Cellular Immunology 264 (2010) 32–41

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Bronchial epithelial cells produce IL-5: Implications for local immune responses in the airways

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ARTICLE INFO

Article history:
Received 22 February 2010
Accepted 26 April 2010
Available online 5 May 2010

Keywords:
IL-5
Asthma
Mucous metaplasia
Airway eosinophilia
IgA

ABSTRACT

IL-5 is a pleiotropic cytokine that promotes eosinophil differentiation and survival. While naïve bronchial epithelial cells (BEC) produce low levels of IL-5, the role of BEC-derived IL-5 in allergic airway inflammation is unknown. We now show that BEC, isolated from mice with OVA-induced allergic airway disease (AAD), produced elevated levels of IL-5 mRNA and protein as compared to BEC from naïve mice. To determine the contribution of BEC-derived IL-5 to effector responses in the airways, IL-5 deficient bone marrow chimeric mice were generated in which IL-5 expression was restricted to stromal (e.g. BEC) or hematopoietic cells. When subjected to AAD, IL-5 produced by BECs contributed to mucous metaplasia, airway eosinophilia, and OVA-specific IgA levels. Thus, IL-5 production by BEC can impact the microenvironment of the lung, modifying pathologic and protective immune responses in the airways.

1. Introduction

Epithelial cells form a continuous lining in the airways, providing a protective barrier between the external and internal environment. Comprised of ciliated cells, goblet cells, and basal cells, the epithelium functions in preventing the passage of airborne irritants, allergens, and pathogens into the lung. In addition to providing a barrier function, the epithelium possesses the ability to generate a wide range of mediators that can modulate inflammatory responses, either helping to maintain homeostasis or enhance inflammation. Among the cytokines and chemokines produced by airway epithelial cells are several key factors that influence pathologies associated with asthma. For example, IL-13, a mediator in goblet cell metaplasia and airway remodeling, is elevated in patients with asthma [1,2]. Goblet cell metaplasia with increased mucus plugging is markedly increased in fatal asthma [3–5]. IL-13 also contributes to eosinophil recruitment through eotaxin-1- and IL-5-dependent pathways [6,7]. In addition, recent studies show that IL-13 release by epithelial cells occurs in rapid response to injury, facilitating repair and repopulation of the damaged area [8]. Granulocyte–macrophage colony stimulating factor (GM-CSF) is produced by the airway epithelium and plays a role in prolonging the survival of eosinophils [9,10]. Elevated levels of GM-CSF are present in the bronchoalveolar lavage fluid (BALF) collected from individuals with asthma [11]. The airway epithelium is a major source of eotaxin, an eosinophil specific chemokine that recruits eosinophils from the blood into the lung and elevated concentrations of eotaxin are found in the BALF and sputum from atopic asthmatic individuals [12–14]. Finally, increased expression of IL-5 has been reported in BALF from individuals with asthma and correlates with increased asthma severity [15–17]. IL-5 is a pleiotropic cytokine, regulating eosinophil proliferation, differentiation, and recruitment in parasitic diseases and allergic asthma [18–22]. While eosinophils are not present in all individuals with asthma, the eosinophilic phenotype is associated with an increased risk of exacerbations and higher risk of fatal outcomes in individuals with severe disease [15].

In the ovalbumin (OVA)-induced murine model of allergic airway disease (AAD), several different approaches have been taken to establish a pivotal role for IL-5 in the development of airway eosinophilia. Anti-IL-5 antibody treatment inhibits eosinophilic lung inflammation, but has no effect on IgE levels or Th2 cell function [23]. IL-5 null mice fail to display increased levels of eosinophils during the acute [22] or chronic stages of disease [24,25]. Together, these results define a role for IL-5 in the generation of airway eosinophilia. Eosinophils are associated with the development of lung dysfunction and to better understand the role of eosinophils in AAD, two separate laboratories generated an eosinophil null mouse. Characterization of C57BL/6 PHIL mice by Lee et al. [26] shows that eosinophils are integral to mucus accumulation and airway hyperreactivity. In contrast, Humbles et al. [27] observe structural changes associated with airway remodeling in Balb/c ΔdblGATA mice, deficient in eosinophils, but no differences

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doi:10.1016/j.cellimm.2010.04.008

in mucus hypersecretion or airway hyperreactivity are noted. The disparity in these outcomes likely involves strain differences specific to the mice [28]. Recent studies further define the role of eosinophils in AAD, showing they are critical in the recruitment of local T effector cells to the lung, by modulating chemokine production [28,29].

IL-5 may also influence other aspects of asthma as demonstrated by studies of IL-5 transgenic mice. Here, constitutive expression of IL-5 in the lung, in the absence of antigen challenge, results in mucous metaplasia, expansion of bronchus-associated lymphoid tissue (BALT), enhanced collagen deposition, and extensive airway eosinophilia [30]. Thus, an activated airway epithelium can dictate changes in the local inflammatory milieu that may result in exacerbation or resolution of disease. In this study, we isolated bronchial epithelial cells (BEC) from mice with AAD and characterized their production of Th2 cytokines, focusing on IL-5. To investigate the function of BEC-derived IL-5, a series of bone marrow (BM) chimeric mice were generated where IL-5 production was limited to stromal cells (e.g. BEC) or hematopoietic cells. This study provides evidence that IL-5 production by airway epithelial cells during AAD can influence local immune responses and induce pathologic changes in the lung.

2. Methods

2.1. Animals and the generation of bone marrow chimeric mice

Seven-week old female C57BL/6J wild-type (CD45.1 or CD45.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in accordance with institutional and Office of Laboratory Animal Welfare guidelines. A colony of IL-5 C57BL/6J null mice [21], gift of Dr. T.V. Rajan (University of Connecticut Health Center; originally purchased from The Jackson Laboratory), was maintained in our animal facility. All animal protocols were approved by the Animal Care Committee at the University of Connecticut Health Center.

Bone marrow (BM) cells were isolated from the femurs and tibias of donor mice by crushing the bone in a mortar with pestle in Hank's balanced saline solution (HBSS) supplemented with HGPG (5 mM Hepes with 50 mg/ml gentamicin, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine). The cells were collected, suspended in Tris–ammonium-chloride (TAC; 13 mM Tris, 135 mM NH_4Cl, pH 7.2) for 3 min at 37 °C to lyse RBC, washed, and resuspended in PBS. Lethally irradiated mice (exposed to a single dose of 1000 rads) were reconstituted with 3–6 $\times$ 10^6 BM cells by tail vein injection. After 4 and 8 weeks, tail vein bleeds were performed and the percent of donor versus host T cells was determined by flow cytometry using the Ly5.1 (CD45.2) and Ly5.2 (CD45.1) congenic markers. Twelve weeks after reconstitution, when repopulation of chimeric mice by donor BM cells was >99%, OVA-sensitization and -challenge was initiated, as described below.

2.2. OVA study protocol

OVA sensitization and aerosol challenge of mice has been previously described for our model of AAD [31]. Briefly, mice were given three weekly intraperitoneal injections of a suspension containing 25 mg of OVA (grade V; Sigma, St. Louis, MO) and 2 mg of aluminum hydroxide in 0.5 ml saline. One week after the last injection, mice were exposed to 0.2% aerosolized OVA generated by a BANG nebulizer (CH Technologies, Inc., Westwood, NJ), delivered in a nose-only inhalation chamber (In-Tox Production, Moriarty, NM). This procedure was repeated daily for 1 h per day for 1–42 days.

Twenty-four hours after the final OVA-aerosol challenge, the lungs from each animal were lavaged *in situ* with sterile saline. The total number of viable leukocytes was determined by trypan blue exclusion using a hemocytometer and leukocyte differentials were determined by May–Grunwald/Giemsa staining of cytocentrifuged cell preparations. BALF was concentrated 10-fold using a Centriplus YM-10 filtration device (Amicon, Beverly, MA), aliquoted, and stored at −80 °C until use. The presence of IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) [Pierce-Endogen, Rockford, IL] and IL-13, eotaxin-1 (CCL11), and TGF-β1 [R & D Systems, Minneapolis, MN] was assayed by ELISA, testing an undiluted and 1:2 dilution of concentrated BALF.

2.3. Isolation of bronchial epithelial cells (BEC)

BEC were isolated using a modified version of the protocol published by Davidson et al. [32]. Briefly, bronchi were removed by gross dissection from 5 AAD or 5 naïve mice and incubated in dissociation medium [Ca_2-free, Mg_2-free MEM with 0.14% pronase (Roche Applied Science, Indianapolis, IN) and 0.01% DNase I (Sigma–Aldrich, St. Louis, MO)]. After 1 h at 37 °C, the tubes were inverted 12–15 times to dissociate epithelial cells from the airways. One milliliter of fetal calf serum (FCS) was added to stop further digestion and cells were collected by centrifugation, then suspended in airway medium [DMEM-F12 supplemented with 10% FCS, 1.2 U/ml insulin (Gibco/Invitrogen, Carlsbad, CA) and pen/strep] and rested for 2 h at 37 °C. RBC and debris were separated from BEC and other mononuclear cells by centrifugation through Histopaque-1077. Cells collected from the interface were washed and resuspended in airway medium for analysis. Approximately 1 $\times$ 10^6 cells were recovered from bronchi harvested from 5 mice.

BEC populations were cyto-centrifuged onto slides and stained with DAPI and a monoclonal antibody specific for cytokeratin (clone PCK-26; Sigma–Aldrich, St. Louis, MO). Typically, 95% of the cells stained positive for cytokeratin, an epithelial cell marker not expressed by lymphocytes [33]. During our initial characterization, slides were also stained with monoclonal antibodies specific for CD4 (clone RM4-5; eBioscience, San Diego, CA), CD11b (clone M1/70; BD Bioscience, San Jose, CA), and CD8 (clone 53-6.7; BD Bioscience), as well as with anti-major basic protein serum (gift from Dr. Jamie Lee, The Mayo Clinic, Phoenix, AZ), followed by the appropriate secondary antibodies.

2.4. Intracellular IL-5 expression

BEC from AAD mice and IL-5 KO mice were isolated in the presence of 10 mg/ml Brefeldin A and incubated for 6 h at 37 °C. BEC were washed, incubated overnight at 4 °C in Fixation/Permeabilization buffer (eBioscience), washed, and cyto-centrifuged onto slides. Cells on slides were double stained with IL-5-PE (clone TRFK-5; eBioscience) and cytokeratin-FITC (clone C-11; Sigma–Aldrich), followed by rabbit anti-PE (Biomeda Corp., Foster City, CA) and biotin-conjugated sheep anti-FITC (AbD Serotec, Raleigh, NC), and finally Alexa Fluor® 546-conjugated goat anti-rabbit IgG (H + L) (Invitrogen Life Technologies, Carlsbad, CA) and Alexa Fluor® 488-conjugated streptavidin (Invitrogen Life Technologies). To estimate the percentage of cells that express IL-5, fields of cells were randomly selected for study under either bright field or Hoffman optics. These images were captured and stored in a computer file. The cells were then observed under epi-fluorescence using either the FITC filter set to detect BEC or TRITC filter set to detect IL-5 staining. Images were obtained under both filter sets and collected for over 100 BEC from AAD and IL-5 KO mice. Using iVision software (version 4.0 BioVision Technologies, Exton, PA), the mean fluorescent intensity of each cell was determined. In addition, the

95% confidence interval for BEC from IL-5 KO mice was determined and the upper value used to estimate non-specific staining. All BEC from AAD mice with a fluorescent intensity greater than the upper 95% confidence level of BEC from IL-5 KO mice were considered positive for IL-5.

2.5. RNA isolation and RNase analysis

BEC, pooled from 5 mice, were resuspended in 1 ml of Ultraspec RNA solution (Biotecx Laboratories, Houston, TX) and stored at −80 °C until use. From the remaining lung tissue, leukocytes were isolated by collagenase treatment with 150 U/ml collagenase (Invitrogen Life Technologies) in RPMI supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% FCS, and HGPG at 37 °C for 1 h. $CD4^+$ T cells were positively selected using CD4 microbeads (Miltenyi Biotech, Auburn, CA), resulting in $\sim 2 \times 10^6$ cells from a pool of 5 mice. Total RNA was isolated from BEC or $CD4^+$ T cells and processed for RNase protection assays, as described by the manufacturer using mCK-1 antisense probes (BD PharMingen, San Jose, CA). The pixel density of all Th1/Th2 bands was determined using iVision software and used to calculate the relative band intensity (i.e. average gray scale value for each individual cytokine/total gray scale values for all Th1/Th2 cytokines).

2.6. Determination of OVA-specific Ig levels in serum

OVA-specific Ig levels were measured by ELISA using isotype-specific capture antibodies as described [34]. Briefly, microtiter plates were coated with rat anti-mouse IgE (R35-72), IgG_1 (A85-3) (BD Pharmingen, Franklin Lakes, NJ), or goat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL). After blocking non-specific binding, isotype-specific Abs were captured in duplicate, analyzing 2-fold serial dilutions of serum. Detection of Ag-specific Igs was performed with OVA-digoxigenin conjugates, followed by anti-digoxigenin-peroxidase (Roche Diagnostics, Indianapolis, IN), and development with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

2.7. Lung histology

Unmanipulated, non-inflated lungs were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E) to assess airway inflammation or with periodic acid-Schiff (PAS) to assess mucus accumulation. To determine the percentage of PAS-positive staining airways, digital images of 8–10 randomly selected airways from each mouse were captured using an Olympus BH2 microscope fitted with an Olympus C5050 digital camera (Olympus Imaging, Center Valley, PA). Three mice were analyzed per group for a total of 25–30 medium size airways. The area of the total airway epithelium, as well as the area of PAS-positive cells was determined using ImageJ software created by Wayne Rasband at The National Institute of Mental Health (NIH, Bethesda, MD).

2.8. Statistical analysis

Analysis of data from experiments with three or more groups was performed using a using one-way analysis of variance (ANOVA), followed by a Newman–Keuls *post hoc* test. Statistical comparisons between two groups were made using an unpaired *t* test, except when the data was not normally distributed. The data collected for cytokine production by isolated BEC (Fig. 2C) was not normally distributed. These comparisons were analyzed using the non-parametric Mann–Whitney test. All tests were performed using the software Prism 5.0 (GraphPadSoftware, La Jolla, CA), with a probability of $p < 0.05$ considered to be significant.

3. Results

3.1. Time course of IL-5, IL-13, and eotaxin production during AAD

In the OVA-induced model of AAD, we have shown that airway inflammation increases over time, with enhanced airway inflammation and eosinophilia occurring after 7 days of OVA-aerosol challenge [31]. Upon continued OVA-aerosol challenge, the number of eosinophils wanes and by day 42 no eosinophils can be detected. Histological analysis of lung tissue demonstrates an increase in the number of PAS-staining airways after 7 days of OVA-aerosol challenge with mucus plugging being readily evident up to 21 days [31]. BALF from the same mice was collected after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge and analyzed for IL-5 and eotaxin, two mediators known to influence airway eosinophilia (Fig. 1). Elevated levels of IL-5 were observed after 3 days of OVA-aerosol challenge ($p < 0.01$ as compared to all other time points), and declined rapidly thereafter, whereas increased levels of eotaxin were noted on day 7 ($p = 0.01$ as compared to days 1, 3, 10, and 14). In addition, increased levels of IL-13, a Th2 cytokine associated with mucus hypersecretion, were present on days 3 and 7 ($p = 0.03$ as compared to all other time points).

3.2. Th2 cytokine production by bronchial epithelial cells (BEC) during AAD

To examine the ability of the airway epithelium to produce Th2 cytokines, bronchial epithelial cells (BEC) were isolated from AAD mice after 3 days of OVA-aerosol challenge. Elevated levels of IL-5 and IL-13 were observed in BALF from AAD mice after 3 days of OVA-aerosol challenge (Fig. 1), and prior to the enhanced airway inflammation and eosinophilia observed after 7 days of OVA-aerosol challenge [31]. Therefore, we chose this time point to maximize the detection of Th2 cytokines, while limiting contamination by other inflammatory cell types. Using a modified protocol developed by Davidson et al. [32], we isolated BEC from the lungs of AAD mice. A typical preparation of BEC was comprised of 95% epithelial cells, <3% lymphocytes, <2% macrophages, <2% polymorphonuclear cells (including eosinophils), and no mast cells. Further analysis of BEC preparations confirmed that eosinophils and $CD4^+$ T cells, two known sources of IL-5 and IL-13, comprised <2% and <1%, respectively, of the epithelial cell preparation.

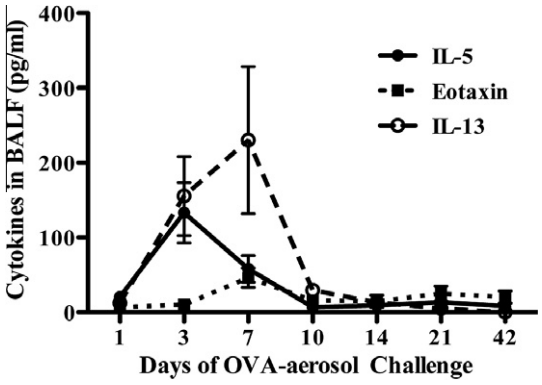

Fig. 1. Time course of IL-5, IL-13, and eotaxin production in AAD mice. BALF was collected from AAD mice after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge and assayed for the presence of IL-5, IL-13, or eotaxin by ELISA. The results are expressed as the means ± SE with $n = 4$–6 mice at each time point. The levels of IL-5 on day 3 were significantly increased as compared to all other time points. The levels of eotaxin on day 7 were significantly increased as compared to days 1, 3, 10, and 14. The levels of IL-13 on day 7 were significantly increased as compared to days 1, 10, 14, 21, and 42.

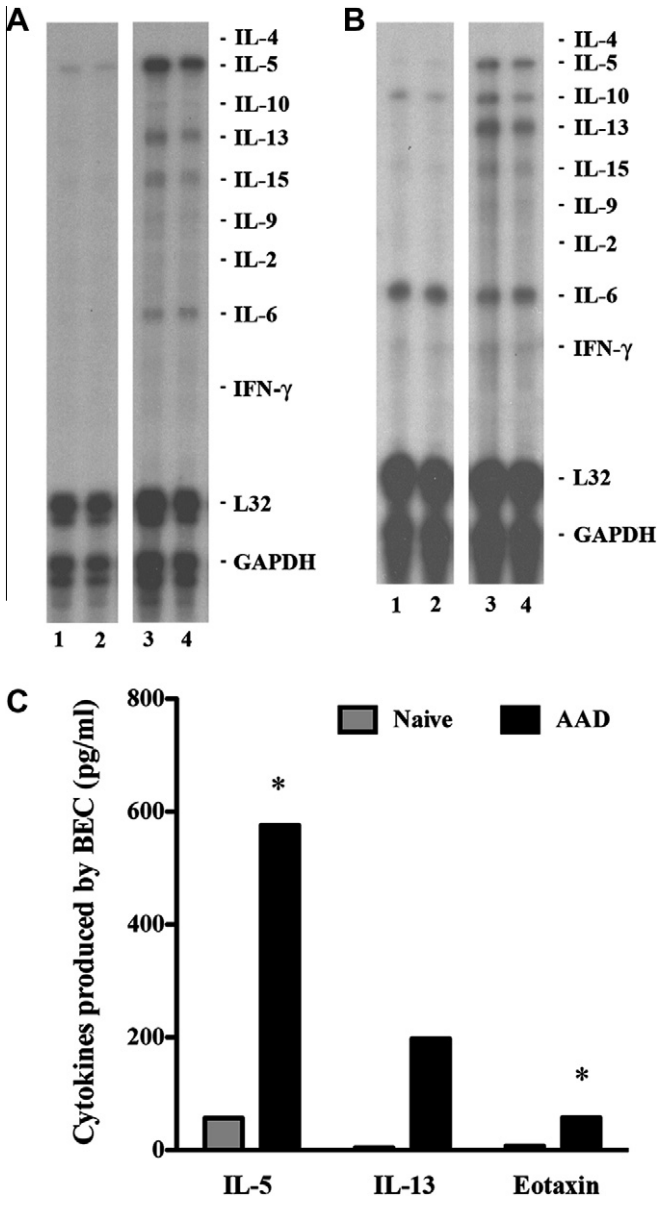

Fig. 2. Th2 cytokine expression and secretion by BEC from AAD mice. BEC were isolated from AAD mice after 3 days of OVA-aerosol challenge (2 pools of 5 mice each) or naïve mice (2 pools of 5 mice each). (A) Total RNA from BEC collected from naïve (lanes 1 and 2) or AAD mice was examined for Th1/Th2 cytokine expression using a RNase protection assay. (B) $CD4^+$ T cells were positively selected from the remaining lung tissue of these mice and total RNA, from naive (lanes 1 and 2) or AAD mice (lanes 3 and 4), was analyzed by RNase protection. (C) Culture supernatants from BEC, isolated from 5 naïve or 5 AAD mice, were assayed for IL-5, IL-13, and eotaxin production by ELISA after 18 h in culture without additional stimulation. Values were expressed as a mean of 3 separate experiments, from naïve (gray bars) or AAD (black bars) mice. Comparisons between groups were performed using the non-parametric Mann–Whitney test. *Significantly increased as compared to naïve ($p < 0.05$).

Using this approach, we isolated BEC from AAD and naïve mice to compare the pattern of Th1/Th2 cytokine mRNA expression. BECs, pooled from 5 mice, were examined in each sample by RNase protection assay (Fig. 2A). After 3 days of OVA-aerosol challenge elevated levels of IL-5 mRNA, and to a lesser extent IL-13, IL-15, and IL-6 mRNAs, were present in BEC from AAD mice (lanes 3 and 4). In contrast, IL-5 mRNA was barely detectable in BEC from naïve mice and expression of other Th2 cytokine mRNAs was not observed (lanes 1 and 2). $CD4^+$ T cells were purified from the lungs of the same mice and analyzed by RNase protection in a similar fashion (Fig. 2B). As expected, the pattern of Th2 cytokine mRNA expression by $CD4^+$ T cells from AAD mice (lanes 3 and 4) was elevated as compared to naïve mice (lanes 1 and 2) with increased levels of IL-5, IL-10, and IL-13 mRNAs being most prominent. By comparing the band intensity of each Th1 or Th2 mRNA, IL-5 mRNA in BEC was determined to be 2-fold greater than that observed in $CD4^+$ T cells, while IL-13 mRNA profiles were similar between the two cell types. Thus BEC, as well as $CD4^+$ T cells, have the potential to contribute unique cytokines to the lung environment during AAD.

Finally, BEC isolated from AAD and naïve mice were examined for Th2 cytokine production by ELISA. As shown in Fig. 2C, the levels of IL-5 and eotaxin present in supernatants from BEC from AAD mice were significantly elevated as compared to naïve mice. An increased level of IL-13 was also observed in BEC from AAD mice, but did not reach significance due to a limited number of samples. In contrast, no difference in the level of GM-CSF was noted between AAD and naïve mice (data not shown). Together, these findings strongly suggested that BEC are a source of Th2 cytokines, particularly IL-5, in the OVA-induced model of AAD.

3.3. BEC as a source of IL-5 in the OVA-induced model of AAD

To directly demonstrate that BEC isolated from AAD mice produced IL-5, intracellular staining was performed. This approach was selected, as single cells suspensions of BEC are difficult to obtain without altering the integrity of the cells, thereby prohibiting the use of fluorescence flow cytometry. Brefeldin A was used during the BEC isolation procedure to block intracellular transport of secreted proteins from the endoplasmic reticulum to the Golgi complex, thereby enhancing intracellular detection [35]. BEC were isolated in the presence of Brefeldin A (10 mg/ml) from AAD mice after 3 days of OVA-aerosol challenge, fixed and permeabilized overnight, and then cyto-centrifuged onto slides. BEC isolated from IL-5 null mice were included as a negative control. As shown in Fig. 3, BEC from AAD mice stained positively for both cytokeratin and IL-5 (panels A–C). In contrast, BEC from IL-5 KO mice stained positively for cytokeratin, but displayed only minimal background staining for IL-5 (panels D–F).

As low levels of background staining were observed in BEC from IL-5 KO mice, we were able to compare the mean pixel intensity from over 100 randomly selected BEC isolated from either AAD or IL-5 KO mice. Significant differences in the mean pixel intensity were observed between the two groups ($p < 0.001$). Using the upper 95% confidence level for staining intensity of BEC from IL-5 KO mice as a cut off for non-specific staining, we estimated the number of BEC from AAD mice that were positive (i.e. scored above background staining) for IL-5. In two separate experiments, 58% and 65% of the BEC from AAD mice were producing IL-5. A representative image is shown in Fig. 4G–H, where intracellular IL-5 was observed in 5 of 9 BEC from AAD mice.

3.4. Absence of IL-5 producing hematopoietic or stromal cells in bone marrow chimeric mice resulted in decreased IL-5 and IL-13 levels in BALF

As IL-5 can influence eosinophil maturation, recruitment, and survival, as well as enhance goblet cell metaplasia, production of IL-5 by BEC may impact multiple features of AAD. To address this issue, we generated a series of IL-5 deficient bone marrow (BM) chimeric mice to dissect the contribution of IL-5 produced by stromal cells (e.g. BEC) from IL-5 produced by hematopoietic cells (e.g. $CD4^+$ T cells, eosinophils, mast cells). We transferred BM cells from IL-5 null donor mice into lethally irradiated C57BL/6 wild-type recipients (KO → WT) or BM cells from C57BL/6 wild-type donors into lethally irradiated IL-5 null recipients (WT → KO), and

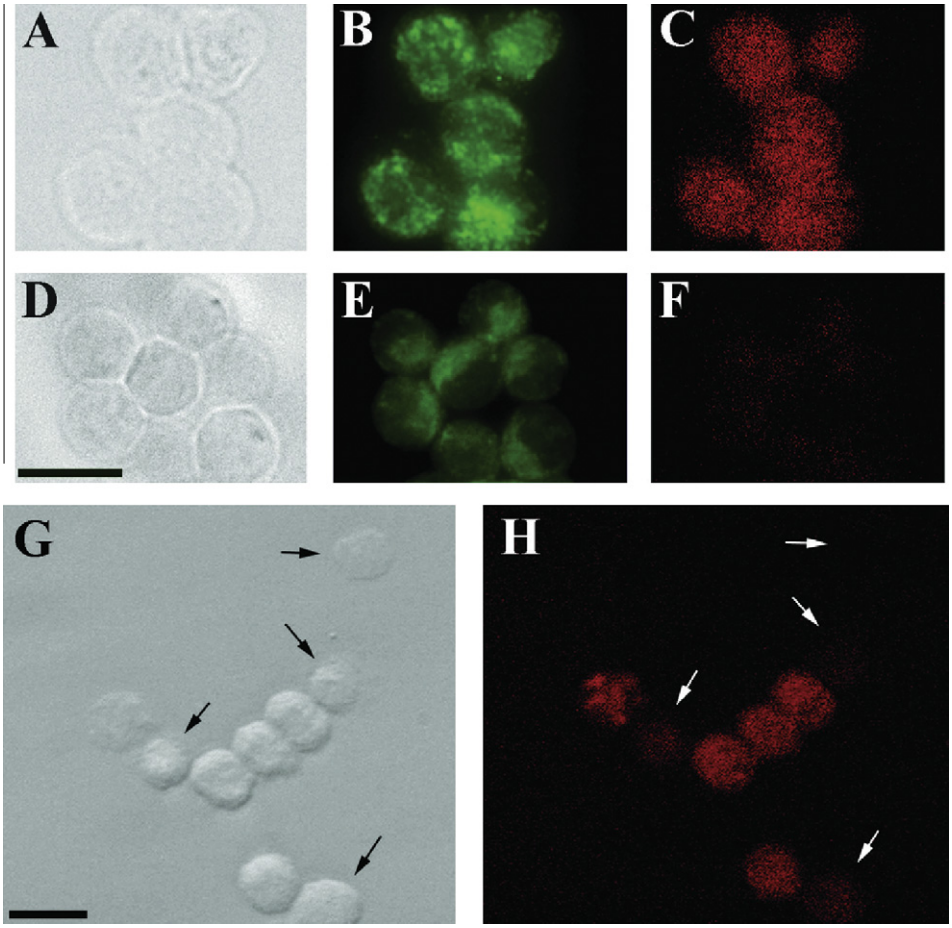

Fig. 3. BEC from AAD mice are a source of IL-5. BEC were isolated in the presence of Brefeldin A from AAD mice after 3 days of OVA-aerosol challenge (panels A-C) or from IL-5 null mice (panels D–F). BEC were stained with mAbs specific for IL-5 (red, panels C and F) and cytokeratin (green, panels B and E) or visualized by phase microscopy (panels A and D). A lower magnification of BEC from AAD mice was observed under Hoffman optics (panel G) or fluorescent microscopy, with intracellular staining for IL-5 (panel H). Arrows indicate BEC not expressing IL-5. Scale bar represents10 μm.

compared them to WT → WT and KO → KO controls. After 12 weeks after reconstitution, when repopulation of chimeric mice by donor BM cells was >99%, the BM chimeric mice were OVA-sensitized, then challenged with OVA-aerosol for 3 or 7 days.

Airway inflammation in the OVA-induced model of AAD is associated with increased Th2 cytokine production (reviewed, [36]). Therefore, we first examined BALF from the IL-5 deficient BM chimeric mice for the presence of the Th2 cytokines IL-5 and IL-13, as well as the chemokine eotaxin. After 3 days of OVA-aerosol challenge, the levels of IL-5 observed in WT → WT BM chimeric mice were significantly elevated as compared to KO → KO ($p < 0.01$), KO → WT ($p < 0.01$), and WT → KO ($p < 0.03$) mice (Fig. 4A). After 7 days of OVA-aerosol challenge, the levels of IL-5 in BALF from all BM chimeric mice were barely detectable (data not shown). These findings were in good agreement with IL-5 production in wild-type AAD mice, where elevated levels of the cytokine are observed at 3 days and decreased thereafter (Fig. 1). As expected, no IL-5 was detected in BALF from KO → KO BM chimeric mice.

Similarly, increased levels of IL-13 were present in BALF from WT → WT BM chimeric mice as compared to KO → KO ($p < 0.02$), KO → WT ($p < 0.04$), and WT → KO ($p < 0.05$) mice after 3 days of OVA-aerosol challenge (Fig. 4A). After 7 days of OVA-aerosol challenge IL-13 levels decreased 10-fold in WT → WT BM chimeric mice, and no differences in IL-13 were observed among the groups (data not shown). Finally, no significant changes in eotaxin expression were noted among the groups after 3 (Fig. 4A) or 7 days of OVA-aerosol challenge.

3.5. The contribution of BEC-derived IL-5 to airway eosinophilia

After 3 days of OVA-aerosol challenge, the numbers of eosinophils in BALF and lung tissue from WT → KO and WT → WT BM chimeric mice were significantly increased as compared to KO → KO and KO → WT mice ($p < 0.005$; data not shown). After 7 days of OVA-aerosol challenge, the numbers of airway eosinophils in BALF and lung tissue were 10- and 4-fold higher in magnitude, respectively, in WT → KO and WT → WT BM chimeric mice. This increase was significant as compared to KO → KO or KO → WT mice (Fig. 4B). Finally, the number of eosinophils in both BALF and lung tissue from KO → WT BM chimeric mice after 7 days of OVA-aerosol challenge was significantly elevated as compared to KO → KO BM chimeric mice (Fig. 4B). Together, these findings suggest that while IL-5 produced by hematopoietic cells (i.e. $CD4^+$ T cells) is a major determinant in airway eosinophilia during AAD, IL-5 produced by BEC or other stromal cells may also contribute to airway inflammation. No differences in the number of macrophages, polymorphonuclear neutrophilic leukocytes, or lymphocytes were observed among the four different groups of BM chimeric mice after 3 or 7 days of OVA-aerosol challenge.

Enhanced levels of OVA-specific IgE are one of the hallmarks of OVA-induced allergic airway inflammation (reviewed, [37]). Therefore, we examined IL-5 deficient BM chimeric mice for the presence of OVA-specific IgE in serum. As shown in Fig. 4C, class switching to OVA-specific IgE was constant among the 4 groups of IL-5 deficient BM chimeric mice. These findings were in good

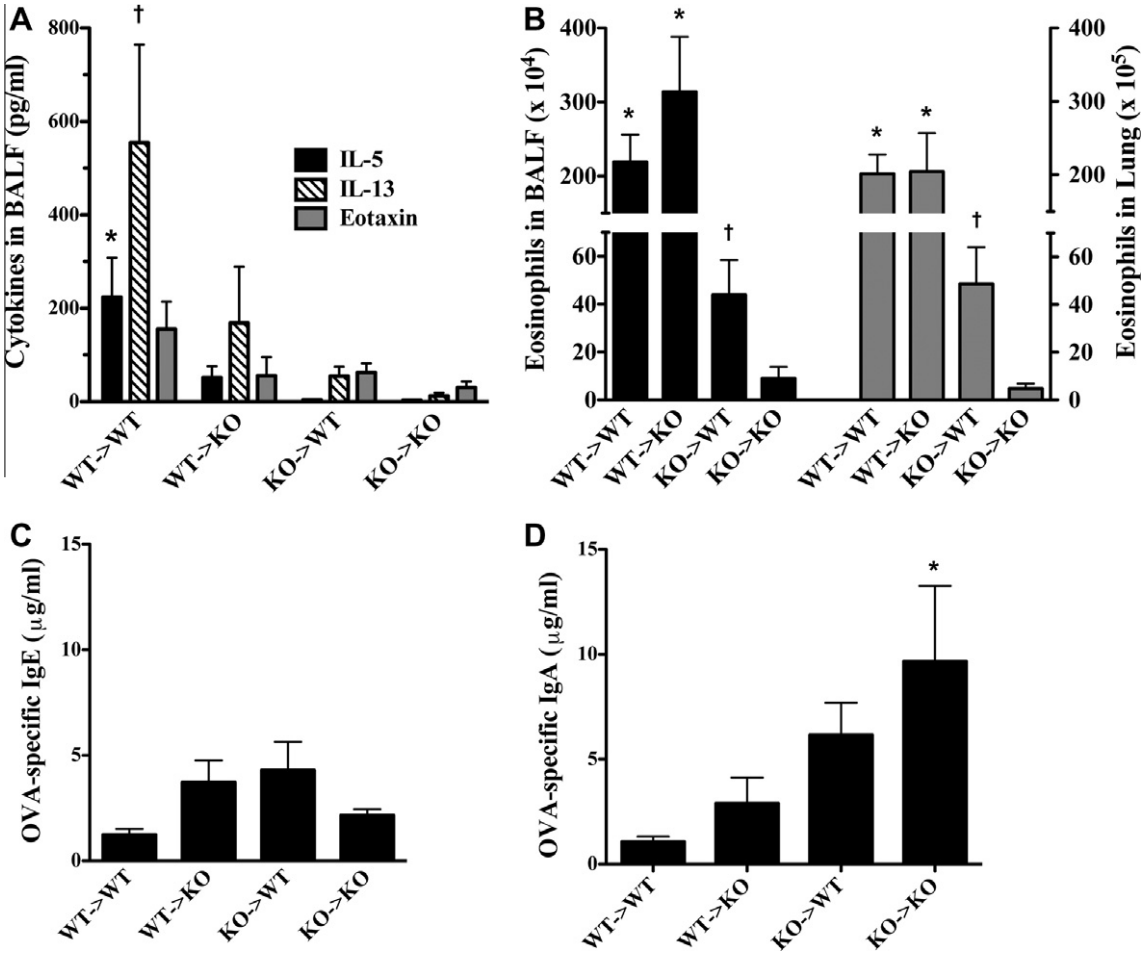

Fig. 4. BEC-derived IL-5 contributes to Th2 cytokine production, airway eosinophilia, and OVA-specific IgA production. A series of IL-5 deficient BM chimeric mice, where IL-5 expression was restricted to stromal cells (KO → WT) or hematopoietic cells (WT → KO), were sensitized and challenged with OVA-aerosol for 3 or 7 days. (A) After 3 days of OVA-aerosol challenge, BALF was collected, concentrated, and analyzed for IL-5, IL-13, or eotaxin by ELISA. *IL-5 levels were significantly elevated as compared to WT → KO ($p < 0.03$), KO → WT ($p < 0.01$), and KO → KO ($p < 0.01$) BM chimeric mice. †IL-13 levels were significantly elevated compared to WT → KO ($p < 0.05$), KO → WT ($p < 0.04$) and KO → KO ($p < 0.02$) BM chimeric mice. (B) After 7 days of OVA-aerosol challenge, leukocytes from BALF and lung tissue were collected and cellular profiles were determined by differential analysis using May–Grunwald/Giemsa staining. *Significantly increased as compared to KO → WT ($p < 0.005$) or KO → KO ($p < 0.0001$) BM chimeric mice. †significantly increased as compared to BALF ($p < 0.05$) and lung tissue ($p < 0.04$) from KO → KO BM chimeric mice. (C and D) After 7 days of OVA-aerosol challenge, serum was collected and assayed for OVA-specific IgE (pg/ml), OVA-specific IgA (pg/ml), and OVA-IgG_1 (ng/ml) by ELISA. Ig switching to OVA-specific IgE (panel C) and OVA-specific IgA (panel D) was determined in 7–8 serum mice per group. *Significantly elevated OVA-specific IgA serum levels as compared to WT → WT BM chimeric mice ($p < 0.03$).

agreement with Foster et al. [22] who observed no differences in serum OVA-specific IgE levels between wild-type and IL-5 null mice during the acute phase of allergic airway inflammation. Next, we examined IL-5 BM chimeric mice for OVA-specific IgA, as IgA is the most common immunoglobulin isotype present at mucosal surfaces, providing a first line of defense against airborne allergens and irritants [38]. Interestingly, when IL-5 production was restricted to either hematopoietic or stromal cells, OVA-specific IgA production was enhanced (Fig. 4D). Moreover, in the absence of IL-5 (i.e. KO → KO BM chimeric mice), this enhancement was further exacerbated. This finding was unexpected, as IL-5 augments OVA-specific IgA production *in vitro* [39–41], although limited analyses have been performed *in vivo*. Nevertheless, the data suggest that both hematopoietic and stromal sources of IL-5 contribute to the control of antigen-specific IgA responses during AAD.

3.6. BEC-derived IL-5 contributes to mucous metaplasia

IL-13 is key component in stimulating mucus hypersecretion [42,43] and differences in both IL-5 and IL-13 were observed in IL-5 deficient BM chimeric mice (Fig. 4A). Therefore, histological evaluation of lung tissue was performed to determine the accumulation of mucus in these mice after 7 days of OVA-aerosol exposure, a time when we had reported an increase in the number of PAS-staining airways in wild-type mice with AAD [31]. Extensive PAS-positive staining, as well as mucus plugging, was evident in airways of WT → WT BM chimeric mice (Fig. 5A). PAS-positive staining of the airway epithelium was also noted in both WT → KO (Fig. 5B) and KO → WT (Fig. 5C) BM chimeric mice, although mucus plugging was limited. In contrast, fewer PAS-positive airways were observed in KO → KO mice (Fig. 5D), as has been reported in IL-5 null mice [44]. Intracellular mucus accumulation was quantified by digital analysis of 8–10 randomly selected airways from each mouse, examining 3 mice per group of IL-5 deficient BM chimeric mice. As shown in Fig. 5E, 22% of the airway epithelium from WT → WT mice were PAS-positive. PAS-positive staining was significantly decreased in WT → KO and KO → WT mice, representing 16.4% and 17.1%, respectively, and further diminished in KO → KO mice (4.9%). Thus, IL-5 production by hematopoietic and stromal cells enhanced mucous metaplasia in airways during AAD.

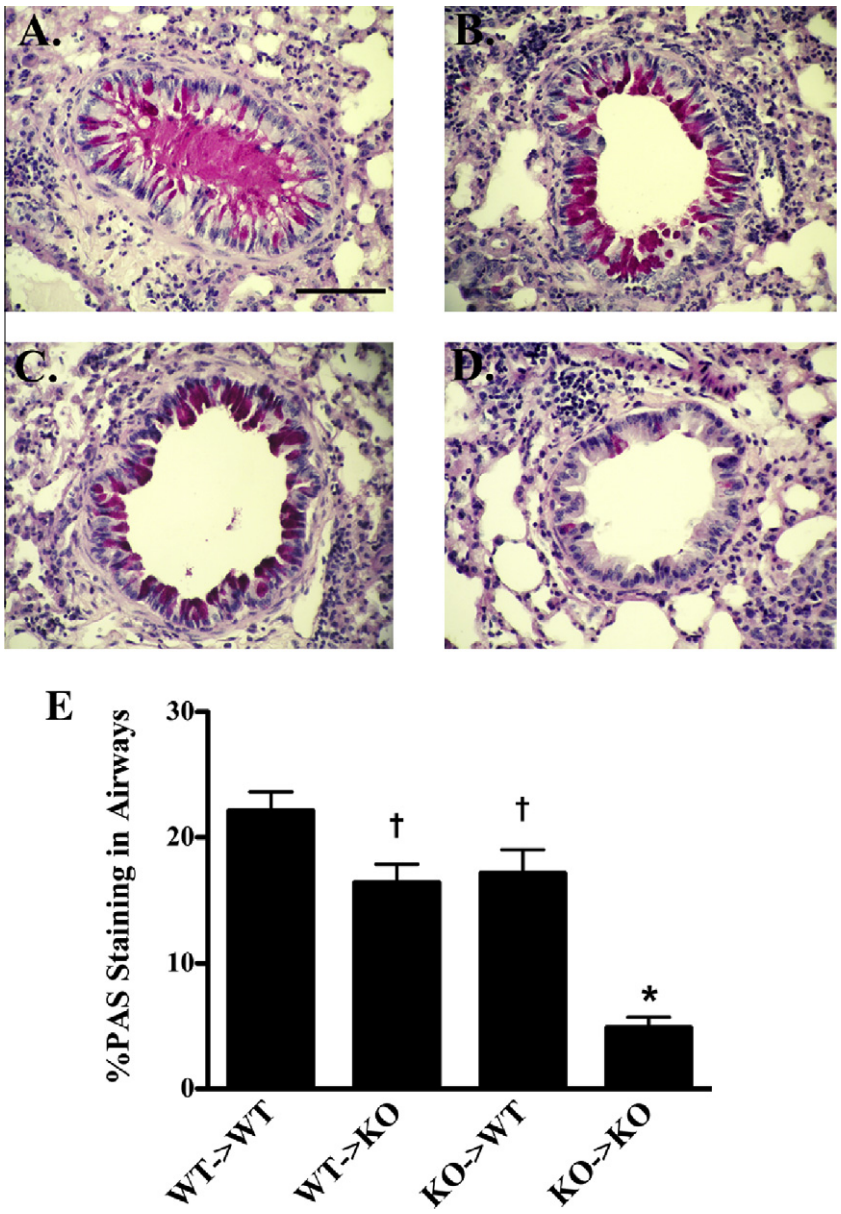

Fig. 5. IL-5 production by both stromal cells and hematopoietic cells contributes to enhanced mucous metaplasia. Representative lung sections stained with PAS from WT → WT (panel A), WT → KO (panel B), KO → WT (panel C) and KO → KO (panel D) after 7 days of OVA-aerosol challenge. The percentage of PAS-positive staining in airways was determined, analyzing 8–10 airways per mouse with 3 mice per group (n = total of 25–30 airways per group). *Significantly decreased as compared to all other groups ($p < 0.001$). †Significantly decreased as compared to WT → WT ($p < 0.05$). Scale bar represents100 μm.

4. Discussion

Detection of IL-5 in the airway epithelium was first reported by Salvi et al. [45] upon examination of endobronchial biopsies from healthy volunteers. Their studies used a highly sensitive RT-PCR ELISA assay to reveal constitutive low-level expression of IL-5 mRNA in primary human bronchial and nasal epithelial cells [45]. Subsequently, others have observed increased IL-5 expression in the bronchial epithelium from healthy volunteers upon nitrogen dioxide exposure [46], as well as from individuals with mild asthma [47]. Upon exposure to air pollutants such as ozone, expression of IL-5 production is heightened in individuals with mild asthma [47]. Similarly, we were able detect low levels of IL-5 production by BEC isolated from naïve mice and this expression was significantly elevated in AAD mice (Fig. 2A and C). These findings validate the use of this animal model in studying BEC-derived IL-5 and provided a means to unravel its function in the development of AAD. Towards this end, we generated a series of IL-5 deficient BM chimeric mice where IL-5 expression was restricted to stromal (e.g. BEC) or hematopoietic cells, then subjected the mice to AAD.

Studies by Lee et al. [30] have shown that over-expression of IL-5 in the airway epithelium in the absence of antigen-challenge resulted in several pathologic outcomes, including goblet cell metaplasia and mucus hypersecretion. While mucus secretion protects the airway epithelium against damage from inhaled pathogens, allergens, and irritants, excessive mucus accumulation in the airways correlates with increased disease severity [3–5]. In our studies, the number of PAS-positive epithelial cells was elevated in WT → WT BM chimeric mice when subjected to AAD, with evidence of mucus plugging in several airways (Fig. 5). We found that the percentage of goblet cell metaplasia (22%), as defined by PAS-staining, was strikingly similar to the percentage of goblet cells found in patients with asthma. Morphometric analysis of autopsied lungs from patients with bronchial asthma reveals 20–25% goblet

cell composition of the epithelium in both central and peripheral airways [48]. A significant decrease in the percentage of PAS-staining epithelial cells was found in both WT → KO and KO → WT mice, suggesting that IL-5 from both stromal cells and hematopoietic cells contributes to mucous metaplasia. The mechanism by which IL-5 influences goblet cell metaplasia and enhanced mucus production is unclear. It is interesting that decreased levels of IL-5 in BALF from WT → KO, KO → WT, and KO → KO mice correlated with decreased levels of IL-13 (Fig. 4A). IL-13 is an important mediator in mucus production and goblet cell hyperplasia [49,50] and evidence that IL-5 can modulate IL-13 production has been reported for murine $CD4^+$ T cells [51] and human eosinophils [52]. Furthermore, mucus accumulation, occurring in IL-5 transgenic mice in the absence of antigen challenge, can be prevented by disrupting the IL-13 signaling pathway [53]. The levels of IL-5 produced by BEC in response to antigen challenge in KO → WT mice were substantially lower (18,000- and 75-fold lower in BALF and serum, respectively), than IL-5 generated by airway epithelium in IL-5 transgenic mice [30]. This provides evidence that under physiologic conditions, BEC-derived IL-5 can impact mucous metaplasia during AAD.

Airway eosinophilia is a hallmark of asthma and a key feature of allergic airway inflammation in animal models of disease. It is known that IL-5, a pleiotropic cytokine that regulates the proliferation, differentiation, and recruitment of eosinophils, is essential for the development of airway eosinophilia during allergic airway inflammation [18–22]. Indeed, IL-5 transgenic mice exhibit a dramatic increase in airway eosinophilia in the absence of antigen challenge [30]. As expected, little to no airway eosinophilia was observed in KO → KO BM chimeric mice which lacked IL-5 when subjected to AAD (Fig. 5B) and these findings are in good agreement with the lack of airway eosinophilia in IL-5 null mice [22]. Furthermore, as expected, eosinophils comprised the majority (>60%) of infiltrating cells in BALF and lung tissue in WT → WT controls, and these findings are in good agreement with our previous studies of wild-type C57BL/6 mice during the development of AAD [31]. The lack of BEC-derived IL-5 in WT → KO BM chimeric mice had no influence on the profile of cells infiltrating the lung or BALF as compared to WT → WT BM chimeric controls, even though a significant decrease in IL-5 production was observed (Fig. 5A). Interestingly, while the number of eosinophils in BALF or lung tissue did not reach the levels observed in WT → WT BM chimeric mice, eosinophils were significantly elevated in KO → WT BM chimeric mice as compared to KO → KO mice (Fig. 5B). The levels of IL-5 in these mice were low, detecting only 6 pg/ml in BALF and 23 pg/ml in serum after 3 days of OVA-aerosol challenge. Yet, such levels of IL-5 in the presence of eotaxin-1 may be sufficient to drive some level of airway eosinophilia. Cooperation between IL-5 and eotaxin-1 is known to enhance eosinophil accumulation, with the suggestion that IL-5 can increase the number of eotaxin responsive cells [54]. Indeed, co-expression of IL-5 with eotaxin-2, which shares the CCRX3 receptor with eotaxin-1, enhances the severity of disease by dramatically increasing airway eosinophilia [55]. Our data supports the idea that hematopoietic cells are the primary source of IL-5 necessary to drive airway eosinophilia during AAD and the contribution of stromal cell-derived IL-5 (e.g. BEC) is modest.

One of the striking pathologies of IL-5 transgenic mice is the presence of bronchus-associated lymphoid tissue (BALT) [30]. Accumulating in typically non-lymphoid areas, these structures are comprised primarily of B cells and may facilitate an efficient local immune response against infection or inflammation (reviewed, [56–58]). However, BALT was not observed in any group of IL-5 deficient BM chimeric mice. In fact, elevated levels of OVA-specific IgA were observed in serum from IL-5 deficient KO → KO BM chimeric mice as compared to WT → WT controls (Fig. 5D), even though IL-5 plays an important role in IgA production. While TGF-β1 is essential for inducing T cell dependent IgA class switching, IL-5 can enhance IgA production by sustaining B cell proliferation, differentiation, and survival [39–41]. Interestingly, using an OVA-induced model of asthma, a recent study has shown that IL-5 null mice produced similar or even higher levels of TGF-β1 in BALF as compared to wild-type controls [59]. In this study, peak levels of TGF-β1 were observed 7 days from the start of OVA-aerosol challenge. However, in our model, TGF-β1 levels in BALF were below the level of detection in all four IL-5 deficient BM chimeric groups at this time point (data not shown). Differences in our model systems (e.g. dose and duration of OVA-aerosol challenge) may have shifted the window of detection for TGF-β1. Furthermore, besides IL-5 and TGF-β1, several other cytokines have been reported to influence IgA production, including IL-2, IL-4, IL-6, and IL-10 (reviewed, [60]). Thus, it is possible that other changes in the local cytokine milieu may explain the elevated levels of IgA observed in KO → KO BM chimeric mice. Finally, we have observed that unlike OVA-specific IgG_1 and OVA-specific IgE, production of OVA-specific IgA in the OVA-induced model of AAD requires both OVA-sensitization and -challenge [34]. This implies that allergen exposure at the mucosal surface of the lung is necessary to trigger an IgA response. While it has been suggested that IgA may augment asthma pathology by disrupting the integrity of the airway epithelium through eosinophil degranulation [61,62], we propose an alternative possibility – that allergen-specific IgA is involved in disease resolution. Indeed, preliminary studies in our laboratories have demonstrated an increase in airway eosinophilia in chronically exposed mice that lack the polymeric Ig receptor, thereby exhibiting little to no secretory IgA on the mucosal surface (data not shown).

The primary function of the airway epithelium is as a sentinel, critical in regulating lung homeostasis. This study demonstrated that BEC are a source of IL-5 and secretion of this cytokine during AAD contributes to mucous metaplasia, airway eosinophilia, and allergen-specific IgA levels. Production of IL-5 by smooth muscle cells has been described [63,64] and we cannot exclude the possibility that these cells, in addition to BEC, contribute to IL-5 production in our KO → WT mice. Still, this study illustrates the ability of Th2 cytokines to influence the local microenvironment of the lung, thereby altering protective and inflammatory immune responses at the mucosal surface. Understanding the mechanisms of airway damage elicited directly or indirectly through IL-5 may provide important insights into the pulmonary pathogenesis associated with asthma and be instrumental in identifying new targets for therapy.

Acknowledgments

We gratefully acknowledge Dr. Leo Lefrançois for help with IL-5 intracellular staining, Dr. Michelle Cloutier for instruction in the isolation of BEC, and Dr. Roger Thrall for continued input on this model.

This work is supported by Grants HL68692 (C.A.W.) and HL080508 (L.P.) from the National Institutes of Health.

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