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Hox5 paralogous genes modulate Th2 cell function during chronic allergic inflammation via regulation of *Gata3*[†]

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Abstract

Allergic asthma is a significant health burden in western countries, and continues to increase in prevalence. T helper 2 (Th2) cells contribute to the development of disease through release of the cytokines interleukin (IL)-4, IL-5 and IL-13, resulting in increased airway eosinophils and mucus hypersecretion. The molecular mechanisms behind the disease pathology remain largely unknown. In this study we investigated a potential regulatory role for the *Hox5* gene family, *Hoxa5*, *Hoxb5* and *Hoxc5*, genes known to be important in lung development within mesenchymal cell populations. We found that *Hox5*-mutant mice show exacerbated pathology compared to wild-type controls in a chronic allergen model, with an increased Th2 response and exacerbated lung tissue pathology. Bone marrow chimera experiments indicated that the observed enhanced pathology was mediated by immune cell function independent of mesenchymal cell Hox5 family function. Examination of T cells grown in Th2 polarizing conditions showed increased proliferation, enhanced *Gata3* expression and elevated production of IL-4, IL-5 and IL-13 in *Hox5*-deficient T cells compared to wild-type controls. Overexpression of FLAG-tagged HOX5 proteins in Jurkat cells demonstrated HOX5 binding to the *Gata3* locus and decreased *Gata3* and IL-4 expression, supporting a role for HOX5 proteins in direct transcriptional control of Th2 development. These results reveal a novel role for *Hox5* genes as developmental regulators of Th2 immune cell function that demonstrates a redeployment of mesenchyme-associated developmental genes.

Introduction

Allergic asthma is characterized by inflammation and bronchiole constriction, which over time leads to adverse remodeling of the airways. In the United States, 1 in 11 children and 1 in 12 adults has asthma, resulting in over 3000 deaths per year with healthcare costs exceeding $50 billion. Annually, there are nearly 500,000 asthma-related hospitalizations and nearly 2 million visits to emergency departments due to asthma, while asthma-related doctor visits approach 9 million (1). Treatment is generally limited to anti-inflammatories

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and inhaled corticosteroids to reduce both the severity and frequency of episodes, without providing a short-term or long-term cure. Allergic asthma is characterized by a Th2 response, which is associated with increased levels of the cytokines IL-4, IL-5 and IL-13 (2–5). The Th2 phenotype is driven by IL-4, which binds to the IL-4 receptor composed of two subunits, IL-4Rα and IL-2Rγ (6, 7). Binding of IL-4 leads to phosphorylation of STAT6, which then homodimerizes and translocates into the nucleus to drive production of the Th2-associated transcription factors, especially GATA3 (8, 9). GATA3 in turn binds to the promoter regions of *Il4*, *Il5* and *Il13* genes. Our lab and others have found that characteristic clinical features of allergic asthma can be replicated in mice following sensitization and challenge with allergen (10–12). While the disease pathologies have been primarily related to immune associated proteins, the regulation of the Th2 responses is incompletely understood.

Hox genes encode transcriptional regulatory factors that are primarily necessary for patterning the body axis and many organ systems (13–15). All mammals have four Hox clusters (HoxA, B, C and D), divided into 13 paralogous groups. Each group has between two and four paralogs, with a total of 39 genes (16). *Hox* genes within each cluster are tightly linked and expressed sequentially from 3′ to 5′. The genes near the 3′ end are generally expressed earlier during development at anterior segments of the embryo, while the genes closer to the 5′ end are expressed later and in more posterior segments of the embryo. All *Hox* genes contain a homeodomain, which is a helix-turn-helix DNA-binding domain composed of 60 amino acids (17–19). This domain is highly conserved among species and preserves function (20). The three members of the *Hox5* paralogous group have been shown to be important in the development of the lung, especially *Hoxa5* (21). Mice with complete loss in the *Hoxa5* gene are minimally viable, with less than 20% of offspring surviving past birth due to defects in the respiratory tract (22), with increased alveolar space and increased numbers of goblet cells in the trachea and primary bronchi (23). Within a paralogous group, there is often significant functional redundancy that allows loss-of-function of one gene to be partially or completely compensated by the remaining members of that group. In the *Hox5* family the *Hoxb5* and *Hoxc5* genes cannot completely compensate for a loss of function of *Hoxa5* with mice lacking all three members of the Hox5 paralogous family have severely affected lungs and are not viable (24).

The role of *Hox* genes in immune cell differentiation and mature immune cell function has not been explored. The majority of research has been focused on *Hoxa9* in leukemia, where translocation of the mixed lineage leukemia (*MLL*) gene leads to overexpression of *Hoxa9* (25). A role for *Hox* genes in hematopoiesis has also been suggested including development of erythrocytes, monocytes and lymphocytes (26, 27). There is less information about the expression of *Hox* genes in mature immune cells. Expression of various *Hox* genes, including the *Hox5* family, has been detected in both naïve and stimulated T lymphocytes, (28, 29). However, the function of these genes in T cells remains unexplored. In this study, we examine the role of *Hox5* genes in the lungs during the development of allergic airway disease. We further explore *Hox5* gene function in Th2 cells, a major driver of allergic disease, and we report a novel role for these genes in T cell differentiation.

Materials and Methods

Mice and allergen challenge

Hox5AabbCc mice and WT controls were bred at the University of Michigan. GATA3-GFP reporter mice (*Gata3*$^{g/+}$) on the C57BL/6 background were provided by Dr. James Douglas Engel (30). The University Committee of Use and Care of Animals (University of Michigan) approved all animal experimental protocols, and experiments were conducted according to the guidelines provided by the committee. Eight to 12-week old mice were sensitized subcutaneously and intraperitoneally with a 1:1 mixture of cockroach antigen (CRA) and incomplete Fruend's adjuvant (IFA). Mice were challenged with 15 ul of CRA (Greer, Lenoir, NC, USA) intranasally at days 14, 18, 22 and 26, then with 50ul of CRA intratracheally on days 30 and 32. Animals were sacrificed either six hours after each challenge or 24 hours after the final challenge.

RNA isolation and qPCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Lung tissue was homogenized in TRIzol prior to extraction. Complementary DNA was synthesized using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and incubated at 37°C for one hour, followed by 95°C for 5 minutes to stop the reaction. Real-time quantitative PCR was multiplexed using Taqman primers with a FAM-conjugated probe and GAPDH with a VIC-conjugated probe (Applied Biosystems) to measure transcription of murine *Hoxa5*, *Hoxb5*, *Hoxc5* and *Gata3* and human *Gata3* and *Il4, IL5* and *IL13*. Fold change was quantified using the $2^{-\Delta\Delta CT}$ method or expression was measured relative to GAPDH using the $2^{-\Delta CT}$ method. Custom primers were designed to measure *Muc5ac* and *Gob5* mRNA levels. All reactions were run on an ABI Prism 7500 Sequence Detection System or ViiA 7 Real Time PCR System (both from Applied Biosystems).

Histolopathology

Lungs were removed after the final allergen challenge. The large left lobe of each lung was inflated by injection with 4% formaldehyde. Lungs were embedded in paraffin, and 5 μm sections were sectioned and stained with hematoxylin and eosin (H&E) to visualize inflammatory cells or with periodic acid-Schiff stain (PAS) to visualize mucus production.

Immunohistochemistry (IHC)

For section IHC, adult lungs were dissected, processed, embedded in paraffin and sectioned as described above for histopathology. Tissue sections were stained with the following antibodies and dilutions: rabbit anti-CCSP (Seven Hills Bioreagents; 1:1000); mouse anti-acetylated tubulin (Sigma-Aldrich; 1:1000); mouse anti-Muc5ac (Novocastra; 1:75). Primary antibodies were detected by using either fluorescently conjugated (Alexafluor; Invitrogen) or Alkaline Phosphatase conjugated (Jackson ImmunoResearch) secondary antibodies (1:500).

Flow cytometry

Lungs and mediastinal lymph nodes were removed and single cells were isolated by enzymatic digestion with 1 mg/ml collagenase A (Roche, Indianapolis, IN, USA) and 20

U/ml DNaseI (Sigma, St. Louis, MO, USA) in RPMI containing 10% FCS. Cells were resuspended in PBS and live cells were identified using LIVE/DEAD Fixable Yellow Dead Cell Stain kit (ThermoFisher Scientific, Waltham, MA, USA), then were washed and resuspended in PBS with 1% FCS and Fc receptors were blocked with purified anti-CD16/32 (clone 93; BioLegend, San Diego, CA, USA). Surface markers were identified using antibodies (clones) against the following antigens, all from BioLegend,: CD3ε (17A2), CD4 (RM4–5), CD69 (H1.2F3), CD8 (53–6.7), and Gr-1 (RB6-8C5). SiglecF (E50–2440) was purchased from BD Biosciences (San Jose, CA, USA). Intracellular staining was performed using an anti-IL-4 antibody (11B11, eBioscience, San Diego, CA, USA) and an anti-Gata3 antibody (TWAJ, eBioscience). Total T cells were gated on $CD3^+$, followed by $CD4^+$ and $CD8^+$ single positive, $CD4^+CD69^+$ to gate activated T cells, or $CD4^+IL\text{-}4^+$ to gate IL-4-producing T cells and $CD4^+Gata3^+$ to gate *Gata3*-producing T cells.

Cytokine production from lymph nodes

Mediastinal lymph nodes were removed and single cells were isolated by enzymatic digestion. 5 x 10^5 cells were plated in 200 μl of complete medium (RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, non-essential amino acids, sodium pyruvate, 2-mercaptoethanol) and were restimulated with CRA for 48 hours. Supernatants were collected and levels of the cytokines IL-4, IL-5, IL-13, IL-17 and IFN-γ were measured by Bioplex assay (Bio-Rad, Hurcules, CA, USA).

Bone marrow transplantation

Hox5AabbCc mice and WT controls were irradiated twice with 500 rad using a ^{137}Cs irradiator, with four hours between each irradiation. Four hours after the second irradiation, mice were injected intravenously with 5x10^6 bone marrow cells from either *Hox5AabbCc* or WT donor mice. Bone marrow cells were allowed to engraft for 12 weeks before mice were used for experimental models.

In vitro skewing of Th subsets

Cells were removed from spleens of naïve mice and a single cell suspension was made by homogenization. Following lysis of red blood cells, $CD4^+$ T cells were isolated using a negative selection kit (Miltenyi Biotec, San Diego, CA, USA). Cells were cultured in complete medium in 96-well plates that had been coated with 2.5 μg/ml of α-CD3 for 2 hours at 37°C. For primary skewing, T cells were activated with 3 μg/ml α-CD3 for 48 hours or 5 days to drive Th0 differentiation. In addition to soluble α-CD28, the following skewing conditions were used: Th1 cells – rIL-12 (100 ng/ml) and α-IL-4 (10 μg/ml), Th2 cells – rIL-4 (100 ng/ml) and α-IL-12 (10 μg/ml) and α-IFN-γ (10 μg/ml), and Th17 cells – rIL-6 (100 ng/ml), rTGF-β (20 ng/ml), α-IL4 (10 μg/ml), α-IL-12 (10 μg/ml) and α-IFN- γ (10 μg/ml). For secondary skewing, cells were rested in complete medium for three days, then restimulated with plate-bound α-CD3 and soluble α-CD28 for 48 hours. For skewing of Jurkat cells, the cells were expanded in RPMI with 10% FCS and penicillin/streptomycin. Following transfection (see following section), cells were cultured on plate-bound α-CD3 in the presence of soluble α-CD28, α-IL-12 and recombinant human IL-4. All antibodies were purchased from eBiosciences, and all recombinant cytokines were purchased from R&D Systems (Minneapolis, MN, USA).

Transfection of Jurkat cells

Jurkat cells were grown in DMEM containing 10% FCS and penicillin/streptomycin. Cells were then transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, cells were plated at 1 x 10^6 cells/ml in 5 ml DMEM with 10% FCS. Lipofectamine 2000 was incubated in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA) for 5 minutes at RT, then 8 ug of DNA was added for an additional 20 minutes. The complexes were then added to the cells and incubated at 37°C for 24 hours.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using an assay kit (Millipore, Billerica, MA, USA) with minor modifications. Briefly, cells were fixed in 1% formaldehyde, then lysed in SDS buffer. Cells were then sonicated using a Branson Digital Sonifier 450 (VWR, West Chester, PA, USA) to create 200–1000 bp fragments. The lysate was clarified by centrifugation, and 5% of the supernatant was saved to measure the input DNA. The remaining chromatin was incubated with 1 μg of anti-FLAG antibody (Abcam, Cambridge, MA, USA) or control IgG (Millipore) and incubated at 4°C with rotation overnight. Immune complexes were precipitated with salmon sperm DNA/protein A agarose beads. Crosslinking was reversed by incubation at 65°C and samples were treated with proteinase K. DNA was purified by phenol:chloroform:isoamyl alcohol separation and ethanol precipitation. Primers for putative STAT6 binding sites on the *Gata3* gene were designed and DNA was amplified by qPCR using SYBR Green buffer (Applied Biosystems). The primers for amplifying DNA are as follows:

STAT6 1: Forward *gcggagaagcatttttcatt* Reverse *gtttctcctgagcccacttg*

STAT6 2: Forward *gcgtcctctaccctgctgt* Reverse *cccctaagacacaaaattcca*

STAT6 3: Forward *tgtgtggatttgcacttgct* Reverse *ataacgtaagccccatgcac*

Statistical analysis

Results are expressed as mean ± SE. Statistical significance was measured by one-way or two-way ANOVA as appropriate followed by post-hoc Student's *t* test as appropriate. A *p* value of <0.05 was considered significant.

Results

Adult Hox5 AabbCc compound mutant mice exhibit morphological defects in epithelial cells in the proximal airway

A previous study has shown that *Hox5* genes (*Hoxa5*, *Hoxb5*, *Hoxc5*) play redundant roles in lung development and that the most severe phenotypes result from the loss of function of all three *Hox5* genes (31). Gene expression analysis by qPCR revealed that all three *Hox5* genes continue to be expressed during adult stages at levels comparable to those observed during embryonic lung development (Fig 1A). Because *Hox5* triple mutants die shortly after birth, we analyzed surviving compound loss-of-function *Hox5* mutants of the genotype $Hoxa5^{+/-};Hoxb5^{-/-};Hoxc5^{+/-}$ (*Hox5 AabbCc*) for possible lung defects at adult stages. IHC analyses demonstrated that the proximal airway of adult *Hox5 AabbCc* mutants have fewer

acetylated tubulin+ ciliated cells compared to controls (Fig 1B). While muc5ac+ goblet cells are normally not observed in the proximal airway of adult wild-type animals, these cells are present in *Hox5 AabbCc* mutants (Fig 1B). Thus, *Hox5* genes continue to be expressed in the adult lung and play important roles in the proper distribution of the epithelial cells of the proximal airway, a mechanism that may impact lung function and airway disease.

Hox5 genes are upregulated in the lungs following allergen challenge

Since *Hox5* genes are critical for lung development and continue to be expressed in the adult lung, we hypothesized that these genes would also be redeployed during chronic disease and are important in the development of allergic airway disease. Our studies initially determined whether *Hox5* gene expression was altered in the lungs of mice exposed to allergen. Mice were sensitized by intraperitoneal and subcutaneous injection of CRA, and challenged into the airway at days 14, 18, 22, 26, 30 and 32 (Fig. 2A). Mice were sacrificed six hours after each challenge, and RNA was extracted from total lung tissue. We found that *Hoxa5*, *Hoxb5* and *Hoxc5* genes were differentially upregulated following allergen challenge, with higher levels of gene expression after the final intratracheal challenges with *Hoxc5* especially prominent (Fig. 2B). The upregulation of *Hox5* gene expression suggests that these genes play a role during the development of disease.

Hox5-deficient mice have increased Th2 inflammation in the lungs

To determine a potential role for *Hox5* genes in the development of chronic allergic disease, we used the *Hox5AabbCc* mice with the CRA model shown in Fig. 2A, and sacrificed mice 24 hours following the final challenge. Hematoxylin and eosin staining of sections was used to visualize inflammation in WT and *Hox5AabbCc* mice, while mucus was visualized by Periodic Acid Schiff staining (Fig. 3A). The observed increase in mucus production was verified by PCR analysis of lung tissue that showed an increase in the mucus-associated genes *Muc5ac* and *Gob5* (Fig. 3B). When treated with allergen, the lungs of these mice demonstrate significantly increased infiltration of inflammatory cells and increased mucus production compared to WT controls. Flow cytometric analyses of the inflammatory cells show a significant increase in the number of $CD4^+$ cells in the lungs, with no change in $CD8^+$ cells (Fig. 3C). As Th2 cells drive allergic inflammation in the lungs, we measured the transcription factor Gata3 in T cells from the lung tissue, and found increased numbers of $Gata3^+CD4^+$ T cells in *Hox5AabbCc* mice (Fig. 3D). In addition, other inflammatory cells associated with allergic airway disease including neutrophils and eosinophils were significantly increased (Fig. 3E). Cells were removed from the mesenteric lymph nodes (MLN) and restimulated *ex-vivo* with CRA, resulting in increased Th2 cytokines IL-4, IL-5 and IL-13 in the *Hox5AabbCc* mice, with no change in IFN-γ production (Fig. 3F). Furthermore, the number of IL-4-producing $CD4^+$ T cells was greater in the MLN from *Hox5AabbCc* mice compared to WT controls, as was the level of IL-4 production from individual T cells as measured by mean fluorescence intensity (MFI) (Fig. 3G). Together, these data suggest that the *Hox5* genes are important in controlling the development of pathology in allergic airway disease.

Increased inflammation in *Hox5AabbCc* mice is due to changes in immune cell function

The alteration of the immune response in the above studies suggests that Hox5 paralogous genes may be contributing to the increased inflammation observed in the *Hox5AabbCc* mice. To address this question, we performed reciprocal bone marrow transplantation experiments, in which bone marrow cells from *Hox5AabbCc* mice were transplanted into irradiated WT mice, and WT bone marrow cells were transplanted into *Hox5AabbCc* mice. Following a 12 week engraftment period, these mice were then sensitized and challenged with CRA as in Fig. 1A. H&E staining of lung sections demonstrated that those mice that received *Hox5AabbCc* bone marrow cells had increased inflammation over mice that received WT bone marrow, regardless of whether the cells were transplanted into WT or *Hox5AabbCc* mice (Fig. 4A). Furthermore, the increased inflammation again corresponded with an increase in $CD4^+$ T cells, including activated $CD4^+CD69^+$ cells (Fig. 4B), while no changes were seen in the numbers of $CD8^+$ T cells or antigen presenting dendritic cells (data not shown). Restimulation of MLN cells with CRA resulted in increased production of IL-4, IL-5 and IL-13, but no changes in IFN-γ (Fig. 4C). Similar to the results from the CRA-treated Hox5AabbCc mice, those mice that received Hox5AabbCc bone marrow had an increase in expression of the mucus-associated genes *Muc5ac* and *Gob5* in the lungs over mice that were injected with WT cells (Fig. 4D), which corresponded to increased mucus in the lungs of these mice (Fig. 4E).

T cells from *Hox5AabbCc* mice produce more Th2 cytokines when skewed *in vitro*

We observed an increase in the number of $CD4^+$ T cells in both the *Hox5AabbCc* mice and WT mice that had been injected with *Hox5AabbCc* bone marrow cells. In addition, many of these cells had an activated phenotype. We therefore asked whether the $CD4^+$ T cells from these mice produced increased levels of Th2 cytokines. We began by investigating whether the expression of the three *Hox5* genes was increased in Th2 cells. We sorted naïve $CD4^+$ T cells from the spleens of WT mice and skewed them *in vitro* toward Th0, Th1, Th2 and Th17 lineages using standard skewing assays (32, 33). Cells were harvested at 2, 6, 24 and 48 hours after skewing, and results showed that *Hox5* gene expression was increased in cells skewed toward the Th2 lineage, but not under other skewing conditions, although this increase was not significant in *Hoxa5*-transfected cells (Fig. 5A). We found that Hoxc5 expression is increased to the greatest degree in these cells, and that gene expression occurs early following IL-4 stimulation, but remains elevated through 48 hours (Fig. 5A). To determine whether the expression of the *Hox5* genes affected Th2 cytokine production, naïve $CD4^+$ T cells were isolated from the spleens of WT and *Hox5AabbCc* mice, and were again skewed to Th2 cells *in vitro*. We found that five days after skewing cells in the presence of IL-4, the levels of IL-4, IL-5 and IL-13 were increased in the cultures from the *Hox5AabbCc* T cells compared to WT T cells (Fig. 5B). The cells were rested for three days, and then restimulated with α-CD3 and α-CD28 to determine whether they maintained increased Th2 cytokine production upon secondary activation. After an additional 48 hours of activation, T cells isolated from *Hox5AabbCc* mice continued to produce increased levels of Th2 cytokines (Fig. 5C). The increase in cytokine production from *Hox5AabbCc* T cells was limited to Th2 cells, as no differences were seen upon primary activation of T cells skewed toward Th1 or Th17 with regard to production of IFN-γ or IL-17, respectively (Fig. S1). Furthermore, the transcription of *Gata3*, a master transcription factor for Th2 cytokine

production, was increased in T cells from *Hox5AabbCc* mice compared to WT controls at both 48 hours and 5 days (Fig. 5D). *Gata3* transcription is activated by the IL-4-induced transcription factor STAT6, which becomes phosphorylated and binds to *Gata3* and other genes. However other STAT6-activated genes such as *c-maf* and *Irf4* were not increased, suggesting that the activation and phosphorylation of STAT6 was not affected (Fig. S2). Finally, to confirm the increased expression of Gata3, we crossed both WT and *Hox5AabbCc* mice with Gata3-GFP reporter mice and skewed splenic naïve $CD4^+$ T cells toward the Th0 and Th2 lineages. We found that Gata3 gene expression was higher in *Hox5AabbCc* mice compared to WT mice 5 days after skewing (Fig. 5E). Representative plots from Th2 skewed cells are shown in Figure 5F.

HOX5 proteins bind to the *Gata3* gene and interfere with STAT6 binding

Our previous results suggest that *Hox5* deficiency results in increased *Gata3* expression that modifies Th2 cytokine production. In order to understand how HOX5 proteins regulate Th2 cells, we used the human Jurkat T cell line to overexpress WT HOX5 proteins. Plasmids expressing FLAG-tagged *Hox5* constructs were transfected into Jurkat cells either individually or together in equal concentrations. We then pulled down the HOX5 proteins by ChIP using an anti-FLAG antibody. We designed primers to amplify putative consensus STAT6 binding sites conserved in humans and mice on the *Gata3* gene (34) at sites that also contain the consensus binding sequence for HOX5 proteins. The location of these sites relative to the transcription start site and gene exons is shown in Fig. 6A. We found that HOX5 proteins bound significantly to two of the three STAT6 binding sites that we assayed (Fig. 6B). When we transfected the *Hox5*-expressing plasmids individually, we found that HOXC5 protein bound more efficiently to the *Gata3* promoter at both STAT6 binding sites relative to HOXA5 and HOXB5 (Fig. 6C). We then confirmed that the binding of the HOX5 proteins resulted in decreased expression of *Gata3* and IL-4. The *Hox5*-expression plasmids were transfected into Jurkat cells and the cells were cultured under Th2 skewing conditions for 24 hours. Decreased *Gata3* expression was observed in cells transfected with *Hoxc5* alone, as well as with all three *Hox5*-expression plasmids (Fig. 6D). Downstream of *Gata3*, we found a similar expression pattern of IL-4 and IL-13 in the transfected cells, although IL-5 was only decreased in cells that were transfected with all three *Hox5*-expressing plasmids (Fig. 6E). Together, these data suggest that the HOX5 proteins bind to the *Gata3* promoter and that HOXC5 binds the most efficiently and thereby affects Th2 cytokine production.

Discussion

Although Hox genes have been identified in some hematopoietic populations, little has been done to describe their function or their role in immune cell populations. In this study, we demonstrate a novel role for the HOX5 paralogous group of proteins in regulating Th2 inflammation in a model of allergic lung disease. These studies highlight the ability of this gene family, primarily described as a mesenchyme associated developmental transcription factor, to be redeployed in T cells for regulation of Th2 cell development. The association of Hox5 proteins with *Gata3* expression is especially key, as it designates the Hox5 proteins to the Th2 lineage, without directly altering any of the other T cell responses or total number of

T cells. The functional significance of this fidelity is not presently clear, however, it appears to be associated with STAT6 binding sites on the *Gata3* gene, an aspect that we continue to explore with the generation of additional tools. Together these studies define several important and novel aspects: 1) Hox5 paralogous proteins provide a discrete regulatory function for the generation of Th2 cells, 2) the absence of Hox5 regulatory control in T cells leads to enhanced Th2 disease pathology *in vivo*, and 3) the function of *Hox5* genes in regulating Th2 cell development is, at least, via the repression of *Gata3* expression. These striking data establish a novel paradigm to be further investigated for the role of HOX proteins in immune cell regulation that controls disease progression.

The HOX5 proteins are critical for lung development, particularly HOXA5. *Hoxa5*$^{-/-}$ mice do not thrive, primarily due to defects in alveolar development due to mesenchyme-associated defects (21–23). The mice used in this study have one mutated *Hoxa5* allele, and these mice thrive without evidence of major defects. While they do have a noticeable defect in alveolarization, this defect does not lead to an altered immune response to CRA as evidenced by our data using bone marrow chimeras. These mice do not have functional *Hoxb5* and previous studies have shown that there is some functional redundancy between *Hoxb5* and *Hoxa5* during lung development (24). While *Hoxc5* has conflicting roles in lung development (24, 31), we found *Hoxc5* to be most highly upregulated in the lung tissue after allergen challenge. We also found that *Hoxc5* was the most highly upregulated in the Th2 cell cultures. And while both *Hoxa5* and *Hoxb5* were able to bind to *Gata3*, our ChIP results show that *Hoxc5* was able to bind at a higher rate. These data may suggest some compartmentalization of *Hox5* paralog function that could separate their function based upon cell specific effects and the maturation state of the host.

Our results suggest a role for the HOX5 proteins in regulating the activation and/or differentiation of Th2 cells specifically, as other subsets of T cells were not altered upon differentiation. In the absence of the HOX5 proteins, an increase in *Gata3* gene expression and Th2 cytokine production was observed. Furthermore, transfecting plasmids expressing Hox5 genes into Jurkat cells results in decreased *Gata3* expression, indicating that HOX5 proteins are able to repress *Gata3*. Several other studies have identified a repressive role for HOX proteins, particularly during development. For example, activation of *Hoxa13* has been shown to down-regulate signaling through the Wnt/β-catenin pathway, which is important in controlling elongation of the anteroposterior axis during development, a result that is replicated by over-expression of *Hoxa13* (35). Other work in both *Drosophila* and mice has shown that the HOX proteins activated during the development of the posterior embryo are able to repress genes that are needed for anterior development (36–38). In addition, different HOX proteins can activate or repress the same gene in different tissues, and this function has been attributed to the non-homeodomain of the protein (39). The mechanism of HOX5 protein function in this study is presently unclear but presents a potentially important and new paradigm.

The mechanism by which HOX proteins affect target gene activation or repression is poorly understood. All HOX proteins recognize a similar AT-rich sequence *in vitro*. Studies in which the homeodomain of one protein was swapped for the domain of another have demonstrated that these domains are at least partially able to confer some degree of

specificity *in vivo* (40, 41). Additionally, the sequences flanking the homeodomain have also been shown to be important in DNA binding (42). Others have shown that the N-terminus of various HOX proteins is important in determining gene specificity in both flies and mammals (39, 43). Furthermore, cofactors that bind to HOX proteins also affect the specificity of the HOX protein for certain target genes and not others (44–46). Finally, the ability of HOX proteins to interact with other transcription factors to target the binding to specific promoter and/or enhancer elements has been suggested to be important for tissue and cell specific functions (47, 48). Our results demonstrate that the HOX5 proteins are able to bind to *Gata3* associated gene elements. While it has been shown that STAT6 binds to the *Gata3* gene in mouse Th2 cells, the activation of *Gata3* in human T cells is not as well defined (34, 49, 50). Our results suggest that HOX5 proteins binding to the *Gata3* gene at STAT6 binding sites downregulate its activation, although whether this is directly due to disrupted STAT6 binding or occurs through indirect mechansims is unknown. It is likely that the HOX5 proteins are acting in concert with other elements to target them to the *Gata3* locus. The identity of these elements remains a subject of investigation and may include STAT6 itself.

This study demonstrates a role for HOX5 proteins in controlling lung inflammation during a model of allergic asthma. The increased inflammation in the *Hox5*-deficient mice was mediated by increased Th2 cell development, which was associated with regulation of *Gata3* expression. These results imply a novel role for the HOX5 paralogs in T cell function leading to regulation of allergic disease and opens up a new area of study in T cell gene regulation. Further studies may lead to not only a better understanding of Th2 associated disease progression but also identify unappreciated roles for HOX proteins beyond development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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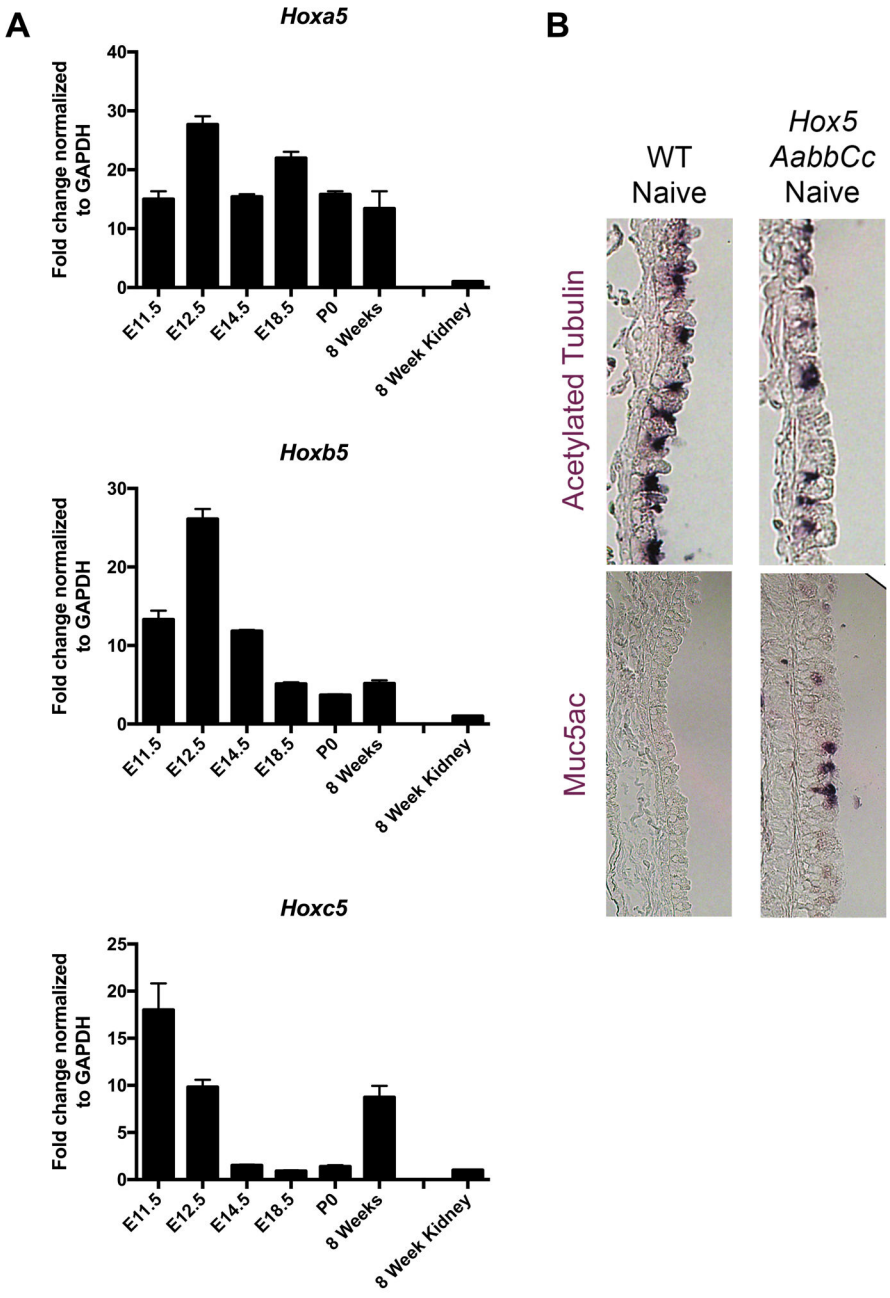

Figure 1. ***Hox5*** **gene expression and proximal airway cell distribution in the adult lung**

(A) Lung expression of Hoxa5, Hoxb5 and Hoxc5 was determined at different stages of embryonic development and at 8 weeks of age by qPCR. Kidney samples were included as a negative control. (B) Sections of adult lung tissue were stained with Muc5ac to measure goblet cells and acetylated tubulin to measure ciliated cells. n = 5 mice/group.

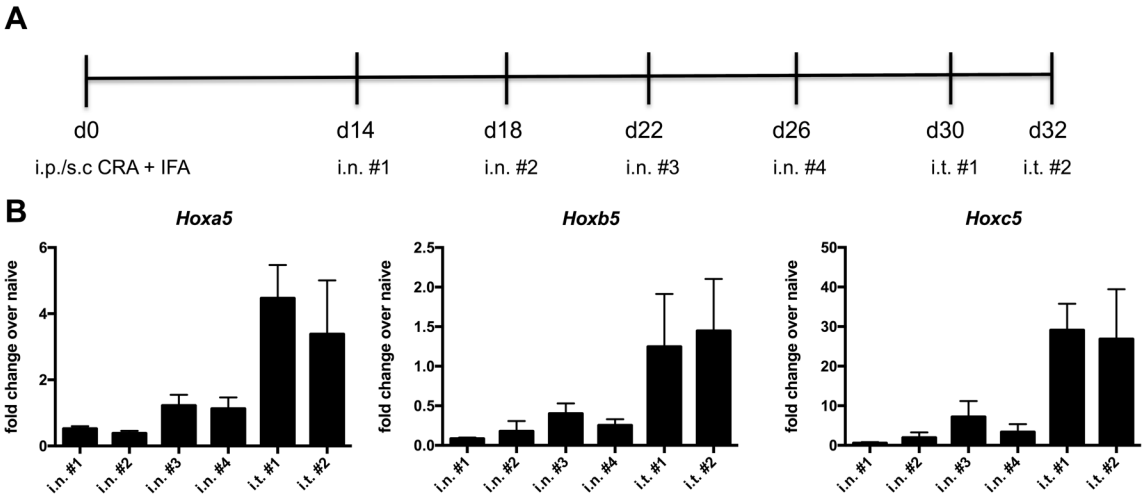

Figure 2. *Hox5* gene expression is upregulated following allergic challenge

(A) Mice were sensitized with cockroach allergen (CRA) mixed with incomplete Fruend's adjuvent by intraperitoneal and subcutaneous injections, then challenged with four intranasal and two intratracheal instillments of CRA. (B) Mice were sacrificed six hours after each challenge, and total RNA was extracted from the lungs. Expression levels of *Hox5* genes was assessed using qPCR. Results are representative from one of two independent experiments with 5 mice/group.

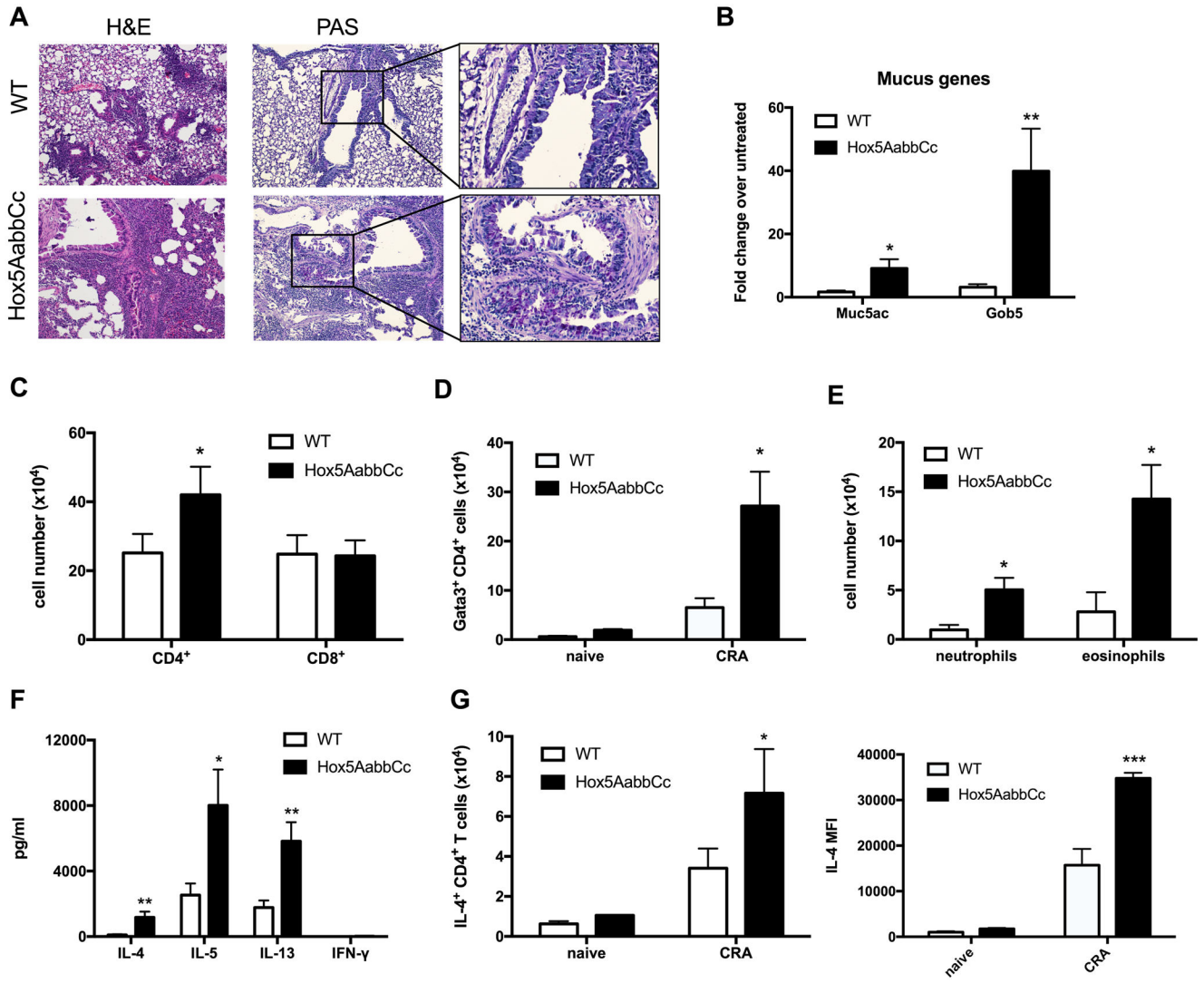

Figure 3. *Hox5AabbCc* mice have increased Th2 inflammation following allergic challenge

(A) Hox5AabbCc mice and WT controls were treated with CRA as in Fig. 1A. Lung sections were stained with hematoxylin and eosin to visualize inflammation and Periodic Acid Schiff to visualize mucus. (B) RNA was extracted from lung tissue and expression of the mucus-associated genes *Muc5ac* and *Gob5* was measured by qPCR. (C) Lung tissue was dissociated using Collagenase A and DNaseI. $CD4^+$ and $CD8^+$ T cells in the lungs were characterized by flow cytometry. (D) Expression of Gata3 in lung $CD4^+$ T cells was measured by intracellular cytokine staining. (E) Neutrophil and eosinophil numbers were measured in lung tissue by flow cytometry following tissue dissociation. (F) Mediastinal lymph nodes were removed and single cells were re-stimulated with CRA. Cytokine production was measured by bioplex after 48 hours. (G) The number of IL-4-producing $CD4^+$ T cells and the mean fluorescence intensity were determined by intracellular flow cytometry. (E) *p<0.05, **p<0.01, ***p<0.001 compared to WT. Results from one of three independent experiments. n= 4–5 mice/group.

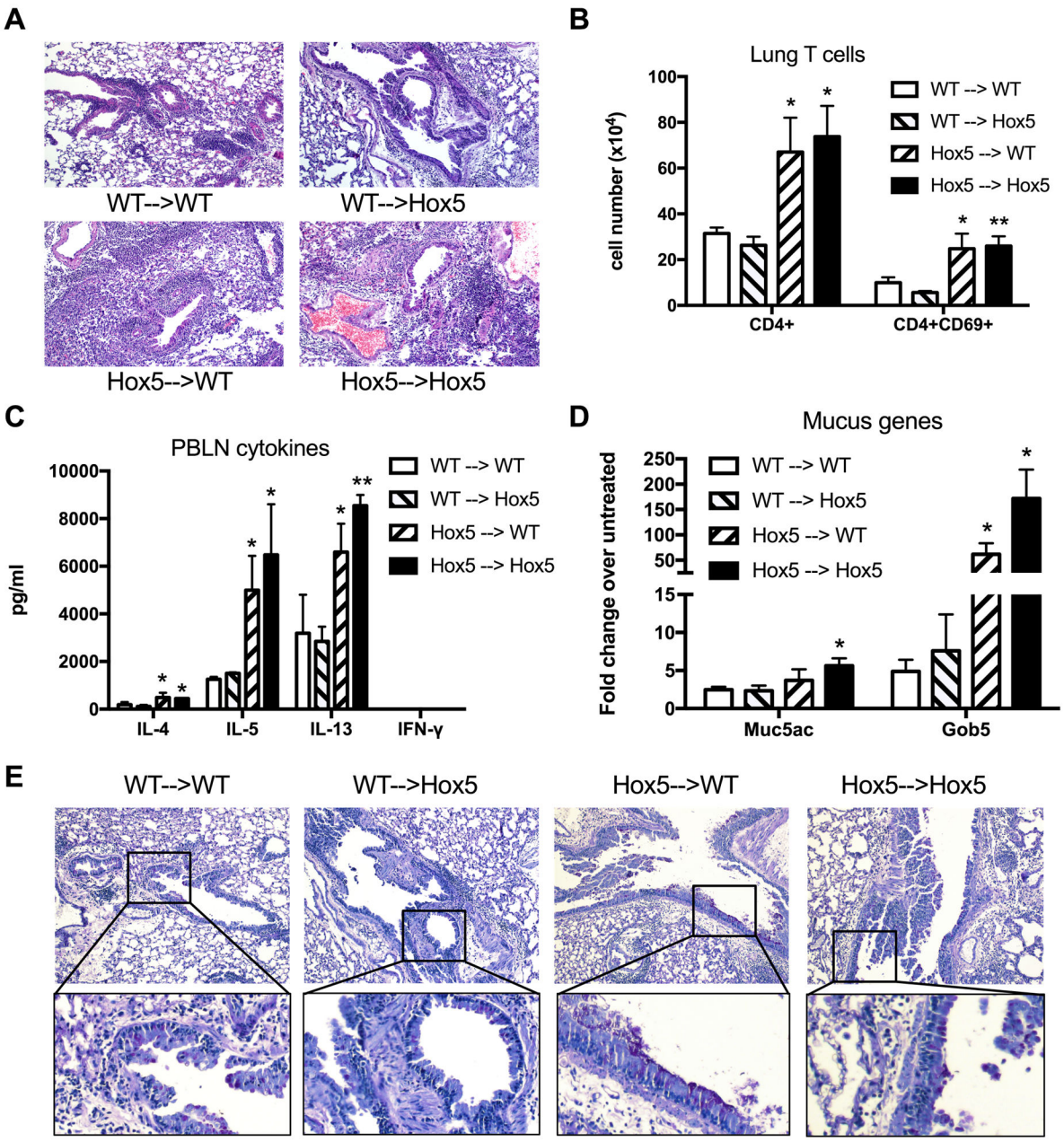

Figure 4. Increased inflammation in *Hox5AabbCc* mice is driven by differences in immune cell function

WT and *Hox5AabbCc* mice were irradiated and reconstituted with bone marrow cells. (A) Inflammation was visualized on lung sections stained with hematoxylin and eosin. (B) Lung cells were analyzed by flow cytometry. (C) Cells from the mediastinal lymph node were re-stimulated in vitro with CRA for 48 hours. Levels of cytokines in the supernatants were measured by bioplex. (D) RNA was extracted from lung tissue and the genes *Muc5ac* and *Gob5* were measured by qPCR. (E) Lung sections were stained with Periodic Acid Schiff staining to visualize mucus. *p<0.05, **p<0.01 compared to WT→ WT mice. Results represent one of two independent experiments. n = 3–5 mice/group.

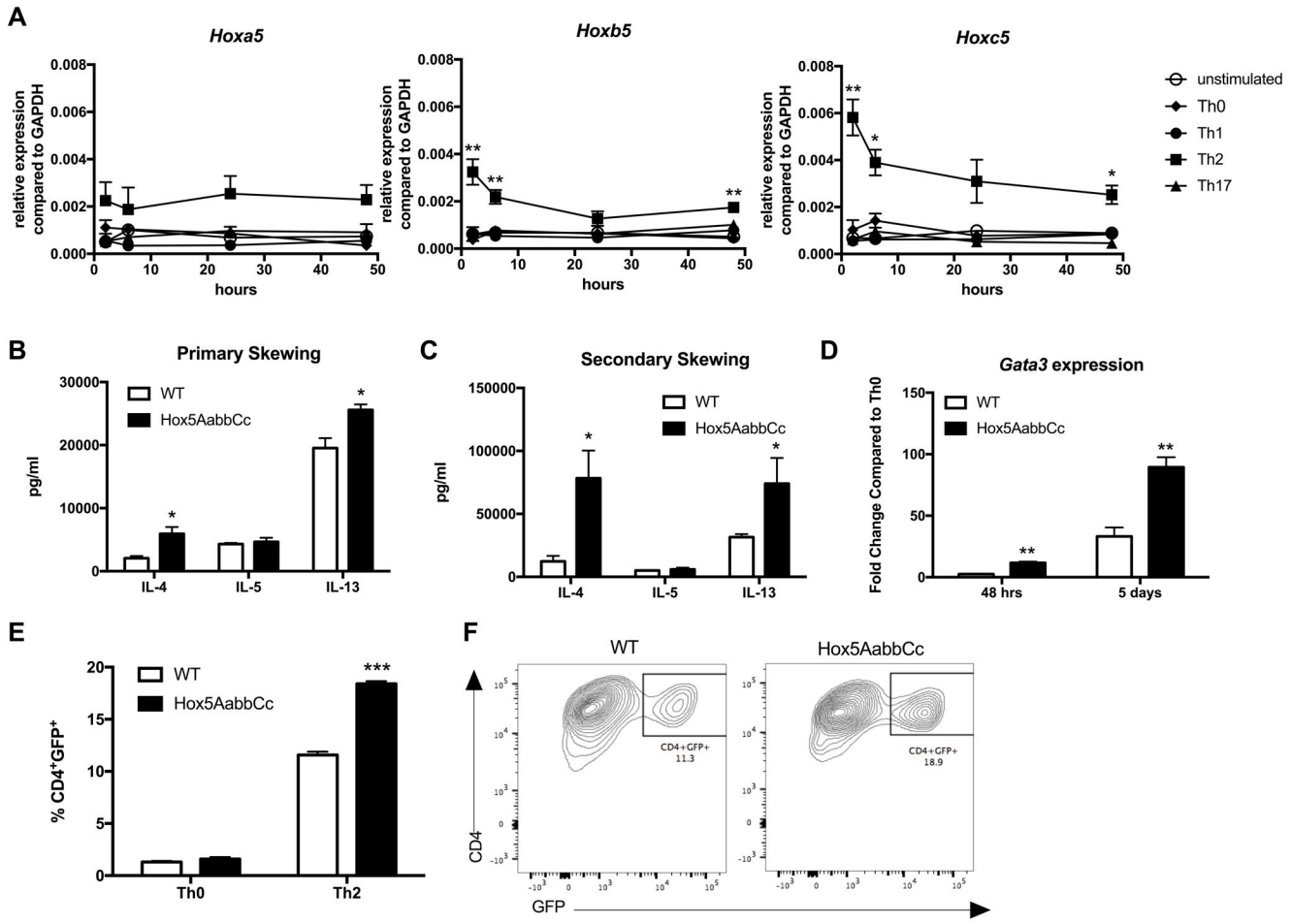

Figure 5. CD4$^+$ T cells from *Hox5AabbCc* mice produce more Th2 cytokines under IL-4 skewing conditions

(A) Naïve T cells were isolated from the spleens of WT mice and were skewed toward Th0, Th1, Th2, Th17 and Treg lineages. Hox gene expression was measured in each lineage by qPCR, and expression was compared to GAPDH using the $2^{-\Delta CT}$ method. Analysis was done by two-way ANOVA, followed by post-hoc *t* test. *p<0.05, **p<0.01 for Th2 compared to Th0 at each time point. (B) Purified T cells from WT or *Hox5AabbCc* mice were cultured under Th2 skewing conditions for five days, and supernatant cytokines were measured by bioplex. *p<0.05 compared to WT cells. (C) T cells were skewed for five days, then washed and rested for three days. The cells were then further stimulated with α-CD3 and α-CD28 for 48 hours to reactivate the cells. Cytokines were measured by bioplex. *p<0.05 compared to WT. (D) T cells were skewed for 48 hours or 5 days in Th2 conditions. RNA was extracted and *Gata3* gene expression was measured by qPCR. **p<0.01 compared to WT cells. Results are representative of three independent experiments. (E) WT and Hox5AabbCc mice were crossed with Gata3-GFP reporter mice, then were cultured under Th2 skewing conditions for five days. GFP expression was measured by flow cytometry. Representative plots are shown in (F). ***p<0.001 compared to WT. Results are from one of two independent experiments. Three samples were run per group, and samples were run in triplicate.

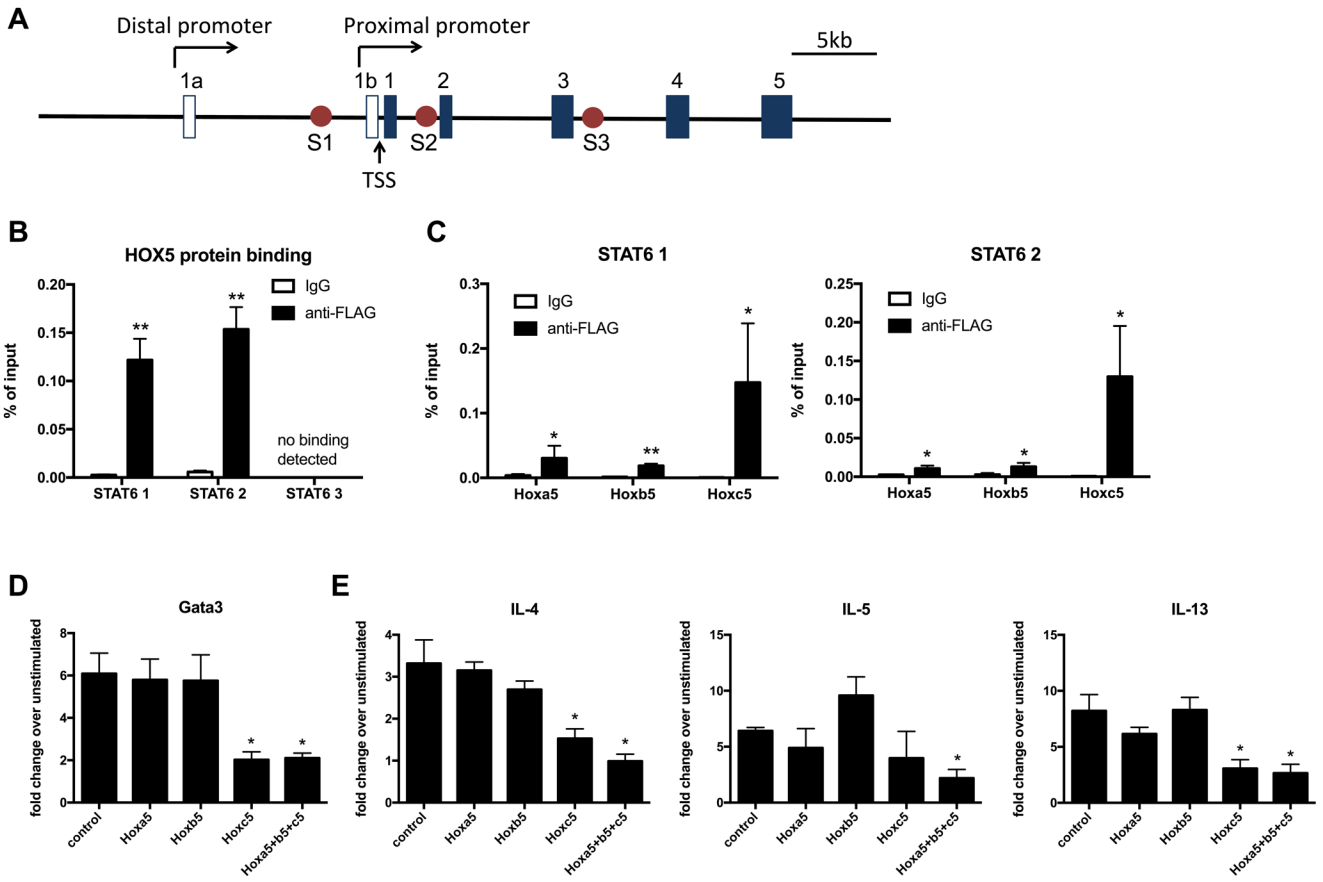

Figure 6. HOX5 proteins interact with the *Gata3* gene at STAT6 binding sites

(A) The Gata3 gene exons are shown in blue boxes and STAT6 binding sites assayed are shown as red circles. The proximal and distal promoters are marked, as well as the transcriptional start site (TSS). (B) Equal concentrations of FLAG-tagged *Hox5*-expressing plasmids were transfected into Jurkat cells for 24 hours, then ChIP was performed using an anti-FLAG antibody. Primers were designed for putative STAT6 binding sites on the *Gata3* gene, and DNA bound to the HOX proteins was amplified by qPCR. (C) Individual Hox5 plasmids were transfected into Jurkat cells and ChIP was done with an anti-FLAG antibody. qPCR was performed on the first two STAT6 binding sites identified in Fig. 5A. *p<0.05, **p<0.01 compared to IgG control. (D) Jurkat cells were transfected with lipofectamine only (control), or with plasmids expressing *Hoxa5*, *Hoxb5*, *Hoxc5* or with all three together (Hoxa5+b5+c5). After 24 hours, cells were then cultured under Th2 skewing conditions. *Gata3* gene expression was measured after a further 24 hours. (E) IL-4, IL-5 and IL-13 gene expression were measured after 24 hours of Th2 skewing conditions. *p<0.05 compared to lipofectamine control. Results are representative of three independent experiments. Three samples were run per group, and samples were run in triplicate.