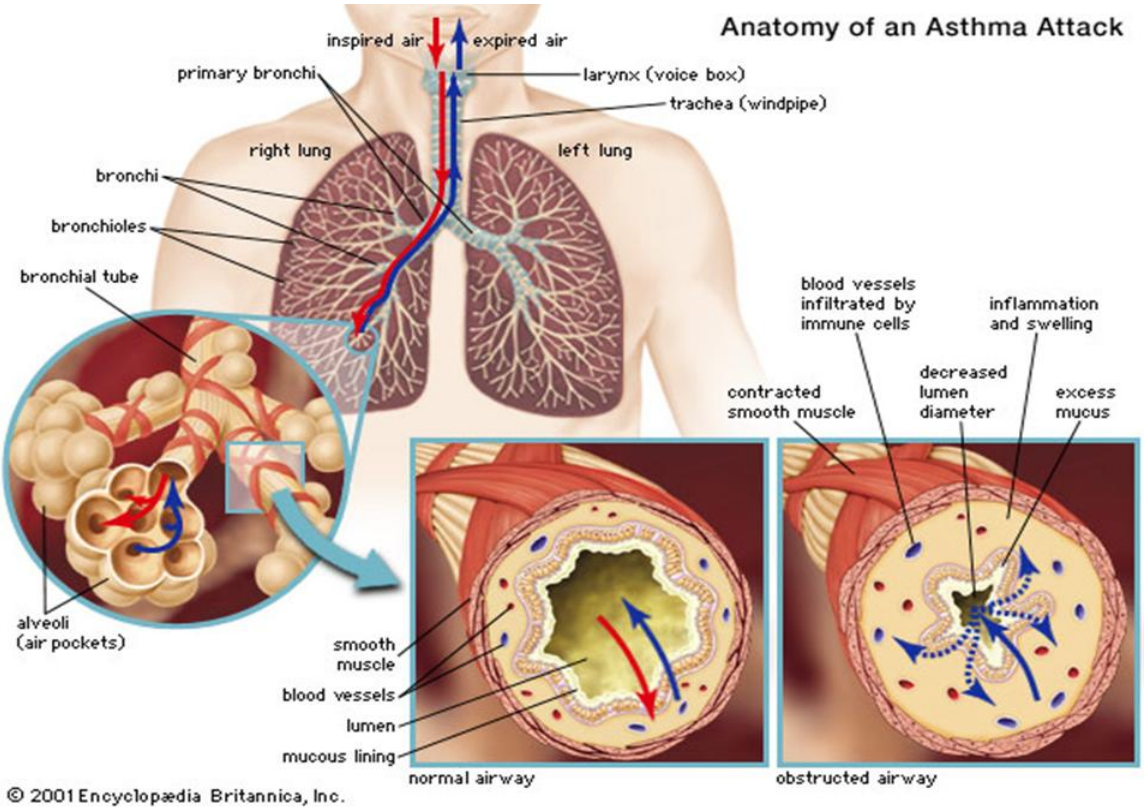
GRADUATE PROGRAM: "MOLECULAR BASIS OF HUMAN DISEASES"

Master Thesis

The Role of Activin-A in Human Allergic Asthma



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ΕΥΧΑΡΙΣΤΙΕΣ

Κατ'αρχάς θα ήθελα να εκφράσω την ευγνωμοσύνη μου στην Δρ.Γεωργία Ξάνθου, η οποία με έκανε με την υπέροχη διδασκαλία της να αγαπήσω την Ανοσολογία και μου έδωσε την ευκαιρία να εκπαιδευτώ σε ένα εργαστήριο-πρότυπο υπό την υποδειγματική καθοδήγησή της. Ο δυναμισμός της και η αφοσίωσή της στην έρευνα με βοήθησαν να ξεπεράσω όλες τις δυσκολίες και υπήρξαν το ουσιαστικό μου στήριγμα.

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CONTENTS

Περίληψη

Το αλλεργικό άσθμα είναι μία χρόνια φλεγμονώδης νόσος των αεραγωγών με σημαντικά αυξημένη συχνότητα εμφάνισης σε βιομηχανοποιημένες χώρες τις τελευταίες δύο δεκαετίες. Το άσθμα χαρακτηρίζεται από ποικίλα και υποτροπιάζοντα κλινικά συμπτώματα, τα οποία περιλαμβάνουν αναπνευστικό συριγμό, βήχα, αίσθημα συσφιγκτικού βάρους στο θώρακα και δύσπνοια. Η εκδήλωση του άσθματος σχετίζεται με τη δημιουργία Τ λεμφοκυτταρικών ανοσολογικών αποκρίσεων τύπου 2 εναντίον αβλαβών εισπνεόμενων περιβαλλοντικών αντιγόνων (αλλεργιογόνα). Οι απορυθμισμένες αλλεργιογονο-ειδικές αποκρίσεις των Τ βοηθητικών λεμφοκυττάρων τύπου 2 στους αεραγωγούς οδηγούν σε ηωσινοφιλική πνευμονική διήθηση, υπερέκκριση βλέννης, αναστρέψιμη σύσπαση των αεραγωγών και βρογχική υπεραντιδραστικότητα. Οι χρησιμοποιούμενες θεραπείες (κυρίως κορτοκοστεροειδή και αδρενεργικοί β-2 αγωνιστές) αδυνατούν να επιφέρουν ίαση και μπορούν μόνο να αντιμετωπίσουν προσωρινά τα κλινικά συμπτώματα. Αξιοσημείωτο είναι το γεγονός ότι, μία σημαντική μερίδα ασθενών πάσχει από σοβαρό άσθμα που ανθίσταται στις παρούσες θεραπευτικές πρακτικές και χρήζει συχνών νοσηλειών, αποτελώντας ένα μείζον ιατρικό πρόβλημα. Συνεπώς, παράγοντες οι οποίοι μπορούν να επάγουν ή/και να ενισχύσουν την ανοσολογική καταστολή σε αυτούς τους ασθενείς αποτελούν σημαντικούς θεραπευτικούς στόχους.

Ανοσολογικοί κατασταλτικοί μηχανισμοί, όπως οι ανοσοκατασταλτικές κυτταροκίνες και τα ρυθμιστικά Τ λεμφοκύτταρα, καταστέλλουν τις απορυθμισμένες Τ ανοσολογικές αποκρίσεις τύπου 2 σε μοντέλα ποντικιών με αλλεργικό άσθμα. Οι ανοσορυθμιστικές κυτταροκίνες ιντερλευκίνη-10 (IL-10) και μετασχηματικός αυξητικός παράγοντας β1 (TGF-β1) είναι ζωτικής σημασίας για την επαγωγή και την κατασταλτική δράση των Τ ρυθμιστικών λεμφοκυττάρων. Παρ'όλα αυτά, η έκφραση ή/και η δράση των Τ ρυθμιστικών λεμφοκυττάρων και των ανοσοκατασταλτικών κυτταροκινών είναι εξασθενημένες στους ασθενείς με αλλεργικό άσθμα.

Η ακτιβίνη-Α είναι μία κυτταροκίνη που ανήκει στην υπεροικογένεια του μετασχηματικού αυξητικού παράγοντα β1 και εμπλέκεται λειτουργικά σε μία ποικιλία σημαντικών βιολογικών διεργασιών. Αυτή η κυτταροκίνη είναι αυξημένη στον ορό ασθματικών ασθενών και ποντικιών κατά τη διάρκεια οξείας και χρόνιας αλλεργικής φλεγμονής των αεραγωγών. Η ομάδα μας έχει πρόσφατα αποδείξει ότι η ακτιβίνη-Α είναι μία ανοσοκατασταλτική κυτταροκίνη καθώς

αναστέλλει τις αλλεργιογονο-ειδικές Τ ανοσολογικές αποκρίσεις τύπου 2 καθώς και την ανάπτυξη βρογχικής υπεραντιδραστικότητας και αλλεργικής φλεγμονώδους νόσου των αεραγωγών στα ποντίκια. Επιπλέον, τα δεδομένα μας έχουν δείξει ότι η ακτιβίνη-Α ασκεί τις ανοσοκατασταλτικές τις ιδιότητες μέσω επαγωγής αλλεργιογονο-ειδικών Τ ρυθμιστικών λεμφοκυττάρων, τα οποία καταστέλλουν τις απορυθμισμένες Τ ανοσολογικές αποκρίσεις τύπου 2 *in vitro* και έπειτα από μεταφορά τους σε μοντέλα ποντικιών με αλλεργικό άσθμα *in vivo*. Ωστόσο, ο ακριβής ρόλος της ακτιβίνης-Α στο ανθρώπινο αλλεργικό βρογχικό άσθμα παραμένει άγνωστος.

Σε αυτή τη μελέτη υποθέσαμε ότι η ακτιβίνη-Α μπορεί να καταστείλει τις ανθρώπινες Τ ανοσολογικές αποκρίσεις τύπου 2 και αντιπροσωπεύει μία κυτταροκίνη-κλειδί για τη ρύθμιση της αλλεργικής φλεγμονής των αεραγωγών και του ανθρώπινου αλλεργικού άσθματος

Οι στόχοι μας ήταν : **1)** να διερευνήσουμε τις επιδράσεις τις ακτιβίνης-Α στην καταστολή των ανθρώπινων αλλεργικών Τ ανοσολογικών αποκρίσεων τύπου 2 στην περιφέρια και τους αεραγωγούς, **2)** να εξετάσουμε το ρόλο της ακτιβίνης-Α στην επαγωγή ανθρώπινων Τ ρυθμιστικών λεμφοκυττάρων και τις επιπτώσεις αυτών στην καταστολή των αλλεργικών Τ ανοσολογικών αποκρίσεων τύπου 2 **3)** να διερευνήσουμε εάν η ακτιβίνη-Α είναι ικανή να ενισχύσει την επαγόμενη από τη χορήγηση των κορτικοστεροειδών ανοσοκαταστολή σε ασθενείς με σοβαρό άσθμα που ανθίσταται στη θεραπεία. Για αυτόν τον σκοπό απομονώσαμε CD4 θετικά Τ λεμφοκύτταρα από το περιφερικό αίμα: α) υγειών εθελοντών, β) ατοπικών, γ)ασθενών με ήπιο/μέτριο άσθμα και δ)ασθενών με σοβαρό άσθμα που ανθίσταται στη θεραπεία καθώς και μονοπύρηνα κύτταρα από το βρογχοκυψελιδικό έκπλυμα ασθενών με σοβαρό, ανθεκτικό στη θεραπεία άσθμα. Εν συνεχεία, καλλιεργήσαμε τα CD4 θετικά Τ λεμφοκύτταρα ή τα μονοπύρηνα κύτταρα μαζί με αντιγονοπαρουσιαστικά κύτταρα και αλλεργιογόνο, υπό την παρουσία ή μη της ακτιβίνης-Α και μελετήσαμε τον πολλαπλασιασμό τους καθώς και την έκκριση κυτταροκινών από αυτά.

Τα δεδομένα μας αποκαλύπτουν ότι η ακτιβίνη-Α είναι ένας σημαντικός ανοσοκατασταλτικός παράγοντας στο ανθρώπινο αλλεργικό βρογχικό άσθμα. Συγκεκριμένα, δείχνουμε ότι καλλιέργεια των CD4 θετικών Τ λεμφοκυττάρων ασθματικών ασθενών παρουσία ακτιβίνης-Α, κατά τη διάρκεια ενεργοποίησής τους με αλλεργιογόνο *in vitro,* μειώνει σε μεγάλο βαθμό τον πολλαπλασιασμό τους και την έκκριση κυτταροκινών από αυτά (ιντερλευκίνη 5/ IL-5,ιντερλευκίνη 13/IL-13 και ιντερφερόνη-γ/IFN-γ). Επίσης, δείχνουμε ότι η ακτιβίνη-Α

καταστέλλει τις αλλεργιογονο-ειδικές αποκρίσεις των μονοπύρηνων κυττάρων του βρογχοκυψελιδικού εκπλύματος ασθενών με σοβαρό άσθμα σε *in vitro* καλλιέργειες. Επιπροσθέτως, τα δεδομένα μας αποδεικνύουν ότι η ανοσοκατασταλτική δράση της ακτιβίνης-Α σχετίζεται με την επαγωγή ανθρώπινων Τ ρυθμιστικών λεμφοκυττάρων τα οποία καταστέλλουν αποτελεσματικά τις προκαλούμενες από το αλλεργιογόνο Τ ανοσολογικές αποκρίσεις τύπου 2 ατοπικών ασθενών *in vitro*. Ακόμη, δείχνουμε ότι η ακτιβίνη-Α ενισχύει σημαντικά την επαγόμενη από δεξαμεθαζόνη ανοσοκαταστολή των αλλεργικών αποκρίσεων σε ασθενείς με σοβαρό, ανθεκτικό στη θεραπεία άσθμα.

Συμπερασματικά, τα δεδομένα μας δείχνουν για πρώτη φορά ότι η ακτιβίνη-Α μπορεί να καταστείλει Τ ανοσολογικές αποκρίσεις τύπου 2 σε ασθενείς με άσθμα και ατοπία, αναδεικνύοντας αυτή την κυτταροκίνη ως ένα νέο θεραπευτικό παράγοντα για το ανθρώπινο αλλεργικό άσθμα. Επιπλέον, τα αποτελέσματά μας αποκαλύπτουν ότι η ακτιβίνη-Α είναι ένας νέος παράγοντας επαγωγής ανθρώπινων Τ ρυθμιστικών λεμφοκυττάρων. Μελλοντικές έρευνες θα πρέπει να εξετάσουν τους μηχανισμούς, μέσω των οποίων η ακτιβίνη-Α επάγει ανθρώπινα Τ ρυθμιστικά λεμφοκύτταρα καθώς και τα μοριακά και κυτταρικά μονοπάτια που εμπλέκονται στην καταστολή των Τ ανοσολογικών αποκρίσεων τύπου 2.

Summary

Allergic asthma is a chronic inflammatory disease of the airways with significantly increased prevalence in industrialized countries the past 2 decades. Asthma is characterized by variable and recurring symptoms, which include wheezing, coughing, chest tightening and breathlessness. It is associated with aberrant T helper type 2 (Th2) immune responses against inhaled harmless environmental antigens (allergens). Dysregulated allergen-specific Th2 responses in the airways lead to pulmonary eosinophillic infiltration, mucus hypersecretion, reversible airway obstruction and airway hyperreactivity (AHR). Current treatments only ameliorate certain clinical features of the disease without providing a cure. Importantly, a significant group of patients has severe, treatment-refractory asthma, requires regular hospitalization and represents a major health-care problem. Hence, factors that can induce and/or enhance immunosuppression are essential therapeutic targets for asthma.

Immunoregulatory mechanisms, including suppressive cytokines and regulatory T cells (Tregs), attenuate aberrant Th2-mediated allergic responses in experimental asthma models. Immunoregulatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor- β1 (TGF-β1), are critical for the induction and suppressive function of Tregs. Nevertheless, the expression and/or function of Tregs and immunosuppressive mediators are impaired in individuals with allergic asthma.

Activin-A is a cytokine member of the TGF-β superfamily involved in a variety of essential biological processes. It is increased in the sera of asthmatics and in mice during acute and chronic allergic airway inflammation. Our group has recently shown that activin-A is an immunosuppressive cytokine as it inhibits allergen-specific Th2 responses and protects against the development of AHR and allergic airway disease in mice. Furthermore, our data have revealed that activin-A exerts suppressive function through induction of allergen-specific Tregs that suppress Th2 responses *in vitro* and upon adoptive transfer *in vivo*. However, the role of activin-A in human allergic asthma remains elusive.

In this study we hypothesized that activin-A can suppress human Th2-mediated allergic responses and represents a key cytokine for the regulation of allergic airway inflammation and human asthma.

Our **specific aims** were: **1)** to investigate the effects of activin-A on the suppression of human Th2 cell-mediated allergic responses in the periphery and the airways **2)** to delineate the role of activin-A in the induction of human Treg cells and their effects on the suppression of allergic Th2 responses and **3)** to examine whether activin-A can enhance steroid-induced immunosuppression in patients with severe, treatment-refractory asthma. For this purpose, we obtained peripheral blood (PB) $CD4^+$ T cells from age-matched: a) healthy individuals, b) atopics, c) mild/moderate asthmatics and d) severe, treatment-refractory, asthmatics and Bronchoalveolar lavage (BAL) mononuclear cells from severe asthmatics. Subsequently, we cultured $CD4^+$ T cells or BAL mononuclear cells with antigen presenting cells APCs and allergen in the presence, or not, of activin-A and we measured their proliferation and cytokine release.

Our data reveal activin-A as a critical immunosuppressive agent for human allergic asthma. More specifically, we demonstrate that activin-A treatment of PB $CD4^+$ T cells from asthmatic individuals during allergen-specific stimulation *in vitro* results in significantly decreased

proliferation and Th effector cytokine (IL-5, IL-13 and IFN-γ) release. Importantly, we show that activin-A also inhibits allergen-specific responses of mononuclear cells in the airways, the active disease site. Notably, our data demonstrate that activin-A-mediated Th suppression is associated with induction of human regulatory T cells that effectively suppress allergen-driven Th2 responses of atopic individuals *in vitro*. In addition, we demonstrate that activin-A greatly enhances dexamethasone-mediated suppression of allergic Th2 responses from individuals with severe, treatment-refractory asthma.

Collectively, our studies show for the first time that activin-A can suppress Th2 allergic responses of atopic and asthmatic individuals, pointing to this cytokine as a critical new therapeutic target for human asthma. Notably, our findings also uncover activin-A as a novel inducer of human Treg cells. Future studies should address the mechanisms through which activin-A induces human Tregs, as well as, the molecular and cellular pathways involved in Th2 cell suppression.

Introduction

Allergic Asthma

Allergic asthma is a major cause of chronic morbidity and mortality with an estimated 300 million individuals affected worldwide during 2009 *[Global Initiative for Asthma (GINA)]. Global strategy for asthma management and prevention: NHLBI/WHO workshop Report: National Institutes of Health, National Heart, Lung and Blood Institute. NIH publication No. 02-3659; updated 2009).* Importantly, its prevalence (up to 29%) and incidence have considerably increased during the last two decades, especially in children. Clinically, asthma is defined by increased AHR and reversible airway obstruction which manifests as coughing, wheezing and intermittent attacks of breathlessness and chest tightening, particularly at night or early in the morning[1].

Asthma is a chronic inflammatory disorder of the airways. Upon airway exposure to various risk factors, inflamed airways become obstructed and air flow becomes limited as a result of bronchoconstriction, mucus hypersecretion and increased recruitment of inflammatory immune cells[2] (**Figure 1**). Common risk factors for asthma symptoms include exposure to allergens

(such as those from house dust mites, animal fur, cockroaches, pollens and molds), occupational irritants, tobacco smoke, respiratory viral infections, exercise, chemical irritants, strong emotional expressions and drugs (such as aspirin and beta blockers) [3].

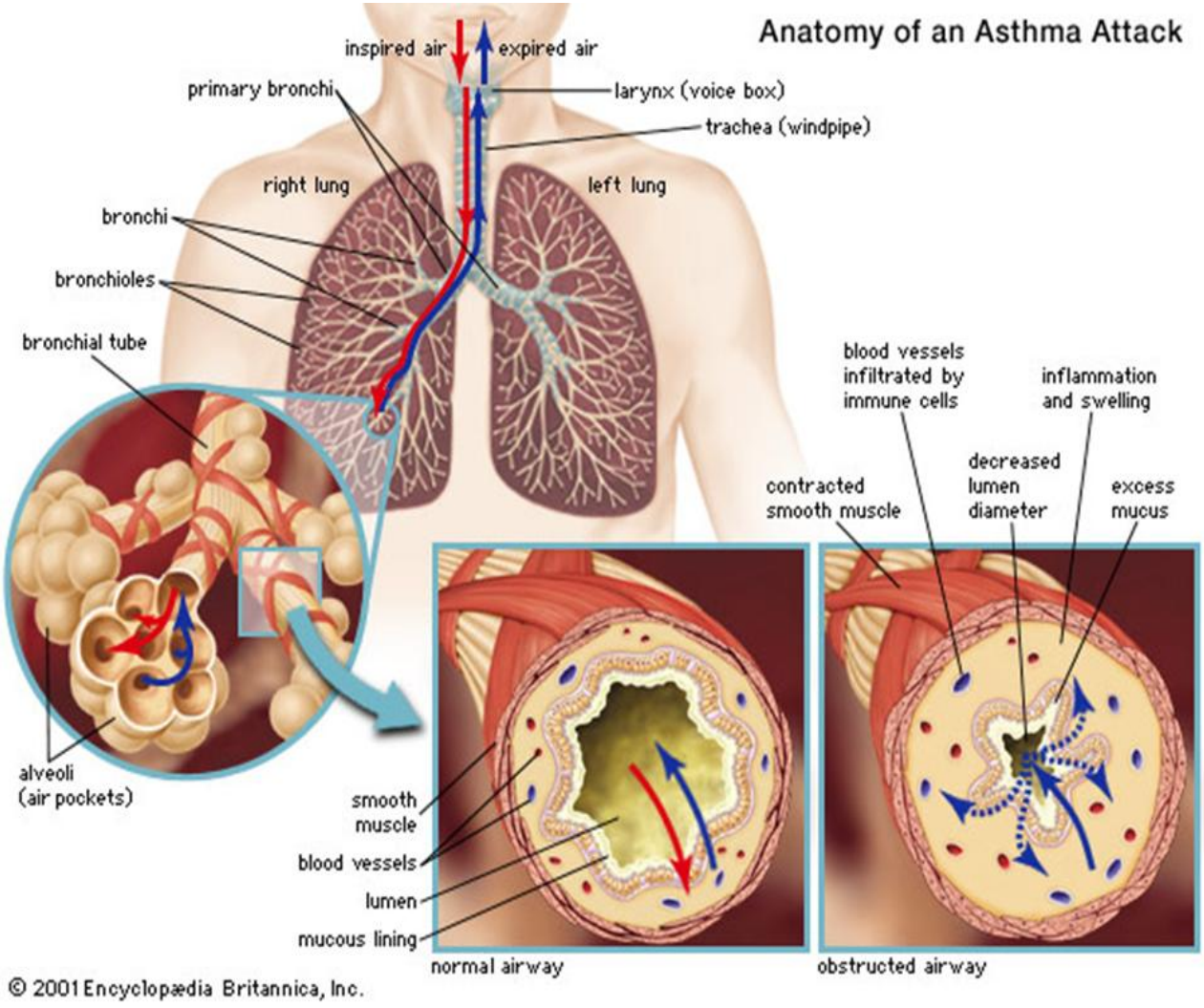

Figure 1.Anatomy of an asthma attack. During an asthma attack inflamed airways become rapidly obstructed and air flow is limited due to increased mucus production, contraction of airway smooth muscle cells and infiltration of inflammatory immune cells *(picture from Encyclopaedia Britanica 2001)*

Individuals with allergic asthma are categorized, according to the incidence, severity of symptoms and response to treatment to four main groups: a) mild, b) mild persistent, c) moderate persistent and d) severe persistent (treatment-refractory). Although individuals with mild and moderate asthma are usually controlled with current therapeutic regimes (including inhaled and per os corticosteroids and long-acting adrenergic beta-2 agonists), individuals with severe persistent asthma often do not respond to therapy and require regular hospitalizations[4]. Importantly, the chronic administration of high doses of certain medications, especially corticosteroids, in individuals with severe asthma often induces severe side effects (such as, osteoporosis, obesity, growth retardation in children, diabetes and hypertension)[4]. These individuals have significantly impaired quality of life and represent a major health care problem for developed countries. Furthermore, allergen-specific immunotherapy, a more targeted therapeutic approach, has important limitations and requires years of administration, while it can also lead to anaphylactic shock in certain individuals[5]. Hence, there is an imperative need to design novel immunotherapies that will be safer and target the cause of the disease.

Allergic Airway Inflammation

Allergic inflammation plays a central role in the development of a wide spectrum of allergic diseases, which have reached epidemic dimensions the last 20 years worldwide and their prevalence is continuing to increase. Asthma, rhinoconjunctivitis, sinusitis, food allergy, atopic dermatitis, angioedema, urticaria, insect and drug allergy are some of the allergic disorders that can occur either alone or in combination. Both genetic predisposition and environmental factors are crucial for the development of allergic airway inflammation[6].

In genetically predisposed individuals, initial exposure to an innocuous environmental allergen, leads to the capture and processing of the allergen by local APCs, mainly dendritic cells (DCs). Subsequently, DCs mature and migrate to regional lymph nodes where they present peptides of the processed allergen in the context of major histocompatibility complex (MHC) class II molecules to naive T cells. This process leads to the activation and differentiation of naove T lymphocytes into Th2 cells[3]. Allergen-specific Th2 cells orchestrate the allergic immune response through the production of Th2-type cytokines. More specifically, IL-4 is crucial for the differentiation of Th2 cells and IgE production, IL-5 governs eosinophilic

differentiation and mobilization, IL-9 controls the recruitment and proliferation of mast cells and IL-13 has an important role in Th2 cell activation and induction of airway remodelling[7].

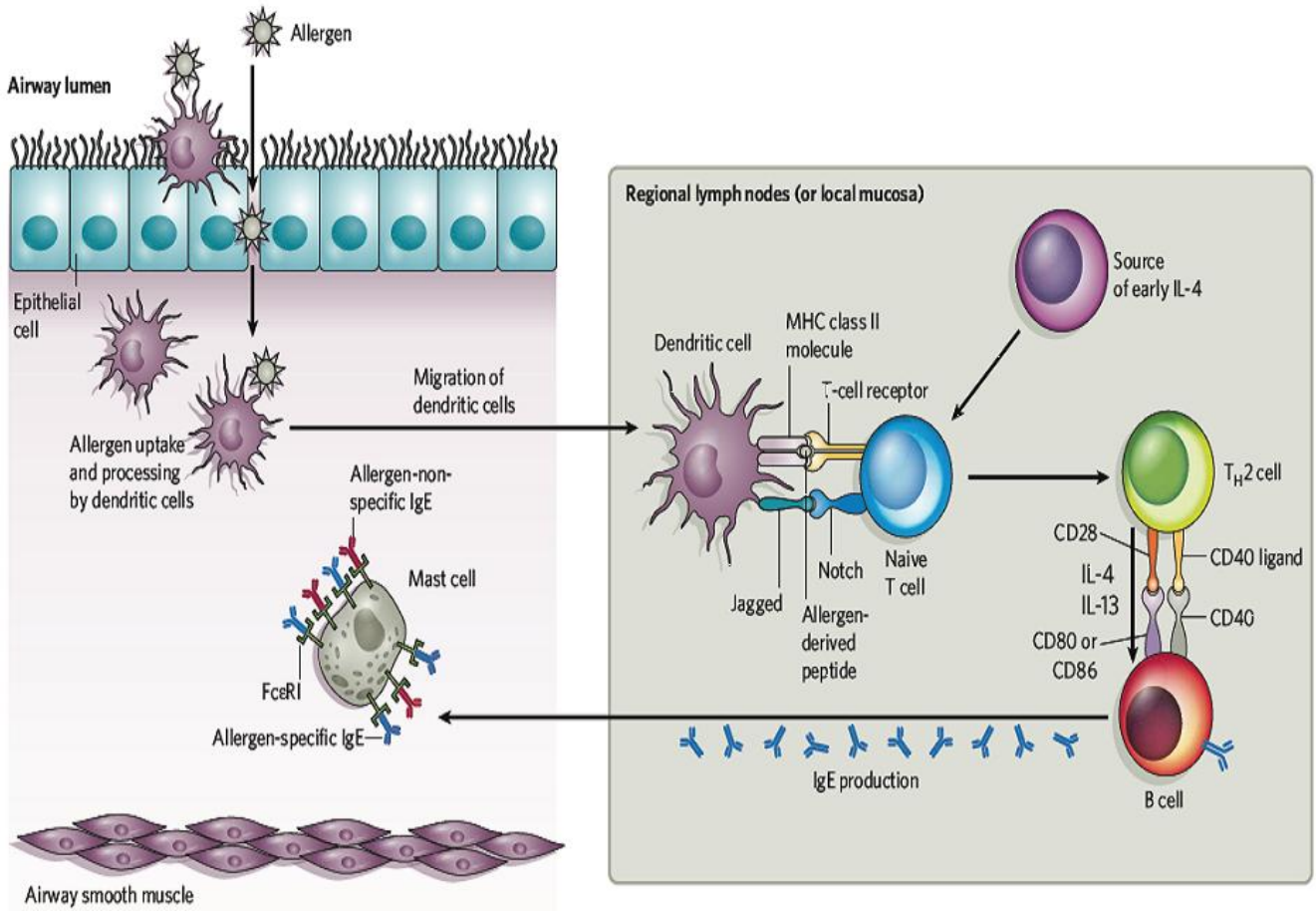

Figure 2.Allergen sensitization phase. Initial contact with an inhaled innocuous allergen leads to the development of the sensitization phase in atopic individuals. Local APCs capture the allergen, process it and present it to naïve T lymphocytes into the regional lymph nodes resulting in their differentiation into allergen-specific Th2 cells *(picture from the article "The development of allergic inflammation", Galli SJ et al, Nature 2008)*

In addition, Th2 lymphocytes mediate isotype switching in B cells, through the production of IL-4 and IL-13 and the ligation of co-stimulatory molecules [CD28 on T cells binds on CD80 or CD86 on B cells and CD40ligand (CD40L) on T cells binds on CD40 on B cells] , in the

germinal centre of the lymph nodes[8]. This procedure, which is termed allergen sensitization, results in the generation of allergen-specific Th2 cells and IgE producing B cells (**Figure 2**). Allergen-specific IgE diffuses into the efferent lymphatic vessel and then enters the circulation, as well as, the interstitial fluid. Subsequently, allergen-specific IgE binds to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils localized in the airways. During the allergen sensitization phase there are no evident clinical symptoms denoting this aberrant immune process[3].

Subsequent exposure to the same allergen eventuates, after a few minutes, to the development of the early phase response that is governed by the crosslinking of allergen-specific IgE bound to high-affinity receptors (FcεRI) on basophils and mast cells. IgE-mediated activation of these innate immune cells drives the rapid release of preformed mediators (i.e.histamine) or the slower secretion of newly synthesized factors (i.e. leukotrienes, prostanoids), as well as, chemokines, cytokines and growth factors. The rapidly secreted mediators induce vascular permeability, vasodilation, smooth-muscle cells contraction and increased mucus production in the airways. The release of these inflammatory mediators contributes substantially to the appearance of the acute clinical signs and symptoms of allergic asthma[9].

The newly synthesized mediators (i.e. chemokine (C-C motif) ligand 2 CCL2, leukotriene B4(LTB_4), IL-8, tumour necrosis factor-α (TNF-α) of mast cells are released in a slower rate and lead to the recruitment of Th2 cells and eosinophils. The generation of the late phase reaction is based on this recruitment and results in the further release of inflammatory mediators (eosinophilic cationic protein, major basic protein etc) and cytokines (IL-4, IL-5, IL-9, IL-13 etc)[10]. The development of the late-phase reaction begins 2-6 hours after the exposure to the allergen and peaks after 6-9 hours. During this phase, innate and adaptive immune cells, as well as, tissue-resident cells contribute to the establishment of cardinal features of allergic airway inflammation. For example, in a late phase reaction, recruited neutrophils release elastase that promotes the activation of matrix metalloproteinases (MMPs) and the degradation of collagen. Moreover, recruited eosinophils secrete basic proteins leading to the

injury of bronchial epithelial cells while several other mediators produced by tissue-resident cells induce bronchoconstriction[11].

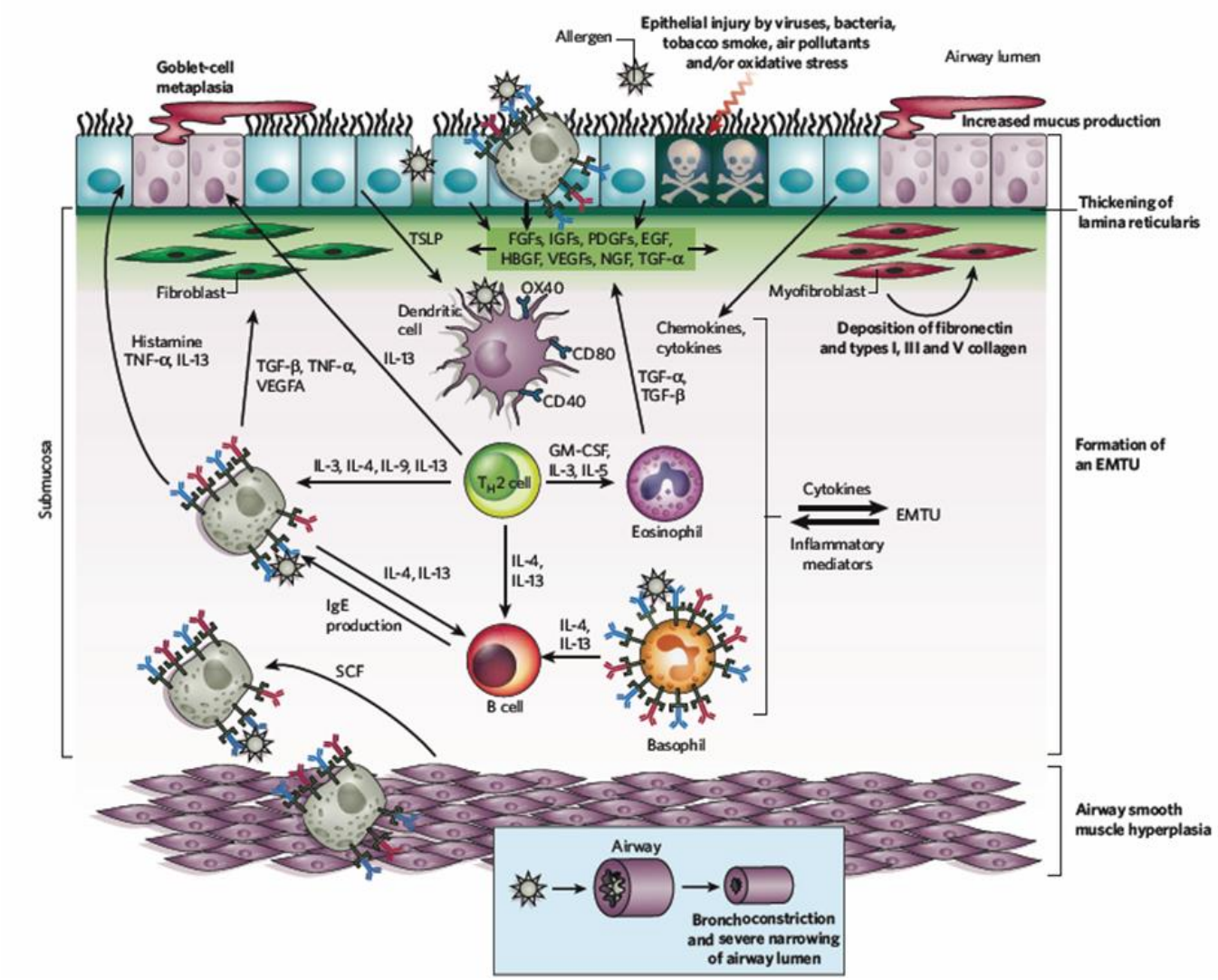

Figure 3.Chronic allergic airway inflammation. Repetitive and/or persistent exposure to the allergen results in the generation and establishment of chronic allergic airway inflammation which is mainly characterized by mucus hypersecretion, airway smooth muscle cell hyperplasia, goblet cell metaplasia and thickening of the lamina reticularis *(picture from the article "The development of allergic inflammation", Galli SJ et al, Nature 2008)*

Repetitive and/or persistent exposure to the allergen gradually leads to the development of chronic allergic airway inflammation and tissue remodelling (**Figure 3**). Inflammation affects then all the layers of the airway wall and both the structure and the function of the tissue-resident cells change dramatically. Furthermore, innate and adaptive immune cells are widely distributed in the airways and develop complicated interactions with a variety of local cells (i.e. epithelial cells, fibroblasts, airway smooth muscle cells)[3, 9] .

Apart from Th2 cells other Th subsets are also considered important for the development of chronic allergic airway inflammation and related disease. For example, Th1 cells induce apoptosis of airway epithelial cells and activation of smooth muscle cells, while Th17 cells trigger allergic airway inflammation through the activation and recruitment of neutrophils[12]. The consequences of the sustained airway inflammation are many and include increased production of mucus due to goblet-cell hyperplasia, development of myofibroblasts, increased vascularity, hypertrophy and hyperplasia of airway smooth muscle cells, thickening of the airway walls, increased deposition of extracellular matrix molecules beneath the epithelial basement membrane (which is called lamina reticularis), such as collagen and fibronectin, and increased production of cytokines and chemokines[13]. All these changes have as a result the injury of epithelial sites and the subsequent development of repair processes. The complex interactions that take place between the epithelium and the mesenchymal cells [known as the epithelial-mesenchymal trophic unit, (EMTU)] during all these processes promote the establishment of the chronic allergic airway inflammation. Individuals with severe allergic asthma, chronic allergic airway inflammation and remodelling display significantly increased bronchoconstriction, thickening of the airway wall and mucus production, features that lead to obstruction of the air flow and exacerbated asthma symptoms[3]. As dysregulated immune responses represent a central pathologic feature of asthma, identification of factors that can effectively suppress allergic airway inflammation and protect against disease may lead to novel therapeutic discoveries.

Regulation of Allergic Airway Inflammation

Critical controllers of allergic immune responses are Treg cells and immunosuppressive cytokines, such as IL-10 and TGF-β1[6, 14]. The immunoregulatory cytokines IL-10 and TGF-β1 are involved in the induction, as well as, the suppressive function of Tregs and are critical for the maintenance of pulmonary homeostasis[15]. More specifically, IL-10 has broad immunomodulatory properties, including the inhibition of pro-inflammatory cytokine secretion, the inhibition of DCs maturation, the suppression of mast cell and eosinophil activation and the induction of IgE to IgG4 switching[16]. The fact that IL-10 expression is decreased in the airways of severe asthmatic individuals and that certain genetic polymorphisms in the promoter of the IL-10 gene are associated with more severe asthma phenotypes also denotes the central role this cytokines plays in the regulation of allergic airway disease. Moreover, therapies that manage to ameliorate certain clinical features of asthma lead to increased production of IL-10 by T cells[17]. TGF-β1 plays also central role in the induction of $Foxp3^+$ aTregs and is important for the prevention of allergen sensitization[15]. However, IL-10 and TGF-β1 cannot completely inhibit allergen-driven inflammatory responses[18, 19]. In fact, these cytokines in certain context exhibit pro-inflammatory effects[20-23]. For example, TGF-β1 promotes the differentiation of pathogenic Th17 cells and exacerbates airway remodelling[20-22], while IL-10, when produced by Th2 cells along with IL-4 and IL-13, enhances Th2 cell activation[23].

Tregs are essential for the maintenance of immune tolerance to innocuous inhaled antigens (**Figure 4**) and mainly consist of the naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs (nTregs) and the adaptive Tregs (aTregs). The latter subset includes the TGF-β1-induced $Foxp3^+$ T cells and the type 1 regulatory $Foxp3^-$ IL-10-producing T cells (Tr1)[24]. Tregs can suppress allergic airway inflammation and AHR in experimental models of asthma and, importantly, prevent cardinal features of chronic disease, such as airway remodelling, mucus hypersecretion and peribronchial collagen deposition[25]. In addition, mouse Tregs can suppress responses by innate effector cells, such as mast cells and eosinophils[26].

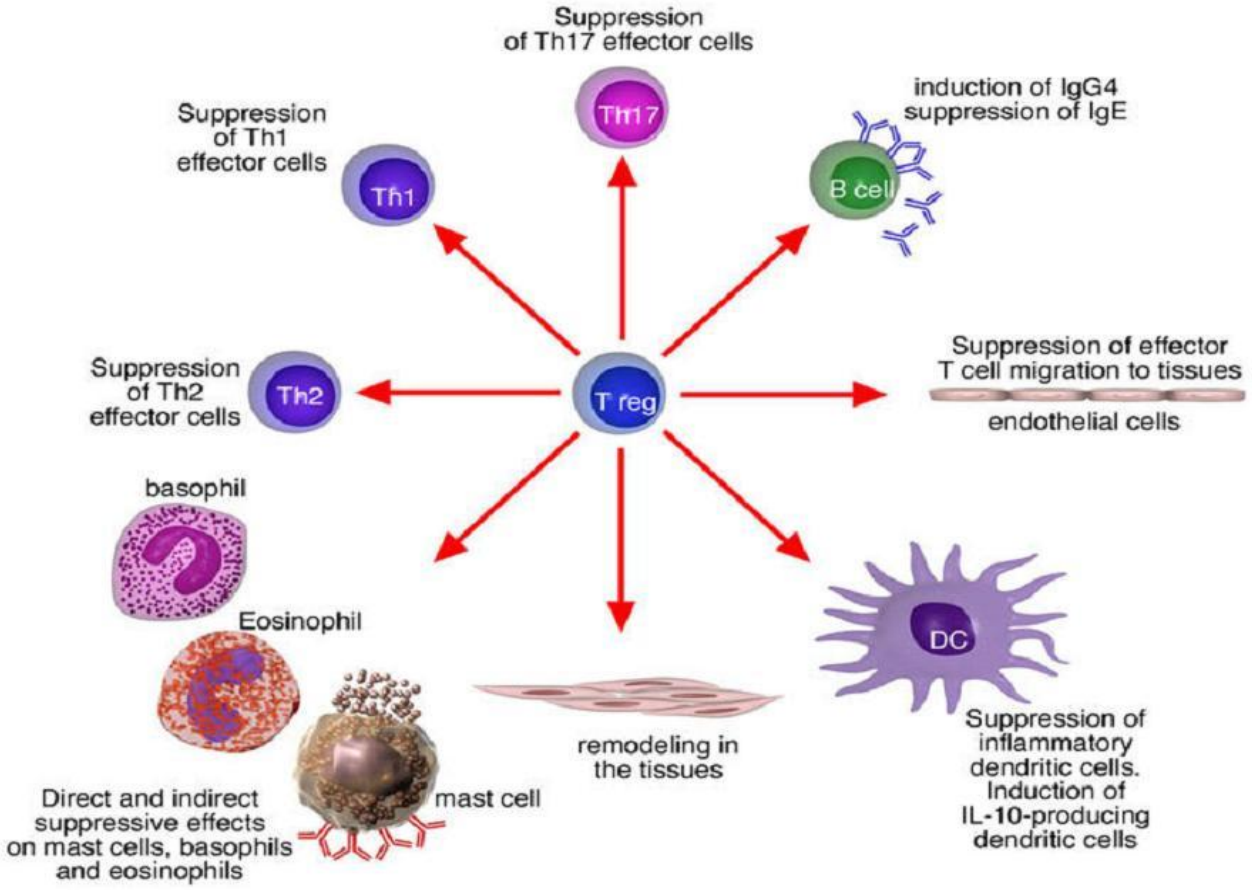

Figure 4.Tregs are essential for the maintenance of immune tolerance to innocuous inhaled antigens. Tregs are critical controllers of allergic airway inflammation since they can suppress a variety of both innate and adaptive immune responses against innocuous inhaled allergens, leading to lung homeostasis. *(picture from the article "Mechanisms and treatment of allergic disease in the big picture of regulatory T cells", Adkis CA et al, J Allergy Clin Immunol 2009)*

Even though there is a plethora of *in vivo* studies investigating the role of Tregs in mouse models of allergic airway inflammation, there are limited studies in humans. Furthermore, the majority of these studies are focused on effects of $CD4^+CD25^+$ T cells without being able to distinguish Tregs from activated effector T cells. These studies have demonstrated that $CD4^+CD25^+$ T cells are reduced in BAL from asthmatic subjects[27]. However, variable results are observed in the PB[28]. Some recent studies have revealed that Foxp3 mRNA expression is decreased in the PB of individuals with asthma as compared with healthy participants[28].

However, the protein levels of Foxp3 were not measured and more importantly Foxp3 expression was not examined in the lung, the active disease site[28]. Moreover, the investigation of the suppressive function of Tregs in patients with asthma is also limited. In a recent study pulmonary and PB $CD4^+CD25^{high}$ T cells from healthy children were able to suppress $CD4^+CD25^-$ T cell responses, in contrast to children suffering from asthma. In the same study pulmonary, but not PB, $CD4^+CD25^{high}$ T cells were decreased in number and less suppressive in asthmatic children[27]. Importantly, their regulatory function was restored following inhalation of corticosteroids[27]. In addition, another study revealed a decrease in the efficacy of the suppressive function of Tregs in Der p1-sensitive asthmatic children[29]. Several groups have shown that administration of corticosteroids results in increased expression of both Foxp3 and IL-10, beneficially influencing Tregs.

Collectively, these studies reveal that Tregs are impaired in patients with ashma. In addition, human aTregs induced by TGF-β1 exhibit an unstable phenotype and, more importantly, do not effectively suppress T cell responses. Moreover, induction of human Tr1 cells requires several rounds of antigenic stimulation and results in few numbers of suppressive cells containing also pathogenic Th effectors. Hence, from a therapeutic standpoint, there is an imperative need for identification of factors that can increase the numbers and/or function of human Treg subsets in asthma[17, 27].

Activin-A

Structure and Signaling Pathway of Activin-A

Activin-A is a pleiotropic cytokine that belongs to the TGF-β superfamily consisting of more than 40 members. It was initially described as a gonadal protein stimulating the biosynthesis and secretion of follicle-stimulating hormone (FSH) from the pituitary[30]. Since then activin-A has been found to play a critical role in a variety of essential biological processes such as mesoderm induction, promotion of neuron survival, stimulation of erythropoiesis, wound repair and maintenance of stem-cell self-renewal and pluripotency[31]. Importantly, activin-$A^{-/-}$ mice are neonatally lethal due to severe craniofacial defects (cleft palate and loss of whiskers, upper

incisors, lower incisors and lower molars) [32]. Moreover, 75% of activin receptor type IIA (actRIIA)$^{-/-}$ mice reach adulthood but have major deficiencies in their reproductive systems (infertility in females due to folliculogenesis defect and delayed fertility in males), while 25% of these mice die at birth due to mandible defects[33]. Recent studies have revealed that activin-A is a true cytokine with crucial role in responses mediated by innate and adaptive immune cells.

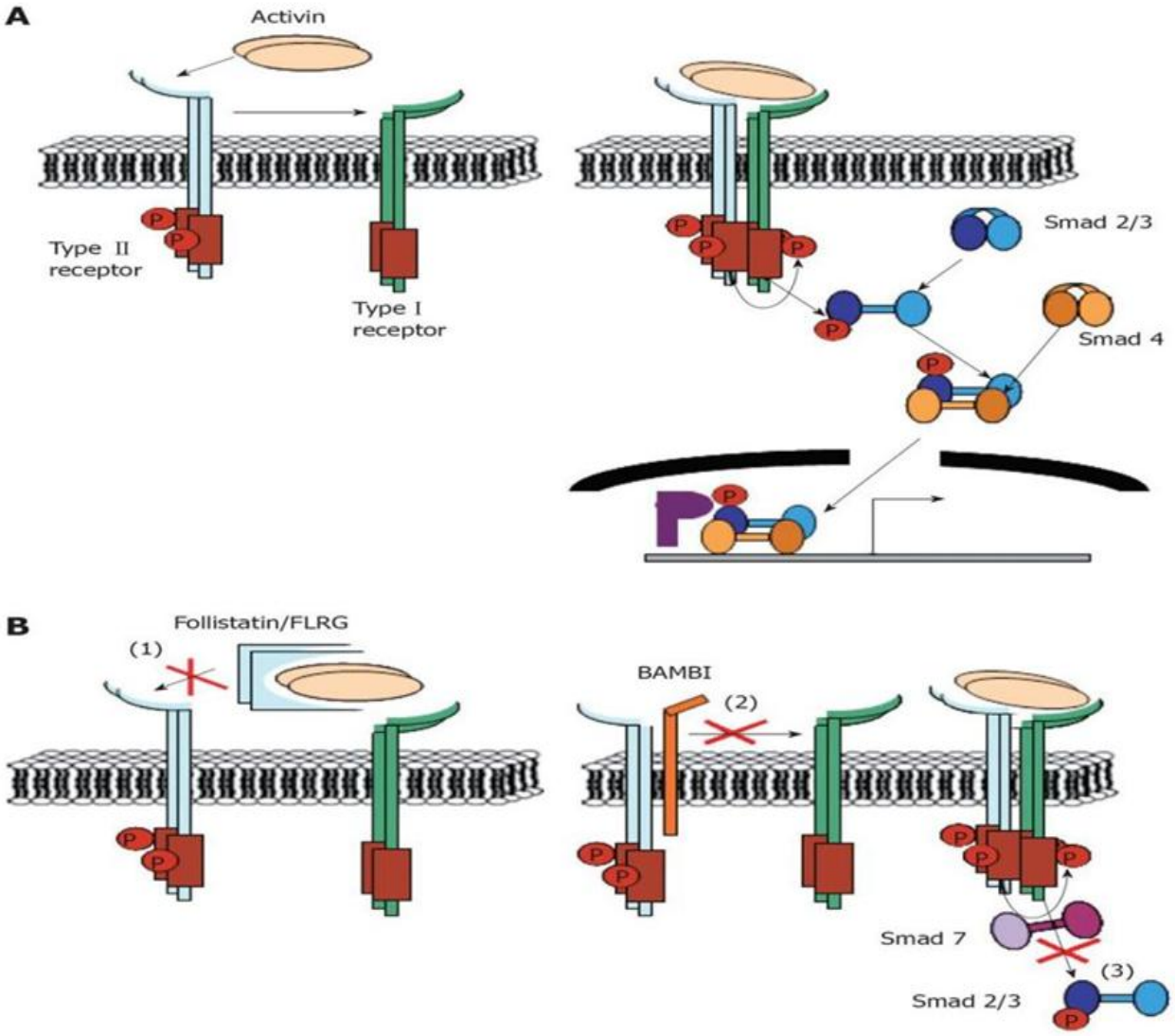

Figure 5.Signalling pathway and regulation of activin-A. A. Signalling of activin-A is mediated through a membrane heterodimeric receptor complex. Initially it binds to the

constitutively active ActR-II, which then recruits and phosphorylates ActR-I. Activated ActR-I phosphorylates then the main intracellular mediators of activin-A's signalling, Smad2/3. pSmad2/3 form a complex with Smad4 and translocate to the nucleus, where they regulate the transcription factor of target genes in cooperation with several transcription co-factors. **B.** Activin-A is highly regulated at both the intracellular and extracellular level by a variety of molecules. Follistatin binds to activin-A, preventing the interaction with its type II receptors, while Smad7 binds stably to ActR-I and prevents the recruitment and phosphorylation of Smad2/3.

Activin-A is a homodimeric protein consisting of two βA-subunits (βAβA), connected by a disulfide bond. Homodimeric activins-B, C and E (consisting of two βB, βC and βE subunits respectively), and the heterodimeric activin-AB (consisting of one βA and one βB subunit) have been also described in mammals. The characteristic feature of all the β subunits is the cysteine knot in their carboxyl-terminus, which is a folding domain, containing nine conserved cysteines, important for the stabilization and dimerization of the ligands. More specifically, the sixth cysteine is essential for dimerization, while the other eight form intramolecular disulfide bonds which determine the three-dimensional structure of the peptides. Transcripts of the βA and the βB subunits are detectable in almost all tissues with especially high expression in reproductive organs, while βC and βE subunits are mainly expressed in the liver[34]. Activin-A is the most extensively investigated ligand and even though activins-B and AB are bioactive, their functions as well as expression patterns are not clearly defined.

In contrast to TGF-β1, activin-A is not produced or stored in an inactive form but is secreted as a glucosylated pre-proligand. Although, the cleavage procedure leading to the generation of the ligand remains elusive, it is possible that the endoprotease furin is involved in the enzymatic cleavage of the 426 pre-proligand to a 20 amino acid signal sequence, 290 propeptide sequence and 116 amino acid activin subunit[35].

Activin-A signalling is mediated by a membrane heterodimeric receptor complex consisting of two types of activin receptors (**Figure 5**), type I [(ActR-I or activin-like kinase receptor 4 and 7 (ALK4 and ALK7)] and type II (ActR-IIA and ActR-IIB)[31]. TGF-β1 signals through distinct to

activin-A receptors, while other members of this superfamily, such as nodal, growth and differentiation factor 11 (GDF11) and myostatin, can use the same receptors as activin-A. These single-pass transmembrane receptors are structurally similar, containing a small cysteine-rich extracellular domain and an intracellular serine/threonine kinase domain. However, only the ActR-I possess a glycine and serine-rich domain, the GS domain, near the intracellular juxtamembrane region, important for signal transduction. Initially, activin-A binds to the constitutively active ActR-II, which then recruits ActR-I and phosphorylates its GS domain. Subsequently, activated ActR-I phosphorylates the main intracellular mediators of activin-A signalling, the Smad proteins[36]. Eight Smad proteins have been identified. The receptor-regulated Smads (R-Smads), which are Smad -1, Smad-2, Smad-3, Smad-5 and Smad-8, are phosphorylated and thus activated by the kinase domains of the receptors. Smad-2 and Smad-3 are the phosphorylation substrates for TGF-β1 and activin-A type I receptors and after their activation they form a complex with the common partner of all Smads (Co-Smad), the Smad-4. Subsequently, this complex translocates to the nucleus, with the help of a nuclear localization signal (NLS) of Smad-4, and in cooperation with several transcription co-factors, such as CBP/p300 and TGIF, regulates the transcription of target genes[36]. Inhibitory Smads (I-Smads), which are Smad-6 and Smad-7, are upregulated after activin-A ligation to its receptors and bind stably to the activated type I receptor, inhibiting the further propagation of its signals, in an autocrine negative feedback loop. Recent studies have shown that apart from the main Smad-mediated signaling pathway, activin-A can transduce its signals through additional, Smad-independent, pathways including mitogen activated protein kinase extracellular signal regulated kinase (MAPK/ERK) and c-Jun N-terminal kinase (JNK) pathways[37].

Regulation of Activin-A

The actions of activin-A are regulated by a variety of molecules at both the extracellular and intracellular level (**Figure 5**). Follistatin (FS) is one of the major inhibitors of activin-A that neutralises its actions by binding with high affinity to activin-A and, thus, preventing the interaction with its type II receptors[38]. Furthermore, FS induces rapid endocytic internalisation and subsequent proteolytic degradation of activin-A. Alternative splicing leads to the formation of two isoforms of FS. The FS288 isoform binds heparan sulphate proteoglycans with high

affinity and is considered to be a local regulator of activin-A's actions, while the FS315 isoform does not bind cell-surface proteoglycans and is the predominant circulating form of the protein. Notably, FS can also inhibit the effects mediated by all activins, as well as, the functions of other TGF-β superfamily members, including myostatin and bone morphogenetic proteins (BMPs)[39]. Inhibins are circulating heterodimeric proteins consisting of the inhibin α subnit and the activin βA (inhibin A, α βA) or βB subunit (inhibin B, α βB). Inhibins downregulate the signalling of all activins mainly through competition for binding to type II receptors but also by binding to activins with variable affinity and, thus, antagonizing their functions[38]. Betaglycan is a protein that binds inhibins and increases their affinity to activin-A type II receptors, leading to functional inhibition of activins[38]. In addition, the pseudoreceptor BAMBI inhibits activin-A, TGF-β and BMPs signaling by interacting with their type I receptors and interrupting the formation of the active receptor signaling complex. Moreover, the co-receptor for nodal ligands, Cripto facilitates nodal signaling by binding to both nodal and activin receptors, inhibiting activin-A's signaling when overexpressed[38]. At the intracellular level, I-Smads bind stably to ALK4 and prevent the recruitment and phosphorylation of R-Smads resulting to the inhibition of the activin-A signaling. Moreover, I-Smads promote the binding of Smad ubiquitin regulatory factors 1 and 2 (Smurf 1 and 2) to activin-A receptors and result in the ubiquitin-dependent degradation of the receptors[40].

The Role of Activin-A in Immunity and Inflammation

Recent studies have revealed activin-A as an important immunoregulatory factor. Nearly every cell type in the body, including immune cells, is capable of synthesizing and secreting activin-A. The secretion of activin-A by monocytes, macrophages, DCs, T cells, B cells, mast cells and neutrophils is highly regulated and dependent on the microenvironment cytokine milieu and the activation status of each cell. A wide range of human cells produce activin-A after *in vitro* or *ex vivo* stimulation with inflammatory mediators. For example, human bone marrow stromal cells express activin-A after *in vitro* stimulation with IL-1α, lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) [41]. Moreover, human DCs upregulate activin-A, ActR-I and ActR-II expression following *in vitro* stimulation with CD40L, LPS, a Toll-like receptor 4 ligand (TLR4) or CpG oligodeoxynucleotides which are TLR9 ligands [42] (**Figure 6**). Except from immune cells, a variety of tissue-resident cells, including epithelial cells,

endothelial cells, intestinal cells, cardiomyocytes, fibroblasts and microglia, can also secrete activin-A, mainly upon stimulation with inflammatory factors, such as LPS and IFN-γ [41, 43]. In addition, human synoviocytes stimulated *ex vivo* with IL-1, IL-8, IFN-γ and TGF-β1 rapidly secrete activin-A [44].

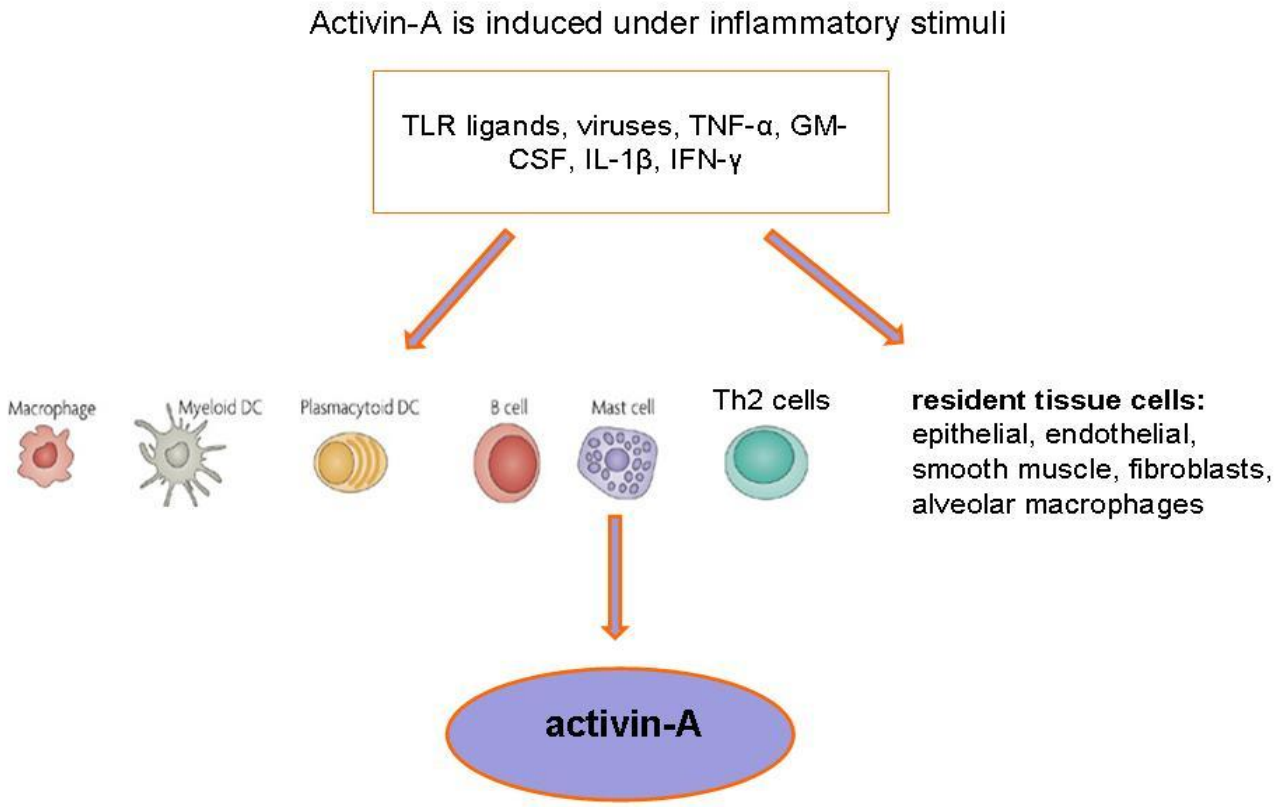

Figure 6. Activin-A is induced under inflammatory stimuli. TLR ligands, viruses, TNF-α, GM-CSF, IL-1β and IFN-γ induce the expression of activin-A in a variety of innate and adaptive immune cells, as well as, in tissue cells.

In vivo studies using an animal model of LPS-induced acute systemic inflammation, demonstrated that activin-A is rapidly released as part of the circulatory cytokine cascade[45]. In

fact, the secretion of activin-A occurs as early as 30 minutes after *in vivo* LPS administration, concurrent with that of TNF-α and prior to IL-6 release[46]. These data are in accordance with the substantially increased serum levels of activin-A in patients with septicemia. Furthermore, activin-A has been found increased in serum and cerebrospinal fluid of patients with bacterial and viral meningitis and was localized to microglia and infiltrating macrophages in the meningitic brain[47]. Importantly, accumulating evidence suggests that activin-A is a critical neuroprotective factor[48].

Activin-A concentration is also elevated in the serum and synovial fluid of patients with inflammatory arthropathies, such as gout, and rheumatoid arthritis (RA) but not in osteoarthritis, which is a degenerative disease[49]. The main cellular sources of activin-A in the synovial fluid are infiltrating macrophages and synoviocytes. In addition, a recent study has also demonstrated that activin-A is increased in the serum of patients with systemic lupus erythematosus (SLE). Importantly, increased serum levels of activin-A correlated with disease activity parameters of both RA and SLE[49]. Furthermore, activin-A as well as its receptors are upregulated in patients with ulcerative colitis and Crohn's disease[50, 51]. Interestingly, the highest levels of activin-A expression are detected in areas of the greatest inflammation and are restricted to the mucosa and sub-mucosa regions. On the contrary, activin-A levels were shown to be reduced in the BAL fluid of patients with the autoimmune pulmonary alveolar proteinosis (PAP), which is a rare lung disease characterized by abnormal accumulation of surfactant within the alveoli and impaired gas exchange[52].

Activin-A is also implicated in tissue repair processes and fibrosis. In fact, activin-A over-expression in the skin of transgenic mice enhances the wound healing but also augments the scarring response[53]. On the other hand, over-expression of the activin antagonist, FS, causes a severe delay in wound repair, but the quality of the healed wound is improved. Recently, the Mad1 transcription factor that inhibits proliferation and induces differentiation of various cell types was identified as a direct target of activin-A in keratinocytes, supporting the essential role that activin-A plays in wound healing[54]. However, activin-A is also involved in the progression of fibrotic diseases, such as interstitial fibrotic lung disease[55]. Activin-A induces migration of fibroblasts and differentiation to myofibroblasts following *in vitro* culture. In

addition, *in vivo* administration of FS in the mouse lung ameliorates bleomycin-induced fibrosis in a bleomycin-induced lung fibrosis mouse model[56].

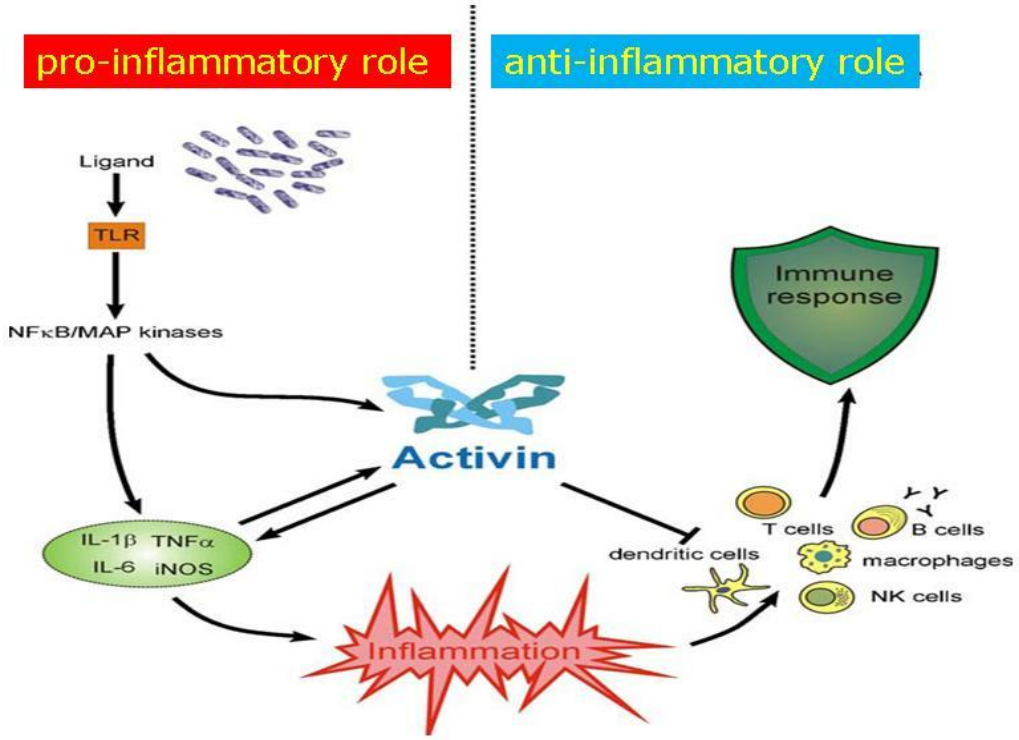

Figure 7.Dual role of activin-A in immune responses. Activin-A exerts distinct effects on immune responses ranging from enhancing to inhibitory, depending on the cell type, the existing cytokine milieu and the context of the response

An intriguing feature of activin-A is that it exerts both pro- and anti-inflammatory actions, depending on the the cytokine milieu and the context of the immune response (**Figure 7**). Regarding the pro-inflammatory effects of activin-A, treatment of bone-marrow derived macrophages with activin-A stimulates the release of the pro-inflammatory mediators TNF-α and IL-1β as well as the production of prostaglandin E2 (PGE2), thromboxane and inducible nitric oxide synthase (iNOS) [57]. In accordance, activin-A increases nitric oxide (NO), IL-1 β and CD14/CD68 expression on resting mouse macrophages and promotes their phagocytic

capacity[58]. Activin-A also influences the biology of DCs. In fact, activin-A promotes the *in vitro* production of CXC chemokine ligands 12 and 14 (CXCL12 and CXCL14) by immature human and murine DCs inducing their directional migration[59]. Moreover, treatment with activin-A *in vitro* stimulates the differentiation of human monocytes to langerhans cell (LC) and enhances LC migration through IL-12p70 induction[60]. Furthermore, *in vitro* pre-treatment of resting B cells with activin-A prior to LPS stimulation results in significantly increased LPS-induced proliferation and IgG production[61]. However, activin-A has no effect on activated B cells. Interestingly, in human pregnancy membranes, activin-A has been shown to exert a dual effect on the production of IL-6 and IL-8, being stimulatory at lower concentrations and inhibitory at higher concentrations[62].

Accumulating evidence reveals also important anti-inflammatory effects of activin-A. Indeed, activin-A inhibits IL-6-driven murine B cell proliferation and monocyte phagocytosis *in vitro*[44]. Moreover, activin-A inhibits phytohemagglutinin (PHA)-induced proliferation of adult rat thymocytes *in vitro*[63]. In addition, activin-A decreases CD14 and MHC-II expression and NO release in cultures of LPS-activated mouse peritoneal macrophages, and suppresses their phagocytic capacity both *in vitro* and *in vivo*[64]. In accordance, a recent study demonstrated that activin-A decreases the secretion of IL-1β and NO, the mRNA levels of IL-1β and iNOS and also reduces the expressions of CD68, CD14 and TLR4 on mouse macrophage cell line RAW264.7 activated by LPS *in vitro*[65]. Murine peritoneal macrophages also express lower IL-1β, IL-6 and higher IL-1 receptor antagonist (IL-1Ra) during LPS stimulation *in vitro* in the presence of activin-A. Very recent studies have also shown that activin-A increases TGF-β1-induced Foxp3 expression on murine $CD4^+$ T cells *in vitro* and enhances conversion of peripheral naive T cells to $Foxp3^+$ Tregs *in vivo*[66]. DC-derived activin-A down-regulates *in vitro* the production of IFN-γ by human natural killer (NK) cells. In addition, activin-A treatment of human NK cells suppresses their proliferation *in vitro*[67]. Treatment with activin-A *in vitro* also reduces non-specific proliferation of human T cells and attenuates CD40L–dependent cytokine and chemokine release by human monocytes and DCs[68].

The Role of Activin-A in the Regulation of Allergic Responses

Several groups have recently demonstrated that activin-A is induced during allergic Th2-mediated responses. Activin-A is highly expressed in the airways of mice after ovalbumin (OVA)-induced acute allergic airway inflammation and submucosal mast cells represent a major source of activin-A *in vivo*[69-70]. In fact, activin-A mRNA is rapidly induced in bone marrow-derived mast cells following IgE cross-linking[70]. Moreover, mouse and human mast cells express activin-A receptors and mouse Th2 cells and macrophages also produce activin-A during allergic airway inflammation[70]. Yet, the most potent producers of activin-A have been shown to be the elastase positive neutrophils that are implicated in the generation and persistence of severe allergic asthma. On the contrary, eosinophils do not produce activin-A. Importantly, the Th2-associated transcription factor c-Maf interacts with the activin-A promoter and is involved in the regulation of βA subunit gene transcription[71]. Furthermore, the expression of activin-A is upregulated in bronchial epithelial cells after induction of experimental asthma[69]. On the contrary, another study demonstrated that activin-A expression is downregulated in the bronchial epithelial cells, while it is increased in the BAL after pulmonary allergen challenge[72]. These discrepancies may be due to the differences between the experimental models used as well as to the distinct time points that were investigated. Regarding the signalling pathway of activin-A in allergic airway inflammation, it has been shown that ALK4 and ActR-IIA along with phosphorylated Smad2 are increased in submucosal fibroblasts, pointing to effects of activin-A on airway repair and remodelling[69].

Recent studies by our group have uncovered activin-A as a key suppressive cytokine in allergic airway inflammation and linked disease in mice[73]. In fact, we showed that endogenously produced activin-A protects against allergic airway disease in mice, since its neutralization during pulmonary allergen challenge leads to significantly exacerbated Th2-mediated allergic responses[73]. Interestingly, we demonstrated that activin-A exerts its inhibitory effects through induction of $CD4^+CD25^-Foxp3^-$ IL-10-producing Treg cells that effectively suppress allergen-driven Th2 responses *in vitro* and upon adoptive co-transfer *in vivo* and confer protection against cardinal features of allergic airway disease[73]. Activin-A-mediated suppression is dependent on both IL-10 and TGF-β1 release, pointing to synergistic effects between these immunosuppressive factors[73]. The effects of neutralization of activin-A

during allergic responses were observed even in the presence of endogenous TGF-β1, indicating that these two cytokines, as also shown by other several groups [70, 71], should not be considered as redundant cytokines during inflammatory processes. In fact, activin-A and TGF-β1 operate through distinct suppressive mechanisms and induce different Treg subsets under the Th2 setting. Even though these cytokines belong to the same superfamily and signal through similar pathways, they often exert distinct functions, possibly due to the use of different receptors and Smad-independent signalling pathways.

Apart from the experimental models of asthma, several studies have uncovered a role for activin-A in human allergic asthma as well. A recent study has demonstrated that activin-A is elevated in the serum of individuals with severe asthma as compared to mild asthmatics and healthy participants. In the same study PB $CD4^+$ T cells from moderate asthmatics were shown to be a potent source of activin-A[74]. Of clinical relevance, therapeutic administration of immunosuppressive corticosteroids in individuals with asthma results in increased expression of activin-A in PB $CD4^+$ T cells. Our group has demonstrated that activin-A expression is increased in bronchial biopsies from patients with mild asthma as compared to healthy airway[73]. Moreover, in agreement with other studies[70, 74, 75], we also showed that activin-A is produced in the airways by T cells, macrophages and mast cells[73]. Interestingly, neutrophils account for the highest number of airway infiltrating cells expressing activin-A in human asthma[75]. Furthermore, activin-A is highly expressed in the human asthmatic epithelium and the expression of activin-A by bronchial epithelial cells is induced by IL-13 and TNF-α[75, 76].

Even though activin-A levels are increased in human asthmatic airways, our recent studies have demonstrated that expression of both ALK4 and ActR-IIA is significantly decreased in airway submucosal cells of mild asthmatics[73]. ActR-IIA levels are minimal also in airway epithelium, while ActR-IIB is almost absent. However, we did not observe increased phosphorylated Smad2 (pSmad2) [73] levels in the airway of mild asthmatics, in contrast to a previous study[75] wherein more symptomatic and severe asthmatics were included. Interestingly, allergen inhalational challenge and, thus, disease provocation induces a rapid (within 24 hours) increase in pSmad2 expression in the airway of mild asthmatics. Both activin-A and TGF-β1 signal through phosphorylation of Smad-2. Importantly, ALK4 and ActR-IIA

expression is increased in bronchial epithelium post-allergen challenge, whereas ALK5 levels are decreased.

In addition, our functional *in vitro* studies demonstrate that r-activin-A significantly increases the proliferation of human bronchial epithelial cells and promotes the epithelial repair process[75]. Moreover, *in vitro* FS treatment of human bronchial epithelial cell greatly enhances TNF-α and IL-13 -induced chemokine expression by these cells, pointing to a potent anti-inflammatory role of activin-A in airway epithelial cell responses. Collectively, it can be postulated that rapid induction of ALK4 and ActR-IIA expression, along with pSmad2, in the airway upon allergen inhalational challenge, may be part of an inherent protective mechanism to respond to activin-A signalling in order to control inflammation and enhance epithelial repair.

Considering the suppressive effects of activin-A in experimental asthma models as well as the findings from human studies, a role for this cytokine in the regulation of human Th2-driven allergic responses is highly plausible. However, the exact role of activin-A in the suppression of human allergic Th2-mediated responses and the induction of human Treg cells remains elusive.

Hypothesis

We hypothesized that activin-A can suppress human Th2-mediated allergic responses and represents a key cytokine for the regulation of allergic airway inflammation and asthma. We also hypothesized that activin-A induces human Treg cells that can inhibit human Th2-mediated allergic responses both in the periphery and the airways of asthmatic individuals.

Specific Aims of the Project

Our specific aims were:

1) To investigate the effects of activin-A on the suppression of human Th2 cell-mediated allergic responses in the periphery and the airways

2) To delineate the role of activin-A in the induction of human Treg cells and their effects on the suppression of allergic Th2 response and

3) To examine whether activin-A can enhance steroid-induced immunosuppression in patients with severe, treatment- refractory, asthma.

Subjects and Methods

Subjects:

PB was obtained from age-matched (range 18-60): **a)** healthy individuals (with no history of atopy or asthma), **b)** atopics (with no history of asthma), **c)** mild/ moderate persistent asthmatics, and **d)** treatment-refractory severe asthmatics (n = 5-10/group). Asthma was defined according to the latest GINA guidelines. All atopic and asthmatic individuals had positive skin prick test (wheal size ≥ 3mm) to allergens and/or increased levels of IgE specific to allergens (the same allergens that were used in ex vivo stimulation cultures). BAL and lung biopsies were obtained from severe asthmatic individuals (n = 2) during their evaluation and follow-up at "Sotiria" Athens Chest Hospital. On entering the study, subjects were stable. Individuals with mild/moderate asthma received inhaled long-acting adrenergic beta-2 agonists and occasionally inhaled corticosteroids, while participants with severe asthma received per os corticosteroids and inhaled long-acting adrenergic beta-2 agonists. Individuals with no clinical features of infection for at least four weeks before the start of the study and none throughout the study period were included. Patients with other immunological disorders (i.e. autoimmune diseases), cancer and/or other respiratory disorder (i.e. COPD) were excluded from the study. All donors signed an informed consent form approved by the "Sotiria" Athens Chest Hospital Ethics Committee**.**

Analysis of BAL:

Bronchoscopy was performed using local anaesthesia according to standard techniques. BAL was performed by sequential instillation and aspirations of three aliquots of 60 mL of warmed sterile 0.9% saline. BAL fluid was placed and transported in ice before analysis. The BAL fluid was strained through a 100μm Dacron net and centrifuged at 400g 10min at 4^{o}C. BAL cells were

counted and analysed for viability by Trypan Blue exclusion. BAL fluid was kept at -80^{o}C until use. BAL mononuclear cells were used for *in vitro* cultures.

Cell isolation and in vitro stimulation:

Peripheral blood mononuclear cells (PBMCs) were obtained by Lymphoprep gradient centrifugion and $CD4^+$ T cells were isolated using Dynal $CD4^+$ isolation kit. $5x10^4$ $CD4^+$ T cells were stimulated for 3 days with: a) 0,5μg/ml soluble anti-CD3 (OKT3 clone) or b)1000SQ/ml allergen (mixed grass extract) in the presence of 10^5 autologous mitomycin-treated CD3-depleted PBMCs (MACS CD3 negative isolation kit). Cells were treated with either PBS or 50ng/ml recombinant activin-A (r-activin-A) (R&D). The well-known immunomodulatory factor dexamethasone served as a positive control for suppression of proliferation at a concentration of 10^{-7}M. Dose response studies in order to define the optimal concentrations of r-activin-A, aCD3, allergen and dexamethasone, were carried out. The effects of r-activin-A on T cell proliferation using ^{3}[H]-Thymidine incorporation were examined.

In order to examine the effects of r-activin-A on allergen-specific responses of airway mononuclear cells, $5x10^5$ BAL mononuclear cells were isolated from individuals with severe asthma and stimulated for 3 days with 1000SQ/ml allergen (mixed grass extract) in the presence of PBS, 50ng/ml r-activin-A or 10^{-7}M dexamethasone.

The ability of r-activin-A to enhance dexamethasone-induced immunosuppression was also examined. Briefly, $5x10^4$ PB $CD4^+$ T cells obtained from individuals with moderate and severe, treatment-refractory, asthma were stimulated for 3 days with 1000SQ/ml allergen (mixed grass extract) and 10^5 autologous mitomycin-treated CD3-depleted PBMCs in the presence of PBS, 50ng/ml r-activin-A, dexamethasone at the concentrations of 10^{-7}M, 10^{-8}M and 10^{-9}M or dexamethasone plus r-activin-A (at the same concentrations).

In other experiments we examined whether r-activin-A-treated $CD4^+$ T cells were anergic, a characteristic feature of Treg cells. For this purpose, $5x10^4$ PB $CD4^+$ T cells were treated with PBS, r-activin-A or r-activin-A plus rIL-2 in the presence of 1000SQ/ml allergen and 10^5

autologous mitomycin-treated CD3-depleted PBMCs. Effects on T cell proliferation were examined as above.

In vitro Suppressive Assays

Subsequently, we investigated the suppressive function of r-activin-A-treated T cells using an *in vitro* suppression assay. Briefly, we initially isolated $5x10^4$ PB naove $CD4^+CD25^-$ $CD45RA^+CD45R0^-$ T cells using the MACS naove $CD4^+$ T cell isolation kit II from atopic participants and cultured them for 7 days with 10^5 autologous mitomycin-treated CD3-depleted PBMCs in the presence of 1000SQ/ml allergen (mixed grass extract). Cells were treated with PBS, r-activin-A or dexamethasone. After 7 days, PBS, r-activin-A or dexamethasone-treated $CD4^+$ T cells were isolated from the initial cultures and co-cultured with freshly isolated, autologous mitomycin-treated CD3-depleted PBMCs and freshly isolated, autologous CFSE labelled (or not) naove $CD4^+$ responder T cells, in different ratios, in the presence of 1000SQ/ml allergen (mixed grass extract). Responder T cell proliferation, using ^{3}[H]-Thymidine incorporation and CFSE staining analysis, was measured after 4 days. The suppressive effects of dexamethasone-induced aTregs on Th responses served as positive controls.

Cytokine analysis

Cytokines were measured in culture supernatants using commercially available ELISA kits for IL-2, IFN-γ, IL-10, IL-5 and IL-13 (R&D). *[For the measurement of IL-13 and IFN-γ r-activin-A- or PBS-treated Th cells were stimulated for 48 hours with 0,1μg/ml soluble anti-CD3(OKT3 clone)].*

Flow-cytometric analysis

$5x10^4$ $CD4^+$ T cells isolated from the PB of atopic individuals (n=2) were cultured with 10^5 autologous mitomycin-treated CD3-depleted PBMCs in the presence of 1000SQ/ml allergen (mixed grass extract) for 3 days. Cells were treated with PBS or r-activin-A. On day 3 cells were collected, washed and stained with Annexin-V-PE and 7AAD-PC5 (Beckman Coulter) in order to measure apoptotic and necrotic cells. To perform the FACS analysis, we used the cytometer Cytomics FC500 (Beckman Coulter).

Statistical Analysis

We used Graph Pad Prism Version 5 (Graph Pad Software Inc, San Diego, Calif) in order to analyze our data. To calculate differences between groups, we used Unpaired Student t test, and data were logarithmized before any Student t tests were performed to calculate absolute values. All results are presented as mean +/- SEM. We considered any difference with a p value of 0.05 or less to be statistically significant.

Results

Activin-A suppresses allergen-specific Th2 cell-mediated responses of atopic individuals in vitro.

Activin-A is expressed by a variety of immune and tissue-resident cells during Th2-driven allergic responses in the human airways. Effector Th2 cell-mediated responses are essential for the development of allergic airway inflammation and human asthma. Importantly, several groups have shown that activin-A is involved in allergic airway inflammation and modulates the function of a wide range of cells during allergic responses. As activin-A and its receptors are expressed by infiltrating Th cells in the human airways, we decided to investigate its the effects on human allergen-specific Th2 cell-mediated responses.

We initially focused our studies on the effects of activin-A on the regulation of Th2 responses from the PB of atopic individuals. Our preliminary studies have demonstrated that the predominant activin-A type I receptor, ALK4, is expressed by human PB $CD4^+$ T cells. Activin-A treatment of PB $CD4^+$ T cells, from atopic subjects, during *ex vivo* stimulation with their specific allergen resulted in significantly decreased ($p<0,05$) proliferation of Th2 cells as compared to PBS treatment. In addition, activin-A treatment resulted in greatly reduced ($p<0,05$) secretion of the Th2-type cytokines IL-5, IL-13 and IL-10. Interestingly, activin-A treatment led to significantly decreased ($p<0,05$) levels of the Th1-type cytokine IFN-γ (**Figure 8**). IFN-γ-producing Th1 cells are considered to play also important role in the pathogenesis of human allergic airway inflammation and related disease.

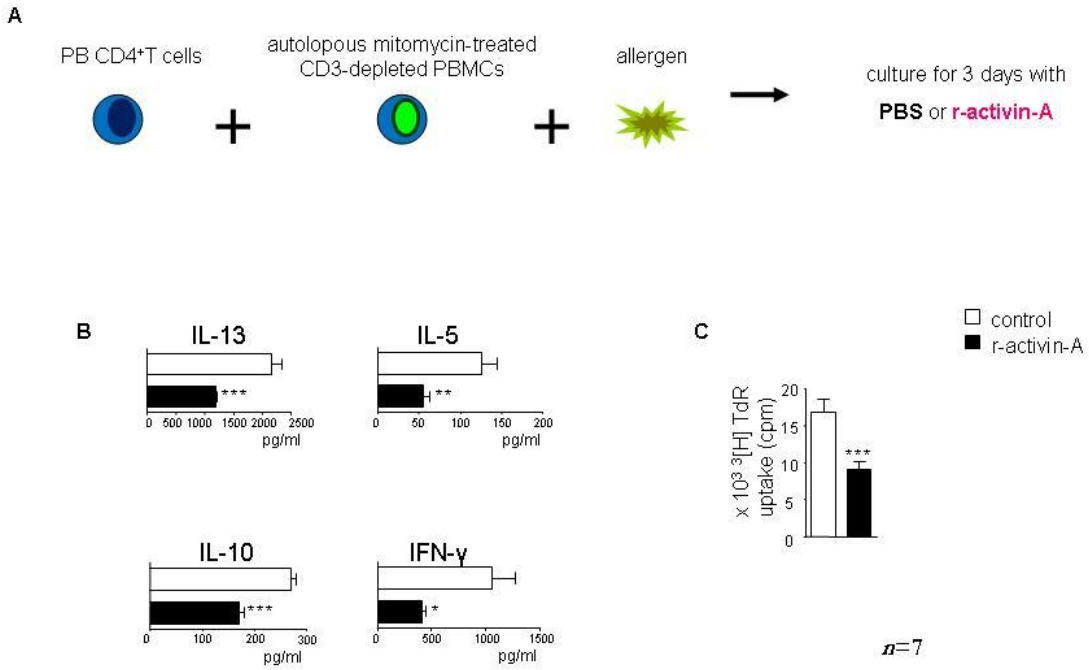

Figure 8.Activin-A suppresses allergen-specific Th2 cell-mediated responses of atopic individuals in vitro. A. Experimental protocol used to evaluate the effects of activin-A on responses mediated by PB $CD4^+$ T cells of atopics in the presence of allergen. $CD4^+$ T cells were cultured with autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen and treated with either PBS or r-activin-A for 3 days. **B.** Treatment of cells with r-activin-A significantly reduced the release of IL-5, IL-13, IL-10, IFN-γ and **C.** greatly decreased the proliferation of these cells**.**

Activin-A suppresses allergen-specific Th2 cell-mediated responses of individuals with mild/moderate persistent and severe, treatment-resistant asthma in vitro.

Subsequently, we investigated whether activin-A exerts similar suppressive effects on Th2 cell-mediated allergic responses from individuals with mild/moderate and severe, treatment-resistant asthma. For this purpose we treated, PB $CD4^+$ T cells with activin-A or PBS during their stimulation with their specific allergen *in vitro*. Indeed, activin-A treatment led to significantly reduced ($p<0,05$) proliferation of the $CD4^+$ T cells isolated from both mild/moderate and severe, treatment-resistant asthmatics, as compared to PBS-treatment. In addition, activin-A greatly decreased ($p<0,05$) the production of the cytokines IL-5, IL-13, IL-10 and IFN-γ as compared to PBS control (**Figure 9**). Taken together, our studies suggest that activin-A is a critical regulator of allergic responses mediated by human Th2 cells, key effectors in the pathogenesis of human allergic asthma.

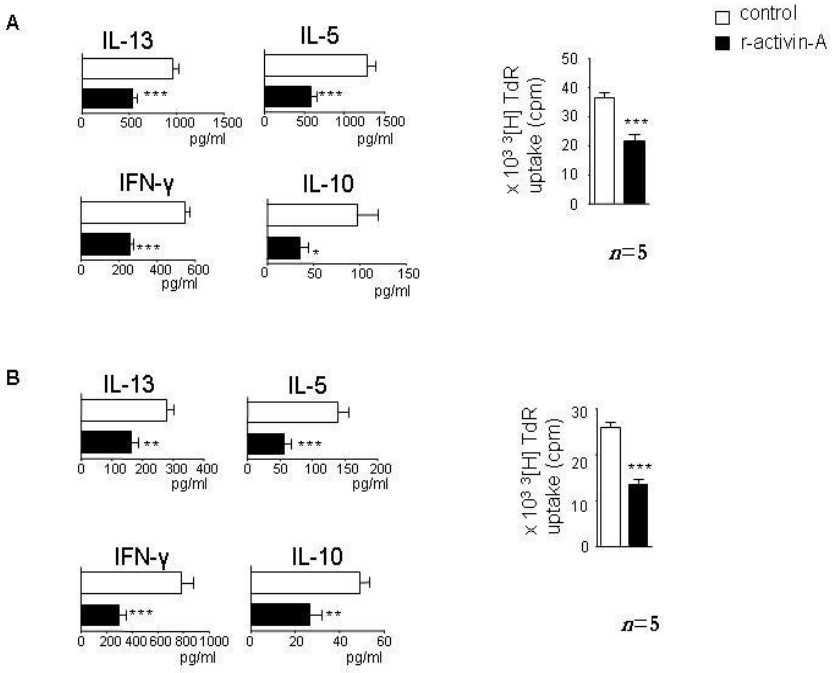

Figure 9.Activin-A suppresses allergen-specific Th2 cell-mediated responses of individuals with mild/moderate persistent and severe, treatment-resistant asthma *in vitro*. A.$CD4^+$ T cells were isolated from the PB of individuals with mild/moderate asthma and cultured with

autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen. Cells were treated with PBS or r-activin-A. r-activin-A treatment significantly reduced the secretion of IL-5, IL-13, IL-10, IFN-γ as well asthe proliferation of these cells. **B.** The same experiments were performed using PB $CD4^+$ T cells of individuals with severe, treatment-refractory asthma. R-activin-a treatment resulted as well in greatly reduced cytokine release and proliferation.

Activin-A inhibits mononuclear cell responses in the airways.

Subsequently, we investigated the role of activin-A in the suppression of allergic responses in the active site of the disease, the airways. For this, we isolated airway mononuclear cells from individuals with severe, treatment-refractory, asthma and studied the effects of activin-A treatment on the proliferation of these cells. Interestingly, activin-A was capable of significantly ($p<0,05$) reducing allergen-induced proliferation of BAL mononuclear cells as compared to PBS treatment (**Figure 10**).These data extent our previous findings and reveal that activin-A can exert suppressive effects on human allergen-specific responses not only in the periphery, but also in the airways of severe asthmatics, the most important patient cohort.

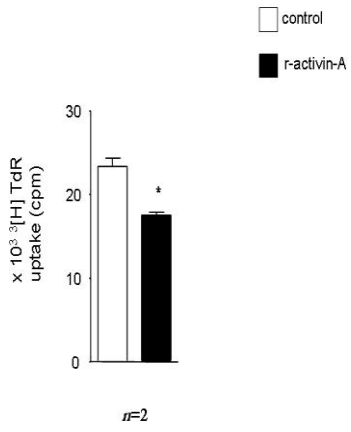

Figure 10.Activin-A inhibits mononuclear cell responses in the airways. BAL mononuclear cells were obtained from individuals with severe treatment-resistant asthma. Cells were cultured with allergen in the presence of PBS or r-activin-A. Treatment with r-activin-A greatly decreased the allergen-driven proliferation of BAL mononuclear cells

Activin-A enhances dexamethasone-mediated suppression of allergic responses from individuals with moderate and severe, treatment-refractory asthma.

Dexamethasone belongs to the category of immunosuppressive corticosteroid drugs, which currently represent the mainstay of therapeutic approach for human allergic asthma. Even though steroids manage to ameliorate the clinical features of asthma, they are unable to target the cause of the disease. Furthermore, a subgroup of patients develops severe, steroid-refractory asthma. The long-term and high dose of per os corticosteroids these patients receive lead to several and often severe side effects. Considering, the immunoregulatory effects that activin-A exerts on human Th2-driven allergic responses of asthmatic individuals, we next examined whether activin-A could enhance the dexamethasone-induced suppression of allergic responses of individuals with moderate and severe, steroid-resistant, asthma.

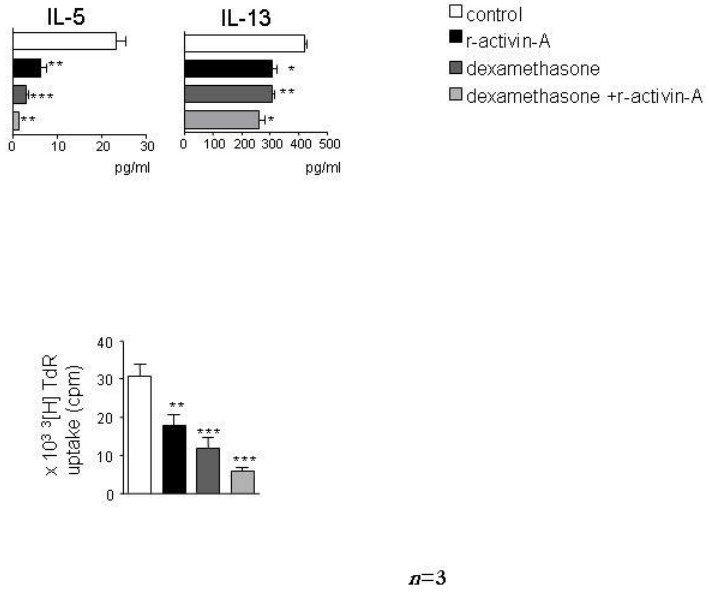

Figure 11.Activin-A enhances dexamethasone-mediated suppression of allergic responses from individuals with moderate and severe, treatment-refractory asthma. PB $CD4^+$ T from individuals with mild/moderate and severe, treatment-refractory asthma were cultured with autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen.

Cells were treated with PBS, r-activin-A, dexamethasone or dexamethasone plus r-activin-A. r-activin-A addition in dexamethasone-cultures greatly enhanced **A.**the dexamethasone-mediated reduction of IL-5 and IL-13 production as well as **B.** the proliferation of these cells

Our data reveal that addition of activin-A in cultures of allergen-stimulated PB $CD4^+$ T cells from moderate and severe, steroid-resistant asthmatic individuals significantly increased ($p<0,05$) dexamethasone-induced suppression of T cell proliferation as compared to treatment with dexamethasone alone. Similarly, addition of activin-A resulted in greatly enhanced ($p<0,05$) dexamethasone-induced suppression of IL-5 and IL-13 release by these cells as compared to dexamethasone alone (**Figure 11**). Importantly, addition of activin-A in dexamethasone-treated cultures nearly abolished Th2-driven allergic responses. These findings suggest that activin-A is a novel effective immunosuppressive agent for human allergic asthma. Follow-up studies may ultimately lead to the design of new therapeutic protocols that will combine the use of activin-A with reduced doses of corticosteroids in order to achieve higher efficacy and safety for the treatment of severe asthmatics.

Activin-A suppresses aCD3-driven human $CD4^+$ T cell responses in vitro.

We next investigated whether activin-A exerts a suppressive role also during non-specifc Th stimulation *in vitro.* Treatment of $CD4^+$ T cells, obtained from the PB of healthy participants with anti-CD3 and autologous mitomycin-treated CD3-depleted PBMCs in the presence of r-activin-A resulted in significantly decreased ($p<0,05$) Th cell proliferation. Similarly, activin-A treatment greatly reduced ($p<0,05$) the release of IL-2 and IFN-γ, while it significantly increased ($p<0,05$) the secretion of IL-10, as compared to PBS treatment (**Figure 12**).

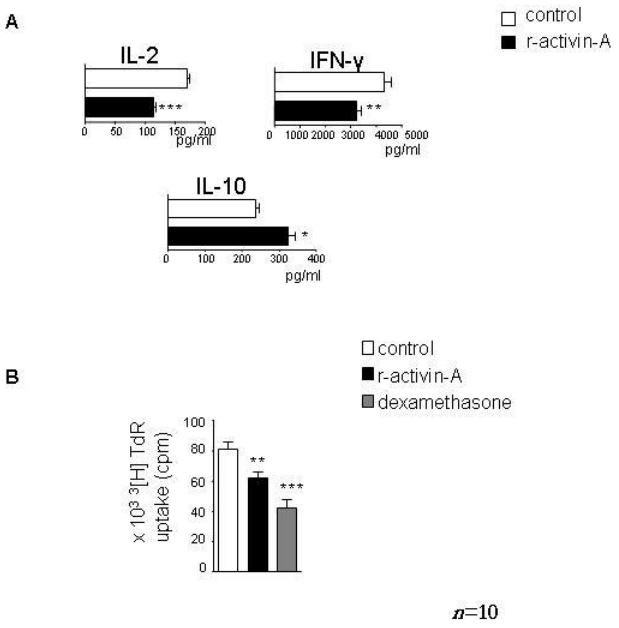

Figure 12.Activin-A suppresses aCD3-driven human CD4$^+$ T cell responses in vitro. PB CD4$^+$ T cells from healthy participants were cultured with autologous mitomycin-treated CD3-depleted PBMCs in the presence of aCD3. Cells were treated with PBS, r-activin-A or dexamethasone. **A.**Treatment with r-activin-A significantly decreased the release of IL-2 and IFN-γ, while it importantly increased the secretion of IL-10 **B.** R-activin-A also greatly reduced the non-specific proliferative response of these cells.

Our data uncover active-A as a cytokine with a broad immunosuppressive function for human Th responses and suggest that it could be used for the inhibition of a variety of immune responses and related diseases.

Activin-A renders human CD4$^+$ T cells anergic

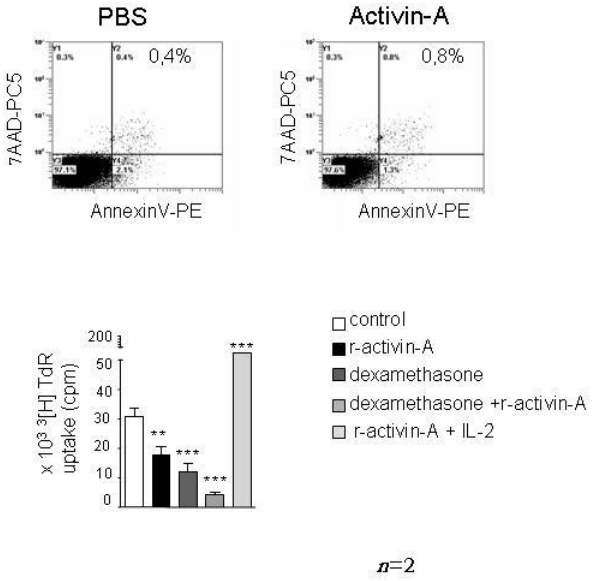

Figure 13. Activin-A renders human CD4$^+$ T cells anergic. A.PB CD4$^+$ T cells from atopics were cultured with autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen and PBS or activin-A. After 3 days of culture cells were stained using Annexin-V-PE and 7-AAD-PC5 and analysed with flow cytometry to evaluate apoptotic and/or necrotic cells. **B.** Addition of rhIL-2 into the cultures of PB CD4$^+$ T cells treated with r-activin-A reversed the r-activin-A-induced suppression of proliferation and resulted in highly proliferating cells.

Susequently, we investigated whether the suppressive effects of activin-A on human Th responses were due to induction of apoptosis and/or necrosis of those cells. For this purpose, after 3 days of culture of PB CD4$^+$ T cells with autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen and PBS or activin-A, we stained cells using the Annexin-V-PE and 7-Aminoactinomycin D (7-AAD)-PC5 that reveal apoptosis and necrosis respectively in flow cytometric analysis. Our data showed that activin-A treatment did not induce increased numbers of Annexin-V$^+$ and/or 7AAD$^+$ T cells as compared to PBS treatment (**Figure 13**)

suggesting that activin-A suppression of Th responsesis not mediated through induction of apoptosis nor necrosis.

We next examined whether activin-A rendered the PB $CD4^+$ T cells anergic. For this, r-activin-A or PBS-treated Th cells were stimulated with allergen in the presence of recombinant human IL-2 (rhIL-2). Indeed, addition of rhIL-2 into cell cultures reversed r-activin-A-induced suppression of T cell proliferation and resulted in highly proliferating cells (**Figure 13**) Our data suggest that activin-A treatment renders human $CD4^+$ T cells anergic.

Activin-A suppresses human Th2 allergic responses through induction of regulatory T cells.

Subsequently, we investigated whether activin-A-mediated suppression of human Th2 cell responses was associated with induction of T cells with regulatory/suppressive function. To address this, we performed an *in vitro* suppression assay, wherein we co-cultured activin-A-or PBS control-treated PB $CD4^+$ T cells from atopic individuals with freshly isolated, autologous mitomycin-treated CD3-depleted PBMCs and freshly isolated, autologous naïve $CD4^+$ responder T cells, in the presence of allergen. Activin-A-treated $CD4^+$ T cells greatly decreased ($p<0,05$) the proliferation and IL-5 release of autologous naïve $CD4^+$ responder T cells (**Figure 14**). Importantly, our data reveal that activin-A exerts its inhibitory effects through induction of human regulatory T cells that effectively suppress allergen-driven T effector responses.

Collectively, our findings uncover, for the first time, activin-A as an essential protective cytokine against human allergic Th2 responses.

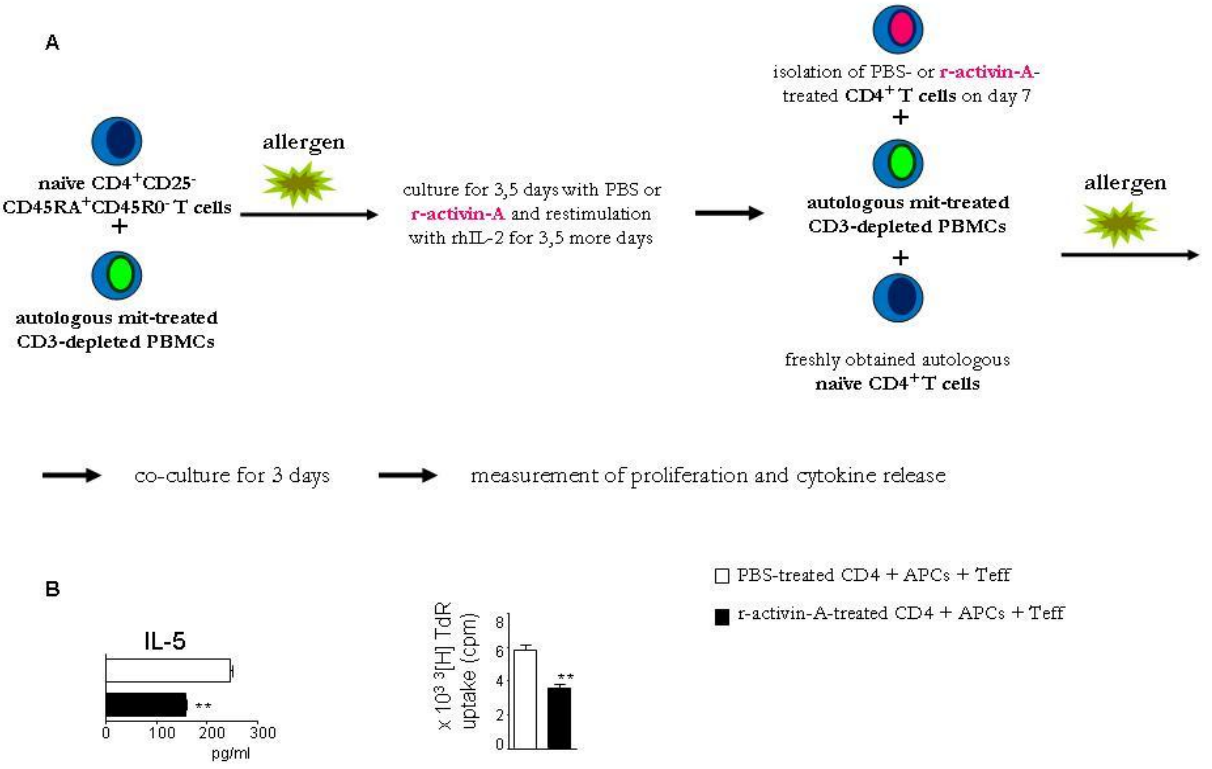

Figure 14.Activin-A suppresses human Th2 allergic suppression through induction of regulatory T cells. A. Experimental protocol of the suppressive assay. Naïve $CD4^+CD25^-$ $CD45RA^+CD45R0^-$ T cells from atopics were cultured with autologous mitomycin-treated CD3-depleted PBMCs in the presence of allergen. Cells were treated with PBS or r-activin-A for 3,5 days and then were restimulated with rhIL-2 for 3,5 more days. On day 7 PBS- or r-activin-A treated $CD4^+$ T cells were obtained from the initial cultures. Treated $CD4^+$ T cells were then co-cultured with freshly isolated autologous mitomycin-treated CD3-depleted PBMCs and naïve $CD4^+CD25^-CD45RA^+CD45R0^-$ T cells in the presence of allergen for 3 days. **B.** r-activin-A-treated $CD4^+$ T cells greatly decreased the IL-5 release and proliferation of autologous naïve $CD4^+$ responder T cells.

Discussion

Activin-A was initially considered as a hormone involved mainly in the regulation of the female reproductive system[30]. Recent studies have revealed activin-A as a pleiotropic cytokine that has important functions in diverse biological procedures. The roles of activin-A in development, fibrosis, stem-cell biology and neuroprotection have been intensively studied the last years[31]. Importantly, our previous studies have shown that activin-A is also implicated in the control of allergic airway inflammation and related disease in mice through the induction of $CD4^+CD25^-$ $Foxp3^-$ IL-10-secreting Treg cells[73].

In the present study, our findings demonstrate for the first time to our knowledge, that activin-A exerts suppressive effects on human allergen-specific Th2 responses and represents a protective cytokine against allergic asthma. Specifically, our data show that activin-A inhibits Th2-driven allergic responses of atopic, mild/moderate asthmatics and more importantly of individuals with severe, treatment-resistant asthma during stimulation with a clinically relevant allergen *in vitro*. Notably, activin-A treatment results in decreased secretion of the Th2-type cytokines IL-5 and IL-13 that actively participate in the orchestration of allergic airway inflammation. In addition, activin-A treatment reduces the release of the Th1-type cytokine IFN-γ, pointing to a general suppressive role in Th-mediated responses. Apart from its effects on these pro-inflammatory cytokines, activin-A treatment also leads to reduced release of IL-10. This is in accordance with the general suppression that activin-A exerts on Th2 cells, which are a major source of IL-10, and cannot exclude the fact that activin-A may also be able to induce IL-10 secreting regulatory T cells, which contribute to the IL-10 poll but to a much lesser degree.

Importantly, we demonstrate that the suppressive effects of activin-A are exerted not only in the periphery, but also at the active disease site, the airways. In fact, addition of activin-A in cultures of airway mononuclear cells of severe asthmatics results in greatly reduced proliferation of these cells. Airway mononuclear cells play predominant role in the induction and progression of human allergic airway inflammation and asthma. Notably, these cells considered to be less susceptible to suppression, possibly as a result of the highly proinflammatory cytokine milieu in the airways.Furthermore, inflammatory cells in the airways of severe, treatment-refractory asthmatics are resistant to suppression. Hence, our findings showing that activin-A can effectively suppress

airway inflammatory responses in this, difficult to suppress, patient cohort have major clinical importance.

Our data also show that activin-A can enhance dexamethasone-induced immune suppression in cultures of $CD4^+$ T cells obtained from patients with moderate and severe steroid-resistant asthma and stimulated with their specific allergen. Notably, the addition of activin-A in dexamethasone cultures nearly abolished allergen-driven Th2 proliferation and cytokine release, indicating that activin-A can be a promising therapeutic target for severe asthmatics. Importantly, a combination of activin-A with the currently administered corticosteroids may lead to reduced doses of these broad immunosuppressive drugs and result in safer and more effective therapeutic approaches.

Examination of the effects of activin-A on non-specific Th cell responses shows that activin-A treatment leads significantly reduced anti-CD3 driven proliferation and cytokine secretion of PB $CD4^+$ T cells. Notably, even though activin-A treatment results in greatly decreased production of IL-2 and IFN-γ by these cells, it has the opposite effect on the secretion of IL-10. In fact, activin-A results in significantly enhanced release of the immunosuppressive cytokine IL-10 by $CD4^+$ T cells stimulated with aCD3. Hence, it is possible that activin-A is a potent inducer of IL-10 secretion in this context and may contribute to the generation of IL-10-secreting aTregs.

In order to investigate the mechanisms that mediate the suppressive function of activin-A, we examined whether activin-A induces cell death and/or apoptosis to the cultures of PB $CD4^+$ T cells. Interestingly, our findings demonstrate that activin-A treatment does not lead to apoptosis nor necrosis of human Th cells, but, on the contrary, renders T cells anergic, a characteristic feature of Tregs. Subsequently, considering the ability of activin-A to induce mouse Tregs, we decided to explore the potency of activin-A to induce the generation of human, suppressive regulatory T cells. Indeed, our findings show that activin-A induces the generation and/or expansion of human suppressive Treg cells that effectively control responder T cell proliferation and cytokine release during stimulation with a clinically relevant allergen *in vitro*.

Collectively, our data uncover activin-A as a critical immunosuppressive agent for human allergic asthma. We anticipate that our data may provide an important step forward to the prospective

clinical application of activin-A, and activin-A-induced allergen-specific Tregs, as a novel therapeutic approach for allergic asthma. For example, PB $CD4^+$ T cells obtained from asthmatic individuals could be treated *ex vivo* with activin-A in order to induce the generation of allergen-specific Tregs. These Tregs could be then re-infused to the patient to prevent or, even, reverse allergic inflammatory responses against the specific allergen. Our findings may also lead to the development of novel clinical protocols that will involve the use of activin-A, or activin-A-induced Tregs, in combination with steroids and/or immunotherapy to improve efficacy, specificity and safety of current treatments. Finally, our findings may aid the design of novel vaccination protocols (using r-activin-A induced allergen-specific Tregs) for the prevention of allergic diseases and particularly asthma in patients at risk.

Future Plans

Part of our future plans is also to investigate the role of activin-A-induced Treg cells in the suppression of human allergic Th2 responses *in vivo* using a humanized mouse model. In order to assess whether activin-A-treated human $CD4^+$ T cells can protect against allergic human diseases, it is essential to demonstrate that these cells can suppress allergen-specific Th2-mediated responses in humans *in vivo*. As ethical and technical constraints do not allow the investigation of the suppressive effects of activin-A-treated human CD4+ T cells into humans *in vivo*, we will utilize the well established humanized, immunodeficient Non Obese Diabetic/Severe Combined Immuno Defficient) (NOD/SCID) mouse model of allergic airway disease[77](**figure 15**).

Briefly, PBMCs obtained from atopic individuals will be treated with r-activin-A or PBS, in the presence of APCs and allergen. PBMCs will be isolated from the cell cultures and labelled, or not, with CFSE to examine their recruitment and proliferation *in vivo*. On day 0 the CFSE-labeled PBMCs will be adoptively transferred [intraperitoneally (i.p.)] alone or along with freshly isolated autologous CFSE-labeled PB PBMCs responder cells into recipient into NOD/SCID mice. On day 7 we will immunize the mice with an i.p. injection using the same allergen. At days 14 and 15intratracheal (i.t.) injection of the allergen will be performed in these immunodeficient mice. On day 16 AHR in response to increased doses of inhaled metacholine will be measured. At day 21 mice will be sacrificed and the suppressive effects of activin-A-induced Tregs on cardinal features of allergic airway disease in mice will be also assessed, as previously described[73]. Moreoner, the

effects of activin-A-induced Tregs on the suppression of allergen-specific proliferation and intracellular cytokine release by CFSE-labeled human PBMCs responder cells will be measured.

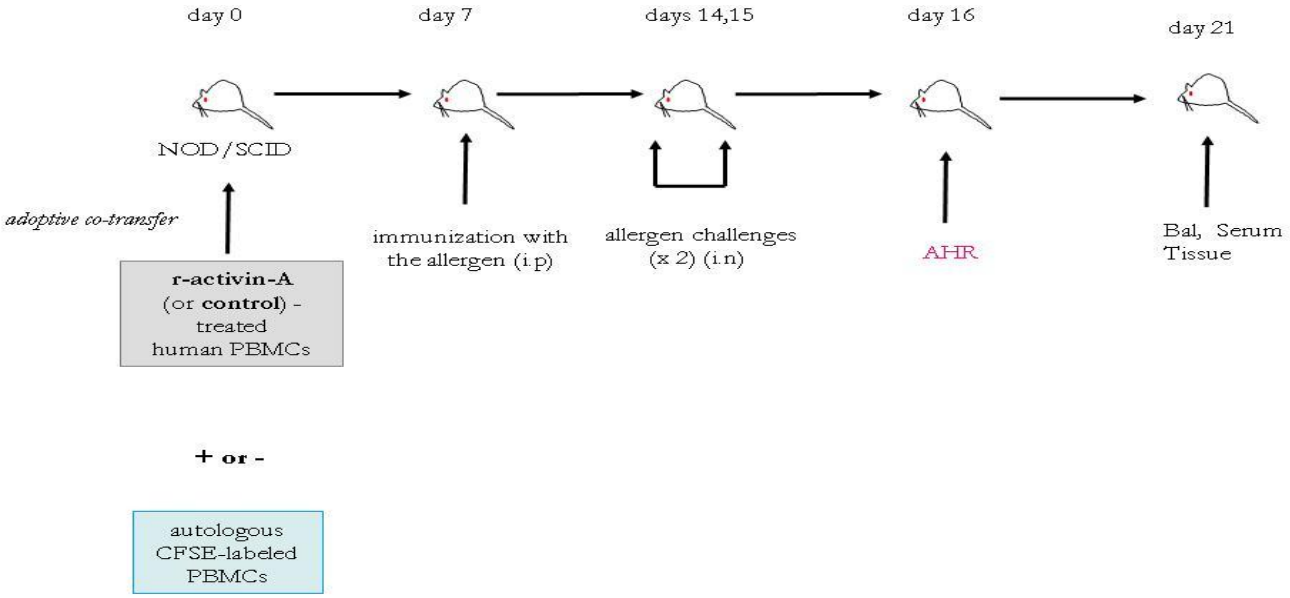

Figure 15. Experimental protocol for the investigation of the role of activin-A-induced Treg cells in the suppression of human allergic Th2 responses in a humanized NOD/SCID mouse model of allergic airway inflammation.

We anticipate that these experimens will aid the translation of our recent findings on the protective role of activin-A in experimental asthma in mice as well as in humans *in vitro* to its effects on the regulation of human disease. Furthermore, we anticipate that the findings obtained from the study on the humanized mouse model, will provide a step forward to the use of activin-A-induced Tregs as a cellular therapy for the suppression of human allergen-specific Th2 mediated responses in asthmatic individuals. Finally, data obtained from the proposed studies will offer critical new insight into the cellular and molecular mechanisms involved in immune suppression in human asthma and expand the current scientific knowledge.

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