β-Arrestin-2 regulates the development of allergic asthma

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Asthma is a chronic inflammatory disorder of the airways that is coordinated by Th2 cells in both human asthmatics and animal models of allergic asthma. Migration of Th2 cells to the lung is key to their inflammatory function and is regulated in large part by chemokine receptors, members of the seven-membrane-spanning receptor family. It has been reported recently that T cells lacking β-arrestin-2, a G protein–coupled receptor regulatory protein, demonstrate impaired migration in vitro. Here we show that allergen-sensitized mice having a targeted deletion of the β-arrestin-2 gene do not accumulate T lymphocytes in their airways, nor do they demonstrate other physiological and inflammatory features characteristic of asthma. In contrast, the airway inflammatory response to LPS, an event not coordinated by Th2 cells, is fully functional in mice lacking β-arrestin-2. β-arrestin-2–deficient mice demonstrate OVA-specific IgE responses, but have defective macrophage-derived chemokine–mediated $CD4^+$ T cell migration to the lung. This report provides the first evidence that β-arrestin-2 is required for the manifestation of allergic asthma. Because β-arrestin-2 regulates the development of allergic inflammation at a proximal step in the inflammatory cascade, novel therapies focused on this protein may prove useful in the treatment of asthma.

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Introduction

Asthma is a complex inflammatory disease that afflicts nearly 15 million Americans. Despite research advances, the worldwide prevalence, morbidity, and mortality of asthma have increased over the last two decades (1–3). In humans, the hallmark feature of allergic asthma is the abnormal expansion in the lung of Th cells that produce Th2 cytokines. This pathological event leads to the symptoms of asthma including airway inflammation, airway hyperresponsiveness, reversible airflow obstruction, and airway remodeling.

Like other immune cells, T cells are functionally dependent on their ability to migrate, localize within tissues, and interact with other immune cells (4). Chemotaxis, the process by which immune cells migrate, is mediated by chemokine activation of chemokine receptors (5).

Chemokine receptors are part of the enormous family of heptahelical cell surface receptors known as G protein–coupled receptors (GPCRs) (6). These receptors transduce extracellular signals into intracellular events by activating heterotrimeric G proteins. The dissociation of these G protein subunits activates cell signaling systems such as adenylate cyclases, phospholipases, and ion channels, which ultimately results in a physiological response. In the case of chemokine receptors, at least one of these physiological responses is cell migration.

Like other GPCRs, chemokine receptor function is regulated by β-arrestin proteins. β-arrestins, members of the arrestin family of proteins, are designated β-arrestin-1 or β-arrestin-2, are ubiquitously expressed, and regulate GPCR function through multiple mechanisms (7–9). As their name suggests, β-arrestin proteins were originally discovered to "arrest" G protein–mediated cell signaling events (10). Since that time, our understanding of the mechanisms by which β-arrestin modulates GPCR function has expanded considerably. In addition to their classical role, β-arrestin proteins also act as adapters that couple GPCRs to a clathrin-coated pit endocytic mechanism and as scaffolds that link GPCRs to a second wave of cell signaling via MAPK and other signaling pathways.

In vitro studies have shown that lymphocytes devoid of β-arrestin-2 and human embryonic kidney 293 cells with suppressed expression of β-arrestin-2 demonstrate impaired migration toward the chemotactic factor stromal cell–derived factor-1α (SDF-1α), also known as CXCL12 (11, 12). Although β-arrestin-2 is essential to the normal migration of immune cells in vitro, the ability of β-arrestin-2 to mediate immune cell chemotaxis in vivo has not been tested.

Because chemotaxis is crucial to the process of inflammation, we theorized that mice lacking β-arrestin-2 might be protected from developing allergic-asthmatic inflammation. To model allergic asthma in mice we

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Nonstandard abbreviations used: G protein–coupled receptor (GPCR); stromal cell–derived factor-1α (SDF-1α); β-arrestin-2–deficient (*βarr2–/–*) macrophage-derived chemokine (MDC); honestly significant difference (HSD).

used a standard method consisting of sensitization and challenge to OVA (13). This mouse model of allergic asthma mimics several features of human asthma.

Methods

Animals. Male and female β-arrestin-2–deficient (*βarr2*$^{-/-}$) (14) and littermate WT mice (backcrossed for six generations onto the C57BL/6 background) were used for all experiments at 8–12 weeks of age. All experiments were conducted in accordance with NIH guidelines for the care and use of animals and with approval from the Duke University Animal Care and Use Committee.

Immunization and airway challenge. Mice were immunized intraperitoneally on days 0, 7, and 14 with 10 μg grade V OVA (Sigma-Aldrich, St. Louis, Missouri, USA) adsorbed to 200 μg of alum adjuvant (Pierce Biotechnology Inc., Rockford, Illinois, USA) diluted in saline. Sham-immunized animals received 200 μg of saline-diluted alum intraperitoneally. Secondary challenge consisted of a 60-minute exposure to 1% (wt/vol) OVA in saline on days 21, 22, and 23. All mice were exposed to aerosol in a 60-l exposure chamber connected to the outlet of a six-jet atomizer that delivered an aerosol of particles with a mean diameter of 0.3 μm (TSI Inc., St. Paul, Minnesota, USA). The term "OVA treatment" is used hereafter to refer to mice treated according to the OVA sensitization and OVA aerosol challenge protocol described above.

Endotoxin exposures. LPS for aerosolization was purchased as lyophilized purified *Escherichia coli* 0111:B4 (Sigma-Aldrich). LPS was solubilized in sterile saline to a concentration of 5 mg/ml, stored at –20°C, and diluted further in saline to the appropriate concentration on the day of the experiment. LPS was aerosolized with a six-jet atomizer (TSI Inc.) that generated particles with a mean diameter of 0.3 μm, and directed into a 60-l exposure chamber for 2.5 hours. At regular intervals, LPS concentrations were determined by sampling the aerosol through a side port on the chamber. Endotoxin concentrations were assayed with the chromogenic *Limulus* amebocyte lysate assay (BioWhittaker Inc., Walkersville, Maryland, USA) as previously described (15). The average endotoxin concentration used was 5.53 ± 0.5 μg/m^3.

Airway responsiveness. The day after the final aerosol challenge, airway responsiveness to methacholine was measured as previously described (16). In brief, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) diluted 50% with saline and then surgically prepared with a tracheal cannula and a jugular vein catheter. Mice were paralyzed with doxacurium chloride (0.25 mg/kg) and ventilated with 100% oxygen at a constant volume of 8–10 ml/kg and a frequency of 125 breaths/min. These ventilator settings resulted in an average resting peak airway pressure of 7.8 ± 0.2 cm H_2O and have been previously shown to provide normal arterial blood gases. Measurement of airway pressure was made at a side port of the tracheal cannula connected to a Validyne differential pressure transducer (Validyne Engineering, Northridge, California, USA). The time-integrated change in peak airway pressure, or airway pressure time index (17), was calculated for a 30-second period beginning immediately after methacholine injection.

Whole-lung lavage. After in vivo measurements, mice were sacrificed by sodium pentobarbital overdose. Lungs were lavaged as previously described (15). Differential cell counts were performed on cytospin preparations (Cytospin 2; Thermo Shandon, Pittsburgh, Pennsylvania, USA) stained using Hema-3 staining kit (Fisher Scientific, Springfield, New Jersey, USA) and 200 cells were classified using standard morphologic criteria. Lavage fluid cytokine levels were determined in lavage fluid using commercially available cytokine ELISA kits (Quantikine; R&D Systems Inc., Minneapolis, Minnesota, USA). The detection limits were as follows: IL-4 (2 pg/ml), IL-5 (7 pg/ml), IL-13 (1.5 pg/ml), IL-12 (< 4 pg/ml), IFN-γ (< 2 pg/ml), and macrophage-derived chemokine (MDC) (< 5 pg/ml).

Serum Ig titers. At three timepoints in the sensitization protocol (day 10, day 17, and after in vivo measurements on day 24), blood was harvested from the inferior vena cava of *βarr2*$^{-/-}$ and WT mice. Serum was separated by centrifugation and stored at –20°C until analyzed. OVA-specific serum IgE, IgG_1, and IgG_{2a} levels were determined using ELISA methodology and geometric mean titer analysis. Briefly, 96-well plates (NUNC MaxiSorp; Nalge Nunc International, Neerijse, Belgium) were coated with 100 μl/well of 100 μg/ml OVA (Sigma-Aldrich) in 0.1 M carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. The wells were washed with 0.5% Tween 20/PBS and blocked with 1% BSA/PBS, which also served as the assay diluent. After 1.5 hours, wells were washed three times and samples (serially diluted 1:2 twelve times, beginning with 1:64) were added across the plate and incubated for 1.5 hours at 37°C. After washing, the primary antibody (sheep anti-mouse IgE from Calbiochem-Novabiochem Corp., San Diego, California, USA) was added and samples were incubated a further 1.5 hours, washed, and incubated a second time with the detection antibody (peroxidase-conjugated rabbit anti-sheep IgG from Calbiochem-Novabiochem Corp.). For detection of OVA-IgG_1, the primary antibody was biotin-conjugated anti-mouse IgG_1 with avidin-HRP at 2.5 μg/ml (A3151; Sigma-Aldrich). For detection of OVA-IgG_{2a}, the primary antibody was HRP-conjugated anti-mouse IgG_{2a} (BD Pharmingen, San Diego, California, USA). After a further wash, all plates were developed with tetramethylbenzidine substrate solution, stopped with 2 N H_2SO_4, and read at 450 nm. Naive serum from nonsensitized C57BL/6J mice was assayed on each plate to calculate a standard curve, and titers were calculated at twofold over the asymptote of the naive curve.

Isolation of lymphocytes. All lymphocyte isolation was carried out in polypropylene tubes. Spleens were isolated from OVA-treated mice and disrupted in a dounce homogenizer in 1 ml RPMI 1640 medium (Invitrogen Corp., Gaithersburg, Maryland, USA). Following centrifugation at 370 *g* for 10 minutes, rbc's were lysed in lysis buffer (0.14 M NH_4Cl and 0.017 M Tris, pH 7.4) and the cells were pelleted. The cells were washed in RPMI, filtered through a 70-μm BD Falcon strainer (Becton, Dickinson and Co.), and counted.

Lungs were isolated and disrupted in HBSS containing 0.5 U/ml heparin through a 200-gauge stainless steel mesh. Lymphocytes from either OVA-treated WT or OVA-treated *βarr2*$^{-/-}$ mice were pooled. The cells were centrifuged at 370 *g* for 10 minutes at 4°C and resuspended in 15 ml of a 33% Percoll solution (Sigma-Aldrich) diluted in HBSS containing 100 U/ml heparin. The cells were centrifuged at 500 *g* for 15 minutes at room temperature. The pelleted cells were resuspended in lysis buffer to remove red blood cells. The cells were then washed in HBSS, resuspended in RPMI containing 10% FBS, and counted.

Chemotaxis assays. Splenocytes (1×10^6) or lung lymphocytes (1×10^5) were placed in 100 μl RPMI containing 10% FBS in the upper chamber of a Transwell insert (6.5 mm diameter, 5-μm pore size; Corning Costar Corp., Acton, Massachusetts, USA). The lower chamber contained 100 nM MDC (R&D Systems Inc.) in 600 μl RPMI prepared with 10% FBS. After incubation at 37°C for 90 minutes, the migrated cells were collected from the lower chamber. The cells were stained with the following antibodies: 17A2-FITC (anti-CD3, BD Pharmingen), GK1.5-phycoerythrin (anti-CD4, BD Pharmingen), and anti-CD45R–tricolor (Caltag Laboratories Inc., Burlingame, California, USA). Cells were then analyzed using a Coulter Epics XL flow cytometer (Coulter Electronics Ltd., Hialeah, Florida, USA). The chemotactic index was calculated as the number of cells migrating toward MDC/number of cells migrating to medium alone. All assays were performed in duplicate. *βarr2*$^{-/-}$ mice and their littermate-matched controls were tested simultaneously.

Histopathology. Immediately after collection of lavage fluid, ice-cold 4% paraformaldehyde (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) in 1× PBS (pH 7.4) was instilled through the tracheal cannula at a constant pressure of 25 cm H_2O to inflation-fix the lung. Specimens were immersed in 4% paraformaldehyde at 4°C overnight and dehydrated in a graded series of ethanol solutions. Tissue was embedded in paraffin. Sections were cut at 5 μm thickness and mounted onto positively charged slides (Super Frost Plus; Fisher Scientific Co.). For determination of inflammatory cells, H&E-stained slides were semiquantitatively scored in a blinded fashion. For determination of $CD3^+$ T cells, tissue sections underwent a high-temperature antigen-unmasking procedure as follows. After dewaxing and rehydration, the sections were immersed in 1.6 l of 1 mM EDTA, pH 8.0, in a pressure cooker that was then closed and slowly, over a period of 3–4 minutes, brought to boiling temperature. After a total time of 5 minutes, the pressure cooker was cooled under running tap water and opened, and the slides were transferred to PBS (pH 7.5) and rested at room temperature for 5 minutes. Endogenous peroxidase was destroyed with 0.3% H_2O_2 for 30 minutes at room temperature, followed by a rinse in blocking normal rabbit serum. The primary antibody used was a rat monoclonal anti-CD3 antibody from Novocastra Laboratories Ltd. (Newcastle, United Kingdom). Slides were incubated with either active antibody (1:250) or with normal rat IgG as a negative control. The secondary antibody was biotin-conjugated rabbit anti-rat IgG (Vector Laboratories Inc., Burlingame, California, USA), addition of which was followed by treatment with standard avidin-biotin HRP. Peroxidase activity was detected with 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc.) and hydrogen peroxide, after which a hematoxylin counterstain was performed.

Statistical analysis. Multivariate ANOVA was used to determine differences in airway responsiveness. A Student *t* test was used to determine differences when only two experimental groups existed. Otherwise, a one-way ANOVA, in combination with a Tukey honestly significant difference (HSD) post-hoc test, was used to determine differences among groups. For all tests, $P < 0.05$ was considered significant.

Results

To examine the role of β-arrestin-2 in the pathogenesis of asthma, we used an OVA model of allergic asthma and an endotoxin model of nonallergic asthma. We measured physiological and biological variables including airway responsiveness, airway inflammatory cell infiltration, T cell chemotaxis, cytokine levels in whole-lung lavage fluid, and serum Ig levels.

Airway responsiveness. OVA treatment resulted in a dramatic increase in airway responsiveness in WT mice (Figure 1) as measured by airway pressure time index. In marked contrast, *βarr2*$^{-/-}$ mice treated with OVA (*βarr2*$^{-/-}$-OVA mice) showed no such increase in airway responsiveness compared with either WT-alum or *βarr2*$^{-/-}$-alum (control) mice.

Lung inflammation. Histological analysis showed that only mild extravasation of inflammatory cells, including $CD3^+$ T lymphocytes, occurred in the airways of *βarr2*$^{-/-}$ mice treated with OVA, whereas severe cellular infiltration occurred in the interstitium of WT-OVA mice (Figure 2, a and b). Quantitation of $CD3^+$ cells per mm of basement membrane or $CD4^+$ cells harvested from lung show that *βarr2*$^{-/-}$ mice experience significantly reduced T lymphocyte infiltration into the lung (Table 1). Differential cell counts from lavage fluid supported the histological data revealing that lymphocyte and eosinophil infiltration into the

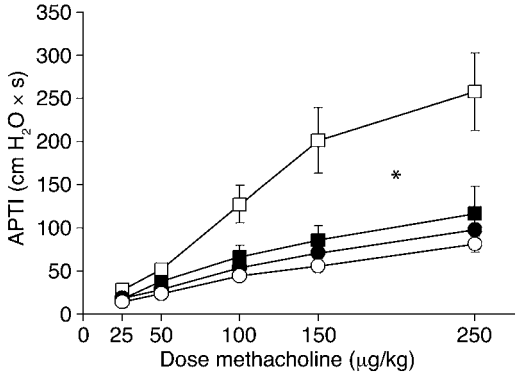

Figure 1
Effect of OVA treatment on airway responsiveness. Airway responsiveness to methacholine, defined by the time-integrated change in peak airway pressure, or airway pressure time index (APTI), was measured for *βarr2*$^{-/-}$ (circles) and WT (squares) mice treated with either alum (filled symbols) or OVA (open symbols). Data are mean ± SEM; n = 9–12 mice per group. *Effect of OVA treatment was significantly different between WT and *βarr2*$^{-/-}$ mice. $P < 0.05$.

lungs of *βarr2*$^{-/-}$-OVA mice was markedly reduced compared with WT-OVA mice and was not different from that in alum-treated control mice of either genotype (Figure 2c). The lack of T lymphocytes in the lung of *βarr2*$^{-/-}$-OVA mice suggests that the migration of T cells to the lung is impaired in the absence of β-arrestin-2.

Cytokine levels. Activated T cells migrate to the lungs and release inflammatory cytokines to orchestrate the allergic inflammatory response. Consistent with the demonstrated lack of lymphocytes in the airways of *βarr2*$^{-/-}$-OVA mice was their diminished level of Th2 cytokines. Analysis of lavage fluid from WT-OVA mice showed a significant increase in the levels of IL-4, IL-5, and IL-13 relative to the respective cytokine level in lavage fluid from *βarr2*$^{-/-}$-OVA mice and alum controls (Figure 3a). To determine whether *βarr2*$^{-/-}$ mice have a Th1-skewed response that might inhibit the development of Th2 lymphocytes, we measured Th1-type cytokines in lung lavage fluid (18). Levels of IL-12 and IFN-γ were not different between WT and *βarr2*$^{-/-}$ mice, and the level of these cytokines was unaffected by OVA treatment (Figure 3b).

Ig production. The absence of the classic features of a Th2-mediated asthmatic response in OVA-treated *βarr2*$^{-/-}$ mice indicates that β-arrestin-2 exerts its regulatory effect early in the progression of allergic asthma. β-Arrestin-2 may regulate the handling of aeroallergen, a series of events that occurs prior to, and is necessary for, T cell stimulation and allergen-specific Ig production. However, the serum levels of OVA-specific IgE and OVA-specific IgG_1 were significantly elevated in *βarr2*$^{-/-}$-OVA mice, and the magnitude of this antigen-specific antibody production was not significantly different from the response observed in WT-OVA mice (Figure 4, a and b). Thus, *βarr2*$^{-/-}$ mice are competent in their ability to present antigen, to generate antigen-specific T cell responses, and to undergo Ig isotype switching. Measurement of IgG_{2a}, a serum Ig typical of a Th1-type response, was not different between WT and *βarr2*$^{-/-}$ mice (Figure 5, a and b). These data show that the lack of a Th2-type response in *βarr2*$^{-/-}$ mice is not due to enhanced induction of the Th1 arm of the Th cell pathway for development.

CD4$^+$ *T cell chemotaxis*. In allergic asthma, MDC is a potent Th2 cell chemoattractant produced by activated lung macrophages, airway smooth muscle cells,

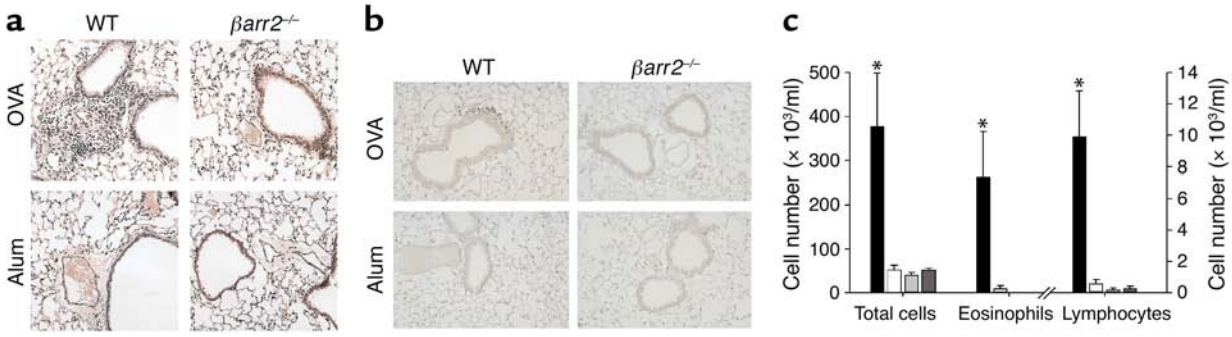

Figure 2
Effect of OVA treatment on airway inflammation. (**a**) Effect of genotype and OVA treatment on lung inflammation evaluated by histological analysis of lung sections. Lung sections from WT-alum (lower left) and *βarr2*$^{-/-}$-alum mice (lower right) appeared normal with no inflammatory cell infiltration. Lung sections from WT-OVA mice (upper left) showed severe cellular infiltration in the interstitium. Lung sections from *βarr2*$^{-/-}$-OVA mice (upper right) showed mild extravasation of inflammatory cells in the interstitium. Representative histological sections are shown (n = 8–11 mice per group). (**b**) Representative images of cross-sectioned airways together with peribronchovascular connective tissue are shown. $CD3^+$ T cells were counted in the subtended bronchovascular interstitium. OVA-treated WT mice showed an increased number of $CD3^+$ T cells in the peribronchovascular zone relative to alum-treated mice. No such infiltration of $CD3^+$ T cells was observed in OVA-treated *βarr2*$^{-/-}$ mice. (**c**) Effect of genotype and OVA treatment on lung inflammation assessed by identification of cells harvested from whole-lung lavage. Black bars represent WT-OVA mice; white bars represent *βarr2*$^{-/-}$-OVA mice; light gray bars represent WT-alum mice; dark gray bars represent *βarr2*$^{-/-}$-alum mice. Data are mean ± SEM; n = 9–12 mice per group. *$P < 0.05$ versus all other groups.

Table 1
Quantitation of T lymphocytes in the lung

	$CD3^+$ cells per mm basement membrane	$CD4^+$ T cells harvested from lung
WT-OVA	31.04 ± 1.36*	21,400/lung
βarr2$^{-/-}$-OVA	2.08 ± 1.57	6,900/lung
WT-alum	2.96 ± 0.85	
βarr2$^{-/-}$-alum	2.21 ± 0.74	

Using calibrated digital images, the perimeter of the bronchiolar basal lamina of each histological profile (represented in Figure 2b) was traced and the length was measured. The results were expressed as the number of $CD3^+$ T cells per mm of airway basal lamina for each profile. To quantitate the number of $CD4^+$ T cells, lungs from OVA-treated mice were processed and cells were identified using GK1.5-phycoerythrin (anti-CD4) and FACS analysis. The mean ± SEM for each treatment/genotype group is shown; n = 7–10 mice per group. *P < 0.05 versus all other groups.

bronchiolar epithelial cells, and by Th2 cells themselves (5). To investigate whether $CD4^+$ T cell migration to the lung is impaired in *βarr2*$^{-/-}$ mice, we tested the ability of splenocytes, thoracic lymph node cells, and lung lymphocytes from OVA-treated WT and *βarr2*$^{-/-}$ mice to respond to MDC. Only lung lymphocytes, and not spleen or lymph node T cells (data not shown), migrated toward MDC. While $CD4^+$ T cells harvested from lung of OVA-treated WT mice migrated well toward MDC, $CD4^+$ T cells lacking β-arrestin-2 were significantly impaired in their migration toward MDC (Figure 6a). MDC levels in lavage fluid were significantly elevated in OVA-treated WT mice, but not in similarly treated *βarr2*$^{-/-}$ mice (Figure 6b). These data support the notion that β-arrestin-2 positively regulates MDC-mediated $CD4^+$ T cell migration to the lung in OVA-treated mice. However, β-arrestin-2 may alternatively, or additionally, regulate $CD4^+$ T cell migration in response to a chemokine other than MDC.

Nonallergic asthma model. To further investigate the specificity of the impaired asthmatic response in *βarr2*$^{-/-}$ mice, we subjected mice to a model of endotoxin-mediated asthma, or nonallergic asthma. Endotoxin, an LPS, is a major component of the outer cell membrane of all gram-negative bacteria, and even small amounts of this molecule are immune-stimulatory (19). Subjects with endotoxic asthma demonstrate lung neutrophilic inflammation and airway hyperresponsiveness, and this response is coordinated by alveolar macrophages and epithelial cells rather than T cells (20). Both WT and *βarr2*$^{-/-}$ mice exposed to aerosolized LPS developed lung neutrophilic inflammation and increased airway responsiveness (Figure 7, a and b). Thus, there is no general impairment of lung inflammatory processes or in the ability of airways to respond to methacholine in *βarr2*$^{-/-}$ mice.

Discussion

When treated with OVA, WT mice develop symptoms of allergic asthma. In stark contrast, these symptoms, including airway inflammation and airway hyperresponsiveness, do not appear in similarly treated *βarr2*$^{-/-}$ mice. These results suggest that β-arrestin-2 is essential to the development of allergic asthma and that it exerts its regulatory effect at a proximal step in the inflammatory cascade.

Numerous immune cell types, including APCs, T lymphocytes, B lymphocytes, eosinophils, and mast cells, interact in a highly coordinated fashion to respond to allergens. Although the nature and sequence of these interactions are not entirely delineated, the infiltration of activated Th2 cells into the lung is a primary pathological event underlying allergic asthma. Without these cells, the symptoms of asthma, including airway inflammation, airway hyperresponsiveness, and reversible airflow obstruction, do not occur (21–23).

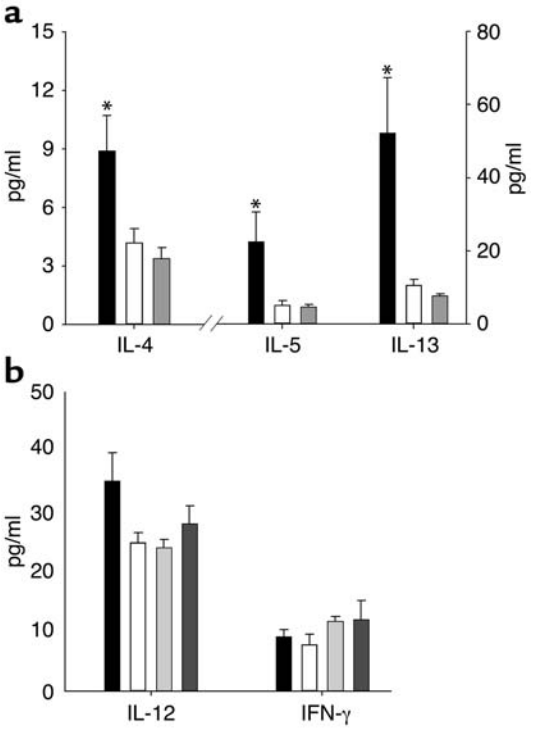

Figure 3
Effect of genotype and OVA treatment on lung cytokine release in whole-lung lavage fluid. (**a**) Cytokines associated with a Th2-type response were significantly elevated in WT-OVA mice relative to *βarr2*$^{-/-}$-OVA mice and alum-treated mice of either genotype. Black bars represent WT-OVA mice; white bars represent *βarr2*$^{-/-}$-OVA mice. Cytokine levels in alum-treated WT and alum-treated *βarr2*$^{-/-}$ mice were not different and therefore were combined as shown by gray bars. Data are mean ± SEM calculated from three independent experiments; n = 11–19 mice per group. *P < 0.05 versus all other groups. (**b**) Cytokines associated with a Th1-type response were not significantly elevated by OVA treatment and were similar for WT and *βarr2*$^{-/-}$ mice. Black bars represent WT-OVA mice; white bars represent *βarr2*$^{-/-}$-OVA mice. Light gray bars represent alum-treated WT mice; dark gray bars represent *βarr2*$^{-/-}$ mice. Data are mean ± SEM calculated from two to three independent experiments; n = 7–13 mice per group.

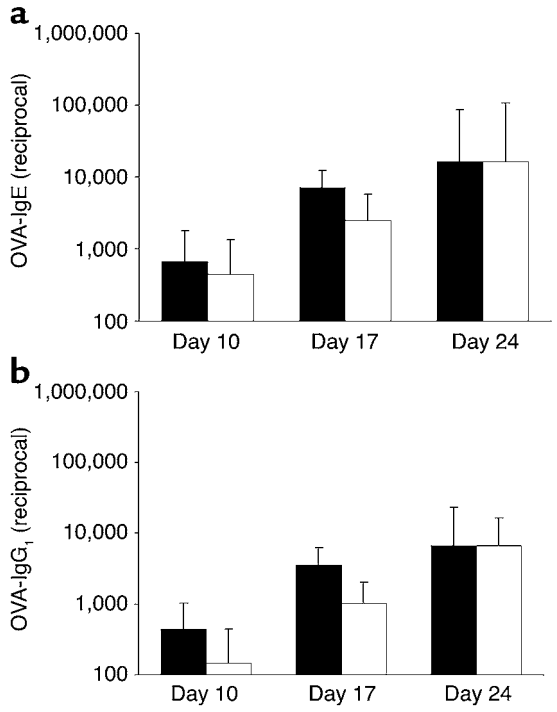

Figure 4
Effect of OVA treatment on serum Ig production. Although OVA-specific IgE (**a**) and IgG_1 (**b**) levels changed significantly over time, serum Ig levels in WT-OVA mice (black bars) were not significantly different from those in *βarr2*$^{-/-}$-OVA mice (white bars) when compared at each timepoint (days 10, 17, and 24). These OVA-specific Ig's were not detected in alum-treated mice of either genotype. Measurements were made using the endpoint titer method. Data are mean ± SD calculated from two independent experiments; *n* = 3–8 mice per group. One-way ANOVA and the Tukey HSD post-hoc test were used.

The initial event in the development of allergic asthma is the processing of inhaled allergens. This involves the capture, modification, and presentation of allergen to T and B cells (24). Once activated, T cells proliferate and differentiate to a Th2 phenotype defined by the release of type 2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13). Release of these cytokines at germinal centers within peripheral lymphoid tissue supports B cell–mediated production of allergen-specific antibodies (25, 26). In addition, type 2 cytokines released from T cells that have migrated to the lung activate and recruit mast cells and eosinophils, the primary effector cells of the allergic asthmatic response (1).

Our study shows that antigen processing, presentation, and activation of T cells are intact in *βarr2*$^{-/-}$ mice since their serum levels of OVA-specific IgE and IgG_1 are comparable to those in WT mice and are significantly elevated compared with alum-treated mice. Although there is a trend for a slight delay in the kinetics of the Ig response in OVA-treated *βarr2*$^{-/-}$ mice, the impact of this on the physiological and biological measurements made on day 24, when OVA-IgE levels are the same, is unknown. Despite the significant production of OVA-IgE and OVA-IgG_1 in OVA-treated *βarr2*$^{-/-}$ mice, the classic features of a Th2-mediated asthmatic response, including airway inflammation, airway hyperresponsiveness, and elevated Th2 cytokines, are absent in these mice. Therefore, the defect in the allergic asthmatic response in *βarr2*$^{-/-}$ mice lies between the generation of a Th2 response and airway inflammation. Our data showing defective $CD4^+$ T cell migration to the Th2 chemoattractant MDC and reduced levels of MDC in lavage fluid suggests that the defect is present at the level of Th2 cell trafficking to the lung.

Other potential mechanisms underlying the phenotype observed in *βarr2*$^{-/-}$ mice have been evaluated but ruled out. Other studies have shown that enhanced induction of Th1-type cytokines may inhibit the development of a Th2-type response (18). In our study we find no evidence that *βarr2*$^{-/-}$ mice experience Th1-type inhibition of their Th2-type response since IgG_{2a}, IL-12, and IFN-γ (factors

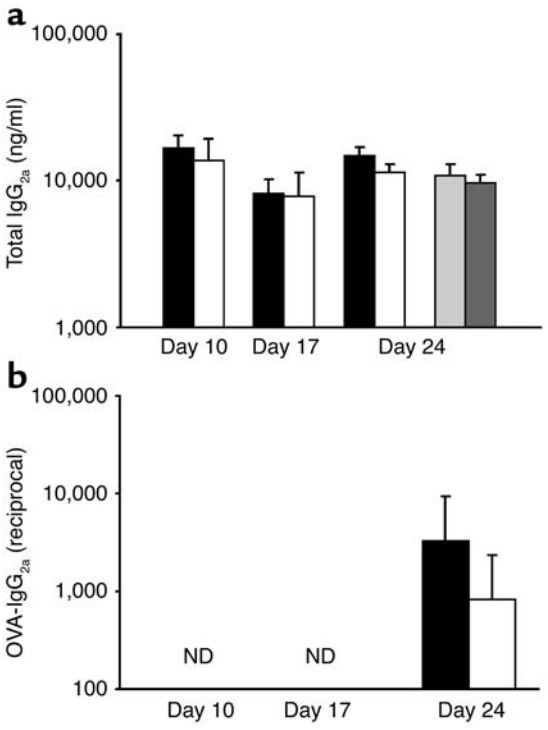

Figure 5
IgG_{2a} production. (**a**) Total serum IgG_{2a} levels were not different between WT-OVA mice (black bars) and *βarr2*$^{-/-}$-OVA mice (white bars) at day 10, 17, or 24. Similarly, there was no difference in IgG_{2a} levels in serum from alum-treated WT (light gray bars) and alum-treated *βarr2*$^{-/-}$ mice (dark gray bars). Data are mean ± SEM calculated from three independent experiments; *n* = 4–14 mice per group. (**b**) OVA-specific IgG_{2a} measurements were not different between WT-OVA mice (black bars) and *βarr2*$^{-/-}$-OVA mice (white bars) at day 24. Measurements were made using the endpoint titer method. Data are mean ± SD calculated from two experiments; *n* = 3 mice per group. ND, non-detectable.

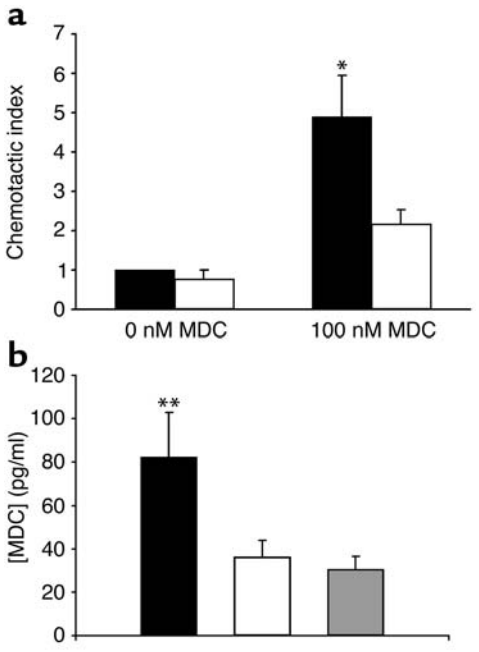

Figure 6
Chemotactic responses to MDC and release of MDC in lavage fluid. (**a**) Lung CD4+ T cells from OVA-treated *βarr2*$^{-/-}$ mice (white bars) exhibit decreased migration toward MDC. CD4+ T cells were isolated on day 24 of the OVA treatment protocol and tested for their ability to move chemotactically toward 100 nM MDC. Shown is the mean chemotactic index and SE from three independent experiments. *$P < 0.05$ vs. *βarr2*$^{-/-}$-OVA mice. (**b**) Release of MDC into whole-lung lavage fluid was significantly induced in OVA-treated WT mice (black bars) but not OVA-treated *βarr2*$^{-/-}$ mice (white bars). MDC levels in alum-treated WT and alum-treated *βarr2*$^{-/-}$ mice were not different and therefore were combined (gray bars). Data are mean ± SEM calculated from two independent experiments; $n = 9$ mice per group. **$P < 0.05$ versus combined alum-treated mice.

indicative of a Th1 response) are not elevated in *βarr2*$^{-/-}$ mice relative to WT mice.

Another possible explanation for the failure of *βarr2*$^{-/-}$ mice to develop asthma is mast cell dysfunction. Antigen crosslinking of cell-surface IgE receptors stimulates mast cell release of mediators including histamine, prostaglandins, leukotrienes, and cytokines. Collectively, these agents perpetuate airway inflammation and airway hyperresponsiveness and thus appear pivotal to the development of allergic asthma (27). However, the ability to respond to OVA and to develop allergic asthma is preserved in mast cell–deficient mice (28, 29). Thus, the failure of *βarr2*$^{-/-}$ mice to develop asthma cannot be explained by mast cell dysfunction.

T cell function is crucial to the development of allergic asthma. When T cells are nonfunctional or absent, allergic inflammation, airway hyperresponsiveness, and lung cytokine production are prevented in response to OVA treatment (21–23). These results are nearly identical to the dramatically impaired allergic asthmatic response we observed in *βarr2*$^{-/-}$ mice. Because *βarr2*$^{-/-}$ mice have normal levels of CD3+ T splenocytes (11) and normal architecture of spleen follicles (data not shown), we suggest that T cell dysfunction, rather than reduction in T cell number, is at the root of the impaired allergic asthmatic response in *βarr2*$^{-/-}$ mice. Since the manifestation of allergic asthma requires T cell proliferation, differentiation, and migration, any one of these processes may be regulated by β-arrestin-2 and thus dysregulated in mice completely lacking that protein.

We hypothesize that T cell migration is the process regulated by β-arrestin-2 in allergic asthma. Multiple in vitro reports demonstrate that β-arrestin regulates chemokine receptor signaling and internalization (28, 30, 31). Recently, others in our group used the chemokine SDF-1α (or CXCL12) to show that CXCR4-mediated in vitro migration of nonactivated splenic T cells is impaired in cells lacking β-arrestin-2 (11). In two separate studies, Gonzalo et al. showed that lung inflammation and airway hyperresponsiveness are significantly reduced when SDF-1α or MDC are neutralized by antibodies (32, 33). Thus, reduced migration of activated T cells to SDF-1α may

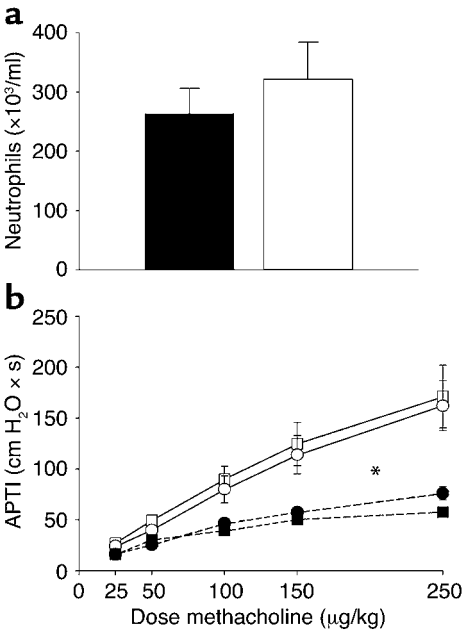

Figure 7
Effect of LPS treatment on airway responsiveness and inflammation. (**a**) Effect of genotype and LPS treatment on lung inflammation assessed by identification of cells harvested from whole-lung lavage. Black bars represent WT LPS-treated mice; white bars represent *βarr2*$^{-/-}$ LPS-treated mice. Data are mean ± SEM; $n = 13–15$ mice per group. (**b**) Airway responsiveness to methacholine, defined by the time-integrated change in peak airway pressure was measured for *βarr2*$^{-/-}$ (circles) and WT (squares) mice treated with LPS (open symbols) or untreated (filled symbols). Data are mean ± SEM; $n = 13–15$ mice per group. *LPS treatment caused a similar significant increase in APTI in WT and *βarr2*$^{-/-}$ mice. $P < 0.05$.

contribute to the phenotype we observed in *βarr2*$^{-/-}$ mice. However, in our study, lung SDF-1α was not significantly induced by OVA treatment in either *βarr2*$^{-/-}$ or WT mice (data not shown). Thus, MDC is more likely to play a significant role in the development of asthma in our model. Indeed, we showed that not only is the MDC signal reduced in *βarr2*$^{-/-}$ mice, but the chemotactic response to this signal is also impaired. Thus, β-arrestin-2 positively regulates MDC-mediated Th2 cell migration in the context of allergic asthma. We hypothesize that diminished Th2 cell migration to the lung is the primary impairment in *βarr2*$^{-/-}$ mice and is largely responsible for their protection against the development of allergic asthma.

The mechanisms by which β-arrestin-2 may regulate T cell chemotaxis in vivo or in vitro are not presently defined. β-Arrestin proteins act as scaffolds to link GPCR activation to at least three MAPK cascades: the extracellular signal–regulated kinase (ERK) cascade (34), the JNK-3 cascade (35), and the p38 MAPK cascade (12). A recent report indicates that β-arrestin-2 activation of the p38 MAPK cascade is required for CXCR4-mediated migration of human embryonic kidney 293 cells to SDF-1α (12). β-Arrestin-2 may similarly regulate CCR4, the receptor for MDC, through mechanisms involving MAPK or other signaling pathways. Although MAPKs regulate the phosphorylation of nuclear transcription factors, recent studies indicate that β-arrestin–MAPK scaffolds are preferentially targeted to cytosolic substrates (34–36). Such cytosolic substrates may be proteins involved in cell chemotaxis. Thus, β-arrestin-2, through its action as a scaffold protein, may positively regulate T cell chemotaxis.

Alternatively, in keeping with their classically described role, β-arrestins may regulate chemotaxis through termination of chemokine receptor signaling (9). This signal termination, or chemokine receptor desensitization, may be crucial to the directional sensing, and thus migration, of T cells.

Although we feel that β-arrestin-2 regulation of T cell migration is the major mechanism responsible for the profound protection against allergic asthma observed in the *βarr2*$^{-/-}$ mice, β-arrestin-2 regulation of other T cell functions, or modulation of cells other than lymphocytes, may make a contribution to the dramatic phenotype observed in these mice.

β-arrestin-2 could regulate nonhematopoietic cells in the lung such as airway smooth muscle or airway epithelial cells. Indeed, levels of MDC in lavage fluid are reduced in *βarr2*$^{-/-}$ mice. This may indicate a positive role for β-arrestin-2 in regulating chemokine production/secretion by either of these lung cell types. Alternatively, reduced MDC levels in lavage fluid may be explained by altered aeroallergen handling in *βarr2*$^{-/-}$ mice or may be a by-product of impaired T cell migration, since Th2 cells are known to produce MDC. Airway responses to methacholine challenge were not different between *βarr2*$^{-/-}$ and WT mice when treated with alum or LPS, suggesting that β-arrestin-2 does not regulate airway smooth muscle cell contraction.

β-arrestin-2 may regulate T cell differentiation. Yamashita et al. showed that T cell antigen receptor–mediated activation of the Ras-ERK1/2 pathway enhances IL-4 receptor signaling and is required for the differentiation of naive T cells into Th2 cells (37). Although neither T cell receptors nor IL-4 receptors are GPCRs, there is evidence in the literature of β-arrestin-2 modulation of non–GPCR-mediated events through transactivation of nonheptahelical receptors or through intracellular modulation of downstream signaling pathways resulting from antigen or cytokine receptor stimulation (38). Although differentiation of T cells to the Th2 lineage may be reduced in *βarr2*$^{-/-}$ mice, our results suggest that this does not occur during the antigen sensitization phase since the release of IL-4 from Th2 cells is required for the production of allergen-specific IgE by B cells (39) and this function is not impaired in *βarr2*$^{-/-}$ mice.

The fact that LPS-exposed *βarr2*$^{-/-}$ mice developed neutrophilic lung inflammation and increased airway responsiveness comparable to similarly treated WT mice demonstrates that there is no universal impairment in inflammatory cell migration in *βarr2*$^{-/-}$ mice. This demonstration of a functionally intact innate immune system is critical to the pursuit of β-arrestin-2 as a potential therapeutic target for treating allergic asthma.

The absence of β-arrestin-2 prevents the development of allergic asthma without compromising the function of the innate immune system. Regulation of T cell migration to the lung by β-arrestin-2 is thought to be the primary mechanism underlying this protection. Because β-arrestin-2 exerts its regulatory effect proximal to the recruitment of activated Th2 cells into the lung it is an attractive therapeutic target for treating allergic asthma.

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