiated splenocytes or Hb7-E² cells. ELISPOT measurement of cytokine levels was performed by 24-h T cell stimulation with at least three replicates in 96-well Multiscreen Immobilon-P plates (Millipore, Bedford, MA) that had been coated overnight with anti-IL-4 (clone 11B11; eBioscience), anti-IFN-γ (clone R4-6A2; eBioscience, San Diego, CA), or anti-IL-2 (clone JES5-5H4; BioXCell, West Lebanon, NH), and labeled with biotinylated anti-IL-4 (BV6-2G2; eBioscience), anti-IFN-γ (XMG12; eBioscience), or anti-IL-2 (JES5-5H4; BioLegend, San Diego, CA) and streptavidin-conjugated HRP (Southern Biotechnology Associates, Birmingham, AL). Plates were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich, St. Louis, MO) and scanned on a CTL Immunospot reader. Data was analyzed using CTL Immunospot 4.0 (Cellular Technology, Shaker Heights, OH).

Gene expression analysis

For microarray analysis, RNA was isolated using RNeasy kits (Qiagen, Valencia, CA), and gene expression analysis was performed using mouse expression 430 2.0 arrays (Affymetrix, Santa Clara, CA). Data were normalized and expression values were modeled using DNA-Chip Analyzer (www.dchip.org) (22). 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 manufacturer’s instructions. The SYBR Green PCR master mix and an ABI7000 machine (Applied Biosystems, Foster City, CA) 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.

NFAT1 nuclear localization

T cells were washed and incubated with 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 H7 cells (1 μM Hb64–76) for 20 min at 37°C in 8-chamber coverglass slides (Lab-Tek; Nalgene Nunc International). Cells were fixed with 100 μl BD Cytofix (BD Biosciences, San Jose, CA) for 20 min at room temperature and washed twice with PBS. Cells were permeablized for 10 min at room temperature in 2% Triton X-100, and then cells were stained (1:100) for 1 h at room temperature in 100 μl permeabilization buffer with 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 CaCl₂, 2 mM MgCl₂) and viewed using the Amaxa mouse T cell Nucleofector kit (Lonza, Walkersville, MD). Briefly, six million T cells were resuspended in 100 μl Nucleofector solution and placed in a cuvette with 4 μg 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 h and then washed and grown in IMDM. Electroporated cells were used in experiments 48 h after the siRNA transfection.

Data analysis

Fluorescent images were analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA) and the 340:380 ratio is displayed on a pseudocolor scale,
Results

**Trpm4 expression levels are higher in Th2 cells relative to Th1**

Th 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 with Th1 cells. Differences in Th 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 DN form of Trpm4 is missing the first 177 aa at the N terminus (13). Trpm4 requires multimerization to form functional channels, and this DN Trpm4 mutant has been shown to success-fully inhibit Trpm4 in Jurkats (13). We expressed the DN mutant using the pMI-DsRed retroviral vector, allowing us to sort a cell surface protein not involved with calcium influx and its levels and oscillations were all significantly higher than controls (Fig. 1E, 1F).

APCs (L cells transfected with I-E\(_k\) and ICAM genes) that had been pulsed with 1 \(\mu\)M HB64–76 peptide overnight (Fig. 2). Ca\(^{2+}\) influx was increased in the Trpm4 siRNA cells compared with control Th2 cells (Fig. 2A, 2B, Supplemental Videos 1, 2).

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 (Figs. 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–H).

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...
FIGURE 2. Trpm4 siRNA of Th2 cells results in increased peak, mean, and oscillatory Ca²⁺ levels. A, Th2 control (Thy1 siRNA) intensity profile of single-cell Ca²⁺ analysis at 10- and 25-min time points. Scale bar, 100 μm. B, Th2 intensity profile (Trpm4 siRNA) of single-cell Ca²⁺ analysis at 10- and 25-min time points. Scale bar, 100 μm. C, Representative Ca²⁺ profiles of control Th2 cells (Thy1 siRNA). D, Representative Ca²⁺ profiles of Th2 cells with inhibition of Trpm4 expression by siRNA. E, Curves showing the average Ca²⁺ levels (340:380 ratio) at each time point over a 25-min time span (n = 20). Values are displayed as the mean ± SEM. Error bars are shown every 5 min 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 Ca²⁺ levels (p < 0.05). 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 Ca²⁺ levels (p < 0.0001). Values are displayed as scatter plots with a line marking the mean. Data (n = 20) are from three independent experiments (see also Supplemental Videos 1, 2).
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 \(\sim 3\)-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 with control cells (Fig. 4A,4B, Supplemental Videos 3, 4). 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 with the control (Fig. 4F–H).

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 has 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 Th 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 that cells have with Ag 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 with Th1 cells (10). Our analysis showed that there is a decrease in Th2 cell track length when Trpm4

**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- and 25-min time points. Scale bar, 100 μm. B, Th1 Trpm4 intensity profile of single-cell Ca\(^{2+}\) analysis at 10- and 25-min time points. Scale bar, 100 μm. 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-min time span (n = 20). Values are displayed as the mean ± SEM. Error bars are shown every 5 min 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 < 0.0001). 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 < 0.0001). 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 < 0.0001). Values are displayed as scatter plots with a line marking the mean. Data (n = 20) are from three independent experiments (see also Supplemental Videos 3, 4).
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 with the control group (Fig. 6C, 6D). Further analysis showed that inhibition of Trpm4 expression in Th1 cells caused significant differences in velocity and motility.

**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-min time span (n = 20). Values are displayed as the mean ± SEM. Error bars are shown every 5 min and omitted elsewhere for clarity. Data are from three independent experiments. D, Comparison of Th1 vector and DN Trpm4 peak Ca²⁺ levels (p < 0.05). 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 < 0.01). 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 < 0.01). 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 cell 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 < 0.05). Values are displayed as the mean ± SEM. F, Histogram showing the motility coefficient (µm²/min) from three separate experiments (n = 20; p < 0.05). Values are displayed as the mean ± SEM. G, Histogram showing the meandering index from three separate experiments (n = 20; NS). Values are displayed as the mean ± SEM.
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 affect 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 Th 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 whether inhibiting Trpm4 expression with siRNA resulted in specific transcriptional regulation of a gene or set of genes, we performed gene chip analysis. Comparison of the Th1 siRNA gene chips (Thy1 versus Trpm4 siRNA) at a 2-fold cutoff level only revealed one overexpressed gene besides the expected difference in Thy1 (Table I). 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 gene chips at a 2-fold cutoff level did not result in any overexpressed genes besides the expected difference in Thy1 (Table I).

We also examined the effect of the DN Trpm4 on gene expression at both 4- and 12-h stimulation time points. At the 2-fold cutoff level for Th1 cells, there were seven over- or underexpressed genes besides Trpm4 (Table I). None of the genes had a direct connection with Ca\(^{2+}\) regulation. For Th2 cells, there were 12 over- or underexpressed genes when using a 2-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 2-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 Th cell function and development, we determined to examine their protein expression levels when Trpm4 expression was inhibited. Th cell polarization changes in T-bet and GATA-3 levels result in changes in chromatin structure at the IFN-γ and IL-4 genes (24). Comparison of GATA-3 levels in Th2 cells were the same when comparing Thy1 and Trpm4 siRNA samples (Fig. 8A). Th1 T-bet levels also showed no difference between Thy1 and Trpm4 siRNA samples (Fig. 8D). Thus, the Th cell transcription factors GATA-3 and T-bet showed no detectable differences when Trpm4 expression levels are inhibited and suggest that Trpm4 inhibition is not causing a lack of polarization to Th subtypes.

**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 < 0.05) or DN vector (p < 0.05). Values are displayed as the mean ± SEM 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 < 0.05) or DN vector (p < 0.05). Values are displayed as the mean ± SEM 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 ± SEM 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 < 0.05) or the DN Trpm4 vector (NS). Values are displayed as the mean ± SEM and 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 (NS) or the DN Trpm4 vector (NS). Values are displayed as the mean ± SEM 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 ± SEM and from three independent experiments.
NFAT localization differentially regulated by Trpm4

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 (25). NFAT proteins are known to be regulated by Ca^{2+} and can bind to both the IFN-γ or GATA-3 silencing of the Th1 or Th2 locus, and then NFAT predominantly binds to either the IFN-γ or IL-4 promoters in early stages of naive T cell activation. Th cell differentiation results in T-cell signaling and can bind to both the IFN-γ or IL-4 promoters in Th1 and Th2 cells (24, 26). To determine whether the differences in cytokine production levels were due to changes in NFAT, we examined Th1 and Th2 NFAT nuclear localization.

In Th2 cells, comparison of the Thy1 siRNA group with Trpm4 siRNA group revealed a significant increase in NFAT localization differentially regulated by Trpm4 and was initially identified as an inducible factor that could bind the IL-2 promoter (25). NFAT proteins are known to be regulated by Ca^{2+} and can bind to both the IFN-γ or IL-4 promoters in early stages of naive T cell activation. Th cell differentiation results in T-cell signaling and can bind to both the IFN-γ or IL-4 promoters in Th1 and Th2 cells (24, 26). To determine whether the differences in cytokine production levels were due to changes in NFAT, we examined Th1 and Th2 NFAT localization.

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Discussion

Activation of Th1 and Th2 cells with identical peptide and APCs 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 (27). 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 Th cell functional changes in motility and cytokine production (IL-2, IL-4, and IFN-γ).

Two reported explanations for the differences normally seen in Th cell Ca^{2+} influx patterns are 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 have 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 $K_o$ 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. Th 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 with Th1 cells (8–11). Using microarray, RT-PCR, and Western blot, we discovered that Trpm4 was expressed higher in Th2 cells compared with Th1 cells, stimulating our interest in the role Trpm4 plays in Ca\(^{2+}\) influx in polarized Th cells stimulated by APCs.

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 Th subsets, increased functional $K_o$, 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 that would increase the Ca\(^{2+}\) differences between subsets, but the evidence for a Th2 voltage-gated Ca\(^{2+}\) channel remains controversial (1, 28, 29).

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, 30, 31). In dendritic cells, Trpm4 has been shown to prevent high levels of Ca\(^{2+}\) and is essential for proper dendritic cell migration (17). Consistent with these studies, we found inhibition of Trpm4 expression resulted in an increase in Th1 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 dendritic cell migration (17) 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 (32). 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 (33–36). 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 (26, 37–39). Of interest in this study, we have found that the inhibition of Trpm4 expression results in altered Ca\(^{2+}\) influx patterns and nuclear localization of NFATc1 in Th1 cells and significant alterations in the levels of IL-2 and Th 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 activa-

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**FIGURE 8.** Transcript 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 is 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 ± SEM) are from three independent experiments. D, 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. E, Histogram of overlap index in Th1 cells with Thy1 or Trpm4 inhibited by siRNA. Results are significantly different, and data (mean ± SEM) are from three independent experiments (n = 115).

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- **A**
  - Gata-3
  - Th2
  - No Stim, Stm
  - (Thy1, Trpm4)

- **B**
  - NFAT
  - Nuclear
  - Overlay
  - 3 µm
  - 1 µm

- **C**
  - Histogram of overlap index in Th2 cells with Thy1 or Trpm4 inhibited by siRNA. Results are significantly different, and data (mean ± SEM) are from three independent experiments (n = 175).

- **D**
  - T-bet
  - Th1
  - No Stim, Stm
  - (Thy1, Trpm4)

- **E**
  - NFAT
  - Nuclear
  - Overlay
  - 3 µm
  - 1 µm

- **F**
  - Histogram of overlap index in Th1 cells with Thy1 or Trpm4 inhibited by siRNA. Results are significantly different, and data (mean ± SEM) are from three independent experiments (n = 115).
tion and NFAT-dependent IL-2 production are well studied and have been shown to be closely tied to the shape and intensity of the Ca" influx (40). Changes in IL-2 levels influence T cell proliferation, memory cell formation, and regulation of inflammatory immune response (41). 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, in this study we report for the first time a divergent role for Trpm4 regulation of Th2 and Th1 function by altering Ca" signaling and NFATc1 nuclear localization. We found that Trpm4 expression levels were higher in Th2 cells compared with Th1 cells. Two independent methods, siRNA and a DN construct, were used to inhibit Trpm4 expression. We found that inhibition of Trpm4 expression resulted in increases in Ca" 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" 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 Th cell Ca" influx and NFATc1 localization by Trpm4 provide a novel means in which subsequent biological functions are controlled.

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Disclosures
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