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Foa2 Programs Th2 Cell-Mediated Innate Immunity in the Developing Lung

Gang Chen,* Huajing Wan,† Fengming Luo,‡ Liqian Zhang,* Yan Xu,* Ian Lewkowich,§ Marsha Wills-Karp,¶ and Jeffrey A. Whitsett*

After birth, the respiratory tract adapts to recurrent exposures to pathogens, allergens, and toxicants by inducing the complex innate and acquired immune systems required for pulmonary homeostasis. In this study, we show that Foxa2, expressed selectively in the respiratory epithelium, plays a critical role in regulating genetic programs influencing Th2 cell-mediated pulmonary inflammation. Deletion of the Foxa2 gene, encoding a winged helix/forkhead box transcription factor that is selectively expressed in respiratory epithelial cells, caused spontaneous pulmonary eosinophilic inflammation and goblet cell metaplasia. Loss of Foxa2 induced the recruitment and activation of myeloid dendritic cells and Th2 cells in the lung, causing increased production of Th2 cytokines and chemokines. Loss of Foxa2-induced expression of genes regulating Th2 cell-mediated inflammation and goblet cell differentiation, including IL-13, IL-4, eotaxins, thymus and activation-regulated chemokine, Il33, Ccl20, and SAM pointed domain-containing Ets transcription factor. Pulmonary inflammation and goblet cell differentiation were abrogated by treatment of neonatal Foxa2Δ/Δ mice with mAb against IL-4Rα subunit. The respiratory epithelium plays a central role in the regulation of Th2-mediated inflammation and innate immunity in the developing lung in a process regulated by Foxa2. The Journal of Immunology, 2010, 184: 6133–6141.

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The sequences presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/query/) under accession number GSE19204.

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; a.u., arbitrary units; BALF, bronchoalveolar lavage fluid; Chia, acidic chitinase; DC, dendritic cell; E, embryonic day; EGFP, enhanced GFP; Eos, eosinophil; GO, gene ontology; Lym, lymphocyte; Mac, macrophage; mDC, myeloid dendritic cell; Neu, neutrophil; pDC, plasmacytoid dendritic cell; PN, postnatal day; qRT-PCR, quantitative RT-PCR; Spdef, SAM pointed domain-containing Ets transcription factor; Tarc, thymus and activation-regulated chemokine.

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differentiation (14, 17, 18). Recent studies demonstrated that goblet cells induced by pulmonary allergens are derived from the differentiation of resident Clara cells that serve as progenitor cells in the bronchiolar epithelium (18, 19). In initial studies, in which Foxa2 was deleted in the respiratory epithelium prior to birth, mucous cell metaplasia, alveolar remodeling, and inflammation were observed (14). The characteristics and mechanisms underlying lung inflammation caused by loss of Foxa2 in airway epithelial cells are unclear at present.

In this study, we demonstrate that respiratory epithelial cell-specific deletion of Foxa2 caused spontaneous Th2 cytokine/chemokine-mediated inflammation and goblet cell metaplasia. The hypothesis, that the inflammatory response was mediated by the spontaneous activation of IL-4R signaling, was tested using a mAb to block IL-4R signaling in neonatal mice. Deletion of Foxa2 induced expression of a network of genes influencing or associated with Th2-cell mediated inflammation and DC recruitment and activation, indicating the role of respiratory epithelial cell in programming inflammatory responses in the developing lung in a process regulated by Foxa2 and requiring IL-4R signaling.

Materials and Methods

Transgenic mice and animal husbandry

Animals were maintained according to protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Research Foundation (Cincinnati, OH). Mice were housed in a pathogen-free barrier facility in humidity- and temperature-controlled rooms on a 12:12 h light/dark cycle and were allowed food and water ad libitum. SFTPC-rtTetO-CRE/Foxa2Loxp/FlxLoxp compound transgenic mice were generated as previously described (14) and used to permanently delete Foxa2 in the fetal lung. Pregnant dams were maintained on doxycycline from embryonic day (E) 6.5 to E12.5 to delete Foxa2 from respiratory epithelial cells during fetal development, producing SFTPC/Foxa2Δ/Δ mice.

Conditional expression of Foxa2 in respiratory epithelial cells was achieved by producing tetO-Foxa2-ires-EGFP transgenic mice that were then mated to Scgb1a1-rta (line II) mice (20). Full-length rat Foxa2 coding sequence together with the 5′ untranslated region and 3′ untranslated region was isolated from pRC/CMV-Foxa2 (14) at EcoRI sites and cloning into the BamHI site of the pPetE-ires-EGFP vector (21) (the latter provided by Dr. Kenneth Campbell, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH) via blunt-end ligation. Transgenes were identified by PCR using the primer set: 5′-AGC AAA GAC CCC AAC GAG AAG C-3′ and 5′-CAA ACA ACA GAT GCC TGG CAA C-3′. Histology and immunohistochemistry

Immunohistochemistry was performed on 5-μm lung sections using rabbit anti-Foxa2 (1:2000–1:3000), guinea pig anti-Spdef (1:4000) generated in this laboratory, goat anti-Foxa3 (1:100; SC-5361, Santa Cruz Biotechnology, Santa Cruz, CA), mouse mAb against Muc5ac (1:50, ab3649, Abcam, Cambridge, MA) (18). Sections were processed with Ag retrieval using heat and citrate buffer. Anti-mouse IL-4Rα mAb (lot number 9382-25B, 11/19/02) (50 μg/ml body weight) or equal volume of sterile saline. On PN15, mice were anesthetized and the lungs lavaged five times with 0.3 ml saline, and the cells were observed (14). The characteristics and mechanisms underlying lung inflammation caused by loss of Foxa2 in airway epithelial cells are unclear at present.

Cytokine and quantitative RT-PCR assays

to determine cytokine levels, BALF was collected as previously described (22). BALF from each adult mouse was concentrated using 0.5 ml (VIVA SPIN4, catalog number VS0413, Sartorius, Bohemia, NY) and subjected to ELISA to determine the concentrations cytokines using the mouse IL-4, IL-5, IL-13, and IFN-γ ELISA kits from eBioscience, San Diego, CA (catalog numbers 88-7044, 88-7054, 88-7137, 88-7104, and 88-7384, respectively). The Cd17 ELISA kit was purchased from R&D Systems (MCC170, Minneapolis, MN). The ELISA was performed with an n of 6–8 mice of each genotype (Foxa2Δ/Δ mice and their littermate controls) at PN15. Whole-lung total RNA was purified by RNeasy Mini Kit (catalog number 74104, Qiagen, Valencia, CA) and reverse transcribed into cDNA by Verso cDNA kit (Applied Biosystems, Foster City, CA) with the TaqMan probes for Foxa2 (Mm00839704_m1), Il4 (Mm00445259_m1), Il5 (Mm00801778_m1) and normalized to Gusb (Mm9999915e_m1), Hprt (Mm9999915f_m1), 18s (Mm00445259_m1), Iil (Mm99999190_m1), Ccl11 (Mm00445259_m1), Ccl12 (Mm00445259_m1), Ccl17 (Mm01248426_m1), Il33 (Mm01195874_m1), Il6 (Mm00839704_m1), Ccl20 (Mm01268753_m1), and Tnf-α (Mm00081778_m1) and normalized to endogenous 18s RNA for control (probe part number 4352930E). qRT-PCR was performed with an n of 3 mice for each genotype at PN11 and PN15.

Flow cytometry

Lung cell suspensions were incubated with anti-CD16/32 (clone 2.4G2) for 30 min and then staining reactions were performed at 4°C. May-Grünwald (mDCs; CD11c+, CD11b−, Gr1−, and CD11b+Gr1−) and plasmacytoid DCs (pDCs; CD11c−, CD11b−, Gr1−, and CD11b+Gr1+) were identified using FACSCalibur (Becton Dickinson, San Jose, CA). For intracellular T cell activation, staining, lung cells were cultured overnight in the presence of PMA (100 ng/ml) and ionomycin (1 μg/ml). Cytokine secretion was blocked with a combination of brefeldin A and monensin (eBioscience) for 4 h. mRNA microarray analysis

Lung RNA was hybridized to the murine genome MOE430A chips (consisting of ≈45,000 gene entries, Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol. The RNA quality and quantity, probe preparation, labeling, hybridization, and image scans were carried out in the CCHMC Affymetrix Core using standard procedures. Affymetrix Microarray suite 5.0 (Affymetrix) was used to scan and quantitate the gene expression under default scan settings. Normalization was performed using the Robust Multichip Average model. Data were further analyzed using affylmGUI (http://bioinf.wehi.edu.au/affylmGUI) from R/Bioconductor package (www.bioconductor.org). Differentially expressed genes were selected with the threshold of p value of <0.01, fold change ≥1.5, and a minimum of two present calls in three samples with relatively higher expression. Gene ontology (GO) analysis was performed using the web-based tool DAVID (Database for annotation, visualization, and integrated discovery).

Overrepresented pathways were identified by comparison of the overlap of differentially expressed genes identified postdeletion of Foxa2 and all genes in MOE430A mouse genome. Genes sets associated with known pathways and disease states were identified from the Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg/), GenMAPP (www.genmapp.org/), and GEAarrays (www.sobiosciences.com/microarrays.php). A pathway was considered to be overrepresented when a probability p value was ≤0.01 and gene hits ≥5. Potential protein/protein or protein/DNA interactions were identified using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA). Ingenuity Pathway Analysis software maps the differentially expressed genes identified from the microarray experiment onto the interactome according to Ingenuity Pathway Knowledge Base, a large curated database of published literature findings related to mammalian biology. Genetic networks preferentially enriched in the sets of mRNAs were generated based on their connectivity. Statistical scores were calculated to rank the resulting networks and pathways using Fisher’s right-tailed exact test. The score indicates the degree of match to the network of a gene set, taking into account the number of network-eligible genes and the size of the network.
Cells were stained with PE-Cy7-conjugated anti-CD4 (RM4-5) and PE-conjugated anti-IL-13 (eBio13A). All mAbs were purchased from eBioscience. Data were acquired with an LSR II flow cytometer (BD Biosciences, San Jose, CA) equipped with lasers tuned to 488 nm, 633 nm, and 405 nm. Spectral overlap was compensated using the FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Plasmid and luciferase reporter assay**

The control plasmid used in luciferase reporter assay expressing enhanced GFP (EGFP) was made by isolation of the IRES-EGFP fragment from pIRE2-EGFP (catalog number 6029-1, Clontech, Mountain View, CA) by BamHI and NotI sites and subcloned into the BamHI site of the p3XFLAG-Myc-CMV plasmid (catalog number E6401, Sigma-Aldrich, St. Louis, MO) via blunt-end ligation. The Foxa2 expression plasmid was made by cloning rat Foxa2 cDNA from pRC/CMV-rFoxa2 using PCR primers: 5'-ATG AAT TCA ATG CTG GGA GCC GTG AAG-3' and 5'-ATG AAT TCG CGG AGC AGT TCA TAA TAGG-3' and inserting into the EcoRI site of the control vector. AccuPrime Taq DNA Polymerase (catalog number 12339-016, Invitrogen, Carlsbad, CA) was used for amplification of DNA fragments. The mouse Ccl17-pGL3 plasmid was cloned by amplification of the 2-kb promoter region from C57/B6 mouse genomic DNA using primers: 5'-GGA CCT GAA ATA GTC AGC ATCC-3' and 5'-CTG AGG TGA AGG TCT TCA TGGG-3' and cloned into the pGL3-basic plasmid (catalog number E1751, Promega, Madison, WI). The luciferase assay was performed by transfection of MLE-15 cells with the mCcl17-pGL3 plasmid along with either control plasmid-expressing EGFP or Foxa2-EGFP at a 1:1 ratio using the transfection reagent Lipofectamine 2000 (catalog number 11668-019, Invitrogen). CMV-βGal plasmid was cotransfected to serve as an internal control, and the luciferase activity was normalized to β-galactosidase activity after 24 h of transfection.

**Statistics**

Statistical differences in cell counts and concentrations of proteins assessed by ELISA were determined using Student t test (two-tailed and unpaired). The statistic method applied to analyze qRT-PCR was the Mood’s Median test. Difference between two groups was considered significant when the p value was <0.05 for all tests.

**Results**

**Conditional deletion of Foxa2 caused Th2 cell-mediated pulmonary inflammation**

To delete Foxa2 expression in respiratory epithelium, dams of SFTPC-rtTA/tetO7-CMV-Cre/Foxa2loxP/loxP transgenic embryos were treated with doxycycline from E6.5 to E12.5. Approximately 30% of the mutant mice died in the postnatal period as previously reported (14, 16). Surviving mice develop spontaneous pulmonary eosinophilic inflammation, goblet cell metaplasia, and airspace enlargement in the first 2 wk of life (Fig. 1A). On PN15, pulmonary inflammation was seen histologically and supported by quantitation of inflammatory cells in BALF. Numbers of eosinophils, lymphocytes, and macrophages were increased in BALF from Foxa2Δ/Δ mice (Fig. 1B). Deletion of Foxa2 was associated with increased expression of Th2 cytokines and chemokines, including IL-13, IL-4, IL-5, and Ccl17 (thymus and activation-regulated chemokine [Tarc]) as measured in BALF (Fig. 1C). CD4+ T cells that were recruited into the lung were further analyzed by flow cytometry, revealing a significant increase of the median fluorescent intensity of IL-13 and IL-17a and abundance of Th2 and Th17 cell populations in the lungs of Foxa2Δ/Δ mice. Expression mRNA of IL-6, a cytokine required for Th17 cell differentiation (23, 24), was significantly induced. In contrast, Ifn-γ mRNA was not altered (Supplemental Fig. 1). Taken together, Foxa2Δ/Δ mice develop spontaneous pulmonary inflammation that is typical of enhanced Th2 cytokine/chemokine activity during the postnatal period of development.

**FIGURE 1.** Deletion of Foxa2 in respiratory epithelium caused pulmonary eosinophilic inflammation and goblet cell differentiation. The Foxa2Δ/Δ mice spontaneously developed airway inflammation, eosinophilic infiltration, and goblet cell metaplasia at PN15. Loss of Foxa2 expression was confirmed by immunohistochemical staining. Increased eosinophils in BALF were revealed by Diff-Quik staining (A, original magnification ×400). Numbers of total cells, eosinophils, macrophages, lymphocytes, and neutrophils in BALF were assayed. Increased numbers of total cells and eosinophils were found in BALF from Foxa2Δ/Δ mice (B). Expression of Th2 cytokines (IL-4, IL-5, and IL-13) and chemokine Ccl17 (Tarc) was increased in BALF of Foxa2Δ/Δ mice as determined by ELISA (C). Inserts in A are the higher magnification of the regions indicated by arrows. The graphs represent means ± SE; n = 6 for each genotype. Scale bar, 50 μm. *p < 0.05, versus controls using Student t test (two-tailed, unpaired). Eos, eosinophil; Lym, lymphocyte; Mac, macrophage; Neu, neutrophil.
Inhibition of IL-4R–mediated signaling inhibited eosinophilic inflammation and goblet cell differentiation induced by Foxa2 deletion

A key pathway that regulates allergic inflammation and tissue remodeling in the airways involves the Th2 cytokines IL-4 and IL-13 and the activation of the IL-4Rα subunit (IL-4Rα) (25). IL-4 is primarily involved in promoting the differentiation and proliferation of Th2 cells and the synthesis of IgE, whereas IL-13 has a critical role in mediating airway hyperresponsiveness (AHR), goblet cell metaplasia, and mucus hypersecretion, the elements that are most closely linked to clinical manifestations of asthma. Blocking IL-4R signaling pathway with neutralizing Ab against IL-4Rα or mutagenesis of IL-4Rα was sufficient to inhibit responses to both IL-4 and IL-13 cytokines in vivo and in vitro (26–28). Specifically, loss of IL-4Rα in Clara cells inhibited mucus production after OVA intrapulmonary exposure in adult mice (29), and loss of IL-4Rα significantly decreased spontaneously induced mucus cells and eosinophilia in neonatal mice (PN10) (30). To test whether pulmonary inflammation and goblet cell differentiation caused by conditional deletion of Foxa2 in the airway epithelium was dependent upon IL-4R–mediated signaling, Foxa2Δ/Δ mice were treated with IL-4Rα mAb at PN2 and PN9. Eosinophilic inflammation was significantly inhibited by anti–IL-4Rα Ab, as shown by the reduction of inflammatory cells and eosinophils in BALF (Fig. 2A). Consistent with inhibition of Th2 inflammation, goblet cell metaplasia and mucus hyperproduction in Foxa2Δ/Δ mice were inhibited by the IL–4Rx Ab. Goblet cell markers, including Alcian blue, and Spdef, Foxa3, and Muc5ac staining were also inhibited by neutralizing Ab against IL-4Rx (Fig. 2B), demonstrating that the goblet cell metaplasia was dependent upon activated IL-4Rx signaling caused by deletion of Foxa2.

Loss of Foxa2 in respiratory epithelium enhances mDC recruitment and activation

Th2-cell mediated inflammation is dependent on activation and migration of pulmonary DCs to the respiratory epithelium (31). As professional APCs, DCs are uniquely capable of fully activating naive T cells. In the lung, two distinct phenotypes of DCs have been identified that have markedly different effects on T cell function. mDCs (defined as CD11c+, CD11b+, Gr1neg, and CD317neg cells) promote robust T cell activation and efficiently promote allergen-induced AHR (32). In contrast, pDCs (defined as CD11clow, CD11bneg, Gr1+, and CD317+ cells) promote the development of Foxp3+ regulatory T cells, preventing allergen-induced AHR (33). Thus, to test the hypothesis that excessive Th2 cell activation observed in mice lacking Foxa2 in the respiratory epithelium was preceded by dysregulation in pulmonary DC recruitment or activity, we assessed mDC and pDC recruitment and activation at PN8, PN11, and PN15 by flow cytometry. Comparing the

![FIGURE 2. Inhibition of IL-4R–suppressed goblet cell differentiation and pulmonary inflammation in Foxa2Δ/Δ mice. IL-4Rx mAb was administered i.p. at PN2 and PN9. Eosinophilic inflammation was inhibited, shown by reduced total cell and eosinophil numbers (A). Goblet cell differentiation was inhibited by Ab treatment, indicated by lack of Spdef, Muc5ac, and Foxa3 staining in Foxa2Δ/Δ mice (B). Inserts show the higher magnification (×46) of eosinophils in perivascular regions pointed by arrows (left panel insets). B, H&E, Spdef and Muc5ac panels were taken at ×100 and Foxa3 panels were taken at ×400. Scale bar, 50 μm. Graphs represent means ± SD. n = 6 for each genotype. *p < 0.01, versus controls using Student t test (two-tailed, unpaired).](http://www.jimmunol.org/bwz/0170136727a.pdf)
frequencies of DC subsets in control and Foxa2Δ/Δ mice revealed a significant increase in the frequency of both mDCs and pDCs found in the lung of Foxa2Δ/Δ mice (Fig. 3A). Although both subsets of DCs were found with greater frequency in the lungs of Foxa2Δ/Δ mice, there was a substantial increase in the mDC/pDC ratio in Foxa2Δ/Δ mice, particularly at PN15, suggesting an environment better suited to T cell activation in Foxa2Δ/Δ mice. Comparing the expression of DC activation-associated costimulatory molecule expression on mDCs from control and Foxa2Δ/Δ mice demonstrated significantly elevated frequencies of mDCs expressing CD80 (Fig. 3B), B7-H1 (Fig. 3C), and CD86 (Fig. 3D). mDCs expressing CD80 were also increased in Foxa2Δ/Δ mice, although changes were not statistically significant. MHC class II was not different from control and Foxa2Δ/Δ mice (Supplemental Fig. 2). Interestingly, although pDCs were also generally more activated in Foxa2Δ/Δ mice than in control mice, by PN15, a time when there was robust activation of T cell cytokine production, the levels of pDC activation had decreased and were indistinguishable from controls (Fig. 3B–D). Collectively, these data demonstrate that concomitant with the increased T cell activation, the recruitment and activation of pulmonary mDCs was increased in Foxa2Δ/Δ mice. Expression of the cytokine mRNAs, including Il4 and Il13, was significantly increased in the lungs of Foxa2Δ/Δ mice at PN11 and PN15 (Fig. 4B, Supplemental Fig. 3). Mechanisms underlying the recruitment and activation of DCs in the lung are not well established at present. The IL-7–like cytokine stem cell factor (SCF), produced by epithelial cells, is known to influence DCs expressing CXCL12, causing differentiation of naive CD4+ T cells to DCs that produce IL-4, IL-13, and TNF but not IL-10 (34, 35). Similarly, CCL20 is a chemokine produced in epithelial cells and capable of inducing DC migration via interaction with CCR6 expressed on the immature DCs (36, 37). Ccl20, but not Tslp mRNA expression, was induced in the lungs of Foxa2Δ/Δ mice at PN15 (Supplemental Fig. 4), suggesting a potential mechanism by which DCs are recruited and activated in the lungs of Foxa2Δ/Δ mice. Although Tslp mRNA was not increased in whole lung at PN11 or PN15 (data not shown), it remains possible that time-dependent or focal changes in its expression would not be detected in the current study design.

**FIGURE 3.** Loss of Foxa2 in respiratory epithelium enhanced recruitment and activation of mDCs. The percentages of both mDCs and pDCs were increased in the lung of Foxa2Δ/Δ mice at PN11 to PN15 (A). Although both subsets of DCs were found with greater frequency in the lungs of Foxa2Δ/Δ mice, there was a substantial increase in the mDC/pDC ratio in Foxa2Δ/Δ mice. To compare the activation of pulmonary mDCs and pDCs in control and Foxa2Δ/Δ mice, expression of costimulatory molecules was assessed in gated populations by flow cytometry. Frequencies of mDCs expressing B7-DC (B), B7-H1 (C), and CD86 (D) were increased in Foxa2Δ/Δ mice at PN11 to PN15. Collectively, these data demonstrate that, concomitant with the increased T cell activation observed in Foxa2Δ/Δ mice, there was enhanced recruitment and activation of pulmonary mDCs compared with control mice. n = 8 at PN8; n = 5 at PN11; and n = 4 at PN15 of each genotype. Graphs represent means ± SE. *p < 0.05; **p < 0.01; ***p < 0.001, versus controls as determined by Student t test (two-tailed, unpaired).
Epithelial cells lining the respiratory and gastrointestinal tract play a pivotal role in initiation, regulation, and resolution of innate and adaptive immune response by expressing a wide range of immune response genes including costimulatory molecules, chemokines, cytokines, and PGs (38). Of particular interest, respiratory epithelium expresses IL-33, a cytokine that promotes Th2 cytokine production (39) and CCL17 (40, 41) that chemoattracts Th2 cells via interactions with CCR4 that is selectively expressed on the Th2 cells (42). Expression of \textit{Il33} and \textit{Ccl17} mRNAs was significantly induced in the \textit{Foxa2}Δ/Δ mice at PN15 (Fig. 4B). CCL17 (Tarc), a potent T cell chemoattractant induced in bronchiolar epithelial cells of asthmatics (40, 41), likely plays an important role in the pulmonary inflammation seen in the \textit{Foxa2}Δ/Δ mice. The effects of \textit{Foxa2} on Tarc gene expression were assessed in MLE-15 cells in vitro. \textit{Foxa2} inhibited the Tarc promoter by \∼40\% (Supplemental Fig. 5).

\textbf{Conditional expression of Foxa2 in the respiratory epithelium inhibited allergen-induced goblet cell differentiation}

A transgenic mouse in which \textit{Foxa2} was conditionally expressed in the respiratory epithelium was produced to test whether increased expression of \textit{Foxa2} was sufficient to inhibit allergen-induced inflammation or goblet cell differentiation (Supplemental Fig. 6). Double-transgenic mice \textit{Scgb1a1-rtTA/tetO7-Foxa2-IRES-EGFP} (\textit{Scgb1a1/Foxa2-IE}) were treated with doxycycline, and the induction of \textit{Foxa2} and EGFP expression was confirmed by immunohistochemistry and fluorescence microscopy (Fig. 5A, Supplemental Fig. 6). Goblet cells, although normally rare in the surface epithelium lining the conducting airways of adult mice, are usually present in young mice (30, 43). Expression of \textit{Foxa2} from PN4-PN18 by doxycycline treatment blocked the normal postnatal goblet cell differentiation (Supplemental Fig. 7). Conditional expression of \textit{Foxa2} in the respiratory epithelium in adult mice inhibited goblet cell differentiation (Fig. 5B, Supplemental Fig. 7).

\textbf{FIGURE 4.} Deletion of \textit{Foxa2} in respiratory epithelial cells influenced expression of mRNAs mediating Th2-like inflammation and goblet cell differentiation. A, Lung cRNAs were produced from \textit{Foxa2}Δ/Δ and control littermate at PN15 (\(n = 3\) of each genotype) and hybridized to murine genome MOE430 chips. Affymetrix microarray analysis (Affymetrix) revealed increased mRNAs delete expression of Th2 cytokines, chemokines including \textit{Il4}, \textit{Il13}, \textit{Ccl17}, \textit{Ccl22}, \textit{Ccl11}, and \textit{Ccl24}, as well as goblet cell-associated genes \textit{Clca3} and \textit{Agr2} postdeletion of \textit{Foxa2}. B, Expression of mRNAs of Th2 cytokines (\textit{Il4}, \textit{Il5}, \textit{Il13}, and \textit{Il33}) and chemokines (\textit{Ccl11}, \textit{Ccl12}, and \textit{Ccl17}), were increased in \textit{Foxa2}Δ/Δ mice at PN15 as detected by qRT-PCR. Graphs represent mean ± SEM at a.u.s. \(n = 3\) for each genotype for qRT-PCR assay. *p < 0.05, versus control determined by Mood’s Median test. a.u., arbitrary unit.
postnatal development, but does not directly control Th2-mediated 
Foxa2 also plays an important role in modulating Th2 immunity in 
differentiation (14, 16). The present findings demonstrate that 
observations supporting the role of Foxa2 in regulating epithelial cell 
mDCs and Th2 lymphocytes were increased in 
chemokines, and their receptors. Recruitment and activation of 
plasia and enhanced expression of Th2 cell-associated cytokines, 
neous pulmonary inflammation associated with goblet cell meta-
serts are the higher magnification (3). Scale bar, 50 

FIGURE 5. Foxa2 inhibited allergen-induced goblet cell differentiation. Scgb1a1/Foxa2-IE transgenic mice were treated with doxycycline for 3 d, increasing Foxa2 expression as detected by immunohistochemistry using a high dilution of Foxa2 Ab at 4 wk of age (A, original magnification ×100). Foxa2 was not prominent at this Ab dilution in single transgenic Scgb1a1-rtTA or Scgb1a1/Foxa2-IE (without doxycycline) transgenic mice. Doxycycline was administered to Scgb1a1-rtTA and Scgb1a1/Foxa2-IE transgenic mice 1 d before intranasal OVA exposure. Foxa2 markedly in-
hhibited goblet cell differentiation as shown by decreased Alcian blue, 
Muc5ac, Spdef, and Foxa3 staining (B, original magnification ×100). In-
serts are the higher magnification (×4) of the regions indicated by arrows. 
Scale bar, 50 μm. Figures are representative of n = 3 for each genotype.

Fig. 8). However, expression of Foxa2 in respiratory epithelial 
cells of the mature lung prior to OVA sensitization did not alter 
Th2 cytokine production or inflammation. Inflammatory cell 
counts (total or differential), as well as IL-4, IL-5, IL-13, IL-10, 
and IFN-γ concentrations, were similar in BALF from Foxa2-
expressing and control mice after OVA exposure (Supplemental 
Fig. 9A, 9B). These finding are consistent with previous ob-
servations supporting the role of Foxa2 in regulating epithelial cell 
differentiation (14, 16). The present findings demonstrate that 
Foxa2 also plays an important role in modulating Th2 immunity in 
postnatal development, but does not directly control Th2-mediated 
inflammatory responses in the mature lung.

Discussion

Deletion of Foxa2 in respiratory epithelial cells caused sponta-
neous pulmonary inflammation associated with goblet cell meta-
plasia and enhanced expression of Th2 cell-associated cytokines, 
chemokines, and their receptors. Recruitment and activation of 
mdCs and Th2 lymphocytes were increased in Foxa2Δ/Δ mice lungs and associated with increased Th2 cytokines (IL-4, IL-5, 
and IL-13) and Ccl17 (Tarc) production and Foxa2 inhibiting Tarc 
gene promoter activity in vitro. The inflammatory effects of Foxa2 
deletion were inhibited by blocking IL-4R signaling in the de-
veloping mouse lung. Taken together, the respiratory epithelium, 
via Foxa2, plays a critical role in programming the innate immune 
system during postnatal development of the lung and serves to 
inhibit the development of Th2-dominated innate immunity.

Th2 cytokine-dominated inflammation postdeletion of Foxa2 in 
the lung

Allergic airway inflammation is characterized by recruitment and 
avtivation of Th2 lymphocytes and eosinophils, goblet cell meta-
plasia, and mucus hyperproduction in a process regulated by 
a number of cytokines and chemokines. A number of the com-
ponents of the IL-4R signaling pathway (e.g., IL-4, IL-13, and 
IL-4Rα) were increased in Foxa2Δ/Δ mice. IL-13 is produced 
primarily in Th2 cells and regulates many asthma-related pro-
cesses, including mucus hyperproduction, eosinophil recruitment 
and survival, and airway hyperreactivity (44). IL-13 blockade abrogated many of the features of asthma (45), demonstrating that 
IL-13 is an important mediator in Th2 responses and asthma 
pathogenesis. The present observation, that inhibition of IL-4Rα 
signaling substantially inhibited pulmonary eosinophilic inflam-
mation and goblet cell metaplasia postdeletion of Foxa2, supports 
the important role of Foxa2 in inhibiting development of Th2-
mediated innate immunity that is dependent upon IL-4R signaling. 
Consistent with the observed Th2-mediated inflammation in 
F Foxa2Δ/Δ mice, a number of factors involved in T cell and eosi-

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mRNAs associated with eosinophilic inflammation were induced in lungs of Foxa2Δ/Δ mice, including Ccl11, Ccl24, Epx, Ifggb, Kng1, Lgtas3, Lycs4, Alox5, Aryl1, Il1rn, and Rag1. Among these, eosinophil-specific chemokines Ccl11 and Ccl24 were increased 7- to 12-fold, respectively. Epx mRNA (eosinophil peroxidase), a heme-containing glycoprotein that may contribute to the pathogenesis of epithelial damage and bronchial hyperreactivity in human asthma (47), was markedly increased in Foxa2Δ/Δ mice. Eosinophil major basic protein (Prg2, 11-fold) and eosinophil-associated R-Nase (Earl1, 90-fold) mRNAs were also dramatically increased.

Th2-associated inflammatory mediators, including acidic chitinase (Chia), chitinase 3-like 1 (Chi3l1), chitinase 3-like 3 (Chi3l3), and chitinase 3-like 4 (Chi3l4) mRNAs, were increased in Foxa2Δ/Δ mice. Chitinases and chitinase-like proteins are believed to play a key role in the innate immunity to parasites and other infectious agents and may play an important role in the pathogenesis of allergy and/or asthma and regulation of eosinophilia and ectaion induction (48–50). Chia is induced via a Th2-specific, IL-13-mediated pathway in lung epithelial cells and macrophages and is increased in lungs of asthematics (48). Chia stimulates chemokine production by pulmonary epithelial cells (50).

Deletion of Foxa2 from lung epithelium disrupted Th1-Th2 balance, inducing a large number of Th2 cytokines and associated signaling pathways. Il6 mRNA, a cytokine regulating Th1, Th2, and Th17 cell differentiation (51, 52), was increased 4.6-fold in Foxa2Δ/Δ mice. Recent studies demonstrated that Foxa2 bound to the Il6 promoter and suppressed Il6 expression in liver (53). Because multiple Th1-associated genes are altered in the Foxa2Δ/Δ mice, it is presently unclear which play the dominant roles in the observed phenotype. Of genes expressed in the respiratory epithelium, both Il33 and the chemokine Ccl17 were significantly induced postdeletion of Foxa2. IL-33 and CCL17 both promote Th2 cytokine responses (38, 39) and are expressed in respiratory epithelial cells. CCL17 attracts Th2 lymphocytes to mucusosal sites (54) and is increased in the bronchial epithelium of patients with asthma (55). The present finding, that Tarc promoter activity in MLE-15 cells was inhibited by Foxa2 in vitro, provides a potential mechanism by which Foxa2 influences Th2 cell recruitment and activation in the lung.

Role of Foxa2 in the regulation of pulmonary DCs

A network of DCs is closely associated with the respiratory epithelium (56). Both mDCs and pDCs are present in the lung, but mDC subsets generally dominate the airway mucosa (57). In the current study, both mDCs and pDCs were detected in the lungs of normal neonatal mice, although mDCs were typically more abundant than pDCs. We previously demonstrated that the numbers of mDCs present typically exceed pDCs by 10–25-fold in the adult mouse lung (58). Thus, in neonatal mice, the lung appears to be a more tolerogenic environment, capable of promoting the development of T regulatory rather than activated T effector cells. In further support of this concept, we observed that although the level of mDC activation increased from PN8 to PN15 in control mice, the activation status of pulmonary pDCs peaked at PN11 and decreased thereafter, suggesting that between PN11 and PN15, there is a shift toward the activation of a population of pulmonary DCs better suited to promote T cell activation. Thus, in the absence of overt immunization, the neonatal lung may represent a generally suppressive environment, favoring the development of tolerance rather than overt immune responses. Collectively, these data demonstrate that, concomitant with the increased T cell activation observed in Foxa2Δ/Δ mice, there is also greater recruitment and activation of pulmonary mDCs in the absence of Foxa2. Fig. 6 summarizes the present findings regarding mechanisms by which Foxa2 influences lung inflammation in the developing mouse.

Goblet cell differentiation caused by deletion of Foxa2 in the developing lung seen in the current study was associated with Th2 cell activation and the induction of Spdef and Foxa3 that appear to function in a transcriptional network in the respiratory epithelium (18). Consistent with recently published studies (59), we demonstrated that forced expression of Foxa2 in the adult mouse inhibited goblet cell differentiation, blocking Spdef and Foxa3 but did not influence allergen-induced inflammation or eosinophilic infiltration. These findings support the role of Foxa2 in the instruction of innate immunity during development rather than a direct role for Foxa2 in suppressing inflammation during OVA sensitization in the mature mouse.

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References


