

Severely blunted allergen-induced pulmonary Th2 cell response and lung hyperresponsiveness in type 1 transient receptor potential channel-deficient mice

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Yildirim E, Carey MA, Card JW, Dietrich A, Flake GP, Zhang Y, Bradbury JA, Reboloso Y, Germolec DR, Morgan DL, Zeldin DC, Birnbaumer L. Severely blunted allergen-induced pulmonary Th2 cell response and lung hyperresponsiveness in type 1 transient receptor potential channel-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 303: L539–L549, 2012. First published July 13, 2012; doi:10.1152/ajplung.00389.2011.—Transient receptor potential channels (TRPCs) are widely expressed and regulate Ca²⁺ entry in the cells that participate in the pathophysiology of airway hyperactivity, inflammation, and remodeling. In vitro studies point to a role for TRPC1-mediated Ca²⁺ signaling in several of these cell types; however, physiological evidence is lacking. Here we identify TRPC1 signaling as proinflammatory and a regulator of lung hyperresponsiveness during allergen-induced pulmonary response. TRPC1-deficient (*Trpc1*^{−/−}) mice are hyposensitive to methacholine challenge and have significantly reduced allergen-induced pulmonary leukocyte infiltration coupled with an attenuated T helper type 2 (Th2) cell response. Upon in vitro allergen exposure, *Trpc1*^{−/−} splenocytes show impaired proliferation and T cell receptor-induced IL-2 production. A high number of germinal centers in spleens of *Trpc1*^{−/−} mice and elevated levels of immunoglobulins in their serum are indicative of dysregulated B cell function and homeostasis. Thus we propose that TRPC1 signaling is necessary in lymphocyte biology and in regulation of allergen-induced lung hyperresponsiveness, making TRPC1 a potential target for treatment of immune diseases and asthma.

calcium entry; transient receptor potential; cation channel; store-operated channel; allergy

BRONCHIAL HYPERRESPONSIVENESS, airway obstruction, and bronchial wall thickening due to smooth muscle hypertrophy and airway inflammation are common features of asthma (4, 21, 26–28). Several members of the transient receptor potential channel (TRPC) family of cation channels (TRPC1, TRPC3, TRPC4, and TRPC6) are expressed in the cell types that participate in these responses and may be important for the understanding of the asthmatic state and allergic inflammation (reviewed in Ref. 9). Under ovalbumin-induced allergic conditions, loss of TRPC6 in mice results in increased agonist-induced contractility of tracheal rings and reduced allergen-induced T helper (Th) type 2 (Th2) cell response with reduced

eosinophil infiltration and Th2 cytokine release in the lungs (44). In cultured human airway smooth muscle cells, TRPC3 is necessary for TNF- α -mediated Ca²⁺ signaling (58). In endothelial cells, loss of TRPC4 results in loss of thrombin-mediated actin-stress fiber formation and reduced retraction of lung endothelial cells, which is coupled with reduction in lung microvascular permeability (8, 55).

In general, TRPCs regulate cellular processes by mediating Ca²⁺ influx in response to various stimuli in nonexcitable cells (i.e., leukocytes and endothelial cells) or in response to membrane potential collapse and activation of voltage-gated Ca²⁺ channels in excitable cells (i.e., smooth muscle cells and neurons) (reviewed in Ref. 3). TRPC1, the founding member of the TRPC family (57, 64), was proposed to be the plasma membrane ion channel that regulates Ca²⁺ entry into cells through activation of G protein-coupled receptors (GPCRs), B cell receptors (BCRs), or store-operated Ca²⁺ entry (SOCE) (36, 37, 53). Although TRPC1 has been investigated extensively in various cell culture systems, in vivo studies related to its potential pathophysiological role in pulmonary function and inflammation are lacking. In DT-40 avian B cells, TRPC1 was linked to nuclear factor of activated T cell (NFAT) signaling (37), a signaling cascade that leads from increases in intracellular Ca²⁺ to subsequent transcriptional activation of various cytokine and chemokine genes [i.e., IL-2 (45), IL-4 (29), IL-13 (13), IL-6, and IFN- γ (reviewed in Ref. 19)]. In addition to TRPC4 and TRPC6, TRPC1 also participates in signaling events that control endothelial cell permeability (30, 35, 54, 56), which results in regulation of vascular tone (15, 60) and leukocyte migration to the site of inflammation during disease. In smooth muscle cells, on the other hand, TRPC1 forms heterotetramers with TRPC5 and TRPC6 (49) and is proposed to be critical for pulmonary smooth muscle cell proliferation and function (24, 46, 52, 53).

In this study we explored the role of TRPC1 in lung function using a murine ovalbumin-induced allergic inflammation and hyperresponsiveness model, which allowed us to examine different aspects of allergen-induced pulmonary inflammation as seen in TRPC1-deficient mice. Under allergic conditions, loss of TRPC1 resulted in airway hyposensitivity to methacholine challenge and blunting of the allergic Th2 cell response, as well as reduced eosinophil infiltration into the lungs, suggesting that TRPC1 is crucial for allergen-induced lung function and is proinflammatory. Data collected on B cell activation and

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function revealed that TRPC1 signaling is similarly a critical regulator of B cell homeostasis.

MATERIALS AND METHODS

Mice

Trpc1^{-/-} (11, 34) and age-matched wild-type (*Trpc1*^{+/+}) mice in pure 129SvEv background were used at 10–16 wk of age. Equal numbers of female and male animals were used. All studies were conducted in accordance with principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences.

Ovalbumin Sensitization, Airway Challenge, and Lung Function Measurements

Mice receiving aluminum hydroxide adjuvant (Alhydrogel, Accurate and Scientific; 3–4 animals per genotype) and ovalbumin (grade V, Sigma) plus adjuvant (8–12 mice per genotype) were studied by the allergic airway inflammation method described by Zeldin and colleagues (6, 7). On day 0, mice were injected intraperitoneally with 20 µg of ovalbumin emulsified in 0.2 ml of aluminum hydroxide adjuvant or saline emulsified in 0.2 ml of adjuvant. On 5 consecutive days (days 14–18), all mice were challenged via the airways in a nose-only exposure chamber for 30 min/day with an aerosol of 1% ovalbumin-in-saline solution. On day 19, invasive analysis of lung function was performed with the FlexiVent mechanical ventilator system (SCIREQ, Montreal, PQ, Canada), as described in detail elsewhere (6). After acquisition of baseline data in the absence of aerosol challenge, airway responsiveness to aerosolized methacholine dissolved in 1× PBS was assessed with the single-compartment and constant-phase models of respiratory mechanics. For the single-compartment model, total respiratory system resistance and total elastance were determined essentially as described elsewhere (16). For the constant-phase model, Newtonian resistance (an indicator of central airway resistance), tissue resistance (an indicator of peripheral airway and parenchymal resistance), and tissue elastance (an indicator of peripheral airway and parenchymal elastance) were determined essentially as described elsewhere (31).

Bronchoalveolar Lavage and Tissue Collection

After assessment of bronchoconstriction, mice were anesthetized by injection of pentobarbital sodium (80 mg/kg ip). Lungs were lavaged with two 1-ml aliquots of sterile pyrogen-free HBSS, which were subsequently combined. Approximately 90% of the total injected volume was consistently recovered. After bronchoalveolar lavage (BAL), the left lungs were inflated with 0.4 ml 4% paraformaldehyde (PFA), which was injected slowly over ~10 s via a blunt needle that was inserted into the trachea. The tracheas were immediately tied off with sutures, and the lungs were submersed in 4% PFA and used for preparation of slides for histopathological evaluation. The right lung was snap-frozen on dry ice and stored at -80°C. Bronchoalveolar lavage fluid (BALF) was placed on ice, and the BALF cells were collected by centrifugation at 360 g for 10 min at 4°C. The 3 ml of supernatant (BALF) were separated into three aliquots: one 100-µl aliquot was kept at 4°C for lactate dehydrogenase assay, a second 100-µl aliquot was frozen at -80°C for total protein analysis, and the remaining 2.8-ml aliquot, to which 1% FBS was added, was frozen at -80°C to be used for analysis of cytokine and chemokine protein levels.

Analysis of the BALF

Total cell counts and BALF cell differentials. The BALF cells were suspended in 1 ml of HBSS (+1% FBS; Hyclone), washed twice with

HBSS (+1% FBS), and resuspended in 1 ml of the same buffer and counted (Coulter Electronics, Hialeah, FL). BALF cells were spun (Cytospin-3, Shandon, Pittsburgh, PA) and stained with Wright-Giemsa (Fisher Scientific, Pittsburgh, PA). A manual white blood cell differential count based on morphological criteria was done for each sample.

Cytokine and chemokine levels in BALF and splenocyte culture medium. ELISA kits were used for measurement of murine eotaxin, thymus- and activation-regulated chemokine (TARC), IL-13, and IL-5 (R & D Systems, Minneapolis, MN) in cell-free BALF following the manufacturer's instructions. Plates were read on a microplate reader. Cytokine and chemokine concentrations were calculated on the basis of standard curves. For analyses of the IL-2, IL-4, IL-5, IL-17, and granulocyte-macrophage colony-stimulating factor protein levels in cell culture, 50 µl of the splenocyte culture medium were used, and protein levels were measured using mouse cytokine panels (Bio-plex mouse cytokine Th1/Th2, IL-5, and IL-17; Bio-Rad) according to the manufacturer's instructions.

Total Immunoglobulin Level Measurements in Serum

Levels of total IgE, IgG1, and IgG2a from each animal group were measured in 0.5 µl of serum samples using ELISA assays (BD Biosciences) following the manufacturer's instructions. Anti-mouse IgE (BD Biosciences), anti-mouse IgG1 (Bethyl Laboratories), and anti-mouse IgG2a (BD Biosciences) were used as capture antibodies. The absorbance from each plate was read at 450 nm. The serum levels of IgG2b, IgG3, IgA, and IgM were quantitated using the Beadlyte mouse immunoglobulin isotyping kit according to the manufacturer's instructions (Upstate).

Isolation of Splenocytes

Spleens from wild-type and *Trpc1*^{-/-} mice were placed in 5 ml of complete RPMI medium at room temperature and passed through a 70-µm cell strainer (BD Falcon) into a 50-ml conical tube. Splenocyte suspension was centrifuged at 500 g for 5 min at 4°C, supernatant was discarded, and red blood cells were lysed by resuspension of the cell pellet in 3 ml of 1× Pharm Lyse reagent (BD Biosciences). After the cells were incubated for 5 min at room temperature, 27 ml of complete RPMI medium were added to the suspension. The cells were centrifuged at 500 g for 5 min at room temperature, the pellet was resuspended in complete RPMI medium, and cell numbers were quantitated.

MTT Assay to Assess Cell Proliferation

Splenocytes (1×10^5 /well) from control and allergen-sensitized wild-type and *Trpc1*^{-/-} mice were cultured in sextuples in 200 µl of complete RPMI medium in the absence or presence of ovalbumin (100 and 1,000 µg) on 96-well plates for 72 h at 37°C. The culture medium was then collected and used to measure cytokine and chemokine levels. Cell proliferation was measured using the 3-(4,5-diphenylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (1, 38).

For measurement of T cell receptor (TCR)-induced cell proliferation, splenocytes (1×10^5 /well) that were isolated from naïve wild-type and *Trpc1*^{-/-} mice were cultured in 200 µl of complete RPMI medium in the presence or absence of plate-bound 10 µg/ml anti-mouse CD3ε antibody (clone 145-11C, BD Pharmingen) and/or 1 µg/ml soluble anti-mouse CD28 antibody (clone 37.51, BD Pharmingen) on 96-well plates for 48 h at 37°C. The culture medium was collected and used to measure IL-2 protein levels by ELISA and TCR-induced proliferation of splenocytes by MTT assay.

Quantitative RT-PCR

Lungs from age-matching naïve wild-type ($n = 3$) and *Trpc1*^{-/-} ($n = 3$) (11) mice were used to extract total RNA using the RNeasy Midi Kit with on-column DNase treatment (Qiagen). Reverse tran-

scription was performed at 42°C for 15 min followed by 5 min at 95°C using RT First Strand Kit reagents (SABiosciences). Real-time PCR was performed in a final volume of 25 µl with an iCycler (Bio-Rad) as follows: initial step for 10 min at 95°C followed by 40 cycles of amplification for 15 s at 95°C and 60 s at 60°C, using SYBR Green qPCR master mix (Qiagen) in triplicates. Results were normalized by GAPDH. Primers [TRPC1 (PPM31640A), TRPC2 (PPM38130A), TRPC3 (PPM29904A), TRPC4 (PPM04057A), TRPC5 (PPM04617A), TRPC6 (PPM04056A), TRPC7 (PPM04062A), and GAPDH (PPM02946E)] were obtained from SABiosciences. mRNA levels were calculated using the following equation: $2^{[C_{i(GAPDH)} - C_{i(TRPC)}]}$, where C_i is cycle threshold.

Lung Histopathology

Serial lung sections (5 µm thick) from the vehicle- and ovalbumin-sensitized mice were stained with hematoxylin-eosin and Alcian blue-periodic acid-Schiff (PAS) stains. A semiquantitative scoring system was employed to evaluate degree of inflammation and goblet cell numbers (criteria and scores are shown in Fig. 2D).

Lung Immunocytochemistry and Western Blotting

ICAM and VCAM expression in the lungs was detected by immunohistochemistry and Western blotting using anti-ICAM-1 (M-19) and anti-VCAM-1 (C-19) antibodies (Santa Cruz Biotechnology). ICAM-1 and VCAM expression detected by immunohistochemistry was evaluated using the scoring criteria described in Fig. 3C.

Spleen Histopathology

Serial cross sections (5 µm thick) through the center of the spleen from vehicle- and ovalbumin-sensitized mice were stained with hematoxylin-eosin and examined histologically. Numbers of active germinal centers, total germinal centers, and plasmacytoid clusters (plasma cells) in hematoxylin-eosin-stained central sections of spleen from control and ovalbumin-sensitized mice were scored. Briefly, the number of germinal centers in each section was first counted with the ×10 objective. The same section was reexamined with the ×20 objective for evidence of mitotic activity or apoptotic debris in these germinal centers; if mitotic or apoptotic material was present, the germinal center was considered to be activated, and the total number of activated germinal centers was tabulated. The ratio of activated germinal centers to total germinal centers in each section was then determined. The same section was reexamined with the ×20 objective for the identification and tabulation of plasmacytoid clusters in the marginal zone of the lymphoid follicles or periarterial lymphoid sheaths. A plasmacytoid cluster was defined as a group of three or more plasma cells or plasmacytoid cells. Each cluster was counted as one cluster, regardless of the number of cells in the cluster. Results of scoring are shown in Fig. 7C.

Statistical Analysis

Values are means ± SE. Immunohistochemical data were analyzed for significance using one-way ANOVA and the nonparametric Kruskal-Wallis test. All other data were analyzed by one-way ANOVA and the Newman-Keuls post test. Values were considered significantly different if $P < 0.05$. The tests were performed using GraphPad Prism4 software.

RESULTS

Blunted Allergen-Induced Airway Responsiveness

Trpc1^{-/-} mice (10, 11) were used to investigate the pathophysiological significance of TRPC1 in allergen-induced lung dysfunction. The conditions that would induce such dysfunction were generated using a well-characterized mouse model of allergic airway inflammation and hyperresponsiveness (6, 7).

As shown in Fig. 1A, the protocol for this model requires that groups of wild-type and *Trpc1*^{-/-} mice undergo a 14-day sensitization period with the allergen (ovalbumin) or the adjuvant, in the case of control animals, followed by 5 consecutive days of challenge with ovalbumin. After 19 days, lung function was evaluated using a FlexiVent system to measure lung function. The results showed that, among the allergen-sensitized group, *Trpc1*^{-/-} mice were hyposensitive to methacholine challenge and had lower total resistance, total elastance, tissue resistance, and tissue elastance (Fig. 1B). We detected no compensatory changes in the expression level of the other six TRPCs in the lungs due to loss of TRPC1 as quantitated by RT-PCR (Fig. 1C), making it unlikely that lung dysfunction in *Trpc1*^{-/-} mice was due to a compensatory change in the transcript level of another TRPC. Thus the absence of TRPC1 resulted in a blunting of allergen-induced airway responsiveness to methacholine and suggests that TRPC1 is a significant modulator of the functional response of the lung to allergen exposure. In accordance with blunted airway responsiveness, BALF from ovalbumin-sensitized *Trpc1*^{-/-} mice showed significantly lower total protein and lactate dehydrogenase levels than control animals, suggesting less epithelial cell injury in the lungs of these mice (Fig. 1D).

Reduced Eosinophils and Attenuated Infiltration of Leukocytes in the Bronchoalveolar Space

Allergens induce Th2 cell-mediated airway inflammation, in which release of cytokines and chemokines from Th2 and endothelial cells induces migration of other leukocytes (e.g., eosinophils) to the site of inflammation. To determine whether TRPC1 contributes to signaling events during allergen-induced pulmonary inflammation, we analyzed the number of leukocytes in the BALF. As expected, the allergen-sensitized control animals had a typical allergic inflammatory response, characterized by an increase in eosinophils to as much as ~85% of the total number of BALF cells. In contrast, the number of total leukocytes, eosinophils, and macrophages in the BALF of allergen-sensitized *Trpc1*^{-/-} mice was reduced by ~67% compared with their wild-type counterparts, whereas no difference in the number of lymphocytes was detected in these animals (Fig. 2A).

We next examined the lung pathology to determine defects in inflammatory response and lung remodeling. Vehicle-treated wild-type and *Trpc1*^{-/-} mice showed no inflammatory infiltrates (Fig. 2B, images a and b). Among the allergen-sensitized group, wild-type and *Trpc1*^{-/-} animals (Fig. 2B, images c and d) showed a severe, but similar, degree of inflammation in the perivascular and peribronchial tissues (Fig. 2C) of the lungs. Interestingly, closer examination of the infiltrates at these sites revealed that, unlike control animals, which developed a eosinophil-predominant pulmonary inflammation (Fig. 2B, image c, inset) with a high number of micronodules formed from eosinophils, macrophages, and giant cells (Fig. 2, B, image e, and C), *Trpc1*^{-/-} mice presented a leukocyte infiltration that was mainly lymphoid in origin (Fig. 2B, image d, inset), with fewer micronodules with giant cells (Fig. 2B, image f, and C). These results suggest a misregulation in Th2 cell response, possibly due to a defect in activation or proliferation of leukocytes, in particular eosinophils. We next examined PAS-stained lung sections for changes in degree of mucus secretion

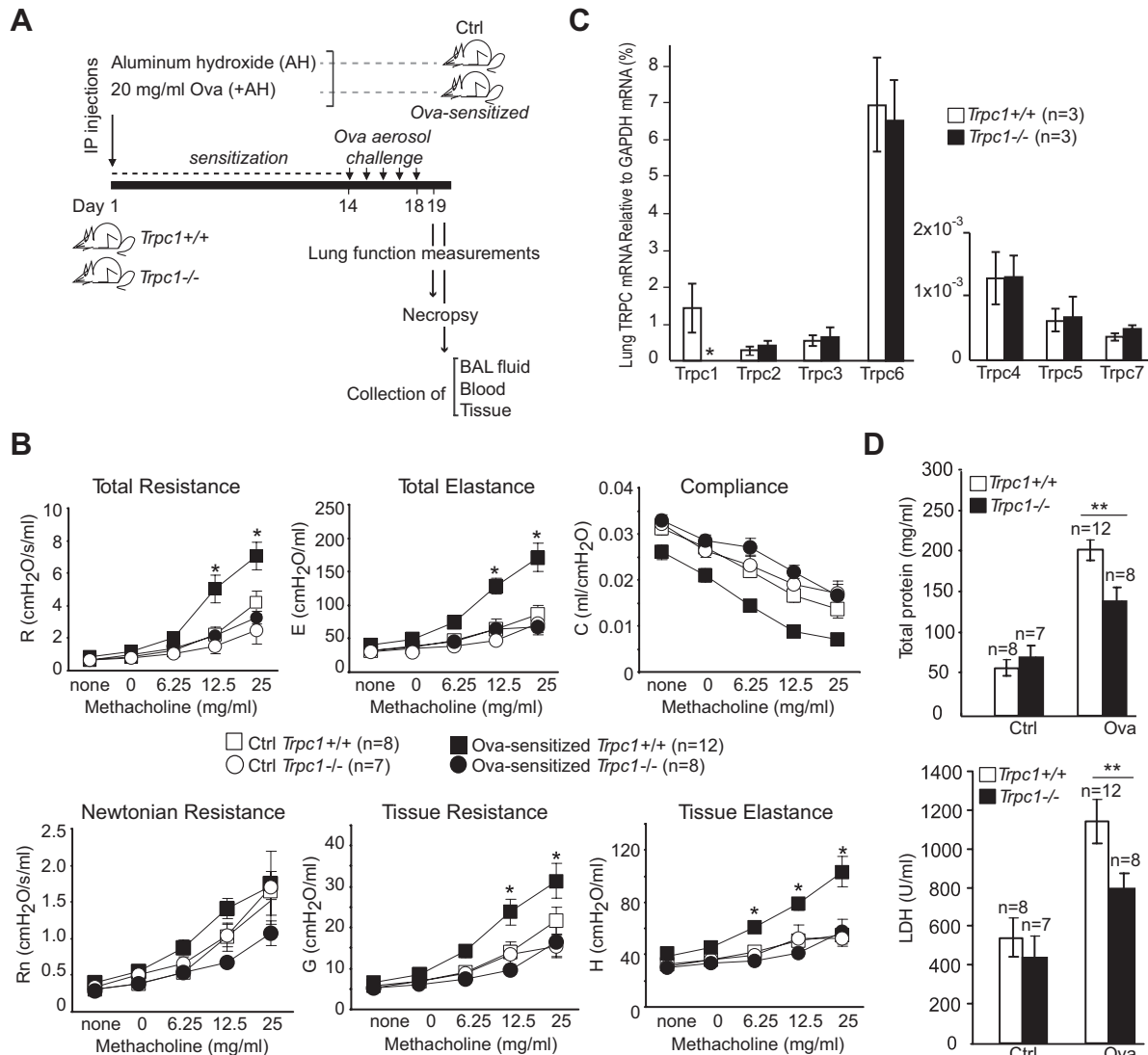


Fig. 1. Allergen-sensitized transient receptor potential channel type 1 (TRPC1)-deficient (*Trpc1*^{-/-}) mice are hyposensitive to methacholine. **A:** schematic showing ovalbumin (Ova) sensitization, airway challenge, and downstream sample processing in wild-type (*Trpc1*^{+/+}) and *Trpc1*^{-/-} mice. BAL, bronchoalveolar lavage. **B:** airway responsiveness [total resistance (R), total elastance (E), compliance (C), Newtonian resistance (R_n), tissue resistance (G), and tissue elastance (H)] to methacholine. *Trpc1*^{-/-} mice were hyposensitive to methacholine challenge compared with their wild-type (Ctrl) counterparts. Values are means $\pm$ SE. **P* < 0.05. **C:** no compensatory changes in gene expression profile of TRPC2, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 in lungs of *Trpc1*^{-/-} animals compared with wild-type mice, as detected by quantitative RT-PCR. Note loss of TRPC1 transcript in lungs of *Trpc1*^{-/-} mice (*). Values are means $\pm$ SE. **D:** reduced levels of total protein and lactate dehydrogenase (LDH) in BAL fluid of allergen-induced *Trpc1*^{-/-} mice suggest lower endothelial cytotoxicity. Values are means $\pm$ SE. ***P* < 0.01.

from the goblet cells and found that mucus secretion from allergen-sensitized *Trpc1*^{-/-} mice was indistinguishable from that from their wild-type counterparts (Fig. 2, *C* and *D*), possibly accounting for the partial development of pulmonary inflammation as seen by lymphocyte infiltrates. Taken together, these observations indicate a role for TRPC1 in eosinophil chemotaxis and/or infiltration of lymphocytes during the allergic response.

Expression of adhesion molecules such as ICAM-1 and VCAM-1 was shown to be upregulated in lung epithelial and vascular endothelial cells upon allergen exposure. This response enables the leukocytes to roll and migrate to the site of inflammation (5, 59). To determine whether changes in expression of these two adhesion molecules influenced reduced and

lymphocyte-predominant inflammatory response in *Trpc1*^{-/-} animals, we analyzed ICAM-1 and VCAM-1 expression by immunohistochemistry and Western blotting and found no significant difference between allergen-induced control and *Trpc1*^{-/-} animals (Fig. 3). These results eliminated the possibility that loss of leukocyte infiltration to the site of inflammation in *Trpc1*^{-/-} mice is caused by dysregulation in the expression of ICAM-1 or VCAM-1.

Attenuated Th2 Cell Response

Allergen-induced pulmonary Th2 cell response involves release of cytokines, the transcription of which is regulated by transcription factors NF κ B and AP-1 (42) and the Ca²⁺-

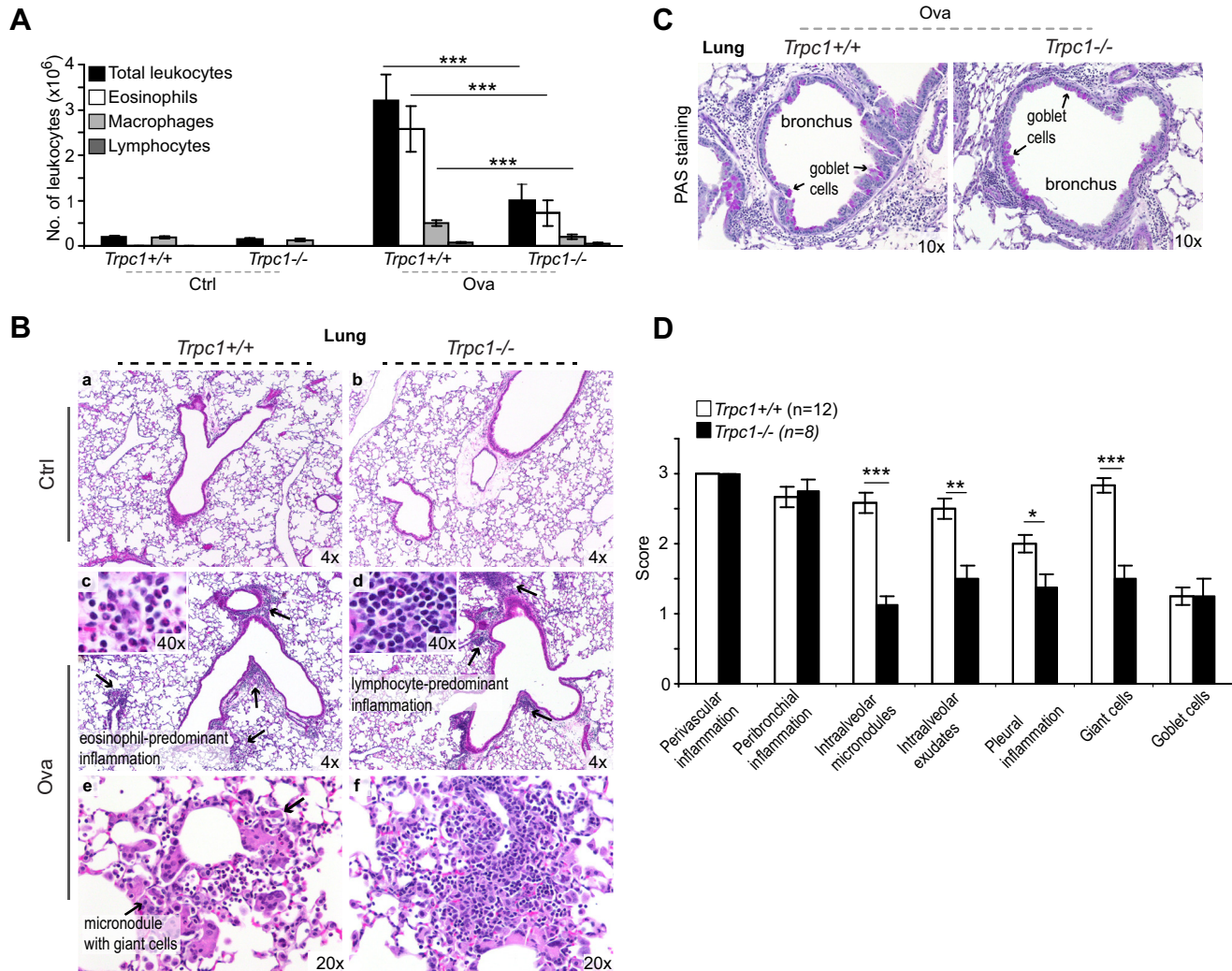


Fig. 2. Deletion of TRPC1 blunts allergen-induced eosinophil infiltration in lungs with reduced number of intra-alveolar micronodules. **A**: quantitation of BAL fluid cells shows significant decrease in eosinophils in allergen-sensitized *Trpc1*^{-/-} mice. Values are means $\pm$ SE. *** P < 0.001. **B**: representative micrographs showing absence of inflammation [control (*images a* and *b*)] in wild-type (*Trpc1*^{+/+}, *a*) and *Trpc1*^{-/-} (*b*) mice, severe eosinophil-predominant (*images c* and *d*, insets) inflammation in perivascular and peribronchial spaces of lungs in allergen-sensitized wild-type animals (*c*) compared with indistinguishable degree of allergen-induced perivascular and peribronchial inflammation in *Trpc1*^{-/-} mice (*d*). Note lymphocyte origin of infiltrates in lungs of *Trpc1*^{-/-} mice (*image d*, inset). Also note reduced number of micronodules with giant cells in alveolar spaces of allergen-sensitized *Trpc1*^{-/-} mice (*image f*) compared with wild-type animals (*image e*). **C**: representative periodic acid-Schiff (PAS)-stained lung micrographs from allergen-sensitized mice showing similar degree of goblet cell staining (indicative of mucus secretion) in lungs of wild-type and *Trpc1*^{-/-} animals. Control groups had undetectable levels of goblet cell staining (data not shown). **D**: semi-quantitative evaluation (scoring) of PAS-stained goblet cells) of pulmonary inflammation and mucus secretion in lungs of allergen-sensitized animals. Values are means $\pm$ SE. * P < 0.05; ** P < 0.01; *** P < 0.001.

dependent transcription factor NFAT (reviewed in Ref. 20). The absence of eosinophils and reduced overall inflammatory response (Fig. 2, A–C) in the lungs of allergen-sensitized *Trpc1*^{-/-} mice suggest that, in the absence of TRPC1, one or more of the regulatory components of the inflammatory pathway is impaired. To determine if loss of TRPC1 affected cytokine release in leukocytes during the Th2-type response, we measured BALF protein levels for cytokines and chemokines that are known to play a role in the differentiation of naïve T cells into Th2 lymphocytes (IL-5 and IL-13), in the recruitment of Th2 cells to the site of inflammation (IL-13 and TARC), in the growth, differentiation, and proliferation of eosinophils (IL-5), and in the chemotaxis of eosinophils (eotaxin). Among the allergen-sensitized group, IL-5, IL-13, and eotaxin levels were significantly reduced in the *Trpc1*^{-/-} mice

compared with wild-type animals (Fig. 4). These data suggest that TRPC1 is proinflammatory and plays a crucial role in the Th2 cell response. These results also confirm earlier in vitro findings suggesting that TRPC1-induced Ca^{2+} signaling is upstream of NFAT signaling in lymphocytes (37).

Reduced Allergen-Induced Proliferation and Release of Th2 Signaling Molecules in Splenocytes In Vitro

We next examined direct effects of the allergen on the *Trpc1*^{-/-} splenocytes and followed subsequent changes in cytokine release and cell proliferation in vitro. We sensitized wild-type and *Trpc1*^{-/-} mice with an intraperitoneal injection of ovalbumin or the adjuvant alone for 19 days followed by another intraperitoneal injection of ovalbumin on day 19. We

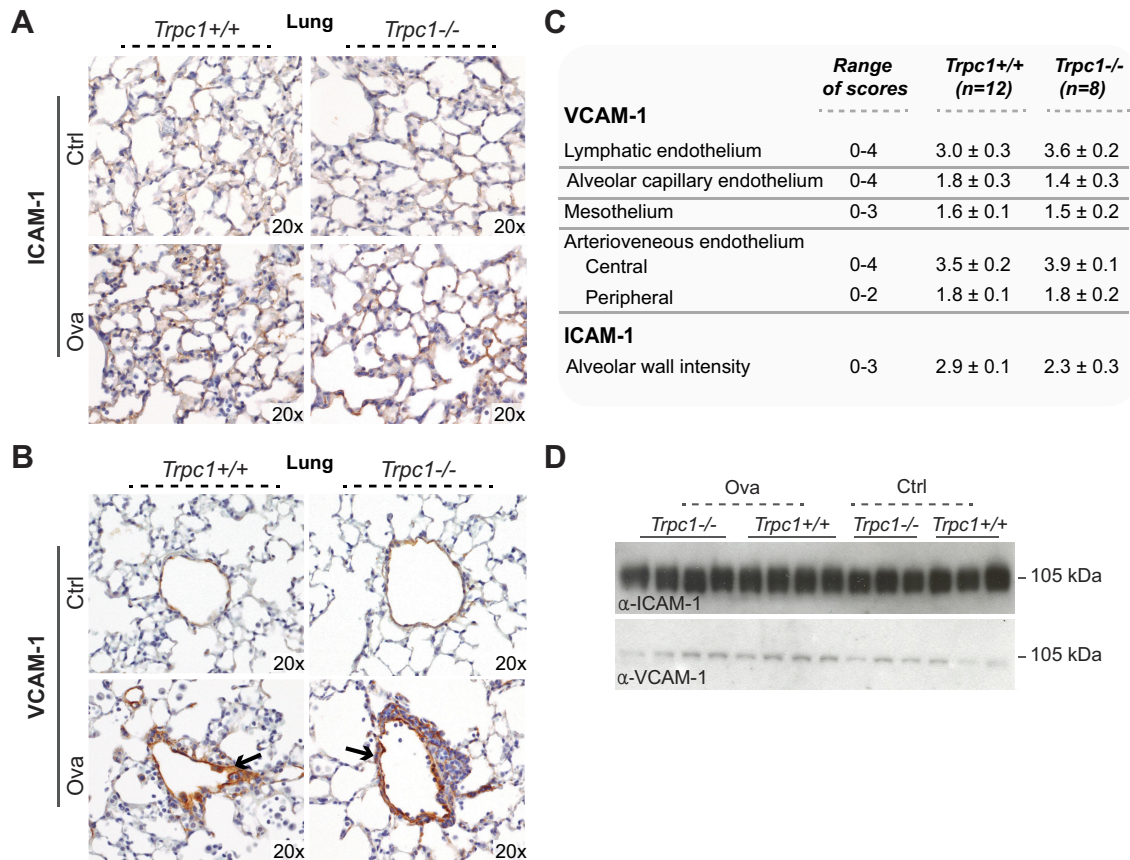


Fig. 3. Change in adhesion molecule expression is not a factor in dysfunctional T helper type 2 (Th2) cell-mediated pulmonary inflammation in *Trpc1*^{-/-} mice. *A* and *B*: similar degree of adhesion molecule expression in lungs of control and allergen-sensitized wild-type and *Trpc1*^{-/-} animals in blinded review of ICAM-1-stained (note linear staining of alveolar pneumocytes and capillaries; *A*) and VCAM-1-stained (note endothelium staining, arrows; *B*) lung sections. *C*: semiquantitative evaluation of ICAM-1 and VCAM-1 immunohistochemistry. *D*: Western blot of whole cell extracts from lung samples from animals with indicated genotypes ($n = 3-4$ animals/group) and conditions showing no significant change in adhesion molecule expression in *Trpc1*^{-/-} compared with wild-type mice.

isolated splenocytes 7 days later, cultured the cells in the absence or presence of increasing concentrations of ovalbumin for 72 h, and measured the rate of cell proliferation using an MTT assay. In the presence of ovalbumin, *Trpc1*^{-/-} splenocytes proliferated less than their wild-type counterparts (Fig. 5A). In accordance with the BALF cytokine profiles, *Trpc1*^{-/-} splenocytes produced significantly less IL-2 (Fig. 5B), IL-4, IL-5, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 5C). These data support our *in vivo* observation that TRPC1 plays a crucial role in transcriptional regulation of key Th2 cytokines.

Impaired TCR-Induced IL-2 Release in Splenocytes

IL-2 expression is a primary signal for the clonal expansion of T cells at the initiation of the Th2 response and is regulated by activation of Ca²⁺-regulated NFAT transcription factor (45). The reduced production of IL-4, IL-5, and, in particular, IL-2 by lymphocytes from allergen-sensitized *Trpc1*^{-/-} mice suggests that NFAT signaling and T cell activation might be altered in these cells. To test this hypothesis, we stimulated TCR signaling in splenocytes from the naïve control and *Trpc1*^{-/-} mice by culturing the cells in the presence of plate-bound anti-CD3 antibody alone or in combination with soluble anti-CD28 antibody for 48 h. Despite the absence of a reduc-

tion in proliferation rate of the *Trpc1*^{-/-} splenocytes in the presence of anti-CD3 antibody or anti-CD3 antibody plus anti-CD28 antibody (Fig. 6A), the production of IL-2 in response to anti-CD3 antibody by *Trpc1*^{-/-} splenocytes was significantly reduced (Fig. 6B). However, IL-2 production by the *Trpc1*^{-/-} and wild-type splenocytes was indistinguishable when the cells were cultured in the presence of anti-CD3 plus anti-CD28 antibodies (Fig. 6B). These results support earlier findings in B cells (37) and provide additional evidence that TRPC1 is downstream of TCR-mediated signaling. It is also likely that TCR-induced proliferation of *Trpc1*^{-/-} lymphocytes was masked upon CD28 costimulation because of a parallel pathway in which NFAT signaling is potentiated upon dissociation of Homer, a cytoplasmic scaffolding protein that participates in receptor-operated Ca²⁺ entry by interacting with the TRPC1:inositol 1,4,5-trisphosphate receptor 3 complex (63), from NFAT, improving its accessibility to calcineurin (19, 22).

Hyperactive B Cells With High Level of Immunoglobulin Release

Given the severe blunting of eosinophilia and diminished Th2 cell response in the allergen-sensitized *Trpc1*^{-/-} mice, we questioned whether B cell responses might also be affected in

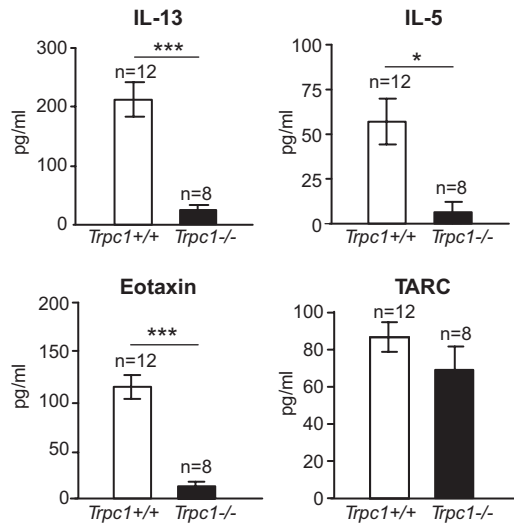


Fig. 4. Reduced expression of Th2-type cytokines and chemokines in lungs of allergen-sensitized *Trpc1*^{-/-} mice. Significantly reduced IL-13, IL-5, and eotaxin and unchanged thymus- and activation-regulated chemokine (TARC) levels in cell-free BAL fluid of *Trpc1*^{-/-} animals compared with control mice. Levels for the proteins were undetectable in control groups (data not shown). Values are means $\pm$ SE. * P < 0.05; *** P < 0.001.

these mice. Spleen-to-body weight ratio of the naïve wild-type and *Trpc1*^{-/-} mice showed readily apparent splenomegaly in the *Trpc1*^{-/-} mice (Fig. 7A). We next examined the pathology of spleen sections for the number of total and active (characterized by the presence of tingible body macrophages) germinal centers and the total number of plasmacytoid foci in the marginal zone of the lymphoid tissue (Fig. 7B). In the white pulp (lymphoid) area, the percentage of active germinal centers was nearly twofold larger in vehicle-treated *Trpc1*^{-/-} mice (Fig. 7B, image b) than wild-type animals (Fig. 7B, image a). The germinal centers of these animals were frequently more rounded and contained more tingible body macrophages, which are indicative of activation. Additionally, segmental expansion of the marginal zone of the lymphoid tissue was apparent in the lymphoid follicles. This expansion was often antipodal to the germinal centers, and the site was enriched for plasma cells and plasmacytoid and monocytoid cells. Semi-quantitative analysis of these spleen sections showed nearly fourfold more plasmacytoid cell clusters in the vehicle-treated *Trpc1*^{-/-} mice (Fig. 7B, image b, inset) than their wild-type controls (Fig. 7B, image a, and C). The hyperactive B cell phenotype that was observed in the vehicle-treated *Trpc1*^{-/-} mice was more pronounced in the spleens of the allergen-sensitized *Trpc1*^{-/-} mice. More plasma cell clusters (Fig. 7B, images c and d) and significantly larger germinal centers (Fig. 7B, images e and f, and C) were detected in the spleens of the allergen-sensitized *Trpc1*^{-/-} mice.

These results were further strengthened by the analysis of total serum immunoglobulin titers by ELISA. In accordance with the hyperactive B cell phenotype, the vehicle-treated *Trpc1*^{-/-} mice had significantly higher serum IgM, IgG2a, and IgG2b levels than wild-type mice (Fig. 7D). The immunoglobulin titers were different in the allergen-sensitized *Trpc1*^{-/-} mice, in that all the immunoglobulins (IgA, IgM, IgE, IgG1, IgG2a, IgG2b, and IgG3) were significantly upregulated compared with their wild-type counterparts (Fig. 7D). Collectively,

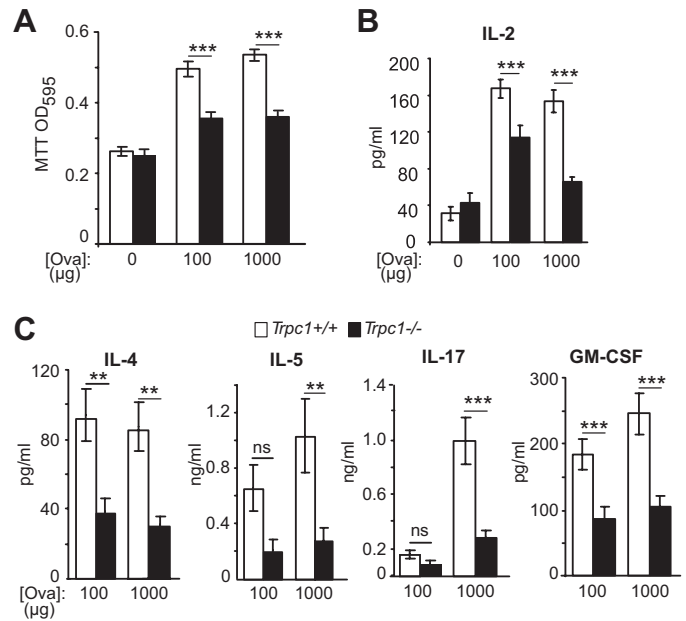


Fig. 5. Allergen-sensitized *Trpc1*^{-/-} splenocytes proliferate less and produce lower levels of Th2 cytokines in response to allergen in vitro. A: attenuated proliferation of splenocytes from allergen-sensitized *Trpc1*^{-/-} animals compared with wild-type mice in the presence of the allergen (ova) in vitro. Proliferation of splenocytes from control (vehicle-treated) mice was indistinguishable between the groups (data not shown). MTT, 3-(4,5-diphenylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD₅₉₅, optical density at 595 nm. B and C: attenuated release of IL-2, IL-4, IL-5, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF) from allergen-sensitized *Trpc1*^{-/-} splenocytes to the culture medium upon stimulation with the allergen in vitro. Levels of immunoreactive IL-4, IL-5, IL-17, and GM-CSF were undetectable in control groups (data not shown). Values are means $\pm$ SE. * P < 0.05; ** P < 0.01; *** P < 0.001. ns, Not significant.

these data provide the first evidence that, directly or indirectly, TRPC1 is a negative regulator of B cell activation.

DISCUSSION

In this study, we provide the first physiological evidence that TRPC1 channels are important regulators of lung function and

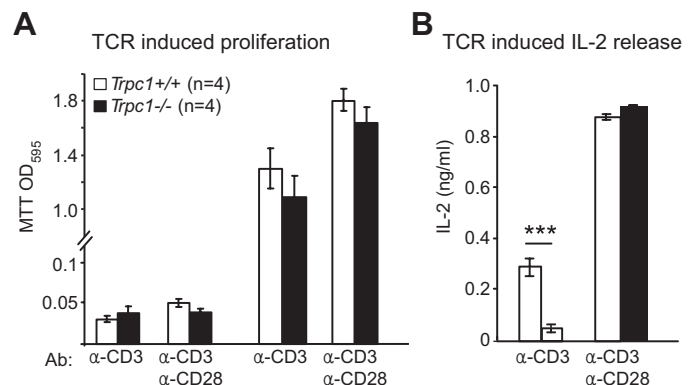


Fig. 6. T cell receptor (TCR)-induced IL-2 release is reduced in *Trpc1*^{-/-} splenocytes. A: loss of TRPC1 did not affect TCR-induced proliferation of splenocytes upon stimulation of TCR signaling by plate-bound anti-CD3 (10 µg/ml) and/or soluble anti-CD28 (1 µg/ml) antibodies. B: reduced TCR-mediated IL-2 secretion from cultured *Trpc1*^{-/-} splenocytes compared with their wild-type counterparts. Values are means $\pm$ SE from a representative of 5 independent experiments. *** P < 0.001.

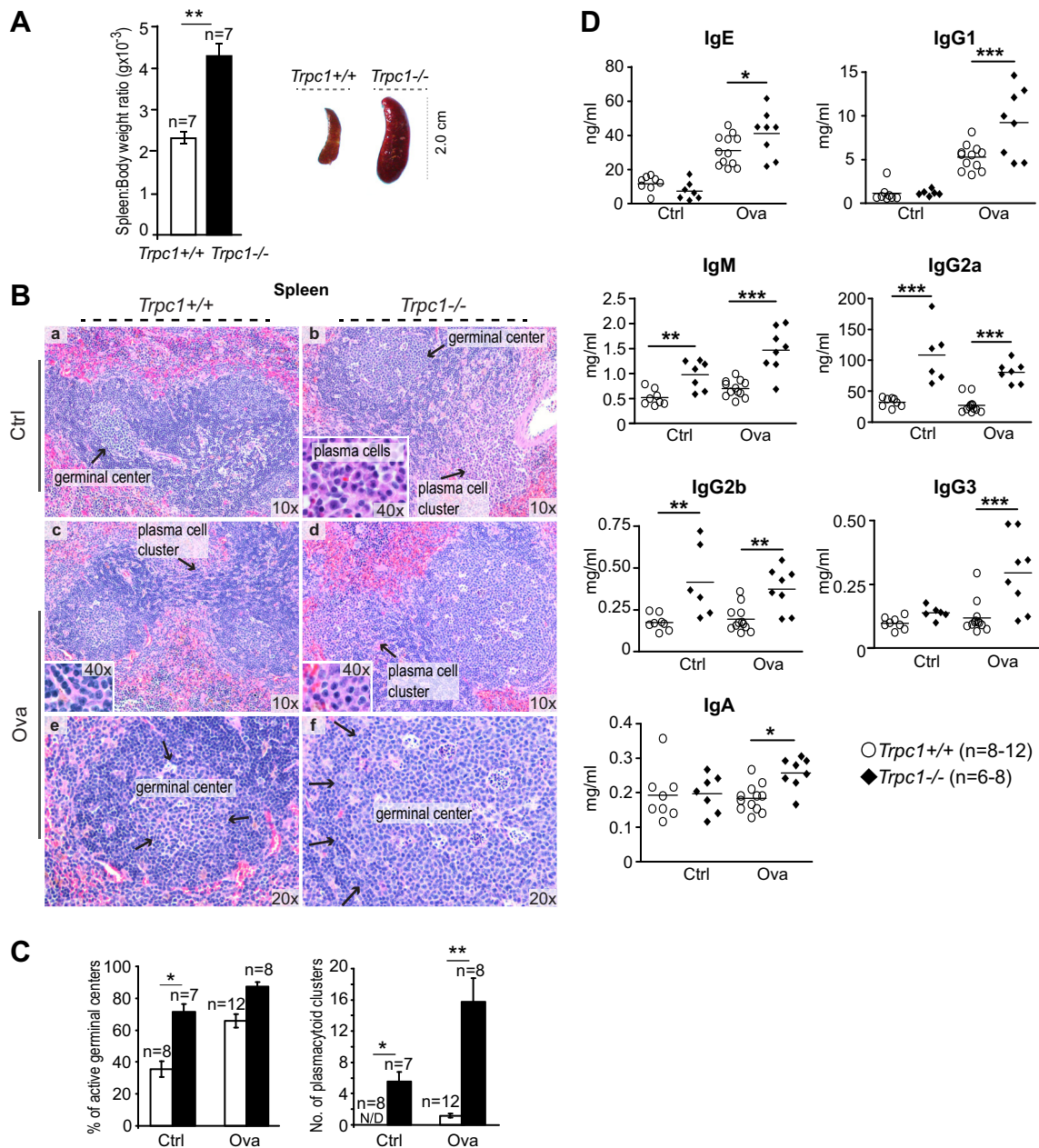


Fig. 7. *Trpc1*^{-/-} mice have splenomegaly with increased plasma cells and activated germinal centers. **A**: higher spleen-to-body weight ratio in naïve *Trpc1*^{-/-} mice. Values are means $\pm$ SE. * P < 0.05; ** P < 0.01. Representative spleen images are shown for wild-type and *Trpc1*^{-/-} mice. **B**: hematoxylin-eosin-stained central sections of spleen from control (n = 6–8 animals/genotype) and allergen-sensitized (n = 8–12 animals/genotype) wild-type and *Trpc1*^{-/-} mice were semiquantitatively analyzed for activated germinal centers and number of plasmacytoid clusters. In images *a* and *b*, note more plasmacytoid clusters and larger activated germinal centers in *Trpc1*^{-/-} than wild-type animals. *Insets*: plasma cells shown at higher magnification. In images *c* and *d*, note larger and more numerous clusters of plasma cells and larger germinal cells with more tingible body macrophages in spleens from allergen-sensitized *Trpc1*^{-/-} mice. In images *e* and *f*, note larger and more active germinal centers of allergen-sensitized *Trpc1*^{-/-} mice than their wild-type counterparts. **C**: percentage of active germinal centers and number of plasmacytoid clusters. Values are means $\pm$ SE. **D**: control and allergen-induced *Trpc1*^{-/-} animals show higher levels of total serum immunoglobulin levels (indicative of hyperactive plasma cells) than their wild-type counterparts. * P < 0.05; ** P < 0.01; *** P < 0.001.

immune cell response. In the lungs, airway smooth muscle cells are the main effector cells governing the constricted or relaxed state of airways, processes that require activation of different G protein-coupled receptors and subsequent induction of inositol 1,4,5-trisphosphate receptor-induced Ca^{2+} release followed by Ca^{2+} entry from the extracellular space (reviewed in Ref. 18). In vitro evidence from antisense oligonucleotide experiments suggests that TRPC1-dependent Ca^{2+} influx is an

important modulator of bronchial constriction and bronchial smooth muscle proliferation (52). Furthermore, TRPC1, in association with TRPC4 and stromal interaction molecule 1 (STIM1) (48), was also reported to regulate lung endothelial cell permeability (8, 50). Our mouse model is in agreement with these findings at the physiological level and suggests that TRPC1 might be involved in signaling pathways to regulate lung function as detected by loss of hyperresponsiveness to

methacholine challenge. It is also important to note that genetic linkage studies in humans have associated asthma to several loci, one of which is 3q22-q24 (17), the locus encoding TRPC1. Therefore, hyposensitivity to allergen exposure in TRPC1 may also be due to decreased genetic susceptibility to allergen-induced bronchoconstriction.

We also report that hyposensitivity to allergen exposure in *Trpc1*^{-/-} animals was accompanied by severely diminished Th2 cell response. In allergen-induced *Trpc1*^{-/-} mice, we detected significantly low levels of IL-4 and IL-13, which are key cytokines for differentiation of naïve T cells into Th2 cells (51) and promote isotype switching in B cells from IgM to IgE and IgG1 for the clonal expansion of Th cells (40, 48). Interestingly, a similar TRPC1-dependent defect was detected with IL-5, a cytokine necessary for growth, differentiation, proliferation, and survival of eosinophils, as well as their priming, to respond to chemokines such as eotaxin (61), suggesting that TRPC1 is proinflammatory. These data might also explain lack of eosinophil-dependent inflammatory response in the airways and lack of typical increase in the levels of the potent eosinophil chemoattractant eotaxin in BALF of *Trpc1*^{-/-} animals upon allergen exposure.

At the cellular level, T and B cells, in particular, demonstrated multiple dysfunctions in signaling pathways. In splenocytes, we measured IL-2 levels as a means to examine changes in anti-CD3-induced activation of TCR signaling, which was significantly diminished in mutant cells, placing TRPC1 signaling upstream of IL-2 transcription in T lymphocytes. This finding complements earlier findings in *Trpc1*^{-/-} avian DT-40 B cells, in which inositol trisphosphate-mediated Ca²⁺ release in response to B cell receptor stimulation and thapsigargin-induced SOCE were attenuated and restored by expression of human TRPC1 (37). Moreover, activity of NFAT was significantly reduced in avian *Trpc1*^{-/-} B cells, which provides evidence for participation of TRPC1 in SOCE in NFAT-mediated gene expression.

In B cells of *Trpc1*^{-/-} mice, we detected apparent spontaneous hyperactivity, as it was possible to detect significant levels of IgM, IgG2a, and IgG2b in the vehicle-treated *Trpc1*^{-/-} mice, in contrast to the undetectable levels of any immunoglobulins in the serum of their wild-type counterparts. Moreover, levels of almost all the immunoglobulins tested were significantly higher in the serum of the allergen-sensitized *Trpc1*^{-/-} mice than control animals. Histological analysis showing more plasma cell accumulation and larger germinal centers in spleen sections of mutant animals supports these findings. High serum levels of various immunoglobulins, in particular IgE and IgG1, normally would induce release of vasoconstrictive mediators (i.e., histamine, prostaglandins, and leukotrienes) (reviewed in Ref. 43) by activating mast cells and, subsequently, cause bronchoconstriction, mucus production, and lymphocyte infiltration in the lungs. High immunoglobulin levels failed to cause higher bronchoconstriction in the lungs of *Trpc1*^{-/-} animals than control mice. However, it is plausible to think that high immunoglobulin levels are responsible for the progression of partial lymphocyte-predominant inflammatory response and mucus secretion (determined by PAS staining), which was indistinguishable between allergen-sensitized mutant and wild-type animals. In many aspects, the immunological phenotype of *Trpc1*^{-/-} mice resembles the phenotypes observed in NFAT-deficient mice, in particular

NFATc1/NFATc2. As in the case of *Trpc1*^{-/-} mice, under Th2-inducing conditions, NFATc1/NFATc2 double-knockout mice show impaired Th cell effector functions, such as release of cytokines, and have hyperactivated B cells, as seen by elevated serum IgG1 and IgE levels (41).

Diminished, but not full, loss of allergen-induced inflammatory response in the allergen-induced *Trpc1*^{-/-} mice also suggests that TRPC1 channels might be functioning in conjunction with other molecules, including other TRPCs (i.e., TRPC3, TRPC4, and TRPC6) and STIM1 Ca²⁺ release-activated Ca²⁺ channels (23, 25, 32, 33, 62). In support of this hypothesis, a reduced allergic airway response has also been found in *Trpc6*^{-/-} mice, to the extent that they were measured, with many of the same characteristics seen in *Trpc1*^{-/-} mice, albeit not as noticeable (44). However, because of the marked upregulation of TRPC3 mRNA in some lung tissues of *Trpc6*^{-/-} mice (10, 12), it has been impossible to ascribe a role for the TRPC6 gene product. Notably, TRPC3 channels have a high degree of spontaneous activity and mediate unregulated Ca²⁺ entry into cells (47), thus mimicking the role we ascribe to TRPC1 in regulation of the allergic Th2 response and B cell homeostasis. We propose that, in all cases, the role of TRPCs is to supply the Ca²⁺ required in inflammatory processes, such as shown for TRPC4 in mediating activation of NFκB in response to thrombin in mouse lung endothelial cells (2).

Even though the mechanism whereby TRPC1 regulates these events is yet to be determined, we propose that TRPC1-coupled Ca²⁺ signaling is necessary in lymphocyte biology, as are Ca²⁺ release-activated Ca²⁺ channels (14, 39), and participates in signaling events for regulation of allergen-induced lung function. Therefore, the *Trpc1*^{-/-} mouse provides a unique model to study different stages of allergen-induced pulmonary inflammation and changes in lung function and presents TRPC1 as a potential drug target for asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.Y., M.A.C., J.W.C., D.C.Z., and L.B. are responsible for conception and design of the research; E.Y., M.A.C., J.W.C., A.D., Y.Z., J.A.B., Y.R., and D.L.M. performed the experiments; E.Y., M.A.C., J.W.C., G.P.F., Y.Z.,

D.C.Z., and L.B. analyzed the data; E.Y., M.A.C., G.P.F., Y.Z., D.R.G., D.L.M., D.C.Z., and L.B. interpreted the results of the experiments; E.Y., J.W.C., and L.B. prepared the figures; E.Y. and L.B. drafted the manuscript; E.Y., A.D., G.P.F., and L.B. edited and revised the manuscript; E.Y., M.A.C., J.W.C., A.D., G.P.F., Y.Z., J.A.B., Y.R., D.R.G., D.L.M., D.C.Z., and L.B. approved the final version of the manuscript.

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