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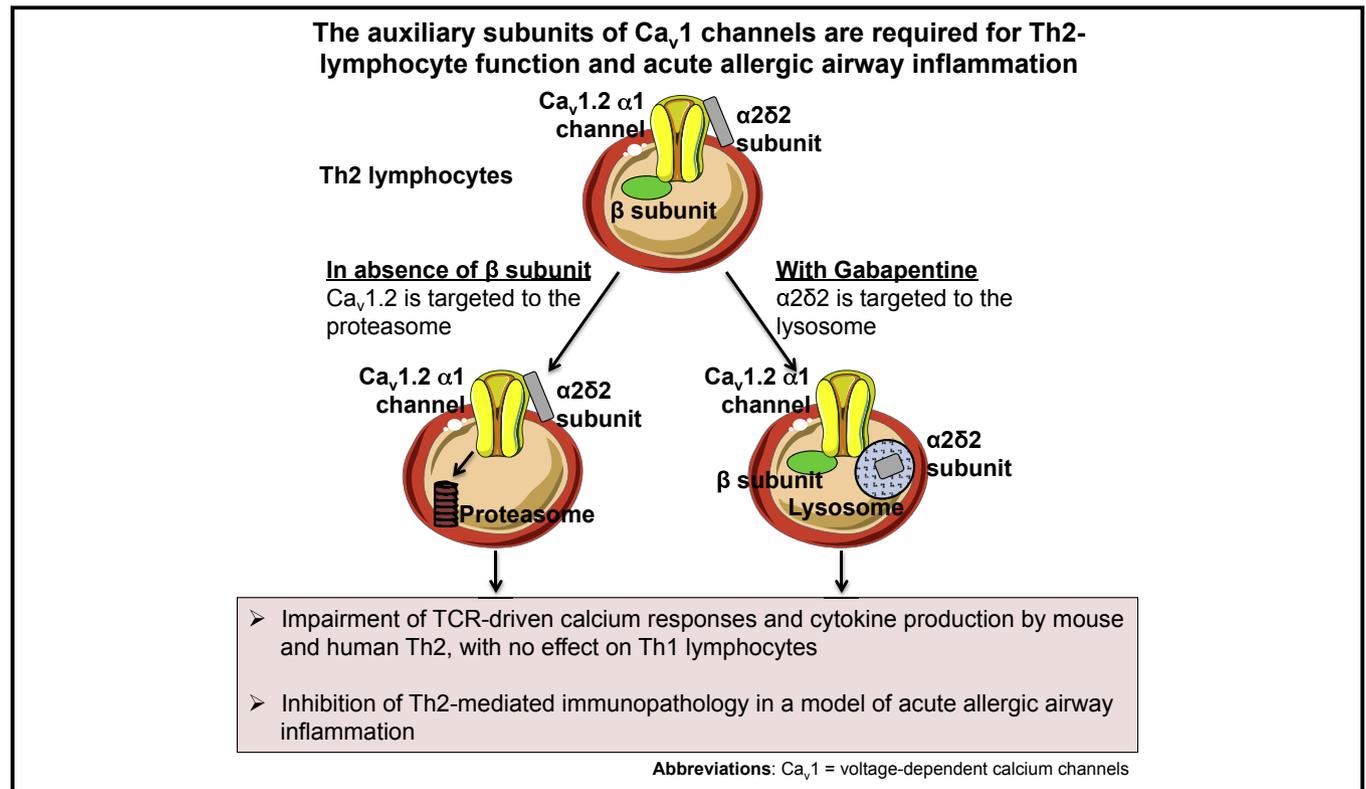
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# The $\beta$ and $\alpha 2\delta$ auxiliary subunits of voltage-gated calcium channel 1 ( $Ca_v1$ ) are required for $T_H2$ lymphocyte function and acute allergic airway inflammation

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## GRAPHICAL ABSTRACT



**Background:** T lymphocytes express not only cell membrane ORAI calcium release-activated calcium modulator 1 but also voltage-gated calcium channel ( $Ca_v$ ) 1 channels. In excitable cells these channels are composed of the ion-forming pore  $\alpha 1$  and auxiliary subunits ( $\beta$  and  $\alpha 2\delta$ ) needed for proper trafficking and activation of the channel. Previously, we

disclosed the role of  $Ca_v1.2 \alpha 1$  in mouse and human  $T_H2$  but not  $T_H1$  cell functions and showed that knocking down  $Ca_v1 \alpha 1$  prevents experimental asthma.

**Objective:** We investigated the role of  $\beta$  and  $\alpha 2\delta$  auxiliary subunits on  $Ca_v1 \alpha 1$  function in  $T_H2$  lymphocytes and on the development of acute allergic airway inflammation.

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**Methods:** We used  $\text{Ca}_v\beta$  antisense oligonucleotides to knock down  $\text{Ca}_v\beta$  and gabapentin, a drug that binds to and inhibits  $\alpha 2\delta 1$  and  $\alpha 2\delta 2$ , to test their effects on  $\text{T}_\text{H}2$  functions and their capacity to reduce allergic airway inflammation.

**Results:** Mouse and human  $\text{T}_\text{H}2$  cells express mainly  $\text{Ca}_v\beta 1$ ,  $\beta 3$ , and  $\alpha 2\delta 2$  subunits.  $\text{Ca}_v\beta$  antisense reduces T-cell receptor-driven calcium responses and cytokine production by mouse and human  $\text{T}_\text{H}2$  cells with no effect on  $\text{T}_\text{H}1$  cells.  $\text{Ca}_v\beta$  is mainly involved in restraining  $\text{Ca}_v 1.2 \alpha 1$  degradation through the proteasome because a proteasome inhibitor partially restores the  $\alpha 1$  protein level. Gabapentin impairs the T-cell receptor-driven calcium response and cytokine production associated with the loss of  $\alpha 2\delta 2$  protein in  $\text{T}_\text{H}2$  cells.

**Conclusions:** These results stress the role of  $\text{Ca}_v\beta$  and  $\alpha 2\delta 2$  auxiliary subunits in the stability and activation of  $\text{Ca}_v 1.2$  channels in  $\text{T}_\text{H}2$  lymphocytes both *in vitro* and *in vivo*, as demonstrated by the beneficial effect of  $\text{Ca}_v\beta$  antisense and gabapentin in allergic airway inflammation. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

**Key words:** Asthma,  $\text{T}_\text{H}2$ , voltage-gated calcium channel 1, calcium, cytokines

Allergic diseases, including rhinitis, atopic dermatitis, asthma, and food allergies, are induced by  $\text{T}_\text{H}2$  lymphocytes.  $\text{T}_\text{H}2$ -type responses are characterized by production of IL-4, IL-5, and IL-13, which contribute to mucus production, eosinophilia, and high levels of antigen-specific IgE. At present, treatments for allergic asthma are often symptomatic, even if in some cases specific allergenic immunotherapy and treatments targeting the cytokines (or their receptors) involved in type 2 inflammation can be beneficial.<sup>1,2</sup> The main immunosuppressants (eg, cyclosporine and tacrolimus) have also been proposed for use in patients with severe asthma resistant to glucocorticoids. However, because they decrease the activity of T cells and therefore the overall immune response by acting on calcium signaling, they have adverse effects.

Calcium is a second messenger that plays specific and key roles in various cellular functions, such as activation, differentiation, proliferation, and death. The role of store-operated  $\text{Ca}^{2+}$  entry is well described, implicating the sensing of T-cell receptor (TCR)-driven endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  depletion by stromal interaction molecule 1, its oligomerization, and its localization in the vicinity of calcium release-activated calcium modulator 1 (ORAI1) channels at the plasma membrane, permitting sustained  $\text{Ca}^{2+}$  entry.<sup>3</sup>

In addition to these channels, the role of voltage-gated calcium channel ( $\text{Ca}_v$ ) 1 channels (defined as voltage activated in excitable cells) in T lymphocytes is now accepted.<sup>4-11</sup>

In excitable cells,  $\text{Ca}_v 1$  channels are composed of the ion-forming pore  $\alpha 1$  and auxiliary  $\beta$  and  $\alpha 2\delta$  subunits, with each subunit being encoded by 4 genes.  $\text{Ca}_v 1.1$  to  $\text{Ca}_v 1.4 \alpha 1$  form the ion pore and support the biophysical and pharmacologic properties of the channel,<sup>12-14</sup> whereas auxiliary  $\beta$  and  $\alpha 2\delta$  subunits increase  $\text{Ca}_v$  currents by enhancing the number of channels at the cell membrane and favoring channel opening.<sup>15-19</sup>  $\text{Ca}_v\beta$  would act by facilitating the correct folding of  $\alpha 1$  and promoting its exit from the ER,<sup>20</sup> whereas  $\text{Ca}_v\alpha 2\delta$  increases insertion of the channel into the cell membrane by favoring the trafficking of the channel from the post-Golgi apparatus and decreasing its turnover.<sup>21-23</sup>

While  $\text{Ca}_v\beta 2$  deletion<sup>24</sup> inhibited thymocyte development,  $\text{Ca}_v\beta 3$  and  $\text{Ca}_v\beta 4$  were found to be important for calcium influx,

#### Abbreviations used

BAL:	Bronchoalveolar lavage
$[\text{Ca}^{2+}]_i$ :	Intracellular $\text{Ca}^{2+}$ concentration
$\text{Ca}_v$ :	Voltage-gated calcium channel
$\text{Ca}_v\beta\text{AS}$ :	$\text{Ca}_v\beta$ antisense
$\text{Ca}_v\beta\text{S}$ :	$\text{Ca}_v\beta$ scrambled
CRTH2:	Chemoattractant receptor-homologous molecule expressed on $\text{T}_\text{H}2$ cells
ER:	Endoplasmic reticulum
HPRT:	Hypoxanthine-guanine phosphoribosyltransferase
ORAI1:	ORAI calcium release-activated calcium modulator 1
OVA:	Ovalbumin
TCR:	T-cell receptor

nuclear factor of activated T cells (NFAT) nuclear translocation, and cytokine production by peripheral  $\text{CD}4^+$  T lymphocytes. Previously, we reported that  $\text{Ca}_v 1.2$  was expressed and functional in human and mouse  $\text{T}_\text{H}2$  cells.<sup>7,9,25</sup> In contrast,  $\text{T}_\text{H}1$  and  $\text{T}_\text{H}17$  lymphocytes in both species lacked  $\text{Ca}_v 1.2$  expression.

Here we address whether  $\text{Ca}_v$  auxiliary subunits are required for channel functions in  $\text{T}_\text{H}2$  lymphocytes. We show that  $\text{Ca}_v\beta 1$ ,  $\text{Ca}_v\beta 3$ , and  $\alpha 2\delta 2$  subunits are expressed in mouse and human  $\text{T}_\text{H}2$  cells. Knocking down  $\text{Ca}_v\beta$  promoted degradation of  $\text{Ca}_v 1.2 \alpha 1$ , which was at least partly rescued by adding MG132, a proteasome inhibitor, whereas gabapentin, an inhibitor of  $\alpha 2\delta 1/2$  subunits, decreased  $\alpha 2\delta 2$  protein levels in  $\text{T}_\text{H}2$  lymphocytes. In both cases, it was associated with a decreased TCR-dependent intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) increase and  $\text{T}_\text{H}2$  cytokine production. In accordance with our *in vitro* results, targeting either  $\text{Ca}_v\beta$  or  $\alpha 2\delta$  *in vivo* was beneficial in a model of acute allergic airway inflammation.

## METHODS

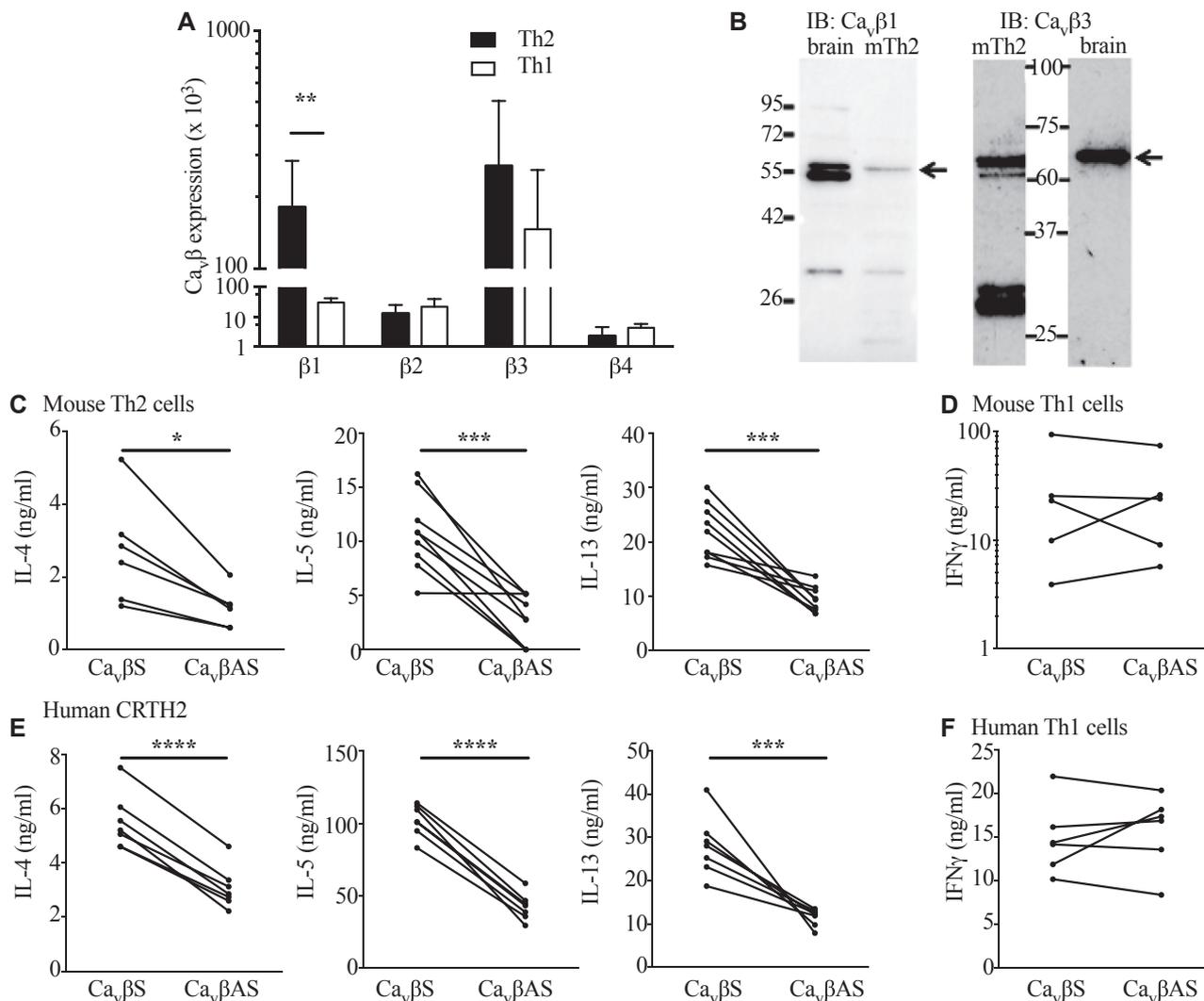
More details are provided in the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Mice and model of acute airway allergic inflammation

Eight- to 12-week-old female BALB/c mice were obtained from Janvier (Le Genest St Isle, France), and TCR ovalbumin (OVA) transgenic DO11.10 mice were maintained in our pathogen-free animal facility. The INSERM U1043 Institutional Review Board for animal experimentation approved protocols. BALB/c mice immunized intraperitoneally with OVA (100  $\mu\text{g}$ ) in alum (2 mg) were 15 days later administered intranasal OVA (50  $\mu\text{g}/\text{d}$ ) in PBS for 5 days, as previously described,<sup>7</sup> with or without  $\text{Ca}_v\beta$  scrambled ( $\text{Ca}_v\beta\text{S}$ ) or  $\text{Ca}_v\beta$  antisense ( $\text{Ca}_v\beta\text{AS}$ ) oligonucleotides (200  $\mu\text{g}/\text{d}$ ) or gabapentin (400 mg/L in drinking water) that was renewed every other day.<sup>26</sup> For  $\text{T}_\text{H}2$  transfer experiments, BALB/c mice (Janvier) were transferred intravenously with  $3 \times 10^6$  DO11.10  $\text{T}_\text{H}2$  cells transfected with  $\text{Ca}_v\beta\text{S}$  or  $\text{Ca}_v\beta\text{AS}$  and given intranasal OVA (50  $\mu\text{g}/\text{d}$ ) for 5 days. Twenty-four hours after the final OVA administration, serum, bronchoalveolar lavage (BAL) fluid, lungs, and draining lymph nodes were collected and processed, as described in the [Methods](#) section in this article's Online Repository.

## Cell culture

Mouse  $\text{T}_\text{H}1$  and  $\text{T}_\text{H}2$  cells were generated by weekly stimulation of DO11.10  $\text{CD}4^+$  T cells with antigen-presenting cells and the 323-339 OVA peptide plus appropriate differentiation cocktails: IL-12 (5 ng/mL) and anti-IL-4 antibody (11B11, 10  $\mu\text{g}/\text{mL}$ ) for  $\text{T}_\text{H}1$  and IL-4 (5 ng/mL) and



**FIG 1.** Ca<sub>v</sub>βAS diminishes cytokine production in mouse and human T<sub>H2</sub> but not in T<sub>H1</sub> cells. **A** and **B**, OVA-specific DO11.10 T<sub>H2</sub> and T<sub>H1</sub> cells were tested for Ca<sub>v</sub>β at the transcript (n = 4; Fig 1, A) or protein level (Fig 1, B). **C-F**, Mouse DO11.10 T<sub>H2</sub> (Fig 1, C), mouse T<sub>H1</sub> (Fig 1, D), human CRTH2<sup>+</sup> (Fig 1, E), or human T<sub>H1</sub> (Fig 1, F) cells were transfected with Ca<sub>v</sub>βS or Ca<sub>v</sub>βAS for 72 hours, then stimulated for 24 hours with anti-CD3/C28 antibodies, and cytokine production was measured. Each pair represents a separate experiment in Fig 1, C and D, and a donor in Fig 1, E and F. \*P < .02, \*\*P < .01, \*\*\*P < .005, and \*\*\*\*P < .001.

anti-IFN-γ antibody (XMG1.2, 10 μg/mL) for T<sub>H2</sub>. PBMCs were obtained from Etablissement Français du Sang, and all human participants provided written informed consent. Human memory chemoattractant receptor-homologous molecule expressed on T<sub>H2</sub> cells (CRTH2)<sup>+</sup> CD4<sup>+</sup> T and human T<sub>H1</sub> cells were obtained and expanded, as previously reported.<sup>9</sup> Cells were transfected with 8 μmol/L oligodeoxynucleotides (Eurogentec, Angers, France) by using TurboFect transfection reagent (Fermentas, Waltham, Mass) and collected 3 days after transfection.

### Single-cell calcium imaging

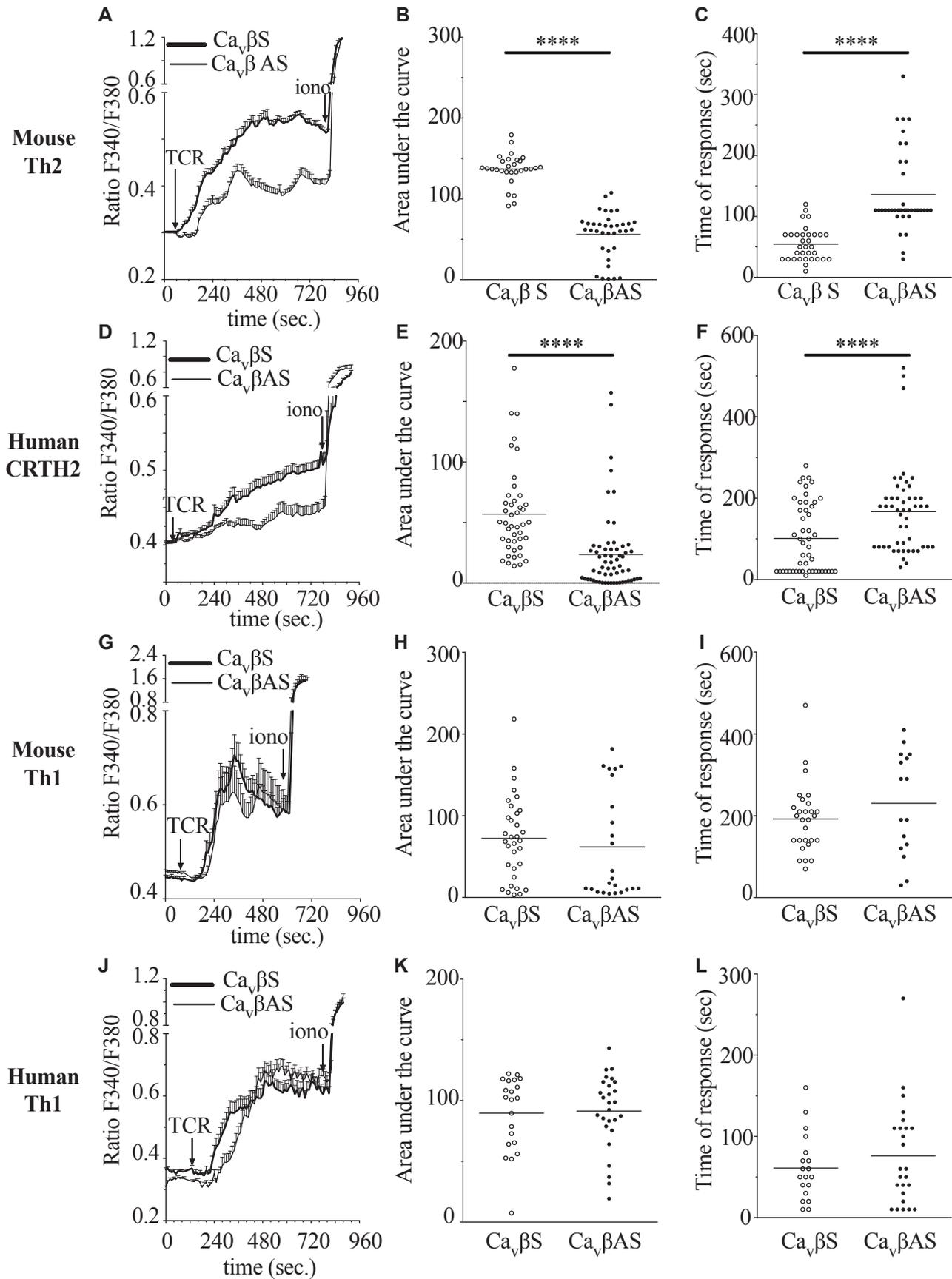
Single-cell calcium imaging was done, as previously described,<sup>7,9</sup> in cells loaded with Fura-2 AM before and after stimulation with biotinylated anti-CD3 cross-linked with streptavidin or anti-CD3/CD28-coated beads (1 bead per cell) for mouse or human T cells, respectively.

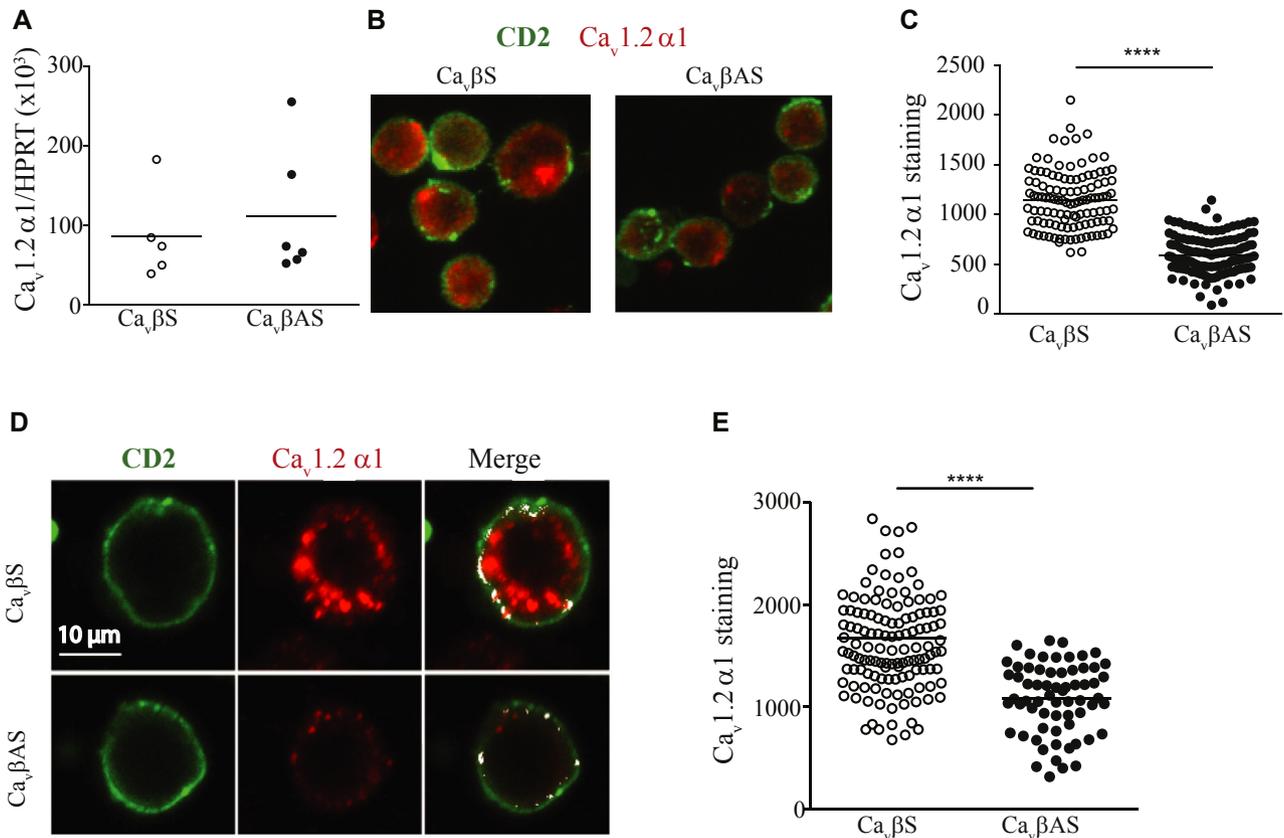
### Confocal microscopy

Cells were stained with anti-CD2 rat IgG antibody (Abcam, Cambridge, United Kingdom) or anti-α2δ2 rabbit antibody (Alomone Labs, Jerusalem, Israel) fixed, permeabilized and the Ca<sub>v</sub> channel subunit staining was performed by using anti-Ca<sub>v</sub>1.2 mouse IgG<sub>2b</sub> (NeuroMab, Davis, Calif), anti-Ca<sub>v</sub>β1 (Alomone Labs), or anti-Ca<sub>v</sub>β3 rabbit IgG (Santa Cruz Labs, Santa Cruz, Calif).

### Real-time quantitative PCR

RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif), and mRNA transcripts were measured by using real-time quantitative PCR with a LightCycler 480 Instrument (Roche, Mannheim, Germany).





**FIG 3.** Knocking down  $Ca_v\beta$  subunits decreases  $Ca_v1\alpha1$  expression in mouse and human  $T_H2$  cells. Cells were transfected with  $Ca_v\beta S$  or  $Ca_v\beta AS$  for 72 hours. **A**, Amount of  $Ca_v1.2\alpha1$  transcripts in mouse D011.10  $T_H2$  cells. **B-E**, Staining with anti-CD2 (green) and anti- $Ca_v1.2\alpha1$  (red) mAbs in mouse D011.10  $T_H2$  cells (Fig 3, **B** and **C**) and human  $CRTH2^+$  cells (Fig 3, **D** and **E**). The  $Ca_v1.2$  cell amount over all the Z-stack sections is quantified in Fig 3, **C** and **E**. One representative experiment of 3 is shown. \*\*\*\* $P < .001$ .

### Immunoblotting

$T_H2$  lymphocytes or brain cells were lysed in RIPA buffer containing protease inhibitors (Roche), and 30  $\mu\text{g}$  per lane for T-cell extracts and 10  $\mu\text{g}$  per lane for brain lysate were submitted to immunoblotting.

### ELISA

For *in vitro* cytokine assays,  $5 \times 10^4$   $T_H1$  and  $T_H2$  cells were seeded onto plates coated with anti-CD3 antibodies (3  $\mu\text{g}/\text{mL}$ ) and stimulated with soluble anti-CD28 antibodies (1  $\mu\text{g}/\text{mL}$ ). Supernatants were collected 24 hours later. For *in vivo* IgE assays, sera were isolated from blood collected into the mouse retro-orbital sinus. Cytokine and immunoglobulin levels were quantified by using the sandwich ELISA method.

### Statistical analysis

Groups were compared by using the paired or unpaired *t* test as needed with GraphPad Prism software (GraphPad Software, La Jolla, Calif).

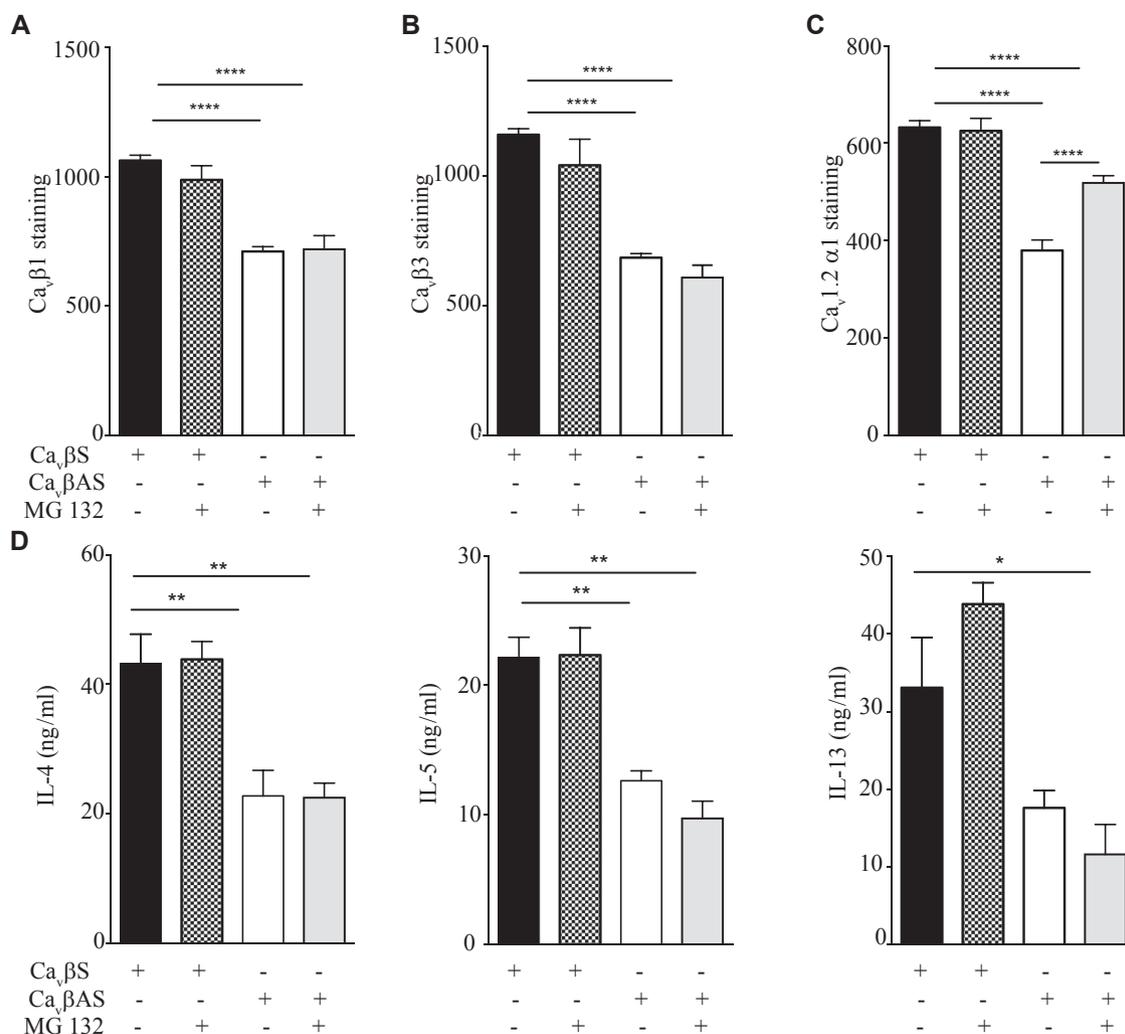
## RESULTS

### Knocking down $Ca_v\beta$ subunits decreases cytokine production induced by TCR engagement in mouse and human $T_H2$ cells

Murine  $T_H2$  cells expressed principally  $Ca_v\beta1$  and  $Ca_v\beta3$  transcripts (Fig 1, **A**), as did human  $T_H2$  cells.<sup>9</sup> The proteins  $Ca_v\beta1$  and  $Ca_v\beta3$  were detected by Western blotting with the same apparent molecular weights as the control neuronal forms (Fig 1, **B**) and by immunofluorescence (see Fig E1, **A** and **B**, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although  $T_H1$  cells did not express  $Ca_v\alpha1$  subunits, they did express  $Ca_v\beta1$  and  $Ca_v\beta3$  at both the transcriptional (Fig 1, **A**) and protein levels, with lower amounts compared with those in  $T_H2$  cells (see Fig E1, **A** and **B**).

To investigate whether  $\beta$  subunits were important for  $T_H2$  functions, we used antisense oligonucleotides targeting the

**FIG 2.** Knocking down  $Ca_v\beta$  subunits reduces the TCR-driven  $[Ca]_i$  increase in mouse and human  $T_H2$  cells without any effect on  $T_H1$  cells. Mouse D011.10  $T_H2$  (**A-C**), human  $CRTH2^+$  (**D-F**), mouse  $T_H1$  (**G-I**), or human  $T_H1$  (**J-L**) cells were transfected with  $Ca_v\beta AS$  or  $Ca_v\beta S$  and loaded with Fura-2 AM. The fluorescence ratio at 340- and 380-nm excitation wavelengths was recorded at the single-cell level before and after TCR stimulation; ionomycin (*iono*) was added at the end of the experiment. Results in Fig 2, **A**, **D**, **G**, and **J** are expressed as the mean of at least 30 cells, and bars represent SEMs. The area under the curve between the time of stimulation and the time at which ionomycin is added is shown in Fig 2, **B**, **E**, **H**, and **K**. The time of response (delay between stimulation and the time showing an increase in ratio over the mean ratio plus 1 SD recorded before stimulation) is shown in Fig 2, **C**, **F**, **I**, and **L**. One representative experiment of 4 to 6 is shown. \*\*\*\* $P < .001$ .



**FIG 4.** MG132 partially restores Ca<sub>v</sub>1.2 α1 in T<sub>H</sub>2 cells knocked down for Ca<sub>v</sub>β without an effect on T<sub>H</sub>2 cytokine production. Mouse DO11.10 T<sub>H</sub>2 cells were transfected with Ca<sub>v</sub>βS or Ca<sub>v</sub>βAS for 48 hours before addition of MG132 (0.5 μmol/L) or vehicle for an additional 24 hours. Cells were then recovered and analyzed by immunofluorescence (**A-C**) or stimulated for 24 hours with anti-CD3/C28 antibodies (**D**). **A-C**, T<sub>H</sub>2 cells were labeled with anti-Ca<sub>v</sub>β1 (Fig 4, **A**), anti-Ca<sub>v</sub>β3 (Fig 4, **B**), or anti-Ca<sub>v</sub>1.2 α1 (Fig 4, **C**) mAbs. Immunofluorescence was summed over all Z-sections. Results are expressed as the mean of 50 to 200 cells. Bars represent SEMs. One representative experiment of 3 is shown. **D**, Cytokine production was measured by ELISA. Results are expressed as the mean of 4 experiments, and bars represent SDs. \**P* < .05, \*\**P* < .01, and \*\*\*\**P* < .001.

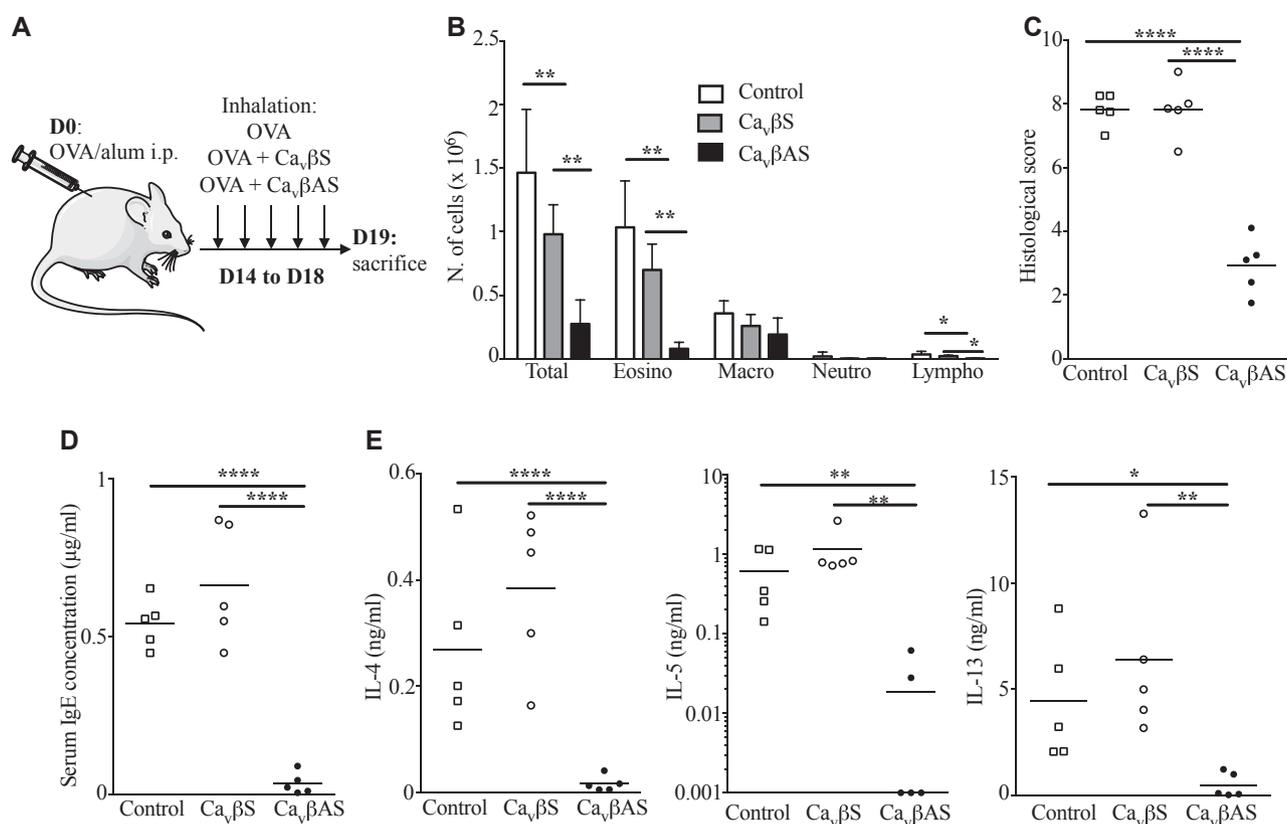
4 Ca<sub>v</sub>β subunits in mice and human subjects.<sup>27</sup> Transfection of mouse T<sub>H</sub>2 cells with Ca<sub>v</sub>βAS decreased the amount of Ca<sub>v</sub>β1 and Ca<sub>v</sub>β3 subunits by Western blotting (see Fig E1, **C** and **D**) and by immunofluorescence (see Fig E1, **E** and **F**), with no effect on expression of ORAI1, a calcium channel important for T-cell function (see Fig E1, **C** and **D**). Ca<sub>v</sub>βAS decreased IL-4, IL-5, and IL-13 production by 50% to 70% in mouse T<sub>H</sub>2 cells compared with Ca<sub>v</sub>βS-transfected T<sub>H</sub>2 cells (Fig 1, **C**) without any effect on IFN-γ production by T<sub>H</sub>1 cells (Fig 1, **D**).

Human memory T<sub>H</sub>2 cells were selected based on expression of the cell membrane marker CRTH2 and were expanded with anti-CD3/anti-CD28-coated beads in the presence of IL-4 and IL-2. Transfection of human T<sub>H</sub>2 lymphocytes with Ca<sub>v</sub>βAS reduced the amount of Ca<sub>v</sub>β subunits at the mRNA level (see Fig E1, **G**) associated with significant diminution of T<sub>H</sub>2 cytokine

production (Fig 1, **E**) without any effect on IFN-γ production by human T<sub>H</sub>1 cells (Fig 1, **F**).

### Knocking down Ca<sub>v</sub>β subunits diminishes TCR-driven calcium response in mouse and human T<sub>H</sub>2 cells

To show that Ca<sub>v</sub>β subunits play a role in Ca<sup>2+</sup> signaling of T<sub>H</sub>2 cells, we analyzed the TCR-driven [Ca<sup>2+</sup>]<sub>i</sub> increase at the single-cell level. Mouse T<sub>H</sub>2 cells transfected with Ca<sub>v</sub>βAS displayed a decreased and delayed calcium response compared with cells transfected with Ca<sub>v</sub>βS (Fig 2, **A**). Indeed, Ca<sub>v</sub>βAS-transfected T<sub>H</sub>2 cells presented a significantly reduced area under the curve compared with control T<sub>H</sub>2 cells (Fig 2, **B**). A significant number of Ca<sub>v</sub>βAS-transfected T<sub>H</sub>2 cells (21% ± 5%) did not show any increase in [Ca<sup>2+</sup>]<sub>i</sub> levels after TCR stimulation, whereas most



**FIG 5.** Ca<sub>v</sub>βAS administration strongly diminishes allergic airway inflammation. **A**, BALB/c mice were immunized with OVA in alum and challenged 15 days later with intranasal OVA only or OVA plus scrambled Ca<sub>v</sub>β (Ca<sub>v</sub>βS) or OVA plus Ca<sub>v</sub>β antisense (Ca<sub>v</sub>βAS) every day for 5 days. *i.p.*, Intraperitoneal. **B**, Number of cells in BAL fluid. **C**, Hematoxylin and eosin–stained sections from mice were scored on a 0- to 12-point scale. **D**, Serum IgE concentrations were measured at the time of death. **E**, Mediastinal lymph nodes were collected, cell suspensions were recalled for 72 hours with OVA, and cytokine production was measured by ELISA. Results in Fig 5, B–E, are means of 5 mice and representative of 2 independent experiments. \**P* < .05, \*\**P* < .01, and \*\*\*\**P* < .001.

control T<sub>H</sub>2 cells (98% ± 2%) did (*P* < .005). Moreover, among responsive cells, Ca<sub>v</sub>βAS-transfected T<sub>H</sub>2 cells had a delayed calcium response compared with control cells (Fig 2, C). However, ionomycin application at the end of the experiment induced a similar [Ca<sup>2+</sup>]<sub>i</sub> increase in T<sub>H</sub>2 cells, irrespective of whether they were transfected with Ca<sub>v</sub>βS or Ca<sub>v</sub>βAS (Fig 2, A), indicating that Ca<sub>v</sub>βAS did not affect loading with the Fura-2 probe.

Ca<sub>v</sub>βAS had the same effect on the TCR-driven [Ca<sup>2+</sup>]<sub>i</sub> increase of human T<sub>H</sub>2 lymphocytes with a delayed and reduced [Ca<sup>2+</sup>]<sub>i</sub> increase (Fig 2, D–F). Conversely, transfection of mouse (see Fig 2, G–I) or human (Fig 2, J–L) T<sub>H</sub>1 cells with Ca<sub>v</sub>βAS had no effect on the shape and the amplitude of the calcium response induced by TCR stimulation, which is in agreement with the lack of an effect of Ca<sub>v</sub>βAS on IFN-γ synthesis by T<sub>H</sub>1 cells (Fig 1, D and F).

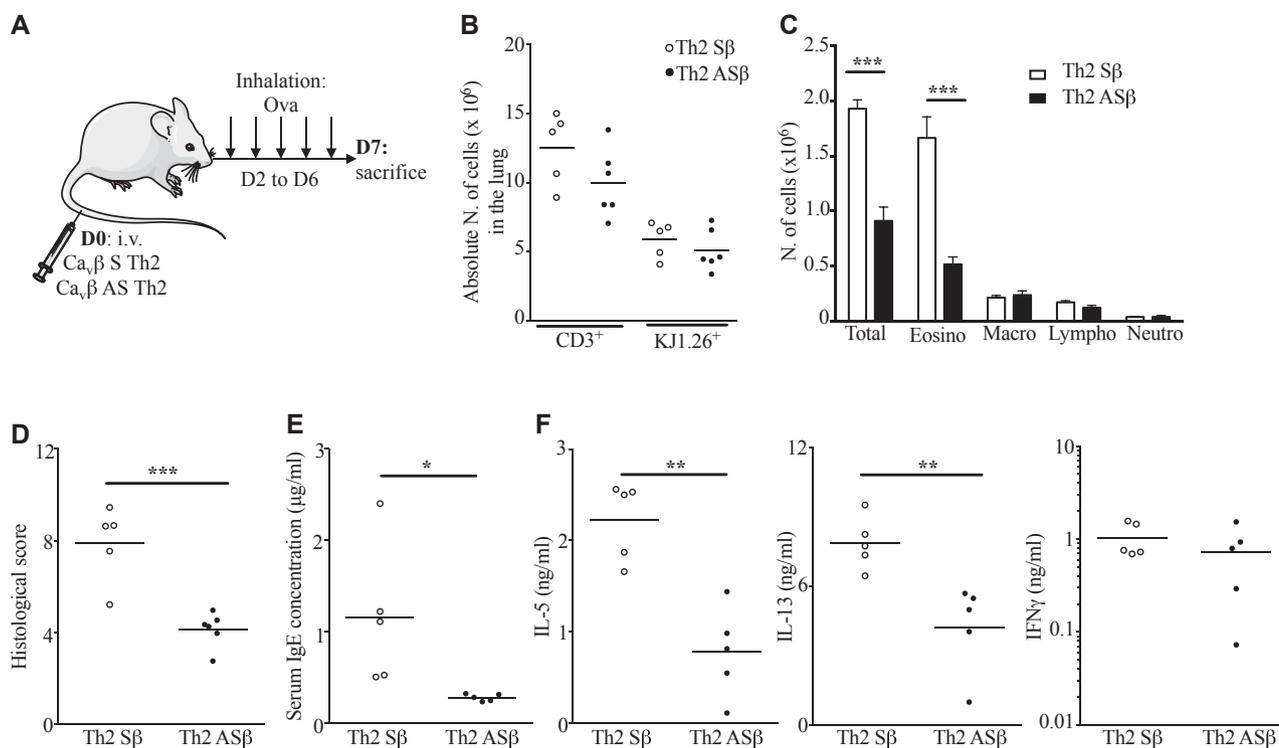
### An inhibitor of the proteasome protects the Ca<sub>v</sub>1.2 α1 subunit from degradation in the absence of Ca<sub>v</sub>β but does not restore cytokine production

One possible role of Ca<sub>v</sub>β subunits could be to prevent Ca<sub>v</sub>1.2 degradation by the proteasome.<sup>28</sup> In agreement with this hypothesis, we showed that transfection of T<sub>H</sub>2 cells with

Ca<sub>v</sub>βAS did not affect levels of Ca<sub>v</sub>1.2 α1 transcripts (Fig 3, A) but strongly reduced the amount of Ca<sub>v</sub>1.2 α1 protein in mouse (Fig 3, B and C) and human (Fig 3, D and E) cells. The proteasome inhibitor MG132 had no effect on amounts of Ca<sub>v</sub>β1 (Fig 4, A) and Ca<sub>v</sub>β3 (Fig 4, B) proteins, but it partially restored Ca<sub>v</sub>1.2 α1 protein expression in murine T<sub>H</sub>2 cells (Fig 4, C). Analysis of colocalization of Ca<sub>v</sub>1.2 α1 with CD2 used as a cell membrane marker shows that Ca<sub>v</sub>βAS decreases the amount of protein localized at the cell membrane, which was partially restored by MG132 (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). However, MG132 treatment did not allow restoration of cytokine production by T<sub>H</sub>2 cells transfected with Ca<sub>v</sub>βAS (Fig 4, D), suggesting that the Ca<sub>v</sub>β subunit could also contribute to regulate channel function, as previously reported.<sup>29–31</sup>

### Intranasal delivery of Ca<sub>v</sub>βAS alleviates T<sub>H</sub>2-dependent airway inflammation

With the goal to investigate the role of Ca<sub>v</sub>β *in vivo*, we evaluated whether Ca<sub>v</sub>βAS modified the course of allergic airway inflammation. Immunization with OVA in alum followed by inhalation of OVA (Fig 5, A) triggered T<sub>H</sub>2-dependent airway inflammation marked by predominant eosinophil infiltration



**FIG 6.** T<sub>H</sub>2 cells transfected with Ca<sub>v</sub>βAS have impaired ability to induce type 2 airway inflammation. **A**, T<sub>H</sub>2 cells transfected with Ca<sub>v</sub>βS or Ca<sub>v</sub>βAS were injected into BALB/c mice given intranasal OVA. *i.v.*, Intravenous. **B**, Lung-infiltrating cells were purified and analyzed by means of flow cytometry to enumerate CD3<sup>+</sup> and CD3<sup>+</sup>KJ1.26<sup>+</sup> cells. **C**, Inflammatory cells were enumerated in BAL fluid. **D**, Lung sections were scored for inflammation. **E**, Serum IgE concentrations were measured at the time of death. **F**, Lung draining lymph node cells were recalled with OVA for determination of cytokine production. Each group comprised 5 mice. \**P* < .05, \*\**P* < .01, and \*\*\**P* < .005.

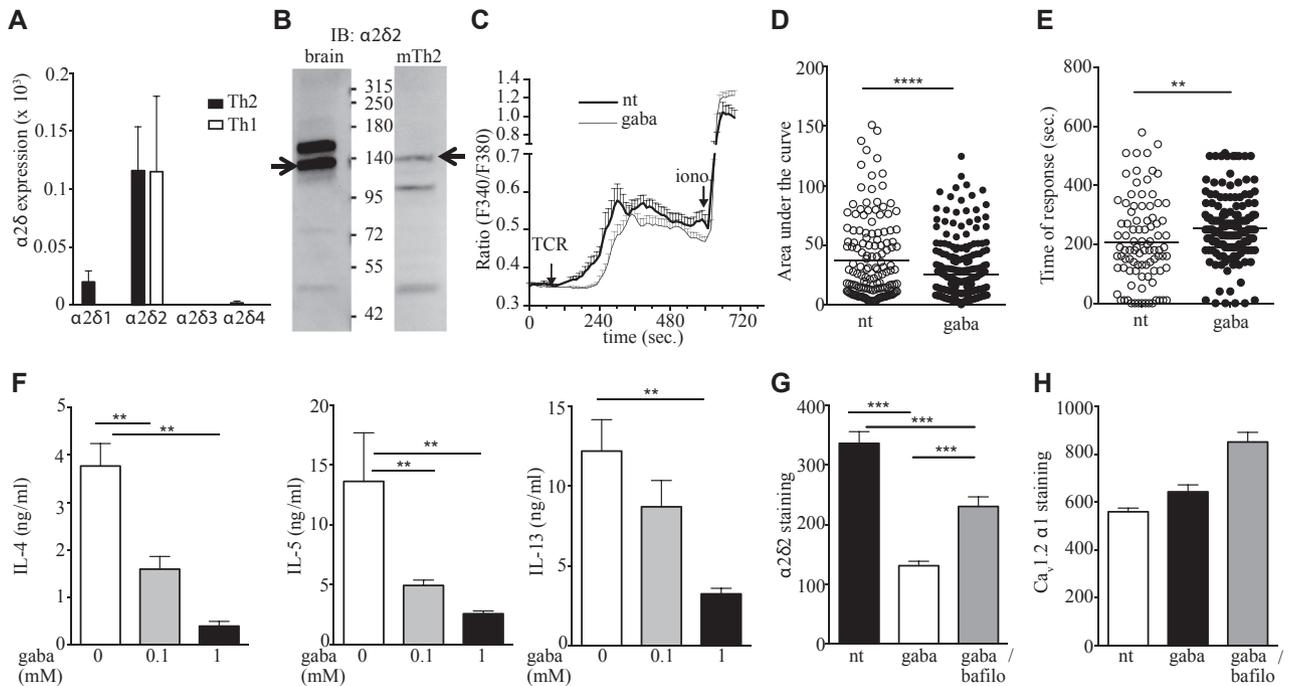
(Fig 5, B). Ca<sub>v</sub>βAS administration decreased strongly the content of inflammatory cells in BAL fluid, including eosinophils and lymphocytes, compared with that seen in control mice treated or not with Ca<sub>v</sub>βS (Fig 5, B). Consistently, histologic examination of lung tissues showed that Ca<sub>v</sub>βAS administration inhibited inflammatory infiltrates (Fig 5, C, and see Fig E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). This protection was associated with defective T<sub>H</sub>2 responses, as shown by the dramatic reduction in serum IgE concentration (Fig 5, D) and the reduced synthesis of IL-4, IL-5, and IL-13 by OVA-stimulated T cells from lung draining lymph node cells (Fig 5, E). In accordance with the effects of Ca<sub>v</sub>βAS on airway inflammation, mice displayed reduced airway hyperresponsiveness (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Interestingly, CD4<sup>+</sup> T cells infiltrating the lungs in Ca<sub>v</sub>βAS-treated mice expressed reduced amounts of Ca<sub>v</sub>1.2 α1 proteins compared with CD4<sup>+</sup> T cells from mice treated with Ca<sub>v</sub>βS (see Fig E3, B-D), suggesting that Ca<sub>v</sub>β was required for the maintenance of Ca<sub>v</sub>1.2 α1 protein expression in lung-infiltrating CD4<sup>+</sup> T cells, as shown for T<sub>H</sub>2 cells *in vitro*.

To investigate whether Ca<sub>v</sub>β expression in T<sub>H</sub>2 cells was necessary and sufficient to control allergic asthma, we transferred OVA-specific TCR transgenic DO11.10 T<sub>H</sub>2 cells transfected with Ca<sub>v</sub>βAS or Ca<sub>v</sub>βS in mice that were then challenged with intranasal OVA (Fig 6, A). Ca<sub>v</sub>βAS- and Ca<sub>v</sub>βS-transfected T<sub>H</sub>2 cells localized equally well in the lungs (Fig 6, B). However, Ca<sub>v</sub>βAS-transfected T<sub>H</sub>2 cells had reduced ability to promote the

T<sub>H</sub>2 response on airway challenge, as shown by the reduction in lung inflammation (Fig 6, C and D, and see Fig E3, E), the lower serum IgE concentration (Fig 6, E), and the impaired T<sub>H</sub>2 cytokine production after recall of antigen-specific draining lymph node T cells with OVA (Fig 6, F). This was not associated with a shift toward a T<sub>H</sub>1 response, as shown by the similar IFN-γ production by OVA-stimulated draining lymph node cells from both groups (Fig 6, F).

### Gabapentin, an inhibitor of Ca<sub>v</sub>α2δ, impairs T<sub>H</sub>2 function and reduces allergic airway inflammation

Mouse T<sub>H</sub>2 cells expressed mainly Ca<sub>v</sub> α2δ2 (Fig 7, A), as did human T<sub>H</sub>2 cells.<sup>9</sup> Blotting and probing T<sub>H</sub>2 cell or brain lysates with anti-α2δ2 antibodies showed similar bands of around 140 kDa (Fig 7, B). To investigate the role of α2δ in Ca<sub>v</sub>1-dependent T<sub>H</sub>2 function, we used gabapentin, a ligand binding to Ca<sub>v</sub> α2δ1 and α2δ2 but not to the other α2δ subunits.<sup>32</sup> Chronic *in vitro* treatment with this drug is known to inhibit Ca<sub>v</sub> currents. Accordingly, treatment of T<sub>H</sub>2 cells with gabapentin for 72 hours decreased and delayed the TCR-driven calcium response (Fig 7, C), as shown by quantification of the area under the curve and time of response, respectively (Fig 7, D and E). This decrease in calcium signaling was associated with reduced T<sub>H</sub>2 cytokine production (Fig 7, F). However, gabapentin had no effect on calcium response and IFN-γ production in T<sub>H</sub>1 lymphocytes (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).



**FIG 7.** Gabapentin (*gaba*), an inhibitor of  $\text{Ca}_v\alpha2\delta$  auxiliary subunits, reduces calcium signaling and cytokine production by mouse  $\text{T}_\text{H}2$  cells. **A**, Mouse DO11.10  $\text{T}_\text{H}2$  cells were analyzed for expression of  $\alpha2\delta$  transcripts ( $n = 4$ ). **B**,  $\text{T}_\text{H}2$  and brain (positive control) lysates were blotted and stained with anti- $\alpha2\delta2$  antibodies. *IB*, Immunoblotting.  $\text{T}_\text{H}2$  cells were preincubated with gabapentin (1 mmol/L) for 72 hours in Fig 7, *C-H*. **C-E**, Cells were then loaded with the calcium probe Fura-2 AM, and the fluorescence ratio at 340- and 380-nm excitation wavelengths was recorded at the single-cell level. Results are expressed as the mean of at least 50 cells, and *bars* represent SEMs. Fig 7, *D* and *E*, show, respectively, the area under the curve and the time of response for each cell defined as in Fig 3. Data are representative of 3 experiments. **F**,  $\text{T}_\text{H}2$  cells were stimulated in the presence or not of gabapentin for an additional 24 hours with anti-CD3/anti-CD28 antibodies. Cytokine content was then measured. **G** and **H**,  $\text{T}_\text{H}2$  cells were incubated or not with bafilomycin (*bafilo*; 0.5  $\mu\text{mol/L}$ ) for the last 24 hours. Cells were stained with anti- $\alpha2\delta2$  (Fig 7, *G*) or anti- $\text{Ca}_v1.2 \alpha1$  (Fig 7, *H*) antibodies. Immunofluorescence was summed all over the Z-stack sections. One representative experiment of 2 is shown. \*\* $P < .01$ , \*\*\* $P < .005$ , and \*\*\*\* $P < .001$ .

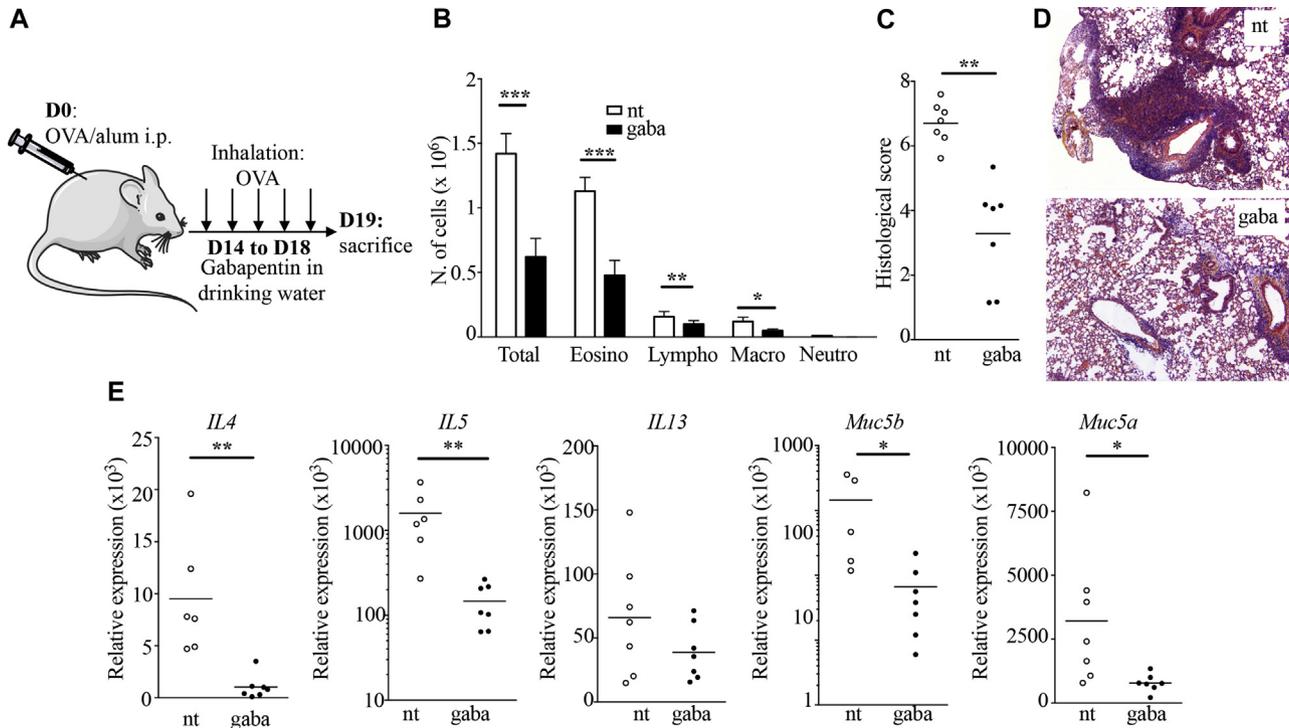
Because gabapentin was reported to induce loss of the  $\text{Ca}_v2$  complex at the cell membrane,<sup>33,34</sup> we tested its effect on  $\text{Ca}_v1.2$  protein expression in  $\text{T}_\text{H}2$  cells. The amount of  $\alpha2\delta2$  protein was strongly reduced in gabapentin-treated  $\text{T}_\text{H}2$  cells (Fig 7, *G*, and see Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), with no effect on  $\text{Ca}_v1.2$  expression (Fig 7, *H*, and see Fig E6). Moreover, Bafilomycin A1, an inhibitor of the vacuolar  $\text{H}^+$  ATPase that inhibited acidification and protein degradation in lysosomes, partially restored the amount of  $\alpha2\delta2$  in gabapentin-treated  $\text{T}_\text{H}2$  lymphocytes (Fig 7, *G*, and see Fig E6), demonstrating that gabapentin targeted  $\alpha2\delta2$  in lysosomes for its degradation.

We then examined the effect of gabapentin administration on the development of allergic airway inflammation (Fig 8, *A*). Administration of gabapentin during OVA challenge partially reduced airway inflammation, as shown by the reduction in the number of inflammatory cells, including eosinophils in BAL fluid (Fig 8, *B*) and histologic examination of lung sections (Fig 8, *C* and *D*). Expression of  $\text{T}_\text{H}2$  cytokines and mucins 5ac and 5b, which are the major components of mucus in the lungs,<sup>35</sup> was decreased in the lungs of gabapentin-treated mice (Fig 8, *E*), supporting a beneficial effect of gabapentin on  $\text{T}_\text{H}2$ -mediated inflammation.

## DISCUSSION

Previously, we reported that knocking down  $\text{Ca}_v1.2$  and  $\text{Ca}_v1.3 \alpha1$  subunits in  $\text{T}_\text{H}2$  cells impaired their functions and protected against asthma.<sup>7</sup> These subunits are voltage-gated calcium ion-forming pores in excitable cells. However, we and other groups showed that they are not voltage gated in lymphocytes. We then investigated whether auxiliary subunits, such as  $\text{Ca}_v\beta$  and  $\text{Ca}_v\alpha2\delta$ , are required for  $\text{T}_\text{H}2$  cell functions and can be targeted beneficially in acute allergic airway inflammation. Our results now show that (1) knocking down  $\text{Ca}_v\beta$  expression with  $\text{Ca}_v\beta\text{AS}$  designed to target the 4  $\beta$ -subunits reduces the TCR-driven  $[\text{Ca}^{2+}]_i$  increase and  $\text{T}_\text{H}2$  cytokine production by mouse and human  $\text{T}_\text{H}2$  cells while sparing  $\text{T}_\text{H}1$  cell responses; (2) this is associated with the loss of  $\text{Ca}_v1.2 \alpha1$  protein, which can be partially overcome by a proteasome inhibitor; (3) intranasal delivery of  $\text{Ca}_v\beta\text{AS}$  strongly diminishes  $\text{T}_\text{H}2$ -mediated immunopathology in a model of acute allergic airway inflammation; and (4) the  $\text{Ca}_v\alpha2\delta1/2$  inhibitor gabapentin impairs calcium signaling and reduces OVA-induced asthma.

Although  $\text{Ca}_v\beta3$  can regulate calcium signaling independently of  $\alpha1$  by limiting inositol 1, 4, 5-triphosphate generation in pancreatic cells,<sup>36</sup> this is unlikely in  $\text{T}_\text{H}2$  cells because knocking down  $\text{Ca}_v\beta$  decreases and not enhances  $[\text{Ca}^{2+}]_i$  level. In addition,



**FIG 8.** Gabapentin (*gaba*), an inhibitor of  $Ca_v\alpha 2\delta$  subunits, decreases type 2 airway inflammation. **A**, BALB/c mice were immunized with OVA in alum and then challenged with intranasal OVA for 5 days. Gabapentin was or was not given in drinking water for the last 5 days. *i.p.*, Intraperitoneal. **B**, The next day, mice were killed, and BAL fluid was collected and analyzed for inflammatory cell content. **C**, Hematoxylin and eosin histologic sections were scored on a 0- to 12-point scale. **D**, Lung inflammation in mice given control (*nt*) and gabapentin. **E**, Expression of *Il4*, *Il5*, *Il13*, *Muc5ac*, and *Muc5b* was quantified in lungs by using quantitative PCR and normalized to housekeeping gene expression. Each experiment includes 6 to 7 mice per group. One representative experiment of 2 is shown. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

$Ca_v\beta$ AS does not affect the calcium signal in  $T_H1$  cells that express  $Ca_v\beta 3$  but no  $Ca_v1\alpha 1$  subunits. Moreover, our results favor a role of  $Ca_v\beta$  related to its interaction with  $Ca_v1\alpha 1$ , as supported by the loss of expression of  $Ca_v1.2\alpha 1$  in  $T_H2$  cells transfected with  $Ca_v\beta$ AS. In  $T_H2$  cells,  $Ca_v\beta$  prevents the degradation of  $Ca_v1.2\alpha 1$ , which is consistent with findings showing that  $\beta 1b$  or  $\beta 2a$  might be required for  $Ca_v1.2$  or  $Ca_v2.1\alpha 1$  protein expression.<sup>28,37</sup> Surprisingly, transfection of ventricular myocytes with the same  $Ca_v\beta$ AS we used in our study resulted in  $Ca_v1\alpha 1$  sequestration without increased cardiac  $Ca_v1.2\alpha 1$  degradation.<sup>38</sup> This apparent discrepancy could be related to differences in  $Ca_v\beta$  subunit equipment and/or regulation in these cell types. Along this line, it has been shown that the absence of  $Ca_v\beta 4$  resulted in decreased expression of  $Ca_v1.1$  but not  $Ca_v1.2\alpha 1$  protein in  $CD4^+$  T cells.<sup>6</sup>

Two mechanisms have been proposed to explain how  $Ca_v\beta$  can protect the  $\alpha 1$  subunit from degradation. First, interaction of  $\beta 2$  with  $Ca_v1.2\alpha 1$  might limit degradation of the channel in lysosomes.<sup>39</sup> Second, interaction of  $\beta 1$  or  $\beta 2$  with  $Ca_v1.2$  or  $Ca_v2.2\alpha 1$  prevents  $\alpha 1$  being addressed to and degraded by the proteasome.<sup>28,37</sup> This second mechanism is in agreement with our data in  $T_H2$  cells. Indeed, we show that knocking down  $Ca_v\beta$  diminished the total amount of endogenous  $Ca_v1.2\alpha 1$ , which was partially restored on proteasome inhibition in  $T_H2$  cells. Whether  $\beta$  subunits are also required for the export of  $\alpha 1$  subunits to reach the cell surface is controversial.<sup>23,28,40</sup> Knocking down  $Ca_v\beta$  resulted in lower expression of  $Ca_v1.2\alpha 1$

at the cell membrane, which was restored in part by the proteasome inhibitor, indicating that  $Ca_v\beta$  was not absolutely required for the channel to exit from the ER or that the remaining  $Ca_v\beta$  subunits were sufficient to promote trafficking of the channel at the cell membrane. The absence of  $Ca_v\beta$ , even in the presence of proteasome inhibitor, did not allow restoration of cytokine production by  $T_H2$  cells, which could be due to the incomplete restoration of  $Ca_v1.2\alpha 1$  and/or a role of  $Ca_v\beta$  on function of the channel at the cell membrane.

Mouse  $T_H2$  cells express predominantly both  $\beta 1$  and  $\beta 3$  subunits, as do human  $T_H2$  cells.<sup>9</sup> It was reported previously that the  $\beta 1$  variant preferentially interacts with  $Ca_v1.1\alpha 1$ , allowing interaction of  $Ca_v1$  with ER-localized type 1 ryanodine receptors<sup>41</sup> and leading to calcium depletion in the ER. It would be interesting to investigate the respective roles of  $Ca_v\beta 1$  and  $Ca_v\beta 3$  in  $Ca_v1.2$  function. Interestingly,  $Ca_v\beta$  can interact with G proteins, scaffolding proteins,<sup>42</sup> and especially AHNAK, which might be required for plasma membrane localization of the channel, as described for  $Ca_v1.1\alpha 1$  channels in T cells.<sup>43</sup>

We found predominantly  $\alpha 2\delta 2$  at the mRNA level in  $T_H2$  cells. We also detected the protein by using Western blotting with the same apparent weight as in neurons and by means of immunofluorescence. Nerve injury can be responsible for chronic pain caused by  $\alpha 2\delta 1$  overexpression in injured sensory neurons.<sup>44,45</sup> Gabapentin is beneficial because this drug, which binds to  $\alpha 2\delta 1$  and  $\alpha 2\delta 2$  but not the other  $\alpha 2\delta$  isoforms,<sup>34</sup> might decrease expression of  $Ca_v2$  channels at the cell

membrane,<sup>33,34,46</sup> leading to a reduction in  $\text{Ca}_v$  calcium currents. However, the authors did not report an effect of gabapentin on the total amount of  $\alpha 2\delta$  and  $\text{Ca}_v \alpha 1$  subunits. Unexpectedly,  $\text{T}_H2$  cells treated with gabapentin show reduced total amounts of  $\alpha 2\delta 2$ , which are restored by an inhibitor of the vacuolar  $\text{H}^+$  ATPase. This suggests that gabapentin favors  $\alpha 2\delta 2$  degradation in an acidic compartment, such as lysosomes. Along this line, gabapentin was shown to prevent recycling of  $\alpha 2\delta 2$  from Rab11-positive recycling endosomes to the plasma membrane.<sup>47</sup> We show here that gabapentin neither affects the total amount of  $\text{Ca}_v 1.2$  protein nor its localization at the plasma membrane in  $\text{T}_H2$  cells. However, the calcium response was decreased on TCR activation, suggesting that  $\alpha 2\delta 2$  can contribute to the function of  $\text{Ca}_v 1.2 \alpha 1$ , as described for  $\alpha 2\delta 1$ .<sup>48</sup> Altogether, our results obtained with gabapentin and knockdown of  $\text{Ca}_v \beta$  in the presence of MG132 indicate that the level of  $\text{Ca}_v 1.2 \alpha 1$  is not the only parameter limiting the calcium response and suggest that both auxiliary subunits are required in addition to  $\text{Ca}_v 1.2 \alpha 1$  for  $\text{T}_H2$  functions.

Altogether, these data stress the role of auxiliary subunits ( $\text{Ca}_v \beta$  and  $\alpha 2\delta$ ) in  $\text{Ca}_v 1.2$  channel function in  $\text{T}_H2$  cells and show that the auxiliary subunits of  $\text{Ca}_v 1$  channels represent potential new therapeutic targets in allergy. Consistently,  $\text{Ca}_v \beta \text{AS}$  has a marked effect on  $\text{T}_H2$  functions, airway inflammation, and hyperreactivity.  $\text{CD4}^+$  T cells infiltrating the lungs express the  $\text{Ca}_v 1.2 \alpha 1$  channel, and this expression is diminished in mice during  $\text{Ca}_v \beta \text{AS}$  treatment, suggesting that  $\text{Ca}_v \beta \text{AS}$  acts in a similar way *in vitro* and *in vivo*. Chronic administration of gabapentin decreased  $\text{T}_H2$  functions and inflammation. Because  $\text{Ca}_v \beta \text{AS}$  and gabapentin act in different ways to decrease  $\text{Ca}^{2+}$  signaling and  $\text{T}_H2$  function, it is tempting to propose a combination of drugs targeting  $\text{Ca}_v \beta$  and  $\text{Ca}_v \alpha 2\delta$ . In that respect, gabapentin is already used for the treatment of chronic cough,<sup>49</sup> a symptom that is currently found in asthmatic patients. Interestingly, peptides stapling the  $\text{Ca}_v \alpha 1$  domain interacting with  $\text{Ca}_v \beta$  subunits and shown to inhibit  $\text{Ca}_v$  functions have been recently described<sup>50</sup> and could be tested for their efficacy in allergic diseases.

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**Clinical implications: The demonstration that auxiliary subunits are involved in calcium signaling through  $\text{Ca}_v 1$  channels and the function of mouse and human  $\text{T}_H2$  lymphocytes supports their potential beneficial effect on allergic asthma.**

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## METHODS

### Oligonucleotide sequences

The sequences for Ca<sub>v</sub>βS (5'-gaagtaggtcttgggtgg-3') and Ca<sub>v</sub>βAS (5'-accagcttccgatccaccagtcatt-3'; Eurogentec) were chosen from Bichet et al.<sup>E1</sup> Ca<sub>v</sub>βAS has 92% complementarity to mouse or human β1 and β3 mRNA.

### Model of acute allergic airway inflammation

BAL fluid was collected, and the content in inflammatory cells was determined, as previously described.<sup>E2</sup> Lung tissue was fixed in 10% buffered formalin for 24 hours and then placed in 70% ethanol before embedding in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. Histologic disease scores of 0 to 3 were attributed based on the severity of peribronchial, perivascular, and interstitial immune cell infiltration and thickening of peribronchial epithelium, resulting in a maximum score of 12. Lung draining lymph nodes were harvested, and 2 × 10<sup>5</sup> cells were stimulated with OVA (100 μg/mL) for 3 days. Cytokine concentrations were measured in supernatants by means of ELISA. In some experiments CD4<sup>+</sup> T cells were purified from the lungs, stained with anti-Ca<sub>v</sub>1.2 antibodies, and analyzed by using confocal microscopy.

### Airway hyperresponsiveness

Anesthetized and ventilated mice were connected through an endotracheal cannula to a whole-body plethysmograph for ventilation (Emka Technologies, Paris, France). After initiating mechanical ventilation, the mouse was paralyzed with an intraperitoneal injection of 0.1 mL of a 10 mg/mL solution of rocuronium bromide. The animal was ventilated at a respiratory rate of 150 breaths/min and a tidal volume of 10 mL/kg against a positive end-expiratory pressure of 3 cm of H<sub>2</sub>O. Then mice were exposed to increasing doses of methacholine aerosol generated through a nebulizer containing 0 to 20 mg/mL methacholine in PBS. Dynamic lung resistance was measured with iox software (Emka Technologies).

### Cell culture

Cells were cultured 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 μg/mL streptomycin, and 50 μmol/L β-mercaptoethanol).

### Single-cell calcium imaging

Cells were loaded with Fura-2 AM, as previously described,<sup>E2,E3</sup> in culture medium containing 5% heat-inactivated FBS for 30 minutes at 37°C. Cells were then washed, plated, and excited in 10-second intervals by using 340- and 380-nm excitation filters. Emission was recorded with 510/540-nm bandpass filters by using a CCD camera before and after stimulation with biotinylated anti-CD3 cross-linked with streptavidin- or anti-CD3/CD28-coated beads (1 bead per cell) for mouse or human T cells, respectively. Fluorescence ratio images at wavelengths of 340 and 380 nm were then calculated with Metafluor software. The curve represents the mean + SEM of fluorescence ratio of 40 to 60 cells. Each experiment was repeated 3 or 4 times. For each cell, we calculated the area under the curve between TCR stimulation and ionomycin application with the GraphPad Prism software. The time of response was defined as the delay for which the fluorescence ratio becomes superior to the mean basal value + 1 SD.

### Confocal microscopy

Cells were seeded onto glass slides coated with poly-L-lysine (Sigma-Aldrich, St Louis, Mo) and then fixated with 4% paraformaldehyde. Staining was performed with anti-CD2 rat IgG antibody (Abcam) or anti-α2δ2 rabbit antibody (Alomone Labs) and revealed with the appropriate secondary antibodies (Molecular Probes, Eugene, Ore). Cells were then permeabilized with PBS containing 0.1% saponin and 0.5% BSA. Ca<sub>v</sub> channel subunit

staining was performed with anti-Ca<sub>v</sub>1.2 mouse IgG<sub>2b</sub> (NeuroMab), anti-Ca<sub>v</sub>β1 (Alomone Labs), or anti-Ca<sub>v</sub>β3 rabbit IgG (Santa Cruz Labs) overnight and revealed with the appropriate secondary antibodies (Molecular Probes). Slides were analyzed by using an LSM 710 confocal microscope (Zeiss, Oberkochen, Germany). Staining with control isotypes was constantly negative. The total fluorescence intensity of each cell was determined on the sum of overall Z sections with ImageJ software (National Institutes of Health, Bethesda, Md). For membrane Ca<sub>v</sub>1.2 staining, a membrane surface was created by using CD2 staining, and this surface was duplicated on Ca<sub>v</sub>1.2 staining to measure the intensity of Ca<sub>v</sub>1.2 at the membrane with the Imaris software (8.4.1; Bitplane) module called Surface. Each measurement was done for each cell individually.

### Real-time quantitative PCR

T<sub>H</sub>1 and T<sub>H</sub>2 cells were lysed, and RNA was extracted with the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with the SuperScript III Reverse Transcriptase (Invitrogen). Transcripts were measured by using real-time quantitative PCR with a LightCycler 480 Instrument (Roche) and the LightCycler 480 SYBR Green I Master (Roche). We used the following primers: Ca<sub>v</sub>β1, 5'-gcagagcgcaggcctta-3' and 5'-tgatggcactccctgtaca-3'; Ca<sub>v</sub>β2, 5'-cgaacagagagccaagcaa-3' and 5'-gggcactatgtcaccaaa-3'; Ca<sub>v</sub>β3, 5'-tgtggcatttctctgtgagga-3' and 5'-ccgatccaccagtcattgc-3'; Ca<sub>v</sub>β4, 5'-gagcga gaacagcaagcag-3' and 5'-tcaaagagatggctgtgc-3'; Ca<sub>v</sub>α2δ1, 3' acgccaactggt gaaattg 5' and 3' ctgcaaaatctccctcca 5'; Ca<sub>v</sub>α2δ2, 3' atttccagaagccatcg 5' and 3' gcttggatcccctctcca 5'; Ca<sub>v</sub>α2δ3, 3' cattttggtggacgtcagtg 5' and 3' ttccgttcagacaaggtcc 5'; Ca<sub>v</sub>α2δ4, 3' ggaagtcgaggctgttgag 5' and 3' ctccagtagttgcccctgt 5'; and hypoxanthine-guanine phosphoribosyltransferase (HPRT), 5'-ctggtgaaaaggacctctcg-3' and 5'-tgaagtactcattatagcaaggcca-3'. Primers for *Il4*, *Il5*, *Il13*, *Muc5ac*, and *Muc5b* were purchased from Qiagen. Quantification of target gene expression was calculated by normalizing values relative to expression of the housekeeping gene HPRT. Amounts of mRNA were expressed as arbitrary units relative to HPRT as follows:

$$\left(2^{-[\text{Ct interest gene} - \text{Ct HPRT}]}\right) \times 10^{-3}$$

### Immunoblotting

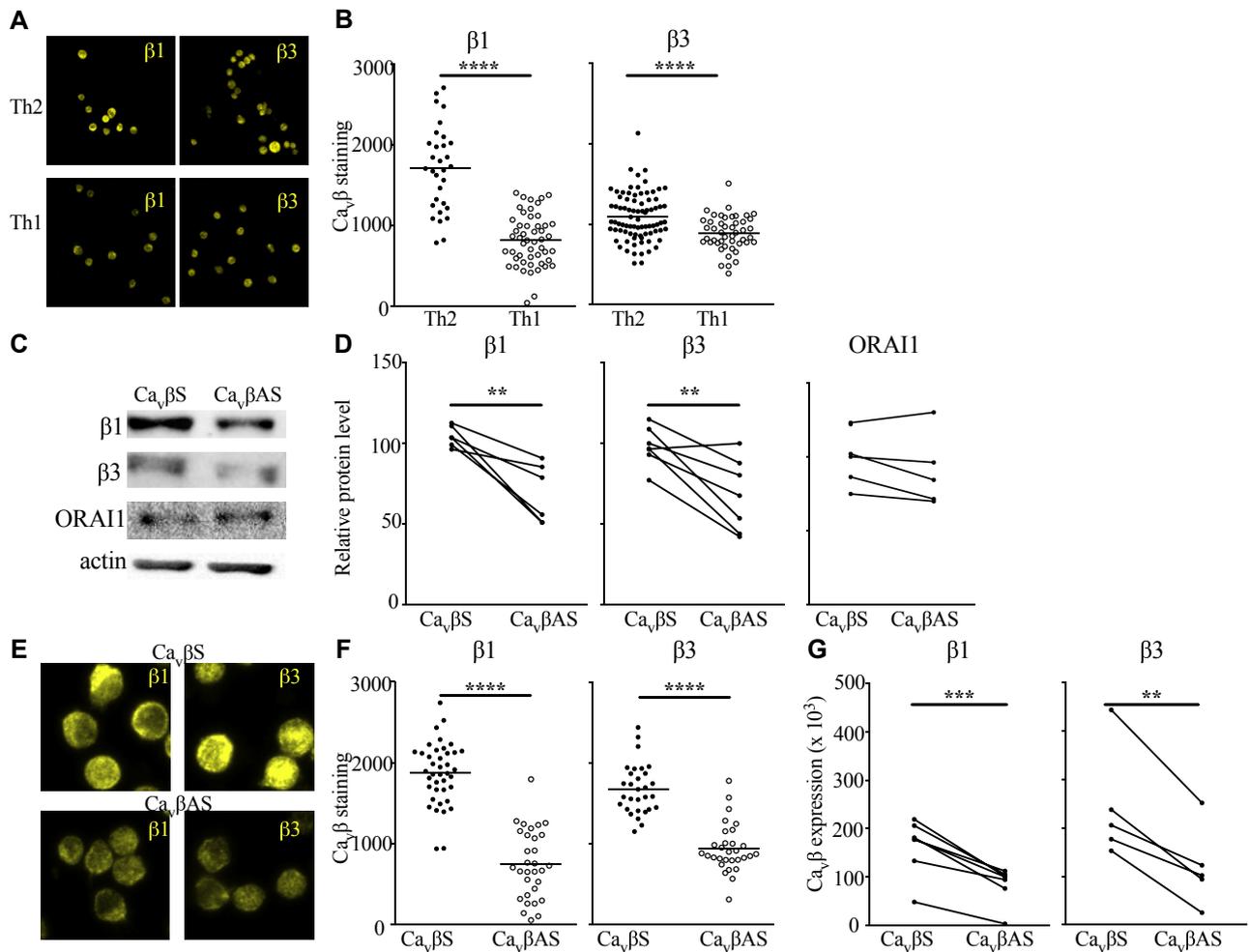
T<sub>H</sub>2 lymphocytes or brain cells were lysed in RIPA buffer containing protease inhibitors (Roche). Lysates (30 μg/lane for T-cell extracts and 10 μg/lane for brain lysates) were submitted to 10% SDS electrophoresis, transferred to Hybond-C Extra nitrocellulose membranes (Amersham, Piscataway, NJ), and then blotted with anti-Ca<sub>v</sub>β1 (Alomone Labs), anti-Ca<sub>v</sub>β3 (Santa Cruz Biotechnology, Santa Cruz, Calif), or anti-α2δ2 (Alomone Labs) primary antibodies. We then stained blots with horseradish peroxidase-coupled secondary antibodies (Cell Signaling Technology, Danvers, Mass) before detection and visualization with the ECL Prime (Amersham) using a ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, Calif). The specificity of anti-Ca<sub>v</sub>β1 and β3 antibodies was verified on human embryonic kidney cells transfected with the corresponding plasmids using Western blotting.

### ELISA

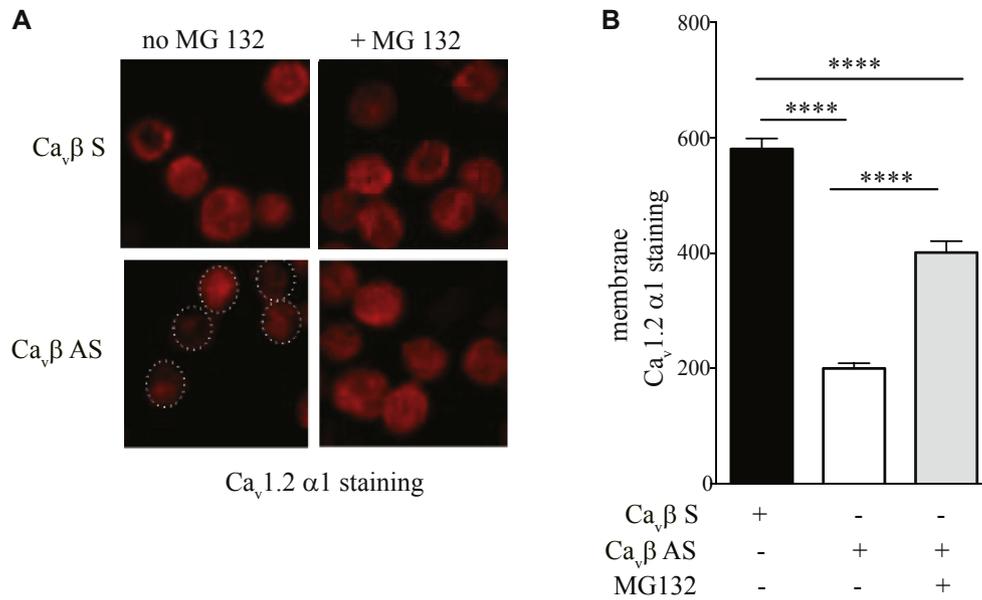
Supernatants or sera were incubated into 96-well plates coated with anti-IL-4, anti-IL-5, anti-IL-13 (eBioscience, San Diego, Calif), anti-IFN-γ, or anti-IgE (Serotec). Bound cytokines and IgE were then labeled with biotinylated anti-IL-4 (eBioscience), anti-IL-5 (eBioscience), anti-IL-13 (eBioscience), anti-IFN-γ (eBioscience), or anti-IgE (BD Pharmingen, San Jose, Calif). Biotinylated antibodies were revealed by means of incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pa) and subsequent addition of the alkaline phosphatase substrate pNPP disodium salt hexahydrate (Sigma-Aldrich). Absorbance was measured at 405 to 650 nm by using an EMax Microplate Reader (Molecular Devices, Sunnyvale, Calif). Cytokine and immunoglobulin concentrations were calculated from standard curves generated by means of titration of recombinant mouse cytokines and purified IgE. Human cytokine ELISAs were performed, as previously described.<sup>E3</sup>

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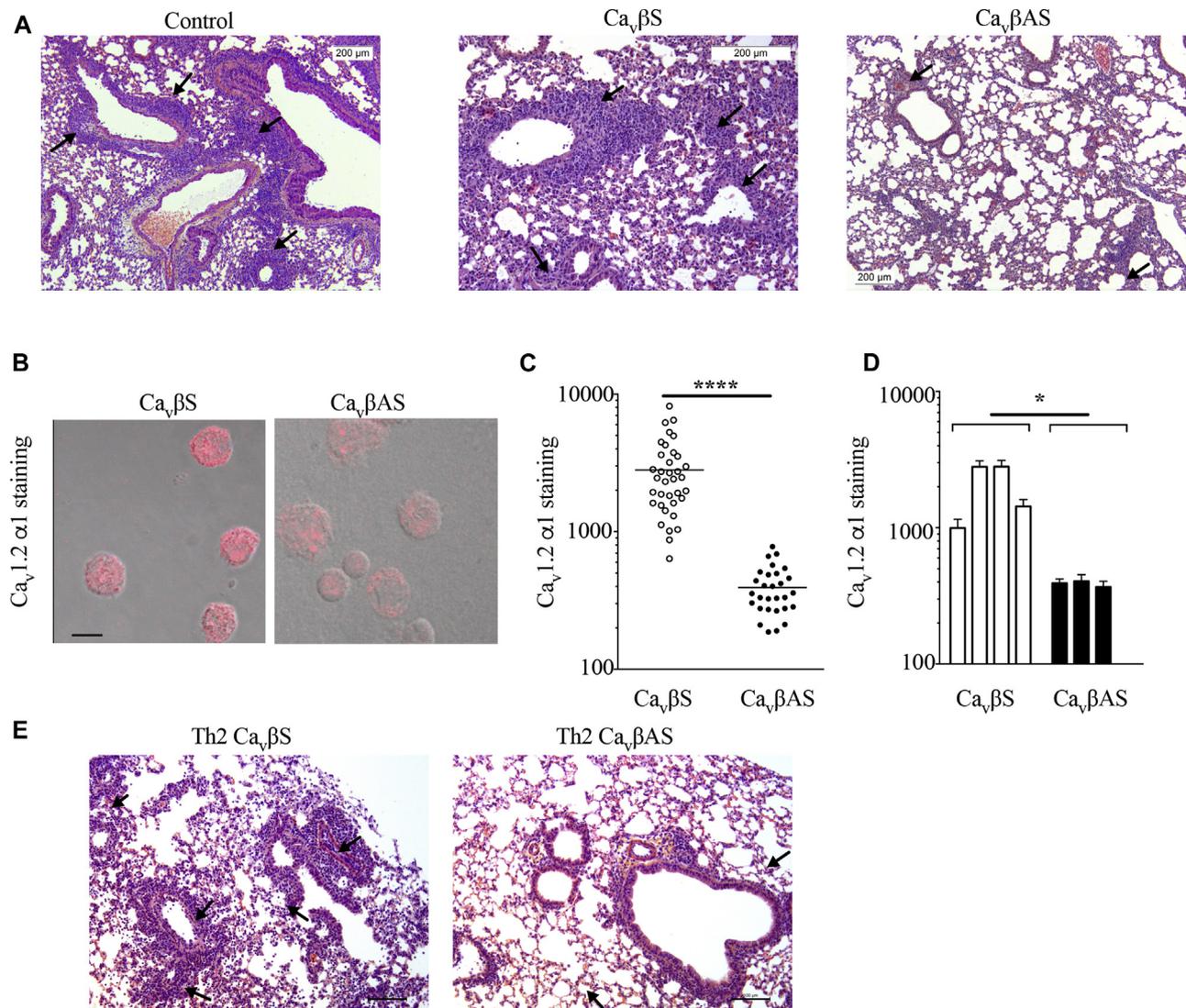
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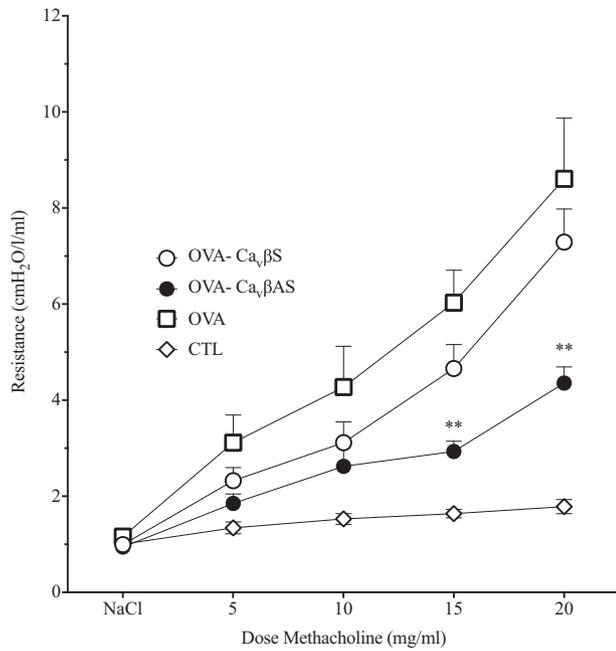
**FIG E1.**  $Ca_v\beta$ AS targets  $Ca_v\beta$ 1 and  $Ca_v\beta$ 3 subunits in mouse and human  $T_H2$  cells. **A**, OVA-specific DO11.10  $T_H2$  and  $T_H1$  cells were stained with anti- $Ca_v\beta$ 1 and anti- $Ca_v\beta$ 3 antibodies. **B**, Expression of  $Ca_v\beta$  per cell. One experiment of 3 was depicted, and each *point* represents 1 cell. **C**, Cell lysates were prepared from mouse  $T_H2$  cells transfected with  $Ca_v\beta$ S or  $Ca_v\beta$ AS after 3 days of transfection. One representative blot is shown. **D**, Protein bands were quantified by using Image Lab software. The ratio between the protein of interest and  $\beta$ -actin was calculated. Results are expressed as the relative protein level to  $Ca_v\beta$ S and shown as separate paired experiments. **E** and **F**,  $T_H2$  cells transfected with  $Ca_v\beta$ S or  $Ca_v\beta$ AS were stained with anti- $Ca_v\beta$ 1 and anti- $Ca_v\beta$ 3 antibodies. Expression of  $Ca_v\beta$  per cell is shown in Fig E1, **F**. One experiment of 3 is depicted, and each *point* represents 1 cell. **G**, CRTH2<sup>+</sup> cells transfected with  $Ca_v\beta$ S or  $Ca_v\beta$ AS were analyzed for  $Ca_v\beta$ 1 and  $Ca_v\beta$ 3 transcript expression. Results are shown as separate paired experiments. \*\* $P < .01$ , \*\*\* $P < .005$ , and \*\*\*\* $P < .0001$ .



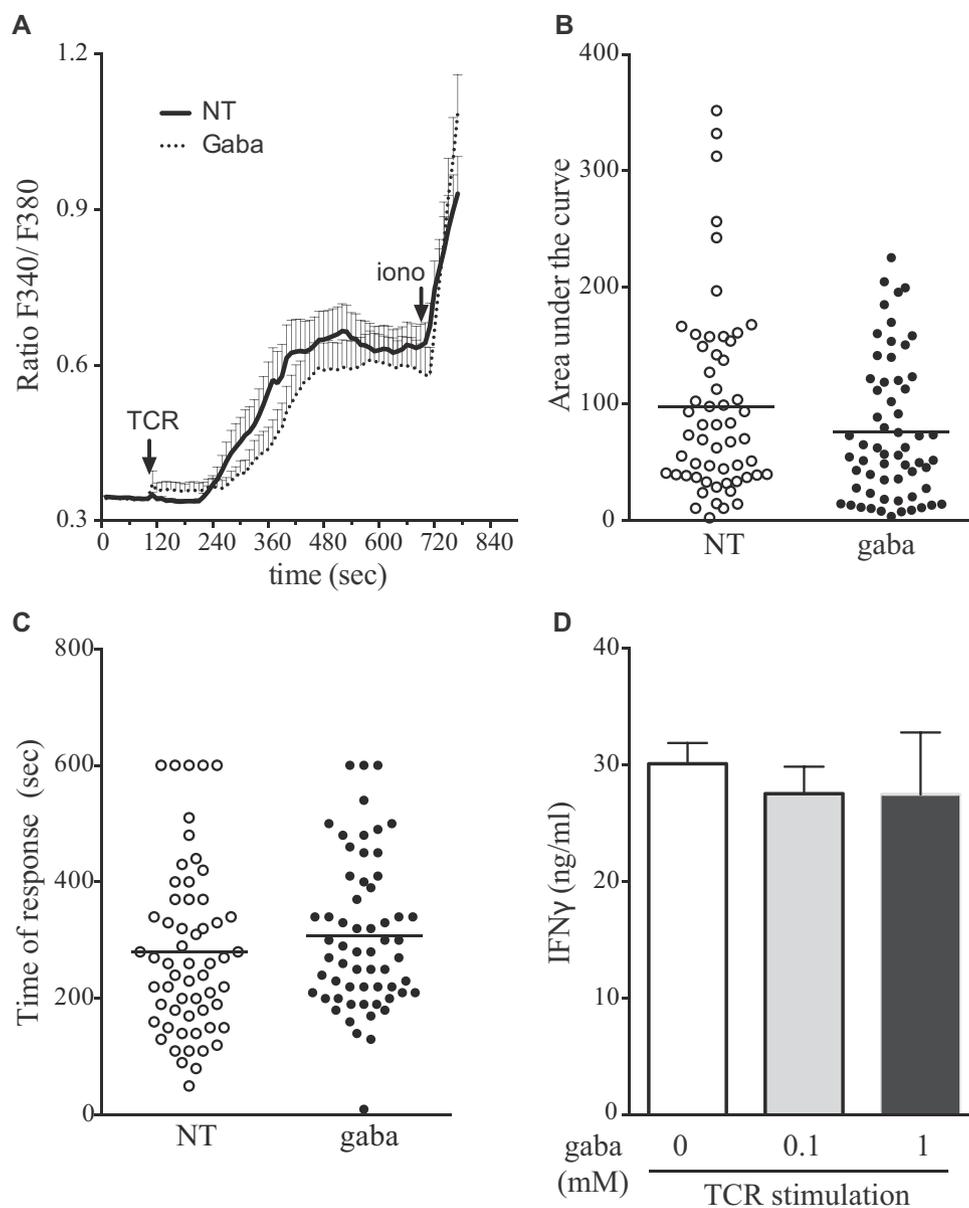
**FIG E2.** The proteasome inhibitor MG132 partially restores total Ca<sub>v</sub>1.2 α1 levels and its expression at the cell membrane. **A**, Mouse DO11.10 T<sub>H</sub>2 cells were transfected with control (Ca<sub>v</sub>βS) or antisense Ca<sub>v</sub>βAS oligonucleotides for 48 hours before addition of MG132 (0.5 μmol/L) or vehicle for an additional 24 hours. Cells were then recovered and labeled with anti-Ca<sub>v</sub>1.2 α1 mAb. *Dotted lines* indicate cells. **B**, T<sub>H</sub>2 cells treated as in Fig E2, **A**, were labeled with both anti-CD2 and anti-Ca<sub>v</sub>1.2 α1 mAbs, and colocalization determined with Imaris software is shown. Results are expressed as means of at least 50 cells, and *bars* represent SEMs. \*\*\*\**P* < .001.



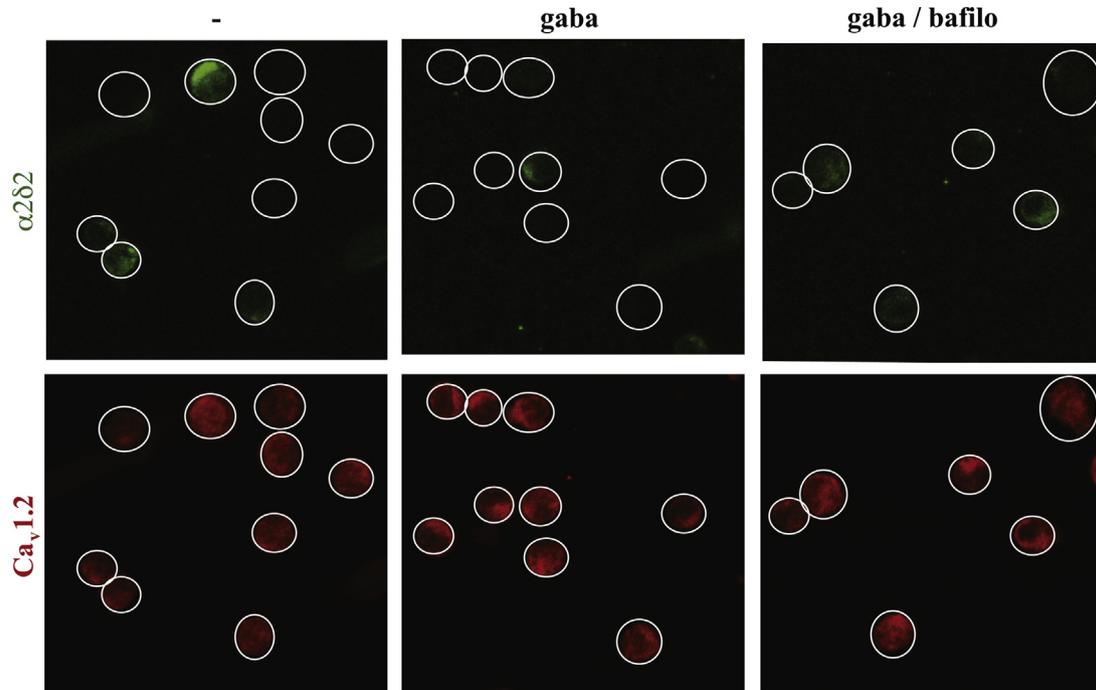
**FIG E3.**  $Ca_v\beta AS$  alleviates type 2 airway inflammation. **A**, BALB/c mice were immunized with OVA in alum and challenged 15 days later with intranasal OVA only (control), OVA plus  $Ca_v\beta S$ , or OVA plus  $Ca_v\beta AS$  every day for 5 days. Representative hematoxylin and eosin–stained sections are shown, and *arrows* indicate inflammatory infiltrates. **B–D**,  $CD4^+$  T cells were purified from lung tissue and stained with anti- $Ca_v1.2 \alpha 1$  mAb. Optical sections are shown in Fig E3, *B*. Quantification was done by collecting data from all the Z-stack sections of each cell. Examples are given in Fig E3, *C*, where each point corresponds to a cell from 1 representative  $Ca_v\beta S$ - or  $Ca_v\beta AS$ -treated mouse. Fig E3, *D*, summarized results obtained from different mice. Each *bar* represents the mean plus 1 SD of at least 30 cells from 1 mouse. \* $P < .05$  and \*\*\*\* $P < .001$ . **E**, Representative hematoxylin and eosin–stained lung sections from mice injected with  $T_H2$  cells transfected with  $Ca_v\beta S$  or  $Ca_v\beta AS$  and given intranasal OVA.



**FIG E4.**  $\text{Ca}_v\beta\text{AS}$  decreased airway hyperreactivity in a mouse model of allergic airway inflammation. BALB/c mice were immunized with OVA in alum (*OVA*) or PBS (*CTL*) and challenged 15 days later with intranasal OVA only, OVA plus  $\text{Ca}_v\beta\text{S}$  or OVA plus  $\text{Ca}_v\beta\text{AS}$  every day for 5 days. Dynamic lung resistance was measured with iox software (Emka Technology). All data are expressed as means + SEMs of 10 mice per group. \*\* $P < .01$ .



**FIG E5.**  $T_H1$  cell functions are not affected by gabapentin (*gaba*) treatment.  $T_H1$  cells were preincubated with gabapentin (1 mmol/L) for 72 hours before loading with the calcium probe Fura-2 AM (**A-C**) or stimulated in the presence or not of gabapentin for an additional 24 hours with anti-CD3/anti-CD28 antibodies (**D**). Fig E5, **A**, Fluorescence ratio at 340- and 380-nm excitation wavelengths was recorded at the single-cell level before and after TCR stimulation. Results are expressed as the mean of at least 50 cells, and *bars* represent SEMs. Fig E5, **B** and **C**, Area under the curve and time of response for each cell, respectively, defined as in Fig 3. Data are representative of 2 experiments. Fig E5, **D**, Cytokine content.



**FIG E6.** Gabapentin (*gaba*) reduces protein levels of the  $\alpha 2\delta 2$  auxiliary subunit in  $T_H2$  cells.  $T_H2$  cells were incubated with gabapentin (1 mmol/L) for 96 hours with or without bafilomycin (*bafilo*; 0.5  $\mu$ mol/L) for the last 24 hours. Cells were stained with anti- $\alpha 2\delta 2$  or anti- $Ca_v1.2$   $\alpha 1$  antibodies. Immunofluorescence was summed over all the Z-stack sections. One representative experiment of 2 is shown. A *white line* surrounds each cell.