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Article type : Original Article: Food Allergy and Gastrointestinal Disease

Separation of the Ca_v1.2-Ca_v1.3 calcium channel duo prevents type-2 allergic airway inflammation

Manuscript Acceptance Date: 16-May-2021

Short title

Ca_v1.2 and Ca_v1.3, a pas de deux in Th2 cells

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/all.14993](#)

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Acknowledgments

We acknowledge support from F. L'Faqihi, A.L. Iscache, and V. Duplan at the flow cytometry facility (INFINITY); from S. Allart and A. Canivet at the imaging facility (INFINITY); R. Romieu and P.E. Paulet at the immunomonitoring facility (INFINITY); Pr F. Brémont, F. Auriol and M. Mus from the Centre d'Investigation Clinique from Purpan Hospital; the animal facility staff (INSERM UMS06, Toulouse); S. Milia and T. Durand-Plavis from the histology facility (INSERM UMS06, Toulouse) and Dr S. Guerder that gave us Plat-E cells and Cre-IRES-GFP bicistronic vector. We thank Dr S. Laffont for critical reading of the manuscript. We would like to thank the Therassay platform for the use of respiratory equipment (Flexivent system) and Stallergenes Greer for kindly providing the HDM total extract. We also thank C. Cenac and A. Mandonnet for technical assistance. Dr. Giang reports personal fees from Ministère de l'éducation nationale, de la recherche et de la technologie, during the conduct of the study; Dr. Mars, Dr. Moreau and Dr. Mejia have nothing to disclose; Dr. Bouchaud and Dr. Magnan report grants from national agency and future investment under then program ANR-16-IDEX-0007, grants from Région Pays de la Loire, grants from Cluster LUNG innOvatiOn (LUNG O2) during the conduct of the study; Dr. Ronsin and Dr. Murphy have nothing to disclose; Dr. Striessnig reports grants from Austrian Science Fund (FWF, P27809) during the conduct of the study; Dr. Guery reports grants from The Foundation for Medical Research (FRM DEQ20180339187) during the conduct of the study; Dr. Pelletier has nothing to disclose; Dr. Savignac reports grants from French Society of Allergology, grants from Translational medical research grant (INSERM-Hospital, A11013BS) during the conduct of the study.

Abstract

Background: Voltage-gated calcium (Ca_v1) channels contribute to T-lymphocyte activation. $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels are expressed in Th2 cells but their respective roles are unknown, which is investigated herein. **Methods:** We generated mice deleted for $\text{Ca}_v1.2$ in T cells or $\text{Ca}_v1.3$ and analyzed TCR-driven signaling. In this line, we developed original fast calcium imaging to measure early elementary calcium events (ECE). We also tested the impact of $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ deletion in models of type-2 airway inflammation. Finally, we checked whether the expression of both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in T cells from asthmatic children correlates with Th2-cytokine expression. **Results:** we demonstrated non-redundant and synergistic functions of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in Th2 cells. Indeed, the deficiency of only one channel in Th2 cells triggers TCR-driven hypo-responsiveness with weakened tyrosine phosphorylation profile, a strong decrease in initial ECE and subsequent reduction in the global calcium response. Moreover, $\text{Ca}_v1.3$ has a particular role in calcium homeostasis. In accordance with the singular roles of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in Th2 cells, deficiency in either one of these channels was sufficient to inhibit cardinal features of type-2 airway inflammation. Furthermore, $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ must be co-expressed within the same CD4^+ T cell to trigger allergic airway inflammation. Accordingly with the concerted roles of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, the expression of both channels by activated CD4^+ T cells from asthmatic children was associated with increased Th2-cytokine transcription. **Conclusions:** Thus, $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ act as a duo and targeting only one of these channels would be efficient in allergy treatment.

Keywords

asthma, Calcium channels, Ca_v1 , cytokines, Th2 lymphocytes, signaling

Introduction

Intracellular Ca^{2+} store mobilization and Ca^{2+} entry into the cell is required for most T-cell functions but intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) needs to be finely tuned since Ca^{2+} overload triggers cell death. Classically, T-cell activation induces a cascade of phosphorylation, the generation of IP_3 releasing endoplasmic reticulum Ca^{2+} stores sensed by STIM1 Ca^{2+} sensor and ORAI Ca^{2+} channel opening¹. However, ORAI channels are not the only Ca^{2+} channels at play, as Ca_v1 channels initially studied in excitable cells are expressed and functional in T lymphocytes^{2,3}.

Ca_v1 channels are formed by the pore-forming $\alpha 1$ subunit ($\text{Ca}_v1.1$ to 1.4 , encoded by *CACNA1S*, *C*, *D*, *F* respectively) and auxiliary subunits (β and $\alpha 2\delta$)⁴, and their contribution in immune cells is attested as gene mutations in *CACNA1F*⁵ and *CACNA1C*⁶ are associated with immunodeficiency. In addition, a SNP located within the second intron of *CACNA1D* is associated with higher risk of developing bacterial meningitis⁷. The role of Ca_v1 channels in T lymphocytes has also been evidenced using mice knocked out for $\alpha 1$ and β subunits forming Ca_v1 channels⁸⁻¹⁰. The expression of Ca_v1 channels is plastic depending on the activation and differentiation status of T cells. $\text{Ca}_v1.4$ predominates in naive human T cells and its expression is reduced as soon as T cells are activated^{10,11}. Conversely, human and mouse Th2 cells selectively up-regulate $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels, while Th1 cells lose Ca_v1 channel expression¹¹⁻¹³. Using Ca_v1 channel blockers or antisense strategy, we previously reported that $\text{Ca}_v1.2/\text{Ca}_v1.3$ channels are necessary for TCR-driven Th2 functions and type 2-mediated airway inflammation¹¹⁻¹⁴.

Since both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are detected in Th2 cells, we investigated their respective roles in Th2-mediated allergic inflammation using knockout mice for each channel. We show that the absence of only one Ca_v1 channel is sufficient to alter very early calcium movements at the cell membrane of Th2 cells after TCR engagement. Although each channel has singular roles, they act as a duo to permit optimal calcium signaling and effector functions. Indeed, we provide evidence for a non-redundant role of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in Th2 cells in allergic airway inflammation. These results may be extrapolated to humans since efficient Th2-cytokine transcription is associated with the detection of both *CACNA1C* and *CACNA1D* mRNAs in activated CD4^+ T cells of asthmatic children.

Methods

Mice

Mice expressing floxed alleles of *Cacna1c* ($\text{Ca}_v1.2^{\text{fl/fl}}$)¹⁵ and $\text{Ca}_v1.3^{-/-}$ mice¹⁶ were given by GG. Murphy and J. Striessnig, respectively and were backcrossed onto the C57BL/6 background for 10 generations in our facility. Deletion of *Cacna1c* in T lymphocytes (hereafter called $\text{Ca}_v1.2^{\text{T-/-}}$), was obtained by crossing $\text{Ca}_v1.2^{\text{fl/fl}}$ mice with mice expressing the Cre under the CD4 promoter control¹⁷. C57BL/6 OT-II mice were crossed to $\text{Ca}_v1.2^{\text{T-/-}}$ or $\text{Ca}_v1.3^{-/-}$ mice to generate OVA-specific Th2 cells lacking $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$. Bone-marrow chimeras were achieved as previously described¹⁸. Mice were housed in specific pathogen-free conditions and handled according to the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Research (study approval APAFIS number 3816). Controls (hereafter called WT) were littermates from Ca_v1 -deficient mice and included $\text{Ca}_v1.2^{\text{fl/fl}}$ Cre-, $\text{Ca}_v1.2^{+/+}$ (Cre+ or Cre-) and $\text{Ca}_v1.3^{+/+}$ mice that behaved similarly in all the assays. Genomic *Cacna1c* and *Cacna1d* DNA and RNA were quantified as described in supplementary material.

T-cell cultures and transduction experiments

Naïve CD4^+ T cell purification, generation of OTII Th2 and Th1 cells, flow cytometry staining, phosphotyrosine staining and cytokine production were described in supplementary material. Supernatant of Plat-E cells transfected with Cre-IRES-GFP bicistronic vector or a plasmid encoding GFP only was used to transduce $\text{Ca}_v1.2^{\text{fl/fl}}$ Th2 cells stimulated by polyclonal activation with coated anti-CD3 and soluble anti-CD28 antibodies in Th2 conditions.

Calcium response analysis

Single-cell intracellular calcium measurements were done as previously described^{11,13} and in supplementary material. Total internal reflection fluorescence microscopy (TIRFM) is presently the best technique to image Ca^{2+} at the mouth of a calcium channel¹⁹. An excitation light with a critical angle is sent to the specimen, triggering a total internal reflection at the interface glass/medium. An evanescent wave is generated over less than 100 nm from the interface, sufficient to excite fluorophores located in this region. Th2 cells were loaded with 5 μM Fluo-5 AM washed and excited at 488 nm wavelength with Nikon 60X TIRF objective (NA 1.49). Emitted light was collected at 510 nm and recorded with a cooled (-80°C) back illuminated EMCCD camera (AndoriXon). Images were recorded every 2 ms for 10 s (5000 images) before stimulation and for 2 min (60000 images after stimulation with biotinylated anti-CD3 cross-linked with streptavidin at 10 $\mu\text{g/mL}$). More details and image processing were described in supplementary

material. In order to determine whether the deletion of Ca_v1 channels in Th2 cells modifies the opening frequencies, we determined them over a period of 4 s (2000 consecutive images) as soon as a channel opens (30 sec after stimulation), and results were expressed in events/second. All these results were robust because they were repeated in four (WT versus Ca_v1.2^{-/-}) and three (WT versus Ca_v1.3^{-/-}) *in vitro* Th2 differentiated independent experiments.

Models of allergic airway inflammation

Mice were sensitized by two intraperitoneal injections of OVA (100 µg) in alum (2 mg) at day 0 and 7. Fourteen days later, mice were given intranasal OVA (50 µg/day) in PBS for 5 days. For CD4⁺ T cell transfer experiments, we injected 5 x 10⁶ naive CD4⁺ T cells 24h before the first immunization. Inflammation induced by OTII Th2 cells was realized by intravenous injection in C57BL/6 mice (Janvier, Le Genest St. Isle, France) with 3 x 10⁶ Th2 cells, followed by intranasal OVA exposure (50 µg/day) for 5 days. As a control, OTII Th2 cells induced no inflammation in mice given PBS with a histological score inferior to 1 (not shown). Mice were analyzed 24h after the last challenge. House dust mice (HDM)-induced lung inflammation was induced as previously described¹⁸ and mice were sacrificed 6 days after the last challenge, except when mentioned otherwise. All parameters of airway inflammation were analyzed at time of sacrifice as previously described^{13,14,20,21} and in supplementary material.

Subjects and blood samples

The characteristics of asthmatic children were reported in **Table Suppl. 1** and samples were processed as described in supplementary material.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 7.0 (Graph-Pad Software, Inc, La Jolla, CA). The estimation analysis in Figure 1E was done in the R computing environment using *dabestr*²².

Results

***Cacna1c* or *Cacna1d* deletion does not affect T-cell development**

As expected, we observed that the genomic deletion of *Cacna1c* (encoding for Ca_v1.2) exon 2 was associated with reduced expression of the corresponding mRNA in Th2 cells from Ca_v1.2^{T-/-} mice (**Figure S1A-D**). Similar results were found for *Cacna1d* (encoding for Ca_v1.3) at genomic and mRNA levels in Th2 cells from Ca_v1.3^{-/-} mice (**Figure S1A-D**). *Orai1*, *Stim1* and *Stim2* expressions were similar irrespective of the genotype (**Figure S1E-F**).

Ca_v1.2 or Ca_v1.3 deficiency did not significantly impact the number and distribution of T cells in the thymus. In periphery, the numbers of CD4⁺, CD8⁺, regulatory and memory/naïve T cells were also similar (**Figure S2 and not shown**). Likewise, the numbers of CD4⁺ and CD8⁺ were not affected by the double deficiency (Ca_v1.2 and Ca_v1.3) in the periphery (**New Figure S3**). As Ca_v1.2 and Ca_v1.3 are selectively expressed in Th2 cells, we analyzed the frequency of these cells in the blood. The frequency of memory Th2 cells based on ST2 expression was similar in WT, Ca_v1.2^{T-/-} or Ca_v1.3^{-/-} mice (**New Figure S4**). This suggests that Ca_v1.2 and Ca_v1.3 are not required for Th2-differentiation in non-manipulated mice.

Ca_v1.2 and Ca_v1.3 calcium channels differentially regulate the TCR-driven initial calcium events in Th2 cells

In order to investigate the respective role of each channel in Th2-cell functions, we cultured Ca_v1.2^{T-/-} and Ca_v1.3^{-/-} OTII CD4⁺ T cells in Th2-polarizing conditions. The absence of Ca_v1.2 or Ca_v1.3 had no effect on Th2-cell differentiation regarding the expression of GATA-3 (**Figure S5A**) and the ability to produce Th2 cytokines (**Figure S5B**) in response to PMA/ionomycin (that bypasses TCR stimulation). However, the global [Ca²⁺]_i rise induced by cross-linked anti-CD3 mAb at the single-cell level was lower in Ca_v1.2^{T-/-} and in Ca_v1.3^{-/-} Th2 cells compared to WT Th2 cells, regarding the curve shape, the area under the curve and the time of response (**Figure 1A-B**). Ca_v1.2 and Ca_v1.3 did not contribute to intracellular calcium store mobilization (**Figure S6**). In Th1 cells, TCR engagement induced similar calcium responses regardless of their genotype (**Figure S7A-B**), demonstrating Ca_v1.2 and Ca_v1.3 play a selective role in Th2 cells.

In order to assess the roles of Ca_v1 channels in the very early calcium responses after TCR activation, we developed TIRFM to record rapid Ca²⁺ changes close to the plasma membrane. Thus, we imaged peaks corresponding to spatiotemporal single elementary calcium events (ECE) likely due to the activity of one or a cluster of Ca²⁺ channels¹⁹. In resting conditions, Th2 cells displayed few ECE and their numbers were similar regardless the genotype (**Figure 1C**). As early as 30 s following stimulation by anti-CD3-biotin/streptavidin complexes, the numbers of ECE increased in WT (p<0.0001, Wilcoxon test) but not in Ca_v1.2^{T-/-} or Ca_v1.3^{-/-} Th2

cells (**Figure 1C-D**). Stimulation of WT but not of $\text{Ca}_v1.2^{\text{T-/-}}$ or $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells also increased calcium channel opening frequency (**Figure 1E**). In $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells, TCR stimulation even led to a significant decrease relative to the resting condition (**Figure 1E**). The ECE opening frequencies were different in resting conditions and after stimulation between $\text{Ca}_v1.2^{\text{T-/-}}$ and $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells (**Figure 1E**). We then analyzed distribution of opening frequencies by ranking them in continuous subclasses (from lower than 7.5 to higher than 22.5 in bins of 2.5 events/s), (**Figure 1F**) and plotted the results as cumulative Gaussian distributions fitting to our data (**Figure 1G**). Stimulation of WT Th2 cells induced a displacement of the opening frequency classes toward higher frequencies with a significant shift of the cumulative Gaussian (**Figure 1F-G**). Stimulation of $\text{Ca}_v1.2^{\text{T-/-}}$ Th2 cells did not modify the class distribution (**Figure 1F-G**). In $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells, we observed a loss of low opening frequency classes in resting conditions compared to WT ($p<0.0001$) and $\text{Ca}_v1.2^{\text{T-/-}}$ ($p<0.0001$) Th2 cells suggesting that $\text{Ca}_v1.3$ shapes the type and/or the properties of the channels fluxing in baseline conditions. Moreover, TCR stimulation in $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells, induced a shift toward lower opening frequencies (**Figure 1F-G**), which is significantly different compared to stimulated WT or $\text{Ca}_v1.2^{\text{T-/-}}$ Th2 cells ($p<0.0001$), showing that $\text{Ca}_v1.3$ also impacts TCR-dependent calcium responses (**Figure 1F-G**) and suggesting that the absence of $\text{Ca}_v1.3$ brakes TCR signaling.

$\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ channel deficiency in Th2 cells renders them hypo-functional with a decreased ability to cause allergic inflammation

Protein phosphorylation is one of the main post-translational modifications driving T-cell activation^{23,24}. Very few calcium ions entering the cell can modify the cell membrane properties and promote early TCR signaling events^{25,26}, which prompted us to investigate the impact of Ca_v1 deficiency in TCR-driven tyrosine phosphorylation. The profile of tyrosine phosphorylation was similar in the groups before stimulation (**Figure 2A and New Figure S8A**), while the overall protein tyrosine phosphorylation was significantly reduced in Th2 cells lacking $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ channels after TCR stimulation (**Figure 2A**). The decreased calcium response and proximal signaling in $\text{Ca}_v1.2$ - or $\text{Ca}_v1.3$ -deficient Th2 cells was associated with a partial but statistically significant impairment of TCR-mediated IL-5 and IL-13 production (**Figure 2B and New Figure S8B**). On the contrary, Th1 cells produced the same amount of IFN γ irrespective of the genotype (**Figure S7C**) showing that effects of Ca_v1 channel-deficiencies on cytokine production were selective of Th2 cells.

We took advantage of the Cre recombinase-induced deletion model in $\text{Ca}_v1.2^{\text{fl/fl}}$ mice to perform an acute deletion of *Cacna1c* in already differentiated Th2-cells. We observed a deletion of the targeted locus of *Cacna1c* gene at DNA level, which correlated with reduced mRNA

expression (**Figure 2C-D**) and marked reduction of Th2-cytokine production in Cre-GFP⁺ Th2 cells as compared to GFP transduced control cells (**Figure 2E**). This decreased cytokine production associated with acute deletion of Ca_v1.2 in differentiated Th2 cells was more marked than in Ca_v1.2^{T-/-} Th2 cells suggesting that compensatory mechanisms take place during T-cell ontogeny when Ca_v1.2 is constitutively absent.

Finally, Ca_v1-deficient OTII Th2 lymphocytes caused less lung inflammation and mucus production than WT Th2 lymphocytes, when injected in WT recipients that were given intranasal OVA (**Figure 2G-I**) although they migrated as efficiently as WT OTII Th2 cells into the lungs (**Figure 2F-H**). This suggests that the reduction in allergic airway inflammation was due to the altered effector functions of adoptively transferred Ca_v1.2- or Ca_v1.3-deficient Th2 cells.

Specific deletion of *Cacna1c* in T lymphocytes dampens type-2 mediated airway diseases

We exposed WT and Ca_v1.2^{T-/-} mice to HDM, a known aeroallergen (**Figure 3A**) and showed that Ca_v1.2 deficiency in T cells was beneficial with reduced numbers of inflammatory cells in the BALF, histological score, mucus production and AHR (**Figure 3B-D**). The trend of decrease of Th2 cytokines, at both transcript expression in the lungs (**Figure 3E**) and production by lung draining lymph node cells after HDM-recall *in vitro* (**New Figure 3F**), as well as the lower total serum IgE (**Figure 3G**) and Derf1-specific IgE (**Figure 3H**) concentrations were supportive of the role of Ca_v1.2 channels in Th2 effector functions during airway inflammation. To assess whether Ca_v1.2 deficiency affected the course of the disease, we sacrificed mice earlier after the last challenge (day 3 instead of day 6). We also observed lower airway inflammation, as attested by the decreased BAL cell numbers and histological score (**New Figure S9**). Ca_v1.2^{T-/-} mice were also protected against airway inflammation induced by OVA immunization and OVA challenge (**Figure S10**). Altogether, these data demonstrate that Ca_v1.2-specific deletion in T lymphocytes hinders the development of Th2-mediated airway inflammation.

***Cacna1d* deletion in the hematopoietic compartment restrains the development of type-2 mediated airway inflammation in mice**

In order to demonstrate that selective deletion of Ca_v1.3 in hematopoietic compartment was sufficient to dampen airway inflammation, we generated irradiated bone marrow chimeras. Chimeric mice with a Ca_v1.3-deficient hematopoietic compartment were protected from airway inflammation, as the numbers of BALF inflammatory cells, the histological score, the mucus production and the AHR were reduced compared to the mice reconstituted with WT bone marrow cells (**Figure 4A-C**). The Th2-response was also decreased in Ca_v1.3-deficient compared to WT chimeras, as indicated by the curtailed *Il5* and *Il13* mRNA expression in lung tissues (**Figure 4D**),

the reduced Th2-cytokine production after HDM recall of lung draining lymph node cells (**New Figure 4E**) and the lower total serum IgE and Derf1-specific IgE concentrations (**Figure 4F-G**). Serum IgE concentration was statistically lower in $\text{Ca}_v1.3^{-/-}$ than in WT mice each time point and even before immunization (**Figure 4F**). Similar results were obtained in the OVA model (**New Figure S11**). Allergic airway inflammation was also decreased in the HDM model when BAL and lung histology were analyzed 3 days after the last challenge (**New Figure S9**). Thus, deletion of $\text{Ca}_v1.3$ in the hematopoietic compartment is sufficient to impede all cardinal features of allergic airway inflammation.

We showed non-redundant roles of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in models of Th2-dependent airway inflammation. We then analyzed chimeric mice reconstituted with bone marrows from mice lacking both $\text{Ca}_v1.2$ in T cells and $\text{Ca}_v1.3$. We showed these mice were protected from airway inflammation induced by HDM or OVA, to the same extent as those deficient for only one channel (**New Figure S12**).

T-cell intrinsic $\text{Ca}_v1.3$ defect is sufficient to alleviate allergic airway inflammation

In order to determine whether the protective action of $\text{Ca}_v1.3$ deficiency was T-cell intrinsic, we performed complementation studies as previously described²⁷. We injected naive WT CD4^+ T cells in $\text{Ca}_v1.3^{-/-}$ chimeras, before immunization with OVA to assess whether the expression of $\text{Ca}_v1.3$ restricted to the injected WT T cells was sufficient to induce airway inflammation. Injection of WT CD4^+ T cells, but not of $\text{Ca}_v1.3^{-/-}$ T cells, restored the numbers of cells in the BALF (eosinophilia), the lung histological score and mucus production in $\text{Ca}_v1.3^{-/-}$, to the same extent as in WT recipients (**Figure 5A-B**). Therefore, intrinsic expression of $\text{Ca}_v1.3$ channel in CD4^+ T cells is sufficient to promote Th2-mediated lung inflammation.

$\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ must be co-expressed within the same CD4^+ T cells for the development of allergic airway inflammation

Our results at the single-cell level showed that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels have non-redundant roles and that these channels have distinct effects on early calcium regulation in Th2 cells suggesting these channels cooperate within the same Th2 cell. Thus, we expected the expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ on different T cells would not allow the development of airway inflammation. To test this, we injected naïve $\text{Ca}_v1.2^{\text{T-/-}}$ CD4^+ T cells (that express $\text{Ca}_v1.3$ but not $\text{Ca}_v1.2$) into $\text{Ca}_v1.3^{-/-}$ chimeric mice (the recipient T cells express only $\text{Ca}_v1.2$) prior to OVA immunization and intranasal OVA challenge. Actually these mice did not develop airway inflammation (**Figure 5A-B**), while $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels are expressed but on different T cells. We also did the reciprocal experiment in $\text{Ca}_v1.2^{\text{T-/-}}$ chimeric mice. As non-chimeric $\text{Ca}_v1.2^{\text{T-/-}}$

mice (Figure 3), $Ca_v1.2^{T-/-}$ chimeric mice were protected from OVA-induced inflammation and the transfer of WT naive $CD4^+$ T cells prior immunization restores airway inflammation (**Figure 5C-D**). However, the injection of $Ca_v1.3$ -deficient naive $CD4^+$ T cells ($Ca_v1.2^+Ca_v1.3^-$) into $Ca_v1.2$ -deficient mice ($Ca_v1.2^-Ca_v1.3^+$) failed to induce airway inflammation (**Figure 5C-D**). Altogether, these results demonstrate that $Ca_v1.2$ and $Ca_v1.3$ co-expression within the same $CD4^+$ T cell is required to induce allergic airway inflammation.

The expression of both $Ca_v1.2$ and $Ca_v1.3$ channels in T cells is associated with higher Th2-cytokine transcription in asthmatic children

Following T-cell stimulation, we detected *CACNA1C* transcripts with similar frequencies in asthmatic and controls (**Figure 6A**; $p=0.35$, χ^2 test), but *CACNA1D* transcripts were detected more frequently in asthmatic than in control children (**Figure 6A**; $p=0.0044$, χ^2 test). The distribution of subjects that express *CACNA1C* and/or *CACNA1D* was similar between asthmatics and controls (**Figure 6A**) before stimulation, even if the proportion of subjects with double expression of *CACNA1C* and *CACNA1D* was superior in asthmatics (54% in asthmatic children versus 36% in controls, **New Figure 6B**). Thus, after TCR-stimulation, *CACNA1C* and/or *CACNA1D* were detected in all the $CD4^+$ samples from asthmatic children but none of them was found in 15.4% of the control donors (**New Figure 6B**). Moreover, both channels were detected in 69.2% of patients versus 30.8% in controls in stimulated conditions (**New Figure 6B**). We then investigated whether the detection of both channels correlated with Th2 cytokine expression in human $CD4^+$ T cells. We observed higher expression of *IL4* and *IL13* transcripts in TCR-stimulated $CD4^+$ T cells from asthmatic children in which both calcium channels (both: *CACNA1C*⁺*CACNA1D*⁺) were detected than in those expressing only one channel (single: *CACNA1C*⁺*CACNA1D*⁻ or *CACNA1C*⁻*CACNA1D*⁺), (**New Figure 6C**). This correlation was consistent with our results in mice and suggested that both channels were also required for optimal type-2 cytokine production in human $CD4^+$ T cells. Only few controls expressed both *CACNA1C* and *CACNA1D* channels, but the same trend was observed regarding the ability to express Th2 cytokines after stimulation.

Discussion

Our study supports the original concept that concerted actions of Ca_v1.2 and Ca_v1.3 are required for optimal Th2-functions and the development of airway allergic inflammation. We show that each channel exerts specific functions in TCR-dependent early ECE in Th2 cells and that both Ca_v1.2 and Ca_v1.3 channels must be expressed in the same CD4⁺ T cell to induce allergic airway inflammation. Finally, the correlation of the detection of both these channels with high Th2-cytokine gene expression in CD4⁺ T cells from asthmatic children further supports a synergistic action of Ca_v1.2 and Ca_v1.3 required for full human Th2-cell activation.

Calcium signaling is necessary for T-cell development in the thymus^{28,29} but Orai channels are not involved³⁰. The role of Ca_v channels is suspected regarding the partial effect of Ca_v1.4 channel¹⁰ and auxiliary Ca_vβ₃³¹ subunit deficiency. Normal T-cell development in Ca_v1.2- or Ca_v1.3-deficient mice precludes a role of these channels in T-cell ontogeny, which is also supported by normal T-cell development in the absence of both Ca_v1.2 and Ca_v1.3.

The selective deletion of Ca_v1.2 in T cells or Ca_v1.3 in the hematopoietic compartment is sufficient to deeply reduce type 2-mediated airway inflammation and hyper-reactivity in two active models of allergic airway inflammation. In both models of Ca_v1-deficiency, airway inflammation was only restored by injection of naive WT CD4⁺ T cells, but not by CD4⁺ T cells lacking either Ca_v1.2 or Ca_v1.3 demonstrating that T-cell intrinsic Ca_v1.2 and Ca_v1.3 expression within the same cell was required to promote the development of allergic airway inflammation. This supports the notion that synergistic actions of Ca_v1.2 and Ca_v1.3 are necessary for optimal Th2-cell functions. Such concerted effect of these channels has never been described before in any cell type. Numerous excitable cells co-express Ca_v1.2 and Ca_v1.3³² but their respective roles remain elusive due to the lack of Ca_v1.3- or Ca_v1.2-selective drugs³³, of antibodies specific for each channel³⁴ and some compensatory effects due to other Ca_v channels observed in knockout Ca_v1.2 or Ca_v1.3 mice³⁵.

Serum IgE concentration, that depends on Th2 cells, is lower in the absence of Ca_v1.2 or Ca_v1.3 channels, even in non-immunized animals, attesting to a physiological role of these channels in steady state Th2-responses. IL5 and IL13 production is decreased in Ca_v1-deficient mice compared to WT mice, however, we do not exclude the absence of Ca_v1 in Th2 cells could also alter the interactions between Th2 and its environment in the lungs, limiting inflammation development.

Ca_v1.2^{T-/-} and Ca_v1.3^{T-/-} Th2 cells present a lower rise of [Ca²⁺]_i observed at single-cell level compared to WT Th2 cells. The residual calcium response in Ca_v1.2^{T-/-} or Ca_v1.3^{T-/-} Th2 cells could be due to other calcium channels such as STIM/ORAI. Contrary to Ca_v1.4 channels that would regulate the intracellular Ca²⁺ stores in naive T lymphocytes¹⁰, Ca_v1.2 or Ca_v1.3 deficiency does

not modify the functionality of STIM/ORAI since the response to thapsigargin or TCR-induced intracellular calcium store mobilization is normal. The deficiency of only one of the two channels results in Th2 hyporesponsiveness evidenced by reduced global tyrosine phosphorylation. This is coherent with the fact that differential sensitivity of protein kinase and protein phosphatase to Ca^{2+} increase conditions synapse activity in neurons³⁶. In addition, calcium signaling is not only downstream of T-cell activation³⁷ but very few calcium ions entering the cell modify the cell membrane properties and promote TCR signaling events^{25,26}. In this line, $\text{Ca}_v1.4$ was shown to associate with Lck and Vav in CD8^+ T cells⁹ suggesting that Ca_v1 channels might be direct partners of TCR signaling. We show a calcium influx at the vicinity of the plasma membrane early after TCR stimulation in WT Th2 cells, associated with both more opened channels and higher opening frequencies. This is not the case in $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ -deficient Th2 cells, indicating that each channel is crucial to trigger the early ECE preceding global calcium influx. In baseline conditions, only $\text{Ca}_v1.3$ deficiency affects calcium fluxes near the cell membrane. Since TIRFM does not provide information on the molecular identity of the channels, we can only suggest that $\text{Ca}_v1.3$ channels have a low opening frequency or that they impact the type or properties of calcium channels involved in basal calcium influxes. These data demonstrate that $\text{Ca}_v1.3$, but not $\text{Ca}_v1.2$, channels shape the baseline calcium homeostasis in Th2 cells. Moreover, the opening frequency distribution in $\text{Ca}_v1.3^{-/-}$ Th2 cells after TCR stimulation is marked by an enrichment in low frequency opening classes compared to WT or $\text{Ca}_v1.2^{\text{T-/-}}$ Th2 cells, suggesting that $\text{Ca}_v1.3$ channels remove a brake counteracting the initiation of calcium influx.

Due to the requirement of both channels within the same lymphocyte to induce inflammation and the different impact of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ on ECE, we favor the hypothesis of cooperative actions of these Ca_v1 channels. Whether and how $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ can cooperate is still unknown. Activated $\text{Ca}_v1.2$ can facilitate neighbouring $\text{Ca}_v1.2$ channel openings^{38,39} and similar reports are described with $\text{Ca}_v1.3$ ⁴⁰. $\text{Ca}_v1.3$ activates at about 10–20 mV more negative potentials than $\text{Ca}_v1.2$ channels^{34,41}, and has an activation threshold near the resting membrane potential. Thus, it is conceivable that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ act sequentially with $\text{Ca}_v1.3$ activation resulting in early Ca^{2+} entry inducing local membrane depolarization, facilitating the opening of neighboring $\text{Ca}_v1.2$ channels. It is also possible that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels act by recruiting distinct sets of adapters, kinases and other channels, as reported in hippocampal neurons⁴², that would generate a functional signaling platform upon TCR activation leading to full Th2-functions.

Our results in mice are also in accordance with data from circulating blood CD4^+ T cells from asthmatic children since both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels were detected more frequently in asthmatic compared to control children and the expression of both channels was associated with higher Th2-cytokine gene expression compared to samples in which only one channel was

detected. TCR activation upregulates Ca_v1 expression (particularly *CACNA1D* expression) which may unmask the bias toward Th2-committed cells in asthmatics. Noteworthy, Ca_v1.2 is often detected in CD4⁺ T cells from both healthy and asthmatics while Ca_v1.3 discriminates asthmatics upon TCR activation suggesting that Ca_v1.3 is the limiting factor required for proper Th2 functions.

Antibodies directed against one or the other Th2 cytokine (or their receptor) are currently used in asthmatic patients with some benefits but they do not treat all the features of pathology⁴³. Targeting Ca_v1 channels in the treatment of allergy is of interest since they are implicated in the elementary calcium events and control Th2-cell biology. A lot of effort is currently devoted to the development of drugs targeting one or the other Ca_v1 channels especially Ca_v1.3 to improve neuropsychiatric diseases. Such an approach could be useful in allergic diseases since affecting only one Ca_v1 channel might inhibit the pathogenic potential of Th2 cells limiting potential adverse side effects and sparing Th1-cell functions, required to fight pathogens.

Declaration of interest

The authors declare no competing interests

Author contributions

NG, MMA and MS performed all the experiments. JEM designed PCR primers and performed the violin and estimation plots. GB and AM performed and interpreted airway hyperresponsiveness experiments. JS and GGM gave the mice with deletion of *Cacna1d* and mice with floxed *Cacna1c*, respectively and criticized the results. MMo and LP performed TIRF experiments. MMo and BR developed software for TIRFM analysis and MMo analyzed the number of open channels and their opening frequency. JCG contributed to the design of experiments and criticized the results. NG, LP and MS did the experimental design, did the figures and wrote the paper. MS supervised the work.

References

- 1 Feske, S. *et al.* A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* **441**, 179-185 (2006).
- 2 Pelletier, L. & Savignac, M. Involvement of ion channels in allergy. *Curr Opin Immunol* **52**, 60-67, doi:10.1016/j.coi.2018.04.006 (2018).
- 3 Feske, S., Skolnik, E. Y. & Prakriya, M. Ion channels and transporters in lymphocyte function and immunity. *Nat Rev Immunol* **12**, 532-547, doi:10.1038/nri3233 (2012).
- 4 Alexander, S. P. H. *et al.* THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Ion channels. *Br J Pharmacol* **176 Suppl 1**, S142-S228, doi:10.1111/bph.14749 (2019).
- 5 Fenninger, F. *et al.* Mutation of an L-Type Calcium Channel Gene Leads to T Lymphocyte Dysfunction. *Front Immunol* **10**, 2473, doi:10.3389/fimmu.2019.02473 (2019).
- 6 Splawski, I. *et al.* Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* **119**, 19-31, doi:10.1016/j.cell.2004.09.011 (2004).
- 7 Liao, P. & Soong, T. W. CaV1.2 channelopathies: from arrhythmias to autism, bipolar disorder, and immunodeficiency. *Pflugers Arch* **460**, 353-359, doi:10.1007/s00424-009-0753-0 (2010).
- 8 Badou, A. *et al.* Critical role for the beta regulatory subunits of Cav channels in T lymphocyte function. *Proc Natl Acad Sci U S A* **103**, 15529-15534 (2006).
- 9 Jha, M. K. *et al.* Defective survival of naive CD8⁺ T lymphocytes in the absence of the beta3 regulatory subunit of voltage-gated calcium channels. *Nat Immunol* **10**, 1275-1282 (2009).
- 10 Omilusik, K. *et al.* The Ca(v)1.4 calcium channel is a critical regulator of T cell receptor signaling and naive T cell homeostasis. *Immunity* **35**, 349-360 (2011).
- 11 Robert, V. *et al.* Protein kinase C-dependent activation of CaV1.2 channels selectively controls human TH2-lymphocyte functions. *J Allergy Clin Immunol* **133**, 1175-1183, doi:10.1016/j.jaci.2013.10.038 (2014).
- 12 Gomes, B. *et al.* Calcium channel blocker prevents T helper type 2 cell-mediated airway inflammation. *Am J Respir Crit Care Med* **175**, 1117-1124 (2007).
- 13 Cabral, M. D. *et al.* Knocking down Cav1 calcium channels implicated in Th2 cell activation prevents experimental asthma. *Am J Respir Crit Care Med* **181**, 1310-1317 (2010).
- 14 Rosa, N. *et al.* The beta and alpha2delta auxiliary subunits of voltage-gated calcium channel 1 (Cav1) are required for TH2 lymphocyte function and acute allergic airway inflammation. *J Allergy Clin Immunol* **142**, 892-903 e898, doi:10.1016/j.jaci.2017.09.045 (2018).
- 15 White, J. A. *et al.* Conditional forebrain deletion of the L-type calcium channel Ca V 1.2 disrupts remote spatial memories in mice. *Learn Mem* **15**, 1-5 (2008).
- 16 Platzer, J. *et al.* Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca²⁺ channels. *Cell* **102**, 89-97 (2000).
- 17 Sawada, S., Scarborough, J. D., Killeen, N. & Littman, D. R. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. *Cell* **77**, 917-929, doi:10.1016/0092-8674(94)90140-6 (1994).

- 18 Laffont, S. *et al.* Androgen signaling negatively controls group 2 innate lymphoid cells. *J Exp Med* **214**, 1581-1592, doi:10.1084/jem.20161807 (2017).
- 19 Demuro, A. & Parker, I. "Optical patch-clamping": single-channel recording by imaging Ca²⁺ flux through individual muscle acetylcholine receptor channels. *J Gen Physiol* **126**, 179-192, doi:10.1085/jgp.200509331 (2005).
- 20 Bouchaud, G. *et al.* Prevention of allergic asthma through Der p 2 peptide vaccination. *J Allergy Clin Immunol* **136**, 197-200 e191, doi:10.1016/j.jaci.2014.12.1938 (2015).
- 21 Bihouee, T. *et al.* Food allergy enhances allergic asthma in mice. *Respir Res* **15**, 142, doi:10.1186/s12931-014-0142-x (2014).
- 22 Ho, J., Tumkaya, T., Aryal, S., Choi, H. & Claridge-Chang, A. Moving beyond P values: data analysis with estimation graphics. *Nat Methods* **16**, 565-566, doi:10.1038/s41592-019-0470-3 (2019).
- 23 Locard-Paulet, M. *et al.* LymphoAtlas: a dynamic and integrated phosphoproteomic resource of TCR signaling in primary T cells reveals ITSN2 as a regulator of effector functions. *Mol Syst Biol* **16**, e9524, doi:10.15252/msb.20209524 (2020).
- 24 Castro-Sanchez, P., Teagle, A. R., Prade, S. & Zamoyska, R. Modulation of TCR Signaling by Tyrosine Phosphatases: From Autoimmunity to Immunotherapy. *Frontiers in cell and developmental biology* **8**, 608747, doi:10.3389/fcell.2020.608747 (2020).
- 25 Shi, X. *et al.* Ca²⁺ regulates T-cell receptor activation by modulating the charge property of lipids. *Nature* **493**, 111-115, doi:10.1038/nature11699 (2013).
- 26 Yi, J., Balagopalan, L., Nguyen, T., McIntire, K. M. & Samelson, L. E. TCR microclusters form spatially segregated domains and sequentially assemble in calcium-dependent kinetic steps. *Nat Commun* **10**, 277, doi:10.1038/s41467-018-08064-2 (2019).
- 27 Mionnet, C. *et al.* CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung. *Nature medicine* **16**, 1305-1312, doi:10.1038/nm.2253 (2010).
- 28 Oukka, M. *et al.* The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* **9**, 295-304, doi:10.1016/s1074-7613(00)80612-3 (1998).
- 29 Aifantis, I., Gounari, F., Scorrano, L., Borowski, C. & von Boehmer, H. Constitutive pre-TCR signaling promotes differentiation through Ca²⁺ mobilization and activation of NF-kappaB and NFAT. *Nat Immunol* **2**, 403-409, doi:10.1038/87704 (2001).
- 30 Feske, S. ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca²⁺ entry in the immune system and beyond. *Immunol Rev* **231**, 189-209, doi:10.1111/j.1600-065X.2009.00818.x (2009).
- 31 Jha, A. *et al.* Essential roles for Cavbeta2 and Cav1 channels in thymocyte development and T cell homeostasis. *Sci Signal* **8**, ra103, doi:10.1126/scisignal.aac7538 (2015).
- 32 Zamponi, G. W., Striessnig, J., Koschak, A. & Dolphin, A. C. The Physiology, Pathology, and Pharmacology of Voltage-Gated Calcium Channels and Their Future Therapeutic Potential. *Pharmacol Rev* **67**, 821-870, doi:10.1124/pr.114.009654 (2015).
- 33 Ortner, N. J. & Striessnig, J. L-type calcium channels as drug targets in CNS disorders. *Channels (Austin)* **10**, 7-13, doi:10.1080/19336950.2015.1048936 (2016).

- 34 Liss, B. & Striessnig, J. The Potential of L-Type Calcium Channels as a Drug Target for Neuroprotective Therapy in Parkinson's Disease. *Annu Rev Pharmacol Toxicol* **59**, 263-289, doi:10.1146/annurev-pharmtox-010818-021214 (2019).
- 35 Poetschke, C. *et al.* Compensatory T-type Ca²⁺ channel activity alters D2-autoreceptor responses of Substantia nigra dopamine neurons from Cav1.3 L-type Ca²⁺ channel KO mice. *Sci Rep* **5**, 13688, doi:10.1038/srep13688 (2015).
- 36 Lee, H. K. Synaptic plasticity and phosphorylation. *Pharmacology & therapeutics* **112**, 810-832, doi:10.1016/j.pharmthera.2006.06.003 (2006).
- 37 Feske, S., Giltman, J., Dolmetsch, R., Staudt, L. M. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol* **2**, 316-324 (2001).
- 38 Dixon, R. E. *et al.* Graded Ca(2+)/calmodulin-dependent coupling of voltage-gated CaV1.2 channels. *Elife* **4**, doi:10.7554/eLife.05608 (2015).
- 39 Ghosh, D. *et al.* Dynamic L-type CaV1.2 channel trafficking facilitates CaV1.2 clustering and cooperative gating. *Biochim Biophys Acta Mol Cell Res* **1865**, 1341-1355, doi:10.1016/j.bbamcr.2018.06.013 (2018).
- 40 Moreno, C. M. *et al.* Ca(2+) entry into neurons is facilitated by cooperative gating of clustered CaV1.3 channels. *Elife* **5**, doi:10.7554/eLife.15744 (2016).
- 41 Lipscombe, D., Helton, T. D. & Xu, W. L-type calcium channels: the low down. *J Neurophysiol* **92**, 2633-2641, doi:10.1152/jn.00486.2004 (2004).
- 42 Zhang, H. *et al.* Ca_v1.2 and Ca_v1.3 neuronal L-type calcium channels: differential targeting and signaling to pCREB. *Eur J Neurosci* **23**, 2297-2310, doi:10.1111/j.1460-9568.2006.04734.x (2006).
- 43 Peters, M. C. & Wenzel, S. E. Intersection of biology and therapeutics: type 2 targeted therapeutics for adult asthma. *Lancet* **395**, 371-383, doi:10.1016/S0140-6736(19)33005-3 (2020).

Figure legends

Figure 1: Original and singular roles of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels in initial plasma membrane calcium events upon TCR activation

WT, $\text{Ca}_v1.2^{\text{T-/-}}$ and $\text{Ca}_v1.3^{\text{-/-}}$ CD4^+ T cells were cultured in Th2-polarizing conditions. **(A-B)** Cells were loaded with Fura-2 AM. The fluorescence ratio at 340 and 380 nm excitation wavelengths was recorded at the single-cell level before (F_0) and after stimulation (F) with cross-linked anti-CD3 mAb (anti-CD3). Ionomycin (Iono) was added at the end of the experiment. Results are expressed as the mean of $F/F_0 \pm \text{SEM}$ (130 to 256 cells per genotype) and are representative of differentiated Th2 cells from 4-5 mice per genotype. The area under the curve between the time of stimulation and the time of ionomycin addition was depicted. Data from all the analyzed cells were pooled. The time of response corresponds to the delay between stimulation and the first time point showing an increase in fluorescence ratio over the mean ratio recorded before stimulation + 1 SD. Results from all the responder cells were pooled. Results were compared by unpaired Student's t-test. **(C-G)** Cells were loaded with Fluo5-AM and stimulated or not with cross-linked anti-CD3 mAb. **(C)** Representative 3-D TIRFM images of elementary Ca^{2+} events (ECE) integrated during 10 ms on a surface of $3 \mu\text{m} \times 3 \mu\text{m}$ of Th2 lymphocyte membrane before (Unstimulated) and after 30 s of stimulation (Stimulated). The inserts show the fluorescence on the cell surface analyzed by TIRFM. **(D)** Results are expressed as the number of ECE after stimulation relative to before stimulation. Each point corresponds to one cell and results were pooled from 4 mice per genotype. Results were compared with Mann-Whitney test. **(E)** Estimation plot of representative measurements of calcium event opening frequencies before and after stimulation (Stim). Top, raw data groups with the median and interquartile range (IQR) shown as a gapped line. Each point represents one calcium event, and 5 ECE/cell were analyzed on $n=20$ WT, $n=13$ $\text{Ca}_v1.2^{\text{T-/-}}$ and $n=9$ $\text{Ca}_v1.3^{\text{-/-}}$ Th2 cells. The effect size of stimulation is plotted underneath for each genotype as the mean of pairwise differences its 95% CI estimated by bootstrap resampling; the shaded curve denotes the resampling distribution. Results were compared with paired Student's t-test between before and after stimulation (Stim) and by unpaired Student's t-test between two genotypes. **(F)** ECE were stratified based on their frequency of opening in classes ranking from low (<7.5 events/s) to high (>22.5 events/s) in bins of 2.5 events/s. The distributions were compared by a chi-square test. **(G)** The cumulative distributions of frequency openings were plotted on graphs and the fitting cumulative Gaussian distribution was indicated. Results are expressed as the mean of 3 to 5 groups of 3-4 cells for each genotype $\pm 1 \text{ SEM}$. * $p<0.05$; ** $p<0.01$, *** $p<0.005$, **** $p<0.0001$.

Figure 2: Ca_v1.2- and Ca_v1.3-deficient Th2 cells both have impaired effector functions and reduced ability to induce inflammation

WT, Ca_v1.2^{T-/} and Ca_v1.3^{-/-} CD4⁺ T cells were cultured in Th2-polarizing conditions. **(A)** Th2 cells were stimulated with biotinylated anti-CD3/anti-CD4 crosslinked with streptavidin for 2 min (Stim) or not (Unstim). Equal amounts of lysate proteins were blotted and probed with antibody against phosphorylated tyrosine (P-Tyr) or β -actin (Actin, loading control). Graphs show the quantification of staining intensities relative to actin intensity for stimulated relative to unstimulated samples. **(B)** Cytokine production in *in vitro* differentiated Th2-cells after 24h of cross-linked anti-CD3 mAb (anti-CD3) stimulation was determined by ELISA. Results were normalized to WT Th2 cells from the same experiment and were pooled from 6-17 mice of 3-5 independent experiments. **(C-E)** *Cacna1c*^{fl/fl}Cre- naive CD4⁺ T cells were differentiated in Th2 cells and then transduced with either GFP alone (GFP) or Cre-GFP (Cre-GFP) retroviral particles. After 4 days of transduction, GFP⁺ cells were cell sorted. qPCR on gDNA **(C)** and RT-qPCR on mRNA **(D)** for detection of *Cacna1c* levels in sorted GFP⁺ cells. Data were normalized to *Rpl13* and *Hprt* gene expression, respectively, as described in supplementary material. Data represent one experiment representative of three. **(E)** Th2-cytokine production of sorted GFP⁺ cells was quantified after 24 hours of coated anti-CD3 and soluble anti-CD28 stimulation by ELISA. Data represent the mean + SEM of 3-5 experiments. **(F-I)** *In vitro* differentiated OTII Th2 cells from WT, Ca_v1.2^{T-/} and Ca_v1.3^{-/-} mice were injected into C57BL/6 mice that were given intranasal OVA. **(F and H)** Lung-infiltrating cells were purified and analyzed by flow cytometry to quantify CD4⁺ TCR β ⁺ and V α 2⁺ OTII cells. **(G and I)** Lung sections were scored for inflammation after HE staining. PAS staining was performed to visualize mucus in histological sections. Each group included 7 mice. One experiment representative out of two is shown. Results are expressed as mean + SEM. Results were compared by Wilcoxon matched-pairs test **(A and E)**, unpaired Student's t-test **(B)**, and Mann-Whitney test **(G-I)**. *p<0.05; **p<0.01; ***p<0.005.

New Figure 3: Ca_v1.2^{T-/} mice develop less severe HDM-induced allergic airway inflammation than WT mice

(A) Mice were given intranasal HDM (1 μ g at day 0 and 10 μ g each day between days 7-11) and sacrificed at day 17. **(B)** Quantification of leukocyte populations in the BAL fluid after MGG staining. The total numbers of each leukocyte subset are shown. **(C)** Lung tissue sections stained with HE or PAS staining and histological score. **(D)** Enhanced pause (Penh) and dynamic lung resistance were measured. Results are expressed as means + SD of 7-8 mice and compared with Tukey's multiple comparison test. **(E-new F)** Th2-cytokine expression in lung quantified by qPCR **(E)** and in lung draining lymph node after HDM stimulation by ELISA **(new F)**.

Quantification of total (**G**) and Derf1-specific (**H**) serum IgE concentration by ELISA at the indicated time points. Results are means + SEM of 4-7 mice and representative of 2-3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ and **** $p < 0.001$ (Mann-Whitney test).

New Figure 4: $Ca_v1.3$ deletion in hematopoietic compartment results in decreased susceptibility to HDM-induced allergic airway inflammation

C57BL/6 mice were irradiated the day before reconstitution with bone marrow cells from WT or $Ca_v1.3^{-/-}$ mice. Eight weeks after reconstitution, HDM-specific allergic airway inflammation was induced. (**A**) Quantification of leukocyte populations present in the BAL fluid using MGG staining. (**B**) Lung tissue sections stained with HE and PAS coloration and histological scores. (**C**) Enhanced pause (Penh) and dynamic lung resistance were measured. Results are expressed as means + SD of 7-8 mice and compared with Tukey's multiple comparison test (**** $p < 0.001$). (**D-new E**) Th2 cytokine expression in lung quantified by qPCR (**D**) and in lung draining lymph node after HDM stimulation by ELISA (**new E**). Total (**F**) and Derf1-specific (**G**) IgE serum concentrations by ELISA. Results are expressed as means + SEM of 5-7 mice and representative of 5 (A, B, C, F and G) and 2 (D and E) independent experiments. * $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney test).

Figure 5: The presence of both $Ca_v1.2$ and $Ca_v1.3$ channels on the same $CD4^+$ T cells is required for the development of allergic airway inflammation

Naive $CD4^+$ T cells from the different genotypes were injected into chimeric mice reconstituted with $Ca_v1.3^{-/-}$ (**A-B**) or $Ca_v1.2^{T-/-}$ (**C-D**) bone marrow cells 24 hours before induction of OVA-mediated airway inflammation. (**A and C**) Quantification of BALF cells after MGG staining. (**B and D**) Representative microscopic images of HE- and PAS-staining of lung sections and quantification of histology score. Results are expressed as means + SEM of 6-9 mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ (Mann-Whitney test).

New Figure 6: The detection of both $Ca_v1.2$ and $Ca_v1.3$ in $CD4^+$ T cells from asthmatic children is associated with high Th2-cytokine expression

We purified $CD4^+$ T cells from blood samples of 26 asthmatic children and 17 age-matched control donors and we stimulated them or not with anti-CD3/CD28 coated beads for 6 hours before RNA extraction. Transcripts for *CACNA1C* (encoding for $Ca_v1.2$), *CACNA1D* (encoding for $Ca_v1.3$), *IL4* and *IL5* were quantified by qPCR. Data were normalized to *GAPDH* gene expression. (**A**) Violin plots represent the expression of *CACNA1C* and *CACNA1D* transcripts, in control and asthmatic children. Each dot or triangle represents one donor and the horizontal bar denotes the

median. Values below the dotted lines were considered as undetectable. **(B)** Control and asthmatic children were classified in four groups depending on *CACNA1C* and *CACNA1D* detection in unstimulated or in TCR-stimulated CD4⁺ T cells. The distribution was analyzed with chi-square test. **(C)** Comparison of the expression of *IL4* and *IL13* after stimulation in CD4⁺ T cells from control and asthmatic children in which we detected *CACNA1C* and *CACNA1D* (both: *CACNA1C*⁺ *CACNA1D*⁺), or only one channel (single: *CACNA1C*⁺ *CACNA1D*⁻ or *CACNA1C*⁻ *CACNA1D*⁺). **p<0.01, and ***p<0.005 (Mann Whitney test).

Figure 1

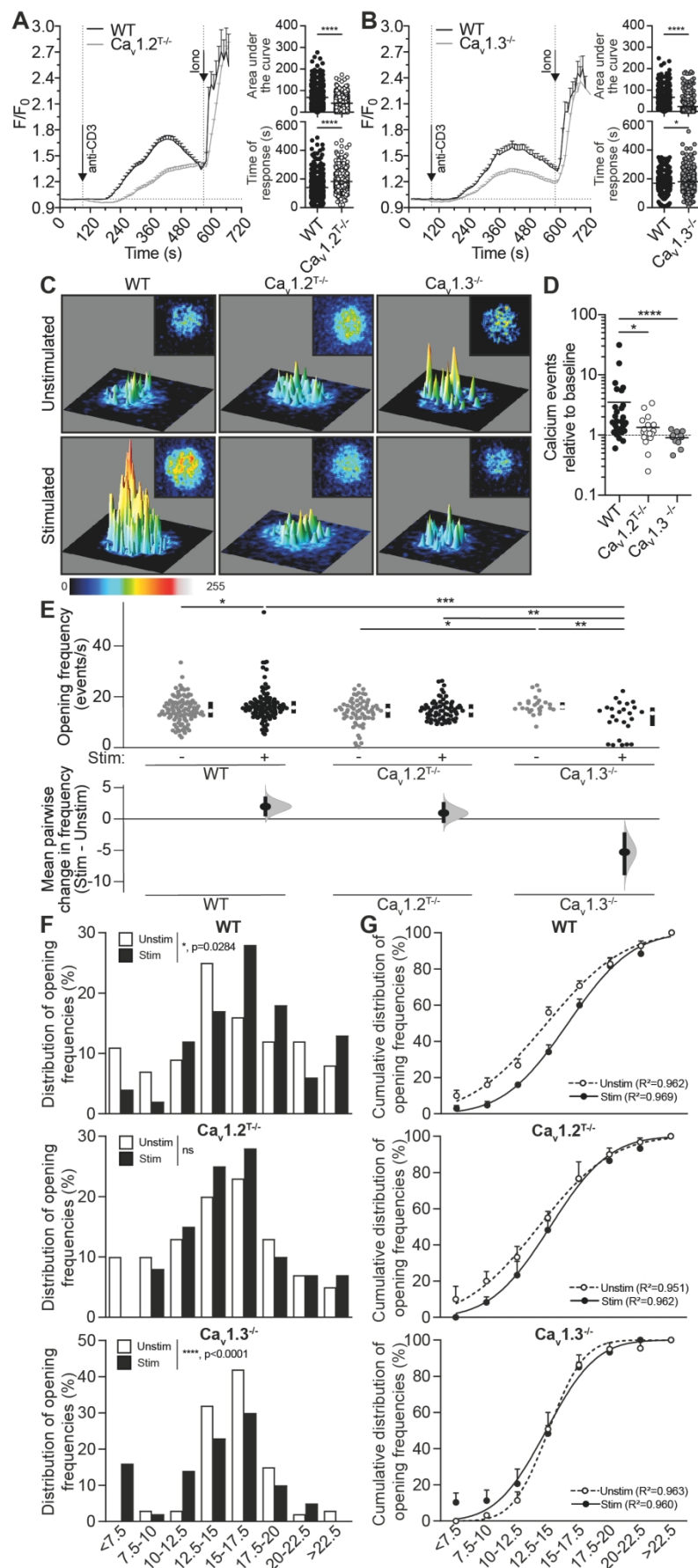
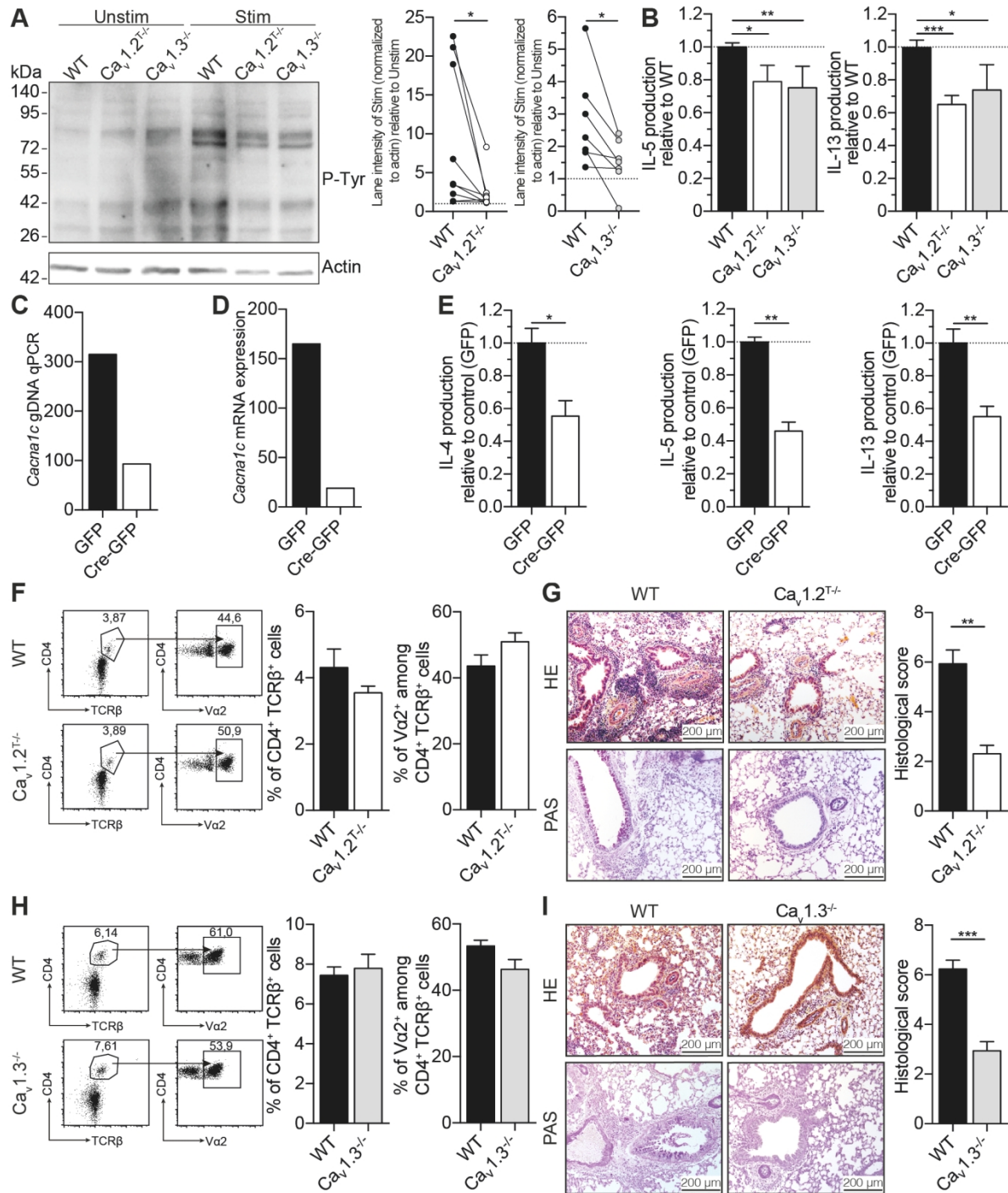
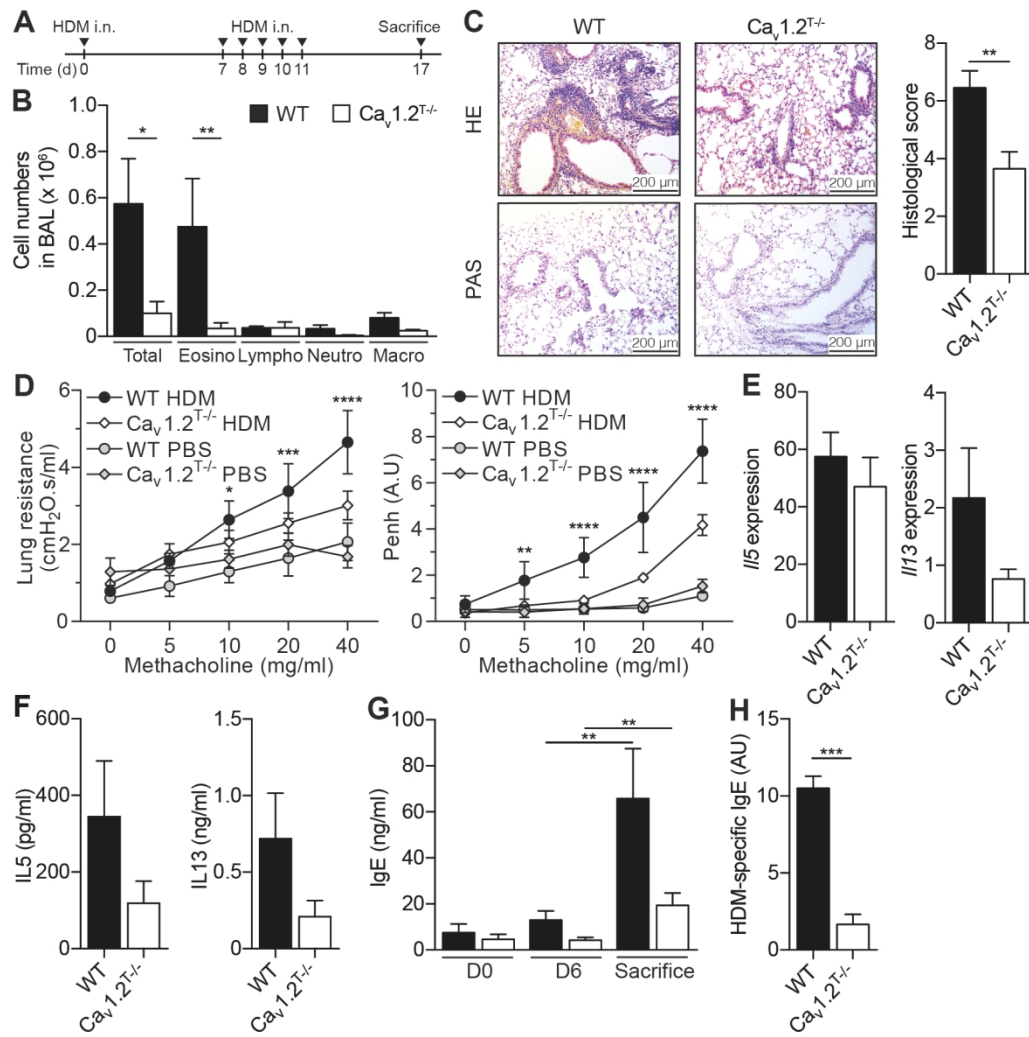


Figure 2



New Figure 3



New Figure 4

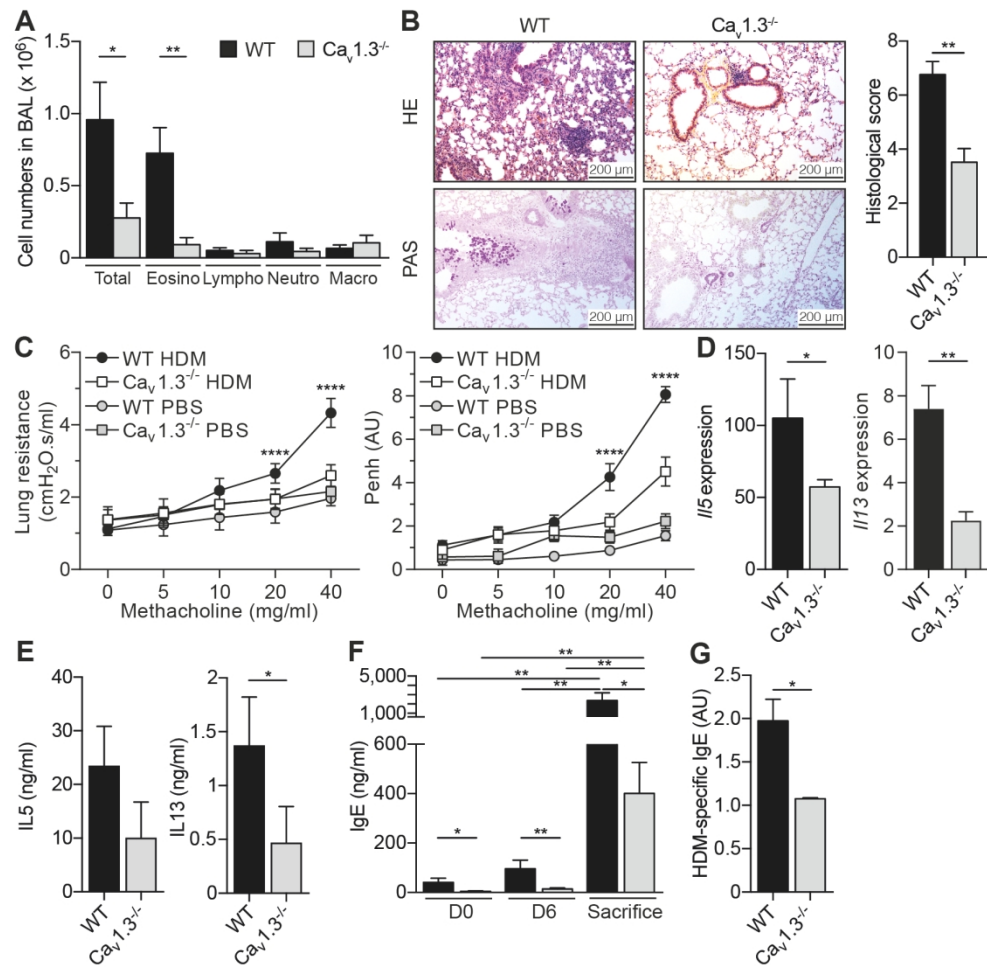
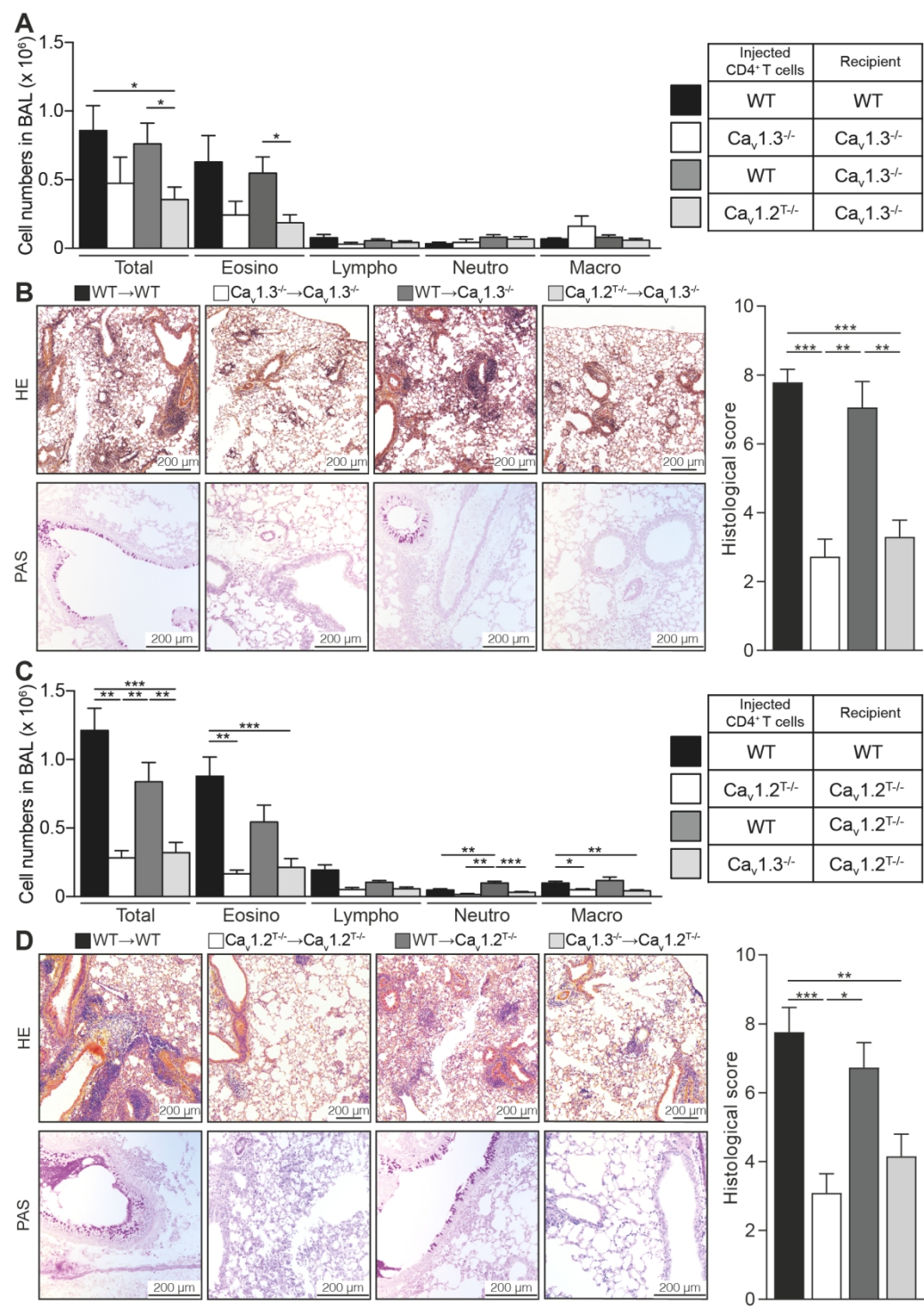
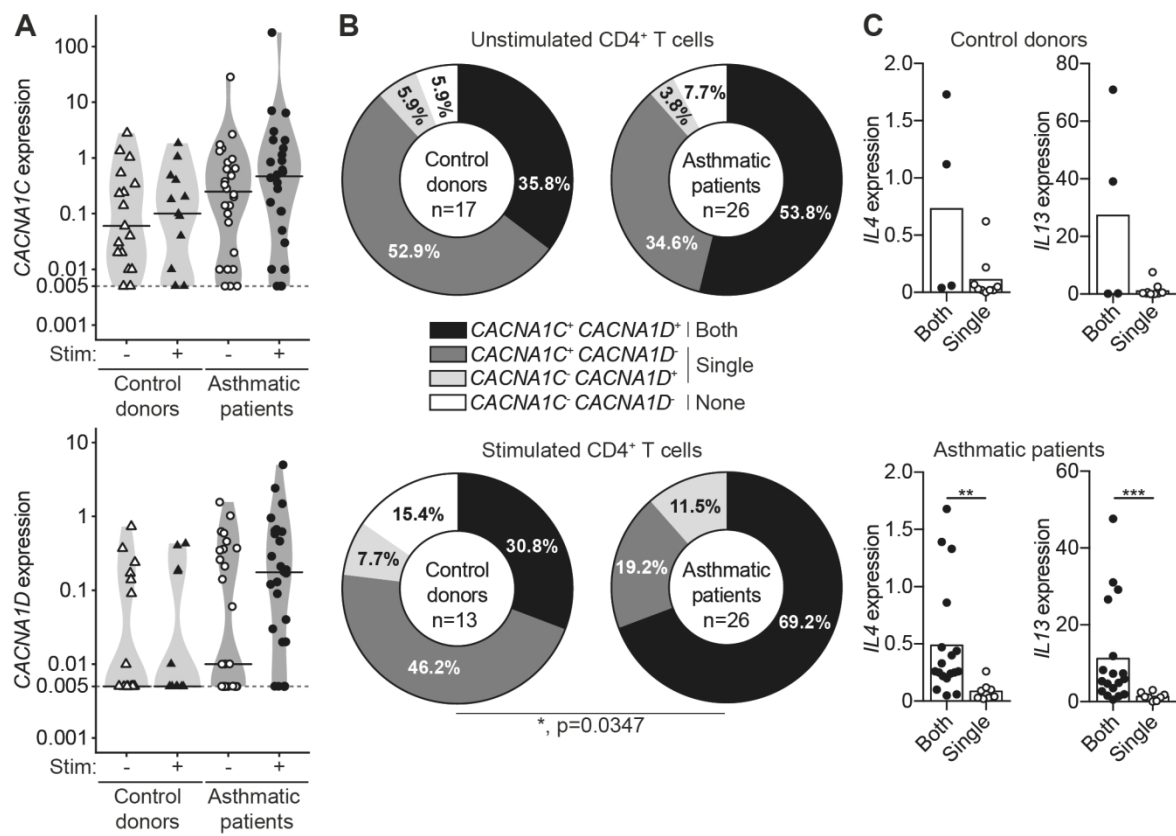
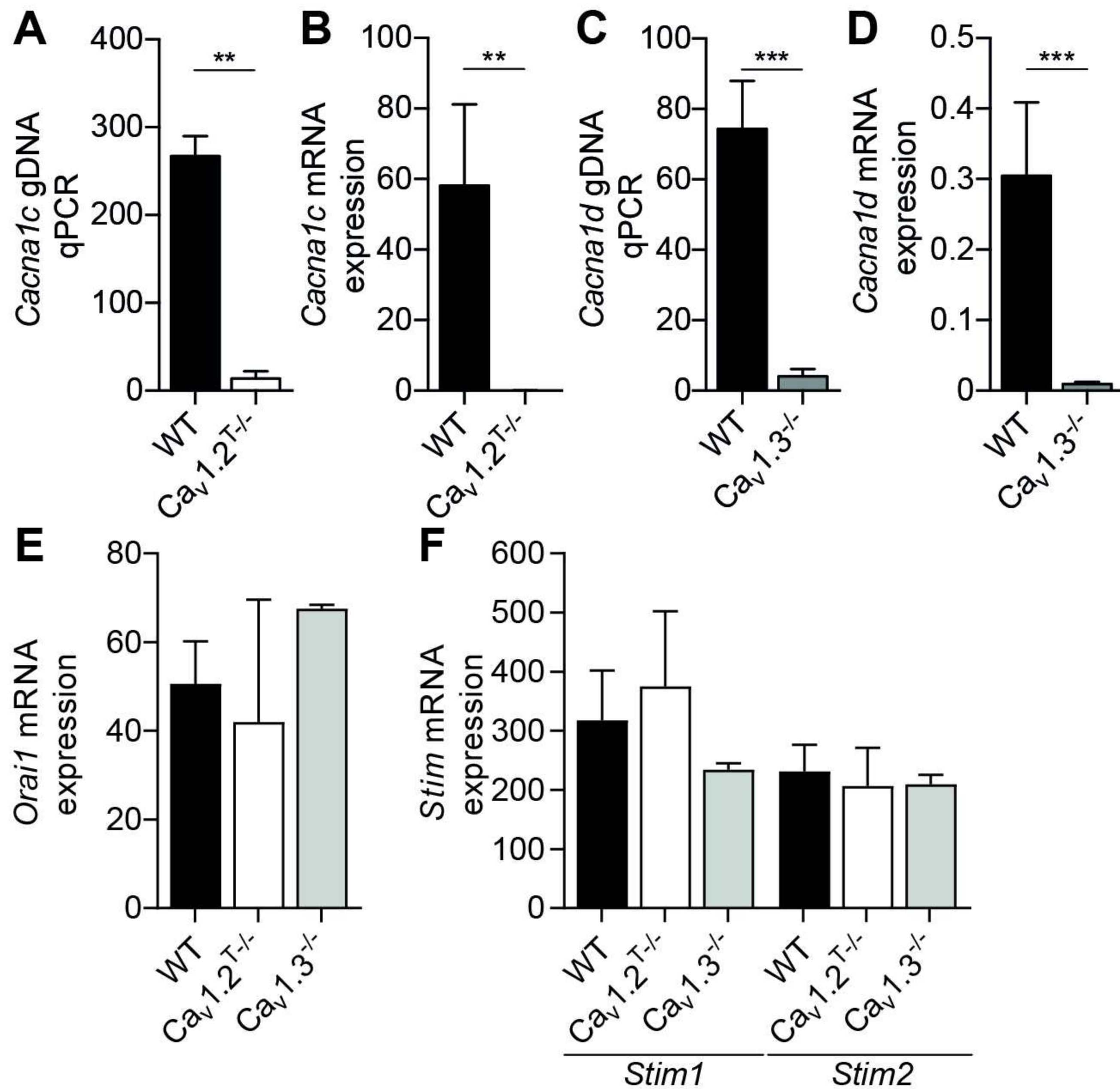


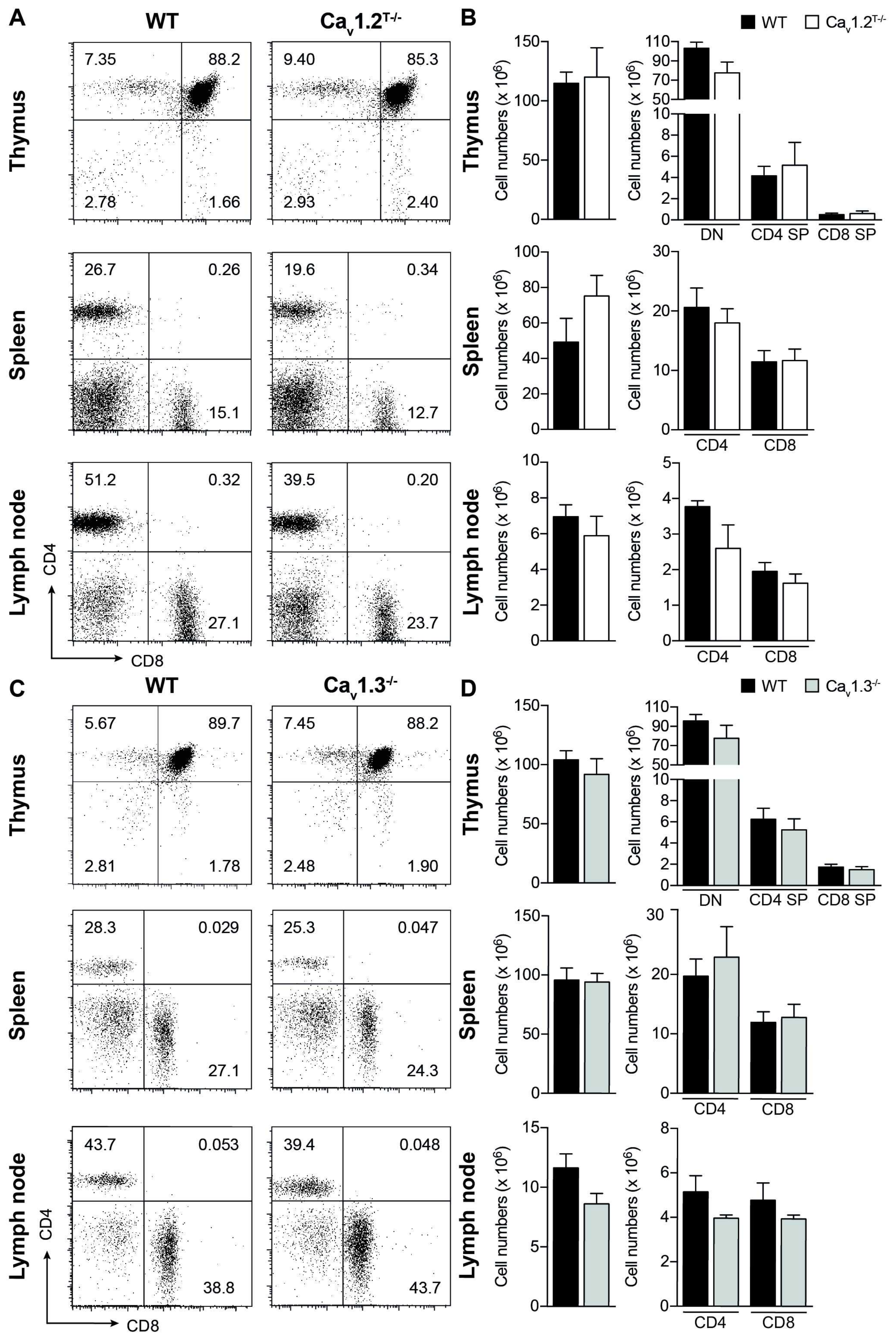
Figure 5

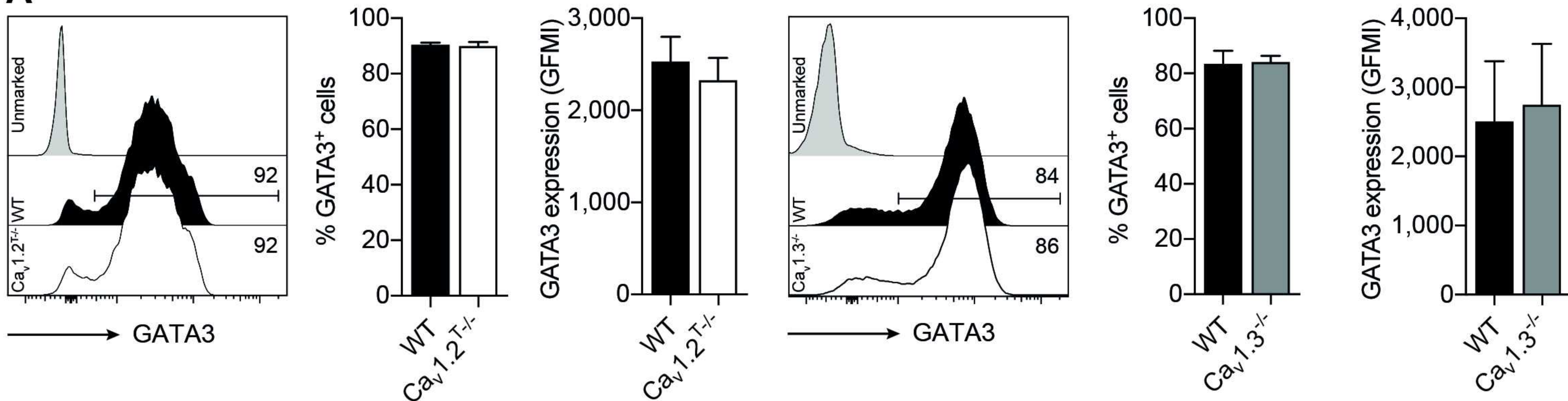
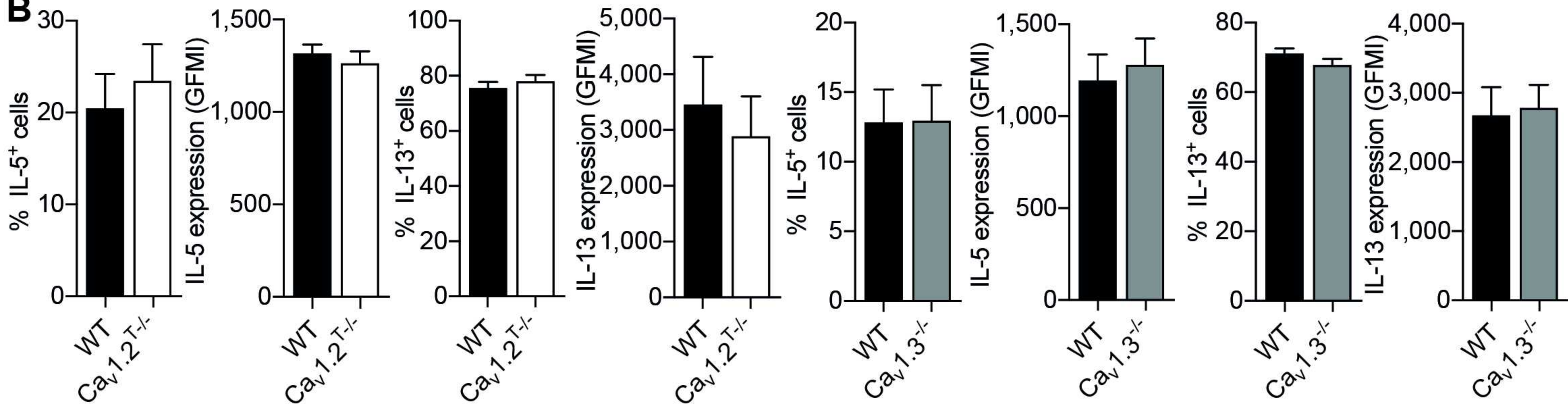


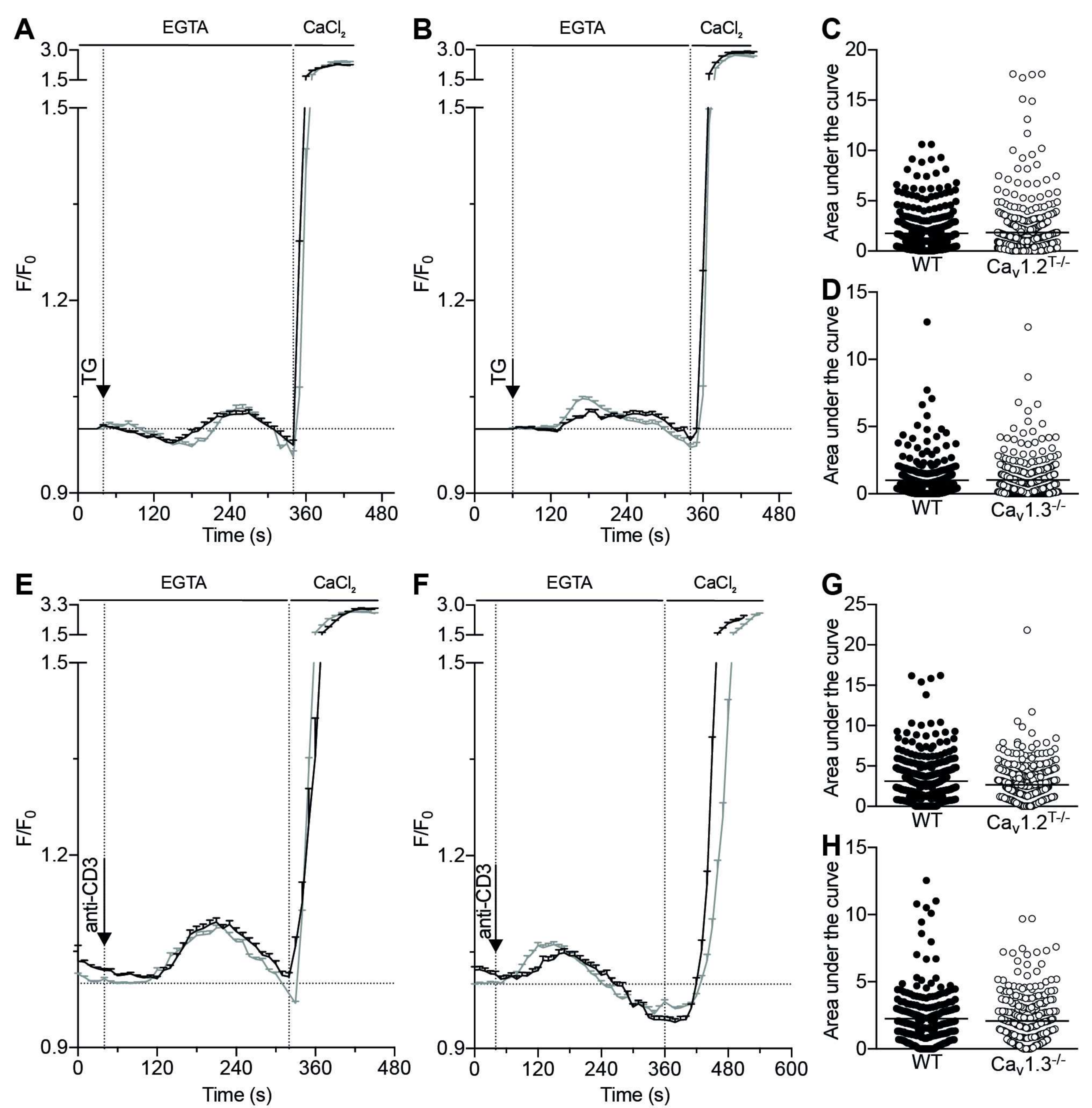
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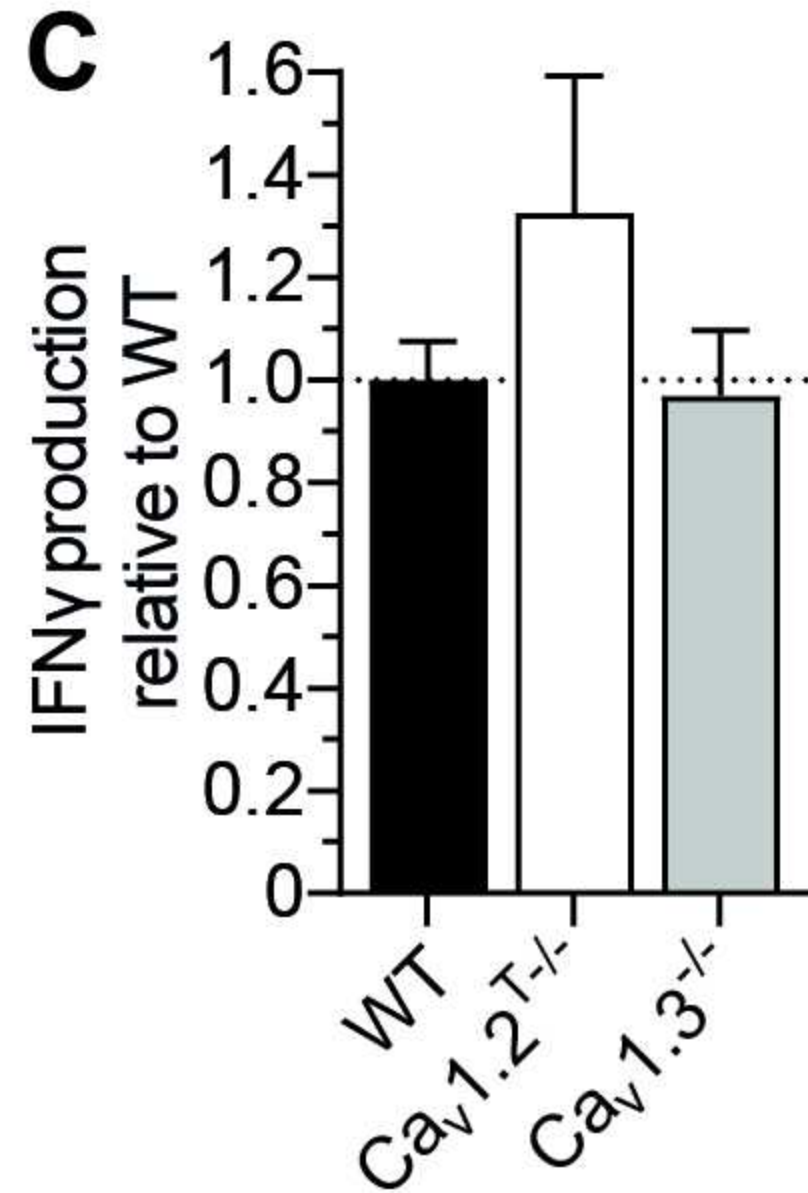
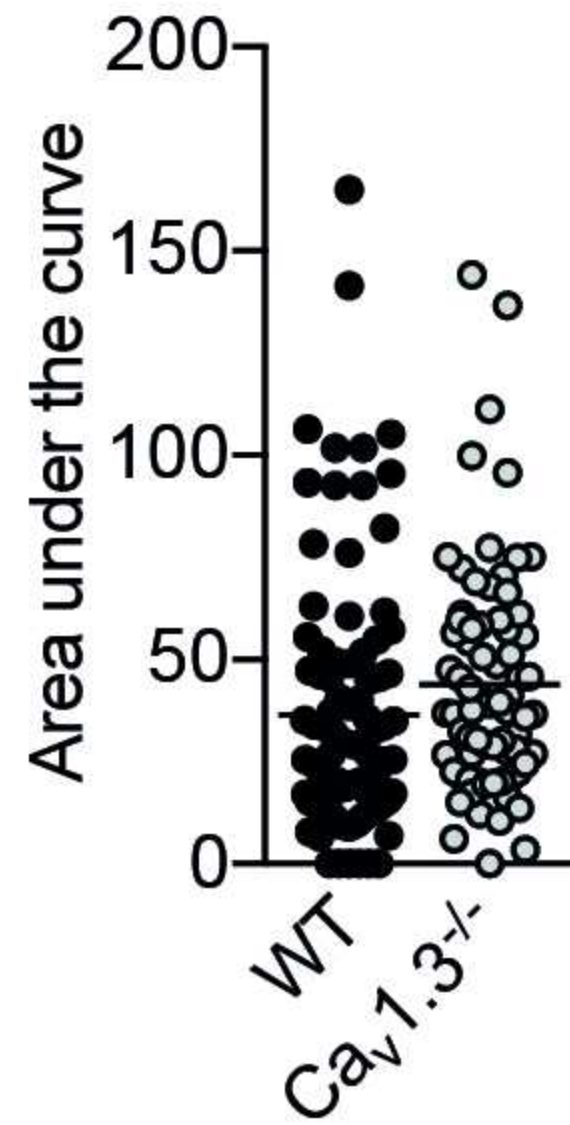
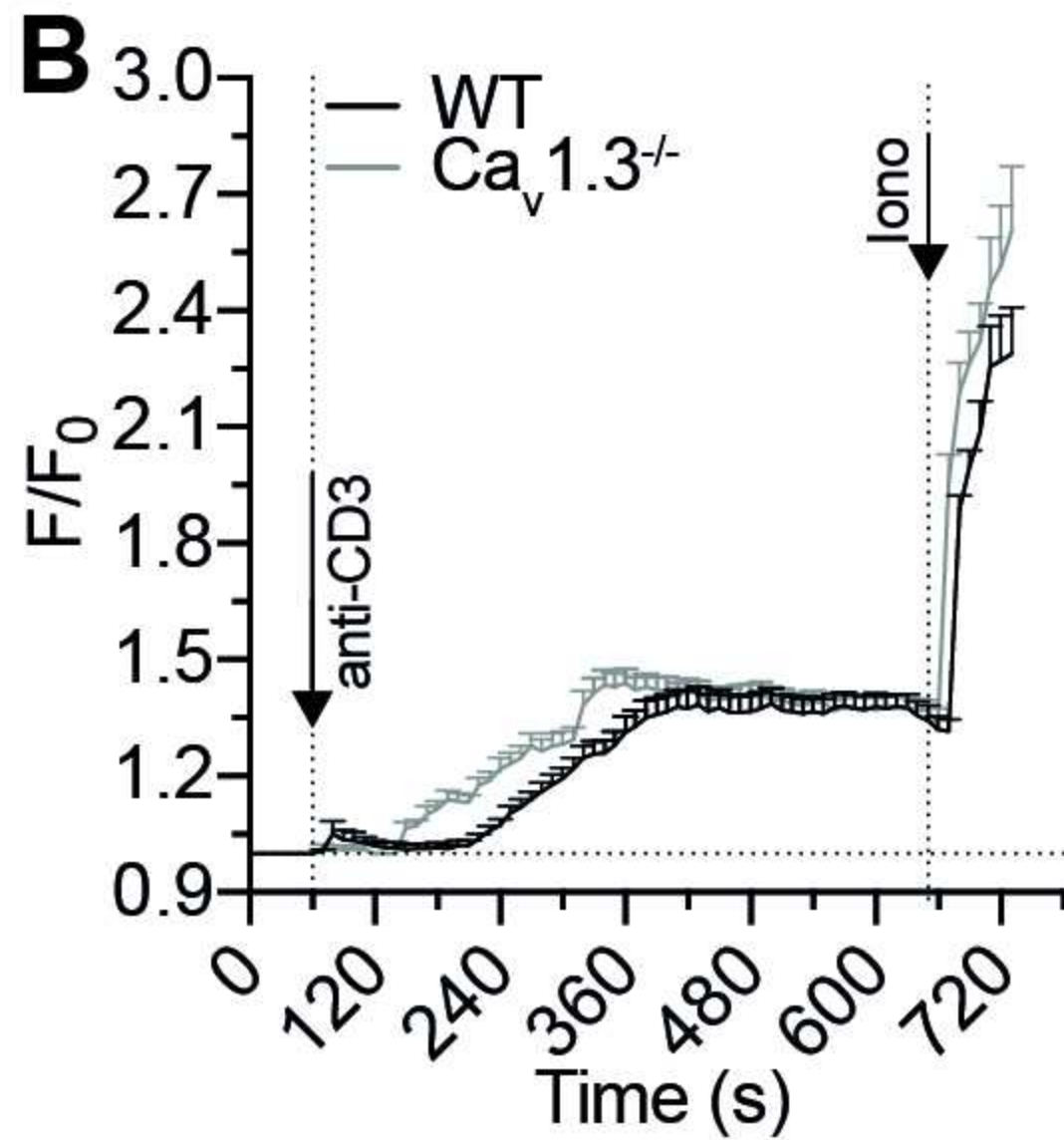
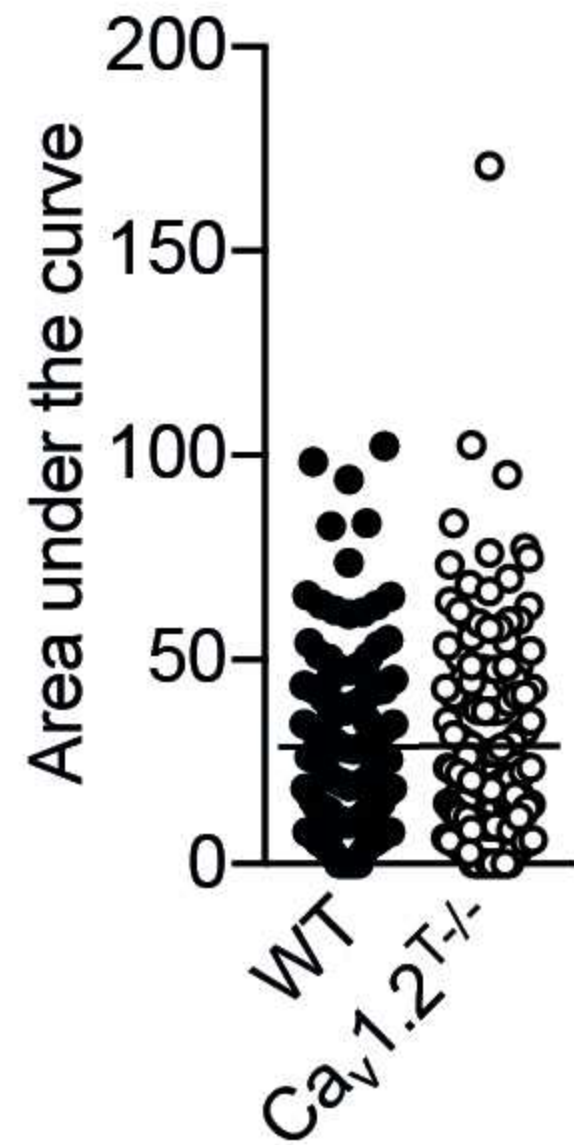
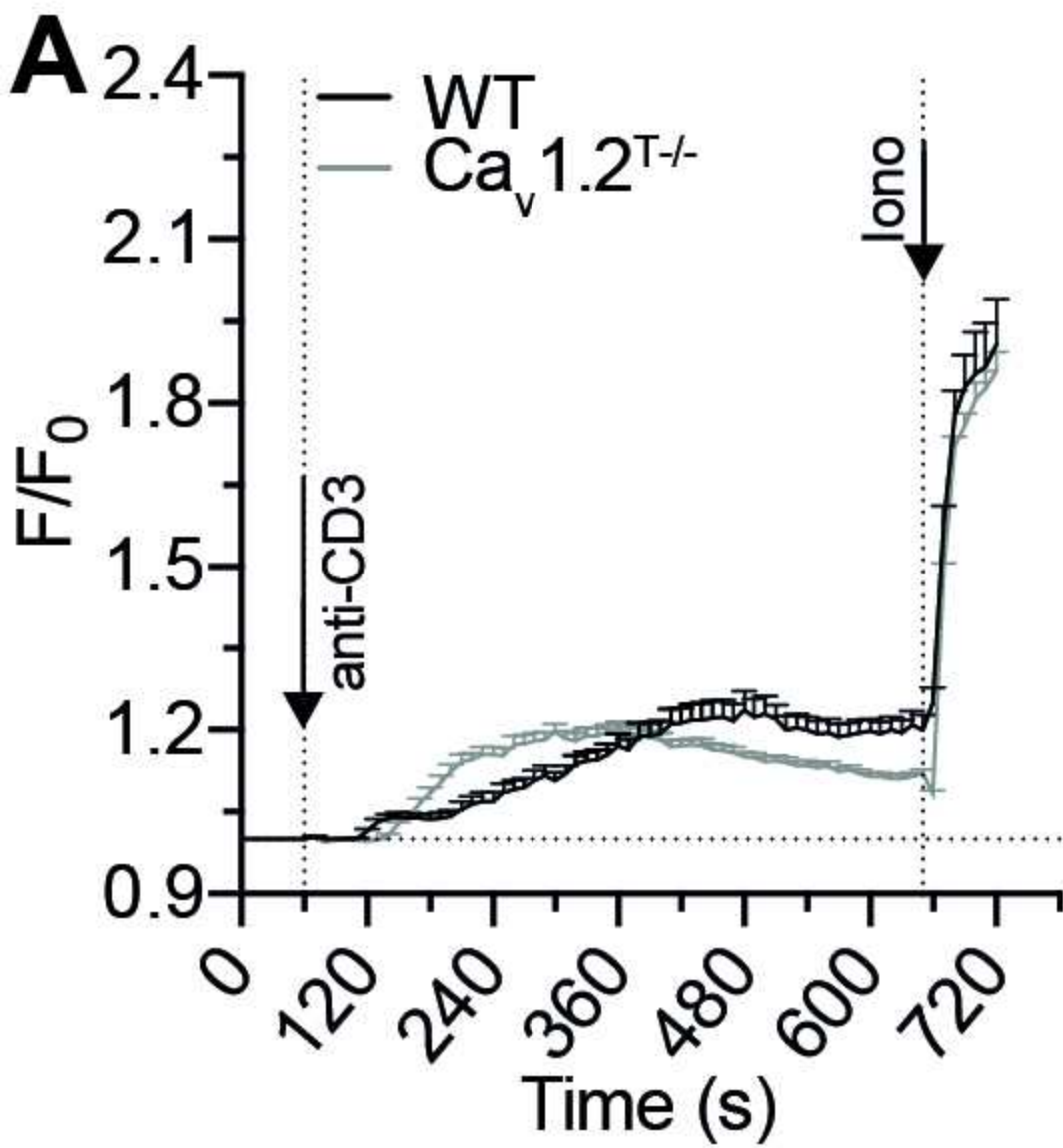


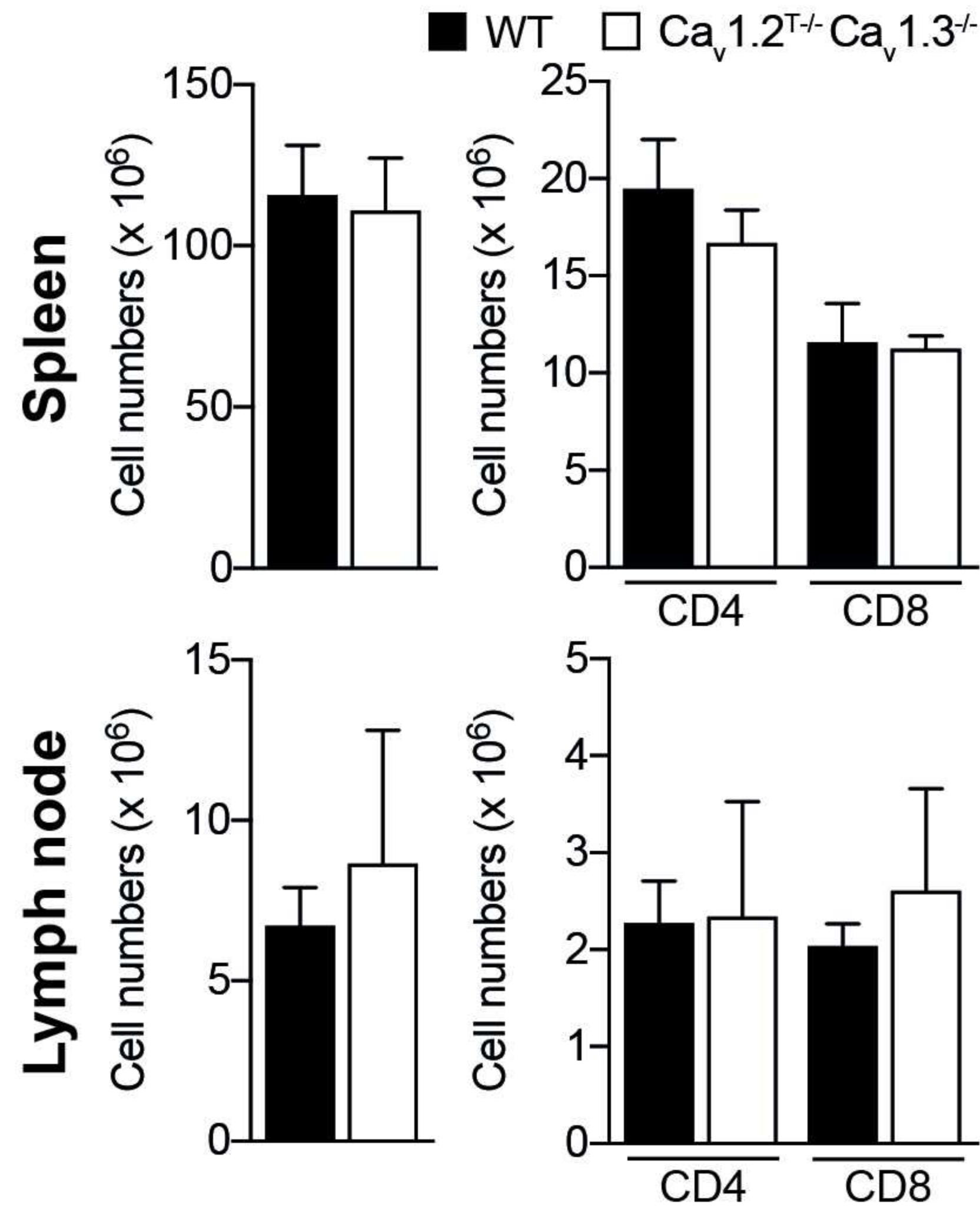


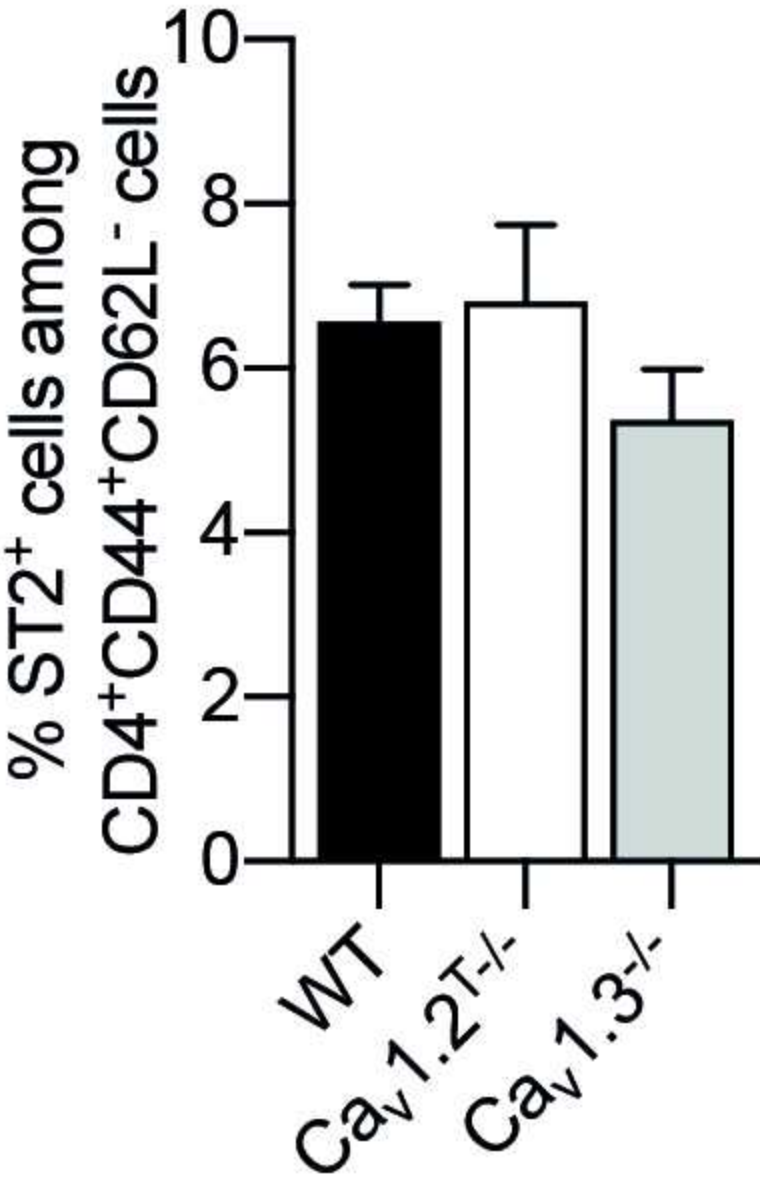


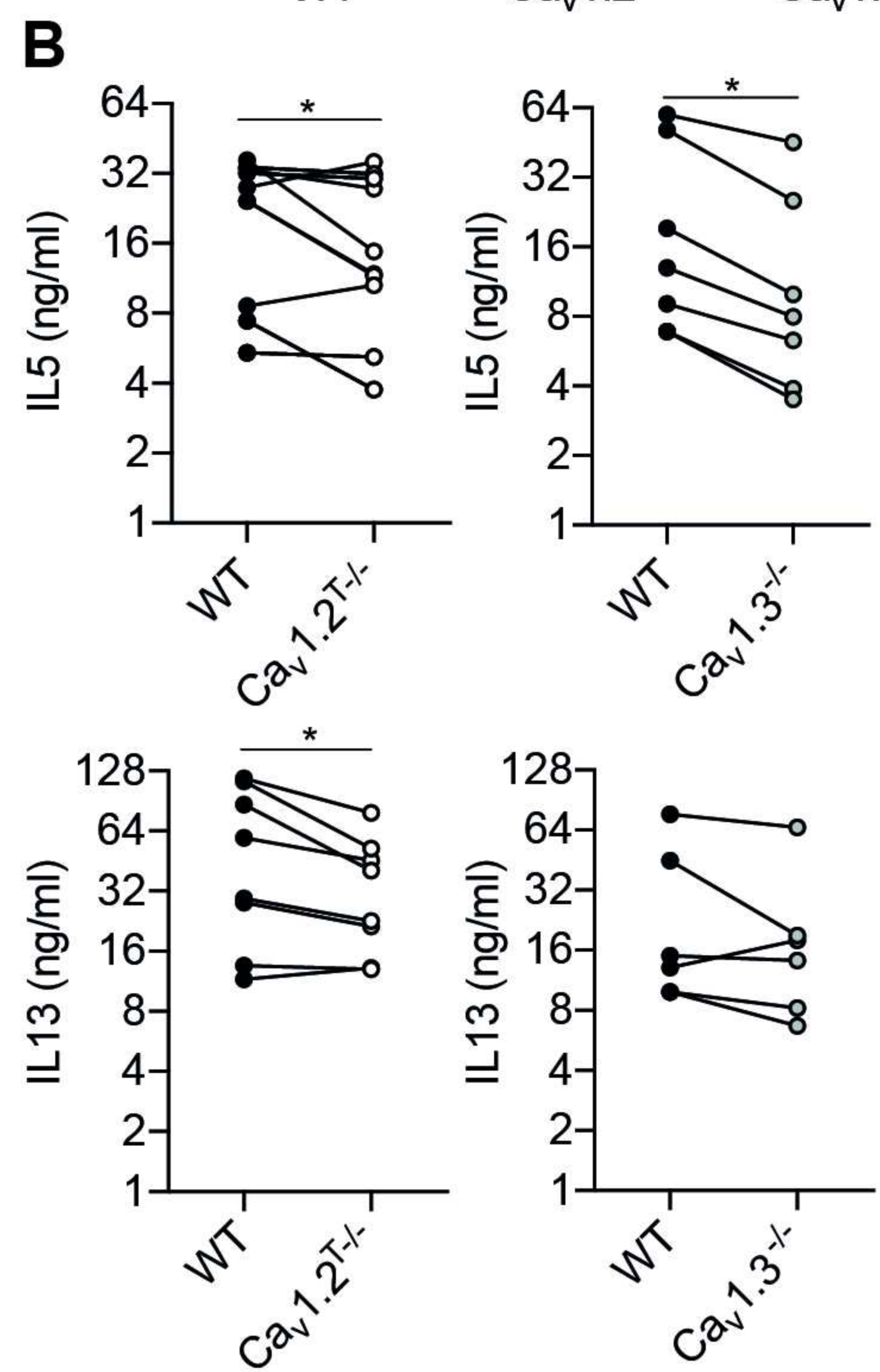
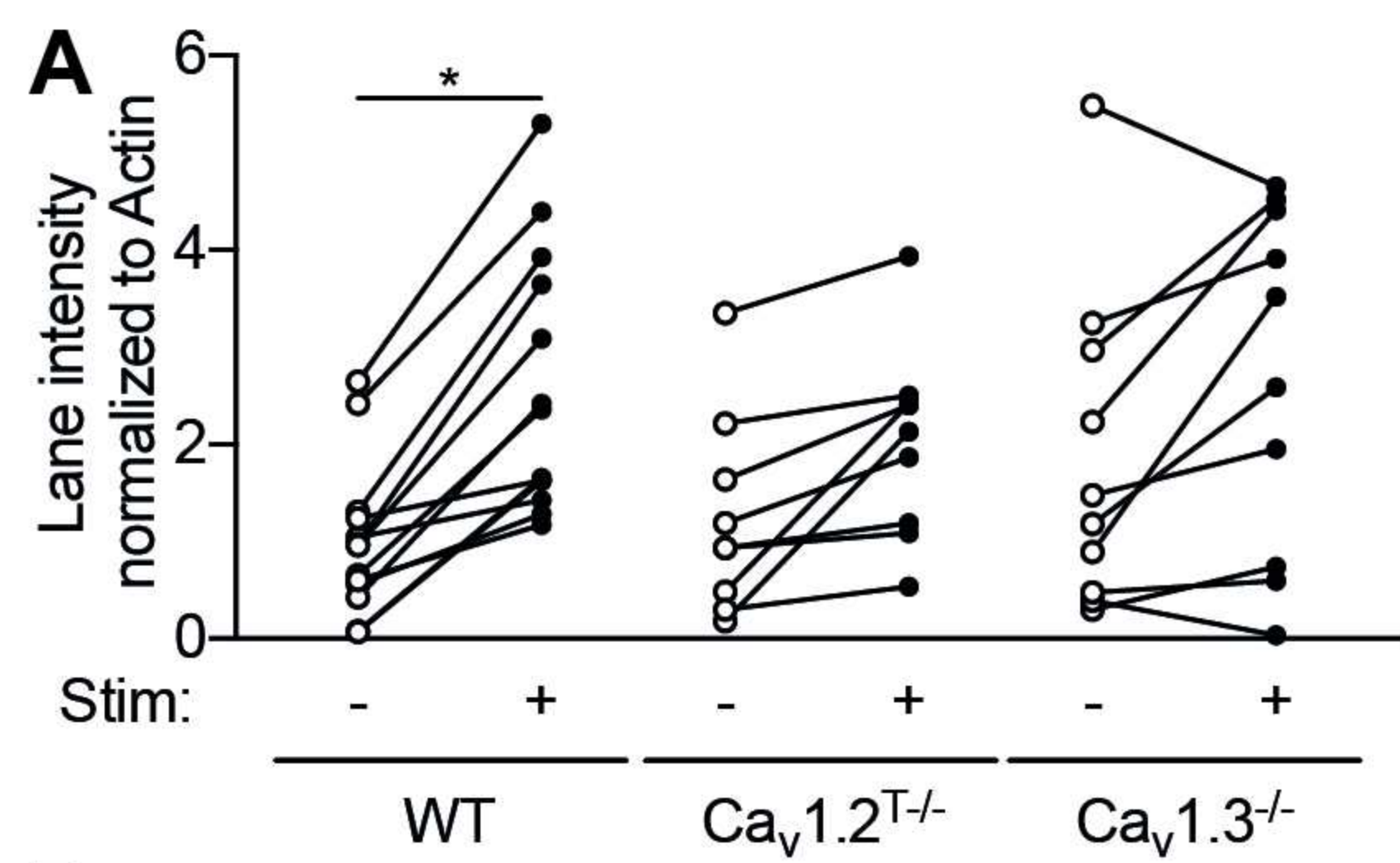
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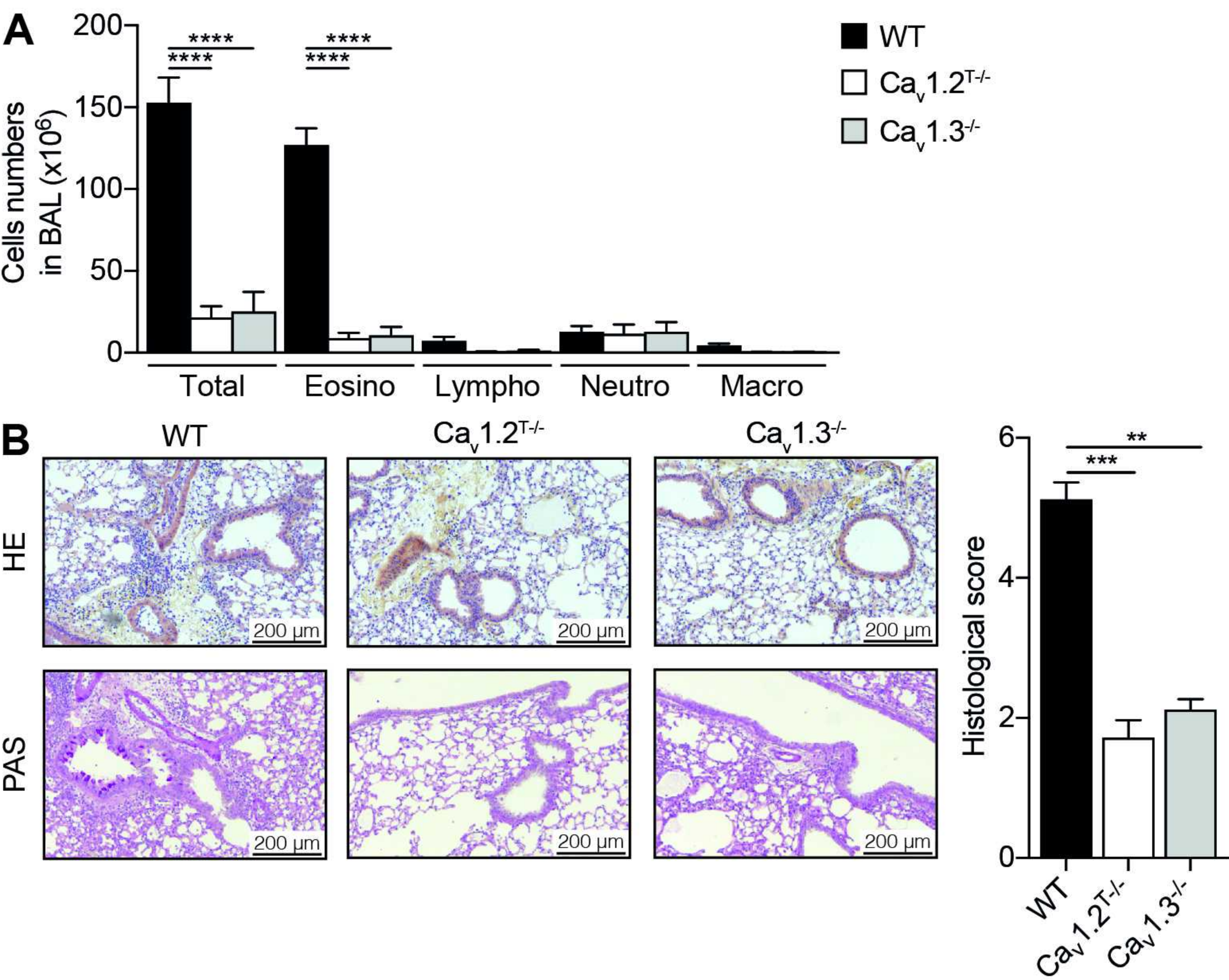


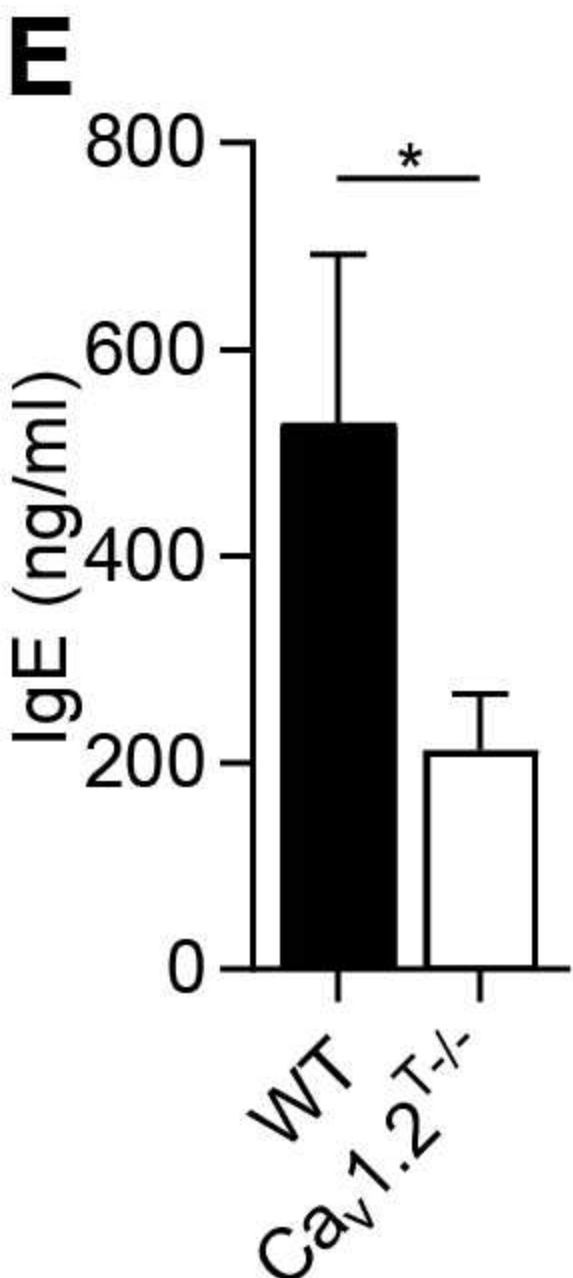
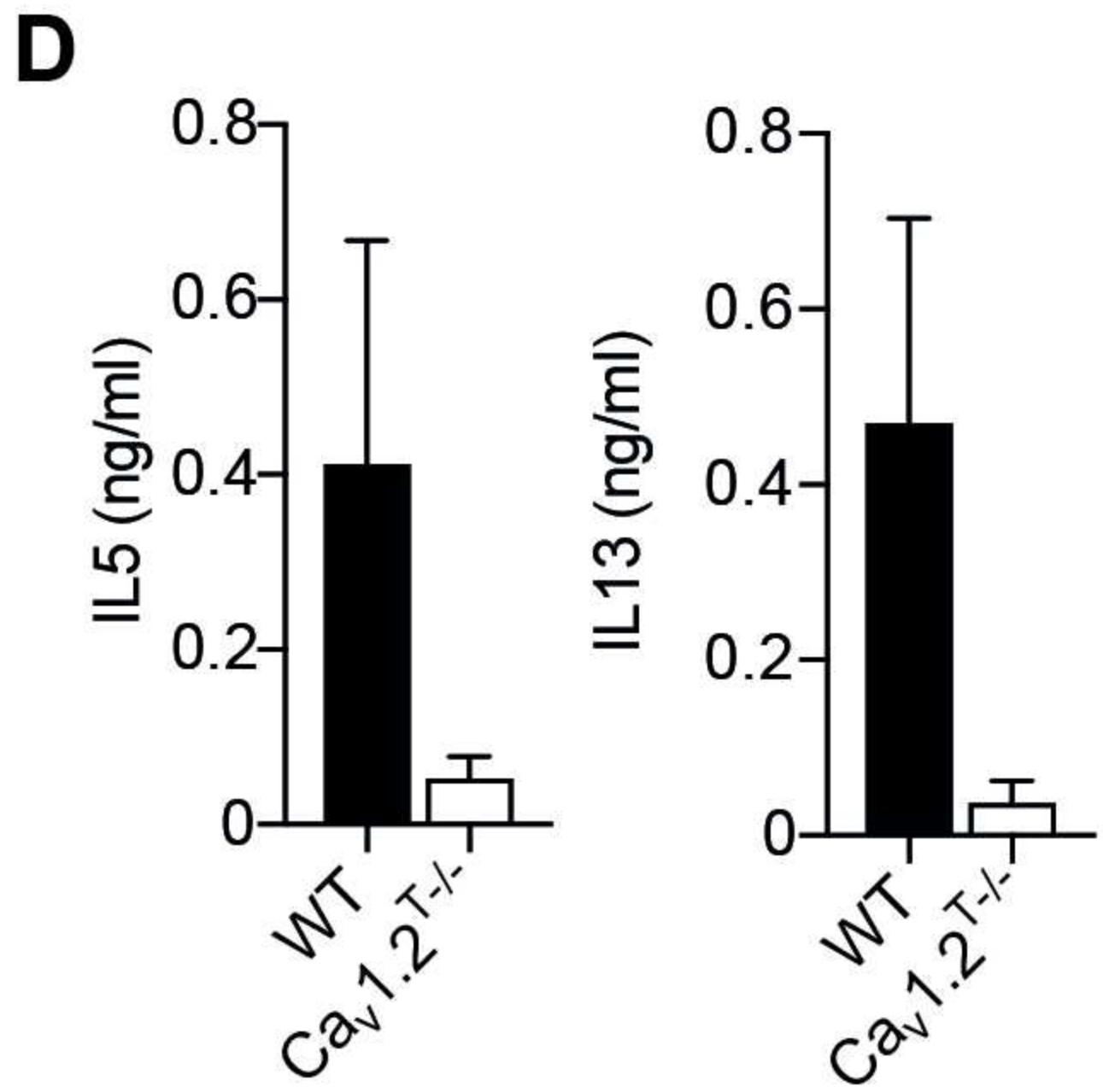
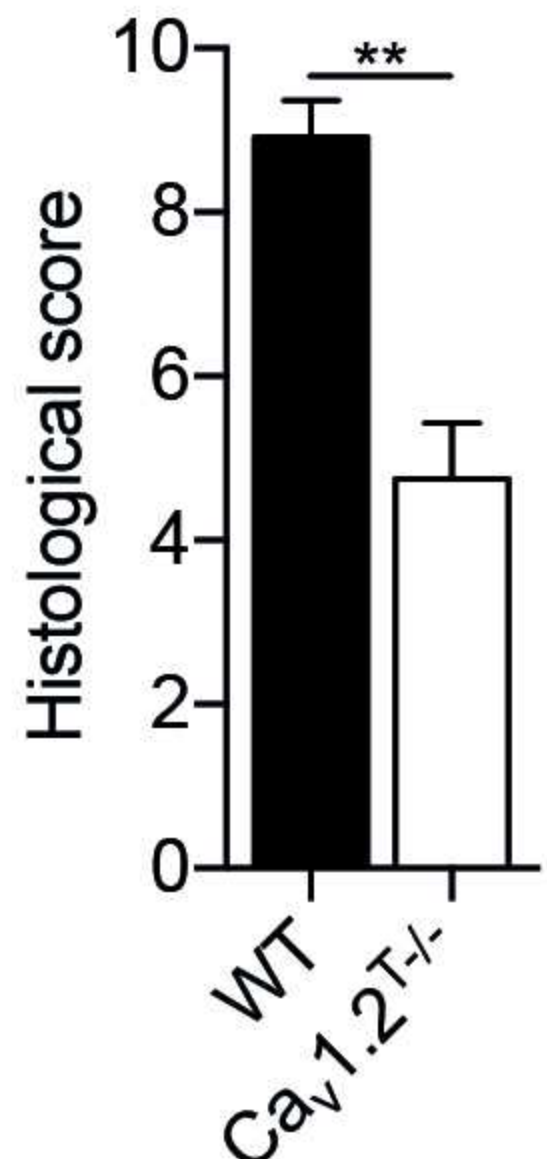
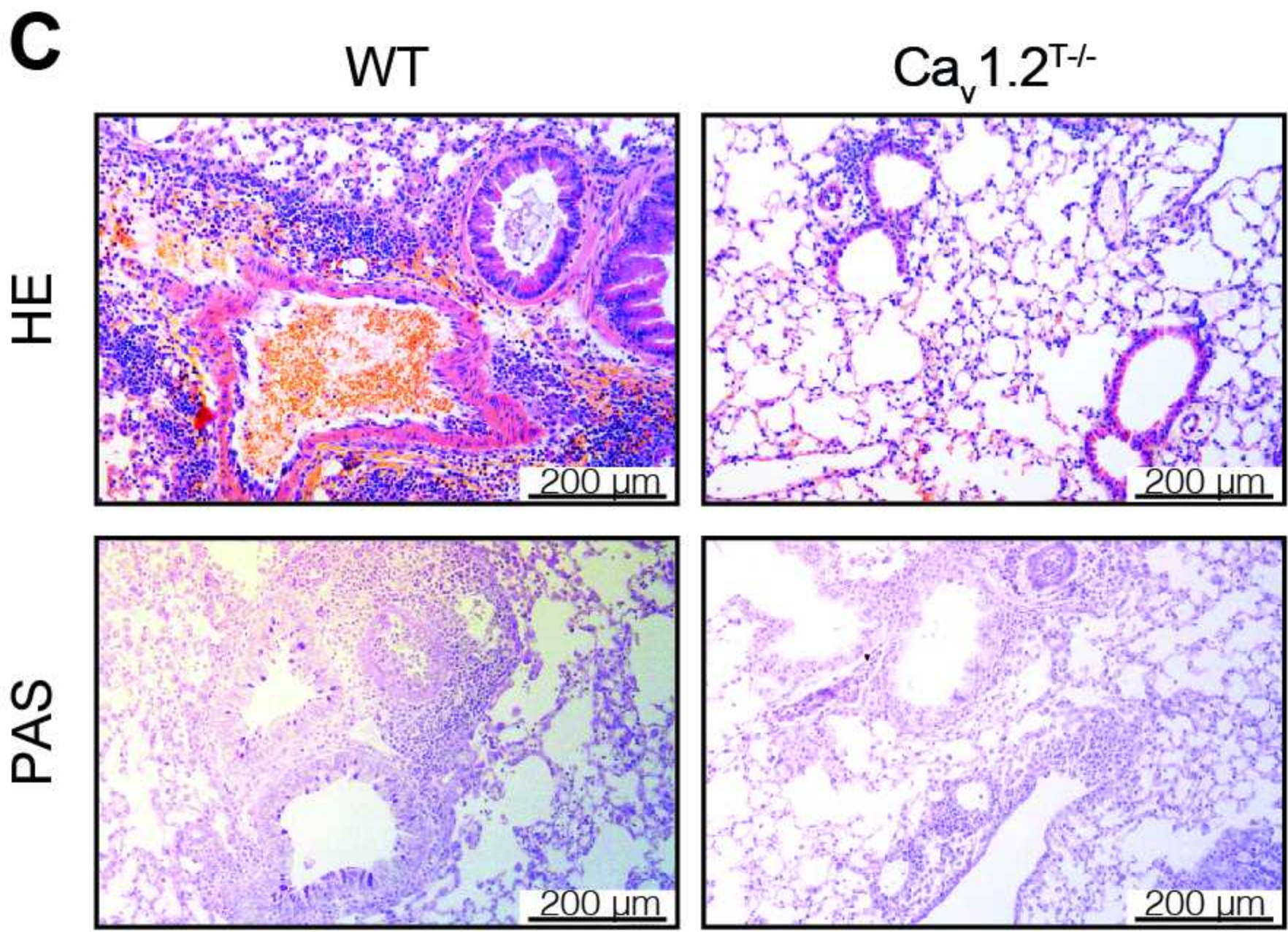
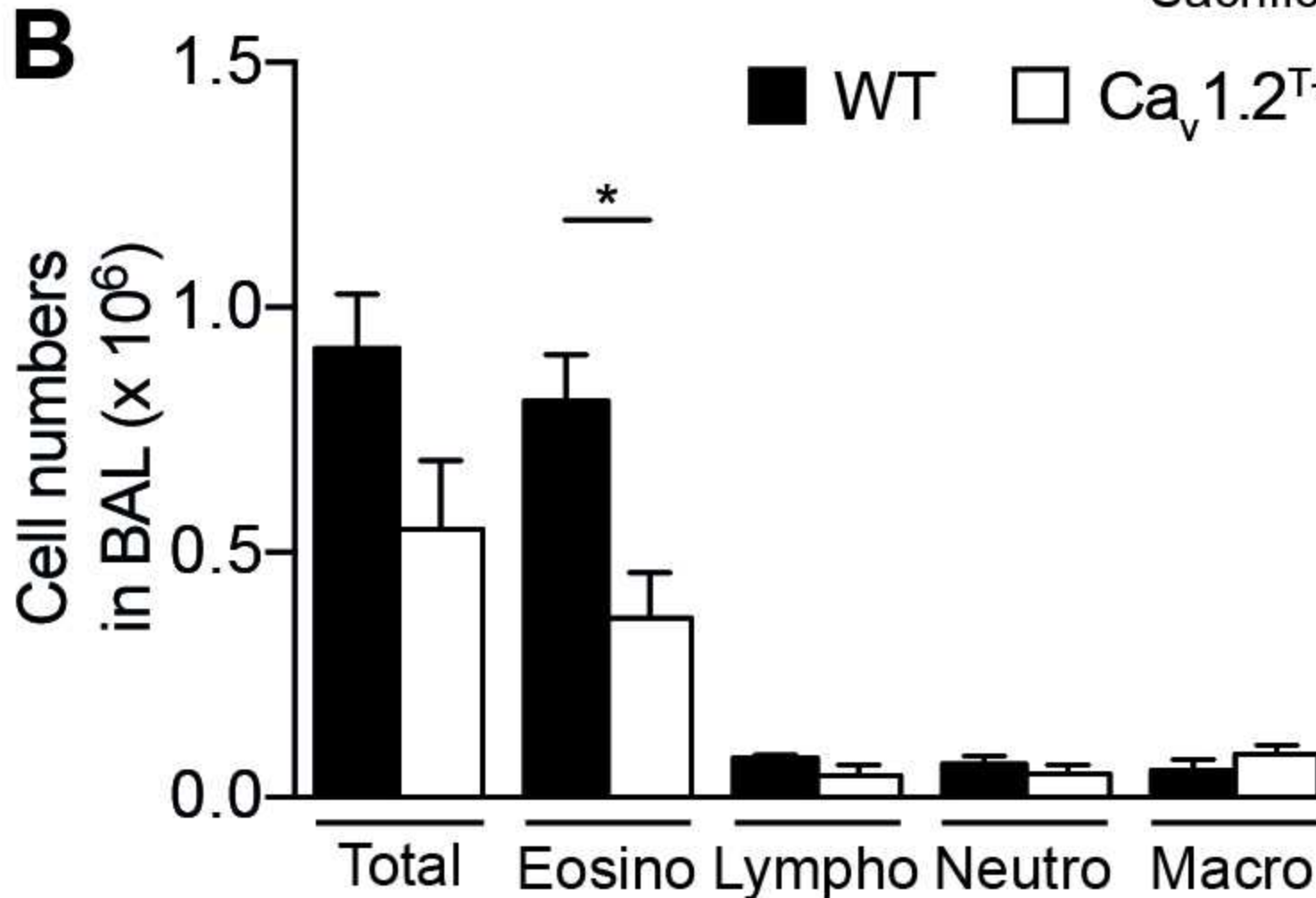


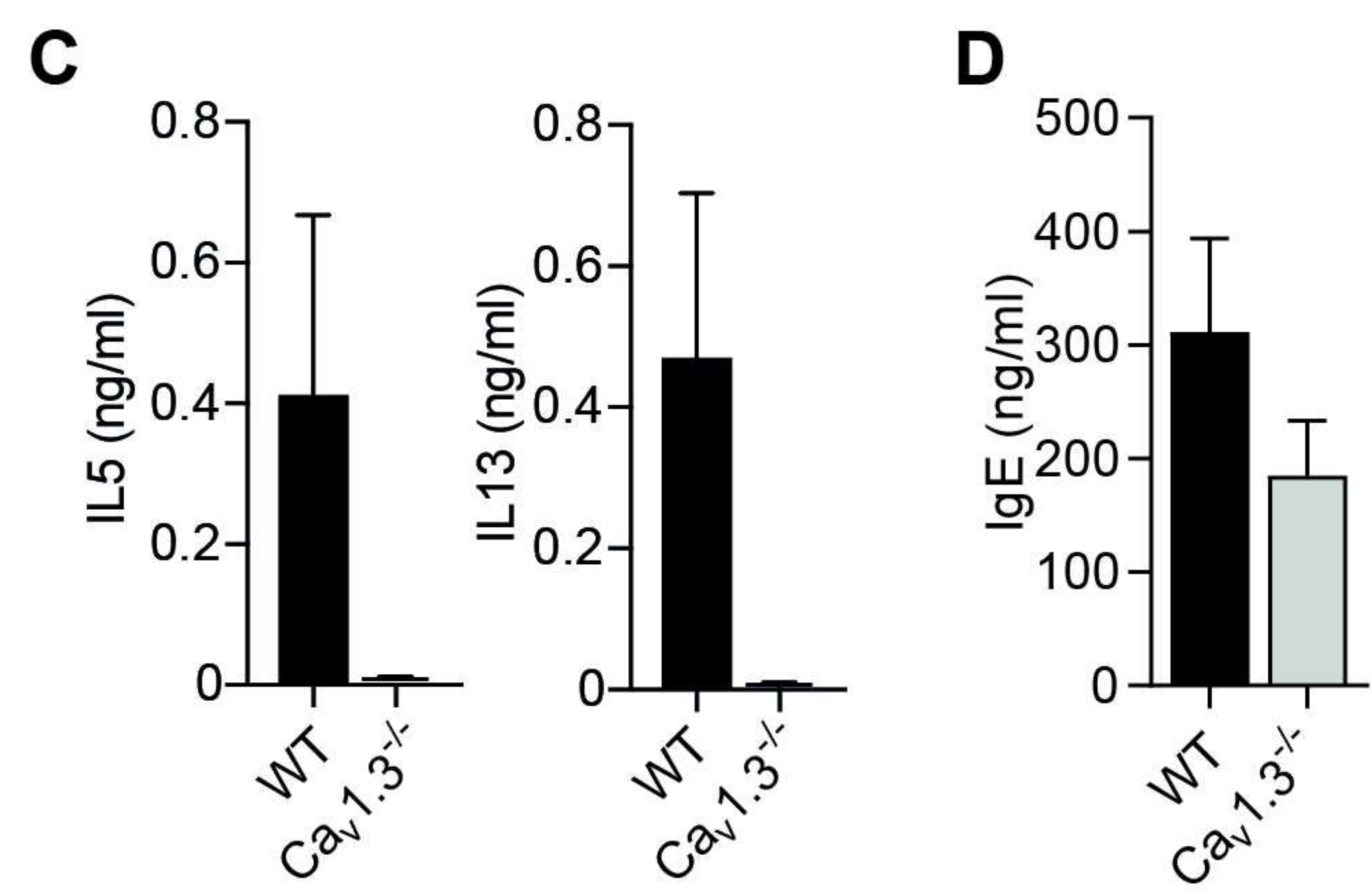
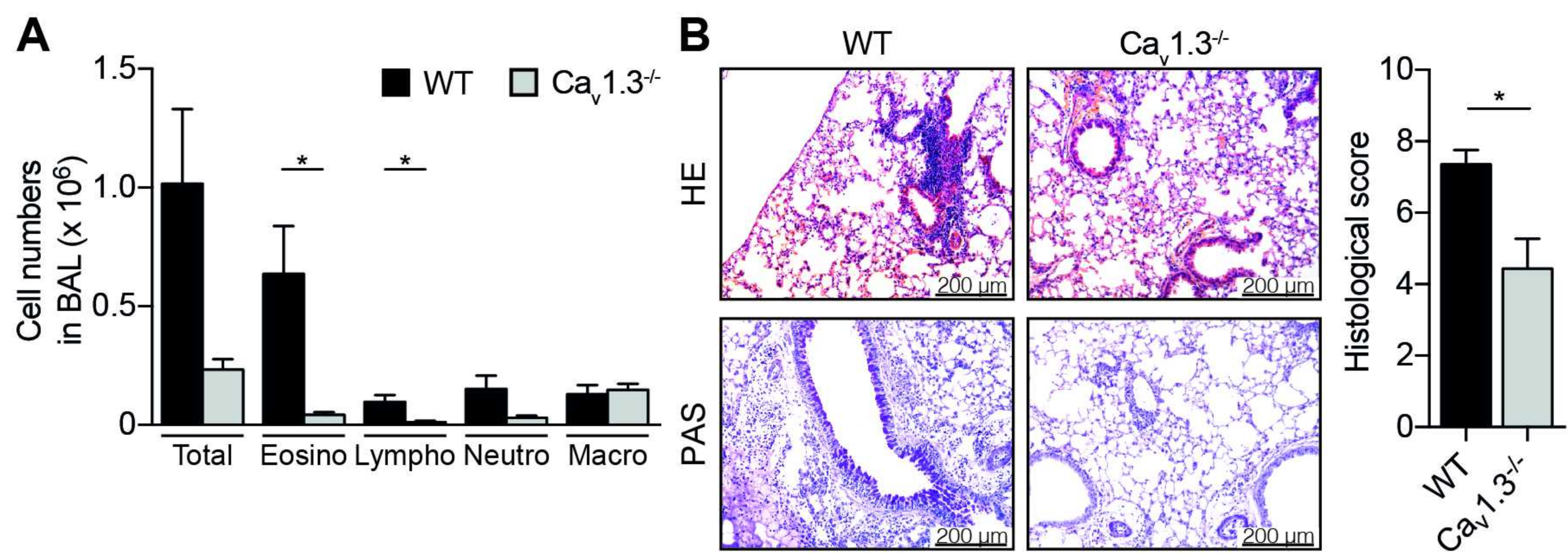


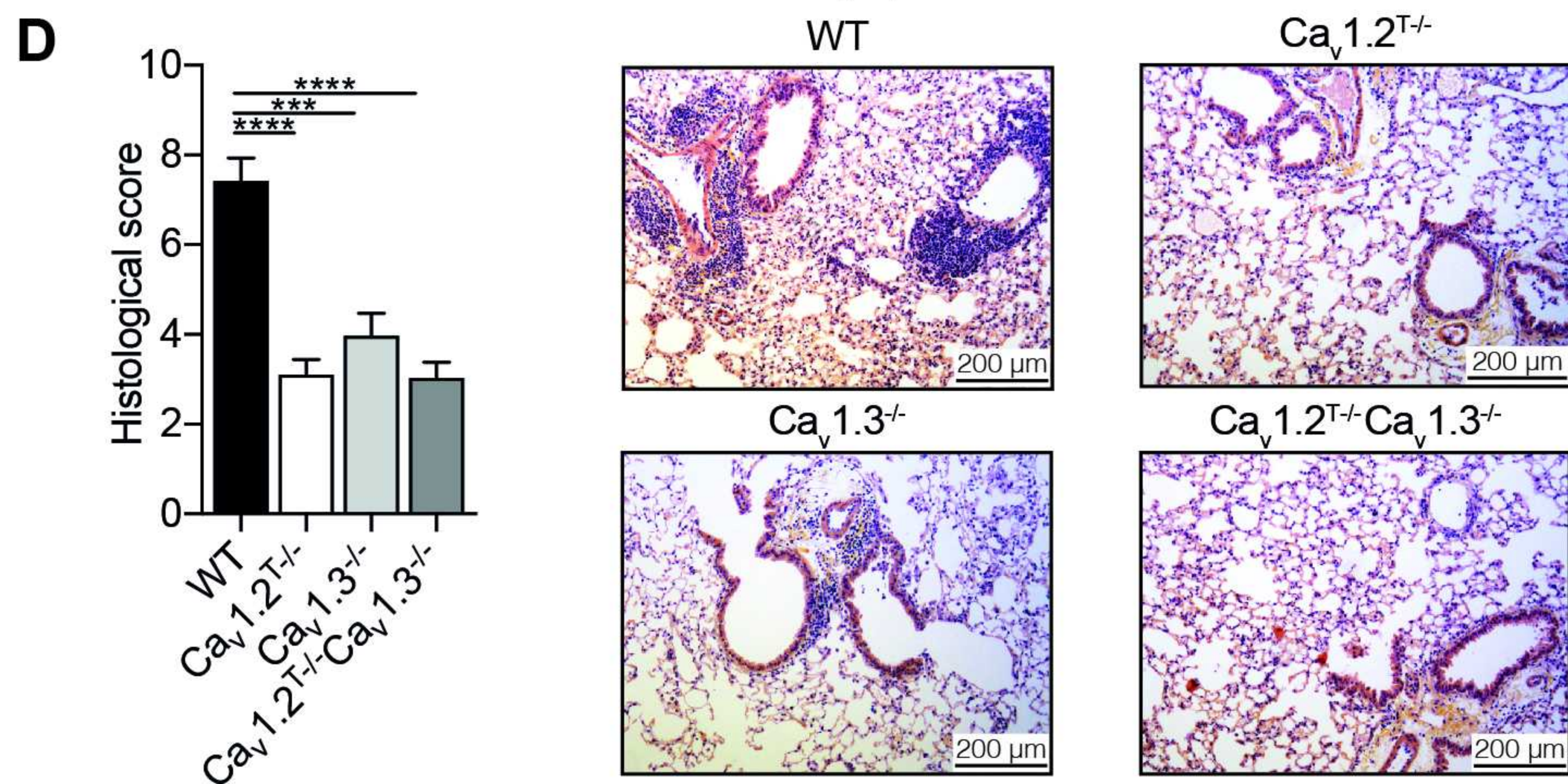
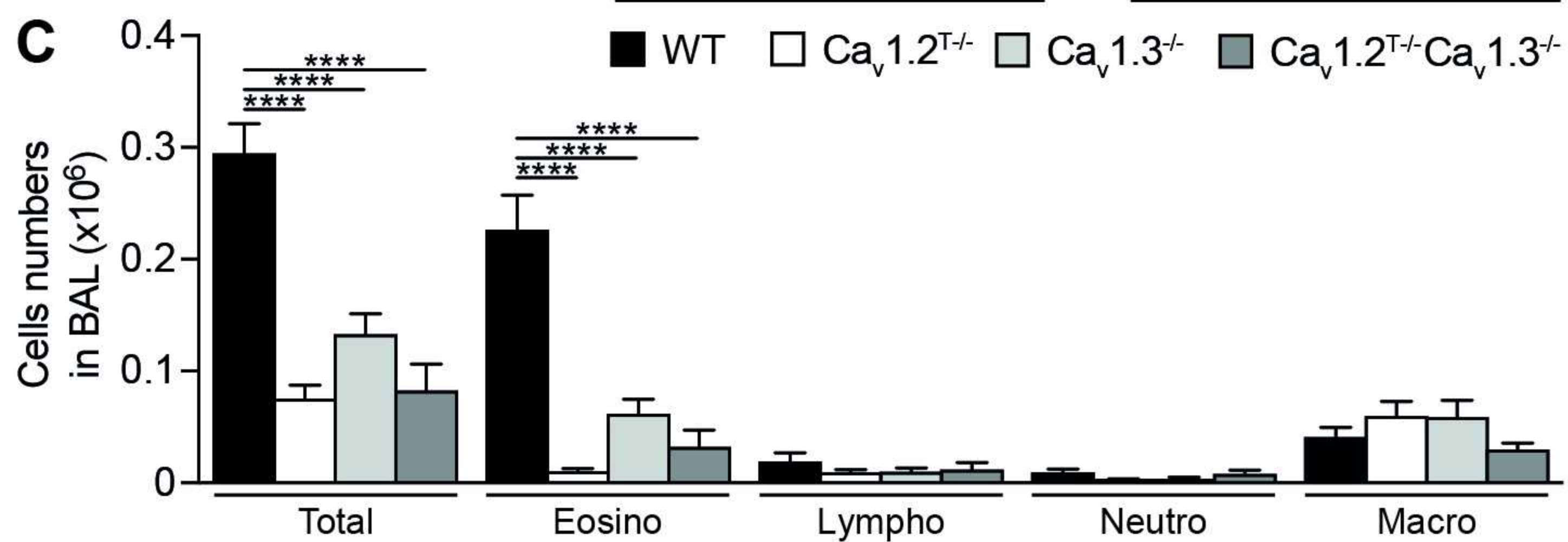
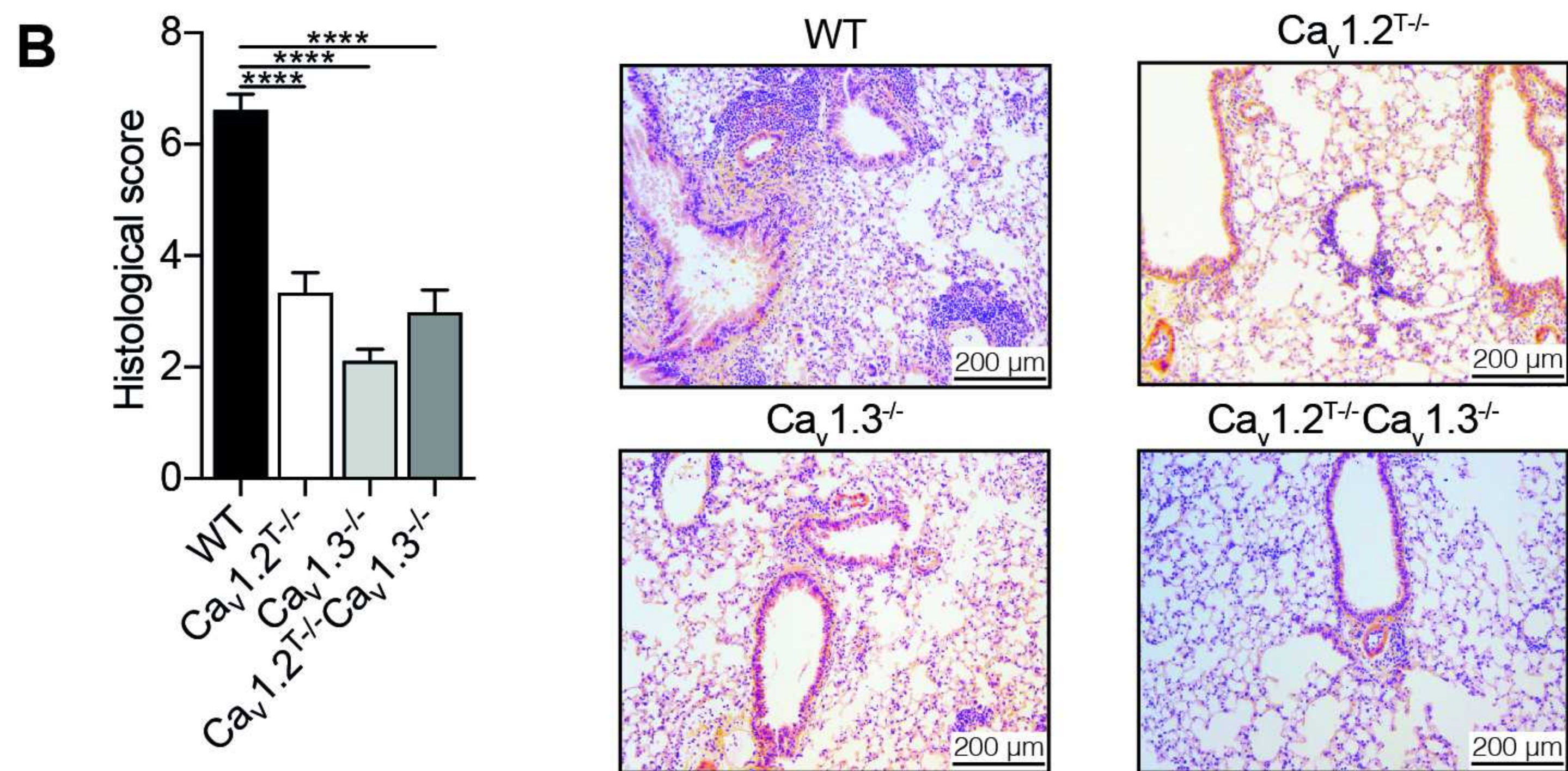
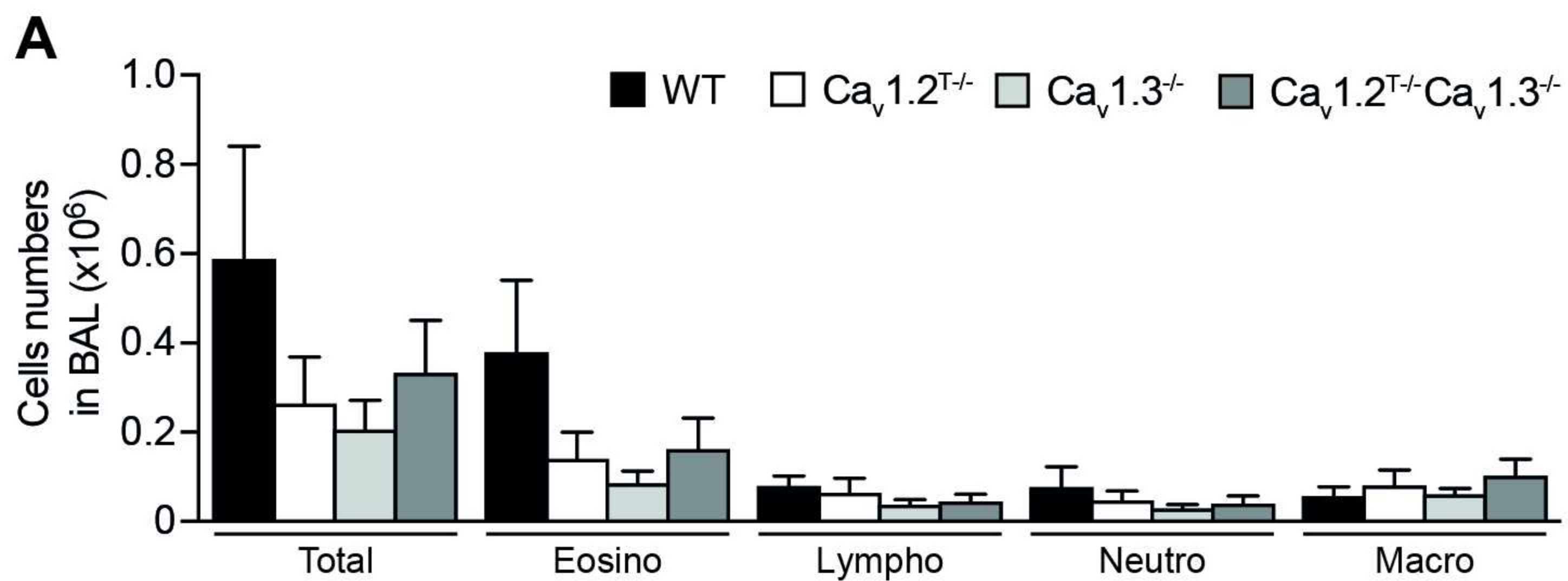












1 **Separation of the Ca_v1.2-Ca_v1.3 calcium channel duo prevents type-2 allergic airway**
2 **inflammation**

3
4 **Short title**

5 Ca_v1.2 and Ca_v1.3, a pas de deux in Th2 cells

6

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28**MATERIAL**

29**Mice**

30 Mice lacking both Ca_v1.2 (in T cells) and Ca_v1.3 (in all the body) were generated by crossing
31 Ca_v1.2^{T-/-} and Ca_v1.3^{-/-} mice between them.

32

33**Airway hyperresponsiveness**

34 Anesthetized and ventilated mice were connected through an endotracheal cannula to a
35 flexiVent system to measure respiratory mechanics (Emka Technologies, Paris, France).
36 After initiating mechanical ventilation, the mouse was paralyzed with an intraperitoneal
37 injection of 0.1 mL of a 10 mg/mL solution of rocuronium bromide. The animal was ventilated
38 at a respiratory rate of 150 breaths/min and a tidal volume of 10 mL/kg against a positive
39 end-expiratory pressure of 3 cm of H₂O. Then mice were exposed to increasing doses of
40 methacholine aerosol generated through a nebulizer containing 0 to 20 mg/mL methacholine
41 in PBS. Dynamic lung resistance was measured with FlexiWare software (Emka
42 Technologies) as previously described¹.

43

44**Cytokine production by lymph node cells**

45 Lung draining lymph nodes were harvested and 6 x 10⁵ cells were stimulated with OVA (300
46 µg/mL) or HDM (100 µg/mL) for 3 days. Cytokine concentrations were measured in the
47 supernatants by ELISA.

48

49**In vitro Th1- and Th2-cell differentiation**

50 Mouse Th1 and Th2 cells were generated by weekly stimulations of OTII CD4⁺ T cells
51 (purified from spleens with Dynabeads® Untouched™ Mouse CD4 Cells Kit, Invitrogen) in
52 the presence of antigen-presenting cells and the 323-339 OVA peptide. Cells were cultured
53 in complete medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Lonza,
54 Allendale, NJ), 1% pyruvate, 1% nonessential amino acids, 2 mmol/L glutamine, 100 U/mL
55 penicillin, 100 mg/mL streptomycin, and 50 µmol/L β-mercaptoethanol) plus appropriate
56 differentiation cocktails: IL-12 (5 ng/mL), IL-2 (5 ng/mL) and anti-IL-4 antibody (11B11, 10 µg/
57 mL) for Th1 and IL-4 (5 ng/mL), IL-2 (5 ng/mL) and anti-IFNγ antibody (XMG1.2, 10 µg/mL)
58 for Th2. Th2 cells were manipulated after two rounds of stimulation when Ca_v1 channels are
59 clearly expressed.

60

61**Naive CD4⁺ T-cell purification**

62 Naive CD4⁺CD25⁻CD62L⁺ T cells were isolated from spleens and lymph nodes by negative
63 selection of CD4⁺CD25⁻ cells using Dynabeads® Untouched™ Mouse CD4 Cells Kit
64 (Invitrogen), accordingly to the manufacturer's instruction. CD4⁺CD62L⁺ T cells were then

65purified by positive selection using CD62L MicroBeads (Miltenyi).

66

67**Retroviral production, transduction of Th2 cells**

68Plat-E cells cultured in DMEM-glutaMax-1 media supplemented with 10% FCS (Lonza), 100
69U/mL penicillin, 100 mg/mL streptomycin, 1mM sodium pyruvate and 50 μ M β -
70mercaptoethanol were harvested from a 60 to 70% confluent culture and transfected with
71either a Cre-IRES-GFP bicistronic vector encoding the Cre recombinase and a GFP reporter
72(referred to as Cre-GFP)², or a plasmid encoding GFP alone (GFP) using Lipofectamine
732000 transfection reagent (Invitrogen). Three days later, the supernatant was harvested,
74centrifuged and filtered on 0.45 μ m before being added to Th2 cell cultures. CD4⁺ T cells
75from Ca_v1.2^{fl/fl} mice were seeded in 96-well plates coated with anti-CD3 antibody (10 μ g/mL)
76plus soluble anti-CD28 antibody (1 μ g/mL) in Th2 differentiation medium. 18 hours after
77stimulation, cells were transduced using Plat-E supernatant complemented with Polybrene
78(10 μ g/mL), IL-4 (5 ng/mL) plus anti-IFN γ (10 μ g/mL) and anti-CD28 (1 μ g/mL) antibodies,
79then plates were centrifuged at 2500 rpm for 90 minutes at 32°C. After 3 additional hours of
80incubation at 32°C, the Plat-E supernatant was discarded and replaced by Th2 differentiation
81medium. Cells were again transduced the day after in a similar way. One day after the 2nd
82transduction, the cells were harvested and sorted based on GFP expression by the FACS
83Aria cell sorter (BD Biosciences) using a 100 μ m nozzle. GFP⁺ cells were put back into
84culture for expansion in Th2 condition medium in 96-well plates coated with anti-CD3
85antibody (10 μ g/mL) plus soluble anti-CD28 antibody (1 μ g/mL) during 4 days to expand
86them. Cytokine production was then assessed after stimulation with coated anti-CD3
87antibody (3 μ g/mL) plus soluble anti-CD28 antibody (1 μ g/mL) for 24h in absence of
88exogenous cytokine.

89

90**Cytometry**

91Single-cell suspensions from thymus, spleen and lymph nodes were obtained by mechanical
92disruption. Red blood cells were lysed with ACK before blood cell staining. Cells were
93blocked with PBS containing 2 mM EDTA, 1% FCS and 5 μ g/mL anti-CD16/CD32 (2.4G2)
94and stained for 30 min on ice with fluorophore-conjugated antibodies. The following
95antibodies were used for phenotyping: anti-TCR- β (H57-597), anti-CD4 (GK1.5), CD8 (56-
966.7), CD19 (1D3), CD44 (IM7), CD62L (MEL-14) from BD Biosciences; Foxp3 (FJK-16s),
97ST2 (RMST2-2) from eBiosciences; CD25 (PC61.5.3) from Thermo Fisher Scientific.
98Intracellular staining of GATA-3 was performed using the Transcription Factor Staining Buffer
99Set (eBioscience) and monoclonal antibodies against GATA-3 (TWAJ, Thermo Fisher
100Scientific). Intracellular cytokine staining for IL-13 (eBio13A, Thermo Fisher Scientific) and IL-
1015 (TRFK5, BD Biosciences) was performed after 4 hours of stimulation with 50 ng/mL PMA

102and 100 ng/mL ionomycin in the presence of 1 µg/mL Brefeldin for the last two hours. In
103passive asthma induced by injection of OVA-transgenic OTII Th2 cells, lungs were cut into
104small fragments and digested for 30 min at 37°C with 1 mg/ml Collagenase III (Worthington
105Biochemical Corporation) and 200 µg/mL DNase I (Sigma-Aldrich). Red blood cells were
106lysed by treatment with hypotonic buffer and then filtered. Single-cell suspensions were
107blocked with PBS containing containing 2 mM EDTA, 1% fetal calf serum and 5 µg/mL anti-
108CD16/CD32 (2.4G2) and stained for 30 min on ice with fluorophore-conjugated antibodies
109following: Fixable Viability dye from Thermo Fischer, CD4 (GK1.5) from eBiosciences, CD45
110(30-F11), TCRβ (H57-597) from BD Biosciences, and Vα2 (B20.1) from Biolegend. Cells
111were analyzed using a Fortessa (BD), and FlowJo software (Tree Star) was used for
112analysis.

113

114**Extraction of genomic DNA and RNA and quantification by qPCR**

115To analyze the deletion of the exon 2 in $Ca_v1.2^{-/-}$ mice³ and the neomycin resistance gene
116insertion in reverse direction into the exon 2 of $Cacna1d^4$ of $Ca_v1.3^{-/-}$ mice at genomic and
117mRNA level, *in vitro* differentiated Th2 cells were lysed and genomic DNA (gDNA) was
118extracted with Tissue spin kit (Macherey-Nagel) and RNA with the RNeasy Mini Kit (Qiagen,
119Hilden, Germany). RNA was extracted from lung tissues using TRIzol (Invitrogen). Reverse
120transcription was performed with the SuperScriptIII Reverse Transcriptase (Invitrogen,
121Carlsbad, Calif). gDNA amplification and transcripts were measured by using real-time
122quantitative PCR with a LightCycler 480 Instrument and the LightCycler 480 SYBR Green I
123Master (Roche, Carlsbad, Calif). Primers for mouse *Il5* and mouse *Il13* were purchased from
124Qiagen and the other primer sequences are given in Table S2. Amounts of gDNA
125amplification were expressed as arbitrary units relative to *Rpl13* as follows: $(2^{-[Ct \text{ interest gene}-Ct$
126 $Rpl13]}) \times 10^{-3}$ and those of mRNA were expressed as arbitrary units relative to HPRT as
127follows: $(2^{-[Ct \text{ interest gene}-Ct \text{ HPRT}]}) \times 10^{-3}$.

128

129**ELISA**

130For *in vitro* cytokine assays, 5×10^4 *in vitro* differentiated Th1 and Th2 cells were seeded
131onto plates and stimulated with biotinylated anti-CD3 antibodies crosslinked with streptavidin
132for 30 minutes at 37°C prior to stimulation (10 µg/mL). Supernatants were collected 24 hours
133later and sandwich ELISA for cytokine quantification was done as previously described⁵. The
134serum IgE quantification was done as previously described⁶. Specific anti-Derf1 IgE were
135assessed by indirect ELISA as previously described⁷.

136

137**Single-cell calcium imaging Intracellular calcium measurements**

138Cells were loaded with 5 µM Fura-2 AM, as previously described⁸ in culture medium

139containing 5% heat-inactivated FBS for 30 minutes at 37°C. Cells were then washed, plated,
140and excited in 10-second intervals by using 340 and 380 nm excitation filters. Emission was
141recorded with 510/540 nm band pass filters by using a CCD camera at the single-cell level.
142To analyze intracellular calcium stores, cells were seeded in culture medium containing 5%
143heat-inactivated FBS and 0.5mM of EGTA and stimulated with thapsigargin (2μM) or
144biotinylated anti-CD3 cross-linked with streptavidin (10 μg/mL). At the end of recording,
145ionomycin (10μM) or CaCl₂ (2mM) was added. The F340/F380 ratio calculated with
146MetaFluor imaging software (Molecular Devices) was indicative of intracellular calcium
147([Ca²⁺]_i). Data are expressed as the F/F₀ ratio (F= F340/F380 in stimulated T cells and F₀ =
148F340/F380 under basal conditions). For each cell, we calculated the area under the curve
149between the stimulation and ionomycin or CaCl₂ application with the GraphPad Prism
150software. The time of response was defined as the delay for which the fluorescence ratio
151becomes superior to the mean basal + 1 SD.

152

153**Total internal reflection fluorescence (TIRF) microscopy**

154TIRFM is presently the best technique to image Ca²⁺ at the mouth of the calcium channel. In
155this technique an excitation light with a critical angle is sent to the specimen, triggering a total
156internal reflection at the interface glass/medium. An evanescent wave is generated over less
157than 100 nm from the interface, sufficient to excite fluorophores located in this region. This
158provides a powerful tool to study spatiotemporal single elementary calcium events (ECE)
159likely due to the activity of one or the cluster of Ca²⁺ channels. This technique mainly
160developed by Demuro and Parker⁹ was used here to study the impact of Ca_v1 deficiency in
161Th2 lymphocytes. Cells were loaded with 5 μM Fluo-5 AM in culture medium containing 5%
162heat-inactivated FBS and 100 μM EGTA-AM buffering intracellular calcium increase at the
163mouth channel for 30 minutes at 37°C. Cells were then washed, plated (5x10⁵/mL, 200
164μL/well in Labtek chambers) in non-fluorescent, CO₂-independent medium for Ca²⁺ imaging
165(pH 7.3), containing 20 mM CaCl₂, 0.81 mM MgSO₄, 5.37 mM KCl, 0.44 mM KH₂PO₄, 137
166mM NaCl, 0.34 mM Na₂HPO₄, 5.55 mM D-glucose, 2 mM L-glutamine, 2 mM sodium
167pyruvate, 20 mM Hepes (adapted from¹⁰). We have recorded elementary Ca²⁺ events using
168Nikon TIRF microscope (Nikon Eclipse Ti) system equipped with a Nikon 60X TIRF objective
169(NA 1.49). Excitation was produced by an expended beam of an Argon ion laser (488 nm).
170Emitted light was collected at 510nm and recorded with a cooled (-80°C) back illuminated
171EMCCD camera (Andor iXon). The full frame was 128 x 128 pixels with 1 pixel = 0.3 μm at
172the lymphocyte membrane and we considered only the central part (64 x 64 pixels) of the
173lymphocyte that was "TIRFed" for analysis (which represents around 30% of the Th2-
174lymphocyte surface). The frame rate was 500 Hz. Images were recorded every 2 ms for 10
175sec (5000 images) before stimulation and for 2 min (60000 images) after stimulation with

176biotinylated anti-CD3 cross-linked with streptavidin at 10 µg/mL). After subtraction of the
177background on the stack of images and treatment with a low-pass filter (4 x 4 median filter),
178the analysis of elementary Ca^{2+} events is performed with a macro written on ImageJ. The
179whole surface of the specimen is scanned in the 2 dimensions by displacement of a window
180of 2 x 2 pixels. The increase in fluorescence is taken positive if it was above a threshold
181corresponding of 2 SD of the intrinsic noise and stored in a table. This procedure is done for
182each image of the stack, giving the individual fluctuations of fluorescence as function of time.
183We took into account only changes observed during at least two consecutive recordings. The
184kinetic in fluorescence was given as F/F_0 where F_0 corresponds to the mean of fluorescence
185recorded on the 100 first images before stimulation. In order to determine whether the
186deletion of Ca_v1 channels in Th2 cells modifies the opening frequencies, we determined them
187over a period of 4 s (2000 consecutive images) as soon as a channel opens (30 s after
188stimulation), and results were expressed in events/second. All these results were robust
189because they were repeated in four (WT versus $\text{Ca}_v1.2^{-/-}$ Th2 cells) and three *in vitro* Th2
190differentiated independent experiments (WT versus $\text{Ca}_v1.3^{-/-}$ Th2 cells).

191

192Phosphotyrosine profile

193Th2 cells (5×10^6) were stimulated or not at 37 °C with with biotinylated anti-CD3 (145-2C11,
194Biolegend) and biotinylated anti-CD4 (GK1.5, Biolegend) cross-linked with streptavidin
195(Thermo scientific) (30 µg/mL). Stimulation was stopped by adding cold PBS and
196centrifugation. Protein extract is prepared with lysis buffer (20 mM Tris, pH 7.5, 150 mM
197NaCl, 4 mM EDTA, 1% Triton X100) supplemented with phosphatase inhibitors (2 mM
198 Na_3VO_4 and 10 mM NaF) and protease inhibitors (Roche Diagnostic). After 30 min of
199incubation on ice, cell lysates were centrifuged at 16000 g for 15 min at 4°C. Postnuclear
200lysates were submitted to SDS-PAGE on precast 4-15% gradient gels (Bio-Rad) and
201transferred to Amersham Hybond 0.45 µm PVDF membranes (GE Healthcare), and then
202blotted with phosphorylated tyrosine (PY20; BD Biosciences) primary antibody. We then
203stained blots with horseradish peroxidase-coupled secondary antibodies (Cell Signaling
204Technology, Danvers, Mass) before detection and visualization with the ECL Prime
205(Amersham) using a ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, Calif). The
206membrane was stripped and incubated with an anti-actin antibody (clone AC-15;
207MilliporeSigma). Densitometric analysis was performed with the Image Lab 5.0 software
208(Bio-Rad).

209

210Subjects and blood samples

211This study enrolled 26 children with mild to moderate asthma and no asthmatic symptoms at
212the time of inclusion (mean age: 9.4 ± 2.1 years) and 17 age-matched controls with no

history of allergy or respiratory disease (mean age: 10.2 ± 1.6 years old). We have chosen to study asthmatic children and not adults to avoid the context of long-term illnesses. All the parents or guardians of the children signed an informed consent. The study protocol was approved by the hospital Ethics Committee. The characteristics of asthmatic children are reported in **Table Suppl. 1**. Asthmatic children had a forced expiratory volume in 1 second (FEV) without treatment of 89 ± 16.7%. A hyper eosinophilia >0.5 G/L was measured in 19/26 patients. Total serum IgE concentration was found high (125-3489 kUI/L) in 25/26 asthmatic children. Specific IgE concentrations directed against at least one of the allergens tested (D1, dermatophagoides pteronyssinus; G3, Dactylis glomerata and M6, Alternaria alternata) were detected in 24/26 asthmatics. PBMC were isolated from the blood samples and CD4⁺ T-cells were isolated with the CD4-untouched isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). After 6 hours of stimulation with anti-CD3/anti-CD28-coated beads (1 bead per 4 cells), total RNA was isolated with a kit from Qiagen (Hilden, Germany) and retrotranscribed in cDNA by means of reverse transcription with SuperScript III (Invitrogen). The primer sequences are given in Table S2. Amounts of mRNA, measured by using real-time quantitative PCR with a LightCycler 480 Instrument and the LightCycler 480 SYBR Green I Master (Roche, Carlsbad, CA), were expressed as arbitrary units relative to *GAPDH* as follows: $(2^{-[Ct \text{ interest gene} - Ct \text{ GAPDH}]}) \times 10^{-3}$.

231

232References

- 2331 Bouchaud, G. *et al.* Prevention of allergic asthma through Der p 2 peptide
 234 vaccination. *J Allergy Clin Immunol* **136**, 197-200 e191,
 235 doi:10.1016/j.jaci.2014.12.1938 (2015).
- 2362 Viret, C., Mahiddine, K., Baker, R. L., Haskins, K. & Guerder, S. The T Cell
 237 Repertoire-Diversifying Enzyme TSSP Contributes to Thymic Selection of
 238 Diabetogenic CD4 T Cell Specificities Reactive to ChgA and IAPP Autoantigens. *J*
 239 *Immunol* **195**, 1964-1973, doi:10.4049/jimmunol.1401683 (2015).
- 2403 White, J. A. *et al.* Conditional forebrain deletion of the L-type calcium channel Ca V
 241 1.2 disrupts remote spatial memories in mice. *Learn Mem* **15**, 1-5 (2008).
- 2424 Platzter, J. *et al.* Congenital deafness and sinoatrial node dysfunction in mice lacking
 243 class D L-type Ca²⁺ channels. *Cell* **102**, 89-97 (2000).
- 2445 Savignac, M. *et al.* Protein kinase C-mediated calcium entry dependent upon
 245 dihydropyridine sensitive channels: a T cell receptor-coupled signaling pathway
 246 involved in IL-4 synthesis. *Faseb J* **15**, 1577-1579 (2001).
- 2476 Laffont, S. *et al.* Androgen signaling negatively controls group 2 innate lymphoid cells.
 248 *J Exp Med* **214**, 1581-1592, doi:10.1084/jem.20161807 (2017).

2497 Bihouee, T. *et al.* Food allergy enhances allergic asthma in mice. *Respir Res* **15**, 142,
250 doi:10.1186/s12931-014-0142-x (2014).

2518 Rosa, N. *et al.* The beta and alpha2delta auxiliary subunits of voltage-gated calcium
252 channel 1 (Cav1) are required for TH2 lymphocyte function and acute allergic airway
253 inflammation. *J Allergy Clin Immunol* **142**, 892-903 e898,
254 doi:10.1016/j.jaci.2017.09.045 (2018).

2559 Demuro, A. & Parker, I. "Optical patch-clamping": single-channel recording by
256 imaging Ca²⁺ flux through individual muscle acetylcholine receptor channels. *J Gen*
257 *Physiol* **126**, 179-192, doi:10.1085/jgp.200509331 (2005).

25810 Nauli, S. M. *et al.* Polycystins 1 and 2 mediate mechanosensation in the primary
259 cilium of kidney cells. *Nat Genet* **33**, 129-137, doi:10.1038/ng1076 (2003).

260 **Figure legends**

261

262 **Figure S1. Disruption of *Cacna1c* and *Cacna1d* genes in Th2 cells**

263 WT (black bars), $\text{Ca}_v1.2^{\text{T-/-}}$ (white bars) and $\text{Ca}_v1.3^{\text{-/-}}$ (grey bars) and CD4^+ T cells were
264 cultured in Th2-polarizing conditions. **(A-D)** The deletion of *Cacna1c* and *Cacna1d* genes
265 was assessed at the genomic (gDNA, **A and C**) and mRNA (**B and D**) levels in *in vitro*
266 differentiated Th2 lymphocytes by qPCR. **(E-F)** Expression of transcripts encoding *Orai1* (**E**),
267 *Stim1* and *Stim2* (**F**) was determined by RT-qPCR. The results are expressed as the mean +
268 SEM of 6-7 mice. ** $p < 0.01$, and *** $p < 0.005$ (Mann-Whitney test).

269

270 **Figure S2. The development of immune cells is normal in $\text{Ca}_v1.2^{\text{T-/-}}$ and $\text{Ca}_v1.3^{\text{-/-}}$ mice**

271 Flow-cytometric analysis of cells from thymus, spleen and lymph nodes of WT (black bars),
272 $\text{Ca}_v1.2^{\text{T-/-}}$ (white bars) and $\text{Ca}_v1.3^{\text{-/-}}$ (grey bars) mice. Representative dot-plots **(A and C)** as
273 well as the absolute numbers of each subpopulation **(B and D)** are shown. Data are
274 representative of three independent experiments and the results are expressed as mean +
275 SEM of 4-7 mice.

276

277 **New Figure S3. The development of immune cells is normal in double ($\text{Ca}_v1.2^{\text{T-/-}}$ and 278 $\text{Ca}_v1.3^{\text{-/-}}$) deficient mice**

279 Flow-cytometric analysis of cells from spleen and lymph nodes of WT (black bars) and
280 double deficient ($\text{Ca}_v1.2^{\text{T-/-}}$ and $\text{Ca}_v1.3^{\text{-/-}}$) (white bars) mice. Absolute numbers of each
281 subpopulation are shown. The results are expressed as mean + SEM of 3 mice.

282

283 **New Figure S4. The frequency of ST2^+ memory CD4^+ T cells is normal in $\text{Ca}_v1.2^{\text{T-/-}}$ and 284 $\text{Ca}_v1.3^{\text{-/-}}$ mice**

285 Flow-cytometric analysis of cells from blood of WT (black bars), $\text{Ca}_v1.2^{\text{T-/-}}$ (white bars) and
286 $\text{Ca}_v1.3^{\text{-/-}}$ (grey bars) mice. Histograms show the frequency of ST2-expressing cells among
287 $\text{CD62L}^-\text{CD44}^+\text{CD4}^+$ T cells. Data are representative of two independent experiments and the
288 results are expressed as mean + SEM of 4-6 mice.

289

290 **Figure S5. The absence of $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ in CD4^+ lymphocytes does not preclude T- 291 cell development and Th2-cell differentiation**

292 WT (black bars), $\text{Ca}_v1.2^{\text{T-/-}}$ (white bars) and $\text{Ca}_v1.3^{\text{-/-}}$ (grey bars) CD4^+ T cells were cultured in
293 Th2-polarizing conditions. **(A)** Expression of GATA3 was determined by flow cytometry.
294 Histograms show the frequency of GATA3-expressing cells and the geometric mean
295 fluorescence intensity of GATA3 on cells. Data are expressed as the mean + SEM of 4-9
296 mice and representative of 2-3 independent experiments. **(B)** Intracellular production of IL-13

297and IL-5 after PMA/ionomycin stimulation from the indicated mice, as determined by flow
298cytometry. Frequency of cells producing IL-5 and IL-13 and the geometric mean fluorescence
299intensity of each cytokine are shown. Data are expressed as the mean + SEM of 7-12 mice
300and representative of 3-4 independent experiments.

301

302**Figure S6. Intracellular calcium store mobilization is normal in $Ca_v1.2$ or $Ca_v1.3$ -**
303**deficient Th2 cells**

304 $Ca_v1.2^{T/-}$ (**A, C, E and G**) and $Ca_v1.3^{-/-}$ (**B, D, F and H**) OTII T-cells were differentiated in Th2
305cells and loaded with Fura-2 AM. $[Ca^{2+}]_i$ variations were determined by recording the
306fluorescence ratio (F340/F380) at 340 and 380 nm excitation wavelengths at the single-cell
307level in Fura2-AM-loaded WT (black lines and circles), $Ca_v1.2^{T/-}$ (grey lines and white circles)
308and $Ca_v1.3^{-/-}$ (grey lines and white circles) Th2 cells in medium containing EGTA before (F0)
309and after (F) addition of thapsigargin (TG) (**A-D**) or cross-linked anti-CD3 (anti-CD3) mAb (**E-**
310**H**). $CaCl_2$ was then added to permit store operated calcium entry. Data are expressed as the
311mean + SEM (150 cells per genotype) and are representative of Th2 cells differentiated from
3123 mice per genotype. (**C-D and G-H**) Area under the curve was measured between the time
313of TG or anti-CD3 stimulation and time of $CaCl_2$ addition.

314

315**Figure S7. TCR-induced calcium response and cytokine production are not affected in**
316***in vitro* differentiated $Ca_v1.2$ - or $Ca_v1.3$ -deficient Th1 cells**

317WT, $Ca_v1.2^{T/-}$ and $Ca_v1.3^{-/-}$ $CD4^+$ T cells were cultured in Th1-polarizing conditions. (**A-B**) *In*
318*vitro* differentiated Th1 cells were loaded with Fura-2 AM. The fluorescence ratio at 340 and
319380 nm excitation wavelengths was recorded at the single-cell level before (F0) and after
320stimulation (F) with cross-linked anti-CD3 mAb (anti-CD3). Ionomycin (Iono) was added at
321the end of the experiment. Data are expressed as the mean of the F/F0 + SEM (90-150 cells
322per genotype) and are representative of differentiated Th1 cells from 3 mice per genotype.
323The area under the curve between the time of stimulation and the time of ionomycin addition
324was depicted. Data from all the analysed cells were pooled. (**C**) Cytokine production in *in*
325*vitro* differentiated Th1 cells after 24h of cross-linked anti-CD3 mAb (anti-CD3) stimulation
326was determined by ELISA. Results were normalized to WT Th1 cells from the same
327experiment and were pooled from 3-10 mice of 3-5 independent experiments. Results were
328compared by unpaired Student's t test (**A-B**) and Wilcoxon matched-pairs test (**C**).

329

330**New Figure S8. $Ca_v1.2$ - and $Ca_v1.3$ -deficient Th2 cells are hypo-responsive**

331WT, $Ca_v1.2^{T/-}$ and $Ca_v1.3^{-/-}$ $CD4^+$ T cells were cultured in Th2-polarizing conditions. (**A**)
332Th2 cells were stimulated with biotinylated anti-CD3/anti-CD4 crosslinked with streptavidin
333for 2 min (+) or not (-). Equal amounts of lysate proteins were blotted and probed with

antibody against phosphorylated tyrosine (P-Tyr) or β -actin (Actin, loading control). Graphs show the quantification of staining intensities normalized to actin intensity. **(B)** Cytokine production in *in vitro* differentiated Th2 cells after 24h of cross-linked anti-CD3 mAb (anti-CD3) stimulation was determined by ELISA. Results were pooled from 6-14 mice of 3 independent experiments. * $p < 0.05$ (Wilcoxon matched-pairs test).

339

New Figure S9. HDM-specific allergic airway inflammation is diminished in $Ca_v1.2$ - or $Ca_v1.3$ -deficient bone marrow chimeras at early time points

C57BL/6 mice were irradiated the day before reconstitution with bone marrow cells from WT (black bars), $Ca_v1.2^{T-/}$ (white bars) or $Ca_v1.3^{-/-}$ (light grey bars) mice. Eight weeks after, HDM-specific allergic airway inflammation was induced and mice were sacrificed 3 days after the last challenge. **(A)** Number of cells in the BAL fluid. **(B)** Representative HE and PAS-stained sections are shown. The histological score was determined. Results are expressed as means + SEM of 7-9 mice. **** $p < 0.001$, *** $p < 0.005$, ** $p < 0.01$ (ANOVA followed by Kruskal-Wallis multiple comparisons test).

349

Figure S10. OVA-specific allergic airway inflammation is strongly diminished in $Ca_v1.2^{T-/}$ mice

(A) WT (black bars), $Ca_v1.2^{T-/}$ (white bars) mice were immunized with OVA in alum intraperitoneally (ip) at days 0 and 14 and challenged 15 days later with intranasal OVA every day for 5 days. **(B)** Number of cells in the BALF. **(C)** HE-stained sections from mice were scored and mucus was visualized by PAS staining. **(New D)** Th2 cytokine expression in lung draining lymph node after OVA stimulation by ELISA. **(E)** Serum IgE concentrations were measured by ELISA at day of sacrifice. Results are means $\pm$ SEM of 4-7 mice and representative of 4 independent experiments. * $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney test).

359

Figure S11. Deficiency of $Ca_v1.3$ in the hematopoietic compartment protects from OVA-induced type-2 airway inflammation

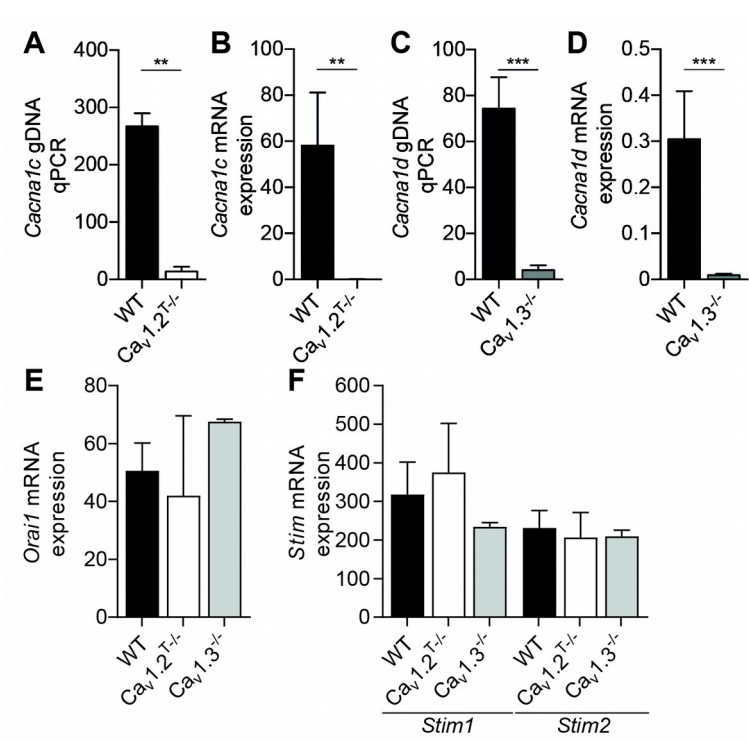
C57BL/6 mice were irradiated the day before reconstitution with bone marrow cells from WT (black bars) or $Ca_v1.3^{-/-}$ (grey bars) mice. Eight weeks after, OVA-specific allergic airway inflammation was induced. **(A)** Number of cells in the BAL fluid. **(B)** Representative HE- and PAS-stained sections are shown. The histological score was determined. **(New C)** Th2 cytokine expression in lung draining lymph node after OVA stimulation by ELISA. **(D)** Serum IgE concentrations were measured by ELISA. Results are expressed as means + SEM of 4-7 mice and representative of 2 independent experiments. * $p < 0.05$ (Mann-Whitney test).

369

**370New Figure S12. HDM- and OVA-specific allergic airway inflammation is strongly
371diminished in mice deficient for both $Ca_v1.2$ and $Ca_v1.3$**

372C57BL/6 mice were irradiated the day before reconstitution with bone marrow cells from WT
373(black bars), $Ca_v1.2^{T-/-}$ (white bars) or $Ca_v1.3^{-/-}$ (light grey bars) or $Ca_v1.2^{T-/-}$ and $Ca_v1.3^{-/-}$ (dark
374grey bars) mice. Eight weeks after, HDM- (**A-B**) or OVA- (**C-D**) specific allergic airway
375inflammation was induced. (**A-C**) Number of cells in the BAL fluid. (**B-D**) Representative HE-
376stained sections are shown. The histological score was determined. Results are expressed
377as means + SEM of 4-7 mice. *** $p < 0.005$, **** $p < 0.001$ (ANOVA followed by Tukey's multiple
378comparisons test).

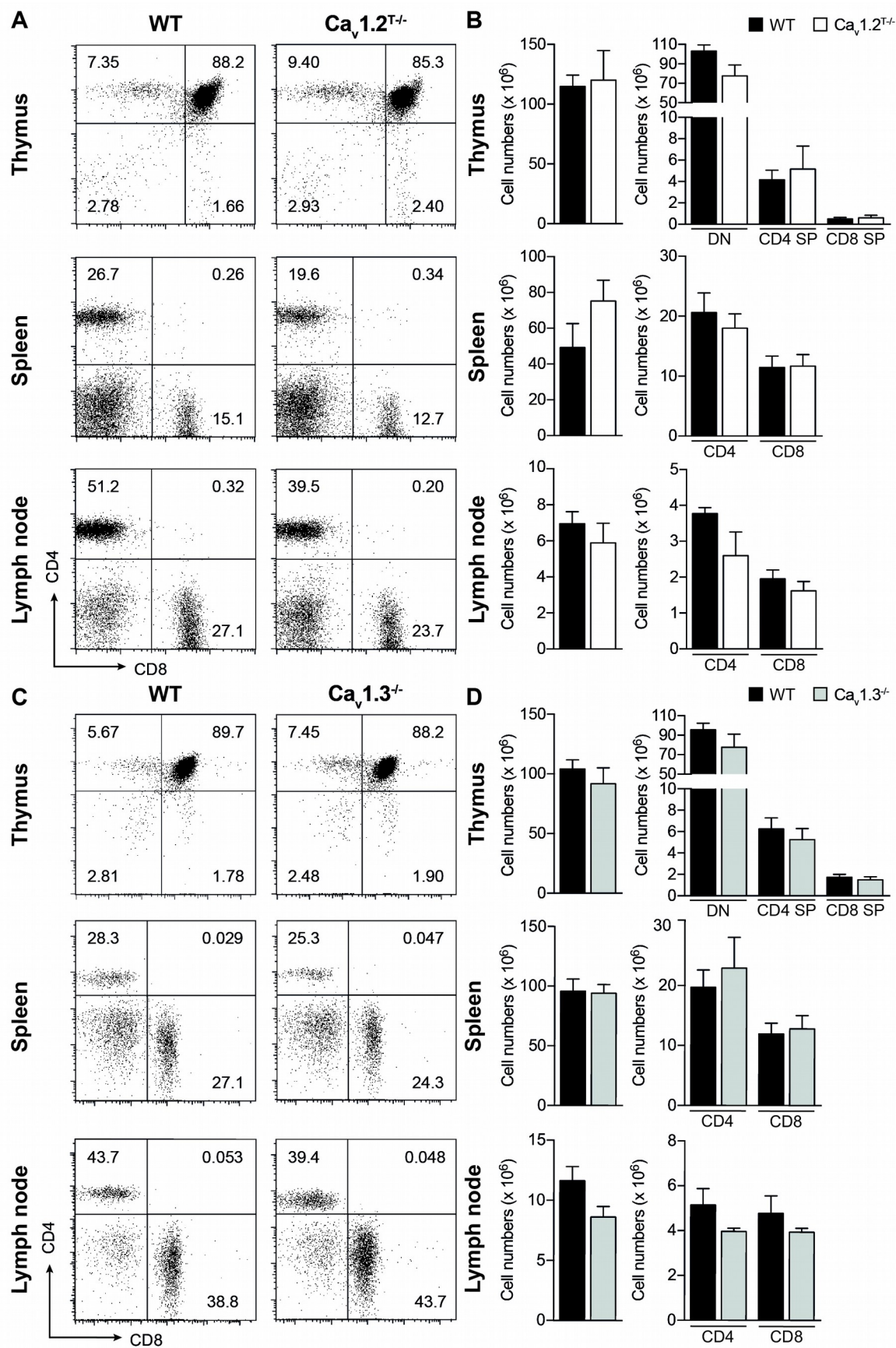
379**Figure S1**



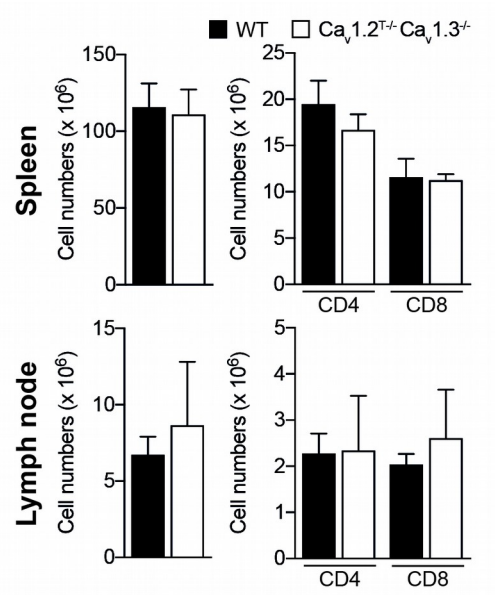
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382Figure S2



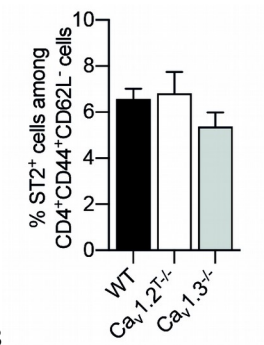
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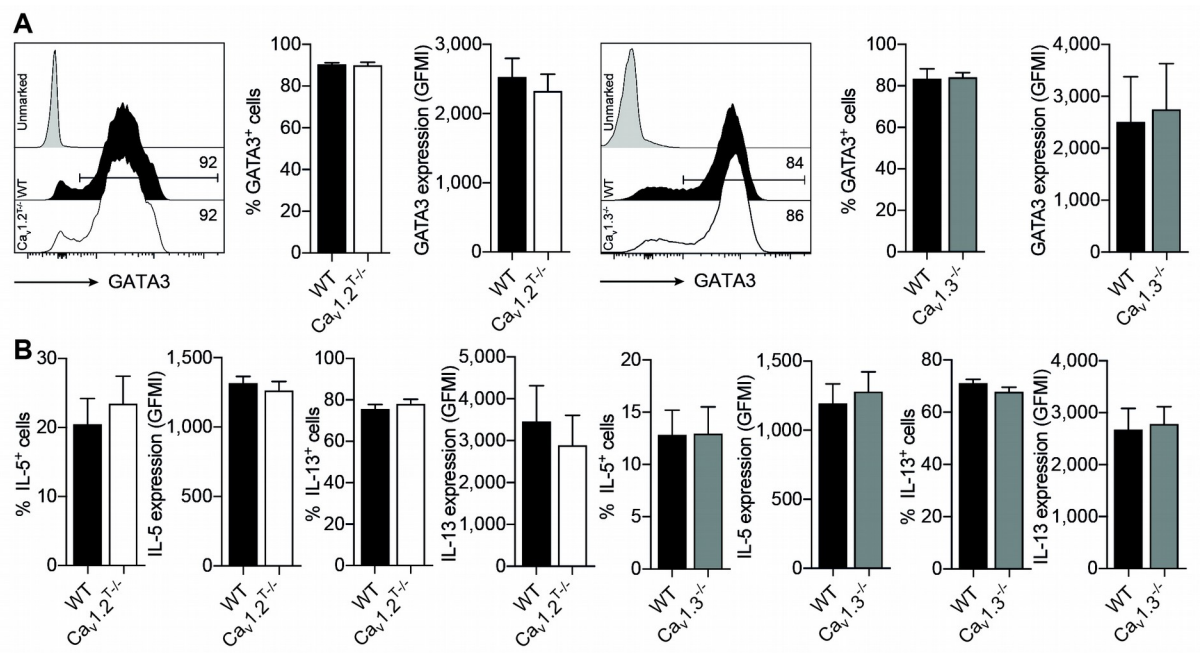
387New Figure S4



388

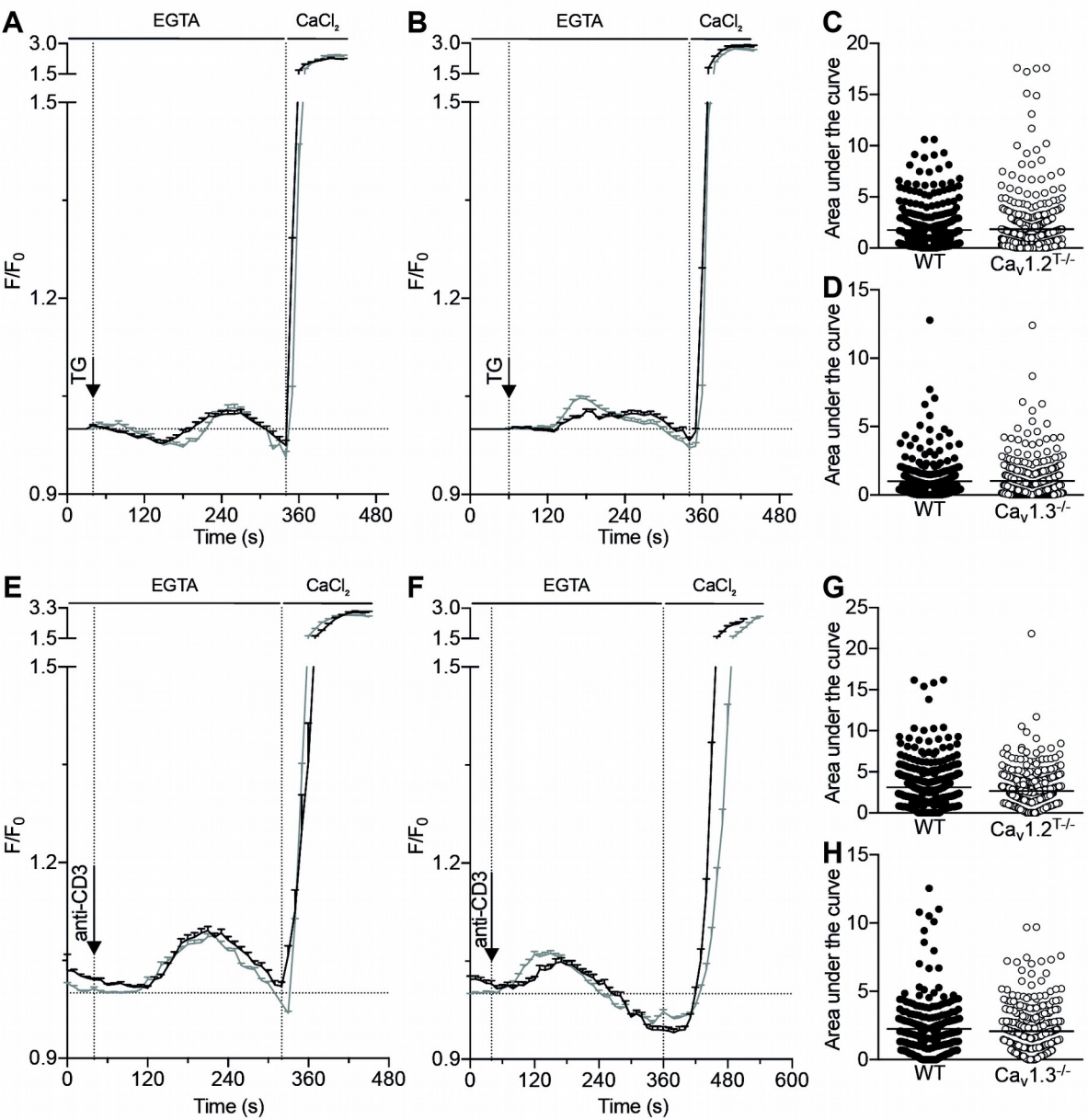
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390Figure S5



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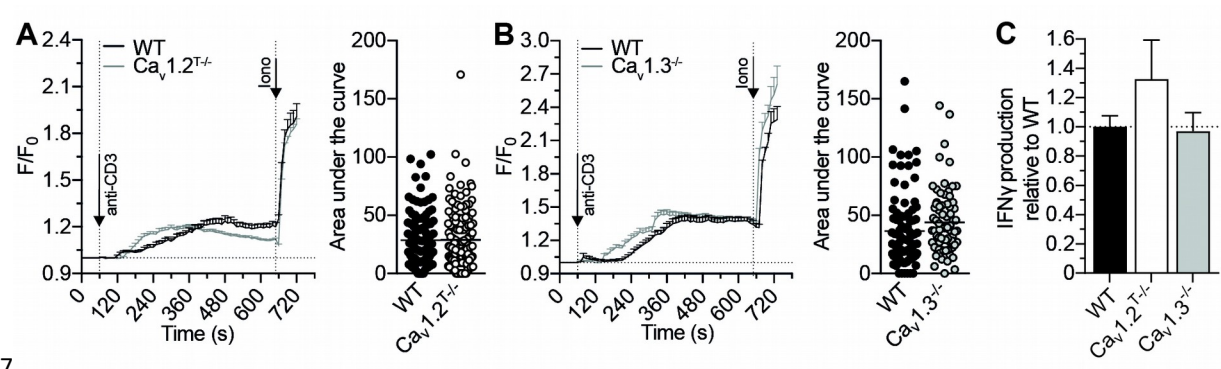
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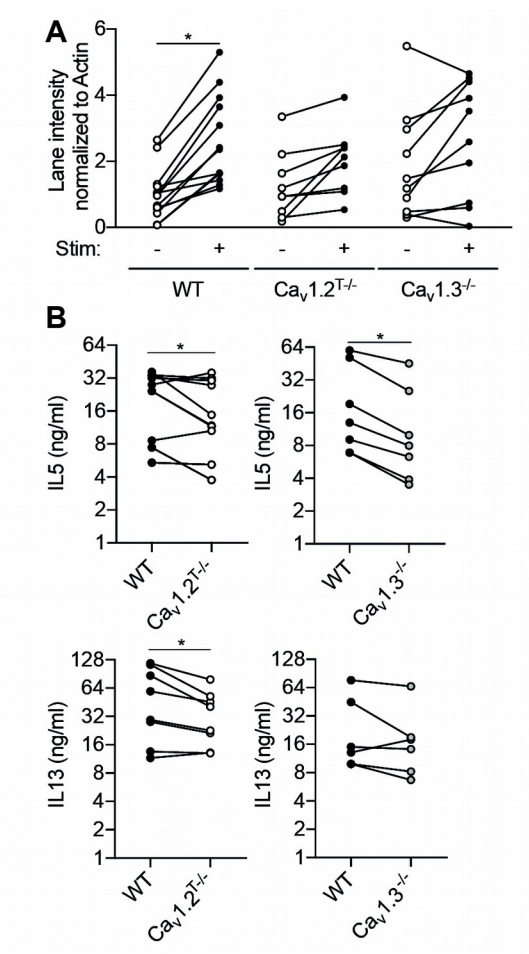
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396Figure S7



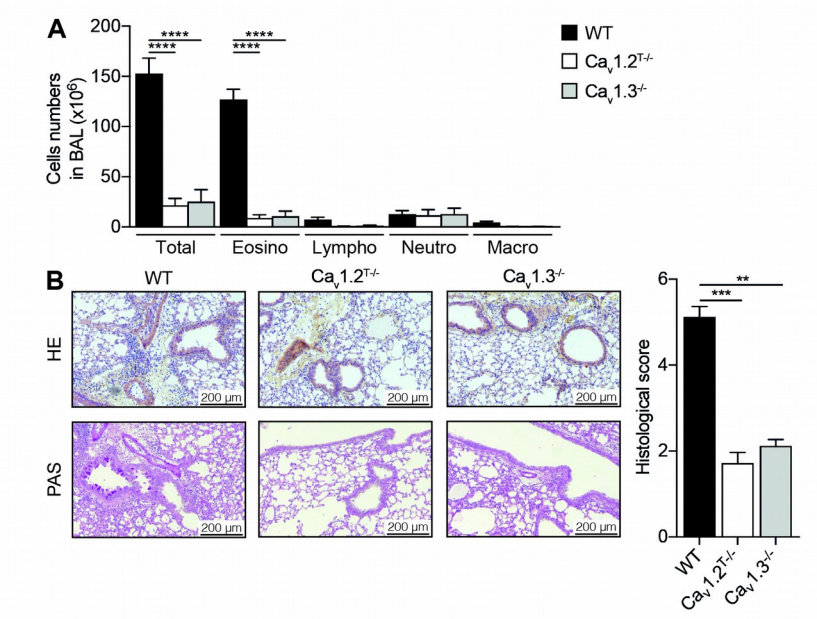
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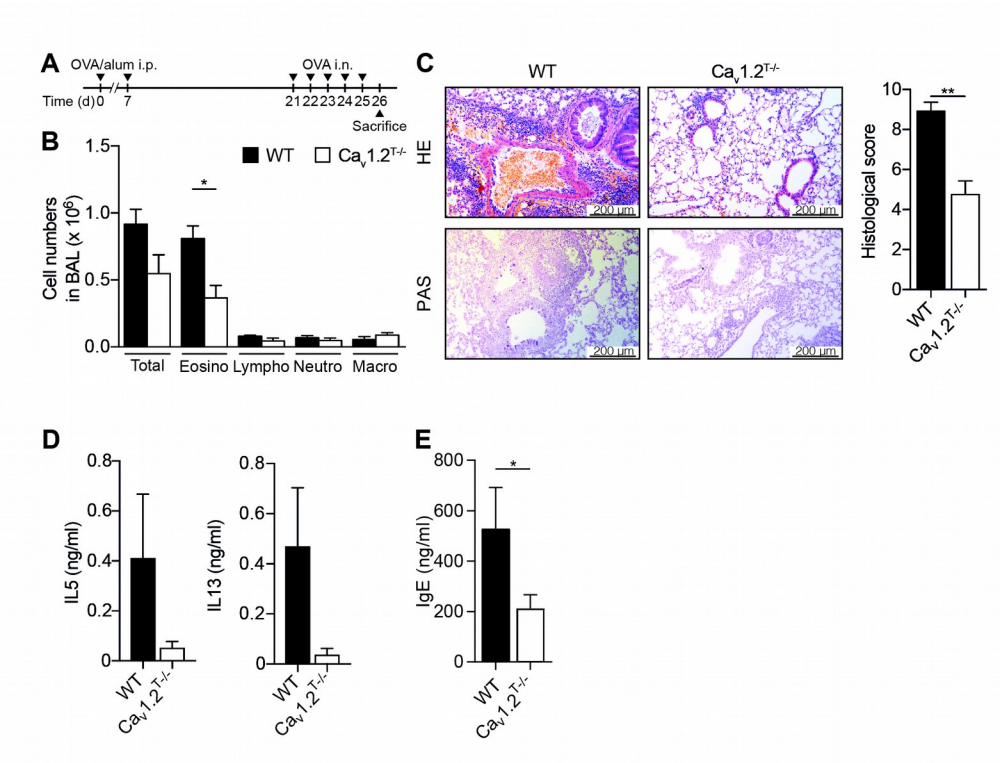
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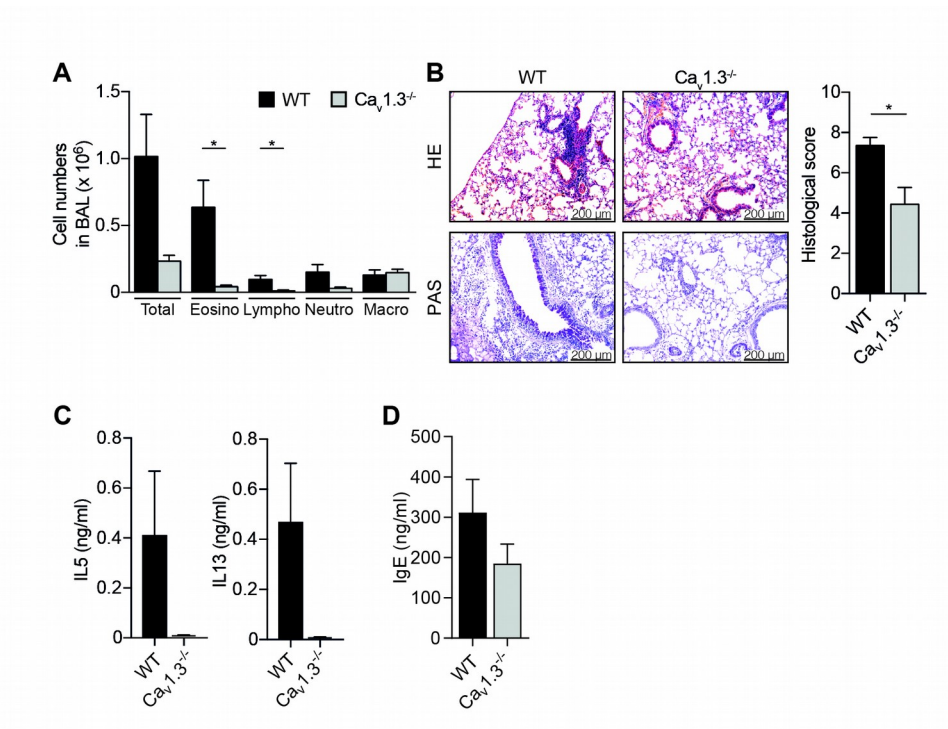
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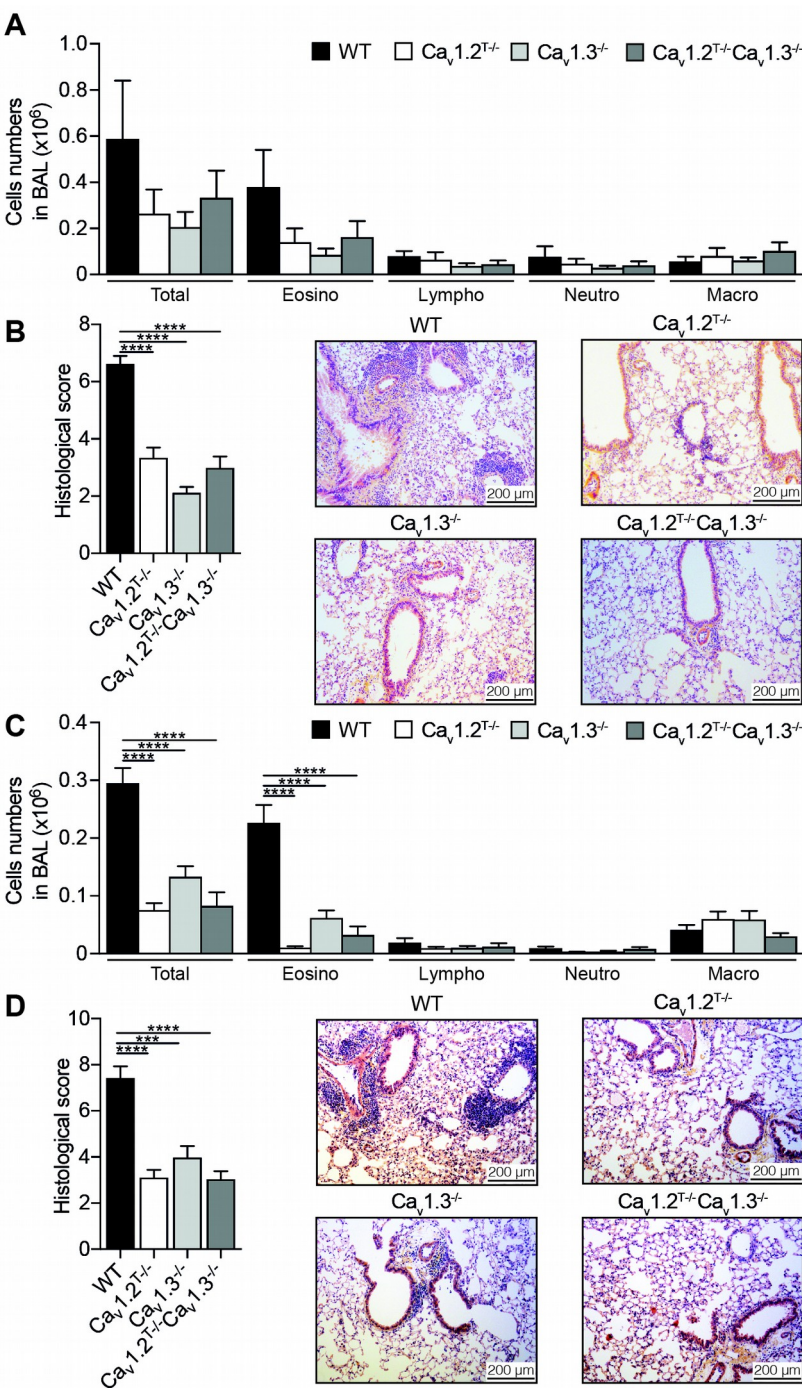


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404







410

411**Table S1. Characteristics of the asthmatic children**

412

Age (years)	Sex	FEV* (%)	Eosinophils G/l	Total IgE (kUI/L)	D1-specific IgE (kUA/L)	G3-specific IgE (kUA/L)	M6-specific IgE (kUA/L)
9.9	m	98	0.3	2005	54.4	>100	0.37
9	f	103	0.7	711	0.89	1.67	2.3
9.1	f	86	0.5	1080	/	/	/
6.8	m	125	0.1	125	0.17	<0.1	31.4
10.2	m	89	1.1	/	1.5	>100	0.22
11.9	f	83	0.3	1077	>100	4.83	<0.1
12.6	m	88	0.9	2248	>100	33.3	0.16
8.2	m	100	0.8	825	>100	41.6	0.22
6.8	f	72	2.6	829	/	/	/
10.8	f	66	0.5	941	>100	0.13	0.1
8.8	m	83	0.5	224	<0.1	39.2	18.7
7.8	m	85	0.2	513	98,6	41.1	<0.1
12.4	m	72	0.8	609	98,8	16	<0.1
7	m	90	0.9	474	>100	<0.1	<0.1
6.6	m	80	0.8	813	81.5	22.4	0.13
7.1	m	112	0.5	816	86.7	0.74	<0.1
11	m	61	0.7	1064	>100	0.15	<0.1
6.6	m	98	0.8	410	83.9	<0.1	<0.1
7.2	m	96	1	465	>100	0.24	<0.1
10.6	m	115	0.8	1969	0.14	0.28	0.11
9.1	m	90	1.4	3489	>100	32.5	0.18
12.5	m	66	0.6	1328	>100	2.43	<0.1
13.5	m	94	0.3	859	1,95	99.8	25.4
9.2	f	75	0.4	464	>100	<0.1	<0.1
11.7	m	67	0.3	515	72	59.9	<0.1
7.6	m	119	0.6	580	65.4	3.7	12.4

413

414*FEV=forced expiratory volume in 1 second

415 **Table S2. List of primers used**

416

Gene	Forward (5'-3')	Reverse (5'-3')
Mouse		
<i>Cacna1c</i> gDNA	ggcaacgcaaccatctctac (exon 2)	gatagggttcttcagggtcaga (exon 2)
<i>Cacna1d</i> gDNA	gctccaagcaaactgtcctatc (exon 2)	tttgctcttggcgtattgct (exon 2)
<i>Rpl13</i>	gaggtcgggtggaagtacca	tgcactctggccttttcctt
<i>Hprt</i>	ctggtgaaaaggacctctcg	tgaagtactcattatagtcaaggga
<i>Cacna1c</i> mRNA	tgacctgaagaaccctatcctcta (exon 2)	tattccctttccggaagac (exon 3)
<i>Cacna1d</i> mRNA	caccaagaggacctatgcgaa (exon 1)	cttggcctgtctagcagcat (exon 2)
<i>Orai1</i>	cctggcgcaagctctactta	ccctgggtgggtagtcattg
<i>Stim1</i>	gtggatgacatggatgaggag	ggaatgggtcaaataccctct
<i>Stim2</i>	gatgacctgatgaagacacac	ctgagactgtggggaggatg
Human		
<i>GAPDH</i>	gccaaggatcatccatgacaac	gtcttctgggtggcagtgat
<i>CACNA1C</i>	cccctctttccagggatgt	cccctctttccagggatgt
<i>CACNA1D</i>	catccgaagagcctgcatta	ggccacacaattggcaaaa
<i>IL4</i>	acggagatggatgtgccaaa	gaagcaccttgaagcccta
<i>IL13</i>	gcttattgaggagctgagcaa	cagggtccacactccatacca

417