Cavβ1 regulates T cell expansion and apoptosis independently of voltage-gated Ca\(^{2+}\) channel function

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TCR stimulation triggers Ca\(^{2+}\) signals that are critical for T cell function and immunity. Several pore-forming \(\alpha\) and auxiliary \(\beta\) subunits of voltage-gated Ca\(^{2+}\) channels (VGCC) were reported in T cells, but their mechanism of activation remains elusive and their contribution to Ca\(^{2+}\) signaling in T cells is controversial. We here identify Cavβ1, encoded by Cacnb1, as a regulator of T cell function. Cacnb1 deletion enhances apoptosis and impairs the clonal expansion of T cells after lymphocytic choriomeningitis virus (LCMV) infection. By contrast, Cacnb1 is dispensable for T cell proliferation, cytokine production and Ca\(^{2+}\) signaling. Using patch clamp electrophysiology and Ca\(^{2+}\) recordings, we are unable to detect voltage-gated Ca\(^{2+}\) currents or Ca\(^{2+}\) influx in human and mouse T cells upon depolarization with or without prior TCR stimulation. mRNAs of several VGCC \(\alpha\) subunits are detectable in human (Ca\(_{v3.3}\), Ca\(_{v3.2}\)) and mouse (Ca\(_{v2.1}\)) T cells, but they lack transcription of many 5’ exons, likely resulting in N-terminally truncated and non-functional proteins. Our findings demonstrate that although Cavβ1 regulates T cell function, these effects are independent of VGCC channel activity.
Chances in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), are essential for signal transduction in all eukaryotic cells including T lymphocytes. The best characterized Ca$^{2+}$ influx pathway in T cells is store-operated Ca$^{2+}$ entry (SOCE) mediated by Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels encoded by ORAI1 and its homologue ORAI2. TCR stimulation results in the generation of the second messenger inositol 1,4,5-trisphosphate (IP$_3$), the opening of IP$_3$ receptor channels and Ca$^{2+}$ release from the endoplasmic reticulum (ER). Ca$^{2+}$ influx from the ER causes the activation of STIM1 resulting in its opening of CRAC channels. CRAC channels are critical for T cell function and immunity to infection as evidenced by the immunodeficiency of CRAC-deficient human patients and mice.

Other Ca$^{2+}$ channels that have been proposed to mediate Ca$^{2+}$ influx in T cells are voltage-gated Ca$^{2+}$-channels (VGCCs). They are critical for Ca$^{2+}$ signaling in excitable cells such as neurons, cardiomyocytes, skeletal muscle and secretory cells, but their function in T cells is less well established. VGCCs are divided into three groups: high-voltage activated (L-Type: Cav1.1, Cav1.2, Cav1.3, Cav1.4), N-Type: Cav2.2; P/Q-Type: Cav2.1), intermediate-voltage activated (R-Type: Cav2.3) and low-voltage activated (T-Type: Cav3.1, Cav3.2, Cav3.3) channels. VGCCs are composed of a Ca$^{2+}$ conducting, pore-forming α1 subunit and several auxiliary β, δ, ε- and γ-subunits. The α1 subunits are composed of four domains (I–IV), each consisting of 6 α-helical transmembrane domains (S1–S6). S1–S4 form the voltage sensing domain (VSD) with S4 containing positively charged amino acids that sense changes in membrane potential, while the S5–S6 subunits constitute the ion conduction pore and selectivity filter. Each VGCC has one β-subunit, which binds via its α-domain to the cytosolic α-interacting domain (AID) in the linker region between domains I and II of the α1 subunit. The four β subunit homologues (β1–β4) increase the plasma membrane expression of α1 subunits, enhance Ca$^{2+}$ currents and modulate the voltage-dependence and kinetics of activation and inactivation of VGCCs. The α1 subunit further binds to the extracellular αδ-subunit and the transmembrane γ-subunit consisting of four transmembrane domains. Mutations in VGCCs and altered Ca$^{2+}$ influx are associated with a plethora of human diseases including cardiac arrhythmias and psychiatric diseases (Cav1.2), autism spectrum disorder and primary aldosteronism (Cav1.3), various X-linked retinal disorders (Cav1.4), familial hemiplegic migraine (Cav2.1), epilepsy (Cav1.3, Cav2.1, Cav3.2) and several forms of ataxia (Cav2.1, Cav3.1)9–11. Similarly, deletion or mutation of α1 and auxiliary VGCC subunits in mice has been reported to cause a large spectrum of neurological, cardiovascular, musculoskeletal and endocrinological phenotypes. Ca$^{2+}$ channel blockers targeting VGCCs such as nimodipine, verapamil and diltiazem are in wide clinical use for the treatment of arterial hypertension. A common denominator of human diseases associated with VGCC mutations, phenotypes of genetically altered mice and the effects of Ca$^{2+}$ channel blockers is that they originate from the altered function of excitable cell types such as neurons, cardiomyocytes or secretory cells including pancreatic β cells or adrenal chromaffin cells.

Several studies have reported that VGCCs are modulating immune responses and Ca$^{2+}$ signaling in T cells by using Ca$^{2+}$ channel blockers, RNA interference (RNAi) and genetic deletion of various α1 and β subunits in mice. RNAi-mediated deletion of Cav1.2 and Cav1.3, or β subunits with antisense oligonucleotides, showed reduced TCR-induced Ca$^{2+}$ influx, cytokine production and experimental asthma in CD4+ T cells polarized into Th helper 2 (Th2) cells. T cells from Cacna1f−/− mice lacking Cav1.4, which is highly expressed in the retina, had reduced Ca$^{2+}$ influx and Ba$^{2+}$ currents in T cells and showed a defect in the function, development and survival of naïve T cells and in T cell responses to intracellular pathogens in vivo. Genetic deletion of the T-type VGCC Cav3.1 in mice had no effect on TCR-induced Ca$^{2+}$ influx in T cells despite reduced low-voltage activated Ca$^{2+}$ currents. However, Cav3.1-deficient mice were protected from experimental autoimmune encephalomyelitis (EAE), which was associated with reduced numbers of IFN-γ and GM-CSF producing T cells in vivo and defects in Th17 cell function in vitro including Ca$^{2+}$ influx, NFAT activation, and the expression of RORγt and IL-17A. In addition, several studies have implicated β subunits of VGCCs in T cell function. For example, T cell-specific deletion of Cavβ2 resulted in a severe defect in T cell development due to impaired thymocyte proliferation and survival. T cells from Cacnb2−/− (Cavβ2 knockout) mice had moderately reduced TCR-induced Ca$^{2+}$ influx, which was associated with loss of Cav1.2 and Cav1.3 protein expression. Similarly, T cells of lethargic mice with a spontaneously occurring mutation in Cacnb4 (encoding Cavβ4) exhibited spastic and athetoid-like behavior with reduced TCR-induced Ca$^{2+}$ influx was moderately reduced in T cells of Cavβ4 mutant mice and those from mice with targeted deletion of Cavβ3. T cells of Cavβ3−/− mice had moderately reduced TCR-induced Ca$^{2+}$ influx and the survival of naive CD8+ T cells was profoundly impaired due to altered expression of pro- and antiapoptotic genes. Cavβ3 deficiency was associated with loss of Cav1.4 protein expression, suggesting that Cavβ3 may regulate Ca$^{2+}$ influx in T cells through Cav1.2. Collectively, these studies suggest that VGCCs contribute to T cell development and function, potentially by regulating TCR-stimulated Ca$^{2+}$ signaling. VGCC as Ca$^{2+}$ channels in T cells, however, are not universally accepted, and biophysical evidence of VGCC currents in T cells is limited. Given the non-excitable nature of T cells it also remains unclear how VGCCs are activated in the context of T cell activation.

In this study, we identify Cacnb1 (Cavβ1) as a regulator of T cell function. Although Cavβ1 is well-studied in skeletal muscle, where it modulates excitation/contraction coupling, its function in T cells has not been reported. Using a pooled shRNA screen to identify ion channels that regulate T cell responses to viral infection in vivo, we find that deletion of Cacnb1 impairs the clonal expansion of antigen specific T cells after viral infection in vivo by enhancing T cell apoptosis. Cavβ1 deletion does not affect TCR-induced Ca$^{2+}$ signaling and production of Ca$^{2+}$-regulated cytokines, suggesting that its function in T cells differs from its canonical one in excitable cells modulating the function of VGCCs. Indeed, a detailed search for voltage-gated Ca$^{2+}$ currents and Ca$^{2+}$ signals in human and mouse T cells fails to provide evidence for the existence of functional VGCCs in T cells. While mRNAs of several VGCC α1 subunits are detectable in T cells by RNA-Seq (Cav3.3, Cav3.2 and Cav2.1), these transcripts are incomplete, and lack expression of multiple 5’ exons that encode the first two (of four) Ca α domains. We conclude that full-length transcripts of α1 subunits of VGCCs are not expressed in T cells, providing an explanation for the absence of VGCC currents and Ca$^{2+}$ influx upon depolarization in T cells.

Results

shRNA screen in vivo identifies Cacnb1 as a VGCC subunit required for clonal expansion of T cells during LCMV infection. To identify ion channels and transporters (ICTs) that regulate T cell function and T cell-mediated immunity during viral infection in vivo, we generated a library of 658 ICTs and regulatory factors, of which 602 ICTs were annotated in both mouse and human genomes. These ICTs were analyzed for their mRNA
expression levels in immune cells using the Immunological Genome Project (ImmGen)\(^27\) and Fantom\(^28,29\) databases, respectively (Supplementary Fig. 1A). We identified 154 ICTs that are expressed at least twofold above the population average in both mouse and human CD4\(^+\) T cells (Supplementary Fig. 1B, C). Similar analyses were conducted in 11 other immune cell populations, resulting in a total of 223 ICTs with >2-fold above average expression across all cell types (Supplementary Fig. 1B). We used this information to generate a customized, pooled shRNA ICT library targeting 223 mouse ICT genes. To delete ICTs, CD4\(^+\) CD45.1\(^+\) T cells were isolated from SMARTA mice that express a transgenic TCR specific for the LCMV GP\(^{51,80}\) epitope\(^30\) and transduced with the shRNA library. shRNA-transduced (Ametrine\(^-\)) T cells were sorted and injected into CD45.2\(^+\) congenic WT host mice, which were next infected with LCMV\(^{\text{ARM}}\) (LCMV\(^{\text{ARM}},\) Fig. 1A). LCMV\(^{\text{ARM}}\) causes an acute viral infection and a well-characterized CD4\(^+\) T cell response\(^31\). 7 days later, donor T cells were isolated from the spleens of host mice, enriched for CD4\(^+\) T cells and injected into SMARTA mice were transduced with a pooled shRNA library targeting 223 ICTs (1342 shRNAs including control shRNAs), enriched by cell sorting for transduced individual healthy donors (HD) and a patient with a STIM1 null mutation (STIM1null). RNA-Seq for mouse T cells and for human and mouse heart, skeletal muscle, brain, frontal cortex, and retina were extracted from GEO datasets (Supplementary Table 2).
cell signaling, survival and function (CD3ε, CD4, Lck, Rpa3, Zap70, Btk). By contrast, suppression of Prdm1, which encodes BLIMP1 and inhibits the differentiation of follicular T helper (Tfh) cells after viral infection, resulted in significant enrichment (P < 0.05, Log2-FC > 2) of T cells (Fig. 1B, and Supplementary Fig. 1D).

Among the ICTs whose shRNA-mediated knockdown significantly depleted T cells in vivo was Cacnb1, which encodes the auxiliary Cavβ1 subunit of VGCCs (Fig. 1B, and Supplementary Fig. 1D). Given the importance of β subunits for VGCC function in excitable cells and the reported function of β2, β3 and β4 in T cells, we first analyzed the expression levels of all Cavβ subunits in mouse and human T cells using the ImmmGen27 and Fantom 52829 gene expression databases, respectively. Compared to other immune cells, mouse T cells express higher levels of Cavβ (Cacnb1), whereas human T cells express both β1 and β3 (encoded by CACNB1 and CACNB3, Fig. 1C, D). We next analyzed absolute mRNA expression levels of β subunits in mouse and human T cells compared to reference tissues with known VGCC function using our own and published RNA-Seq data. In T cells of wildtype (WT) mice and healthy human donors (HDs), β1 and β3 were the only Cavβ subunits showing robust mRNA levels (Fig. 1E, F). Whereas β1 and β3 mRNA levels in human T cells remained high after TCR stimulation, their expression decreased in mouse CD4+ and CD8+ T cells at 12–24 h after stimulation. Analysis of other auxiliary subunits of VGCCs showed that although γ subunits are generally expressed at very low levels, αδδ is robustly expressed in both human and mouse T cells (Fig. 1E, F). It is noteworthy that expression levels of all four β subunits and α subunits did not markedly differ between human CD4+ and CD8+ T cells (Supplementary Fig. 1E, F). The shRNA screen and expression data suggest that Cavβ1 may have a non-redundant function in T cells during LCMV infection.

Cavβ1 is required for T cell expansion and survival in vitro and in vivo. Because Cavβ1 is highly and selectively expressed in mouse and human T cells compared to other β subunits, and because β2, β3 and β4 were reported to regulate T cell development and function20,23,24, we further investigated the function of Cavβ1 in T cells. To delete Cacnb1 expression in mouse T cells we used two approaches: (1) CRISPR/Cas9 gene editing by retrovirally transducing CD4+ T cells of Cas9-SL-GFP Cd4Cre knock-in mice with small guide (sg) RNAs targeting Cacnb1, and (2) shRNA-mediated knockdown by transducing mouse CD4+ T cells with individual shRNAs targeting Cacnb1. Both approaches achieved ~50–70% reduction of Cavβ1 mRNA and protein levels (Fig. 2A, B and Supplementary Fig. 2A, B). Deletion of Cavβ1 reduced the numbers of transduced (Amt+) CD4+ T cells after TCR stimulation in vitro relative to T cells transduced with control sgRNAs or shRNAs under co-culture conditions (Fig. 2C and Supplementary Fig. 2C). No significant defects in CD4+ T cell proliferation were detectable after Cacnb1 deletion by either sgRNAs or shRNAs (Fig. 2D and Supplementary Fig. 2D). By contrast, we observed a significant increase in apoptosis in Cavβ1-deficient CD4+ T cells compared to control T cells (Fig. 2E and Supplementary Fig. 2E) suggesting that Cavβ1 in T cells contributes to T cell survival.

We next investigated the ability of Cacnb1-deficient T cells to expand after infection of mice with LCMVArm. CD4+ T cells from Cas9-SL-GFP Cd4Cre knock-in mice that had been crossed to SMARTA mice were transduced with sgRNAs against Cacnb1 (Ametrine+) and mixed at a 1:1 ratio with CD4+ T cells transduced with control sgRNAs (GFP+), allowing us to investigate the effects of Cavβ1 deletion in CD4+ T cells compared to mock-transduced T cells in the same host mice. As an additional control, CD4+ T cells were transduced with control sgRNAs encoded by vectors expressing either Ametrine or GFP reporters that were also mixed at a 1:1 ratio (sgCtrlAmet, sgCtrlGFP). Following T cell transfer WT host mice were infected with LCMVArm (Fig. 2F). 7 days post-infection, we observed a significant ~2.4-fold decrease of CD4+ T cells transduced with sgCacnb1 (Amt+) compared to sgCtrl (Amt+) when normalized to sgCtrl (GFP+) transduced T cells (Fig. 2F). Similar observations were made using an orthogonal approach by transducing CD4+ T cells from SMARTA mice with shCacnb1 (Amt+) and shCtrl (GFP+) followed by transfer of T cells at a 1:1 ratio and infection with LCMVArm (Supplementary Fig. 2F). Whereas the ratio of shCtrl (Amt+) to shCtrl (GFP+) transduced CD4+ T cells remained unchanged 7 days after infection, we found a strong, ~sixfold reduction in the ratio of shCacnb1 (Amt+) to shCtrl (GFP+) transduced T cells (Supplementary Fig. 2F). Together, these data indicate that Cavβ1 is required for the clonal expansion of CD4+ T cells in vitro and in vivo after viral infection by regulating T cell survival.

Cavβ1 expression in T cells is dispensable for TCR-mediated Ca2+ influx and cytokine production. The major canonical function of Cavβ subunits is to regulate the function of VGCCs and thereby Ca2+ signaling in excitable cells2. Ca2+ signals are critical for many T cell functions including cell proliferation, survival and cytokine production1,2. Because previous studies had shown that Cavβ3 and β4 subunits regulate Ca2+ influx in T cells and T cell function23,24, we next investigated the effects of Cavβ1 deletion on Ca2+ signaling and T cell function. Following deletion of Cavβ1 by transducing CD4+ T cells with sgCacnb1 or shCacnb1, T cells were stimulated by CD3 crosslinking and analyzed for cytosolic Ca2+ concentrations. Cavβ1 deletion in T cells by either sgRNA or shRNA did not impair TCR-induced Ca2+ influx (Fig. 3A, B and Supplementary Fig. 3A, B). Likewise, SOCE induced by 1 μM ionomycin (to deplete ER Ca2+ stores) was not affected by suppression of Cacnb1 expression. By contrast, deletion of Stim1 to suppress CRAC channel activation strongly suppressed TCR-mediated Ca2+ influx. We next analyzed if the expression of cytokines that are known to be regulated by Ca2+ is impaired in Cacnb1-deficient T cells. Deletion of Cavβ1 in CD4+ T cells by transduction with sgRNAs and shRNAs had no effect on the production of IL-2, TNF and IFN-γ in response to PMA/ionomycin stimulation compared to T cells transduced with control sgRNAs and shRNAs (Fig. 3C, D and Supplementary Fig. 3C, D). By contrast, deletion of STIM1 strongly suppressed the production of all three cytokines. Collectively, these data demonstrate that Cavβ1 is dispensable for TCR-induced Ca2+ signaling and cytokine production in T cells.

Depolarization does not evoke Ca2+ influx or Ca2+ currents in T cells. Whether VGCCs are functional as Ca2+ channels in T cells and regulate Ca2+ signaling has remained controversial. The normal Ca2+ signals in Cavβ1-deficient T cells despite altered T cell function prompted us to investigate whether VGCC function is detectable in human and mouse T cells. To this end, we measured Ca2+ signals in T cells following exposure to high extracellular concentrations of K+. K+ ([K+]o) to depolarize the membrane potential (Vm) as was first demonstrated in lymphocytes by Deutsh et al. and shown to activate VGCCs in excitable cells33, 60 mM and 150 mM [K+]o, were predicted (using the Goldman–Hodgkin–Katz equation) to depolarize the Vm of T cells from ~−55 mV to −24 mV and 0 mV, respectively. Exposure to 60 or 150 mM [K+]o did not induce an increase in intracellular [Ca2+] in mouse (Fig. 4A) or human CD4+ T cells (Fig. 4B). By contrast, depletion of ER Ca2+ stores with ionomycin induced robust SOCE in mouse and human T cells at
Fig. 2 Deletion of Cav1.1 impairs viability of CD4+ T cells and their expansion after viral infection. A mRNA expression of Cacnb1 in CD4+ T cells of LSL-Cas9; Cd4Cre mice transduced with control sgRNA (sgCtrl) and sgRNA targeting Cacnb1. mRNA levels were measured in transduced (Ametrine+) T cells by qPCR at day 3 post-transduction. Rplp2 was used as housekeeping control. sgCacnb1 samples were normalized to sgCtrl. B Representative Western blot (left) and quantification (right) of Cav1.1 protein in CD4+ T cells transduced with sgCtrl or sgCacnb1. After 4-5 days, Cav1.1 was detected using a monoclonal antibody recognizing aa 19-34 in the N-terminus of Cav1.1. Data in (A, B) are the mean ± SEM of n = 3 mice from independent experiments. C-E CD4+ T cells from LSL-Cas9; Cd4Cre mice were transduced with sgCtrl or sgCacnb1 and at day 4 restimulated with anti-CD3 + CD28. C Cell counts shown as the ratio of sgCacnb1/sgCtrl transduced T cells normalized to non-transduced T cells. D Representative flow cytometry plots (left) and quantification (right) of CFSE dilution at 1 and 3 days after re-stimulation. E Representative flow cytometry plots (left) and quantification (right) of apoptosis measured by annexin V staining at 3 days after re-stimulation. Data in (C-E) are the mean ± SEM of n = 6 mice (in C, D) and n = 8 mice (in E). F Adoptive transfer of CD4+ T cells from SMARTA LSL-Cas9; Cd4Cre mice that has been transduced with sgCtrl or sgCacnb1 followed by LCMV Armstrong infection. Transduced donor T cells were mixed at 1:1 ratio before injection. At day 7 post-infection, the ratios of sgCacnb1/sgCtrl T cells (and sgCtrl/sgCtrl) were analyzed. Representative flow cytometry plots (bottom left) and quantification (bottom right) of T cell ratios. Data are the mean ± SEM from n = 3 independent experiments pooled from n = 3 donor SMARTA; LSL-Cas9; Cd4Cre mice and n = 10 WT host mice per group. Statistical analysis was conducted by two-tailed, unpaired Student’s t test. **p < 0.01, ***p < 0.001.

Physiological extracellular [K+]o (4.5 mM), which was suppressed by depolarization of Vm in 60 mM [K+]o (Fig. 4B). This reduction is expected because depolarization of Vm collapses the electrical gradient required for Ca2+ influx through store-operated CRAC channels. To demonstrate that depolarization of Vm by application of high extracellular K+ is able to activate VGCCs, we transfected HEK293 cells, which are not excitable, with the α1 subunit of Cav1.2 together with β, γ and δ subunits and subjected these cells to the same depolarization protocol. Addition of 150 mM extracellular K+ evoked Ca2+ influx in Cav1.2 transfected cells, but not in untransfected cells (Supplementary Fig. 4A). As expected, Ca2+ influx in Cav1.2 expressing cells could be blocked by the Ca2+ channel blocker nimboline (8 μM) (Supplementary Fig. 4B). Moreover, we tested if depolarization of Vm by high extracellular K+ induces voltage-dependent Ca2+ influx in PC12 cells which endogenously express VGCCs34. Exposure of PC12 cells to 150 mM [K+]o induced a robust, transient increase in intracellular Ca2+ levels (Supplementary Fig. 4C).

To more precisely and dynamically control the membrane potential in T cells and to measure voltage-dependent Ca2+ influx and Ca2+ currents in T cells, we investigated VGCC channel function by patch-clamp electrophysiology. Human T cells from healthy donors (HD) were loaded with the Ca2+ sensitive dye Indo-1 to measure [Ca2+]i concentrations and patch-clamped to record VGCC currents. The perforated-patch configuration was chosen to minimize run-down of VGCC currents that commonly occur during whole-cell recordings. Simultaneous measurements of [Ca2+]i and VGCC currents provided two independent ways to measure depolarization-evoked Ca2+ signals. In separate experiments, we measured VGCC currents in the presence of 110 mM Ba2+ as the charge carrier, which conducts through the channel about twofold better than Ca2+ and confirms the added advantage that Ba2+ currents, unlike Ca2+ currents, do not inactivate, thereby optimizing detection of even small VGCC currents in T cells. To activate VGCCs, human T cells of LSL-Cas9; Cd4Cre mice transduced with sgCtrl or sgCacnb1 were subjected to the same depolarization protocol. Addition of 150 mM extracellular K+ evoked Ca2+ influx in Cav1.2 transfected cells, but not in untransfected cells (Supplementary Fig. 4A). As expected, Ca2+ influx in Cav1.2 expressing cells could be blocked by the Ca2+ channel blocker nimboline (8 μM) (Supplementary Fig. 4B). Moreover, we tested if depolarization of Vm by high extracellular K+ induces voltage-dependent Ca2+ influx in PC12 cells which endogenously express VGCCs34. Exposure of PC12 cells to 150 mM [K+]o induced a robust, transient increase in intracellular Ca2+ levels (Supplementary Fig. 4C).
Similarly, applying depolarizing steps in PC12 cells from +2 mM Ca$^{2+}$ transfected with Cav1.2 channels from −evoked robust voltage-gated Ca$^{2+}$ through CRAC channels (assessed by integrating the Ca$^{2+}$ currents, we analyzed voltage-gated Ca$^{2+}$ sensitivity of our recording system. To further exclude the contour plots (C) and quantification (D) of IL-2$, TNF$ and IFN-γ$ CD4$+ T cells. Data in (A, B, D) are the mean ± SEM of n = 7 independent experiments performed in duplicates. Statistical analysis by two-tailed, unpaired Student’s t test. **p < 0.01, ***p < 0.001.

−100 mV delivered every 1 s. Under these conditions we observed a robust rise in [Ca$^{2+}$], that peaked at ~500 nM (Fig. 4F, H). This rise in [Ca$^{2+}$], was paralleled by Ca$^{2+}$ cur- rents with an inwardly rectifying current-voltage relationship typical of CRAC channels (Fig. 4G, H). In these latter experiments, a 6 nM rise in [Ca$^{2+}$], immediately following readi- tion of extracellular Ca$^{2+}$ could be detected with the 170 I Ca$^{2+}$ influx that flowed through CRAC channels (assessed by integrating the Ca$^{2+}$ current charge over the 200 ms duration of the step-ramp pulse) (Fig. 4G), demonstrating that the inability to observe increases in [Ca$^{2+}$], after depolarization of T cells was not due to a low sensitivity of our recording system. To further exclude the possibility that recording conditions are not sensitive enough to detect VGCC currents, we analyzed voltage-gated Ca$^{2+}$ currents in HEK293 cells transfected with Cav1.2 and PC12 cells endogenously expressing VGCCs using the whole-cell patch clamp configuration. Stepwise depolarization of HEK293 cells transfected with Cav1.2 channels from −80 mV to +80 mV evoked robust voltage-gated Ca$^{2+}$ currents that could be blocked completely with 8 μM nimodipine (Supplementary Fig. 5A). Similarly, applying depolarizing steps in PC12 cells from −80 mV to +60 mV induced obvious voltage-gated Ca$^{2+}$ currents in these ne- uroendocrine cells (Supplementary Fig. 5B). Collectively, these experiments demonstrate that even under sensitive recording conditions neither voltage-activated Ca$^{2+}$ influx nor Ca$^{2+}$ currents consistent with the presence of functional VGCCs can be detected in human T cells.

**Fig. 3 Cav1.1 is not required for Ca$^{2+}$ influx and cytokine production by T cells.** CD4$^+$ T cells of LSL-Cas9; Cd4Cre mice were transduced with sgCtrl, sgCacnb1 or sgStim1. A, B After 3 days, Amt$^+$ T cells were enriched by cell sorting, recovered for one day in medium containing IL-2 and IL-7 and analyzed. Cytosolic Ca$^{2+}$ levels were measured following stimulation of T cells by anti-CD3 (TCR) cross-linking and ionomycin (Iono) in Ringer’s solution containing 2 mM Ca$^{2+}$. Average Ca$^{2+}$ traces (A) and quantification (B) of the area under the curve (AUC) in the time periods indicated by the dotted lines. C, D Cytokine production by CD4$^+$ T cells was measured at day 4 after transduction and restimulation for 6 h with PMA and ionomycin. Representative contour plots (C) and quantification (D) of IL-2$, TNF$ and IFN-γ$ CD4$^+$ T cells. Data in (A, B, D) are the mean ± SEM of n = 7 independent experiments performed in duplicates. Statistical analysis by two-tailed, unpaired Student’s t test. **p < 0.01, ***p < 0.001.

**TCR stimulation fails to evoke depolarization-induced Ca$^{2+}$ influx and VGCC currents in mouse and human T cells.** Although membrane depolarization is sufficient to activate VGCCs and Ca$^{2+}$ influx in excitable cells, we hypothesized that in non-excit- able T cells an additional stimulus may be required to activate VGCCs. To test this hypothesis, we measured Ca$^{2+}$ influx in mouse CD4$^+$ T cells that were stimulated by CD3-crosslinking prior to depolarization with high [K$^+$]$_o$. TCR stimulation in the presence of physiological [K$^+$]$_o$ resulted in Ca$^{2+}$ influx, which could be further amplified by inducing SOCE with ionomycin (Fig. 5A). TCR stimulation followed by depolarization with 60 or 150 mM [K$^+$]$_o$ did not increase Ca$^{2+}$ influx; instead 150 mM [K$^+$]$_o$ significantly reduced [Ca$^{2+}$], (Fig. 5A, B). Likewise, SOCE induced by ionomycin stimulation was decreased by 150 mM [K$^+$]$_o$. Similar results were observed in human T cells that were stimulated with anti-CD3 antibody (OKT3) after membrane depolarization with either 60 mM or 150 mM [K$^+$]$_o$, (Supplementary Fig. 6A, B). The fact that membrane depolarization decreases, rather than increases, Ca$^{2+}$ signals in T cells is consistent with the requirement for a negative V$\text{m}$ to provide the electrical gradient for Ca$^{2+}$ influx through CRAC and other Ca$^{2+}$ channels.
To directly evaluate whether functional VGCC channels are present in mouse T cells, we carried out measurements of whole-cell Ca\(^{2+}\) currents using patch-clamp electrophysiology in response to depolarizing voltage steps. CD4\(^{+}\) T cells isolated from the spleen of C57BL/6 mice were kept overnight in culture medium supplemented with IL-7 or stimulated for 2 days with anti-CD3/CD28 antibodies before analysis of channel function. To activate VGCCs, T cells were depolarized stepwise from \(-80\) mV to \(+60\) mV from a holding potential of \(-70\) mV. These recordings failed to induce any inward currents in naïve unstimulated or stimulated mouse T cells (Fig. 5C, D). As a control for our recording conditions, we measured K\(^{+}\) currents resulting from the voltage dependent activation of Kv1.3, which is well known to regulate the membrane potential of T cells and T.
Fig. 4 Depolarization of T cells fails to evoke Ca2+ influx and Ca2+ currents. A Cytosolic Ca2+ levels in mouse CD4+ T cells. T cells were stimulated with anti-CD3 + CD28, cultured for 3 days and exposed to 60 mM (top) and 150 mM (bottom) KCl followed by stimulation with ionomycin (Iono). B Cytosolic Ca2+ levels in human T cells from a healthy donor (HD) cultured for 10 days in vitro, exposed to 60 mM KCl and stimulated with ionomycin. Averaged Ca2+ traces (left) and quantification (right) of the mean F340/F380 ratio during the time periods indicated by dotted lines. Data shown are the mean ± SEM of n = 3–4 (A) and n = 7 (B) independent experiments conducted in duplicates. C–H No voltage-gated Ca2+ currents and signals in human T cells. C Membrane currents in HD T cells were recorded in 110 mM Ba2+ in response to voltage steps from −60 to +60 mV for 200 ms from a holding potential of −80 mV. Current traces were leak-subtracted using the P/8 method with steps from −100 mV. D Current-voltage (I–V) plots (top) and [Ca2+]i concentrations (bottom) measured using Indo-1 in the same cell stimulated in the presence of 20 mM extracellular Ca2+ using the voltage protocol shown in (C). E [Ca2+]i, was measured in Indo-1 loaded HD T cells held at −80 mV for 20–30 s to establish the baseline [Ca2+]i, followed by application of 40–50 depolarizing steps to +10 mV every 1 s. F, G Simultaneous measurements of [Ca2+]i and ICRAC. F T cells pretreated with TG were exposed to a step-ramp voltage protocol comprising a −100 mV step (50 ms) followed by a ramp from −100 to +100 mV (50 ms) every 1 s. The holding potential was +60 mV to prevent Ca2+ influx during the interpulse interval. G Representative I–V plot typical of ICRAC recorded during the −100 mV pulse from the experiments shown in (F). H [Ca2+]i, rises (left) and current densities (right) in response to either depolarizing steps (+10 mV) or TG treatment. For details see Methods. Data in (C–H) represent the mean ± SEM from n = 5–6 cells per condition. Statistical analysis by two-tailed, unpaired Student’s t test. *p < 0.01, ***p < 0.001.

The problem we investigated whether VGCC currents are unmasked in the absence of functional CRAC channels. To this end, we used T cells from a patient with a loss-of-function (LOF) mutation in ORAI1 (p.R91W), which abolishes CRAC channel function and SOCE42. We activated VGCCs in ORAI1-deficient T cells by depolarizing the membrane stepwise from −60 to +60 mV from a holding potential of −80 mV using the perforated patch configuration and simultaneously measured [Ca2+]i with Indo-1 (Fig. 6A). No inward Ca2+ currents or depolarization-evoked Ca2+ signals could be detected with this protocol using either external solutions containing 20 mM Ca2+ (Fig. 6B). To again exclude the possibility that activation of VGCCs in T cells requires TCR signaling in addition to depolarization, ORAI1-deficient T cells were first stimulated with OKT3 followed by stepwise depolarization. TCR cross-linking in the absence of CRAC channels failed to unmask depolarization-evoked membrane currents and Ca2+ signals (Fig. 6C). We reasoned again that we might not be able to detect small VGCC currents if T cells expressed only a few functional VGCCs per cell. We therefore measured [Ca2+]i in Indo-1-loaded T cells that were repeatedly depolarized every 1 s to produce a buildup of cytosolic Ca2+. Neither ORAI1-deficient T cells that were left untreated nor cells stimulated with OKT3 showed increases in [Ca2+]i upon repeated depolarization (Fig. 6D, E). By contrast, stimulation of ORAI1-deficient T cells with a high dose of ionomycin to bypass Ca2+ influx through CRAC channel resulted in the robust elevation of [Ca2+]i (Fig. 6F). Collectively, these data demonstrate that the absence of functional CRAC channels does not result in compensatory activity of VGCCs.

Previous reports have shown that activation of STIM1, which is essential for the activation of CRAC channels by binding to ORAI143, inhibits Ca2+ influx through L-type Cav1.2 channels in response to depolarization44,45. These studies suggested that STIM1 reciprocally activates CRAC channels while suppressing Cav1.2. To test the hypothesis that STIM1 suppresses VGCC function in T cells, we first analyzed Ca2+ influx in T cells from a patient with a null mutation in STIM1 (c.497 + 776 A > G) that abolishes STIM1 protein expression. Application of 60 mM KCl to depolarize Vm cells did not evoke a rise in [Ca2+]i in T cells from either a HD or the STIM1-deficient patient (Fig. 6G). Whereas ionomycin induced SOCE in HD T cells, no increase in [Ca2+]i was observed in the absence of STIM1 (Fig. 6G). We next analyzed Ca2+ signals in T cells from WT and Stim1(lof)/Ca2+Cre mice with conditional deletion of STIM1 in T cells. Depolarization of Stim1-deficient mouse T cells by application of 60 mM or 150 mM KCl failed to induce a rise in [Ca2+]i (Fig. 6H, I). In WT T cells, depolarization with high [K+]o, suppressed SOCE induced by ionomycin stimulation (Fig. 6H, I). To exclude the possibility that VGCC function in
T cells in the absence of STIM1 requires both TCR stimulation and depolarization, we stimulated T cells from a HD and the STIM1-deficient patient with OKT3 after depolarization with 60 mM or 150 mM \([K^+]_o\). Depolarization suppressed the TCR induced increase in \([Ca^{2+}]_i\), observed in HD T cells, and failed to evoke a rise in \([Ca^{2+}]_i\) in STIM1-deficient T cells (Supplementary Fig. 8A, B). Similar observations were made in mouse T cells from WT and Stim1Cre mice. TCR crosslinking followed by depolarization with 60 mM or 150 mM \([K^+]_o\), did not evoke an increase in \([Ca^{2+}]_i\), in STIM1-deficient T cells (Supplementary Fig. 8C, D). In WT T cells, depolarization suppressed \([Ca^{2+}]_i\) influx following TCR and ionomycin stimulation as expected. Collectively, these data demonstrate that STIM1 deletion in either human or mouse T cells fails to induce voltage-gated \(Ca^{2+}\) channel activity.

Several \(a1\) pore subunits of VGCCs are expressed in T cells but lack the N terminus. Several studies have reported mRNA and/or
protein expression in T cells of L- and T-type VGCCs including Cav1.2, Cav1.3, Cav1.4 and Cav3.1. Because we were unable to detect evidence for the presence of functional VGCCs in T cells, this raises the question if the α1 pore subunits of VGCCs are expressed in human and mouse T cells. To address this question, we first investigated the protein levels of the L-type Ca\(^{2+}\) channels Cav1.2, Cav1.3 and Cav1.4, which had previously been reported in T cells. For Cav1.2, no protein band was detectable in lysates of human T cells and only a very weak band in mouse T cells (Supplementary Fig. 9A) suggesting nearly complete or complete absence of Cav1.2 in T cells. The specificity of the anti-Cav1.2 antibody used was confirmed using HEK293 cells transfected with either Cav1.2 or Cav1.3. Some putative Cav1.3 protein expression was observed in mouse, but not human, T cells although the identity of the observed
bands at the expected 230 kDa size is not certain in part because they migrated slightly faster than the reference band in HEK293 cells overexpressing Cav1.3 and because we also detected an (albeit weaker) band in HEK293 cells transfected with Cav1.2 (Supplementary Fig. 9A). Cav1.4 protein was readily detectable in mouse retina, but was absent in thymocytes (Supplementary Fig. 9B). The specificity of the anti-Cav1.4 antibody was confirmed using retina from Cav1.4−/− mice. Cav1.4 expression in human T cells was difficult to assess because of a very strong band detected by the Cav1.4 antibody in human T cells that ran just below the Cav1.4 reference band in retina along with some weaker bands above the Cav1.4 reference band.

Given the limited specificity of most antibodies for all but some VGCC α1 subunits, we also investigated their expression by analyzing RNA-Seq data of human and mouse T cells from a HD or WT mice, respectively, that were left untreated or stimulated by TCR crosslinking. We focused on VGCCs whose TPM or WT mice, respectively, that were left untreated or stimulated by TCR crosslinking. We focused on VGCCs whose TPM were detectable in mouse CD4+ T cells by using an shRNA screening approach to identify ion channel candidates. We conducted an exon-level alignment of RNA-seq data for those VGCCs that we had found to be expressed in human and mouse T cells. mRNA for Cav3.3 is the most abundant of all VGCCs in human T cells. However, only transcripts of exons 12-37 were detectable in human T cells, which was in contrast to brain (frontal cortex) where all 37 exons of Cav3.3 (encoded by CACNAII) are expressed (Fig. 7C). We found two putative transcription start sites (TSS) 5′ of exon 12 in human T cells by searching the refTSS database. We made similar observations for Cav3.2 (CACNAI), which is the second highest expressed VGCC in human T cells. Transcript levels of exons 1-12 were undetectable or very low in human T cells, whereas all exons 1-35 were expressed in brain tissue (Fig. 7E). We detected a putative TSS in exon 13, which may initiate mRNA expression in T cells (Fig. 7F). Cav2.1 (encoded by CACNAI) is the most highly expressed α1 subunit in mouse T cells at the transcriptional level. Exon usage analysis demonstrated that exons 1-33 (of 49) were not or weakly expressed in T cells (Fig. 7G). We detected 3 putative TSS in exon 34 of mouse CACNAI, which may initiate transcription of a truncated mRNA (Fig. 7H). Collectively, these data demonstrate that although mRNAs for several VGCCs can be detected in mouse and human T cells, the transcripts are incomplete and result in N-terminally truncated proteins. For example, the non-transcribed exons 1-11 of CACNAI encode amino acids (aa) 1-715 of the Cav3.3 protein, which form the N terminus of Cav3.3, its first channel domain (I), the I–II linker including the α-interaction domain (AID) and TM1-3 of the second channel domain (II) (Fig. 7H). Our data predict similar N-terminally truncated proteins for human Cav3.3 and mouse Cav2.1. Even if these proteins were stable and properly located in the plasma membrane, they would very likely not be functional Ca2+ channels, providing an explanation for the absence of VGCC currents and Ca2+ influx upon depolarization in T cells.

**Discussion**

We here identified Cavβ1 as a regulator of clonal expansion of T cells. By using an shRNA screening approach to identify ion channels that control T cell-mediated immunity, we found that Cavβ1 was required for the clonal expansion of CD4+ T cells after LCMV infection in vivo. Whereas deletion of Cavβ1 did not affect the proliferation of T cells, it was required to prevent T cell apoptosis following TCR stimulation in vitro. Three other Cavβ

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**Fig. 6 Lack of ORAI1 or STIM1 does not induce voltage-gated Ca2+ current or Ca2+ influx in T cells. A-C** Perforated patch recordings of human T cells from a patient with a loss-of-function (LOF) mutation (p.R91W) in ORAI1 (ORAI1LOF). T cells were left untreated (B) or stimulated with OKT3 (C) for 5-25 min prior to measurements. To record Ca2+ current and cytosolic Ca2+ levels, T cells were stepped from −60 to −60 mV for 200 ms from a holding potential of ~80 mV. Displayed are membrane currents (top), I-V plots (middle) and [Ca2+]i traces (bottom) measured simultaneously in the same cells in 20 mM Ca2+ solution. Currents were leak-subtracted using the P/8 method. Data shown in (B, C) are representative of n = 7 and n = 3 cells, respectively. D, E T cells of the ORAI1LOF patient were loaded with Indo-1 and either left unstimulated (D) or stimulated with OKT3 (E). T cells were stepped to −10 mV for 200 ms every second from a holding potential of ~80 mV. Ca2+ traces in (D, E) are representative of n = 5 and n = 4 cells, respectively. F Quantification of [Ca2+]i, at −10 mV in unstimulated and OKT3-stimulated T cells and after treatment with 5 μM ionomycin (Iono). Δ[Ca2+]i was measured as the difference between the [Ca2+]i prior to and at the end of 30 depolarization pulses. Data shown are the mean ± SEM from n = 4-5 cells. G Cytosolic Ca2+ levels in human T cells from a patient with a STIM1 c.497 + 776 A > G null mutation (STIM1LOF). T cells were cultured for 10 days in vitro, loaded with Fura-2 and depolarized with Ringer’s solution containing 60 mM KCl. Averaged Ca2+ traces (left) and quantification (right) of the peak F340/F380 ratios during the time periods indicated by dotted lines. Data are the mean ± SEM from n = 5 independent experiments. (Note that Ca2+ traces of the HD T cells are as those shown in Fig. 4B; HD T cells were analyzed together with STIM1LOF T cells and are shown for comparison). H, I Cytosolic Ca2+ levels in CD4+ T cells from wildtype (WT) and Stim1−/−/Cac4Cre mice. T cells were activated for 3 days with anti-CD3/CD28 and then depolarized with Ringer’s solution containing 60 mM or 150 mM KCl. H Averaged Ca2+ traces (middle) and I quantification of the mean (left) and AUC (right) of F340/F380 ratios during the indicated time periods. Data represent the mean ± SEM from n = 5 independent experiments. Statistical analysis by two-tailed Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001.
subunits have also been implicated in T cell function. Lack of functional Cavβ2 and Cavβ4 was associated with a severe defect in T cell development, thymic and splenic involution and lymphocytopenia. Cavβ3 deficiency resulted in impaired survival of naive CD8+ T cells. Together, these studies and our data indicate that Cavβ subunits regulate T cell function, especially T cell survival. In contrast to the Cavβ1 deficiency phenotype described here, deletion or mutation of Cavβ2, Cavβ3 or Cavβ4 subunits resulted in moderately reduced TCR-induced Ca2+ influx in T cells, which was explained as arising from reduced Cav1.2/Cav1.3 and Cav1.4 protein expression in Cavβ2 and Cavβ3 deficient T cells, respectively. It is noteworthy that we only observed robust expression of Cavβ1 and Cavβ3 in mouse and human T cells by RNA-Seq, but not that of Cavβ2 and Cavβ4.
Cavβ4. Because we analyzed T cells from secondary lymphoid organs and blood of mice and humans, respectively, it is possible that Cavβ1 and Cavβ3 regulate the function of mature T cells such as survival, whereas Cavβ2 and Cavβ4 are critical in immature T cells during their development.

The mechanisms by which Cavβ1 controls apoptosis remain unclear. In excitable cells Cavβ subunits regulate VGCC function by promoting the cell surface expression of α subunits and controlling channel activation and inactivation. Reduced surface expression or activation of α subunits in the absence of Cavβ1 might therefore result in impaired Ca2+ influx in T cells and explain impaired T cell survival. However, Cavβ1 deletion in T cells did not impair TCR-induced Ca2+ influx, suggesting that Cavβ1 function is independent of regulating VGCC channels. Ca2+ signaling is an important regulator of apoptosis in T cells with both pro- and anti-apoptotic effects observed. However, our observation that Ca2+ influx in T cells is unaffected by the loss of Cavβ1 excludes the possibility that increased apoptosis is caused by effects of Cavβ1 on Ca2+ signals. Cavβ1-deficient T cells also showed normal production of cytokines such as IFN-γ, TNF and IL-2 whose transcription is dependent on Ca2+ signaling, further demonstrating that Cavβ1 is not required for Ca2+ influx in mouse T cells.

Although Cavβ proteins have mostly been thought of in terms of auxiliary subunits of VGCCs, a significant body of evidence demonstrates that they interact with many other proteins and have many VGCC-independent functions. For instance, Cavβ proteins interact with other ion channels including ryanodine receptors, membrane receptors, Ras-related monomeric small GTP-binding (RGK) proteins, dynamin, actin and the scaffolding protein AHNAK. One of the most intriguing functions of Cavβ proteins is their function in controlling gene expression in the nucleus. A Cavβ4 splice variant was shown to interact with heterochromatin protein 1 (HP1γ), which mediates gene silencing. Full-length Cavβ3 interacts with Pax6(S), an isoform of the transcription factor Pax6, to repress its transcriptional activity. Moreover, overexpression of Cavβ4 in HEK293 cells was shown to modulate gene expression. The Cavβ1a isoform was shown to localize to the nucleus of muscle progenitor cells (MPC) and bind to the myogenin promoter. Deletion of Cavβ1a altered MPC expansion in vitro and in vivo, and changed global gene expression. It is possible that Cavβ1 also controls gene expression in T cells, which would be distinct from its canonical purpose of regulating VGCC function in excitable cells.

Because we identified Cavβ1 as a regulator of T cell function and previous reports had implicated other α and β subunits in Ca2+ influx and the function of T cells, we investigated the contribution of VGCCs to Ca2+ signaling in T cells. Evidence supporting a function of VGCCs in T cells comes from the use of DHP Ca2+ channel blockers, RNAi mediated knockdown of VGCC expression and knockout mice. Using a variety of measurement protocols in both human and mouse T cells, we were unable, however, to detect any evidence of functional VGCCs in T cells. Depolarization of T cells failed to evoke Ca2+ influx and VGCC currents even under electro-physiological recording conditions greatly optimized to detect small currents. Similar results were obtained in T cells simultaneously activated by TCR stimulation based on the hypothesis that depolarization may not be sufficient to activate VGCCs in T cells and that additional stimuli may be required for their activation. Moreover, VGCC currents were undetectable in T cells lacking CRAC channel function. Because CRAC channels are the dominant Ca2+ influx pathway in T cells after TCR stimulation, we reasoned that lack of CRAC channel function may result in a compensatory upregulation of VGCC currents, which was not the case. Lastly, deletion of STIM1, which was reported to inhibit Cav1.2 ectopically expressed in HEK293 or Jurkat cells, did not evoke Ca2+ influx upon depolarization of mouse or human T cells. Together, our studies fail to provide evidence for the existence of functional VGCCs in T cells.

How can these findings be reconciled with reports of VGCC function in T cells? Initial evidence supporting VGCC function in T cells came from the use of VGCC blockers including DHPs (amilodipine, nicardipine, nimodipine) and non-DHPs (verapamil, diltiazem), which were reported to inhibit Ca2+ influx in T cells. Micromolar concentrations of VGCC blockers, however, also inhibit several K+ channels, notably Kv1.3 in T cells, thereby reducing Vm and the electrical driving force for Ca2+ influx through CRAC channels. In fact, selective blockade of Kv1.3 and KCa3.1 channels in T cells inhibits Ca2+ influx. It is noteworthy that there is no compelling evidence showing that patients treated with VGCC blockers have impaired immune responses despite the widespread clinical use of these drugs for the treatment of many cardiovascular conditions. More specific evidence supporting VGCC function in immunity comes from studies in mice. Targeted disruption of Cacna1f encoding Cav1.4 resulted in reduced CD4+ and CD8+ T cell numbers and function, most strikingly impaired T cell responses after infection with L. monocytogenes and murine gamma-herpesvirus 68 (MHV-68). T cells of Cacna1f−/− mice had reduced TCR stimulation-induced Ca2+ influx and voltage-dependent Ba2+ currents. We were not able to observe Ba2+ currents upon depolarization of human peripheral blood T cells. Although this discrepancy could be due to a specific function of Cav1.4 in mouse but not human T cells, we failed to observe depolarization-induced Ca2+ influx in both human and mouse T cells. Another study showed that deletion of Cacna1g encoding Cav3.1 impairs the production of cytokines by Th17 cells in vitro
and renders mice resistant to EAE, a mouse model of multiple sclerosis. CD4+ T cells of Caenalg−/− mice had reduced voltage-activated Ca2+ currents and Ca2+ influx when exposed to extracellular Ca2+. This defect was more prominent in Th17 cells, which expressed higher levels of Caenalg mRNA than Th1 or Th2 cells. By contrast, no defect in Ca2+ influx was observed in Caenalg−/− T cells after TCR stimulation. This study suggested that Cav3.1 channels mediate Ca2+ influx at the resting membrane potential in T cells, especially in Th17 cells.

These findings regarding Cav1.4 and Cav3.1 functions in mouse T cells are in apparent contrast to our failure to detect VGCC currents in human peripheral blood T cells. A potential explanation for this discrepancy could be that mouse T cells have functional VGCCs whereas human T cells do not. Arguing against this explanation is the fact that we failed to detect voltage-dependent Ca2+ influx in mouse T cells under any of the conditions tested. To activate VGCCs, we depolarized human and mouse T cells to ~−24 mV and −0 mV (with 60 mM and 150 mM [K+]o, respectively), which would be sufficient to activate L-type (Cav1.4) and T-type (Cav3.1) channels that open at membrane potentials between −40 to −10 mV and −60 to −70 mV, respectively. Depolarization should, therefore, have induced voltage-dependent Ca2+ currents and Ca2+ influx in our experiments. An intriguing feature of T-type VGCCs such Cav3.1 that may explain their function in T cells is their activation at low voltages in the −60 to −70 mV range, which coincides with the resting Vm of T cells (−53 to −59 mV). Window currents at these membrane potentials result from the overlap of activation and inactivation of T-type channels, and in the CNS were found to be important for the regulation of neuronal arousal. In T cells, they may contribute to Ca2+ influx and T cell function at resting Vm and explain the effects of Cav3.1 deletion on T cell function in Caenalg−/− mice. Notwithstanding the potential importance of window currents for T cell function, if functional T-type VGCCs were expressed in T cells, we should have been able to detect Ca2+ currents and Ca2+ influx upon depolarization. Our inability to detect T-type VGCC currents in mouse T cells is consistent with the lack of Cav3.1 mRNA expression in mouse or human T cells.

Our RNA-Seq data demonstrated transcription of several genes encoding α1 pore subunits in human and mouse T cells, but the α1 pore subunits we detected were different from those reported before, namely Cav1.2, Cav1.3, Cav1.4 and Cav3.1. The most abundant α1 subunits we detected were Cav3.3 and Cav2.1 in human and mouse T cells, respectively, whereas transcripts encoding Cav1.2, Cav1.3, Cav1.4 and Cav3.1 were not or barely detectable. Moreover, we were unable to detect Cav1.2 or Cav1.4 proteins in either mouse or human T cells. The analysis of protein expression of other α1 subunits was limited by the quality of available antibodies, which included the validation of Cav3.3 protein expression. A potential explanation for our inability to detect VGCC function and mRNA of previously reported Cav α1 subunits is that their expression is restricted to particular T cell subsets. Cav1.2 levels were reported to be increased in human Th2 cells compared to Th1 or Th0 cells, and Cav3.1 mRNA levels were transiently increased in Th17 cells. Our analysis of published RNA-Seq data of mouse CD4+ T cells confirms relatively higher mRNA levels for Caenalc (Cav1.2) and Caenalg (Cav3.1) in Th2 and Treg cells, respectively, compared to naïve T cells, Th1 and Th17 cells. However, physiological evidence of functional VGCCs in Th2 or Treg cells is currently lacking.

Intriguingly, neither Cav3.3 and Cav2.1 have been implicated in T cell function before. To understand why their robust mRNA expression was not associated with VGCC function, we took advantage of the fact that RNA-seq experiments can be read to individual exons to determine exon usage and potential splice variants in T cells. Whereas mRNA corresponding to all 37 exons of human CACNA1I was detected in the brain, where Cav3.3 function has been reported, only mRNA corresponding to exons 12–37 of CACNA1I was detectable in human T cells. We observed a similar lack of transcription of 5′ exons for human CACNA1H (Cav3.2) and mouse Caenala1 (Cav2.1). We detected two TSS preceding exon 12 of CACNA1I and a TSS in exon 13 of CACNA1H, which coincide with the 5′ end of the mRNA transcripts in T cells. These 5′ truncated mRNAs are predicted to encode proteins that lack the NH2 terminus of the α1 subunits; they encode including the entire channel domain I, the cytosolic I–II linker and part of domain II. It is unlikely, however, that the truncated proteins are stable or properly localize in the plasma membrane. While it is theoretically conceivable that a protein comprising domains III and IV of Cav3.3 is expressed and may assemble into a homomeric or heteromeric Ca2+ channel that is gated by a voltage-independent mechanism, we consider this possibility to be remote.

Alternative splicing of VGCC α1 subunits is well documented and an important mechanism to produce channels with distinct functional properties. However, alternative splicing typically occurs in the C-termirnus, and involves the alternative usage of individual exons. For instance, alternative splicing of Cav3.3 was shown to involve exon 9 encoding the I–II linker domain and exons 33 and 34 encoding part of the C-terminus of the channel, thereby giving rise to variants with distinct biophysical properties. Alternative splicing of VGCCs has also been reported in T cells. These include two variants of Cav1.4 in human T cells that lack expression of exons 31–34 & 37, and 32 & 37, respectively, predicted to delete the VSD of domain IV and its DHP binding site, which may render the channel insensitive to depolarization. The alternative exon usage of human CACNA1I and CACNA1H as well as mouse Caenala1 in T cells we demonstrate here has not been reported before. Of note, Man et al. recently reported that human and mouse cardiomyocytes do not express full-length transcripts of the neuronal voltage-gated sodium channel Nav1.8 (Scn10a), but instead express a short transcript (Scn10a-short) comprising only the last 7 exons. Transcription of this short variant occurred from an intronic enhancer-promoter complex. Overexpression of Nav1.8-short protein was shown to modulate the function of the main cardiac sodium channel Nav1.5 and heart rhythm. Whether truncated Cav3.3 protein is expressed in human T cells and suppresses VGCC function remains to be elucidated.

While our study does not support the existence of functional VGCCs in T cells, it suggests that Cavβ1 has alternative, VGCC-independent functions in T cells. These findings have implications for the assessment of the safety of VGCC channel blockers that are in wide clinical use for cardiovascular diseases, which based on our data are not expected to result in suppression of immune function. This interpretation is consistent with a lack of clinical evidence for immunosuppression in patients treated with Ca2+ channel blockers.

Methods

Mice. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the New York University Grossman School of Medicine. Stim3ββ Cre/Cre, Caenalg-mutant, congenic CD45.1 SMARTA; 21 have been described previously; Congenic CD45.2 strain 000664, Cd4Cre (strain 017336) and Rosa26-LSL-Cas9 knock-in (strain 024857) mice were purchased from the Jackson laboratory (Bar Harbor, ME). SMARTA; LSL-Cas9; Cd4Cre mice were generated by crossing SMARTA, Cd4Cre and LSL-Cas9 mice. All animals were on a pure C57BL/6 genetic background. Male and Female mice were used between 8 and 16 weeks of age. Mice were maintained under specific pathogen-free conditions with a 12 h dark/light cycle, at 22–25 °C and 50–60% humidity with water and food provided ad libitum.

Human cells. Experiments using human cells were conducted in accordance with protocols approved by the Institutional Review Board of the New York University
Grossman School of Medicine. Informed consent for the studies was obtained from the patients’ parents and HDs in accordance with the Declaration of Helsinki. T cells from HDs were cultured in the presence of IL-2 (20 IU/ml) and IL-7 (2.5 ng/ml) in complete RPMI medium (Corning, 10-040-CV) containing 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin and 0.1% 37.5 μg/ml polybrene (EMD Millipore, TR-1003-G). Retroviral supernatant from T cells was diluted 1:2 with complete RPMI containing 10 IU/ml rh-IL-2 and 2.5 μg/ml IL-7. 16 h later, the T cell supernatant was replaced with fresh complete RPMI containing IL-2 and IL-7 16 h later. 3 days after transduction, transduced T cells (Amt+) were enriched by cell sorting using a ST2001 (HA951) cell sorter (Stanford University). The retroviral supernatant was collected 36 and 48 h after transfection. To produce pseudotyped retrovirus for CRISPR/Cas9 gene editing (pMIR-Amt, pMRI-GFP)81 and small guide RNAs (sgRNAs), 97-mer oligonucleotides (IDT Ultramers) were cotransfected with the ecotropic packaging vector pCL-Eco using GenJet lipofection (SignaGen, SL100489). Retroviral supernatant was collected 3 days after spin infection.

In vivo shRNA screen. LCMV-specific CD4+ T cells isolated from the spleens of CD45.1+ SMARTA mice were purified using the Magnisort Mouse CD4+ T cell Enrichment Kit and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. T cells were transduced 24 h after stimulation with the shRNA library packaged in pseudotyped retroviral particles at ~0.3 multiplicity of infection in the presence of 8 μg/ml polybrene. 30 min after spin infection, the retroviral supernatant was diluted 1:2 with complete RPMI containing 20 IU/ml rh-IL-2 and 2.5 μg/ml IL-7. 16 h later, the T cell supernatant was replaced with fresh complete RPMI containing IL-2 and IL-7 16 h later. 3 days after transduction, transduced T cells (Amt+) were enriched by cell sorting using a ST2001 (HA951) cell sorter (Stanford University). The retroviral supernatant was collected 36 and 48 h after transfection. To produce pseudotyped retrovirus for CRISPR/Cas9 gene editing (pMIR-Amt, pMRI-GFP)81 and small guide RNAs (sgRNAs), 97-mer oligonucleotides (IDT Ultramers) were cotransfected with the ecotropic packaging vector pCL-Eco using GenJet lipofection reagent (SignaGen, SL100489). Retroviral supernatant was collected 36 and 60 h after transfection.

T cell culture. Human T cells were separated from whole blood by density gradient centrifugation using Ficoll-Paque plus (GE Amersham) and expanded in vitro as previously described82. Mouse CD4+ T cells were purified from splenocytes using the Magnisort Mouse CD4+ T cell Enrichment Kit (Invitrogen, MS22-7762-74) according to manufacturer’s protocol. CD4+ T cells were stimulated in flat-bottom 12-well plates (1 x 10^6 cells/ml per well) with 1 μg/ml plate-bound anti-CD3 (Bio X cell, clone 2C11, 14-0331-85) and 1 μg/ml anti-CD28 antibodies (Bio X cell, clone 37.5, Bio X cell). T cells were cultured in complete RPMI medium (Corning,10-040-CV) containing 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin and 0.1% β-mercaptoethanol. After 3 days of stimulation, T cells were detached and transferred to a new plate by phenol extraction and using the QIAEX II Gel Extraction Kit (Qiagen, 20021). For electrophoresis, cells were plated onto poly-L-lysine coated coverslips for patch clamp experiments. HEK293 cells were maintained in suspension at 37°C with 5% CO₂, plated at 25 × 10⁵ cells/mL. Supernatants were collected 36, 48 h after transfection. To produce pseudotyped retrovirus for transduction of T cells, Plat E cells were transduced with retroviral supernatant and 8 μg/ml anti-CD3/anti-CD28 antibodies by spin-infection (1.450 × g, 90 min, 32°C) in the presence of retroviral supernatant and 8 μg/ml anti-CD3/anti-CD28 antibodies. T cells were transduced 24 h after stimulation with the shRNA library packaged in pseudotyped retroviral particles at ~0.3 multiplicity of infection in the presence of 8 μg/ml polybrene. 30 min after spin infection, the retroviral supernatant was diluted 1:2 with complete RPMI containing 20 IU/ml rh-IL-2 and 2.5 μg/ml IL-7. 16 h later, the T cell supernatant was replaced with fresh complete RPMI containing IL-2 and IL-7 16 h later. 3 days after transduction, transduced T cells (Amt+) were enriched by cell sorting using a ST2001 (HA951) cell sorter (Stanford University). The retroviral supernatant was collected 36 and 48 h after transfection. To produce pseudotyped retrovirus for CRISPR/Cas9 gene editing (pMIR-Amt, pMRI-GFP)81 and small guide RNAs (sgRNAs), 97-mer oligonucleotides (IDT Ultramers) were cotransfected with the ecotropic packaging vector pCL-Eco using GenJet lipofection reagent (SignaGen, SL100489). Retroviral supernatant was collected 36 and 60 h after transfection.

Transfections and production of pseudotyped retrovirus. HEK293 cells were transfected with pCAV1.2 WT (150 ng), pcDNA3.1 a + β (75 ng) and Cherry-C1 (20 ng) kindly provided by Dr. R.W. Tsien (NYU) using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were used for electrophysiology 24–48 h after transfection. To produce pseudotyped retrovirus for transduction of T cells, Plat E cells were transduced with retroviral expression plasmids encoding shRNAs (plMPd-Amt, plMPd-GFP)81 and small guide RNAs for CRISPR/Cas9 gene editing (pMIR-Ant, pMRI-GFP)81. Plat E cells were cotransfected with the ectropic packaging vector pC3-Lipo using GenJet lipofection reagent (SignaGen, SL100489). Retroviral supernatant was collected 36 and 60 h after transfection.

T cell culture. Human T cells were separated from whole blood by density gradient centrifugation using Ficoll-Paque plus (GE Amersham) and expanded in vitro as previously described82. Mouse CD4+ T cells were purified from splenocytes using the Magnisort Mouse CD4+ T cell Enrichment Kit (Invitrogen, MS22-7762-74) according to manufacturer’s protocol. CD4+ T cells were stimulated in flat-bottom 12-well plates (1 x 10^6 cells/ml per well) with 1 μg/ml plate-bound anti-CD3 (Bio X cell, clone 2C11, 14-0331-85) and 1 μg/ml anti-CD28 antibodies (Bio X cell, clone 37.5, Bio X cell). T cells were cultured in complete RPMI medium (Corning,10-040-CV) containing 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin and 0.1% β-mercaptoethanol. After 3 days of stimulation, T cells were detached and transferred to a new plate by phenol extraction and using the QIAEX II Gel Extraction Kit (Qiagen, 20021). For electrophoresis, cells were plated onto poly-L-lysine coated coverslips one day before transfection and grown in a medium containing DMEM/F12 (Corning: 0-990-CV), 10% fetal bovine serum (Corning, 35-011-CV), 2 mM glutamine, 50 U penicillin and 50 μg/ml streptomycin.

Design and cloning of shRNAs and sgRNAs. shRNA target sequences against mouse Cacnb1 were extracted from the shRNA library, sgRNA target sequences were designed using Benchling software (https://www.benchling.com/crispr/). In addition, shRNA or sgRNA targeting a non-mammalian gene (Renilla luciferase) or human VEGF were used as controls. shRNA and sgRNA were cloned into the plMPD81 and pMRI80 retroviral expression vectors, respectively, that encode Ametrine or GFP reporter. shRNA and sgRNA sequences are provided in Supplementary Table 1.

RNA-sequencing. RNA-Seq data derived from human and mouse tissues were extracted from the Gene Expression Omnibus (GEO) database and are listed in Supplementary Table 2. FASTQ files were trimmed with Trimmomatic v0.3616 and aligned with STAR/2.6.189 to the corresponding human GRCh38/hg38 or mouse GRCH38/mm10 genome assembly. BigWig files were generated using deepTools v2.2.2.1 and normalized using the PyCondor library to calculate per-megabase mapped (RPKM). Human transcripts were counted using Salmon v0.14.19 with annotation from Gencode Gene Transfer M21 (GTF file), and mouse transcripts were aligned and counted using the featureCounts function in the subread package v1.6.37 with annotation from Genecode Gene Mtx21 (GTF file) and a subread format file. The final data to generate heatmaps of gene expression for human and mouse samples was TPM normalized to allow comparison of expression levels between different genes. The final heatmap visualization was done in python using the washsk script: v0.8.1 package83.

Additional RNA-Seq data were generated using human CD4+ T cells from a SMARTA (Amlt) patient homogenized for a separate RNA expression study in human immune cells using the Immunological Genome Project (Immgen)27 and Fantoms58 databases. 223 ITCs were determined to be expressed at least twofold above the population average across all immune cell types and included in the shRNA library. Each ITC was targeted by five shRNAs. Also included in the pooled shRNA library were 34 positive controls (genes known to regulate T-cell proliferation and survival) and 13 negative controls (genes not expressed in mammalian cells nor in T cells). shRNAs were designed as described in84. Briefly, shRNAs targeting 223 ITCs were designed using the DSIR algorithm82 and further filtered to select shRNAs with effective shRNA mir processing and potent knockdown. For the new generation of shRNA plasmids, 97-mer oligonucleotides (IDT Ultrimers) coding for the respective shRNAs were synthesized on 55k arrays (Agilent) and cloned into the plMPD recipient vector, which is based on miR-E vector and encodes Ametrine (Amt) as fluorescent reporter81. The pooled shRNA library containing 3432 shRNAs was sequenced by HiSeq 2500 (Illumina) to confirm equal representation of shRNAs and subsequently used to transfect Plat E cells for the production of pseudotyped retroviruses.
5 μg/ml plate-bound anti-CD3 (clone OKT3, 14-0037-82) and 10 μg/ml soluble anti-CD28 (clone CD28.2, 14-0289-82, both eBioscience) antibodies for 6 h in RPMI1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, 74004) and RNA quality was analyzed using a Bioanalyzer 2100 (Agilent) using PICO chips. RNA-Seq libraries were prepared using the TruSeq RNA sample prep v2 kit (Illumina TrueSeq Stranded mRNA, RS-122-2001) and 10 ng total RNA following the manufacturer’s instructions. The amplified libraries were purified using AMPure beads (Beckman Coulter, A68381), quantified by Qubit 2.0 fluorometer (Life Technologies), and visualized using an Agilent Tapestation 2200. The libraries were pooled equimolarly, loaded on the HiSeq 2500 DNA Sequencer (Illumina) and run as single 50 nucleotide reads. For analysis of VGCC expression, data from the human (GRCh38.p12) Gencode v30 GTF (M25 GTF) and the human (GRCh38.p12) Gencode v30 GTF (refTSS51). ICT mRNA expression in human T cells was performed using a MoFlo XDP cell sorter (Beckman Coulter).

**Flow cytometry.** Cells from tissue culture or isolated from mouse spleens were washed in cold PBS containing 3% FBS and 2 mM EDTA (“FACS buffer”). Staining of cell surface molecules with fluorescently labelled antibodies was performed at RT for 10 min in the dark. Intracellular (IC) cytokine staining was performed using the IC staining buffer kit (Biolegend, 344704) at 1:200 dilution in the last 10 min of incubation, plated onto UV-sterilized coverslips pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich, P4700). A20 cells were stained for 30 min with 1 μg/ml anti-CD4 (clone RPA-T4, Sigma-Aldrich, B7358), anti-CD68 (clone OX-6, Sigma-Aldrich, A1690), anti-ICAM-1 (clone 1A15-1E6, Sigma-Aldrich, BRL1171), anti-CD11b (clone M1/70, Sigma-Aldrich, M1778), and anti-CD123 (clone 2H7, Sigma-Aldrich, M7221). A20 cells were incubated with 1 μg/ml biotin-conjugated anti-CD3e Ab (Ab-22/31; BD pharmaingen, 553059) at the time of Fura-2 loading and stained with 1 μg/ml of the cell-permeable dye DAPI (4′,6-diamidino-2-phenylindole, Sigma) for 5 min before being analyzed.

**Intracellular (IC) cytokine staining was performed using the ProPrep (Invitrogen, I-24222) and 20 nM phorbol myristate acetate (PMA, Calbiochem, 727836) as recommended by the manufacturer’s instructions. All antibodies were used at 1:200 dilution and the complete list of antibodies used can be found in Supplementary Table 3. Briefly, T cells transduced with sgRNAs or shRNAs were stimulated with 1 μM monophosphate (8-Br-cAMP, Sigma-Aldrich, B7880) for 10 min before Ca²⁺ measurements. Human T cells were stimulated with 5 μg/ml anti-CD3 monoclonal antibody OKT3 during Ca²⁺ measurements. At the end of some experiments, T cells were stimulated with 1 μM ionomycin. For some experiments, T cells were pre-incubated with 200 nM PMA or 1 μM 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, Sigma-Aldrich, B7880) for 10 min before Ca²⁺ measurements.

**Patch-clamp electrophysiology.** Patch-clamp experiments were conducted in the standard whole-cell recording configuration at room temperature using an Axopatch 1D+ Axoclamp amplifier (Axon Instruments) interfaced to a Macintosh G3 computer. Recording electrodes were pulled from 100 μl pipettes coated with Silgard and fire-polished to a final resistance of 2–5 MΩ. Stimulation and data acquisition and analysis were performed using in-house routines (R. Lewis, Stanford University) developed on the Igor Pro platform (Wavemetrics). Currents were filtered at 1 or 3 kHz and displayed on an EYFs (F340/380) ratios or the area under the curve (AUC) for a specific time period as indicated in each experiment. For the Δ(Ca²⁺), measurements using a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices), T cells were loaded with Fura-2 as described above, recorded every 3 s using a train of depolarizing pulses (20 mM Cs-methanesulfonate, 8 MgCl2, 10 Cs-HEPES (pH 7.2), L-type channel blocker (Instrutech) and a Macintosh G3 computer. Recording electrodes were pulled from 100 μl pipettes coated with Silgard and fire-polished to a final resistance of 2–5 MΩ. Stimulation and data acquisition and analysis were performed using in-house routines (R. Lewis, Stanford University) developed on the Igor Pro platform (Wavemetrics). Currents were filtered at 1 or 3 kHz and displayed on an EYFs (F340/380) ratios or the area under the curve (AUC) for a specific time period as indicated in each experiment.
numbers, including accession numbers, cell types, species and PMID were downloaded from GEO and are listed in Supplementary Table 2 with information.

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Author contributions
S.E., A.R.C., R.S.L., M.P., and S.F. designed the research. S.E., A.R.C., W.L., B.L., M.Y., A.Y.T., I.S., and P.P.R. performed bioinformatic analyses. R.G., A.L., and J.W.H. provided reagents. S.E., A.R.C., I.S., A.Y.T., W.L., P.P.R., A.L., J.W.H., M.Y., R.S.L., M.P., and S.F. analyzed the data. S.E., A.R.C., M.P., and S.F. wrote the paper with additional input by J.W.H.

Competing interests
S.F. is a cofounder of CalciMedica. All other coauthors declare no competing interests.

Additional information
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