HHS Public Access

Author manuscript

*J Immunol*. Author manuscript; available in PMC 2025 September 15.

Published in final edited form as:

*J Immunol*. 2024 September 15; 213(6): 823–830. doi:10.4049/jimmunol.2300635.

# Activin A promotes differentiation of a pathogenic multi-cytokine IL-9-secreting CD4+ T population

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## Abstract

The development of Th subsets results from cellular and cytokine cues that are present in the inflammatory environment. The developing T cell integrates multiple signals from the environment that sculpt the cytokine-producing capacity of the effector T cell. Importantly, T cells can discriminate similar cytokine signals to generate distinct outcomes and that discrimination is critical in T helper subset development. IL-9-secreting Th9 cells regulate multiple immune responses, including immunity to pathogens and tumors, allergic inflammation, and autoimmunity. In combination with IL-4, TGFβ or Activin A promote IL-9 production, yet it is not clear if both TGFβ family members generate Th9 cells with identical phenotype and function. We observed that in contrast to TGFβ that efficiently represses Th2 cytokines in murine Th9 cultures, differentiation with Activin A produced a multi-cytokine T cell phenotype with secretion of IL-4, IL-5, IL-13, IL-10, in addition to IL-9. Moreover, multi-cytokine secreting cells are more effective at promoting allergic inflammation. These observations suggest that although TGFβ and IL-4 were identified as cytokines that stimulate optimal IL-9 production, they might not be the only cytokines that generate optimal function from IL-9-producing T cells in immunity and disease.

## Introduction

As a component of adaptive immunity, the T helper (Th) compartment maintains the flexibility to support the appropriate and specific immune response to a vast number of pathogens. Cytokines as signaling molecules present in the inflammatory environment that activate transcription factors allowing the acquisition of restricted cytokine-secretion profiles. Each Th subset is defined by a signature cytokine production profile, immune function, and pathogenic potential.

Th9 cells produce Interleukin(IL)-9 as a signature cytokine (1–3). Th9 cells and IL-9 have beneficial effects in eradicating both tumor and helminthic infections (4, 5). In contrast,

inappropriate production of IL-9 and Th9 cells have been linked to pathology in colitis and atopic inflammation (6–8). Immune responses and inflammatory disorders are increasingly recognized as having a Th9 cell component that significantly contributes to the disease process.

The Th9 subset was first described *in vitro* after a Th2 cell population was cultured with TGFβ (2). Further work found direct differentiation through stimulation with IL-4 and TGFβ (9) that required multiple transcription factors including PU.1, Irf4, Etv5, Batf, and Gata3 to promote the Th9 phenotype (10–13). Alternative cytokines have been demonstrated to support the differentiation of an IL-9-secreting T cell. Activin A (ActA), a Smad activator similar to TGFβ has also been used to generate Th9 cells (14). Blockade of TGF-β or activin A during primary allergen exposure in vivo in mouse models reduces Th9 differentiation and ameliorates allergic inflammation suggesting some redundancy in the generation of these effectors (14).

Pathogenic Th2 (pTh2) cells have been described across several reports examining distinct human allergic diseases (15–21). In several of these reports, IL-9 is one of the pTh2 cytokines (15, 19, 20) that include IL-13 and IL-5 but not IL-4 (22). Using a mouse model to define pathogenic subsets in an allergen recall model we identified tissue resident IL-9-secreting Th subset similar to pTh2 cells (23). This population of cells, which we termed Th9rm (for Th9 resident memory), was transcriptionally and chromatin-structurally distinct from Th2 cells (that secreted IL-5 and IL-13, but not IL-9) or Th9 cells that secreted IL-9 but not other Th2 cytokines (23). While the combination of TGFβ and IL-4 promote the classic Th9 phenotype by repressing Th2 cytokine production, the cytokines that promote the development of cells with a pTh2 phenotype are unknown.

In this study, we identified cytokine combinations that generate multi-cytokine producing Th9 cells *in vitro*. Moreover, and distinct from the TGFβ + IL-4 combination, culture with Activin A and IL-4 can maintain IL-9 production through multiple rounds of *in vitro* culture. The multi-cytokine phenotype of Th9 cells is similar to what has been described for “pathogenic” Th2 and the Activin A and IL-4-primed cells are capable of promoting robust airway inflammation.

## Material and Methods

### Mice

C57BL/6 mice were bred in house at the Indiana University School of Medicine, (Indianapolis, IN) or purchased from Jackson Laboratory (Bar Harbor, Maine). *Il9*-INFER reporter mice have been previously described (4). All mice were used with the approval of the Indiana University Institutional Animal Care and Use Committee.

### Naive CD4+ T cell isolation and in vitro culture

CD4+CD62L+ T cells were isolated from the spleens of the indicated mice using magnetic separation following the supplier’s protocol (Miltenyi Biotec, Auburn, CA). Cells were cultured at $10^6$ cells/ml in complete RPMI 1640 on plates coated with anti-CD3 and soluble anti-CD28 as previously described (24). Furthermore, cells cultured under Th9 conditions

were supplemented with human TGF-β1 (2 ng/ml) or activin A (5 ng/ml), IL-4 (20 ng/ml), and anti-IFN-γ (10 mg/ml). Th2 cells were cultured identically to Th9 cells, but in 10 ng/ml IL-4 and in the absence of TGF-β1. After the initial 3 days in culture, cells were removed from anti-CD3–coated plates and expanded with 3 volumes of fresh media containing the same concentrations of IL-4 and TGF-β1 or activin A. For the second rounds of culture, cells were cultured and expanded as per the initial round of differentiation.

### Intracellular cytokine staining

After the indicated times in culture, the frequency of cytokine-producing T cells was determined by intracellular cytokine staining. Briefly, 0.5–1.0 $10^6$ cells were stimulated in media containing PMA and ionomycin or plate-bound anti-CD3 as previously described (24). After 2–3 h, monensin (2 mM) was added to stimulated cells, and 3 h later, cells were stained with a fixable viability dye (eBioscience) and fixed with 4% formaldehyde at room temperature for 10 min. After fixation, cells were permeabilized with permeabilization buffer (eBioscience) and stained for intracellular cytokines (IL-4: 11B11 [BioLegend], IL-9: RM9A4 [BioLegend], IL-10: JES5-16E3 [BioLegend], and IL-17A: eBio17B7 [eBioscience]) in the same buffer.

### Adoptive transfer experiments

Briefly, in vitro differentiated ovalbumin (OVA)–specific Th2 or Th9 cells from day 4 cultures were adoptively transferred intravenously into wild-type (WT) recipient mice (25). Twenty-four hours after cell transfer, mice were challenged intranasally with 100 mg of OVA for 5 days. Mice were then euthanized 24 hours after the last challenge for further analysis.

### Bronchoalveolar lavage and lung histology

The trachea was cannulated, and lungs underwent lavage 2 times with 1mL of PBS to collect bronchoalveolar lavage (BAL) fluid cells. Cells recovered in BAL fluid were counted with a hemocytometer. Eosinophils, neutrophils, and mononuclear cells in BAL fluid were distinguished by cell size and expression of CD3, B220, CCR3, CD11c, and MHC class II and analyzed by means of flow cytometry, as previously described (26). After lavage, lung tissues were fixed in neutral buffered formalin. Paraffin embedded lung tissue sections were stained with hematoxylin and eosin, periodic acid–Schiff, or toluidine blue to evaluate the infiltration of inflammatory cells, mucus-producing cells, and mast cells, respectively, by means of light microscopy.

### Real-time PCR

RNA was harvested from cells at the indicated time points in TRIzol reagent (Life Technologies). cDNA was produced by reverse transcribing mRNA via the manufacturer's directions (Invitrogen). Real-time PCR was carried out with TaqMan primers (Life Technologies) using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, Calif). RNA was normalized to expression levels of β2-microglobulin, and relative expression was calculated by using the -ΔΔCt method.

### Heatmap Visualization

Heatmap data visualization was generated using the program heatmapper (http://www.heatmapper.ca/expression/). Relative gene expression from real-time PCR data from mouse whole lung samples compared chronic allergic airway disease module to naïve mice through a log base 2-fold-change ratio. Each column is the comparison between individual mice randomized between each group.

### Statistical analysis

All statistics were done using Prism software version 8 (GraphPad Software) using a student *t* test unless otherwise stated. Flow cytometry data were collected using an Attune flow cytometer (Life Technologies) and were analyzed using FlowJo version 10 (Tree Star).

## Results

### Allergic airway IL-9-secreting T helper cells have a multi-cytokine profile

To examine Th9 cells from the allergic lung we used an established chronic model of *Aspergillus fumigatus* challenge (23). Briefly, C57BL/6 were challenged intranasally 3 times a week for 6 weeks (Fig. 1A). CD4+ T cells were magnetically sorted to prevent paracrine non-T cell cytokine production from altering the cytokine profile during re-stimulation. The cytokine profile from allergic airway T cells demonstrated a robust IL-9-secreting population. Interestingly, the IL-9 producing cells coproduced a number of additional Th2 cytokines including IL-5, IL-10, and IL-13 (Fig. 1B). Cells producing IL-2 and IL-4, were largely exclusive of IL-9 (Fig. 1B). The majority of IL-9-producers were IL-17A-negative, although there was a smaller population of double positive cells (Fig. 1B). IL-9 was almost exclusively produced by cells that were positive for other cytokines, with very little IL-9 produced from cytokine negative cells (Supplemental Fig. S1A–B). When gating on the IL-9-secreting cell population, the majority of the cells expressed at least 2 additional cytokines with IL-5 and IL-13 were the most commonly co-expressed cytokines (Fig. 1C and Fig. S1C). Next, we examined the transcriptional signature of these allergic airway IL-9-secreting cells. We used the INFER-IL-9 reporter mouse to flow sort IL-9-secreting cells expressing the GFP marker for competency to produce IL-9 (27). Similar to the protein data, the INFER+ cells demonstrated high expression levels of *Il9, Il5, Il13.* As expected, INFER+ cells expressed low amounts of *Il17a* or *Ifng* (Fig. 1D).

We then examined expression of surface receptors that might impact IL-9 production comparing INFER+ cells isolated from mice having acute (2 week) or chronic (6 week) challenge protocols (Fig. 1A). Expression of the ActA receptor chain, *Acvr1b*, decreased in INFER-negative cells but was maintained in INFER+ cells from chronically challenged lung (Fig. 1E). In microarray and functional data, surface markers of receptors for epithelial-derived cytokines IL-25 (IL17RB) (28), IL-33 (ST2) (16), and TSLP (TSLPR) (19) have been identified on “pathogenic” Th2 cells. We found the gene expression for all these markers to be increased in the INFER+ population particularly in the chronic challenge model (Fig. 1F). *Il1rl1*, the gene for the ST2 receptor was expressed at a high level and increased between the acute and chronic timepoints (Fig. 1F). *Gata3* also had greater expression in the INFER+ population at the chronic timepoint (Fig. 1G). While it is possible

that INFER+ cells contain some cells that are not producing IL-9, they do generate a significant enrichment for IL-9-producing cells (23, 27).

## Activin A expression is specifically induced in allergic airway disease

While ActA was previously found to support Th9 differentiation, a comprehensive screen of all TGFβ superfamily members and related genes in allergic airway disease had not been performed. mRNA levels from whole lung single cell suspensions were compared between the 6-week *A.f.* chronic model and naïve mice never receiving any challenges. Of the 46 TGFβ superfamily genes examined, expression of only 2 were found to be significantly increased in response to chronic airway allergen exposure (Fig. 2A–B). These included *Inhba* (inhibin subunit βA) that encodes the monomeric form of ActA, and *Gdf3* (growth differentiation factor 3) a TGFβ superfamily member that has been shown to modulate other members' activity. *Tgfb1* encoding an isoform of TGFβ was not increased in allergic airway disease, consistent with a number of asthma studies, although this does not reflect TGFβ activity (29, 30) (Fig. 2A–B). To further define differential expression patterns between *Tgfb1* and *Inhba* we screened primary lymphoid organs, secondary lymphoid organs, and peripheral tissues. While *Tgfb1* expression was high in secondary lymphoid organs and peripheral tissues, *Inhba* was observed primarily in peripheral tissues suggesting that it may be more relevant at sites of inflammation (Fig. 2C).

## Activin A induces IL-9 without repressing Th2 cytokines

Naïve CD4+ T cells cultured under Th9 polarizing conditions with the cytokines TGFβ and IL-4 produce IL-9 and IL-10, but rapidly lose their capacity to produce IL-9 after extended culture or multiple rounds of differentiation (24). In short-term cultures, ActA was less potent at inducing IL-9 production as compared to TGFβ1. However, cells that had been cultured with multiple rounds of IL-4 and ActA better maintained IL-9 production, compared to TGFβ1+IL-4 controls (Fig. 3A–B). The effects of culture with GDF3 were also examined over multiple rounds of culture but no induction of IL-9 was detected (not shown). ActA and IL-4 produced CD4+IL9+ T cells that also produced IL-13 and IL-10 (Fig. 3A–B). In an additional round of ActA culture the percentage of IL-13 and IL-10 double positive cells increased (Fig. 3C). Parallel experiments were performed with IL-9 reporter-expressing (INFER) cells cultured under these conditions. Similar to results with wild type cells, T cells cultured with ActA + IL-4 produced more Th2 cytokines than TGFβ1+IL-4 cultures, and more IL-9 than Th2 cultures (Supplemental Fig. S2). When the cytokine profile of IL-9+CD4+ T cells is examined based on IL-10 and IL-13 production, the pattern most similar to allergic airway Th9 cells was cells cultured with ActA (Fig. 3D). We examined the complete intracellular cytokine profile of cultures with TGFβ or ActA in addition to IL-4. Following a second round of culture, ActA was able to induce IL-9 to similar levels as TGFβ without the inhibition of other Th2 cytokines (Fig. 3E).

Since ActA results in more stable IL-9-secreting cells, we wondered whether maintained ActA was required, or if TGFβ1+IL-4 could alter the phenotype. We tested additional conditions where both ActA and TGFβ1+IL-4 were added together. Switching ActA cultured cells to culture with TGFβ1 or both cytokines, resulted in increased IL-9 and IL-10 production, but decreased IL-13 (Supplemental Fig. S3). In contrast, cultures incubated

first with TGFβ1+IL-4, and then switched to ActA +IL-4 did not increase IL-9, and had no impact on IL-10, but did increase IL-13 production (Supplemental Fig. S3). Together, these data suggest that primary exposure to ActA results in the optimal polyfunctional phenotype, and that TGFβ1 exposure, even at a secondary time point, represses Th2 cytokine production, sculpting the phenotype to primarily IL-9 secretors.

## Activin A does not inhibit the early expression of Gata3

GATA3 binding to promoter regions is critical for the production of IL-5 (31) and IL-13 (32), but not necessarily IL-4 (33). IL-4 promotes the expression of *Gata3* (31, 34–36). Furthermore, TGFβ inhibits the early expression and function of GATA3 (37) through a mechanism that is still unclear. Using a similar approach, we examined how the transcription of *Gata3* and GATA3 target genes were impacted by culture with IL-4, and TGFβ (subsequently referred to as Th9T cells) or ActA (subsequently referred to as Th9A cells), early during activation (20 hours) and after the differentiation period (5 days). Similar to previous results, Th9T cultures at 20 hours showed no induction of *Gata3* above Th0 cultures (Fig. 4A). In contrast, Th9A cultures had *Gata3* transcription at least equivalent to Th2 cultures. The expression of Gata3 was similar at day 5 except for an increase in all conditions over Th0 conditions (Fig. 4B). Unstimulated IL-9 transcription was low at 20hrs, and highest in Th9T cultures on day 5 (Fig. 4A–B). *Fos* and *Foxp3* were examined as downstream targets that are directly up-regulated and down-regulated by *Gata3*. Th9T cultures demonstrated decreased *Fos* and increased *Foxp3* transcription when compared to Th9A, consistent with lower expression of *Gata3* in these cells. GATA3 intracellular transcription factor staining and geometric mean fluorescence intensity confirmed decreased Gata3 following culture with TGFβ compared to ActA (Fig. 4C–E).

We have also identified the IL-33/ST2 pathway as important in IL-9 production from resident memory cells in an allergen recall model (23). Moreover, GATA3 is an important transcription factor supporting the expression of ST2, the IL-33 receptor (38). Th9A cultures demonstrated significantly greater levels ST2 when compared to Th9T (Fig. 4F). There was a similar pattern of CD69 staining in the cultures (Fig. 4F). To test the function of the ST2 receptor we compared responsiveness to IL-33 between Th9A and Th9T cultures. At the end of round 2 of culture, cells were washed and restimulated on anti-CD3 coated plates in the presence or absence of IL-33 for 24 hours to detect IL-9 secretion by ELISA. In cultures stimulated in the absence IL-33, IL-9 was only detected in the Th9T culture condition (Fig. 4G). When stimulated with IL-33 however, there was a 60-fold increase in IL-9 production from both the condition with IL-4 and the Th9A condition, while Th9T cells were not responsive to IL-33 (Fig. 4H). Together, these studies suggest that the ability of TGFβ1 to suppress *Gata3* expression is central for limiting the development of the multi-cytokine-secreting Th9 phenotype whereas ActA allows for greater GATA3 expression and a polyfunctional Th9 response.

## Activin A-primed Th9 cells promote allergic airway disease

As we observed that TGFβ1 and ActA-cultured Th9 cells had different polyfunctional characteristics, we wanted to compare the in vivo inflammatory potential between these two populations. Naïve CD4 cells were isolated from ovalbumin-specific TCR-transgenic

DO11.10 mice and differentiated Th2, Th9T or Th9A conditions for 4 days. Two million cells in each respective culture condition were adoptively transferred to naïve BALB/c mice via tail vein injection. The average percentage of IL-9+ cells in the Th9T and Th9A conditions were 50% and 18%, respectively. Mice were intranasally challenged with OVA daily for 5 days prior to analysis. Total lung cellularity was increased in the mice that received Th9A cells, resulting in significantly increased numbers of eosinophils, but not neutrophils, or macrophages (Fig. 5A). Th2 and Th9A DO11.10 recipient mice had increased cellular infiltrate around the conducting airways and greater goblet cell metaplasia compared to Th9T recipients (Fig. 5B).

We thought that it was possible that transfer of two million cells generated overwhelming inflammation making it harder to discriminate effects between the populations. We therefore performed additional experiments and demonstrated that transferring 0.5 million differentiated Th9T or Th9A T cells was useful for distinguishing pro-inflammatory activity. Lung tissue from the Th9A recipient mice had increased inflammation scores by H&E staining and mucus producing score by periodic acid–Schiff staining of lung tissue sections, compared to Th9T recipient tissue or tissue from mice that received only PBS (Fig. 5C). This suggested that the Th9A had increased cellular infiltrate and greater goblet cell metaplasia compared to Th9T recipients (Fig. 5C). We then analyzed gene expression of the mucus-associated gene *Clca1*, and eosinophil-recruiting chemokines *Ccl5*, *Ccl11*, and *Ccl24* using qRT-PCR of lung tissue (Fig. 5D). We observed that the Th9A cells had a greater capacity for induction of each of the genes providing further evidence for goblet cell metaplasia and providing a potential mechanism for eosinophil recruitment. Together, these data demonstrate that TGFβ and Activin A priming generated IL-9-secreting cells with distinct phenotypes and pro-inflammatory potential.

## Discussion

While Th subsets that are polarized in vitro have been an important tool for defining the development and function of cytokine-secreting populations, there have long been concerns that they do not represent cells derived in vivo in various contexts (39). The most pathogenic subsets of Th cells produce multiple cytokines, often combinations of cytokines associated with more than one subset. Pathogenic Th2 cells have been identified in multiple diseases in humans and in many studies, produce IL-9 in addition to the standard Th2 cytokines. This contrasts Th9 cultures that have extensively been characterized to produce IL-9 with very little expression of other Th subset cytokines (2, 9, 12, 40). In this report we defined Activin A as a cytokine that generates Th cells with a phenotype more closely approximating pathogenic Th2 cells in vivo.

In a screen of all TGFβ superfamily members, activin A was specifically induced in allergic airway inflammation and promoted a multi-cytokine-secreting phenotype. Although TGFβ was not increased at the gene expression level, it is possible that post-translational modifications may contribute to varying TGFβ activity (41, 42). Activin A was previously shown to promote IL-9-secreting T cells and allergic inflammation was most effectively blocked by simultaneous blockade of TGFβ and Activin A. The difference between the TGFβ and ActA signals that is the basis for these distinct outcomes in phenotype are not

fully defined but may depend on the ability of TGFβ but not ActA to activate ERK signaling that is known to impact GATA3 stability (43–45). Mechanistically, the maintenance of Th2 cytokine expression in IL-9-promoting cultures correlated with the inability of Activin A to repress GATA3 as efficiently as TGFβ (37). This also correlates with the responsiveness to IL-33, since IL-33R/ST2 is a GATA3-regulated gene (38). IL-33 has been shown to support Th9 differentiation (46) and support IL-9 production from CD4+ T cells isolated from ulcerative colitis models (6). We previously observed a significant role for IL-33 in T cell IL-9 production in an allergic airway recall model (23). In this study, we found that allergic airway Th9 cells and activin A Th9 cells secreted 30-fold and 60-fold more IL-9 in the presence of IL-33 respectively. In contrast, Th9 cells differentiated with TGFβ did not have increased IL-9 in the presence of IL-33 and expressed limited ST2 receptor. IL-33 is thought to be produced from damaged tissue that occurs in helminth infection and allergen exposure, but can also be produced by dendritic cells (47–50). It is still unclear if IL-33 plays an obligate role in the generation and maintenance of pathogenic Th2 cells.

The ability of Activin A-primed Th9 cells to generate robust allergic airway inflammation while also producing less IL-9 than TGFβ-primed Th9 cells supports the contribution of the multi-cytokine secreting phenotype. The disease relevance of multi-cytokine-secreting cells is supported by two reports that highlighted the potential role of IL-9-expressing, multi-cytokine-secreting T cells in human asthma (15, 21). Our previous studies indicated that the pro-inflammatory function of polarized Th9 cells relies on IL-9 and other Th2 cytokines (25, 51). This report expands on that point by suggesting that T cell secreting more cytokines have a greater influence on the development of allergic airway inflammation, recruiting more cell to the lung and promoting more mucus gene expression and goblet cell metaplasia. This approach should lead to further insights on the function of multi-cytokine secreting T cells.

The generation of IL-9-secreting T cells in vitro is dependent on integrating multiple cytokine signals. The balance of TGFβ and IL-4 signals is critical; a shift towards TGFβ results in greater Treg differentiation, and while the IL-4 signal is absolutely required for optimal IL-9 production, a shift towards IL-4 signaling in the absence of TGFβ results in more Th2 cells and less IL-9 production (2, 9, 13, 40). There are many other signals that can promote IL-9 production, including IL-1β, that can function in the absence of TGFβ (52), and IL-25 (53). It is not clear if these cytokines also impact a multi-cytokine-promoting phenotype. We have recently shown that the TNF superfamily member TL1A promotes a multi-cytokine producing Th9 cell that is important for cytokine production in vivo (54) and it is not yet tested whether TL1A might also work in concert with ActA. Blockade of ActA alone was not sufficient to impact allergic inflammation (14), although blockade of Activin A receptors has not been tested in vitro for these studies or in vivo. All of these combinations will be interesting to test for in vitro and in vivo function in the future.

The description of pathogenic Th2 cells highlighted important complexity in the development of human disease. While in vitro models of Th differentiation might be insufficient to capture the complexity observed in vivo, T cell differentiation in vivo is still a cytokine-driven process. Identification of additional cytokines that promote distinct

phenotypes that have altered functions will be useful tools in modeling human disease and defining the contribution of more complex Th cell phenotypes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by Public Health Service grants from the National Institutes of Health (R01 AI057459 and AI129241 to M.H.K.). B.J.U. was supported by National Institutes of Health Grants T32 AI060519 and F30 HL147515. Core facility usage was also supported by Indiana University Simon Cancer Center Support Grants P30 CA082709 and U54 DK106846 from the National Institutes of Health. Support provided by the Herman B Wells Center for Pediatric Research was, in part, from the Riley Children's Foundation.

## Abbreviations:

| | |
|---|---|
| **ActA** | Activin A |
| **Th9T** | Th9 cells derived with TGFβ + IL-4 |
| **Th9A** | Th9 cells derived with Activin A + IL-4 |

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**Key Points:**

IL-9-secreting T cells in chronic inflammation are polyfunctional

Activin A promotes a polyfunctional Th cell phenotype

Activin A-primed Th cells promote robust allergic airway inflammation

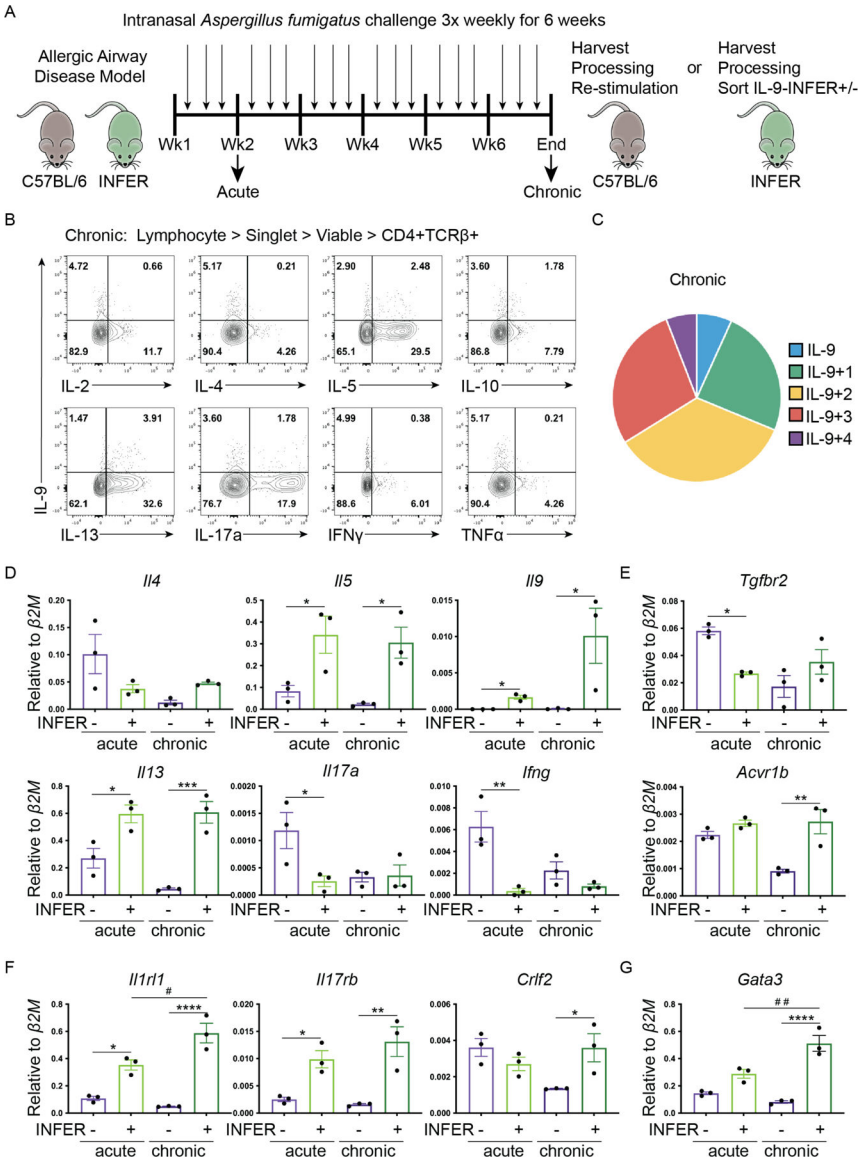


**Figure 1. Th9 cells from allergic airways have a multicytokine profile.**

(A) Schematic of the chronic model of allergic airway disease with isolation strategy of CD4+ INFER-IL-9 reporter cells.

(B) Flow analysis of isolated lung CD4+ cells from the chronic model after restimulation with PMA Ionomycin.

(C) Multi-cytokine positive cells from IL-9+ CD4+ T cells in the lung. The diagram indicates the proportion of cells positive for IL-9 alone, or IL-9 + 1, 2, 3, or 4 additional cytokines that were examined (IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IFNγ).

(D-G) INFER-IL-9 reporter +/− cells were flow sorted after either an acute or chronic challenge and screened for (D) cytokine expression, (E) TGFβ superfamily member receptor expression, (F) innate cytokine receptor expression, and (G) *Gata3* (n=3). Data are presented as mean +/− SEM from 2–3 independent experiments. One-way ANOVA with

post hoc Tukey test was used to generate p values. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****$p<0.0001$.

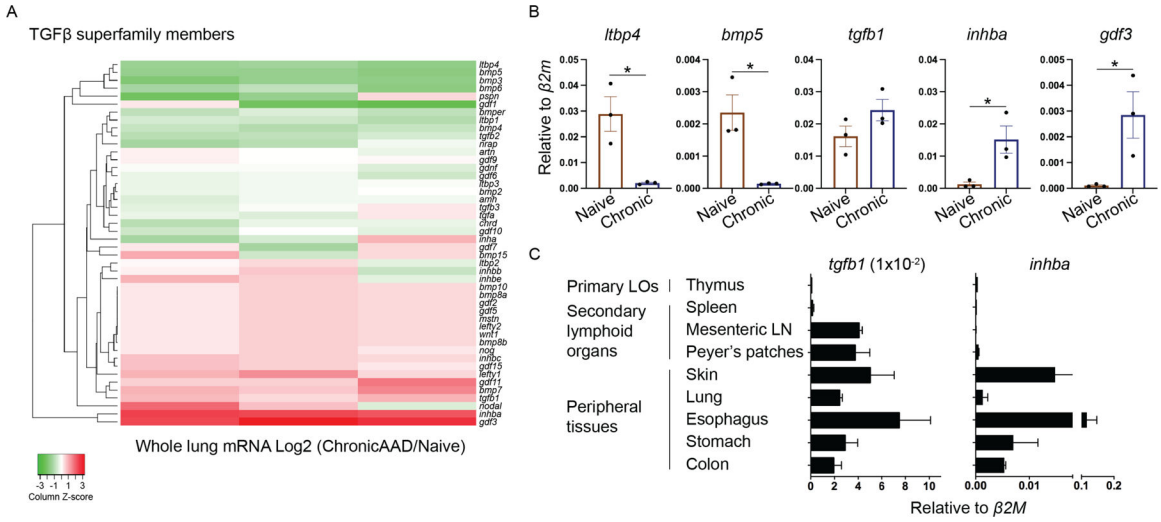


**Figure 2. Activin A is induced in allergic airway disease.**

(A) Heatmap representation of Log2 mRNA expression comparing TGFβ superfamily member ligands, receptors, and related genes in lung tissue from the chronic model (n=3).

(B) mRNA expression from A (n = 3).

(C) mRNA expression of *Tgfb* and *Inhba* throughout immune and peripheral tissues of a naïve mouse. Data are presented as mean +/− SEM from 2–3 independent experiments. Student's unpaired two-tailed t test was used to generate p values. *, $p<0.05$

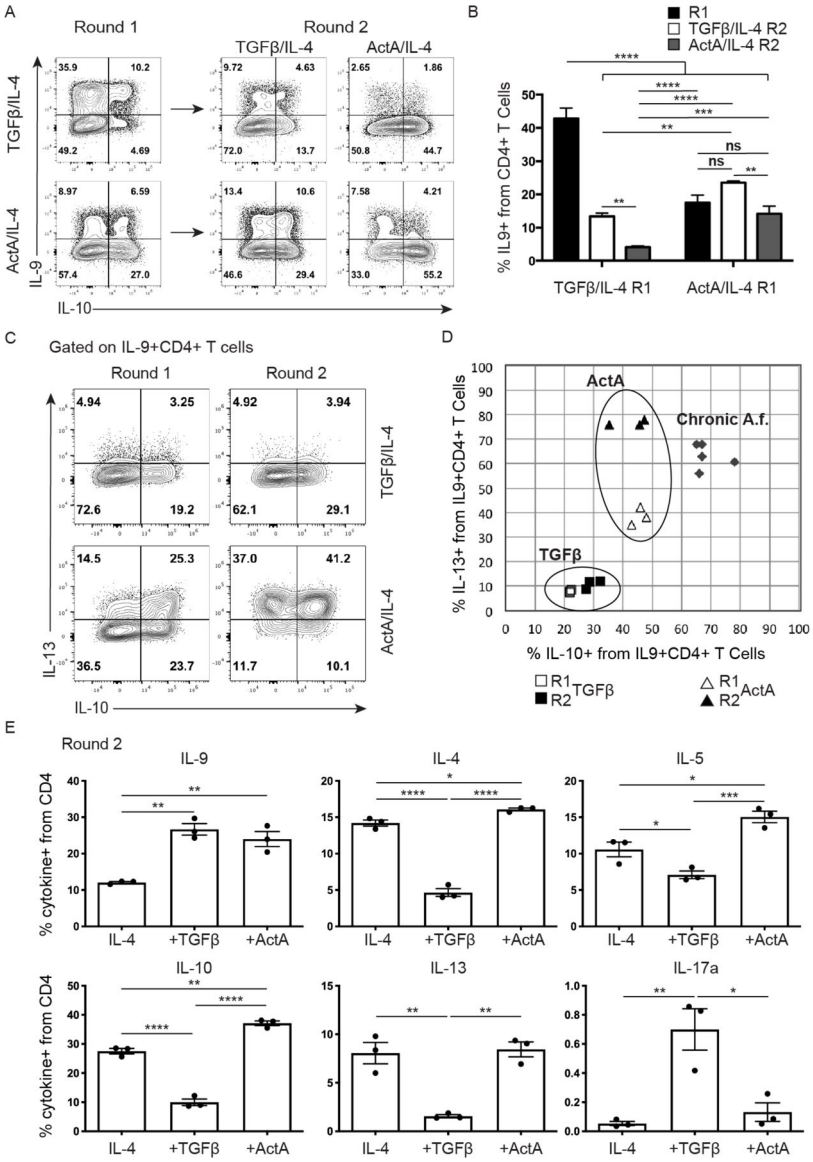


**Figure 3. Activin A induces IL-9-positive cells with a multi-cytokine profile.**

Naïve CD4 T cells were isolated from wild type and cultured with the indicated cytokine combinations for five days (R1) and then cultured for a second round in the indicated cytokine combinations. (A) Representative dot plots showing cytokine expression after stimulation with PMA/ionomycin at the end of R1 and R2. (B) Frequencies of IL-9+ cells from A. (C) Representative dot plots from cells gated upon CD4+IL-9+ showing cytokine co-expression after stimulation with PMA/ionomycin at the end of R1 and R2. (D) Scatter plot of the percentage of IL-13+ and IL10+ cells from CD4+IL-9+ cultures depicted in C. (E) Cytokine profiles were compared between R2 Th9 cultures with either TGFβ or Activin A after restimulation with PMA/Ionomycin or αCD3. One-way ANOVA with a post hoc Tukey test was used to generate p values for multiple comparisons. 3 mice/group and representative of 2–3 experiments. *, p<0.05, **, p<0.01, ***, p<0.001., ****, p<0.0001.

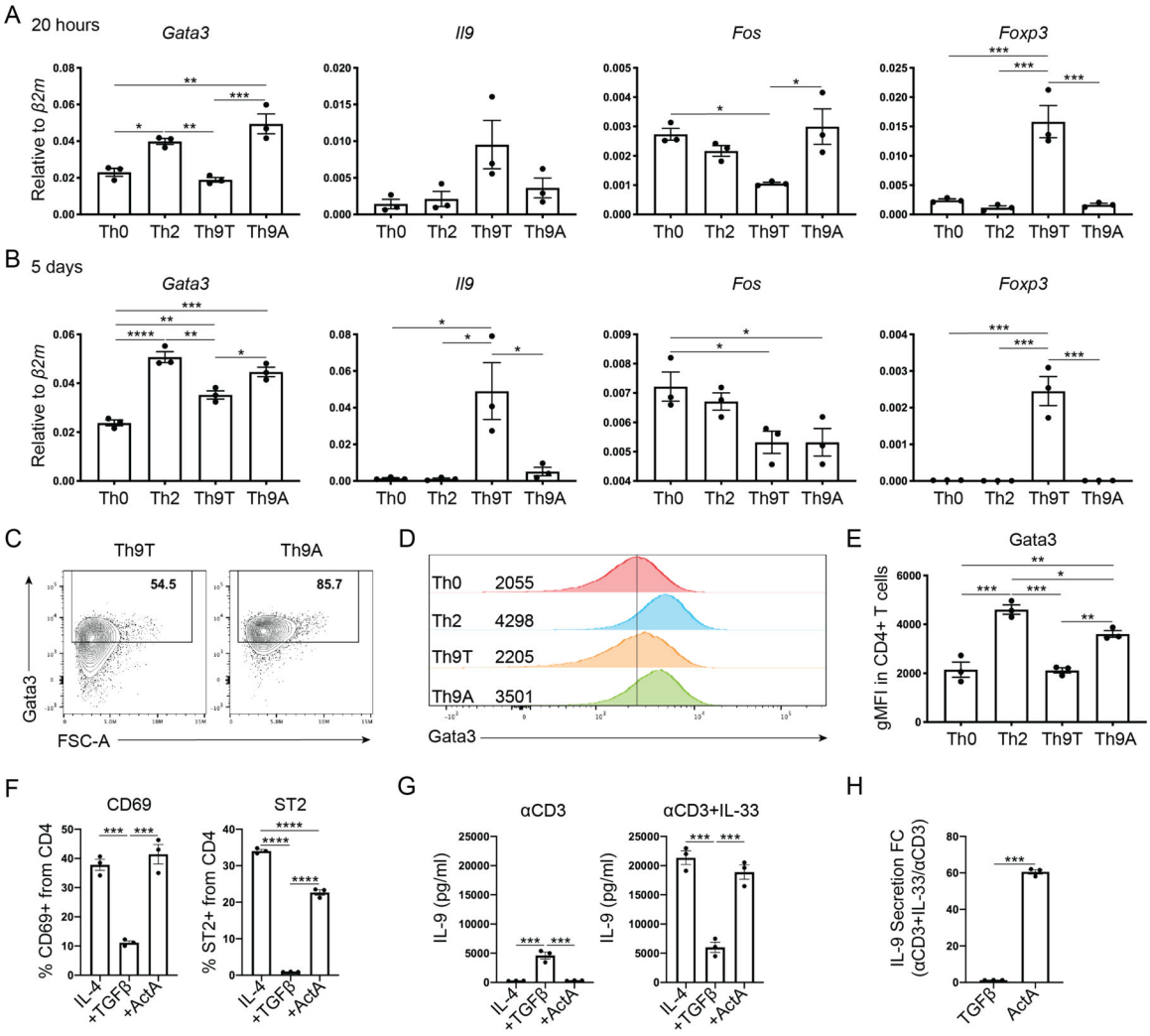


**Figure 4. Activin A does not inhibit Gata3 to the same extent as TGFβ.**

(A) Gene expression after 20 hours of culture in various Th subset conditions. (B) Gene expression after 5 days of culture in various Th subset conditions. (C) Representative dot plot of Gata3 intracellular protein staining on day 5. (D) Representative histograms of geometric mean fluorescence intensity from the various subsets on day 5. (E) Average geometric mean fluorescence intensity from D. (F) Frequencies of CD69 and ST2 positive cells in the second round of culture. (G) IL-9 secretion from round two cultures restimulated for 24 hours with anti-CD3 with and without IL-33. (H) Fold-change in IL-9 production with the addition of IL-33. One-way ANOVA with a post hoc Tukey test was used to generate p values for multiple comparisons. Student's unpaired two-tailed t test was used to generate p values. Average of 3 mice/condition and representative of 2–3 experiments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$., ****, $p<0.0001$.

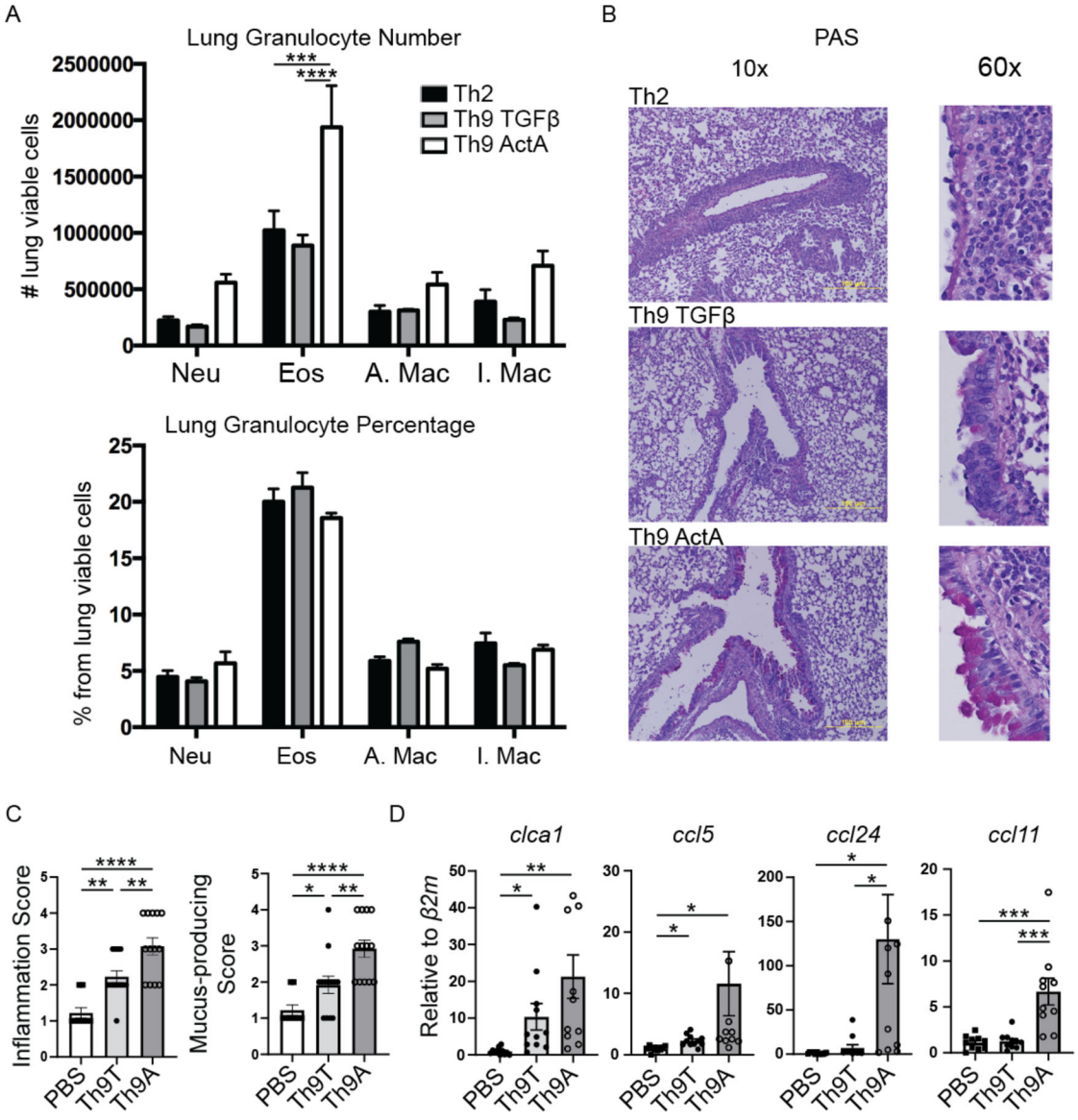


**Figure 5. Adoptive transfer of Activin A IL-9-secreting T cells increased cellularity and mucus production in the lung.**

(A) Lung granulocyte numbers and percentages from viable cell counts. (B) Representative histology sections from lungs at the end of the adoptive transfer. (C) Inflammation score and mucus producing scores of lung tissue scetions from various Th subset adoptive transferred mice. (D) Gene expression of lung tissue after 5 days OVA challenge in various Th subset adoptive transferred mice. One-way ANOVA with a post hoc Tukey test was used to generate p values for multiple comparisons. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$., ****, $p<0.0001$.