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*J Clin Invest.* 2006;116(8):2183-2192. [https://doi.org/10.1172/JCI24767](https://doi.org/10.1172/JCI24767).

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The adipocyte fatty acid–binding protein aP2 is required in allergic airway inflammation

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The adipocyte fatty acid–binding protein aP2 regulates systemic glucose and lipid metabolism. We report that aP2, in addition to being abundantly expressed by adipocytes, is also expressed by human airway epithelial cells and shows a striking upregulation following stimulation of epithelial cells with the Th2 cytokines IL-4 and IL-13. Regulation of aP2 mRNA expression by Th2 cytokines was highly dependent on STAT6, a transcription factor with a major regulatory role in allergic inflammation. We examined aP2-deficient mice in a model of allergic airway inflammation and found that infiltration of leukocytes, especially eosinophils, into the airways was highly dependent on aP2 function. T cell priming was unaffected by aP2 deficiency, suggesting that aP2 was acting locally within the lung, and analysis of bone marrow chimeras implicated non-hematopoietic cells, most likely bronchial epithelial cells, as the site of action of aP2 in allergic airway inflammation. Thus, aP2 regulates allergic airway inflammation and may provide a link between fatty acid metabolism and asthma.

Introduction
Asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness, tissue remodeling, and airflow obstruction (1). The underlying pathogenetic mechanisms in asthma are only partly understood, a situation reflected in the variable and incomplete responses by patients to current therapeutics. The incidence of asthma in Western countries has increased markedly over the last 20 years, and in countries such as the USA and Australia, it now affects approximately 10% of the population (2, 3). Environmental factors must be largely responsible for this sudden increase in incidence. Current thinking is dominated by the hygiene hypothesis, according to which childhood microbial infection is a major determinant of predisposition to atopic asthma (4). Numerous additional environmental factors, such as diet, airborne pollution, and composition of gut commensal microflora are likely involved (5), and further hypotheses that account for the dramatic increase in asthma incidence are required.

Most cases of asthma are associated with characteristic airway immunopathology involving Th2 lymphocytes, eosinophils, mast cells, and goblet cell hyperplasia. The Th2 cytokines IL-4, IL-5, IL-9, and IL-13 initiate and promote asthma pathogenesis (1). IL-4 drives Th2 lymphocyte polarization and also induces IgE class switching. Its importance is highlighted by the attenuated allergic airway phenotype in IL-4-deficient mice (6). IL-5 regulates eosinophil responses by promoting eosinophil differentiation, activation, and survival. In addition to regulating allergic leukocyte responses, Th2 cytokines can directly affect lung parenchymal cells. For example, IL-13 signaling in airway epithelial cells (AECs) mediates airway hyperreactivity and induces airway mucus production (7). Both IL-4 and IL-13 also induce broad phenotypic changes in airway smooth muscle cells (8).

The bronchial epithelium is a crucial barrier to the external environment, providing defense against inhaled particles such as allergens and playing a vital role in asthma pathophysiology by regulating such diverse processes as airway remodeling and mucus production (9). Airway epithelial stimulation by IL-4 and IL-13 results in upregulation of adhesion molecules and chemokines such as eotaxin and monocyte chemoattractant protein–1 (MCP-1) that recruit allergic leukocytes to the airways (10–14). However, the contribution of AECs to allergic airway inflammation and the molecular pathways that regulate epithelial cell responses are yet to be fully understood.

Our laboratory has applied gene profiling to the discovery of novel genes regulating inflammatory disease (15–18). To identify potential allergic regulatory and effector pathways in bronchial epithelium, we generated a gene expression profile of human bronchial epithelial cells (HBEs) upon stimulation with the Th2 cytokine IL-4. Here we present evidence that the adipocyte/macrophage fatty acid–binding protein (FABP) aP2 is expressed in HBEs and is strongly upregulated by both IL-4 and IL-13. Using a mouse model of allergic airway inflammation, we demonstrate a key role for aP2 in regulating eosinophil recruitment and pulmonary inflammatory cytokine production. These results suggest that in addition to its role in type 2 diabetes and atherosclerosis (19–21), aP2 may play an essential role in allergic airway diseases such as asthma and offers an additional intriguing link between the immune and metabolic systems.

Results
IL-4 and IL-13 upregulate aP2 expression in HBEs. To identify new pathways and factors involved in the pathogenesis of asthma, we developed a gene expression profile of HBEs stimulated with the Th2 cytokine IL-4 and found a subset of 38 genes to be IL-4 responsive (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI24767DS1). Surprisingly, the transcript for the FABP aP2 was one of the most highly regulated genes in response to IL-4 treatment (Figure 1). aP2 is the predominant

Nonstandard abbreviations used: AEC, airway epithelial cell; BAL, bronchoalveolar lavage; FABP, fatty acid–binding protein; HBE, human bronchial epithelial cell; 5-LO, 5-lipoxygenase; MCP-1, monocyte chemoattractant protein–1.

Conflict of interest: The authors have declared that no conflict of interest exists.


FABP in adipocytes and has a proposed role in intracellular fatty acid transport trafficking (22, 23). Although αP2 was originally considered to be adipocyte specific, it has also recently been identified in activated macrophages, where it regulates cholesterol metabolism and cytokine production (20, 24). According to real-time PCR, IL-4 and another key Th2 cytokine, IL-13, enhanced αP2 mRNA expression as quickly as 1 hour, with maximal expression detected at 12–48 hours (Figure 2A). αP2 was also expressed and was upregulated by IL-4 and IL-13 in the immortalized AEC lines BEAS-2B and A549 (Figure 2B). Enhanced HBE αP2 protein expression following IL-4 or IL-13 stimulation was also observed by immunocytochemistry (Figure 2C). The related FABP mal1, which is thought to have overlapping functions with αP2 (25), was also modestly upregulated by IL-4 or IL-13 in HBEs (Figure 2A). The array data also showed that no other members of the FABP family were expressed by HBEs (data not shown).

Contrasting regulation of αP2 expression in HBEs and adipocytes. In adipocytes and macrophages, αP2 expression is regulated by PPARγ (26, 27). Interestingly, in HBEs, the synthetic PPARγ agonist rosiglitazone had no effect on αP2 mRNA expression (Figure 3A), although it strongly upregulated expression of the PPARγ-responsive gene CD36 (Figure 3B) (28, 29). In contrast to HBEs, rosiglitazone strongly induced αP2 expression in 3T3-L1 preadipocytes, while treatment with IL-4 had no effect (Figure 3A), thus highlighting fundamental differences between HBEs and 3T3-L1 cells in the regulation of αP2 expression. Furthermore, HBEs express comparable levels of αP2 to THP-1 monocytes but have approximately 10,000-fold less αP2 mRNA than human adipose tissue (Figure 3C), suggesting that the function of FABPs in bronchial epithelium might differ from that in adipocytes. The fact that different signaling pathways regulate αP2 expression in cytokine-stimulated HBEs and differentiating adipocytes along with the marked differences in expression levels in these cell types suggest that αP2 functions differently in the metabolic and immune systems.

αP2 is a STAT6-regulated gene in HBEs. To further investigate signaling pathways leading to αP2 expression in AECs, we investigated the importance of the transcription factor STAT6. This transcription factor is crucial to IL-4 and IL-13 signaling and binds to the promoter of genes important in allergic responses, such as IL-4 receptor α and eotaxin (30–32). Bioinformatics analysis of sequence upstream of the 5′ transcriptional start site in mouse and human genomic αP2 sequences revealed a conserved putative STAT6-binding site at positions −383 and −396, respectively (Supplemental Figure 2A). Since STAT6 signaling is activated by IL-4 in AECs (33) (Supplemental Figure 2B), we focused on this signaling element as a potential regulator of αP2 expression. We initially used overexpression studies to confirm the importance of STAT6. Transfection of BEAS-2B AECs with a plasmid encoding WT STAT6 increased αP2 expression 2.1-fold over that of control transfectants, and expression of a constitutively active STAT6 mutant significantly upregulated αP2 expression 6.1-fold (Figure 3D). Conversely, αP2 upregulation by IL-4 was blocked in transfectants expressing a dominant negative STAT6 isoform (Figure 3E). In WT primary mouse AECs, αP2 expression was increased by treatment with IL-4 plus TNF-α (Figure 3F), but it was unchanged in AECs from STAT6-deficient mice (Figure 3G). Taken together, these studies confirm that IL-4–mediated STAT6 signal transduction in AECs leads to downstream αP2 gene expression and that STAT6 activation alone is sufficient to increase αP2 gene transcription.

The Th1 cytokine IFN-γ downregulates αP2 expression. Given the strong regulation of αP2 expression by Th2-related cytokines, we next tested a range of other cytokines and factors known to affect
bronchial epithelial cell function. In contrast to IL-13 or IL-4, IFN-γ downregulated aP2 expression, while IL-1, IL-3, IL-6, IL-9, MCP-1, TNF-α, or IL-10 had no effect (Figure 4A). Whereas IL-4 and IL-13 exacerbate allergic airway inflammation, the prototypic Th1 cytokine IFN-γ is, in some cases, protective against allergy by antagonizing Th2 effector function (34, 35). Thus, upregulation by Th2 cytokines and downregulation by Th1 cytokines strongly implicate aP2 as a participant in allergic airway inflammation.

Preferential expression of aP2 in allergic tissues. To more rigorously investigate the potential role of aP2 in regulating airway inflammation, we used a mouse model of allergic airway inflammation. We investigated whether aP2 is expressed in airway epithelium of mice with pulmonary allergic inflammation. In lungs from non-allergic control mice, a low level of aP2 staining was observed in AECs (Figure 4B). In mice with allergic airway inflammation, aP2 staining intensity in the AECs was noticeably increased. In lungs from both the nonallergic and allergic groups, aP2 staining was observed almost exclusively in the AECs, although occasional isolated cells within the lung, which may be macrophages or DCs (refs. 20, 27, and Rolph et al., manuscript submitted for publication), were also stained. Very strong aP2 staining was also detected in human turbinate tissue (Supplemental Figure 3) and nasal polyps (data not shown), although there was no correlation between staining intensity and degree of inflammation. Thus, aP2 is regulated at both the transcript and protein levels, in epithelial cell lines as well as bronchial epithelium in vivo.

Regulation of allergic airway inflammation by aP2. We next tested the role of aP2 in allergic airway inflammation by utilizing aP2−/− mice. After OVA sensitization and aeroallergen challenge, a significant increase in total cell number was observed in the bronchoalveolar lavage (BAL) fluid of WT (aP2+/+) mice (Figure 5A). The cellular infiltrate was composed largely of eosinophils (Figure 5B), as is typical for this model. In contrast, the total number of BAL leukocytes was significantly decreased in aP2−/− mice (Figure 5A), and this was associated with reductions in the number of eosinophils and neutrophils (Figure 5B). Lung histology also revealed a significantly reduced peribronchial and perivascular inflammatory infiltrate in allergic aP2−/− mice, compared with allergic aP2+/+ controls (Figure 5C, upper panels) (P < 0.01, Mann-Whitney U test). However, mucus overproduction in allergic mice was unaffected by aP2 deficiency (Figure 5C, lower panels).

To further understand the role of aP2 in allergic airway inflammation, we measured cytokine levels in the BAL fluid. In accordance with the marked attenuation in airway eosinophilia, aP2−/− mice had significantly less IL-5 and IL-13 in BAL fluid, while a trend toward lower eotaxin levels was observed (Figure 6A). Similarly, in comparison to aP2−/− mice, we detected a significant reduction in IL-5 production by OVA-stimulated cells from the draining lymph nodes of aP2−/− mice (Figure 6B). Gene profiling of lung tissue from allergic aP2+/+ and aP2−/− mice also revealed a broad reduction in many genes already implicated in Th2 inflammation, such as the chemokines eotaxin-1 and -2, MCP-1, and matrix metalloproteinases MMP-12 and -9 and arginase I (15, 36, 37) in aP2−/− mice (Supplemental Figure 5) (38).

Antigen-specific T cell proliferation is unimpaired in aP2−/− mice. A logical assumption based on the above findings is that aP2 deficiency in AECs might compromise airway Th2 responses. To exclude the possibility that the initial T cell priming contributed to the phenotype of the aP2−/− mice in the allergic airway inflammation model, mice were immunized with OVA in alum, and antigen specific-responses in the spleen were measured. OVA-specific proliferation (Figure 6C) and production of IL-5 and IFN-γ in supernatants (unpublished observations) were equivalent from WT and aP2−/− spleen cells. Thus, T cell priming in the
Non-hematopoietic cells regulate allergic lung inflammation in aP2−/− mice. To more rigorously test whether hematopoietic or non-hematopoietic cells were responsible for ameliorated lung inflammation in aP2-deficient mice, bone marrow transfer experiments were conducted. WT and aP2−/− mice were lethally irradiated and reconstituted with WT or aP2−/− bone marrow to generate WT→WT, aP2−/−→WT, and WT→aP2−/− chimeric mice. The mice were then sensitized to OVA and challenged with aerosolized OVA to induce allergic lung inflammation. After antigen challenge, WT→WT and aP2−/−→WT mice had similar total numbers of leukocytes infiltrating the airway lumen, whereas aP2−/− mice that received WT bone marrow had significantly fewer leukocytes (Figure 7A). Differential cell counts of lavaged cells revealed a significant reduction in the number of eosinophils from WT→aP2−/− chimeras compared with WT→WT and aP2−/−→WT animals, while monocyte/macrophage numbers remained unchanged (Figure 7B). Allergic cytokine levels were also altered in allergic aP2-knockout mice with WT immune cells. In these mice, eotaxin and IL-5 levels were significantly decreased in lung lavage fluid (Figure 7C), while IL-5 and IL-13 levels were significantly reduced in culture supernatants from lung-draining lymph node cells restimulated with OVA antigen (Figure 7, D and E). These results clearly indicate that non-hematopoietic cells are the site of action of aP2 in allergic airway inflammation.

The expression of aP2, and its regulation by IL-4 and IL-13, in AECs strongly suggests that these cells are the site of action of aP2. To obtain further evidence in support of this, the response of aP2-deficient mice was tested in a model of allergic inflammation independent of AECs. Mice were sensitized to OVA in alum by 2 subcutaneous injections, followed by intraperitoneal OVA challenge. Twenty-four hours following OVA challenge, this protocol induced a

allergic airway inflammation model was not noticeably affected by aP2 deficiency, suggesting that the defect is likely to be mediated locally within the lung.
localized peritoneal allergic inflammation, with substantial influx of multiple leukocyte subsets, particularly eosinophils and macrophages, into the peritoneal cavity. There was no difference between WT and aP2−/− mice in the magnitude and nature of the inflammatory infiltrate (Figure 8, A and B). These results indicate there is not a general defect in allergic responses in the aP2−/− mice and give further support to our proposal that the airway epithelium is the site of action of aP2 in allergic airway inflammation.

Discussion
Lipids have diverse and complex cellular functions, acting as membrane phospholipid constituents, metabolic substrates, precursors for signaling molecules, and mediators of gene expression. Highly complex molecular pathways are required to regulate these activities and maintain intra- and extracellular lipid homeostasis. FABPs are considered to play a critical role in lipid homeostasis, although their precise molecular mode(s) of action are incompletely understood. The adipocyte FABP, aP2, has long been utilized as a specific adipocyte marker and has recently also been identified in macrophages (39). aP2 is thought to regulate fatty acid uptake, release, and storage in adipocytes and participates in systemic glucose homeostasis and in macrophage responses in atherosclerosis (20, 40). Our findings now identify aP2 as an IL-4/IL-13–regulated gene in bronchial epithelial cells and demonstrate a key role for this FABP in Th2 cytokine-mediated airway inflammation.

The factors regulating aP2 expression have been widely studied in adipocytes, particularly during adipocyte differentiation. In adipocytes, aP2 expression is controlled predominantly by fatty acids and particularly by PPARγ agonists (41). Despite the fact that they have a functional PPARγ signaling pathway, we found no evidence...
for PPARγ-mediated regulation of aP2 expression in HBEs. In airway epithelium, STAT6 was shown to be largely responsible for upregulation of aP2 by IL-4. The other major stimulus regulating aP2 expression in HBEs was the type 1 cytokine IFN-γ, which induced a significant downregulation. Upregulation by Th2 cytokines and downregulation by Th1 cytokines provided a strong indication that aP2 was involved in Th2 inflammation. This was further supported by the observation of increased aP2 expression in AECs of mice undergoing allergic airway inflammation.

The bronchial epithelium plays an active role in the asthmatic inflammatory response. In particular, STAT6-dependent responses to IL-4 and IL-13 in epithelial cells are thought to

**Figure 6**
Allergic cytokine production is decreased in allergic aP2-deficient mice. (A) Detection of IL-5, IL-13, and eotaxin in BAL fluid from nonallergic (n = 10) and allergic (n = 12–15) aP2+/+ or aP2–/– mice. Data represent mean values ± SEM. *P < 0.05; **P < 0.005. (B) IL-5 was measured in culture supernatants from lung-draining lymph node cells restimulated in vitro with OVA or PBS only (Ctrl). Data represent mean ± SEM of 3–4 cultures in triplicate wells with cells pooled from 3–4 mice per culture. *P < 0.0005. (C) Unimpaired T cell proliferation in aP2–/– mice. Splenocytes from OVA/alum-immunized or PBS/alum control mice were stimulated with OVA antigen in vitro and cellular proliferation assayed. Data represent mean ± SEM of triplicate wells with 5 and 2 mice per genotype for OVA/alum and PBS/alum groups, respectively.

**Figure 7**
Non-hematopoietic cells are responsible for reduced allergic airway inflammation in aP2–/– mice. WT or aP2–/– (KO) bone marrow was transferred into irradiated mice to generate KO→WT, WT→KO, or WT→WT (donor→recipient) chimeras, and their responses to allergic airway inflammation assessed. Total (A) and differential (B) cell counts from BAL of bone marrow chimeric mice. Data represent mean values ± SEM for n = 13 KO→WT mice, n = 11 WT→KO mice, and n = 6 WT→WT mice. *P < 0.05 compared with KO→WT mice. (C) Detection of IL-5 and eotaxin in BAL fluid from allergic chimeric mice. Data represent mean ± SEM for n = 13 KO→WT mice and n = 11 WT→KO mice. *P < 0.05; **P < 0.005. (D and E) Detection of IL-5 and IL-13 from culture supernatants of allergic chimeric mouse lung-draining lymph node cells restimulated in vitro with OVA. Data represent mean ± SEM of 3 cultures from cells pooled from 4–5 mice per condition. ND, not detected.
The identification of a FABP that regulates allergic airway inflammation emphasizes the importance of lipids in the inflammatory response, and our findings contribute to the emerging theme of overlap between inflammatory and metabolic pathways (39). ap2 is a member of a larger family of FABPs with distinct patterns of tissue distribution (23). The ability of ap2 to regulate inflammation may thus represent a general feature of FABP biology, implying a role for FABPs in a broad range of inflammatory diseases. Finally, our findings suggest blocking ap2 function as a novel approach for treatment of asthma and other inflammatory lung diseases.

Methods

Cell culture. Normal HBEs (Cambrex) were maintained in Bronchial Epithelial Growth Media (BEGM), which consisted of Clonetics Bronchial Epithelial Basal Media (BEBM) supplemented with 52 mg/l bovine pituitary extract, 0.5 mg/l hydrocortisone, 0.5 mg/l human recombinant epidermal growth factor, 0.5 mg/l epinephrine, 10 mg/l transferrin, 5 mg/l insulin, 0.1 mg/l retinoic acid, 6.5 mg/l triiodothyronine, 50 mg/l gen- tamicin, and 50 mg/l amphotericin B. All media components were from Cambrex. When 80% confluent, cells were subcultured at a ratio of 1:3 with 0.025% trypsin-EDTA (Invitrogen). Prior to stimulation, HBEs were rested overnight in BEBM containing 0.1% low-endotoxin BSA (Sigma-Aldrich). HBEs were treated for 18 hours for all experiments except where noted and used at no later than passage 8.

3T3-L1 preadipocytes and A549 and BEAS-2B cells were used at 80% confluency and maintained in complete DMEM or DMEM/Ham’s F-12 (1:1) (Invitrogen). THP-1 cells were cultured in complete RPMI 1640. Complete media were those supplemented with 10% heat-inactivated FCS, 2 mM glutamine, and 100 U/ml penicillin/streptomycin and were all purchased from Invitrogen.

Primary mouse AECs from mice 8–12 weeks old were cultured as described previously (52) with the following modifications. Enzymatic dissociation of tracheal cells was performed for 45 minutes, and cells from 2 trachea were cultured in 1 well of a 24-well plate coated with type I collagen (Sigma-Aldrich) in DMEM/Ham’s F-12 (1:1) media supplemented with insulin (120 IU/l), 5% FCS, 2 mM glucose, and 100 U/ml penicillin/streptomycin. Cell purity was typically greater than 98% as assessed by staining cells grown on glass coverslips with anti-cytokeratin antibodies (Sigma-Aldrich). Prior to cytokine treatment, AECs were rested in unsupplemented AEC growth media with 0.1% BSA overnight.

Overexpression studies. BEAS-2B cells were cultured in T75 flasks (Nunc) and transiently transfected 72 hours with Lipofectamine 2000 (Invitrogen) with 10 μg plasmid DNA. Mammalian STAT6 and constitutively active STAT6 (STAT6VT) constructs were a kind gift from H. Nakajima (Chiba University, Chiba, Japan) and dominant negative STAT6 (STAT6DN) plasmid was from A. Masuda (Nagoya University, Nagoya, Japan). For dominant negative studies, cells were transfected 6 hours and media replaced with DMEM with 0.1% BSA overnight prior to cytokine stimulation.

Reagents. HBEs were treated with the following human recombinant cytokines from BD Biosciences—Pharmlingen: IL-3 (20 ng/ml), IL-4 (10 ng/ml), IL-6 (5 ng/ml), IL-10 (10 ng/ml), IL-13 (10 ng/ml), and IFN-γ (100 U/ml); or from PeproTech: IL-1 (10 ng/ml), TNF-α (10 ng/ml), and IL-1β (10 ng/ml). PBS was used as a control.

The Journal of Clinical Investigation

http://www.jci.org

Volume 116
Number 8
August 2006

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murine IL-4 (20 ng/ml). PMA and LPS (Sigma-Aldrich) were added for a final concentration of 50 ng/ml and 100 ng/ml, respectively. Rosiglitazone was purchased from Cayman Chemicals.

RNA extraction and GeneChip hybridizations. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) or TRIzol (Invitrogen) as per the manufacturer’s instructions. Two HBE lines, lot 8F1142 and 7F1482, that originated from white males aged 18 months and 32 years, respectively, were gene profiled using U133plus2 GeneChips (Affymetrix). The full microarray data set is available online at http://gspring.garvan.unsw.edu.au. For mouse experiments, lung RNA was pooled from n = 3 WT and n = 2 ap2−/− animals. cRNA and GeneChips (Affymetrix) were prepared as described previously (53). MicroArray Analysis Suite 5.0 software (Affymetrix) was used to generate “signal to log ratio” values from gene “signal” intensities and from the fold change that was calculated (fold change = $2^\text{signal} \times \text{log ratio}$). A gene was considered to be differentially regulated with a fold change of at least 2.

Animals. ap2−/− mice (40) were backcrossed for 12 generations to C57BL/6 mice (21) and used in airway inflammation studies. ap2−/− littermates or C57BL/6 mice from the Garvan Institute were used as controls. STAT6−/− mice were from the John Curtin School of Medical Research, Australian National University. BALB/c mice were purchased from Animal Resource Center (Perth, Western Australia, Australia) and used as controls for STAT6−/− mice and other airway inflammation studies. Animals were housed under conventional barrier protection and experiments performed with approval of the Garvan Institute/St. Vincent’s Hospital Animal Experimentation and Ethics Committee, which complies with the Australian National Health and Medical Research Council (NH&MRC) code of practice for the care and use of animals for scientific purposes.

Mouse model of allergic airway inflammation. Mice (15–23 weeks of age in one experiment that observed allergic inflammation in ap2-deficient mice and 8–10 weeks in all other experiments) were sensitized by intraperitoneal injection of 100 μg OVA (Sigma-Aldrich) or PBS adsorbed on 100 μl alum (Progen) on days 0 and 12 and challenged i.p. days 0, 2, 4, 7, 9, and 11, and days 14, 16, 18, 20, and 22. In a second experiment, mice were i.p. on days 0 and 12 and challenged i.p. on days 14, 16, 18, and 20. In all experiments, mice were sacrificed on day 21, and after cannulation of the trachea, BAL was performed with 1 ml PBS per mouse. Total cell counts were performed, and cytospin preparations of BAL cells were stained with modified Wright-Giemsa stain (Sigma-Aldrich) to permit differential leukocyte counts based on cellular morphology and staining characteristics. For lung histology, mouse lungs were fixed in 10% phosphate buffered formalin and embedded in paraffin. Sections were cut and stained with H&E for general histology or with Alcian blue–PAS for evaluation of mucus production. The extent of the inflammatory response was graded in a blinded fashion as 0, minimal or no inflammation; 1, mild inflammation, only perivascular or peribronchial; 2, moderate inflammation, some parenchymal involvement; 3, marked inflammation, widespread parenchymal involvement; 4, severe inflammation, little normal parenchyma. The extent of mucous cell change in the largest visible airway was graded according to a semilogarithmic scale and is described elsewhere (56).

Bone marrow chimeras. Mice aged 5–7 weeks were irradiated with 9.50 Gy and 24 hours later reconstituted i.v. with 7 × 10^6 total bone marrow cells from C57BL/6 or ap2−/− mice. Allergic lung inflammation was induced in mice 6 weeks after engraftment with i.p. immunization of 100 μg OVA in alum on day 0 and four 30-minute 1% OVA aerosol challenges on days 14, 16, 18, and 20. Allergic lung inflammation was assessed as described above. Hematopoietic cells from peripheral blood of chimeric mice were genotyped to ensure successful reconstitution of donor bone marrow (data not shown).

Mouse model of allergic peritonitis. Mice aged 6–8 weeks were immunized s.c. with 100 μg OVA or PBS in alum on days 0 and 12 and challenged i.p. with 50 μg OVA in 200 μl PBS, or PBS only, every second day from day 24.
Twenty-four hours after the last challenge, mice were lavaged once with 1.5 ml and then twice with 3 ml ice-cold PBS. Cells from all lavages were pooled and prepared for cell counts as described above.

**Lymph node cell culture and cytokine measurement.** Lymph-draining lymph node pools were pooled into groups, gently forced through a 70-μm nylon cell-strainer, and resuspended in complete RPMI 1640. Cells were cultured at 4 × 10^6 cells/ml in 96-well plates with 10, 50, or 100 ng OVA per well for 72 hours and cell-free culture supernatants collected and stored at −80°C until cytokine levels were assayed by ELISA according to the manufacturer’s protocol: IL-5 (BD Biosciences—PharMingen), IL-13 (PeproTech), and eotaxin (R&D Systems).

**T cell proliferation assay.** Mice were sensitized with OVA as per the bone marrow chimera allergic airway inflammation experiments and sacrificed on day 14. The spleen was disaggregated through a 70-μm nylon cell-strainer and erythrocytes lysed. Splenocytes containing 1 × 10^6 T cells/200 μl were cultured in complete RPMI 1640 for 72 hours, with 10 or 50 μg OVA. Cultures were pulsed with [3H]thymidine (1 μCi/well) 18 hours before harvesting and quantified using a β-scintillation counter.

**Statistics.** For continuous data, 2-tailed Student’s t test was utilized, and P < 0.05 was considered significant. Grading of histological changes was compared using a nonparametric Mann-Whitney U test.

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**Acknowledgments**

We thank J. Thatcher, L.G. Ng, T. So, and Z. Commandeur for technical assistance; P. Foster and M. Yang for discussion about the allergic inflammation model; R. Gallagher for human tissue; and the staff at the Garvan Institute Biological Testing Facility for animal care. This work was supported by grants from the Cooperative Research Centre for Asthma and Airways and the NH&MRC of Australia (to C.M. Mackay and M.S. Rolph) and the Sandler Program of Asthma Research (to G.S. Hotamisligil).

Received for publication February 15, 2005, and accepted in revised form May 23, 2006.

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