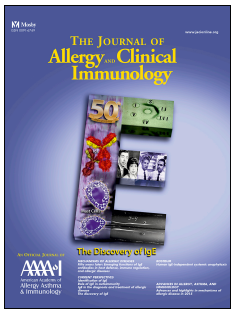
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The β and α2δ auxiliary subunits of Ca_V1 channels are required for Th2-lymphocyte function and acute allergic airway inflammation

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PII: S0091-6749(17)31740-2

DOI: 10.1016/j.jaci.2017.09.045

Reference: YMAI 13103

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 11 April 2017

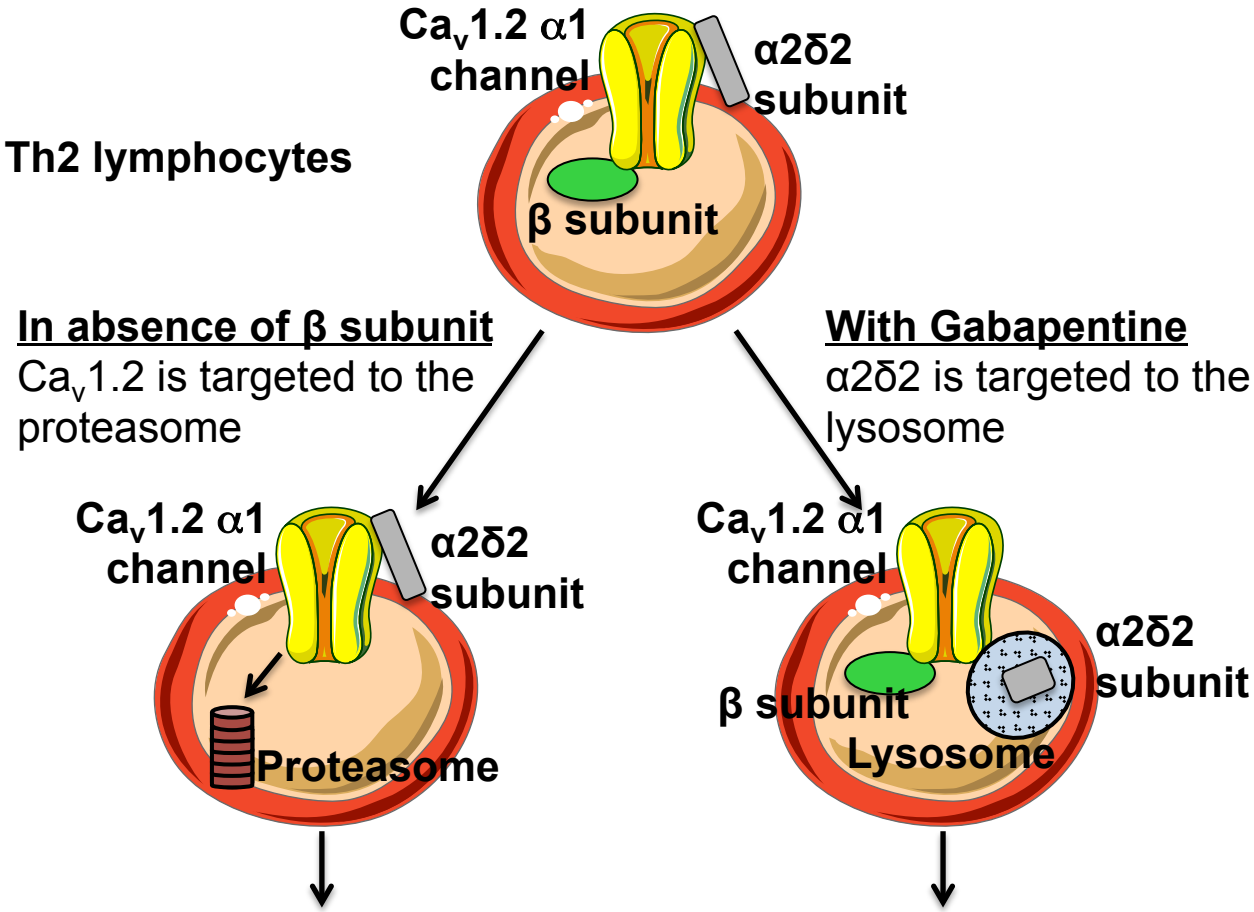
Revised Date: 4 August 2017

Accepted Date: 8 September 2017

Please cite this article as: Rosa N, Triffaux E, Robert V, Mars M, Klein M, Bouchaud G, Canivet A, Magnan A, Guéry J-C, Pelletier L, Savignac M, The β and α2δ auxiliary subunits of Ca_V1 channels are required for Th2-lymphocyte function and acute allergic airway inflammation, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.09.045.

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The auxiliary subunits of Ca_v1 channels are required for Th2-lymphocyte function and acute allergic airway inflammation



- Impairment of TCR-driven calcium responses and cytokine production by mouse and human Th2, with no effect on Th1 lymphocytes
- Inhibition of Th2-mediated immunopathology in a model of acute allergic airway inflammation

Abbreviations: Ca_v1 = voltage-dependent calcium channels

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The β and α2δ auxiliary subunits of Ca_v1 channels are required for Th2-lymphocyte function and acute allergic airway inflammation

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Acknowledgements: This work was supported by the French National Institute for Health and Medical research (INSERM) and by grants from the French Society of Allergology and from the Conseil Régional Midi-Pyrénées. N.R. was supported by a fellowship from Conseil Regional of Midi-Pyrénées and by INSERM.

Abstract

Background: T-lymphocytes express not only the cell membrane calcium ORAI1 but also voltage-dependent Ca_v1 channels. In excitable cells, these channels are composed of the ion forming pore α1 and auxiliary subunits (β and α2δ) needed for proper trafficking and activation of the channel. We previously disclosed the role of $Ca_v1.2$ α1 in mouse and human Th2- but not Th1-cell functions and showed that knocking-down Ca_v1 α1 prevents experimental asthma

Objective: We investigated the role of β and α2δ auxiliary subunits on Ca_v1 α1 function in Th2 lymphocytes and on the development of acute allergic airway inflammation.

Methods: We used antisense oligonucleotides (Ca_vβAS) to knockdown Ca_vβ and gabapentin, a drug that binds to and inhibits α2δ1 and α2δ2, to test their effects on Th2 functions and their capacity to reduce allergic airway inflammation.

Results: Mouse and human Th2-cells express mainly Ca_vβ1, β3 and α2δ2 subunits. Ca_vβAS reduces TCR-driven calcium responses and cytokine production by mouse and human Th2, with no effect on Th1-cells. Ca_vβ is mainly involved in restraining $Ca_v1.2$ α1 degradation through the proteasome as a proteasome inhibitor partially restores the α1 protein level. Gabapentin impairs TCR-driven calcium response and cytokine production associated with the loss of α2δ2 protein in Th2-cells.

Conclusions: These results stress the role of Ca_vβ and α2δ2 auxiliary subunits in the stability and activation of $Ca_v1.2$ channels in Th2 lymphocytes both *in vitro and in vivo* as demonstrated by the beneficial effect of Ca_vβAS and gabapentin in allergic airway inflammation.

Clinical implications

The demonstration that auxiliary subunits are involved in calcium signaling through Ca_v1 channels and function of mouse and human Th2-lymphocytes supports their potential beneficial effect on allergic asthma.

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Capsule summary

This work demonstrates that $Ca_v\beta$ and α2δ auxiliary subunits of Ca_v1 calcium channels are required for proper calcium signaling in Th2 lymphocytes and their targeting is beneficial in allergic asthma.

Key words: asthma; Th2; Ca_v1 calcium channel; calcium; cytokines

Abbreviations

AS ONs: antisense oligonucleotides

BAL: broncho-alveolar lavage

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

Ca_v: voltage-gated calcium channels

CRTH2: Chemoattractant receptor-homologous molecule expressed on Th2 cells

ER: endoplasmic reticulum

FBS: fetal bovine serum

HEK: Human embryonic kidney

IFN: interferon

Ig: immunoglobulin

IL: interleukins

NFAT: nuclear factor of activated T cells

ONs: oligonucleotides

OVA: ovalbumin

TCR: T-cell receptor

Th2: T-helper type 2

vacuolar H^+ ATPase : v-ATPase

WB: western blotting

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Introduction

Allergic diseases that include rhinitis, atopic dermatitis, asthma and food allergies, are induced by T-helper type 2 (Th2) lymphocytes. Th2-type responses are characterized by the production of interleukins (IL-) 4, 5 and 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 inflammation of the type 2 can be beneficial (discussed in [1, 2]). The main immunosuppressants (ciclosporin A, tacrolimus...) have also been proposed in severe asthma resistant to glucocorticoids. However, as 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 Ca^{2+} entry is well described, implicating the sensing of TCR-driven endoplasmic reticulum (ER) Ca^{2+} depletion by STIM-1, its oligomerization and its localization in the vicinity of ORAI-1 calcium channels at the plasma membrane permitting sustained Ca^{2+} entry [3]. Besides these channels, the role of Ca_v1 channels (defined as voltage activated in excitable cells) in T-lymphocytes is now accepted [4-11].

In excitable cells, Ca_v1 channels are composed of the ion forming pore α1 and auxiliary β and α2δ subunits, each subunit being encoded by four genes. $Ca_v1.1$ to $Ca_v1.4$ α1 form the ion pore and support the biophysical and pharmacological properties of the channel [12-14], while auxiliary β and α2δ subunits increase Ca_v currents by enhancing the number of the channels at the cell membrane and favoring channel opening [15-19]. Ca_vβ would act by facilitating the correct folding of α1 and promoting its exit from the endoplasmic reticulum (ER) [20], while Ca_vα2δ increases the insertion of the channel into the cell membrane by favoring the trafficking of the channel from the post Golgi apparatus and decreasing its turnover [21-23].

Whereas $Ca_v\beta2$ deletion [24] inhibited thymocyte development, $Ca_v\beta3$ and $Ca_v\beta4$ were found important for calcium influx, NFAT nuclear translocation and cytokine production by peripheral $CD4^+$ T lymphocytes. We previously reported that $Ca_v1.2$ was ~~selectively~~ expressed and functional in human and mouse Th2-cells [7, 9, 25]. In contrast, Th1 and Th17 lymphocytes in both species lacked $Ca_v1.2$ expression. Herein, we now address whether Ca_v auxiliary subunits are required for channel functions in Th2-lymphocytes. We show that $Ca_v\beta1$, $Ca_v\beta3$ and α2δ2 subunits are expressed in mouse and human Th2-cells. Knocking down $Ca_v\beta$ promoted degradation of $Ca_v1.2$ α1, which was at least partly rescued by adding MG132, a proteasome inhibitor whereas gabapentin, an inhibitor of α2δ1/2 subunits decreased α2δ2 protein level in Th2-lymphocytes. In both cases, it was associated with decreased TCR-dependent $[Ca^{2+}]_i$ increase and Th2 cytokine productions. In accordance with our *in vitro* results, targeting either $Ca_v\beta$ or α2δ *in vivo* was beneficial in a model of acute allergic airway inflammation.

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Materials and Methods

More details are given in Supplemental methods.

Mice and model of acute airway allergic inflammation

Eight- to 12-wk-old female mice BALB/c mice originated from Janvier (Le Genest St.Isle, France) and TCR 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 µg) in alum (2 mg), were given 15 days later intranasal OVA (50 µg/day) in PBS for 5 days as previously described [7] with or without scrambled ($Ca_v\beta S$) or $Ca_v\beta$ antisense specific ($Ca_v\beta AS$) oligonucleotides (200µg/day) or received or not Gabapentin (400 mg/l in drinking water) that was renewed every other day according to [26]. For Th2 transfer experiments, BALB/c mice (Janvier, Le Genest St.Isle, France) were intravenously transferred with 3 x 10^6 D011.10 Th2 cells transfected with $Ca_v\beta S$ or $Ca_v\beta AS$ and given intranasal OVA (50 µg/day) for 5 days. Twenty-four hours after the final OVA administration, serum, bronchoalveolar lavage fluid (BALF), lungs and draining lymph node were collected and processed as described in supplemental methods.

Cell culture

Mouse Th1 and Th2-cells were generated by weekly stimulation of DO11.10 $CD4^+$ T-cells with antigen presenting cells (APC) and the 323-339 OVA peptide plus appropriate differentiation cocktails: IL-12 (5 ng/ml) and anti-IL-4 antibody (11B11, 10 µg/ml) for Th1 and IL-4 (5 ng/ml) and anti-IFNγ antibody (XMG1.2, 10 µg/ml) for Th2. PBMCs were obtained from ‘‘Etablissement Français du Sang,’’ and all human participants provided written informed consent. Human memory $CRTH2^+$ $CD4^+$ T- and human Th1-cells were obtained and expanded as previously reported [9]. Cells were transfected with 8 µM oligodeoxynucleotides (Eurogentec) using TurboFect transfection reagent (Fermentas) and collected three days after transfection.

148

149 **Single cell calcium imaging**

150 These measurements were done as previously described[7, 9] in cells loaded with Fura-2AM
151 before and after stimulation with biotinylated anti-CD3 cross-linked with streptavidin or anti-
152 CD3/CD28 coated beads (1 bead per cell), for mouse or human T cells respectively.

153

154 **Confocal microscopy**

155 Cells were stained with anti-CD2 rat IgG antibody (Abcam), anti-α2δ2 rabbit antibody
156 (Alomone Labs) before the permeabilization and the Ca_v channel subunit staining was
157 performed using anti-Ca_v1.2 mouse IgG2b (NeuroMab), anti-Ca_vβ1 (Alomone) or anti-Ca_vβ3
158 rabbit IgG (Santa Cruz Lab).

159

160 **Real-time quantitative PCR**

161 RNAs were extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription was
162 performed with the SuperScript III Reverse Transcriptase (Invitrogen) and mRNA transcripts
163 were measured by real-time quantitative PCR using a LightCycler 480 Instrument (Roche).

164

165 **Immunoblotting**

166 Th2 lymphocytes or brain cells were lysed in RIPA buffer containing protease inhibitors
167 (Roche) and 30 µg/ lane for T-cell extracts and 10 µg/ lane for brain lysate were submitted to
168 immunoblotting.

169

170 **ELISA**

171 For *in vitro* cytokine assays, $5x10^4$ Th1 and Th2 cells were seeded into plates coated with
172 anti-CD3 antibodies (3 µg/mL) and stimulated with soluble anti-CD28 antibodies (1 µg/mL).
173 Supernatants were collected 24 hours later. For *in vivo* IgE assays, sera were isolated from
174 blood collected into the mouse retro-orbital sinus. Cytokines and immunoglobulins were
175 quantified using the sandwich ELISA method.

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176

177 **Statistical analysis**

178 Groups were compared using the paired or unpaired t test as needed thanks to the

179 Graphpad Prism software.

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Results

Knocking down $Ca_v\beta$ subunits decreases cytokine production induced by TCR engagement in mouse and human Th2-cells

Murine Th2-cells expressed principally $Ca_v\beta1$ and $Ca_v\beta3$ transcripts (Fig. 1A), as human Th2 cells did [9]. 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. 1B) and by immunofluorescence (Supplemental Fig. 1A and B). Although Th1-cells did not express $Ca_v\alpha1$ subunits, they did express $Ca_v\beta1$ and $Ca_v\beta3$ at both transcriptional (Fig. 1A), and protein level with lower amounts compared to Th2-cells (Supplemental Fig. 1A and B).

To investigate whether β subunits were important for Th2 functions, we used antisense ONs targeting the four $Ca_v\beta$ subunits in mice and humans [27]. Transfection of mouse Th2-cells with $Ca_v\beta$AS decreased the amount of $Ca_v\beta1$ and $Ca_v\beta3$ subunits by western blotting (new supplemental Fig. 1C and D) and by immunofluorescence (new supplemental Fig. 1E and F), with no effect on the expression of ORAI1, a calcium channel important for T-cell functions (new supplemental Fig. 1C, D). $Ca_v\beta$AS decreased IL-4, IL-5 and IL-13 production by 50 to 70% in mouse Th2-cells compared to $Ca_v\beta$S-transfected Th2-cells (Fig. 1C) without any effect on IFNγ production by Th1-cells (new Fig. 1D).

Human memory Th2-cells were selected on the basis of the expression of the cell membrane marker CRTH2 and expanded with anti-CD3/anti-CD28-coated beads in the presence of IL-4 and IL-2. Transfection of human Th2-lymphocytes with $Ca_v\beta$AS reduced the amount of $Ca_v\beta$ subunits at the mRNA level (new supplemental Fig. 1G) associated with significantly diminution of Th2-cytokine production (Fig. 1E) without any effect on IFNγ production by human Th1-cells (Fig. 1F).

Knocking down $Ca_v\beta$ subunits diminishes TCR-driven calcium response in mouse and human Th2-cells

In order to show that $Ca_v\beta$ play a role in Ca^{2+} signaling of Th2-cells, we analyzed the TCR-driven intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase at the single cell-level. Mouse

Th2-cells transfected with $Ca_v\beta AS$ displayed a decreased and delayed calcium response compared to cells transfected with $Ca_v\beta S$ (Fig. 2A). Indeed, $Ca_v\beta AS$-transfected Th2-cells presented a significant reduced area under the curve compared to control Th2-cells (Fig. 2B). A significant number of $Ca_v\beta AS$-transfected Th2-cells (21 ± 5 %) did not show any increase in $[Ca^{2+}]_i$ after TCR stimulation while most control Th2-cells (98 ± 2 %) did it ($p< 0.005$). Moreover, among responsive cells, $Ca_v\beta AS$-transfected Th2-cells had a delayed calcium response as compared to control cells (Fig. 2C). However, ionomycin application at the end of the experiment induced similar $[Ca^{2+}]_i$ increase in Th2-cells whether they were transfected with $Ca_v\beta S$ or $Ca_v\beta AS$ (Fig. 2A), indicating that $Ca_v\beta AS$ did not affect the loading with the Fura-2 probe.

$Ca_v\beta AS$ had the same effect on TCR-driven $[Ca^{2+}]_i$ increase of human Th2-lymphocytes with a delayed and reduced $[Ca^{2+}]_i$ rise (Fig. 2D-F). Conversely, transfection of mouse (new Fig.2 G-I) or human (Fig.2 J-L) Th1-cells with $Ca_v\beta AS$ had no impact on the shape and the amplitude of the calcium response induced by TCR stimulation, in agreement with the lack of $Ca_v\beta AS$ effect on IFNγ synthesis by Th1-cells (new Fig. 1D and 1F).

An inhibitor of the proteasome protects the $Ca_v1.2$ α1 subunit from degradation in the absence of $Ca_v\beta$, but does not restore cytokine production

One possible role of $Ca_v\beta$ subunits could be to prevent $Ca_v1.2$ degradation by the proteasome [28]. In agreement with this hypothesis, we showed that transfection of Th2-cells with $Ca_v\beta AS$ did not affect the levels of $Ca_v1.2$ α1 transcripts (Fig. 3A) but strongly reduced the amount of $Ca_v1.2$ α1 protein in mouse (Fig 3B-C) and in human (Fig. 3D-E) cells. The proteasome inhibitor MG132 had no effect on the amounts of $Ca_v\beta 1$ (Fig. 4A) and $Ca_v\beta 3$ proteins (Fig. 4B), but it partially restored $Ca_v1.2$ α1 protein expression in murine Th2-cells (Fig. 4C). The analysis of co-localization of $Ca_v1.2$ α1 with CD2 used as a cell membrane marker shows that $Ca_v\beta AS$ decreases the amount of the protein localized at the cell membrane, which was partially restored by MG132 (Supplemental Fig. 2). However, MG132 treatment did not allow restoration of cytokine production by Th2-cells transfected with

Ca_vβAS (Fig. 4D), suggesting that Ca_vβ subunit could also contribute to regulate channel function as reported [29-31].

Intranasal delivery of Ca_vβAS alleviates Th2-dependent airway inflammation

With the goal to investigate the role of Ca_vβ *in vivo*, we evaluated if Ca_vβAS modified the course of allergic airway inflammation. Immunization with OVA in alum followed by inhalation of OVA (Fig. 5A) triggered Th2-dependent airway inflammation marked by predominant eosinophil infiltration (Fig. 5B). Ca_vβAS administration strongly decreased the content of inflammatory cells in the broncho-alveolar lavage (BAL) fluid including eosinophils and lymphocytes compared to control mice treated or not with Ca_vβS (Fig. 5B). Consistently histological examination of lung tissues showed that Ca_vβAS administration inhibited inflammatory infiltrates (Fig. 5C, and Supplemental Fig. 3A). This protection was associated with defective Th2 responses as shown by the dramatic reduction in serum immunoglobulin (Ig) E concentration (new Fig. 5D) and the reduced synthesis of IL-4, IL-5 and IL-13 by OVA-stimulated T-cells from lung draining lymph node cells (Fig. 5E). In accordance with the effects of Ca_vβAS on airway inflammation, mice displayed reduced airway hyper-responsiveness (new Supplemental Fig. 4). Interestingly, $CD4^+$ T-cells infiltrating the lungs in Ca_vβAS-treated mice expressed reduced amounts of $Ca_v1.2$ α1 proteins compared to $CD4^+$ T-cells from mice treated with Ca_vβS (Supplemental Fig. 3B-D), suggesting that Ca_vβ was required for the maintenance of $Ca_v1.2$ α1 protein expression in lung infiltrating $CD4^+$ T cells as shown for Th2-cells *in vitro*.

To investigate whether Ca_vβ expression in Th2-cells was necessary and sufficient to control allergic asthma, we transferred OVA-specific TCR transgenic DO11.10 Th2 cells transfected with Ca_vβAS or Ca_vβS in mice that were then challenged with intranasal OVA (new Fig.6A). Ca_vβAS- and Ca_vβS-transfected Th2-cells localized equally well in the lungs (new Fig.6B). However, Ca_vβAS-transfected Th2-cells had reduced ability to promote Th2 response upon airway challenge, as shown by the reduction in lung inflammation (new Fig. 6C, D, new supplemental Fig.3E), the lower serum IgE concentration (new Fig. 6E) and the

impaired Th2 cytokine production after recall of antigen-specific draining lymph node T-cells with OVA (new Fig. 6F). This was not associated with a shift towards Th1 response as shown by the similar IFNγ production by OVA-stimulated draining lymph node cells from both groups (new Fig. 6F).

Gabapentin, an inhibitor of Ca_vα2δ impairs Th2 functions and reduces allergic airway inflammation

Mouse Th2 cells mainly expressed Ca_v α2δ2 (Fig. 7A), as human Th2-cells did [9]. Blotting and probing Th2-cell or brain lysates with anti-α2δ2 antibodies showed similar bands around 140 kDa (Fig. 7B). To investigate the role of α2δ in Ca_v1-dependent Th2 function, we used gabapentin, a ligand binding to Ca_v α2δ1 and α2δ2 but not to the other α2δ subunits [32]. Chronic *in vitro* treatment with this drug is known to inhibit Ca_v currents. Accordingly, treatment of Th2-cells with gabapentin for 72 hours decreased and delayed the TCR-driven calcium response (Fig. 7C), as shown by the quantification of the area under the curve and the time of response, respectively (Fig. 7D and E). The decrease in calcium signaling was associated with reduced Th2-cytokine production (Fig. 7F). However, gabapentin had no effect on calcium response and IFNγ production in Th1-lymphocytes (Supplemental Fig. 5).

Since gabapentin was reported to induce the loss of Ca_v2 complex at the cell membrane [33, 34], we tested its effect on Ca_v1.2 protein expression In Th2 cells. The amount of α2δ2 protein was strongly reduced in gabapentin treated Th2 cells (Fig. 7G and Supplemental Fig. 6), with no effect on Ca_v1.2 expression (Fig. 7H and Supplemental Fig. 6). Moreover, Bafilomycin A1, an inhibitor of the vacuolar H^+ ATPase (v-ATPase) that inhibited acidification and protein degradation in lysosomes, partially restored the amount of α2δ2 in gabapentin-treated Th2-lymphocytes (Fig. 7G), demonstrating that gabapentin targeted α2δ2 in lysosomes for its degradation.

We then examined the effect of gabapentin administration on the development of allergic airway inflammation (new Fig. 8A). Administration of gabapentin during the challenge with OVA partially reduced airway inflammation as shown by the reduction in the number of

inflammatory cells, including eosinophils in the BAL fluid (Fig. 8B), and histological examination of lung sections (Fig. 8C-D). The expression of Th2 cytokines and of mucins 5ac and 5b that are the major components of mucus in the lungs [35] was decreased in the lungs of gabapentin-treated mice (new Fig. 8E), supporting a beneficial effect of gabapentin on Th2-mediated inflammation.

Discussion

We previously reported that knocking down $Ca_v1.2$ and $Ca_v1.3$ α1 subunits in Th2-cells impaired their functions and protected against asthma [7]. 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 asked if auxiliary subunits as $Ca_v\beta$ and $Ca_v\alpha2\delta$ are required for Th2-cell functions and can be beneficially targeted in acute allergic airway inflammation. Our results now show that i) knocking down $Ca_v\beta$ expression with $Ca_v\beta$AS designed to target the four beta subunits reduces TCR-driven $[Ca^{2+}]_i$ increase and Th2-cytokine production by mouse and human Th2-cells, while sparing Th1-cell responses; ii) it is associated with the loss of $Ca_v1.2$ α1protein, which can be partially overcome by a proteasome inhibitor; iii) intranasal delivery of $Ca_v\beta$ AS strongly diminishes Th2-mediated immunopathology in a model of acute allergic airway inflammation; iv) the $Ca_v\alpha2\delta1/2$ inhibitor gabapentin impairs calcium signaling and reduces OVA-induced asthma.

Although $Ca_v\beta3$ can regulate calcium signaling independently of α1 by limiting IP3 generation in pancreatic cells [36], this is unlikely in Th2-cells because knocking down $Ca_v\beta$ depresses and not enhances $[Ca^{2+}]_i$. In addition $Ca_v\beta$AS do not affect the calcium signal in Th1-cells that express $Ca_v\beta3$ but no Ca_v1 α1 subunits. Moreover, our results favor a role of $Ca_v\beta$ related to its interaction with $Ca_v1\alpha1$ as supported by the loss of expression of $Ca_v1.2$ α1 in Th2-cells transfected with $Ca_v\beta$AS. In Th2-cells, $Ca_v\beta$ prevents the degradation of $Ca_v1.2$ α1, which is consistent with findings showing that β1b or β2a may be required for $Ca_v1.2$ or $Ca_v2.1$ α1 protein expression [28, 37]. Surprisingly, transfection of ventricular myocytes with the same $Ca_v\beta$AS we used in our study resulted in Ca_v1 α1 sequestration

without increased cardiac $Ca_v1.2$ α1 degradation [38]. This apparent discrepancy in T-lymphocytes and cardiac myocytes could be related to differences in $Ca_vβ$ subunit equipment and/or regulation in these cell types. Along this line, it has been shown that the absence of $Ca_vβ4$ resulted in decreased expression of $Ca_v1.1$ but not $Ca_v1.2$ α1 protein in $CD4^+$ T-cells [6].

Two mechanisms have been proposed to explain how $Ca_vβ$ can protect the α1 subunit from degradation. First, the interaction of β2 with $Ca_v1.2$ α1 might limit the degradation of the channel in the lysosomes [39]. Second, the interaction of β1 or β2 with $Ca_v1.2$ or $Ca_v2.2$ α1 prevents α1 to be addressed to and degraded by the proteasome [28, 37]. The second mechanism is in agreement with our data in Th2-cells. Indeed, we show that knocking-down $Ca_vβ$ diminished the total amount of endogenous $Ca_v1.2$ α1, partially restored upon proteasome inhibition in Th2-cells. Whether β subunits are also required for the export of α1 subunits to reach the cell surface is controversial [23, 28, 40]. Knocking down $Ca_vβ$ resulted in lower expression of $Ca_v1.2$ α1 at the cell membrane, which was partially restored by the proteasome inhibitor indicating that $Ca_vβ$ were not absolutely required for the channel to exit from the ER or that the remaining $Ca_vβ$ subunits were sufficient to promote the trafficking of the channel at the cell membrane. The absence of $Ca_vβ$ even in the presence of proteasome inhibitor did not allow the restoration of cytokine production by Th2-cells, which could be due to the incomplete restoration of $Ca_v1.2$ α1 and/or a role of $Ca_vβ$ on the function of the channel at the cell membrane.

Mouse Th2-cells express predominantly both β1 and β3 subunits as human Th2 cells do [9]. It was previously reported that β1 variant preferentially interacts with $Ca_v1.1$ α1 allowing the interaction of Ca_v1 with ER-localized type 1 Ryanodine receptors (RyR) [41] leading to calcium depletion in the ER. It would be interesting to investigate the respective roles of $Ca_vβ1$ and $Ca_vβ3$ in $Ca_v1.2$ function. Interestingly, $Ca_vβ$ can interact with G proteins, scaffolding proteins (reviewed in [42]) and especially AHNAK, which might be required for plasma membrane localization of the channel as described for $Ca_v1.1$ α1 channels in T-cells [43].

We evidenced predominantly α2δ2 at the mRNA level in Th2-cells. We also detected the protein by WB with the same apparent weight as in neurons and by immunofluorescence. Nerve injury can be responsible for chronic pain due with α2δ1 overexpression in injured sensory neurons [44, 45]. Gabapentin is beneficial because this drug that binds to α2δ1 and α2δ1 but not the other α2δ isoforms [34] may decrease the expression of the Ca_v2 channels at the cell membrane [33, 34, 46] leading to a reduction in Ca_v calcium currents. However, the authors did not report an effect of gabapentin on the total amount of α2δ and of Ca_v α1 subunits. Unexpectedly, Th2-cells treated with gabapentin show reduced total amounts of α2δ2, which is restored by an inhibitor of the vacuolar H^+ ATPAse. This suggests that gabapentin favors α2δ2 degradation in an acidic compartment as lysosomes. Along this line, gabapentin was shown to prevent the recycling of α2δ2 from Rab11-positive recycling endosomes to the plasma membrane [47]. We show here that gabapentin neither affects the total amount of $Ca_v1.2$ protein nor its localization at the plasma membrane in Th2-cells. However the calcium response was decreased upon TCR activation suggesting that α2δ2 may contribute to the function of $Ca_v1.2$ α1 as described for α2δ1 [48]. Altogether our results obtained with gabapentin and knockdown of Ca_vβ in the presence of MG132 indicate that the level of $Ca_v1.2$ α1 is not the only parameter limiting the calcium response and suggest that both auxiliary subunits are required in addition to $Ca_v1.2$ α1 for Th2 functions.

Altogether, these data stress the role of auxiliary subunits (Ca_vβ and α2δ) in $Ca_v1.2$ channel functions in Th2-cells and show that the auxiliary subunits of Ca_v1 channels represent potential new therapeutic targets in allergy. Consistently, Ca_vβAS has a marked effect on Th2 functions, airway inflammation and hyper-reactivity. $CD4^+$ T-cells infiltrating the lungs express $Ca_v1.2$ α1 channel and this expression is diminished in mice during Ca_vβ AS treatment suggesting that Ca_vβAS act in a similar way *in vitro* and *in vivo*. Chronic administration of gabapentin decreased Th2 functions and inflammation. Since Ca_vβAS and gabapentin act by different ways to decrease Ca^{2+} signaling and Th2 function, it is tempting to propose a combination of drugs targeting Ca_vβ and Ca_vα2δ. In that respects gabapentin is already used for the treatment of chronic cough [49], a symptom, which is currently found in

asthmatic patients. Interestingly, peptides stapling the Ca_v α1 domain interacting with Ca_vβ subunits and shown to inhibit Ca_v functions have been recently described [50] and could be tested for their efficacy in allergic diseases.

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Acknowledgements: We thank FE L'Faqihi-Olive, V. Duplan-Eche and AL. Iscache for technical assistance at the Flow cytometry facility of INSERM UMR 1043, CPTP and also the imaging platforms (INSERM U1043, CPTP), the animal facility and the histology platforms (Anexplo Toulouse). We also thank Joris Hien-Turpin for his technical assistance and P. Lory for the gift of plasmids that encode $Ca_v\beta$ subunits. This work has been supported by the BM1406 COST Action thanks to helpful discussions.

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Figure legends

New Fig. 1: Ca_vβAS diminish cytokine production in mouse and human Th2 but not in Th1-cells.

OVA-specific DO11.10 Th2 and Th1-cells were tested for Ca_vβ at level transcript in **A** (n=4), at protein level by immunoblotting in **B.** Mouse DO11.10 Th2 (**C**), Th1 **(D)**, human $CRTH2^+$ (**E**) or Th1-cells (**F**) were transfected with Ca_vβS or Ca_v βAS for 72h, stimulated for 24 hours with anti-CD3/C28 antibodies and cytokine production was measured. Each pair represents a separate experiment in **C,D** and a donor in **E,F**. * p<0.02; ** p< 0.01; ***p<0.005 and ****p<0.001.

New Fig. 2: Knocking down Ca_vβ subunits reduces TCR-driven $[Ca]_i$ increase in mouse and human Th2-cells without any effect on Th1-cells.

Mouse DO11.10 Th2 (**A-C**), human $CRTH2^+$ (**D-F**), mouse Th1 (new **G-I**) or human Th1 (**J-L**) cells were transfected with Ca_vβAS or Ca_vβS and loaded with Fura2-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 (**A, D, G, J**) are expressed as the mean of at least 30 cells and bars represent the SEM. The area under the curve between the time of stimulation and the time where iono is added is shown in **B, E, H and K**. The time of response (delay between stimulation and the time showing an increase of the ratio over the mean ratio plus 1 SD recorded before stimulation) is shown in **C, F, I and L**. One representative experiment of 4 to 6 is shown. ****p<0.001.

Fig. 3: Knocking down Ca_vβ subunits decreases Ca_v1 α1 expression in mouse and human Th2-cells.

Cells were transfected with Ca_vβS or Ca_vβAS, for 72 hours. **A**) shows the amount of Ca_v1.2 α1 transcripts in mouse D011.10 Th2-cells. **B-E**) show the staining with anti-CD2 (green) and anti-Ca_v1.2 α1 (red) monoclonal antibodies in mouse D011.10 Th2 cells (**B-C**) and in human

555 $CRTH2^+$ (**D-E**). The $Ca_v1.2$ cell amount over all the Z stack sections is quantified in (**C, E**).
556 One representative experiment out of three is shown. **** p <0.001.
557
558 **Fig. 4: MG132 partially restores $Ca_v1.2$ α1 in Th2 cells knocked down for $Ca_v\beta$ without**
559 **impact on Th2 cytokine production.**
560 Mouse DO11.10 Th2 cells were transfected with $Ca_v\beta S$ or $Ca_v\beta AS$ for 48 hours before
561 addition of MG 132 (0.5 µM) or vehicle for additional 24 hours. Cells were then recovered
562 and analyzed by immunofluorescence (**A-C**) or were stimulated for 24 hours with anti-
563 CD3/C28 antibodies. Cytokine production was measured by ELISA (**D**). Th2-cells were
564 labeled with anti-$Ca_v\beta 1$ (**A**) anti-$Ca_v\beta 3$ (**B**) or anti-$Ca_v1.2$ α1 mAb (**C**). Immunofluorescence
565 was summed over all Z sections. (**A-C**) Results are expressed as the mean of 50-200 cells.
566 The bars represent the SEM. One experiment representative of 3 is shown. (**D**) Results are
567 expressed as the mean of 4 experiments and the bars represent the SD. * p<0.05; **p<0.01;
568 ****p<0.001.
569
570 **New Fig. 5: $Ca_v\beta AS$ administration strongly diminishes allergic airway inflammation.**
571 **A**) BALB/c mice were immunized with OVA in alum and were challenged 15 days later with
572 intranasal OVA only or OVA plus scrambled $Ca_v\beta$ ($Ca_v\beta S$) or OVA plus $Ca_v\beta$ antisense ($Ca_v\beta$
573 AS) every day for 5 days. **B**) Number of cells in the broncho-alveolar lavage fluid (BAL). **C**)
574 H&E sections from the mice were scored on a 0 to 12 scale. **D**) Serum IgE concentration was
575 measured at the time of sacrifice. **E**) Mediastinal lymph nodes were collected and cell
576 suspensions were recalled for 72 hours with OVA and cytokine production was measured by
577 ELISA. Results in B-E are the mean of 5 mice and are representative of 2 independent
578 experiments. * p<0.05; **p<0.01; ****p<0.001.
579
580
581

New Fig. 6: Th2-cells transfected with Ca_vβAS have impaired ability to induce type 2-airway inflammation.

A) Th2-cells transfected with Ca_vβS (Th2 Sβ) or Ca_vβAS (Th2 ASβ) were injected into BALB/c mice given intranasal OVA. **B**) Lung-infiltrating cells were purified and analyzed by flow cytometry to enumerate $CD3^+$ and $CD3^+KJ1.26^+$ cells. **C**) Inflammatory cells were enumerated in the BAL fluid. **D**) Lung sections were scored for inflammation. **E**) Serum IgE concentration was measured at the time of sacrifice. **F**) Lung draining lymph node cells were recalled with OVA for determination of cytokine production. Each group comprised five mice. *p< 0.05; **p<0.01; ***p<0.005.

Fig. 7: Gabapentin, an inhibitor of Ca_vα2δ auxiliary subunits reduces calcium signaling and cytokine production by mouse Th2-cells.

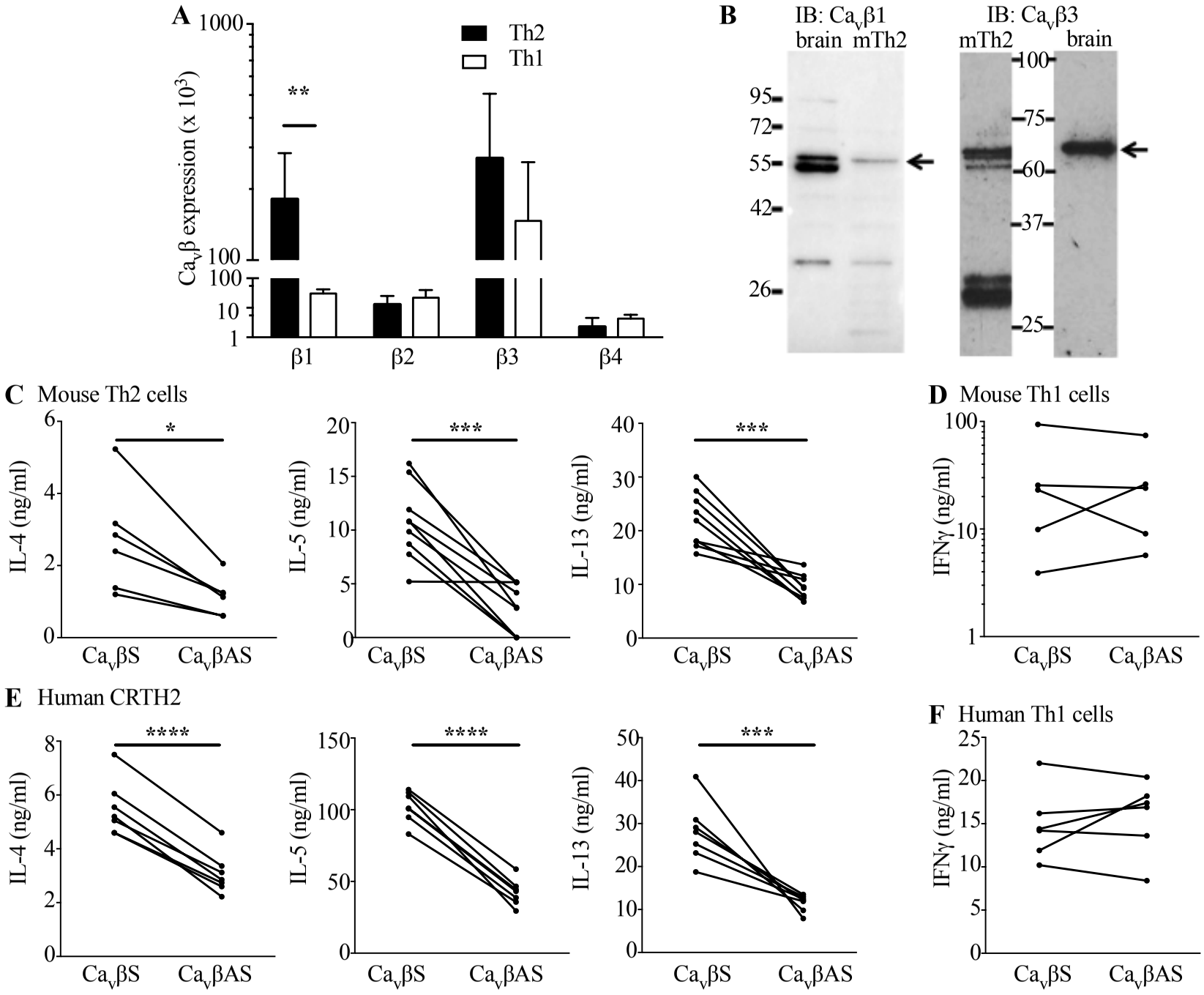
A) Mouse DO11.10 Th2 cells were analyzed for the expression of α2δ transcripts (n=4). **B**) Th2 and brain (positive control) lysates were blotted and stained with anti-α2δ2 antibodies. Th2-cells were pre-incubated with gabapentin (1mM) for 72 hours (**C-H**). **C-E**) Cells were then loaded with the calcium probe Fura2-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 the SEM. **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**) Th2-cells were stimulated in the presence or not of gabapentin for additional 24 hours with anti-CD3/anti-CD28 antibodies. The cytokine content was then measured. **G-H**) Th2-cells were incubated or not with bafilomycin (0.5 μM) the last 24 hours. Cells were stained with anti-α2δ2 (**G**) or anti-Ca_v1.2 α1 (**H**) Ab. Immunofluorescence was summed all over the Z stack sections. One representative experiment out of 2 is shown. **p<0.01; *** p<0.005 and ****p<0.001.

New Fig. 8: Gabapentin, an inhibitor of Ca_vα2δ subunits decreases type 2 airway inflammation.

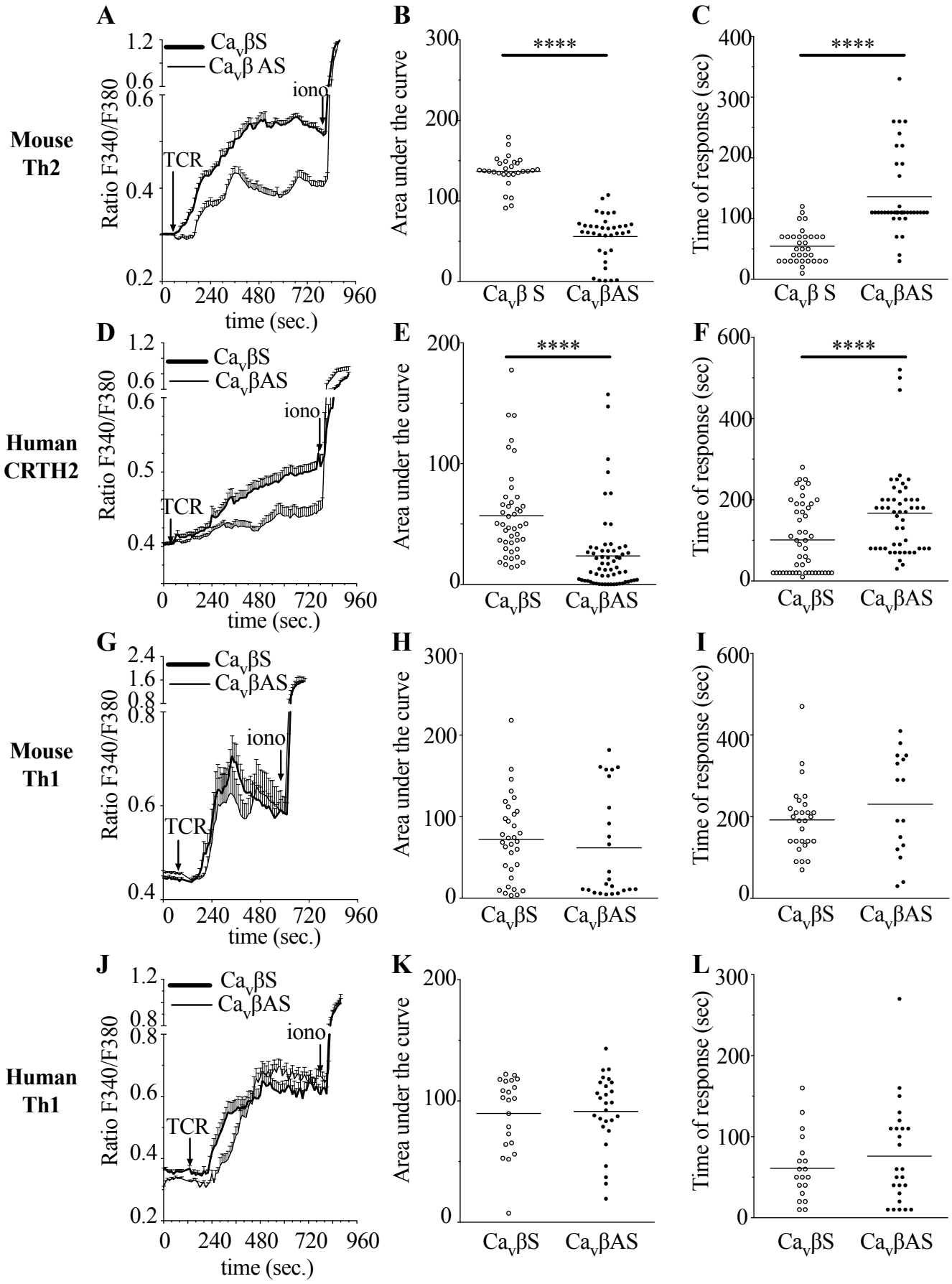
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610 **A**) BALB/c mice were immunized with OVA in alum and then challenged with intranasal OVA
611 for 5 days. Gabapentin was or not given in drinking water for the last 5 days. The next day
612 mice were killed and the broncho-alveolar lavage fluid was collected and analyzed for the
613 inflammatory cell content (**B**). **C**) H&E histological sections were scored on a 0-12 scale. **D)**
614 exemplifies lung inflammation in control (nt) and gabapentin given mice (gaba). **E**) The
615 expression of *il4*, *il5*, *il13*, *Muc5ac* and *Muc5b* was quantified in lungs by qPCR and
616 normalized to housekeeping gene expression. Each experiment includes 6 to 7 mice per
617 group. One representative experiment of 2 is shown. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.
618

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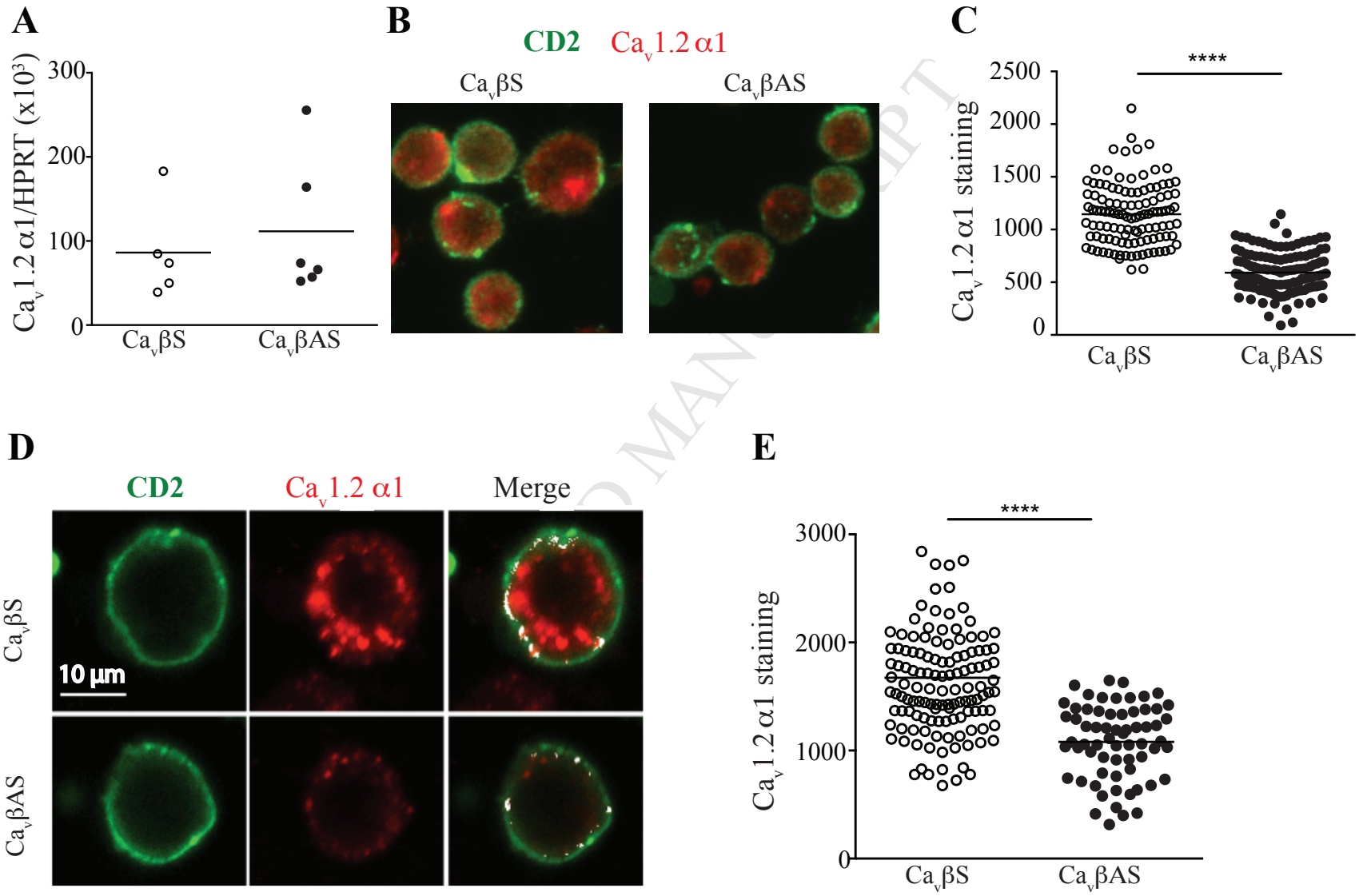

A
Th2
Th1
Cavβ expression (x 10^3)
1000
100
10
1
**
β1
β2
β3
β4
B
IB: Cavβ1
brain
mTh2
IB: Cavβ3
mTh2
brain
95
72
55
42
26
100
75
60
37
25
C Mouse Th2 cells
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
*
***
***
CavβS
CavβAS
D Mouse Th1 cells
IFNγ (ng/ml)
E Human CRTH2
****
****
***
F Human Th1 cells

new Fig. 1


A
B
C
D
E
F
G
H
I
J
K
L
Mouse Th2
Human CRTH2
Mouse Th1
Human Th1
Ratio F340/F380
time (sec.)
TCR
iono
Ca$_v$βS
Ca$_v$β AS
Ca$_v$βAS
Area under the curve
Time of response (sec)
****
0 240 480 720 960

new Fig. 2

Figure 3


A
Ca$_v$1.2 α1/HPRT (x10^3)
300
200
100
0
Ca$_v$βS
Ca$_v$βAS
B
CD2
Ca$_v$1.2 α1
Ca$_v$βS
Ca$_v$βAS
C
Ca$_v$1.2 α1 staining
2500
2000
1500
1000
500
0
****
Ca$_v$βS
Ca$_v$βAS
D
CD2
Ca$_v$1.2 α1
Merge
Ca$_v$βS
Ca$_v$βAS
10 µm
E
Ca$_v$1.2 α1 staining
3000
2000
1000
0
****
Ca$_v$βS
Ca$_v$βAS

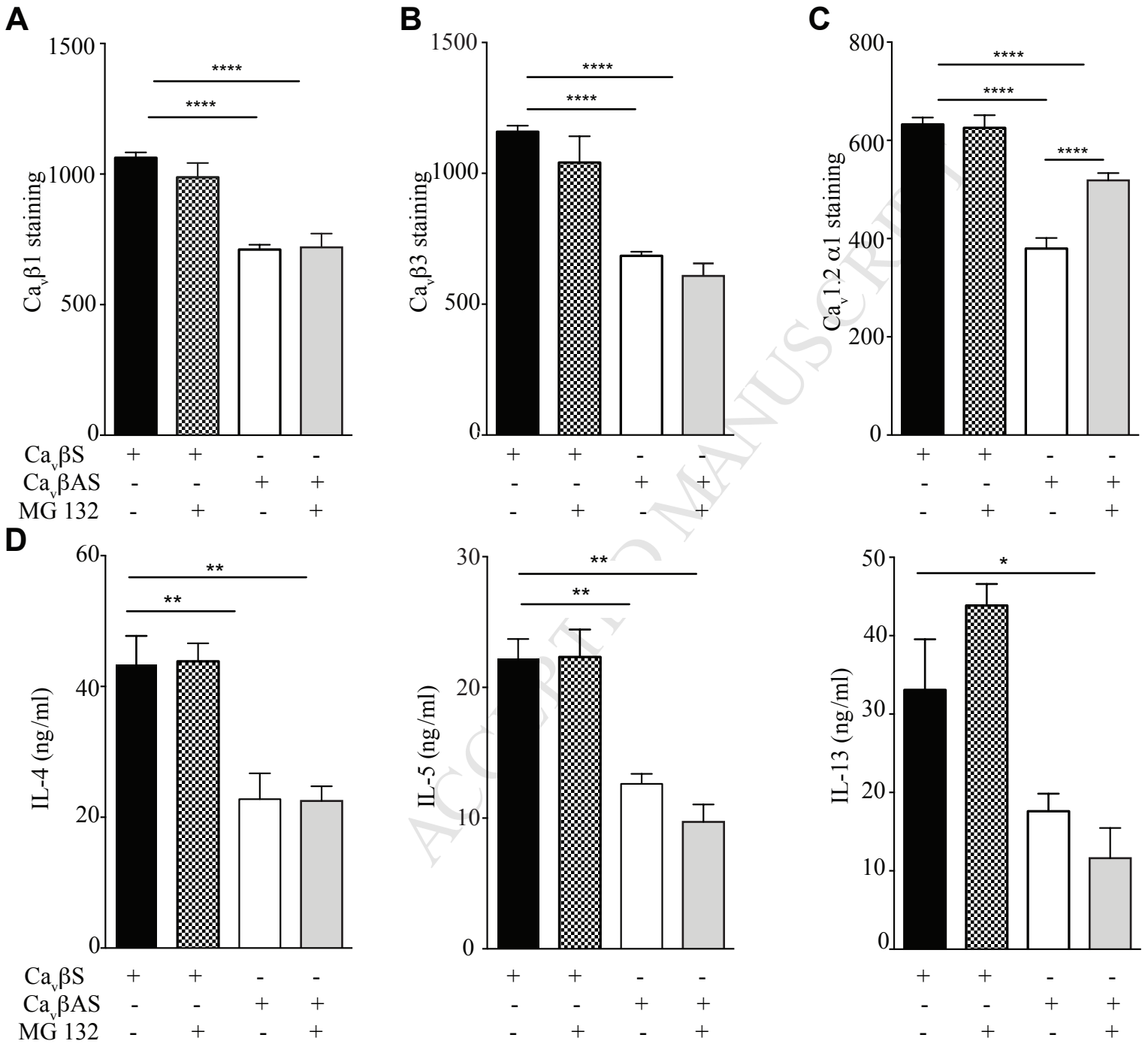
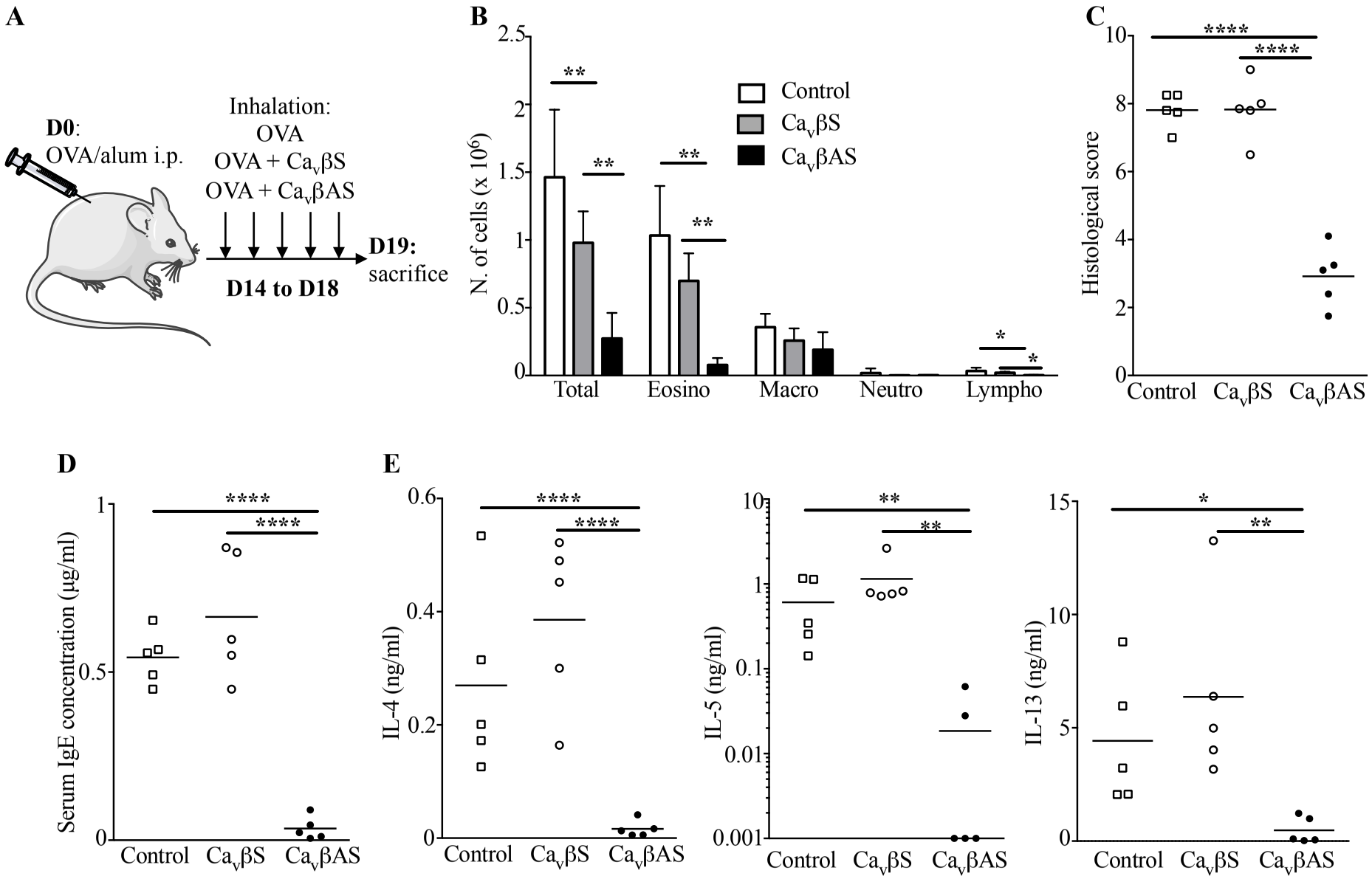
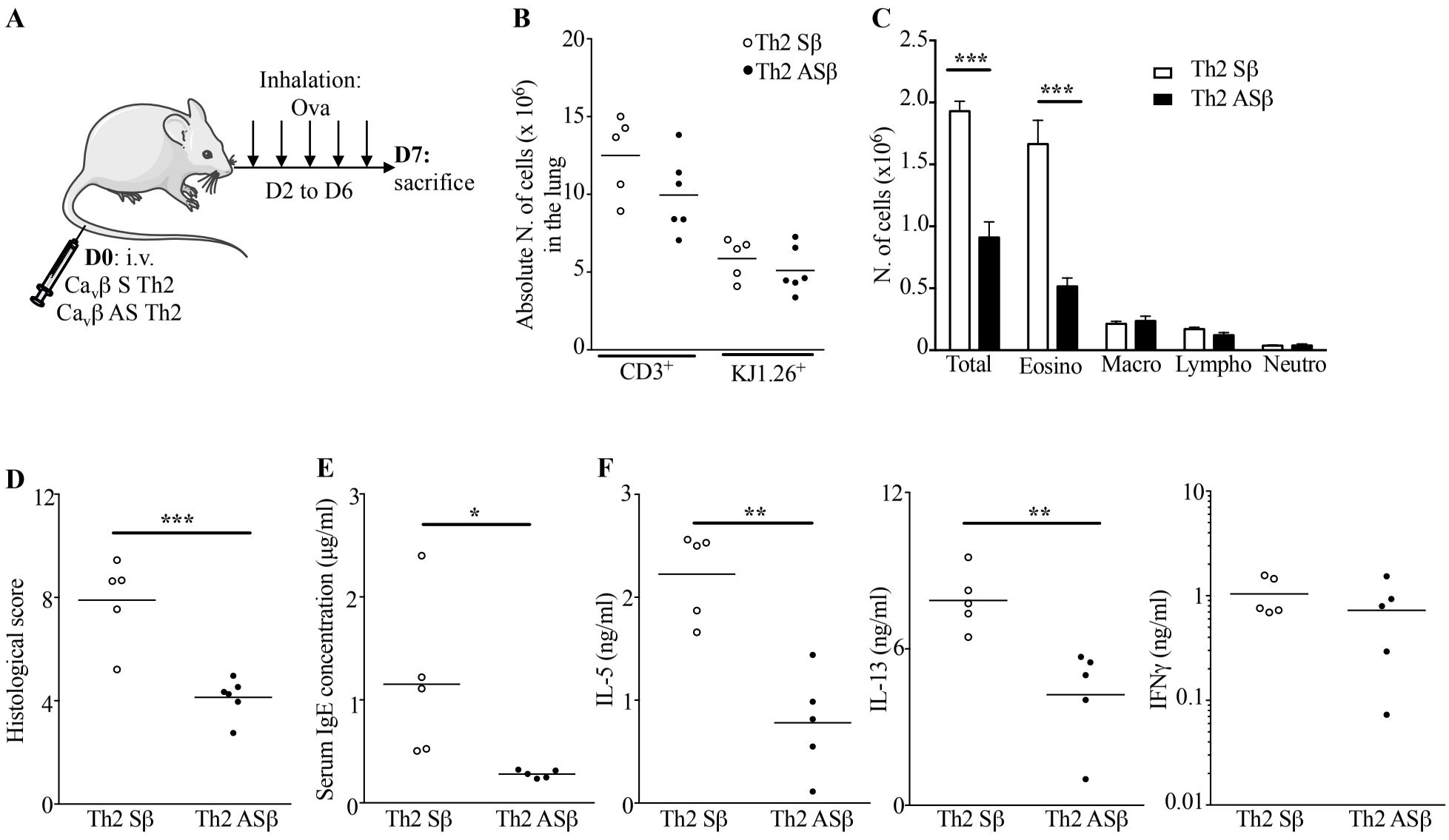

A
B
C
D
Ca$_v$β1 staining
Ca$_v$β3 staining
Ca$_v$1.2 α1 staining
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
Ca$_v$βS
Ca$_v$βAS
MG 132
****
**
*

Figure 4

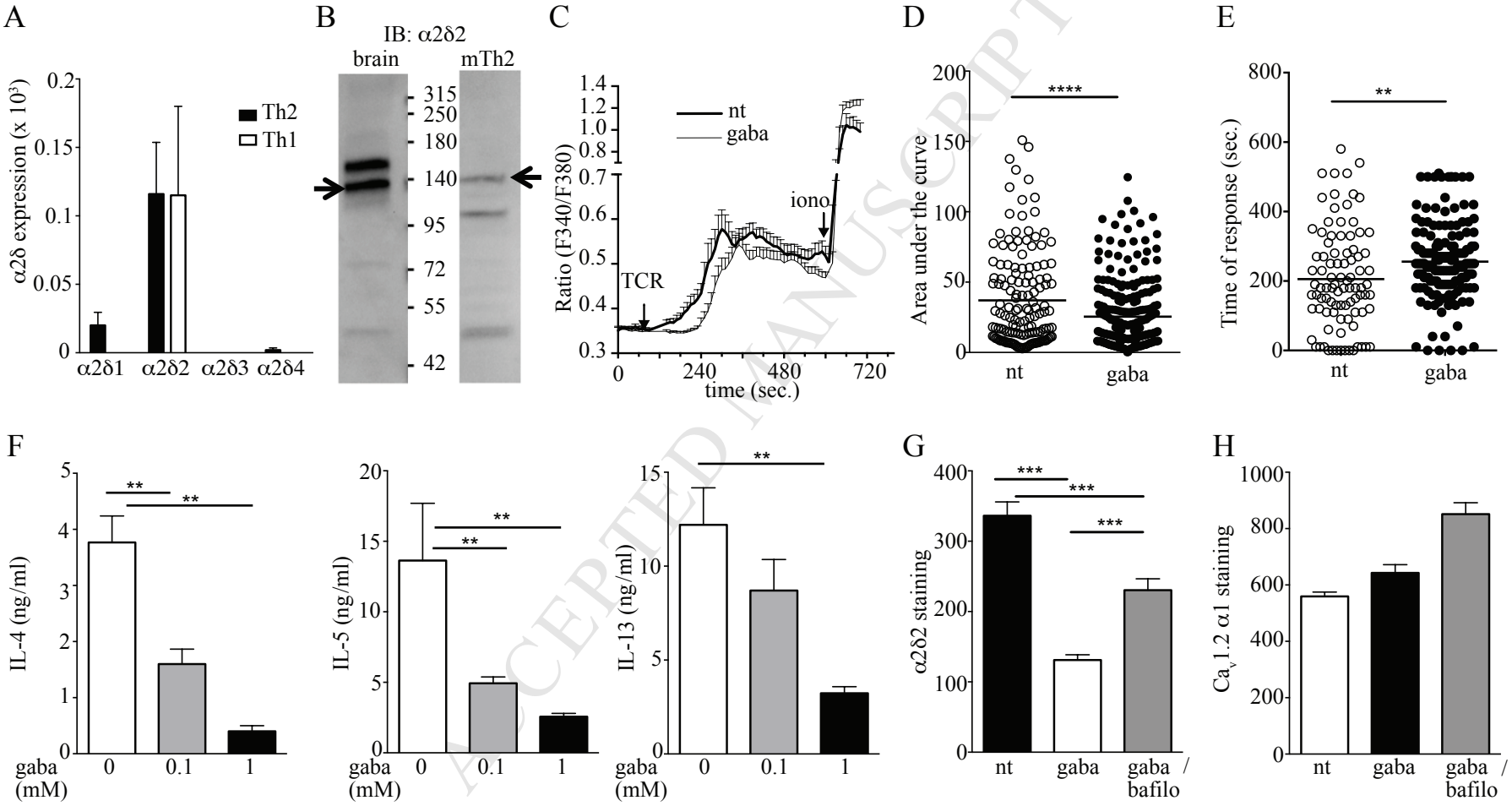

A
D0:
OVA/alum i.p.
Inhalation:
OVA
OVA + CavβS
OVA + CavβAS
D14 to D18
D19:
sacrifice
B
N. of cells (x 10^6)
Control
CavβS
CavβAS
Total
Eosino
Macro
Neutro
Lympho
C
Histological score
Control
CavβS
CavβAS
D
Serum IgE concentration (µg/ml)
E
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)

new Fig. 5

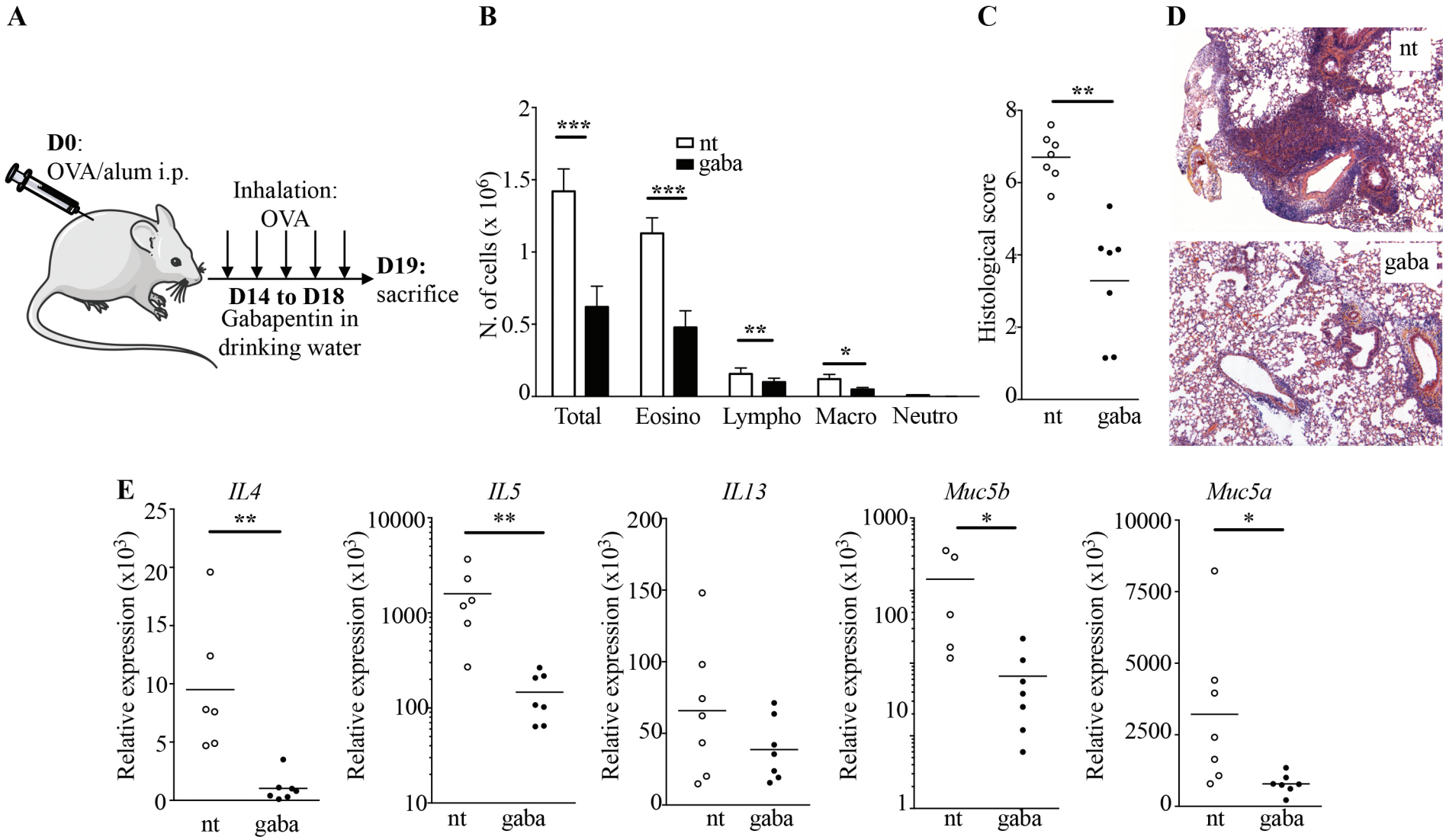

A
Inhalation:
Ova
D2 to D6
D7:
sacrifice
D0: i.v.
Cavβ S Th2
Cavβ AS Th2
B
Th2 Sβ
Th2 ASβ
Absolute N. of cells (x 10^6) in the lung
CD3+
KJ1.26+
C
Th2 Sβ
Th2 ASβ
N. of cells (x10^6)
***
***
Total
Eosino
Macro
Lympho
Neutro
D
Histological score
***
Th2 Sβ
Th2 ASβ
E
Serum IgE concentration (µg/ml)
*
Th2 Sβ
Th2 ASβ
F
IL-5 (ng/ml)
**
Th2 Sβ
Th2 ASβ
IL-13 (ng/ml)
**
Th2 Sβ
Th2 ASβ
IFNγ (ng/ml)
Th2 Sβ
Th2 ASβ

new Fig. 6

Figure 7



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A
D0:
OVA/alum i.p.
Inhalation:
OVA
D14 to D18
Gabapentin in
drinking water
D19:
sacrifice
B
N. of cells (x 10^6)
nt
gaba
***
***
**
*
Total
Eosino
Lympho
Macro
Neutro
C
Histological score
**
nt
gaba
D
nt
gaba
E
IL4
IL5
IL13
Muc5b
Muc5a
Relative expression (x10^3)
**
**
*
*
nt
gaba

new Fig. 8

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Supplemental methods

Oligonucleotide sequences

The sequences $Ca_v\beta S$: 5'-gaagtaggtcttggtggtgg-3' and $Ca_v\beta AS$: 5'-accagccttccgatccaccagtcatt-3' (Eurogentec, Angers France) were chosen from [20]. $Ca_v\beta AS$ have 92% complementarity to mouse or human β1 and β3 mRNA.

Model of acute airway allergic inflammation

The BALF was collected and the content in inflammatory cells was determined as previously [6]. Lung tissue was fixed in 10% buffered formalin for 24 h and then placed in ethanol 70% before embedding in paraffin. Sections (4 µm) were stained with hematoxylin an eosin. Histological disease scores from 0 to 3 were attributed based on the severity of peribronchial, perivascular and interstitial immune cell infiltration, and thickening of peri-bronchial epithelium, resulting in a maximum score of 12. Lung draining lymph nodes were harvested and $2x10^5$ cells were stimulated with OVA (100µg/ml) for 3 days. Cytokine concentrations were measured in the supernatants by ELISA. In some experiments, $CD4^+$ T cells were purified from the lungs, stained with anti-$Ca_v1.2$ antibodies and analyzed by confocal microscopy.

Airway Hyperresponsiveness

Anesthetized and ventilated mice were connected via an endotracheal cannula to a whole body plethysmography for ventilated subject system (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 3cm H2O. 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 using iox software (Emka).

Cell culture

Cells were cultured in complete medium (RPMI 1640 supplemented with 10% FCS (Lonza, Allendale, NJ), 1% pyruvate, 1% nonessential amino acids, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM β-mercaptoethanol.

Single cell calcium imaging

Cells were loaded with Fura-2AM as previously described [6, 8] in culture medium containing 5% heat-inactivated FBS for 30 min at 37℃. Cells were then washed, plated and excited in 10 second intervals using 340 and 380 nm excitation filters. Emission was recorded using 510/540 nm band pass filters by 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 340 and 380 nm wavelengths were then calculated using the Metafluor software. The curve represents the mean of fluorescence ratio of 40-60 cells + SEM. Each experiment was repeated three or four 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) and were then fixated with 4% paraformaldehyde. Staining was performed with anti-CD2 rat IgG antibody (Abcam), anti-α2δ2 rabbit antibody (Alomone Labs) and revealed with the appropriate secondary antibodies (Molecular Probes). Cells were then permeabilized with PBS containing 0.1% saponine and 0.5% bovine serum albumin. Ca_v channel subunit staining was performed using anti-Ca_v1.2 mouse IgG2b (NeuroMab), anti-Ca_vβ1 (Alomone) or anti-Ca_vβ3 rabbit IgG (Santa Cruz Lab) overnight and revealed with the appropriate secondary antibodies (Molecular Probes). Slides were analyzed using a LSM 710 confocal microscope

(Zeiss). Staining with control isotypes was constantly negative. The total fluorescence intensity of each cell was determined on the sum of over all Z sections with ImageJ software. For membrane $Ca_v1.2$ staining, a surface of the membrane was created using CD2 staining and this surface was duplicated on $Ca_v1.2$ staining in order to measure the intensity of the $Ca_v1.2$ at the membrane using Imaris 8.4.1 (Bitplane) module called Surface. Each measurement was done for each cell individually.

Real-time quantitative PCR

Th1 and Th2 cells were lysed and RNA extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed with the SuperScript III Reverse Transcriptase (Invitrogen). The transcripts were measured by real-time quantitative PCR using a LightCycler 480 Instrument (Roche) and the LightCycler® 480 SYBR Green I Master (Roche). We used the following primers: $Ca_v\beta1$: 5'-gcagagcgccaggcctta-3' and 5'-tgatggccactccctgtaca-3'; $Ca_v\beta2$: 5'-cgaacagagagccaagcaa-3' and 5'-gggcactatgtcacccaaa-3'; $Ca_v\beta3$: 5'-tgtggcatttgctgtgagga-3' and 5'-ccgatccaccagtcattgc-3'; $Ca_v\beta4$: 5'-gagcgagaacagcaagcag-3' and 5'-tcaaaggagatggctgtgc-3'; $Ca_v\alpha2\delta1$: 3' acgccaactggttgaaattg 5' and 3' cttgcaaaatcttccctcca 5'; $Ca_v\alpha2\delta2$: 3' atttccagaaagcccatcg 5'and 3' gtcttggatcccctctcca 5'; $Ca_v\alpha2\delta3$: 3' cattttggtggacgtcagtg 5' and 3' ttccgttcagacaaggttcc 5'; $Ca_v\alpha2\delta4$: 3' ggaaagtcgaggctgttgag 5' and 3' ctccacgtagttgcccttgt 5'; HPRT (hypoxanthine-guanine phosphoribosyltransferase): 5'-ctggtgaaaaggacctctcg-3' and 5'-tgaagtactcattatagtcaagggca-3'. The primers for il4, il5, il13, Muc5ac and Muc5b are purchased from Qiagen. Quantification of target gene expression was calculated by normalizing the values relative to the expression of the housekeeping gene HPRT. The amounts of mRNA were expressed as arbitrary units relative to HPRT ($2^{-(Ct\ interest\ gene\ -\ Ct\ HPRT)}$) x 10^{-3}.

Immunoblotting

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Th2 lymphocytes or brain cells were lysed in RIPA buffer containing protease inhibitors (Roche). The lysates (30 µg/ lane for T-cell extracts and 10 µg/ lane for brain lysate) were submitted to 10% SDS electrophoresis, transferred to Hybond-C Extra nitrocellulose membranes (Amersham) and then blotted with anti-Ca_vβ1 (Alomone Labs), anti-Ca_vβ3 (Santa Cruz Biotechnology) or anti-α2δ2 (Alomone labs) primary antibodies. We then stained blots with horseradish peroxidase coupled secondary antibodies (Cell Signaling Technology), before detection and visualization with the ECL Prime (Amersham) using a ChemiDoc XRS System (Bio-Rad). The specificity of anti-Ca_vβ1 and β3 antibodies was verified on HEKs transfected with the corresponding plasmids by western blotting.

ELISA

Supernatants or serums were incubated into 96 wells plates coated with anti-IL-4, anti-IL-5, anti-IL-13 (eBioscience), anti-IFNγ 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-IFNγ (eBioscience), or anti-IgE (BD Pharmingen). Biotinylated antibodies were revealed by incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch) and subsequent adding of the alkaline phosphatase substrate pNPP disodium salt hexahydrate (Sigma-Aldrich). Absorbance was measured at 405-650 nm using an EMax Microplate Reader (Molecular Devices). Cytokines and immunoglobulins concentrations were calculated from standard curves generated by titration of recombinant mouse cytokines and purified IgE. Human cytokine ELISA were done as previously described in [8].

Supplemental Figure legends

New Supplemental Fig. 1: Ca_vβAS target Ca_vβ1 and Ca_vβ3 subunits in mouse and human Th2-cells. OVA-specific DO11.10 Th2 and Th1-cells were stained with anti-Ca_vβ1 and anti-Ca_vβ3 antibodies as exemplified in **A**). The expression of Ca_vβ per cell was shown

in **B**. One experiment out of 3 was depicted and each point represented one cell. **C**) Cell lysates were prepared from mouse Th2-cells transfected with $Ca_v\beta S$ or $Ca_v\beta AS$ after 3 days of transfection. One representative blot was shown. **D)** Protein bands were quantified using Image Lab. The ratio between the protein of interest and β-actin was calculated. The results were expressed as the relative protein level to $Ca_v\beta S$ and shown as separate paired experiments. **E-F)** Th2-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. The expression of $Ca_v\beta$ per cell was shown in F. One experiment out of 3 was depicted and each point represented one cell. **G**) $CRTH2^+$ 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 were shown as separate paired experiments. **p<0.01; ***p<0.005 and ****p<0.0001.

Supplemental Fig. 2: The proteasome inhibitor MG132 partially restores total $Ca_v1.2$ α1 level and its expression at the cell membrane. A) Mouse DO11.10 Th2-cells were transfected with control ($Ca_v\beta$ S) or antisense $Ca_v\beta$ AS oligonucleotides for 48 hours before addition of MG 132 (0.5 µM) or vehicle for additional 24h. Cells were then recovered and labeled with anti-$Ca_v1.2$ α1 mAb. The dotted lines indicate the cells. **B**) Th2-cells treated as in A were labeled with both anti-CD2 and anti-$Ca_v1.2$ α1 mAbs and the co-localization determined with the Imaris software is shown. Results are expressed as the mean of at least 50 cells and bars represent the SEM.

New Supplemental Fig. 3: $Ca_v\beta AS$ alleviate type 2 airway inflammation. A) BALB/c mice were immunized with OVA in alum and were challenged 15 days later with intranasal OVA only (control) or OVA plus $Ca_v\beta S$ or OVA plus $Ca_v\beta AS$ every day for 5 days. Representative H&E sections were shown and the arrows indicated the inflammatory infiltrates. **B-D**) $CD4^+$ T-cells were purified from lung and stained with anti-$Ca_v1.2$ α1 mAb. Optical sections were shown in **B**. Quantification was done by collecting data from all the Z stack sections of each cell. Exemples were given in **C** where each point corresponds to one cell from one representative $Ca_v\beta S$ or $Ca_v\beta AS$-treated mouse. **D**) summarized results obtained from

different mice. Each bar represents the mean plus 1SD of at least 30 cells from one mouse. * p<0.05; ****p<0.001. E) showed representative H&E lung sections from mice injected with Th2-cells transfected with $Ca_v\beta S$ or $Ca_v\beta AS$ and given intranasal OVA.

New Supplemental Fig. 4: $Ca_v\beta AS$ decreased airway hyper reactivity in a mouse model of allergic airway inflammation.

BALB/c mice were immunized with OVA in alum (OVA) or with PBS (ctl) and were challenged 15 days later with intranasal OVA only or OVA plus scrambled $Ca_v\beta$ ($Ca_v\beta S$) or OVA plus $Ca_v\beta$ antisense ($Ca_v\beta AS$) every day for 5 days. Dynamic lung resistance was measured using iox software (Emka). All data are expressed as the mean + SEM of 10 mice per group. **p<0.01

Supplemental Fig. 5: Functions of Th1-cells are not affected by gabapentin treatment.

Th1-cells were pre-incubated with gabapentin (1mM) for 72 hours before loading with the calcium probe Fura2-AM (**A-C**) or stimulated in the presence or not of gabapentin for additional 24 hours with anti-CD3/anti-CD28 antibodies (**D**). **A**) The fluorescence ratio at 340 and 380 nm excitation wavelengths was recorded at the single cell level before and after TCR stimulation. Results were expressed as the mean of at least 50 cells and bars represented the SEM. **B and C** showed respectively the area under the curve and the time of response for each cell, defined as in Fig. 3. Data were representative of 2 experiments. **D**) The cytokine content was measured.

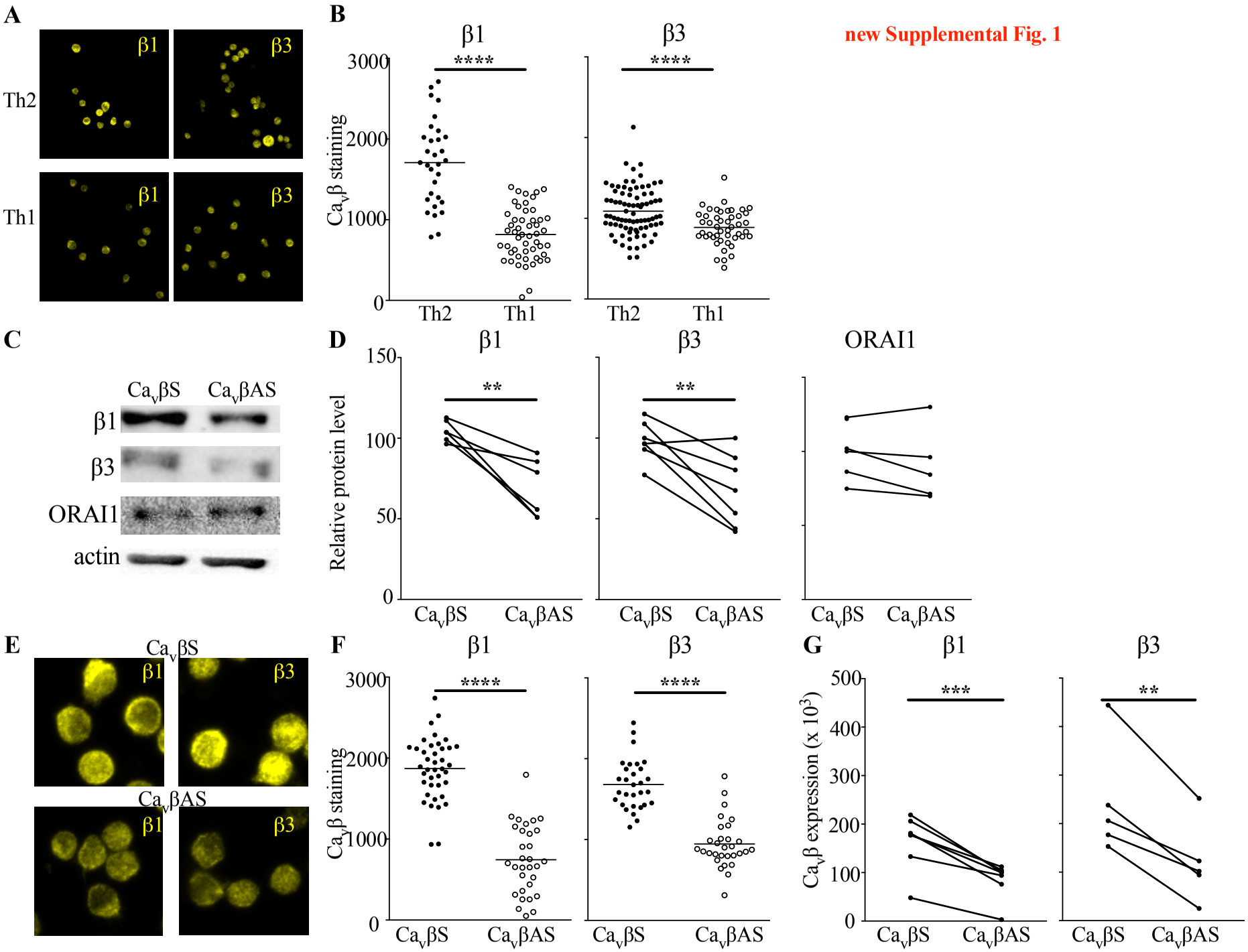
Supplemental Fig. 6: Gabapentin reduces the protein level of the α2δ2 auxiliary subunit in Th2-cells.

Th2-cells were incubated with gabapentin (1mM) for 96 hours and with or not bafilomycin (0.5 µM) the last 24 hours. Cells were stained with anti-α2δ2 or anti-$Ca_v1.2$ α1 Ab.

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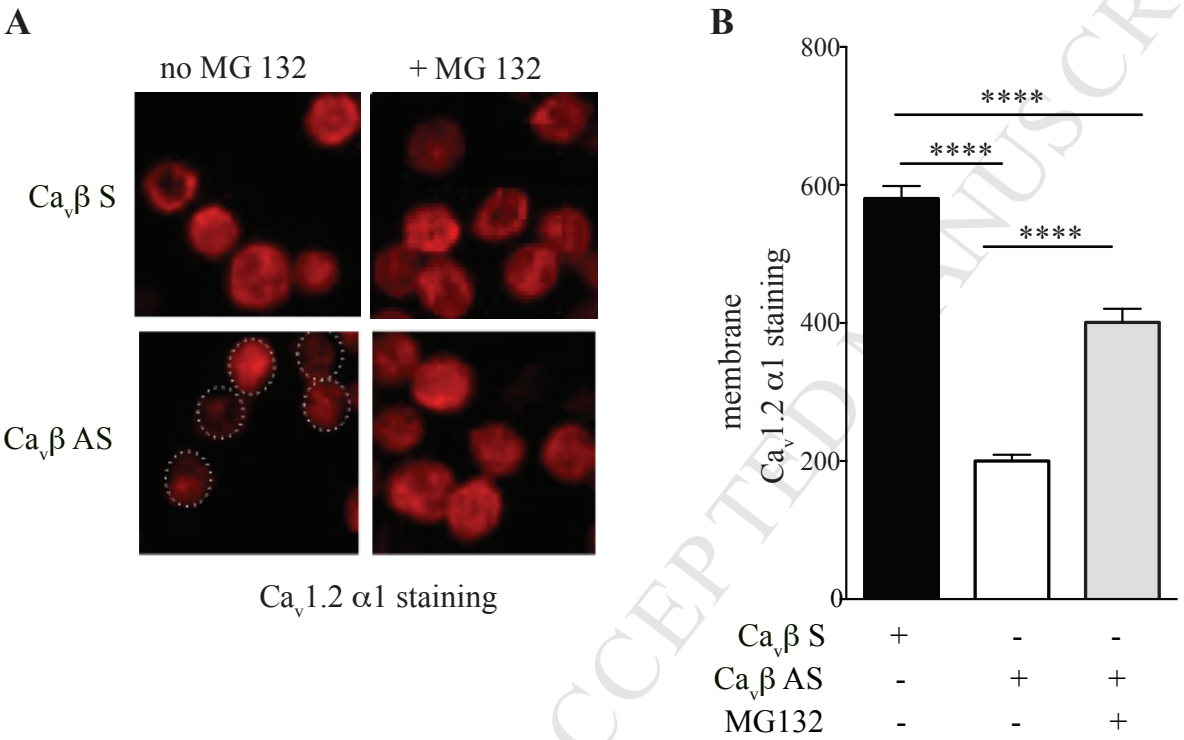
Immunofluorescence was summed all over the Z stack sections. One representative experiment out of 2 was shown. A white line surrounds each cell.

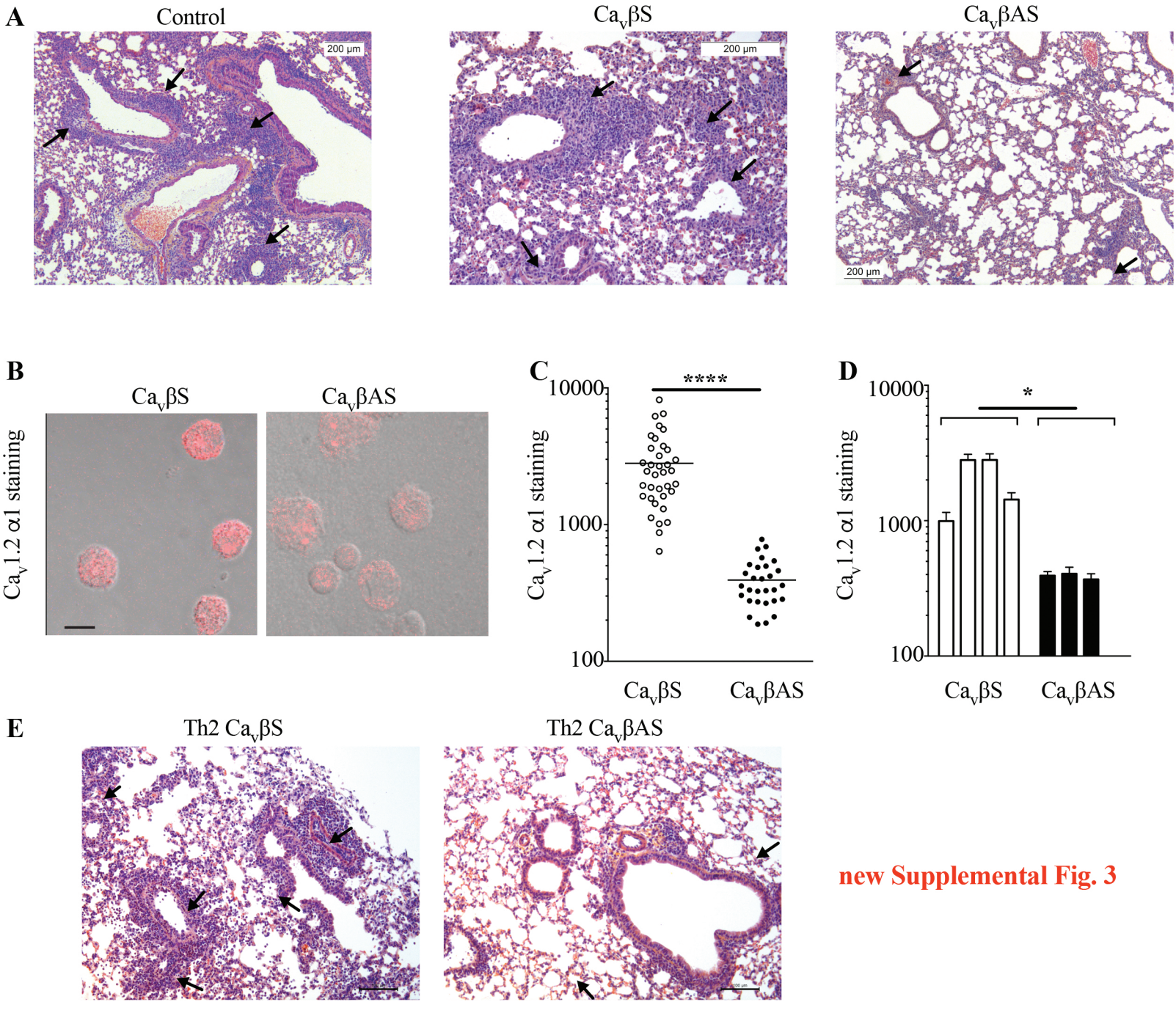
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A
β1
β3
Th2
Th1
B
β1
β3
****
****
3000
2000
1000
0
Ca$_v$β staining
Th2
Th1
Th2
Th1
new Supplemental Fig. 1
C
Ca$_v$βS
Ca$_v$βAS
β1
β3
ORAI1
actin
D
β1
β3
ORAI1
**
**
150
100
50
0
Relative protein level
Ca$_v$βS
Ca$_v$βAS
Ca$_v$βS
Ca$_v$βAS
Ca$_v$βS
Ca$_v$βAS
E
Ca$_v$βS
β1
β3
Ca$_v$βAS
β1
β3
F
β1
β3
****
****
3000
2000
1000
0
Ca$_v$β staining
Ca$_v$βS
Ca$_v$βAS
Ca$_v$βS
Ca$_v$βAS
G
β1
β3
***
**
500
400
300
200
100
0
Ca$_v$β expression (x 10^3)
Ca$_v$βS
Ca$_v$βAS
Ca$_v$βS
Ca$_v$βAS

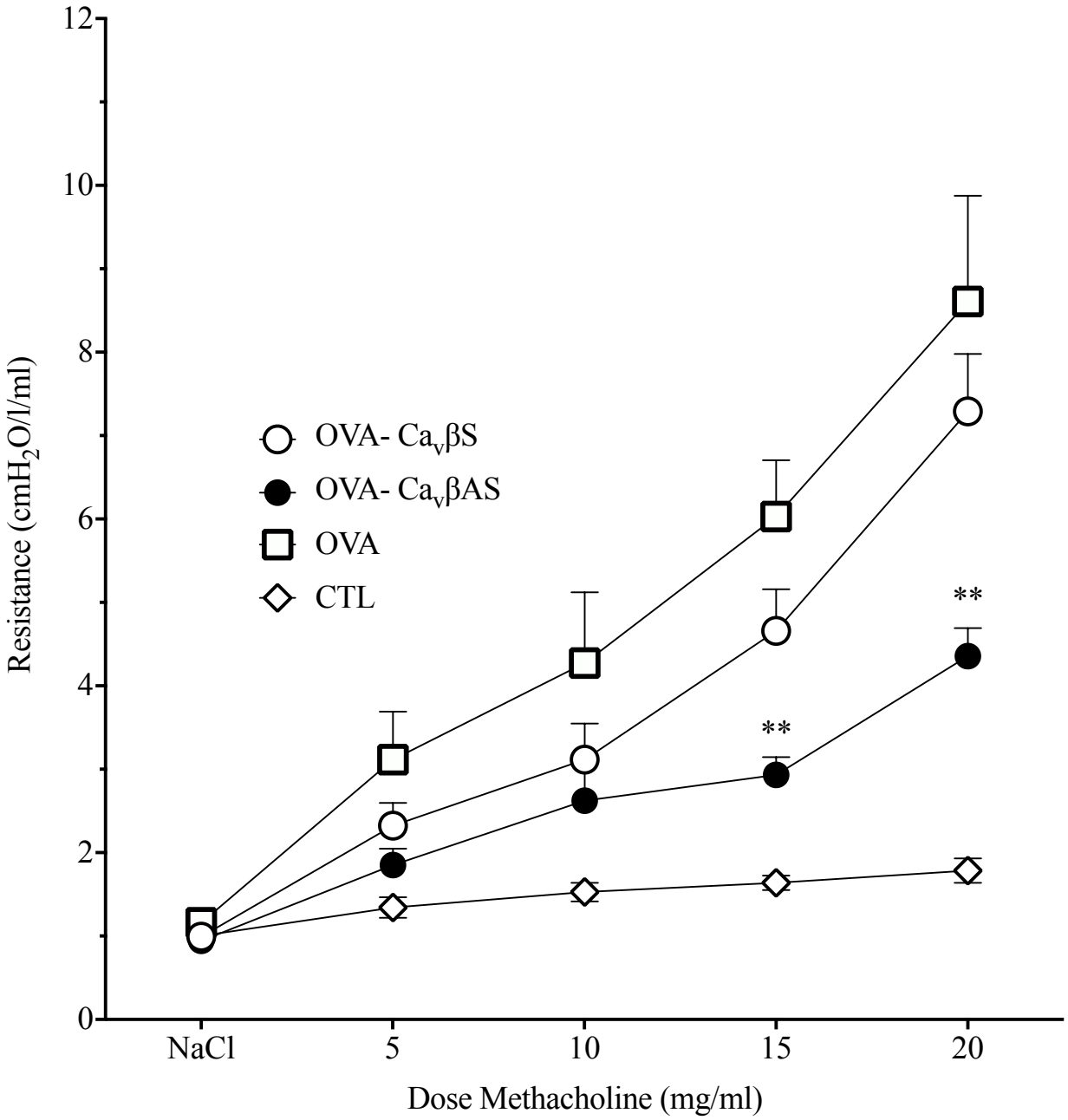
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Supplemental Figure 2





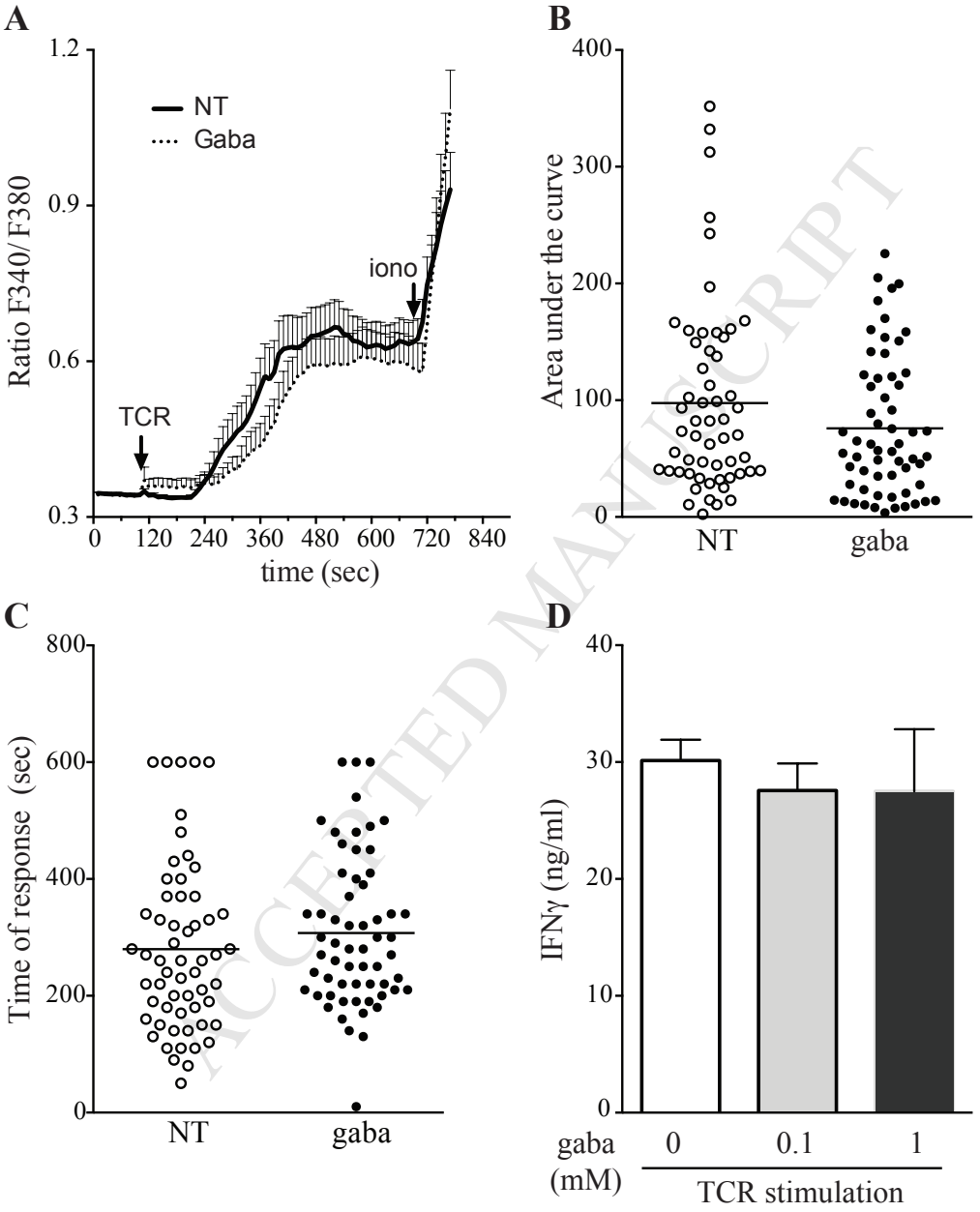
new Supplemental Fig. 3



new Supplemental Fig. 4

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Supplemental Figure 5



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Supplemental Figure 6

