Published in final edited form as:

J Allergy Clin Immunol. 2012 April ; 129(4): 1000–10.e3. doi:10.1016/j.jaci.2011.12.965.

Activin A and TGF- β promote T_H9 cell-mediated pulmonary allergic pathology

Carla P. Jones, PhD*, Lisa G. Gregory, PhD*, Benjamin Causton, BSc, Gaynor A. Campbell, PhD, and Clare M. Lloyd, PhD

Leukocyte Biology Section, Faculty of Medicine, National Heart and Lung Institute, Imperial College, London, United Kingdom

Abstract

Background—IL-9-secreting (T_H9) T cells are thought to represent a distinct T-cell subset. However, evidence for their functionality in disease is uncertain.

Objective—To define a functional phenotype for T_H9-driven pathology *in vivo*.

Methods—We used fluorescence-activated cell sorting to identify circulating T_H9 cells in atopic and nonatopic subjects. In mice we utilized a model of allergic airways disease induced by house dust mite to determine T_H9 cell function *in vivo* and the role of activin A in T_H9 generation.

Results—Allergic patients have elevated T_H9 cell numbers in comparison to nonatopic donors, which correlates with elevated IgE levels. In a murine model, allergen challenge with house dust mite leads to rapid T_H9 differentiation and proliferation, with much faster kinetics than for T_H2 cell differentiation, resulting in the specific recruitment and activation of mast cells. The TGF- β superfamily member activin A replicates the function of TGF- β 1 in driving the *in vitro* generation of T_H9 cells. Importantly, the *in vivo* inhibition of T_H9 differentiation induced by allergen was achieved only when activin A and TGF- β were blocked in conjunction but not alone, resulting in reduced airway hyperreactivity and collagen deposition. Conversely, adoptive transfer of T_H9 cells results in enhanced pathology.

Conclusion—Our data identify a distinct functional role for T_H9 cells and outline a novel pathway for their generation *in vitro* and *in vivo*. Functionally, T_H9 cells promote allergic responses resulting in enhanced pathology mediated by the specific recruitment and activation of mast cells in the lungs.

Keywords

Activin A; T_H9 cells; IL-9; asthma; allergy; mast cells; house dust mite; TGF- β

IL-9, a signature cytokine involved in asthma, is secreted by a range of resident and infiltrating cells and has been described as a candidate gene during genetic linkage analysis.^{1,2} IL-9 is important for the development of disease pathology, particularly for the survival and differentiation of mast cells.^{3–5} Although originally described as a T_H2 cytokine, IL-9 is produced by a distinct subset of effector T cells, termed T_H9 cells.^{3,6–8} These cells have been generated *in vitro* in an environment of IL-4 and TGF- β 1,^{7,8} but

direct evidence for their existence *in vivo* is lacking. Although T_H9 cells lack expression of either T bet or GATA-3, the defining transcription factors for T_H1 or T_H2 lineage development, respectively, PU.1 and interferon regulatory factor 4 have both been associated with T_H9 cell differentiation.^{9,10} IL-25 maximizes T_H9 differentiation, due to the expression of its receptor, interleukin-17 receptor B (IL-17RB), on the cell surface.^{11,12} Importantly, this pathway was shown to be important for the regulation of IL-9 expression during allergic airway inflammation, since IL-25 deficiency leads to reduced IL-9 expression and airway inflammation.¹³

There is a wealth of evidence to show that IL-9 is involved in the development of allergic responses.^{5,14,15} Many cells are able to secrete IL-9, but the specific cellular source has not been determined *in vivo*.^{16,17} T_H9 cells have been implicated in the development of disease in the central nervous system,³ the lung,¹⁰ and the eye¹⁸; however, it is not clear how they actually contribute to pathology in these organs. In particular, the specific contribution that T_H9 cells might make during the development of allergic immune responses remains unclear.

The TGF- β superfamily member activin A plays a vital role during allergic responses, promoting enhanced remodeling and airway hyperreactivity (AHR) via interactions with IL-25.¹⁹ This interaction between activin A and IL-25 coupled with the fact that IL-25 maximizes TGF- β 1-mediated differentiation of T_H9 cells prompted us to investigate the role of activin A in T_H9 development. We demonstrate that activin A is able to replicate the ability of TGF- β 1 to drive T_H9 polarization *in vitro* and *in vivo*. Critically, effective suppression of T_H9 function *in vivo* is entirely reliant on the blockade of both TGF- β and activin A. Our studies show that T_H9 cells have an *in vivo* function that is distinct, but complementary to that of T_H2 cells, and reveal a novel pathway to T_H9 differentiation that has direct consequences for the development of allergic pathology.

METHODS

Mice

BALB/c and severe combined immunodeficiency mice (6-8 weeks) were purchased from Charles River (Morgate, United Kingdom [UK]). All experiments were performed in accordance with UK Home Office guidelines.

Induction and analysis of allergic airway inflammation

Mice received 15 μ g of house dust mite (HDM) extract (*Dermatophagoides pteronyssinus* in saline; Greer Laboratories, Lenoir, NC) or saline intranasally 3 days a week for 1 or 3 weeks. In adoptive transfer experiments, 1×10^6 *in vitro* generated T_H9 cells (more than 90% CD4⁺IL-9⁺IL-13⁻IFN- γ ⁻) were injected intraperitoneally. In blocking experiments, mice received 20 μ g of neutralizing antibody to murine activin A (R&D Systems, Abingdon, UK) and/or anti-TGF- β (5 mg/kg, clone 1D11 pan neutralizing TGF- β 1-3; Genzyme Corporation, Cambridge, Mass) or isotype control intraperitoneally. Serum, bronchoalveolar lavage fluid, lung homogenates, and draining lymph nodes were harvested as previously described.¹⁹ AHR and airway remodeling were assessed as described previously.¹⁹

T_H9 cell differentiation

CD3⁺CD4⁺ cells from lymph nodes and spleens were stimulated with plate-bound anti-CD3 (1 μ g/mL; BD Pharmingen, Oxford, UK) plus soluble anti-CD28 (2 μ g/mL; BD Pharmingen) in the presence of 5 ng/mL of rIL-4 and 1 ng/mL of rTGF- β 1 or 20 ng/mL of activin A with or without rIL-25 (10 ng/mL) in IMDM (Sigma, Gillingham, UK)

supplemented with 10% FCS (Life Technologies, Carlsbad, Calif). T_H2 cells were differentiated with 10 µg of anti-IFN γ and 10 ng/mL of IL-4. After 4 days, cells were restimulated with 500 ng/mL of ionomycin and 50 ng/mL of phorbol 2-myristate 13-acetate in the presence of brefeldin (BD Pharmingen). Cells were stained for CD4, PU.1 (Cell Signalling Technology, Beverly, Mass), IL-13, IL-10, IL-17, IFN- γ (BD Pharmingen), or IL-9 (Biolegend, San Diego, Calif). T_H9 cells are defined as CD4⁺IL-9⁺IL-13⁻IFN- γ ⁻ cells. T_H2 cells are defined as CD4⁺IL-13⁺. For the detection of IL-17RB surface expression, T cells were stained with anti-IL-17RB, followed by anti-rabbit IgG Alexa488 (Invitrogen, Paisley, UK). Cells from the lung were additionally stained with the following antibodies: anti-GR1, anti-SiglecF (BD Pharmingen), anti-mouse T1/ST2 (Morwell Diagnostics, Zurich, Switzerland), or relevant isotype controls. Labeled cells were acquired on the BD fluorescence-activated cell sorting Aria (BD Bioscience, Oxford, UK) and further analyzed by using FlowJo (Treestar, Ashland, Ore). An equal number of total events were collected for analysis.

Cytokine analysis

Paired antibodies for murine IL-4, IL-5, TGF- β 1, and IFN- γ (BD Pharmingen) and IL-25, activin A, IL-33, IL-13 thymic stromal lymphopoietin, CCL20, and IgE (R&D Systems, Minneapolis, Minn) were used in standardized sandwich ELISAs. Human IL-9 ELISA kit was purchased from Peprotech (Rocky Hill, NJ). All presented data have been normalized for tissue weight. Mouse mast cell protease-1 (Moredun, Penicuik, UK) was used according to the manufacturer's protocol.

Immunohistochemistry

Paraffin sections were stained with rabbit anti-mouse tryptase beta 1 (Abcam, Cambridge, UK). Intraepithelial mast cells were scored in the whole lung section (normalized for area), and median of the numbers of intraepithelial mast cells/section was reported.

Quantification of total lung collagen

Recently synthesized acid-soluble collagens were measured in lung tissue by biochemical assay according to the manufacturer's instructions (Sircol collagen assay; Biocolor, Belfast, UK).

Human T_H9 cells

The study was approved by the ethics committee of the Royal Brompton and Harefield Hospitals NHS Trust and was performed with the subjects' written informed consent. Whole blood (50 mL) was collected for the isolation of PBMCs and the measurement of serum IgE level by ELISA. Atopic subjects had self-reported allergies to either pollen or HDM or both. Only those individuals who had not used medication to treat symptoms of allergy for 4 weeks prior to blood sampling were included in the study. Atopic status was confirmed by elevated serum IgE levels. PBMCs were stimulated as before and analyzed for the expression of PU.1 (Cell Signaling Technology), IL-13, INF- γ , and IL-9 (BD Pharmingen). T_H9 cells are defined as CD4⁺IL-9⁺IL-13⁻IFN- γ ⁻ cells.

Statistical analysis

Data were analyzed by using Prism 4 (GraphPad Software Inc, La Jolla, Calif). Data shown represent means $\pm$ SEMs of at least 2 independent experiments (n = 6-16). Multiple comparisons were performed by using the Kruskal-Wallis test for nonparametric data, and where statistical differences ($P < .05$) were observed the data sets were further analyzed by using a paired Mann-Whitney *U* test. Correlation analysis was performed by using the Spearman test.

RESULTS

T_H9 cells are generated in the lungs following exposure to allergen and are associated with atopy in patients

PBMCs isolated from asthmatic children generate greater numbers of T_H9 cells *in vitro* when compared with nonasthmatic controls.⁹ However, direct evidence for the existence of T_H9 cells during an allergic response *in vivo* is lacking. In order to directly evaluate the T_H9 subset in human subjects, we isolated PBMCs from allergic or nonallergic donors, and quantified them by flow. We found significantly higher numbers of circulating T_H9 cells, defined as CD4⁺IL-9⁺IL-13⁻IFN- γ ⁻, in allergic donors than in nonallergic donors (Fig 1, A). Interestingly, circulating numbers of T_H9 cells correlated with levels of IgE in the plasma from these subjects ($P = .045$; $r^2 = 0.50$; Fig 1, B). The ETS-family transcription factor PU.1 is reported to be required for the development of T_H9 cells.⁹ However, although a proportion of T_H9 cells from atopic donors do express PU.1, this was not true for all cells (median 52%; range 5%-90% of T_H9 cells) (Fig 1, C).

The number of T_H9 and T_H2 cells isolated from the lungs of mice exposed to inhaled HDM extract was also determined. CD4⁺IL-9⁺IL-13⁻IFN- γ ⁻IL-17⁻ cells were only rarely observed in lungs from naive mice; however, inhalation of the environmental allergen HDM led to the early *in vivo* differentiation of T_H9 cells (Fig 1, D; see Fig E1, A, in this article's Online Repository at www.jacionline.org for flow plots). We detected maximal numbers of T_H9 cells in both the lungs and airways after 1 week, and numbers were maintained during allergen exposure. In contrast, the kinetics of T_H2 cell generation was slower, with numbers increasing over time and continued allergen challenge (Fig 1, D). T_H9 cells generated *in vivo* do not coexpress IL-10 or T_H2 or T_H1 cytokines (Fig 1, E). Moreover, T_H9 cells did not express T1/ST2 (a surrogate marker for T_H2 cells). T_H9 cells were observed in lung cell suspensions isolated from HDM-exposed mice following ex vivo restimulation with HDM, indicating that T_H9 cells are antigen specific (Fig 1, F). Analysis of other lymphocyte populations showed that T_H9 cells are the major source of IL-9 at this time point (see Fig E1, B) in response to allergen challenge. A proportion of murine CD4⁺IL-9⁺IL-13⁻ cells express the transcription factor PU.1 (42.9 ± 3.9), but this was not true for all T_H9 cells (Fig 1, G).

These data show for the first time that T_H9 cells can be observed directly in the circulation of atopic people. Moreover, T cells differentiate *in vivo* into a T_H9 phenotype in response to exposure to an environmental allergen. At early time points, they are more prevalent than T_H2 cells in the lungs, indicating that they are an important effector T-cell population immediately following allergen exposure and are involved in the development of the initial immune response.

Activin A, a TGF- β family member, drives T_H9 differentiation *in vitro*

The cytokine activin A is a member of the TGF- β superfamily, and we have previously observed that activin A is important in driving allergic pathology *in vivo*, in close association with IL-25.¹⁹ IL-25 has been shown to enhance the expression of IL-9 in T_H9 cells *in vitro*.¹³ We therefore sought to determine whether activin A could affect T_H9 cell generation. CD3⁺CD4⁺ cells were isolated from naive mice and activated *in vitro* in the presence of IL-4 in conjunction with either TGF- β 1 or activin A. Addition of activin A to *in vitro* cultures results in the development of T_H9 cells (Fig 2, A), replicating the effect of TGF- β 1. The addition of IL-25 to IL-4/activin A-driven cultures enhanced the generation of T_H9 cells, in a similar manner to that observed in IL-4/TGF- β 1-driven cultures (Fig 2, A and B; see Fig E2, A, in this article's Online Repository at www.jacionline.org). Activin A-driven T_H9 differentiated cells shared similar phenotypic characteristics to those generated

in cultures with TGF- β 1, in that a proportion of them expressed the transcription factor PU.1 (Fig 2, *C*). Interestingly, activin A-driven T_H9 cells secrete IL-9 only in the presence of suboptimal doses (0.1 ng/mL) of TGF- β 1 (Fig 2, *D*). *in vitro* generated T_H9 cells did not coexpress IL-10 or IL-17, but they did show expression of the IL-25 receptor IL-17RB on the cell surface (Fig 2, *C*). These data have implications for the generation of T_H9 cells in the lung following exposure to allergen, since TGF- β 1, activin A, and IL-25 are all generated in the lung following allergen exposure *in vivo* and might collaborate to promote differentiation of T_H9 cells *in vivo* (see Fig E2, *B*). These data imply that the promotion of T_H9 cells is not solely restricted to TGF- β 1 but may be a function of the wider TGF- β superfamily.

Transfer of T_H9 cells *in vivo* promotes allergic inflammation with a mast cell phenotype

Although IL-9 is thought to play a role in the development of allergic immune responses, we wanted to determine the specific functional role that T_H9 cells exert *in vivo*. Therefore, we generated T_H9 cells *in vitro* in the presence of IL-4, IL-25, and TGF- β 1 or activin A and transferred them to naive mice before exposure to HDM extract (Fig 3, *A*). *In vitro* generated T_H9 cells expressed IL-9 but not IL-13 or IL-10, and were observed in the lungs following intraperitoneal injection (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org). Transfer of either activin A or TGF- β 1-driven T_H9 cells to mice followed by exposure to allergen resulted in heightened allergic pathology *in vivo* with increased cell recruitment to the airways and lung in comparison to mice given just HDM (Fig 3, *B*) or T_H2 cells as a control (see Fig E3, *C*). Analysis of SiglecF⁺ granulocytes revealed that these cellular infiltrates were composed of eosinophils (Fig 3, *C*). As expected, numbers of T_H2 cells were not significantly modulated by T_H9 cell transfer (Fig 3, *D*).

IL-9 is thought to be important for the recruitment of mast cells to the lung and influencing the development of allergic pathology^{4,15,20,21}; therefore, we measured serum levels of mast cell protease. Transfer of activin A-driven or TGF- β 1-driven T_H9 cells significantly increased levels of mouse mast cell protease-1 following HDM challenge, signifying an increase in mast cell activation in these groups (Fig 3, *E*). Histological analysis also revealed an increase in the numbers of intraepithelial mast cells within the lungs of mice following T_H9 cell transfer (Fig 3, *F*). Transfer of T_H9 cells to HDM-treated mice was also associated with enhanced levels of serum IgE (Fig 3, *G*). Moreover, *ex vivo* restimulation of lymphocytes with HDM revealed enhanced IL-13 and IL-9 levels in cultures from mice transferred with T_H9 cells, compared with those treated with HDM alone (Fig 3, *H* and *I*). Pulmonary levels of T_H2 and innate cytokines and chemokines (IL-4, IL-5 and IL-25, IL-33 thymic stromal lymphopoietin or CCL20) commonly associated with allergic responses did not change following transfer of T_H9 cells in comparison with HDM controls (data not shown).

In order to determine the specific effect of T_H9 cell transfer on the development of allergic immune responses in the absence of endogenous T_H9 differentiation, we transferred T_H9 cells into mice with severe combined immunodeficiency. T_H9 cell transfer and HDM exposure still elicited lung inflammation (Fig 3, *J*), specifically the recruitment and activation of mast cells to the lung (Fig 3, *K* and *L*). We also observed that T_H9 cells injected intraperitoneally traffic to the lungs and draining lymph nodes after exposure to HDM (see Fig E3, *D*). These data show that T_H9 cells differentiated *in vitro* by either TGF- β 1/IL-4 or activin A/IL-4 do not differ in their function *in vivo* and demonstrate unequivocally that T_H9 cells can promote immune responses to allergen, leading to enhanced pathology, specifically the recruitment and activation of mast cells in the lungs.

Blockade of TGF- β and activin A during primary allergen exposure *in vivo* reduces T_H9 differentiation and ameliorates allergic inflammation

Since we determined that TGF- β 1 and activin A levels are elevated after allergen exposure and both are able to drive the differentiation of T_H9 cells *in vitro*, we set out to determine whether these 2 cytokines are able to affect T_H9 generation *in vivo*. Therefore, we used neutralizing antibodies specific for TGF- β or activin A either alone or in combination during acute allergen challenge (Fig 4, *A*). The combination of anti-TGF- β and anti-activin A antibodies significantly reduced the *in vivo* differentiation of T_H9 cells (Fig 4, *B*). In contrast, neither anti-TGF- β nor anti-activin A antibodies used alone affected the development of T_H9 cells (Fig 4, *B*). Reduction of T_H9 cells by combined anti-TGF- β /activin A antibody treatment was accompanied by a general decrease in cell recruitment to the lung (Fig 4, *C*), particularly eosinophils (Fig 4, *D*). Conversely, numbers of T_H2 cells were not affected by blocking TGF- β and activin A (alone or in combination) (Fig 4, *E*). Levels of serum mouse mast cell protease-1 were reduced to baseline by treatment with anti-TGF- β /activin A, and similarly mast cell recruitment to the lung was significantly decreased (Fig 4, *F* and *G*). Combined anti-TGF- β /activin A antibody treatment considerably reduced the levels of IL-9 and IL-13 released from antigen-stimulated lymphocytes isolated from lung draining lymph nodes of allergic mice (Fig 4, *H* and *I*). Intriguingly, IL-25 levels in the lung were reduced to baseline values following treatment with either anti-TGF- β /activin A antibodies in combination or after anti-activin A alone (Fig 4, *J*), underscoring the particular relationship between activin A and IL-25. There was no effect on levels of other proallergic cytokines and chemokines in the lung, including IL-33, thymic stromal lymphopoietin or CCL20, or classical T_H2-associated cytokines such as IL-4 and IL-5 (data not shown).

These experiments emphasize the specific role that T_H9 cells play during allergic immune responses, mediating the recruitment and activation of mast cells in the lung, and underscore the importance of the role that both TGF- β and activin A play in effecting their generation *in vivo* and the ensuing downstream pathology.

Long-term blockade of TGF- β and activin A *in vivo* leads to a reduction in mast cell-driven pathology in the lung

IL-9 is involved in the development of airway remodeling; therefore, in order to determine how blockade of the T_H9 population *in vivo* would affect HDM-induced AHR and airway remodeling following long-term allergen exposure, we administered a combination of anti-TGF- β and anti-activin A antibodies over 3 weeks (Fig 5, *A*). Exposure of mice to inhaled HDM extract results in eosinophilic airway inflammation and AHR in association with airway remodeling, the cardinal features of asthma in humans.¹⁹ The particular combination of anti-TGF- β and anti-activin A antibodies modulated features of airway remodeling but not inflammation. Decreased mucus production (Fig 5, *B* and *C*) and intraepithelial mast cell numbers and activation (Fig 5, *B*, *D*, and *E*) were observed in antibody-treated mice when compared with isotype-treated control mice. Collagen deposition was also reduced to basal levels by long-term treatment with anti-TGF- β /activin A antibodies (Fig 5, *B* and *F*). In contrast, there was no effect on cell recruitment to the lung (Fig 5, *G*) or on the production of cytokines associated with the allergic response such as IL-4, IL-5, IL-13, IL-33, and CCL20 (data not shown). However, there was again a specific effect on IL-25, which was significantly reduced in the lung (Fig 5, *H*). Importantly, there was a moderate but significant reduction in airway resistance in mice treated with combined anti-TGF- β /activin A antibodies (Fig 5, *I*).

These data suggest that manipulating T_H9 cells via TGF- β /activin A affects key features of airway remodeling and AHR following allergen exposure.

DISCUSSION

Asthma is a complex heterogeneous disease thought to occur as a consequence of aberrant T_H2 immunity to innocuous environmental particles such as dust and animal dander. However, it is clear that a range of other T cells contribute to disease pathology, which might account for this heterogeneity.²² We demonstrate here that T_H9 cells are present in human peripheral blood, since previous studies have shown IL-9 levels only in plasma or T_H9 cells generated *in vitro* from PBMCs. Importantly, allergic donors have higher circulating numbers of T_H9 cells when compared with nonallergic controls, which correlates with levels of plasma IgE. T_H9 cells have a key role in the development of allergic immune responses that is distinct to that played by T_H2 cells. T_H9 cells are generated early after allergen exposure and specifically promote mast cell recruitment and activation within the lung.

Activin A plays a key role in a diverse array of biological processes including fetal growth and development as well as during inflammation and tissue fibrosis.²³⁻²⁵ Activin A is upregulated in the pulmonary epithelium and in peripheral blood CD4 cells following allergen challenge, in both mouse models and in allergic patients.^{19,24,25} Expression of ALK-4, the receptor for activin A, is also increased after allergen challenge in T cells in lung biopsies from asthmatic patients.²⁵ Moreover, like TGF- β 1, activin A exhibits both pro- and antiinflammatory properties depending on the cytokine milieu. Activin A/IL-4 elicited *in vitro* differentiation of T_H9 cells to a similar degree as TGF- β 1, and resulted in T_H9 cells with similar characteristics. Interestingly, *in vitro* differentiated T_H9 cells require both TGF- β 1 and activin A for optimal IL-9 secretion as previously demonstrated for IL-10 secretion from regulatory T cells.^{26,27} T_H9 cells differentiated in the presence of activin A promote the same augmentation of pathology as observed when TGF- β 1-driven T_H9 cells were injected *in vivo*. TGF- β 1 has an acknowledged role in the differentiation of a number of T-cell subsets including regulatory T cells, T_H17 cells, and T_H9 cells.²² The data presented here indicate that activin A has a similar role, suggesting that T-cell plasticity *in vivo* is dependent on the wider TGF- β superfamily.

Interestingly, we have previously shown that allergen-induced expression of activin A in the epithelium is associated with IL-25 production, and the 2 cytokines cooperate to drive allergic pathology *in vivo*.¹⁹ These data have implications for the generation of T_H9 cells *in vivo* since IL-25 augments the differentiation of T_H9 cells *in vitro*.¹³ In the present study we show that IL-25 also enhances T_H9 generation driven by activin A and that blockade of activin A results in the suppression of IL-25. Our data emphasize the relationship between activin A and IL-25 in the allergic lung and outlines a novel pathway for the generation of T_H9 cells during allergen exposure.

IL-9 has been associated with the development of pathology during helminth infections and allergic inflammation,^{8,10} and antigen-specific T_H9 cells have been shown to induce experimental autoimmune encephalomyelitis³ and promote moderate ocular inflammation.¹⁸ We now provide direct evidence for the presence of T_H9 cells within the lungs. T_H9 and T_H2 cell differentiation in response to allergen challenge *in vivo* follows very distinct kinetics. We determined that T_H9 cells are rapidly generated *in vivo* following exposure of mice to inhaled HDM. In contrast, T_H2 cell differentiation and recruitment to the lung is slower, peaking 3 weeks after initial allergen exposure. Similarly, eosinophil numbers in the lung are greatest following 3 weeks of allergen challenge. This is supported by *in vitro* kinetic data showing that following activation, IL-9 production by polarized T_H9 cells is rapid in comparison to the production of IL-4 or IL-17 from T_H2 or T_H17 lines, respectively.¹⁸ Taken together these results suggest that in the lung T_H9 cells may develop independently from T_H2 cells following allergen exposure and have distinct functions *in*

vivo. Indeed, following transfer of T_H9 cells to immunocompetent or immunodeficient mice, the most striking pathologic feature of the inflammatory response was the recruitment and activation of mast cells. Adoptively transferred T_H9 cells generated in the presence of either TGF- β 1 or activin A were recruited to the lung on exposure to inhaled allergen, and promoted mast cell activation as well as increased IgE levels in the serum. Similarly, numbers of circulating T_H9 cells correlated with IgE levels in human serum. IL-9 potentiates IL-4-induced IgE release from B cells^{28,29} and has been associated with mast cell function.¹⁶

PU.1 is required for the development of IL-9-secreting T cells in mouse and human cultures *in vitro*.⁹ However, PU.1 expression is not restricted to the T_H9 lineage and has been described as a central regulator of all hematopoietic cell lineages.³⁰ We found that only a proportion of *in vivo* generated T_H9 cells express PU.1. This was particularly evident in allergic donors where exposure to allergen was not controlled. *in vivo*, it is likely that while PU.1 might be essential to generate T_H9 cells, once polarized the cells do not have a requirement for continued expression. Thus, the expression of this transcription factor may fluctuate according to allergen exposure, and is not a defining feature of the T_H9 lineage.

In order to investigate the functional contribution of activin A to the *in vivo* development of T_H9 cells, we performed blocking studies with neutralizing antibodies to TGF- β or activin A following allergen challenge *in vivo*. Combined TGF- β and activin A blockade resulted in the complete abrogation of T_H9 differentiation early after allergen exposure. During prolonged allergen exposure, dual TGF- β /activin A blockade resulted in the attenuation of features of airway remodeling including mucus production and collagen deposition. AHR was also reduced as were the number of mast cells in the lung, although there was no effect on pulmonary eosinophilia following chronic HDM challenge. This reflects our recent study with an IL-9-blocking antibody, which resulted in the abrogation of mast cell recruitment with a concomitant reduction in airway remodeling, partial resolution of AHR, but no effect on classical T_H2 pathological features such as IL-13, IL-5, and eosinophilia.⁴ It is important to note that these parameters were not affected by treatment with anti-TGF- β or anti-activin A antibodies given alone as previously described.^{19,31}

TGF- β 1 and activin A have overlapping functions *in vitro* and *in vivo*, but activin A has not previously been associated with T_H9 development. Interestingly, they share a signaling pathway via Smad3, which was identified as an asthma susceptibility gene during genomewide association studies.³² Although long-term blockade of activin A alone had no impact on indices of inflammation, remodeling, or AHR, levels of IL-25 in the lung were significantly reduced. These data suggest a model for early events following allergen challenge with local TGF- β cooperating with epithelial-derived activin A and IL-25 to promote T_H9 generation and development of allergic pathology. Activin A has previously been postulated to be a regulator of mast cell activity *in vivo*.³³ Our data now indicate that the regulation of T_H9 activity by activin A may be a key driver of mast cell recruitment and activation during allergic inflammation, particularly during the early stages of the response.

In conclusion, we have shown that T_H9 cells are increased during allergic responses both in a murine model of allergic airways disease and in atopic human subjects. Our results define for the first time a functional phenotype for T_H9-driven pathology *in vivo* that is different but complementary to that elicited by T_H2 cells. Moreover, we provide *in vitro* and *in vivo* evidence that activin A is able to drive the differentiation of T_H9 cells. Taken together our data outline a novel pathway for T_H9 generation and identify a key functional role for T_H9 cells as a vital component of the early allergic immune response driving mast cell recruitment and activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Wellcome Trust (grant nos. 057704 and 085851/Z/08/Z).

We thank Alison Humbles and Catherine Hawrylowicz for critical discussion of the results and Simone Walker for assistance with the lung function measurements. Anti-TGF- β was a kind gift from Genzyme Corporation.

Abbreviations used

AHR	Airway hyperreactivity
HDM	House dust mite
IL-17RB	Interleukin-17 receptor B
UK	United Kingdom

REFERENCES

1. Nicolaides NC, Holroyd KJ, Ewart SL, Eleff SM, Kiser MB, Dragwa CR, et al. Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci U S A*. 1997; 94:13175–80. [PubMed: 9371819]
2. Doull IJ, Lawrence S, Watson M, Begishvili T, Beasley RW, Lampe F, et al. Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. *Am J Respir Crit Care Med*. 1996; 153:1280–4. [PubMed: 8616554]
3. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol*. 2009; 183:7169–77. [PubMed: 19890056]
4. Kearley J, Erjefalt JS, Andersson C, Benjamin E, Jones CP, Robichaud A, et al. IL-9 governs allergen-induced mast cell numbers in the lung and chronic remodeling of the airways. *Am J Respir Crit Care Med*. 2011; 183:865–75. [PubMed: 20971830]
5. Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med*. 1998; 188:1307–20. [PubMed: 9763610]
6. Soroosh P, Doherty TA. Th9 and allergic disease. *Immunology*. 2009; 127:450–8. [PubMed: 19604299]
7. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(–) effector T cells. *Nat Immunol*. 2008; 9:1347–55. [PubMed: 18997793]
8. Veldhoen M, Uytendhoe C, van SJ, Helmby H, Westendorf A, Buer J, et al. Transforming growth factor-beta “reprograms” the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*. 2008; 9:1341–6. [PubMed: 18931678]
9. Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol*. 2010; 11:527–34. [PubMed: 20431622]
10. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010; 33:192–202. [PubMed: 20674401]
11. Angkasekwinai P, Chang SH, Thapa M, Watarai H, Dong C. Regulation of IL-9 expression by IL-25 signaling. *Nat Immunol*. 2010; 11:250–6. [PubMed: 20154671]
12. Neill DR, McKenzie AN. TH9 cell generation. TH9: the latest addition to the expanding repertoire of IL-25 targets. *Immunol Cell Biol*. 2010; 88:502–4. [PubMed: 20309011]

13. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med*. 2007; 204:1509–17. [PubMed: 17562814]
14. Erpenbeck VJ, Hohlfeld JM, Volkmann B, Hagenberg A, Geldmacher H, Braun A, et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol*. 2003; 111:1319–27. [PubMed: 12789235]
15. McLane MP, Haczk A, van de Rijn M, Weiss C, Ferrante V, MacDonald D, et al. Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. *Am J Respir Cell Mol Biol*. 1998; 19:713–20. [PubMed: 9806735]
16. Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol*. 2010; 10:683–7. [PubMed: 20847745]
17. Nowak EC, Noelle RJ. Interleukin-9 and T cell subsets. *Cell Cycle*. 2009; 8:3798–9. [PubMed: 19934658]
18. Tan C, Aziz MK, Lovaas JD, Vistica BP, Shi G, Wawrousek EF, et al. Antigen-specific Th9 cells exhibit uniqueness in their kinetics of cytokine production and short retention at the inflammatory site. *J Immunol*. 2010; 185:6795–801. [PubMed: 20971929]
19. Gregory LG, Mathie SA, Walker SA, Pegorier S, Jones CP, Lloyd CM. Overexpression of Smad2 drives house dust mite-mediated airway remodeling and airway hyperresponsiveness via activin and IL-25. *Am J Respir Crit Care Med*. 2010; 182:143–54. [PubMed: 20339149]
20. Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C, et al. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med*. 2008; 205:897–913. [PubMed: 18378796]
21. Matsuzawa S, Sakashita K, Kinoshita T, Ito S, Yamashita T, Koike K. IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. *J Immunol*. 2003; 170:3461–7. [PubMed: 12646606]
22. Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just T(H)2 cells. *Nat Rev Immunol*. 2010; 10:838–48. [PubMed: 21060320]
23. Chen YG, Lui HM, Lin SL, Lee JM, Ying SY. Regulation of cell proliferation, apoptosis, and carcinogenesis by activin. *Exp Biol Med* (Maywood). 2002; 227:75–87. [PubMed: 11815670]
24. Karagiannidis C, Hense G, Martin C, Epstein M, Ruckert B, Mantel PY, et al. Activin A is an acute allergen-responsive cytokine and provides a link to TGF-beta-mediated airway remodeling in asthma. *J Allergy Clin Immunol*. 2006; 117:111–8. [PubMed: 16387593]
25. Kariyawasam HH, Pegorier S, Barkans J, Xanthou G, Aizen M, Ying S, et al. Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. *J Allergy Clin Immunol*. 2009; 124:454–62. [PubMed: 19733294]
26. Huber S, Schramm C. Role of activin A in the induction of Foxp3+ and Foxp3- CD4+ regulatory T cells. *Crit Rev Immunol*. 2011; 31:53–60. [PubMed: 21395511]
27. Huber S, Stahl FR, Schrader J, Luth S, Presser K, Carambia A, et al. Activin A promotes the TGF-beta-induced conversion of CD4+ J Immunol. 2009; 182:4633–40. [PubMed: 19342638]
28. Fawaz LM, Sharif-Askari E, Hajoui O, Soussi-Gounni A, Hamid Q, Mazer BD. Expression of IL-9 receptor alpha chain on human germinal center B cells modulates IgE secretion. *J Allergy Clin Immunol*. 2007; 120:1208–15. [PubMed: 17919707]
29. Petit-Frere C, Dugas B, Braquet P, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. *Immunology*. 1993; 79:146–51. [PubMed: 8509135]
30. Carotta S, Wu L, Nutt SL. Surprising new roles for PU.1 in the adaptive immune response. *Immunol Rev*. 2010; 238:63–75. [PubMed: 20969585]
31. Fattouh R, Midence NG, Arias K, Johnson JR, Walker TD, Goncharova S, et al. Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am J Respir Crit Care Med*. 2008; 177:593–603. [PubMed: 18174546]
32. Moffat MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. 2010; 363:1211–21. [PubMed: 20860503]

33. Funaba M, Ikeda T, Murakami M, Ogawa K, Tsuchida K, Sugino H, et al. Transcriptional activation of mouse mast cell Protease-7 by activin and transforming growth factor-beta is inhibited by microphthalmia-associated transcription factor. *J Biol Chem.* 2003; 278:52032–41. [PubMed: 14527958]

Key messages

- Circulating T_H9 cells in allergic subjects correlate with plasma IgE levels.
- Allergen induces early *in vivo* differentiation of pulmonary T_H9 cells.
- Novel role for activin A in driving the generation of T_H9 cells.

FIG 1.

Identification of *in vivo* generated T_H9 cells. **A**, Number of T_H9 cells in PBMCs from nonallergic and allergic donors. **B**, Correlation between the number of circulating T_H9 cells and plasma IgE levels in humans. **C**, Percentage of freshly isolated human T_H9 cells expressing PU.1. **D**, T_H9 and T_H2 cells in the lungs of mice challenged with PBS or HDM for up to 3 weeks. **E**, Phenotype of murine *in vivo* generated T_H9 cells after 1 week of HDM challenge. **F**, Allergen specificity of *in vivo* generated pulmonary T cells. **G**, Percentage of T_H9 cells expressing PU.1 following 1-week HDM challenge in mice. Data shown represent means $\pm$ SEMs. * $P < .05$.

FIG 2.

Induction of T_H9 differentiation *in vitro* by activin A. **A**, T_H9 cells generated in the presence of IL-4 alone or in association with TGF- β 1, activin A, and/or IL-25. **B**, Percentage of T_H9 cells plotted in a histogram. **C**, Phenotype of T_H9 cells differentiated in the presence of IL-4 with either TGF- β 1 or activin A. **D**, IL-9 secretion from activin A-derived T_H9 cells after 4 days in culture in the presence of 0.1 ng/mL of TGF- β 1. Data shown represent means $\pm$ SEMs.

FIG 3.

T_H9 Adoptive transfer induces mast cell recruitment to the lungs. **A**, Schematic of experimental protocol. **B**, Total cells recovered from the lung 1 week after T_H9 cell transfer and challenge with either PBS or HDM. Eosinophils (Siglec F⁺) (**C**) and T_H2 cells (**D**) recovered from the lung. Serum mMCP-1 levels (**E**) and intraepithelial mast cell numbers (**F**) scored in lung sections expressed as total number of mouse tryptase beta 1 positive cells per lung section. **G**, Serum IgE levels. IL-13 (**H**) and IL-9 (**I**) levels in supernatants from HDM-stimulated LN cell cultures. **J**, Total cells recovered from the lungs of SCID mice adoptively transferred with T_H9 cells and treated with either PBS or HDM. Serum mMCP-1 levels (**K**) and intraepithelial mast cell numbers (**L**) in SCID mice. Data shown represent means $\pm$ SEMs. **P* < .05. LN, Lymph node; mMCP-1, mouse mast cell protease-1; NS, not significant; SCID, severe combined immunodeficiency.

FIG 4.

Acute blockade of TGF- β and activin A inhibits T_H9 differentiation. **A**, Schematic of experimental protocol. **B**, T_H9 cells recovered from the lung after treatment with either anti-TGF- β and/or anti-activin A in mice challenged with either PBS or HDM. Total cells (**C**), lung eosinophils (**D**), and T_H2 cells (**E**) recovered from the lung. Serum mMCP-1 levels (**F**) and intraepithelial mast cells (**G**) per lung section. IL-9 (**H**) and IL-13 (**I**) levels in supernatants from lymph node cell cultures. **J**, IL-25 levels in the lungs. Data shown represent means $\pm$ SEMs. * P < .05. mMCP-1, Mouse mast cell protease-1.

FIG 5.

Chronic blockade of TGF- β and activin A reduces airway remodeling. **A**, Schematic of experimental protocol. **B**, Peribronchial and perivascular cellular infiltrate (*H&E*), purple-colored mucin-containing cells in the epithelium (*PAS*), and perivascular and peribronchiolar collagen (sirius red) and brown stained intraepithelial mast cells. Original magnification $\times 40$. Scale bar = 50 μ m. **C**, Quantification of mucus positive cells. **D**, Intraepithelial mast cells per lung section. **E**, Serum mMCP-1 levels. **F**, Total lung collagen. **G**, Total cells recovered from BAL and lung. **H**, IL-25 levels in the lung. **I**, Airway resistance (*RI*) following 3-week HDM challenge. Data shown represent means $\pm$ SEMs. $*P < .05$. *BAL*, Bronchoalveolar lavage; *MCPT7*, mouse tryptase beta 1; *mMCP-1*, mouse mast cell protease-1; *PAS*, periodic acid-Schiff.