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Abr, a Negative Regulator of Rac, Attenuates Cockroach Allergen–Induced Asthma in a Mouse Model

Dapeng Gong,¹ Fei Fei, Min Lim, Min Yu,² John Groffen, and Nora Heisterkamp

Abr deactivates Ras-related C3 botulinum toxin substrate (Rac), a master molecular switch that positively regulates many immune cell functions, by converting it to its GDP-bound conformation. In this article, we report that, in the absence of Abr function, cockroach allergen (CRA)-immunized mice experienced a fatal asthma attack when challenged with CRA. The asthma in *abr*^{−/−} mice was characterized by increased pulmonary mucus production, elevated serum IgE, and leukocyte airway infiltration. Decreased pulmonary compliance was further documented by increased airway resistance upon methacholine challenge. Peribronchial and bronchoalveolar lavage eosinophils, key cells associated with allergic asthma, were increased in *abr*^{−/−} mice, but adoptive transfer of this cell type from immunized mice to naive controls, followed by CRA challenge, showed that eosinophils are not primarily responsible for differences in airway resistance between controls and *abr*-null mutants. CD4⁺ T cell numbers in the airways of CRA-challenged *abr*^{−/−} mice also were significantly increased compared with controls, as were the Th2 T cell–secreted cytokines IL-4 and IL-5 in total lung. Interestingly, when control and *abr*^{−/−} CD4⁺ T cells from CRA-immunized mice were transferred to wild-type animals, airway resistance upon challenge with CRA was significantly higher in mice transplanted with T cells lacking Abr function. CD4⁺ T cells from CRA-immunized and challenged *abr*^{−/−} mice contained elevated levels of activated GTP-bound Rac compared with wild-type controls. Functionally, *abr*^{−/−} CD4⁺ T cells from CRA-exposed mice showed significantly enhanced chemotaxis toward CCL21. These results identify Abr-regulated CD4⁺ T cell migration as an important component of severe CRA-evoked allergic asthma in mice. *The Journal of Immunology*, 2013, 191: 000–000.

The human *ABR* gene on chromosome 17p13.3 encodes a 98-kDa protein that consists of four modular domains (1). Of these, the function of the domain with homology to GTPase-activating proteins (GAPs) for the Rho family of GTPases has been investigated in most detail (2, 3). Members of the Rho family, including Ras-related C3 botulinum toxin substrate (Rac), act as molecular switches, cycling from an active GTP-bound state to an inactive GDP-bound state. The active and inactive states have different conformations, with the GTP-bound active form of Rac responsible for the interaction with and activation of downstream effectors. For example, binding of RacGTP to a major effector, the p21 protein (Cdc42/Rac)–activated kinase 1 (Pak1), induces a conformational change in Pak1 that activates its kinase activity (4). RacGTP activates glycogen phosphorylase and stimulates T cell proliferation by a similar mechanism (5).

Rac activity is crucial for the positive activation of numerous processes involving reorganization of the actin cytoskeleton in immune cells (diapedesis, chemotaxis, phagocytosis) and for activation of the NADPH oxidase expressed by myeloid and other cells (6). There are only three Rac proteins that perform these numerous functions. The specificity of these small G-proteins is accomplished through tightly controlled regulation of their activation and deactivation cycle. A family of proteins called G-nucleotide exchange factors (GEFs) is responsible for activation, and >69 human Rho family GEFs are known to exist. Deactivation is catalyzed by the GAPs, of which ~70 have been identified, including Abr (7, 8). The various GAPs and GEFs differ from each other in tissue or cell type–specific expression, subcellular location, and upstream activation. Therefore, a major field of research is to determine which GAPs and GEFs regulate the various functions of Rac in different cells, tissues, and pathologies (9).

Studies by us (10) and other investigators (3) using purified proteins established that Abr has a specificity for deactivating Rac proteins, although it also was active on cell cycle control protein 42 homolog (Cdc42) in vitro. To definitively determine the function of Abr in vivo, we generated mice lacking *abr*. Experiments with these mice, as well as transfection of Abr into cultured cells and assays for deactivation of Rac or cell cycle control protein 42 homolog, were all consistent with this GAP only catalyzing the deactivation of Rac and not of other Rho family members in vivo (11). Mice lacking Abr are phenotypically normal. However, when challenged in experimental models of sepsis (12) and pulmonary hypertension (13), the consequences of the lack of a functional Abr protein became evident in the form of significantly exacerbated pathology.

Asthma is a serious health problem that appears to be increasing in incidence. Acute asthma attacks are responsible for many emergency room visits and can cause death. In 2009, 24.6 million patients with asthma were reported in the United States alone, of which ~8 million were children (14). Studies indicate that expo-

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; CRA, cockroach allergen; EPO, eosinophil peroxidase; GAP, GTPase-activating protein; GEF, G-nucleotide exchange factor; i.n., intranasal(ly); Pak1, p21 protein (Cdc42/Rac)–activated kinase 1; PNU, protein nitrogen unit; Rac, Ras-related C3 botulinum toxin substrate; wt, wild type.

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sure to cockroach allergen (CRA) plays an important role in asthma (15–17). CRA consists of proteins derived from cockroach saliva, feces, exoskeleton, and dead bodies. Because of the ubiquitous existence of cockroaches and their widespread household infestation in urban dwellings, CRA poses a serious risk for allergic asthma (18).

A murine model for human atopic asthma has been developed on the basis of sensitization and exposure to CRA (19, 20). Because CRA is associated with human asthma, the CRA-induced asthma model in mice is clinically relevant (19–21). In this study, using this model in mice lacking ABR, we demonstrate that ABR is responsible for keeping severe pathological manifestations of asthma in check through regulation of the influx of CD4⁺ T cells.

Materials and Methods

Animals

abr^{−/−} mice were generated previously (22) and were maintained on an FVBJ inbred background. All animal studies were approved by the Institutional Animal Care and Use Committee of the Saban Research Institute, Children's Hospital Los Angeles. For each set of experiments, only age- and gender-matched mice were compared.

CRA sensitization and challenge

Mice (7–9 wk old) were sensitized and challenged with CRA (Holister Stier Laboratories, Spokane, WA), according to guidelines established by previous studies (19, 23, 24). For the experiment in Fig. 1, age-matched control (compound heterozygotes, *abr*^{+/−}, *bcr*^{+/−}, phenotypically wild-type [wt]) and *abr*^{−/−} mice were immunized by i.p. (50-μl) and s.c. (50-μl) injections of a 1:1 dilution of CRA (120,000 protein nitrogen units [PNU]/ml) in IFA (Sigma-Aldrich, St. Louis, MO) followed by intranasal (i.n., 20 μl) instillation of a 1:2 dilution of CRA in PBS on days 7, 14, 20, and 22. For all other experiments, mice were immunized i.p. and s.c. with 50 μl emulsion, consisting of a 1:1 mixture of CRA (20,000 PNU/ml) and IFA. On day 14, mice were anesthetized i.p. with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and given 20 μl CRA (10,000 PNU/ml) i.n. to localize the systemic response to the airways of the lung. After six additional days (on day 20 from initial sensitization), mice were challenged intratracheally with 40 μl CRA (10,000 PNU/ml), followed by a second dose 48 h later (day 22). All measurements were performed (or samples were taken) 24 h following the second challenge (day 23).

Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured using a mouse plethysmograph (flexiVent; SCIREQ USA, Tempe, AZ). Briefly, mice were weighed and anesthetized with sodium pentobarbital (90 mg/kg body weight) by i.p. injection to achieve deep anesthesia. Trachea were exposed and intubated with an 18-gauge metal tube, through which mice were connected to the precalibrated plethysmograph and quasi-sinusoidally ventilated with a computer-controlled ventilator with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min to achieve a mean lung volume close to that of normal breathing. Once the anesthesia and ventilation were stabilized (no spontaneous breathing, because it interferes with the measurement by the flexiVent system), a “snapshot” perturbation, which is a sinusoidal wave of inspiration and expiration controlled by the ventilator, was applied to measure airway resistance using the computer software that comes with the flexiVent system. After the baseline levels of airway resistance were measured, mice were challenged with methacholine via an in-line nebulizer. The optimal methacholine dose (10 mg/ml, via the flexiVent nebulizer) for AHR in our asthma model was determined by a dose-response curve (1–160 mg/ml) and used throughout the experiments. After the methacholine challenge via the nebulizer, the airway resistance was measured by the flexiVent software following the “snapshot” perturbation. Airway resistance levels (following methacholine challenges), as indices for airway hyperactivity, were plotted and compared among different groups of mice to indicate the severity of AHR in our asthma model.

Cytokine and serum IgE measurements

Blood was collected through retro-orbital bleeding and centrifuged at 2500 × *g* for 10 min. Serum was separated and stored at −80°C until analysis. Total serum IgE levels were measured using an ELISA kit (BioLegend, San Diego, CA), according to the manufacturer's instructions. Lungs were

collected on day 23 following the sensitization protocol. Samples were homogenized, and an aliquot of lung homogenates was centrifuged at 2000 rpm for 5 min. Supernatants were collected for IL-4 and IL-5 measurement using mouse ELISA kits (BioLegend), according to the manufacturer's protocol. CCL21 levels in total lung and bronchoalveolar lavage fluid (BALF) IL-4 and IL-5 levels were determined using murine ELISA kits for 6Ckine/CCL21 (Sigma-Aldrich) and for IL-4 and IL-5 (BioLegend).

Bronchoalveolar lavage cells

To prepare for bronchoalveolar lavage, tracheas were exposed and cannulated with an 18-gauge angiocath. Lungs were lavaged five times with 0.8 ml cold sterile PBS containing 25 nM EDTA. For studies of cytokines, the first wash was collected separately and clarified by centrifugation, and the supernatant was stored at −80°C. Total BALF cells were pooled from the five washes, and total cell counts were recorded. BALF cells were analyzed by flow cytometry or cytospin. For cytospins, 80–100,000 cells were dispersed using a cytospin centrifuge (Shandon Scientific) and differentially stained with Kwik-Diff stain (Fisher Scientific; similar to Wright-Giemsa). The cell types (alveolar macrophages, monocytes, lymphocytes, neutrophils, and eosinophils) were differentially counted and expressed as a percentage of the total leukocytes. Eosinophils were identified morphologically, based on their distinct nuclei and cytoplasmic granules. We used flow cytometry analysis to confirm alveolar macrophage quantification. BALF cells were stained with FITC-Ly6G, PerCP-CD45, and allophycocyanin-CD11b. Because of the autofluorescence (into the FITC channel) of alveolar macrophages, they form a unique population Ly6G^{med}CD11b^{med}, which distinguishes them from neutrophils (Ly6G^{hi}CD11b^{hi}) and from monocytes/inflammatory macrophages (Ly6G^{lo}CD11b^{hi}).

Flow cytometry and intracellular staining

Cells were obtained from blood, spleen, and lung. To prepare single-cell lung suspensions, tissues were cut into small pieces (1–2 mm) and digested with collagenase B (2 mg/ml) and DNase I (0.5 mg/ml; both from Roche) at 37°C for 40 min. The suspension was ground through a 70-μm cell strainer (Fisher Scientific) using a syringe plunger to disrupt residual tissue. The cells were pelleted and resuspended in RBC Lysis Buffer (Pharm Lyse; BD). Cells were collected after a PBS wash and stained with fluorochrome-labeled mAbs. Data were acquired on an Accuri flow cytometer and analyzed using Accuri software (BD Bioscience). FITC-Ly6G, FITC-CD4, PerCP-CD45, allophycocyanin-CD11b, PE-IL-4, and PE-CD8 were from BioLegend. For intracellular staining of IL-4, spleen cells from naive mice were isolated and cultured in complete DMEM medium at 2 × 10⁶ cells/ml/well in a 24-well plate precoated with anti-CD3 and anti-CD28 (2 μg/ml) for 48 h. For Th2 polarization, cells were primed in the presence of IL-4 (10 μg/ml), with anti-IFN-γ (10 μg/ml), and anti-IL-12 (10 μg/ml)-blocking Abs added. For Th0 cells, no cytokines or Abs were added. Cells were washed and analyzed after 2–3 d of priming. Aliquots of 10⁶ cells/ml were incubated for 4–6 h with brefeldin A (1 μg; GolgiPlug; BD Biosciences), PMA (50 ng/ml), and ionomycin (0.5 μg/ml) for analysis of cytokine production. Cells were pelleted, resuspended in PBS with 2% FCS, stained with surface marker Abs, fixed, permeabilized with BD Cytotfix/Cytoperm, and stained intracellularly with Abs to IL-4.

Measurement of peroxidase

Cell-free BALF supernatants were collected and frozen at −80°C. A total of 50 μl each sample was mixed with 100 μl substrate (0.2 mg/ml *O*-phenylenediamine in Tris [pH 8], containing 0.1% Triton and 0.02% H₂O₂) in 96-well plates. The reaction was allowed to progress for 30 min before quenching with 50 μl 2 M sulfuric acid. ODs were read at 490 nm using an ELISA plate reader. The cellular components of the BALF preparation contained 1.23 × 10⁶ eosinophils/0.06 × 10⁶ neutrophils (for wt) and 1.82 × 10⁶ eosinophils/0.092 × 10⁶ neutrophils (for *abr*^{−/−}). Thus, neutrophil numbers were 5% of eosinophil numbers, and the difference observed in these assays primarily represents eosinophil peroxidase (EPO) release with a small contribution of neutrophil myeloperoxidase in BALF.

Lung histopathology and peribronchial eosinophil quantification

Twenty-four hours following the final CRA challenge, lungs were fixed with 10% buffered formalin for 10 h (or overnight) and then transferred to 70% ethanol. The fixed lungs were embedded in paraffin, and multiple 5-μm sections were cut. Sections were stained with H&E for light microscopic analysis. Eosinophils in the peribronchial region were counted in 50 high-power fields under 400× magnification. Other sections were stained with periodic acid-Schiff to examine mucin production.

Measurement of T cell activation

To measure CD4⁺ T cell activation, we purified T cells from the spleens of naive mice ($n = 3/\text{genotype}$) using a MACS column (Miltenyi Biotec). T cells were stimulated for 48 h by plating at 2×10^6 cells/ml on plates coated with 2 $\mu\text{g}/\text{ml}$ anti-CD3/CD28 Abs. Controls were incubated on noncoated plates. Cell surface expression of the T cell activation markers CD25 and CD69 (BioLegend) was compared using FACS. Supernatants of CD4⁺ T cells from two wt and *abr*^{-/-} mice/genotype that had been activated with anti-CD3/CD28 were compared for cytokine secretion using a mouse cytokine Ab array (cat. no. ARY006; R&D Systems, Minneapolis, MN), according to the supplier's protocols.

Adoptive transfer

CD4⁺ T cells were isolated from spleen using a MACS column (Miltenyi Biotec), whereas eosinophils (SSC^{hi}CD11b⁺Ly6G⁻) were flow sorted from single-cell lung suspensions using a BD FACSaria sorter on day 21, following the protocol in Fig. 2A. Postsort purity of eosinophil preparations was ~90%, as assessed by characteristic morphology after examination of stained cytospin preparations. FACS analysis of postpurification T cells showed that both isolates were >95% CD4⁺ T cells (data not shown). A total of 5×10^6 CD4⁺ T cells (or 0.5×10^6 eosinophils) in 0.1 ml PBS was injected via the tail vein into naive wt mice, followed by intratracheal CRA challenge at 24 and 72 h after adoptive transfer (0.1 ml PBS alone was injected as procedure control). AHR was measured 24 h after the final CRA challenge.

T cell migration assay

CD4⁺ T cells were isolated from spleen on day 21 following the protocol in Fig. 2A. A total of 1×10^5 CD4⁺ T cells was seeded in the upper chamber of 5- μm pore size Transwells (Corning). CCL21 (200 ng/ml) was added to the lower chamber, and the Transwell plates were incubated for 2 h at 37°C. Cells in the lower chambers were counted and expressed as the percentage of the total cells.

Rac activation assay and Western blotting

CD4⁺ T cells were isolated using a MACS column (Miltenyi Biotec) from spleens of CRA-challenged mice on day 23, following the protocol in Fig. 2A. Cells were lysed in Mg lysis/washing buffer (11) on ice. Lysates were incubated with recombinant GST-Pak1-binding domain that had been precoupled with glutathione-agarose beads at 4°C for 1.5 h with constant rotation. Beads were washed with Mg lysis/washing buffer and resuspended in SDS sample buffer. Lysates for total Rac1 measurement were collected prior to the incubation with pull-down tool. Western blots were incubated with anti-Rac1 Abs (Cytoskeleton). We used previously described rabbit polyclonal Abs (22) to investigate Abr expression in different cell types. Mouse embryonic endothelial cells were isolated and immortalized using polyoma middle-T, as described (25). Human pulmonary artery endothelial cells were from Invitrogen (Carlsbad, CA). Mouse alveolar type II cells were isolated as described (26).

Statistical analysis

Data are expressed as mean $\pm$ SEM and were analyzed by the unpaired Student *t* test or one-way ANOVA using Prism software (GraphPad Software, CA), unless indicated otherwise. The *p* values < 0.05 were considered statistically significant.

Results

Increased asthma-associated mortality in CRA-challenged *abr*^{-/-} mice

To examine whether *abr*^{-/-} mice differ from control wt mice in their reaction to CRA, age-matched control wt and *abr*^{-/-} mice were immunized with CRA, followed by i.n. CRA challenges (Fig. 1A; *Materials and Methods*). As shown in Fig. 1B, five of six wt mice survived sensitization and rechallenge with this dose of CRA. However, none of the mice lacking Abr survived after the fourth CRA challenge on day 22. This indicates that Abr function provides protection to mice against a fatal asthma attack subsequent to repeated exposure to high-dose CRA.

Lack of Abr leads to exacerbated asthma pathology

To generate a milder outcome, we reduced the intensity of CRA sensitization and challenge (Fig. 2A; *Materials and Methods*).

Previous studies demonstrated that this procedure elicits a strong Th2-mediated asthmatic response (19, 23, 24). Mice challenged with this protocol were then analyzed for pathophysiology.

A prominent feature of asthma development is the overproduction of mucin, which leads to narrowing of the lumen of the airways and obstruction of breathing (27). As shown in Fig. 2B, upon CRA challenges, infiltration of leukocytes was markedly elevated in the lungs of *abr*^{-/-} mice, particularly around the peribronchial and perivascular regions. Moreover, the airway lumen of *abr*^{-/-} mice were reduced as a result of epithelial hyperplasia. Mucin production was also increased in the lungs of *abr*^{-/-} mice (Fig. 2C). Clinically, airway constriction is assessed by measurement of AHR after administration of methacholine (24). As shown in Fig. 2D, the airway resistance was significantly greater in CRA-sensitized and challenged *abr*^{-/-} mice than in control mice. Serum IgE levels, another hallmark of asthma, were also substantially elevated in *abr*^{-/-} mice (Fig. 2E). Nonimmunized *abr*^{-/-} and wt mice had similar airway resistance and IgE levels (Fig. 2D, 2E). This shows that Abr function reduces typical pathological characteristics associated with asthma in the nonlethal CRA model.

Increased eosinophil recruitment in CRA-challenged *abr*^{-/-} mice

The inflammation in chronic asthma is associated with leukocyte infiltration and accumulation in the airways (28). Nonimmunized *abr*^{-/-} and wt mice had comparable numbers of CD45⁺ lymphocytes and CD19⁺ B cells in lung and spleen (data not shown). As expected, upon CRA challenge, the airways of both wt and *abr*^{-/-} mice were infiltrated by eosinophils and lymphocytes. Interestingly, the absolute number of eosinophils was markedly higher in the airways of *abr*^{-/-} mice (Fig. 3A). Absolute neutrophil numbers, consisting of ~5% of that of eosinophils, were comparable between genotypes. Moreover, peroxidase activity, of which EPO most probably constituted the major fraction, was elevated in the cell-free BALF of *abr*^{-/-} mice (Fig. 3B). Because EPO is a marker of eosinophil degranulation, this further supports the concept that the absence of Abr leads to an increased accumulation of eosinophils in the airways. Quantitation of eosinophils in peribronchial regions also revealed a significant increase in their infiltration in lungs of CRA-challenged *abr*^{-/-} mice compared with controls (Fig. 3C). Because eosinophil degranulation is directly correlated with airway function (29), this result is consistent with the increased airway resistance in *abr*^{-/-} mice, as shown in Fig. 2D.

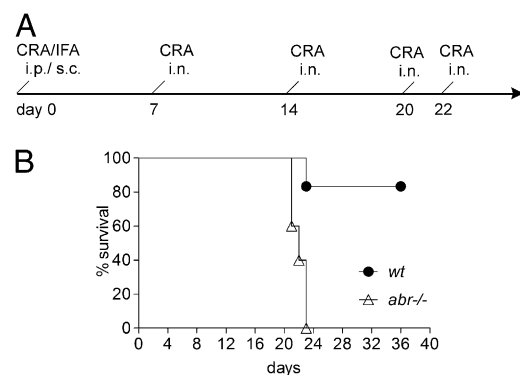
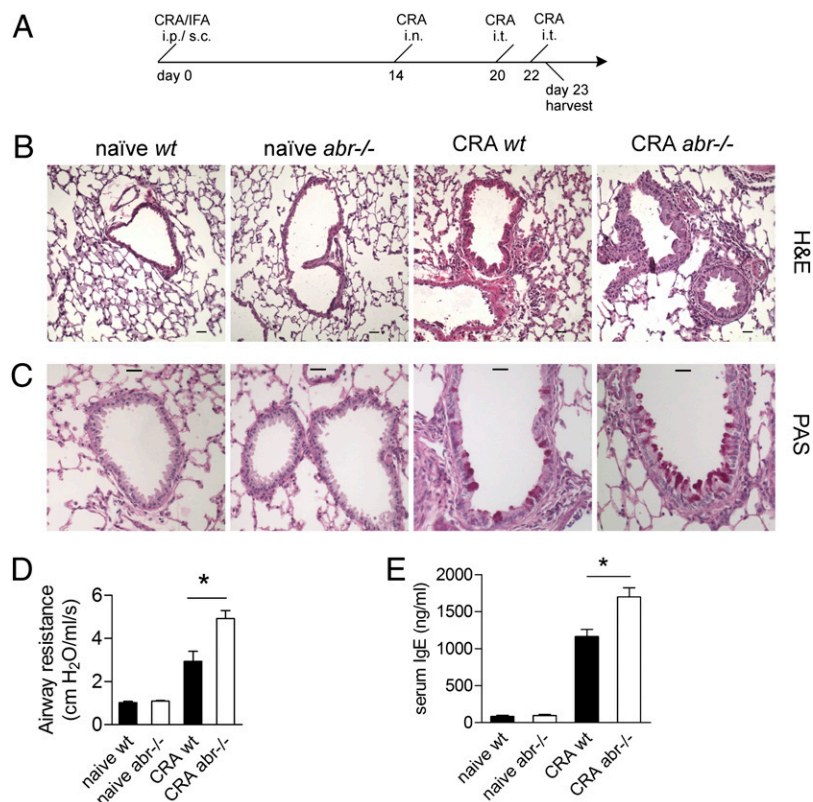


FIGURE 1. Increased asthma-associated mortality in CRA-challenged *abr*^{-/-} mice. Schematic CRA-challenge protocol (**A**) and survival (**B**) in wt ($n = 6$) and *abr*^{-/-} ($n = 5$) mice upon CRA challenges. Kaplan–Meier survival curves were analyzed by the log-rank test ($p < 0.01$). Data are representative of two independently performed experiments.

FIGURE 2. Lack of *Abr* leads to exacerbated asthma pathology. **(A)** Schematic timeline for the CRA sensitization and challenge **(B, C)**. Representative histology of lung sections stained by H&E **(B)** and periodic acid–Schiff **(C)**. Mucin stains as a purple-red color. Sections were made at comparable depth on lungs oriented in a similar fashion. Scale bars, 25 μ m. **(D)** Airway resistance (cm H₂O/ml/s) measurements on day 23. **(E)** Serum IgE levels on day 23 assayed by ELISA. Blood [for serum samples in **(E)**] was collected from the same mice from which lungs were subsequently harvested for histology staining **(B, C)**. Data in **(D)** and **(E)** are mean $\pm$ SEM ($n = 3$ –4 mice/group). Results in **(B)**–**(E)** are representative of two independent experiments. * $p < 0.05$ one-way ANOVA.



Because eosinophils can drive asthma development, their abnormal reactivity could explain the more severe phenotype in *abr*^{-/-} mice challenged with CRA. To investigate this, we flow-sorted eosinophils from lungs of CRA-challenged wt and *abr*^{-/-} mice on day 21, following the CRA challenge protocol in Fig. 2A, and injected them via the tail vein into naive wt mice. As shown in Fig.

3D, upon challenge of the recipient mice with CRA, AHR was significantly increased in comparison with control mice that received only PBS (no eosinophils), confirming that transfer of eosinophils conferred airway hypersensitivity. However, the responses of mice transplanted with wt or *abr*^{-/-} eosinophils were not significantly different, indicating that eosinophils lacking *Abr* are not responsible for the increased reaction of these animals to CRA compared with eosinophils with *Abr* function.

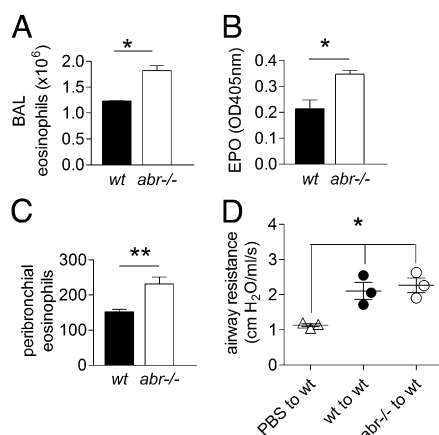


FIGURE 3. Increased eosinophil recruitment in CRA-challenged *abr*^{-/-} mice. **(A)** Eosinophil counts in BALF. **(B)** EPO levels in cell-free BALF. Data from one experiment are shown in **(A)** and **(B)**. **(C)** Total peribronchial eosinophil counts from 50 high-power fields (original magnification $\times 400$) of H&E-stained sections. Results are representative of two independent experiments. Data in **(A)**–**(C)** are mean $\pm$ SEM ($n = 3$ –4 mice/genotype). * $p < 0.05$, ** $p < 0.01$, unpaired Student *t* test. **(D)** Adoptive transfer of eosinophils. Eosinophils (SSC^{hi}CD11b⁺Ly6G⁺) flow-sorted from single-cell suspensions of lungs on day 21, following the immunization/challenge protocol in Fig. 2A, were transferred into naive wt mice and subjected to intratracheal CRA challenge 24 and 72 h later. AHR was measured 24 h after the final CRA challenge ($n = 3$ mice/group). Data from one experiment were analyzed. * $p < 0.05$, one-way ANOVA.

Increased T cell involvement in CRA-challenged *abr*^{-/-} mice

To examine whether naive (non-CRA-challenged) mice lacking *Abr* differ from their wt counterparts with regard to T cell populations, we compared these in spleen and lung cell suspensions using FACS. As shown in Fig. 4A, the numbers of CD4⁺ T cells and CD8⁺ T cells in lung were comparable between genotypes in the absence of CRA sensitization. A similar result was obtained with populations from spleen (data not shown).

However, upon CRA challenge, the airways of *abr*^{-/-} mice showed significantly elevated levels of T cells, with CD4⁺ T cell numbers ~ 10 -fold higher than that of CD8⁺ T cells (Fig. 4B, data not shown). CD4⁺ T cells produce IL-4 and IL-5, cytokines that are critical to the development of asthma. IL-4 is crucial for the class switch of B cells to produce IgE, whereas IL-5 promotes the production and maturation of eosinophils (30, 31). In naive mice, IL-4 production, as indicated by the percentage of splenic Th0- or Th2-polarized CD4⁺ T cells that were IL-4⁺, was not significantly different between *abr*^{-/-} and wt controls (Fig. 4C). However, consistent with a role for increased numbers of CD4⁺ T cells in CRA-challenged *abr*^{-/-} mice, we measured significantly elevated levels of both cytokines in lung homogenates of these *abr*^{-/-} mice compared with wt controls (Fig. 4D). Although levels of these cytokines were also higher in the BALF of *abr*^{-/-} mice, these differences were not statistically significant (Fig. 4E).

We next examined whether CD4⁺ T cells could be responsible for the more severe asthma development in mice lacking *Abr*

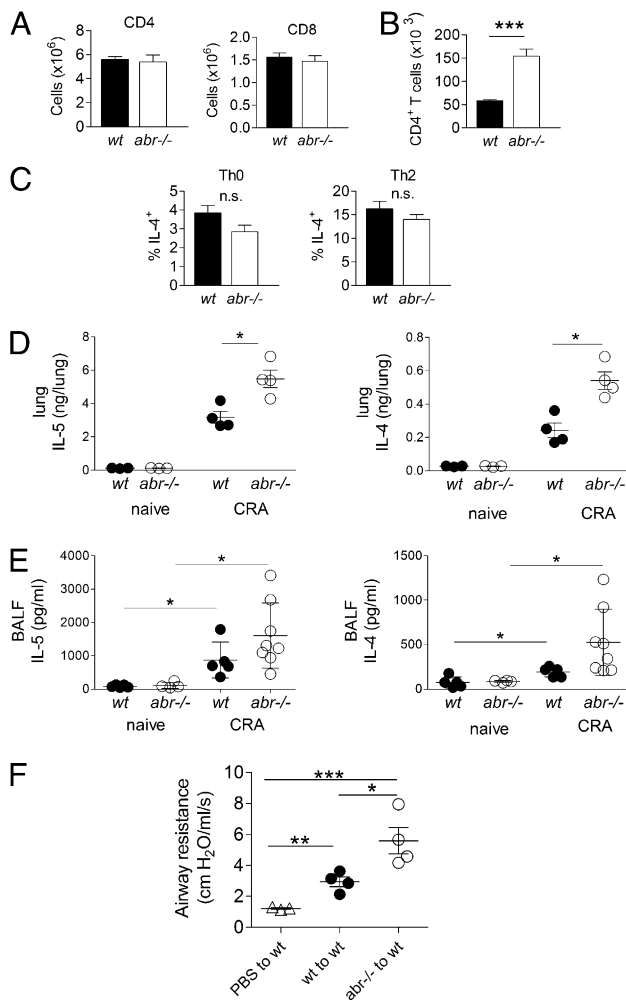


FIGURE 4. Increased CD4⁺ T cell involvement in asthmatic response of CRA-challenged *abr*^{-/-} mice. **(A)** Single-cell suspensions of total lung of naive *abr*^{-/-} and control mice evaluated by FACS for numbers of CD4⁺ or CD8⁺ T cells. **(B)** CD4⁺ T cell counts in BALF of CRA-immunized and challenged mice. Data from one experiment are shown in (A) and (B) ($n = 3-4$ mice/genotype). **(C)** Percentage of IL-4-producing CD4⁺ T cells in naive wt and *abr*^{-/-} mice. Splenic T cells cultured under Th0 or Th2 conditions for 2 d were incubated with PMA and ionomycin for 4 h in the presence of brefeldin A. IL-4 was measured by intracellular staining ($n = 4$ mice/genotype). For (A)–(C), data (mean $\pm$ SEM) were collected from three or four mice/genotype. *** $p < 0.001$, unpaired Student t test (B). **(D)** IL-4 and IL-5 in lung homogenates of naive and CRA-immunized/challenged wt and *abr*^{-/-} mice. **(E)** IL-4 and IL-5 in BALF of naive control and CRA-immunized and challenged mice. **(F)** Airway resistance measurements after adoptive transfer of CD4⁺ T cells from CRA-challenged *abr*^{-/-} mice into naive wt mice. On day 21, splenic CD4⁺ T cells (protocol in Fig. 2A) were transferred into naive wt mice, followed by intratracheal CRA challenge 24 and 72 h later. AHR was measured 24 h after the final CRA challenge. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA. In (E) data are pooled from two experiments with four or five control naive mice/group and five wt and eight *abr*^{-/-} mice/treatment group. For (C) and (D), one of two experiments with similar results is shown ($n = 3-4$ mice/group). n.s., Not significant.

function. Splenic CD4⁺ T cells were isolated from CRA-sensitized wt and *abr*^{-/-} mice. FACS analysis of postpurification T cells showed that both isolates consisted of >95% CD4⁺ T cells (data not shown). Upon adoptive transfer of these CD4⁺ T cells into naive wt mice, followed by CRA challenge, CD4⁺ T cells from *abr*^{-/-} mice were clearly significantly more active in causing airway resistance than were their wt counterparts (Fig. 4F), showing that lack

of Abr function in CD4⁺ T cells is a major cause of the exacerbated asthma development in *abr*^{-/-} mice.

To address the possibility that T cells lacking Abr are intrinsically more sensitive to activation, we activated splenic CD4⁺ T cells from naive *abr*^{-/-} and wt mice for 48 h with Abs against CD3 and CD28. However, FACS analysis for CD25 and CD69 activation markers yielded similar results, and there were no statistically significant differences in cytokine secretion between the activated wt and *abr*^{-/-} cells (Supplemental Fig. 1).

CD4⁺ T cells from CRA-challenged *abr*^{-/-} mice show increased migration and elevated GTP-Rac1 levels

Our data showed that absolute CD4⁺ T cell numbers were increased in CRA-challenged *abr*^{-/-} mice. Because Abr can regulate actin cytoskeletal reorganization needed for motility (11), increased cell numbers could be caused by increased migration of these cells to the lung. To investigate this, we compared chemotaxis of activated CD4⁺ T cells isolated from the spleens of wt and *abr*^{-/-} CRA-sensitized mice toward CCL21, a chemokine that

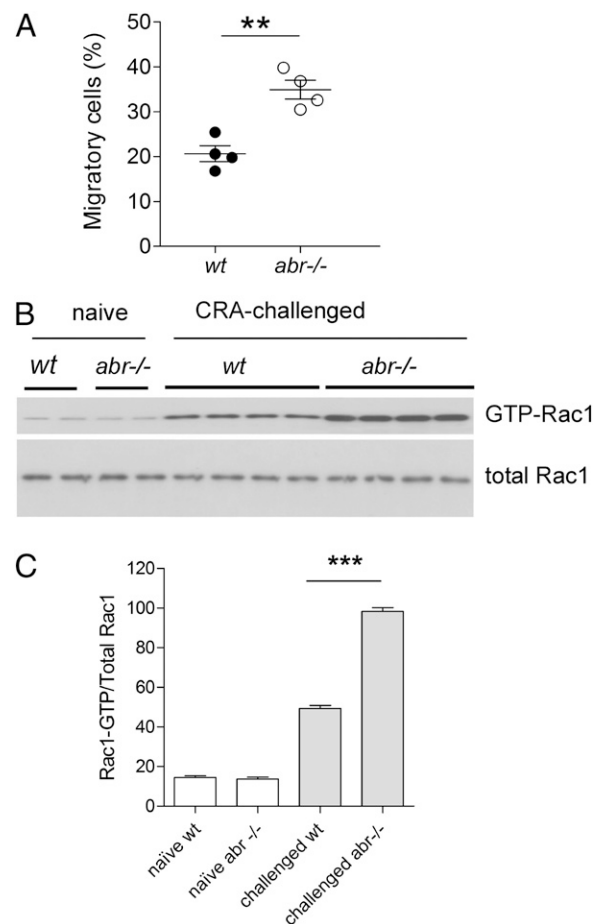


FIGURE 5. CD4⁺ T cells from CRA-challenged *abr*^{-/-} mice show increased migration and elevated GTP-Rac1 levels compared with wt controls. **(A)** CD4⁺ T cells were isolated from spleen on day 21, following the protocol in Fig. 2A. A total of 1×10^5 CD4⁺ T cells was seeded in the upper chamber of Transwells. Cells migrated to CCL21 in the lower chamber for 2 h at 37°C. Migrating cells in the lower chambers were counted and expressed as the percentage of the total cells. Data are mean $\pm$ SEM ($n = 4$ mice/group). ** $p < 0.01$, Student t test. **(B)** Detection of activated Rac by Western blot in CD4⁺ T cells isolated from spleen on day 21, following the protocol in Fig. 2A. **(C)** Quantification of activated Rac1. The relative levels of activated Rac1 were calculated by normalizing the ratio of GTP-Rac1/Total Rac1. *** $p < 0.001$, one-way ANOVA. For (A)–(C), results were similar in two independent experiments.

promotes chemotaxis of activated T cells to lymph nodes (32). Interestingly, in vivo CRA-activated *abr*^{-/-} CD4⁺ T cells exhibited significantly enhanced chemotaxis compared with wt CD4⁺ T cells (Fig. 5A). Because Rac activity mediates T cell migration, and Rac-deficient T cells exhibit defective chemokine-induced chemotaxis (33), we examined the activation state of Rac1 protein in CD4⁺ T cells from CRA-challenged wt and *abr*^{-/-} mice. As shown in Fig. 5B and 5C, the levels of GTP-Rac1 in CD4⁺ T cells of CRA-sensitized *abr*^{-/-} mice were substantially higher than were those of wt control mice, consistent with a role for Abr in negatively regulating the pool of activated, GTP-bound Rac in CD4⁺ T cells that have been sensitized by CRA.

Discussion

Asthma is an economically significant health problem of which the underlying causes are incompletely understood. The identification of molecular mechanisms that promote asthma development may be an important component in the search for more effective therapies. In the current study, we used a mouse model to investigate the development of asthma caused by cockroach exposure. This allergen has not been studied as extensively as allergen in mouse models, although it is directly relevant to human asthma. We identify Abr as the first Rac-specific GTPase-activating protein that mitigates the development of lethal, CRA-evoked allergic asthma.

In the model used in this study, as in human asthma, inflammation is an essential component. Fatal asthma in humans is associated with luminal obstruction caused by exudate composed of mucus and cells (34). The finding that our nonlethal model was characterized by increased mucus production and infiltrates of immune cells suggests that obstruction caused by similar masses may have contributed to death in the lethal model.

Asthma pathogenesis is a complicated process that involves multiple cell types of the immune system. Eosinophils and CD4⁺ T cells are known to play major roles in the pathology of Ag-induced allergic asthma (35). Eosinophils are the central effector cells for asthma symptoms, because they produce proteases that degrade and remodel tissue extracellular matrix, promote mucus production and airway constriction, and secrete proinflammatory factors that propagate inflammation and recruit other immune cells (36). CD4⁺ T cells, particularly Th2-polarized CD4⁺ T cells, produce IL-4 that stimulates B cell IgE class switch and IL-5 that causes eosinophil maturation and recruitment. Thus, intrinsic abnormalities of either eosinophils or CD4⁺ T cells in the absence of Abr could have resulted in the exacerbated asthma pathology observed in CRA-challenged *abr*^{-/-} mice. We used an adoptive-transfer approach to clearly establish that *abr*^{-/-} CD4⁺ T cells, not *abr*^{-/-} eosinophils, are responsible for the worsened asthma pathology. This indicates that CD4⁺ T cells have intrinsic abnormalities in the absence of Abr.

Because the levels of IL-4 and IL-5 were increased in the lungs of *abr*^{-/-} mice that developed CRA-evoked asthma, it was possible that Abr deficiency would increase the production of these cytokines by individual activated CD4⁺ T cells. However, upon in vitro activation, CD4⁺ T cells of naive *abr*^{-/-} mice produced similar levels of IL-4 on a per-cell basis as did their wt counterparts. Thus, the increased levels of IL-4 and IL-5 are likely the result of the significantly increased absolute numbers of CD4⁺ T cells in the lungs of mice deficient for Abr.

De novo migration of T cells into the lung upon allergen challenge contributes significantly to the increased numbers of peribronchial and airway infiltrates. Indeed, imaging of CD4⁺ T cells in mice showed that this is a very rapid process; within 6 h of allergen inhalation, focus formation of CD4⁺ T cells occurs in the lung, which precedes eosinophil infiltration (37). Thus, we

examined migration of CD4⁺ T cells from CRA-sensitized and challenged *abr*^{-/-} mice toward CCL21. This chemokine promotes CD4⁺ T cell migration to lymphoid tissue (38). To our knowledge, our results show for the first time that CD4⁺ T cells activated in vivo by CRA contain increased levels of activated Rac and, moreover, that activated CD4⁺ *abr*^{-/-} T cells migrate more rapidly toward CCL21. However, it is unlikely that this cytokine is responsible for CRA-evoked migration of CD4⁺ T cells into the lung, because CCL21 levels did not increase in lungs of wt or *abr*^{-/-} CRA-challenged mice compared with controls (Supplemental Fig. 2). These results are in agreement with those of Ploix et al. (39), who did not find evidence for involvement of CCL21 in the pathology of asthma using mouse models.

Our overall results are consistent with studies on T cells of mice with conditional ablation of Rac1 and complete knockout of Rac2. These Racs were shown to be critical for T cell migration to and within lymph nodes (33). Because Abr is a GAP specific for Rac, we conclude that Abr is a major GAP in CD4⁺ T cells that regulates T cell mobility through suppression of Rac activation, thus attenuating the CRA-induced asthma. Interestingly, the concept of targeting Th2 cell migration into lungs as a therapeutic target for asthma has been proposed (40), and inhibition of SDF-1 α -CXCR4-mediated T cell migration in a mouse model of OVA-evoked asthma was shown to have beneficial effects (41).

Asthma has a prominent involvement of immune cells, but other cell types also contribute to its pathology. The characteristic airway remodeling involves increases in smooth muscle cell mass and angiogenesis (42). We showed previously that Abr is expressed in pulmonary artery smooth muscle cells (13), and Western blot analysis indicates that Abr is expressed in other cell types, including endothelial cells (Supplemental Fig. 3). Thus, it is possible that expression of Abr in nonimmune cell types contributes to the exacerbated pathology exhibited by *abr*^{-/-} mice immunized and challenged with CRA. Overall, our study identified a new component of the regulatory pathway that is in place to prevent lethal asthma attacks in reaction to an allergen that is relevant to human asthma. Abr and Rac represent new potential therapeutic targets that could be explored for asthma treatment.

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Disclosures

The authors have no financial conflicts of interest.

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Figure S1

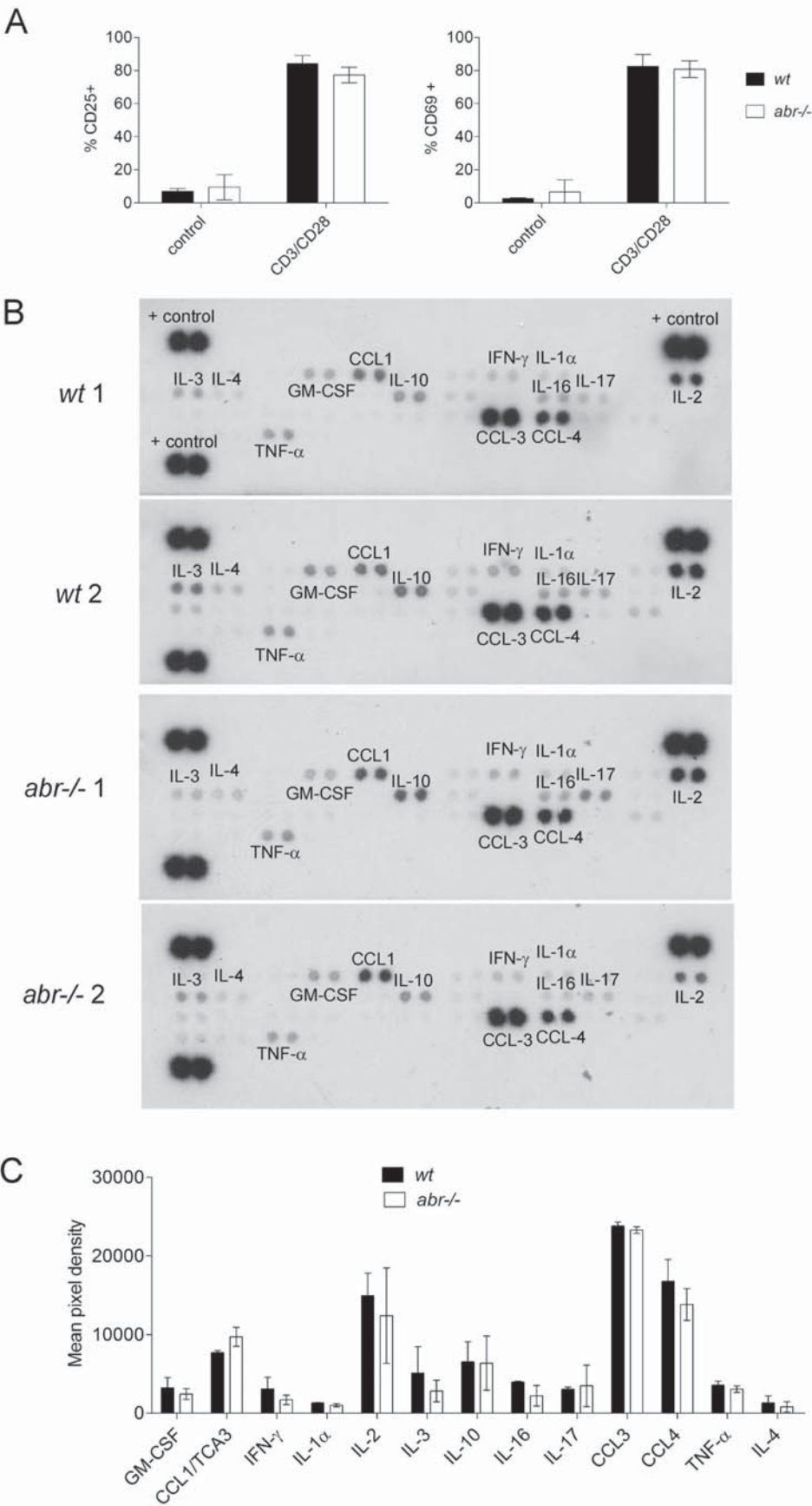


Figure S2

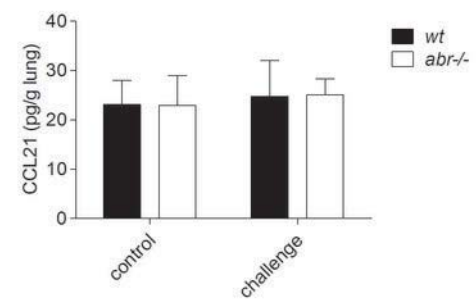


Figure S3

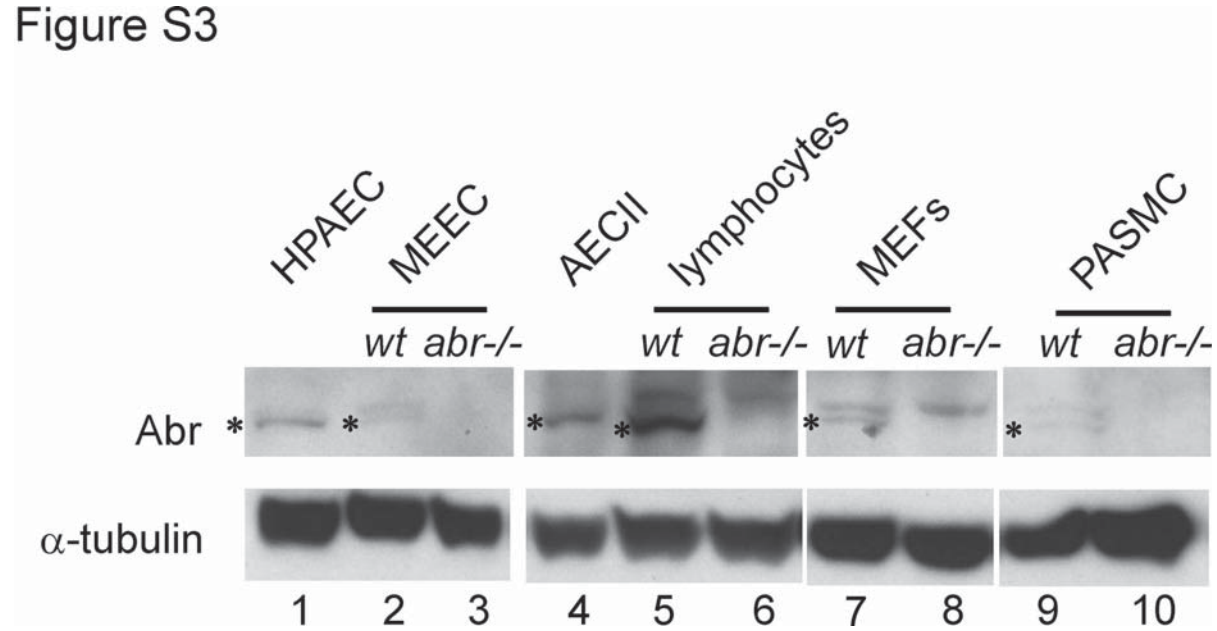


FIGURE S1. CD4⁺ T cells from *abr*^{-/-} mice are not constitutively hyperactivated. CD4⁺ T cells of the indicated genotypes activated for 2 days by plate-bound anti-CD3/CD28 antibodies were examined for expression of cell surface activation markers CD25 and CD69 using FACS (**A**) or for secretion of cytokines/chemokines into the cell culture medium (**B, C**) using an antibody array. (**A**) The percentage of CD25⁺ or CD69⁺ CD4⁺ T-cells is as indicated. The mean fluorescent intensity of control and activated *wt* and *abr*^{-/-} CD4⁺ T cells was also comparable (not shown); n=3/genotype. (**B**) Duplicate antibody arrays reacted with supernatant of the indicated genotype CD4⁺ T cells with identity of detected cytokines as indicated. (**C**), mean pixel intensity of scanned filters. (**B, C**), n =2/genotype.

FIGURE S2. CCL21 levels in control and CRA-immunized and challenged *wt* and *abr*^{-/-} lungs. Lung homogenates on d23 of mice immunized and challenged with CRA as indicated in Fig. 2A were assayed for CCL21. *wt* control n=5; *abr*^{-/-} control n=4; *wt* CRA-challenged n=5; *abr*^{-/-} CRA-challenged n=8.

FIGURE S3. Western blot analysis of Abr expression in different cell types. Western blots were reacted with perviously described (ref 22) polyclonal rabbit anti-Abr antibodies. The Abr-specific 91 kDa band is indicated with an *. These polyclonal antibodies also detect apecific (cross-reacting) proteins as reported (ref 22). Samples include endothelial cells (HPAEC, human pulmonary artery endothelial cells; MEEC, mouse embryonic endothelial cells) lanes 1-3; epithelial cells (AECII, *wt* mouse alveolar type II cells) lane 4; lymphocytes (spleen) lanes 5-6; and fibroblasts (MEFs, mouse embryonic fibroblasts; PASMC, pulmonary artery smooth muscle cells) lanes 7-10. All images from the same exposure of the same Western blot. One of three experiments. α -tubulin, loading control.