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Separation of the $Ca_V1.2$-$Ca_V1.3$ calcium channel duo prevents type-2 allergic airway inflammation

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Short title

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

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Abstract

Background: Voltage-gated calcium (Ca_v1) channels contribute to T-lymphocyte activation. $Ca_v1.2$ and $Ca_v1.3$ channels are expressed in Th2 cells but their respective roles are unknown, which is investigated herein. Methods: We generated mice deleted for $Ca_v1.2$ in T cells or $Ca_v1.3$ 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 $Ca_v1.2$ or $Ca_v1.3$ deletion in models of type-2 airway inflammation. Finally, we checked whether the expression of both $Ca_v1.2$ and $Ca_v1.3$ in T cells from asthmatic children correlates with Th2-cytokine expression. Results: we demonstrated non-redundant and synergistic functions of $Ca_v1.2$ and $Ca_v1.3$ 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, $Ca_v1.3$ has a particular role in calcium homeostasis. In accordance with the singular roles of $Ca_v1.2$ and $Ca_v1.3$ in Th2 cells, deficiency in either one

of these channels was sufficient to inhibit cardinal features of type-2 airway inflammation. Furthermore, $Ca_v1.2$ and $Ca_v1.3$ must be co-expressed within the same $CD4^+$ T cell to trigger allergic airway inflammation. Accordingly with the concerted roles of $Ca_v1.2$ and $Ca_v1.3$, the expression of both channels by activated $CD4^+$ T cells from asthmatic children was associated with increased Th2-cytokine transcription. Conclusions: Thus, $Ca_v1.2$ and $Ca_v1.3$ 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 ($[Ca^{2+}]_i$) needs to be finely tuned since Ca^{2+} overload triggers cell death. Classically, T-cell activation induces a cascade of phosphorylation, the generation of IP3 releasing endoplasmic reticulum Ca^{2+} stores sensed by STIM1 Ca^{2+} sensor and ORAI Ca^{2+} channel opening[1]. 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 α1 subunit ($Ca_v1.1$ to 1.4, encoded by *CACNA1S*, *C*, *D*, *F* respectively) and auxiliary subunits (β and α2δ)[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 meningitis[7]. The role of Ca_v1 channels in T lymphocytes has also been evidenced using mice knocked out for α1 and β subunits forming Ca_v1 channels[8-10]. The expression of Ca_v1 channels is plastic depending on the activation and differentiation status of T cells. $Ca_v1.4$ 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 $Ca_v1.2$ and $Ca_v1.3$ channels, while Th1 cells lose Ca_v1 channel expression[11-13]. Using Ca_v1 channel blockers or antisense strategy, we previously reported that $Ca_v1.2$/$Ca_v1.3$ channels are necessary for TCR-driven Th2 functions and type 2-mediated airway inflammation[11-14].

Since both $Ca_v1.2$ and $Ca_v1.3$ 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 $Ca_v1.2$ and $Ca_v1.3$ 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* ($Ca_v1.2^{fl/fl}$)[15] and $Ca_v1.3^{-/-}$ mice[16] 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 $Ca_v1.2^{T-/-}$), was obtained by crossing $Ca_v1.2^{f/f}$ mice with mice expressing the Cre under the CD4 promoter control[17]. C57BL/6 OT-II mice were crossed to $Ca_v1.2^{T-/-}$ or $Ca_v1.3^{-/-}$ mice to generate OVA-specific Th2 cells lacking $Ca_v1.2$ or $Ca_v1.3$. Bone-marrow chimeras were achieved as previously described[18]. 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 $Ca_v1.2^{fl/fl}$Cre-, $Ca_v1.2^{+/+}$ (Cre+ or Cre-) and $Ca_v1.3^{+/+}$ 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 $Ca_v1.2^{fl/fl}$ 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 channel[19]. 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 µg/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 $Ca_v1.2^{T-/-}$) 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 10^6 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 10^6 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 described[18] 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/naive 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 $[Ca^{2+}]_i$ 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 Ca^{2+} 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 Ca^{2+} channels[19]. 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 $Ca_v1.2^{T-/-}$ or $Ca_v1.3^{-/-}$ Th2 cells also increased calcium channel opening frequency (**Figure 1E**). In $Ca_v1.3^{-/-}$ 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 $Ca_v1.2^{T-/-}$ and $Ca_v1.3^{-/-}$ 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 $Ca_v1.2^{T-/-}$ Th2 cells did not modify the class distribution (**Figure 1F-G**). In $Ca_v1.3^{-/-}$ Th2 cells, we observed a loss of low opening frequency classes in resting conditions compared to WT ($p<0.0001$) and $Ca_v1.2^{T-/-}$ ($p<0.0001$) Th2 cells suggesting that $Ca_v1.3$ shapes the type and/or the properties of the channels fluxing in baseline conditions. Moreover, TCR stimulation in $Ca_v1.3^{-/-}$ Th2 cells, induced a shift toward lower opening frequencies (**Figure 1F-G**), which is significantly different compared to stimulated WT or $Ca_v1.2^{T-/-}$ Th2 cells ($p<0.0001$), showing that $Ca_v1.3$ also impacts TCR-dependent calcium responses (**Figure 1F-G**) and suggesting that the absence of $Ca_v1.3$ brakes TCR signaling.

$Ca_v1.2$ or $Ca_v1.3$ 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 $Ca_v1.2$ or $Ca_v1.3$ channels after TCR stimulation (**Figure 2A**). The decreased calcium response and proximal signaling in $Ca_v1.2$- or $Ca_v1.3$-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 IFNγ 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 $Ca_v1.2^{fl/fl}$ 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 $Ca_v1.3^{-/-}$ 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 $Ca_v1.3$ in the hematopoietic compartment is sufficient to impede all cardinal features of allergic airway inflammation.

We showed non-redundant roles of $Ca_v1.2$ and $Ca_v1.3$ in models of Th2-dependent airway inflammation. We then analyzed chimeric mice reconstituted with bone marrows from mice lacking both $Ca_v1.2$ in T cells and $Ca_v1.3$. 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 $Ca_v1.3$ defect is sufficient to alleviate allergic airway inflammation

In order to determine whether the protective action of $Ca_v1.3$ deficiency was T-cell intrinsic, we performed complementation studies as previously described[27]. We injected naive WT $CD4^+$ T cells in $Ca_v1.3^{-/-}$ chimeras, before immunization with OVA to assess whether the expression of $Ca_v1.3$ restricted to the injected WT T cells was sufficient to induce airway inflammation. Injection of WT $CD4^+$ T cells, but not of $Ca_v1.3^{-/-}$ T cells, restored the numbers of cells in the BALF (eosinophilia), the lung histological score and mucus production in $Ca_v1.3^{-/-}$, to the same extent as in WT recipients (**Figure 5A-B**). Therefore, intrinsic expression of $Ca_v1.3$ channel in $CD4^+$ T cells is sufficient to promote Th2-mediated lung inflammation.

$Ca_v1.2$ and $Ca_v1.3$ 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 $Ca_v1.2$ and $Ca_v1.3$ 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 $Ca_v1.2$ and $Ca_v1.3$ on different T cells would not allow the development of airway inflammation. To test this, we injected naïve $Ca_v1.2^{T-/-}$ $CD4^+$ T cells (that express $Ca_v1.3$ but not $Ca_v1.2$) into $Ca_v1.3^{-/-}$ chimeric mice (the recipient T cells express only $Ca_v1.2$) prior to OVA immunization and intranasal OVA challenge. Actually these mice did not develop airway inflammation (**Figure 5A-B**), while $Ca_v1.2$ and $Ca_v1.3$ channels are expressed but on different T cells. We also did the reciprocal experiment in $Ca_v1.2^{T-/-}$ chimeric mice. As non-chimeric $Ca_v1.2^{T-/-}$ 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 involved[30]. The role of Ca_v channels is suspected regarding the partial effect of $Ca_v1.4$ channel[10] and auxiliary $Ca_v\beta$□[31] 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 $Ca_v1.3$[32] but their respective roles remain elusive due to the lack of $Ca_v1.3$- or $Ca_v1.2$-selective drugs[33], of antibodies specific for each channel[34] and some compensatory effects due to other Ca_v channels observed in knockout $Ca_v1.2$ or $Ca_v1.3$ mice[35].

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 $Ca_v1.3^{-/-}$ Th2 cells present a lower rise of $[Ca^{2+}]_i$ observed at single-cell level compared to WT Th2 cells. The residual calcium response in $Ca_v1.2^{T-/-}$ or $Ca_v1.3^{-/-}$ Th2 cells could be due to other calcium channels such as STIM/ORAI. Contrary to $Ca_v1.4$ channels that would regulate the intracellular Ca^{2+} stores in naive T lymphocytes[10], $Ca_v1.2$ 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 neurons[36]. In addition, calcium signaling is not only downstream of T-cell activation[37] but very few calcium ions entering the cell modify the cell membrane properties and promote TCR signaling events[25,26]. In this line, $Ca_v1.4$ was shown to associate with Lck and Vav in $CD8^+$ T cells[9] 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 $Ca_v1.2$ and $Ca_v1.3$-deficient Th2 cells, indicating that each channel is crucial to trigger the early ECE preceding global calcium influx. In baseline

conditions, only $Ca_v1.3$ 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 $Ca_v1.3$ 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 $Ca_v1.3$, but not $Ca_v1.2$, channels shape the baseline calcium homeostasis in Th2 cells. Moreover, the opening frequency distribution in $Ca_v1.3^{-/-}$ Th2 cells after TCR stimulation is marked by an enrichment in low frequency opening classes compared to WT or $Ca_v1.2^{T-/-}$ Th2 cells, suggesting that $Ca_v1.3$ 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 $Ca_v1.2$ and $Ca_v1.3$ on ECE, we favor the hypothesis of cooperative actions of these Ca_v1 channels. Whether and how $Ca_v1.2$ and $Ca_v1.3$ can cooperate is still unknown. Activated $Ca_v1.2$ can facilitate neighbouring $Ca_v1.2$ channel openings[38,39] and similar reports are described with $Ca_v1.3$[40]. $Ca_v1.3$ activates at about 10–20 mV more negative potentials than $Ca_v1.2$ channels[34,41], and has an activation threshold near the resting membrane potential. Thus, it is conceivable that $Ca_v1.2$ and $Ca_v1.3$ act sequentially with $Ca_v1.3$ activation resulting in early Ca^{2+} entry inducing local membrane depolarization, facilitating the opening of neighboring $Ca_v1.2$ channels. It is also possible that $Ca_v1.2$ and $Ca_v1.3$ channels act by recruiting distinct sets of adapters, kinases and other channels, as reported in hippocampal neurons[42], 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 $Ca_v1.2$ and $Ca_v1.3$ 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 pathology[43]. 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.

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555 **Figure legends**

556

557 **Figure 1: Original and singular roles of $Ca_v1.2$ and $Ca_v1.3$ channels in initial plasma**
558 **membrane calcium events upon TCR activation**

WT, $Ca_v1.2^{T-/-}$ and $Ca_v1.3^{-/-}$ $CD4^+$ 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 (F0) 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/F0 + 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 µm x 3 µm 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 $Ca_v1.2^{T-/-}$ and n=9 $Ca_v1.3^{-/-}$ 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 +1 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$^+$ TCRβ$^+$ and Vα2$^+$ 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 µg at day 0 and 10 µg 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

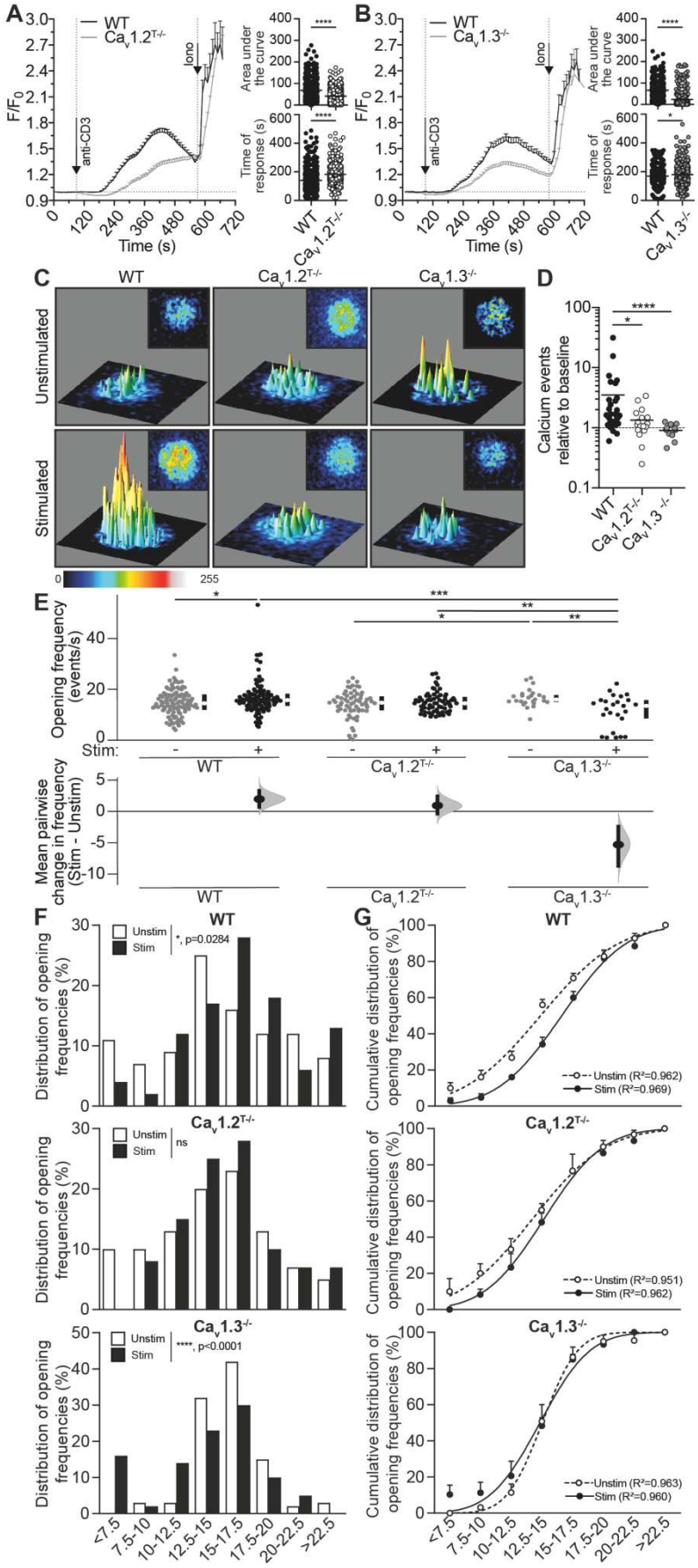
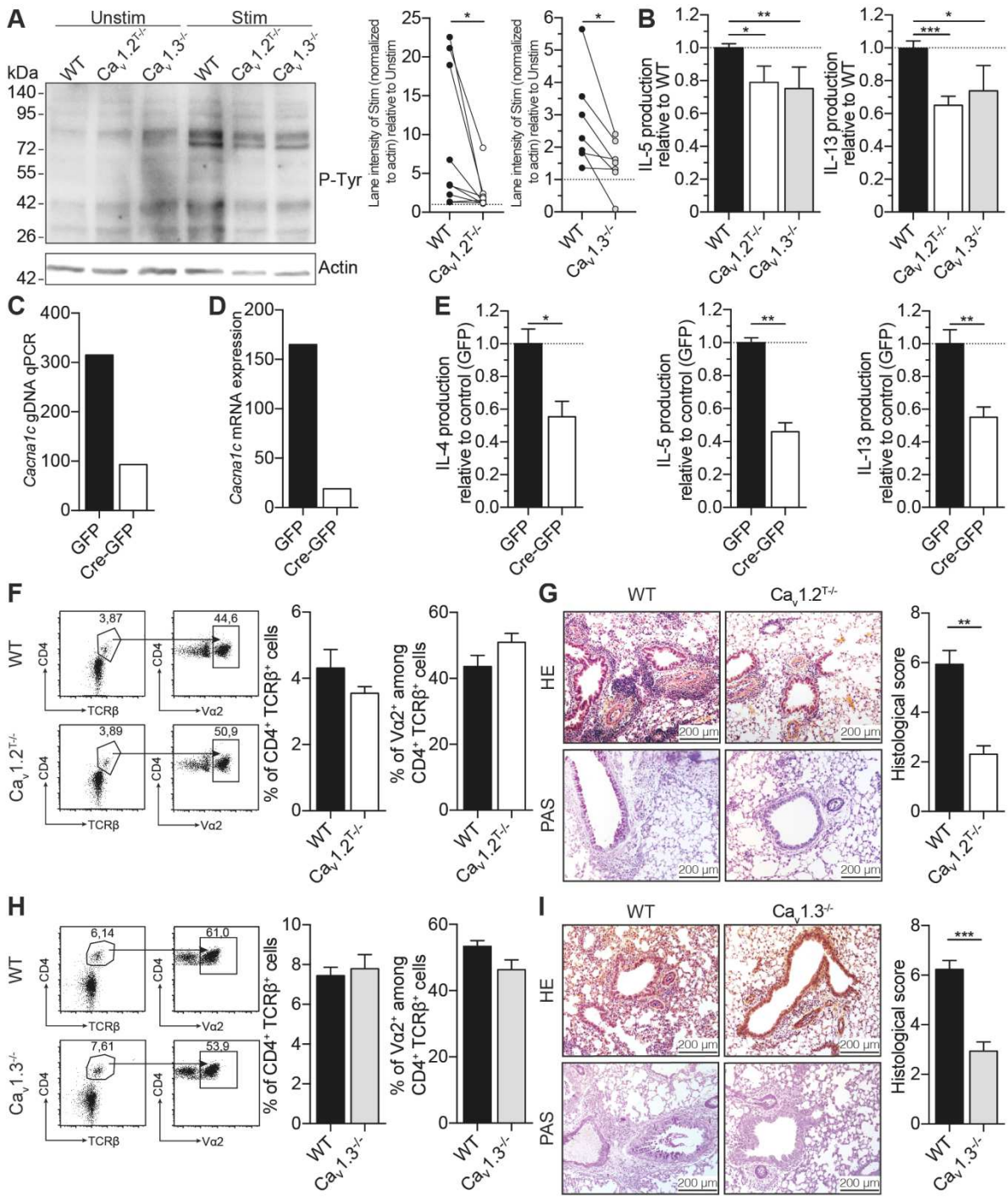


Figure 2



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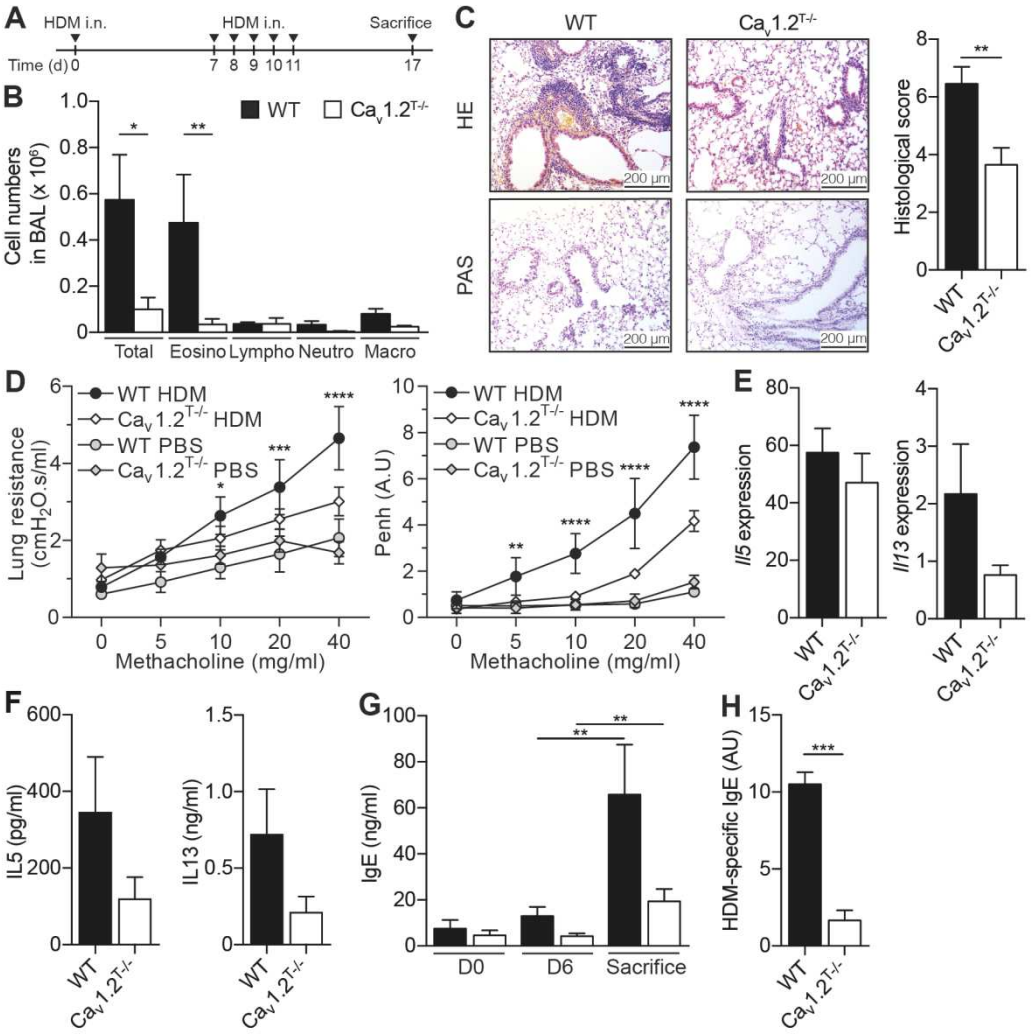
676 **New Figure 3**



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679 **New Figure 4**

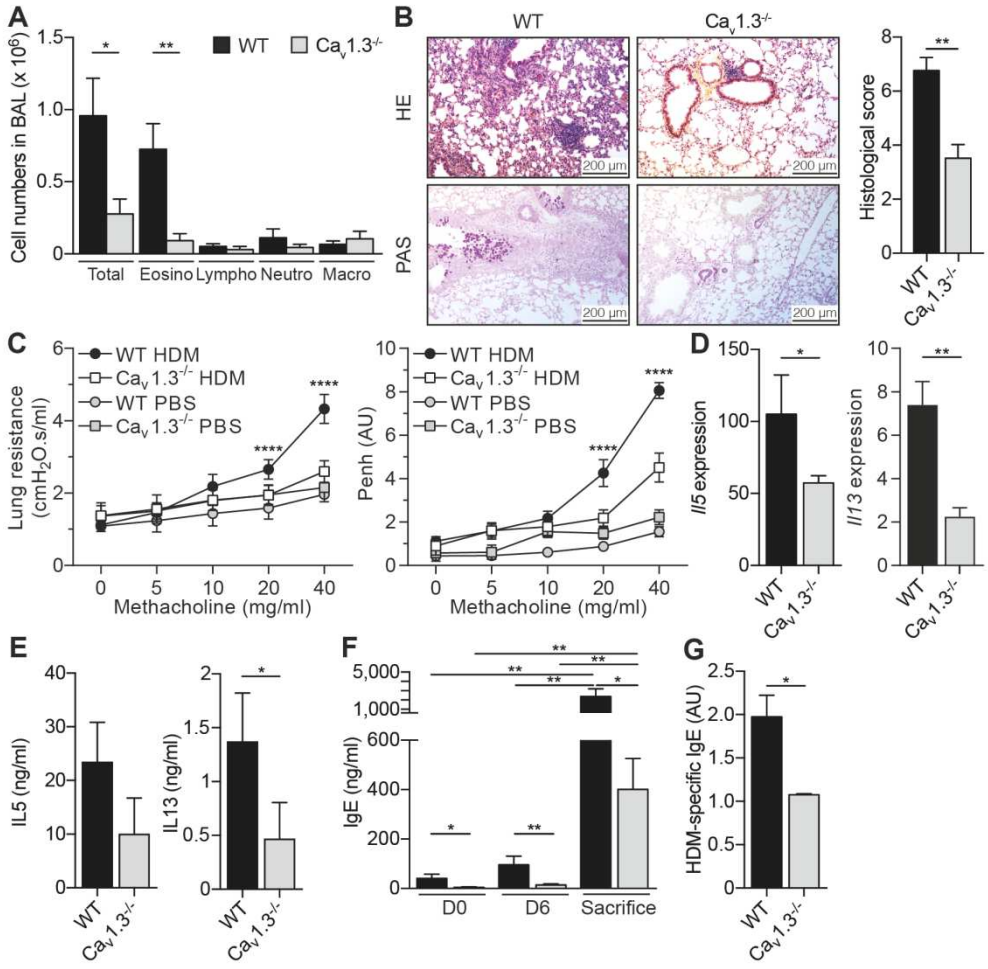
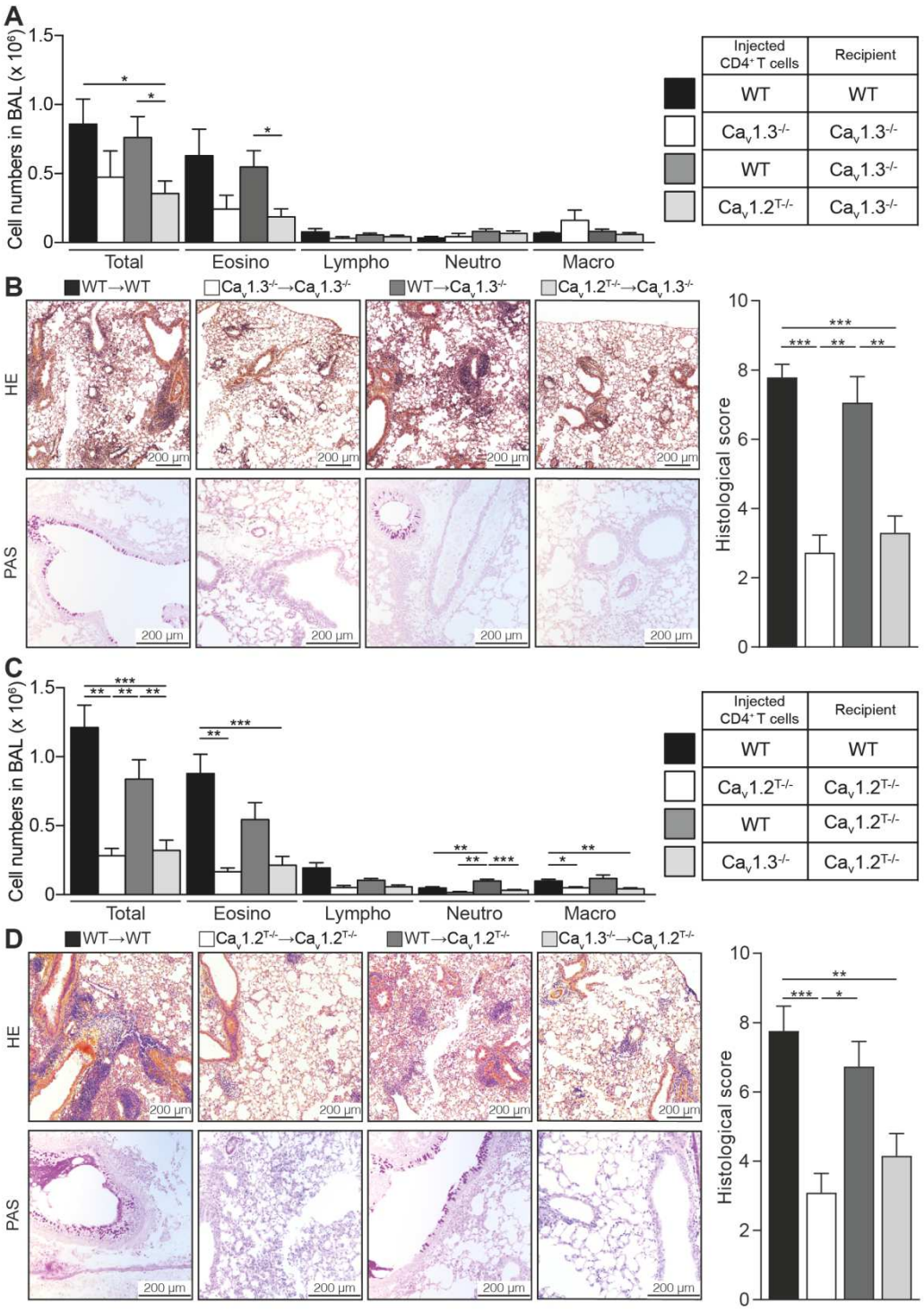


Figure 5



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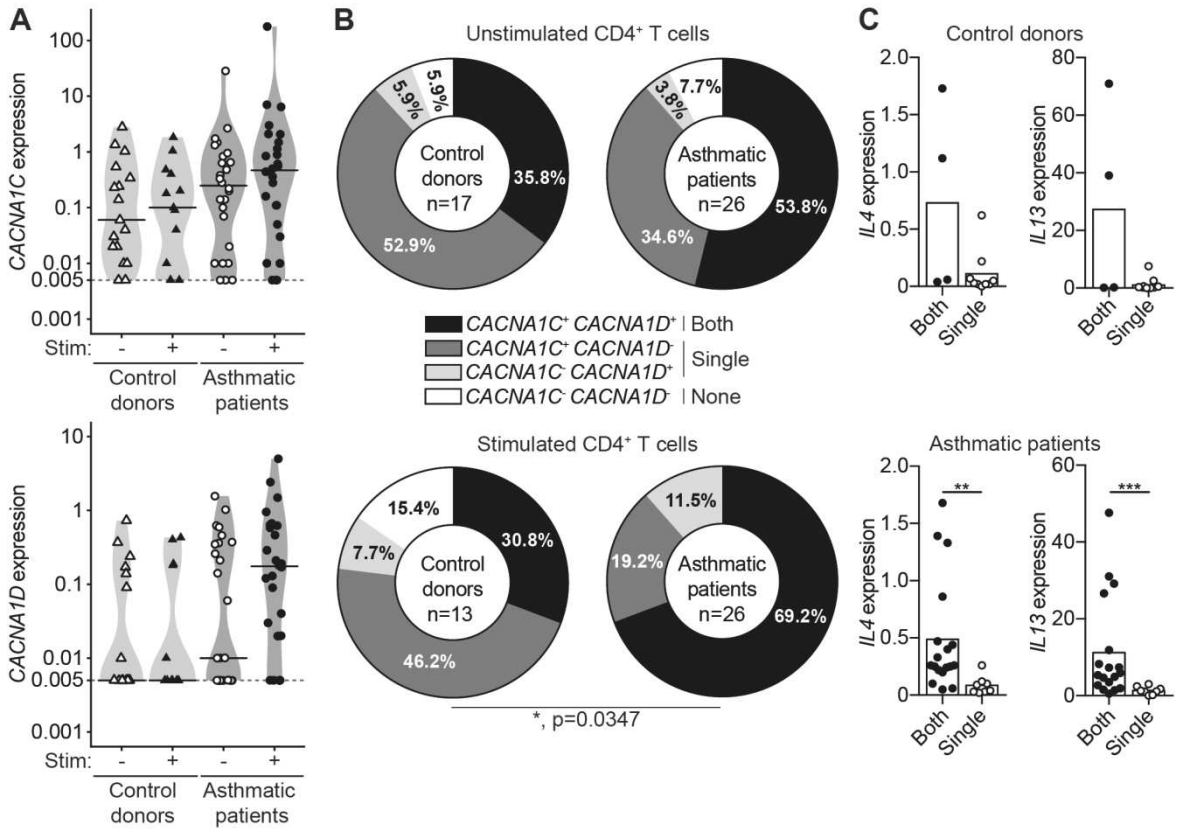
685 **New Figure 6**



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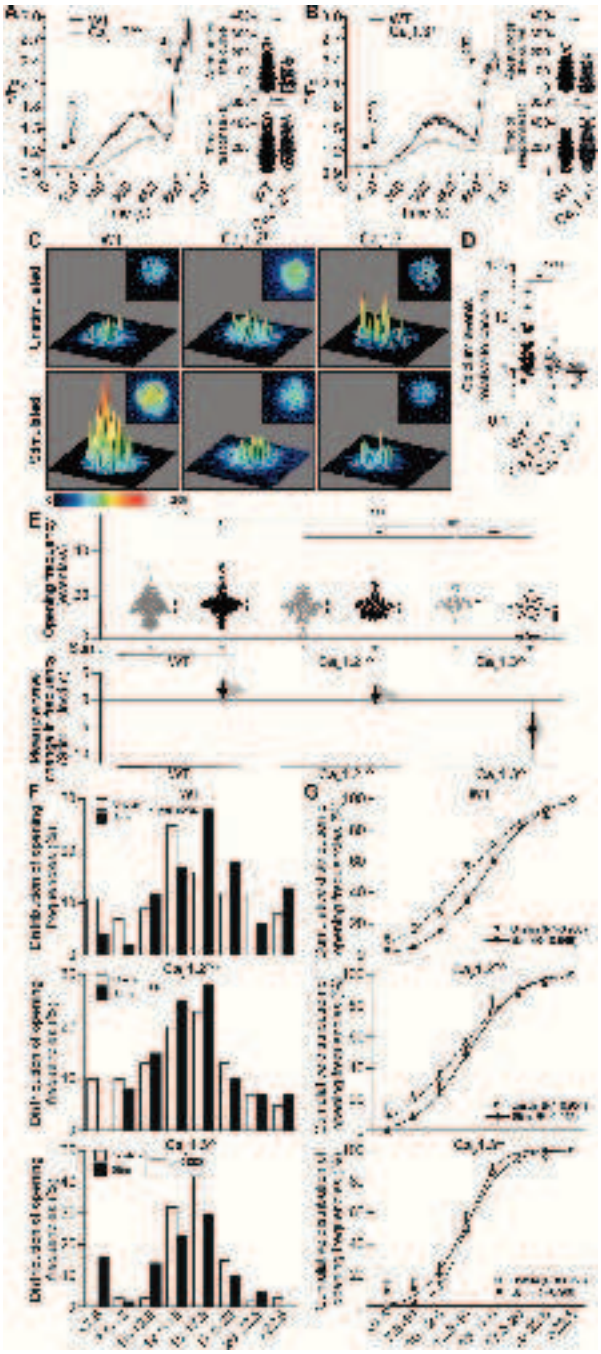

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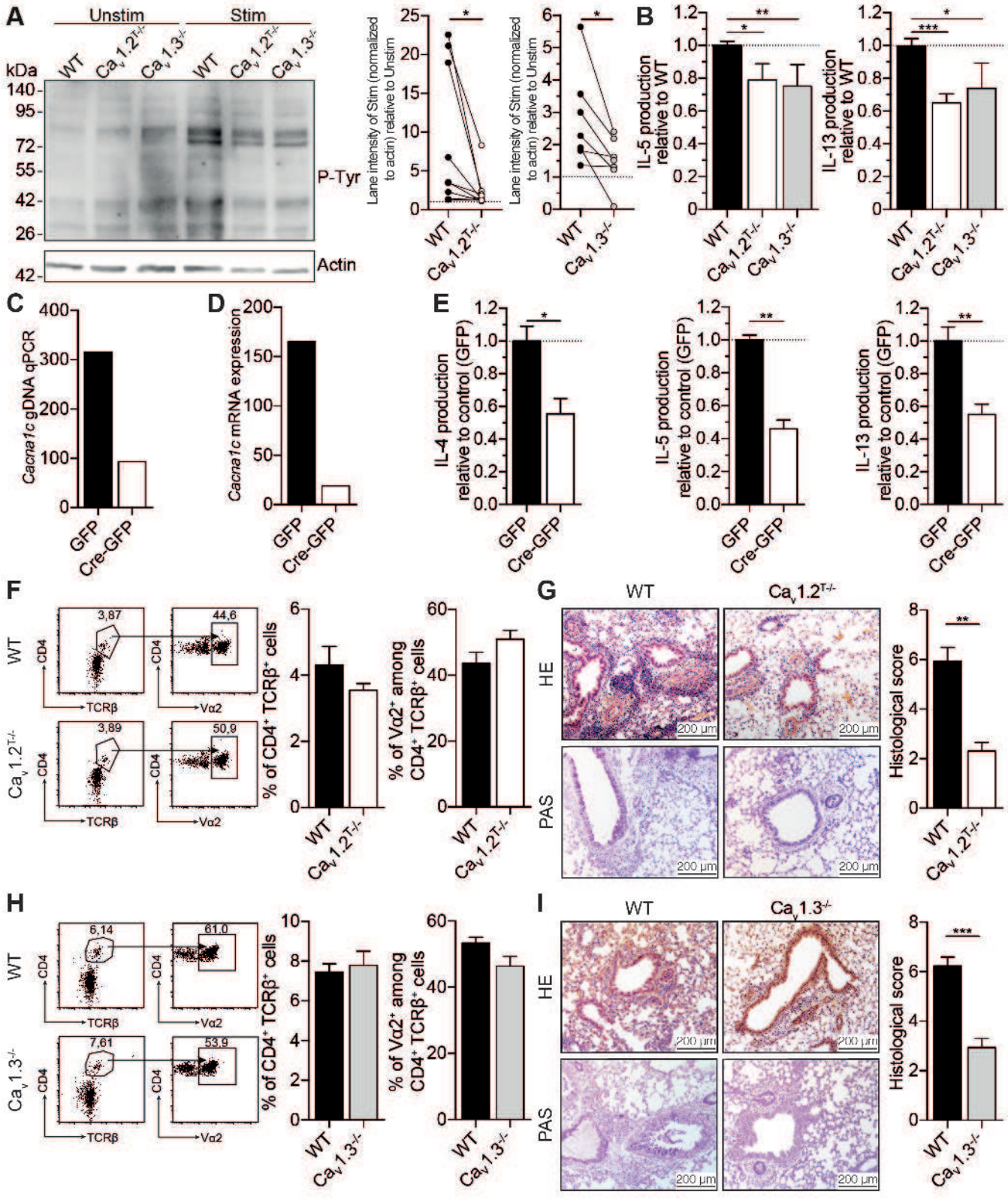

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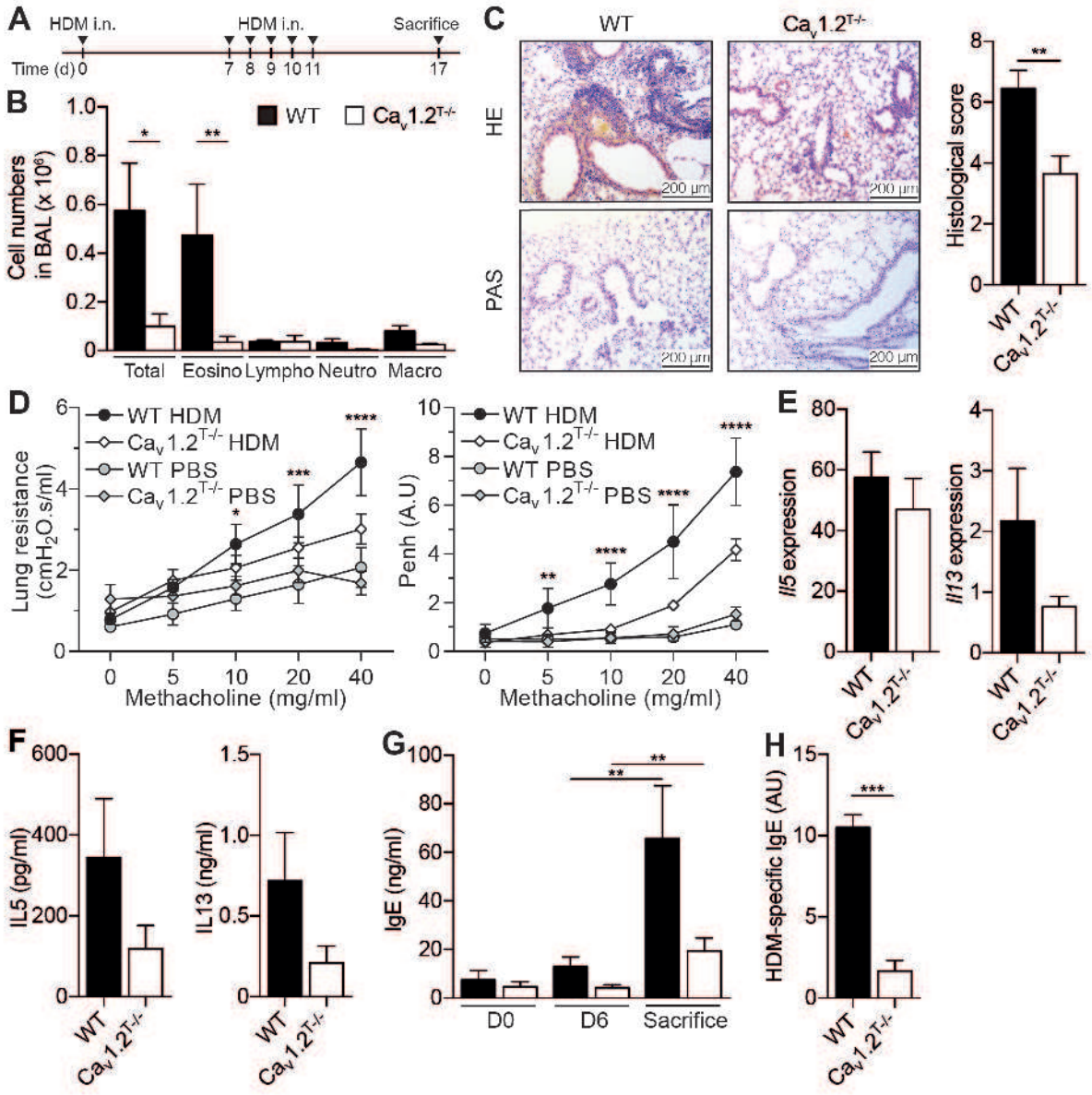

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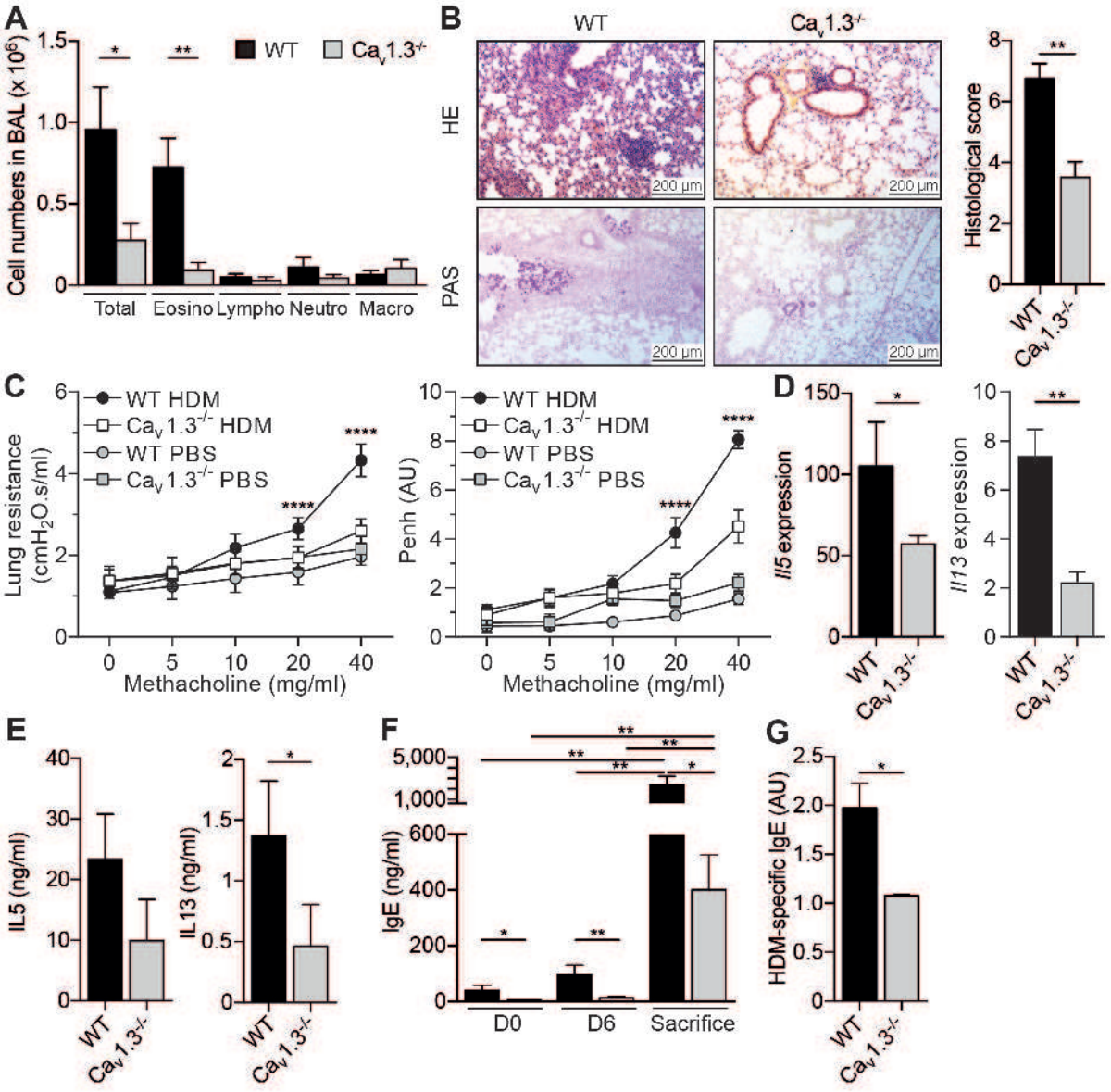

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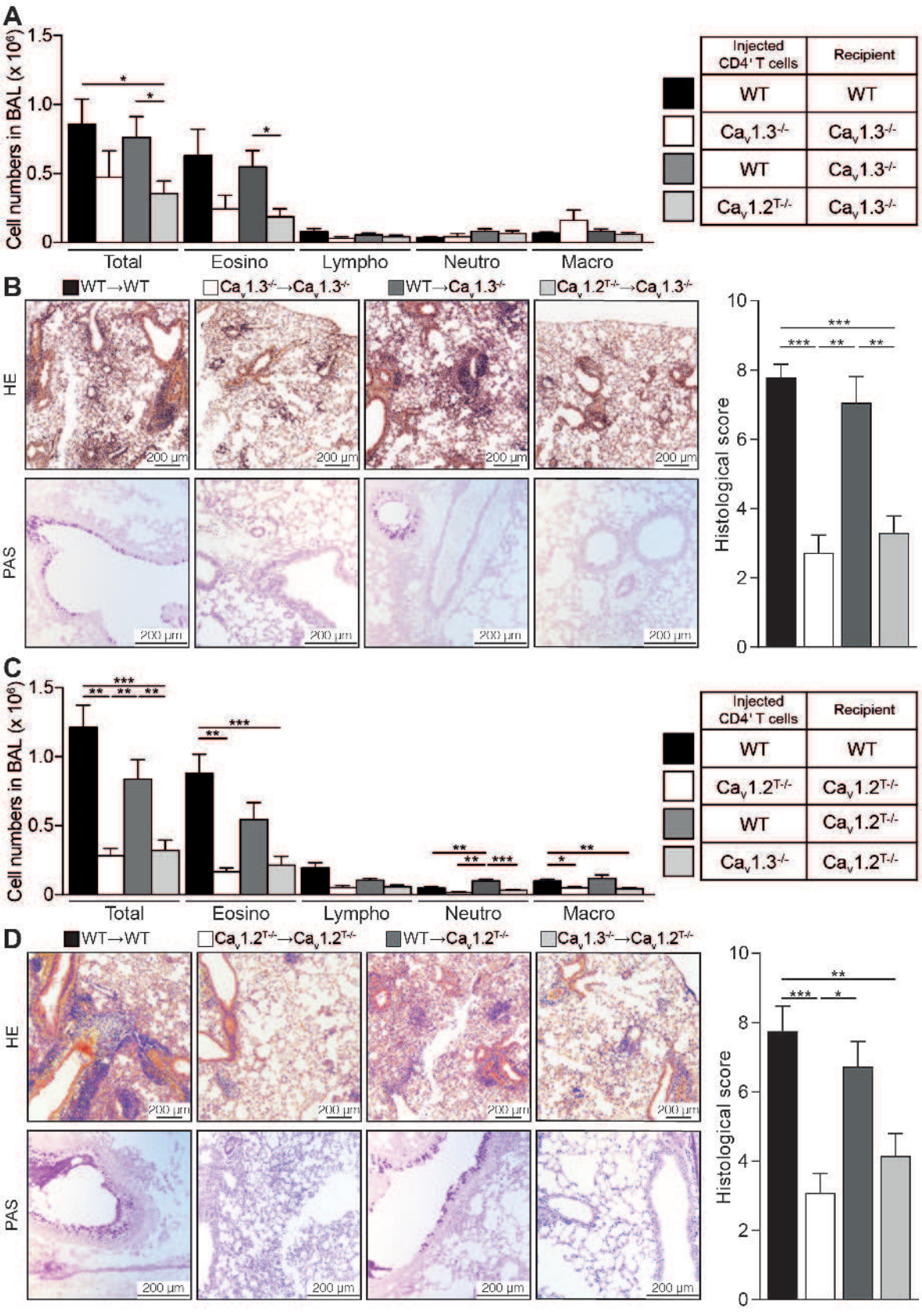

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