

Protein kinase C-dependent activation of Ca_v1.2 channels selectively controls human T_H2-lymphocyte functions

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Background: In addition to calcium release-activated calcium channel/ORAI calcium channels, the role of voltage-gated calcium (Ca_v1) channels in T-cell calcium signaling is emerging. Ca_v1 channels are formed by α 1 (Ca_v1.1 to Ca_v1.4) and auxiliary subunits. We previously demonstrated that mouse T_H2 cells selectively overexpressed Ca_v1.2 and Ca_v1.3 channels. Knocking down these channels with Ca_v1 antisense (AS) oligonucleotides inhibited T_H2 functions and experimental asthma.

Objective: We investigated the expression profile and role of Ca_v1 channels in human T-cell subsets, with a focus on T_H2 cells. **Methods:** We compared the profile of Ca_v1 channel subunit expression in T-cell subsets isolated *ex vivo* from the blood of healthy donors, as well as *in vitro*-polarized T-cell subsets, and tested the effect of the Ca_v1 inhibitors nicardipine and Ca_v1.2AS on their functions.

Results: Ca_v1.4 expression was detectable in CD4⁺ T cells, *ex vivo* T_H1 cells, and T_H17 cells, whereas Ca_v1.2 channels predominated in T_H2 cells only. T-cell activation resulted in Ca_v1.4 downregulation, whereas Ca_v1.2 expression was selectively maintained in polarized T_H2 cells and absent in T_H1 or T_H9 cells. Nicardipine and Ca_v1.2AS decreased Ca²⁺ and cytokine responses in T_H2, but not T_H1, cells. Protein kinase C (PKC) α/β inhibition decreased Ca²⁺ and cytokine responses, whereas both calcium and cytokine responses induced by PKC activation were inhibited by nicardipine or Ca_v1.2AS in T_H2 cells.

Conclusion: This study highlights the selective expression of Ca_v1.2 channels in human T_H2 cells and the role of PKC-dependent Ca_v1.2 channel activation in T_H2 cell function. Blocking PKC or Ca_v1.2 channel activation in T_H2 cells might represent new strategies to treat allergic diseases in human subjects. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Chemoattractant receptor-homologous molecule expressed on T_H2 cells, asthma, calcium, voltage-dependent calcium channels, T_H2 cytokines, protein kinase C α/β

T-cell receptor (TCR) engagement induces the formation of inositol 1, 4, 5-triphosphate¹ binding to its receptor at the membrane of the endoplasmic reticulum (ER), leading to the release of Ca²⁺ from the ER into the cytosol. The ER Ca²⁺ sensor stromal interaction molecules sense the decreased ER Ca²⁺ concentration and activate the ORAI calcium channels at the plasma membrane.² ORAI channels then open and sustain the Ca²⁺ influx needed for full-blown T-cell activation.³⁻⁵ However, other calcium channels¹ are likely to contribute to Ca²⁺ entry in T lymphocytes, including voltage-dependent Ca_v1 channels also defined as dihydropyridine receptors. These consist of the Ca²⁺-forming pore α 1 subunit encoded by 4 genes (Ca_v1.1 to Ca_v1.4) and the Ca_v β (Ca_v β 1 to Ca_v β 4) and Ca_v α 2- δ (Ca_v α 2- δ 1 to Ca_v α 2- δ 4) auxiliary subunits.⁶ The inhibitory effect of dihydropyridine antagonists on the Ca²⁺ response, the detection of Ca_v1 mRNA-related products in T cells, and the immune phenotype observed in mice with genetic ablation of Ca_v1.4,⁷ Ca_v β 3, or Ca_v β 4 subunits^{8,9} support a role for Ca_v1 channels in T-cell function.^{10,11} Ca_v1.1-, Ca_v1.2-, and/or Ca_v1.3-related channels were also reported as implicated in calcium signaling of T lymphocytes, raising the question of the respective contribution of each isoform in human and murine T-cell activation.¹²⁻¹⁴ Furthermore, patients with Timothy syndrome caused by mutated Ca_v1.2, which is responsible for a gain of function of the channel and display, among other disorders, increased susceptibility to recurrent infections.^{15,16} Correct cell functions depend on a tight regulation of intracellular Ca²⁺ levels, and both loss and gain of function of Ca_v1 were shown to induce alterations in cell function.¹⁷ Our group previously showed that mouse T_H2 lymphocytes producing IL-4, IL-5, and IL-13 and orchestrating allergic diseases overexpress Ca_v1.2 and Ca_v1.3 channels compared with T_H1 or CD4⁺ T cells.¹⁸ T_H2 cells transfected with Ca_v1 antisense (AS) oligodeoxynucleotides (ODNs; Ca_v1AS) displayed impaired TCR-driven Ca²⁺ responses and cytokine production. In addition, Ca_v1AS inhalation prevented the development of experimental asthma.¹⁸ We then looked for the expression profile of Ca_v1 channel isoforms in human CD4⁺ T-cell subsets with a focus on T_H2 cells. We show that human T_H2, but not T_H1, T_H17, or T_H9, cells overexpress Ca_v1.2 channels, which play a critical role in calcium signaling and T_H2 cytokine production.

METHODS

Methods for human blood cell subset isolation, generation of T_H subset cells, RNA preparation and reverse transcription, cell proliferation, intracellular cytokine staining, cytokine determination, and fluorescence microscopy are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

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Abbreviations used

AS:	Antisense
[Ca] _i :	Intracellular calcium concentration
Ca _v 1:	Voltage-dependent calcium channels
CFSE:	Carboxyfluorescein succinimidyl ester
CRTH2:	Chemoattractant receptor-homologous molecule expressed on T _H 2 cells
ER:	Endoplasmic reticulum
FITC:	Fluorescein isothiocyanate
NFAT:	Nuclear factor of activated T cells
ODN:	Oligodeoxynucleotide
PE:	Phycoerythrin
PKC:	Protein kinase C
PMA:	Phorbol 12-myristate 13-acetate
qPCR:	Quantitative PCR
TCR:	T-cell receptor

Real-time quantitative PCR

Primer sequences are listed in [Table E1](#) in this article's Online Repository at www.jacionline.org. Transcripts were measured by using real-time quantitative PCR (qPCR) with the LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany) and expressed as arbitrary units relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase ($2^{-[\text{Ct interest gene} - \text{Ct GAPDH}]}$) $\times 10^{-6}$ or 10^{-5} . Ct is defined as the numbers of cycle for which fluorescence is detectable, and a value of less than 1.5 was the limit of PCR product detection.

Antisense transfection experiments

Cells were transfected with 8 $\mu\text{mol/L}$ Ca_v1.2AS or sense oligonucleotides in the presence of Turbofect (Fermentas; Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's recommendations.

Intracellular calcium measurements

The fluorescence (F_{340}/F_{380}) was measured at an emission wavelength of 510 nm for excitation at wavelengths of 340 and 380 nm at the single-cell level in Fura2-AM-loaded cells, as previously described.¹⁹ The F_{340}/F_{380} ratio was indicative of intracellular calcium ([Ca]_i). Resting cells were recorded for approximately 1 minute before adding α -CD3/ α -CD28-coated beads (5 beads per cell) or phorbol 12-myristate 13-acetate (PMA; 200 ng/mL). The response was then recorded for 15 minutes before the addition of ionomycin (10 $\mu\text{mol/L}$), which was used as a positive control. Images were analyzed with MetaFluor imaging software. Data are always expressed as the F/F₀ ratio ($F = F_{340}/F_{380}$ in stimulated T cells and $F_0 = F_{340}/F_{380}$ under basal conditions) and represent the mean of at least 25 cells. The area under the curve over the mean + 2 SDs of the baseline ratio was measured with GraphPad Prism (GraphPad Software, La Jolla, Calif). Cells for which the F_{340}/F_{380} ratio was superior to the mean + 2 SDs of basal levels were considered responsive cells.

Statistical analysis

Results were expressed as the mean + 1 SD. The significance of differences was calculated by using paired or unpaired nonparametric or parametric tests depending on the number of samples. A *P* value of less than .05 was considered significant.

RESULTS**Resting and activated human T lymphocytes express all the subunits required to form functional Ca_v1 channels**

Analyzing the expression of Ca_v1 α 1 subunits in immune cells from the peripheral blood of healthy donors revealed that Ca_v1.4

predominated in lymphocytes at steady state ([Fig 1, A](#)). Ca_v1.2 transcripts were expressed in CD4⁺ and, to a lesser degree, CD8⁺ T lymphocytes. Ca_v1.3 was detected in neutrophils and CD4⁺ and CD8⁺ T lymphocytes and highly expressed in eosinophils ([Fig 1, A](#)). Ca_v1.1 transcripts were not detected in any hematopoietic cell subsets (data not shown).

Among CD4⁺ T cells, expression of Ca_v1.4 was higher in CD4⁺CD45RA⁺ naive than in CD4⁺CD45RA⁺ memory T cells, whereas Ca_v1.2 and Ca_v1.3 were weakly expressed in both subsets ([Fig 1, B](#)). The stimulation of CD4⁺ T cells with α -CD3/ α -CD28-coated beads downregulated Ca_v1.4 transcripts. By contrast, the expression of Ca_v1.2 and Ca_v1.3 transcripts was maintained or even upregulated on TCR stimulation ([Fig 1, C](#)).

Resting CD4⁺ T lymphocytes expressed mainly transcripts coding for β 1, β 3, and α 2 δ 2 auxiliary subunits ([Fig 1, D](#)), and their expression tends to increase after TCR engagement ([Fig 1, D](#)). These data indicate that all the subunits required to compose functional Ca_v1 channels are expressed in resting and activated CD4⁺ T lymphocytes and that TCR engagement controls their expression levels.

Nicardipine decreases Ca²⁺ response and cytokine production without affecting CD4⁺ T-cell proliferation

Nicardipine, a dihydropyridine antagonizing Ca_v1 channels, affects neither the survival (data not shown) nor the proliferative response induced by TCR stimulation of CD4⁺ cells, as shown by using a carboxyfluorescein succinimidyl ester (CFSE) dilution assay ([Fig 2, A](#)) or based on thymidine uptake (data not shown). TCR cross-linking induced a rapid [Ca]_i increase, followed by a plateau in CD4⁺ T cells, which was strongly diminished by nicardipine ([Fig 2, B](#)). The residual TCR-driven [Ca]_i increase in nicardipine-treated cells was only 26% of the values observed in untreated CD4⁺ cells (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). Nicardipine also reduced the frequency of IL-2-, IFN- γ -, and IL-4-producing cells ([Fig 2, C and D](#)) and cytokine secretion ([Fig 2, E](#)) after TCR stimulation. These data suggest that Ca_v1 channels participate in the calcium response and cytokine production of CD4⁺ T cells.

Ex vivo sorted T_H1, T_H2, and T_H17 cell subsets express distinct sets of Ca_v1 channels that might contribute to T-cell functions

On the basis of the expression of cell-surface markers, T_H1 (CD4⁺CD45RA⁺CXCR3⁺CCR6⁺), T_H17 (CD4⁺CD45RA⁺CCR6⁺), and T_H2 (CD4⁺ chemoattractant receptor-homologous molecule expressed on T_H2 cells [CRTH2]⁺) cells were *ex vivo* sorted from healthy donor PBMCs ([Fig 3, A and B](#)). These subsets represented 8% to 20%, 15% to 30%, and 0.2% to 3% of CD4⁺ T cells and were characterized by the selective expression of *IFNG*, *IL17*, and *IL4* transcripts, respectively ([Fig 3, C](#)). Ca_v1.4 was the sole isoform detected in *ex vivo* T_H1 and T_H17 subsets ([Fig 3, D](#)). Interestingly, CD4⁺CRTH2⁺ cells predominantly expressed Ca_v1.2 and, to a lesser degree, Ca_v1.3 channels, which were not detected in T_H1 and T_H17 lymphocytes ([Fig 3, D](#)). Nicardipine reduced subset-specific cytokine production by CRTH2⁺, CCR6⁺ T_H17, and CXCR3⁺CCR6⁺ T_H1 ([Fig 3, E](#)) cells, suggesting a functional role of Ca_v1 channels in these cells.

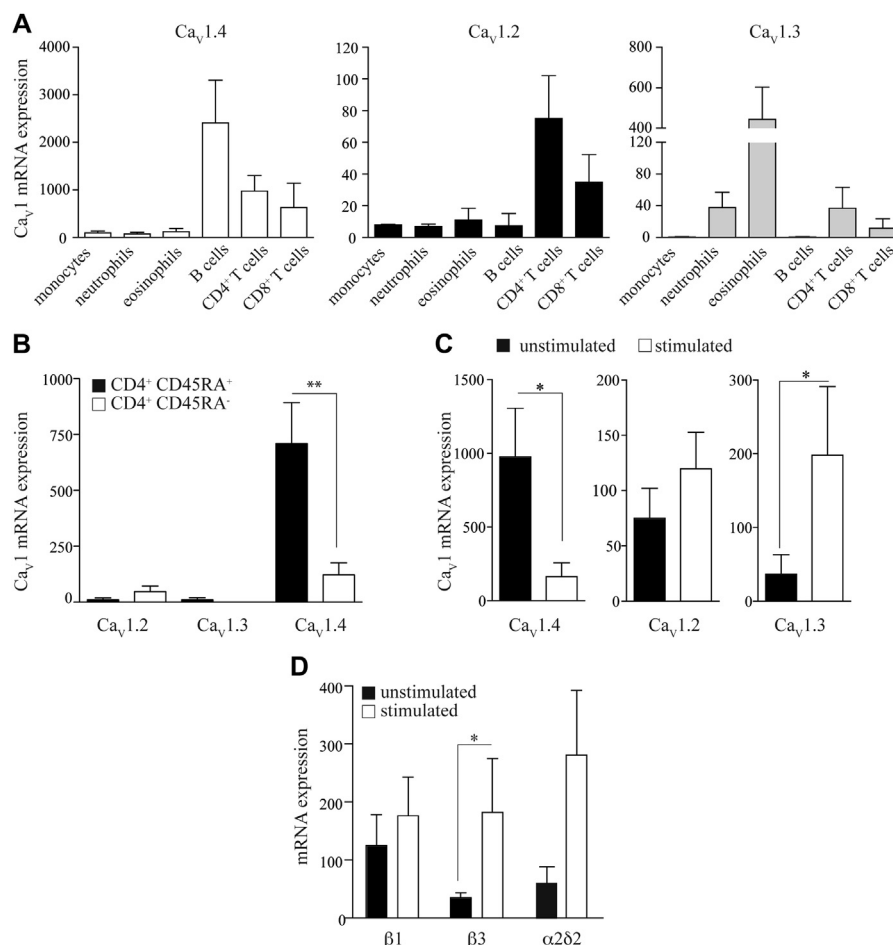


FIG 1. Ca_v1 subunit expression in human leukocytes. $Ca_v1\alpha1$ (A–C) and auxiliary subunit (D) transcripts were quantified in leukocytes from healthy blood donors ($n = 6-8$; Fig 1, A), in $CD4^+CD45RA^+$ and $CD4^+CD45RA^-$ sorted cells ($n = 5$; Fig 1, B), and in purified $CD4^+$ cells ($n = 5$) stimulated with anti-CD3/anti-CD28-coated beads for 4 hours (Fig 1, C and D). * $P < .05$ and ** $P < .01$.

$Ca_v1.2$ channel expression is selective of human expanded T_H2 cells relative to other polarized T-cell subsets

We activated $CD4^+$ T cells under neutral (T_H0) and polarizing conditions. Tables E2 and E3 in this article's Online Repository at www.jacionline.org show that TCR-activated T_H0 and T_H1 cells produce IFN- γ , whereas T_H2 and T_H9 cells selectively express *IL4* and *IL9* mRNA, respectively. The intracellular cytokine staining confirms the appropriate polarization of T_H1 and T_H2 cells (see Fig E2 in this article's Online Repository at www.jacionline.org). T_H0 cells expressed some amounts of $Ca_v1.4$ transcripts (Fig 4, A) at the same level as activated $CD4^+$ T cells (Fig 1, C). In T_H1 cells the expression of all the $Ca_v1\alpha1$ subunits was almost completely suppressed (Fig 4, A). The $Ca_v1.2$ isoform predominated in T_H2 cells, and this expression was selective relative to the other T-cell subsets (Fig 4, A), which is similar to results seen in *ex vivo*-purified CRTH2 $^+$ cells (Fig 3, D). The auxiliary subunits $\beta1$, $\beta3$, and $\alpha2\delta2$ were detected in both T_H1 and T_H2 cells (Fig 4, B). T_H9 cells, a lineage-specific subset recently shown to induce asthma,²⁰ expressed $Ca_v1.4$ and $Ca_v1.3$, but no $Ca_v1.2$, transcripts (Fig 4, A). Altogether, these results show that the expression profile of Ca_v1 channels depends on the state

of differentiation, with the $Ca_v1.2$ isoform being selectively expressed in T_H2 cells.

$Ca_v1.2$ and $Ca_v1.3$ expression in T_H2 cells corresponded to approximately 20% and 105% of the expression levels found in control neuroblastoma cells (see Fig E3 in this article's Online Repository at www.jacionline.org), showing that Ca_v1 transcript levels in T_H2 and neural cells were in the same range of expression. Exons 1/1a, 8/8a, 9a, 41a, and 45 discriminate between the cardiac and neuronal $Ca_v1.2$ isoforms.^{16,21} Sequencing PCR products from human T_H2 cells showed expression of exons 1 and 8, whereas exons 1a, 8a, 9a, 41a, and 45 were not found. These data indicated that human T_H2 cells expressed the neuronal isoforms of $Ca_v1.2$. Sequence analysis of exons 5, 13, 24, and 34 (not shown) demonstrated that $Ca_v1.2$ expressed in human T_H2 cells was not structurally devoid of the voltage sensors in agreement with our findings in mice.¹⁸ Consistent with mRNA data (Figs 1, C, and 4, A), staining for $Ca_v1.2$ protein was more intense in polarized T_H2 cells compared with that seen in resting $CD4^+$ T cells, whereas T_H1 cells were not labeled (Fig 4, C, and see Fig E4 in this article's Online Repository at www.jacionline.org). Therefore $Ca_v1.2$ transcripts and protein were present in bulk $CD4^+$ T cells, upregulated in T_H2 cells, and barely detectable in differentiated T_H1 and T_H9 cells.

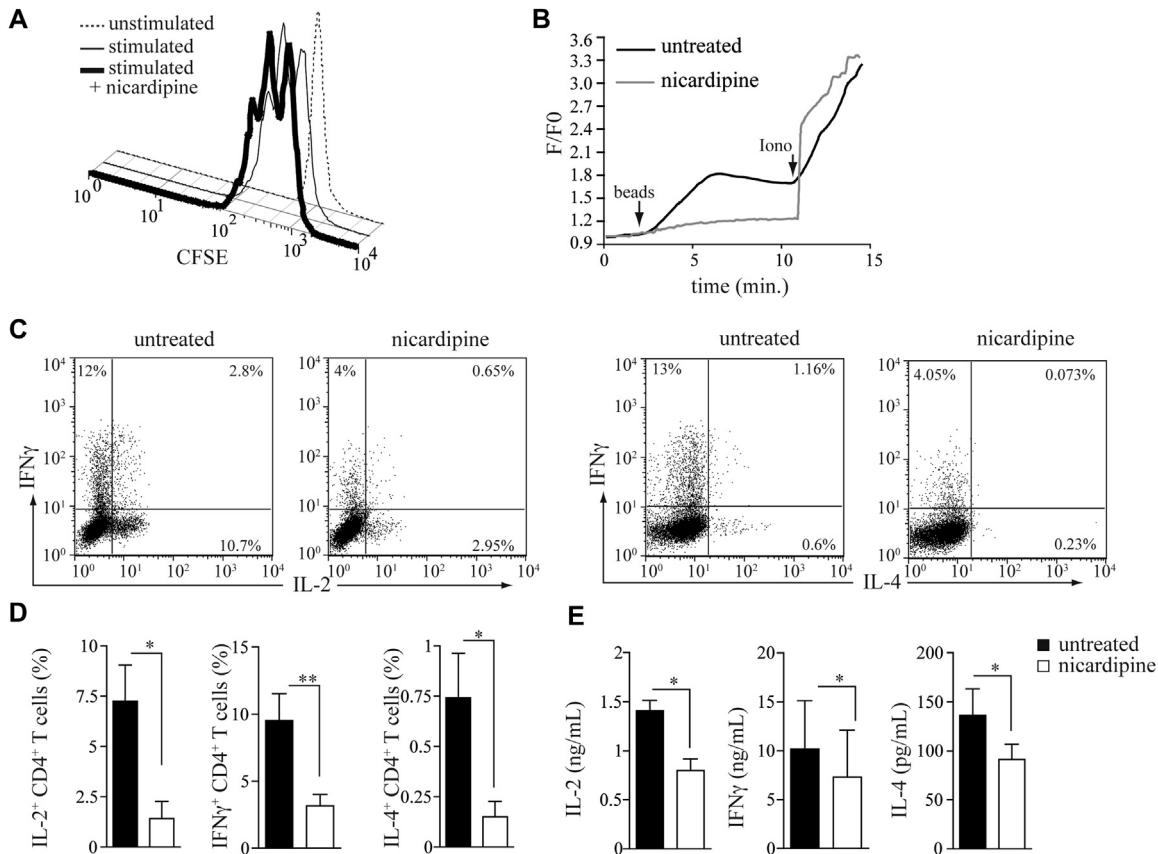


FIG 2. Nicardipine, a Ca_v1 antagonist, inhibits TCR-driven calcium and cytokine responses in human CD4^+ T cells. We assessed the proliferation (CFSE dilution; **A**), $[\text{Ca}]_i$ variations (1 experiment of 4) (**B**), cytokine-producing cell frequency (**C** and **D**), and cytokine production (**E**) by CD4^+ T cells stimulated with anti-CD3/anti-CD28-coated beads with or without nicardipine. Results are from 4 to 5 experiments. * $P < .05$ and ** $P < .01$.

Nicardipine strongly reduces TCR-driven $[\text{Ca}]_i$ increase and cytokine production in $\text{T}_\text{H}2$ cells without any effect on $\text{T}_\text{H}1$ cells

Nicardipine altered neither $\text{T}_\text{H}2$ or $\text{T}_\text{H}1$ cell viability (date not shown) nor their TCR-dependent proliferative response (Fig 4, D). By contrast, nicardipine dramatically reduced the TCR-driven increase in $[\text{Ca}]_i$ in $\text{T}_\text{H}2$ cells (Fig 4, E, and see Fig E1, B) but not in $\text{T}_\text{H}1$ cells (Fig 4, F, and see Fig E1, C). The residual TCR-driven $[\text{Ca}]_i$ increase in nicardipine-treated $\text{T}_\text{H}2$ cells was only 23% of the value observed in untreated $\text{T}_\text{H}2$ cells (see Fig E1, B). Consistent with the reduced TCR-driven Ca^{2+} response, nicardipine diminished levels of nuclear translocation of nuclear factor of activated T cells (NFAT), the major Ca^{2+} -dependent transcription factor in $\text{T}_\text{H}2$ cells (Fig 4, G, and see Fig E5, A, in this article's Online Repository at www.jacionline.org). This was associated with impaired $\text{T}_\text{H}2$ cytokine gene transcription (see Fig E5, B) and decreased frequency of IL-4-producing cells, from $28\% \pm 10\%$ to $17\% \pm 6\%$ ($n = 7$, $P < .02$). Nicardipine and diltiazem, which belong to chemically distinct classes of Ca_v1 channel blockers, inhibited $\text{T}_\text{H}2$ cytokine production in a dose-dependent manner (Fig 4, H, and see Fig E6, A and B, in this article's Online Repository at www.jacionline.org). These inhibitions were partial compared with cyclosporine A, an inhibitor of calcineurin (Fig 4, H), suggesting that Ca_v1 -independent but Ca^{2+} -dependent

(cyclosporin A-sensitive) pathways also contribute to $\text{T}_\text{H}2$ cytokine production.

In contrast with its effect on $\text{T}_\text{H}2$ cells, nicardipine affected neither IFN- γ secretion by *in vitro*-differentiated $\text{T}_\text{H}1$ cells (Fig 4, I) or $\text{T}_\text{H}0$ cells (26 ± 4 vs 25 ± 3 ng/mL in nicardipine-treated and control cells, respectively) nor *IL9* gene transcription in $\text{T}_\text{H}9$ cells (data not shown) on TCR stimulation, indicating differential effects of nicardipine on the types of effectors.

Knocking down $\text{Ca}_v1.2$ channels strongly impairs TCR-driven $[\text{Ca}]_i$ increase and cytokine production in $\text{T}_\text{H}2$ cells without any effect on $\text{T}_\text{H}1$ cells

Because $\text{T}_\text{H}2$ cells selectively express $\text{Ca}_v1.2$ channels, we tested the effect of $\text{Ca}_v1.2$ AS on $\text{T}_\text{H}2$ cell functions. First, we showed that transfection of $\text{T}_\text{H}2$ cells with different $\text{Ca}_v1.2$ -specific antisense ODNs (see Table E4 in this article's Online Repository at www.jacionline.org) reduced the ability to secrete $\text{T}_\text{H}2$ cytokines on TCR stimulation (see Fig E7, A, in this article's Online Repository at www.jacionline.org). Using the most effective $\text{Ca}_v1.2$ AS, AS6, we showed that the inhibition of $\text{T}_\text{H}2$ cytokine production was dose dependent (see Fig E7, B). The concentration of 8 $\mu\text{mol/L}$ AS ODN was chosen for further experiments. $\text{Ca}_v1.2$ AS decreased $\text{Ca}_v1.2$ mRNA and protein expression (see Fig E7, C and D) but affected neither T-cell viability

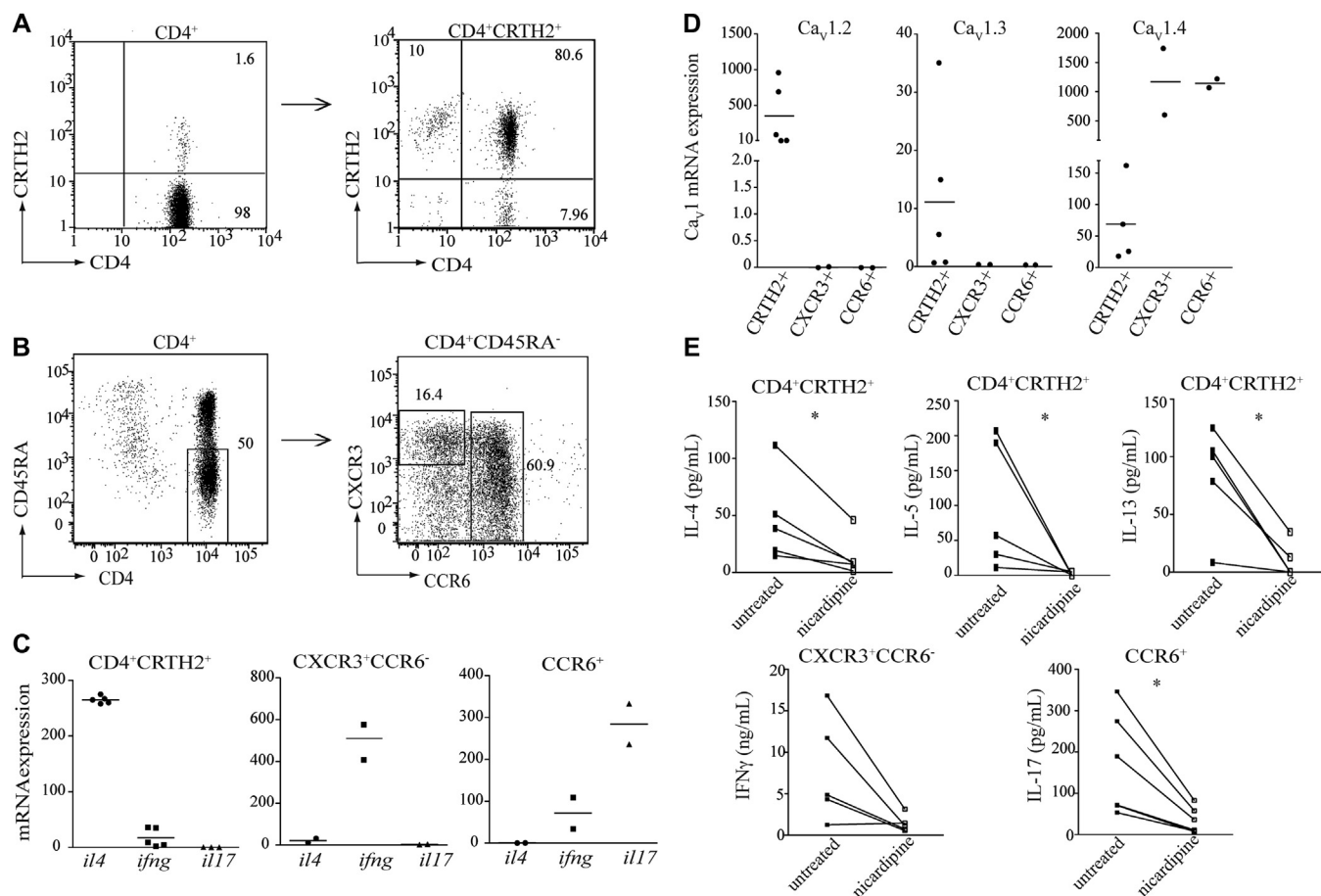


FIG 3. Profile of Ca_v1 expression by *ex vivo*-sorted T_H1, T_H17, and T_H2 cells and effect of nicardipine on cytokine production. Freshly purified CD4⁺CRTH2⁺ cells (**A**) and sorted CD4⁺CD45RA⁻CCR6⁺ (T_H17) cells and CD4⁺CD45RA⁻CXCR3⁺CCR6⁻ (T_H1) cells (**B**) were analyzed for subset-specific cytokine transcripts (**C**) and Ca_v1 (**D**) expression and TCR-induced cytokine production in the presence of nicardipine (**E**). Each symbol represents a donor. **P* < .02.

(data not shown) nor proliferation (Fig 5, A). Ca_v1.2AS decreased the calcium response on TCR stimulation (Fig 5, B, and see Fig E1, D) and cytokine production (Fig 5, C) in T_H2 cells. Consistent with the lack of Ca_v1.2 expression in T_H1 cells, Ca_v1.2AS had no effect on IFN-γ production by T_H1 cells (Fig 5, D).

Protein kinase C-dependent activation of Ca_v1.2 channels controls calcium response and cytokine production in T_H2 cells

Protein kinase C (PKC) α can activate Ca_v1.2 channels in smooth arteriolar cells independently of cell membrane potential variations and even at the resting cell membrane potential.²² Gö6976, an inhibitor of PKCα/β, was tested at concentrations ranging from 10 nmol/L to 1 μmol/L without a deleterious effect on cell viability (data not shown) and T_H1 or T_H2 cell proliferation (Fig 6, A). Interestingly, Gö6976 reduced TCR-driven [Ca]_i increases (Fig 6, B, and see Fig E1, E) and strongly suppressed cytokine production by T_H2 (Fig 6, C, and see Fig E6, C) but not T_H1 (Fig 6, B and D, and see Fig E1, F) cells.

PMA, a phorbol ester activator of PKC, induced a weak and rapid Ca²⁺ response that was reduced by nicardipine (Fig 6, E, and see Fig E1, G). Moreover, PMA induces IL-5 and IL-13

production, which were inhibited by Ca_v1.2AS (Fig 6, F). These data demonstrate that PKC activates Ca_v1.2 channels, which is required for Ca²⁺ response and cytokine production on TCR stimulation in T_H2 cells.

DISCUSSION

Herein we characterized the expression profile of Ca_v1 channels in human effector CD4⁺ T cells, and we provided evidence for a critical role of Ca_v1.2 channels in the TCR-driven Ca²⁺ response and cytokine production by human T_H2 lymphocytes.

We showed that among circulating blood cells, lymphocytes and eosinophils were the only cells to express Ca_v1α1 transcripts. In the steady state Ca_v1.4 subunit expression characterized resting T and B lymphocytes. In addition, CD4⁺ T cells also expressed the Ca_vβ and Ca_vα2δ auxiliary subunits required for optimal trafficking and functions of Ca_v1 channels.²³⁻²⁵

Nicardipine decreased TCR-elicited [Ca]_i increases and cytokine production in CD4⁺ T cells, arguing for a participation of Ca_v1 channels in the calcium response in these cells. Nicardipine did not inhibit proliferation, suggesting that the residual calcium response that persisted in the presence of nicardipine was sufficient to promote some degree of T-cell activation. Indeed, 10%

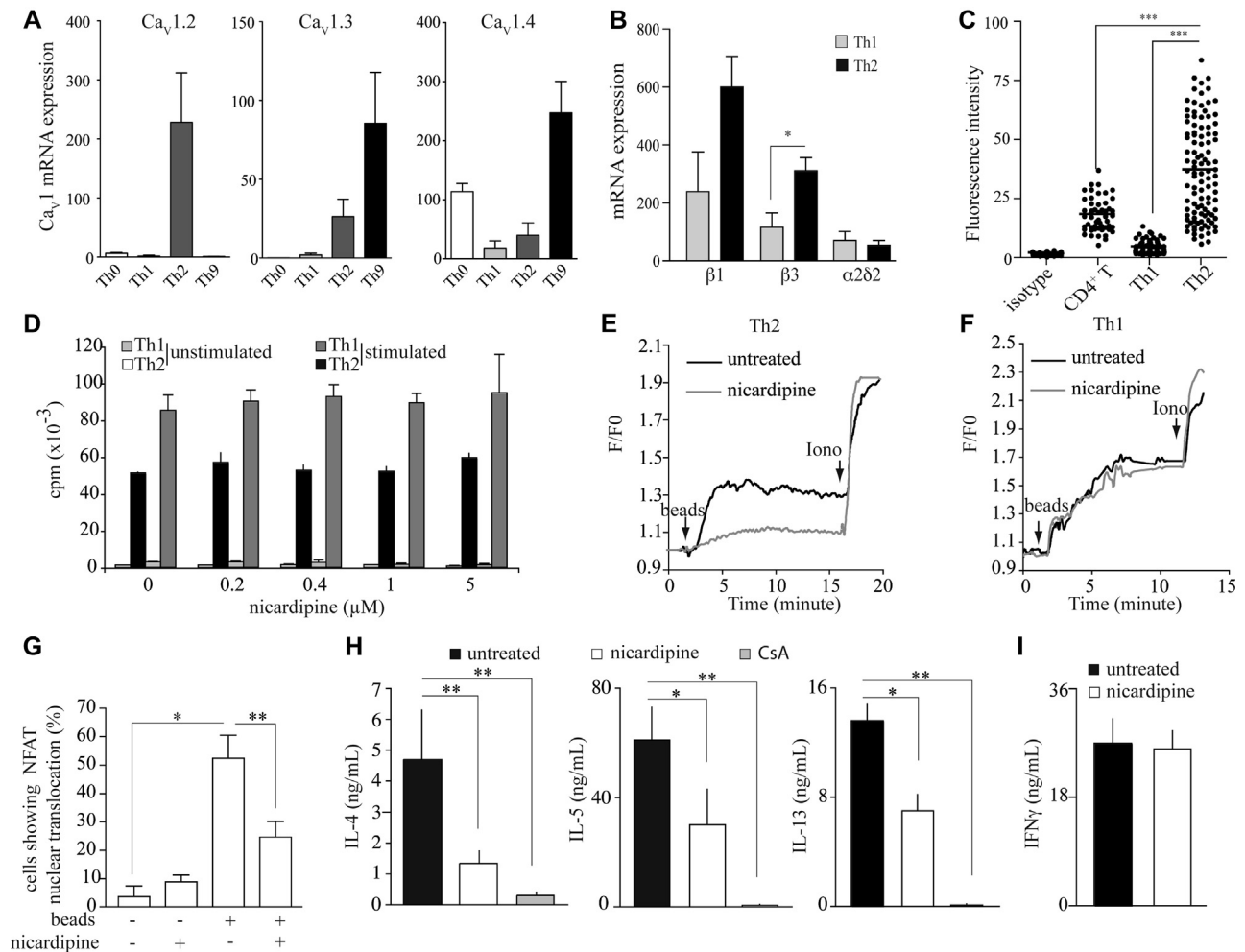


FIG 4. Ca_v1 expression and effect of nicardipine on *in vitro*-polarized T-cell functions. **A** and **B**, $Ca_v1\alpha1$ (Fig 4, A) and auxiliary (Fig 4, B) subunit expression in polarized T cells ($n = 4-5$). **C**, Intensity of $Ca_v1.2$ staining (1 experiment of 3). **D-I**, Effect of inhibitors on TCR-elicited proliferation (Fig 4, D), $[Ca]_i$ (Fig 4, E and F), NFAT nuclear translocation (Fig 4, G), and cytokine production (Fig 4, H and I) by Th2 (Fig 4, D, E, G, and H) and Th1 (Fig 4, D, F, and I) cells ($n = 5$). * $P < .05$, ** $P < .01$, and *** $P < .001$.

of the calcium response elicited by TCR engagement has been shown to be sufficient for T-cell proliferation.^{26,27} The predominance of $Ca_v1.4$ in T cells is in accordance with results in $Ca_v1.4$ null mice,⁷ which harbor defective proliferation of naive T cells, diminished $[Ca]_i$ stores, and reduced TCR-dependent calcium entry.

$Ca_v1.4$ was expressed in naive CD4⁺CD45RA⁺, CD4⁺CD45RA⁻CXCR3⁺ Th1, and CD4⁺CD4RA⁻CCR6⁺ Th17 cells, which could explain the inhibitory effect of nicardipine on cytokine production by these subsets. TCR-mediated activation of T cells induced a fast decrease in $Ca_v1.4$ expression, and accordingly, $Ca_v1.4$ was mostly expressed in *ex vivo*-isolated naive relative to memory CD4⁺ T cells, suggesting that $Ca_v1.4$ might play a more prominent role in T-cell survival or during the initial phase of T-cell activation. Contrary to CXCR3⁺CD4⁺ T cells, Th1 cells polarized *in vitro* expressed no detectable Ca_v1 transcripts, and nicardipine did not affect their IFN- γ production. CXCR3⁺CD4⁺ T cells are a heterogeneous population containing both central memory cells that do not produce effector cytokines but spontaneously differentiate to Th1 and effector

memory Th1 cells.²⁸ Long-term stimulated Th1 cells cultured in the presence of IL-12 could represent fully differentiated Th1 cells encountered, such as during inflammatory processes. The requirement of Ca_v1 channels for T-cell survival, function, or both could be different in these distinct populations. It will be interesting to assess whether Ca_v1 expression is also lost in Th1 cells *in vivo* on activation in inflammatory conditions.

Expanded Th2 cells overexpressed the $Ca_v1.2$ isoform as freshly isolated CRTH2⁺ cells compared with that seen in Th0, Th9, and Th1 polarized cells. The reasons for such differences in the expression pattern of Ca_v1 channels between polarized Th2 cells and other effector T cells could be related to the disparity in the TCR-driven calcium signature required to turn on appropriate genetic programs and effector functions in a given effector subtype. Accordingly, the calcium response is clearly different between Th1, Th2, and Th17 cells.²⁹⁻³¹ We believe that lineage-specific transcription factors might be involved in the regulation of Ca_v1 expression. Accordingly, we showed that the expression of GATA3 precedes the overexpression of $Ca_v1.2$ channels in mouse Th2 cells.¹⁸

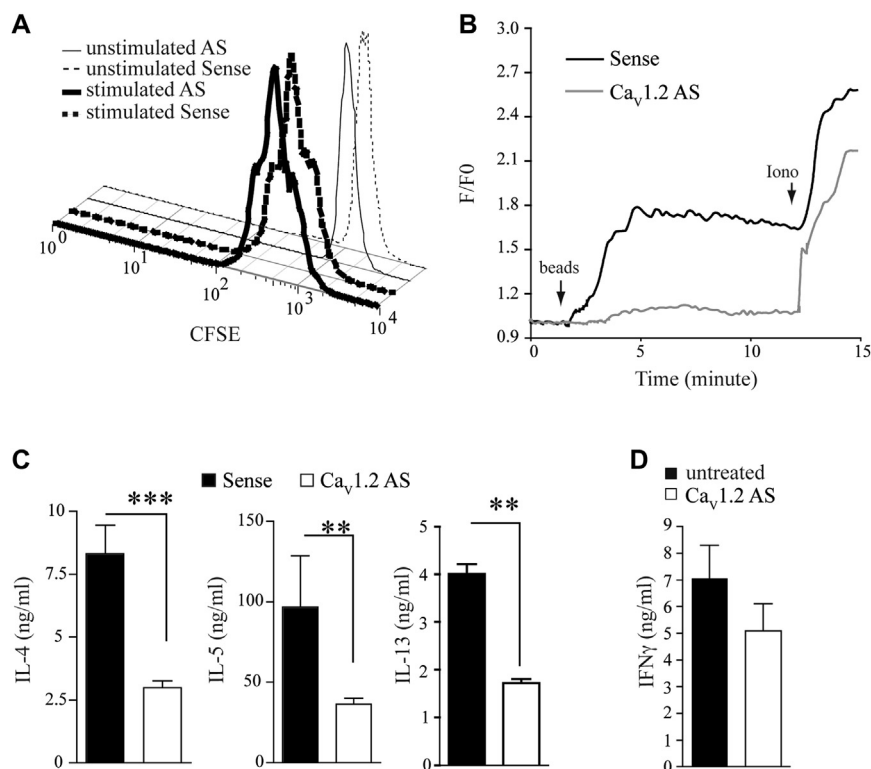


FIG 5. Knocking down Ca_v1.2 reduces TCR-driven calcium response and cytokine production by T_H2 cells with no effect on T_H1 cells. **A-C**, Proliferation (Fig 5, *A*), [Ca]_i variations (Fig 5, *B*), and cytokine production (Fig 5, *C*) in T_H2 cells transfected with Ca_v1.2AS activated with anti-CD3/anti-CD28-coated beads. **D**, IFN-γ production on TCR stimulation in T_H1 cells transfected with Ca_v1.2AS. Results are from 4 to 8 experiments. ***P* < .01 and ****P* < .001.

Two structurally distinct Ca_v1 channel inhibitors, the dihydropyridine antagonist nicardipine and the benzothiazepine antagonist diltiazem, inhibited IL-4, IL-5, and IL-13 production by T_H2 cells. Nicardipine acted by reducing the TCR-driven calcium response and T_H2 cytokine gene transcription without any effect on T_H1 cell functions, suggesting that the inhibitory effect of calcium blockers was related to their effects on Ca_v1 channels. Knocking down Ca_v1.2 with different Ca_v1.2 AS suppressed TCR-driven calcium response and T_H2 cytokine production in a dose-dependent manner, strongly suggesting that the inhibitory effect of nicardipine on T_H2 cells was related to its effect on Ca_v1.2. Transfection of T_H2 cells with Ca_v1.2AS inhibited approximately 50% of Ca_v1.2 mRNA and protein in T_H2 cells, suggesting that the partial loss of Ca_v1.2 molecules is sufficient to hamper calcium signaling and cytokine production in T_H2 cells. Interestingly, only a small portion of the Ca_v1.2 channels seem to be localized near the cell membrane, suggesting that a partial decrease in the total amount of Ca_v1.2 strongly affects the number of Ca_v1 channels required for the TCR-driven calcium response in T_H2 cells. Transfection with Ca_v1.2AS did not affect human T_H1 cell functions, which is consistent with the absence of Ca_v1.2 in these cells and is in agreement with our previous data in mice.¹⁸ The sequences of overlapping Ca_v1.2-specific PCR products from human T_H2 cells were compatible with expression of neuronal forms of the channels, as in mouse T_H2 cells.¹⁸ Moreover, the sequences coding the 4 putative voltage sensors that characterize classical Ca_v1 channels were found in human T_H2 cells, indicating that absence of

voltage sensitivity was not related to structural alterations in the lymphocyte-associated Ca_v1 form.

PKCα can activate Ca_v1 channels independently of cell membrane potential, even at resting cell membrane potential, in excitable^{32,33} and nonexcitable³⁴ cells. We previously reported that PKC contributed to Ca_v1 channel-dependent calcium entry in IL-4-producing T-cell hybridoma.³⁵ Herein we show that Gö6976, an inhibitor of PKCα/β, decreases the calcium response and cytokine production in human T_H2 lymphocytes. Moreover, direct activation of PKC by phorbol ester triggers [Ca]_i increase *per se* and cytokine production by T_H2 cells in a Ca_v1.2-dependent manner. The more pronounced effect of the PKCα/β inhibitor on T_H2 cell functions compared with the effect of nicardipine or Ca_v1.2AS might suggest additional effects of Gö6976 on other targets in T_H2 cells. In striking contrast, Gö6976 had no effect on T_H1 functions, indicating that PKCα/β-independent signaling pathways are responsible for calcium entry and IFN-γ production in this subset.

We identified Ca_v1.2 as a critical regulator of the Ca²⁺ response and cytokine production in human highly polarized T_H2 cells, whereas this channel was not expressed in others subsets, including T_H1, T_H9, and T_H17 cells. Nicardipine is broadly used in the treatment of hypertensive patients, although at lower doses than those required to induce an effect on T_H2 cells (approximately 1-5 μmol/L), which could be due to the fact that these drugs have a better affinity for dihydropyridine at depolarized cell membrane potentials.³⁶ This could in part explain why dihydropyridine antagonist treatments administered to hypertensive

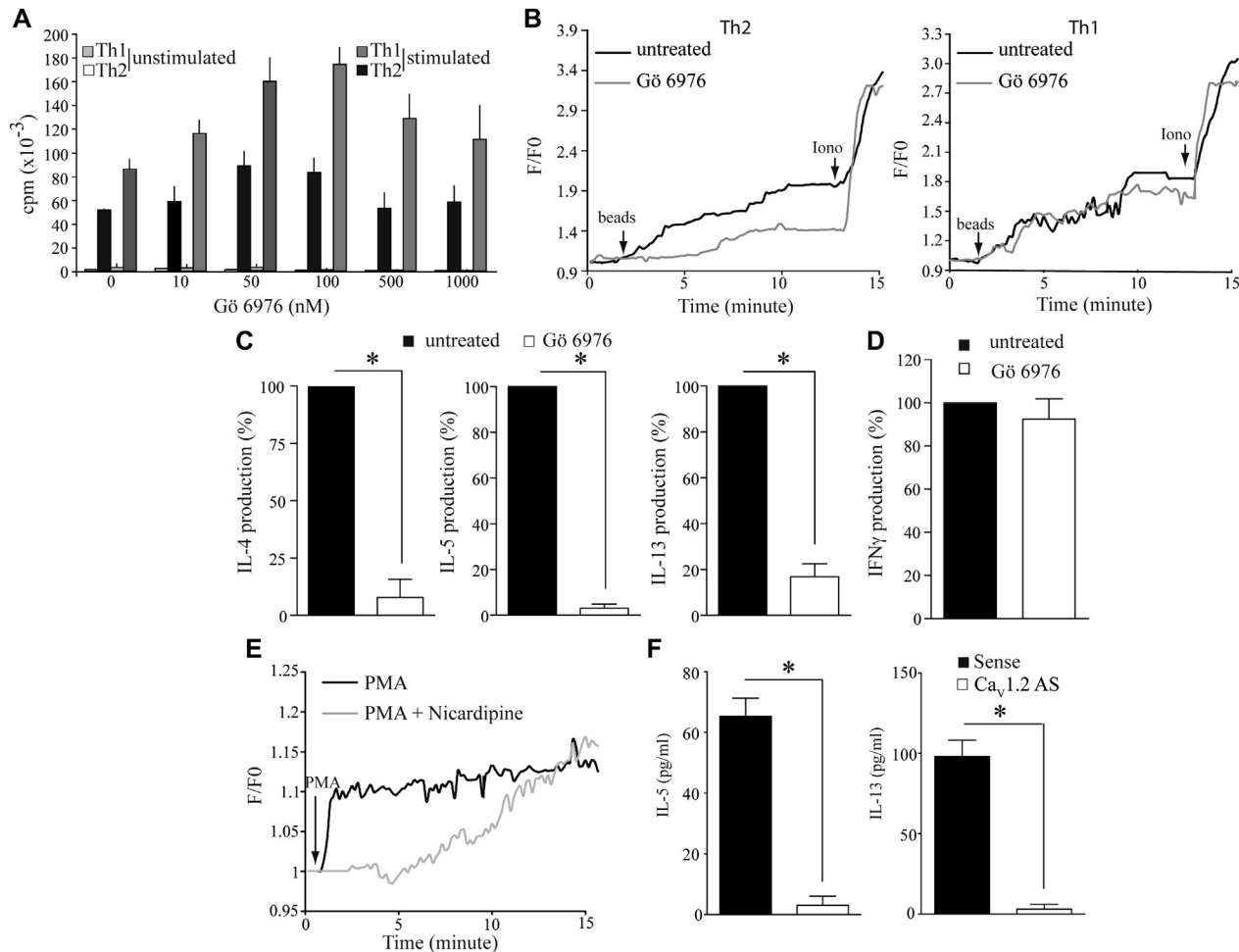


FIG 6. PKC-dependent $\text{Ca}_v1.2$ regulation in T_H2 cells. **A-D**, Effect of Gö6976 on TCR-driven proliferation (Fig 6, A), $[\text{Ca}]_i$ increase (1 experiment of 4; Fig 6, B), and cytokine production (Fig 6, C and D) by T_H2 (Fig 6, A-C) and T_H1 (Fig 6, A, B, and D) cells ($n = 5$). **E** and **F**, Effect of PMA on $[\text{Ca}]_i$ (1 experiment of 3; Fig 6, E) and cytokine (Fig 6, F) production by T_H2 cells with or without nicardipine (Fig 6, E) or after transfection with $\text{Ca}_v1.2\text{AS}$ (Fig 6, F; $n = 4$). * $P < .05$.

patients have not been reported thus far to be beneficial in the treatment of allergies. The development of more effective dihydropyridine antagonists or AS ODNs targeting $\text{Ca}_v1.2$ channels expressed by T_H2 cells might offer new opportunities in the treatment of T_H2 -mediated allergic diseases, preserving the responses of subsets, such as T_H1 lymphocytes, that are important effectors of the adaptive immune responses against pathogens and tumors.

We thank Dr B. Constantin (UMR CNRS 6187, University of Poitiers, France) for providing cDNA samples from human skeletal muscle, Sophie Allart and Astrid Canivet (Cellular Imaging, CPTP), and Fatima L'Faqihi and Valérie Duplan (Cytometry platform, CPTP).

Clinical implications: The $\text{Ca}_v1.2$ channel subunit and $\text{PKC}\alpha/\beta$ might provide potential therapeutic targets selectively involved in Ca^{2+} signaling and cytokine regulation by human T_H2 cells.

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METHODS

Reagents

Nicardipine and cyclosporin A were purchased from Novartis (Basel, Switzerland), and Gö6976, the inhibitor of PKC α/β , was purchased from Calbiochem (San Diego, Calif). Diltiazem and PMA were from Sigma (St Louis, Mo). Except when otherwise mentioned, we used nicardipine at 5 $\mu\text{mol/L}$, cyclosporin A at 0.1 $\mu\text{g/mL}$, Gö6976 at 1 $\mu\text{mol/L}$, and PMA at 200 ng/mL. The following antibodies were purchased from BD Bioscience (San Jose, Calif): anti-CD8–phycoerythrin (PE), anti-CD4–fluorescein isothiocyanate (FITC), anti-CD19–PE, anti-CD4–Pacific blue, anti-CD45RA–FITC, anti-CCR6–PE, anti-CXCR3–allophycocyanin, anti-IL-4–allophycocyanin, anti-IL-2–PE, and anti-IFN- γ –FITC. Anti-IL-5–PE and anti-IL-13–PerCP-Cy5.5 were purchased from BioLegend (Ozyme, Saint Quentin en Yvelines, France).

Purification of human leukocyte populations

PBMCs were obtained from “Etablissement Français du Sang,” and all human participants provided written informed consent. Monocytes were purified with the human monocyte enrichment kit (Stem Cell, Grenoble, France). Eosinophils and neutrophils were purified from Ficoll-Paque PLUS gradient pellets with the eosinophil negative selection kit (Stem Cell). The negative fraction contained eosinophils, and the positive fraction contained neutrophils.

Purification of CD4⁺, CRTH2⁺, T_H1, and T_H17 cells

CD4⁺ T cells were isolated with the CD4-untouched isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). CD4⁺CRTH2⁺ cells (80% $\pm$ 5% pure) enriched in memory T_H2 cells^{E1,E2} were isolated with the CRTH2 positive isolation kit (Miltenyi Biotech). Isolated CXCR3⁺CCR6[−] (T_H1) and CCR6⁺ (T_H17) cells were from CD4⁺CRTH2[−] fractions labeled with anti-CCR6 and anti-CXCR3 antibodies and sorted on a FACSAria (BD Biosciences).

Generation of human T_H0, T_H1, T_H2, and T_H9 effector lymphocytes

We expanded CD4⁺CRTH2⁺ cells (2 $\times$ 10⁵ in a 96-well plate) by means of stimulation with anti-CD3/anti-CD28–coated beads (1 bead for 4 cells; Invitrogen, Carlsbad, Calif) for 15 days in complete medium (RPMI 1640 supplemented with 10% FCS (Lonza, Allendale, NJ), 1% pyruvate, 1% nonessential amino acids, 2 mmol/L glutamine, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 50 $\mu\text{mol/L}$ β -mercaptoethanol containing IL-2 (5 ng/mL; PeproTech, London, United Kingdom), IL-4 (5 ng/mL, PeproTech), and anti-IFN- γ antibodies (5 $\mu\text{g/mL}$, BD Bioscience), which resulted in a 4- to 7-fold expansion of T_H2 cells. T_H1 cells were obtained by stimulating CD4⁺CRTH2[−] cells for 7 days with anti-CD3/anti-CD28–coated beads (1 bead per 4 cells) in complete medium containing IL-12 (5 ng/mL, PeproTech), IL-2 (5 ng/mL), and anti-IL-4 (5 $\mu\text{g/mL}$, BD Bioscience) antibodies. T_H0 and T_H9 cells were obtained by stimulating CD4⁺CD45RA⁺ T cells for 7 days with anti-CD3/anti-CD28–coated beads (1 bead per 4 cells) in complete medium alone or in medium supplemented with IL-2 (5 ng/mL), IL-4 (25 ng/mL), TGF- β (5 ng/mL, PeproTech), and anti-IFN- γ antibody (5 $\mu\text{g/mL}$), respectively.

RNA preparation and reverse transcription

Total RNA was isolated with a kit from Qiagen (Hilden, Germany) and retrotranscribed in cDNA by means of reverse transcription with SuperScript III (Invitrogen).

Cell proliferation

Cell division was assessed in cells (0.5 $\times$ 10⁶ cells per well in 24-well plates) stained with 2.5 $\mu\text{mol/L}$ CFSE (Invitrogen) for 10 minutes and stimulated with anti-CD3/anti-CD28–coated beads for 72 hours before analysis by means of flow cytometry. Resting CFSE-labeled cells were used to determine the initial labeling. Proliferation was also assessed by means of measurement of tritiated thymidine uptake. Cells were pulsed during the last 8 hours of culture with 1 μCi (37 KBq) of tritiated thymidine (40 Ci/nmol; Radiochemical Center, GE Healthcare, Little Chalfont, United Kingdom). Cells were harvested onto glass fiber filter membranes, and tritiated TdR incorporation was measured with a MicroBeta Trilux luminescence counter (PerkinElmer, Waltham, Mass).

Intracellular cytokine staining

Cells (10⁶) were activated with PMA (50 $\mu\text{g/mL}$, Sigma-Aldrich) and ionomycin (50 $\mu\text{g/mL}$, Sigma-Aldrich) for 4 hours with brefeldin A (5 $\mu\text{g/mL}$; eBioscience, San Diego, Calif) added for the final 2 hours or with anti-CD3/anti-CD28–coated beads (1 bead for 4 cells) for 6 hours with brefeldin A added for the final 3 hours. Cells were then fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin for cytokine staining. Intracellular cytokine staining obtained on a FACSCalibur (BD Biosciences) was analyzed by using FlowJo software (TreeStar, Ashland, Ore).

Cytokine determination

T cells were seeded (5 $\times$ 10⁴ cells per well) into 96-well flat-bottom plates and stimulated with anti-CD3/anti-CD28–coated beads (1 bead per 4 cells) for 24 hours, and cytokine production was quantified by means of ELISA. Plates were coated with anti-IL-4 or anti-IFN- γ (3 $\mu\text{g/mL}$, Ozyme), IL-5 (2 $\mu\text{g/mL}$), anti-IL-13 (1 $\mu\text{g/mL}$), or anti-IL-17 A (1 $\mu\text{g/mL}$, eBioscience). Cytokines were revealed by means of incubation with biotinylated anti-IL-4 (2.5 $\mu\text{g/mL}$), IFN- γ (3 $\mu\text{g/mL}$), IL-5 (0.5 $\mu\text{g/mL}$), IL-13 (1.5 $\mu\text{g/mL}$), and IL-17 (0.25 $\mu\text{g/mL}$), followed by alkaline phosphatase–streptavidin and its PA substrate (Sigma). Absorbance was read at 650 nm. The sensibility for all ELISAs was 50 pg/mL. IL-2 production was measured by means of bioassay with the CTLL-2 cell line.

Fluorescence microscopy

For NFAT translocation measurement, T_H2 cells (10⁶ cell per well in 24-well plates) were stimulated with α -CD3/ α -CD28–coated beads for 24 hours, transferred onto poly-D-lysine–coated slides, and fixed with 4% paraformaldehyde. After permeabilization, cells were stained with anti-NFATc1 antibody (BD Bioscience) and then with goat anti-mouse IgG1 antibody (Molecular Probes, Eugene, Ore). Nuclei were stained with 5 $\mu\text{g/mL}$ 4'-6-diamidino-2-phenylindole dihydrochloride (Invitrogen). For intracellular Ca_v1.2 staining, cells were labeled with anti-Ca_v1.2 mAb (Neuromab, Davis, Calif) and Alexa Fluor 555–labeled goat anti-mouse IgG₂ b. Images were acquired with an LSM 710 inverted confocal microscope (Carl Zeiss AG, Jena, Germany) equipped with a Plan Apochromat X63 (1.4 oil). Staining with control isotype did not result in detectable fluorescence. The fluorescence intensities were determined with ImageJ software. Cells whose NFAT was translocated in the nucleus were defined as having a nuclear/cytosol ratio superior to the mean + 2 SD of values in resting cells.

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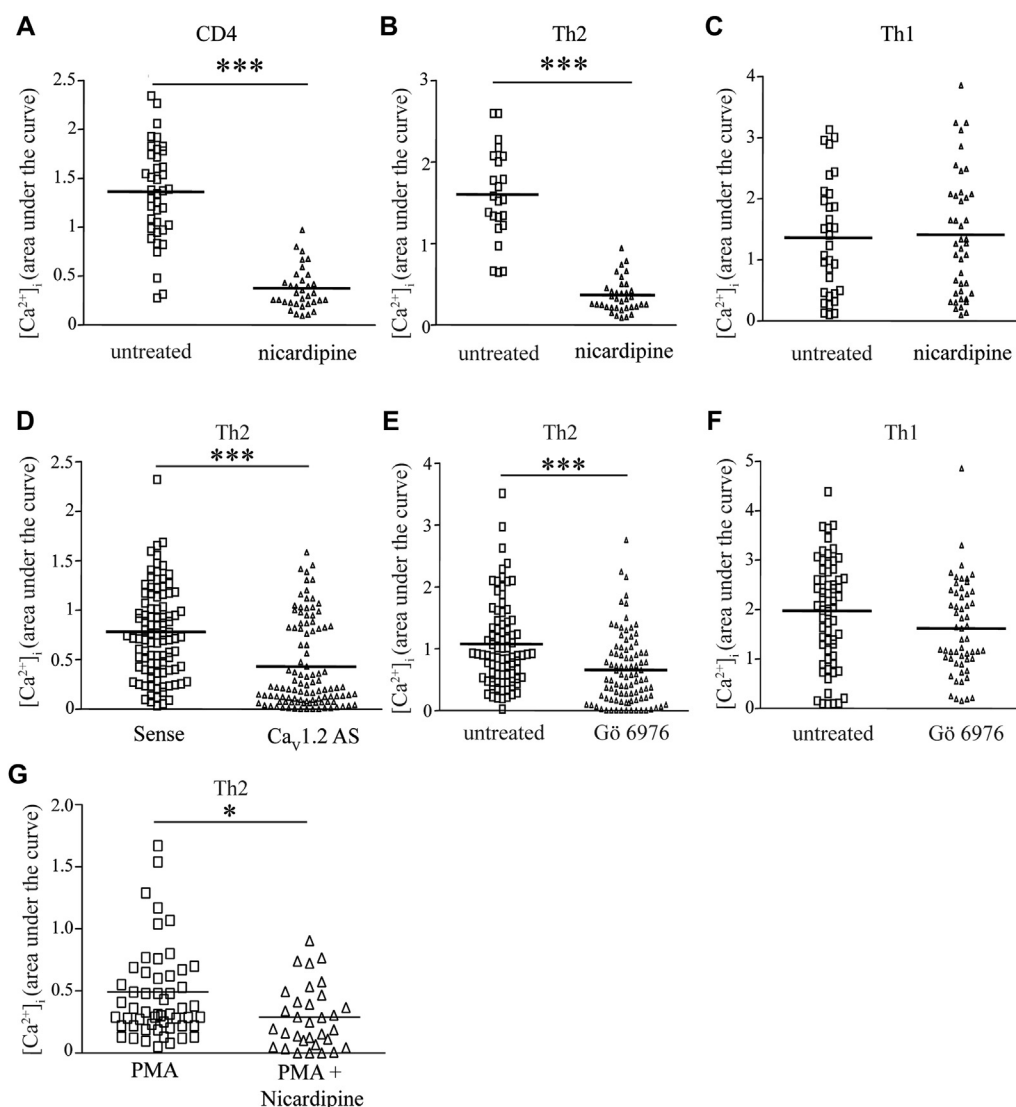


FIG E1. Ca_v1.2- and PKC-dependent regulation of [Ca]_i concentrations in human T_H2 cells. **A-C** and **E-G**, CD4⁺ (Fig E1, A), T_H2 (Fig E1, B, E, and G), or T_H1 (Fig E1, C and F) cells were preincubated or not with nicardipine (5 μmol/L; Fig E1, A-C and G) or PKCα/β inhibitor (1 μmol/L; Fig E1, E and F). **D**, T_H2 cells were transfected with Ca_v1.2 AS or sense ODNs. All the cells were loaded with Fura-2AM and recorded under baseline conditions and after stimulation with anti-CD3/anti-CD28-coated beads (Fig E1, A-F) or with PMA (200 ng/mL; Fig E1, G). Results were expressed as the area under the curve after stimulation, as described in the [Methods](#) section. Each symbol represents 1 cell, and the results are representative of 4 to 8 experiments from 2 to 4 donors (Fig E1, A-F) and 2 experiments from 2 donors (Fig E1, G). **P* < .05 and ****P* < .0001.

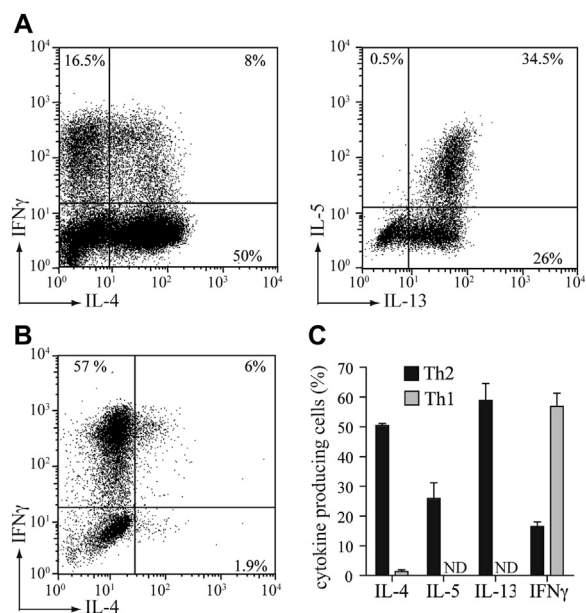


FIG E2. Characterization of human expanded CD4⁺CRTH2⁺ and differentiated T_H1 cells. CRTH2⁺ and CRTH2⁻ cells purified from CD4⁺ cells were expanded with anti-CD3/anti-CD28-coated beads in the presence of IL-4, IL-2, and anti-IFN-γ antibody (for 15 days) and in the presence of IL-12, IL-2, and anti-IL-4 antibody (for 7 days), respectively. Cells were then stimulated with PMA and ionomycin for 4 hours. Brefeldin A was added for the last 2 hours. **A** and **B**, Intracellular IL-4, IL-5, IL-13, and IFN-γ staining of CRTH2⁺ (Fig E2, **A**) and CRTH2⁻ (Fig E2, **B**) is shown. **C**, The graph represents means + SDs of the percentages of cytokine-producing cells from 6 donors. ND, Not done.

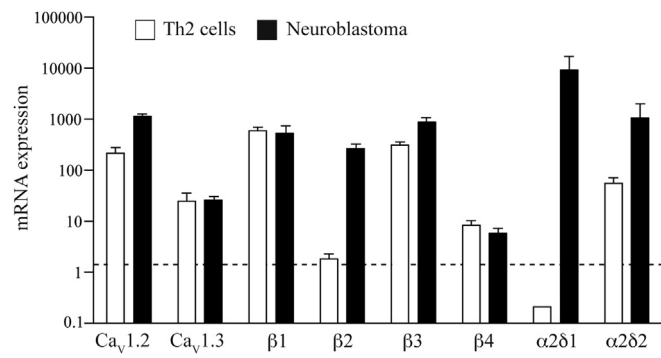


FIG E3. Comparative expression of Ca_v1α1 and auxiliary subunits between T_H2 cells and the neuroblastoma. RNA was extracted from expanded T_H2 cells and from the SHY5Y neuroblastoma cell line used as a positive control. They were retrotranscribed in cDNA, and the expression of Ca_v1.2 and Ca_v1.3 subunits, as well as the expression of auxiliary β1, β2, β3, β4, α2δ1, and α2δ2 subunits, was quantified by using qPCR. The histogram represents the mean + SD of 3 experiments. The *dotted line* indicates the limit of qPCR product detection.

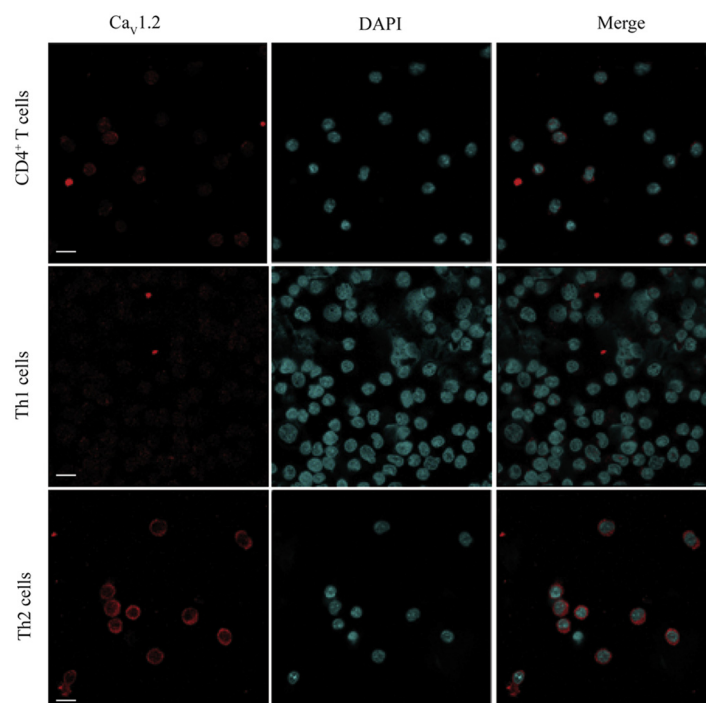


FIG E4. $\text{Ca}_v1.2$ protein levels are selectively upregulated in $\text{T}_\text{H}2$, but not $\text{T}_\text{H}1$, cells. CD4^+ , $\text{T}_\text{H}1$, and $\text{T}_\text{H}2$ cells were permeabilized and stained with anti- $\text{Ca}_v1.2$ mAb, followed by incubation with secondary Alexa Fluor 555 goat anti-mouse IgG_{2b} antibody. Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed by means of confocal microscopy. One experiment representative of 2 donors is shown. Bars = 10 μm .

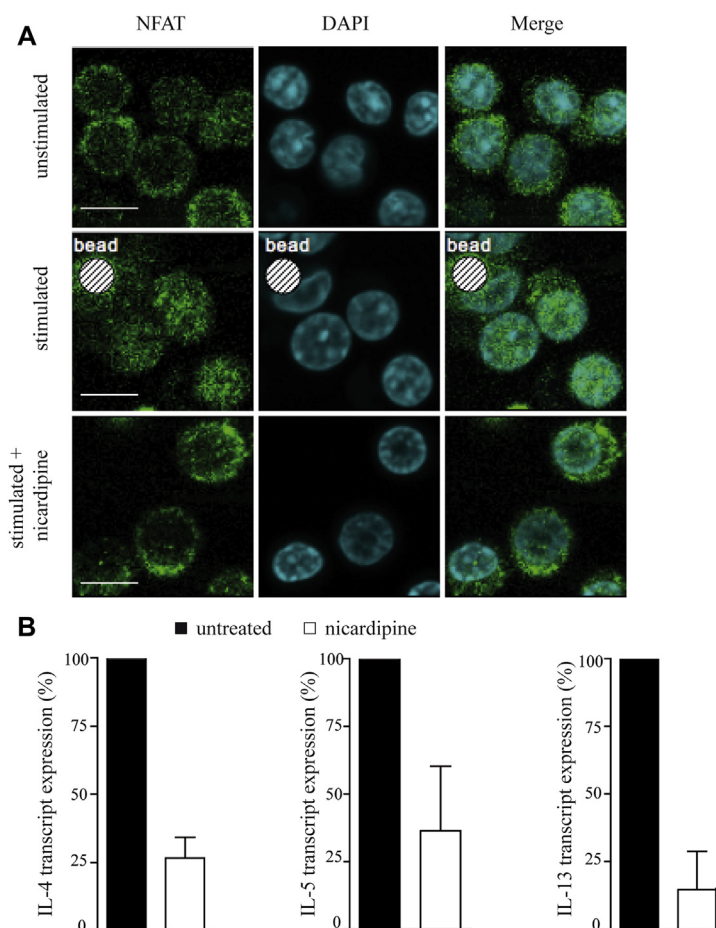


FIG E5. Nicardipine reduces NFAT nuclear translocation and cytokine expression in T_H2 cells. **A**, T_H2 cells were stimulated or not with anti-CD3/anti-CD28-coated beads in the presence or not of nicardipine ($5 \mu\text{mol/L}$) for 24 hours. Then these cells were permeabilized and stained with anti-NFATc1 antibody and secondary Alexa Fluor 488 goat anti-mouse IgG₁ antibody. Nuclei were distinguished by means of 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cells were analyzed by using confocal microscopy. One experiment representative of 2 donors is shown. Bars = $10 \mu\text{m}$. **B**, T_H2 cells were stimulated or not with anti-CD3/anti-CD28-coated beads in the presence or not of nicardipine ($5 \mu\text{mol/L}$) for 4 hours. Cytokine mRNA expression was assessed by using qPCR. Results were expressed as means + SDs of 3 donors.

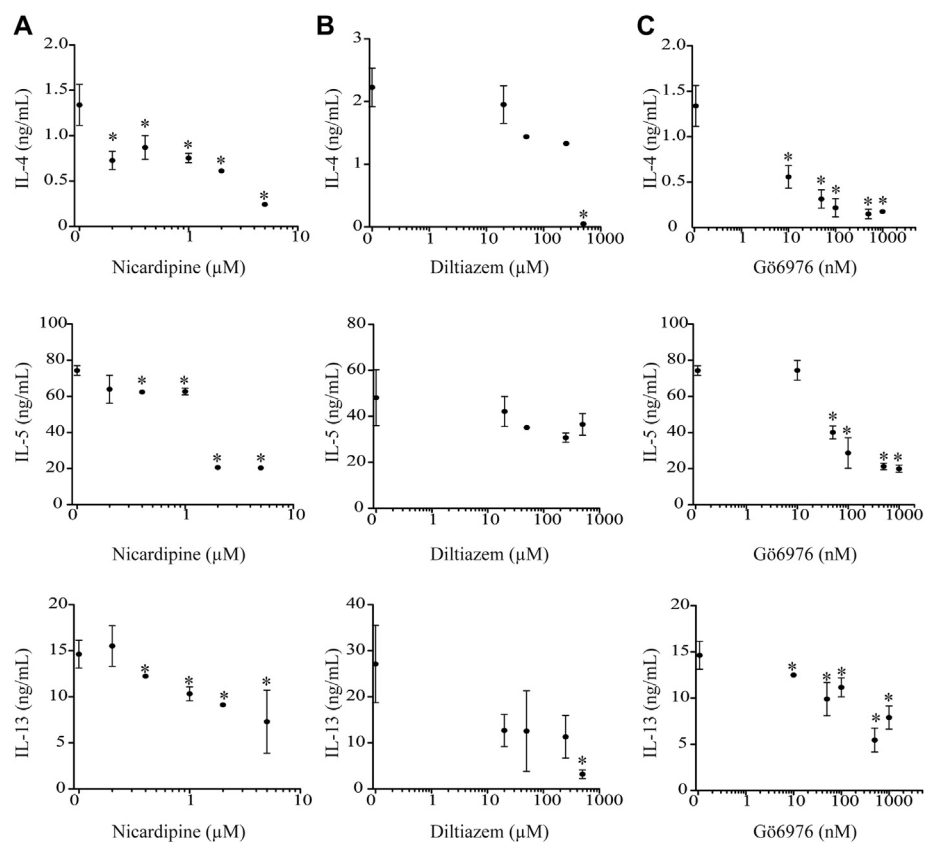


FIG E6. Nicardipine, diltiazem, and Gö6976 reduce T_H2 cytokine production in a dose-dependent manner. Human T_H2 cells were collected after expansion, washed, and stimulated for 24 hours with anti-CD3/anti-CD28-coated beads without any cytokine in the presence of increasing concentrations of nicardipine (**A**), diltiazem (**B**), or Gö6976 (**C**). Cytokine content was measured by means of ELISA. Results were expressed as means + SDs of 4 to 6 cultures. * $P < .02$.

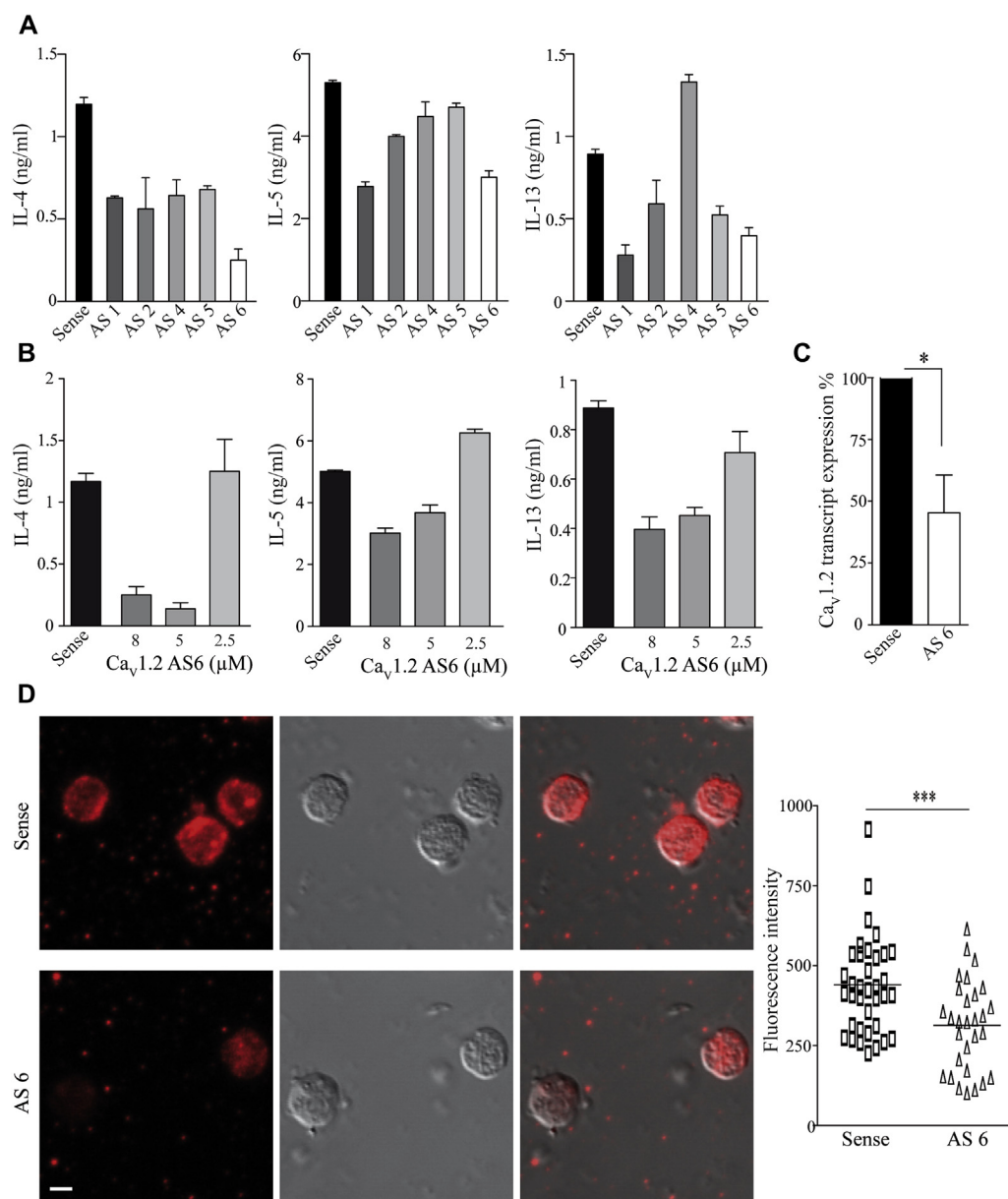


FIG E7. Ca_v1.2AS decreases cytokine production and Ca_v1.2 expression at the mRNA and protein levels in T_H2 cells. **A** and **B**, T_H2 cells were transfected with different Ca_v1.2AS (8 μmol/L) or sense ODNs for 72 hours. Transfected T_H2 cells were then assessed for cytokine production after 24 hours of TCR stimulation. Results are expressed as means + SDs of 3 to 6 cultures and are representative of 2 donors. **C**, Transfected T_H2 cells were assessed for Ca_v1.2 mRNA expression by means of qPCR. Results are expressed as means + SDs of 6 donors. **D**, Intracellular Ca_v1.2 staining was performed in sense and Ca_v1.2AS transfected T_H2 cells. The histogram shows quantification of the total fluorescence intensity. Each symbol represents 1 cell. One experiment representative of 4 from 2 donors is shown. Bars = 5 μm. **P* < .05 and ****P* < .001.

TABLE E1. List of primers used

Genes	Forward (5'-3')	Reverse (5'-3')
Ca ν 1.1	CAG-GCT-TGA-ACA-AAA-TCA-TCC-A	CTT-CAC-GAT-GTC-ATG-GCA-CTT-C
Ca ν 1.2	GGC-TAC-CTG-GAT-TGG-ATC-ACT-C	GTG-AGA-CCG-AGT-CCG-TCA-ACA
Ca ν 1.3	AAG-TCT-CTG-GTG-CTG-GTG-GA	GCC-CTA-CAT-CTT-CTG-CGA-TT
Ca ν 1.4	GTT-CCA-TGA-CAG-AGA-CCC	GCG-GCA-GAC-TCT-GGT-TTT-CA
β 1	TCG-AGC-CCA-AAG-ACT-TCC-T	CGA-AGG-CTG-TCC-AGT-TTG-A
β 2	GCC-ATC-TCA-TTC-GAA-GCA-A	GCT-GCA-GCC-TCA-TGT-TTT-C
β 3	GCC-CAT-CTC-TGG-ACT-CAG-AC	AGC-TGA-CAT-TGG-TCC-TCA-CC
β 4	CGG-ATC-CAG-CAA-GAA-CAA-A	GGG-AGT-TGC-TCG-GAA-TGT-C
α 2 δ 1	AAT-TGC-AGC-CAG-GGA-TAT-TG	CAC-TGG-TGA-GCT-GCT-TGA-AC
α 2 δ 2	TGC-TGC-AGA-GAA-CTT-CCA-GA	TCC-TCA-CTC-TCA-GGG-TCG-C
α 2 δ 3	TGA-CGT-CCA-AGT-ACC-AAC-GA	GAT-GGG-TCA-CGG-TCA-AAG-TT
α 2 δ 4	GAG-GAC-ATG-GAG-AAC-ATG-CTG	TTC-TCG-TCCTC-TCG-TTG-AT
<i>IL4</i>	TCT-GTG-CAC-CGA-GTT-GAC-CGT-AAC-A	AGC-CCG-CCA-GGC-CCC-AGA
<i>IL5</i>	GCT-TCT-GCA-TTT-GAG-TTT-GCT-AGC-T	TGG-CCG-TCA-ATG-TAT-TTC-TTT-ATT-AAG
<i>IL13</i>	AAC-CAG-AAG-GCT-CCG-CTC-TGC-AA	AGT-TGA-GTC-CCT-CGC-GAA-AAA
<i>IL9</i>	CAA-ACA-AGA-TAC-CCA-CTG-ATT-TTC-A	TTG-GTT-GCA-TGG-CTG-TTC-AC
<i>IL17</i>	CAC-CTC-ACC-TTG-GAA-TCT-CCA	TGG-CGG-CAC-TTT-GCC-T
<i>IFNG</i>	GCA-TCC-AAA-AGA-GTG-GGA-GAC-CAT-CA	CTG-GGA-TGC-TCT-TCG-ACC-TTG-AAA-CA
<i>GAPDH</i>	AGC-ACC-AGG-TGG-TCT-CCT-CT	CCA-AAT-TCG-TTG-TCA-TAC-CAG

GAPDH, Gluceraldehyde-3-phosphate dehydrogenase.

TABLE E2. Profile of cytokine production by T_H0, T_H1, and T_H2 cells on *in vitro* expansion

Cell type	IFN- γ (ng/mL)		IL-4 (ng/mL)	
	Unstimulated	Stimulated	Unstimulated	Stimulated
T _H 0	0.5 $\pm$ 0.2	18 $\pm$ 2.4*	ND	0.05 $\pm$ 0.02
T _H 1	0.33 $\pm$ 0.2	20 $\pm$ 2.4*	ND	0.12 $\pm$ 0.02
T _H 2	0.1 $\pm$ 0.2	0.5 $\pm$ 0.15	ND	4.6 $\pm$ 0.18*

After 7 days of culture in polarizing conditions, CD4⁺ T cells were washed and stimulated or not with anti-CD3/anti-CD28-coated beads for 24 hours. The cytokine content was measured by means of ELISA. Results are expressed as means $\pm$ SDs of 6 experiments.

ND, Not detected.

* $P < .005$ when compared with resting cells.

TABLE E3. Profile of cytokine expression in generated T_H9 cells

T _H 9	<i>IFNG</i>	<i>IL4</i>	<i>IL9</i>
Resting	5, 0	1, 1	212, 440
Stimulated	1, 5	4, 2	2636, 3244

After 7 days of culture in T_H9-polarizing conditions, cells were washed and stimulated or not with anti-CD3/anti-CD28-coated beads for 6 hours before mRNA extraction. Cytokine expression was measured by means of qPCR. Results were expressed as $2^{-\Delta Ct} \times 10^{-5}$ (relative to gluceraldehyde-3-phosphate dehydrogenase expression). Individual values from 2 differentiations are shown.

TABLE E4. Sequences of Ca_v1.2-specific antisense ODNs

Oligonucleotides	Sequences (5'-3')	NM_001129839	Exon targeted
AS1	TCC GTG CTG TTG CTG GGC TCA	4599-4581	37
AS2	ACT CTG GGG CAC ACT TCT TG	4648-4577	37
AS4	TCC TCT CTC CCA AAC CCA CCT	7891-7871	50
AS5	CCT TCT CCT CTT CCT CCT CCT	2633-2613	16
AS6	CCT CGT ATT CTC ATT GAC CAT	334-314	1