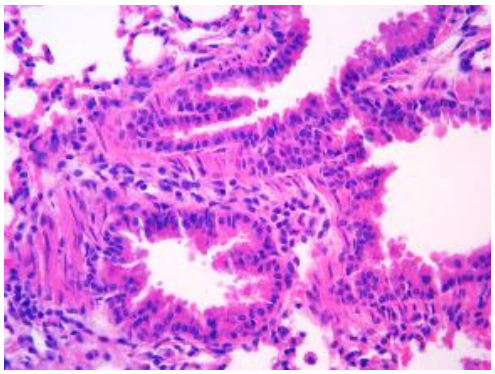
ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
UNIVERSITY OF CRETE
ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ
FACULTY OF MEDICINE

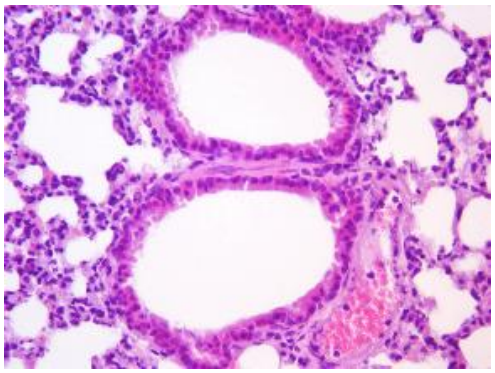
ACADEMY OF ATHENS
BRFAA
Biomedical Research Foundation
Academy of Athens

Διδακτορική Διατριβή

“Ο ρόλος της ακτιβίνης-Α στην καταστολή των ανθρώπινων αλλεργικών αντιδράσεων”

“The role of activin-A in the suppression of human allergic responses”





Σοφία Τούσα

Η εργασία εκπονήθηκε στο εργαστήριο κυτταρικής ανοσολογίας του Ιδρύματος Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών, υπό την επίβλεψη της Δρ. Γεωργίας Ξάνθου. Μέρος των πειραμάτων πραγματοποιήθηκε στο εργαστήριο κυτταρικής ανοσολογίας του Ινστιτούτου Βϊοιατρικών Ερευνών της Μπελινζόνα, στην Ελβετία, υπό την επίβλεψη της Δρ. Federica Sallusto. Τα αποτελέσματα της διδακτορικής διατριβής δημοσιεύτηκαν στο περιοδικό Proceedings of the National Academy of Sciences of the United States of America (PNAS), στο πρωτότυπο άρθρο “Activin-A co-opts IRF4 and AhR signaling to induce human regulatory T cells that restrain asthmatic responses” (doi: 10.1073/pnas.1616942114).

Τριμελής Συμβουλευτική Επιτροπή:

1. Πρόδρομος Σιδηρόπουλος, Καθηγητής Ρευματολογίας, Ιατρική Σχολή Πανεπιστημίου Κρήτης.
2. Γεωργία Ξάνθου-Τσιγκόγλου, Ερευνήτρια ΄Β, Ίδρυμα Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών.
3. Γεώργιος Γουλιέλμος, Καθηγητής Μοριακής Γενετικής του Ανθρώπου με έμφαση στην Παθολογία, Ιατρική Σχολή Πανεπιστημίου Κρήτης.

Επταμελής Εξεταστική Επιτροπή:

1. Πρόδρομος Σιδηρόπουλος, Καθηγητής Ρευματολογίας, Ιατρική Σχολή Πανεπιστημίου Κρήτης.
2. Γεωργία Ξάνθου-Τσιγκόγλου, Ερευνήτρια ΄Β, Ίδρυμα Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών.
3. Γεώργιος Γουλιέλμος, Καθηγητής Μοριακής Γενετικής του Ανθρώπου με έμφαση στην Παθολογία, Ιατρική Σχολή Πανεπιστημίου Κρήτης.
4. Ευσταθία Καψογεώργου, Αναπληρώτρια Καθηγήτρια Ανοσολογίας, Ιατρική Σχολή Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών.
5. Γεώργιος Θυφρονίτης, Καθηγητής Ανοσολογίας, Τμήμα Βιολογικών Εφαρμογών & Τεχνολογιών Πανεπιστημίου Ιωαννίνων
6. Κώστας Βεκρέλλης, Ερευνητής ΄Β, Ίδρυμα Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών.
7. Γεώργιος Μπερτσιάς, Αναπληρωτής Καθηγητής Ρευματολογίας-Κλινικής Ανοσολογίας, Ιατρική Σχολή Πανεπιστημίου Κρήτης.

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στο εργαστήριο της, στο Ινστιτούτο Βιοϊατρικών Ερευνών στην Μπελινζόνα της Ελβετίας, ώστε να πραγματοποιήσω μέρος των πειραμάτων στα πλαίσια δύο τριμηνιαίων ερευνητικών υποτροφιών που έλαβα. Σε αυτό το σημείο θα ήθελα να ευχαριστήσω την Ευρωπαϊκή Ακαδημία Αλλεργιολογίας και Κλινικής Ανοσολογίας για την ερευνητική υποτροφία που μου έδωσε το 2013 αλλά και την Ευρωπαϊκή Πνευμονολογική Εταιρία για την ερευνητική υποτροφία που μου απένειμε το 2014. Επίσης ευχαριστώ θερμά το Πανεπιστήμιο Κρήτης που με τίμησε με την απονομή της υποτροφίας του κληροδοτήματος «Μαρίας Μιχαήλ Μανασσάκη» κατά το ακαδημαϊκό έτος 2011-2012. Ένα ξεχωριστό ευχαριστώ οφείλω στην Δρ. Tess Melinda Brodie, η οποία με δίδαξε νέες ανοσολογικές τεχνικές και με βοήθησε σε όλη τη διάρκεια της παραμονής μου στο Ινστιτούτο. Ευχαριστώ από καρδιάς την Δρ. Yagmur Farsakoglu και τον Δρ. Jérémie Goldstein για την συνεχή στήριξη που μου παρείχαν αλλά κυρίως για την πολύτιμη φιλία τους. Επιπροσθέτως ευχαριστώ πολύ τον Δρ. Ανέστη Παπαδόπουλο για τη βοήθειά του κατά τη συγγραφή της διατριβής μου και την ουσιαστική στήριξη που μου παρείχε κατά τη διάρκεια της ειδικότητάς μου στο Γενικό Νοσοκομείο Έδεσσας.

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Στα αγγελούδια της ζωής μου,
Μαρία-Μιχαέλα και Άρη-Γεώργιο

CONTENTS

ΠΕΡΙΛΗΨΗ

Το άσθμα είναι μία ετερογενής νόσος, η οποία χαρακτηρίζεται από υπεραντιδραστικότητα και χρόνια φλεγμονή τόσο των μικρών όσο και των μεγάλων αεραγωγών. Καθορίζεται από ένα ιστορικό αναπνευστικών συμπτωμάτων, τα οποία περιλαμβάνουν εκπνευστικό συριγμό, βήχα, αίσθημα συσφιγκτικού βάρους στο θώρακα και δύσπνοια. Τα κλινικά συμπτώματα του άσθματος είναι υποτροπιάζοντα, ποικίλλουν σε διάρκεια και ένταση και συνοδεύονται από ελάττωση του εκπνεόμενου όγκου αέρα. Μία πληθώρα γενετικών, δημογραφικών και κλινικών παραγόντων καθώς και παθοφυσιολογικών μηχανισμών εμπλέκεται στην ταξινόμηση του άσθματος σε διαφορετικούς «φαινοτύπους». Στις κατευθυντήριες οδηγίες GINA (Global Initiative for Asthma) του 2022 αναφέρονται οι πέντε πιο συχνοί φαινότυποι του άσθματος: 1. Αλλεργικό άσθμα. 2. Μη αλλεργικό άσθμα. 3. Άσθμα με έναρξη των συμπτωμάτων στην ενήλικη ζωή. 4. Άσθμα με εμμένουσα μείωση του εκπνεόμενου όγκου αέρα. 5. Άσθμα σχετιζόμενο με παχυσαρκία. Επιπλέον, με βάση την ανοσολογική απόκριση η οποία επικρατεί στην ανάπτυξη και εξέλιξη της νόσου, το άσθμα ταξινομείται σε δύο ενδοτύπους: α) το τύπου 2/αλλεργικό άσθμα, που χαρακτηρίζεται από φλεγμονή διαμεσολαβούμενη κυρίως από βοηθητικά τύπου 2 Τ λεμφοκύτταρα και ηωσινοφιλία, και β) το μη-τύπου 2 άσθμα, το οποίο σχετίζεται με φλεγμονή διαμεσολαβούμενη κυρίως από βοηθητικά τύπου 1 ή/και τύπου 17 Τ λεμφοκύτταρα και αυξημένο αριθμό ουδετερόφιλων κυττάρων. Στην παρούσα διατριβή ασχοληθήκαμε με τον συχνότερο φαινότυπο και ενδότυπο του άσθματος, το αλλεργικό/τύπου 2 άσθμα.

Το αλλεργικό άσθμα αποτελεί την πιο συχνή νόσο των αεραγωγών, επηρεάζοντας περισσότερους από 300 εκατομμύρια ασθενείς σε όλο τον κόσμο. Ο σημαντικά αυξημένος επιπολασμός της νόσου τις τελευταίες δεκαετίες, ιδίως στα παιδιά των αναπτυγμένων χωρών, καθιστά το αλλεργικό άσθμα ένα παγκόσμιο κοινωνικο-οικονομικό πρόβλημα. Πολλοί τύποι κυττάρων (επιθηλιακά κύτταρα, αντιγονοπαρουσιαστικά κύτταρα, ηωσινόφιλα, μαστοκύτταρα, Β και Τ λεμφοκύτταρα κ.α.) εμπλέκονται στην παθογένεια του αλλεργικού άσθματος και αρκετές μελέτες έχουν αναδείξει την σπουδαιότητα της αλληλεπίδρασης μεταξύ των επιθηλιακών κυττάρων των βρόγχων και των κυττάρων του ανοσοποιητικού συστήματος κατά την είσοδο ενός εισπνεόμενου περιβαλλοντικού

αντιγόνου (αλλεργιογόνο) στους αεραγωγούς. Βασικός παθογενετικός μηχανισμός κατά την ανάπτυξη του αλλεργικού άσθματος είναι η επαγωγή Τ βοηθητικών λεμφοκυτταρικών ανοσολογικών αποκρίσεων τύπου 2 εναντίον εισπνεόμενων αλλεργιογόνων. Τα Τ βοηθητικά λεμφοκύτταρα τύπου 2 παράγουν συγκεκριμένες πρωτεΐνες, που ονομάζονται κυτταροκίνες και συμβάλλουν δραστικά στην ανάπτυξη της αλλεργικής φλεγμονής στους αεραγωγούς. Οι κυτταροκίνες αυτές είναι κυρίως οι ιντερλευκίνες (IL) 4, 5, 9 και 13 (IL-4, IL-5, IL-9, IL-13). Οι απορρυθμισμένες αλλεργιογόνο-ειδικές αποκρίσεις των Τ βοηθητικών λεμφοκυττάρων τύπου 2 στους αεραγωγούς οδηγούν σε ηωσινοφιλική πνευμονική διήθηση, υπερέκκριση βλέννης, αναστρέψιμη σύσπαση των λείων μυϊκών ινών των αεραγωγών και βρογχική υπεραντιδραστικότητα. Οι περισσότερες χρησιμοποιούμενες θεραπείες αδυνατούν να επιφέρουν ίαση προσφέροντας μόνο προσωρινή ανακούφιση από τα κλινικά συμπτώματα. Αξιοσημείωτο είναι το γεγονός ότι μία μερίδα ασθενών πάσχει από σοβαρό άσθμα που ανθίσταται στις παρούσες θεραπευτικές πρακτικές και χρήζει συχνών νοσηλειών, αποτελώντας ένα μείζον ιατρικό και κοινωνικο-οικονομικό πρόβλημα. Συνεπώς, παράγοντες οι οποίοι μπορούν να επάγουν ή/και να ενισχύσουν την στοχευμένη ανοσολογική καταστολή των αλλεργικών Τ λεμφοκυτταρικών αποκρίσεων τύπου 2 σε αυτούς τους ασθενείς αποτελούν σημαντικούς θεραπευτικούς στόχους.

Ανοσολογικοί κατασταλτικοί μηχανισμοί, όπως οι ανοσοκατασταλτικές κυτταροκίνες και τα ρυθμιστικά Τ λεμφοκύτταρα (Tregs), καταστέλλουν τις απορρυθμισμένες Τ ανοσολογικές αποκρίσεις τύπου 2 σε μοντέλα ποντικών με αλλεργικό άσθμα. Πολλές μελέτες έχουν επισημάνει ως βασικούς ρυθμιστές των αλλεργικών Τ λεμφοκυτταρικών αποκρίσεων τύπου 2 δύο υποπληθυσμούς των ρυθμιστικών $CD4^+$ Τ λεμφοκυττάρων, τα ρυθμιστικά $CD4^+$ Τ λεμφοκύτταρα που ωριμάζουν και διαφοροποιούνται στο θύμο αδένα και εκφράζουν τον μεταγραφικό παράγοντα Foxp3 (Forkhead box P3)(natural regulatory T cells/nTregs) και τα επαγόμενα στην περιφέρεια από αθώα $CD4^+$ Τ λεμφοκύτταρα που εκκρίνουν IL-10 (τύπου 1 ρυθμιστικά Τ λεμφοκύτταρα/ Tr1 cells). Επιπροσθέτως, οι ανοσορρυθμιστικές κυτταροκίνες IL-10, IL-35 και μετασχηματικός αυξητικός παράγοντας β (TGF-β) είναι ζωτικής σημασίας για την κατασταλτική δράση των Τ ρυθμιστικών λεμφοκυττάρων. Εντούτοις, μια πληθώρα ερευνών αποκαλύπτει ότι τόσο ο αριθμός όσο και η δράση των Τ ρυθμιστικών λεμφοκυττάρων είναι εξασθενημένη στους ασθενείς με αλλεργικό άσθμα.

Η ακτιβίνη-Α είναι μία κυτταροκίνη που ανήκει στην υπεροικογένεια του TGF-β 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 ερευνητική ομάδα της Δρ. Γεωργίας Ξάνθου έχει αποδείξει ότι η ακτιβίνη-Α είναι μία ανοσοκατασταλτική κυτταροκίνη καθώς αναστέλλει τις ειδικές έναντι αλλεργιογόνου Τ ανοσολογικές αποκρίσεις τύπου 2, την ανάπτυξη βρογχικής υπεραντιδραστικότητας και την αλλεργική φλεγμονή στους αεραγωγούς των ποντικών με πειραματικό αλλεργικό άσθμα. Η ακτιβίνη-Α ασκεί τις ανοσοκατασταλτικές της ιδιότητες μέσω της επαγωγής αλλεργιογόνο-ειδικών Τ ρυθμιστικών λεμφοκυττάρων, τα οποία καταστέλλουν τις απορρυθμισμένες Τ ανοσολογικές αποκρίσεις τύπου 2 *in vitro* και έπειτα από μεταφορά τους σε μοντέλα ποντικών με αλλεργικό άσθμα *in vivo*. Ωστόσο, ο ακριβής ρόλος της ακτιβίνης-Α στο ανθρώπινο αλλεργικό βρογχικό άσθμα παρέμενε άγνωστος.

Σε αυτή τη μελέτη υποθέσαμε ότι η ακτιβίνη-Α μπορεί να επάγει τη διαφοροποίηση ανθρώπινων $CD4^+$ Τ ρυθμιστικών λεμφοκυττάρων και να καταστείλει τις ανθρώπινες Τ ανοσολογικές αποκρίσεις τύπου 2. Βασισμένοι στα δημοσιευμένα αποτελέσματα της ερευνητικής ομάδας της Δρ. Γεωργίας Ξάνθου, υποθέσαμε ότι η ακτιβίνη-Α αντιπροσωπεύει μία κυτταροκίνη-κλειδί για τη ρύθμιση της αλλεργικής φλεγμονής των αεραγωγών και του ανθρώπινου αλλεργικού άσθματος.

Οι στόχοι μας ήταν :

α) Να διερευνήσουμε τις επιδράσεις της ακτιβίνης-Α στην καταστολή των ανθρώπινων αλλεργικών Τ ανοσολογικών αποκρίσεων τύπου 2 και

β) Να εξετάσουμε το ρόλο της ακτιβίνης-Α στην επαγωγή ανθρώπινων $CD4^+$ Τ ρυθμιστικών λεμφοκυττάρων καθώς και τις επιπτώσεις αυτών στην καταστολή των αλλεργικών Τ ανοσολογικών αποκρίσεων τύπου 2.

Τα δεδομένα μας αποκαλύπτουν ότι η ακτιβίνη-Α είναι ένας σημαντικός ανοσοκατασταλτικός παράγοντας στο ανθρώπινο αλλεργικό άσθμα.

Συγκεκριμένα, δείχνουμε ότι:

1) Η ακτιβίνη-Α επάγει την διαφοροποίηση ανθρώπινων ρυθμιστικών $CD4^+$ Τ λεμφοκυττάρων, τα οποία παράγουν την ανοσοκατασταλτική κυτταροκίνη IL-10 και εκφράζουν στην επιφάνειά τους τα μόρια inducible T-cell costimulator (ICOS), lymphocyte activation gene 3 protein (LAG-3) και CD49b. Συνεπώς, τα επαγόμενα από την ακτιβίνη-Α ρυθμιστικά $CD4^+$ Τ λεμφοκύτταρα παρουσιάζουν κοινά βασικά χαρακτηριστικά με τα επαγόμενα ρυθμιστικά $CD4^+$ Τ λεμφοκύτταρα τύπου 1 (Tr1 cells).

2) Τα επαγόμενα από την ακτιβίνη-Α ανθρώπινα ρυθμιστικά $CD4^+$ Τ λεμφοκύτταρα (act-A-iTr1 κύτταρα) εμφανίζουν έντονη *in vitro* κατασταλτική δράση έναντι ανθρώπινων αλλεργικών Τ αποκρίσεων διαμεσολαβούμενων τόσο από αθώα όσο και από ενεργοποιημένα $CD4^+$ Τ βοηθητικά λεμφοκύτταρα τύπου 2. Οι κυτταροκίνες IL-10 και TGF-β και ο παράγοντας συνενεργοποίησης των Τ λεμφοκυττάρων ICOS εμπλέκονται στην κατασταλτική δράση των act-A-iTr1 κυττάρων.

3) Στο επίκεντρο των μοριακών μηχανισμών της επαγωγής των ανθρώπινων $CD4^+$ Τ ρυθμιστικών λεμφοκυττάρων από την ακτιβίνη-Α βρίσκεται η επαγωγή της έκφρασης των μεταγραφικών παραγόντων aryl hydrocarbon receptor (AhR) και interferon regulatory factor 4 (IRF4). Αυτοί οι μεταγραφικοί παράγοντες μετακινούνται στον πυρήνα των κυττάρων, που καλλιεργούνται παρουσία ακτιβίνης-Α, δημιουργούν ένα πολυμερές μεταγραφικό σύμπλεγμα μαζί με την πρωτεΐνη πρόσδεσης του AhR [AhR nuclear translocator (ARNT)] και συνεργάζονται ώστε να επάγουν την μεταγραφή του γονιδίου *ICOS*, το οποίο εμφανίζει αυξημένη έκφραση, τόσο σε επίπεδο mRNA όσο και σε πρωτεϊνικό επίπεδο, στα act-A-iTr1 κύτταρα.

4) Η *in vivo* μεταφορά των act-A-iTr1 κυττάρων σε ανθρωποποιημένα μοντέλα πειραματικού αλλεργικού άσθματος ποντικών, σταματάει την ανάπτυξη και την εξέλιξη της νόσου ενώ μπορεί να καταστείλει εγκατεστημένη αλλεργική φλεγμονή μέσω της έκκρισης IL-10.

5) Προσθήκη ακτιβίνης-Α σε *in vitro* καλλιέργεια ενεργοποίησης με αλλεργιογόνο $CD4^+$ Τ λεμφοκυττάρων που απομονώθηκαν από ασθενείς με ατοπία αλλά και μέτριο και

σοβαρό άσθμα (επαγόμενο από το συγκεκριμένο αλλεργιογόνο), μειώνει σε μεγάλο βαθμό τον πολλαπλασιασμό τους και την έκκριση προφλεγμονωδών κυτταροκινών.

Συμπερασματικά, τα δεδομένα μας αναδεικνύουν για πρώτη φορά την ακτιβίνη-Α ως έναν νέο παράγοντα επαγωγής ανθρώπινων, λειτουργικών $CD4^+$ Τ ρυθμιστικών λεμφοκυττάρων που εκκρίνουν την ανοσορρυθμιστική κυτταροκίνη IL-10 και εκφράζουν τα επιφανειακά μόρια που χαρακτηρίζουν τα Tr1 κύτταρα, ICOS, LAG-3 και CD49b. Τα ανθρώπινα act-A-iTr1 κύτταρα εμφανίζουν έντονη κατασταλτική δράση έναντι αλλεργικών αποκρίσεων διαμεσολαβούμενων τόσο από αθώα όσο και από ενεργοποιημένα $CD4^+$ Τ βοηθητικά λεμφοκύτταρα τύπου 2 μέσω μηχανισμών στους οποίους εμπλέκονται οι κυτταροκίνες IL-10 και TGF-β καθώς και ο επαγόμενος παράγοντας συνενεργοποίησης των Τ λεμφοκυττάρων ICOS. Στο επίκεντρο των μοριακών μηχανισμών, αποδεικνύουμε ότι η ενεργοποίηση και συνεργασία των μεταγραφικών παραγόντων IRF4 και AhR διαδραματίζουν καταλυτικό ρόλο στην διαφοροποίηση των act-A-iTr1 κυττάρων καθώς σχηματίζουν ένα μεταγραφικό σύμπλεγμα που επάγει την έκφραση των γονιδίων *IL10* και *ICOS*. Η *in vivo* μεταφορά των act-A-iTr1 κυττάρων σε ανθρωποποιημένα μοντέλα πειραματικού αλλεργικού άσθματος ποντικών αποκαλύπτει ότι τα act-A-iTr1 κύτταρα διατηρούν τις ανοσορρυθμιστικές τους ιδιότητες *in vivo* καθώς σταματάνε την ανάπτυξη του πειραματικού αλλεργικού άσθματος και καταστέλλουν εγκατεστημένη αλλεργική φλεγμονή στους αεραγωγούς των ποντικών μέσω της έκκρισης της κυτταροκίνης IL-10. Ιδιαίτερη κλινική σημασία έχουν τα αποτελέσματα μας που δείχνουν ότι η προσθήκη ακτιβίνης-Α σε *in vitro* καλλιέργειες $CD4^+$ Τ λεμφοκυττάρων, που απομονώθηκαν από το περιφερικό αίμα ατόμων με ατοπία και άσθμα ποικίλης βαρύτητας, παρουσία αλλεργιογόνου, καταστέλλει τον πολλαπλασιασμό και την έκκριση προφλεγμονωδών κυτταροκινών.

Συνεπώς, η μελέτη μας αναδεικνύει την ακτιβίνη-Α ως ένα νέο πιθανό θεραπευτικό στόχο στο ανθρώπινο αλλεργικό άσθμα. Μελλοντικές μελέτες θα μπορούσαν να διερευνήσουν την δυνατότητα χρησιμοποίησης των act-A-iTr1 κυττάρων ως θεραπευτική μέθοδο για την ανάπτυξη ανοσολογικής ανοχής στους αεραγωγούς των ασθενών με αλλεργικό άσθμα.

SUMMARY

Asthma is a heterogeneous disease, characterized by airway hyperresponsiveness and chronic inflammation of the small-conducting and the large-conducting airways. It is defined by the history of recurrent respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough which vary over time and in intensity, accompanied by variable expiratory airflow limitation. Recognizable clusters of genetic, demographic, clinical and pathophysiological characteristics are often called “asthma phenotypes”. GINA (Global Initiative for Asthma) 2022 guidelines report the five most common “phenotypes” of asthma: 1. Allergic asthma. 2. Non-allergic asthma. 3. Adult-onset (late-onset) asthma. 4. Asthma with persistent airflow limitation. 5. Asthma with obesity. On the other hand, a pathogenetic classification of asthma in two endotypes is based on the type of the immune cell response that mediates disease development and progression. Hence, asthma is classified as: (a) type 2/allergic asthma, characterized predominantly by T helper type 2 (Th2) cell-mediated inflammation and eosinophilia, and (b) non-type 2 asthma associated mainly with Th1 and/or Th17 cell-mediated inflammation and increased numbers of neutrophils. Our research was focused on the most frequent asthma endotype/phenotype, the type 2/allergic asthma.

Allergic asthma represents the most common disorder of the airways, affecting more than 300 million people worldwide. The significantly increased prevalence of the disease, especially in the children of industrialized countries the last decades, makes asthma a substantial global social-economic burden. Many cell types (epithelial cells, antigen presenting cells, eosinophils, basophils, mast cells, B and T cells e.t.c.) are involved in the pathogenesis of allergic asthma while several studies depict the importance of the interaction between bronchial epithelial cells and immune cells following inhaled allergen encounter during the initiation of asthmatic responses. Main pathogenetic factor of the disease is the generation of aberrant Th2 immune responses against allergens. Th2 cells produce specific proteins, named cytokines that are critically involved in the development of allergic airway inflammation. The major Th2-type cytokines are the interleukins 4, 5, 9 and 13 (IL-4, IL-5, IL-9, IL-13). Dysregulated allergen-specific Th2 responses in the airways lead to pulmonary eosinophilic infiltration, mucus hypersecretion, reversible airway

obstruction and airway hyperresponsiveness (AHR). Most of the current treatments only ameliorate certain clinical features of the disease without providing a cure. Of note, a group of patients has severe, treatment-refractory asthma, requires regular hospitalization and represents a major health-care and socioeconomic problem. Therefore, factors that can induce and/or enhance immunosuppression represent essential therapeutic targets for human allergic asthma.

Immunoregulatory mechanisms, including suppressive cytokines and regulatory T cells (Tregs), attenuate aberrant Th2-mediated allergic responses in experimental asthma mouse models. A large body of evidence has uncovered as key regulators of aberrant allergic Th2 responses two subtypes of Tregs, the thymus-derived natural Tregs (nTregs), that express the transcription factor Foxp3 and the peripherally-induced by naive $CD4^+$ T cells, type 1 regulatory T cells (Tr1 cells) that secrete the cytokine IL-10. Moreover, immunoregulatory cytokines, such as IL-10, IL-35 and transforming growth factor- β (TGF-β), are critical for the induction and suppressive function of Tregs. Nevertheless, accumulating evidence supports the notion that the numbers and function of Tregs and immunosuppressive mediators are severely impaired in individuals with allergic asthma.

Activin-A is a cytokine, member of the TGF-β superfamily that is critically implicated 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. Studies by Dr. Xanthou's group have revealed 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. Activin-A exerts its regulatory functions through the induction of allergen-specific Treg cells that suppress Th2 responses *in vitro* and upon adoptive transfer *in vivo*. However, the exact role of activin-A in human allergic asthma remained elusive.

In the present study we hypothesized that activin-A can induce the differentiation of human regulatory $CD4^+$ T cells and can suppress human Th2-mediated allergic responses. Based on previously published findings by Dr. Xanthou's group, we hypothesized that activin-A represents a key cytokine for the regulation of allergic airway inflammation and human allergic asthma.

Our specific aims were:

a) To investigate the effects of activin-A on the suppression of human Th2 cell-mediated allergic responses and

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

Our data reveal activin-A as a critical immunosuppressive agent for human allergic asthma.

More specifically, we demonstrate that:

1) Activin-A drives the generation of human $CD4^+$ T cells that produce copious amounts of the immunoregulatory cytokine IL-10 and express the surface molecules ICOS, LAG-3 and CD49b. Hence activin-A-induced human $CD4^+$ T cells possess key feature characteristics of human Tr1 cells.

2) Activin-A-induced human Tr1-like cells (act-A-iTr1 cells) display strong suppressive functions toward allergen-driven responses induced by naive and *in vivo*-primed human Th2 cells. Act-A-iTr1 cells exert their regulatory functions through IL-10-, TGF-β- and ICOS-mediated pathways.

3) Activin-A signalling induces the activation of the transcription factors IRF4 and AhR in human $CD4^+$ T cells, which bind in *ICOS* promoter elements and control gene expression. Furthermore, IRF4 along with AhR and its binding partner, ARNT, form a transcription factor complex that is essential for effector molecule expression by human act-A-iTr1 cells.

4) *In vivo* adoptive transfer of human act-A-iTr1 cells in humanized mouse models of experimental asthma, can prevent, and even reverse, established allergic airway inflammation and confer protection against cardinal asthma manifestations in an IL-10–dependent manner.

5) Addition of activin-A in *in vitro* stimulatory cultures with a clinically-relevant allergen of human $CD4^+$ T cells isolated from atopic and asthmatic individuals with distinct disease severities, leads to the significant reduction of $CD4^+$ T cell proliferation and effector cytokine release.

Collectively, our studies uncover for the first time activin-A as a novel inducer of human IL-10-producing regulatory $CD4^+$ T cells that express the Tr1-cell-associated markers ICOS, LAG-3 and CD49b. Human act-A-iTr1 cells display robust suppressive functions against naive and effector T-cell responses to allergen through IL-10–, TGF-β–, and ICOS– mediated mechanisms. Regarding the underlying molecular mechanism, we reveal that the transcription factors IRF4 and AhR play pivotal role in the generation of human act-A-iTr1 cells since they form a transcriptional complex essential for the expression of *IL10* and *ICOS* genes. Using a humanized mouse model of experimental asthma, we demonstrate that human act-A–iTr1 cells retain their immunosuppressive properties upon adoptive transfer *in vivo* and can prevent, and even reverse, established allergic airway inflammation and confer significant protection against cardinal asthma manifestations in an IL-10–dependent pathway. Of clinical relevance, our research illustrates that activin-A is able to restrain allergen-driven responses by $CD4^+$ effector T cells obtained from asthmatic individuals across the spectrum of disease severity.

Hence our studies may mark activin-A as a potentially attractive new therapeutic target for human asthma. Future studies could address the utilization of human act-A-iTr1 cells in adoptive-transfer cell therapy regimes aiming at re-establishing tolerance in the human asthmatic airways.

ABBREVIATIONS

Ab: Antibody
Act-A-iTr1 cells: Activin-A-induced human Tr1-like cells
ActRII: Activin-A receptors type II
AHR: Airway hyperresponsiveness
AhR: Aryl hydrocarbon receptor
AIT: Allergen-specific immunotherapy
ALK: Activin-like kinase
ALPP: Alkaline phosphatase, placental
APCs: Antigen presenting cells
ARNT: Aryl hydrocarbon receptor nuclear translocator
ASM: Airway smooth muscle cells
BALF: Bronchoalveolar lavage fluid
BAMBI: BMP pseudoreceptor and activin membrane-bound inhibitor homolog
Bcl-6: B-cell lymphoma 6
BM: Basement membrane
BMPs: Bone morphogenetic proteins
CCL: C-C motif chemokine ligand
CCR: C-C motif chemokine receptor
CD: Cluster of differentiation
CFSE: Carboxyfluorescein succinimidyl ester
ChIP: Chromatin immunoprecipitation assay
CLRs: C-type lectin receptors
CM: Conditioned medium
COPD: Chronic obstructive pulmonary disease
Co-Smad: Common Smad
CBP: CREB protein binding protein
CREB: cAMP response element-binding protein
CRTH2: Chemoattractant receptor-homologous molecule expressed on Th2 cells
CS: Cigarette smoke
CTLA-4: Cytotoxic T lymphocyte antigen-4
CXCR: C-X-C family chemokine receptor

CYP1A1: Cytochrome P450 family-1 subfamily-A polypeptide-1

DCs: Dendritic cells

DLNs: Draining lymph nodes

DP: Double positive

DR5: Death receptor 5

EAR: Early phase allergic response

ECM: Extracellular matrix

EGF: Epidermal growth factor

ERK: Extracellular signal-regulated kinase

EMTU: Epithelial-mesenchymal trophic unit

ET-1: Endothelin-1

EVs: Extracellular vesicles

FcεRI: High-affinity IgE Fc immunoglobulin receptor

FeNO: Fractional exhaled nitric oxide

Foxp3: Forkhead box protein 3

FS: Follistatin

GATA-3: GATA-binding protein 3

GDFs: Growth and differentiation factors

GINA: Global Initiative for Asthma

GITR: Glucocorticoid-induced tumor necrosis factor receptor- related protein

GM-CSF: Granulocyte macrophage colony-stimulating factor

GVHD: Graft-versus-host disease

GZMB: Granzyme B

HLA: Human leukocyte antigen

ICOS: Inducible T cell costimulator

ICOSL: Inducible T cell costimulator ligand

ICSs: Inhaled corticosteroids

IDO: Indoleamine 2,3-dioxygenase

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IGF-1: Insulin growth factor-1

IL: Interleukin

IL-Rα: IL receptor α chain

ILCs: Innate lymphoid cells
iNOS: inducible Nitric oxide synthase
IP: Immunoprecipitation
I.p.: Intraperitoneally
IPEX: Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF4: Interferon regulatory factor 4
I-Smads: Inhibitory Smads
ISRE: Interferon-stimulated response elements
iTregs: i*n vitro* induced Treg cells
JNK: c-Jun N-terminal kinase
LABAs: Long-acting β2-adrenergic receptor agonists
LAG-3: Lymphocyte activation gene 3
LAMAs: Long-acting muscarinic antagonists
LAR: Late phase allergic response
LC: Langerhans cells
LPS: Lipopolysaccharide
LT: Leukotriene
LTRAs: Leukotriene receptor antagonists
MAF: c-MAF protoongogene
MAPK: Mitogen activated protein kinase
MARE: MAF-recognition element
MHC: Major Histocompatibility Complex
MLNs: Mediastinal lymph nodes
MMPs: Matrix metalloproteinases
NFAT: Nuclear factor of activated T cells
NGF: Nerve growth factor
NK: Natural killer cell
NKT: Natural killer T cell
NLRs: NOD (nucleotide-binding oligomerization domain) like receptors
NOD-SCID: Non-obese diabetic severe combined immunodeficient
Nrp-1: Neuropilin-1
nTregs: Natural regulatory T cells
OCSs: Oral corticosteroids

ORMDL3: ORMDL Sphingolipid Biosynthesis Regulator 3

OVA: Ovalbumin

OX40L: OX40 ligand

PAMPs: Pathogen-associated molecular patterns

PAP: Pulmonary alveolar proteinosis

PB: Peripheral blood

PBMCs: Peripheral blood mononuclear cells

PD-1: Programmed cell death protein-1

PenH: Enhanced pause

PG: Prostaglandin

PHA: Phytohemagglutinin

PKN2: Protein kinase N2

PRRs: Pattern recognition receptors

pSmad: phosphorylated Smad

pTregs: peripherally-induced Tregs

PTK2: Protein tyrosine kinase 2

RBM: Reticular basement membrane

RORa: Retinoic acid receptor-related orphan receptor alpha transcription factor

RORC: Retinoic acid receptor-related orphan receptor gamma gene

R-Smads: Receptor-regulated Smads

RSV: Respiratory syncytial virus

SABAs: Short-acting β2-adrenergic receptor agonists

shRNA: Short hairpin RNA

sIgE: Serum specific IgE

Smad: Mothers against decapentaplegic

SP: Single positive

STAT: Signal transducer and activator of transcription

ST2: Interleukin 1 receptor-like 1/ Interleukin 33 receptor

TBX21/Tbet: T-box transcription factor TBX21/Tbet

Tconv: Control-treated/ PBS-treated T cells/ Conventional T cells

TCR: T cell receptor

Teff: Effector T cells

TFBSs: Transcription factor-binding sites

$T_{FH:}$ T follicular helper

TGF-β: Transforming growth factor β

TGFBR: Transforming growth factor β receptor

TGIF: Transforming growth interacting factor

Th: T helper

TIGIT: T-cell immunoreceptor with Ig and ITIM domains

TIM-3: T-cell immunoglobulin and mucin domain-3

TLRs: Toll-like receptors

TNF: Tumor necrosis factor

TNFRSF8: TNF Receptor Superfamily Member 8/CD30

TRAIL: TNFrelated apoptosis-inducing ligand

Tregs: Regulatory T cells

tTregs: Thymus-derived Tregs

TRPV1: Transient receptor potential vanilloid 1

TSLP: Thymic stromal lymphopoietin

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

XRE: Xenobiotic response element

1. INTRODUCTION

1.1 Allergic Asthma

Asthma is an “ancient” disease. Scriptures from China (2.600 B.C.) and Egypt (1.500 B.C.) report symptoms of dyspnea while the term asthma (άσθμα in Greek) was initially mentioned in Iliad, an ancient Greek epic poem written by Homer in 800 B.C. About 400 years later, Hippocrates defined the word asthma for the medical community as severe shortness of breath and he linked this symptom to environmental triggers and specific professions, such as metalwork (1). However, Hippocrates only saw asthma as a symptom, and it was not until around 100 A.C. when another Greek physician, Aretaeus the Cappadocian composed a more detailed definition of asthma, similar to the modern understanding of how the disease develops (1). The ancient Romans also explored the condition and around 50 A.C., Pliny the Elder described links between pollen and dyspnea and recommended a predecessor of epinephrine, a beta2-agonist common in current quick-relief asthma treatment, as a therapy (1).

In the 19th century, Dr. Henry Hyde Salter was the first to identify eosinophils in the sputum of individuals with asthma and achieved acclaim for his accurate descriptions and medical drawings of pathophysiological conditions in the lungs during asthma attacks (1). In 1892, Sir William Osler noted the similarities between asthma and allergic conditions, such as hay fever, as well as asthma’s propensity to develop in families. Nevertheless, he assumed that contraction of airway smooth muscle cells was the central feature of asthma paving the way for the establishment of bronchodilators as the hallmark of disease therapy. The increased mortality from asthma through the 1960s and 1980s (2) prompted researchers to reshape their understanding of the pathogenesis of asthma. In 1967, the identification of immunoglobulin E (IgE) as the 5th immunoglobulin class by Johansson and Ishizaka (3) provided the crucial link between asthma and allergic airway inflammation. Thus, by the 1980s, asthma was linked to inflammation in the airways and the clinical trials conducted in the 1970s establishing that inhaled corticosteroids, notably beclomethasone dipropionate (4), was a highly effective controller drug for individuals with asthma, further supported this notion.

A breakthrough in the understanding of the underlying mechanisms controlling the development of asthma came in 1992 with the identification of a special subset of T cells capable of secreting cytokines that selectively interacted with mast cells, basophils and eosinophils in the bronchoalveolar lavage fluid (BALF) of individuals with asthma, the T helper type 2 cells (Th2) (5). Even though, the concept of Th2-cell-mediated immunity driving asthma pathogenesis has dominated over the last 30 years, a significant amount of research focusing on both individuals with asthma and mouse models of experimental asthma has uncovered asthma as a much more complex syndrome with various pathophysiological mechanisms/endotypes that requires personalized therapeutic approaches. Our research was focused on the most prevalent asthma endotype, the type 2/allergic asthma.

A remarkable power of the immune system lies on its ability to induce protective immunity against invading pathogens, while maintaining tolerance towards self and environmental antigens. Impaired respiratory tolerance against inhaled innocuous antigens (allergens) may lead to allergic diseases, such as allergic asthma, a serious health problem worldwide (6). Allergic asthma is a chronic inflammatory disorder of the airways and represents a major cause of chronic morbidity with over 300 million individuals affected worldwide (7). Notably, its prevalence and incidence have considerably increased during the last three decades, especially in industrialized countries, in which allergic asthma is now the most prevalent chronic disease in childhood (7). Clinically, asthma is defined by attacks of airway hyperresponsiveness (AHR) and reversible airway obstruction which manifest as coughing, wheezing and intermittent attacks of breathlessness and chest tightening, particularly at night or early in the morning (**figure 1**) (8). According to the latest global initiative for asthma (GINA) criteria, individuals with allergic asthma are categorized, depending on the incidence, severity of clinical symptoms and response to treatment to three main groups: a) mild asthma which is well-controlled with step 1 or 2 treatments, b) moderate asthma that is well-controlled with step 3 or 4 treatments, and c) severe asthma that remains uncontrolled despite adherence with maximal optimized doses of step 4 treatments or that worsens once high doses are decreased (8). Of note, approximately 3-10% of people with asthma suffer from severe disease and type 2 inflammation is found in the majority of patients with severe asthma (8).

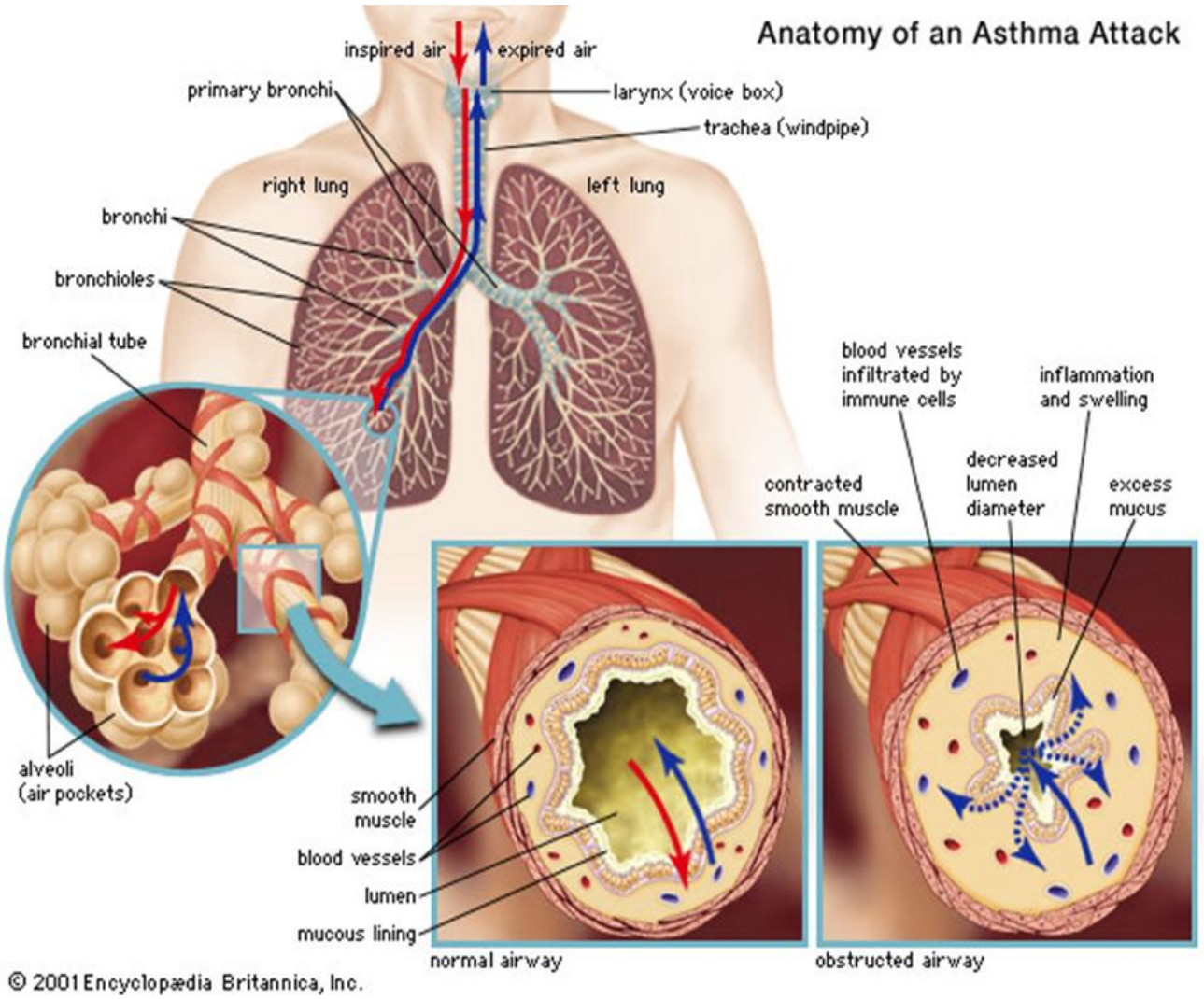

Figure 1. Anatomy of an asthma attack. During an asthma attack inflamed airways become rapidly obstructed and air flow is limited due to plasma extravasation, oedema, infiltration of inflammatory cells and contraction of airway smooth muscle cells. Persistent airflow obstruction occurs by mucus plugs deposition in subsegmental airways and airway remodelling processes such as goblet cell metaplasia, excessive subepithelial collagen deposition, airway smooth muscle hyperplasia, and angiogenesis. Characteristic clinical features of asthma attacks are wheeze, shortness of breath, chest tightness and cough. (*Picture adapted from Encyclopaedia Britannica 2001*).

There is a strong genetic association of allergic asthma with atopy, the predisposition of an individual to produce IgE antibodies against inhaled environmental allergens (9). Moreover, a plethora of genetic factors are associated with increased risk of developing

asthma (10). *IL4, IL13, RAD50, HLA-DRB1, HLA-DQA1, TSLP, IL25, IL33* (11)*, DENND1B* (12)*, ORMDL3* (13), *PKN2*, *PTK2*, *ALPP* (14), *ST2* (15), *ADAM33* (16) gene variants seem to be some of the most important asthma susceptibility factors while variants of the *TGFBR1* and *TNFRSF8* genes are associated with decreased asthma prevalence (17). In addition to genetic predisposition, a plethora of environmental factors has been shown to affect the risk of developing asthma as well as the disease progression. More specifically, inhaled allergens (18, 19), cigarette smoke (20), air pollutants especially with a diameter smaller than 10 μm (21), indoor air contaminants with endocrine-disrupting chemicals (22) and inhaled ligands of the aryl hydrocarbon receptor (AhR) environmental sensor (23) have been proven to provoke asthma attacks and exacerbate allergic airway inflammation. Furthermore, bacterial and viral respiratory infections, especially with respiratory syncytial virus (RSV) or rhinovirus, exercise and stress represent key trigger factors of asthma manifestations (24).

Numerous epidemiological risk factors have also been identified such as ethnicity, low socioeconomic status, obesity, caesarean section, maternal tobacco smoking, prematurity and antibiotic and paracetamol use during the first year of life (25, 26). In fact, prematurity is associated with a fourfold increase in the risk of developing asthma, the largest effect of any known epidemiological risk factor for this disease (26), suggesting that low lung function is critically implicated in the induction of allergic airway inflammation. Interestingly, a growing body of evidence indicates a significant role of lung and gut microbiota in the development and progression of allergic asthma since dysregulation of the composition and function of the microbiome has a great impact on asthma pathogenesis (27). Moreover, accumulating data suggests a role for neurogenic inflammation in chronic allergic asthma (28). Transient receptor potential vanilloid 1 (TRPV1), that is implicated in a variety of neuronal inflammatory pathways, has been proven to modulate inflammatory and structural changes in chronic asthma (29) while nerve growth factor (NGF), a neurotrophin that mediates neuroplasticity, has been shown to enhance the activity of eosinophils and promote airway hyperreactivity (30).

Diagnosis of allergic asthma is predominantly based on a detailed personal and family history, physical examination, performance of spirometry/peak expiratory flow and bronchodilator responsiveness/reversibility test (8). Additionally, the identification of

several molecular biomarkers, that have been developed using the omics technologies, assists in the diagnosis, classification and monitoring of the disease control during therapy (31). The most pertinent biomarkers of type 2 asthma are serum specific IgE (sIgE) against inhaled allergens, fractional exhaled nitric oxide (FeNO), blood and/or sputum eosinophilia, periostin, type 2 cytokines [interleukin-4, -5 and -13 (IL-4, IL-5 and IL-13)] and innate/epithelial cytokines [(IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)] (31). Even though biomarkers are crucial measurable indicators of the presence, severity and prediction of disease prognosis, they are not precise in selecting the patients that will respond to a targeted therapeutic intervention. For instance, although levels of blood eosinophils are used as a selection criterion for the administration of specific treatment, they are downregulated by all currently available treatments in individuals with severe asthma (32).

A significant challenge regarding allergic asthma diagnosis is the recognition of co-morbidities that often accompany the disease including multi-organ allergies, such as allergic rhinitis, sinus conjunctivitis, atopic dermatitis and food allergy, as well as non-allergic disorders, such as obesity, gastro-esophageal reflux, obstructive sleep apnea and psychiatric disorders (8, 33). Identification and concomitant treatment of these comorbidities are of great importance for the improvement of all clinical features developed by individuals with allergic asthma. The use of patient reported outcome measures (PROMs), which are self-completed questionnaires, measure the health related quality of life before and after an intervention and can help clinicians understand the burden of disease experienced by the patient thus identifying the significance of comorbidities (34).

For the selection of initial treatment and continuation of disease management, individuals with allergic asthma are grouped into one of five categories/steps that are used to determine requirements with controller drugs. According to the latest GINA report, the mainstay therapy of allergic asthma for steps 1 - 4 is the administration of inhaled corticosteroids (ICSs) alone or more often in combination with long-acting β2-adrenergic receptor agonists (LABAs) and potentially the use of a short-acting β2-adrenergic receptor agonist (SABA) as a reliever therapy whenever is needed (8). Corticosteroids are the most potent and consistently effective anti-inflammatory agents for long-term management of allergic asthma. Still, it is proven that these drugs cannot offer a cure neither alter the

natural history of the disease (35, 36). Additional controller drugs include SABAs, LABAs, long-acting muscarinic antagonists (LAMAs), leukotriene receptor antagonists (LTRAs) and, for the most severe disease, low dose azithromycin and oral corticosteroids (OCSs) (8). All current therapies that are used in steps 1-4 as well as OCSs used in step 5 target the downregulation of inflammation without affecting the underlying disease mechanism, therefore they cannot prevent long-term decline in lung function. Although mild and moderate asthma is usually controlled with these pharmacological interventions, approximately 3% to 10% of individuals with allergic asthma develop difficult-to-treat, severe disease that does not respond to standard therapy and leads to regular hospitalizations (37). Requiring high-dose ICSs and multiple other controllers or even OCSs to remain in control of their symptoms or even uncontrolled despite these therapies, the burden of severe asthma to these patients and to healthcare systems is extremely high (37). Of note, the chronic administration of high doses of certain medications, especially corticosteroids, in individuals with severe asthma often induces severe systemic side effects such as, growth retardation in children, adrenal suppression, osteoporosis, skin thinning, cataract, hypertension and secondary diabetes mellitus (38, 39). These individuals have significantly impaired quality of life and represent a major health care and socioeconomic problem for developed countries.

Six biologic interventions that suppress key inflammatory type 2 mediators are available nowadays for the control of severe allergic asthma (8). These are the monoclonal antibodies omalizumab, targeting the Fc part of free IgE, mepolizumab and reslizumab, targeting IL-5, benralizumab, blocking IL-5 receptor α chain (IL-5Rα), dupilumab, blocking IL-4Rα and tezepelumab which binds circulating TSLP (8, 40). Biologicals are utilized as add-on treatments at step 5 and were shown to have steroid-sparing effects and reduce asthma exacerbations, as well as hospital admissions, in randomized control trials (41). However, the limitations of their use in children younger than 12 years old (only omalizumab, mepolizumab and dupilumab administration is approved for ≥6 years old), the absence of reliable biomarkers for the appropriate selection of patients that could respond well following treatment with specific monoclonal antibodies, the considerable cost, the lack of sufficient proof that biologicals could affect the natural history of allergic asthma, their rare but life-threatening side-effects as well as the unknown long-term efficacy and safety represent significant obstacles for their use (42 - 44). In addition, so far, none of the

aforementioned monoclonal antibodies has shown a long-lasting effect and termination of administration usually results in a reoccurrence of symptoms. Indeed, stopping after even 5 years of omalizumab therapy resulted in an increase in exacerbations compared with patients who stayed on anti-IgE treatment (45). Given the established significance of type 2 immunity in anti-helminth defense, as well as its emerging importance in tissue repair processes, metabolism and cancer (46, 47), there is an imperative need for continued pharmacological surveillance to monitor toxicity signals and ascertain whether long-term administration is safe.

In contrast to the majority of the current therapeutic interventions that only seems to dampen certain aspects of allergic inflammation, allergen specific immunotherapy (AIT) is the only causative treatment available, which can cure and prevent allergic responses and linked disease. Successful AIT is associated with a variety of changes at the cellular level and the induction of tolerogenic responses, such as induction of allergen-specific regulatory T cells (Tregs), eradication of allergen-specific Th2 cells, regulation of allergen-specific IgE and IgG production, modification of dendritic cell (DC) phenotypes and mast cell and basophilic activation thresholds (48). Nevertheless, duration of AIT is between 2 and 5 years, and a large number of subcutaneous or sublingual administrations of escalating doses of allergen are essential to reach efficacy (49). Of note, uncontrolled asthma is the main contraindication to AIT while the direct and late IgE-mediated side effects of AIT can be life-threatening, thus requiring medical surveillance in a specialized centre (50). Hence, there is an imperative need to design novel immunotherapies that will be safer and target the cause of the disease, which is the underlying aberrant immune response to the allergen.

1.2 Allergic Airway Inflammation

Allergic airway inflammation that leads to asthma manifestations is initiated at mucosal surfaces where environmental inhaled allergens contact airway epithelial cells. Both genetic predisposition and environmental factors are crucial for the development and progression of aberrant allergic airway responses against innocuous aeroallergens (10 - 23). Accumulating evidence indicates that, following allergen encounter, the interplay between bronchial epithelial cells and DCs is fundamental for the initiation of a pathogenic immunological cascade leading to the establishment of allergic airway inflammation (51). Many airborne allergens are characterized by properties which endow them with the capacity to penetrate mucus and epithelial barriers, such as stability, proteolytic activity and/or molecular mimicry (52, 53). On the other hand, airway DCs can capture and process allergens that cannot enter the mucus layer, through their dendrites, which cross the epithelial barrier and are in contact with the airway lumen (**figure 2**) (54).

Importantly, during this procedure, epithelial cells of the airways, which are equipped with pattern recognition receptors (PRRs), produce the “alarmins” cytokines lL-25 (55), IL-33 (56) and TSLP as well as the granulocyte-macrophage colony-stimulating factor (GM-CSF) and the C-C family chemokines, CCL5, CCL11, CCL17 and CCL22 in response to environmental stimuli, such as inhaled allergens, viruses and air pollutants (**figure 2**) (57 – 59). In fact, numeral allergens carry pathogen-associated molecular patterns (PAMPs) that interact with the PRRs of the bronchial epithelial cells and DCs, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) and serve as “danger signals” for the host immune response (**figure 2**) (60). Upon allergen encounter, these “danger signals” together with cytokines and chemokines provided by the airway epithelium, augment the maturation of immature DCs which then migrate to the mediastinal lymph nodes (MLNs) in order to present epitopes of the processed allergens in the context of the major histocompatibility complex (MHC) class II molecules to naive $CD4^+$ T cells (**figure 2**) (61).

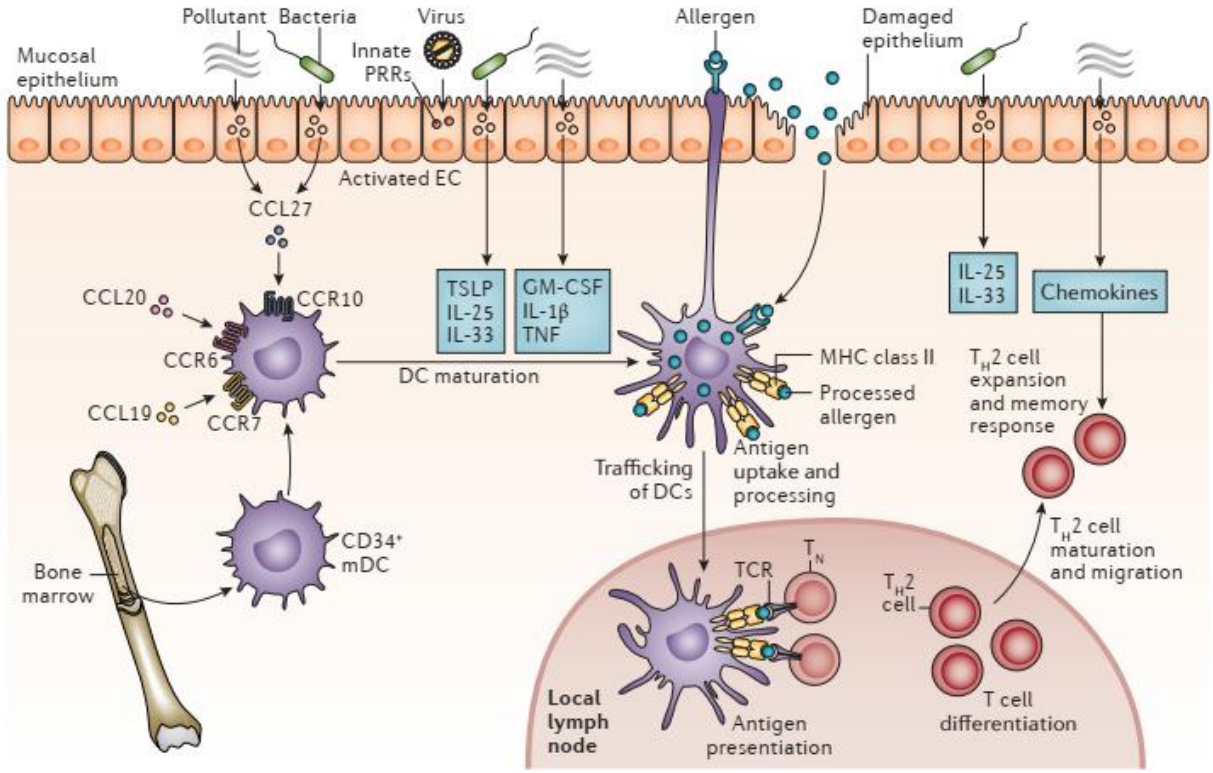

Figure 2. Allergen sensitization phase. Airborne allergens with proteolytic capacities, viruses, bacteria and pollutants perturb the airway epithelial layer leading to initial "danger signalling" through activation of PRRs expressed by the airway epithelial cells and DCs. In response to these danger signals, cytokines and chemokines that are released by the airway epithelium result in the maturation of DCs. Allergen-loaded mature DCs migrate to the mediastinal lymph nodes where they interact with naive $CD4^+$ T cells (TN) through the T cell receptor (TCR), MHC class II and co-stimulatory molecules in order to drive T cell differentiation. Then, additional epithelial-derived cytokines and chemokines, such as IL-25, IL-33, TSLP, CCL17 and CCL22 promote the maturation and migration of Th2 cells into the mucosa. (*Picture adapted from Holgate S. T. Innate and adaptive immune responses in asthma. Nat. Med. 2012*).

Depending on the allergen type and dose, the cytokine micromilieu and the interaction of specific surface molecules, the allergen presentation by mature DCs instructs naive $CD4^+$ T cells to differentiate into Th1, Th2, Th9, Th17, Th22, Treg or T follicular helper cells (T_{FH}) (62). Under Th2-related cytokines predominance, naive $CD4^+$ T cell polarization is skewed toward a Th2 phenotype, a procedure dominated by the activation of the master regulator of Th2 cells, the transcription factor GATA binding protein 3 (GATA-3) (63). Allergen-specific Th2 cells display a characteristic surface-molecule signature, the

IL-33R, the C-C family chemokine receptor 4/CCR4 and the chemoattractant receptor-homologous molecule expressed on Th2 cells/CRTH2, which allows them to traffic to the airways, in response to CCL17 and CCL22 (64). In lung tissue, allergen-specific Th2 effector cells orchestrate the allergic immune response through the production of specific Th2-type cytokines that promote the generation of various characteristic features of allergic airway inflammation and linked disease. Specifically, IL-4 is crucial for IgE isotype switching in B lymphocytes, induces goblet cell metaplasia and promotes the generation of more Th2 cells in a positive feedback loop (65), IL-5 is strongly involved in the recruitment and survival of eosinophils (66), IL-9 facilitates goblet cell metaplasia and mast cell hyperplasia (67) while IL-13 promotes mucus production and AHR and, in synergy with IL-4, induces the expression of key adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), to allow for eosinophil extravasation (**figure 3**) (65, 68).

Except from Th2 cells, during allergen presentation by mature DCs, naive $CD4^+$ T cells can differentiate into T_{FH} cells, a specialized $CD4^+$ T cell population that expresses the C-X-C family chemokine receptor type 5 (CXCR5) and is uniquely regulated by the transcription factor B-cell lymphoma 6 (Bcl-6) (69). Generation of human T_{FH} cells seems to be accomplished in the presence of transforming growth factor-β (TGF-β) together with IL-12 and IL-23 (70). Recent studies show that T_{FH} cells, rather than Th2 cells, predominantly produce IL-4 and IL-21 in B cell follicles and are essential mediators of IgE class switching, especially during severe asthma development, in both mice and humans (71). In fact, T_{FH} cells, can access B cell follicles and regulate the germinal center response through marked expression of inducible co-stimulator molecule (ICOS), programmed cell death 1 (PD-1), and CD40-ligand (CD40L), which are essential for interacting with B cells (**figure 3 and 4**) (72).

Even though adaptive immunity, mediated predominantly by Th2 cells, is essential for allergic airway inflammation, a growing body of evidence supports an important role for the innate immune responses both in the initiation and establishment of allergic asthma. Eosinophils, basophils, mast cells, macrophages, unconventional T cells and the recently described group 2 of innate lymphoid cells (ILC2s) are critical mediators of the development of aberrant allergic immune responses against airborne allergens (73, 74). Lung-resident ILC2s, which have emerged as crucial players in the generation of type 2

responses and allergic airway inflammation, arise from common lymphoid progenitors in the bone marrow and are dependent mainly on the transcription factor GATA-3, while they also express the transcription factor RORa (75). Phenotypically, they lack the expression of conventional lineage marker and re-arranged antigen-specific receptors, while they express ICOS, CRTH2, CD90, CD45, CD161, CD127/IL-7Ra, CD25 and the IL-33 receptor ST2 (76). Notably, once activated by the epithelial-derived “alarmins” and by eicosanoids, prostaglandin D2 (PGD2) and leukotrienes, produced by mast cells, macrophages and eosinophils, ILC2s provide a potent innate source of the type 2 cytokines IL-5, IL-9, IL-13 and IL-4 (77, 78) (**figure 3 and 4**). Interestingly, activated ILC2s also secrete amphiregulin, a member of the epidermal growth factor (EGF) family, which is critically involved in airway remodelling and repair processes following tissue damage (79) (**figure 3**).

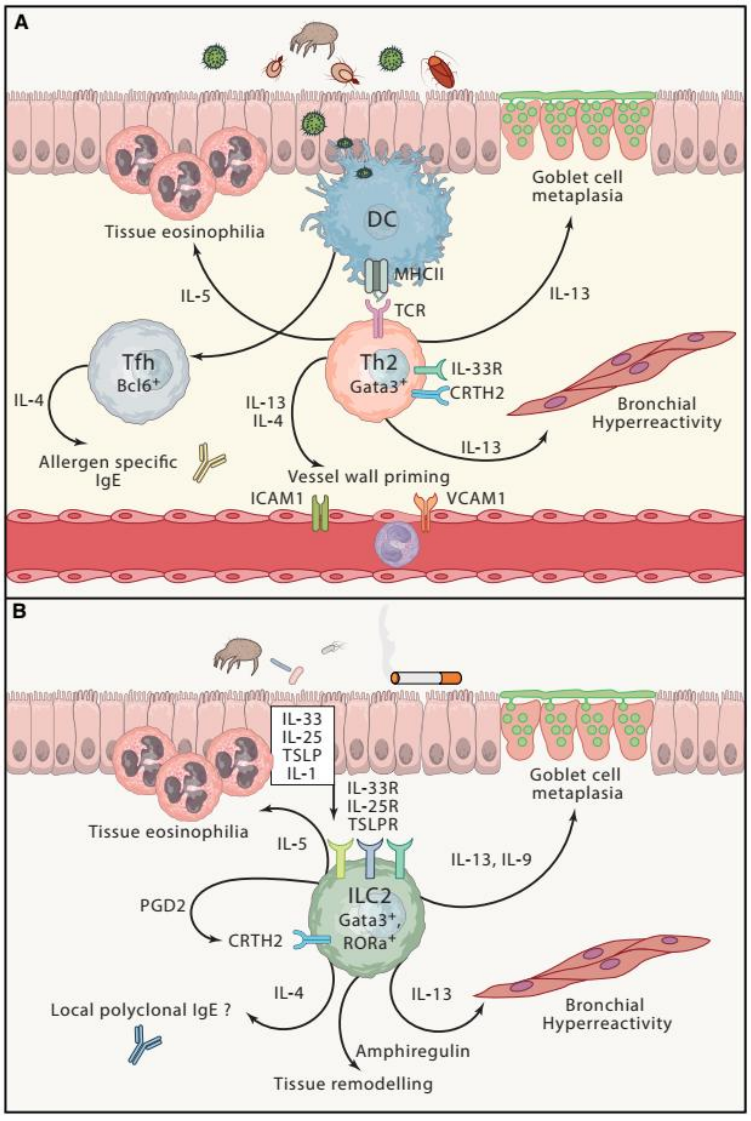

Figure 3. The origin and functions of type 2 cytokines in allergic airway inflammation. (**A**) **Th2-derived cytokines orchestrate allergic airway inflammation**. DCs capture and process inhaled allergens to present them to naïve $CD4^+$ T cells on MHC II molecules to the TCR. Simultaneously, airway epithelial cells produce cytokines which induce the maturation of DCs and the polarization of Th2 cells which express the transcription factor GATA3, cytokine receptor IL-33R, CCR4 and CRTH2. Th2 cells secrete type 2 cytokines including IL-4, IL-5 and IL-13 promoting key features of allergic airway inflammation. IL-4 and IL-13 induce the expression of adhesion molecules such as VCAM-1 to allow for eosinophil extravasation. Some T cells develop into T_{FH} in the lung-draining lymph nodes and control IgE class switching in allergen-specific B cells via abundant production of IL-4. (**B**) **ILC2-derived cytokines regulate innate type 2 inflammation**. In response to environmental stimuli the epithelial cells secrete IL-25, IL-33 and TSLP and activate ILC2s, which express the transcription factors GATA3 and ROR-a. These cells produce large amounts of PGD2 that causes ILC2 activation in an autocrine manner by acting on the CRTH2 receptor. ILC2s share many characteristics with Th2 cells but are unique in their high secretion of IL-9 that can promote goblet cell metaplasia and mast cell growth and survival. ILC2s also produce amphiregulin which induces tissue repair following damage. Whereas IL-4-producing T_{FH} cells stimulate allergen-specific IgE synthesis, ILC2s might be able to promote polyclonal IgE synthesis via secretion of IL-4. (*Picture adapted from Lambrecht B.N., et al. The Cytokines of Asthma. Immunity 2019*).

Apart from activating ILC2s, IL-25, IL-33 and TSLP promote allergic inflammation in the airways by orchestrating both innate and adaptive immunity (**figure 2, 3 and 4**). IL-33, which is the most rapidly released “alarmin” into the airways following allergen exposure, binds to ST2 and stimulates receptor-bearing cells to produce and secrete cytokines and growth factors that promote allergic responses (80). Hence, it acts on a plethora of pro-inflammatory cells, including mast cells, basophils, eosinophils, macrophages, NK cells, NKT cells, T_{FH} cells, Th2 cells and DCs (81) (**figure 3 and 4**). Moreover, IL-33 acts as a chemoattractant for Th2 cells (82) and promotes IL-5 producing Th2 cells and airway inflammation (83). “Alarmins”, most profoundly TSLP, target DCs and mediate mobilization, activation, and induction of the tumor necrosis factor (TNF) superfamily member OX40 ligand (OX40L) (84 - 86) (**figure 4**). In several human studies, OX40L-positive DCs stimulate naive $CD4^+$ T cells in a way that results to an IL-4-competent state, consistent with an important priming step in Th2 cell differentiation (86, 87). Furthermore, IL-33 and IL-25 which are very well-known activators of ILC2 proliferation and cytokine production, induce OX40L expression on ILC2s, and even boost cytokine production by

effector T cells (Teff) in a non-cognate manner (88, 89). In addition, TSLP acts directly on T cells by binding to its receptor TSLPR, in the presence of $CD4^+$ T cell activation through TCR stimulation, and promotes the differentiation and proliferation of Th2 cells via induction of *IL4* gene transcription (90). Secreted IL-4 upregulates the expression of TSLPR on Th2 cells in a positive feedback loop, further amplifying Th2 responses (90). Except from direct effects on Th2 cells, TSLP induces secretion of CCL17 by DCs, indirectly promoting $CCR4^+$ Th2 cell migration to the active site of inflammation (91). Furthermore, TSLP acts on $TSLPR^+$ B cell progenitors and facilitates their lymphopoiesis and terminal differentiation (92). Moreover, a variety of innate immune cells which express the TSLPR, including eosinophils (93), mast cells (94) and alternatively activated macrophages (95), get activated and increase cytokine production, in response to TSLP stimulation, a process that may also be influenced by epithelial-derived TGF-β (96). IL-25 mediates its effects by binding to IL-25 receptor (IL-17RB) and amplifies established TSLP-driven Th2 responses by enhancing expression of genes encoding Th2-associated transcription factors (GATA3, c-MAF and JUNB), increasing expression of Th2 signature surface markers (CCR4 and IL-4R) and maintaining CRTH2 expression (97). IL-25 acts also directly on naive human $CD4^+$ T cells to induce Th2 differentiation and enhance the expression of its own receptor (98).

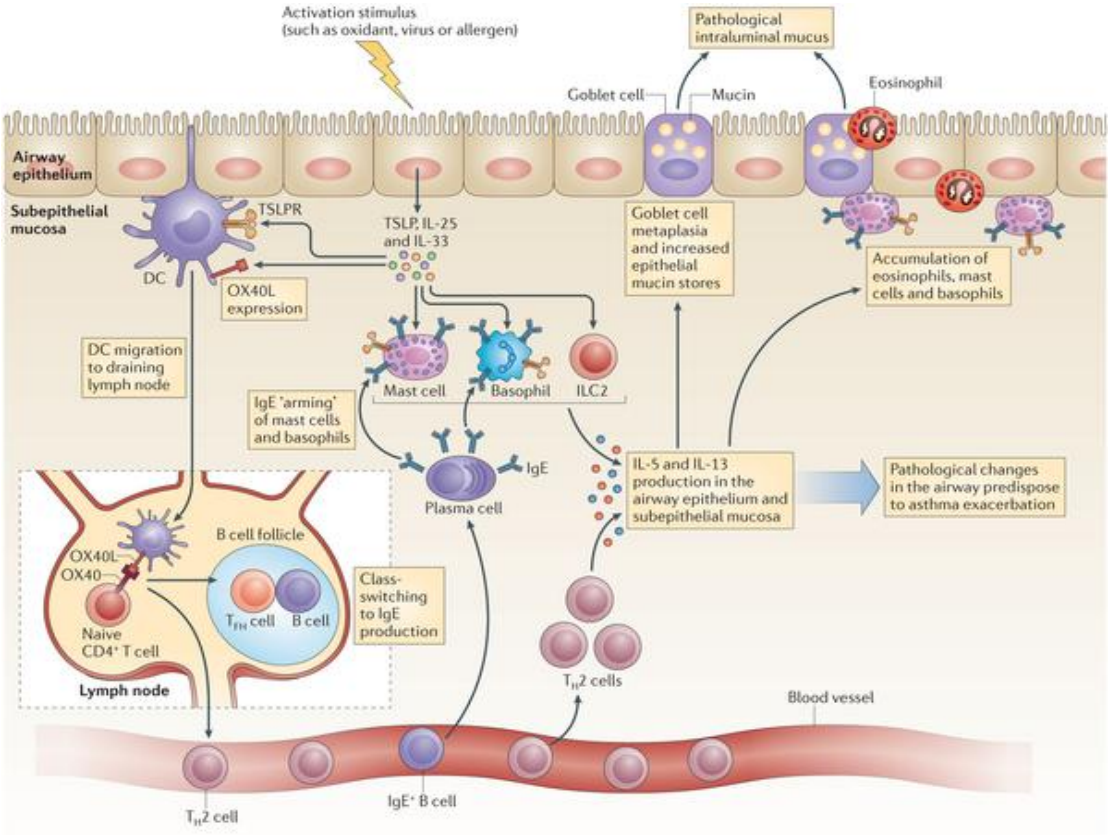

Figure 4. Development of allergic airway inflammation. Alarmins, which are the epithelial cell-derived cytokines IL-25, IL-33 and TSLP induce the expression of OX40L on DCs and promote their migration to the MLNs where they activate naive $CD4^+$ T cells towards an IL-4-competent state. Once activated, IL-4-competent T cells migrate to the B cell zones of DLNs and differentiate into T_{FH} cells or exit into the draining lymph and the circulation to complete maturation as Th2 cells. IL-4-secreting T_{FH} cells in parafollicular B cell areas mediate IgE switching and conjugate with B cells to drive affinity maturation within germinal centers, whereas Th2 cells migrate to the airways where they secrete IL-5 and IL-13 to mediate inflammatory and remodelling changes in the airway mucosa. In addition, IL-25, IL-33 and TSLP activate lung-resident ILC2s which then provide a potent innate source of the type 2 cytokines further promoting the initiation of allergic responses in the airways. (*Picture adapted from Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nat Immunol 2015*).

All the aforementioned events constitute the initial procedure termed allergen sensitization phase which results in the generation of allergen-specific Th2 effector and memory cells and IgE producing plasma cells (**figure 2 and 4**). During this phase there are no evident clinical signs and symptoms denoting aberrant immune responses. However, once sensitized, further exposure of the airways to allergen initiates within minutes the development of the early phase allergic response (EAR) which reaches a maximum within 30 min, resolves within 2-3 hours and is characterized by the key clinical features of allergic asthma, wheeze, cough, chest tightening and dyspnea (99) (**figure 5**). Mechanistically, subsequent exposure to the original or a cross-reactive airborne allergen results in the crosslinking of pre-formed IgE-allergen complexes with the high-affinity receptor for IgE, FcεRI, which is predominantly expressed by mast cells and basophils and has a tetrameric structure, consisting of one IgE-binding α-chain, one β-chain, which spans the plasma membrane and functions as a signal amplifier and two identical and largely intracellular γ chains (100) (**figure 5**). Interestingly, IgE binding to FcεRI also stabilizes the receptor's expression on the surface of the mast cells and basophils (101), thereby increasing the numbers of FcεRI on the cell surface (102). When the FcεRI aggregation is of sufficient strength and duration, it triggers complex signalling events in activated mast cells and basophils instructing them to secrete a diverse group of highly active biological mediators (103). Some of these inflammatory mediators are released within minutes of allergen re-exposure, such as histamine, proteases and heparin, which are stored preformed in the cytoplasmic granules as well as newly, though rapidly produced, lipid-

derived mediators, including PGD2, sphingosine-1-phosphate, leukotrienes (LTB4, LTC4, LTD4 and LTE4) and the preformed TNF-α (104, 105) (**figure 5**). These mediators promote vascular permeability, vasodilation, smooth-muscle cell contraction and enhanced mucus production in the airways, processes that contribute substantially to the appearance of the acute clinical signs and symptoms of allergic asthma (99) (**figure 5**).

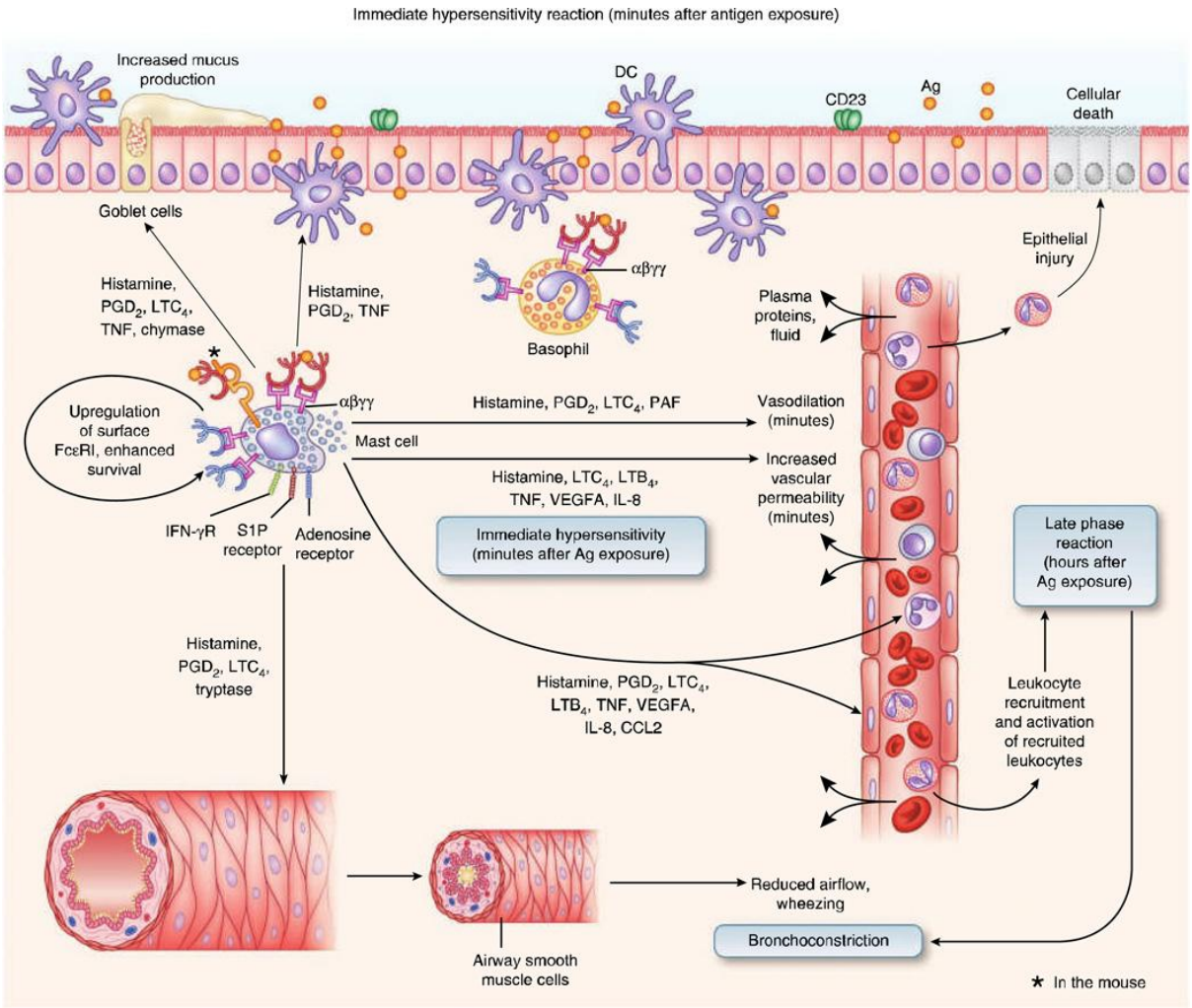

Figure 5. Early phase allergic response. Allergen-specific IgE antibodies bind to the FcεRI on mast cells and basophils to initiate an immediate early phase response within minutes of allergen re-exposure. Mast cells get activated, release preformed mediators and enhance the synthesis of cytokines, chemokines and growth factors. The rapidly secreted inflammatory products promote bronchoconstriction, vasodilation, vascular permeability and mucus production. (*Picture adapted from Galli S.J. et al. IgE and mast cells in allergic disease. Nat Med 2012*).

Other products, including a diverse spectrum of cytokines, chemokines and growth factors, are produced by mast cells from new transcripts and therefore secreted over a period of hours after the initial mast cell activation. The newly synthesized mediators, such as type 2 cytokines (IL-4, IL-5, IL-13), IL-6, TNF-α, TGF-β, vascular endothelial growth factor (VEGF), GM-CSF and chemokines (CCL1, CCL2, CCL3, CCL4, CCL5, CCL17, CCL22 etc) are released in a slower rate and lead to the recruitment of Th2 cells, eosinophils, basophils, monocytes and neutrophils (106) (**figure 6**). Interestingly, almost half of the patients re-exposed to the inhaled allergen will experience within 4–6 hours the generation of the late phase allergic reaction (LAR), which is mainly based on the aforementioned recruitment of effector innate and adaptive immune cells and can last for several days (107) (**figure 6**). The exact mechanisms underlying the differences between the pathways leading to the dual response and those leading to the early allergic response alone are poorly understood. Emerging evidence suggests that, even thought the early recruitment of eosinophils is of known high impact on the initiation of LAR (108), complex interactions between tissue-resident and infiltrated immune cells are crucial for the progression of the procedure (109, 110). In addition, recent publications have shown that LAR can be associated with increased AHR to several non-allergic stimuli (107).

During LAR tissue-resident cells as well as recruited immune cells release various mediators that influence the biology of structural cells, including vascular endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and nerve cells. For instance, elastase released by neutrophils promotes the activation of matrix metalloproteinases (MMPs) and the degradation of type III collagen (111), while basic proteins secreted by eosinophils (eosinophilic cationic protein, major basic protein etc) can injure epithelial cells (112) (**figure 6**). In addition, type 2 cytokines produced by effector Th2 cells further promote vascular permeability, goblet cell metaplasia, bronchoconstriction and AHR (113). As a result, accelerated inflammation, marked vascular permeability and oedema, prolonged airway narrowing, sustained smooth muscle cell contraction and enhanced mucus production contribute to the establishment of the characteristic clinical features of allergic asthma (114) (**figure 6**).

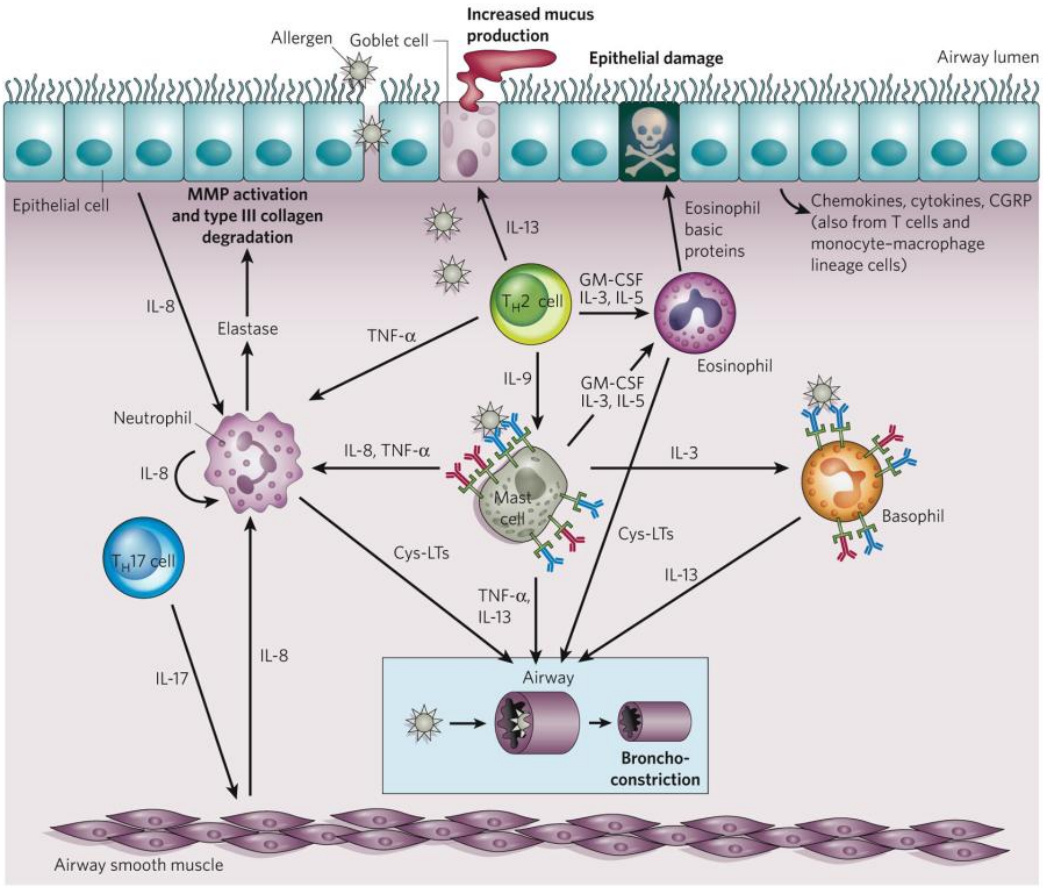

Figure 6. Late phase allergic response. LAR typically occurs 4-6 hours following allergen inhalation and reflects the actions of innate and adaptive immune cells that have been recruited from the circulation, as well as the secretion of inflammatory mediators by tissue-resident cells. Activated mast cells by IgE- and allergen-dependent FcεRI aggregation secrete several inflammatory mediators. Basic proteins released by activated eosinophils damage epithelial cells and elastase released by neutrophils promotes the degradation of type III collagen through MMPs activation. Cytokines and chemokines secreted by tissue cells and Th2 cells further promote inflammatory cell infiltration and enhance mucus production and AHR. (*Picture adapted from Galli SJ et al. The development of allergic inflammation. Nature 2008*).

Repetitive and/or persistent exposure to allergen gradually leads to the development of chronic allergic airway inflammation characterized by prolonged aberrant innate and adaptive immunological responses and recurrent injuries of the tissue-resident cells (114). Every tissue of the human body has the capacity of restoration to the physiological architecture following damage. Although a quiescent organ at homeostatic conditions,

human lung possesses a significant reparative capacity in response to injury (115). Irregular or defective repair mechanisms, termed remodelling, are increasingly linked to the pathophysiology of several airway diseases including allergic asthma (116). Indeed, airway remodelling is established as a central pathogenetic procedure taking place in the airways of individuals with asthma and is often associated with more severe phenotypes of the disease and refractoriness to treatment (117). Still, the precise underlying mechanisms that control airway remodelling remain poorly understood.

For many years remodelling of the asthmatic airways was largely considered to be a consequence of chronic tissue inflammation, a hypothesis recently challenged by several studies conducted in children even before the clinical diagnosis of allergic asthma, depicting that airway wall remodelling often occurs prior to the detection of any inflammation (118 – 120). Moreover, in bronchial specimens of children with moderate and severe asthma aberrant structural cell activation and thickened reticular basement membrane (RBM) are evident in the absence of significant eosinophilic inflammation (121). In agreement, emerging evidence has shown that in adult asthma, airway wall remodelling is independent of inflammation and occurs following repeated challenges of volunteers with allergens or methacholine (122), pointing to frequent constriction of the tissue as a key factor for the development of remodelling. Interestingly, early epigenetic events condition the lung to develop chronic inflammatory diseases later in life, while childhood infections, genetic and environmental factors, especially air pollution, affect lung function and render the airways vulnerable to disease generation (123, 124). Hence, it is supported that central for the generation of remodelling processes is an intrinsic propensity of the asthmatic airways for damage and irregular restoration, which gets exacerbated by allergen-driven aberrant immune responses (125).

In homeostatic conditions, the epithelium layer of the trachea and the conducting airways is arranged predominantly as a pseudostratified layer, with a variety of cells including, ciliated, goblet, neuroendocrine, tuft and basal cells that are adherent to the basement membrane (BM) (126). BM is composed of the lamina densa, on which the epithelial cells are attached to, an upper layer called the basal lamina (true basement membrane) and the RBM derived from the attenuated fibroblast sheath located below (127). The lamina propria is situated underneath BM and is constituted by several

mesenchymal cells such as fibroblasts, endothelial cells, and airway smooth muscle (ASM) cells and by the extracellular matrix (ECM) proteins, such as polysaccharides, collagens, fibronectin and water (128). Importantly, ECM is pivotal for tissue structure, cell migration, proliferation, viability, and morphology (129) and the balance between the expression of MMPs and their inhibitors (TIMP) is crucial for the transition of its structure (130).

Since 1999 it has been proposed that communication between the airway epithelial and mesenchymal cells, especially attenuated fibroblasts, termed epithelial-mesenchymal trophic unit (EMTU) plays a major role in lung development, repair and homeostasis (131). Accumulating evidence suggests that abnormal activation of the EMTU pathway in response to chronic mucosal injury is fundamental for the development of airway wall remodelling (132) and may, at least partly, explain the identification of remodelling processes in bronchial biopsies of children in the absence of inflammation. Mechanistically, following tissue damage, epithelial cells get activated and release several mediators, including insulin-like growth factor (IGF)-1 (133), endothelin (ET) -1 (133, 134), MMPs (especially MMP9) (135), VEGF (136), TGF-β superfamily members (133, 137, 138), IL-13 (138) and osteopontin (139) all of which activate the underlying mesenchymal cell unit and, ultimately lead to RBM thickening, smooth muscle hyperplasia, myofibroblasts proliferation and subepithelial fibrosis. In addition, induction of the EGF receptor (EGFR) expression in both injured and intact epithelial cells suggests a spreading of the epithelial damage and impaired repair procedures (140).

In healthy individuals, inflammation is appropriately resolved and the architecture of the airway wall is properly restored, through the development of a plethora of physiological procedures, such as proliferation and self-renewal of epithelial cells, reconstitution of the impaired ECM components, clearance of activated immune cells, autophagy and production of repair-promoting mediators, including lipoxins, resolvins and protectins (125, 141). On the other hand, in individuals with asthma both the resolution of inflammation and the tissue repair processes are severely defective and histopathological studies of airway remodelling in human bronchial biopsies have shown alterations in the airway epithelium (loss of epithelial barrier function, goblet cell hyperplasia), the ECM (thickening of the basement membrane and subepithelial fibrosis) and the mesenchyme (ASM hypertrophy

and hyperplasia, fibroblasts to myofibroblasts transformation and angiogenesis) (142) (**figure 7**).

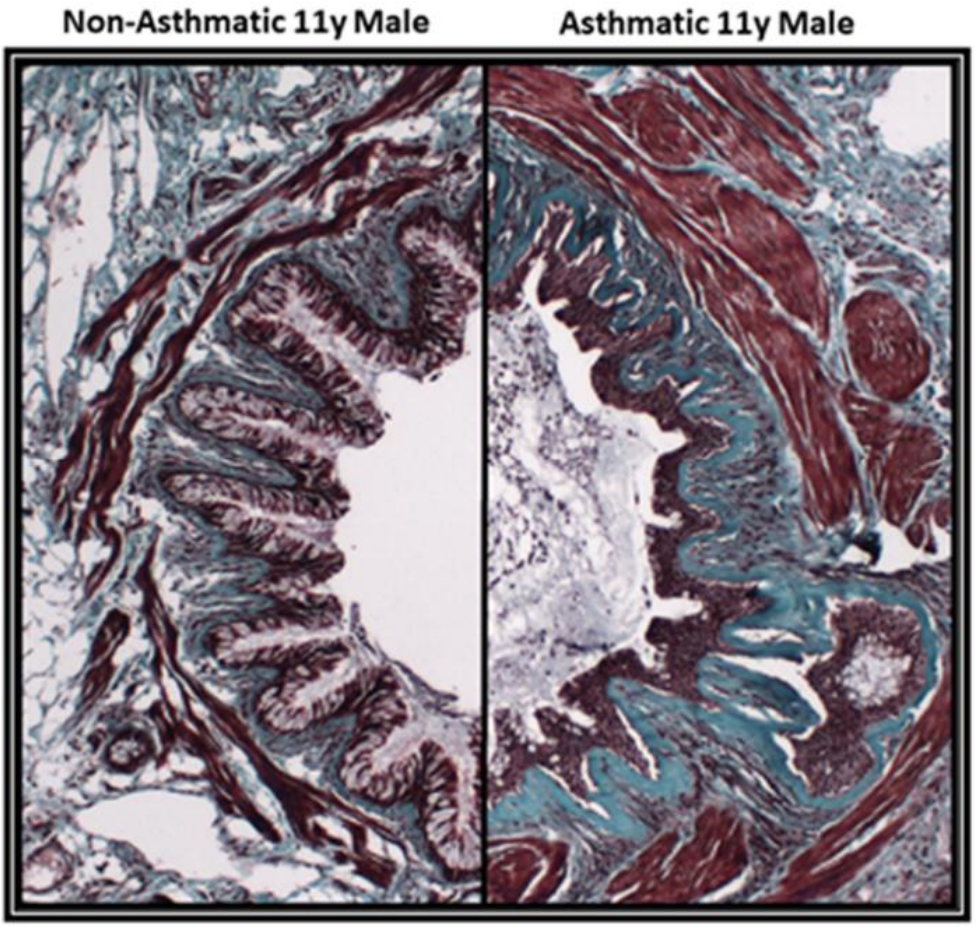

Figure 7. Features of airway wall remodelling. Bronchial sections of a large airway from a normal control individual (left) and an age- and sex-matched large airway from an asthmatic individual (right). Depicted characteristics of wall remodelling are increased smooth muscle mass, damaged airway epithelium, basement membrane thickening, mucus plugging of the airway lumen and subepithelial fibrosis. (*Picture adapted from Osei E.T., et al. What Have In Vitro Co-Culture Models Taught Us about the Contribution of Epithelial-Mesenchymal Interactions to Airway Inflammation and Remodelling in Asthma? Cells 2020*).

Sustained Th2-mediated inflammation has been shown to cause goblet cell metaplasia, RBM thickening and AHR in transgenic mice overexpressing the type 2 cytokines IL-4, IL-5 and IL-9 in the airways (143, 144), while IL-4 and IL-13 induced

transformation of fibroblasts into myofibroblasts and increased production of type III collagen (145). Furthermore, in humans increased expression of IL-13 has been associated with epithelial dysfunction and enhanced release of mucus (146). Osteopontin is another cytokine with elevated expression in the asthmatic airway which is implicated in fibroblast and ASM proliferation (147). In addition, TGF-β superfamily members, activin-A, TGF-β1 and bone morphogenetic proteins (BMPs) are well recognized mediators of airway wall remodelling processes, including epithelial cell proliferation, subepithelial fibrosis and ASM hypertrophy (148, 149). Still, activin-A and TGF-β can also ameliorate tissue remodelling pathways through direct suppression and/or indirect regulation of allergic airway inflammation. In fact, recent studies by our group have revealed that activin-A inhibits VEGF-induced proliferation and cytokine secretion by human pulmonary endothelial cells through IL-18- and VEGFR1- dependent pathways, pointing to this cytokine as a novel anti-angiogenic factor for human asthma (138).

Except from the contribution of cytokines, allergen-driven IgE formation also promotes airway wall remodelling indirectly, through its significant implication in the initiation and progression of the inflammatory cascade, but also through direct mechanisms. More specifically, soluble IgE binds to both low- and high- affinity IgE receptors expressed on human ASM leading to their contraction and release of CCL-11, IL-4, IL-5 and IL-13 (150). Furthermore, IgE-activated mast cells produce several mediators, such as VEGF (151), TGF-β1 (152) that contribute to airway wall remodelling. Eosinophils also produce and express many fibrogenic factors, particularly TGF-β1 (153) and ablation of eosinophils in mice conferred protection against peribronchiolar collagen deposition and increases in airway smooth muscle following chronic allergen exposure (154). In concordance, anti-IL-5 treatment of individuals with allergic asthma using the monoclonal antibody mepolizumab resulted in a reduction in airway eosinophils and decreased expression of eosinophil-derived TGF-β1 accompanied by reduced expression of tenascin, lumican, and procollagen III in the bronchial mucosal RBM (155). Therefore, it is conceivable that complex and diverse pathways, including allergen-driven aberrant immunological responses and EMTU interactions, act coordinately in order to establish remodelling in the airways of individuals with asthma and further investigation is essential to provide better insights on the precise mechanisms.

1.3 Regulation of Allergic Responses

1.3.1 Regulatory T Cell Subsets and Functions

Immunological tolerance towards self and innocuous environmental antigens represents one of the most crucial factors for the maintenance of homeostasis in every leaving organism (156). Productive immune responses against foreign pathogenic antigens and cancerous cells are generated in order to protect individuals against infections and tumor development. On the same time, a complex network of non-redundant mechanisms is utilized by the immune system to avoid the mounting of detrimental responses against self and foreign harmless antigens, thus preserving the integrity of the individual's own tissues (156). Central tolerance develops during the maturation of T lymphocytes in the thymus and is achieved by the clonal deletion/apoptosis of autoreactive thymocytes through negative selection. In the cortex of the thymus immature $CD4^+CD8^+$ double-positive (DP) T cells whose TCRs recognize self-peptide-self-MHC complexes too strongly undergo clonal deletion whereas DP T cells that recognize low affinity self peptide–MHC complexes are positively selected in order to differentiate to $CD4^+$ or $CD8^+$ single-positive (SP) cells and migrate to the medulla of the thymus (157) (**Figure 8**). In the thymic medulla weak TCR stimulation by a self antigen results in the maturation of SP cells into conventional T cells (Tconv) while strong and persistent TCR stimulation by a self antigen leads to the apoptosis of SP T cells. Intriguingly, high-affinity but transient TCR- self-peptide-self-MHC complexes interactions create signals that promote the differentiation of SP T cells into the forkhead box protein 3 (Foxp3)-expressing Treg lineage (157, 158), resulting in a Treg repertoire skewed toward self-recognition (thymus-derived Tregs/tTregs).

On the other hand, tolerance can also be induced in the periphery once the mature SP T lymphocytes have exited the thymus. Tconv cells that exit the thymus circulate as naive T cells in the bloodstream and peripheral lymphoid tissues where they can recognize agonist antigens. The presentation of an agonist antigen to naive T cells can lead to distinct T cell fates mainly depending on the nature of the antigen (type and dose), the type of the antigen presenting cell and the cytokine milieu. Thus, naive T cells may undergo clonal deletion or may differentiate into mature Teff cells or into peripherally derived Treg

(pTreg) cells (157). Peripheral tolerance mechanisms include the immunological ignorance, the induction of anergy (functional unresponsiveness), the deletion of T lymphocytes from the T cell repertoire following activation and the suppression of aberrant immune responses by regulatory cell subsets, predominantly Tregs (159, 160) (**Figure 8**).

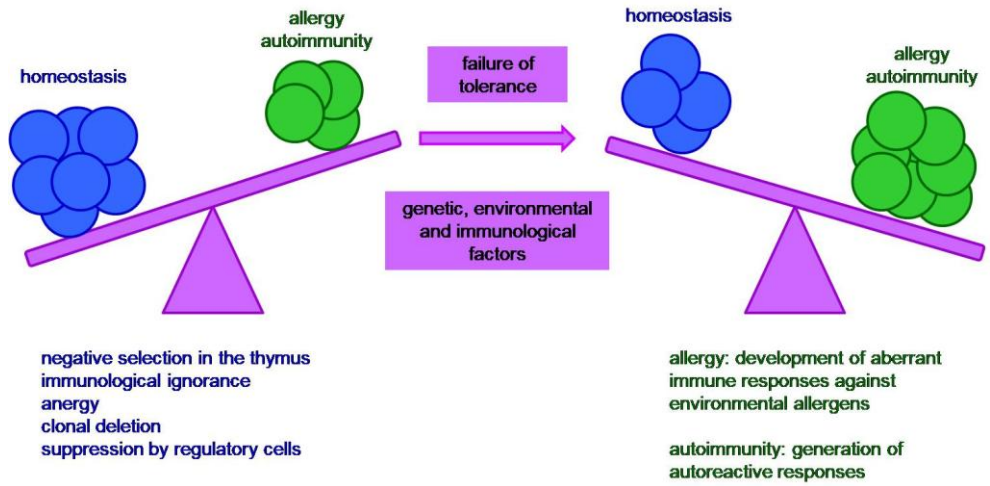

Figure 8. Immunological tolerance. The immune system utilizes multiple mechanisms in order to generate protective immune responses against foreign pathogenic antigens and cancerous cells without mounting detrimental responses against self and foreign harmless antigens. However, the impairment of these mechanisms due to environmental, genetic and immunological factors can lead to the establishment of allergic and/or autoimmune diseases.

The first study which referred to Treg cells as “cells with suppressive capacity deriving from the thymus” was published on 1970 by Gershon and Kondo (161). Unfortunately, investigation on the field of these “suppressor cells” was abandoned for 25 years, mainly due to the lack of markers for their specific identification which would allow their characterization. In 1995, Shimon Sakaguchi et al published an innovative study which reinstated the interest on Treg cells. They introduced IL-2 receptor α chain/CD25 as a molecule preferentially expressed by approximately 10% of $CD4^+$ T cells obtained from the peripheral blood of normal, adult, unimmunized mice (162). $CD4^+CD25^+$ cells exhibited strong suppressive capacity against autoimmune responses and the adoptive transfer of $CD4^+CD25^-$ T cells into athymic nude BALB/c mice resulted in the development of spontaneous multi-organ autoimmune disorders, such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis (162). Of note,

reconstitution of $CD4^+CD25^+$ T cells 10 days following transfer of $CD4^+CD25^-$ T cells suppressed these diseases (162).

Since 1995 Treg cells have been extensively scrutinized and proven to be dedicated suppressor cells which control immunological self-tolerance and immune responses to pathogens, allergens and tumor antigens (163). However, there was still a problem for immunologists in the thorough study of Tregs, since CD25 is also expressed by activated Teff cells and therefore could not be considered as a discriminating marker for Treg cells (164). In 2003, two independent elegant studies by the groups of Sakaguchi and Rudensky identified transcription factor Foxp3 as a bona fide marker of Treg cells that develop in the thymus. They revealed that expression of Foxp3 is essential and sufficient for regulatory function and ectopic transduction of the *FOXP3* gene into conventional T cells induces them to transform into suppressive Treg cells (165, 166). Scurfy mice, which lack the transcription factor Foxp3 are deficient in mature, thymus-derived Treg cells and as a result develop a severe lymphoproliferative autoimmune disease (167). Equivalent in humans, mutations in the *FOXP3* gene give rise to immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, a life-threatening autoimmune disorder (168). Following the discovery of Foxp3 as the master transcription factor for the generation of tTregs, a vast plethora of studies investigated comprehensively the phenotype and function of these cells. tTreg cells, which account for 5%–10% of mature human or mouse $CD4^+$ T cells, express CD4, Foxp3, high levels of CD25 and low levels of CD127/IL-7R α chain (169) and predominantly induce immune tolerance to autoantigens. Still, intensive study has revealed that tTregs are also important in regulating many other immune responses, including responses to pathogenic agents (170), gut microbiota (171), environmental allergens and food (172), tumors (173), and transplanted tissues (174).

In contrast to murine $Foxp3^+$ Treg cells, recent studies imply that human $Foxp3^+$ cells are not phenotypically and functionally homogenous. In fact, Sakaguchi's group suggested a separation of human $CD4^+CD25^+Foxp3^+$ cells based on the expression of CD45RA. The proposed subpopulations were $CD45RA^+FoxP3^{lo}$ resting/naive Treg cells, $CD45RA^-FoxP3^{hi}$ activated/effector Treg cells both of which are potently suppressive and cytokine-secreting $CD45RA^-FoxP3^{lo}$ non-Treg cells, resembling memory/effector $CD4^+$ T cells (175). It is

worth noting that the two subpopulations of human regulatory $CD4^+CD25^+Foxp3^+$ T cells discriminated by the expression of CD45RA stand for distinct stages of Treg cell differentiation so that activated $CD45RA^-FoxP3^{hi}$ Tregs represent terminally differentiated Treg cells which rapidly die whereas resting $CD45RA^+FoxP3^{lo}$ Treg cells proliferate, upregulate Foxp3 expression and downregulate CD45RA expression following *in vitro* and/or *in vivo* stimulation (175). Interestingly, human $Foxp3^+$ Treg cells can also be subdivided into ICOS expressing cells that preferentially produce IL-10 and $ICOS^-$ Tregs that secrete only TGF-β (176). In addition, two surface proteins, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and FcR-like 3 (FcRL3) and the transcription factor Helios have been used together to distinguish human suppressive $TIGIT^+FcRL3^+Helios^+Foxp3^+$ Tregs from $TIGIT^-FcRL3^-Helios^-Foxp3^+$ non-suppressive cells (177).

It is now well-known that the term Treg cells includes a diverse group of cells with immunoregulatory properties. As mentioned above, $CD4^+Foxp3^+$ tTregs are selected in the thymus by transient, high avidity MHC-II-self-antigen-TCR interactions, whereas peripherally generated $CD4^+$ Treg cells/pTregs comprise both Foxp3 positive and negative populations developed in the peripheral lymphoid tissues and mainly regulate immune responses at the mucosal surfaces against non-self and external harmless antigens while they are also fundamental for the maintenance of tolerance during pregnancy (178 - 180). Peripheral induction of Treg cells could represent an important mechanism to generate Treg cells with specificity for exogenous antigens, such as allergens, as well as to maintain Treg cell populations with age. Generation of $CD4^+$ Treg cells from naive conventional $CD4^+Foxp3^-$ T cells following TCR stimulation in the periphery occurs under specific cytokine milieu, such as TGF-β and IL-2, and/or via DCs with immunoregulatory properties, called tolerogenic DCs. Interestingly, *in vitro* differentiated Treg cells, termed iTregs, represent another subset of Tregs, resembling pTregs, and their induction is also based on certain cytokines and naive $CD4^+$ T cells - tolerogenic DCs interactions (181). So far, the most well-investigated Tregs generated outside the thymus are $CD4^+Foxp3^-$ type 1 regulatory T cells (Tr1) that produce substantial levels of IL-10 (182, 183), the $CD4^+$ TGF-β-secreting Th3 cells, that under certain conditions can obtain Foxp3 expression (184, 185), the TGF-β-induced $CD4^+Foxp3^+$ iTregs (186) and the $CD4^+Foxp3^-$ Tregs generated under IL-35 presence (iT_R35), which release IL-35 and IL-10 (187).

In concordance with their phenotypic diversity, the suppressive functions of the different subsets of tTregs, pTregs and iTregs are various and multilevel. There are several proposed mechanisms with experimental support, but it is likely that no single mechanism is responsible for the full range of biological phenomena involving Tregs. Moreover, it seems that in different microenvironments distinct mechanisms and even alternative subsets of regulatory cells are involved in tuning the immune response and conferring tolerance (188). Accumulating evidence suggests that Treg cells exert their regulatory effects on several cell types, including $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, mast cells, eosinophils, basophils, neutrophils, NK cells, natural killer T cells (NKT) and DCs (188, 189).

The basic mechanisms of Treg-mediated immunosuppression can be broadly divided into those that target effector cells and those that primarily target antigen-presenting cells, predominantly DCs. More specifically, Treg cells may secrete suppressor soluble factors, such as IL-10 (190), TGF-β (191) and IL-35 (192) that can directly inhibit the function of responder T cells, myeloid cells and DCs (**figure 9**). Moreover, the functions of the ectoenzymes CD39 (ATP apyrase) and CD73 (ecto-5'- AMP-nucleotidase) expressed by $Foxp3^+$ Tregs result in the formation of adenosine and the activation of the adenosine receptor 2A expressed on Tcells, which mediates inhibitory signals, through cAMP, leading to Teff cell suppression (189, 193) (**figure 9**). In addition, Treg cells that express higher levels of CD25, the IL-2 receptor α subunit, have the capacity to compete with Teff cells for IL-2, a cytokine essential for the survival and proliferation of Teff cells, thus resulting in the induction of Teff cell apoptosis (194) (**figure 9**). Furthermore, Treg cells can directly kill Teff cells through expression of granzymes in a perforin-dependent manner (189, 195) (**figure 9**). In addition, PD-L1 expressed by Tregs interacts with PD-1 expressed by activated Teff cells leading to anergy or even inducing the generation of IL-10-secreting pTregs (188). Activated Treg cells may also upregulate the expression of galectin-1 on their cell surface that can interact with several receptors, such as CD45, CD43 and CD7 on Teff cells leading to cell cycle arrest, apoptosis and inhibition of production of proinflammatory cytokines (196). Activation of Treg is also involved in the upregulation of TRAIL (TNFrelated apoptosis-inducing ligand) molecule expression that binds to death receptor 5 (DR5) expressed by Teff cells inducing their apoptosis via caspase-8 (188).

Besides activation state it is worth noting that Treg subsets expressing transcription factors typical of Teff cell populations may be associated with increased suppressive capacities against them. Thus, in relation to Th2 subpopulation, the Th2-associated transcription factor interferon regulatory factor 4 (IRF4) expressed by Tregs enables them to upregulate ICOS and CTLA-4 molecules which are crucial in limiting aberrant Th2-mediated immune responses (188). Importantly, ICOS expression on Tregs is of great importance since interaction with its ligand ICOS-L enables Tregs survival and efficient suppression (188). A recently described mechanism of Treg-mediated suppression of Teff cell function is the release of extracellular vesicles (EVs) which are cell-derived membranous structures mediating protein, lipid, and genetic material exchange, mediating intercellular communication (189) (**figure 9**). Following TCR stimulation Tregs release EVs which promote the generation of a tolerogenic milieu in a cell-free manner (**figure 9**) and some of the proposed mechanisms are the transmission of enzymes, miRNAs-induced gene silencing (miR-155, Let-7b, Let-7d) and the action of surface proteins. Interestingly, murine Treg-derived EVs have been shown not only to reduce $CD4^+$ Teff cell activation and cytokine release but also to upregulate IL-10 secretion by murine DCs, pointing to an additional role for EVs in Treg-mediated DC suppression (189).

Regarding the suppressive functions of Tregs on DCs, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on the surface of Treg cells downregulates or prevents the upregulation of CD80 and CD86 and the MHC molecules on DCs, resulting in impaired antigen presentation (197) (**figure 9**). In addition, CTLA-4 expressed by Treg cells interacts with CD80- and CD86- expressing DCs to condition them to upregulate indoleamine 2,3-dioxygenase (IDO), which is a potent regulatory molecule that induces the catabolism of the essential for T cell protein synthesis amino acid tryptophan into kynurenine resulting in the inhibition of activation of Teff cells (198) (**figure 9**). Besides CTLA-4, Treg PD-1 and DC PD-L1 interaction inhibits DC maturation and promotes tolerogenic DC generation (188). Another cell surface molecule that plays a role in Treg-mediated suppression of DC function is lymphocyte-activation gene 3 (LAG-3/CD223), a CD4 homolog that binds MHC class II molecules expressed by immature DCs and suppresses DC maturation (199) (**figure 9**). Neuropilin (Nrp-1) is a receptor for class III semaphorins and a coreceptor for VEGF which is preferentially expressed on Treg cells and promotes long interactions between Tregs and immature DCs resulting in restriction of

DCs access to Teff cells (200). Furthermore, the interaction of TIGIT surface molecule, highly expressed on Tregs, with its coreceptor CD155 expressed on DCs induces the later to secrete the inhibitory cytokines IL-10 and TGF-β further promoting Treg-mediated suppression (188, 201).

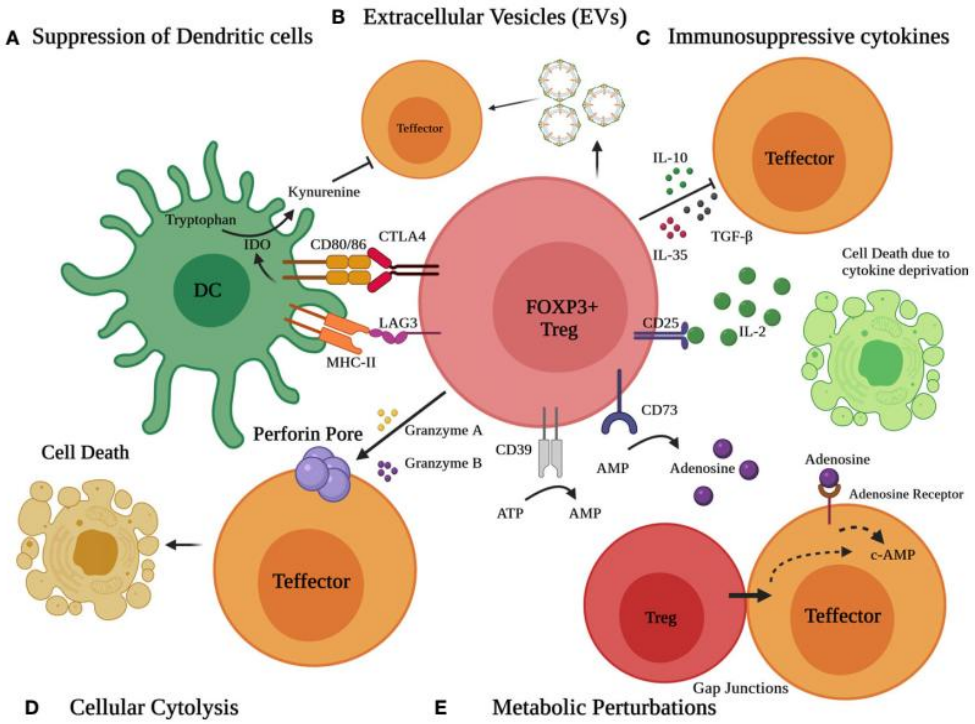

Figure 9. Basic mechanisms of suppressive function mediated by Treg cells. (**A**) Regulation of DCs. **(B)** Release of extracellular vehicles. **(C)** Production of immunoregulatory cytokines. **(D)** Induction of cellular cytolysis. **(E)** Metabolic disruption pathways. *(Picture adapted from Payal Grover, et al. Regulatory T Cells: Regulation of Identity and Function. Front Immunol. 2021).*

1.3.2 Regulation of Allergic Airway Inflammation by Regulatory CD4$^+$ T Cells

The lung represents a unique mucosal environment, which is specialized to meet the requirements for its primary function of gaseous exchange. An equivalent requisite of the pulmonary tract is to maintain tolerance in the face of continuous exposure to potential antigens. Exposal to airborne antigens, many of which, although immunogenic, do not represent a threat to the host, like allergens, stimulates the generation and activation of antigen-specific effector/memory Th2 cells in genetically predisposed individuals. In order

to avoid the persistent induction of immune responses to allergens which would cause chronic inflammation, particular tolerogenic mechanisms exist to facilitate the discrimination between innocuous antigens and pathogens and the suppression of detrimental immune responses. Accumulating evidence supports that $CD4^+$ Treg cells play pivotal role in the suppression of dysregulated immune responses towards inhaled allergens and are essential for the maintenance of respiratory homeostasis (202).

Mouse models of allergic airway disease have long been used to dissect the immunological mechanisms underlying the pathophysiological features of asthma. Early studies have highlighted the fact that antigens entering via the respiratory route generally induce tolerance or weak Th2 cell responses. In the early 1980s, Holt et al. demonstrated that exposure of mice to aerosolized ovalbumin (OVA) intranasally in the absence of adjuvant elicited transient IgE responses, which subsequently declined (203). When the animals were challenged intraperitoneally (i.p.) with OVA, their IgE responses were markedly suppressed relative to controls. Authors postulated a role for relatively long-lived antigen-specific suppressor cells that could transfer inhibition of the IgE response to other animals (203). More recently, tolerance induction in the airways in animal models has been specifically correlated with the induction of $CD4^+$ Tregs. Repeated exposure of mice to low-dose allergen promoted the development of a regulatory $CD4^+$ population that expressed membrane-bound TGF-β and Foxp3. Adoptive transfer of these cells to naive mice prevented allergen sensitization (204). A higher dose of inhaled allergen stimulated the development of a Treg population that produced copious amounts of IL-10 and blocked the development of AHR when they were adoptively transferred into sensitized mice (205). Interestingly, mature pulmonary DCs were implicated in the induction of these Tregs through ICOS-ICOSL interactions (205). In agreement, adoptive transfer of OVA-specific $CD4^+CD25^+$ Tregs to OVA-sensitized mice was found to suppress allergic airway inflammation and AHR through a mechanism dependent upon IL-10 (206). When delivered after the onset of disease, $CD4^+CD25^+$ Tregs were able to downregulate established inflammation and prevent airway remodelling processes (207). Conversely, depletion of $CD4^+CD25^+$ Treg cells before allergen sensitization enhanced the severity of lung inflammation and AHR through induction of airway DCs maturation and enhanced potential to promote Teff cell proliferation (208). Interestingly, intratracheal administration of lung $CD4^+CD25^+$ T cells prior to allergen challenge of sensitized mice suppressed allergic

airway inflammation and AHR and led to decreased Th2 cytokine secretion, higher levels of IL-10 and TGF-β and less severe lung histopathology (209). Pointing again to a significant role for IL-10 in the induction of airway tolerance, when $CD4^+CD25^+$ T cells were isolated from the lungs of IL-$10^{-/-}$ mice were not able to exert the previously described regulatory functions (209).

Even though airway Treg cells do not need to be antigen specific for *in vivo* suppression (210), they appear rapidly after allergen exposure (211). In fact, during chronic exposure of sensitized rats to allergen aerosols, $CD4^+CD25^+Foxp3^+LAG3^+CTLA^+$ $CD45RC^+$ Treg cells appeared in the airway mucosa and regional lymph nodes within 24 hours of initiation of exposal (211). These Tregs prevented Teff cell activation and AHR and their maintenance was absolutely dependent on continuing allergen stimulation (211). On the other hand, iTreg cell adoptive transfer into mouse models of experimental asthma has also been proved to confer protection against cardinal features of the disease. More specifically, polyclonal TGF-β-induced $CD4^+FoxP3^+$ Treg cells adoptively transferred to OVA-sensitized mice prior to intranasal OVA challenge significantly downregulated AHR, eosinophil recruitment, mucus production, IgE levels and airway remodelling, while adoptive transfer during allergen challenge suppressed airway inflammation and improved lung function (212). In concordance, OVA-specific $CD4^+$ T cells genetically engineered to express IL-10 abolished AHR and airway eosinophilia in BALB/c mice sensitized and challenged with OVA and in severe combined immunodeficient (SCID) mice reconstituted with OVA-specific Th2 effector cells (213). Interestingly, neutralization of IL-10 reversed the suppressive effects of these OVA-specific $CD4^+$ T cells (213). Another study depicting the importance of iTregs showed that iTreg cells, but not tTreg cells, effectively suppressed the ILC2-driven production of IL-5 and IL-13 both *in vitro* and *in vivo* through IL-10 and TGF-β secretion and ICOS-ICOSL interactions between iTregs and ILC2s (214). Interestingly, Tregs except from ILC2s can also downregulate other innate immune cells important for the development of allergic airway inflammation. For instance, $CD4^+CD25^+$ Tregs directly inhibit the FcεRI-dependent degranulation of mast cells through cell-cell contact involving OX40-OX40L interactions between Tregs and mast cells, respectively (215). In addition, it seems that IL-10 secreted by allergen-specific Tr1 cells is important for the inhibition of eosinophilic activation and release of IgE by B cells (216).

The aforementioned studies highlight a significant role for tTregs, pTregs and iTregs in the control of allergic airway inflammation and most of them indicate IL-10 as an essential mediator for maintenance of airway immune tolerance. In contrast to TGF-β which seems to exert both pro- and anti-inflammatory effects on allergic responses depending on the cytokine milieu and the characteristics of the effector cells involved (217), IL-10 plays a fundamental and non-redundant role in the induction of immune tolerance. A wide range of cells can produce IL-10, including T cells, B cells, macrophages, DCs, mast cells, and eosinophils (218). In concordance, IL-10 has broad immunosuppressive functions and modulates the activity of several cell subsets involved in allergic reactions, such as mast cells (219), eosinophils (220), Th2 cells (221) and DCs (222) (**figure 10**). Furthermore, IL-10 inhibits IgE and promotes IgG4 production, an immunoglobulin isotope believed to be protective in the context of allergic diseases (223) (**figure 10**). Although IL-10 is secreted by Th2 cells, it has been shown that ablation of IL-10 signalling in Th2 cells augments Th2 survival and exacerbates allergic airway inflammation in a house dust mite murine model, via a granzyme B-dependent pathway, implicating IL-10 in a negative feedback loop of Th2-self regulation (224). In fact, although IL-10 is not required for control of systemic autoimmunity, it has been depicted that it is absolutely required for restraint of deviant immune responses at mucosal surfaces such as gut or lung and Treg-specific deletion of IL-10 promotes allergic airway inflammation (225). Moreover, IL-10 gene transfer to the airways suppresses OVA-induced cellular recruitment and airway inflammation in mice in a dose-dependent manner (226), while intranasal instillation of IL-10 concurrent with OVA challenge inhibits leukocyte recruitment (227).

Studies conducted in humans have also suggested a crucial role for IL-10 in allergic airway inflammation and linked disease. IL-10 expression is decreased in the airways of severe asthmatics and certain genetic polymorphisms in the *IL10* promoter are associated with more severe asthma phenotypes (218). In agreement, there is a substantial increase in the frequency of allergen-specific IL-10-producing T cells in the peripheral blood of healthy, non atopic individuals as compared to allergic patients, who demonstrated reduced IL-10^+ and increased IL-4^+ allergen-responsive T cells (228). Conversely, natural immune tolerance in non atopic individuals, specifically beekeepers in whom seasonal exposure to bee stings is important to maintain tolerance is associated with increased venom allergen-specific $CD4^+$ IL-10-producing T cells (229).

Based on these studies of human IL-10-producing regulatory subsets and pertinent to the significance of IL-10 in the suppression of aberrant allergen-driven immune responses, it is conceivable to assume that Tr1 cells represent a Treg subpopulation fundamental for the maintenance of respiratory tolerance. Tr1 cells are generated in the periphery following antigenic stimulation in the presence of IL-10 (**figure 10)**, lack Foxp3 expression and produce copious amounts of IL-10 while also secreting TGF-β, some IL-5, IFN-γ, low levels of IL-2, but not IL-4 (182, 230). To date no specific transcription factor has been defined as master regulator of Tr1 cell differentiation. Both human and murine memory Tr1 cells have been shown to coexpress the surface molecules CD4, LAG3 and CD49b (231, 232). Even though LAG3 and CD49b can be expressed by other activated and memory T cell populations it seems that when $CD4^{+}LAG3^{+}CD49b^{+}$ memory T cells are purified, the cytokine secretion panel and the suppressive effects expected of Tr1 cells are confined to this population (232). Additional surface markers found on Tr1 cells are PD-1, CTLA-4, ICOS, TIGIT, T-cell immunoglobulin and mucin domain-3 (TIM-3), CD226, CD73 and CD39 (232). The principal mechanism of immune regulation mediated by Tr1 cells is the secretion of IL-10 which inhibits Teff proliferation and pro-inflammatory cytokine production by DCs (182, 231, 232).

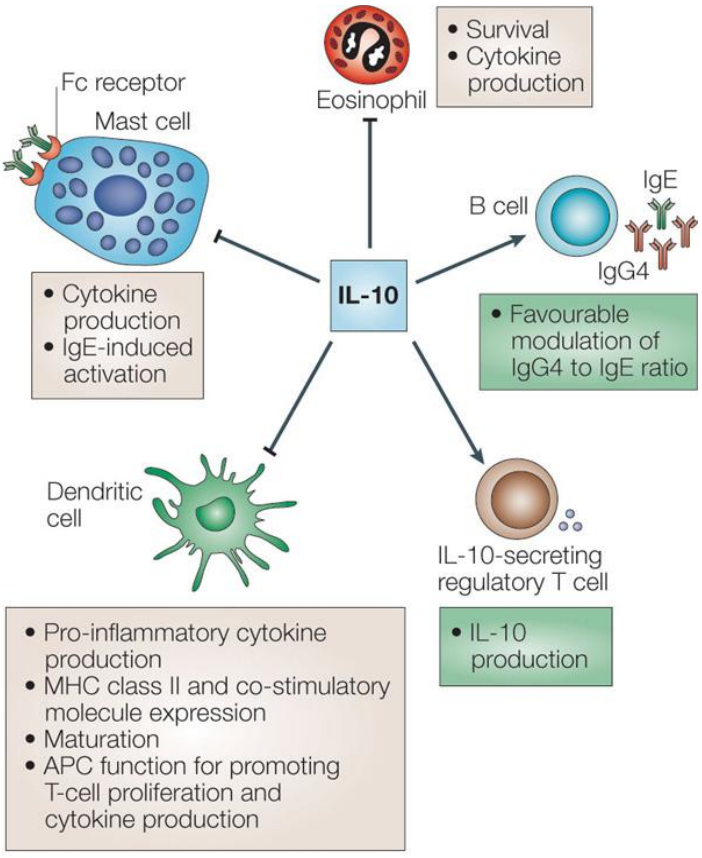

Figure 10. Functions of IL-10. IL-10 is a pleiotropic immunoregulatory cytokine that restrains activation and cytokine secretion by mast cells and suppresses cytokine production and survival of eosinophils. Moreover, IL-10 inhibits the maturation of DCs and the activation of Th2 cells. Furthermore, IL-10 enhances immunoglobulin iGg4 isotype switching in B cells and promotes the induction of Tr1 cells in the periphery. *(Picture adapted from Hawrylowicz C.M. et al. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol. 2005).*

Even though a vast plethora of studies has underlined the central role of Treg cells in the maintenance of immune tolerance in the airways, emerging evidence suggests that under certain inflammatory conditions Tregs obtain the phenotypic and immune characteristics of Teff cells and are no longer immunosuppressive (233). Interestingly, recurrent infections with RSV of infant mice tolerized to OVA through their mother's milk induced allergic airway disease in response to OVA as compared to uninfected tolerized control mice (234). RSV infection induced GATA-3 expression and Th2 cytokine production in $Foxp3^+$ Tregs and compromised their suppressive function (234), unrevealing a possible mechanism through which early viral infections render the airways susceptible to the later development of allergic asthma. This Treg plasticity and instability is one the greatest obstacles encountered in the clinical application of Treg cells, and its underlying mechanism is not fully elucidated. This process seems to be controllable under homeostatic conditions, whereas in chronic inflammation leads to the transition of Tregs into pathogenic Teff cells, such as Th17 (235). Furthermore, mice with enhanced IL-4R signalling exhibit signal transducer and activator of transcription (STAT) 6-dependent impaired generation and function of mucosal allergen-specific Treg cells through acquisition by Treg cells of a Th2-like phenotype (236).

Besides the instability of Treg cells under certain inflammatory conditions, a plethora of studies implies that Treg cell number and functions are severely impaired in individuals with allergic asthma, further supporting a fundamental role for these $CD4^+$ T cells in the suppression of allergic responses. More specifically, it has been reported that patients with mild asthma have fewer $CD4^+CD25^{high}Foxp3^+$ Tregs in the peripheral blood than non-asthma normal individuals (237). Another study has shown that children with allergic asthma have fewer $Foxp3^+$ Tregs than normal children in their lungs, the active disease site (238). Moreover, it has been shown that circulating $CD4^+CD25^+$ Tregs from individuals with hay fever have a reduced ability to inhibit proliferation and IL-5 release by

$CD4^+CD25^-$ Teff cells when compared to healthy non atopic individuals (239). Interestingly, in Dermatophagoides pteronyssinus (Der p)-sensitive asthmatic children the percentage of $CD4^+CD25^{high}$ Treg in peripheral blood was increased but the expression of Foxp3 and its cell-induced suppressive activity were significantly lower when compared to non atopic controls (240). Notably, the expression of Foxp3 and the functional activity of $CD4^+CD25^{high}$ Treg cells were reversed in allergic asthmatics who received AIT (240). In addition, Foxp3 protein expression within $CD4^+CD25^{high}$ Tregs has been found significantly decreased in asthmatic patients versus healthy individuals (241). Another interesting study has depicted that $CD4^+CD25^{high}CD127^{lo}$ Tregs obtained from individuals with allergic asthma show decreased chemotactic responses, specifically to CCL1, in comparison with their healthy control counterparts (242). In addition, decreased CCL1-mediated chemotaxis significantly correlated with asthma severity and decreased pulmonary function in asthmatic patients (242). Importantly, $CD4^+CD25^{high}Foxp3^+$ Treg cells play a prominent role in the prevention of allergic sensitization in early life, as proven by the severe allergic manifestations of children with IPEX syndrome (168).

Collectively, accumulating evidence reveals that Treg cells are impaired in patients with allergic asthma. Of great clinical importance, early-phase clinical trials of Treg cell transfusion therapies have shown feasibility, tolerability and potential efficacy in many disease settings, such as graft-versus-host disease (GVHD) prophylaxis, type I diabetes, and organ transplantation (243). Interestingly, several studies have shown that the transfusion of antigen specific Tregs has more efficacious advantages than the transfusion of polyclonal Tregs and can accurately act on the lesion site (244). To date, no study of transfusion of Tregs in asthma has been conducted. Nevertheless, in a clinical trial, the researchers transfused *in vitro* expanded OVA-specific iTregs into 20 patients with refractory Crohn's disease. These patients generated good tolerance, which had a dose-dependent relationship with the transfusion of OVA-specific iTregs (245), indicating that the transfusion of antigen-specific Tregs may also play important roles in the prevention and control of asthma. Still, generation of stable antigen-specific Tregs and transfusion technique face many difficulties, such as the small number of Tregs that can be collected in the peripheral blood and the lack of reference standards for *in vitro* expansion and transfusion mechanisms. Further investigation may pave the way for the utilization of allergen-specific iTreg cell transfusion therapy for allergic asthma.

1.4 Activin-A

1.4.1 Structure and Signalling Pathway of Activin-A

One of the most well characterized cytokine families that exhibit fundamental roles in immune regulation is the TGF-β superfamily. This superfamily consists of more than 45 members in humans, such as activins, inhibins, myostatin, BMPs, growth and differentiation factors (GDFs) and nodal (246). Activins are found in either homodimer or heterodimer forms that mainly consist of a combination of βA or/and βB subunits, linked with disulfide bonds (**figure 11A**) (247). There are three functional isoforms of activins: activin-A (βAβA), activin-B (βBβB) and activin-AB (βAβB) (247). In addition, the βC and βE subunits have been identified in mammals and the βB in Xenopus laevis (248). 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 (249). 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 proteins. Transcripts of the βA and the βB subunits are detectable in nearly all tissues with high expression in reproductive organs, while βC and βE subunits are mainly expressed in the liver (248). Activin-A (approximately 25kDa) is the most investigated ligand and extensive research over the past decades unraveled activin-A as a significant mediator in fundamental biologic processes, including embryonic development, stem cell maintenance and differentiation, haematopoiesis, cell proliferation and tissue fibrosis (246, 247). Activin-B and –AB are bioactive, whose functions and expression patterns are yet not clearly defined.

Activin-A signalling is mediated by a heterotetrameric receptor complex consisting of two types of activin receptors, the type I [activin-like kinase (ALK)] receptors ALK2/ Activin receptor type IA, ALK4/ Activin receptor type IB and ALK7/Activin receptor type IC and the type II receptors (ActRIIA and ActRIIB) (247, 250) (**figure 11B**). Activin-A favors ALK4 binding while it shows lower affinity for ALK2 and ALK7. TGF-β1 signals through receptors distinct to activin-A receptors, while other members of this superfamily, such as nodal, GDF11 and myostatin, can, in certain context, use the same receptors with activin-A (251). Still, the *Acvr2b* gene (encoding ActRIIB) has been found to produce 4 alternatively spliced

transcripts that exhibit distinct binding affinities for activin-A and crystallography studies of the activin-A - ActRIIB receptor complex have depicted that activin-A exhibits a different binding pattern compared to the rest of the TGF-β superfamily members (252). ActRI and ActRII are homodimeric single-pass transmembrane serine/threonine kinase receptors that are structurally similar and contain a small cysteine-rich extracellular domain and an intracellular serine/threonine kinase domain (250). However, ActRI possesses a glycine and serine-rich domain near the intracellular juxtamembrane region which is important for intracellular signal transduction (250).

Initially, activin-A binds to the constitutively active homodimeric type II receptors and this induces the recruitment and phosphorylation of the homodimeric type I receptors, resulting in the formation of a heterotetrameric signalling complex (250) (**figure 11B**). Activated ActRI phosphorylates the mothers against decapentaplegic (Smad) proteins, the main intracellular mediators of activin-A signalling pathway (**figure 11B**) (253). Eight Smad proteins have been identified. The receptor-regulated Smads (R-Smads), Smad-1, Smad-2, Smad-3, Smad-5 and Smad-8, become phosphorylated and thus activated by the kinase domains of the receptors upon ligand binding (253). Smad-2 and Smad-3 are the phosphorylation substrates for TGF-β1 and activin-A type I receptors and following activation, they form a complex with the common mediator of all Smads (Co-Smad), the Smad-4 (253). Subsequently, this complex translocates to the nucleus facilitated by a nuclear localization signal of Smad-4, and in cooperation with several transcription co-factors, such as the cAMP response element-binding protein (CREB) binding protein (CBP)/p300 and the transforming growth interacting factor (TGIF), regulates the transcription of target genes (251) (**figure 11B**). Inhibitory Smads (I-Smads), which are the Smad-6 and Smad-7, are upregulated upon activin-A binding to its receptors and bind stably to the activated type I receptor, inhibiting further signal propagation, in an autocrine negative feedback loop (254). Recent studies have shown that activin-A apart from the canonical Smad-mediated signalling pathway, can activate other intracellular signalling pathways including mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK), p38 and c-Jun N-terminal kinase, JNK (255) (**figure 11B**). In addition, activin-A, through Smad-2 activation can activate the canonical wingless and int-1 (Wnt) signalling pathway (256). Of note, despite the many structural similarities of the TGF-β

superfamily of cytokines and their receptors and downstream signalling components, they have very different biological activities that belie their common characteristics.

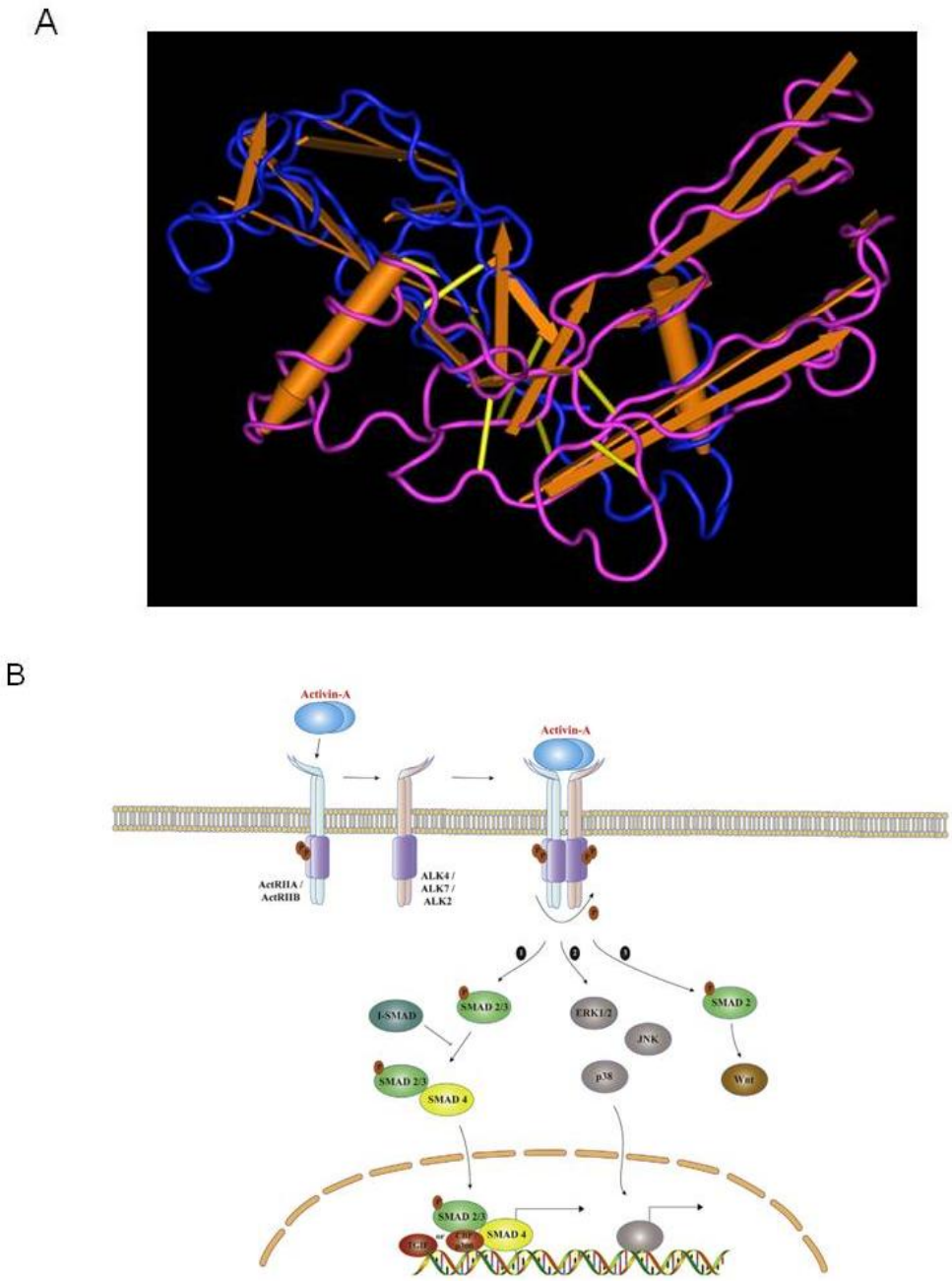

Figure 11. Structure and signalling pathway of activin-A. (**A**) 3-dimensional structure of activin-A homodimer based on the analysis of the PDB file 2ARV with the Cn3D software. Helix and strand objects are depicted, as well as, the virtual disulfide bonds between the cysteine residues (yellow lines). (**B**) Signalling of activin-A is mediated through a membrane heterotetrameric receptor

complex. Activin-A binds to the constitutively active ActRII (ActRIIA or ActRIIB) which then recruits and phosphorylates ActRI (ALK4, ALK7 or ALK2). According to the canonical signal transduction pathway, activated ActRI phosphorylates the main intracellular mediators of activin-A signalling, Smad2/3. pSmad2/3 form a complex with Smad4 and translocate to the nucleus, where they regulate the transcription of target genes in cooperation with several transcription co-factors (e.g CBP/p300 or TGIF) (1). In the non-Smad-mediated signal transduction pathway, ActRI activates the ERK, p38 or JNK kinases which in turn regulate the transcription of target genes (2). Finally, activin-A, through Smad-2 phosphorylation can activate the canonical wingless and int-1 (Wnt) signalling pathway (3). *(Picture A adapted from Tousa S. et al. Activin-A: A New Piece in the Puzzle of Tolerance in Asthma. Clinical Anti-Inflammatory & Anti-Allergy Drugs, 2014. Picture B adapted from Morianos I, et al. Activin-A in the regulation of immunity in health and disease. J Autoimmun. 2019).*

Considering the critical roles of activins in fundamental biological processes, their actions are highly regulated by a variety of molecules at both the extracellular and the intracellular levels. Follistatin (FS) is one of the major inhibitors of activin-A which neutralizes its actions by binding with high affinity to activin-A and thus preventing the interaction with its type II receptors (257) (**figure 12**). Furthermore, FS induces rapid endocytic internalization of activin-A followed by proteolytic degradation. Alternative splicing leads to the formation of two isoforms of FS. The FS288 isoform binds to eparan sulfate proteoglycans with high affinity and is considered to be a local regulator of the actions of activin-A, while the FS315 isoform does not bind to cell-surface proteoglycans and predominantly removes activin-A from the circulation. Notably, FS can inhibit the effects mediated by all activins, as well as, the functions of other TGF-β superfamily members, including myostatin and BMPs.

Inhibins αre circulating heterodimeric proteins consisting of the inhibin α subunit and the activin βA (inhibin A, αβA) or βB subunit (inhibin B, αβB). Inhibins downregulate the signalling of all activins mainly through competition for binding to the type II receptors but also by binding to activins with variable affinity and, thus, antagonizing their functions (257) (**figure 12**). Betaglycan is a protein that binds to inhibins and increases their affinity to ActRII, leading to functional inhibition of activins (257) (**figure 12**). In addition, the pseudoreceptor BMP and activin membrane-bound inhibitor homolog (BAMBI) inhibits activin-A, TGF-β and BMP signalling by interacting with their type I receptors and interrupting the formation of the active receptor signalling complex. Moreover, when

overexpressed, the co-receptor for nodal ligands, Cripto, inhibits activin-A signalling and facilitates nodal signalling by binding to both nodal and activin receptors (257) (**figure 12**). At the intracellular level, the I-Smads bind stably to type I receptors and prevent the recruitment and phosphorylation of R-Smads resulting to the inhibition of activin-A downstream signalling (**figure 12**). Moreover, I-Smads promote the binding of the Smad ubiquitin regulatory factors 1 and 2 to activin-A receptors, resulting in ubiquitin-dependent degradation of the receptors (254).

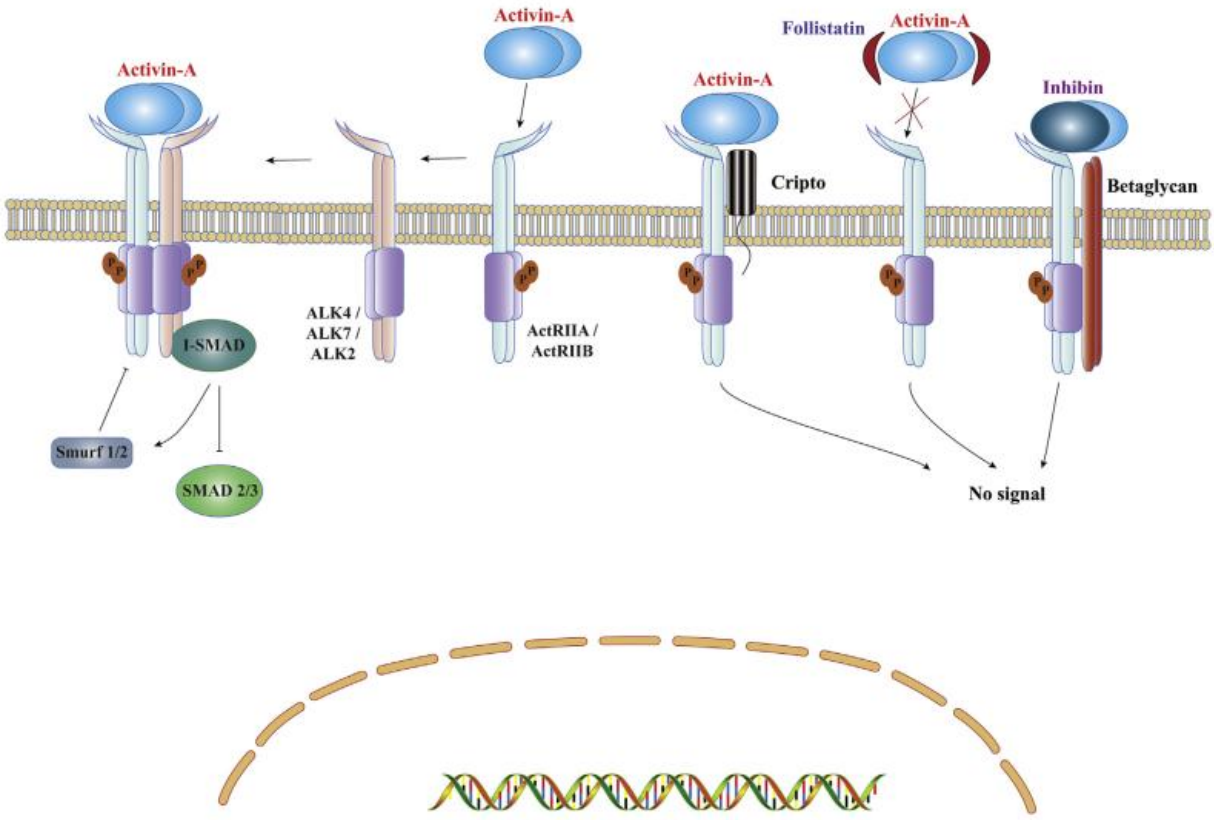

Figure 12. Regulation of activin-A's signalling pathway. I-Smads Smad-6 and -7 are upregulated upon activin-A binding to its receptors and bind to the activated ActRI, inhibiting signal propagation intracellularly. FS is one of the major inhibitors of activin-A which neutralizes its actions by binding with high affinity to activin-A thus preventing its interaction with its type II receptors. Inhibins compete for binding to the type II receptors but can also bind directly to activins with variable affinities. Betaglycan binds inhibins and enhances their affinity for ActRII, leading to the inhibition of the interaction between activins and the type II receptors. Cripto the co-receptor for nodal ligands inhibits activin-A signalling by binding to activin-A receptors. *(Picture adapted from Morianos I, et al. Activin-A in the regulation of immunity in health and disease. J Autoimmun. 2019).*

Activin-A was initially described as a gonadal protein stimulating the biosynthesis and secretion of follicle stimulating hormone from the pituitary (246). Since then, a growing body of evidence has uncovered critical roles for activin-A in a variety of biological processes. Activin-A is highly conserved among vertebrates, sharing over 95% homology between species. Mice lacking the βA subunit of activin-A die within 24 hours after birth due to severe craniofacial defects while, mice lacking the βB subunit exhibit abnormal development and reduced reproductive capacity (258, 259). ActRII deficient mice either die at birth due to mandible defects, or reach adulthood but exhibit major deficiencies in their reproductive systems (259).

1.4.2 The Role of Activin-A in Immunity and Inflammation

Nearly every cell type in the body is capable of synthesizing and secreting activin-A. Embryonic and adult thymic stromal cells and thymocytes express activin-A, along with its signalling components, pointing to an important role during thymus development (260, 261). Moreover, *in vitro* studies showed that activin-A restrains phytohemagglutinin (PHA)-induced proliferation of adult rat thymocytes as well as PB $CD4^+$ T cells (262). Murine microglial cells and peritoneal macrophages secrete activin-A upon *in vitro* TLR stimulation (263) (**figure 13**). Alternatively activated microglial cells (M2) express activin-A and oligodendrocyte progenitor cells express activin-A receptors during remyelination (264). Murine neutrophils secrete activin-A which can be further upregulated upon stimulation with TNF-α *in vitro* (265) (**figure 13**). Mouse NK cells also express ActRIIA and ActRIIB and produce activin-A in an IL-2-dependent manner, while activin-A enhances IL-2 release by NK cells, in a positive feedback loop (266). B cells express activin-A type I and II receptors and secrete activin-A upon *in vitro* lipopolysaccharide (LPS) stimulation (267) (**figure 13**). In addition, activin-A is rapidly released in the circulation during *in vivo* LPS-induced acute systemic inflammation (268). 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 (269). The expression of activin-A by epithelial cells, endothelial cells and infiltrating macrophages is increased in the colon of mice during the acute phase of 2,4,6-trinitrobenzenesulfonic acid colitis (270) (**figure 13**). In $GMCSF^{-/-}$ mice, a model

resembling pulmonary alveolar proteinosis (PAP), activin-A is increased in alveolar macrophages and in the BALF (271). Furthermore, during *in vivo* Leishmania major infection, mice with IL-4Rα-deficient DCs show increased parasite loads and enhanced mRNA levels of IL-23p19 and activin-A (272).

Human cells also produce activin-A following stimulation with inflammatory mediators. Neutrophils secrete activin-A upon stimulation with TNF-α *in vitro*, while DCs upregulate activin-A, ActRI and ActRII expression following stimulation with CD40L and TLR4 and TLR9 ligands (273, 274) (**figure 13**). NK cells express the activin-A type I and II receptors (275) (**figure 13**). Activin-A is expressed by bone marrow stromal cells after *in vitro* stimulation with IL-1, LPS and TNF-α and by bone marrow monocytes and fibroblasts upon co-culture with stimulated $CD4^+$ T cells, a process dependent on CD40-CD40L interactions, GM-CSF and IFN-γ secretion (276 – 278) (**figure 13**). Synoviocytes stimulated *ex vivo* with IL-1, IL-8, IFN-γ and TGF-β1 rapidly secrete activin-A (279). In addition, airway epithelial cells secrete copious amounts of activin-A following infection with rhinovirus *ex vivo* (280).

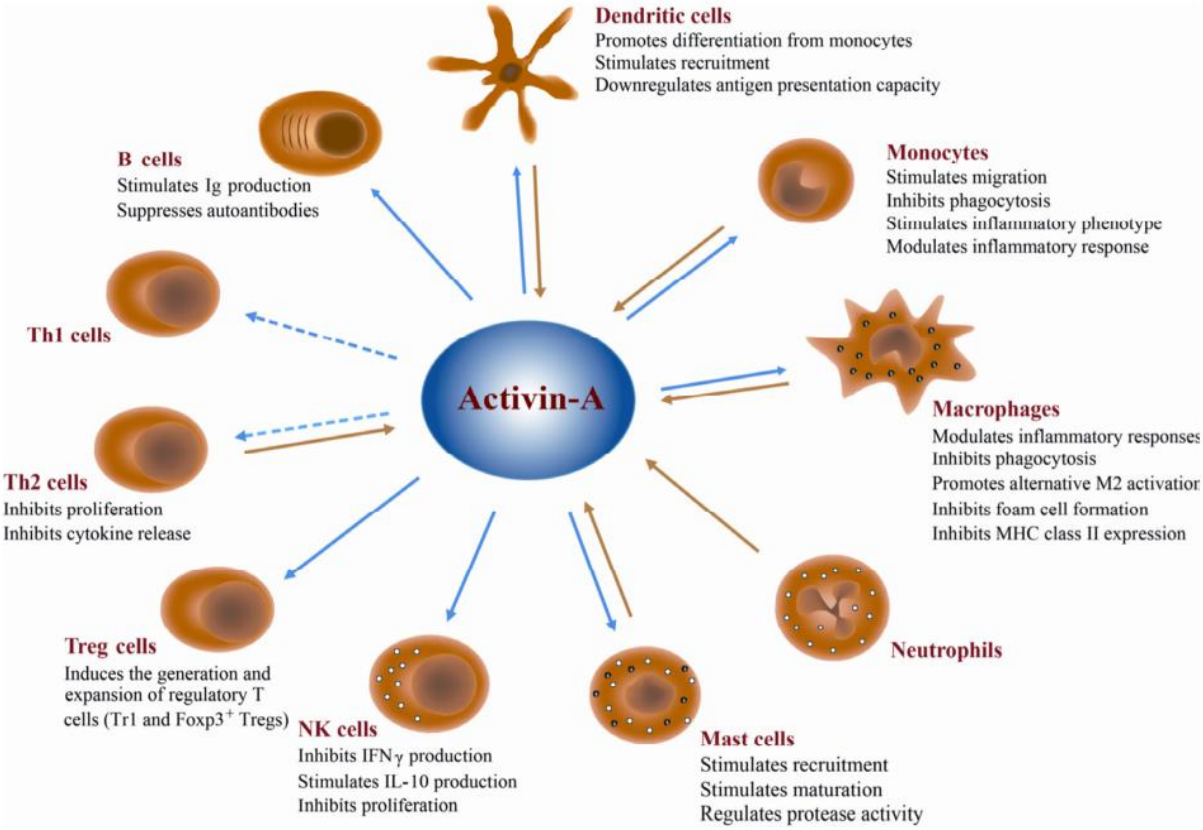

Figure 13. Activin-A effects on innate and adaptive immune cells. Activin-A is produced by monocytes, macrophages, DCs, neutrophils, mast cells and Th2 lymphocytes (orange arrows). The effects of activin-A on distinct immune cell subsets are also depicted (blue arrows). *(Picture adapted from Tousa S. et al. Activin-A: A New Piece in the Puzzle of Tolerance in Asthma. Clinical Anti-Inflammatory & Anti-Allergy Drugs, 2014)*

A plethora of studies suggest that activin-A is involved in human immunological disorders. Activin-A serum levels are increased in patients with septicemia and related to the severity of inflammation (277). Activin-A is also increased in the serum of pre-term septicemic neonates and upregulated by neonatal leukocytes following LPS and PHA stimulation (281). Activin-A is increased in the serum and cerebrospinal fluid of patients with bacterial and viral meningitis and in the serum and synovial fluid of patients with inflammatory arthropathies, such as gout, and rheumatoid arthritis (282 – 284). In addition, activin-A is increased in the serum of patients with systemic lupus erythematosus and its levels correlate with disease activity parameters (284). Furthermore, activin-A and its receptors are upregulated in ulcerative colitis and Crohn's disease (285, 286). Serum activin-A levels are increased in systemic sclerosis and its signalling pathway components are upregulated in affected fibroblasts (287). Activin-A is upregulated in the bronchial epithelium of patients with chronic obstructive pulmonary disease (COPD) compared to never smokers and smokers without COPD (288, 289). In contrast to the animal model, activin-A is decreased in BAL cells and fluid in individuals with PAP (290). These discrepancies may arise from the differences in the experimental settings utilized; in GM-CSF-/- mice there is a total deficiency in GM-CSF, suggesting that compensatory mechanisms may be developed, while in individuals with PAP, GM-CSF levels are not totally absent. Another difference lies on the expression of inducible nitric oxide synthase (iNOS), shown to be increased in murine alveolar macrophages, while it was undetectable in the alveolar macrophages obtained from PAP patients, a finding that could have affected activin-A's expression. Importantly, the animal model does not recapitulate all the features of the autoimmune disease and additional regulatory mechanisms may exist in the human lungs that control activin-A's expression.

Activin-A is also implicated in tissue repair and fibrotic processes, being upregulated, along with its main type I and II receptors, in wounded murine skin. In fact, activin-A overexpression in skin keratinocytes does not only enhance wound healing processes but

also augments the scarring response (291). Activin-A is also involved in the pathogenesis of fibrotic diseases (292). Activin-A is expressed by alveolar macrophages, epithelial and smooth muscle cells in the lungs of patients with interstitial fibrotic lung disease and induces the migration of fibroblasts and their differentiation to myofibroblasts *in vitro* (293). Importantly, *in vivo* administration of FS in the mouse lung ameliorates bleomycin-induced fibrosis (294).

An intriguing feature of activin-A is that it can exert both pro- and anti- inflammatory functions (**figure 13**). Activin-A stimulates the release of TNF-α and IL-1β, as well as, the production of PDGE2, thromboxane and iNOS by mouse bone-marrow derived macrophages (295) (**figure 13**). In accordance, activin-A increases NO, IL-1β and CD14/CD68 expression on resting mouse peritoneal macrophages and promotes their phagocytic capacity (296). Activin-A promotes the *in vitro* alternative activation of murine macrophages towards the M2 phenotype (297) (**figure 13**). Hence, treatment of resting macrophages with activin-A seems to polarize them towards a pro-inflammatory M1-like phenotype. Activin-A also promotes the production of CXCL12 and CXCL14 chemokines by immature human and murine DCs inducing their directional migration *in vitro* (298) (**figure 13**). Activin-A stimulates the differentiation of human monocytes to Langerhans cells (LC) and enhances LC migration through IL-12p70 induction (299) (**figure 13**). Pre-treatment of resting mouse B cells with activin-A results in significantly increased LPS-induced B cell proliferation and IgG production *in vitro* (266) (**figure 13**). Notably, activin-A enhances antigen-driven IFN-γ release by mouse $CD8^+$ T cells (300). Furthermore, cigarette smoke (CS)-exposed human bronchial epithelial cells express increased levels of activin-A, and FS administration during *in vivo* CS exposure results in reduced lung inflammation (289).

Notwithstanding these findings, accumulating evidence reveals important anti-inflammatory functions of activin-A (**figure 13**). Activin-A inhibits IL-6-mediated murine B cell proliferation and monocyte phagocytosis *in vitro* (279) (**figure 13**). In addition, activin-A decreases CD14 and MHC-II expression, as well as, NO release by LPS-activated mouse peritoneal macrophages and suppresses their phagocytic capacity both *in vitro* and *in vivo* (301) (**figure 13**). Activin-A decreases the secretion of IL-1β and NO and reduces CD68, CD14 and TLR4 by the mouse macrophage cell line RAW264.7 during activation with LPS

in vitro (302) (**figure 13**). Activin-A restrains LPS effects on murine peritoneal macrophages *in vivo* through the suppression of TLR4 expression (303). Therefore, in contrast to resting macrophages, activin-A exerts strong anti-inflammatory effects in activated macrophages. Notably, activin-A suppresses the ability of murine NK cells to lyse target cells (266). Moreover, activin-A inhibits PHA-induced proliferation of adult rat thymocytes and peripheral blood T lymphocytes (262). Notably, activin-A increases TGF-β1-induced Foxp3 expression on murine $CD4^+CD25^-$ T cells *in vitro* and enhances the conversion of peripheral $CD4^+CD25^-$ T cells to $Foxp3^+$ Tregs *in vivo* (304) (**figure 13**). Furthermore, activin-A induces IgA secretion by murine B cells upon *in vitro* LPS stimulation (305) (**figure 13**), as well as, by the mesenteric lymph node cells *in vivo*, pointing to effects on the maintenance of gut immune homeostasis (306).

Pertinent to human immune responses, activin-A inhibits the production of IL-1β, while it enhances the production of IL-1 receptor antagonist, in activated THP-1 and U-937 human monocytic cells, resulting in decreased IL-1β biological activity (307) (**figure 13**). In addition, blocking of activin-A signalling in DCs by FS, during stimulation with CD40L *in vitro*, results in increased cytokine and chemokine release (274). Moreover, FS-mediated inhibition of DC-derived activin-A enhances the proliferation of viral-specific $CD8^+$ Teff cells, suggesting a role for activin-A in anti-viral immunity (274). Activin-A downregulates human leukocyte antigen DR on monocyte-derived DCs and diminishes their antigen presenting capacities (308) (**figure 13**). Notably, our group has demonstrated that *in vitro* treatment of neonatal T cells and monocytes with activin-A diminishes PHA and LPS-induced IL-1β, IL-6, and CXCL8, concomitant with an increase in IL-10 (281). Activin-A suppresses the proliferation, the expression of CD25 and T-bet and the secretion of IFN-γ, CCL4, CCL3, CXCL8 and CXCL10 by NK cells *in vitro* (275) (**figure 13**). Moreover, activin-A, inhibits mouse and human NK proliferation, suppresses NK-mediated cytotoxicity and promotes tissue-residency features on NK cells (309). Activin-A restrains PHA-driven proliferation of B cells isolated from healthy individuals and patients with PAP and reduces the production of auto-antibodies against GM-CSF (290). Activin-A decreases the proliferation of $CD4^+$ T cells during polyclonal stimulation (310). A recent study has implicated activin-A in human T_{FH} biology, uncovering activin-A as the most potent inducer of the CXCR5 and PD-1 molecules that characterize T_{FH} cells (311). In addition, activin-A

in synergy with IL-2 seems to modulate T_{FH} gene program and pSmad2/3 complex is important for the activin-A-mediated regulation of T_{FH} cell differentiation (311).

Since activin-A is produced by various immune and tissue cells and exerts distinct effects on them depending on microenvironment and the state of activation of the target immune cells it is conceivable that it may play important roles during several immune-mediated disorders. Indeed, activin-A has been implicated in the pathophysiology of human autoimmune diseases. Patients with systemic lupus erythematosus have elevated serum levels of activin-A while activin-A's signalling pathway is upregulated in patients with rheumatoid arthritis and seem to correlate with disease activity. However, activin-A's *in vivo* effects on the pathogenesis of these autoimmune diseases remains elusive (312). In addition, activin-A has been found to play both pro- and anti -tumorigenic roles in patients with cancer, determined by the context of the immune response and the type of the cancer. In fact activin-A is involved in tumor growth procedures through promotion of the immunosuppressive activities of macrophages and Tregs (312). In contrast, activin-A exerts anti-tumorigenic effects by boosting $CD4^+$ and $CD8^+$ Teff cell responses and DC-mediated antigen presentation (312). Therefore, it is evident that activin-A is a multifaceted cytokine affecting a plethora of innate and adaptive immune responses acting on multiple cell types. It can be considered as a "Janus" cytokine with both pro- and anti- inflammatory functions in an array of immune-mediated disorders.

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

During the past years, it has become increasingly evident that activin-A plays a fundamental role in allergen-driven Th2 cell responses and airway remodelling. Murine differentiated Th2 cells represent a major source of activin-A, while the Th2-related transcription factor c-Maf controls activin-A expression as it cooperates with the nuclear factor of activated T cells (NFAT) and transactivates the activin βA promoter (297). Apart from Th2 cells, mast cells also produce activin-A and mast cell-derived activin-A induces airway smooth muscle cell proliferation *in vitro*, suggesting a key role for activin-A in AHR, as well as, in airway remodelling (313, 314) (**figure 14**). Moreover, IgE-cross-linking on bone marrow-derived mast cells induces a rapid expression of activin-A mRNA (314).

Mouse and human mast cells also express activin-A receptors and activin-A enhances the differentiation and recruitment of bone marrow-derived mast cells *in vitro* (315). Furthermore, activin-A is implicated in the alternative activation of macrophages into the arginase-1+ M2 phenotype, a process involved in Th2-cell associated responses and tissue repair (297).

In line with the *in vitro* studies, findings in experimental models have implicated activin-A in the regulation of allergic responses *in vivo*. Activin-A is expressed in the BAL and submucosal mast cells in a mouse model of OVA-induced acute allergic airway inflammation (314, 316). Furthermore, activin-A mRNA levels are strongly upregulated in the lungs of mice in an experimental asthma model (316). Mouse mast cells express activin-A receptors and mouse Th2 cells and macrophages produce activin-A during allergic airway inflammation *in vivo* (314). Another study revealed that the expression of activin-A is downregulated in the bronchial epithelium, while it is increased in the BAL following pulmonary allergen challenge (317). In addition, intratracheal injection of IL-13, a protocol that induces allergic airway inflammation, significantly increases activin-A in the BAL, associated with enhanced mucus production (318). Of note, activin-A, in the presence of suboptimal doses of TGF-β, induces the generation of Th9 cells *in vitro*, while blockade of activin-A along with TGF-β1, during acute allergen challenge *in vivo*, decreases Th9 cell differentiation and ameliorates allergic airway inflammation (319) (**figure 14**).

Noteworthy, seminal studies, using a model of pulmonary house dust mite administration, have demonstrated that overexpression of Smad-2 in airway epithelial cells, results in a marked upregulation of activin-A and IL-25 in the airway accompanied by enhanced airway remodelling and AHR. *In vivo* blockade of activin-A attenuates airway structural changes, concomitant with a decrease in IL-25 expression, pointing to a novel functional link between activin-A and IL-25 in airway remodelling (320). In agreement, FS attenuates lung fibrosis, a major component of asthmatic airway remodelling, in a mouse model of chronic OVA administration (321). Moreover, following allergen challenge, submucosal fibroblasts express elevated levels of ALK4 and ActRIIA, along with pSmad2, further supporting a role for activin-A in airway repair and remodelling processes (322). On the other hand, recent studies by our group have revealed that activin-A inhibits VEGF-

induced proliferation and cytokine secretion by human pulmonary endothelial cells through IL-18 and VEGFR1-dependent pathways, pointing to this cytokine as a novel anti-angiogenic factor for human asthma (138). Regarding the expression of the signalling pathway components of activin-A during allergic airway inflammation, it has been shown that submucosal fibroblasts express elevated levels of ALK4 and ActRIIA, along with pSmad-2, further supporting a role for activin-A in airway repair and remodelling (316).

Recent studies by our group have uncovered a critical protective role for activin-A in allergic airway inflammation and linked disease in mice (323). 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 cell-mediated allergic responses (323). In corollary, *in vivo* administration of recombinant activin-A, right before allergen challenge, ameliorates all cardinal features of experimental asthma, including AHR. Apart from its direct suppressive effects on Th2 cell responses, we demonstrated that activin-A also induces the generation of $CD4^+CD25^-Foxp3^-$IL-10-producing Treg cells that effectively suppress allergen-driven Th2 cell responses *in vitro* and upon adoptive transfer *in vivo* and confer protection against allergic airway disease (323). Activin-A-mediated Th2 cell suppression is dependent on IL-10 and TGF-β1 release (323). 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 groups should not be considered as redundant during inflammatory processes (267, 275, 324). This could be partly due to the fact that activin-A and TGF-β1 utilize different receptors, as well as, distinct, Smad-independent, signalling pathways (277). Furthermore, activin-A, in contrast to TGF-β1, is secreted as a glucosylated pre-proligand and, therefore, its regulation and expression patterns *in vivo* are different (325). Moreover, activin-A and TGF-β1 operate through distinct suppressive mechanisms and induce different Treg cell subsets under the Th2 setting (186, 323). Importantly, the signalling pathway components of activin-A and the other TGF-β superfamily members are differentially expressed in the lungs, at steady state and following *in vivo* inhalational allergen challenge, emphasizing the distinct regulation of these cytokines in asthma.

Surprisingly, Ogawa et al. have demonstrated that activin-A is not produced by $CD4^+CD25^+$ Treg cells upon polyclonal stimulation *in vitro*, raising concerns pertaining to

the ability of activin-A to induce tolerance to aeroallergens (297). Still, the expression of the Treg cell lineage-specification transcription factor, Foxp3, by $CD4^+CD25^+$ T cells was not examined and, therefore, it is not clear whether these cells represent bona fide natural Treg cells or activated conventional T cells. Moreover, the expression of activin-A by natural Treg cells *in vivo*, at steady state or in an inflammatory setting, was not investigated and cannot be excluded.

In contrast to our findings, a different study showed that pulmonary administration of activin-A inhibitor, FS, decreases Th2 responses, pointing to pro-inflammatory effects of activin-A locally (318). The observed differences could be, at least partly, caused by the use of distinct activin-A inhibitors. We have administered a specific activin-A neutralizing antibody, whereas Hardy C.L. et al. administered FS, an inhibitor that also neutralizes other activins and bone morphogenetic proteins (257, 326). Considering that these TGF-superfamily members are expressed in the allergic airways the observed differences may be related to the inhibition of these proteins (137, 316). Moreover, activin-A may exert distinct *in vivo* functions depending on the route of administration or the site of overexpression, as shown for other immunomodulatory cytokines, such as TGF-β1, IL-10 and IL-27 (327 – 329).

Studies by our group have also revealed that murine activin-A-induced Treg cells induce the generation of DCs with immunoregulatory properties (actTreg-modified DCs). More specifically, through a series of adoptive transfer experiments we discovered that activin-A-induced Treg cells gave rise to DCs with suppressive functions towards experimental asthma (330). Act-Treg-modified DCs showed an immature phenotype expressing low MHC-II and co-stimulatory molecules, had an impaired capacity to uptake antigen and migrate to the draining lymph nodes and exhibited attenuated cytokine release in response to LPS challenge *in vivo* (330). In addition, act-Treg-modified DCs displayed poor immunostimulatory potential, exemplified by a decreased ability to prime allergen-specific T cell responses both *in vitro* and *in vivo* (330). Interestingly, administration of actTreg-modified DCs conferred protection against experimental asthma both in preventive and therapeutic protocols through the de novo generation and expansion of $Foxp3^+$ Tregs (330). Notably, disruption of PD-1 signalling abolished the capacity of activin-A-induced

Treg cells to generate tolerogenic DCs pointing to the involvement of the PD-1/PDL-1 immunoregulatory pathway (330).

Accumulating evidence has also proposed a role for activin-A in the regulation of human allergen-driven immune responses and linked disease. Activin-A is expressed by macrophages in lung tissues of patients with asthma (314) (**figure 14**). Increased activin-A expression is also observed in T cells, macrophages and mast cells in bronchial biopsies from patients with moderate asthma, compared to healthy individuals (310, 314, 323). Interestingly, neutrophils are identified as the most potent providers of activin-A in the airways of asthmatic patients (137) (**figure 14**). Furthermore, elevated activin-A expression is detected in PB CD4+ T cells from individuals with mild/moderate asthma administered with corticosteroids in comparison to untreated mild/moderate asthmatics and healthy individuals (310). Studies by our group and others have shown that activin-A levels are elevated in the serum and BALF of patients with severe asthma as compared to mild-moderate asthmatics and healthy controls (138, 310). Another group has also depicted that activin A expression is higher in the sputum and BALF of patients with severe asthma, compared to mild-moderate asthmatics and correlated with eosinophil numbers and reticular basement membrane thickness (331). *In vitro* studies have shown that activin-A expression is induced in primary human bronchial epithelial cells upon IL-13 administration (318). Moreover, a recent study has revealed that PB eosinophils of allergic individuals produce copious amounts of activin-A upon *in vitro* stimulation with IL-3 and TNF-α (331). Furthermore, increased activin-A mRNA levels are detected in eosinophils isolated from the BALF of allergic individuals after allergen inhalational challenge (331). Studies by our groups have also demonstrated that activin-A is upregulated after allergen inhalational challenge in patients with nasal polyps, while its signalling pathway components are decreased. In addition, *ex vivo* polyclonal stimulation of nasal mucosa cells obtained from patients with nasal polyps enhances activin-A expression which, along with TGF-β1, synergize to increase IL-5 release (332). Still expression of activin-A's receptors ALK4 and ActRIIA is decreased in airway submucosal cells of mild/moderate asthmatic patients as compared to healthy individuals (138, 323).

Notably, a recent study has implicated activin-A in the suppression of Th2-cell mediated allergic responses in the skin. Mice overexpressing activin-A in keratinocytes (K14-Activin tg) exhibit decreased Th2 cell-associated cytokine expression in the skin and lower OVA-specific IgE levels in a model of OVA-induced cutaneous allergic inflammation, pointing to effects of activin-A in the maintenance of skin homeostasis (333).

Many of the aforementioned studies highlight a pivotal role for activin-A in tilting the balance of pathogenic Th2 cells towards anti-inflammatory Treg cells in allergic responses. Still, the possible effects of activin-A on the induction of human regulatory T cells remained undefined.

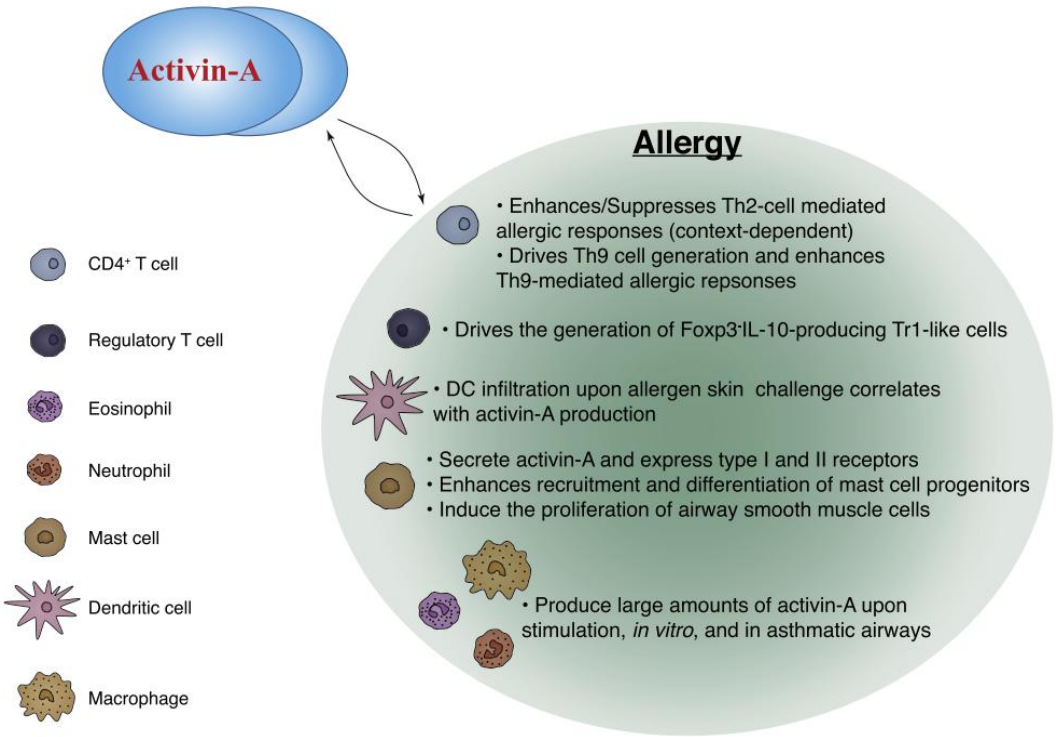

Figure 14. The role of activin-A in allergic responses. Activin-A can exert both pro- and anti-inflammatory roles in allergic diseases, such as asthma and atopic dermatitis, depending on the microenvironment and the type and status of activation of the immune target cells. The fundamental effects of activin-A on distinct immune cells, implicated in the pathophysiology of allergic disorders, are depicted *(Picture adapted from Morianos I, et al. Activin-A in the regulation of immunity in health and disease. J Autoimmun. 2019)*

2. HYPOTHESIS AND SPECIFIC AIMS

In the present study our hypothesis was that activin-A is a critical immunoregulatory factor that controls human allergen-driven Th2 cell responses and restrains allergic airway inflammation.

We also hypothesized that activin-A exerts its regulatory effects through the induction of human regulatory T cells that effectively suppress allergen-specific $CD4^+$ T cell responses in the periphery and the airways of atopic and asthmatic individuals.

Our specific aims were:

a) To investigate the effects of activin-A on the suppression of human Th2 cell-mediated allergic responses in the periphery and the airways of individuals with atopy and allergic asthma and

b) To delineate the role of activin-A in the induction of human regulatory T cells and their effects on the inhibition of allergic Th2 responses.

3. MATERIALS AND METHODS

Study population. Peripheral blood samples were obtained from 13 individuals with atopy without asthma and 22 individuals with atopic asthma (mild, moderate and severe) at the “Sotiria” Athens Chest Hospital, the Allergology department of the “401” General Military Hospital of Athens, the Lugano and the Milano University Hospital. Asthma was defined according to the 2015 guidelines from the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (ginasthma.org/2017-gina-reportglobal-strategy-for-asthma-management-and-prevention). Severe asthma was defined according to the latest American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines. Atopic and asthmatic individuals had a positive skin prick test result (wheal size ≥ 3mm) to the allergen LPS-free mixed grasses extract (ALK-Abello) and/or increased allergen-specific IgE. Subjects did not receive treatment with anti-IgE or anti-IL-5 monoclonal antibodies throughout the study and all blood samples were obtained out of season. On entering the study, all individuals were healthy and stable. 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 autoimmune disorders, cancer and/or other respiratory diseases were excluded. Buffy Coats were obtained from healthy individuals at Lugano and Milano University Hospitals. The study was conducted according to the principles described in the Declaration of Helsinki. All participants signed a written informed consent form approved by the “Sotiria” Athens Chest Hospital Ethics Committee, the Ethics Committee of the “401” General Military Hospital of Athens, the Cantonal Ethical Committee of Cantone Ticino and the Ethics Committee of the Lugano and Milano University Hospitals.

Mice. Female (6-8 weeks old) non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice were obtained from the Jackson Laboratories. Mice were maintained at the Biomedical Research Foundation of the Academy of Athens Animal Facility and were regularly checked for the absence of mouse serum Igs. Animal handling and procedures were in accordance with US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (no. A5736-

01) and with the European Union Directive 86/609/European Economic Community (EEC) on protection of animals used for experimental purposes.

Isolation and culture of peripheral blood T cells. Peripheral blood mononuclear cells (PBMCs) were obtained by Lymphoprep (Axis-Shield Diagnostics) and/or Histopaque (Sigma-Aldrich) density-gradient centrifugation. Naive $CD4^+$ T cells were isolated using magnetic microbeads ($CD4^+CD25^-CD45RO^-$; Miltenyi Biotec) or by sorting with a FACS Aria cells sorter (BD Biosciences). Cell viability staining was performed, and dying cells were excluded from analyses. CD3-depleted PBMCs (Miltenyi Biotec) or $CD14^+$ monocytes (miltenyi Biotec) were treated with mitomycin-C (Sigma–Aldrich) and used as APCs. Similar results were obtained using either APC population and results were pooled for statistical analysis. Naive $CD4^+$ T cells (2×10^4 cells) were stimulated with autologous APCs (2×10^4 cells) loaded with LPS-free mixed grasses extract [ALK-Abello, 1.000 standardized quality-units (SQ-U)/ml; kindly provided by P.Wurtzen, ALK-Abello, Hørsholm, Denmark] in the presence of PBS (Tconv cells) or recombinant activin-A (act-A-iTr1 cells, 50 ng/ml; R&D Systems) for 7-9 days. In all *in vitro* stimulations, mixed grasses extract was used. In certain experiments, naive $CD4^+$ T cells were stimulated with plate-bound anti-human CD3 (1 µg/mL, OKT3; BD Biosciences) and soluble anti-human CD28 (1 µg/mL, CD28.2; BD Biosciences). Cells were cultured in RPMI 1640/GlutaMAX supplement medium with 10% FBS and 100 units/mL penicillin and 100 µg/mL streptomycin (all from Gibco) in 96-well, U-bottomed Nunc MicroWell Plates with Nunclon Delta Surface (Thermo Scientific). In studies examining IL-10 production, cells were stimulated for additional 24–48 h. Dose-response studies were performed to determine the optimal doses of activin-A, allergen, and anti-CD3/CD28 antibodies. Recombinant human (rh) IL-2 (20 international units/mL; R&D Systems); the Smad3 inhibitor SIS3 (10 nM; Sigma–Aldrich); CH-223191 (3 µM; Calbiochem); and neutralizing antibodies against ALK4 (20 µg/mL; R&D Systems), IL-10R (20 µg/mL; R&D Systems), and TGF-β (5 µg/mL; R&D Systems), or the respective Ig controls, were added in cell cultures. To obtain conditioned medium (CM), $CD4^+$ T cells were isolated from activin-A–or control-treated cultures, washed, and stimulated overnight with anti-CD3/CD28 antibodies. CM was then added in cocultures of naive $CD4^+$ T cells and allergen-loaded APCs.

In other experiments, total $CD4^+$ T cells (5 x 10^4 cells) obtained from atopic and asthmatic individuals were isolated using magnetic microbeads (Miltenyi Biotec) or the Dynal CD4 Positive Isolation Kit (Dynabeads; Invitrogen) and stimulated with autologous APCs (2 x 10^4 cells) loaded with mixed grasses extract for 4–5 d.

In experiments investigating T-cell hyporesponsiveness, $CD4^+$ T cells were isolated from primary induction cultures, labeled using a Cell Trace CFSE (carboxyfluorescein succinimidyl ester) Proliferation Kit (Molecular Probes) and restimulated either with fresh allergen-loaded APCs or with anti-CD3/CD28 antibodies for another 5–6 d.

In experiments investigating *in vitro* stability during prolonged culture, $CD4^+$ T cells were isolated from primary cultures as above and stimulated every week with freshly isolated, allergen-loaded APCs for a period of 4 wk and in the presence of rhIL-2.

For evaluation of Ag specificity, naive $CD4^+$ T cells were cultured with allergen-loaded, mitomycin-treated APCs in the presence of control (PBS) or activin-A for two rounds of stimulation (7 d each); then, $CD4^+$ T cells were isolated and cultured with fresh APCs in the presence of tetanus toxoid (5 μg/mL; Alexis Biochemicals) for another 6 d.

For *in vitro* human Tr1-cell generation, naive $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of mitomycin-treated, CD3-depleted PBMCs, rhIL-10 (100 ng/mL; R&D Systems) and rhIFN-α2b (5 ng/mL; Merck). After 7 d, T cells were restimulated under identical conditions for an additional 7 d. At the end of the 14 d of culture, IL-10, CD49b, and LAG-3 expression was analyzed by FACS. The fold expansion of T cells was calculated by dividing the absolute number of cells after 14 d of *in vitro* culture by the absolute number of cells at the start of the culture.

***In vitro* suppression assays.** Tconv or act-A–iTr1 cells (2 x 10^4 cells) generated as above, were stained with CFSE (Molecular Probes, Life Technologies) and cocultured with Cell Trace Violet (Molecular Probes, Life Technologies) -labelled naive, allergen-primed $CD4^+$ T responder cells or sorted Th2 effector/memory cells (at a 1:1 ratio), in the presence of allergen and autologous APCs for 4 days. A transwell system (R&D Systems) was used, wherein naive $CD4^+$ T cells, APCs, and allergen were placed in the bottom

chambers and act-A–iTr1 or Tconv cells, APCs, and allergen were added in the upper chambers. T-cell proliferation was evaluated by ^{3}[H]-thymidine incorporation or CFSE and Cell Trace Violet staining and analyzed by flow cytometry.

In certain experiments, neutralizing antibodies against IL-10Rα (30 μg/ml; R&D Systems), ICOS ligand (ICOSL) (25 μg/ml; eBiosciences), activin-A (20 μg/ml, R&D Systems) and TGF-β1,2,3 (7,5 μg/ml; R&D) or their respective Ig controls were added in secondary suppressive cultures.

In other experiments, Tconv, act-A–iTr1, or IL-10–Tr1 cells were cocultured with naive CFSE-labeled CD4^{+} T responders in the presence of anti-human CD3 (1 μg/mL) and soluble anti-human CD28 (1 μg/mL) for 5 days. The suppression of cell proliferation was calculated by measuring the ^{3}[H]-thymidine incorporation or Cell Trace proliferation dye dilution-based division index of T responders cultured across Tconv or act-A-iTr1 cells in comparison to T responders alone, according to the formula:

$$100 - \frac{\text{Proliferation of T-responder cells cultured across Tconv or act-A} - \text{iTr1}}{\text{Proliferation of T-responder cells alone}} \times 100\%.$$

Knockdown with shRNA. Naive CD4^{+} T cells (2 x 10^{4} cells) were activated with allergen-loaded APCs (2 x 10^{4} cells) or anti-CD3/CD28 antibodies with or without activin-A for 2.5 days. Cells were spun with lentivirus containing GFP-expressing shRNA against human *AHR*, *IRF4*, or a scrambled RNA sequence (Dharmacon) and 8 μg/mL polybrene (Sigma–Aldrich). Following 12 hours of incubation at 37^{o}C, T cells were washed and differentiated for 3 days, as described above. Transduced cells were selected by sorting of live GFP^{+} T cells, using FACS Aria cell sorter.

Cytokine Analysis. Cytokines were measured in culture supernatants and mouse BALF using commercially available ELISA kits for human IL-10, IL-5, IL-4, IL-13, IL-17, and IFN-γ (R&D Systems).

Flow Cytometry analysis. Cells were stained with fluorescently labeled antibodies to CD4, ICOS, PD-1, CTLA-4, GITR, CD25, CD69, CD127, LAG3, CD49b, CCR3, CCR5, and CRTH2 (all from eBiosciences); CCR4 (BD Pharmingen); and CCR6, CCR7, CXCR3, CD14, CD95 and CD45RA (all from Biolegend). The staining for CD49b and LAG3 was performed at 37 °C for 20 min. For intracellular cytokine staining, T cells were restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Sigma–Aldrich) and Golgi-Stop (BD Biosciences), and stained with antibodies against IL-4, IL-5, and IL-13 (all from eBiosciences); IL-17 (Beckman Coulter); and IFN-γ (Beckman Coulter) according to the manufacturers' instructions. Foxp3 (eBiosciences) staining was performed according to the manufacturer's instructions. For IL-10 detection, T cells were restimulated with anti-CD3/CD28 antibodies overnight. FACS acquisition was performed with a Cytomics FC500 cytometer (Beckman Coulter) and an LSRFortessa cytometer (BD Biosciences), and data were analyzed using FlowJo software.

Immunofluorescence microscopy. T-cell cytospins (5 x 10^4 cells per slide) were prepared in poly-L-lysine slides (Thermo Scientific), fixed in 4% (wt/vol) paraformaldehyde at 4°C and incubated in 10% sucrose. Slides were washed twice and blocked in 10% donkey serum and then diluted in 0.1% Triton-X for 30 min. Slides were incubated overnight at 4 °C with primary biotinylated antibodies against human AhR (Santa Cruz Biotechnology), c-Maf (Santa Cruz Biotechnology), IRF4 (R&D Systems), or control IgG (R&D Systems). Slides were then incubated with fluorescently labeled secondary antibodies (R&D Systems). Nuclear staining and mounting of the slides were carried out using ProLong Gold Anti-Fade Reagent with DAPI (Life Technologies). Image acquisition was performed by a confocal laser scanning microscope (Leica TCS SP5), and images were acquired by differential interference contrast optics. AhR^+, c-Maf^+, and $IRF4^+$ T cells were counted in each cytospin by two independent observers and expressed as the percentage of total cells.

Quantitative real-time PCR. Human naive $CD4^+$ T cells were stimulated with allergen-loaded, mitomycin-treated APCs or anti-CD3/CD28 antibodies in the presence of Tconv or act-A–iTr1 cells for 3 days. Total RNA was isolated in TRI Reagent (Molecular Research Center) and then reverse-transcribed using Superscript II (Invitrogen), according to the manufacturer's recommendations. RNA samples were treated with RQ1 RNase-Free

DNase (Promega) before reverse transcription to eliminate contaminating genomic DNA. The expression of human *FOXP3, TBX21, GATA3, RORC, AHR, CYP1A1, IRF4, MAF, GZMB, LAG3, ICOS, IFNG, IL10, IL4, IL13, IL5, IL17A* and *GAPDH* mRNA were analyzed using SYBR Green Master mix and primers (shown in Table 1). The relative expression of human genes over the expression of *GAPDH* was calculated using the method developed by Livak et. al. (334). Primer sequences are presented in table 1.

Genes	Forward primer	Reverse primer
AHR	CCGTGTCGATGTATCAGTGC	GCCTGGCAGTACTGGATTGT
CYP1A1	CAGCTGACTTCATCCCTATTC	AGCTGGACATTGGCGTTCTCA
MAF	ACGAGAAGTTGGTGAGCAGC	TTCCAAAATGTGGCGTATCC
IRF4	CATGACAACGCCTTACCCTT	AAACGTCATGGGACATTGGT
ICOS	GGACCATTCTCATGCCAACT	AAAGGCTGCACATCCTATGG
IL-10	GGCGCTGTCATCGATTTCTT	TTGGAGCTTATTAAAGGCATTCTTC
GZMB	GTGCAGGAAGATCGAAAGTGC	GGGCCACCTTGTTACACACA
LAG3	GGCAATCATCACAGTGACTCC	GAGCTCCACACAAAGCGTTC
FOXP3	GAAACAGCACATTCCCAGAGTTC	ATGGCCCAGCGGATGAG
GATA3	GCGGGCTCTATCACAAAATGA	CTCTCCTGGCTGCAGACAGC
TBX21	GATGCGCCAGGAAGTTTCAT	GCACAATCATCTGGGTCACATT
RORC	GCAGCGCTCCAACATCTTCTC	GCACACCGTTCCCACATCTC
IL-4	CTTTGCTGCCTCCAAGAACAC	GCGAGTGTCCTTCTCATGGT
IL-5	TACGTGTATGCCATCCCCAC	TTCAGTGCACAGTTGGTGATTT
IL-13	TGACAGCTGGCATGTACTGTG	ATGCAAGCTGGAAAACTGCC
IL-17A	CCCGGACTGTGATGGTCAAC	TCTCTTGCTGGATGGGGACA
IFNG	GAGTGTGGAGACCATCAAGGA	CTGTTTTAGCTGCTGGCGAC
GAPDH	GCAAATTCCATGGCACCGT	TCGCCCCACTTGATTTTGG

Table 1. Primer pairs that were utilized for the real-time PCR analysis.

RNA-Sequencing and bioinformatics analysis. RNA-seq libraries were generated using Illumina's TruSeq RNA Library Preparation Kit v2 (Illumina), and the sequencing was implemented with a HiSeq 2000 system (Illumina) by the European Molecular Biology Laboratory. Two biological replicates were used for each condition. For analyzing the sequencing files of Tconv and act-A–iTr1 human cells, the following pipeline was performed: For each biological replicate, fastq quality control was assessed; when required, Illumina adapters were clipped, and bases with quality less than 10 were trimmed from the 3′ end of the reads, allowing reads of a minimum length of 20 bases. The high-quality reads were then aligned against the UCSC Genome Browser hg19 human reference genome build, using TopHat2 with default settings and keeping only uniquely

aligned reads. Alignments were then processed for differential expression analysis using the metaseqR Bioconductor package. Read counts were calculated on the Ensembl exon set version 69, and effective library sizes were estimated to normalize the counts table [DESeq method (335)]. For differential expression statistical testing, DESeq and edgeR (336) bioconductor packages were combined to calculate a “meta P-value,” resulting in 513 significantly differentially regulated genes. To compare gene expression patterns between human act-A–iTr1 cells and Tr1 cells, reads per kilobase per million mapped reads (RPKM) and robust multi-array average (RMA) signal intensity values were scaled to have a mean of 0 and an SD of 1. Expression values of the two platforms were plotted in a heat map for 48 RNA-seq differentially expressed genes also present in the microarray study and sorted increasingly with respect to the RNA-seq comparison log2 (FC) value. Bioinformatics analysis and visualizations were applied through custom Perl and R scripts. Microarray data were downloaded from the Gene Expression Omnibus (accession no. GSM1295189).

Chromatin immunoprecipitation (ChIP). Naive $CD4^+$ T cells were differentiated for 6 days with anti-human CD3/CD28 antibodies in the presence or absence of activin-A. T cells were fixed with 1% formaldehyde, quenched with 0.125M glycine and lysed to release chromatin, which sheared to a mean size of 150- to 300-bp fragments by sonication. Samples were incubated overnight with anti-human IRF4, AhR, and c-Maf antibodies (Santa Cruz Biotechnology). Normal rabbit or goat IgG (Santa Cruz Biotechnology) served as a negative control. Magnetic Dynal G beads (Invitrogen) were incubated with the immunocomplexes. For sequential ChIPs, the initial ChIP was performed with the anti-IRF4 antibody, chromatin was eluted with DTT, and a second immunoprecipitation was performed using the anti-AhR or anti–c-Maf antibody. Immunoprecipitated DNA was extracted by reverse cross-linking at 65 °C and proteinase K treatment, followed by AMPure magnetic beads purification. The relative enrichment of ChIP versus IgG (relative to input DNA) was determined and calculated by quantitative RT-PCR for the regions of interest. The following primer pairs were used: IL10, 5′-GTCTTGGGTATTCATCCCAGGTTGGGG-3′ (forward) and 5′-CTGTGGGTTCTCATTCGCGTGTTCCTA-3′ (reverse); ICOS (−2 kb), 5′-AACCAACTTTCCATGCCCCA-3′ (forward) and 5′-CCATGCCTCGCCTTCTCAAA-3′ (reverse); ICOS (−1 kb), 5′-GCCTCCTCCTTACCTTGCAG-3′ (forward) and 5′-

TCTGAACGCGCTTCCAAGAT-3′ (reverse); and ICOS [transcription start site (TSS)], 5′-AACATGAAGTCAGGCCTCTGG-3′ (forward) and 5′-ACATGAGTGACATGGTGCCC-3′ (reverse).

In silico regulatory region analyses of selected genes. The analyses aimed at the identification of the consensus binding sequences of c-Maf, AhR, and IRF4 in human *ICOS* and *IL10*. The *ICOS* and *IL10* sequences, with the accession numbers ENSG00000163600 and ENSG00000136634, respectively were extracted from the Ensembl database (useast.ensembl.org/index.html). The retrieved regulatory regions extending 3,000 bp upstream to 3,000 bp downstream of every known TSS of each gene were submitted to the MatInspector platform in the Genomatix database (www.genomatix.de/en/index/html) to identify putative transcription factor-binding sites (TFBSs) for all of the transcription factors of Matrix Family Library Version 9.0, which contains 1,381 weight matrices. To filter the output of the algorithm, the experimentally verified TFBSs of the known regulators in IL10 (337) were considered as evidence for the true positive results. Any predicted TFBSs with P values and scores beyond the values of the experimentally verified ones were excluded from the results.

In silico structural study of AhR/IRF4/ARNT interactions. AhR and IRF4 were requested from the Protein Data Bank (PDB) (www.rcsb.org/pdb/home/home.do) and the most relevant solved structures (with PDB ID codes 4ZRB and 3DSH, respectively) sharing sequence similarity greater than 30% with these factors were retrieved. Briefly, we used the solved structure of the heterodimeric HIF-1a/ARNT complex with HRE DNA (PDB ID code 4ZPR) as a template to construct the structure of AhR, based on previous studies showing that AhR can functionally interact with ARNT (338). Homology modeling was carried out through the SWISSMODEL platform (https://swissmodel.expasy.org/) and the models were subsequently evaluated with Anolea, QMEAN6, Gromos, DFire and Procheck (swissmodel.expasy.org/workspace/?func=tools_structureassessment1). The models of AhR and IRF4 were used for molecular docking via the ZDOCK server (zdock.umassmed.edu/), whereas Yet Another Scientific Artificial Reality Application (YASARA; www.yasara.org/) was used to perform the molecular dynamics simulations and the energy minimization of the ZDOCK output complex, as well as the recalculation of its energy. The duration of the molecular dynamics simulation studies was 10 ns.

Superimpositions, rmsd calculations, and visualization of the structures were performed using the DeepView-SwissPdbViewer.

Reconstitution of NOD-SCID mice. In the prevention protocol, NOD-SCID mice were reconstituted on day 0 with 2 x 10^6 Tconv or act-A–iTr1 cells and 2 x10^6 autologous APCs and were immunized intraperitoneally (i.p.) with *Phleum pratense* (5 µg per mouse in 0.2 mL of alum; kindly provided by P. Wurtzen). On day 1, mice received autologous, BrdU-labeled, $CD4^+$ T cells (10^6 cells). On days 9 and 10, mice were challenged with aerosolized P. pratense; AHR was measured the following day, and mice were killed. In other experiments, mice were administered (i.p.) anti-human IL-10Rα (30 µg per mouse; Biolegend) or Ig control prior to and 1 d following act-A–iTr1 cell (or Tconv cell) transfer. $CD4^+$ T-responder cells were incubated with anti–IL-10Rα antibody or Ig control before transfer. In the therapeutic protocol, NOD-SCID mice were reconstituted on day 0 with naive $CD4^+$ T cells (10^6 cells) and autologous APCs (10^6 cells), and were immunized with P. pratense/alum. On day 8, mice received autologous Tconv or act-A–Tr1 cells (2 x 10^6 cells). Mice were challenged and killed as described above.

AHR. Airway hyperresponsiveness was measured in mice 24h after the final allergen challenge by whole-body plethysmography (Buxco Research Systems) to calculate enhanced pause (PenH). Responses to inhaled methacholine at increasing concentrations of 3-100 mg/ml were measured for 1 min, as described (323).

BAL analysis. BAL harvesting was performed, as described (339). Inflammatory cells were obtained by cannulation of the trachea and lavage of the airway lumen with PBS. BAL cells were counted and analysed for viability by Trypan Blue exclusion. BAL fluid was kept at -80^{o}C until use.

Lung histology. 4-µm paraffin-embedded lung sections were stained to evaluate lung cell infiltration and histological scores were obtained as described (323, 339).

Mouse cell culture, proliferation and *ex vivo* suppression assays. Draining lymph node (DLN) cells were isolated at the time of euthanasia and cultured at a concentration of 10^6 cells per well with 2,5µg/mL *P. pratense*. $CD4^+$ T cells were isolated using the Dynal

CD4 positive isolation kit (Dynabeads, Invitrogen). T cell proliferation was measured after restimulation with 2,5µg/ml *Phleum pratense*, as described above. T-cell proliferation was measured by ^{3}[H]thymidine incorporation or BrdU staining, followed by flow cytometry.

Statistical analysis. Data distribution was assessed with the D'Agostino–Pearson normality test. Analyses were performed using the Student's t test and the Mann–Whitney test as appropriate, using GraphPad Prism Version 5. AHR was analyzed by two-way ANOVA for repeated measures, followed by the Student's t test. Any difference with a P value of 0.05 or less was considered as statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

4. RESULTS

Activin-A induces human Tr1-like cells

Our initial intention was to delineate the effects of activin-A on the regulation of human T-cell–driven allergic responses. Therefore, we used our well-established *in vitro* T-cell priming model (340) by sorting naive $CD4^+CD45RA^+CCR7^+CD95^-$ T cells from the peripheral blood of individuals with atopy and stimulating them with allergen-loaded, mitomycin-treated, CD3-depleted antigen-presenting cells (APCs) in the presence of activin-A or control (PBS) (**Fig. 1A**). These specific stimulation conditions have been proven to skew T-cell responses toward Th2 (341). Indeed, control-treated $CD4^+$ T cells (hereafter referred to as Tconv cells) showed increased proliferation, concomitant with high percentages of IL-4^+ and IL-13^+ cells and moderate to low IFN-γ^+ and IL-17^+ cells following allergen exposure (**Fig. 1B, C, E**). On the other hand, T cells stimulated in the absence of allergen did not exhibit discernible proliferation (**Fig. 1D**). Interestingly, activin-A–treated T cells showed a reduced proliferative capacity and significantly decreased expression of the cytokines IL-4, IL-13, IL-17 and IFN-γ (**Fig. 1B, C, E**). Furthermore, activin-A markedly increased the frequencies of $CD4^+$IL-10^+ T cells (**Fig. 1B and 1E**).

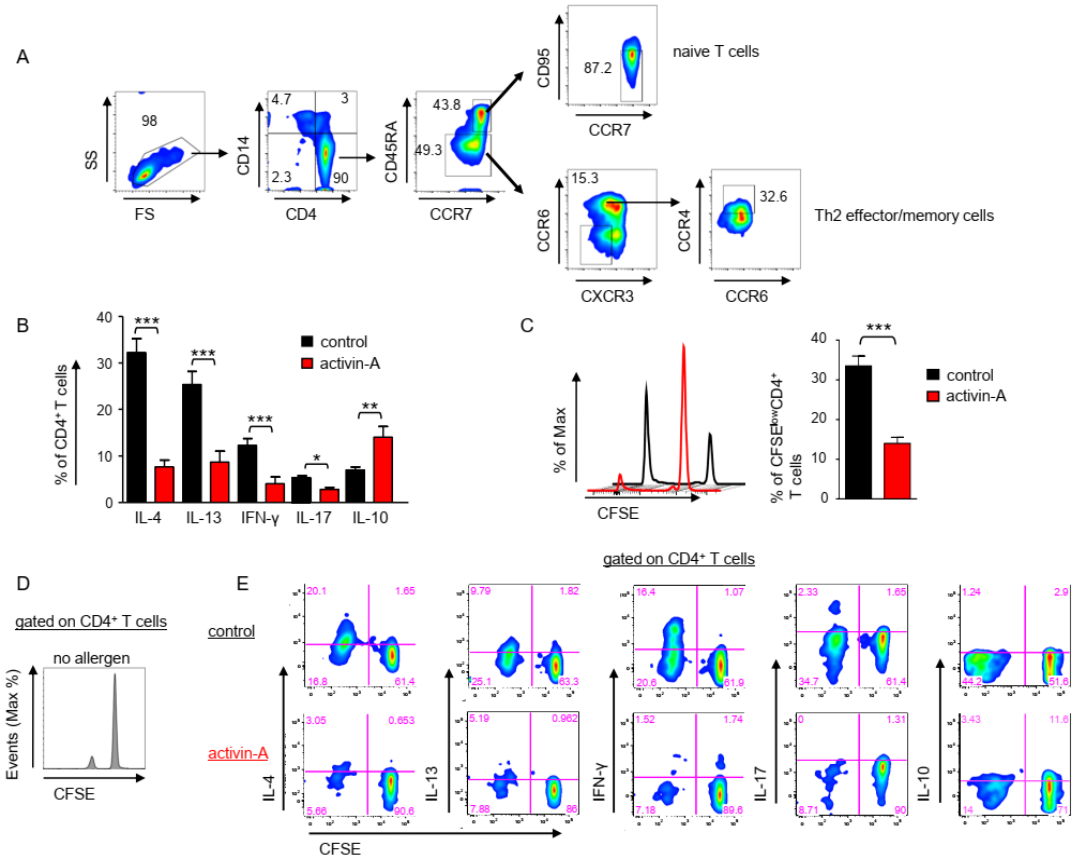

Figure 1. Activin-A generates human T cells that produce copious amounts of IL-10 and reduced levels of Th2-related cytokines during allergen stimulation. (**A**) Experimental protocol used for the isolation of human CD4$^+$ T-cell subsets. Naive and Th2-effector/memory cells were sorted from the PBMC fraction according to the gating strategy depicted. Representative FACS plots are shown. FS, forward scatter; SS, side scatter. (**B**) Human naive CD4$^+$ T cells were sorted from the peripheral blood of atopics, labeled with CFSE and stimulated with allergen (mixed grasses extract)-loaded, mitomycin-treated, CD3-depleted APCs in the presence of PBS (control) or activin-A for 7 d (or 9 d for IL-10) and analyzed by flow cytometry. The percentages of cytokine-producing T cells are shown. Data are expressed as mean ± SEM and are pooled from *n* = 8 independent experiments (*n* = 8 donors). (**C, Left**) Representative FACS plots showing T-cell proliferation gated on CD4$^+$ T cells. Max, maximum. (**C, Right**) Cumulative data pooled from *n* = 6–8 independent experiments (*n* = 8 donors). (**D**) Human naive CD4$^+$ T cells were labeled with CFSE and cultured with mitomycin-treated CD3-depleted APCs. Representative FACS plot of *n* = 8 independent experiments (*n* =8 donors) shows T-cell proliferation. (**E**) Human naive CD4$^+$ T cells were labeled with CFSE and cultured with mitomycin-treated, allergen-loaded, CD3-depleted APCs in the presence of control (PBS) or activin-A for 7 or 9 d (for IL-10). Representative FACS plots depict cytokine-expressing CD4$^+$ T cells and are representative of *n* = 8 independent experiments

(*n* =8 donors). Statistical significance was obtained by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001).

In support of the previous data, activin-A treatment resulted in considerably elevated *IL10* mRNA levels concomitant with low mRNA levels of *IL4*, *IL13*, *IL5*, *IL17* and *IFNγ* (**Fig. 2A**). In addition, IL-10 release was significantly elevated in culture supernatants, whereas IL-4, IL-13, and IFN-γ release was markedly decreased in the presence of activin-A (**Fig. 2B**). Notably, blockade of TGF-β in primary cultures did not restrain the activin-A–mediated increase in the percentages of $CD4^+IL\text{-}10^+$ T cells (**Fig. 2C**). Activin-A–treated T cells remained unresponsive upon secondary restimulation with fresh allergen-loaded APCs (**Fig. 2D and E**), a feature that characterizes Tr1 cells. Interestingly, this activin-A-induced hyporesponsiveness was not associated with T-cell apoptosis/necrosis, as proven by similar frequencies of $7AAD^+Annexin\text{-}V^+CD4^+$ T cells among activin-A–and control-treated T cells (**Fig. 2F**). In fact, addition of IL-2 reversed activin-A antiproliferative effects, similar to recent observations on human T_{FH} cells (311) (**Fig. 2G**). Notably, activin-A–treated $CD4^+$ T cells exhibited Ag specificity since allergen-primed T cells retained their ability to respond upon subsequent exposure to tetanus toxoid, an irrelevant antigen (**Fig. 2H**).

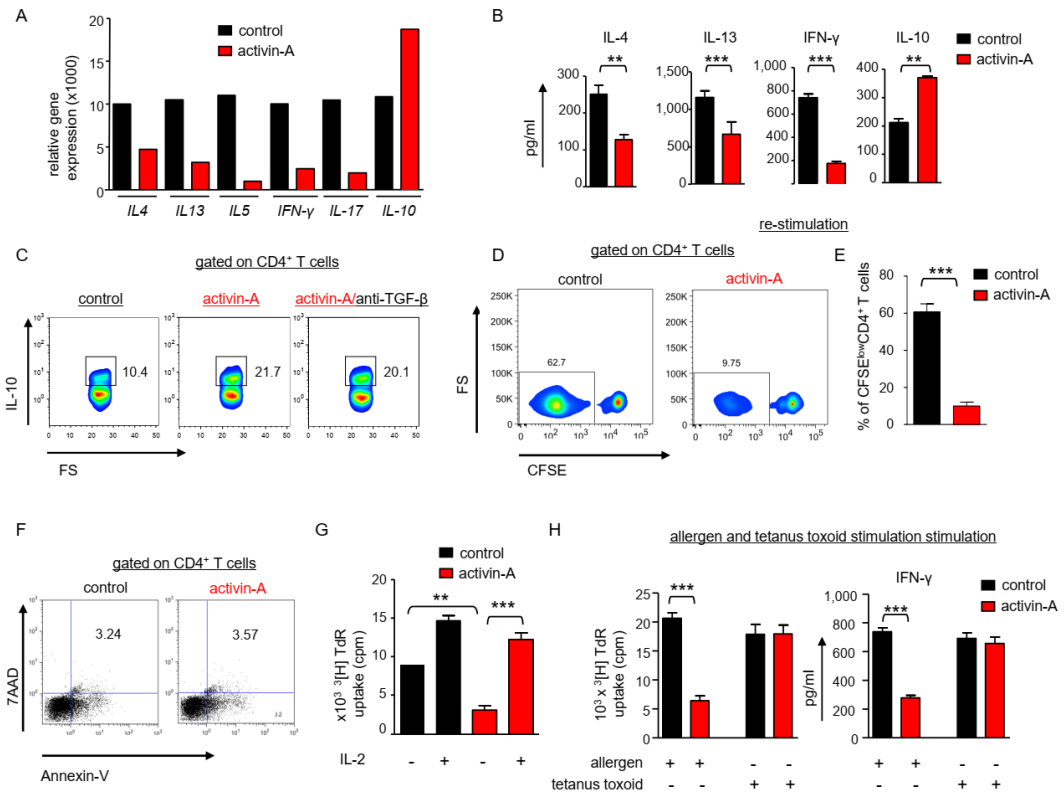

Figure 2. Activin-A-treated T cells remain hyporesponsive following allergen stimulation *in vitro* and exhibit allergen specificity. (**A**) Real-time PCR analysis of cytokine mRNA expression in $CD4^+$ T cells stimulated with mitomycin-treated, allergen-loaded, CD3-depleted APCs in the presence of control or activin-A for 3 d. Results are presented relative to GAPDH and are representative of *n* = 3 separate experiments (*n* = 3 donors). (**B**) Cytokine secretion in culture supernatants is presented. Data are expressed as mean ± SEM of triplicate wells and are pooled from *n* = 8 independent experiments (*n* = 8 donors). (**C**) Human naive $CD4^+$ T cells were stimulated as in A in the presence or absence of an anti–TGF-β antibody. FACS plots are gated on $CD4^+$ T cells and show IL-10–expressing cells. Data are representative of *n* = 3 separate experiments (*n* = 3 donors). (**D**) Tconv or act-A–iTr1 cells were isolated from primary stimulation cultures, labeled with CFSE and restimulated with fresh allergen-loaded, mitomycin-treated APCs for another 7 d. Representative FACS plots are shown. (**E**) Cumulative data, shown as mean ± SEM, from *n* = 3 independent experiments (*n* = 3 donors) are depicted. (**F**) FACS plots showing annexin-V and 7-AAD expressing Tconv or act-A–iTr1 cells. Numbers in plots indicate percentages of annexin-$V^+7AAD^+CD4^+$ T cells (data represent *n* = 2 donors). (**G**) Tconv or act-A–iTr1 cells were restimulated with allergen-loaded, mitomycin-treated APCs and recombinant human IL-2, as indicated. ^{3}[H]Thymidine incorporation is depicted. Data shown are mean ± SEM of triplicate wells and are representative of *n* = 4 independent experiments (*n* = 4 donors). TdR, tritiated thymidine. (**H**) Naive $CD4^+$ T cells were cultured with mitomycin-treated, allergen-loaded APCs in the presence of control (PBS) or activin-A for 14 d, and CD4+ T cells were then isolated and cultured with fresh APCs in the presence of tetanus toxoid for another 6 d. ^{3}[H]Thymidine incorporation and IFN-γ release in secondary cultures are depicted. Data shown are mean ± SEM of triplicate wells and are pooled from *n* = 2 independent experiments (*n* = 2 donors). Statistical significance was obtained by the Student's t test (**P < 0.01; ***P < 0.001).

Activin-A significantly decreased T-cell proliferation and enhanced the frequencies of $CD4^+IL\text{-}10^+$ T cells in the context of anti-CD3/CD28 stimulation in the absence of APCs, pointing to direct effects on $CD4^+$ T cells that are not associated with regulation of APC functions (**Fig. 3A and B**).

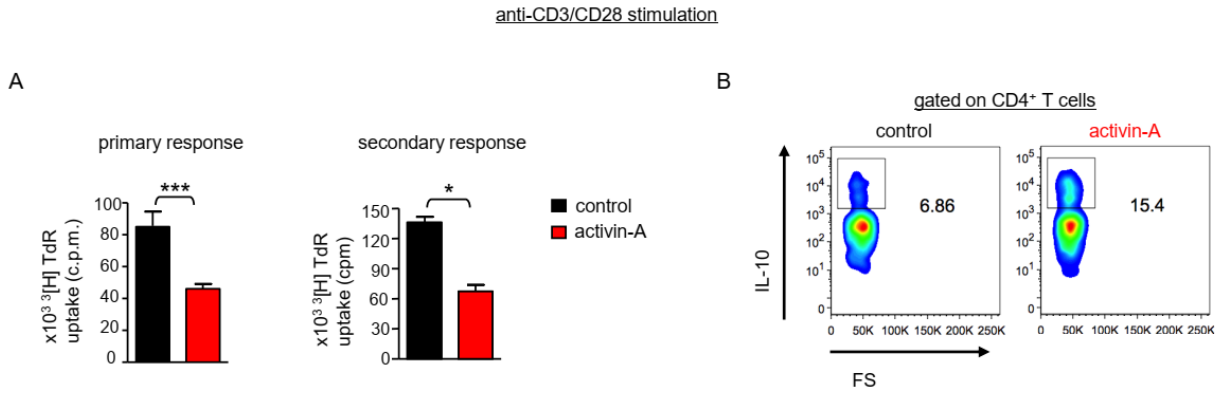

Figure 3. Activin-A suppresses anti-CD3–driven human T-cell responses in an APC-free system. (**A, Left**) Human naive $CD4^+$ T cells were stimulated with anti-CD3/CD28 antibodies in the presence of activin-A (act-A–iTr1 cells) or PBS (Tconv cells) for 7 d. (**A, Right**) Tconv or act-A–iTr1 cells were isolated from primary induction cultures and restimulated with anti-CD3/CD28 antibodies for another 7 d. ^{3}[H]Thymidine incorporation is depicted. Data shown are mean ± SEM of triplicate wells and are pooled from *n* = 4 independent experiments (*n* = 4 donors). (**B**) Representative FACS plots showing IL-10–expressing cells, gated on $CD4^+$ T cells, stimulated as above. Data represent *n* = 4 independent experiments (*n* = 4 donors). Statistical significance was obtained by the Student's t test (*P < 0.05; ***P < 0.001).

To further characterize the immunophenotype of activin-A–treated human T cells, we next investigated the expression of Treg-cell–associated markers. PD-1, GITR, CTLA-4 and Foxp3 were expressed at equivalent levels between activin-A– and control-treated T cells (**Fig. 4A - D**). Still, ICOS levels were markedly increased upon stimulation with activin-A, whereas the T-cell activation markers CD69, CD25, and CD127 were decreased (**Fig. 4A and B**). Regarding the chemokine receptor expression profile, CRTh2, CCR3, CCR4, CCR5, CCR6 and CXCR3 were dampened in activin-A–treated T cells, while CCR7 was increased (**Fig. 5A and B**).

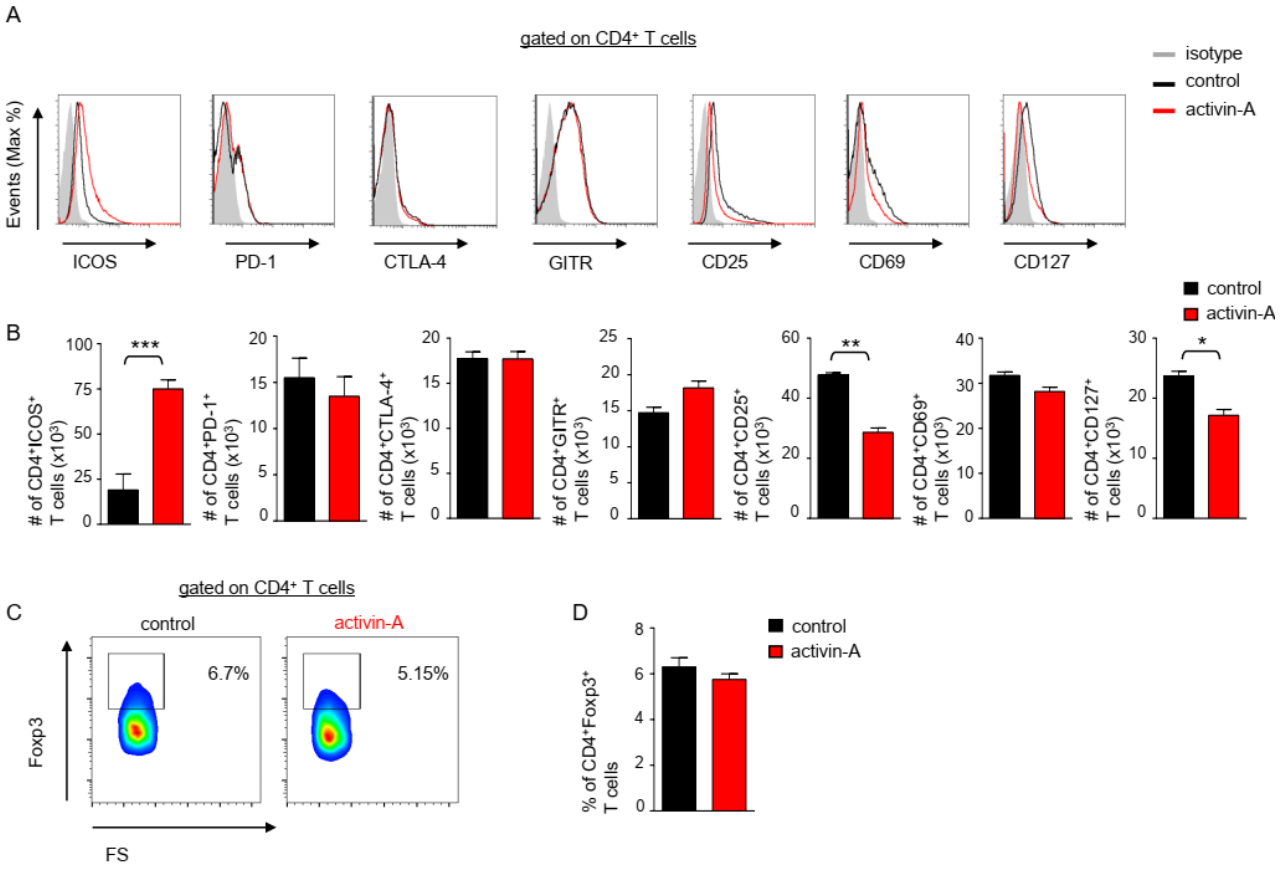

Figure 4. Activin-A-treated T cells express the Treg cell-associated marker ICOS. Representative FACS plots show surface marker expression (**A**) or intracellular Foxp3 levels (**C**) in human $CD4^+$ T cells following stimulation with activin-A or control (PBS) in the presence of allergen-loaded APCs. Cumulative data depict the numbers of Treg-cell markers expressing $CD4^+$ T cells (**B**) or the percentages of $Foxp3^+CD4^+$ T cells (**D**). Data shown are mean ± SEM and are pooled from *n* = 8 independent experiments (*n* = 8 donors). Statistical analysis was performed by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001).

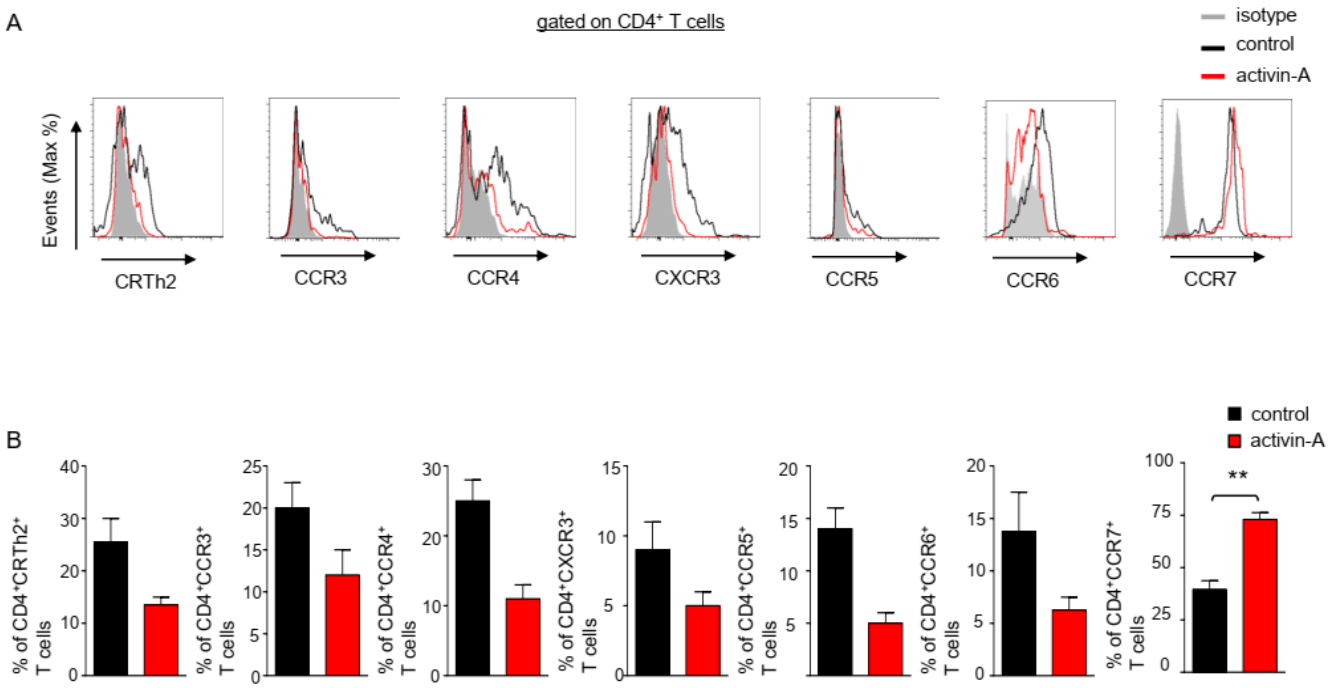

Figure 5. Activin-A-treated T cells express the chemokine receptor CCR7. (**A**) Representative FACS plots showing chemokine receptor levels on human $CD4^+$ T cells following stimulation with activin-A or control (PBS) in the presence of allergen-loaded APCs. (**B**) Cumulative data depict the percentages of chemokine receptor-expressing $CD4^+$ T cells. Data are shown as mean ± SEM and are pooled from *n* = 8 independent experiments (*n* = 8 donors). Statistical significance was obtained by the Student's t test (**P < 0.01)

Interestingly, we observed a striking increase in the percentages of $ICOS^+IL\text{-}10^+CD4^+$ T cells among activin-A–treated T cells, accompanied by markedly elevated *IL10* and *ICOS* mRNA levels (**Fig. 6A and B**). Moreover, a large proportion of $CD4^+ICOS^+IL\text{-}10^+$ T cells among activin-A–treated T cells expressed CD49b and LAG3, recently identified as human Tr1-cell–associated markers (231), concomitant with a significant increase in the mRNAs encoding *LAG3* and *GZMB* (**Fig. 6B and C**). IL-10–induced human Tr1 cells, generated as previously described (231), contained similar percentages of $CD4^+IL\text{-}10^+$ T cells, compared with activin-A–treated $CD4^+$ T cells, whereas LAG-3 and CD49b expression was increased in IL-10–induced Tr1 cells (**Fig. 6D and E**). The expansion of activin-A–treated $CD4^+$ T cells in culture was significantly increased compared with the expansion of IL-10–induced Tr1 cells, which exhibited poor expansion in agreement with previous studies (231) (**Fig. 6F**).

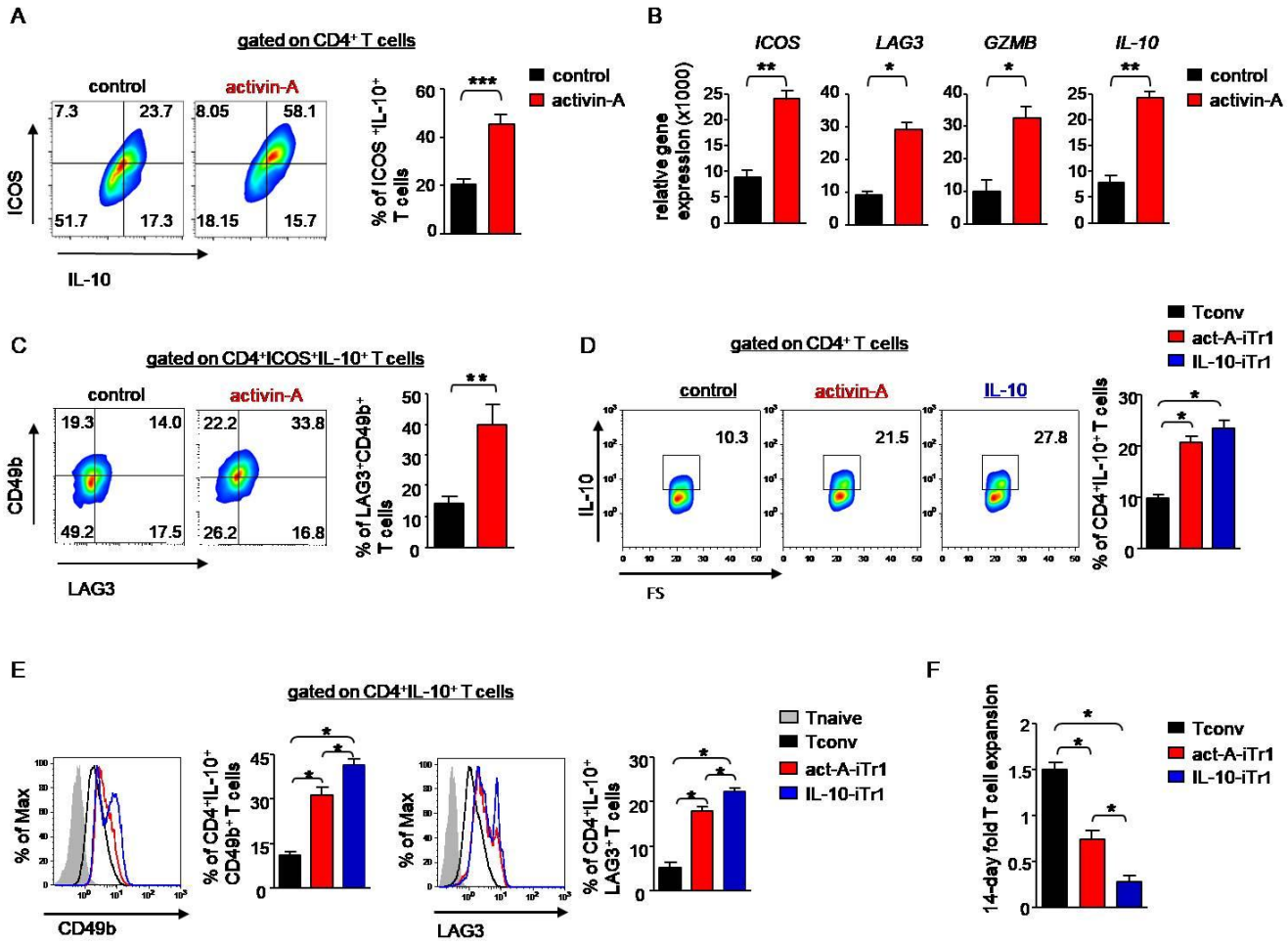

Figure 6. Activin-A drives the differentiation of human Tr1-like cells which show phenotypic similarities with IL-10–induced human Tr1 cells. (**A**) Representative FACS plots showing ICOS and IL-10 expression gated on $CD4^+$ T cells. Cumulative data are pooled from *n* = 6–8 independent experiments (*n* = 8 donors). (**B**) Real-time PCR analysis of *ICOS*, *LAG3*, *GZMB* and *IL10* mRNA levels in $CD4^+$ T cells stimulated as above for 3 d. Results are presented relative to *GAPDH* and are pooled from *n* = 5 separate experiments (*n* = 5 donors). (**C**) Representative FACS plots (Left) and percentages (Right) of $LAG3^+CD49b^+$ cells among $CD4^+IL\text{-}10^+ICOS^+$ T cells are depicted. Data are pooled from *n* = 4–6 separate experiments (*n* = 6 donors). (**D**) Human naive $CD4^+$ T cells were stimulated with anti-CD3/CD28 antibodies in the presence of mitomycin-treated, CD3-depleted APCs and activin-A (act-A–iTr1), PBS (Tconv), or rhIL-10/rhIFN-α (IL-10–iTr1) cells for 7 d. Tconv, act-A–iTr1 or IL-10–iTr1 cells were isolated from primary induction cultures and restimulated as above for another 7 d. (Left) Representative FACS plots show IL-10–expressing cells gated on $CD4^+$ T cells. (Right) Cumulative data are pooled from *n* = 4 independent experiments (*n* = 4 donors). (**E**) Representative FACS plots showing CD49b and LAG3 expression, gated on $CD4^+IL\text{-}10^+$ T cells. Cumulative data are pooled from *n* = 4 independent experiments (*n* =4 donors). (**F**) Fold expansion of cells over 14 d of stimulation. Data are pooled from *n* = 4 independent

experiments (*n* = 4 donors). Statistical significance for figures A, B and C was obtained by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001). Statistical analysis for figures D, E and F was performed by a Mann–Whitney unpaired Student's t test (*P < 0.05).

Notably, even after prolonged (4 weeks) T-cell receptor stimulation, $CD4^+$ T cells stimulated with activin-A did not express Th-effector cytokines, although they maintained high frequencies of $CD4^+IL\text{-}10^+ICOS^+$ T cells (**Fig. 7A–C**). Collectively, these findings reveal that activin-A stimulation restrains allergen-driven human T-cell responses and instructs the differentiation of IL-10–producing Tr1-like cells (act-A-iTr1 cells).

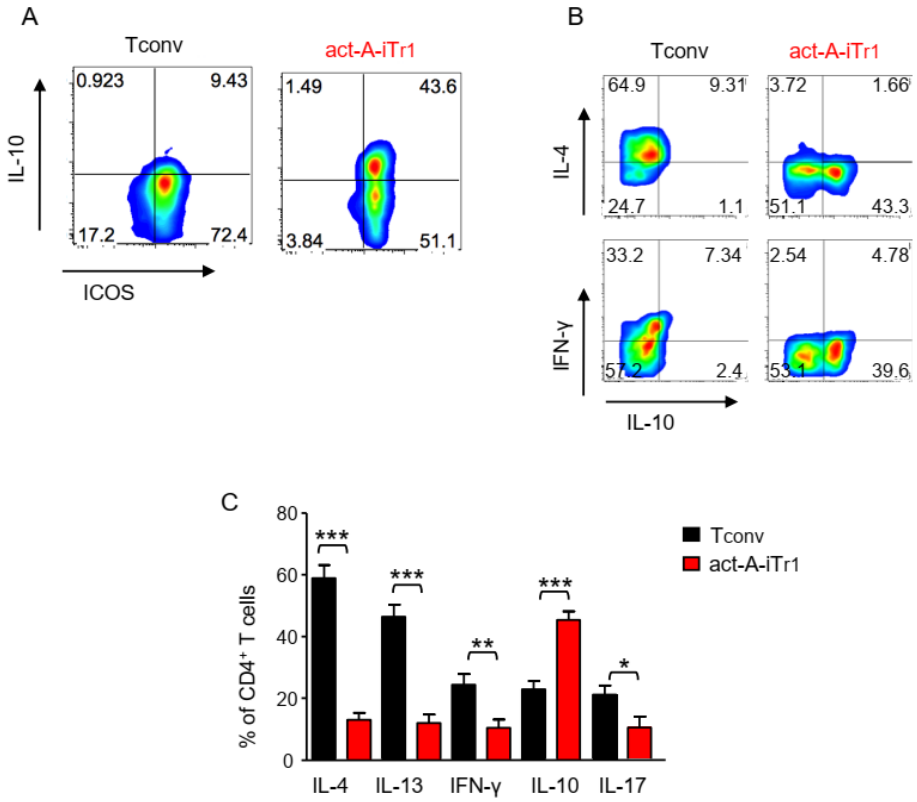

Figure 7. Act-A–iTr1 cells retain their phenotype following prolonged stimulation *in vitro*. Human Tconv or act-A–iTr1 cells were restimulated (once per week) with allergen-loaded APCs and IL-2 for 4 wk. Representative FACS plots show ICOS and IL-10 expression gated on $CD4^+$ T cells (**A**) and intracellular cytokine expression (**B**). Data represent *n* = 5–6 separate experiments (*n* = 6 donors). (**C**) Cumulative data showing the percentages of cytokine-producing $CD4^+$ T cells. Data shown are mean ± SEM and are pooled from *n* = 5–6 independent experiments (*n* = 6 donors). Statistical analysis was performed by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001).

Act-A-iTr1 cells display strong suppressive functions.

On the basis of the aforementioned findings, we next explored whether stimulation with activin-A endows human T cells with immunoregulatory functions. To address this issue, we cocultured CFSE-labeled, activin-A–treated, or control-treated T cells, as potential suppressors, with autologous Cell Trace Violet-labeled naive $CD4^+$ T responders and allergen-primed APCs. Our data showed that activin-A–treated T cells greatly suppressed the proliferation of T responders, even at high T-responder/T-suppressor ratios (**Fig. 8A and B**). Moreover, act-A–iTr1 cells significantly decreased IL-5 and IL-13 release in culture supernatants (**Fig. 8C**). Still, IFN-γ levels remained unaltered, possibly due to activation of a counterbalancing mechanism used by act-A–iTr1 cells to inhibit Th2 cytokine secretion (**Fig. 8D**). In contrast, control-treated T cells did not exhibit discernible suppressor functions (**Fig. 8A–C**). The proliferation of act-A–iTr1 cells remained low in suppressive cultures (**Fig. 8A and B**). Naive T responders cultured with allergen-pulsed APCs alone displayed enhanced proliferative capacity, compared with the other groups (**Fig. 8B**). IL-10–induced human Tr1 cells demonstrated an enhanced suppressive capacity toward T-responder cell proliferation, compared with act-A–iTr1 cells (**Fig. 8E**).

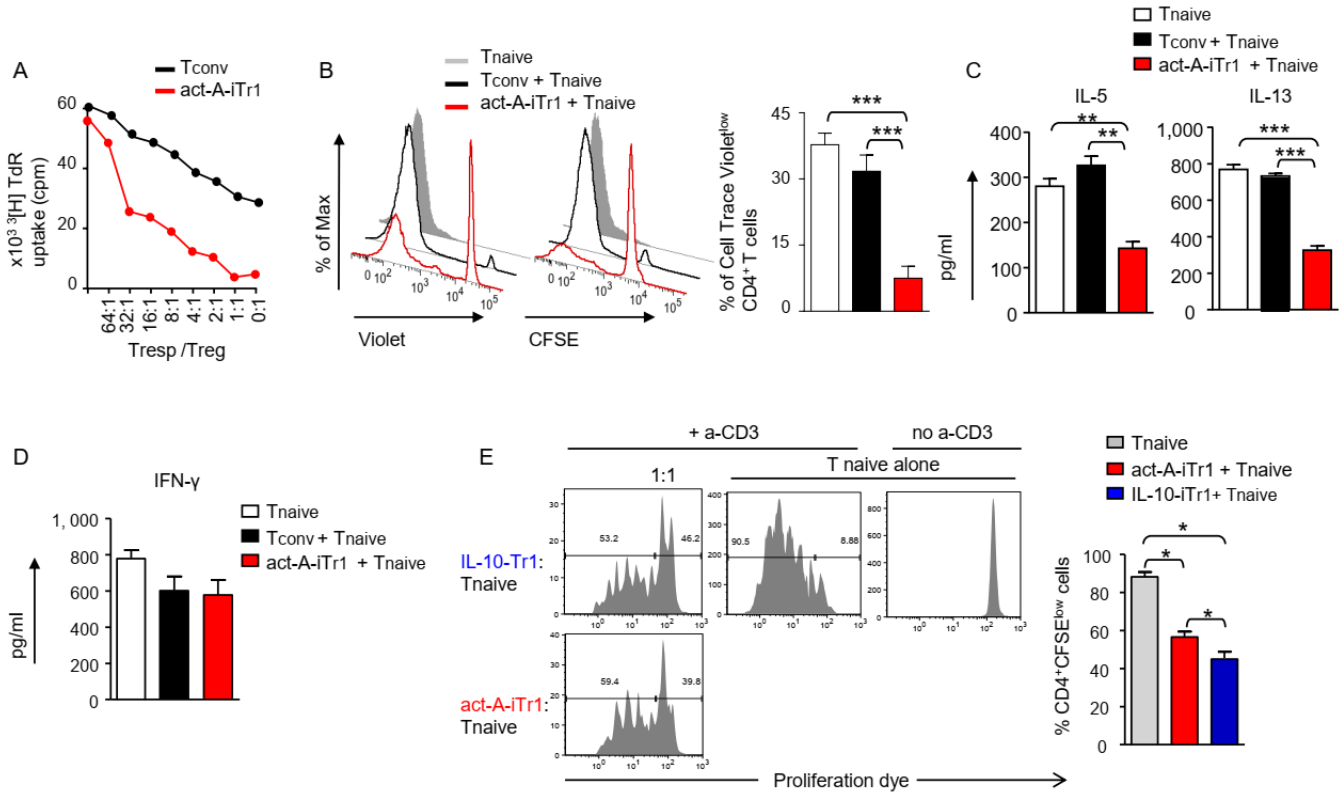

Figure 8. Activin-A–induced human Tr1-like cells display strong suppressive functions. (**A**) Human naive $CD4^+$ T cells were stimulated with allergen-loaded, mitomycin-treated APCs in the presence of PBS (Tconv) or activin-A (act-A-iTr1) cells and T cells were then isolated and cocultured with autologous naive $CD4^+$ T responders (at the indicated ratios) in the presence of allergen-loaded APCs for 4 d. ^{3}[H]Thymidine incorporation is shown. Data are representative of *n* = 3 independent experiments (*n* = 3 donors). TdR, tritiated thymidine. (**B**) Tconv or actA–iTr1 cells were generated as in A, CFSE-labeled, and cocultured with Cell Trace Violet-labeled autologous naive $CD4^+$ T responders and allergen-loaded, mitomycin-treated APCs. Representative FACS plots showing T-cell proliferation (Left) and cumulative data (Right), shown as mean ± SEM, are pooled from *n* = 6–8 separate experiments (*n* = 8 donors). (**C**) Th2 cytokines in culture supernatants are shown. Data are expressed as mean ± SEM of triplicate wells and are pooled from *n* = 6–8 separate experiments (*n* = 8 donors). (**D**) Human naive $CD4^+$ T cells were stimulated with allergen-loaded, mitomycin-treated APCs in the presence of PBS (Tconv cells) or activin-A (act-A–iTr1 cells), and T cells were then isolated and cocultured with autologous naive $CD4^+$ T responders in the presence of allergen-loaded APCs for 4 d. IFN-γ in culture supernatants is shown. Data are expressed as mean ± SEM of triplicate wells and are pooled from *n* = 6–8 separate experiments (*n* = 8 donors). (**E**) Human naive $CD4^+$ T cells were stimulated with anti-CD3/CD28 antibodies in the presence of mitomycin-treated, CD3-depleted APCs and activin-A (act-A–iTr1) or rhIL-10/rhIFN-α (IL-10–iTr1) cells for 7 d. Act-A–iTr1 and IL-10–iTr1 cells were then isolated and cocultured with Cell Trace-CFSE–labeled autologous naive $CD4^+$ T cells in the presence of anti-CD3/CD28 antibodies for 5 d. (Left) Representative FACS plots show T-responder proliferation. (Right) Cumulative data, shown as mean ± SEM, are pooled from *n* = 4 independent experiments (*n* = 4 donors). Statistical analysis for the figures A – D was performed by the Student's t test (A–C and E) (**P < 0.01; ***P < 0.001). Statistical analysis for the figure E was performed by a Mann–Whitney unpaired Student's t test (*P < 0.05).

To elucidate the mechanisms involved in the suppressive functions of human act-A–iTr1 cells, we initially used a transwell system. The suppressive capacity of activin-A–treated $CD4^+$ T cells across a permeable membrane was decreased, but not abolished, in this setting, pointing to the involvement of both cell contact-dependent and independent mechanisms (**Fig. 9A**). Indeed, transfer of the conditioned medium (CM) obtained from the supernatants of act-A–iTr1 cells to fresh $CD4^+$ T-cell/APC cocultures suppressed T-responder proliferation (**Fig. 9B**). Interestingly, IL-10 production was significantly increased in secondary cultures (**Fig. 9B**). Disruption of IL-10 signalling, using an antibody against

the IL-10 receptor (IL-10R) in secondary cultures, partially reversed the inhibitory effects of the CM obtained from act-A–iTr1 cells, emphasizing the involvement of IL-10 in their suppressive functions (**Fig. 9C**). Blockade of IL-10, TGF-β, or ICOS signalling in secondary suppressive cultures partly impaired the suppressive capacity of act-A–iTr1 cells, whereas blockade of activin-A did not significantly affect their functions (**Fig. 9D**). Concomitant blocking of IL-10, TGF-β, and ICOS signalling further reduced act-A–iTr1 cell inhibitory functions, compared with blocking each pathway alone or combinations of two; however, the data did not reach statistical significance (**Fig. 9D**). Overall, these findings reveal that human act-A–iTr1 cells exhibit robust suppressive functions against naive and Teff-cell responses to allergen through IL-10–, TGF-β–, and ICOS-dependent pathways.

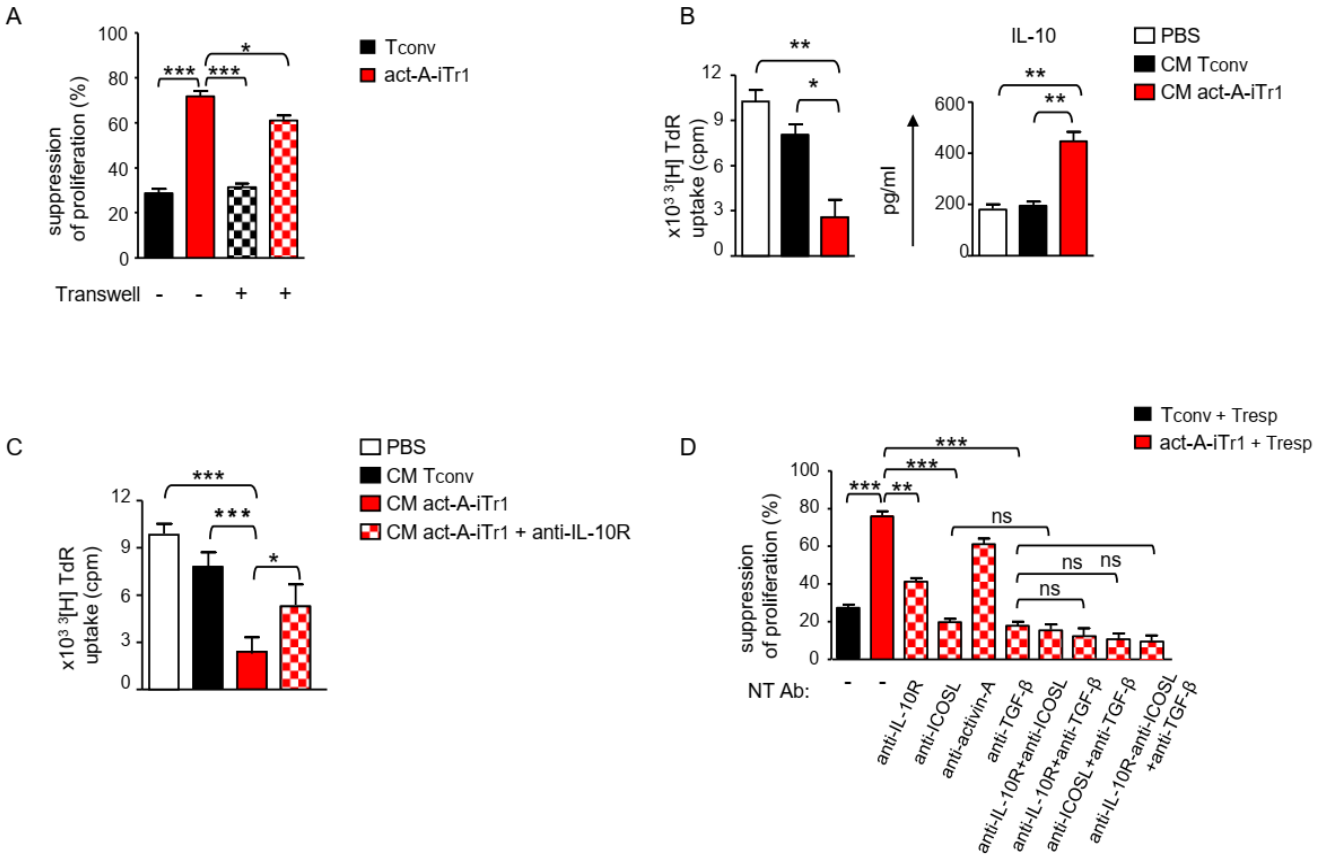

Figure 9. IL-10, ICOS and TGF-β are involved in the *in vitro* suppressive functions of human act-A–iTr1 cells. (**A**) Tconv or act-A–iTr1 cells and allergen-loaded APCs were cultured in a transwell system across from cocultures of autologous naive $CD4^+$ T cells and APCs. Suppression of T-responder cell proliferation is shown. Data are expressed as mean ± SEM of triplicate wells and are pooled from *n* = 3 independent experiments (*n* = 3 donors). (**B**) CM from Tconv or act-A–

iTr1 culture supernatants was added to naive $CD4^+$ T-cell/APC cocultures. ^{3}[H]Thymidine incorporation and IL-10 release are depicted. Data are expressed as mean ± SEM of triplicate wells and are pooled from *n* = 4–6 separate experiments (*n* = 4 donors). (**C**) CM from Tconv or act-A–iTr1 culture supernatants was added to naive $CD4^+$ T-cell/APC cocultures, as indicated. ^{3}[H]Thymidine incorporation is depicted. Data are expressed as mean ± SEM of triplicate wells and pooled from *n* = 3–4 experiments (*n* = 4 donors). (**D**) Tconv or act-A–iTr1 cells were cocultured with autologous T responders and allergen-loaded, mitomycin-treated APCs, as indicated. Suppression of T-cell proliferation is shown. Data are shown as mean ± SEM of triplicate wells and summarize *n* = 5 separate experiments (*n* = 5 donors). Statistical analysis was performed by a nonparametric (Mann–Whitney) unpaired Student's t test (A and C) and the Student's t test (B and D) (*P < 0.05; **P < 0.01; ***P < 0.001). ns, not significant; NT Ab, neutralizing antibody.

IRF4 is activated in human act-A–iTr1 cells and forms multipartite complexes with AhR in the ICOS promoter.

We next attempted to decipher the transcriptional pathways contributing to activin-A–induced human Tr1-cell differentiation. Investigation of the expression of the major Th-cell lineage–associated transcription factors *TBX21*, *GATA3*, and *RORC* at an earlier time point (day 3) during T-cell differentiation demonstrated that mRNA levels were similar or slightly decreased in act-A–iTr1 cells, whereas *FOXP3* was moderately increased, compared with Tconv cells (**Fig. 10A**). Interestingly, act-A–iTr1 cells expressed increased transcripts of the Tr1-cell–associated transcription factors *MAF* (encoding c-Maf) and *AHR* along with its major gene target, xenobiotic metabolizing enzyme cytochrome P450, encoded by *CYP1A1* (337) (**Fig. 10A**). Moreover, activin-A stimulation induced a marked up-regulation in the mRNA levels of *IRF4*, a transcription factor not previously associated with human or mouse Tr1 cells (**Fig. 10A**). Inhibition of activin-A signalling, using an anti-ALK4 antibody or the Smad3 inhibitor (SIS3), decreased activin-A–induced gene transcription, pointing to the involvement of its canonical signalling pathways (**Fig. 10B**). Confocal microscopy studies demonstrated that activin-A stimulation induced an enhanced translocation of IRF4, c-Maf, and AhR into the nucleus of human $CD4^+$ T cells (**Fig. 10C - E**).

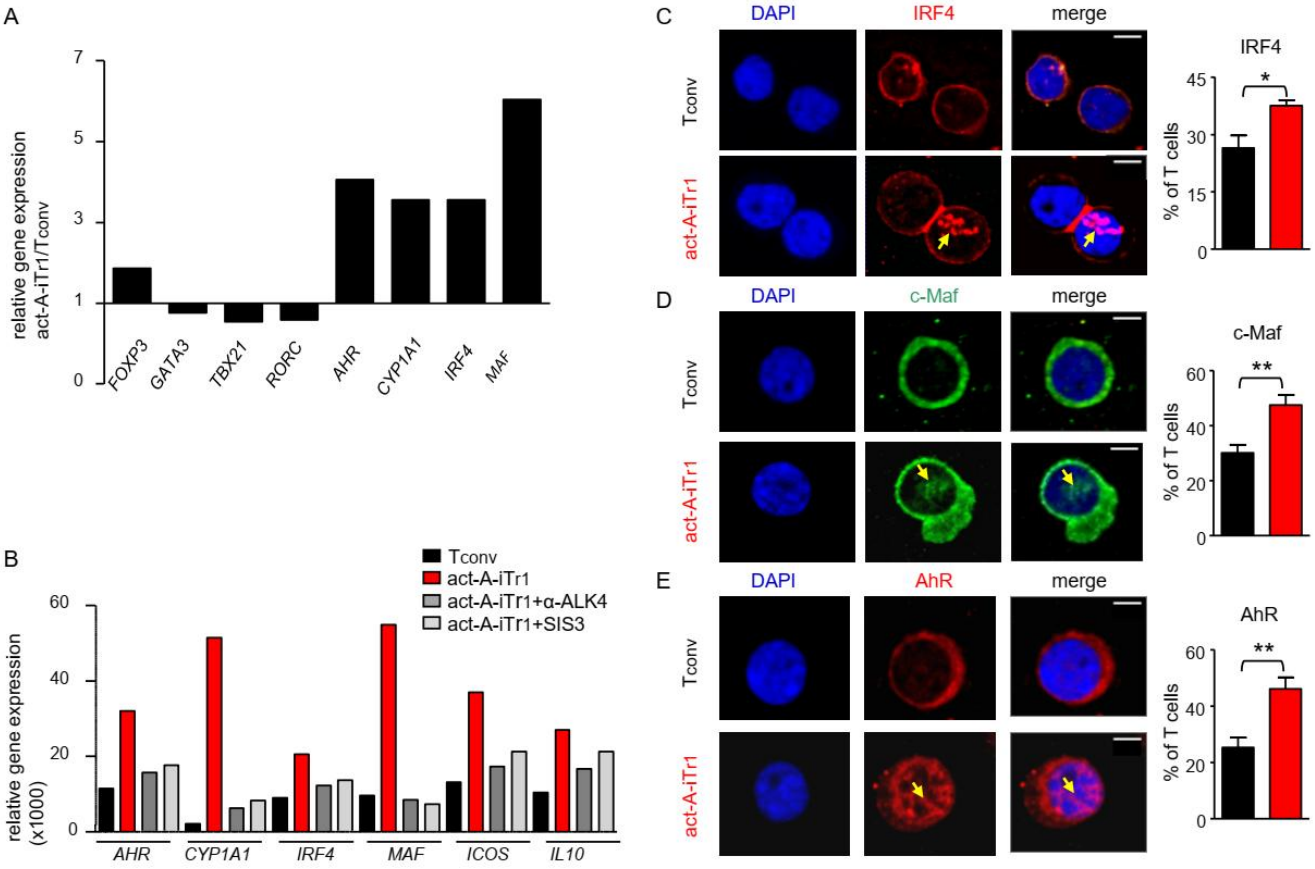

Figure 10. IRF4, AhR, and c-Maf are activated upon stimulation of human CD4$^+$ T cells with activin-A. (**A**) Human naive CD4$^+$ T cells were stimulated with allergen-loaded, mitomycin-treated APCs in the presence of PBS or activin-A for 3 d. Real-time PCR analysis of the indicated genes is shown. Results are representative of *n* = 4 separate experiments (*n* = 4 donors) and were normalized to *GAPDH*. (**B**) Tconv or act-A–iTr1 cells were generated as above with an anti-ALK4 blocking antibody or the Smad3 inhibitor, SIS3. Real-time PCR results are shown relative to *GAPDH*. Data are representative of *n* = 4 independent experiments (*n* = 4 donors). (**C–E**) Confocal microscopy images of Tconv or act-A–iTr1 cells stained with fluorescently conjugated anti-AhR, anti-IRF4, and anti–c-Maf antibodies. Nuclei are stained blue with DAPI. (Scale bars: IRF4 images, 4 μm; AhR and c-Maf images, 2 μm). Cumulative data showing the percentages of AhR$^+$, IRF4$^+$, or c-Maf$^+$ T cells were pooled from *n* = 4 independent experiments (*n* = 4 donors). Statistical analysis was performed by a nonparametric (Mann–Whitney) unpaired Student's t test (*P < 0.05; **P < 0.01).

Comparison of the global gene expression profile of human actA–iTr1 cells with the global gene expression profile of Tconv cells, using RNA-sequencing (RNA-seq) analysis,

revealed notable differences in their transcriptome profile, with 513 genes being significantly differentially regulated, 200 of which were down-regulated and 313 of which were up-regulated (**Fig. 11A and B**). Volcano plot visualization demonstrated that the transcriptional signature of act-A–iTr1 cells was characterized by substantial changes compared with Tconv cells, with nearly 50% of the total differentially expressed genes showing a difference in expression greater than two fold [log2 (fold change [FC]) < −1 or > 1] (**Fig. 11A**). These genes belonged to biologically important gene families, including chemokines/cytokines or their receptors, transcription factors, surface receptors, and Treg-cell markers (**Fig. 11B**). Interestingly, several genes, including *LAG3*, *IRF4*, *GZMB*, *CCL1*, *CSF2*, *DGKI*, *CD70*, and *EBI3*, were enriched in both human actA–iTr1 cells and IL-10–induced Tr1 cells (231), whereas others, such as *CCL18*, *WNT2B*, *MMP15*, and *CD163*, were downregulated in both groups (**Fig. 11C**).

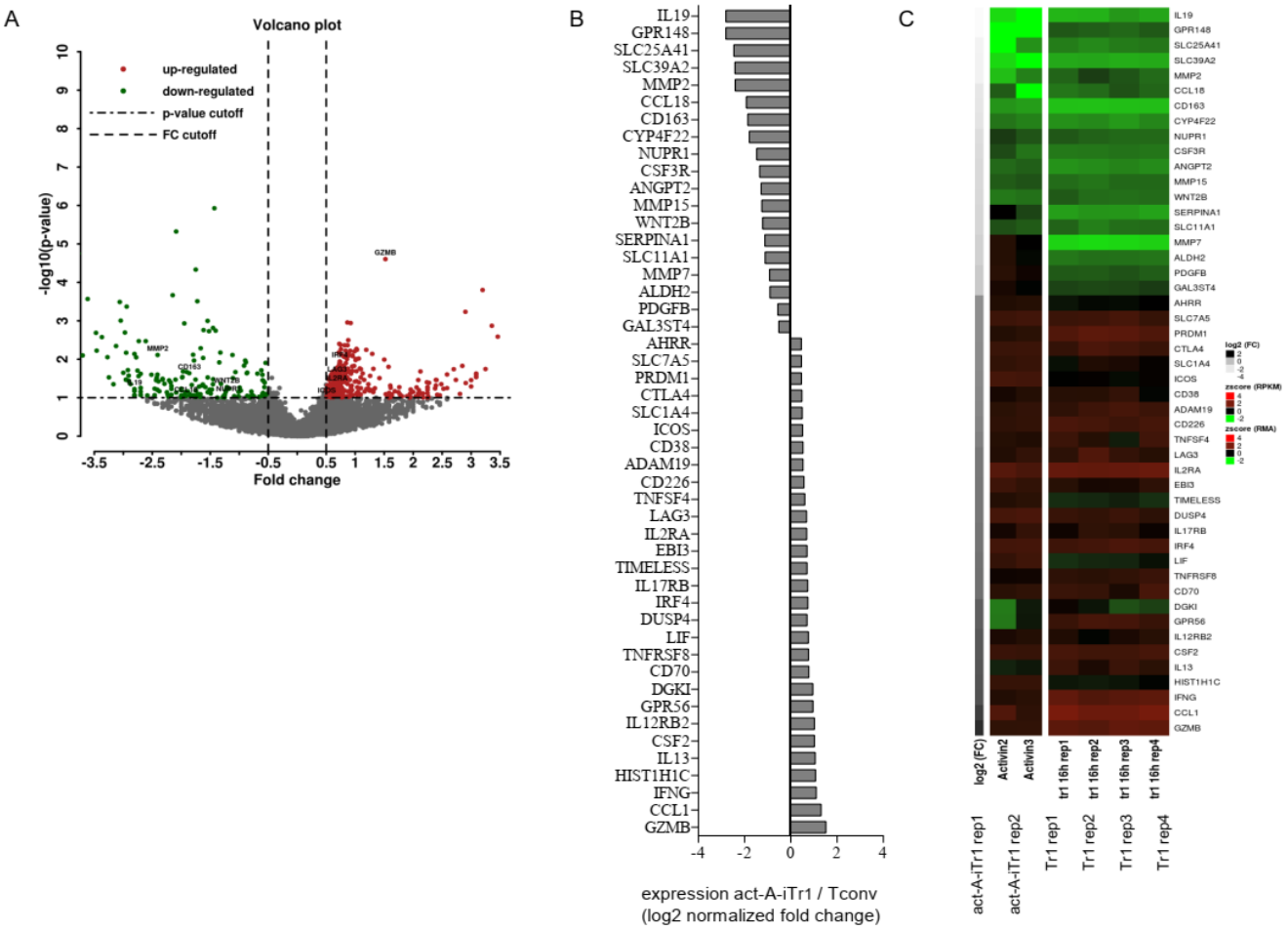

Figure 11. Transcriptional signature of human act-A–iTr1 cells. (**A**) Changes in gene expression (x axis) and statistical significance (y axis) in human act-A–iTr1 cells relative to Tconv cells, presented as a volcano plot [red: P < 0.1 and log2 (FC) > 0.5, green: P < 0.1 and log2 (FC) < −0.5]. (**B**) Differential expression of 48 immune response-related genes in act-A–iTr1 cells relative to Tconv cells. (**C**) Comparison of the transcriptional profile of act-A–iTr1 cells with the transcriptional profile of human Tr1 cells (231). The genes shown in B, which were differentially expressed in act-A–iTr1 cells, were used for the comparison. Genes were sorted by increasing RNA-seq log2 (FC), revealing similar patterns of expression between the two platforms. Regression analysis showed a strong correlation between the average reads per kilobase per million mapped reads (RPKM) values (RNA-seq) and the average RMA values (microarrays) (P < 0.001). All expression values were scaled to have a mean of zero and SD of 1.

In view of the aforementioned findings, we hypothesized that activin-A up-regulates *IL10* and *ICOS* expression in human $CD4^+$ T cells through IRF4-, AhR-, and c-Maf–dependent pathways. To examine this hypothesis, we initially performed bioinformatics analysis to decipher whether these transcription factors have putative binding sites in human *IL10* and *ICOS* gene loci. Indeed, we identified two IRF4-binding sites [IFN-stimulated response elements (ISREs)] in the *IL10* promoter and three ISREs in the *ICOS* locus (**Fig. 12A and B**). In agreement with previous studies, we found a putative AhR-binding site [xenobiotic response element (XRE-1)] and a c-Maf site [MAF-recognition element (MARE-1)] within the *IL10* promoter, which were in high proximity and partially overlapping (337) (**Fig. 12A**). Moreover, we identified a c-Maf–binding site (MARE-2, −150 bp) in the *IL10* promoter and two XRE and five MARE sites in the *ICOS* locus (Fig. **12A and B**).

We next focused on investigating whether activin-A signalling in $CD4^+$ T cells induces the binding of IRF4, AhR, and c-Maf to their response elements in *IL10* and *ICOS* loci. Chromatin immunoprecipitation (ChIP) assays revealed a marked enrichment of IRF4 binding to the ISRE (−318 bp) site in the *IL10* promoter in act-A–iTr1 cells, compared with Tconv cells (**Fig. 12C**). In addition, AhR and c-Maf bound on the XRE-1 (−230 bp) and MARE-1 (−200 bp) sites, respectively, in the *IL10* promoter in human act-A–iTr1 cells (**Fig. 12C**). In contrast, AhR showed no discernible binding to the XRE-1 element in the *IL10* promoter in Tconv cells, whereas c-Maf bound moderately to its MARE-1 site (**Fig. 12C**). Pertinent to the *ICOS* locus, our data showed that IRF4 exhibited great enrichment to its

ISRE (−1.3 kb) site, whereas AhR bound to its respective XRE site (−1,440 bp) in actA–iTr1 cells (**Fig. 12D**). Modest binding of c-Maf to the MARE site (−1,330 bp) in the *ICOS* locus was also observed (**Fig. 12D**). The ability of IRF4 and AhR to bind to the *ICOS* promoter in human T cells in response to activin-A stimulation, and the proximity of their binding sites, prompted us to explore whether they form transcriptional complexes at the *ICOS* locus. To address this issue, we performed sequential ChIP analysis of actA–iTr1 cells or Tconv cells. Remarkably, our findings identified the presence of IRF4/AhR complexes in the *ICOS* promoter in act-A–iTr1 cells, but not in Tconv cells (**Fig. 12E**).

To dissect the molecular interactions of IRF4 with AhR further, we performed in silico analysis of putative protein–protein interactions between the two transcription factors. Because a description of the solved structures of the full-length sequences of these transcription factors was currently lacking, reliable models were constructed and submitted for molecular docking. Interestingly, our studies revealed that IRF4 and AhR can form a tripartite molecular complex together with the AhR-binding partner, ARNT (338) (**Fig. 12F**). This complex was highly stable thermodynamically because after the minimization of its energy, it was characterized by an energy value of −260685.0 KJ/mol. Moreover, this structure remained stable throughout the duration (10 ns) of the molecular dynamics simulation studies. Additionally, when this energetically minimized complex was superimposed with the initial docking output, the rmsd values between the carbon α-atoms were extremely low (Cα rmsd values at 0.52 Å). Together, this evidence supports the notion that the proposed docking result of IRF4/AhR is highly reliable and exhibits high thermodynamic stability.

These results collectively demonstrate that activin-A induces the activation of IRF4, AhR, and c-Maf in human $CD4^+$ T cells, which bind in their response elements in *IL10* and *ICOS* promoters through the formation of multipartite transcriptional complexes.

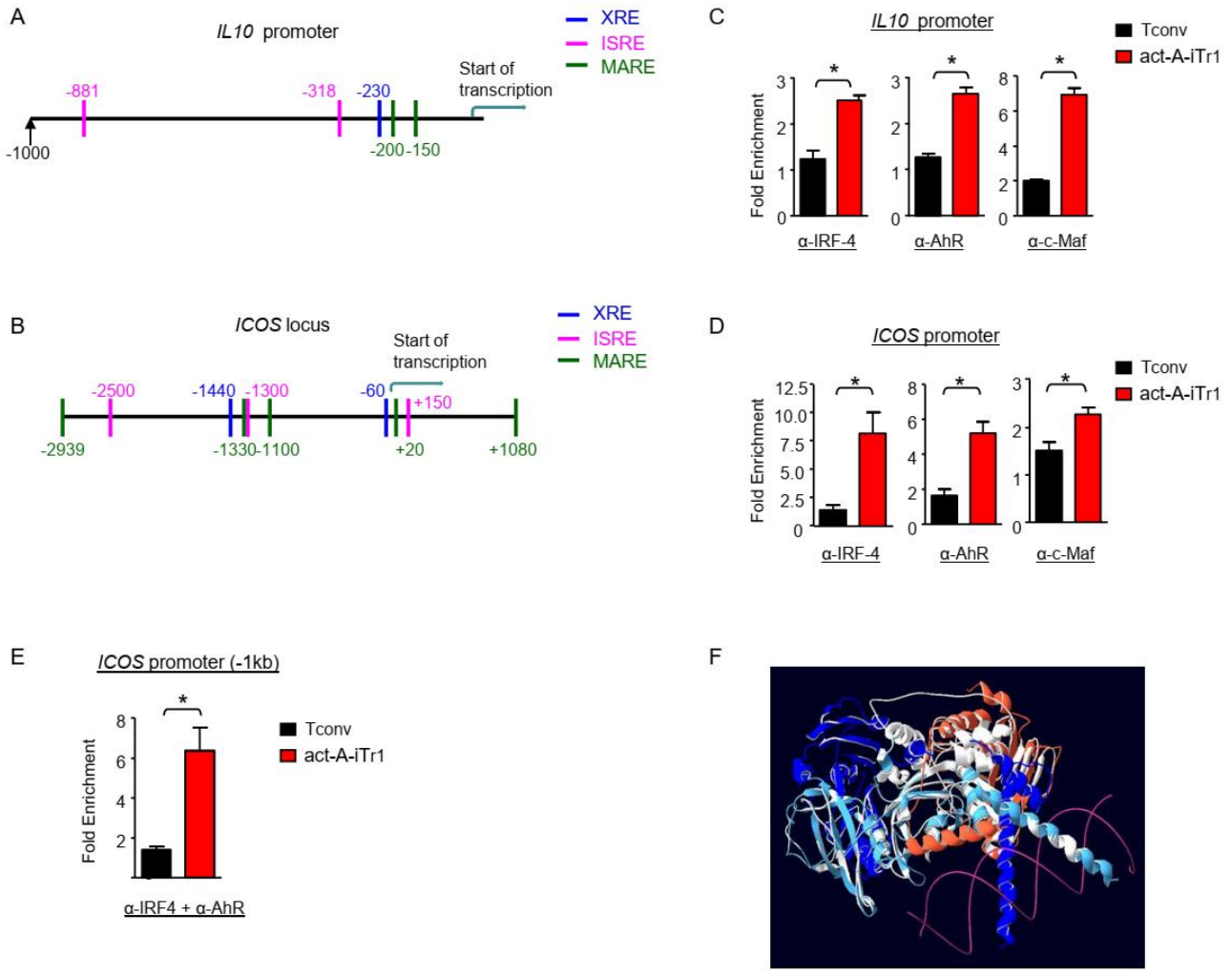

Figure 12. IRF4 assembles into a complex with AhR in the ICOS promoter in human act-A–iTr1 cells. Schematic representation of the human IL10 promoter (**A**) and ICOS locus (**B**); the c-Maf, AhR, and IRF4 putative binding sites are shown. Positions are given in base pairs relative to the transcription start site. (**C and D**) Tconv or act-A–iTr1 cells were generated following stimulation with anti-CD3/CD28 antibodies in the presence of PBS or activin-A, respectively. ChIP analysis of IRF4, AhR, and c-Maf binding in the IL10 (**C**) and ICOS (**D**) promoters is shown. Data are presented as ± SEM and are pooled from two independent experiments (*n* = 4 donors, two donors per experiment). (**E**) Tconv or act-A–iTr1 cells were generated as in C. Sequential ChIP analysis demonstrates IRF4 and AhR co-binding in the ICOS promoter. Data are presented as mean ± SEM and are pooled from two independent experiments (*n* = 4 donors, two per experiment). (**F**) Computational model of the putative protein–protein interactions of IRF4 and AHR is shown in white superimposed with the solved structures used for the homology modelling (PDB ID codes 3DSH and 4ZPR). The template chain of the IRF4 model is shown in orange. In the 4ZPR structure, the ARNT chain is shown in blue, the AHR template chain is shown in light blue, and the

DNA is shown in magenta. Statistical analysis was performed by a nonparametric (Mann–Whitney) unpaired Student's t test (*P < 0.05).

IRF4 and AhR signalling is essential for act-A–iTr1 cell differentiation.

To obtain functional evidence that IRF4 is involved in activin-A–mediated differentiation of human Tr1-like cells, we used *IRF4*-specific shRNA (sh*IRF4*) to target IRF4 expression in T cells directly. Briefly, $CD4^+$ T cells transduced with lentiviral particles containing constructs of GFP-shRNA for IRF4 were sorted based on viability and GFP expression, and used in *in vitro* suppression assays. Indeed, knockdown of IRF4 decreased *IRF4*, *ICOS*, and *IL10* mRNA levels in $CD4^+$ T cells in response to activin-A (**Fig. 13A**). The percentages of LAG-3^+CD49b^+ T cells were also decreased, compared with sh-scrambled–treated, activin-A–stimulated T cells (**Fig. 13B**). *IRF4* silencing also compromised the suppressive functions of act-A–iTr1 cells and partly reversed their hypoproliferative state (**Fig. 13C and D**).

Since IRF4 assembles into a complex with AhR in human T cells in response to activin-A, we next explored the role of AhR in activin-A–induced responses using CH-223191, a potent AhR antagonist (337). Pharmacological blockade of AhR nearly abrogated activin-A–induced AhR activation, as shown by a marked down-regulation of *CYP1A1* mRNA (**Fig. 13E**). Interestingly, inhibition of AhR signalling partly reversed activin-A–induced *IL10*, *ICOS*, and *MAF* up-regulation and significantly decreased the frequencies of $CD4^+ICOS^+IL\text{-}10^+$ T cells (**Fig. 13E**). In addition, disruption of AhR signalling impaired the suppressive capacities of act-A–iTr1 cells, accompanied by a significant reduction in IL-10 release (**Fig. 13F**). In summary, these results provide compelling evidence that IRF4 and AhR are critically involved in activin-A–induced differentiation of human Tr1-like cells.

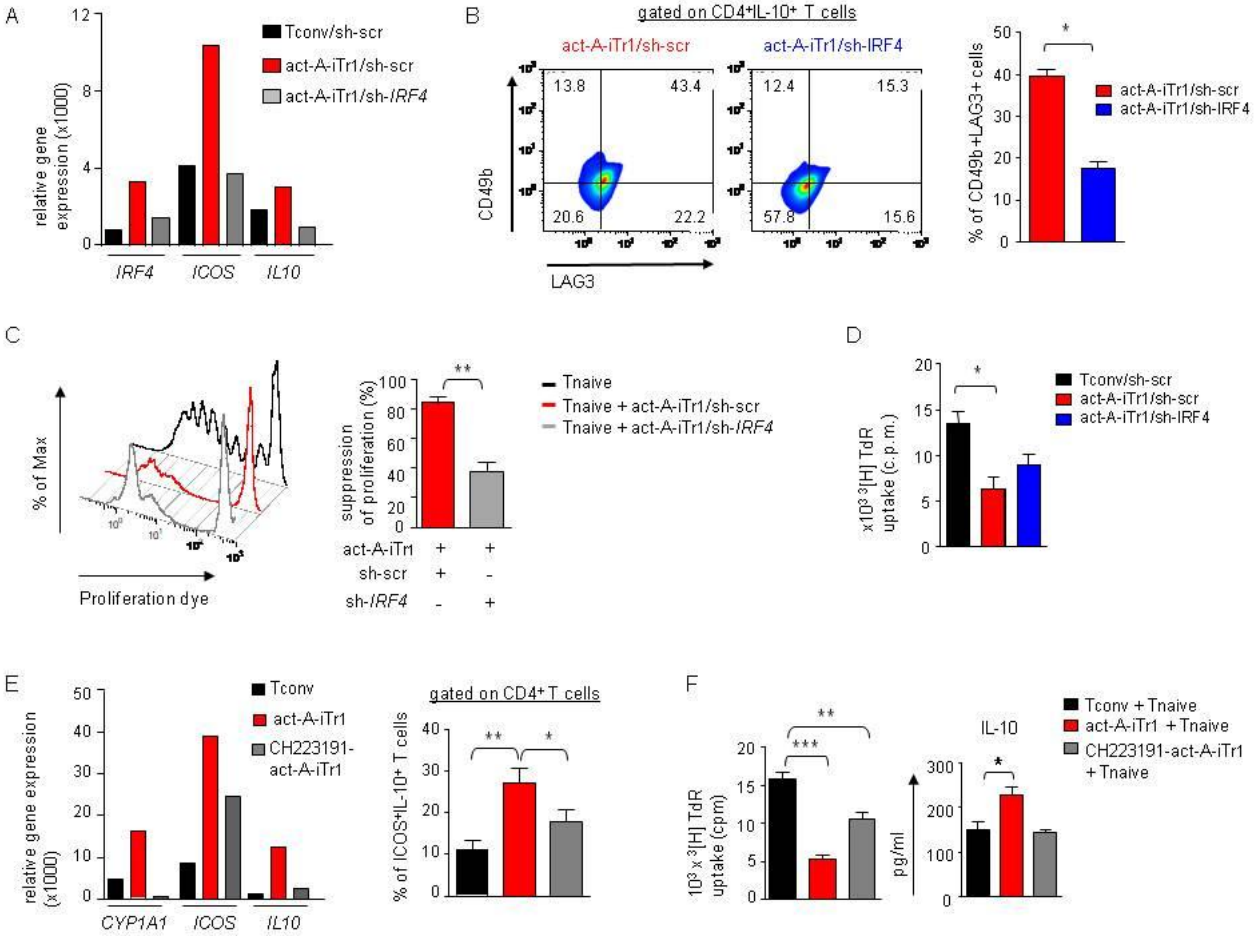

Figure 13. IRF4 and AhR drive activin-A–induced human Tr1 cell differentiation. (**A**) Tconv or act-A–iTr1 cells were stimulated with APCs and allergen, and transduced with shRNA against human IRF4 (sh-IRF4) or a scrambled nontargeting control RNA sequence (sh-scr). Real-time PCR analysis is shown. Data are relative to *GAPDH* and represent *n* = 3 independent experiments (*n* = 3 donors). (**B**) Tconv or act-A–iTr1 cells were stimulated and transduced as above. Representative FACS plots (Left) and cumulative data (Right) show LAG3 and CD49b expression, gated on GFP^+CD4^+ T cells representative of *n* = 4 separate experiments (*n* = 4 donors). (**C**) Transduced act-A–iTr1 cells were cocultured with naive Cell Trace Violet labeled $CD4^+$ T cells and allergen-loaded APCs. Representative FACS plots of T-responder cell proliferation are shown. Cumulative data, shown as mean ± SEM, depict suppression of proliferation (%) and are pooled from *n* = 4 separate experiments (*n* = 4 donors). (**D**) ^{3}[H]Thymidine incorporation is depicted. Data shown are mean ± SEM of triplicate wells and are pooled from *n* = 4 independent experiments (*n* = 4 donors). (**E**) Tconv or act-A–iTr1 cells were generated following stimulation with allergen-loaded APCs in the presence or absence of the AhR antagonist CH223191. (Left) Real-time PCR analysis is shown. Results are relative to *GAPDH* and represent *n* = 4 separate experiments (*n* = 4 donors). (Right) Cumulative data showing the percentages of $CD4^+ICOS^+IL\text{-}10^+$ T cells summarize four

independent experiments (*n* = 4 donors). (**F**) Tconv or act-A–iTr1 cells, generated as in E, were isolated and cocultured with naive $CD4^+$ T cells and allergen-loaded APCs. ^{3}[H]Thymidine incorporation and IL-10 release are shown. Data are mean± SEM of triplicate wells and are pooled from *n* = 4 independent experiments (*n* = 2 donors). Statistical analysis for the figures B and D was performed by a nonparametric (Mann–Whitney) unpaired Student's t test (*P < 0.05). Statistical significance for the figures C, E and F was obtained by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001).

In Vivo administration of human act-A–iTr1 cells prevents the development of allergic airway disease.

Considering ongoing efforts to use adoptive Treg-cell transfers as a therapy for immune-mediated human diseases, the issue of their *in vivo* stability is of critical clinical importance. To explore whether act-A–iTr1 cells can maintain their suppressive functions upon transfer *in vivo*, we used a well-established humanized mouse model of allergic airway inflammation (341, 342). Briefly, we transferred allergen-primed human act-A–iTr1 or Tconv cells, along with autologous APCs, into immunodeficient NOD-SCID mice and examined their responses upon immunization and aerosol challenge with allergen (**Fig. 14A**). Indeed, human $CD4^+$ T cells isolated from the lymph nodes (LNs) of NOD-SCID mice reconstituted with act-A–iTr1 cells were able to suppress the proliferation and Th2 cytokine release by cocultured naive T responders in *ex vivo* suppression assays (**Fig. 14B**). In contrast, $CD4^+$ T cells obtained from the LNs of Tconv-cell–reconstituted mice did not exhibit discernible suppressor functions (**Fig. 14B**). These data indicate that act-A–iTr1 cells can retain their potent inhibitory functions against other T-cell responses even following *in vivo* transfer into a highly inflammatory milieu.

Subsequently, we investigated whether *in vivo* administration of human act-A–iTr1 cells in a preventive protocol could confer protection against features of experimental asthma (**Fig. 14C**). Indeed, adoptive transfer of act-A–iTr1 cells significantly decreased AHR to metacholine, a cardinal asthma manifestation, induced by co-transferred human Teff cells (**Fig. 14D**). Importantly, administration of act-A–iTr1 cells significantly dampened inflammatory cell influx in the BALF and prevented the development of pulmonary inflammation (**Fig. 14E and F**). Tconv cells did not suppress airway inflammation or AHR.

In fact, allergic responses were enhanced compared with recipients of T responders alone (**Fig. 14D-F**). Considering the key role of IL-10 in the suppressive functions of human act-A–iTr1 cells *in vitro*, we examined its effects *in vivo* through administration of an anti–IL-10R antibody (or an isotype-matched control) (**Fig. 14C**). Disruption of IL-10 signalling partly reversed the suppressive effects of act-A–iTr1 cells on AHR and lung inflammation, but did not affect inflammatory cell responses in the BAL, compared with mice that received act-A–iTr1 cells but were treated with the isotype-matched control antibody (**Fig.14D-F**).

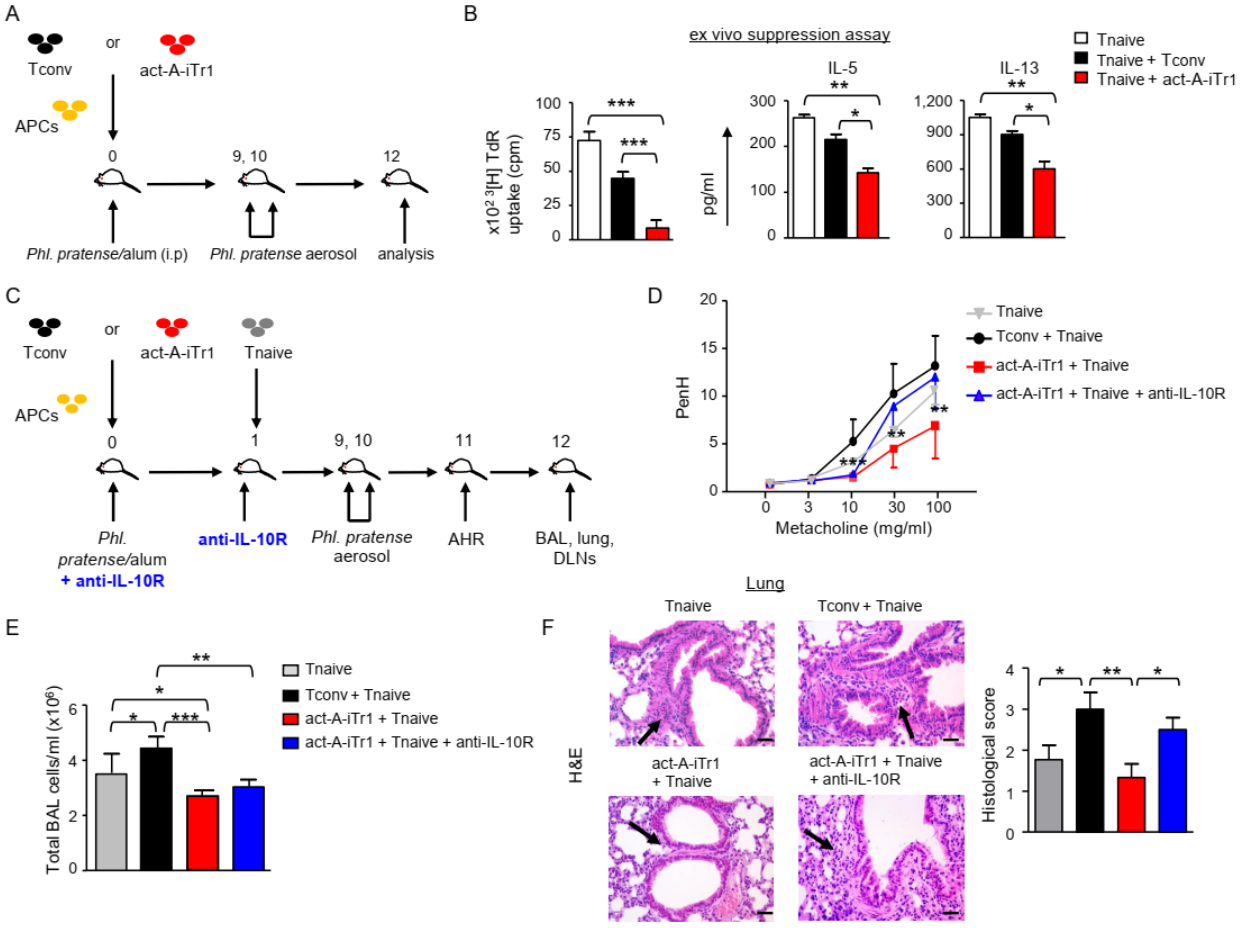

Figure 14. *In vivo* administration of human act-A–iTr1 cells prevents allergic airway disease partly through IL-10-dependent mechanisms. Schematic representation of the humanized mouse models of allergic asthma. (**A**) Experimental protocol used. (**B**) Human $CD4^+$ T cells were isolated from the LNs and spleens of reconstituted mice at the time of euthanasia and cocultured with freshly isolated autologous naive $CD4^+$ T responders, APCs and Phleum pratense.

^{3}[H]Thymidine incorporation and cytokine release are depicted. Data shown are mean ± SEM of triplicate wells and are representative of two to three independent experiments (*n* = 4–6 mice per group). Statistical analysis was performed by the Student's t test (* P < 0.05; **P < 0.01; ***P < 0.001). (**C**) In the prevention protocol, NOD-SCID mice were reconstituted on day 0 with 2 × 10^6 Tconv or act-A–iTr1 cells and 2 × 10^6 autologous APCs, and were immunized (i.p.) with P. pratense. On day 1, mice received autologous naive $CD4^+$ T cells (10^6 cells). On days 9 and 10, mice were challenged with aerosolized P. pratense; AHR was measured the following day, and mice were killed. In other experiments, mice were administered (i.p.) anti-human IL-10Rα or Ig control 1 d prior to and following act-A–iTr1 cell or Tconv cell transfer. (**D**) AHR is depicted. Enhanced pause (PenH) results are expressed as mean ± SEM. Data were analyzed by two-way ANOVA for repeated measures, followed by the Student's t test, and are pooled from two to three independent experiments (*n* = 4–6 mice per group). (**E**) Total BAL cell numbers are shown. (**F**) Representative photomicrographs and histological scores of H&E-stained lung sections are depicted. (Scale bars: 50 μm). Statistical analysis was performed by the Student's t test (*P < 0.05; **P < 0.01).

To explore the effects of act-A–iTr1 cells on the regulation of human Teff responses *in vivo*, we isolated lung and DLN cells and restimulated them with allergen *ex vivo*. In line with the attenuated allergic airway disease severity, the capacity of DLN cells to proliferate and secrete IL-13 was significantly decreased in mice co-transferred with act-A–iTr1 cells, an effect that was partly reversed upon inhibition of IL-10 signalling (**Fig. 15A**). Moreover, the frequencies of $IL\text{-}4^+$ and $IL\text{-}13^+$ $CD4^+$ Teff cells in the DLNs of mice administered act-A–iTr1 cells were significantly decreased, and this effect was also reversed upon IL-10R blockade (**Fig. 15B**). Altogether, these findings reveal that human act-A–iTr1 cells retain their immunosuppressive properties upon transfer *in vivo* and confer significant protection against asthma manifestations, partly through an IL-10–dependent mechanism.

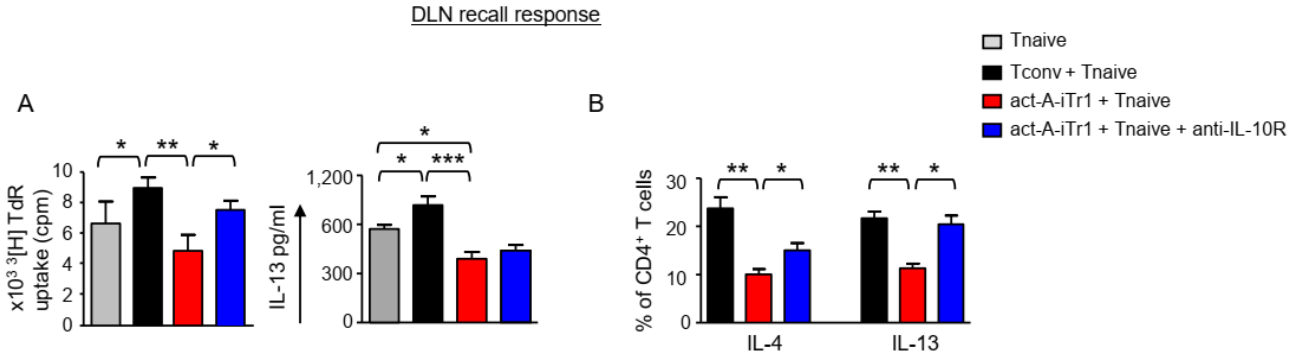

Figure 15. Act-A–iTr1 cells restrain human T-effector cell responses *in vivo*. (**A**) DLN cells were harvested at the time of euthanasia and restimulated with Phl. pratense. ^{3}[H]Thymidine incorporation and IL-13 release in culture supernatants are presented. Data are mean ± SEM of triplicate wells. (**B**) Percentages of cytokine-producing human $CD4^{+}$ T cells in the DLNs are shown. Data are representative of two to three independent experiments (*n* = 4–6 mice per group). Statistical analysis was performed by the Student's t test (*P < 0.05; **P < 0.01; ***P < 0.001).

Therapeutic administration of human act-A–iTr1 cells reverses established allergic responses and ameliorates asthma severity.

To explore whether human act-A–iTr1 cells could reverse ongoing allergic airway inflammation, we adoptively transferred act-A–iTr1 cells, right before aerosol challenge, in mice reconstituted with T cells and sensitized to allergen (**Fig. 16A**). Indeed, administration of act-A–iTr1 cells provoked a significant decrease in AHR induced by human Th2-effector cells and attenuated inflammatory cell infiltration in the lungs (**Fig. 16B and C**), suggesting that this regime may have applications in ameliorating disease manifestations.

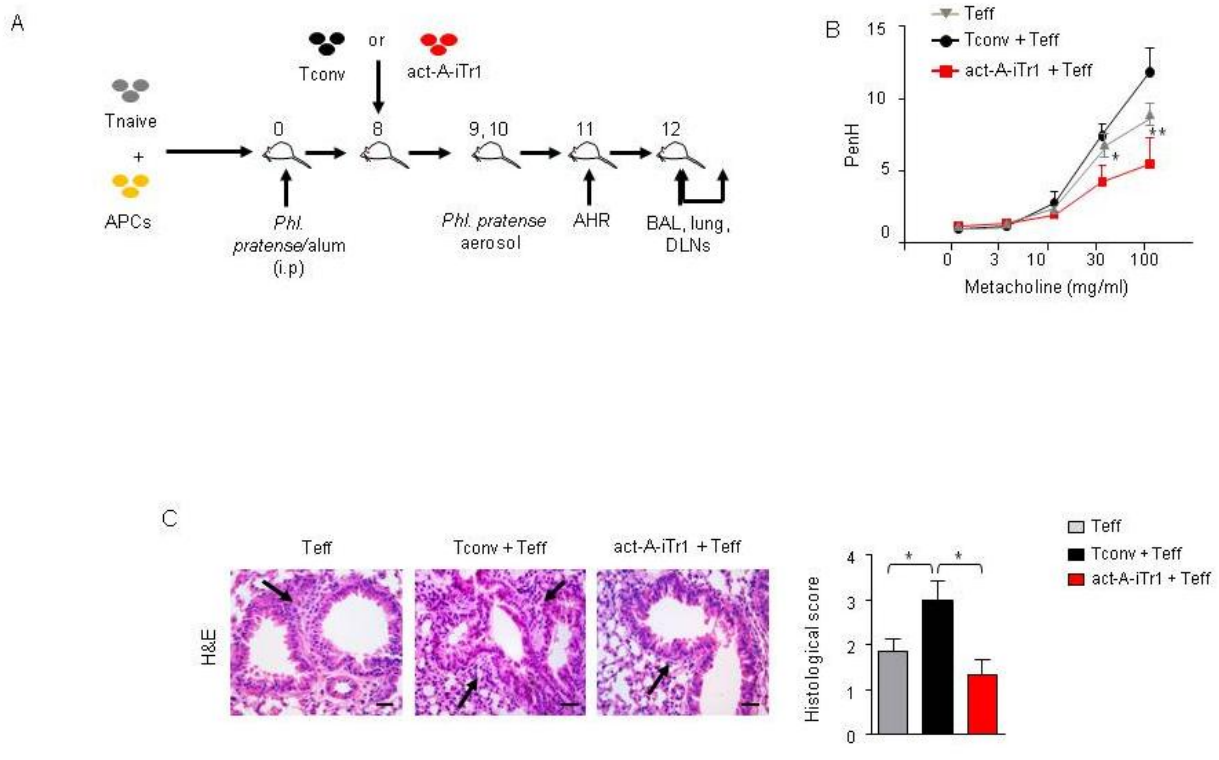

Figure 16. Therapeutic administration of human act-A–iTr1 cells reverses established allergic airway disease. (**A**) Schematic representation of the humanized mouse model of allergic asthma. In this therapeutic protocol, NOD-SCID mice were reconstituted on day 0 with naive $CD4^+$ T cells (10^6 cells) and autologous APCs (10^6 cells), and immunized with Phl. pratense/alum i.p. On day 8, mice received autologous Tconv or act-A–iTr1 cells (2×10^6 cells). (**B**) AHR is depicted. Results are expressed as means ± SEM. PenH results are expressed as mean ± SEM. Data were analyzed by two-way ANOVA for repeated measures, followed by the Student's t test, and are pooled from two to three independent experiments (*n* = 4–6 mice per group). (**C**) Representative photomicrographs and histological scores of H&E-stained lung sections are shown. (Scale bars: 50 μm.). Statistical analysis was performed by the Student's t test (*P < 0.05). Teff, T effector cell.

Activin-A suppresses allergen-specific T-cell responses from atopic and asthmatic individuals.

Finally, to investigate whether our findings had potential clinical relevance, we examined the effects of activin-A on the suppression of T-cell responses to mixed grasses extract, a clinically relevant allergen, from individuals with atopy as well as distinct asthma severities. Interestingly, activin-A induced a significant decrease in robust proliferative responses of $CD4^+$ T cells to allergen in atopic individuals and in subjects with mild/moderate and severe asthma (**Fig. 17A - C**). This decrease was accompanied by a substantial down-regulation in the release of IL-5 and IL-13 (**Fig. 17A, B and D**). Notably, activin-A also restrained T-cell responses, including IL-17 production, in a small group of patients with severe, steroid-refractory asthma (**Fig. 17D and E**).

Hence, altogether our findings point to activin-A as a novel immunoregulatory factor for human allergic asthma and unravel human act-A-iTr1 cells as a population of induced Tregs that expresses key features typical of Tr1 cells and displays robust suppressive capacities against naive and effector allergen-driven T cell responses.

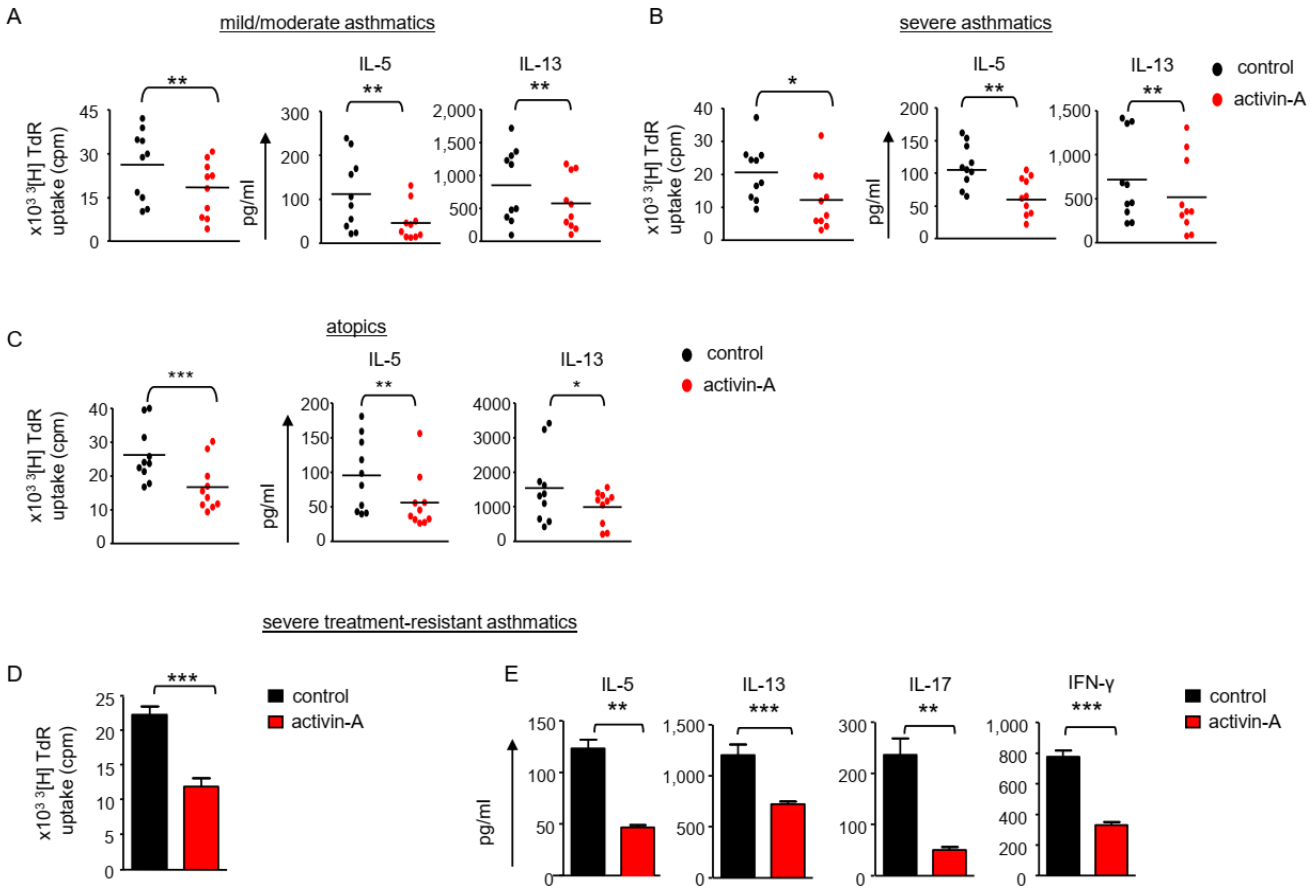

Figure 17. Activin-A restrains allergen-driven T-cell responses from atopic and asthmatic individuals with distinct disease severities. (**A and B**) $CD4^+$ T cells were isolated from the peripheral blood of asthmatics and stimulated with mitomycin-treated, CD3-depleted, mixed grasses extract-loaded APCs, as indicated. ^{3}[H]Thymidine incorporation and cytokine release in culture supernatants are depicted. Data are mean ± SEM of triplicate wells (*n* = 10 donors). (**C**) $CD4^+$ T cells were isolated from the peripheral blood of atopics and stimulated with mitomycin-treated, CD3-depleted APCs and mixed grasses extract. ^{3}[H]Thymidine incorporation and cytokine release are shown. Data are mean ± SEM of triplicate wells and are pooled from *n* = 10 donors. (**D and E**) $CD4^+$ T cells were isolated from the peripheral blood of subjects with severe, steroid-resistant asthma and stimulated as above. ^{3}[H]Thymidine incorporation (**D**) and cytokine release (**E**) are depicted. Data are mean ± SEM of triplicate wells and are pooled from *n* = 5–6 donors. Statistical analysis was performed by a nonparametric (Mann–Whitney) unpaired Student's t test (*P <0.05; **P < 0.01; ***P < 0.001).

5. DISCUSSION

Research during the past 20 years has greatly facilitated the understanding of the biology of activin-A: from acting as an essential factor in reproductive processes, to the realization that activin-A is a multifaceted cytokine affecting a plethora of innate and adaptive immune responses acting on multiple cell types. In the present study, we uncover a novel role for activin-A as an inducer of human Tr1-like cells that exhibit robust suppressive effects against allergen-induced naive and effector Th2 responses. Act-A-iTr1 cells posses key features characteristic of human Tr1 cells since they produce copious amounts of the immunoregulatory cytokine IL-10 and they express the surface molecules LAG3 and CD49b, recently identified as specific markers for human and mouse Tr1 cells (231). In addition, our data underscore a crucial role for IRF4 in human Tr1 cell biology, demonstrating that in response to activin-A, IRF4 assembles into a multipartite molecular complex with AhR and controls Tr1-cell differentiation and effector functions. Of translational importance, we depict that upon therapeutic transfer in a humanized mouse model of allergic asthma act-A–iTr1 cells confer protection against cardinal manifestations of allergic airway inflammation and linked disease. Overall, our studies highlight human act-A–iTr1 cells as potent therapeutic targets that can be used for the design of novel cell-based immunotherapies aiming to control human allergic diseases.

Using our established *in vitro* human T-cell priming model which closely resembles the allergen sensitization phase, we show that activin-A significantly restrains T-cell proliferation and Th2-effector cytokine release. Notably, activin-A treatment renders human T cells hyporesponsive following re-exposure to the same allergen *in vitro* and adoptive transfer into humanized mice *in vivo*. In addition, stimulation with activin-A endows human naive $CD4^+$ T cells with key Tr1-cell–associated characteristics, such as expression of ICOS, LAG3, CD49b, and granzyme-B as well as production of significant levels of IL-10 (231, 343, 344). Moreover, act-A–iTr1 cells exert strong suppressive functions accompanied by enhanced expansion in culture, compared with IL-10–induced Tr1 cells. Interestingly, RNA-seq analysis of the global gene expression profile of human act-A–iTr1 cells, compared with the recently identified $LAG3^+CD49b^+$ Tr1 cells, uncovered several similarities in their transcriptional signatures (231). Seminal studies have documented a

critical role for activin-A in instructing the differentiation of T_{FH} cells in humans and of Th9 and induced Foxp3$^+$ Treg cells in mice (304, 311, 319). Considering that several cytokines induce the differentiation of Th cells into distinct subsets depending on the context and the presence of other cytokines in the micromilieu (345), our findings demonstrating that activin-A can drive the differentiation of human Tr1-like cells extend the findings of previous reports.

An important finding of the present studies is the newly identified role of IRF4 as an essential component of the transcriptional network through which activin-A instructs human Tr1-cell differentiation. Even though the transcription factor IRF4 is a well-known contributor to the generation of several Th subsets, such as Th1, Th2, Th9, Th17 and T_{FH} cells, its involvement in human Tr1 cell biology has not been previously described (346, 347). Our findings demonstrate that IRF4 knockdown impairs activin-A–induced *IL10* and *ICOS* upregulation, decreases the percentages of LAG3$^+$CD49b$^+$ T cells, and compromises the suppressive functions of human act-A–iTr1 cells. Moreover, using sequential ChIP experiments and computational studies, we provide evidence that IRF4 forms a transcriptional complex with AhR and ARNT that seems to be fundamental for activin-A–induced ICOS up-regulation in Tr1 cells. It is worthnoting that another scientific group has confirmed our findings further supporting a central role for the transcription factor IRF4 in the generation and function of both murine and human Tr1 cells (348). Considering that AhR and ARNT activate target gene expression through direct interactions with other transcription factors (338), our data illuminate IRF4 as a key partner in AhR/ARNT-driven ICOS upregulation. Interestingly, ablation of a conditional IRF4 allele in mouse Foxp3+ Treg cells reduces ICOS, GZMB, and IL-10 expression and decreases their inhibitory capacity upon adoptive transfer *in vivo*, also implicating IRF4 in ICOS regulation and Treg-cell functions (347). Taken together, it becomes evident that IRF4 represents a central nodule of the transcriptional machinery that instructs effector molecule expression and suppressive functions in both Foxp3$^+$ Treg and Foxp3$^-$ Tr1 cells.

Our results also reveal that inhibition of AhR signalling abrogates activin-A–induced *IL10* and *ICOS* upregulation and impairs the inhibitory capacities of human act-A–iTr1 cells. Of note, it has been shown that depending on the stimuli, signalling through AhR drives the differentiation of human naive T cells into either Foxp3$^+$ Treg cells or Foxp3$^-$ Tr1

cells (337, 349). Our studies, uncovering activin-A as a novel factor utilizing AhR-induced signals to induce the differentiation of human Tr1 cells, further support this notion. Since it is established that AhR activation in the presence of TGF-β results in the generation of $Foxp3^+$ Treg cells, our findings reinforce the concept that although belonging to the same superfamily, activin-A and TGF-β exert distinct non-redundant functions on human T-cell responses, instructing the differentiation of different Treg-cell subsets (323). In concordance, our results demonstrate that neutralization of TGF-β does not affect the activin-A–mediated increase in the percentages of $CD4^+$IL-10-producing T cells.

A striking finding of our studies is that *in vivo* administration of act-A–iTr1 cells prevents the development of allergen-driven airway inflammation and ameliorates disease severity in a humanized mouse model of allergic asthma. Of high relevance to clinical settings, we also show that mice receiving act-A–iTr1 cells during allergen inhalational challenge and after the establishment of allergic inflammation exhibit dampened Th2-effector responses and attenuated pulmonary inflammation, suggesting that this regime may have therapeutic applications. Notably, the suppressive effects of act-A–iTr1 cells predominantly rely on IL-10 signalling *in vivo*, further emphasizing the fundamental role of IL-10 in the establishment of immune tolerance in the airways (221, 232). It is established that in individuals with severe persistent asthma, $CD4^+$ Teff cells are overactivated and often become resistant to mainstay immunosuppressive therapies, including corticosteroids (37). Hence, our findings revealing activin-A as an efficient controller of allergen-driven responses by $CD4^+$ Teff cells from asthmatic individuals across the spectrum of disease severity, may mark this cytokine as a potentially attractive therapeutic target for asthma.

Along with our study, accumulating evidence underlines a possible protective role for activin-A in aberrant Th2 cell-mediated allergic immune responses and associated diseases, including allergic asthma and atopic dermatitis (138, 323, 330, 333). Nevertheless, considering the pleiotropic effects of activin-A on essential physiologic and pathologic processes, modulation of activin-A or its signalling pathways *in vivo* could have serious repercussions for immunity in terms both of loss of regulation and potential pathogenic activity. Further dissection of the multilevel functions of activin-A during aberrant allergen-driven immune responses and their underlying molecular mechanisms is

essential to obtain a better insight of the exact role of this pleiotropic cytokine in human allergic asthma. For instance, the effects of activin-A on several aspects of innate inflammatory responses, including inflammasome activation and autophagy, have not been explored. Moreover, the role of activin-A in ILC2s generation and function remains elusive. Interestingly, since it has been recently described that Treg cells downregulate ILC2 functions through ICOS-ICOSL interactions (214), it is possible that act-A-iTr1 cells which express ICOS may be capable of regulating ILC2s.

Pertinent to our study, a question that has to be answered is whether interactions between AhR and c-Maf also participate in activin-A-mediated generation of human Tr1 cells. In support, it has been proven that in the presence of IL-27 AhR cooperates with c-Maf to induce *IL10* expression in murine $CD4^+$ T cells (349). Given that for an array of immune-mediated disorders therapeutic regimes based on iTregs adoptive transfer are under clinical trials another important issue is whether act-A-iTr1 cells can retain their immunoregulatory properties following administration in patients with asthma as they did after *in vivo* transfer into humanized mice. Notably, emerging evidence implies that the transfusion of antigen-specific Tregs has more efficacious advantages than the transfusion of polyclonal Tregs and can accurately act on the lesion site (244). Nevertheless, the clinical transformation of antigen-modified Tregs transfusion technique still faces many difficulties, such as the small number of Tregs that can be obtained as well as the lack of reference standards for *in vitro* proliferation and transfusion techniques. Therefore, further investigation on the *in vitro* adequate expansion of act-A-iTr1 cells is of imperative need.

In conclusion, we have identified an activin-A–induced, IRF4/AhR-dependent transcriptional network that drives the generation of human Tr1 cells strongly suppressive against Th2-cell–mediated allergic responses. Considering that adoptive T-cell therapies are underway to control immune-mediated diseases, our findings illuminate act-A–iTr1 cells as targets that can be exploited for the control of asthma and other human diseases associated with dysregulated immunity.

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