

RESEARCH ARTICLE

FABP4 regulates eosinophil recruitment and activation in allergic airway inflammation

Xiao Na Ge,¹ Idil Bastan,¹ Mythili Dileepan,¹ Yana Greenberg,¹ Sung Gil Ha,¹ Kaylee A. Steen,² David A. Bernlohr,² Savita P. Rao,¹ and P. Sriramara¹

¹Laboratory of Allergic Diseases and Inflammation, Department of Veterinary and Biomedical Sciences, Saint Paul, Minnesota; and ²Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Saint Paul, Minnesota

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Ge XN, Bastan I, Dileepan M, Greenberg Y, Ha SG, Steen KA, Bernlohr DA, Rao SP, Sriramara¹ FABP4 regulates eosinophil recruitment and activation in allergic airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 315: L227–L240, 2018. First published April 26, 2018; doi:10.1152/ajplung.00429.2017.—Fatty acid binding protein 4 (FABP4), a member of a family of lipid-binding proteins, is known to play a role in inflammation by virtue of its ability to regulate intracellular events such as lipid fluxes and signaling. Studies have indicated a proinflammatory role for FABP4 in allergic asthma although its expression and function in eosinophils, the predominant inflammatory cells recruited to allergic airways, were not investigated. We examined expression of FABP4 in murine eosinophils and its role in regulating cell recruitment in vitro as well as in cockroach antigen (CRA)-induced allergic airway inflammation. CRA exposure led to airway recruitment of FABP4-expressing inflammatory cells, specifically eosinophils, in wild-type (WT) mice. FABP4 expression in eosinophils was induced by TNF- α as well as IL-4 and IL-13. FABP4-deficient eosinophils exhibited markedly decreased cell spreading/formation of leading edges on vascular cell adhesion molecule-1 and significantly decreased adhesion to intercellular adhesion molecule-1 associated with reduced β 2-integrin expression relative to WT cells. Furthermore, FABP4-deficient eosinophils exhibited decreased migration, F-actin polymerization, calcium flux, and ERK(1/2) phosphorylation in response to eotaxin-1. In vivo, CRA-challenged FABP4-deficient mice exhibited attenuated eosinophilia and significantly reduced airway inflammation (improved airway reactivity, lower IL-5, IL-13, TNF- α , and cysteinyl leukotriene C4 levels, decreased airway structural changes) compared with WT mice. In conclusion, expression of FABP4 in eosinophils is induced during conditions of inflammation and plays a proinflammatory role in the development of allergic asthma by promoting eosinophil adhesion and migration and contributing to the development of various aspects of airway inflammation.

activation; allergic asthma; cell adhesion and migration; eosinophils; fatty acid binding protein 4

INTRODUCTION

Fatty acid binding protein 4 (FABP4) is a member of a family of intracellular lipid-binding proteins that reversibly binds intracellular hydrophobic ligands and transports them throughout cellular compartments, thus playing a role in cel-

lular lipid fluxes, metabolism, and signaling (32). It is predominantly a cytosolic protein, which is abundantly expressed in adipocytes and macrophages (15) and is secreted by adipocytes in response to cAMP (49). In adipocytes, FABP4 serves as a lipid chaperone and facilitates hormone-stimulated lipolysis (10), whereas, in macrophages, the protein stabilizes leukotriene A₄ to hydrolysis, thereby potentiating synthesis of cysteinyl leukotriene C₄ (LTC₄) (29, 72). Studies have also demonstrated FABP4 expression in other cell types, such as dendritic cells, epithelial cells, and endothelial cells under physiological and/or pathological conditions (18). In addition to its well-recognized role in the pathophysiology of metabolic (type 2 diabetes) and cardiovascular (atherosclerosis) diseases (18, 32), FABP4 is known to be involved in biological processes, such as angiogenesis (23), inflammation (32), and tumorigenesis (26, 28).

Previous studies have shown that FABP4 contributes to the pathogenesis of allergic airway inflammation (59); that is an inflammatory disease characterized by the recruitment of large numbers of eosinophils to the airways, elevated Th2 cytokines, and airway hyperresponsiveness (AHR) (30). In the airway epithelium, FABP4 plays a significant role in regulating allergic airway inflammation locally in the lungs. Consistent with this, adoptive transfer experiments using bone marrow (BM) from either wild-type (WT) or FABP4-deficient mice revealed that allergen-challenged WT mice receiving FABP4-deficient or WT BM had similar leukocyte recruitment to the airways, whereas allergen-challenged FABP4-deficient mice that received WT BM had reduced leukocyte recruitment. Although this study showed decreased eosinophil infiltration in allergen-challenged FABP4-deficient mice, whether eosinophils recruited to allergen-challenged lungs in a WT setting express FABP4 and the potential role of eosinophil-expressed FABP4 in contributing to disease pathogenesis were not investigated. Subsequent investigations have shown a proinflammatory role for FABP4 in T cells, dendritic cells (54), and macrophages (69), wherein FABP4-deficient cells have been shown to mount a diminished immune response (lower cytokine production, defective signaling, etc.) upon stimulation. However, there are no studies regarding the potential role of FABP4 in regulating eosinophil function during allergic airway inflammation.

In addition to protein mediators (e.g., IL-5, eotaxins), lipid mediators of inflammation, such as LTC₄, prostaglandin D₂ (PGD₂), and platelet activating factor (PAF), participate in

Address for reprint requests and other correspondence: P. Sriramara¹, Univ. of Minnesota, 1971 Commonwealth Ave., St. Paul, MN 55108 (e-mail: psrao@umn.edu).

eosinophil recruitment and activation and contribute to the pathogenesis of allergic airway inflammation and asthma (43). Given the proinflammatory role of FABP4 in other inflammatory cells and its ability to regulate intracellular signaling events in these cells (35, 45, 54, 66), we examined expression of FABP4 in eosinophils and investigated its role in regulating eosinophil recruitment and function at a cellular level, as well as in the context of the pathophysiology of allergic airway inflammation in a mouse model using cockroach antigen (CRA), an important allergen that contributes to the development of asthma in humans (52). Our studies demonstrate that FABP4 plays an important role in regulating eosinophil trafficking (adhesion and migration) and recruitment to the airways, thus contributing to the development of allergic asthma.

MATERIALS AND METHODS

Mice and model of acute allergic airway inflammation. Male WT and FABP4 whole body knockout (KO) (31) mice on a C57BL/6J background (8–12 wk) bred in house were used for induction of allergic airway inflammation with CRA (Hollister Stier, Spokane, WA) as described previously (21) and outlined in Fig. 1A. Control mice were sensitized with aluminum hydroxide in saline and were challenged with saline instead of CRA. Mice were housed in specific antigen-free conditions and given food (standard laboratory chow)

and water ad libitum during the study. Twenty-four hours after the last allergen challenge, mice were used for collection of samples after euthanasia by CO₂ inhalation or for measurement of airway responsiveness. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Measurement of airway responsiveness. Invasive measurement of airway responsiveness was conducted in anesthetized mice using the FinePointe RC System (Buxco, Wilmington, NC) as described previously (3). Briefly, changes in pulmonary resistance (R_L) were monitored continuously in response to saline followed by increasing concentrations of inhaled methacholine (3–25 mg/ml) nebulized for 18–20 s and expressed as mean R_L values following each dose of methacholine.

Sample collection. Bronchoalveolar lavage fluid (BALF), blood, and BM were collected from mice 24 h after the last allergen challenge. Total cell counts in the BALF were determined using a hemocytometer. Differential cell counts in the BALF, blood, and BM were determined based on morphological criteria from cytospin slides stained with Hema3 staining system (Thermo Fisher Scientific, Pittsburgh, PA). BALF supernatants were stored at -80°C for later analysis. Right lungs were snap frozen and stored at -80°C until further evaluation, whereas left lungs were perfused, fixed in 4% paraformaldehyde, and paraffin embedded.

Measurement of lung cytokines, chemokines, and LTC₄. Th1 (IL-2, IFN- γ)/Th2 (IL-4, IL-5, IL-13) cytokine and TNF- α levels in BALF

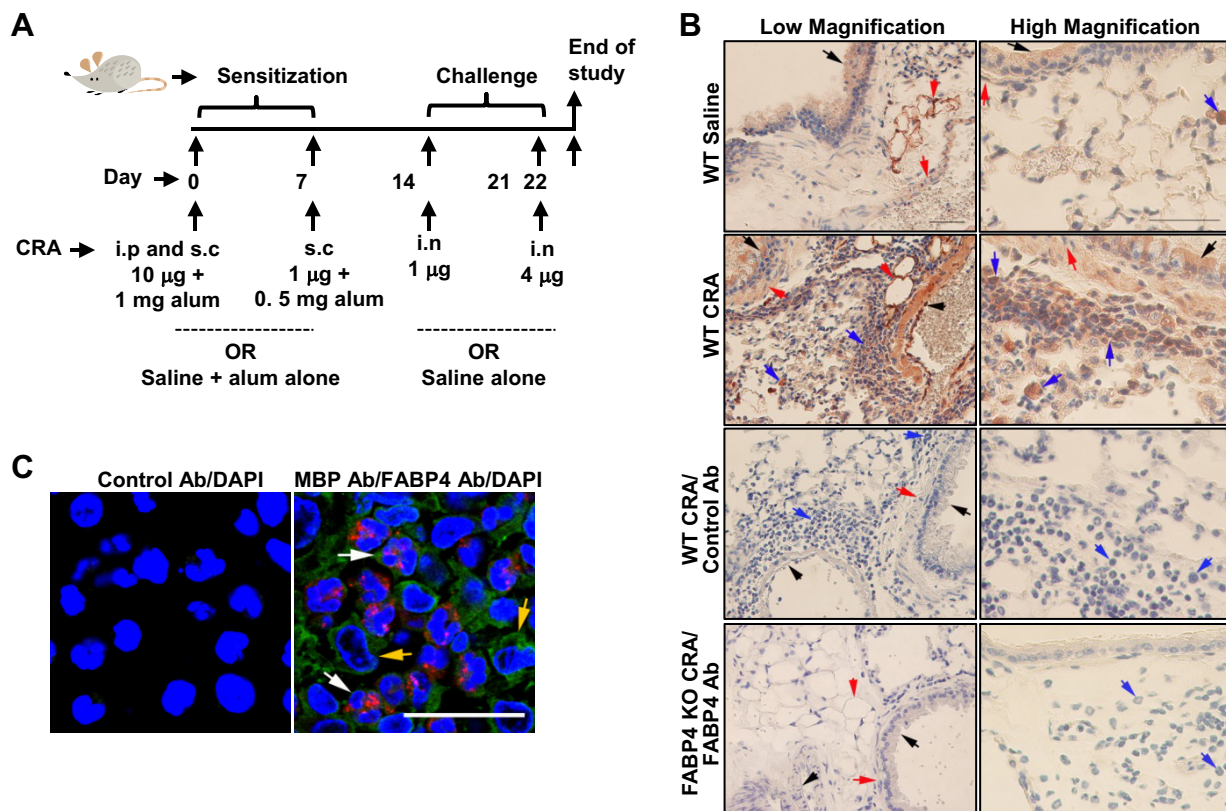


Fig. 1. Allergen exposure leads to increased fatty acid binding protein 4 (FABP4) expression in the lungs. **A:** outline of protocol for cockroach antigen (CRA)-induced allergic airway inflammation in mice. i.p., intraperitoneal; s.c., subcutaneous; i.n., intranasal. **B:** expression of FABP4 in the lungs of wild-type (WT) mice exposed to CRA challenge (*top, middle*) or saline alone (control, *top*) after immunohistochemical staining with anti-FABP4. Airway epithelial cells are shown with black arrows; endothelial cells, black arrowheads; smooth muscle cells, red arrows; adipocytes, red arrowheads; and inflammatory cells, blue arrows. Immunohistochemical staining of lung sections from allergen-challenged WT mice with a control antibody (*bottom, middle*) and from allergen-challenged FABP4 knockout (KO) mice with FABP4 antibody (*bottom*) is shown as negative controls. Scale bar = 50 µm. **C:** dual immunostaining of lung sections from CRA-exposed WT mice with antibodies against FABP4 (green) and eosinophil-specific major basic protein (MBP) (red). Cells positive for FABP4 only are indicated with yellow arrows, and cells positive for FABP4 and MBP are indicated with white arrows. Scale bar = 20 µm. Data representative of 3 mice/group are shown in **B** and **C**.

and supernatant of lung tissue lysates were determined by flow cytometry using Cytometric Bead Array Flex Set Kits (BD Biosciences, San Diego, CA) and expressed as picograms per milliliter (for BALF) or picograms of cytokine per milligram of protein (for lung tissue). Eotaxin-1 (CCL11) and eotaxin-2 (CCL24) were measured using ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. LTC₄ in the lung tissue was assessed by liquid chromatography-mass spectrometry analysis (Shimadzu Prominence 2D high-performance liquid chromatograph coupled to an AB/Sciex API 4000 QTrap MS spectrometer) as described previously (21) and expressed as nanograms per gram of lung tissue.

Lung histology. Paraffin-embedded tissue sections (4 μ m thick) were stained with Harris modified hematoxylin and Shandon instant eosin (H and E, Thermo Fisher Scientific) to determine cellular infiltration. FABP4 expression in the lung tissue was evaluated by immunohistochemical staining. Lung sections were blocked with 3% rabbit serum after antigen retrieval and then stained overnight at 4°C with goat polyclonal antibodies against FABP4 (10 μ g/ml, R&D Systems). VECTASTAIN Elite ABC kit containing biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA) and the peroxidase 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories) were used for detection of bound antibodies. Infiltrated eosinophils in the lung tissue were visualized and quantitated by immunohistochemical staining for eosinophil-specific major basic protein (MBP) using rat mAb against murine MBP and expressed as the average number of MBP-positive cells per field (73). Airway mucus production and airway smooth muscle hypertrophy/hyperplasia were assessed by staining lung sections with periodic acid-Schiff (PAS) reagent (Sigma-Aldrich, St. Louis, MO) and mouse mAb against α -smooth muscle actin (SMA, Sigma-Aldrich), respectively, and quantitated from captured images using ImageJ image analysis software (1) as described in our previous studies (21). In all cases, stained slides were examined using a Nikon Microphot EPI-FL microscope, and images were captured with an Olympus DP71 camera. To detect whether eosinophils recruited to the lung tissue express FABP4, lung sections of CRA-challenged mice were incubated overnight with goat polyclonal antibodies against FABP4 and rat mAb against MBP (5 μ g/ml) at 4°C after blocking. FITC-conjugated donkey anti-goat IgG and rhodamine-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies (1 h, room temperature). DAPI was used to visualize nuclei. Slides were examined at ambient temperature using a FLUOVIEW FV1000/BX61 confocal laser scanning biological microscope equipped with an UPlanSApo lens [$\times 20/0.85$ (oil)] and a PlanApo N lens [$\times 60/1.42$ (oil)]. FV10-ASW 2.0 software was used for image acquisition (Olympus, Melville, NY).

Culture of murine eosinophils and treatment with cytokines. Eosinophils were routinely cultured from BM of naïve male or female WT and FABP4 KO mice as described previously (4). Differentiated cells between days 11 and 14 of culture that were positive for expression of MBP and Siglec-F were used in studies. Number of mature eosinophils derived from BM cultures of WT mice was not significantly different from those of FABP4 KO mice [WT: $24.9 \pm 7.7 \times 10^6$ cells; FABP4 KO: $21.7 \pm 5.0 \times 10^6$ cells (mean $\pm$ SE of 7 cultures)].

To examine the effect of cytokines on FABP4 expression, WT murine eosinophils on day 11 of culture were transferred to 24-well plates containing medium with recombinant murine IL-4, IL-13, TNF- α , IL-3, or granulocyte macrophage colony-stimulating factor (50 ng/ml; all from PeproTech, Rocky Hill, NJ) or medium alone (control) and cultured for an additional 24 h at 37°C in a CO₂ incubator. In some experiments, cells were preincubated with the signal transducer and activator of transcription 6 (STAT6) inhibitor AS 1517499 (100 nM; Axon Medchem, Reston, VA) or vehicle alone (DMSO) in culture medium for 30 min at 37°C before treatment with IL-4 or IL-13 as described above. Cells were then used for qRT-PCR or Western blotting.

qRT-PCR. Total RNA extracted from cells was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). FABP4 and peroxisome proliferator-activated receptor (PPAR)- γ gene expression was examined by qRT-PCR with specific primers [FABP4, forward primer: GATGCCTTTGTGGGAACCT, reverse primer: CTGTCGTCTGCGGTGATTT (58); PPAR- γ , forward primer: GCCATTGAGTGCCGAGTC, reverse primer: TGTGGATC-CGGCAGTTAAG (70)]. For FABP4, qRT-PCR was carried out in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using iTaq Universal SYBR Green Supermix Kit (Bio-Rad). For PPAR- γ , qRT-PCR was carried out in an Mx3000P qPCR System (Agilent, Santa Clara, CA). The relative amount of target mRNA was calculated based on its threshold cycle, Ct, compared with the Ct of the internal standard β -actin. Results were expressed as fold change relative to untreated cells using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) (57).

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with proteinase inhibitors, and protein concentration in cell lysates was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Cell lysates were separated by electrophoresis on 15% polyacrylamide gels under reduced conditions. Western blot analysis was carried out using rabbit polyclonal Ab against FABP4 (0.1 μ g/ml, overnight at 4°C) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (0.16 μ g/ml; Jackson ImmunoResearch). HRP-conjugated anti-mouse β -actin (Santa Cruz Biotechnology, Dallas, TX) was used to detect expression of β -actin as a loading control. WesternBright enhanced chemiluminescence HRP substrate (Advanta, Menlo Park, CA) was used to develop the blots, and bands were visualized on X-ray films. Band intensity was quantitated using ImageJ software, and the expression level of FABP4 was normalized against that of β -actin.

To assess ERK(1/2) activation, WT and FABP4 KO eosinophils were treated with eotaxin-1 (100 nM; PeproTech) for 15 min at 37°C. After being washed, cells were lysed in RIPA buffer. Protein samples were electrophoresed on 12% gels under reduced conditions and analyzed by Western blot with rabbit polyclonal Ab against phospho-ERK(1/2) (0.14 μ g/ml, overnight at 4°C; Cell Signaling Technology, Danvers, MA) followed by HRP-conjugated anti-rabbit IgG (0.16 μ g/ml; Jackson ImmunoResearch). Blots were developed, and bands visualized as described above. Membranes were then stripped and probed with rabbit mAb against total ERK(1/2) (0.08 μ g/ml, overnight at 4°C; Cell Signaling Technology). ImageJ software was used to measure intensity of the detected bands, and expression level of phospho-ERK(1/2) was normalized against that of total ERK(1/2).

Static adhesion and in vitro migration assay. Static adhesion and in vitro migration assays were performed as described previously (22). Briefly, an equal number of WT and FABP4 KO eosinophils were allowed to adhere to recombinant murine (rm) coated vascular cell adhesion molecule (VCAM-1; Leinco Technologies, Fenton, MO)-coated coverslips or rm intercellular adhesion molecule (ICAM-1; Leinco Technologies)-coated coverslips for 30 min at 37°C. After a gentle rinse (2–3 s) in PBS, adherent cells on coverslips were fixed with 4% paraformaldehyde, stained with Alexa Fluor 488 phalloidin (Life Technologies, Carlsbad, CA) and DAPI to visualize cytoskeleton and nuclei, and then examined by confocal microscopy. The number of adherent cells per field in five randomly selected fields ($\times 600$ magnification) was manually determined for each coverslip, and the number of these adherent cells undergoing spreading with formation of membrane protrusions/leading edges was expressed as a percentage of the total number of adherent cells in the field. Migration of WT and FABP4 KO eosinophils in response to eotaxin-1 (100 nM, PeproTech) was assessed using 96-well Corning HTS Transwell chambers as described previously (4). To measure transendothelial migration, STR-12 murine BM endothelial cells were grown to confluence on gelatin-coated Transwell chamber inserts, pretreated with TNF- α [5 ng/ml, 5 h (14)] and then used in the migration assay. The number of cells that migrated in each case was evaluated under an Olympus CK2 inverted microscope ($\times 400$ magnification), and cells

in a fixed number of randomly selected fields of each well were manually enumerated. Results were expressed as percent cell migration relative to WT cells or as the average number of cells/field.

Laminar flow chamber assay. Rolling of WT and FABP4 KO eosinophils on rmVCAM-1 under conditions of flow [wall shear stress 1.0–2.0 dynes/cm², i.e., ~1 ml/min (63)] was evaluated in an in vitro parallel plate flow chamber as described previously (4). Interaction of eosinophils with VCAM-1-coated coverslips for each genotype was observed using an inverted phase-contrast microscope, and images were video recorded for subsequent offline analysis to manually determine the number of interacting cells in 2 min (53). Detachment of WT and FABP4 KO eosinophils from ICAM-1 under flow conditions was evaluated as described previously (62) but with minor modifications. WT and FABP4 KO eosinophils were adhered to rm ICAM-1-coated coverslips (as described for static adhesion assay) and then placed in the laminar flow chamber and exposed to flow conditions (5 dyn/cm², ~5 ml/min). After ~5 min, flow was stopped, and the number of eosinophils still adherent on ICAM-1 per field was manually counted at ×200 magnification in five randomly selected fields per coverslip for each genotype.

Adoptive transfer of eosinophils to allergen-challenged mice. To evaluate difference in migration between WT and FABP4 KO eosinophils in vivo, C57BL/6J WT mice (normal expression of stromal and endothelial adhesion molecules/ligands) were exposed to allergen challenge followed by adoptive transfer of cultured eosinophils as described previously (68) but with minor modifications. Allergic airway inflammation was induced using the fungal allergen *Alternaria alternata* as described previously (27). BM eosinophil culture (described earlier) and induction of allergic airway inflammation were synchronized so that the last challenge with *A. alternata* was performed on day 13 of the eosinophil culture. WT and FABP4 KO eosinophils on day 13 of culture were harvested and labeled with different fluorescent dyes [carboxyfluorescein succinimidyl ester (CFSE) or CellTracker Red, both from Thermo Fisher Scientific] as described previously (36). Labeling of eosinophils was switched when experiments were repeated (e.g., first experiment: WT with CFSE, FABP4 KO with CellTracker Red; second experiment: WT with CellTracker Red, FABP4 KO with CFSE) to account for any nonspecific effect of the dyes. Six hours after the last allergen challenge, a mixture of an equal number of labeled WT and FABP4 KO eosinophils (~10 × 10⁶ cells of each genotype) in PBS was transferred intravenously by retroorbital injection. Twenty-four hours after eosinophil transfer, animals were euthanized, and BALF was collected. Total cell counts were determined using a hemocytometer, and the number of CFSE or CellTracker Red-labeled cells in BALF was determined based on flow cytometry (BD LSRFortessa X-20 equipped with BD FACSDiva software for data acquisition, BD Biosciences). Flow cytometry data was analyzed using FlowJo (version 8.8.2; Tree Star, Ashland OR).

Expression of cell surface receptors. Cell surface adhesion and chemokine receptor expression by WT and FABP4 KO eosinophils was evaluated using mAb against CD49d (α4, clone PS/2), CD11a (αL; eBioscience, San Diego, CA), CD11b (αM, eBioscience), L-selectin (clone MEL-14), or CD18 (β2, clone M18/2, eBioscience) followed by FITC-conjugated goat anti-rat IgG. Rat IgG2b (for CD49d and CD11b) and rat IgG2a (for CD11a, CD18, and L-selectin) were used as isotype controls. All antibodies were used at 10 μg/ml. For C-C chemokine receptor type 3 (CCR3) expression, cells were either untreated or treated with eotaxin-1 (100 nM) and then incubated with FITC-conjugated rat anti-mouse CCR3 (2.5 μg/ml, R&D Systems) with FITC-conjugated rat IgG2a as isotype control. Cells were analyzed using a BD FACScan flow cytometer equipped with BD CellQuest Pro (BD Biosciences) for data acquisition and FlowJo software for data analysis.

Actin polymerization. Actin polymerization in WT and FABP4 KO eosinophils in response to a chemotactic stimulus was assessed as described previously (3). Briefly, cells were adhered on poly-L-lysine

(10 μg/ml, Sigma-Aldrich)-coated chamber slides (Nalgene Nunc International, Rochester, NY) for 30 min at 37°C and then treated with eotaxin-1 (100 nM, 10 s to 10 min). Cells were then permeabilized, stained with Alexa Fluor 488 Phalloidin, counterstained with DAPI, and examined by confocal microscopy.

Calcium imaging. Intracellular calcium [Ca²⁺]_i concentration in BM-derived eosinophils was determined using the permeant Ca²⁺ indicator dye fura-2 AM (Thermo Fisher Scientific) as described previously (4). Briefly, eosinophils attached to poly-L-lysine-coated glass coverslips were incubated with 5 μM fura-2 AM for 30 min at 37°C and 5% CO₂. Coverslips were washed gently, perfused with HBSS containing 10.0 mM HEPES, 11.0 mM glucose, 2.5 mM CaCl₂, and 1.2 mM MgCl₂ (pH 7.4) and mounted on the stage of a Nikon Diaphot inverted microscope (Nikon Instruments, Tokyo, Japan) in an open slide chamber. Fura-2 AM-loaded cells were alternately excited at 340 and 380 nm with a Lambda DG-4 high-speed wavelength switcher (Sutter Instrument, Novato, CA). Basal [Ca²⁺]_i was measured by real-time digital video fluorescence imaging using NIS-Elements imaging software (Nikon Instruments). After 2 min, eosinophils were stimulated with eotaxin-1 (Peprotech) and evaluated for an additional 6 min to assess agonist-induced [Ca²⁺]_i responses. In some experiments, after basal [Ca²⁺]_i was measured for 5–6 min, WT and FABP4 KO eosinophils were exposed to 2 μM ionomycin, free acid (Biovision, Milpitas, CA) as a positive control and monitored for an additional 3 min. The ratio of fluorescence emissions at 340 nm and 380 nm was determined at each time point, which is directly reflective of the amount of [Ca²⁺]_i.

Statistical analysis. Data are presented as means ± SE unless otherwise stated. For in vitro studies, statistical significance between groups was determined using a two-tailed unpaired Student's *t*-test. For in vivo studies, a two-tailed test was used to establish a statistically significant difference in recruitment of total inflammatory cells between allergen-challenged WT and FABP4 KO mice. A one-tailed test was used for all other analyses. A *P* value ≤0.05 was considered significant.

RESULTS

Allergen-exposure induces FABP4 expression in the lungs. In the present study, we found that, in a mouse model of acute allergic airway inflammation induced by CRA (Fig. 1A), expression of FABP4 was markedly increased in the lungs (Fig. 1B). FABP4 expression by immunohistochemistry was observed mostly in airway epithelial cells, adipocytes, endothelial cells, and smooth muscle cells in the lungs of both control and allergen-challenged mice (Fig. 1B, *top* and *top, middle*, respectively). CRA exposure resulted in increased FABP4 expression in the lungs relative to control mice, which was predominantly associated with recruited inflammatory cells (Fig. 1B, *top, middle*). No positive staining in airway epithelial cells, endothelial cells, smooth muscle cells, adipocytes, or inflammatory cells was detected in lung sections from allergen-challenged WT mice stained with a control antibody (Fig. 1B, *bottom, middle*) or in sections from allergen-challenged FABP4-deficient mice stained with antibodies against FABP4 (Fig. 1B, *bottom*). Dual immunostaining of lung sections from these mice with antibodies against FABP4 and eosinophil-specific MBP showed cells that stained positively for FABP4 (green) and MBP (red), i.e., eosinophils (Fig. 1C, white arrows), as well as cells that were positive for FABP4 but negative for MBP and most likely macrophages (Fig. 1C, yellow arrows). These findings indicate that eosinophils, the predominant inflammatory cells in the inflamed lungs of allergen-challenged mice, express FABP4.

FABP4 is an inducible protein in murine eosinophils. To understand the role of FABP4 in eosinophils at a cellular level, we first examined expression of FABP4 in BM-derived murine eosinophils at the level of mRNA and protein. Expression of FABP4 in unstimulated cells was found to be extremely low (data not shown). Considering that eosinophils recruited to allergic airways express FABP4 (Fig. 1C), we investigated whether expression of this protein in eosinophils was induced under conditions of inflammation. Cytokines including IL-4 and IL-13 are known to be associated with allergic asthma and to upregulate FABP4 expression in airway epithelial cells (59). Consistent with this, FABP4 expression was strongly induced by IL-4 and TNF- α and to a lesser extent by IL-13 at the level of mRNA (Fig. 2A). In line with the increased expression at the mRNA level, IL-4- and TNF- α -stimulated cells showed a significant increase in expression of FABP4 relative to untreated cells as indicated by an ~15-kDa band in Western blots corresponding to the established molecular weight of FABP4 (18). Treatment with IL-13 yielded only a modest increase in FABP4 expression (Fig. 2, B and C). Because IL-4 and IL-13 mediate their effects via the signal transduction molecule and transcription factor STAT6 during asthma pathogenesis (25), effect of these cytokines on FABP4 protein expression was examined in the presence of a STAT6 inhibitor. Induction of FABP4 expression by IL-4 and IL-13 was inhibited in the presence of this inhibitor, indicating a STAT6-dependent mechanism (Fig. 2B).

FABP4 promotes eosinophil adhesion and migration. We next investigated whether FABP4 plays a role in regulating eosinophil recruitment at the level of cell adhesion and migration. Adhesion of eosinophils from WT and FABP4-deficient mice on endothelial cell adhesion molecules VCAM-1 and ICAM-1 that support rolling and adhesion *in vivo* (7) was first examined under static conditions. Although no difference was noted in the number of cells adherent on VCAM-1 (Fig. 3A, left), significantly fewer FABP4-deficient eosinophils under-

went cell spreading and formation of membrane protrusions/leading edges (Fig. 3A, right, and B). In contrast to VCAM-1, FABP4-deficient eosinophils exhibited significantly decreased adhesion on ICAM-1 compared with WT eosinophils (Fig. 3C, left). Cells of both genotypes did not show extensive cell spreading on ICAM-1 (Fig. 3C, right, and D) although adherent WT eosinophils showed a marginal increase in phalloidin binding at the cell periphery compared with FABP4-deficient eosinophils. Next, interaction of WT and FABP4-deficient eosinophils with VCAM-1 or ICAM-1 was examined under conditions of flow. Rolling of FABP4-deficient eosinophils on VCAM-1 was similar to that of WT cells (Fig. 3E) consistent with the observation that eosinophils of both genotypes interact with VCAM-1 in equal numbers under static conditions (Fig. 3A, left). Because of the decreased adhesion noted on ICAM-1 relative to WT cells under static conditions (Fig. 3C, left), we examined whether FABP4-deficient eosinophils detach more readily than WT cells under conditions of flow. In these studies, equal numbers of both cell types were allowed to adhere on ICAM-1 and then exposed to flow conditions. Fewer FABP4-deficient eosinophils remained adherent on coverslips relative to WT cells (Fig. 3F). Next, expression of integrin- β 2 (CD18), the major counter-receptor for ICAM-1, as well as expression of other cell adhesion molecules (α 4, α L, α M, L-selectin), was evaluated by flow cytometry. FABP4-deficient eosinophils expressed lower levels of integrin- β 2 relative to WT eosinophils (Fig. 3G), which may be responsible for the decreased adhesion noted on ICAM-1. Expression levels of α 4, α L, α M, and L-selectin were similar in WT and FABP4-deficient eosinophils. Previous studies in macrophages have shown that FABP4 deficiency is associated with increased expression and activity of PPAR- γ (20, 45), a member of the nuclear receptor superfamily of ligand-activated transcription factors. We examined basal expression of PPAR- γ in WT and FABP4-deficient eosinophils. PPAR- γ gene expression in

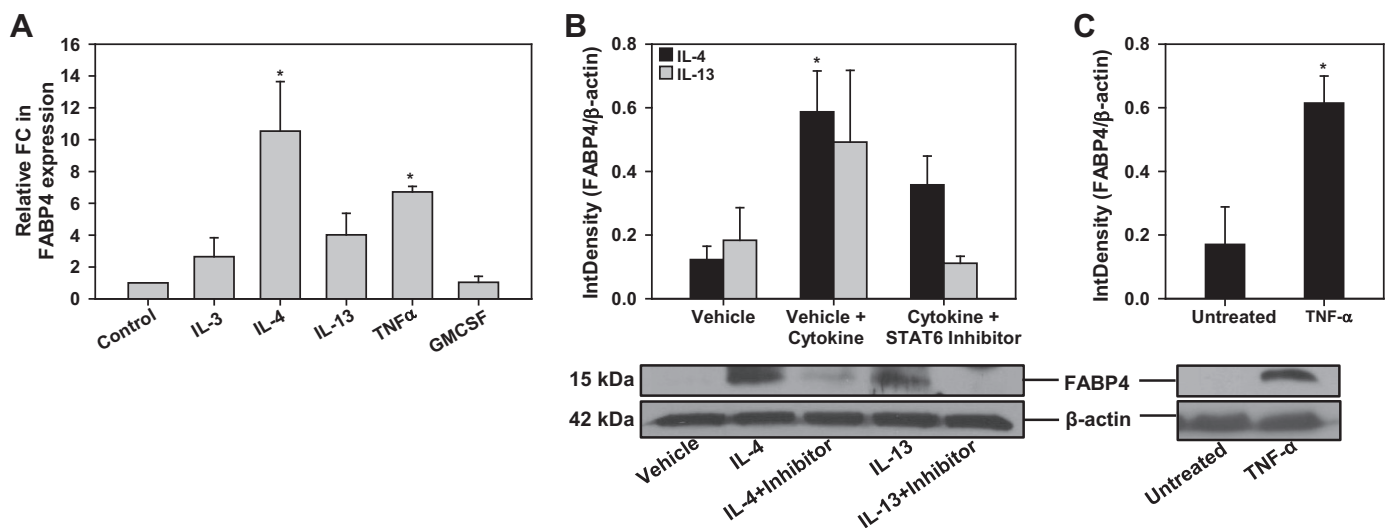


Fig. 2. IL-4, TNF- α , and IL-13 induce fatty acid binding protein 4 (FABP4) expression in murine eosinophils. A: effect of inflammatory cytokines on FABP4 expression in mouse eosinophils by qPCR. GM-CSF, granulocyte macrophage colony-stimulating factor. B: effect of IL-4 and IL-13 on FABP4 protein expression in the presence of signal transducer and activator of transcription 6 (STAT6) inhibitor (100 nM) or vehicle (DMSO) examined by Western blot followed by densitometric analysis. C: effect of TNF- α on FABP4 protein expression by Western blot followed by densitometric analysis. Combined data (means $\pm$ SE) of 3 experiments are shown. Representative data for Western blot are shown below graph in B and C. * P < 0.03 in A, <0.05 in B and C for control/vehicle/untreated vs. cytokine treated.

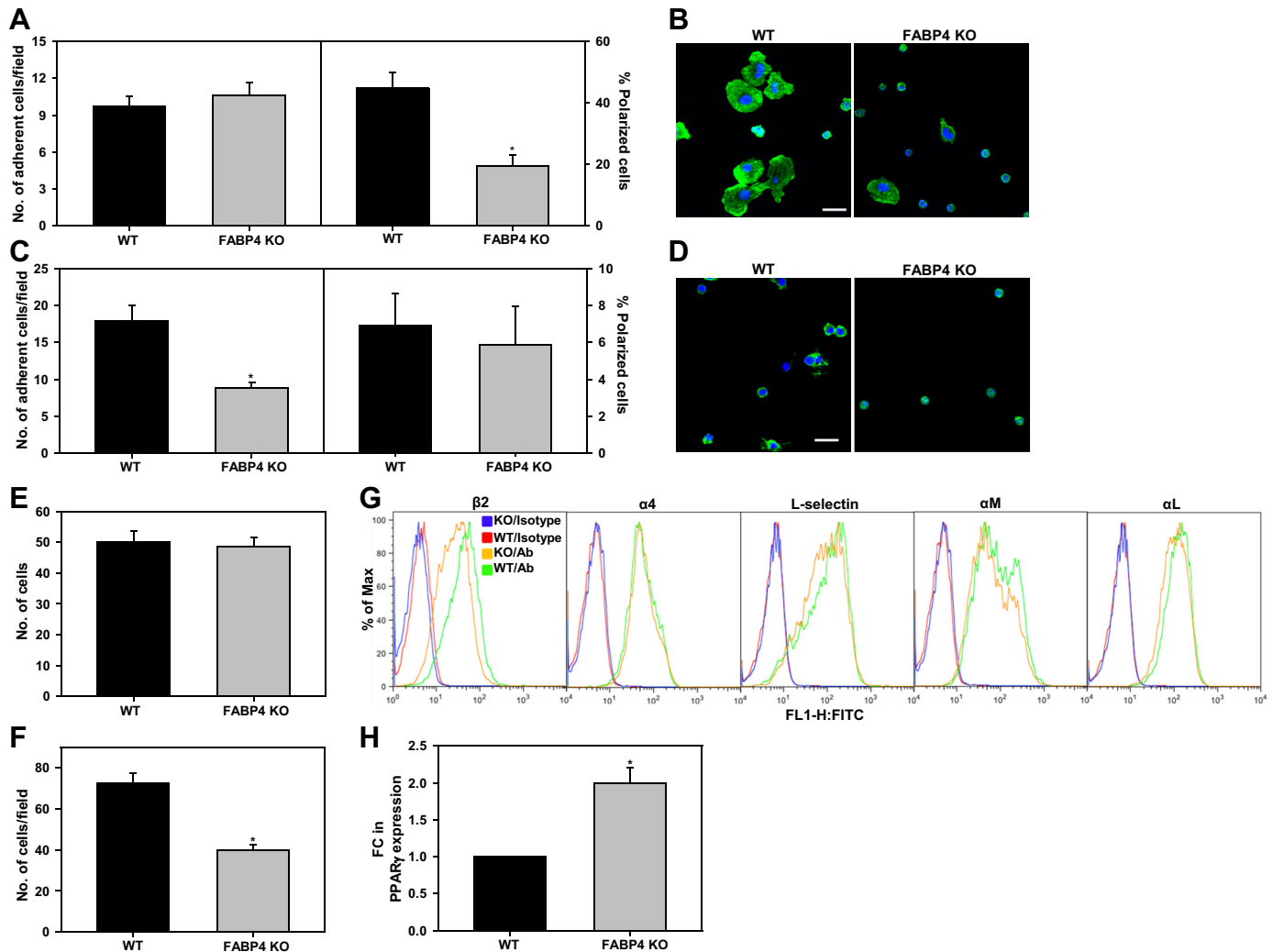


Fig. 3. Fatty acid binding protein 4 (FABP4) promotes cell adhesion via regulation of integrin- β 2 expression. *A* and *B*: adhesion and morphology of wild-type (WT) and FABP4 knockout (KO) eosinophils on recombinant murine (rm) VCAM-1-coated coverslips. *C* and *D*: adhesion and morphology of WT and FABP4 KO eosinophils on rm ICAM-1-coated coverslips. Scale bar = 20 μ m in *B* and *D*. *E*: rolling of WT and FABP4 KO eosinophils on rm VCAM-1-coated coverslips under conditions of flow (wall shear stress 1.0–2.0 dynes/cm²) in a flow chamber. *F*: detachment of WT and FABP4 KO eosinophils from rm ICAM-1-coated coverslips under conditions of flow in a flow chamber. *G*: expression of cell adhesion molecules in WT and FABP4 KO eosinophils by flow cytometry. *H*: expression of peroxisome proliferator-activated receptor- γ (PPAR- γ) in WT and FABP4 KO eosinophils by qPCR. Combined data (means $\pm$ SE) of 3 experiments in duplicate are shown in *A*, *C*, *E*, *F*, and *H*. Representative data of 3 independent experiments with eosinophils from 3 different mice/group are shown in *B*, *D*, and *G*. * P < 0.01 in *A*, *C*, and *F* and <0.05 in *H* for comparison with WT eosinophils.

FABP4-deficient eosinophils was significantly higher than in WT eosinophils (Fig. 3*H*).

In addition to altered adhesion on VCAM-1 and ICAM-1, FABP4-deficient eosinophils showed reduced migration toward eotaxin-1 relative to WT eosinophils in vitro across membranes, as well as endothelial cell monolayers (Fig. 4*A*, *top* and *bottom*), although expression of the eotaxin-1 receptor CCR3 was similar in cells of both genotypes in the absence (baseline, Fig. 4*B*, *top*) and presence of eotaxin-1 (stimulated, Fig. 4*B*, *bottom*). Cytoskeletal reorganization in response to cellular activation, which is dependent on actin polymerization, is critical for cell migration (19). To evaluate the kinetics of actin polymerization in WT and FABP4-deficient eosinophils in response to cell activation, confocal microscopy of cells adherent on poly-L-lysine-coated surfaces was performed (Fig. 4*C*). WT eosinophils demonstrated a defined pattern of membrane-associated F-actin in the absence of eotaxin-1. After

exposure to eotaxin-1, WT cells showed dramatic spreading associated with increased phalloidin binding in the cytoplasm and at the cell periphery at 10 s and 2 min, reverting back to near baseline levels (as in untreated cells) by 10 min. FABP4-deficient eosinophils showed marginally higher baseline phalloidin binding relative to WT cells that was not entirely membrane associated. Interestingly, after exposure to eotaxin-1, FABP4-deficient cells failed to undergo spreading and did not exhibit changes in intensity or pattern of phalloidin binding even at 10 min.

Eotaxin-1 is also known to induce calcium flux in eosinophils (56). Baseline (unstimulated) [Ca²⁺]_i levels were similar in WT and FABP4-deficient eosinophils using real-time digital video fluorescence imaging (Fig. 4*D*, *top*). In response to eotaxin-1, although WT and FABP4-deficient eosinophils demonstrated a similar pattern of an initial increase in free [Ca²⁺]_i within a few seconds followed by reduction, [Ca²⁺]_i

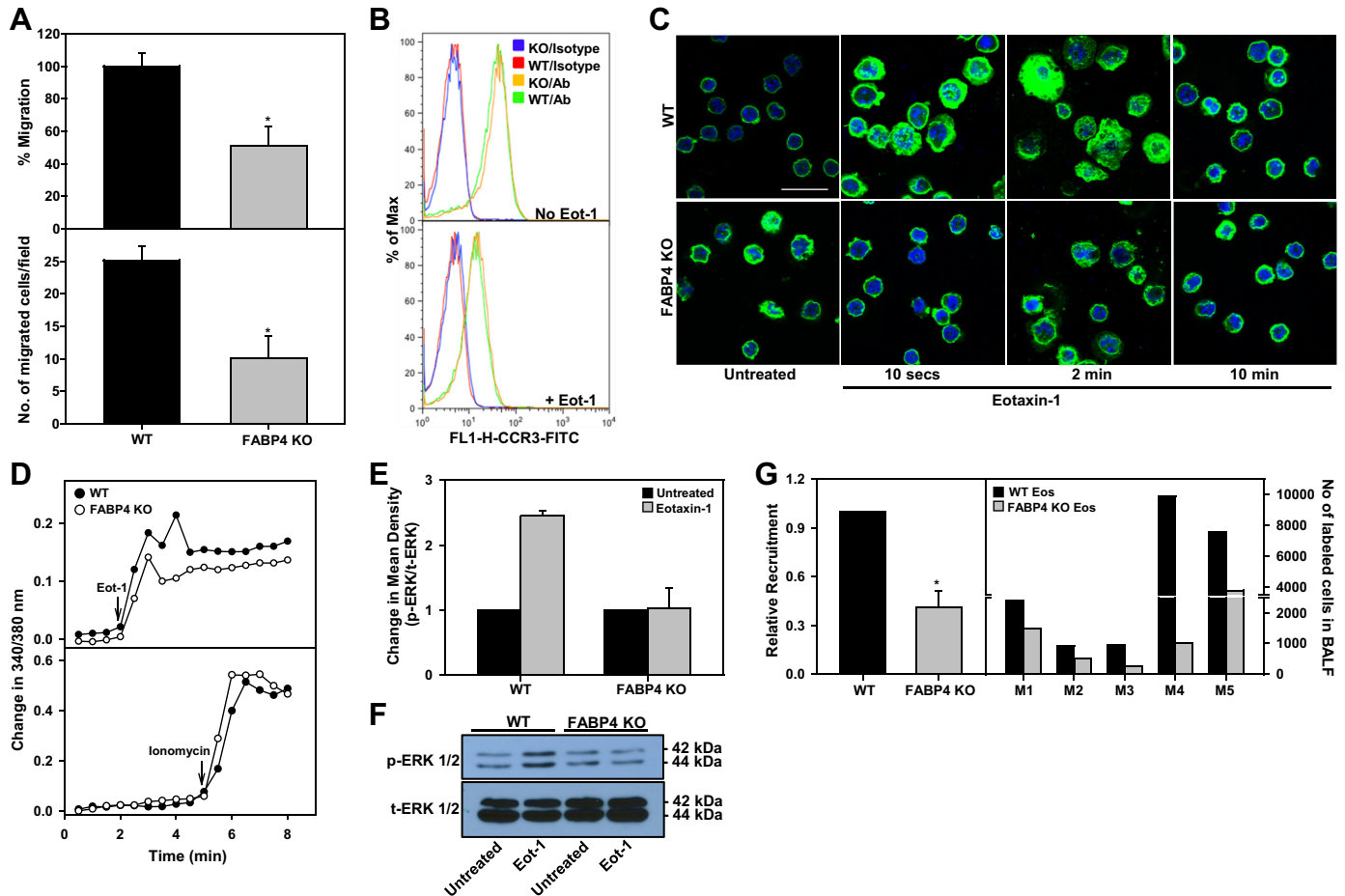


Fig. 4. Fatty acid binding protein 4 (FABP4) is required for efficient eosinophil migration. **A**: in vitro migration of wild-type (WT) and FABP4 knockout (KO) eosinophils toward murine eotaxin-1 (100 nM) across membranes (top) or endothelial cell monolayers (bottom) in Transwell chambers. **B**: expression of C-C chemokine receptor type 3 (CCR3) in WT and FABP4 KO eosinophils without and with eotaxin-1 treatment (100 nM, 30 min) by flow cytometry. **C**: FITC-phalloidin staining of WT and FABP4 KO eosinophils adhered on poly-L-lysine and treated with eotaxin-1 (100 nM) for the indicated time points. Scale bar = 20 μ m. **D**: $[Ca^{2+}]_i$ levels in WT and FABP4 KO before and after stimulation with eotaxin-1 (top) or ionomycin (bottom) by digital videofluorescence imaging using the Ca^{2+} indicator dye fura-2 AM. >150 cells were analyzed for each genotype. **E**: total and phospho-ERK(1/2) expression in WT and FABP4 KO eosinophils treated with eotaxin-1 (100 nM, 15 min) by Western blot analysis followed by densitometric analysis of protein bands. Data represent means $\pm$ SD of 2 experiments. **F**: representative Western blot of total and phospho-ERK(1/2) expression in WT and FABP4 KO eosinophils treated with eotaxin-1 as in **E**. **G**: recruitment of intravenously transferred fluorescently labeled WT and FABP4 KO eosinophils to the lungs of *A. alternata*-challenged WT mice 24 h after infusion evaluated by flow cytometry. Data of individual experiments ($n = 5$ WT mice, M1–M5, right) and recruitment relative to WT eosinophils (left) are shown. BALF, bronchoalveolar lavage fluid. Combined data (means $\pm$ SE) of 3 experiments are shown in **A** and **D**, and data representative of 3 independent experiments with eosinophils from 3 different mice/group are shown in **B** and **C**. * $P < 0.02$ in **A** and **G** for comparison with WT eosinophils.

levels in WT eosinophils remained elevated compared with FABP4-deficient cells up to 8 min (Fig. 4D, top). In contrast, both WT and FABP4-deficient eosinophils demonstrated a similar response to ionomycin, a Ca^{2+} ionophore that directly transports Ca^{2+} across biological membranes to artificially increase $[Ca^{2+}]_i$ (Fig. 4D, bottom), suggesting that eotaxin-1-induced $[Ca^{2+}]_i$ response is altered in FABP4-deficient eosinophils. Activation of ERK(1/2) is an important signaling event elicited in eotaxin-1-exposed eosinophils and is required for cell migration in vitro (6) and recruitment in vivo (13). WT cells exposed to eotaxin-1 for 15 min demonstrated increased activation of ERK(1/2), whereas no activation of this signaling molecule was noted in FABP4-deficient eosinophils (Fig. 4, E and F). Overall, deficiency of FABP4 appears to cause irregular F-actin polymerization, resulting in attenuated cytoskeletal changes along with decreased $[Ca^{2+}]_i$ levels and ERK(1/2) activation in response to eotaxin-1, all of which are likely to

contribute to the reduced migration of these cells. When examined in vivo, recruitment of infused fluorescently labeled FABP4-deficient eosinophils to allergen-challenged lungs was lower than that of labeled WT eosinophils in a WT setting, further supporting the role of FABP4 in regulating eosinophil migration (Fig. 4G).

FABP4 deficiency results in decreased eosinophilia. To evaluate the role of FABP4 in eosinophil recruitment in an experimental model of allergic asthma, CRA, a clinically relevant allergen strongly associated with the development of asthma among children and young adults (52), was utilized. WT and FABP4-deficient mice were exposed to CRA as outlined in Fig. 1A. Allergen-challenged FABP4-deficient mice showed significantly reduced recruitment of inflammatory cells in the lung tissue based on H and E staining and in the BALF compared with corresponding WT mice (Fig. 5, A and B, respectively). Differential cell counts in the BALF

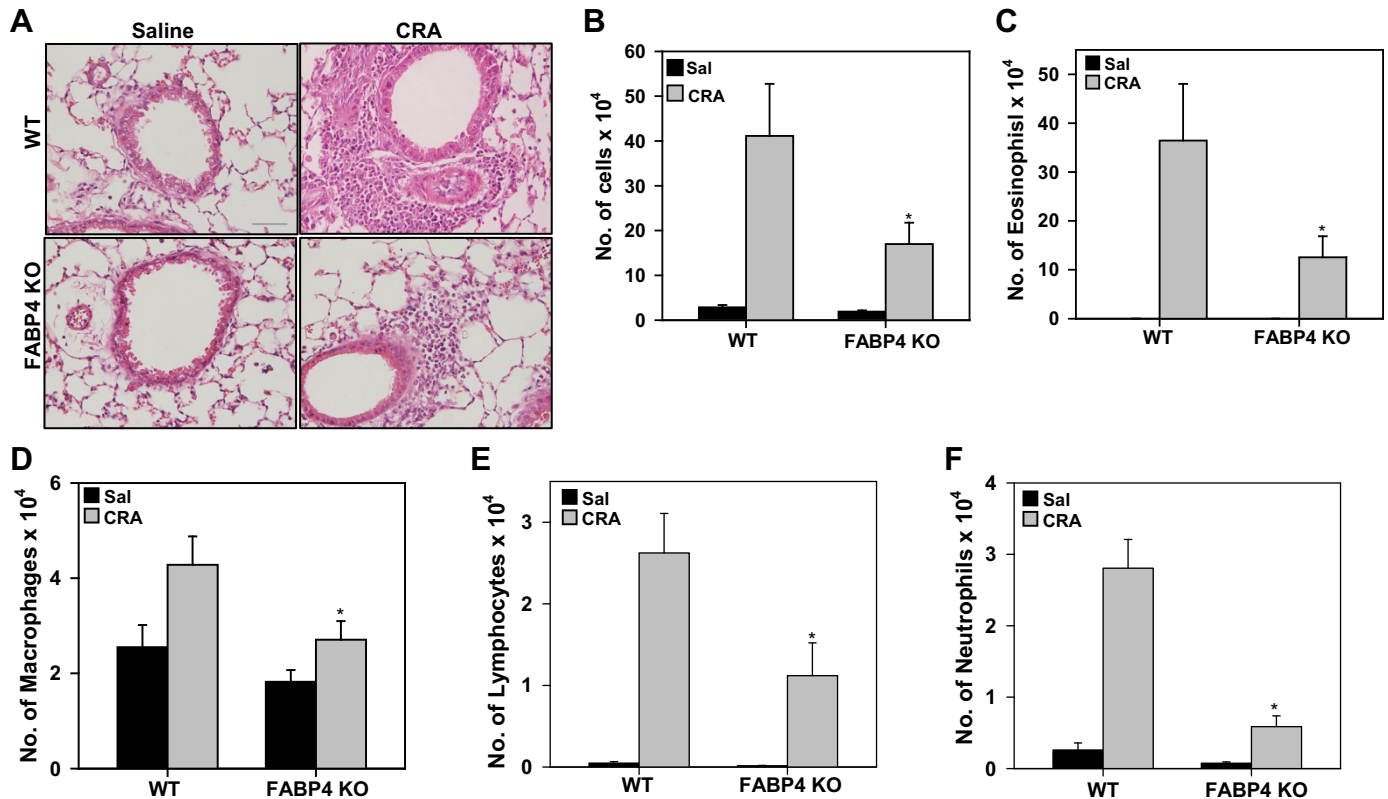


Fig. 5. Fatty acid binding protein 4 (FABP4) deficiency results in attenuation of allergen-induced airway cellular inflammation. **A**: hematoxylin and eosin (H and E) staining of paraffin-embedded lung sections from saline (control) and cockroach antigen (CRA)-challenged wild-type (WT) and FABP4 knockout (KO) mice. Representative image for each group is shown. Scale bar = 50 μ m. **B**: total cell counts in the bronchoalveolar lavage fluid (BALF) of CRA-challenged and control WT and FABP4 KO mice. **C–F**: number of eosinophils, macrophages, lymphocytes, and neutrophils, respectively, in the BALF of CRA-challenged and control WT and FABP4 KO mice. Combined data (means $\pm$ SE) of mice from 2 to 3 independent experiments are shown in **B–F** ($n = 8–12$ mice/group). * $P < 0.05$ in **B–D** and <0.02 in **E** and **F** for comparison of CRA-challenged groups.

indicated that the numbers of eosinophils, macrophages, lymphocytes, and neutrophils were significantly lower in CRA-challenged FABP4 KO mice relative to allergen-challenged WT mice (Fig. 5, **C–F**). These findings are different from that noted previously with ovalbumin challenge (59), in which a reduction in only eosinophils and neutrophils was noted. In the present study, consistent with decreased ability of FABP4-deficient eosinophils to migrate toward eotaxin-1 *in vitro*, eosinophil recruitment to the airways was also distinctly lower in allergen-challenged FABP4-deficient mice. These mice had significantly reduced tissue eosinophils based on detection of MBP-positive cells in lung tissue sections (Fig. 6, **A** and **B**). Furthermore, eosinophils in the peripheral blood and BM of allergen-challenged FABP4-deficient mice were also significantly lower than in WT counterparts (Fig. 6, **C** and **D**).

CRA-induced allergic inflammation is attenuated in FABP4-deficient mice. Elevated levels of Th2 cytokines and eotaxins in the lungs are driving factors of allergic airway inflammation (2, 42). In the present study, CRA challenge did not alter levels of Th1 cytokines (IL-2 and IFN- γ) or the Th2 cytokine IL-4 in WT mice but induced expression of IL-5 and IL-13, as well as TNF- α , in the BALF and lung tissue (Fig. 7, **A** and **B**). Although levels of IL-5, IL-13, and TNF- α in allergen-challenged FABP4-deficient mice were higher than in corresponding controls, they were significantly lower than in allergen-challenged WT mice, specifically in the BALF (Fig. 7A). In

addition, levels of eosinophil chemoattractants eotaxin-1 and -2 were measured in the BALF of WT and FABP4-deficient mice. Level of both eotaxins was higher in allergen-challenged mice relative to the control mice with no significant difference in levels between CRA-challenged WT and FABP4-deficient mice (Fig. 7C). The decreased airway eosinophilia noted in CRA-challenged FABP4-deficient mice (Figs. 5C and 6B), despite the lack of a striking difference in levels of eotaxin-1 and -2 in the lungs between these groups (Fig. 7C), further supports a direct role for FABP4 in promoting eosinophil recruitment independent of chemokine levels. Given the pro-inflammatory role of lipid mediators such as leukotrienes during allergic airway inflammation (41), we examined levels of LTC₄ in the lung tissue of allergen-challenged WT vs. FABP4-deficient mice. Consistent with the asthma phenotype, allergen-challenged WT mice showed elevated levels of LTC₄ relative to control mice (Fig. 7D). Importantly, LTC₄ level in CRA-challenged FABP4-deficient mice was significantly lower than in WT mice.

AHR is a hallmark feature of allergic asthma. To assess airway responsiveness in the FABP4-null mouse, R_L to increasing concentrations of aerosolized methacholine in control and CRA-challenged WT and FABP4-deficient mice was evaluated. Control mice of both genotypes displayed a marginal increase in airway reactivity with increasing doses of methacholine. R_L was markedly higher in allergen-challenged WT

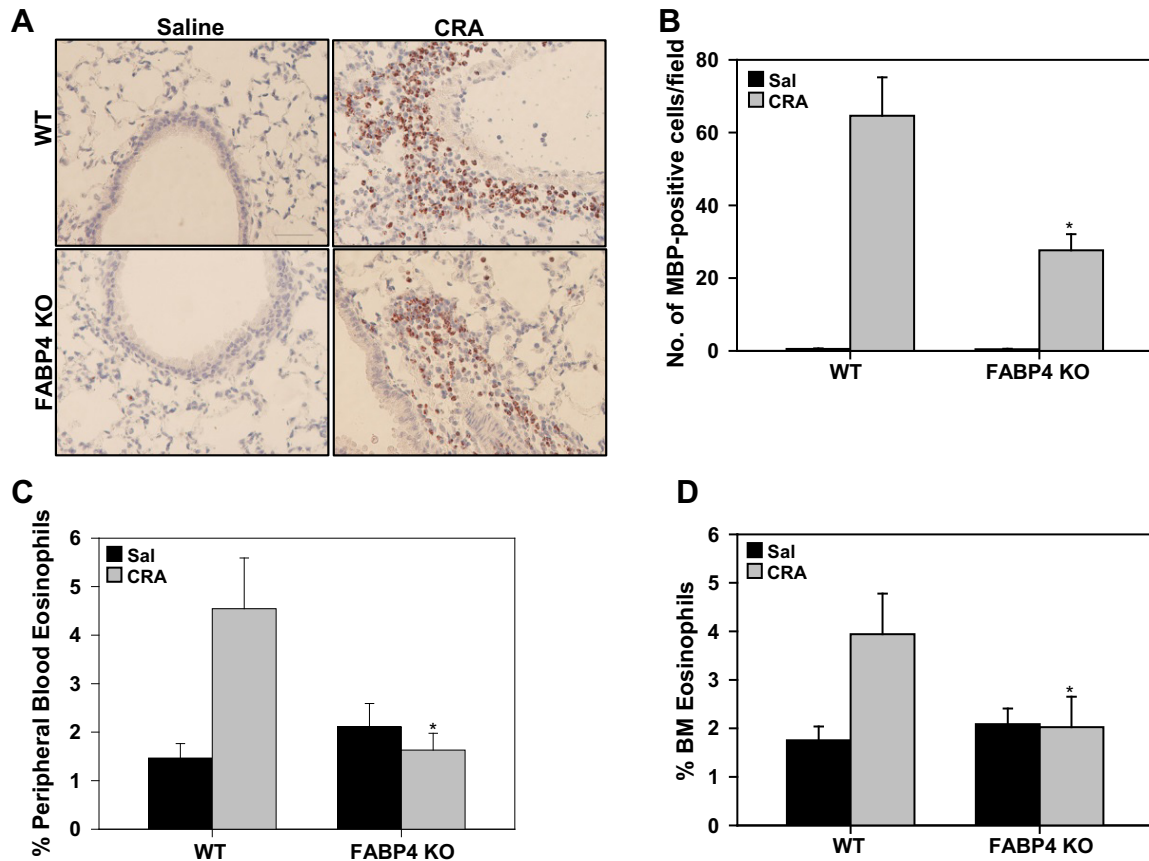


Fig. 6. Mice deficient in fatty acid binding protein 4 (FABP4) exhibit decreased allergen-induced eosinophilia. *A* and *B*: infiltrated eosinophils in lung tissue detected by immunohistochemical staining for major basic protein (MBP) (stained dark brown) and quantitation of immunopositive cells. Representative images for control and cockroach antigen (CRA)-challenged wild-type (WT) and FABP4 knockout (KO) mice are shown in *A*, and the number of positively stained cells in randomly selected nonoverlapping microscopic fields (18 ± 4) counted at $\times 400$ magnification is shown in *B*. Scale bar = 50 μm . *C* and *D*: percentage of eosinophils in peripheral blood and bone marrow (BM), respectively, of control and CRA-challenged WT and FABP4 KO mice determined based on cell morphology after Hema3 staining. Combined data (means $\pm$ SE) of mice from 2 to 3 independent experiments are shown ($n = 8$ –12 mice/group in *B* and *D* and 5 mice/group in *C*). * $P < 0.01$ in *B* and < 0.05 in *C* and *D* for comparison of CRA-challenged groups.

and FABP4-deficient mice compared with corresponding control mice at higher concentrations of inhaled methacholine (> 6 mg/ml) with airway reactivity in allergen-challenged FABP4-deficient mice significantly lower than in WT mice at 25 mg/ml methacholine (Fig. 8A). Allergen exposure also leads to structural changes in the airways caused by increased mucus production and airway smooth muscle hyperplasia/hypertrophy, which in turn can contribute to airflow obstruction (5). WT mice showed increased airway mucus secretion and smooth muscle proliferation based on PAS staining and immunohistochemistry for expression of smooth muscle actin, respectively (Fig. 8, *B–E*). In FABP4-deficient mice, allergen-induced airway mucus production (Fig. 8, *B* and *C*) and smooth muscle mass (Fig. 8, *D* and *E*) were significantly lower relative to WT counterparts, consistent with the decreased AHR noted in these mice. Thus deficiency of FABP4 results in decreased structural changes in allergic airways, leading to improved airway responsiveness.

DISCUSSION

Previous studies have reported a proinflammatory role for FABP4 in ovalbumin-induced allergic airway inflammation, wherein allergen-exposed FABP4-deficient mice exhibited de-

creased airway infiltration of leukocytes, specifically eosinophils (59), suggesting that FABP4 acts locally in the lung, most likely in bronchial epithelial cells. FABP4 is known to be an important mediator of inflammatory processes in T cells, dendritic cells, and macrophages, as well as in experimental inflammation models (32, 34, 48, 69). Although FABP4 clearly promotes allergic airway inflammation, its expression and function in eosinophils, the predominant inflammatory cells recruited to allergic airways, are not known. Our studies indicate that FABP4 expression in response to CRA challenge is increased in the lungs of mice largely because of recruitment of FABP4-expressing inflammatory cells. Importantly, our studies show for the first time that eosinophils recruited to the airways express FABP4. Interestingly, expression of FABP5, a FABP with 52% amino acid identity to FABP4, is induced in the sputum and nasal lavage fluid of allergic asthmatics and thought to play a role in VEGF-mediated airway inflammation (64) similar to FABP4 (23).

FABP4 is an inducible gene, and inflammatory mediators, such as Th2 cytokines, HIF-1 α , and LPS, are known to induce expression of this protein in other cell types (33, 44, 59). In addition, FABP4 has been shown to be induced by growth factors such as VEGF in endothelial cells and to function as a

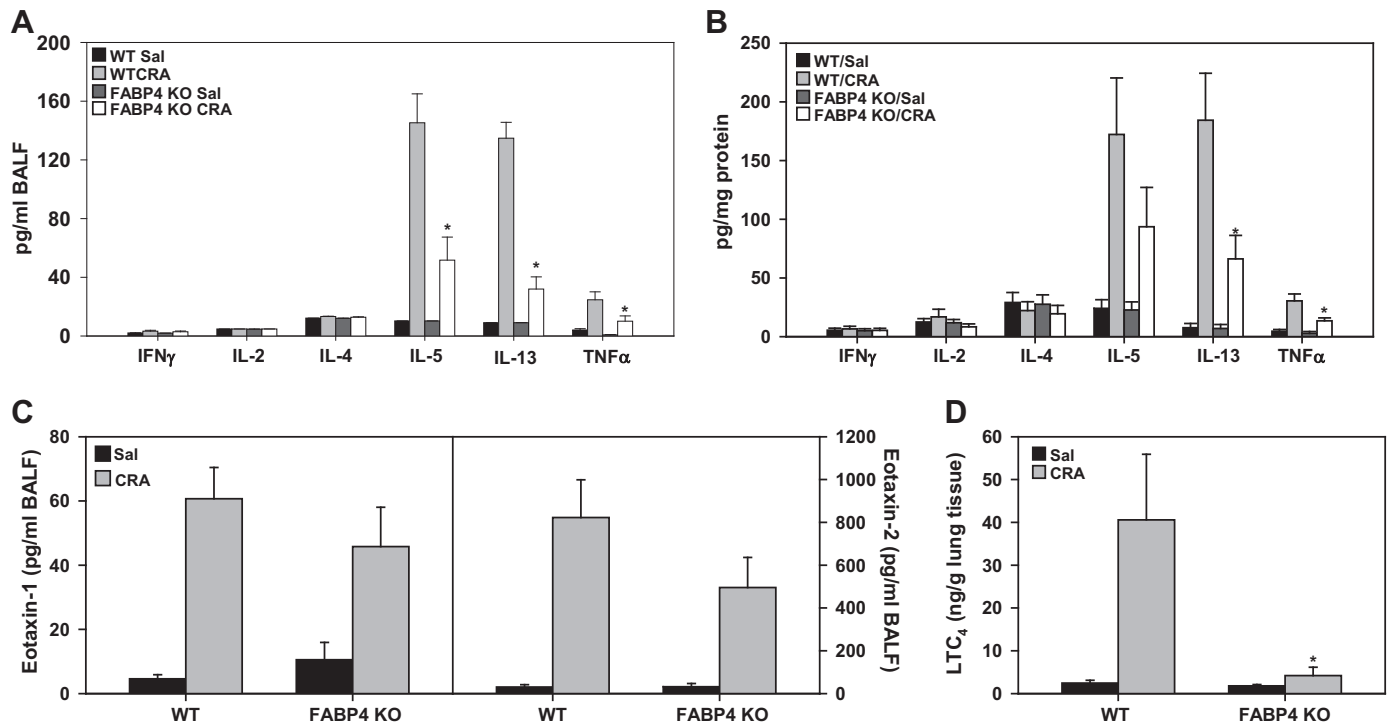


Fig. 7. Decreased levels of proinflammatory mediators in the lungs of allergen-challenged fatty acid binding protein 4 (FABP4)-deficient mice. A and B: Th1-Th2 cytokine and TNF- α levels in bronchoalveolar lavage fluid (BALF) and lung lysates of control and cockroach antigen (CRA)-challenged wild-type (WT) and FABP4 knockout (KO) mice, respectively. C: eotaxin-1 and -2 levels in BALF of above groups of mice. D: cysteinyl leukotriene C₄ (LTC₄) levels in lung tissue of above groups of mice. Combined data (means $\pm$ SE) of $n = 9-10$ mice/group in A, 4-8 mice/group in B, 7-9 mice/group in C, and 3-4 mice/group in D are shown. * $P < 0.03$ in A and B and < 0.05 in D for comparison of CRA-challenged groups.

regulator of cell proliferation and airway angiogenesis (23). In the present study, inflammatory cytokines associated with allergic asthma, such as IL-4, IL-13, and TNF- α , induced FABP4 mRNA and protein expression in eosinophils. Furthermore, treatment with IL-4 or IL-13 in the presence of a STAT6 inhibitor reduced FABP4 expression, suggesting that FABP4 in eosinophils is regulated by STAT6. These findings are similar to that noted in primary murine airway epithelial cells and human bronchial epithelial cell lines, in which STAT6-deficient cells failed to exhibit increased FABP4 expression after IL-4 treatment (59). Additionally, a conserved putative STAT6 binding was identified at the 5' transcriptional start site in the mouse and human FABP4 gene in that study. Our observation of induction of FABP4 by TNF- α in eosinophils is novel and in contrast to the effect of TNF- α on FABP4 expression in white adipose tissue (50). Studies in IL-4-stimulated macrophages and dendritic cells have shown that STAT6 interacts with transcription factor PPAR- γ on promoters of PPAR- γ target genes, including FABP4, to induce its expression at the level of the gene and protein (65). It is likely that a similar mechanism might be responsible for the induction of FABP4 in IL-4-treated eosinophils.

Given that decreased eosinophil recruitment was noted in ovalbumin-challenged FABP4-deficient mice (59), we investigated whether FABP4 plays a role in eosinophil adhesion and migration, cellular events important for recruitment. Eosinophil recruitment to sites of inflammation in vivo involves a multistep paradigm, which includes initial tethering/rolling in inflamed blood vessels followed by activation-dependent firm adhesion and chemoattractant-induced transmigration into ex-

travascular sites [as reviewed earlier (55)]. Although able to interact with VCAM-1 in a manner similar to WT cells under conditions of flow, eosinophils deficient in FABP4 failed to efficiently form membrane protrusion/leading edges upon adhesion to VCAM-1.

Furthermore, FABP4-deficient eosinophils exhibited decreased ability to adhere to ICAM-1 relative to WT cells. This may be due to the fact that FABP4-deficient eosinophils expressed reduced levels of the $\beta 2$ -subunit of integrin- $\alpha M\beta 2$, the counter receptor for ICAM-1. Cell spreading and formation of leading edges are important cytoskeletal changes that are driven by actin polymerization and are essential for stable cell adhesion and directed cell motility movement toward a chemokine gradient (51).

In line with decreased adhesion to ICAM-1, FABP4-deficient eosinophils demonstrated markedly reduced migration, including across endothelial barriers, toward eotaxin-1 compared with WT eosinophils despite similar expression level of the eotaxin-1 receptor CCR3. As noted in the case of adhesion to VCAM-1, FABP4-deficient eosinophils exhibited attenuated cell spreading and decreased actin polymerization in response to stimulation with eotaxin-1. Remodeling of the actin cytoskeleton is critical for cell motility and requires the coordinated polymerization and depolymerization of F-actin. Because the level of expression of CCR3 is not affected by FABP4 deficiency, it is more likely that signaling downstream of CCR3 is impaired in the absence of FABP4. This is further supported by the decreased eotaxin-1-induced Ca²⁺ flux in these cells. Cell polarization and migration are known to be coordinated by Ca²⁺ signaling via regulation of integrin activation (60) and

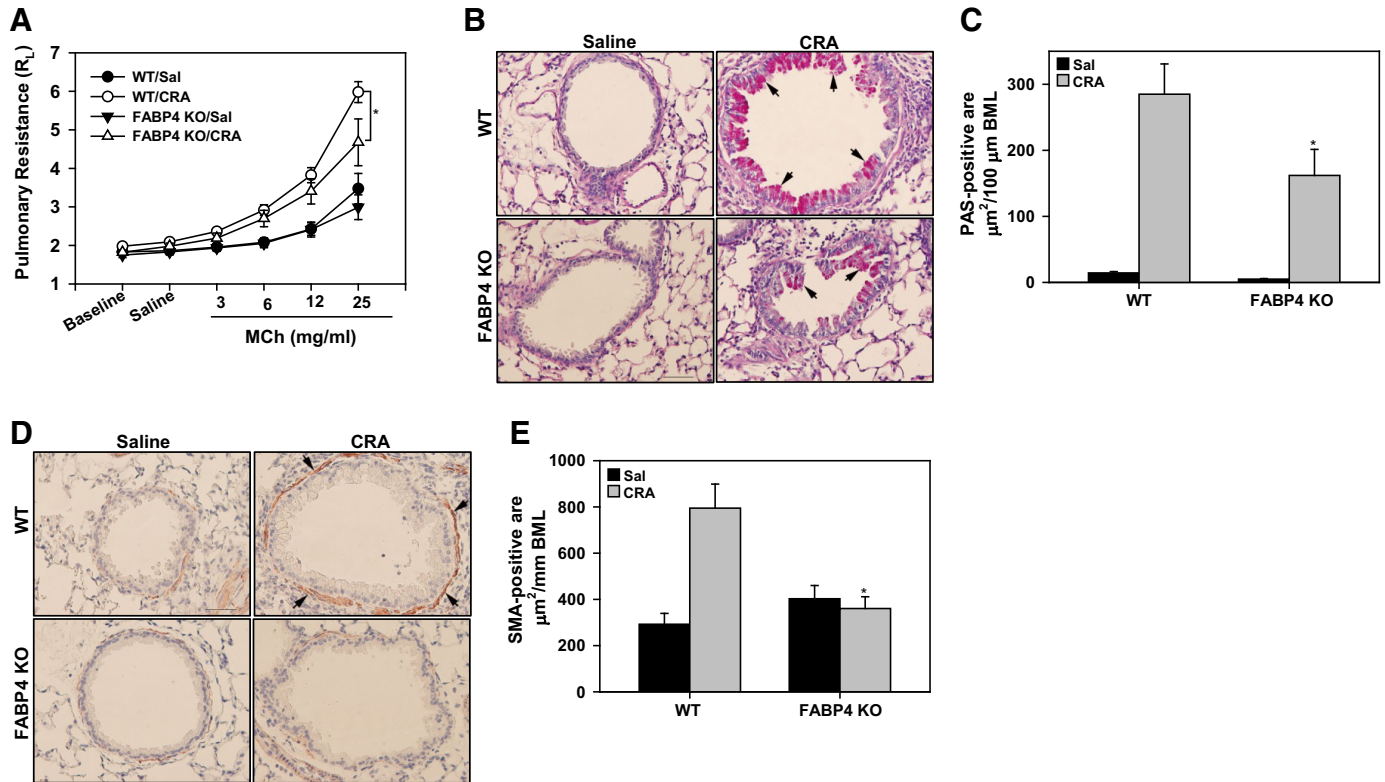


Fig. 8. Airway hyperresponsiveness (AHR), mucus secretion, and smooth muscle thickening are attenuated in allergen-challenged fatty acid binding protein 4 (FABP4)-deficient mice. **A**: invasive measurement of airway responsiveness following exposure to increasing concentrations of aerosolized methacholine (MCh) in mechanically ventilated control and cockroach antigen (CRA)-challenged wild-type (WT) and FABP4 knockout (KO) mice. **B** and **C**: airway mucus secretion in the above groups of mice assessed by periodic acid-Schiff (PAS) staining (stained dark-pink, black arrows) and quantitation of PAS-positive area (4–5 airways/mouse with perimeter $730 \pm 20 \mu\text{m}$). Representative images are shown in **B**. Scale bar = $50 \mu\text{m}$. **D** and **E**: airway smooth muscle thickening in control and allergen-challenged mice assessed by immunohistochemical staining for α -smooth muscle actin (α -SMA) expression (stained brown, black arrows) and quantitation of α -SMA-positive area (3–4 airways/mouse with perimeter $710 \pm 20 \mu\text{m}$). Representative images are shown in **D**. Scale bar = $50 \mu\text{m}$. Combined data (means $\pm$ SE) of $n = 6$ –8 mice/group for AHR, 8–10 mice/group for PAS, and 7–9 mice/group for α -SMA are shown. * $P < 0.04$ in **A**, and <0.025 in **C** and **E** for comparison of CRA-challenged groups.

modulation of actin dynamics through activation of signaling molecules that interact with actin (PKC and calmodulin-dependent kinases) (67). Furthermore, our studies indicate that FABP4-deficient eosinophils fail to undergo activation of ERK(1/2) in response to eotaxin-1. ERK(1/2) activation is an important signaling event required for eotaxin-1-induced migration of eosinophils (6, 8). Studies in other cell types have shown that expression level of FABP4 is linked to ERK(1/2) activation. In cardiomyocytes, overexpression of FABP4 was associated with increased ERK phosphorylation, which in turn upregulated cardiac hypertrophic gene expression (71), whereas FABP4 knockdown in a squamous carcinoma cell line inhibited ERK(1/2) activation and cell growth (37). Additionally, treatment of human vascular smooth muscle cells with FABP4 extracellularly has been shown to induce cell proliferation and migration in an ERK-dependent manner (24). Overall, our novel findings suggest that FABP4 plays an important role in cell adhesion and migration by regulating cytoskeletal changes, β_2 -integrin expression, and key signaling events, such as $[\text{Ca}^{2+}]_i$ flux and ERK(1/2) activation. Furthermore, in vivo confirmation of a role for FABP4 in eosinophil migration comes from studies in which a reduced number of infused FABP4-deficient eosinophils was recruited to allergic airways relative to WT cells in a WT setting. To our knowledge, this is

the first identification of a direct involvement of FABP4 in migration of eosinophils.

In a previous study investigating the role of FABP4 in allergic airway inflammation induced by ovalbumin (59), the airway epithelium was identified as the major site of action of FABP4. In their study, allergic chimeric FABP4 KO mice that received WT BM cells had lower BALF levels of eotaxin-1 and IL-5 compared with WT mice that received FABP4 KO BM cells, suggesting that nonhematopoietic cells are responsible for reduced allergic airway inflammation in FABP4 KO mice. In addition to the role of airway epithelial FABP4 identified in their study, our present studies using mature eosinophils derived from murine BM demonstrate a direct role for eosinophil-expressed FABP4 in regulating cell recruitment in the context of allergic inflammation. These in vitro findings are further supported by our studies in a mouse model of allergic airway inflammation. CRA-challenged FABP4-deficient mice had significantly lower airway cellular inflammation with, not only decreased eosinophils and neutrophils as noted in the previous study with ovalbumin-induced allergic inflammation (59), but also with lymphocytes and macrophages. Furthermore, a significant reduction in peribronchial and peripheral blood eosinophils and BM eosinophils was noted in these mice. Unlike the previous study, which reported lower eotaxin-1 and -2 expres-

sion in the lungs of allergen-challenged FABP4-deficient mice relative to WT mice (59), levels of these two chemokines were not significantly different between CRA-challenged WT and FABP4-deficient mice in our study. This differential observation may be due to differences between the two models of allergen-induced airway inflammation (nature of the allergen, number of challenges, duration of study, etc.) and sample analyzed (lung transcript level vs. BALF protein level).

Overall, the decreased number of eosinophils in the lungs and peripheral blood of CRA-challenged FABP4-deficient mice relative to WT counterparts, despite no significant difference in eotaxin levels, is likely to be in part due to the inherently decreased ability of FABP4-deficient eosinophils to adhere and migrate efficiently. Although the role of FABP4 in regulation of hematopoiesis is still unclear, absence of increased eosinophils in the BM of FABP4-deficient mice after CRA challenge is suggestive of a role for FABP4 in the generation of eosinophils in addition to its role in eosinophil recruitment. Level of IL-5, a cytokine that regulates generation and maturation of eosinophils in the BM (17), was substantially lower in the BALF and lung tissue in CRA-challenged FABP4-deficient mice relative to WT counterparts; however, circulating levels of this cytokine were not determined in the present study, and therefore it is not clear whether this may be a contributing factor for the reduced generation of eosinophils in the BM.

Along with the reduced eosinophilia, CRA-challenged FABP4-deficient mice displayed significantly decreased AHR, mucus secretion, and smooth muscle mass, as well as lower Th2 cytokines (IL-5 and IL-13), TNF- α , and LTC₄ levels relative to WT mice. IL-13 and TNF- α promote AHR (9, 47), and decreased levels of these cytokines may result in decreased mucus secretion and improved airway responsiveness in FABP4-deficient mice. Eosinophils, through the release of various mediators, including leukotrienes, are crucial for mucus secretion in the airways and in the development of AHR (39, 41). FABP4 has been shown to stabilize LTA₄ toward chemical hydrolysis, thus favoring LTC₄ synthesis (29, 72), and studies have shown that LTC₄ induces chemotaxis of eosinophils and expression of the adhesion molecule Mac-1 (α M β 2-integrin) (16). Thus absence of FABP4 is likely to contribute to the reduced levels of LTC₄ in the lungs of allergen-challenged FABP4-deficient mice, which in turn can lead to reduced eosinophil recruitment to the airways. Airway smooth muscle plays a crucial role in the pathophysiology of asthma. Increased smooth muscle mass resulting in airway wall thickening induces bronchoconstriction, AHR, and airway remodeling in asthma (12). Our studies show that airway smooth muscle thickness in CRA-challenged FABP4-deficient mice is reduced to levels observed in corresponding control mice. Previous studies have shown a direct role for exogenous FABP4 in induction of vascular smooth muscle cell migration and proliferation in vitro (24). Although extracellular/circulating levels FABP4 (i.e., BALF or serum/plasma) were not measured in the present study, FABP4 is known to be secreted, and elevated circulating levels of FABP4 have been shown to be associated with metabolic diseases and immune dysfunction (32, 49). On the basis of this, it is not unreasonable to speculate that extracellular/secreted FABP4 in a WT setting may induce airway smooth muscle cell proliferation in a manner similar to

vascular smooth muscle cells and thus increase airway smooth muscle mass, inducing AHR.

FABP4 deficiency is associated with increased expression and activity of PPAR- γ in macrophages (20, 45), and reconstitution of FABP4-deficient macrophages with FABP4 reduces expression of PPAR- γ (20), suggesting the existence of an inverse correlation between the two molecules and suppression of PPAR- γ as an effect of FABP4 in this context. Our studies show that, similar to macrophages, basal expression of PPAR- γ is significantly higher in FABP4-deficient eosinophils relative to WT eosinophils. Additionally, treatment of monocytes with a PPAR- γ agonist has been shown to result in reduction of β 2-integrin expression (11). It is possible that increased basal PPAR- γ expression in FABP4-deficient eosinophils may be responsible for the constitutively reduced β 2-integrin expression by these cells. Enhanced PPAR- γ expression and/or activity has been shown to inhibit eosinophil activation, degranulation (46), and migration (61), as well as attenuate allergic airway inflammation (eosinophilic inflammation, Th2 cytokine release, improved lung function) (40) and airway remodeling (38) in experimental models of allergic asthma. Taken together, absence of FABP4 in eosinophils associated with an increased PPAR- γ expression may result in reduced β 2 expression, leading to decreased eosinophil recruitment and attenuated allergic inflammation, as observed in CRA-challenged FABP4-deficient mice.

In summary, FABP4 is an inducible protein that is expressed by eosinophils in allergen-exposed lungs and plays a proinflammatory role in the development of allergic asthma by regulating eosinophil recruitment and promoting various aspects of allergen-induced airway inflammation. At a cellular level, FABP4 appears to play a direct role in supporting eosinophil adhesion and migration by regulating cytoskeletal changes and β 2-integrin expression in a Ca²⁺-dependent manner. This finding is further supported by decreased eosinophil recruitment to the lungs with markedly reduced airway inflammation (Th2 cytokine, AHR, airway mucus, smooth muscle mass) in an experimental model of allergic asthma. Overall, these studies suggest that suppression of FABP4 may be beneficial in preventing eosinophil migration, resulting in attenuation of allergic asthma.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

X.N.G., I.B., M.D., Y.G., S.G.H., and K.A.S. performed experiments; X.N.G., I.B., M.D., Y.G., S.G.H., and K.A.S. analyzed data; X.N.G., M.D., S.P.R., and P.S. interpreted results of experiments; X.N.G. and S.P.R. drafted manuscript; X.N.G., S.P.R., and P.S. approved final version of manuscript; D.A.B., S.P.R., and P.S. conceived and designed research; D.A.B., S.P.R., and P.S. edited and revised manuscript; S.P.R. prepared figures.

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