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

Eda Yildirim,1* Michelle A. Carey,1* Jeffrey W. Card,1 Alexander Dietrich,2 Gordon P. Flake,1 Yingpei Zhang,1 J. Alyce Bradbury,1 Yvette Rebolloso,1 Dori R. Germolec,1 Daniel L. Morgan,1 Darryl C. Zeldin,1 and Lutz Birnbaumer1

1Intramural Research Program, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina; and 2Department of Anesthesiology, David Geffen School of Medicine, University of California, Los Angeles, California

Submitted 15 December 2011; accepted in final form 11 July 2012

Yildirim E, Carey MA, Card JW, Dietrich A, Flake GP, Zhang Y, Bradbury JA, Rebolloso 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 hyperreactivity, 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 hyporesponsive to methacholine challenge and blunting of the allergic Th2 cell response, as seen in TRPC1-deficient mice. Under allergic conditions, different aspects of allergen-induced pulmonary 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 IFNγ (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 (42, 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 hyporesponsiveness to TNF-α-mediated Ca2+ signaling (58). In endothelial cells, loss of TRPC4 results in loss of thrombomodulin-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 IFNγ (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 hyporesponsiveness 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 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 thrombomodulin-mediated actin-stress fiber formation and reduced retraction of lung endothelial cells, which is coupled with reduction in lung microvascular permeability (8, 55).

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

* E. Yildirim and M. A. Carey contributed equally to this work.

Address for reprint requests and other correspondence: L. Birnbaumer, Laboratory of Neurobiology, National Institutes of Health, Dept. of Health and Human Services, 111 T. W. Alexander Dr., Research Triangle Park, NC 27709 (e-mail: birnbaul@nih.gov).

http://www.ajplung.org
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 by injection of pentobarbital sodium (80 mg/kg ip). Lungs were 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 × 107/well) from control and allergen-sensitized wild-type and Trpc1−/− mice were cultured in sixtuples 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 × 107/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 then 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-
tricle 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 (PPM4057A), TRPC5 (PPM4617A), TRPC6 (PPM4056A), TRPC7 (PPM4062A), and GAPDH (PPM2946E)] were obtained from SABiosciences. mRNA levels were calculated using the following equation: $2^{\Delta\Delta Ct}$, where $Ct$ is cycle threshold.

**Lang 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).

**Lang 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, BAL 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. 2B, 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.
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 NFκB and AP-1 (42) and the Ca2++-
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 Ca2+ 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.
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. 5B). In accordance with the BALF cytokine profiles, Trpc1−/− splenocytes produced significantly less IL-2 (Fig. 5A), IL-4, IL-5, IL-17, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 5C). These data support our in vivo observations 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 reduction 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...
ulin titers were different in the 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 ± 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 macrophage clusters in the vehicle-treated Trpc1−/− mice (Fig. 7B, image b, inset) than their wild-type counterparts. Values are means ± SE. *P < 0.05; ***P < 0.001.

These results were further strengthened by the analysis of total serum immunoglobulin titers by ELISA. In accordance with the hyperreactive 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, 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.
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

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 ± 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. Inset: 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 ± 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.
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 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.

ACKNOWLEDGMENTS

We thank J. Abramowitz for critical reading of the manuscript. We are grateful to all members of the Birnbaumer and Zeldin labs for stimulating discussions and feedback. We acknowledge members of the National Institute of Environmental Health Sciences histology core facility for assistance with histology, S. Ward for assistance with cell differential readings, and T. Sliwa for the maintenance of the mouse colonies.

Present addresses: E. Yildirim, Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, MA 02114; J. W. Card, Cantox Health Sciences International, An Intertek Company, 2233 Argentia Rd., Suite 308, Mississauga, ON, Canada L5N 2X7; A. Dietrich, Walther-Straub Institute for Pharmacology and Toxicology, LM University, Munich, Germany.

GRANTS

This work was supported by the Intramural Research Program of the National Institutes of Health: National Institute of Environmental Health Sciences Grants Z01-ES-101684 (L. Birnbaumer) and Z01-ES-025043 (D. C. Zeldin). This research was conducted in part at the National Institutes of Environmental Health Sciences Inhalation Facility under contract to Alion Science and Technology.

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.,
BLUNTED ALLERGEN-INDUCED PULMONARY RESPONSE IN Trpc2−/− MICE


REFERENCES


6.スピードはないと考えられる。


48. Rosing RJ, Luo C, Hogan PG. Differential regulation of IgG1 by 10.220.33.1 on June 20, 2017 http://ajplung.physiology.org/ Downloaded from