

Knocking Down Ca_v1 Calcium Channels Implicated in Th2 Cell Activation Prevents Experimental Asthma

Marilena Djata Cabral^{1,2,3,7*}, Pierre-Emmanuel Paulet^{1,2,3,7*}, Virginie Robert^{1,2,3,7*}, Bruno Gomes^{1,2,3}, Marie-Laure Renoud^{1,2,3}, Magali Savignac^{1,2,3,5}, Catherine Leclerc^{2,4,5}, Marc Moreau^{2,4,5}, David Lair⁷, Marie Langelot⁷, Antoine Magnan⁷, Hans Yssel⁸, Bernard Mariame^{1,2,3}, Jean-Charles Guéry^{1,2,3,6}, and Lucette Pelletier^{1,2,3,5,6}

¹Institut National de la Santé et de la Recherche Médicale (INSERM), U563, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France; ²Université Paul Sabatier, Toulouse, France; ³Institut Fédératif de la Recherche Bio-Médicale de Toulouse, Toulouse, France; ⁴Centre National de la Recherche Scientifique (CNRS), UMR 5547, Centre de Biologie du Développement, Toulouse, France; ⁵CNRS Groupement de recherche, GDR 2688, France; ⁶Centre Hospitalier Universitaire, Toulouse, France; ⁷INSERM, U915, L'institut du thorax, Equipe AVENIR, Faculté de Médecine, Université de Nantes, CHU Nantes, L'institut du thorax, Service de Pneumologie, Plate-Forme Transversale d'Allergologie, Nantes, France; ⁸INSERM, U844, Montpellier, France

Rationale: Th2 cells orchestrate allergic asthma and the cytokines they produce (IL-4, IL-5, and IL-13) are deleterious in allergy. Therefore, it is important to identify key signaling molecules expressed by Th2 cells that are essential for their function. We have previously shown that dihydropyridines selectively modulate Th2 cell functions.

Objectives: Because dihydropyridines bind to and modulate voltage-dependent calcium (Ca_v1) channel in excitable cells, we aimed at showing that Th2 cells selectively express functional Ca_v1-related channels, the inhibition of which may prevent asthma.

Methods: We looked for Ca_v1 channel expression in Th2 and Th1 cells by real-time polymerase chain reaction and Western blotting. We sequenced the isoforms expressed by Th2 cells and tested whether Ca_v1 antisense oligodeoxynucleotides (Ca_v1AS) affected Ca²⁺ signaling and cytokine production. Finally, we tested the effect of Ca_v1AS in the passive asthma model by injection of ovalbumin-specific Th2 cells transfected with Ca_v1AS into BALB/c mice challenged with intranasal ovalbumin and in the active model of asthma by intranasal delivery of Ca_v1AS together with soluble ovalbumin in BALB/c mice previously immunized with ovalbumin in alum.

Measurements and Main Results: We show that mouse Th2 but not Th1 cells expressed Ca_v1.2 and Ca_v1.3 channels. Th2 cells transfected with Ca_v1AS had impaired Ca²⁺ signaling and cytokine production, and lost their ability to induce airway inflammation on adoptive transfer. Furthermore, intranasal administration of Ca_v1AS suppressed airway inflammation and hyperreactivity in an active model of asthma.

Conclusions: These results indicate that Th2 cells selectively express Ca_v1 channels that may be efficiently targeted in T lymphocytes to prevent experimental asthma.

Keywords: Th2 cells; voltage-dependent calcium channels; allergic asthma; antisense oligonucleotides

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Ca²⁺ is an intracellular second messenger that is differentially regulated in T-cell subsets and especially in Th2 lymphocytes. Whereas store-operated calcium channels CRAC/Orai are now admitted as the channels responsible for Ca²⁺ influx in T cells, the role of other calcium channels including voltage-dependent Ca²⁺ (Ca_v1) channels is still a matter of debate.

What This Study Adds to the Field

We show that Ca_v1.2 and Ca_v1.3 channels are selectively expressed in murine Th2 cells and that Ca_v1-specific antisense oligonucleotides block specifically TCR-driven Ca²⁺ signalling and Th2 cytokine production. Moreover, intranasal delivery of these oligonucleotides prevented the development of asthma, offering a rationale for developing drugs targeting these channels in Th2 lymphocytes.

Effector T-cell subsets produce distinct sets of cytokines and may cause different immune-mediated diseases. Among them, Th2 cells that produce IL-4, -5, and -13 are key mediators of allergic asthma both in experimental models and in asthmatic patients (1, 2). However, the inhibition of one or the other Th2 cytokine was disappointing in the treatment of asthma as shown by the low impact of anti-IL-4 (1) or anti-IL-5 antibody administration on the course of allergic diseases (3).

Intracellular Ca²⁺ is a second messenger essential for numerous T-cell functions (4). The fact that the regulation of intracellular Ca²⁺ concentration ([Ca²⁺]_i) is different in various T-cell effectors (5–9) may offer the opportunity to target key intermediates of Ca²⁺ signaling to inhibit specifically the functions of one given T-cell subset. It is classically admitted that stimulation through the T-cell receptor (TCR) induces inositol triphosphate production that binds to its receptors on the membrane of the endoplasmic reticulum (ER) leading to the release of ER Ca²⁺ stores into the cytosol. Stim-1, considered as the sensor of the ER Ca²⁺ depletion, redistributes toward the cell membrane activating the store-operated Ca²⁺ channels (SOC) CRAC/Orai, resulting in Ca²⁺ entry from the external medium (reviewed in [10]). However, other calcium channels at the cell membrane (11, 12) might contribute to sustained calcium signaling in activated T cells. Among them, several groups reported the expression of some Ca_v1-related Ca²⁺ channels in T cells and showed that dihydropyridines known to antagonize Ca_v1 channels may inhibit

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* These authors contributed equally to this study.

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Correspondence and requests for reprints should be addressed to Lucette Pelletier, M.D., Ph.D., INSERM U563, Bâtiment A, Place du Dr Baylac, BP 028, 31024, Toulouse Cedex 3, France. E-mail: lucette.pelletier@inserm.fr

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T-cell functions (13–15). However, the molecular identity of these channels was not clear and direct evidence for a functional role of these channels in T-cell activation is lacking (13–19).

We have previously shown that Th2 cells expressed dihydropyridine receptors (DHPR) (20, 21) defined as Ca_v1 channels in excitable cells (22) and that inhibitors of DHPR prevented experimental asthma (21). Herein, we show that differentiation in Th2 cells but not in Th1 cells was associated with the up-regulation of Ca_v1.2 and Ca_v1.3 channels both at the mRNA and protein level. The sequences of these channels presented high homology to neuronal isoforms of Ca_v1.2 and Ca_v1.3 channels. In addition, transfection with Ca_v1-specific antisense (Ca_v1AS) oligodeoxynucleotides (ONs) suppressed DHPR expression, calcium signaling, and the production of cytokines by Th2 cells with no impact on Th1-cell functions. Moreover, ovalbumin (OVA)-specific transgenic Th2 cells transfected with Ca_v1AS were no longer able to induce asthma on adoptive transfer in BALB/c mice given intranasal OVA. Finally, intranasal administration of Ca_v1AS at the time of intranasal challenge with OVA was effective in active experimental asthma, preventing airway inflammation, Th2-cell activation in the lung draining lymph nodes, and airway hyperreactivity. Some of the results of these studies have been previously reported in the form of an abstract (23).

METHODS

Mice

Seven- to ten-week-old female BALB/c mice (Janvier Ets, Le Genest St. Isle, France) and DO11.10 BALB/c mice, which express a transgenic TCR specific for the 323–339 OVA peptide, were bred and maintained in our animal facility. Our institutional review board for animal experimentation (Ethical Committee of the “Institut Federatif de Recherche” 50, Toulouse, France) approved all aspects of animal care.

In vitro T-Cell Differentiation

CD4⁺ T cells were isolated from spleens of DO11.10 BALB/c mice. They were stimulated weekly in the presence of irradiated BALB/c as a source of antigen-presenting cells (APCs), 0.3 μM OVA 323–339 peptide, and IL-4 plus anti-IFN-γ antibody or IL-12 and anti-IL-4 antibody to achieve Th2- and Th1-cell differentiation, respectively ([21] *see the online supplement*).

Reverse Transcriptase Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Reverse transcriptase polymerase chain reaction (PCR) and real-time PCR were performed by using various primers listed in the online supplement. PCR products obtained by using primers specific for Ca_v1.2 and Ca_v1.3 encoding genes (*see Table E1 in the online supplement*) were cloned and sequenced.

Immunoblotting

Transfection experiments were performed as indicated in the online supplement. Th1 or Th2 cells specific for OVA peptide were transfected with Ca_v1.2 or Ca_v1.3 sense (S), mismatched, or antisense (AS) ONs the sequences of which are given in Table E2. Because similar results were obtained by using sense or mismatched ONs, only results obtained with T cells transfected with sense ONs were shown. A total of 50 μg of lysates from Th1, Th2, or Th2 cells transfected with control or AS ONs 48 hours before were immunoblotted with anti-Ca_v1.2 or anti-Ca_v1.3 polyclonal or monoclonal antibodies (*see online supplement*).

Confocal Microscopy

Cells were stained with monoclonal anti-Ca_v1.2 antibody, and with Alexa-fluor-555-labeled anti-IgG2b mouse immunoglobulin (Invitrogen; Carlsbad, CA).

Single-Cell Intracellular Ca²⁺ Measurements

These measurements were done as previously described (21, 24) in cells loaded with 5 μM Fura2/AM before being stimulated with anti-TCR plus anti-CD28 antibodies, thapsigargin or ionomycin.

Th1 and Th2 Transfer Experiments

Th1 and Th2 cells transfected as described in the online data supplement were injected intravenously into BALB/c mice (5 × 10⁶ cells/mouse) and the mice were then given intranasal OVA protein (50 μg in phosphate-buffered saline) once per day for 7 days. Bronchoalveolar lavage fluid (BALF) was collected 24 hours after the final OVA administration.

In Vivo Administration of Ca_v1-specific ONs

BALB/c mice primed intraperitoneally with OVA in alum (Sigma-Aldrich; St. Louis, MO) were challenged 15 days later through intranasal route with a mixture of OVA (50 μg in phosphate-buffered saline) and 200 μg of Ca_v1.2 and Ca_v1.3 AS ONs or controls in phosphate-buffered saline for 5 days. The doses of ONs, corresponding to 15 nmol/day, were chosen according to previous works (25). Twenty-four hours after the final OVA administration, the mice were anesthetized and BALF was collected by cannulation of the trachea and lavage with phosphate-buffered saline.

Airway Inflammation and Stimulation Assays

Cells from the BALF were stained with May-Grünwald Giemsa and lung sections with hematoxylin and eosin (21). CD4⁺ T cells were purified from peribronchial lymph nodes and stimulated with OVA and APCs as described in the online supplement.

Airway Hyperresponsiveness

This was assessed 24 hours after the final OVA as previously described in conscious (21) and ventilated animals (*see online supplement*).

Statistical Analysis

Data are presented as means + SD. Data were analyzed by using the Mann-Whitney test or the Kruskal-Wallis test when more than one group was compared. A *P* value < 0.05 was considered significant.

RESULTS

Th2 But Not Th1 Cells Express Ca_v1.2 and Ca_v1.3 Channels

Th2 cells were previously shown to express DHPR (20), defined as Ca_v1 channels in excitable cells, leading us to look for the expression of Ca_v1 channel family members in this T-cell subset. Four genes encode Ca_v1 channels (Ca_v1.1–Ca_v1.4) that make up the Ca²⁺ pore. Whereas PCR products for Ca_v1.2 and Ca_v1.3 were obtained from fully differentiated Th2 cells, Ca_v1 transcripts were undetectable in Th1 cells (Figure 1A). Neither Th1 nor Th2 cells expressed Ca_v1.4 transcripts, whereas they were easily evidenced in retina (not shown). The cDNAs encoding the four β subunits that may associate with the Ca_v1 channels were also present in Th2 cells but not in Th1 cells (Figure 1A). We then used real-time PCR to quantify Ca_v1.2 and Ca_v1.3 expression during T-cell differentiation. Low levels of Ca_v1 products were measured in naive CD4⁺ T cells and expression of both Ca_v1.2 and Ca_v1.3 increased progressively during Th2-cell differentiation from the end of the first stimulation (Figure 1B, *right panel*). Notably, the expression of GATA-3, the Th2 lineage-specific transcription factor, preceded the expression of Ca_v1 channels (Figure 1B, *left panel*), suggesting that commitment to the Th2 lineage is required for induction of Ca_v1 gene expression in T lymphocytes. Conversely, Ca_v1 products became undetectable in differentiating Th1 cells (Figure 1C, *right panel*) that express the transcription factor T-bet, characteristic of Th1 cells (Figure 1C, *left panel*). Notably, the up-regulation of Ca_v1 channels in differentiating Th2 cells paralleled the

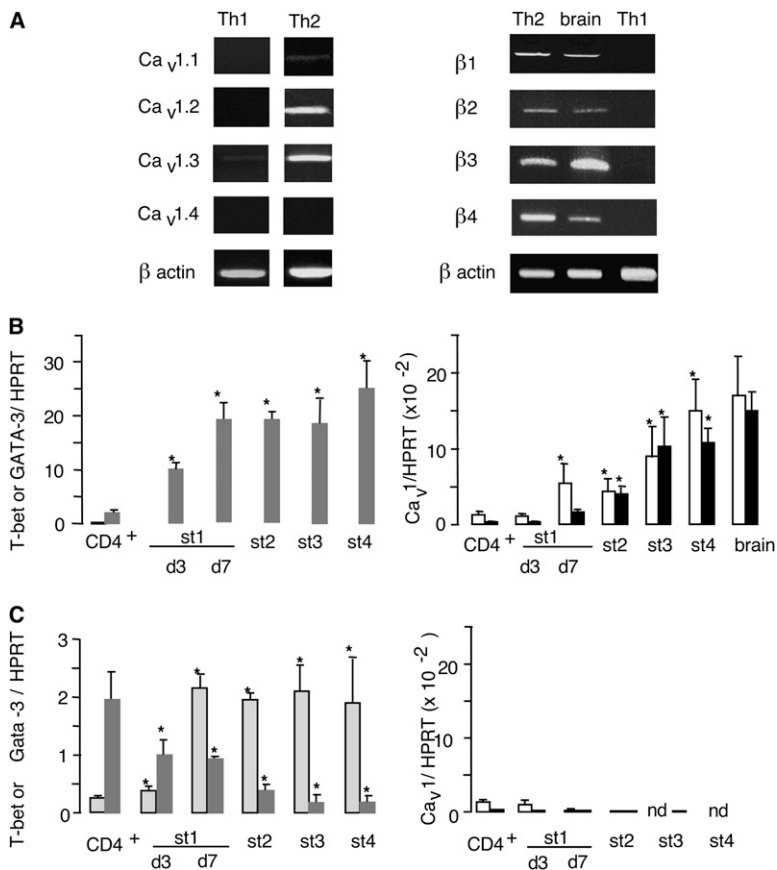


Figure 1. Cav1.2 and Cav1.3 are up-regulated in Th2 but not in Th1 cells. (A) cDNAs from OVA-specific D011.10 Th1 and Th2 cells collected after three rounds of stimulation were tested for the expression of Cav1 isoforms (Cav1.1 to Cav1.4) and β actin (left panel). cDNAs from Th2 cells, Th1 cells, and brain used as a positive control were tested for expression of auxiliary Cav1 β subunits and β actin (right panel). One representative experiment out of the 10 performed. (B and C) We analyzed the expression of transcripts for GATA-3 (dark gray bars) and T-bet (light gray bars) (left panels), and Cav1.2 (white bars) and Cav1.3 (black bars) (right panels) by real-time PCR in CD4⁺ T cells, Th2 cells (B), and Th1 cells (C) collected 3 and 7 days (d) after the first stimulation (st1) and thereafter 3 days after the second (st2), third (st3), and fourth stimulation (st4). Data were normalized to levels of the housekeeping gene HPRT. They represent the mean $\pm$ 1SD from seven experiments; * $P < 0.01$ compared with CD4⁺ T cells. nd = not detectable.

expression of DHPR (Figures 2A and 2C), whereas this expression was barely detectable and undetectable in CD4⁺ and in Th1 cells, respectively (Figures 2B and 2C).

To determine whether differentiated Th2 cells express classical Cav1 channels (for which numerous splice variants

have been described), we performed reverse transcriptase PCR that amplified two overlapping products, each of more than 3 kbp, encompassing the entire cDNA sequences encoding Cav1.2 and Cav1.3. The sequence coding Cav1.3 was identical to the neuronal Cav1.3a (Genbank accession AJ4377291.1).

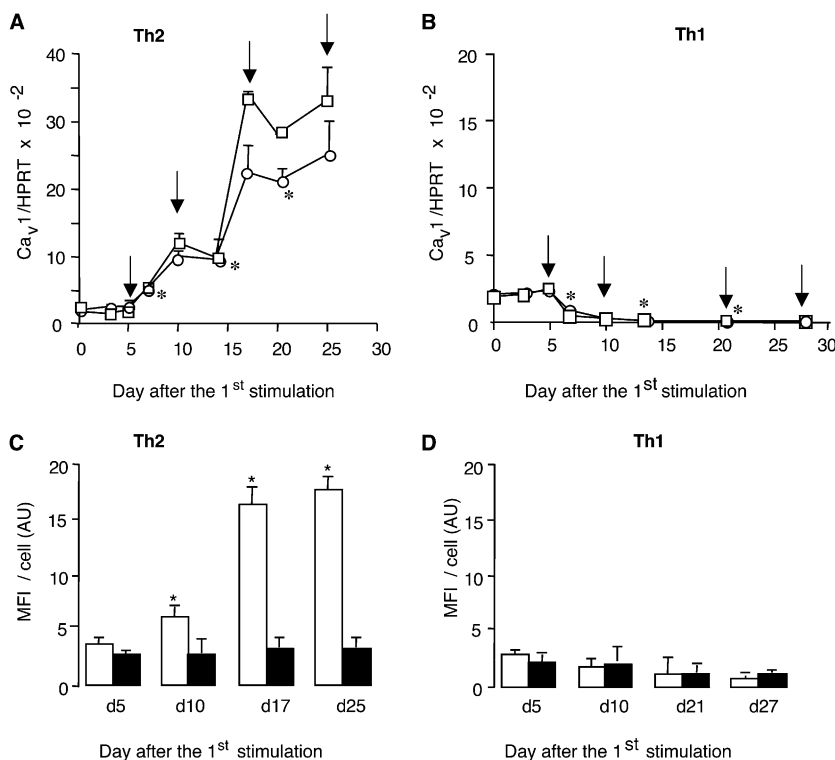


Figure 2. The expression of Cav1 channels correlates with the detection of DHPR in differentiating Th2 cells. cDNAs from OVA-specific D011.10 Th2 (A) and Th1 cells (B) were collected at different days after the first stimulation and tested for the expression of Cav1.2 (open squares) and Cav1.3 (open circles) mRNA by real-time quantitative PCR. Data were normalized to levels of the housekeeping gene HPRT. The stars indicate the time of restimulation with APCs and OVA. At the time-points indicated by the arrows, the cells were labeled with ST-BODIPY-DHP (C and D). The fluorescence intensity was quantified by using the Image J software and the intensity per cell was averaged (open bars). The staining was abolished by preincubation with an excess of unlabelled DHP (black bars) showing the specificity of the staining. * $P < 0.01$ compared with controls.

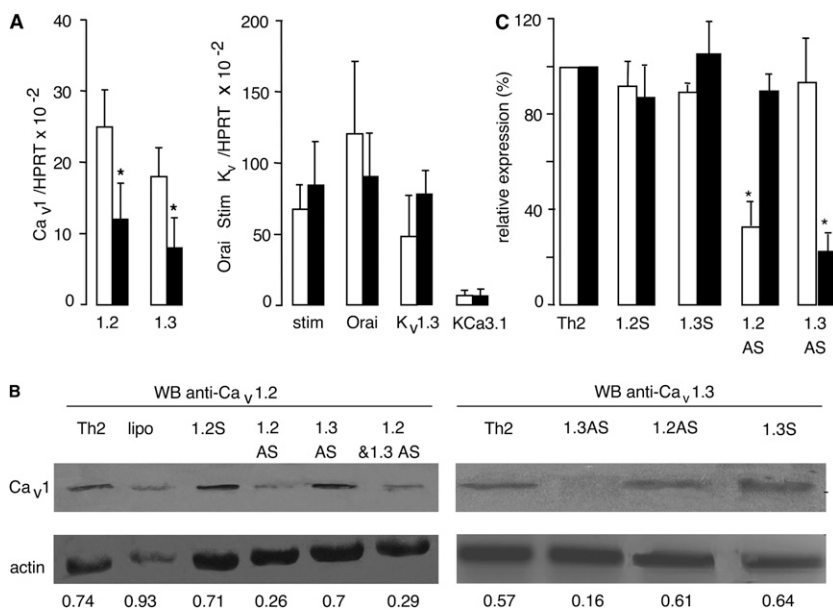


Figure 3. Ca_v1.2 and Ca_v1.3 expression is reduced by transfection with specific antisense oligodeoxynucleotides. (A) Real-time PCR was performed to quantify the expression of Ca_v1.2 (*n* = 7), Ca_v1.3 (*n* = 7), stim1 (*n* = 5), Orai1 (*n* = 5), K_v1.3 (*n* = 3), and Kca3.1 (*n* = 3) in Th2 cells transfected with a mixture of Ca_v1.2 and Ca_v1.3 antisense (black bars) or sense (white bars) ONs. The numbers in parentheses represent the number of experiments performed. Data were normalized to levels of the housekeeping gene HPRT. * *P* < 0.01 compared with controls. (B) Th2 cells treated with lipofectin (lipo), or transfected with Ca_v1.2 (1.2), Ca_v1.3 (1.3) sense (S) or antisense (AS) were analyzed 48 hours after transfection by Western blotting with anti-Ca_v1.2 or Ca_v1.3 monoclonal antibodies. Actin was used as a loading control. The intensity of bands was quantified and normalized to the level of actin. The histograms in C represent the expression of Ca_v1.2 (white bars) and Ca_v1.3 (black bars) in lysates from cells treated as in B relative to the expression in Th2 cells transfected with control ONs (mean + 1SD from six experiments; * *P* < 0.02 compared with Th2 cells transfected with control ONs).

Multiple splice variants may encode Ca_v1.2 channels with some exons that characterize the cardiac form, such as the exon 1a instead of the exon 1b, and the use of exons 34a, 41a, and 45 that were excluded in other forms (26). We found that the sequences of Ca_v1.2 cloned from Th2 cells did not express cardiac-specific exons and differed from the Genbank accession L01776 sequence (cloned from the BALB/c brain) only by the use of the exon 8 instead of the exon 8a (exons that are known to be exclusive) (26) and by the absence of exon 33 (see Figure E1). However, the Th2-cell sequences were found to contain the sequences reported to encode the four voltage sensors in classical Ca_v1 channels (27).

As shown in Figure E2 in the online supplement, Western blots of Th2-cell extracts probed with anti-Ca_v1.2 and anti-Ca_v1.3 polyclonal antibodies revealed polypeptides of about 210

and 240 kD, respectively. These bands corresponded to the expected sizes for classical Ca_v1 channels found in the brain (not shown). Preincubating anti-Ca_v1.2 or anti-Ca_v1.3 antibody with the antigenic peptides markedly decreased the band intensity as expected. By contrast, none of these channels were detected in cell lysates from differentiated Th1 cells (see Figure E2).

Ca_v1AS Alter TCR-dependent Calcium Signaling and IL-4 Production by Th2 Cells With no Impact on Th1 Cell Functions

To investigate the function of Ca_v1 channels in Th2 cells, we used Ca_v1AS ONs to reduce translation of the mRNAs. As shown in Figure 3A, transfection of Th2 cells with a mixture of

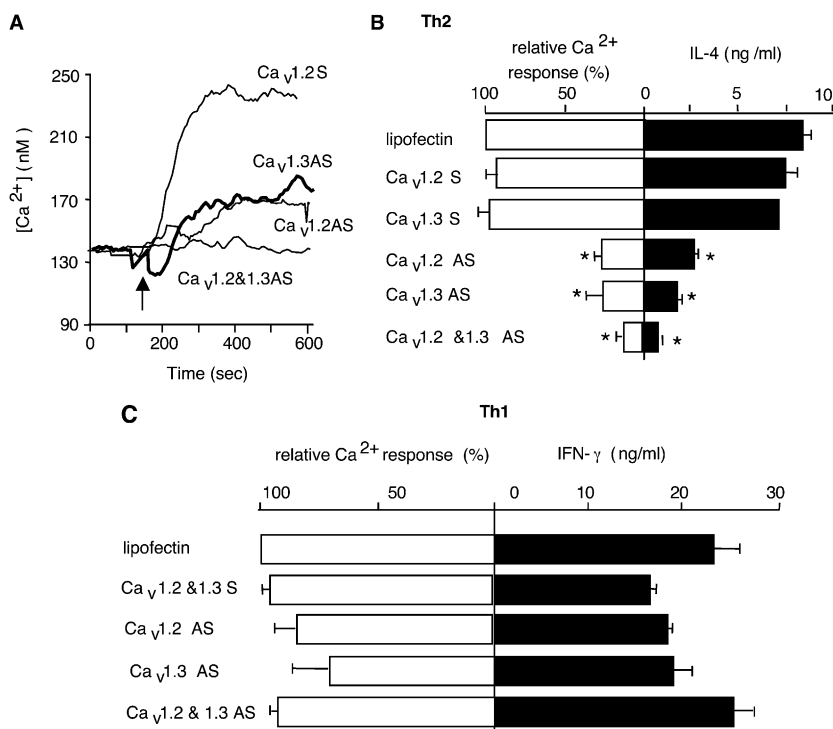


Figure 4. Ca_v1AS reduce the TCR-dependent Ca²⁺ response and cytokine production by Th2 but not by Th1 cells. (A) Th2 cells transfected with Ca_v1.2 sense (S), Ca_v1.3 sense (S), Ca_v1.2 antisense (AS), Ca_v1.3 antisense (AS), or a mixture of Ca_v1.2 and Ca_v1.3 AS ONs were loaded with Fura-2/AM. Intracellular Ca²⁺ levels were recorded for 2 minutes before stimulation with anti-TCR mAb (arrow) and for 7–8 minutes after stimulation. At least 45 cells were recorded and [Ca²⁺]_i calculated for each cell were averaged at each time-point. Standard errors are not indicated, but they were less than 10% of the mean. One representative experiment out of six is shown. (B and C) The mean peak values of [Ca²⁺]_i and cytokine production elicited by TCR stimulation were measured in Th2 (B) and in Th1 (C) cells treated with lipofectin or transfected with Ca_v1.2 or Ca_v1.3 S or AS ONs. The relative Ca²⁺ response represents the percentage of the peak [Ca²⁺]_i value relative to the data obtained from T cells treated with lipofectin alone; they represent the mean + 1SD of six experiments; * *P* < 0.01 compared with controls.

Ca_v1.2 and Ca_v1.3AS reduced Ca_v1.2 and Ca_v1.3 gene expression with no effect on stim1, Orai1, or the potassium channels K_v1.3 and KCa3.1 described as required for Ca²⁺ entry into memory and activated naive T cells, respectively (28). Ca_v1.2 and Ca_v1.3AS also reduced the amounts of proteins as shown by Western blotting (Figures 3B and 3C). Transfection with Ca_v1AS significantly reduced the binding of the Ca_v1 ligand DHP to Th2 cells, as shown by staining cells with the fluorescent dye BODIPY-DHP (see Figure E3). Thus, Ca_v1AS reduce expression of the channels and so reduce the amount of DHP binding to Th2 cells showing the molecular identity of DHPR in Th2 cells.

The [Ca²⁺]_i increase (Figure 4A) and IL-4 synthesis (Figure 4B) that are exquisitely Ca²⁺-dependent (29, 30) were lower on TCR activation in Th2 cells transfected with Ca_v1.2 or Ca_v1.3 AS and especially in Th2 cells transfected with both AS, compared with Th2 cells transfected with control ONs. The analysis of Th2 cells transfected with Ca_v1.2 and Ca_v1.3 AS showed that the [Ca²⁺]_i rise was reduced in about 80% of cells compared with controls as exemplified in Figure E4. Among these cells, an oscillatory pattern without plateau was observed in 15% of cells and 10% of cells did not respond at all. This reduction in Ca²⁺ signaling was not caused by global alterations in Th2-cell functions because the proliferation of Th2 cells transfected with Ca_v1AS was comparable with that of control Th2 cells (see Figure E5). Transfection of Th1 cells with Ca_v1AS modified neither the Ca²⁺ response nor IFN-γ synthesis on TCR stimulation (Figure 4C; see Figure E4). Ca_v1AS also did not affect SOC channels in Th2 cells as indicated by the absence of effect on the Ca²⁺ response induced by thapsigargin, an inhibitor of the intracellular Ca²⁺ pump, SERCA, which activates SOC channels through the depletion of ER Ca²⁺ stores (see Figure E6A). Accordingly, transfection with Ca_v1AS, which

inhibited TCR-triggered IL-4 secretion in Th2 cells, had no effect on IL-4 production induced by PMA plus ionomycin or thapsigargin (see Figure E6B). Thus, Ca_v1AS impedes specifically the increase in [Ca²⁺]_i and cytokine production induced by TCR-mediated stimulation of Th2 cells.

Th2 Cells Transfected with Ca_v1AS Lose Their Ability to Induce Asthma

To investigate whether Ca_v1 is involved in allergic inflammation-inducing activity of Th2 cells *in vivo*, OVA-specific transgenic Th2 cells were transfected with Ca_v1AS (Th2AS) or Ca_v1S (Th2S) and injected into BALB/c mice that were given intranasal OVA to induce airway inflammation. Mononuclear cells were purified from the lungs of mice injected with Th2AS or control Th2 cells and were labeled with T and B lymphocyte-specific antibodies. Similar numbers of transgenic CD4⁺ T cells, identified by staining with anti-KJ1.26 antibodies, were found in the lungs of mice after the transfer of either Th2AS or Th2S, whereas the number of B cells and of CD4⁺ T cells that did not express the transgenic TCR were reduced in the lungs of mice transferred with Th2AS (see Figure E7), suggesting that Th2AS can localize into the lungs but have a reduced capacity to induce the recruitment of other T and B cells. The number of inflammatory cells, in particular eosinophils, was dramatically reduced in the BALF of mice injected with Th2AS compared with the controls (Figure 5A). This was consistent with the absence of or minor lung inflammation in these animals compared with controls (Figure 5B). The lack of lung inflammation in mice injected with Th2AS was accompanied by a decrease in the IL-4 concentration in the BALF (Figure 5C) and a reduction in the number of lymphocytes in the lung-draining lymph nodes (Figure 5D) compared with controls.

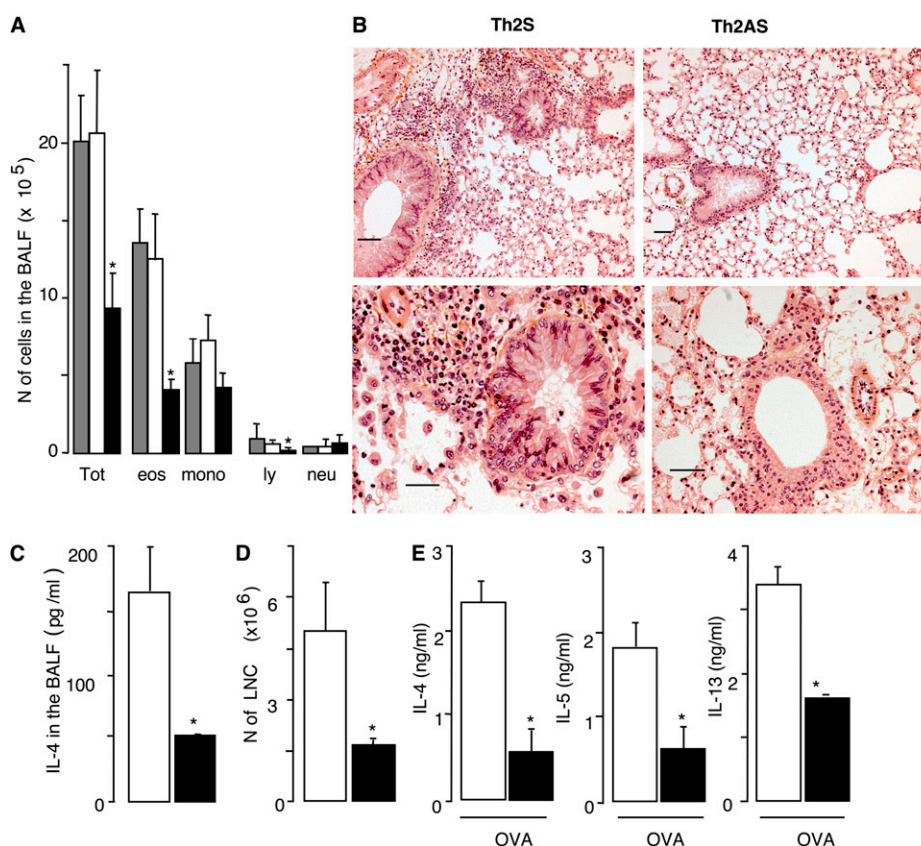


Figure 5. Th2 cells transfected with Ca_v1AS lose their ability to induce asthma. (A and B) OVA-specific Th2 cells transfected or not with Ca_v1.2 and Ca_v1.3 sense (Th2S) or antisense (Th2AS) oligonucleotides were transferred into BALB/c mice that were exposed to intranasal OVA for 7 days. (A) The number of inflammatory cells was reduced in the BALF of mice transferred 7 days before with Th2AS (black bars) compared with mice injected with Th2 (gray bars) or Th2S (white bars) (* *P* < 0.01). Data represent the mean ± 1SD from five mice and are representative of four experiments with five mice per group. Tot = total; eos = eosinophils; mono = monocytes/macrophages; ly = lymphocytes; neu = neutrophils. (B) Unlike untreated Th2 and Th2S, Th2AS did not induce airway inflammation when injected in BALB/c mice given intranasal OVA as shown on lung sections stained with H&E. Bars, 100 μm. (C) IL-4 concentration was reduced in the BALF of mice transferred with Th2AS (black bars) compared with mice injected with Th2 or Th2S (white bars). Data represent the mean ± 1SD from five mice and are representative of five experiments. The number of cells in the lung-draining lymph nodes of mice injected with Th2AS (D) and the capacity of CD4⁺ T cells isolated from these lymph nodes to produce cytokines after *in vitro* stimulation with APCs and OVA (E) was reduced compared with controls (* *P* < 0.01; n = five independent experiments).

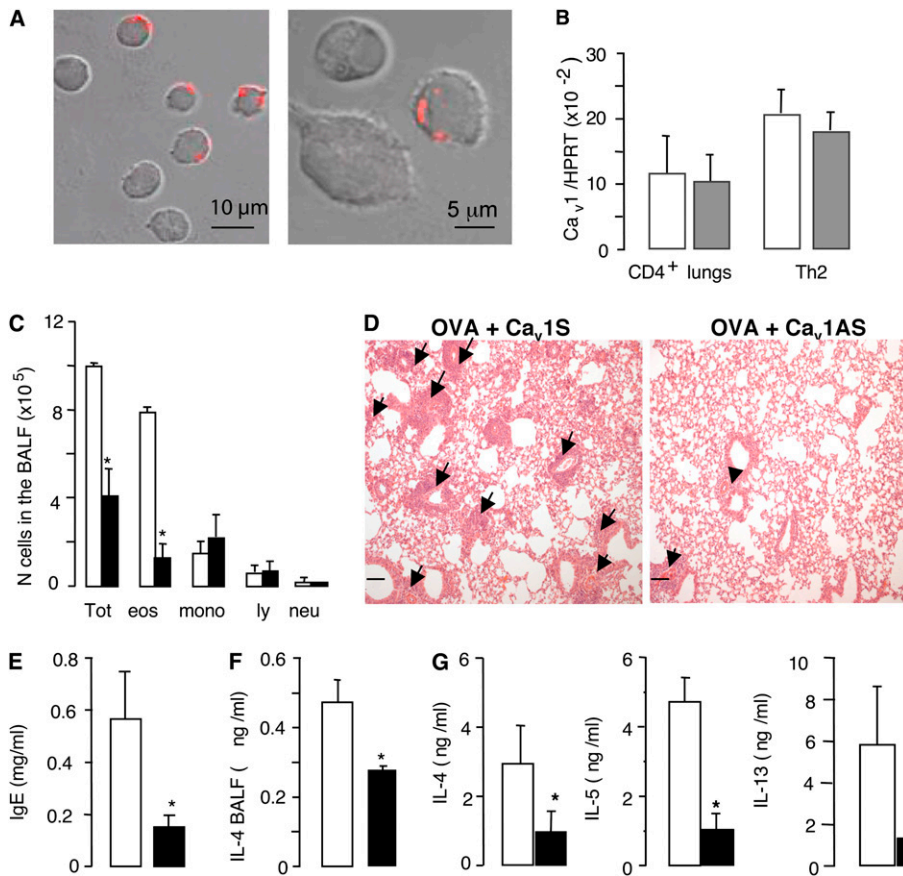


Figure 6. Ca_v1AS impair the development of asthma. (A and B) BALB/c mice immunized with OVA in alum were exposed 15 days later to daily doses of intranasal OVA for 5 days. CD4⁺ T cells were purified from the lungs. An intracellular staining with the monoclonal anti-Ca_v1.2 antibody was performed (A). In parallel, cDNAs were prepared and analyzed for the expression of Ca_v1.2 (white bars) and Ca_v1.3 (gray bars) by real-time PCR (B). Data were normalized to levels of the housekeeping gene HPRT and represent the mean + SD from three independent experiments. Results obtained with cDNAs from Th2 cells obtained after three rounds of stimulation were shown for comparison. (C to G) BALB/c mice were immunized with OVA and were exposed 15 days later to daily doses of intranasal OVA mixed with Ca_v1.2 plus Ca_v1.3 sense (Ca_v1S) (white bars) or antisense (Ca_v1AS) (black bars) for 5 days. (C) Ca_v1AS reduced the number of inflammatory cells in the BALF compared with controls (* *P* < 0.01; five mice per group). Tot = total; eos = eosinophils; mono = monocytes/ macrophages; ly = lymphocytes; neu = neutrophils. One representative experiment out of four is shown. (D) Few signs of lung inflammation were evidenced on lung sections from mice treated with Ca_v1AS (H&E staining), whereas numerous inflammatory cell infiltrates indicated by the arrows were

found in the lungs of controls. Bar, 200 μ m. (E) Serum IgE concentration was measured in mice that received intranasal administration of OVA and Ca_v1 S (open bars) or AS (black bars) ONs for 5 days (five mice per group). IL-4 concentration (F) was reduced in the BALF of mice given Ca_v1AS (black bars) compared with controls (white bars) and the capacity of CD4⁺ T cells isolated from the lymph nodes of mice given Ca_v1AS (G) to produce IL-4, IL-5, and IL-13 after *in vitro* stimulation with APCs and OVA compared with controls (* *P* < 0.01; *n* = 5 independent experiments).

Moreover, CD4⁺ T cells from the draining lymph nodes produced less IL-4, -5, and -13 when stimulated with APCs and OVA compared with controls (Figure 5E). Thus, knocking-down Ca_v1 channels in Th2 cells strongly inhibits their ability to induce airway inflammation on adoptive transfer.

Intranasal Delivery of Ca_v1AS Prevents Experimental Active Asthma

We showed that around 30–50% of CD4⁺ T cells that infiltrated the lungs of asthmatic mice were stained with anti-Ca_v1.2 mAb (Figure 6A), which is in agreement with the expression of Ca_v1.2 and Ca_v1.3 in these cells as assessed by reverse transcriptase PCR (Figure 6B) and with the detection of DHPR on these cells (21). To assess whether inhaled Ca_v1AS might be beneficial directly in experimental asthma, we immunized BALB/c mice with OVA and then challenged them with a mixture of intranasal OVA and Ca_v1AS. The number of inflammatory cells (mainly eosinophils; Figure 6C) was reduced in the BALF of mice treated with Ca_v1AS and these mice developed only mild lung inflammation (Figure 6D). The analysis of cells from the lungs recovered after Ficoll isolation showed a reduction in the number of CD4⁺ T cells and of B cells in the lungs of mice given Ca_v1AS compared with controls, whereas the number of NK, NKT, or CD8⁺ T cells remained unchanged (not shown). In addition, total serum IgE concentration was significantly lower in mice treated with Ca_v1AS ONs compared with controls (Figure 6E). The concentration of IL-4 in the BALF of mice given Ca_v1 AS was reduced (Figure 6F)

and CD4⁺ T cells isolated from their lung-draining lymph nodes produced less IL-4, -5, -13 (Figure 6G), and -10 (not shown) on stimulation *in vitro* with APCs and OVA than did similar cells isolated from control mice. Mice treated with Ca_v1AS showed reduced alterations of the pulmonary functions in response to the acetylcholine analog methacholine compared with the controls (Figure 7; see Figure E8). Thus, intranasal administration of Ca_v1AS prevents antigen-specific recalled Th2 cell responses, IgE production, lung eosinophil infiltrates, and airway hyperreactivity, demonstrating their *in vivo* efficacy to prevent asthma.

DISCUSSION

Ca_v1-related molecules have been detected at the mRNA or protein level in various nonexcitable tissues including dendritic cells (31, 32), mast cells (33), B lymphocytes (34, 35), and T lymphocytes (13–17). However, their molecular identity is not established and the demonstration of their role in calcium signaling is unclear. For some authors, the presence of Ca_v1.4-related sequences (Ca_v1.4 opening at more negative cell membrane potentials than the other Ca_v1 types) (14, 15) or differences in Ca_v1 sequences relative to classical channels, and especially truncated forms (13–15), were presumed to be responsible for the absence of voltage-sensitivity in immune cells. This work demonstrates conclusively that Th2 cells express full-length Ca_v1.2 and Ca_v1.3 channels. Notably, the deletion of exon 33 observed in all the Ca_v1.2 coding sequences

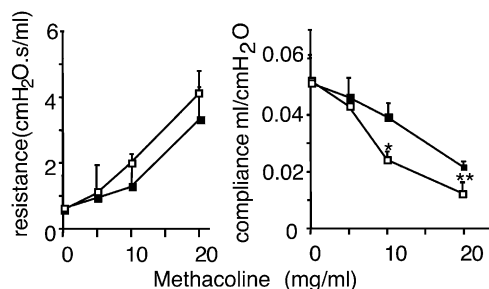


Figure 7. Ca_v1AS improve respiratory functions in an experimental model of active asthma. BALB/c mice were immunized with OVA in alum and were exposed 15 days later to daily doses of intranasal OVA mixed with Ca_v1.2 plus Ca_v1.3 sense (Ca_v1S) or antisense (Ca_v1AS) ONs for 5 days. Mice treated with Ca_v1AS (black squares) displayed better airway compliance and reduced airway resistance on inhalation of increasing concentrations of methacholine compared with mice treated with Ca_v1S (open squares). * $P < 0.005$. ** $P < 0.05$, five mice per group.

cloned from Th2 cells was already reported in epithelial cells (36) and in smooth muscle cells in which this variant would open for more hyperpolarized cell potential than channels that express exon 33 (37). Other laboratories failed to demonstrate the presence of voltage-gated channels in T lymphocytes including Th2 cells (7), which is consistent with our own data (not shown). The absence of voltage sensitivity in Th2 cells could be related to the capacity of Ca_v1 channels to cluster into signaling complexes (38), where they can interact with proteins, such as the scaffolding protein AHNK (17, 39, 40) or protein kinase C (41, 42). Such interactions have already been shown to enhance Ca²⁺ entry and to diminish or suppress the voltage dependence of Ca_v1 channels (38, 41–44). Ca_v1.2 and Ca_v1.3 expression seems to be constitutive in effector Th2 cells. However, an increase in the expression of these channels following T-cell activation cannot be ruled out, which could account for a relatively lower expression at Day 7 after stimulation compared with the level at Day 3 of stimulation, as exemplified in Figure 2. It is also possible that TCR stimulation promotes the localization of Ca_v1 channels at the cell membrane, favoring the interaction with signaling components regulating the opening of the channels.

In our experiments, transfection with Ca_v1AS had a major impact on the shape and amplitude of the TCR-dependent Ca²⁺ response but did not affect the rise in [Ca²⁺]_i induced by thapsigargin, which activates SOC channels. Accordingly, Ca_v1AS did not alter the expression of CRAC/Orai channels in T cells. Likewise, the effect of Ca_v1AS was not the consequence of a nonspecific effect of the antisense ONs on the expression of potassium channels responsible for the K⁺ efflux out of the cell, which maintains the electrochemical driving force required for Ca²⁺ influx. Surprisingly, Ca_v1AS did not alter the capacity of Th2 cells to proliferate suggesting that this process was relatively calcium-insensitive in these cells or that the residual TCR-driven intracellular Ca²⁺ rise observed in Th2AS is sufficient for proliferation. Altogether, we provided evidence that Ca_v1 channels in Th2 cells do not behave like SOC channels. These data are therefore consistent with the sequential involvement of several types of Ca⁺ channels during TCR-driven Th2-cell activation. Our hypothesis is that the activation of Ca_v1 channels would precede the opening of other Ca²⁺ channels, such as CRAC/Orai. Why this would occur in Th2 but not in Th1 cells remains puzzling. We could speculate that Ca_v1 channels contribute to generate a “calcium signature” that is clearly different in Th2 and Th1 cells (as exemplified in

Figure E4), which could contribute to activate distinct gene expression patterns.

Th2 cells are continuously active in the lungs of asthmatics (45), which may play a prominent role in the persistent inflammation and airway remodeling seen in this disease. The suppression of Th2-mediated airway inflammation by Ca_v1AS is likely to result from preventing both *in situ* Th2-cell activation and the amplification of Th2-cell responses from a newly recruited pool of antigen-specific naive CD4⁺ T cells, defined as IL-4-dependent in experimental models (46, 47). Moreover, we cannot exclude that intranasal delivery of Ca_v1AS affects other cells in addition to Th2 cells in the lung. From this point of view, it will be interesting to assess the expression of Ca_v1 channels in lung-infiltrating immune cells, such as eosinophils, basophils, mast cells, and B cells. An effect on bronchial smooth muscle cells might also contribute to the effect on airway hyperreactivity. Whether or not this is the case, targeting Ca_v1 channels in Th2 cells might prove particularly effective in asthma because of the simultaneous blockade of all Th2 effector functions. Preliminary data suggest that Ca_v1 channel expression is detected in human Th2-cell clones and that an antagonist of DHPR decreased calcium entry in these clones (see Figure E9). It will be necessary to identify which Ca_v1 channels are expressed and to demonstrate that they selectively regulate human Th2-cell effector functions. If this is the case, targeting these channels should selectively impair Th2-cell activation without inducing generalized immunosuppression, thus sparing other effector T cells, such as Th1 cells, thereby preserving immune responses against infectious agents.

Conflict of Interest Statement: M.D.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P-E.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.G. has been employed full-time as a veterinarian by Pierre Fabre since 2008. M-L.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.L. is an employee of Stallergenes. A.M. received \$1,001–\$5,000 from Merck and \$1,001–\$5,000 from Novartis for serving on an advisory board, up to \$1,000 from Stallergenes, up to \$1,000 from ALK-Abello, up to \$1,000 from Merck and \$1,001–\$5,000 from AstraZeneca in lecture fees. H.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J-C.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Knocking-down Ca_v1 calcium channels implicated in Th2-cell activation prevents experimental asthma

Marilena Djata Cabral, Pierre-Emmanuel Paulet, Virginie Robert, Bruno Gomes, Marie-Laure Renoud, Magali Savignac, Catherine Leclerc, Marc Moreau, Bernard Mariamé, Jean-Charles Guéry & Lucette Pelletier.

Online Data supplement

METHODS

***In vitro* T-cell activation and differentiation.** CD4⁺ T cells were isolated from spleens of DO11.10 BALB/c mice by negative selection with the Dynal® Mouse CD4 Negative Isolation Kit (Invitrogen Carlsbad, CA). CD4⁺ T cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum (ATGC, Noisy Le Grand, France), 1% pyruvate, 1% non-essential amino acids, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-mercaptoethanol. CD4⁺ T-cells (10⁶/ well) were differentiated along the Th2 pathway by weekly stimulation with 2.5 x 10⁶ irradiated BALB/c spleen cells/well, 0.3 µM OVA 323–339 peptide (Neosystem, Strasbourg, France), 10 ng/ml IL-4 and 10 µg/ml anti-IFNγ (XMG1.2) antibody (both purchased from BD Pharmingen, San Diego, CA). Th1-cell differentiation was achieved by weekly stimulation of 10⁶ CD4⁺ T cells with APCs (5 x 10⁶ /well), 5 ng/ml IL-12 (BD Pharmingen) and 10 µg/ml of anti-IL-4 antibody (11B.11, BD Pharmingen). Cytokine concentration was measured by ELISA (1) in the supernatants of T cells (2.5 x 10⁴ cells/well) stimulated with plate-bound anti-TCR mAb (H57-597, BD Pharmingen, 1 µg/ml) for 24 h.

Sequencing of cDNAs encoding the Ca_v1.2 and Ca_v1.3 subunits in Th2-cells. The primers listed in Supplementary Table E1 enabled us to determine the full-length sequences of the Ca_v1.2 and Ca_v1.3 subunits. The PCR amplification products were cloned and sequenced using an ABI PRISM® 3100 Genetic Analyzer with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). At least ten clones were sequenced for each product.

Detection of Ca_v1 PCR products. Total RNA was isolated from 10⁷ cells by using the SV Total RNA Isolation System (Promega, Madison, WI) and reverse transcribed with poly(dT) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Specific primers were used as follows, for Ca_v1.1: 5'-tgtggtatgtcgtcacttctcc-3' and 5'-cgtaatgatgctgccgatg-3'; for Ca_v1.2: 5'-caagccctcacaaggaatgc-3' and 5'-aaagttgccctgctgtcactc-3'; for Ca_v1.3: 5'-cttcacagccatctttacggtg-3' and 5'-agaggccgcaagaccctca-3'; for Ca_v1.4: 5'-tccacacactccaccagcag-3' and 5'-atggtcaacgtgttgagg-3'; for β 1 subunit: 5'-agcatgccagtgtgcacgagtac-3' and 5'-agccctccagctcattcttattgc-3'; for β 2: 5'-ataaccacagagaggagccaca-3' and 5'-tatacatccctgttccactcgcca-3'; for β 3: 5'-tcctggacttcagaaccagcag-3' and 5'-ttgtggtcatgctccgagtctcg-3'; for β 4: 5'-tgagacacagcaaccattctacagaga-3' and 5'-atgtcgggagtcattggctgcatc-3'; for β actin: 5'-tggaatcctgtggcatccatgaaac-3' and 5'-taaaacgcagctcagtaacagtccg-3'. PCR conditions were 94°C for 45 sec, followed by 55°C (Ca_v1 and β subunits) or 60°C (β -actin) for 45 sec, and 72°C for 1 min over 21 cycles (β -actin) or 40 cycles (Ca_v1 and β subunits).

Real-time quantitative PCR. Ca_v1.2, Ca_v1.3, GATA-3, T-bet, Orai1, Stim1, K_v1.3, KCa3.1 and HPRT (hypoxanthine-guanine phosphoribosyltransferase) transcripts were measured by real-time quantitative PCR using an ABI Prism 7000 Sequence Detection System. PCR was performed with the PCR SYBR Green Sequence Detection System (Perkin Elmer). We used the following primers to quantify mRNA encoding Ca_v1.2: 5'-agacgcgggtggtttttta-3' and 5'-gaagacgctgttccggttacc-3'; Ca_v1.3: 5'-

tgaggccaaaagtaaccccgagg-3' and 5'-cgccatcatagccgaaaggacaat-3'; GATA-3: 5'-gccatgggtagagaggcag-3' and 5'- ttggagactcctcacgcatgt-3'; T-bet: 5'-cggtagcagagcggcaagt-3' and 5'- cagctgacccaagaggaatca-3'; Orai1: 5'-gcttcgccatggtagcgat-3'; and 5'-gctgatcatgagggcaaac-3'; Stim1: 5'-gccacagcttggcctgg-3' and 5'-gctccatcaggctgtgg-3'; K_v1.3: 5'-ccagcacctctcctcttcag-3' and 5'- agggcatacacagaccaagg-3'; KCa3.1: 5'-tgccatttgcgtactgagag-3' and 5'-gacaaaggaggaaggcagtg-3'; HPRT: 5'-ctggtgaaaaggacctctcg-3' and 5'- tgaagtactcattatagtaaggga-3'. Quantification of target gene expression was calculated by normalising the values relative to the expression of HPRT.

Transfection experiments. Th1 or Th2-cells specific for the OVA 323–339 peptide were transfected with Ca_v1.2 or Ca_v1.3 S, mismatched or AS ONs by using Lipofectin® (Invitrogen). The sequences of the ONs are given in Supplementary Table E2. FACS analysis showed that transfection with FITC-conjugated control or AS ONs labeled 60–70% of cells (data not shown).

Immunoblotting. 10⁷ cells Th2 or Th1 cells were lysed for 15 min in lysis buffer containing 4 mM EDTA, 1% Triton, 150 mM NaCl, 20 mM Tris HCl pH 8, protease inhibitor cocktail (1 tablet / 6 ml buffer, Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged at 13,000 rpm to remove insoluble material. The protein content of the supernatant was measured by using a detergent-compatible protein assay (Bio-Rad, Hercules, CA). Samples (50 µg/lane for Th2 and Th1 samples except when otherwise mentioned) were subjected to 7.5% SDS-PAGE and transferred onto

nitrocellulose membranes (Bio-Rad). Membranes were saturated with TBS/Tween containing 1% milk and 1% BSA and incubated overnight with anti-Ca_v1.2 and anti-Ca_v1.3 polyclonal (Alomone Labs, Jerusalem, Israel) or monoclonal antibodies (UC Davis NINDS/NIMH NeuroMab facility, Davis CA). Blots were developed with the appropriate HRP-conjugated secondary antibody and Amersham Biosciences Enhanced Chemiluminescence system (GE Healthcare, Little Chalfont, UK). Membranes were then washed and incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M 2-Mercaptoethanol) at 60°C for 30 min to be reprobed with anti-actin antibodies as a loading control.

Th1 and Th2 transfer experiments. Th1 and Th2-cells transfected as described above were injected intravenously into BALB/c recipient mice (5×10^6 cells per mouse) and the mice were then given intranasal OVA protein (50 µg in PBS) once per day for 7 days. BALF was collected 24 hours after the final OVA administration.

***In vivo* administration of Ca_v1-specific AS.** BALB/c mice primed intraperitoneally with 100 µg OVA protein in 2 mg alum (Sigma) were challenged 15 days later with a mixture of intranasal OVA (50 µg in PBS) and control or Ca_v1.2 and Ca_v1.3 AS (200 µg corresponding to 15 nmoles/ day) in PBS for 5 days. Twenty-four hours after the final OVA administration, the mice were anaesthetized and BALF was collected by cannulation of the trachea and lavage with PBS.

Airway inflammation and stimulation assays. BALF was centrifuged at 300 g. Cell pellets were suspended in PBS and the number of cells was determined by trypan blue exclusion. Cells were then centrifuged onto slides and stained with May-Grunwald Giemsa to identify inflammatory cells. Lung samples were fixed in formalin overnight, stored in 70% ethanol, and 5 µm-thick sections were stained with hematoxylin and eosin. CD4⁺ T cells (25 x 10⁴/ well) were purified from peribronchial lymph nodes and stimulated for 24 h with OVA (100 µg/ml) and irradiated BALB/c spleen cells as a source of APCs (2 x 10⁶/ well).

Airway hyper-responsiveness. It was assessed 24 h after the final OVA inhalation by using a whole body plethysmograph (EMKA Technologies, France). Baseline-enhanced pause (Penh) values were recorded for 3 min before the mice were exposed to intranasal methacholine as previously described (2). The Penh values measured during 20 min sequence were averaged and expressed as the fold increase over baseline values. Alternatively dynamic lung compliance was measured using a Flexivent apparatus (SCIREQ). Mice were anesthetized with a mix solution containing 16 mg/kg Xylazine (Rompun, Bayer) and 40 mg/kg ketamine, (Imalgene, Merial), tracheotomized, paralyzed (0,05 mg/kg rocuronium bromide) and immediately intubated with a catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.3 ml, and a positive-end expiratory pressure of 2 ml H₂O was applied. Increasing concentrations of methacholine (0-20 mg/ml) were administered at the rate of 20 puffs per 10 s, with each puff of aerosol delivery lasting 10 ms, via a nebulizer aerosol system (Aeroneb, Aerogen). Baseline resistance was restored before administering the

subsequent doses of methacholine.

Supplementary Table E1: Primers used to determine the sequences of Th2-cell Ca_v1.2 and Ca_v1.3 cDNAs.

	Primers *
Ca _v 1.2 (L01776)	Fw 1059 - 1081; Rev 4802- 4825 Fw 4344- 4367; Rev 7579- 7608
Ca _v 1.3 (NM-001083616)	Fw 284- 304; Rev 3708-3728 Fw 3519- 3537; Rev 6914- 6932

*Refer to the murine sequences L01776.1 (Ca_v1.2) and NM-001083616 (Ca_v1.3).

Supplementary Table E2: Oligodeoxynucleotides used in transfection experiments

ON	Sequence	Localization
Ca _v 1.2 AS	5'cctcgtgttttcattgaccat ^{3'}	1187-1167 L01776
Ca _v 1.2 S	5'taccagtacttttgtgctcc ^{3'}	
Ca _v 1.2 mis	5'tctcgtgttttacttagccac ^{3'}	
Ca _v 1.3 AS	5'ttagccttctctcttcctttgagaattctccactaaggacacc ^{3'}	1250-1207 AJ4377291.1
Ca _v 1.3 S	5'ccacaggaatcacctcttaagagtttccttccttctctgatt ^{3'}	

AS: antisense; S: sense; mis: mismatched. * L01776 and AJ4377291.1 refer to the murine Ca_v1.2 and Ca_v1.3a sequences respectively.

Supplementary Figure E1: Th2-cells express neuronal Ca_v1.2 isoform. This figure compares the sequences of Ca_v1.2 in Th2-cells to the neuronal isoform (Genbank accession: L01776) between the exons 31 and 34. None of the 12 cDNA clones isolated from Th2-cells expressed the exon 33.

Supplementary Figure E2: Th2- but not Th1-cells express Ca_v1.2 and anti-Ca_v1.3 proteins. Lysates from differentiated Th1 and Th2-cells were resolved by SDS-PAGE and then immunoblotted (50 µg protein/lane) with anti-Ca_v1.2 or anti-Ca_v1.3 polyclonal antibodies. The anti-Ca_v1.2 and anti-Ca_v1.3 antibodies were pre-incubated (Th2-pep) or not with the antigenic peptide before use. The figure shows one representative experiment out of five performed.

Supplementary Figure E3: Ca_v1AS reduce dihydropyridine receptor expression in Th2-cells. Th2-cells collected after three rounds of stimulation were treated with lipofectin alone, or were transfected with Ca_v1.2 (1.2) or Ca_v1.3 (1.3) sense (S) or antisense (AS). Cells were stained 24 h later with ST-BODIPY-labeled DHP. The fluorescence intensity was quantified by using the Image J software and the intensity/per cell was averaged. Results represent the mean of fluorescence intensity/per cell obtained from five independent experiments. *p < 0.01, as compared to Th2-cells transfected with Ca_v1S. The staining was completely abolished by pre-incubation with an excess of unlabelled DHP (not shown).

Supplementary Figure E4: Ca_v1AS impair Ca²⁺ signaling in Th2- but not in Th1-cells. Th2- or Th1-cells transfected with a mixture of Ca_v1.2 and Ca_v1.3 sense (control) or antisense (AS) were loaded with Fura-2/AM. Intracellular Ca²⁺ levels were recorded for 1 min before stimulation with anti-TCR mAb (arrow) and for 5–6 min after stimulation. The picture shows the traces of individual cell recordings in one field. Ionomycin (iono, 1 μM) was used as a positive control. One representative experiment out of five performed.

Supplementary Figure E5: Ca_v1AS do not alter the proliferation of Th2-cells A) Th2-cells were transfected with a mixture of Ca_v1.2 and Ca_v1.3 antisense (Th2AS) or sense (Th2S). The cells were stained 48h later with carboxyfluorescein succinimidyl ester (CFSE) and stimulated on plates coated with anti-TCR mAb in the presence of anti-CD28 mAb. The fluorescence was then analyzed by FACS 48h later and showed that Th2AS and Th2S proliferated equally well. The histogram shows the percentage of dividing cells in Th2, Th2S and Th2AS. Results are expressed as mean + SD of 4 cultures.

Supplementary Figure E6: Ca_v1AS modify neither the calcium response nor IL-4 production induced by stimulation with thapsigargin or PMA/ionomycin. A) Th2-cells were either treated with lipofectin only (lipo) or transfected with Ca_v1.2 (1.2) or Ca_v1.3 (1.3) sense (S) or antisense (AS) ONs and were loaded with Fura2-AM. The [Ca²⁺]_i was recorded before stimulation (Unst.) and after stimulation with thapsigargin (Thapsi). [Ca²⁺]_i was recorded in 60–70 cells for each condition. The peak value for [Ca²⁺]_i was determined for each cell and the mean value calculated for each sample. The

histograms represent the mean + 1SD of the mean peak values of $[Ca^{2+}]_i$ from six independent experiments. **B)** Production of IL-4 by Th2-cells transfected with Ca_v1AS after stimulation with PMA/ionomycin (PMA/iono), thapsigargin (Thapsi), or anti-TCR antibody (a-TCR). Data were expressed relative to the production of Th2-cells transfected with control ONs and represented the mean + 1SD of five experiments. The ranges of IL-4 production induced by stimulation through the TCR, by PMA/iono or thapsigargin were around 11.5, 7.5 and 6 ng/ml respectively.

Supplementary Figure E7: Th2-cells transfected with Ca_v1AS can localize into the lungs of BALB/c mice upon adoptive transfer. 24 h after transfection of Th2-cells with a mixture of Ca_v1.2 and Ca_v1.3 sense (Th2S) or antisense (Th2AS), cells were injected intravenously in BALB/c mice that were given intranasal OVA for 7 days. The lungs were collected, digested and cells were labeled with anti-CD4, anti-CD8, anti-CD19 and anti-KJ1.26 antibodies. Results show the absolute numbers of total mononuclear cells (tot), of CD4⁺, CD8⁺ T-cells and of B-cells (LB) recovered from mice injected with Th2S (open bars) or Th2AS (black bars), (5 mice by group). * $p < 0.05$ when compared to controls.

Supplementary Figure 8. Ca_v1AS suppress airway hyper-reactivity in an experimental model of active asthma. BALB/c mice were immunized with OVA in alum and were exposed 15 days later to daily doses of intranasal OVA mixed with Ca_v1.2 plus Ca_v1.3 sense (Ca_v1S) or antisense (Ca_v1AS) ONs for 5 days. Mice treated with Ca_v1AS (black circles) displayed no or only mild airway hyper-reactivity upon

exposure to increasing concentrations of intranasal methacholine, unlike mice exposed to either OVA alone (Ct, open squares) or mice treated with Ca_v1S (open circles); * p< 0.01, 5 mice per group. One representative experiment out of three is shown. The dashed line represents the results obtained with mice primed with OVA in alum and given intranasal PBS.

Supplementary Fig. E9. Human Th2 cell clones express functional DHPR/Ca_v1 channels. Three Th2 clones derived from the skin of patients suffering from nickel-induced allergic dermatitis, the characteristics of which were previously described (3), were analyzed by RT-PCR for the presence of Ca_v1 channels by using consensual primers (4). An example is shown in **A**). The PCR products were cloned and sequenced confirming the presence of Ca_v1 mRNA in these T-cell clones. **B**) Two of these clones were incubated or not with nicardipine (10 μM) for 1h at 37°C before to be loaded with Fura2-AM. Intracellular Ca²⁺ levels were recorded every 10 sec before and after stimulation with anti-CD3 plus anti-CD28 mAb (arrow). The ratios of the fluorescence due to excitation at 340 nm to that at 380 nm were calculated for each cell and averaged at each time-point. Results were similar for both clones. Data were expressed as the mean ± 1 SD of at least 30 cells. Ionomycin (iono, 1 μM) was used as a positive control. Nicardipine significantly decreased TCR-induced Ca²⁺ rise at each time-point (at least p < 0.01).

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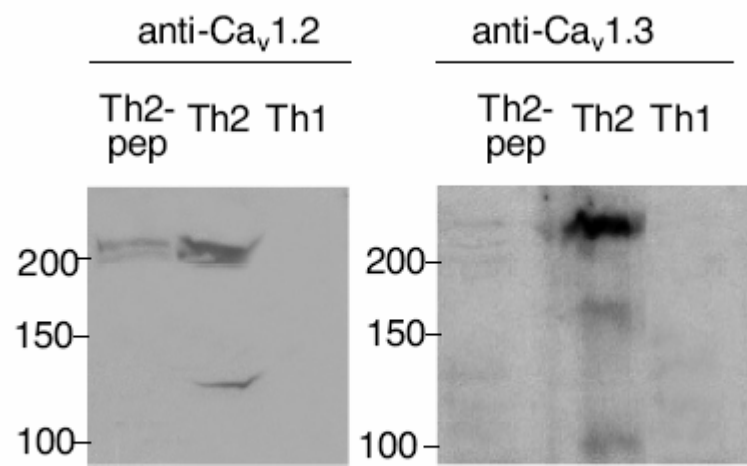
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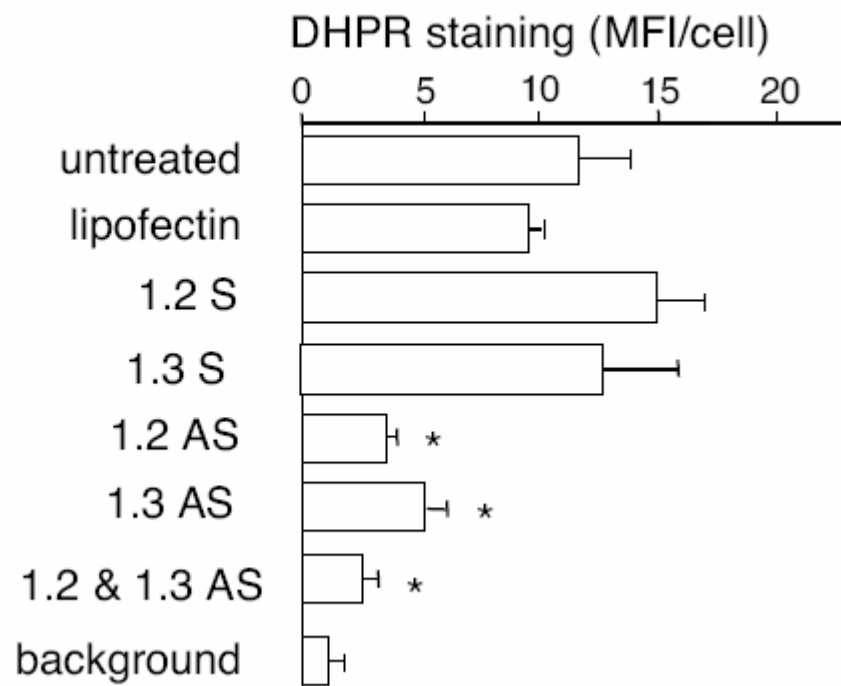
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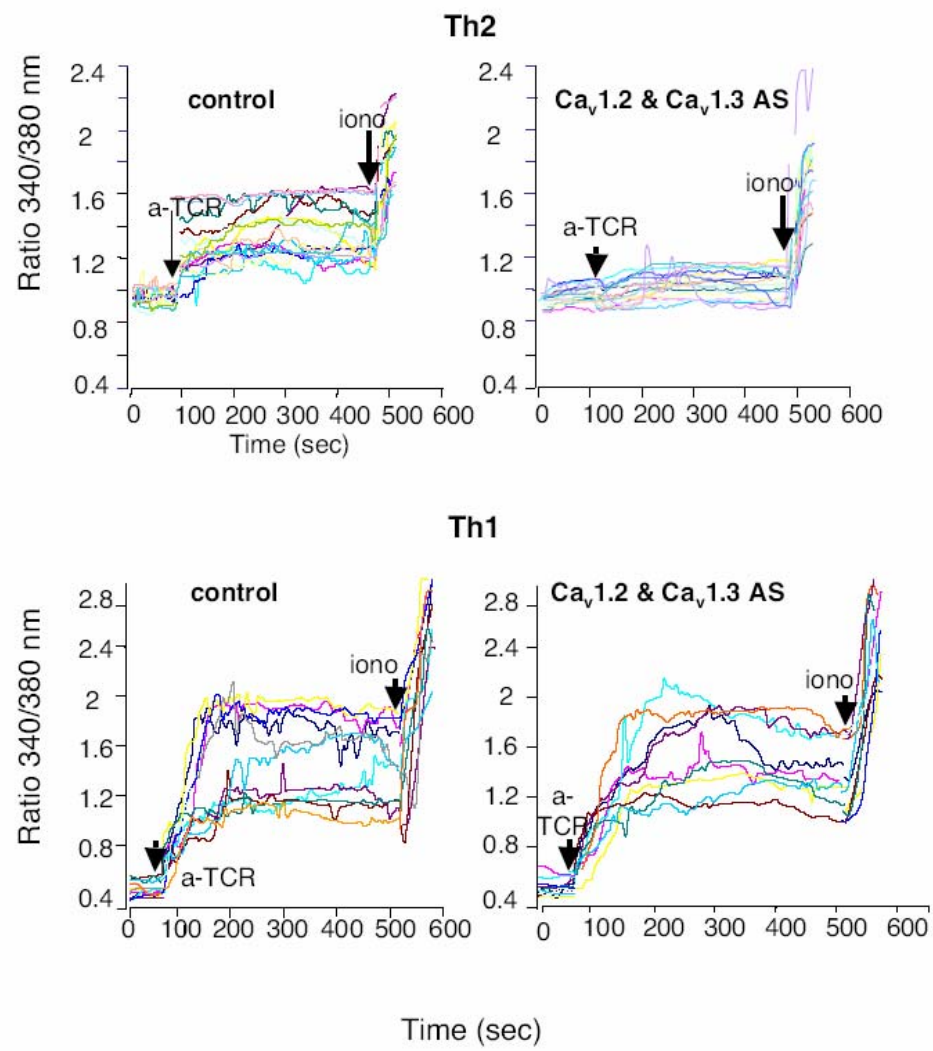
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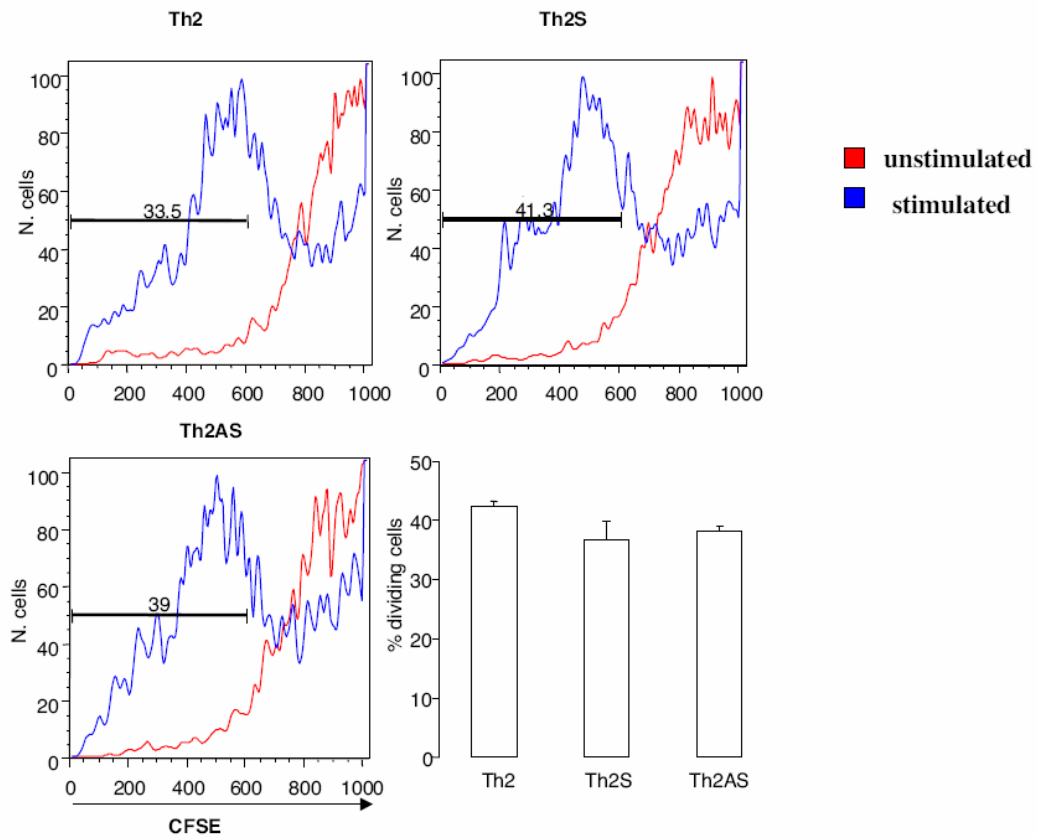
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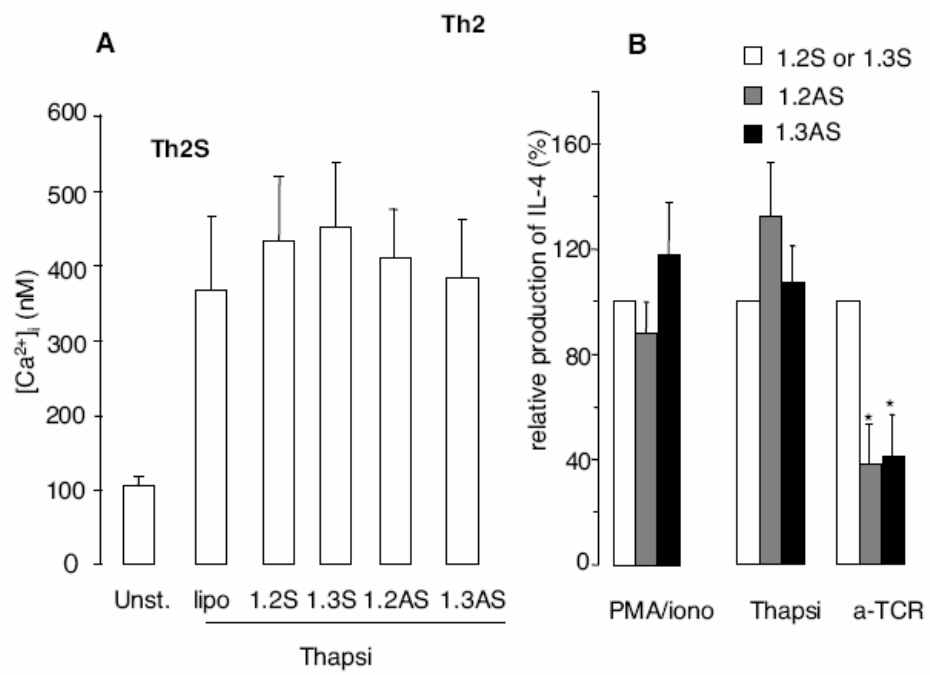
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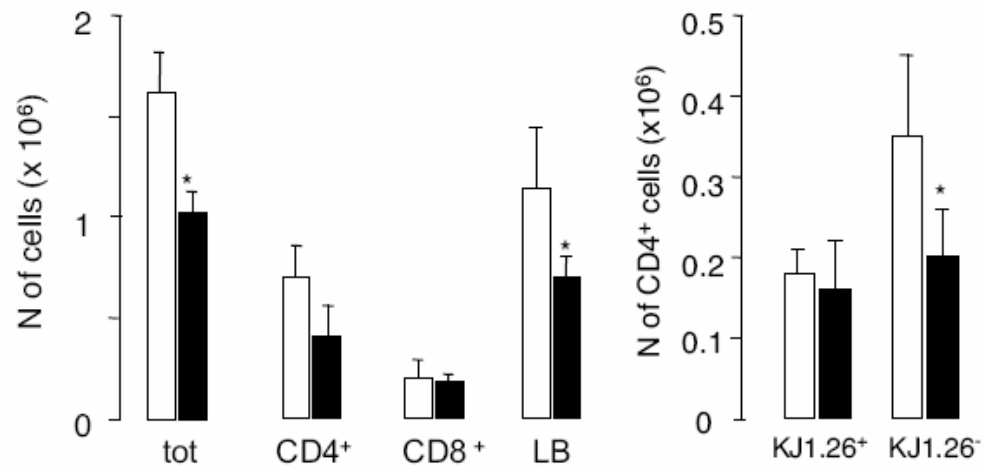
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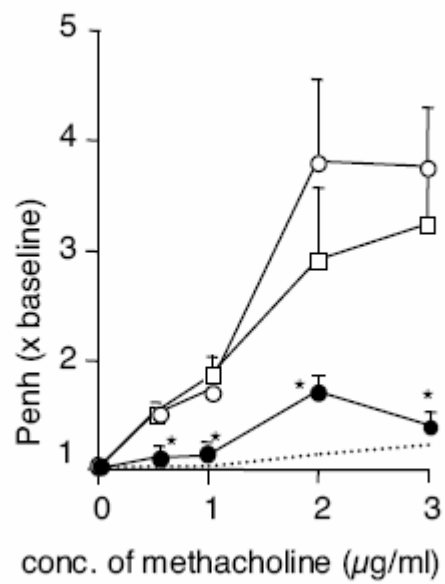
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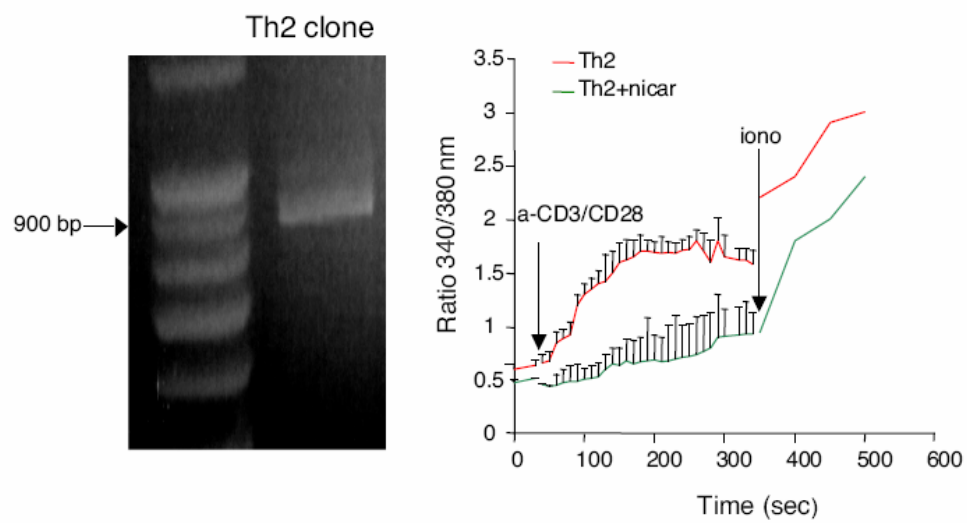
Supplementary Figure E6



Supplementary Figure E7



Supplementary Figure E8



Supplementary Figure E9