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Investigation of the mechanisms of anti-TIGIT antibody protective function in allergic airway disease

MASTER THESIS

Kostopoulou Eleni

Supervisor: Professor Panayotis Verginis

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Διερεύνηση του προστατευτικού μηχανισμού δράσης του αντι-TIGIT στην αλλεργική ασθένεια των αεραγωγών

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Κωστοπούλου Ελένη

Επιβλέπων: Καθηγητής Παναγιώτης Βεργίνης

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ABSTRACT

The increased prevalence of allergic diseases observed in western countries during the last decades has raised the concern over these disorders. The underlying etiology remains unclarified, although genetic and environmental factors, together with modern lifestyle seem responsible. Asthma is considered one of the most common forms of allergic diseases, presenting various phenotypes attributed to different molecular pathways. Thus, the design of efficient therapies targeting asthma is challenging.

Albeit type 2 immune responses (Th2) were initially thought to be solely driving allergic asthma induction, recent evidence supports the involvement of non-Th2-derived factors in the pathogenesis of this disease, highlighting its complexity. Asthmatic responses are suppressed by a population of T lymphocytes, the so-called T regulatory cells (Tregs), which firmly promote immune homeostasis and tolerance induction. Breakage of tolerance against allergens due to impaired Treg cell function can result in the development of allergy and asthma.

Recently, a Treg cell subset exhibiting enhanced immunosuppressive capacity associated with the expression of the TIGIT (T cell immunoglobulin and ITIM domain) receptor was identified. Interestingly, this Treg subpopulation was found to differentially affect distinct Th cell lineages, as Th1 and Th17 responses are negatively regulated, while Th2 responses are not hindered. Moreover, previous research of our lab showed that TIGIT exerts a fostering effect on Th2 responses upon allergen challenges in an OVA-induced allergic asthma model, as blockade of this molecule significantly blunted allergic responses.

The question raised by this study was whether this effect was due to blockade of Th2- or/and Treg-derived TIGIT and, therefore, the objective of the present dissertation was to investigate the putative implication of $TIGIT^+$ Tregs in anti-TIGIT-mediated attenuation of allergic airway inflammation. Results showed that $TIGIT^+$ Tregs are dispensable for the amelioration of allergic responses upon TIGIT blockade during challenges. Further investigation is needed to elucidate the complex molecular pathways leading to TIGIT-mediated aggravation of Th2 responses in asthma. Shedding light on these underlying mechanisms in the Th2 context is of great value, since TIGIT is emerging as a promising therapeutic target not only for asthma treatment, but also for cancer immunotherapy.

Keywords: allergic asthma, Th2 responses, regulatory T cells, Foxp3, TIGIT, DEREG, DT-mediated Treg cell depletion

ΠΕΡΙΛΗΨΗ

Τις τελευταίες δεκαετίες, ο επιπολασμός των αλλεργικών ασθενειών αυξήθηκε ραγδαία στις δυτικές χώρες, προκαλώντας ανησυχία σχετικά με τις διαταραχές αυτές. Η υποκείμενη αιτία δεν έχει διευκρινιστεί πλήρως, αν και γενετικοί και περιβαλλοντικοί παράγοντες, μαζί με τον σύγχρονο τρόπο ζωής μπορούν να θεωρηθούν υπεύθυνοι. Το άσθμα θεωρείται μία από τις πιο κοινές μορφές αλλεργικών ασθενειών, με ποικιλία φαινοτύπων, λόγω των διαφορετικών μοριακών μονοπατιών που μπορούν να οδηγήσουν σε αυτό. Έτσι, ο σχεδιασμός αποτελεσματικών θεραπειών για το άσθμα καθίσταται δύσκολος.

Αν και οι ανοσοαποκρίσεις τύπου 2 (Th2) θεωρήθηκαν αρχικά οι μόνοι υπεύθυνοι για την επαγωγή αλλεργικού άσθματος, πλέον υποστηρίζεται ότι επιπλέον παράμετροι, που δε σχετίζονται με τις Th2 αποκρίσεις, συμμετέχουν στην παθογένεση αυτής της νόσου, υπογραμμίζοντας έτσι την πολυπλοκότητά της. Οι αλλεργικές αποκρίσεις καταστέλλονται από έναν πληθυσμό Τ λεμφοκυττάρων, τα Τ ρυθμιστικά κύτταρα (Tregs), τα οποία προάγουν την ανοσολογική ομοιόσταση και την επαγωγή ανοχής. Η απώλεια ανοχής στα αλλεργιογόνα, λόγω ανεπαρκούς λειτουργικότητας των Tregs, μπορεί να οδηγήσει σε ανάπτυξη αλλεργίας και άσθματος.

Πρόσφατα ταυτοποιήθηκε ένας υποπληθυσμός Tregs με αυξημένη ανοσο-κατασταλτική ικανότητα, σχετιζόμενη με την έκφραση του υποδοχέα TIGIT (T cell immunoglobulin and ITIM domain). Αξιοσημείωτο είναι ότι τα TIGIT^{+} Tregs φαίνεται να ρυθμίζουν διαφορετικά τους διάφορους τύπους Th αποκρίσεων, καταστέλλοντας τις Th1 και Th17, αλλά όχι τις Th2 αποκρίσεις. Επιπλέον, προηγούμενη έρευνα του εργαστηρίου μας έδειξε ότι το TIGIT ενισχύει τις Th2 αποκρίσεις κατά τις αντιγονικές επανεκθέσεις σε μοντέλο αλλεργικού άσθματος επαγόμενο από ωοαλβουμίνη (OVA), καθώς η παρεμπόδιση του TIGIT ελάττωσε σημαντικά τις αλλεργικές αποκρίσεις.

Το ερώτημα που προέκυψε ήταν εάν αυτό το αποτέλεσμα προερχόταν από την καταστολή δράσης του TIGIT των Th2 κυττάρων ή/και του TIGIT των Tregs. Ως εκ τούτου, στόχος της παρούσας διατριβής ήταν η διερεύνηση του πιθανού ρόλου των TIGIT^{+} Tregs στην παρατηρούμενη ύφεση της αλλεργικής ασθένειας των αεραγωγών μετά από χορήγηση αντι-TIGIT αντισώματος. Τα αποτελέσματα έδειξαν ότι τα TIGIT^{+} Tregs δεν απαιτούνται για την παρατηρούμενη ελάττωση των αλλεργικών αποκρίσεων, λόγω της παρεμπόδισης του TIGIT κατά τις αντιγονικές επανεκθέσεις. Περαιτέρω διερεύνηση απαιτείται, προκειμένου να κατανοηθούν τα σύνθετα μοριακά μονοπάτια στα οποία συμμετέχει το TIGIT, οδηγώντας σε επιδείνωση των Th2 αποκρίσεων στο άσθμα. Η αποσαφήνιση των μηχανισμών αυτών στο Th2 πλαίσιο είναι σημαντική,

καθώς το TIGIT θεωρείται πολλά υποσχόμενος θεραπευτικός στόχος όχι μόνο για τη θεραπεία του άσθματος, αλλά και για την ανοσοθεραπεία του καρκίνου.

Λέξεις-κλειδιά: αλλεργικό άσθμα, Th2 αποκρίσεις, Τ ρυθμιστικά κύτταρα, Foxp3, TIGIT, DEREG, απαλοιφή Τ ρυθμιστικών κυττάρων με χορήγηση DT

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ABBREVIATIONS

Ab	Antibody	FITC	Fluorescein Isothiocyanate
AD	Atopic Dermatitis	Foxp3	Forkhead box P3
AEC	Airway Epithelial Cell	GARP	Glycoprotein A repetitions predominant receptor
Ag	Antigen	GATA3	GATA-binding protein 3
AHR	Airway Hyperresponsiveness	G-CSF	Granulocyte Colony-Stimulating Factor
AJC	Apical Junctional Complex	GITR	Glucocorticoid-inducible Tumor Necrosis Factor receptor
Alum	Aluminum hydroxide	GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
ANOVA	Analysis of Variance	Grb2	Growth factor receptor-bound protein 2
APC	Allophycocyanin	GVHD	Graft-Versus-Host Disease
APC	Antigen-Presenting Cell	H_2O_2	Hydrogen peroxide
AR	Allergic Rhinitis	H_2SO_4	Sulfuric acid
Areg	Amphiregulin	HDM	House Dust Mite
ASIT	Antigen-Specific Immunotherapy	H&E	Hematoxylin and Eosin
BAC	Bacterial Artificial Chromosome	Hes1	Hairy and enhancer of split 1
BALF	Bronchoalveolar Lavage Fluid	HRP	Horseradish Peroxidase
BCR	B cell receptor	Hprt1	Hypoxanthine phosphoribosyltransferase 1
BSA	Bovine Serum Albumin	ICER	Inducible cAMP Early Repressor
BV	Brilliant Violet	ICOS	Inducible Co-stimulatory Molecule
cAMP	cyclic Adenosine Monophosphate	IDO	Indoleamine 2,3-dioxygenase
cDC	conventional Dendritic Cell	IFN	Interferon
cDNA	complementary DNA	IL	Interleukin
CEBPα	CCAAT/Enhancer-Binding Protein α	ILC2	Innate Lymphoid Cell 2
CNS2	Conserved Non-coding Sequence 2	IPEX	Immune dysregulation Polyendocrinopathy Enteropathy X-linked syndrome
CTLA-4	Cytotoxic T Lymphocyte Antigen-4	IRF-4	Interferon Regulatory Factor 4
Cy	Cyanine	ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
DC	Dendritic Cell	iTreg	induced Treg cell
DEREG	Depletion of Regulatory T cells	ITT	Ig Tail-Tyrosine
dH_2O	distilled water	LN	Lymph Node
dNTP	deoxyribonucleotide Triphosphate	MAPK	Mitogen-Activated Protein Kinase
DT	Diphtheria Toxin	MBP	Major Basic Protein
DTR	Diphtheria Toxin receptor	MDC	Macrophage-Derived Chemokine
DTT	Dithiothreitol	MHC	Major Histocompatibility Complex
ECP	Eosinophil Cationic Protein	MLN	Mediastinal Lymph Node
EDTA	Ethylendiaminetetraacetic acid	moDC	monocyte-derived Dendritic Cell
eGFP	enhanced Green Fluorescence Protein	NF-κB	Nuclear Factor-κB
ELISA	Enzyme-Linked Immunosorbent Assay	NK	Natural Killer cell
EPO	Eosinophil Peroxidase	NKT	Natural Killer T cell
EPX	Eosinophil Protein X	Nrp-1	Neuropilin-1
FACS	Fluorescence-Activated Cell Sorting	nTreg	natural Treg cell
FBS	Fetal Bovine Serum	O.D.	Optical Density
FcεRI	High-affinity Fcε Receptor 1	O/N	Overnight
Fgl2	Fibrinogen-like protein 2	OVA	Ovalbumin

PAF	Platelet-Activating Factor
PAS	Periodic Acid Schiff
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell Death protein 1
pDC	plasmacytoid Dendritic Cell
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PK	Proteinase K
PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen Recognition Receptor
PVR	Poliovirus Receptor
qPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RIA	Radioimmunoassay
ROR	RAR-related Orphan Receptor
RT	Room Temperature
Runx	Runt-related transcription factor
SEM	Standard Error of the Mean
SHIP1	SH2-containing Inositol Phosphatase 1
SHP2	Src Homology region 2 domain-containing Phosphatase 2
STAT6	Signal Transducer and Activator of Transcription 6
TAE	Tris Acetate-Ethylendiaminetetraacetic acid
TAM2	Tumor-Associated Macrophages type 2
TARC	Thymus- and Activation-Regulated Chemokine
TCR	T cell receptor
TF	Transcription Factor
Tfh	T follicular helper cell
TGF-β	Transforming Growth Factor β
Th2	T helper type 2 cell
TIGIT	T cell Immunoglobulin and ITIM domain
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TME	Tumor microenvironment
TNF-α	Tumor Necrosis Factor α
Tr1	IL-10-producing type 1 Treg cell
Treg	Regulatory T cell
TSLP	Thymic Stromal Lymphopoietin
UV	Ultraviolet
WT	Wild Type

1. INTRODUCTION

1.1 Overview of allergic diseases

The immune system consists of a highly complex cell and molecule network evolved to discriminate between potentially harmful pathogens and self or external innocuous antigens, responding properly in each case[1,2]. Development of immune responses requires numerous mechanisms which, once dysregulated, could result in tolerance-related diseases, such as cancer, graft-versus-host disease (GVHD), autoimmunity, asthma and allergy[1,2]. Over the past decades, an increment in prevalence of allergic diseases has been observed worldwide, but especially in industrialized countries[3,4]. Indeed, allergy affects up to 30% of overall population and is expected that the percentage of patients will reach to 40% worldwide two decades ahead. In addition, the life quality of allergic individuals is greatly impaired and costs regarding healthcare are augmented. In view of the above, allergy is considered as a critical public health problem with socio-economic consequences[1].

The reasons behind this disorder are not completely understood yet[1]. However, genetic predisposition is assumed as a significant risk factor for allergy development[3]. In fact, linkage analysis, initially in mice and afterwards in humans, has led to the discovery of several susceptibility genes, such as *ADAM33* and *HAVCR1 (TIM1)*, which are associated with asthma[5]. However, genetic changes have been unable to justify the increase of incidence and prevalence of allergic disease throughout the last 70 years, which led to the speculation that environmental factors and contemporary lifestyle of western countries also play a role[3,6,7]. In line with that, the so-called "hygiene hypothesis" has emerged, suggesting that diminished microbial exposure due to family size reduction, extensive antibiotic and antipyretic usage and improved hygienic standards of civilized society have contributed to the increased rates of allergy observed over the years[3,7,8]. The early postnatal period has proven critical for establishment and stabilization of intestinal microbiota and changes in its composition resulting from modern lifestyle may affect tolerance induction, thus influencing the risk of allergic disease onset[3,7,8]. A possible explanation of how environmental factors can have an impact on allergy development is epigenetic modifications, that is heritable alterations in DNA not affecting its sequence[6,7]. Indeed, it has been found that exposure to pollen, air pollutants, such as polycyclic aromatic hydrocarbons and NO_2, and smoke,

either prenatally, postnatally or active smoking, have been correlated with epigenetic changes that increase the risk of allergic disorder development[7].

Allergic diseases may exhibit a wide range of clinical manifestations depending on the way an allergen is introduced inside the body[7,9]. Thus, the major forms include allergic asthma, allergic rhinitis (AR), atopic dermatitis (AD), conjunctivitis[9], food allergy and anaphylaxis[1]. All of the above are immunoglobulin E (IgE)-driven and are classified as atopic disorders, but allergic diseases not related to IgE also exist, such as non-IgE-mediated food allergy, allergic contact dermatitis and intrinsic AD[7]. In terms of asthma, the past years has become evident that it represents an immensely heterogeneous disorder with many different phenotypes arising from differing pathogenic mechanisms and immunological parameters[3,5]. Such phenotypes comprise allergic asthma, being the most common type, severe steroid-resistant asthma and asthma provoked by exposure to cigarette smoke, air pollutants, diesel exhaust particles, obesity, exercise and aspirin (Table 1)[5]. It is, therefore, apparent that asthma can be induced by a specific allergen or by non-specific stimuli, involving allergic (atopic asthma) or intrinsic (non-atopic asthma) molecular and cellular pathways, respectively[5,10]. Despite the different pathogenic mechanisms behind these pathways and phenotypes, co-existence of distinct forms of asthma in patients is quite common and interaction of their pathways is not excluded. The complexity and diversity attributed to asthma, renders the development of therapeutic approaches demanding[5].

Table 1: Clinical asthma phenotypes and the pathophysiological mechanisms involved[5].

Clinical phenotype of asthma	Th2 cell requirement	Mechanisms/effector cells
Allergen	+	IL-4, IL-5, IL-9, IL-13, TSLP, IL-25, IL-33, IL-17, $CD4^+$ cells, DCs, eosinophils, mast cells, basophils, NKT cells
Viral infection	-	Th2 cytokines, alveolar macrophages, NKT cells (innate immune cells)
Air pollution, cigarette smoke, diesel particles, smoke	-	IL-17, oxidative stress, small particles, neutrophils, NKT cells
Aspirin	-	Leukotrienes, loss of prostaglandin E_2
Obesity	-	Oxidative stress
Severe, steroid resistant	-	IL-17, neutrophils, NKT cells
Exercise, cold air	-	Heat transfer, change in mucosal osmolality, cytokines

1.1.1 Pathognomonic features of asthma and underlying immune responses

Asthma is generally considered as a long-range pulmonary inflammatory disease of the airways characterized by mucus hyperproduction, airway hyperresponsiveness (AHR), reversible airflow obstruction, bronchoconstriction and airway remodeling[3,10], leading to symptoms, such as coughing, wheezing and short breath[5]. These hallmarks of asthma are long believed to be mediated by type 2 immune responses, as indicated by the infiltration of diverse inflammatory cell types, such as T helper type 2 (Th2) cells, mast cells, eosinophils, neutrophils, dendritic cells (DCs) and B lymphocytes[3]. In accordance with this, elevated numbers of Th2 cells have been found in the lungs of asthma patients[3,5] and animal model studies specify the substantial role of Th2 cells in disease development[3].

A typical Th2 inflammatory response mainly involves Th2 lymphocytes and IgE antibodies (Abs), however, the possibility of a Th2 immune response beyond specific IgE presence has been recently reported. In fact, several allergens, such as house dust mite (HDM) and mold, or environmental factors, like viruses and pollutants are capable of triggering both innate and adaptive type 2 immunity, even when IgE antibodies are absent. Various studies have linked bronchial epithelium dysfunction with such a response, contributing to asthma pathogenesis. Adequacy of epithelial barrier is delineated by both anatomic integrity and, principally, immunological functionality, the latter one having strong relevance with innate immunity. Epithelial dysfunction, anatomically and functionally, could explain non-IgE-mediated Th2 responses. Genetically predisposed individuals that appear to have impaired apical junctional complexes (AJCs), thus forming a defective epithelial barrier, are more prone to become asthmatics in the face of allergen exposure[11].

Airway epithelial cell (AEC) activation through pathogen recognition receptors (PRRs), including toll-like receptors (TLRs), caused by allergens, including HDM and mold, or tissue injury due to environmental stimuli lead to the production of damage-associated cytokines, such as interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) by AECs. These molecules mediate Th2 responses by stimulating Th2 cells and type 2 innate lymphoid (ILC2) cells, which secrete Th2 cytokines[11]. Similar responses are also driven by allergen proteases[11], such as the plant-originated cysteine protease, papain, via tissue injury caused by epithelial barrier disruption[10]. In light of the previous findings, the event of type 2 inflammatory response

should be considered as the result of an elaborate crosstalk involving as much the airway epithelium as innate and adaptive immunity[11] .

1.1.2 Induction of type 2 responses in response to allergens

The first encounter of the organism with an allergen activates immunologic mechanisms, which lead to allergen sensitization. After the allergen has entered the body, it is soon recognized by the PRRs of specific mucosal-resident DCs in the airways, skin or intestine[1]. DCs represent the key link between innate and adaptive immunity, acting as antigen-presenting cells (APCs) to activate naïve T lymphocytes. The main three subsets consist of conventional DCs (cDCs), plasmacytoid (pDCs) and monocyte-derived DCs (moDCs). Under normal conditions the vast majority of lung DCs are cDCs, specifically $CD103^+$ (cDC1s) and $CD11b^+$ cDCs (cDC2s), while only a small percentage of pDCs is detectable[12]. The subset required for induction of Th2 responses is claimed to be $CD11b^+$ cDCs, whereas cDC1s are responsible for Th1 response induction[12] and, in fact, they could even be tolerogenic against inhaled allergens[13].

Despite the fact that allergens can be directly sensed by DCs, it is now believed that DCs can also be activated by the epithelium. Under allergen exposure, epithelial cells, expressing various PRRs, produce a number of epithelial cytokines, like IL-25, IL-33, TSLP, and granulocyte-macrophage colony-stimulating factor (GM-CSF), among of which, TSLP is mainly responsible for DC activation[12].

DCs that have captured the introduced allergen, internalize it, process it and migrate to the draining lymph nodes (LNs), where they present its peptides via major histocompatibility complex (MHC) class II molecules to naïve $CD4^+$ T cells[1]. TSLP-activated $CD11b^+$ cDCs express the tumor necrosis factor (TNF) superfamily member, OX40L, ligand of the co-stimulatory molecule OX40 expressed on T cells, and interactions between these two molecules result in activation of naïve $CD4^+$ T cells to acquire IL-4-competency[5,14]. Following, the activated IL-4-competent $CD4^+$ T cells in lymph nodes differentiate either into T follicular helper (Tfh) cells and transport to B cell zones or into allergen-specific $CD4^+$ Th2 cells, which exit the LN and enter the circulation to complete their maturation[14]. Tfh cells, being specialized helpers of B cells, they secrete IL-4[15], thus mediating isotype class-switching and production of IgE antibodies by antigen-specific B lymphocytes[3]. It is of note that IL-4 derived from Tfh

and not from Th2 cells is critical for these events, as Th2 cells exit LNs[14]. Other cells, such as basophils and natural killer T (NKT) cells may also facilitate as early source of IL-4[1]. Allergen-specific IgE antibodies produced by B cells bind the high-affinity Fcε receptor 1 (FcεRI) on the surface of mast cells and basophils, thus sensitizing those cells[1,10,11]. Moreover, a pool of memory allergen-specific Th2 and B cells is generated following clonal expansion during this stage, which will intervene upon subsequent allergen exposures[1].

Another population participating is the ILC2s, innate cells that lack T cell (TCR) or B cell (BCR) antigen receptors, though producing substantial amounts of Th2 cytokines[12,15] in response to epithelial cell-derived cytokines IL-25 and IL-33[15]. Their implication in sensitization phase lies on the fact that MHC class II has been reported on their surface and its expression is augmented during sensitization, suggesting a role as antigen-presenting cells[10,12,15]. Whether ILC2s are essential for antigen (Ag) presentation and how these cells migrate into the LNs is still unclear, though[10]. Furthermore, ILC2-derived IL-13 has been found important for the migration of $CD11b^+$ cDCs into the draining LN, to promote differentiation of naïve T cells into Th2 cells and, additionally, induces expression of thymus- and activation-regulated chemokine (TARC), also known as CCL17, from DCs, which attracts Th2 cells[10]. ILC2s secrete as well the "instructive" cytokine IL-6, which is crucial for the stimulation of naïve T cells and important growth factor for B cells. Survival and maturation of B cells could also be driven by ILC2s as they express the inducible co-stimulatory molecule, ICOS, which likely interacts with its ligand ICOSL on B cells. Lastly, the capacity to enhance IgE production from B cells has been observed *in vitro*, therefore implying that ILC2s might be involved in IgE-dependent sensitization[15].

The sensitized individuals do not present any clinical symptoms, however they are ready to respond under following allergen encounters[1]. When that happens, the allergen causes cross-linking of the IgE antibody bound to FcεRI on sensitized basophils and mast cells[1,10,11]. This induces activation of these cells, which release through degranulation various inflammatory mediators, such as heparin[1], biogenic amines (histamine and serotonin), proteoglycans, serglycin, lipid mediators (platelet-activating factor [PAF], leukotrienes, prostaglandins, and sphingolipids), mast cell-derived proteases (chymase and tryptase)[11] and chemokines, like IL-8, lymphotactin, CCL1 (TCA-3), CCL5 (RANTES), CCL2 (MCP-1) and CCL3 (MIP1-α), as well as cytokines, like IL-4, IL-5, IL-6, IL-13, IL-25, TNF-α and transforming growth factor (TGF)-β[5].

Release of all these basophil- and mast cell-derived substances drives the immediate phase of Th2 inflammation[1] via recruitment of inflammatory cells, mainly eosinophils, smooth muscle constriction and enhancement of vascular permeability[10,11]. Interestingly, expression of MHC class II on basophils, mast cells and eosinophils is indicative of their potential role as APCs[5].

IL-25 produced by airway structural cells is also promoting eosinophilia and Th2 cell recruitment to the airways[12]. Highly polarized Th2 cells, alveolar macrophages and activated eosinophils have been as well reported to secrete IL-25[5,12]. Aside from its role in allergic airway inflammation promotion, IL-25 is also thought to be involved in airway remodeling via enhancing endothelial cell proliferation and generation of microvessel structures[12]. Another cytokine produced under inflammatory conditions is IL-33, mainly, as previously mentioned, by epithelial cells, but also by DCs, mast cells, macrophages and monocytes. Its major contribution to Th2 responses is the activation and accumulation of Th2 and ILC2 cells, macrophages[12], as well as natural killer (NK) and NKT cells[3]. The latter ones, represent a lymphocyte subset combining features of both classic T cells and NK cells and are also activated by TSLP and IL-25. These cells seem to be specifically important for AHR induction as they produce high levels of IL-4, IL-13, interferon (IFN)-γ and Th2 chemokines[5]. Additional functions of IL-33 include facilitation of basophil and eosinophil degranulation, together with eosinophil survival promotion[5,12]. Finally, chemokines produced by lung DCs, such as CCL17 and CCL22 are responsible for the attraction of Th2 cells, eosinophils and basophils into the lungs[5].

Late phase of the allergic response is marked by the infiltration into the airway of activated Th2 memory cells, as well as newly differentiated effector Th2 cells, which produce keystone Th2 cytokines, particularly IL-4, IL-5, IL-9 and IL-13, thus orchestrating the inflammatory response[1]. Studies have shown that generation of tertiary lymphoid tissue inside the lung, wherein memory Th2 cells reside, is quite significant during chronic allergic inflammation[1]. Polarization of T cells towards Th2 state is achieved through activation of the transcription factors (TFs) signal transducer and activator of transcription 6 (STAT6) and GATA-binding protein 3 (GATA3)[3,10] and requires IL-4, mainly provided by basophils, which produce this cytokine at high levels[10,11]. Activation of both memory and newly differentiated Th2 cells is mediated by DCs, which after allergen exposure express FcεRI receptor[15], and B cells via IgE-facilitated allergen presentation[1]. Th2-derived IL-4 mediates differentiation of naïve

$CD4^+$ T cells into Th2 cells[11], while IL-13 promotes goblet cell proliferation, along with mucin and mucus hyperproduction, IgE production in B cells and AHR development[3,10,11]. Tissue eosinophilia and mast cell hyperplasia are controlled by IL-5 and IL-9[10,11]. IL-5, in particular, is a critical mediator of eosinophil differentiation from precursors in the bone marrow[3] and infiltration into the airways[3,15].

Eosinophils play a pivotal role in the pathogenesis of asthma, not exclusively but mainly in the late-phase response by producing several proinflammatory and cytotoxic mediators[5,11,15]. In particular, four of the major proteins their cytosolic granules contain are major basic protein (MBP) eosinophil cationic protein (ECP), eosinophil protein X (EPX) and eosinophil peroxidase (EPO), all of which exert cytotoxic activity[11]. In addition, eosinophils release lipid mediators (cysteinyl leukotrienes), oxygen radicals and many cytokines, including IL-1β, IL-6, IL-8, IL-4[11], IL5, IL-10, IL-13, TNF-α and also the Th1 cytokines IFN-γ and IL-2[5], that contribute to their proinflammatory capacity. Regarding the proinflammatory role of IL-10, which is generally a suppressor of inflammatory immune responses, Jeannin *et al*. have revealed that this cytokine can act in concert with IL-4 to promote IgE production by B cells upon CD40 ligation on these cells[16]. Production of all these eosinophil-derived substances eventually provokes fibrosis, resulting in AHR and airway remodeling[11]. Importantly, IL-13 produced by eosinophils acts in a way similar to ILC2-stemmed IL-13, promoting migration of $CD11b^+$ DCs towards the draining LN for allergen presentation[10].

Oddly, it has been proposed that eosinophils may promote the maintenance of macrophages, providing IL-4 and IL-13, thus skewing their activation towards the alternatively activated macrophage population, also known as M2 macrophages[15]. These cells hold a controversial role in allergic responses, as they are generally thought to be anti-inflammatory and facilitate immune homeostasis in lungs through impeding antigen presentation by DCs, Th2 cell activation and cytokine production and IgE secretion[5]. However, emerging evidence propose that M2 macrophages might contribute to asthma by producing also proinflammatory Th1, Th2 and Th17 cytokines, as well as IL-33, upon different circumstances[5]. Moreover, depletion of macrophages in lungs leads to obstruction of prolonged AHR induction[5].

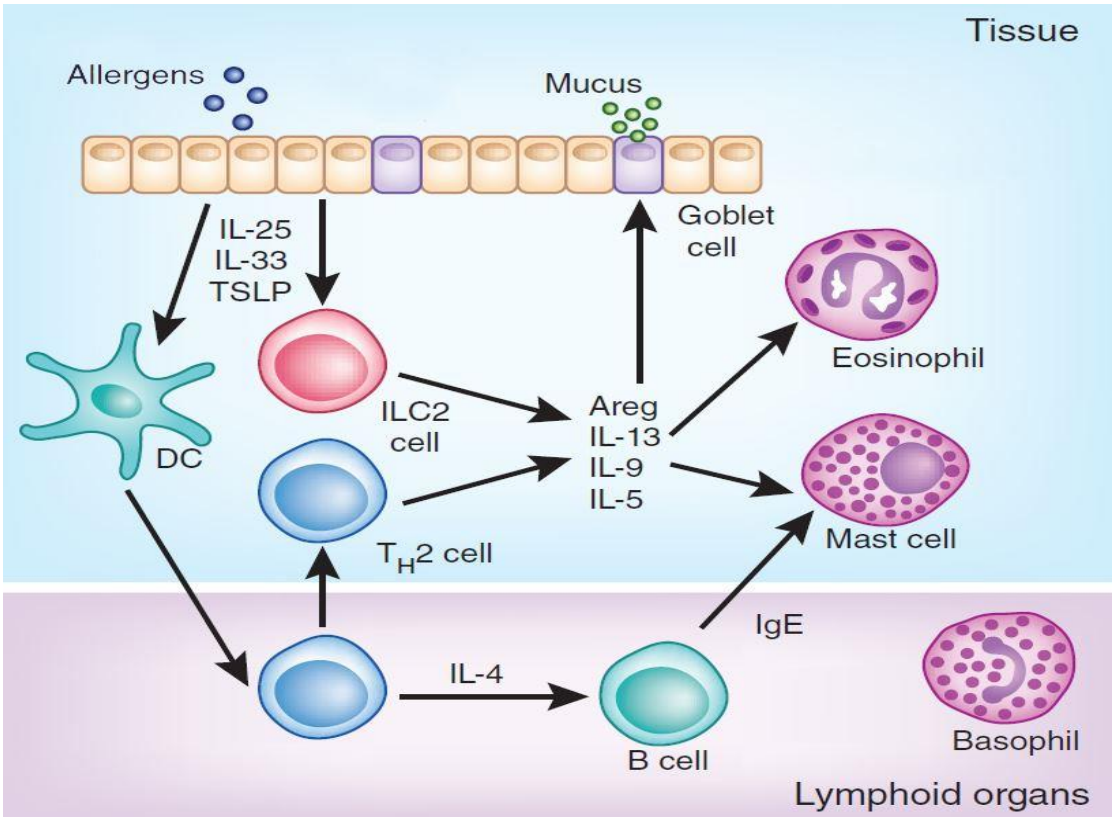
Another innate cell population responsible for eliciting Th2 immune responses regards the ILC2 cells, which provide an early source of IL-5, IL-9, and IL-13 in the lung[17] as a response to protease allergens, chemicals or helminth parasites[10]. Activation of ILC2s is mediated not only by AEC-derived IL-25, IL-33 and TSLP, but also by

lipids, like leukotrienes and prostaglandins[17]. The fact that these cells inhabit specific tissue niches and do not circulate, accounts for their implication in the early phase of the response, as well as in chronic allergic inflammation[10,17]. The presence of ILC2s has also been identified in lung-draining lymph nodes after asthma induction with administration of IL-25 or IL-33, albeit their role there is still unknown[18].

ILC2-produced IL-5 and IL-13 represent a large proportion of the total Th2 cytokine pool in the lungs in different asthma models. More precisely, either in intranasal administration of IL-25 or IL-33, which induces an innate-dominated response, or in ovalbumin (OVA)/aluminum hydroxide (alum) induced asthma, which is mainly T cell-dependent, ILC2s and T cells seem to equally contribute to IL-5 and IL-13 production[18]. Thus, ILC2 cells are considered as strong mediators of mucus production, airway eosinophilia[1], and Th2 polarization[10], therefore promoting asthma induction. Moreover, they seem to be correlated with resistance to steroid treatment, which stems from the fact that TSLP is likely to activate STAT5 in ILC2s, thus rendering them resistant to steroid-induced cell death[12]. Interestingly, evidence supports a role of ILC2s in airway tissue repair in the chronic allergic inflammation, as they constitutively express amphiregulin (Areg), a member of the epidermal growth factor family, which facilitates repairing of epithelium and fibroblasts. Furthermore, ILC2s activate macrophages to produce collagen, which also promotes airway wall remodeling[10].

It is thought that adaptive immunity may also feedback to ILC2s via signals generated from $CD4^+$ T cells. In particular, MHC II-TCR interactions between ILC2s and T cells result in IL-2 production by the latter, which supports proliferation, survival and IL-13 production of ILC2s[12,15]. Hence, there is a reciprocal interface among ILC2s and T cells, raising the possibility that ILC2s could be acting as much before as after the adaptive immunity has been initiated[12]. To what extent ILC2 cells participate in allergic reactions is determined by those interactions with cells of the adaptive compartment, both as inducers of adaptive responses and as responders to T cell-derived molecules[15]. Notably, it has been shown that allergic inflammation in lungs after intranasal administration of IL-25, IL-33 or protease allergens can be induced even in the absence of T and B cells, implying a probable involvement of ILC2s in this reaction[15]. Indeed, in RAG-deficient mice, which lack mature lymphocytes, ILC2 cells represent the major source of IL-5 and IL-13 under papain-induced asthma[19]. These data suggest that ILC2 cells may exert a significant role in the development of non-

atopic asthma, which is IgE-independent and mostly innate cells are involved[10]. Picture 1 illustrates the complexity of type 2 immune responses that orchestrate allergic asthma.



Picture 1: Overview of pulmonary allergic lung inflammation. Th2 responses are initiated when allergens disrupt the epithelium, which in turn secretes IL-25, IL-33 and TSLP. Those cytokines result in ILC2 and DC activation. ILC2s, then, produce directly Th2 cytokines and DCs induce Th2 responses. DCs loaded with allergen migrate to the draining LN, thus priming naïve T cells and mediating their differentiation into either adaptive Th2 cells or Tfh cells. Inside the lymphoid organ Tfh cells assist antigen-specific B cell-driven IgE production. IgE Abs bind to the high-affinity FcεRI on mast cells and basophils upon sensitization and secondary encounter with the same allergen causes cross-linking of FcεRI-bound IgE. This leads to the activation of these innate immune cells, which, then, release a variety of inflammatory mediators. Th2 cells exit the LN and migrate to the inflamed lungs where, after antigen challenge, they produce Th2 cytokines, like ILC2s. IL-5 and IL-9 support tissue eosinophilia and mast cell hyperplasia, while IL-13 promotes goblet cell-mediated mucus production. Amphiregulin (Areg) is involved in tissue remodeling. All of the above are the main orchestrators of allergic inflammation and AHR. Adapted version from reference 15.

1.1.3 Expanding immunologic mechanisms beyond Th2 responses in asthma

In light of the various mechanisms discovered, embedding molecules previously unrelated to asthma, the classic Th2 paradigm needed to be extended[5,20]. Indeed, non-allergic asthma, for example, implicates pathways that are distinct from the conventional Th2 response[20]. Moreover, it seems that, while the Th2 paradigm claims that eosinophilia coming along with airway inflammation should result in AHR, the majority of sensitized patients with allergic rhinitis do not display AHR, hence are not asthmatic. This indicates that, although Th2 responses are critical for atopy, which raises the risk of asthma, additional mechanisms that eventually lead to the development of the disease may be involved[20]. What is more, therapeutic interventions that aim Th2 cells and their cytokines, albeit efficient in animal models, exhibit little or no efficacy in clinical action[12,20]. These observations, together with the fact that neutrophilic inflammation, as well as non-Th2 cytokines, like IFN-γ and IL-17 are often present in the lungs of asthmatic patients indicate that other inflammatory factors might also regulate asthma phenotype[20]. Indeed, over the past decade research has shifted asthma from a Th2 cell and IgE-dependent disease to a more complex and heterogeneous one, where new emerging effector Th cell subsets coupled with their cytokine repertoire participate[12,20]. Those that gained more attention are discussed below.

According to the Th1/Th2 model, it would have been expected that the role of Th1 cells is to diminish the activity of Th2 cells, thus abrogating the allergic inflammation[1,9,20]. Indeed, it has been initially proposed that Th1 cells per se do not provoke any hallmarks of asthma[12]. In fact, IFN-γ produced by these cells is a negative regulator of Th2 lineage differentiation[7,9], IgE production[20], Th2 cell-mediated eosinophilia, mucus production, and AHR[12]. However, adoptive transfer or activation of Th1 cells in animal models of asthma failed to retract the established Th2 responses and in some cases aggravated the airway inflammation[9]. In addition, subcutaneously administered recombinant human IFN-γ had no detectable efficacy in asthmatic patients compared to controls[20]. Interestingly, it has been reported that Th1 cells are abundant in the airways of patients[12] and IFN-γ levels are increased upon disease exacerbation[12,20], suggesting that they may contribute to asthma pathogenesis. Consistent with these, recent studies have proposed that production of IFN-γ during the effector phase, when Th2 responses have been established, can enhance allergic inflammation by mediating apoptosis of the epithelium, thus destructing the epithelial

barrier[1,20]. Similarly, Th1 cells have been shown to promote the recruitment of Th2 cells into the airways in a non-antigen associated mechanism, thereby augmenting inflammation and AHR. Lastly, Th1 cells may also induce neutrophilic inflammation in the lungs, a common feature of severe asthma[1,12].

Neutrophilic inflammation, however, is mainly mediated by IL-17-producing $CD4^+$ T cells, the so-called Th17 cells, which represent a lineage different from Th1 and Th2 cells and, along with their signature cytokine, IL-17A, they also produce IL-17F and IL-22[12]. Indeed, levels of IL-17A and IL-17F are frequently increased in the lungs, bronchoalveolar lavage fluid (BALF) and sputum of asthmatic patients and these elevated levels are correlated with neutrophilic inflammation, enhanced AHR and corticosteroid resistance[5,12,20]. Hence, it is apparent that Th17 cells and their secreted cytokines are substantial drivers of this severe asthma subtype[12]. A possible incriminating factor for such disease phenotype could be the exposure to fungal allergens, that can stimulate not only Th2 immunity, but also the differentiation of Th17 cells[11], achieved by induction of the lineage-specific transcription factors RAR-related orphan receptor (ROR)γt and RORα[5,20].

IL-17A may be also produced by a small subset of lymphocytes termed γδ T cells, NKT cells, macrophages and neutrophils themselves, and is considered a robust neutrophil chemotactic agent[5]. In addition to this property, evidence supports that IL-17A is able to mediate eosinophilic airway inflammation by inducing expression of eotaxin, an eosinophil recruiting chemokine, from airway smooth muscle cells[12,20]. Moreover, IL-17A is particularly involved in smooth muscle cell contraction, promoting antigen-induced AHR[12]. It can also act, in most cases, together with IL-17F, to regulate local inflammation, by inducing the release by smooth muscle cells and fibroblasts of several proinflammatory cytokines, such as IL-1β, IL-6, TNF-α and granulocyte colony-stimulating factor (G-CSF), as well as IL-8 and CXCL1/Gro-α, which are neutrophil chemotactic proteins[20]. Recent studies also highlight the role of IL-17A in goblet cell hyperplasia, as it coordinates with IL-6 to promote secretion of the mucin proteins MUC5B and MUC5AC from bronchial epithelium in humans. Interestingly, IL-17 has been also correlated with chronic asthma, in terms of collagen production and airway remodeling[20].

Regarding its role in allergic asthma, studies in OVA-induced mouse models showed that exogenous IL-17A diminishes airway eosinophilia[21] and the use of neutralizing mouse anti-IL-17A antibody leads to increased eosinophil infiltration and IL-5

detection in BALF[22]. These data imply a suppressive role of IL-17A during established type 2 immune responses. In accordance with the above, no significant beneficial effect was observed in a study where monoclonal antibody brodalumab against IL-17 receptor A (IL-17RA) was administered to patients with moderate to severe asthma[23]. Nevertheless, it has been reported that IL-17A is able to directly induce AHR, as long-term expression of this cytokine in the lung, achieved through adenovirus-mediated gene transfer, proved adequate enough to provoke AHR[24]. Additionally, Pichavant *et al*. demonstrated that NKT cell-derived IL-17 is indispensable for AHR induction in a non-atopic asthma model, generated by exposure to ozone[25]. Overall, it is becoming evident that IL-17 might have a supplementary function in allergic asthma, but a crucial role in non-allergic asthma, where neutrophils are largely implicated[20].

Another cytokine produced by murine Th17 cells is IL-22. In humans, though, the majority of $CD4^+$ T cells that produce IL-22 do not secrete cytokines or transcription factors related to other Th cell subsets, such as Th1, Th2 or Th17, thereby postulating that they comprise an independent lineage, called Th22 cells[12]. In asthmatic patients, elevated levels of IL-22 mRNA in peripheral blood mononuclear cells (PBMCs), along with increased IL-22 in sera have been detected[12]. It has been found that the main population producing IL-22 in lungs of patients with asthma is $CD4^+$ T cells. Among them, 15% do not produce either IFN-γ or IL-17, assuming that Th22 cells play a role in asthma pathogenesis. The anti-inflammatory activity of IL-22 is counteracted by IFN-γ, suggesting that these two cytokines exert reciprocal antagonistic functions, acting on airway epithelial cells[26].

In mouse models, both proinflammatory and anti-inflammatory properties have been attributed to IL-22. Indeed, a study using an airway inflammation model, concluded that IL-22 is anti-inflammatory, when IL-17 is absent, whilst in the presence of IL-17, IL-22 promotes the recruitment of proinflammatory cells[27]. In the context of asthma, several studies report that IL-22 suppresses eosinophilic inflammation, Th2 cytokine production and AHR in models of antigen-induced airway inflammation, proposing a protective role of IL-22 against asthma. However, Besnard *et al*. highlight the essential role of IL-22 in the induction of OVA-driven allergic asthma, albeit it seems to restrain the effector phase. This study also shows that, at least some of the proinflammatory function of IL-17A is dependent on IL-22[28].

An additional Th cell subset involved in allergic airway inflammation are Th9 cells, which largely produce the cytokine IL-9[15,20]. They are considered distinct from Th2

cells[20], although Th2 can be reprogrammed to become Th9 cells under the influence of TGF-β[15]. Notably, $CD4^+$ T cells from BAL fluid of patients with asthma produce IL-9, suggesting a role of this cytokine in allergic responses[15]. Aiming to elucidate this role, studies in mice have shown that, when overexpressed in lungs, IL-9 induces eosinophilia, accumulation of mast cells and AHR, as well as chronic asthma-associated features, like fibrosis and airway remodeling[29]. In IL-9-deficient mice with OVA-induced asthma, though, no changes in eosinophilic infiltration, goblet cell hyperplasia and IgE production were observed compared to wild type mice[30]. It is, therefore, proposed that IL-9 has a redundant role in antigen-specific AHR development, with other Th2 cytokines compensating for allergic response induction[30].

1.2 Regulatory T cells as negative regulators of Th2 immune responses

The effector function of all Th1, Th2 and Th17 helpers is contained by another T cell subset, named regulatory T cells (Tregs)[31], which promote immune homeostasis by regulating the immune responses[3,4,32]. Tregs encompass various subgroups with suppressive potential, thus protecting from excessive immune responses to pathogens and vigorously contribute to immune tolerance induction[2] towards environmental antigens, cancer cells and transplanted organs[1]. Hence, Tregs play a critical role in preventing the development of various disorders, such as autoimmune diseases, graft-versus-host responses and allergy[31,33]. Growing evidence suggests that immune tolerance to allergens provided by Tregs is important for prevention of Th2-mediated responses that lead to airway inflammation and asthma[4,33].

Development of allergic diseases is the outcome of defective tolerogenic immune responses against allergens, due to tolerance maintenance failure by Tregs[8]. Indeed, it seems that Tregs of healthy individuals actively suppress Th2 responses to allergens[9] and elevated risk for allergic disorders is thought to stem from deficits in generation and function of Tregs[4]. It has been reported that frequencies of Tregs in BAL fluid from children with asthma were strikingly lower than healthy controls[4,8,33]. Moreover, it was shown that mild asthmatic patients had less Tregs in the peripheral blood than normal individuals[33]. Insufficient function of Tregs is indicated by *in vitro* studies in Treg cells obtained from blood of allergic and healthy subjects, where atopic Tregs exhibited reduced capability to restrain proliferation of allergen-specific effector cells and Th2 cytokine production in response to allergens[3,8]. Since Treg cells of asthmatic patients

appear to be defective, they may represent an attractive therapeutic target to treat the disease[3].

1.2.1 Treg cell generation and their subsets

The identification of forkhead-winged transcription factor Foxp3 (forkhead box P3) as the master switch transcription factor for differentiation and generation of functional Tregs established them as a distinct $CD4^+$ T cell subset[1,8,32]. Ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells mediates their conversion into cells with suppressive properties[4,9]. Although in mice Foxp3 is exclusively expressed in Tregs, human $CD4^+$ T cells, when activated by TCR stimulation, transiently express low-level FOXP3 without suppressive capacity[32].

The significance of Foxp3 for Treg development was first demonstrated in mice with a deletion in the forkhead domain of *Foxp3* gene, called scurfy mice. These mice exhibit intense multiorgan inflammatory response, atopic-dermatitis skin-like disease and allergic airway inflammation characterized by IgE hypersecretion, eosinophilia and dysregulated production of Th1 and Th2 cytokines[1]. Accordingly, loss-of-function mutations in *FOXP3* in humans lead to development of a multi-organ lymphoproliferative autoimmune disease, known as Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome[3,8]. These patients suffer not only from autoimmunity in endocrine organs, like type I diabetes and thyroiditis[32], but also from allergic inflammation, including food allergy, eczema, high IgE in serum and eosinophilic inflammation arising from infancy[1,33]. These manifestations underscore the importance of functional Tregs in development of healthy immune responses.

Regulation of Treg suppressive activity by Foxp3 involves inhibition of effector cytokine expression, such as IL-4, TNF-α, IFN-γ, IL-17[3] and IL-2[2]. Expression of Foxp3 itself is greatly dependent on the Runt-related transcription factor (Runx)1 and Runx3, whose induction is regulated by TGF-β[1]. On the contrary, a variety of factors are shown to impede TGF-β-mediated Foxp3 expression. For instance, STAT6 and GATA3 induced by IL-4, bind to the promoter region of *Foxp3* and inhibit its expression[1,4], thus representing a possible mechanism of breaking tolerance in Th2 immune responses[1]. It has been recently proposed that immunological characteristics of Tregs are attributed not solely to the expression of Foxp3, but also to epigenetic

alterations significant for Foxp3 expression maintenance, thereby conferring efficient Treg cell differentiation and establishment of stable lineage[1].

An interesting feature of Tregs is that they partially utilize the transcriptional programs of the target Th cells, via expressing major transcription factors, such as T-bet for Th1 cells and interferon regulatory factor (IRF)-4 for Th2 cells[8,31,33]. This is believed to facilitate constriction of the effector T cell function in which the respective factor is expressed[31]. While under normal conditions this procedure remains manageable, in the context of chronic inflammation it becomes uncontrollable, resulting in reprogramming of Tregs into pathogenic effector T cells[8,33]. Thus, in the highly inflammatory environment shaped by allergic diseases, it is possible that Treg suppressive response can be overcome and Tregs can acquire a proinflammatory pathogenic phenotype[8]. This defective Treg generation under inflammatory conditions is accompanied by loss of Foxp3 expression, while transcription factors that regulate differentiation into other Th cell subsets are retained. These so-called exFoxp3 cells secrete proinflammatory cytokines, such as IFN-γ and IL-17, thereby contributing to allergic airway inflammation[8,31]. A mutation linked to this condition in the mouse gene of IL-4 Receptor α chain (*IL-4Rα*) leads to enhanced phosphorylation and activation of the downstream transcription factor STAT6 in response to IL-4 and IL-13. Therefore, Tregs of these mice exhibit deficits in development and function and adopt a Th2 cell-like phenotype. Similarly, a polymorphism in human *IL-4Rα* gene is related to severe asthma in carriers, as it reinforces IL-4Rα-mediated signaling, thus increasing susceptibility to allergic diseases[8].

Tregs can be broadly divided into two general categories: thymus-derived or natural Tregs (nTregs) and induced Tregs (iTregs). Naturally occurring Tregs originate from $CD4^+$ thymocytes in the thymus following high-affinity interactions of their TCR with autoantigen-MHC complexes in thymic stromal cells[3,4]. These cells specifically express Foxp3 and constitute nearly 5 to 15% of peripheral $CD4^+$ T cells in healthy mice and humans[4]. $CD4^+Foxp3^+$ nTregs are crucial for sustaining immune tolerance to self-antigens and also for containing immune responses against foreign antigens[3,8,33]. Consistent with this, studies in humans reveal that nTregs play a nonredundant role in hampering allergen-specific Th2 responses[9].

The second subset of Tregs, inducible Tregs, occurs extrathymically in peripheral lymphoid tissues from naïve $CD4^+Foxp3^-$ T cells after antigenic stimulation[32,33]. iTregs are further grouped into three main subsets: (i) induced $Foxp3^+$ Treg cells, (ii)

$CD4^+Foxp3^-$ IL-10-producing type 1 Treg (Tr1) cells and (iii) $CD4^+Foxp3^+$ TGF-β-producing Th3 cells[1]. Generation of iTregs requires the presence of specific cytokines, like TGF-β, IL-10 and IL-2 or tolerogenic DCs[3,33]. It has been generally proposed that immature or partially mature DCs are responsible for skewing the differentiation of $CD4^+$ T cells towards Tregs, while mature DCs promote polarization into different effector T-cell subsets[2]. However, in the past years it became evident that fully matured DCs have also the capacity to produce functional Tregs under specific circumstances. These include interactions with probiotic or pathogen-derived molecules and $Foxp3^+$ Tregs, as well as exposure to several exogenous signals (vitamin D3 metabolites, retinoic acid [RA], adenosine and histamine) and tolerogenic cytokines, like TGF-β and IL-10[2].

iTregs mostly regulate tolerance towards antigens not expressed in the thymus, such as commensal microbiota, food antigens and autoantigens[3,33]. In spite of their low percentage in $CD4^+$ T cells, they are highly enriched in local tissues, like the gastrointestinal tract and lungs, especially upon chronic inflammation, where they act to promote local immune tolerance and restrict inflammatory responses[8,33]. In the lungs, the major cell population that drives iTreg induction is the resident macrophages, constantly producing TGF-β and retinoic acid[8]. The important role of iTregs in allergy has been underlined by a study, which, using an asthma mouse model, showed that antigen-specific iTregs were able to efficiently hinder Th2 responses in the absence of natural Tregs[34]. However, it appears that $CD4^+Foxp3^+$ iTregs are less stable than nTregs on grounds of being more prone to losing Foxp3 expression in inflammatory environments, thus becoming exTregs. This is attributed to differences in the methylation status of CpG motifs in the conserved non-coding sequence 2 (CNS2) of the *Foxp3* gene[4,8]. CNS2 locus is steadily hypomethylated in nTRegs, whilst in iTregs is incompletely demethylated[8].

Studies elucidating the functions of nTregs versus iTregs face difficulties to distinguish these two populations due to lack of specific unique markers defining each subset[4,8]. The only proposed markers to facilitate this distinction are the transcription factor Helios and the membrane-bound coreceptor Neuropilin-1 (Nrp-1), which are more abundant in nTregs compared to iTregs. Given that Nrp1 could be upregulated under inflammatory conditions, Helios is considered a more reliable marker to be used for such studies[8]. Typical markers found in $CD4^+Foxp3^+$ Tregs include the α chain of the IL-2 receptor (CD25), cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed

cell death protein 1 (PD-1)[1], CD103 (αEβ7 integrin), ICOS[8], CD45RO[33], CD122 (β chain of IL-2 receptor) and glucocorticoid-inducible tumor necrosis factor receptor (GITR)[32]. However, most of these molecules are also expressed by activated $CD4^+$ effector T cells, thus are not Treg-specific[2,9]. On the other hand, the α chain of the IL-7R (CD127) has been suggested as a differentiation marker expressed on activated T effectors, but not on Tregs[1].

A lot of attention is drawn to the comprehension of immune system development during lifetime with the purpose of defining what triggers allergic diseases[31]. There is growing evidence that exposure to environmental microorganisms during early years in life is negatively correlated with the risk of developing allergy later on[3]. It is now believed that Tregs are involved in this phenomenon by inducing tolerance to harmless antigens[3,33]. This tolerance induction mainly takes place at the intestinal mucosa, where commensal microbiota seem to promote the generation of tolerogenic DCs. Secretion of anti-inflammatory molecules, like IL-10, TGF-β and RA by such DCs favors the differentiation of $CD4^+$ T cells to iTregs in the gut[3]. In addition, microflora is also reported to act directly on T cells. For instance, short-chain fatty acids that some bacteria produce after dietary fiber fermentation, signal to $CD4^+$ T cells to convert them into iTregs and enhance their proliferative capacity[35]. Moreover, a study has shown that *Bacteroides fragilis*, a predominant gut commensal, mediates iTreg induction through activation of the TLR2 signaling pathway on $CD4^+$ T cells[36]. Thus, it becomes clear that alterations in the composition of intestinal microbiota may disturb the interactions between microorganisms and immune cells and contribute to the development of allergic diseases[3].

This strict correlation among gut microflora and allergy leads to the speculation that iTregs responsible for maintaining allergen tolerance are mainly induced at the gastrointestinal tract and then migrate to the site of inflammation to exert their suppressive functions. Moreover, the significance of mucosal tolerance establishment against a variety of allergens early in life lies in the fact that induction of Foxp3 expression in naïve $CD4^+$ T cells is impeded by inflammatory cytokines, such as IL-4 and IL-6. Consequently, the efficiency of *de novo* iTreg generation is expected to descend as the immune system matures and generation of effector T cells has occurred after allergen sensitization. Thus, necessary condition for a lifelong tolerance to allergens, which minimizes the possibility of asthma development, is the sufficient induction of allergen-specific Tregs in early life. In line with the above, it has been

reported that in humans and animal models of allergy, where both sensitization and challenge are mediated through inhalation of allergen, induction of iTregs may be constricted due to the restrictive activities of IL-4 and IL-6[31].

However, an interesting finding is that these same cytokines seem to promote nTreg proliferative capacity and maintenance of their Foxp3 expression, thereby explaining the powerful presence of this subset in the airways during allergic inflammation. It has been demonstrated, though, that chronic STAT6 signaling by IL-4 in effector Th2 cells renders them resistant to Treg-driven suppression. Hence, it is suggested that, despite the beneficial effects of this cytokine on nTregs, in the context of chronic inflammation they might be unable to efficiently repress Th2 functions[31].

1.2.2 Treg cell-mediated mechanisms of suppression

In general, the suppressive effects of Tregs include not only cell-cell contact, but also the secretion of soluble molecules[1,8]. Four main mechanisms of suppression have been described, which involve suppressive cytokines (IL-10, TGF-β, and IL-35), membrane-bound inhibitory factors that may mediate suppression of DC activation (CTLA-4, PD-1), metabolic disruption mechanisms (CD25, cAMP, adenosine receptor 2, histamine receptor 2, CD39, and CD73) and cytolysis (granzymes A and B)[1,2]. Utilizing these mechanisms, Tregs exert suppressive functions during both the sensitization and effector phase of allergic reactions[2] by directly or indirectly repressing most of the cell types implicated in the initiation and preservation of those responses[1]. Importantly, they do not only hamper the activation of allergen-specific Th2 cells, but also repress Th1- and Th17-driven responses, which participate in allergic inflammation, too[1].

It has been proposed that suppressive activity of nTregs is mainly manifested through intercellular contact[3]. For example, Tregs strongly interact with DCs, thus restricting interactions between DCs and naïve $CD4^+$ T cells. This is achieved through the higher expression of adhesion molecules, like LFA-1 and ICAM-1, and NRP1, which promote the Treg-DC interplay[32]. Moreover, LAG-3, a homolog of CD4, is quite enriched on Tregs and binds with high affinity to MHC class II molecules on DCs, therefore promoting the down-modulation of DC function by Tregs[3,32]. Hence, activated Tregs can physically limit the access of naïve T cells to APCs[32]. An additional deprivation mechanism, independent of cell-cell contact, refers to that of IL-2. Tregs

are known to produce very small amounts of IL-2, while highly expressing its receptor. This renders them quite dependent on exogenous IL-2 to sustain their proliferation and survival. Therefore, Tregs can deprive IL-2 from naïve $CD4^+$ T cells, thus inhibiting their activation[32].

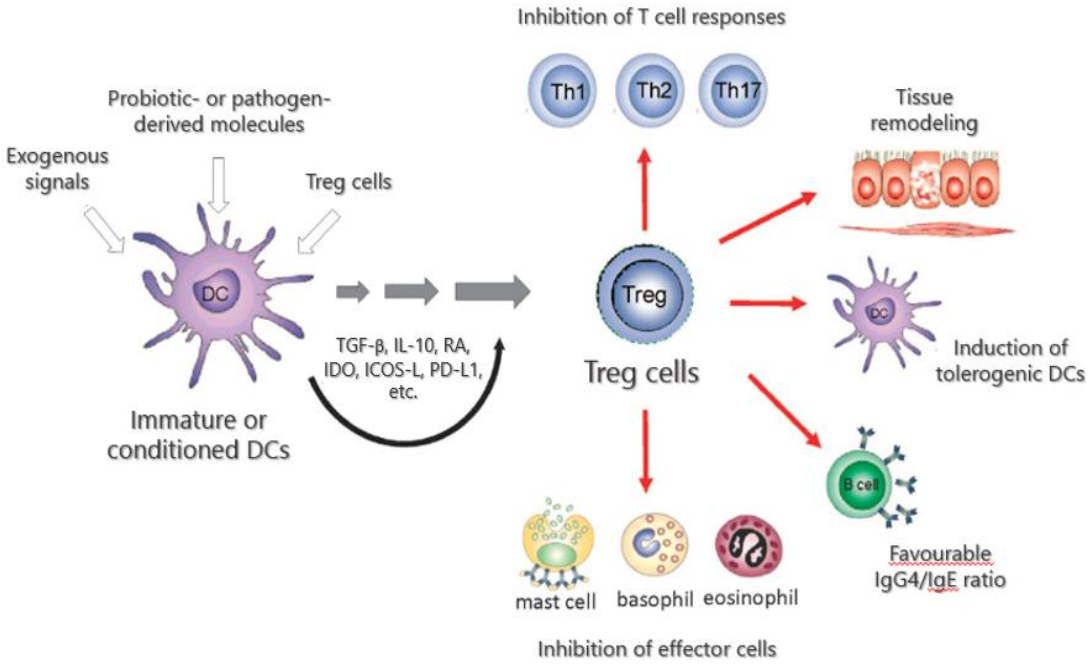
Another surface molecule that confers Treg-mediated suppression is the co-inhibitory receptor CTLA-4. This receptor shares the same ligands, CD80 and CD86 expressed on DCs, with CD28, which is expressed on T and B cells and transduces co-stimulatory signals, essential for activation of these cells[8,32]. However, CTLA-4 presents higher affinity for both DC-expressed ligands, thus outcompeting DC28 for binding to CD80/CD86 and decreasing the immunostimulatory activity of DCs[3,32]. CTLA-4 is also responsible for downregulating MHC class II and co-stimulatory molecule expression on DCs[8,31]. Additional suppressive functions of the CTLA-4-CD80/86 axis is that engagement of CTLA-4 to CD80/86 results in upregulation of the tryptophan catabolizing enzyme molecule indoleamine 2,3-dioxygenase (IDO) in DCs. IDO mediates T cell suppression by promoting pro-apoptotic metabolites and diminishing the available tryptophan in the T cell surroundings[3,32]. Finally, CTLA-4 signaling has been proposed to increase the conversion of naïve T cells to $Foxp3^+$ T cells via TGF-β[32].

Another molecule important for contact-dependent suppression by Tregs is the cyclic adenosine monophosphate (cAMP). It seems that cAMP is diffused from the Treg into the T effector cell through gap junctions and activates the transcriptional repressor inducible cAMP early repressor (ICER), which in turn inhibits T effector proliferation and IL-2 synthesis[3,32]. Tregs are also able to obstruct mast cell degranulation by directly acting on mast cells through OX40. Interactions between OX40 on Tregs and OX40L on mast cells increase the levels of cAMP in the latter, leading to blockage of extracellular $Ca2^+$ influx and, hence, inhibition of cytosolic granule release[8]. Additionally, Tregs are able to downregulate FcεRI expression on mast cells via a mechanism that does not rely on IL-10 and TGF-β and, therefore, indirectly decrease allergen-induced degranulation[1]. IL-33-driven expansion of ILC2s can be also compromised by Tregs with subsequent reduction of IL-4 secreted by these cells[8].

iTreg-mediated suppression, on the other side, is thought to be more dependent on cytokine secretion[3]. IL-10 exerts an important role in both establishing peripheral tolerance and preventing prolonged inflammatory responses[2,9]. Numerous studies have

reported that, regarding allergic responses, IL-10 has emerged as a key cytokine to alleviate allergic inflammation[1]. Beside Tregs, this cytokine is generally produced by other T cells, B cells, DCs, monocytes, macrophages and mast cells[2,32]. The most prominent producers of IL-10, however, are Tr1 cells, which also use TGF-β, as well as cytotoxic molecules, like perforin and granzymes, to mediate their function[1].

Immunosuppressive actions of IL-10 include its capacity to suppress the production of proinflammatory cytokines, chemokines and chemokine receptors, as well as to attenuate $CD4^+$ T cell activation both directly and indirectly[2]. Indirect inhibition is mediated through downregulation of MHC class II molecules and CD80/CD86 on DCs, thus promoting tolerogenicity of these cells, leading to T cell anergy[8,31]. Repression of



Picture 2: Effects of Tregs on type 2 responses. Immature or mature conditioned DCs mediate generation of Treg cells, which can inhibit allergic inflammation through various modes of action. These include both cell–cell contact and secretion of suppressive cytokines, like IL-10 and TGF-β. Hence, Tregs mediate direct or indirect suppression of Th cell activation, the latter including down-modulation of DCs. Moreover, they promote DC tolerogenicity, which further endorses Treg differentiation. Tregs also interfere with survival of effector cells, such as mast cells, eosinophils and basophils and degranulation of mast cells. Regarding B cells, Tregs are able to restrain IgE production, while promoting IgG4 isotype. Lastly, Tregs are implicated in airway tissue repair. Adapted version from reference 2.

T-cell co-stimulation is mediated by IL-10 also through direct action on CD28, ICOS and CD2[2]. In addition, there are indications of direct inhibitory effects on generation and proliferation of Th17 cells, as they express IL-10 receptor[3,32]. B cells are also regulated by IL-10 in terms of Ig isotype, as it promotes switching from IgE into the

anti-inflammatory IgG4[2,9,31]. Lastly, IL-10 per se is capable of inducing $CD4^+$ naïve T cell differentiation into IL-10-secreting cells[32].

TGF-β is another immunosuppressive cytokine with pleiotropic functions, including allergen tolerance maintenance[2,32]. Its implication in asthma is complex and, apart from TGF-β-secreting Th3 cells and nTregs, it is also expressed by several other cell types, like eosinophils and tissue resident cells. Therefore, the concrete contribution of TGF-β produced by Tregs to allergic responses requests further study[2]. The suppressive effects of this cytokine regard the inhibition of both B and T cell proliferation, as well as Th1 and Th2 differentiation and survival[2]. Indeed, it has been proposed that the latent form of TGF-β bound on the surface of Tregs through glycoprotein A repetitions predominant (GARP) receptor[32], activates Notch signaling, leading to induction of hairy and enhancer of split 1 (Hes1) in naïve $CD4^+$ T cells[31]. This protein strongly suppresses gene expression and is associated with T cell activation obstruction[31]. In addition, evidence suggests that histamine increases responsiveness of $CD4^+$ T cells to TGF-β through the abundant on Th2 cells histamine-2 receptor, thus augmenting TGF-β-mediated constraint of Th2 reactions[9]. TGF-β also promotes immature and suppressive phenotypes of macrophages and DCs[2], together with IDO expression in the latter[32]. As mentioned before, TGF-β can induce Foxp3 expression in naïve $CD4^+$ T cells, too, thereby conferring immunosuppressive capacities to these cells[2].

More controversial is the involvement of TGF-β produced by Tregs, eosinophils or lung epithelium in airway remodeling by exerting immunomodulatory and fibrogenic functions, like collagen-I synthesis induction in parallel with collagenase expression inhibition[2]. Thus, TGF-β may represent a significant mediator of lung healing aiming to repair asthmatic airways, albeit this tissue remodeling could result in the opposite effects and contribute to the pathogenesis of asthma[1]. A relatively new cytokine identified as exclusively produced by Tregs is IL-35, which enhances their suppressive effects[31,33]. Functions of IL-35 include promotion of Treg proliferation, while it suppresses Th17 responses. IL-35-producing Tregs, named as "iTr35", albeit not expressing Foxp3, are highly stable *in vivo*[33]. Alongside cytokine production, Tregs also secrete the cytotoxic molecules, perforin and granzyme, in order to kill APCs[1,32]. All in all, the distinct Treg cell subsets seem to be essential negative regulators of allergic diseases by utilizing both contact- and secretion-dependent mechanisms, each at different proportions. Picture 2 summarizes the targets of Treg immunosuppressive functions.

1.2.3 Treg cell-oriented therapeutic interventions

Due to Treg capacity of suppressing inflammatory responses, therapeutic use of these cells is considered as an effective approach for treating asthma[4]. Up until now, the most broadly used medication involves inhaled glucocorticoids and β2-adrenergic agonists, which are primarily efficient in eosinophilic inflammation[10]. These treatments are shown to be related with Treg induction in asthmatic patients and glucocorticoids, in particular, are responsible for inducing IL-10 secretion by human T cells[3]. However, in cases of severe asthma, resistance in glucocorticoids is observed, likely attributed to interactions between genetics, environmental factors and patterns of immune cells implicated in the asthmatic response[37]. Thus, combining glucocorticoids with 1,25-dihydroxyvitamin D3 (Calcitriol), which has been reported to restore IL-10 production in steroid-resistant patients, may provide a more effectual approach[3,4].

Nevertheless, these therapies aim to down-modulate the inflammatory processes leading to asthma and not to regulate the mechanisms that drive this disease[3]. Thus, a more tolerance-inducing focused approach has been introduced, termed as antigen-specific immunotherapy (ASIT)[33]. More precisely, ASIT includes administration of a specific allergic antigen in increasing doses[3] in order to achieve desensitization and long-term tolerance induction towards this antigen[33]. Therefore, through ASIT not only symptoms can be controlled, but also asthma development from allergic rhinitis can be prevented and regression of an established disease is feasible[38]. Tregs are significantly involved in this process as augmented Treg differentiation along with enhanced IL-10 production and low levels of *FOXP3* methylation have been observed in successfully ASIT-treated patients[33]. However, this therapeutic approach still faces limitations and further investigation is needed to overcome them[33].

1.3 TIGIT: a molecule implicated in asthma

1.3.1 Background information and modes of action

With respect to the existence of functionally diverse subtypes of Tregs, a co-inhibitory molecule on the surface of $Foxp3^+$ Treg cells called T cell immunoglobulin and ITIM domain (TIGIT) has been shown to delineate a Treg subpopulation with highly suppressive capacity[39]. TIGIT was initially identified in 2009 through bioinformatics by three independent groups and was also given the names Vstm3 and

WUCAM[40-42]. It is a member of the CD28 family, a subgroup of the Ig superfamily[43,44] and consists of an extracellular Ig variable domain, a type 1 transmembrane region and a cytoplasmic tail that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) motif and an Ig tail-tyrosine (ITT)-like motif[43-45]. Albeit highly conserved between mouse and human, which of these two motifs is required for TIGIT signaling is unclear and may vary between species[43,44]. Cells expressing TIGIT are almost exclusively lymphocytes, such as effector $CD4^+$ and $CD8^+$ T cells, Tregs, Tfh and memory T cells, and beside T cell compartment it is also expressed on NK cells[46]. TIGIT expression on naïve T cells is minimal or absent but readily induced upon activation[45].

TIGIT receptor is considered critical for peripheral tolerance maintenance and seems to control the threshold of T cell activation, as imposed by enhanced T cell proliferation and cytokine production, as well as exacerbation of autoimmunity in the absence of TIGIT *in vivo*[47]. Poliovirus receptor (PVR), also known as Necl5 and CD155, was identified as a high-affinity cognate receptor for TIGIT[42], but it was shown that TIGIT can also bind CD112 (or else PVRL2/nectin2), though with lower affinity[43,45]. Both CD155 and CD112 are expressed on APCs, particularly DCs, and in some other cell types, as well as tumor cells[42,44].

CD226 (or DNAM-1) is a glycoprotein, also member of the Ig superfamily, serving as a co-stimulatory receptor and is expressed on NK cells, monocytes, platelets and activated $CD4^+$ and $CD8^+$ T cells[48]. CD226 binds the same ligands as TIGIT and together form a pathway that resembles CD28/CTLA-4–CD80/CD86 pathway, where both CD28 and CTLA-4 receptors are found on T and NK cells and their ligands are expressed on APCs[43]. Moreover, CD28 binding provides positive, while CTLA-4 negative signaling[49]. Similarly, CD226 competes TIGIT for engagement to CD155 and CD112, with the former being a positive T cell regulator and the latter having an inhibitory role[43,47]. Interestingly, TIGIT binding to CD155 is of much higher affinity, 100-fold actually[42], than CD226-CD155 interactions[43,49], like CTLA-4 has higher affinity for CD80/CD86 than CD28, indicating competitive inhibition of co-stimulation[44]. Collectively, whether the immune response will be elicited or inhibited largely depends on structural differences, dynamic regulation of TIGIT and CD226 co-expression, as well as their differing affinities for the same ligands[44].

TIGIT downstream cell intrinsic signaling through its ITT-like and ITIM motifs has been mostly studied in NK cells[44,45]. Phosphorylation in these motifs induces the

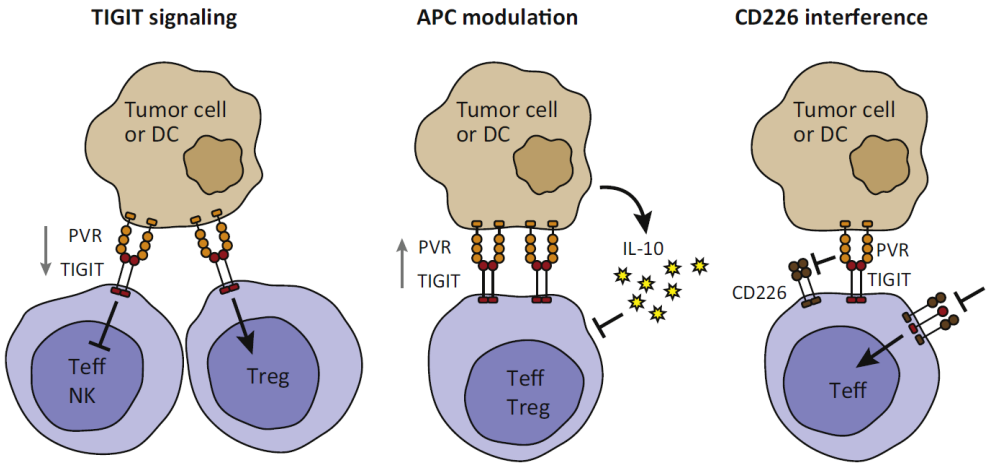
binding of cytosolic adaptor protein Grb2 (growth factor receptor-bound protein 2) to the TIGIT tail, which in turn leads to recruitment of SH2-containing inositol phosphatase 1 (SHIP1). SHIP1, then, hinders the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways[43,44]. Moreover, upon phosphorylation of ITT-like motif, SHIP1 is recruited through β-arrestin 2 and interferes with TRAF6 auto-ubiquitination, therefore restricting nuclear factor (NF)-κB signaling[44]. These effects result in reduced NK cytotoxicity, granule polarization, and cytokine secretion[43].

A few mechanisms of TIGIT inhibitory function towards effector T cells have been proposed. Initial reports revealed a cell-extrinsic regulation by engagement with CD155 on DCs[42]. TIGIT homodimers trans-interact with CD155 homodimers forming heterotetramers, thus leading to CD155 tail phosphorylation and recruitment of Src homology region 2 domain-containing phosphatase 2 (SHP2) to mediate activation of MAPKs Erk[44] and p38 in DCs[45]. TIGIT-CD155 engagement eventually leads to decrease of IL-12p40 production together with increment in production of IL-10, thus endorsing a tolerogenic DC phenotype[42,44,45]. Yu *et al*. suggested that this might be a mechanism through which fully activated DCs could still confer dampening of T cell proliferation and cytokine production[42].

Following studies showed that TIGIT can mediate T cell responses in a cell-intrinsic manner, too[47]. TIGIT signaling in T cells attenuates activation, proliferation and cytokine production by downregulating components of the TCR complex, like TCRα and CD3ε, as well as downstream molecules of the TCR signaling pathway[43,45]. On the other hand, several molecules important for T cell maintenance, such as the anti-apoptotic Bcl-xL and cytokine receptors IL-2R, IL-7R, and IL-15R are found upregulated. In this respect, although TIGIT limits T cell activation and expansion, it seems that this molecule also contributes to their survival, therefore ensuring that they are not deleted from the repertoire[47].

In addition to competing with CD226 for binding their ligands, TIGIT has been also reported to interact with CD266 in cis, thus disrupting its homodimerization and its subsequent co-stimulation. However, the degree of TIGIT and CD226 co-expression on T cells in the context of inflammation needs further investigation[43]. Moreover, a recent study indicated that tolerant state is successively passed from induced tolerant T cells to newly activated cells via the TIGIT/CD155 axis, where TIGIT acts extrinsically as a ligand[50]. In particular, TIGIT expression on tolerant cells interacts with CD155 on

TCR-activated T cells and enhances TIGIT expression in the latter, while suppressing IL-2 production. Therefore, a novel process for long-term maintenance of tolerant state in T cell population was proposed, alternative of the long-lived tolerant T cells[50]. The main inhibitory functions of TIGIT are synopsized in Picture 3.



Picture 3: TIGIT-mediated impeding modes of action. TIGIT signaling through its ITIM and/or ITT motifs leads to NK and T effector cell inhibition upon binding to PVR, while promoting Treg cell function (left). TIGIT binding to PVR can also induce PVR signaling in DCs, resulting in a tolerogenic phenotype (middle). Finally, TIGIT can interfere with CD226-mediated signaling by having higher affinity for binding to PVR or by disrupting homodimerization of CD226 (right). Adapted version from reference 45.

1.3.2 TIGIT differentially affects distinct Th cell subsets

Several co-inhibitory receptors, like PD-1 and CTLA-4, are significant regulators of T cell responses in chronic diseases, including tumor, which renders them attractive targets for therapeutic interventions[43,44]. Despite the notable success of immunotherapies that block CTLA-4 and PD-1 in treating several types of cancer, their efficiency remains quite low in a reasonable number of patients[43,44]. This has drawn attention to other co-inhibitory receptors, including TIGIT, and therapies interfering with these molecules are currently being tested in clinical trials[43,44].

The functional relevance of TIGIT in tumor setting mainly concerns its high enrichment in $CD8^+$ tumor-infiltrating lymphocytes (TILs), where it marks the most dysfunctional subset, co-expressing PD-1, Tim-3 and Lag-3[46]. Suppression of anti-tumor $CD8^+$ T cell responses depends on many of the above-mentioned mechanisms and it has been postulated that TIGIT expressed on tumor-tissue Tregs is the main mediator of suppression unlike TIGIT expressed on $CD8^+$ TILs[46]. Indeed, TIGIT-bearing Tregs exhibit a highly activated and repressive phenotype with increased

secretion of IL-10, perforin and TGF-β[44]. These molecules, together with the induction of tolerogenic DCs through TIGIT-CD155 interactions may contribute to suppression of anti-tumor responses[46]. Moreover, TIGIT could be mediating the differentiation of tumor-associated macrophages type 2 (TAM2), which also produce IL-10 and TGF-β, as well as enzymes, like arginase-1 and IDO, responsible for nutrient depletion in the tumor microenvironment (TME)[44]. Finally, it is apparent that TIGIT on Tregs might limit the availability of CD155 for interactions with CD226 on $CD8^+$ T and NK cells[44].

TAM2 are induced in response to Th2 cytokines, like IL-4, which are present in the TME[44]. Indeed, a study of Joller *et al*. revealed that $TIGIT^+$ Tregs selectively suppress Th1 and Th17 responses, while sparing Th2 responses, therefore suggesting that TIGIT defines not only a highly activated, but also a functionally distinct Treg population[39]. They also showed that the majority of $TIGIT^+$ Tregs are nTregs, as its expression was correlated with markers, like Nrp-1 and Helios[39]. This is consistent with the fact that TIGIT gene is a direct target of Foxp3 and Treg-specific regions in $TIGIT^+$ Tregs have been found highly demethylated compared to their $TIGIT^-$ Treg counterparts, which is associated with enhanced lineage stability[43]. The transcriptional profile of $TIGIT^+$ Tregs justifies their enhanced immunosuppressive function, as indicated by the expression of many co-inhibitory receptors (CTLA-4, PD-1, Lag-3, Tim-3) and effector molecules, including IL-10, granzyme B and fibrinogen-like protein 2 (Fgl2)[39].

Interestingly, this study[39] found that TIGIT ligation promotes *Fgl2* transcription in $TIGIT^+$ Tregs, possibly through induction of the transcription factor CCAAT/enhancer-binding protein (CEBP)α, which then binds to the genomic region of *Fgl2*, thus driving its expression. Fgl2 was identified as the responsible molecule for differential suppression of $TIGIT^+$ Tregs. In addition to its role in diminution of all three Th subset proliferation, it was essential for preventing repression of the Th2 cytokine IL-4 both *in vitro* and *in vivo*, while it was not involved in the impediment of Th1 and Th17 cell cytokine production. Hence, both $TIGIT^+$ Tregs and $TIGIT^-$ Tregs exhibit comparable suppressive capacity regarding Th1/Th17 cell differentiation, however $TIGIT^+$ Tregs selectively spare Th2 responses. On the whole, this study elegantly demonstrated the differential suppression of distinct Th cell lineages and, subsequently, the functional specialization that TIGIT attributes to Tregs, rendering this molecule a target for specific Treg subgroup manipulation, in order for definite immune responses to be controlled[39].

1.3.3 Allergic airway inflammation is reinforced by TIGIT

In line with these results, a previous study of our lab identified the promotive role of TIGIT in Th2 immunity upon allergic asthma induction[51]. More precisely, TIGIT expression was upregulated specifically in Th2 polarized cells after reactivation, in contrast with Th1 and Th17 cell cultures. In addition, TIGIT production on Th2 cells was correlated with enhanced expression of GATA3 and c-Maf, as well as Th2 cytokine production. Allergic mice exhibited increased numbers not only of $TIGIT^+$ $CD4^+$ T cells, but also of $CD155^+$ DCs in draining mediastinal lymph nodes (MLNs) compared to controls and further investigation underscored the importance of TIGIT-CD155 interaction for Th2 differentiation and cytokine secretion *in vitro*. Importantly, TIGIT blockade with a polyclonal anti-TIGIT blocking Ab upon polarization towards the three distinct Th subsets led to significant reduction of $TIGIT^+$ Th2 cells, while Th1 and Th17 polarized cells had unaltered $TIGIT^+$/$TIGIT^-$ T cell balance[51].

In an *in vivo* setting, administration of TIGIT-blocking Ab during allergen (OVA) challenges in mice evolving allergic asthma attenuated disease manifestations, as indicated by diminished infiltration of eosinophils and macrophages in lungs and lower mucus production, as well as OVA-specific IgE production. Accordingly, blockade of TIGIT significantly decreased the numbers of Th2 and Tfh effector cells in MLNs, together with the levels of *Gata3* expression in $CD4^+$ T cells isolated from these lymph nodes. Moreover, production of IL-4, IL-5, IL-13 and IL-10 in Ag-specific responses of MLNs, as well as in BAL fluid was dramatically reduced. Overall, TIGIT seems to serve a promotive role upon Th2 recall responses, as its blockade during antigenic challenge conferred remarkable protection from allergic airway inflammation, while not favoring Th1 or Th17 Ag-specific responses[51].

1.4 Aim of the study

TIGIT obstruction through blocking Ab in the above experiments[51], however, could presumably occur on both Th2 effector cells and Tregs, Therefore, the aim of this dissertation was to elucidate the putative involvement of $TIGIT^+$ Treg cells in anti-TIGIT-mediated suppression of allergic airway disease, thus identifying the TIGIT source that contributes to Th2 inflammatory response. To address this issue, the DEpletion of REGulatory T cells (DEREG) murine model was harnessed for specific depletion of Treg cells[52]. These bacterial artificial chromosome (BAC) transgenic mice

express the diphtheria toxin receptor (DTR) coupled to enhanced green fluorescence protein (eGFP) under the control of an additional Foxp3 promoter. This allows direct analysis of this subset and, upon diphtheria toxin (DT) administration, particular *in vivo* deletion of $Foxp3^+$ Treg cell population is achieved[52]. Tregs were depleted in sensitized mice prior to OVA-mediated asthma induction and anti-TIGIT Ab was administered before OVA challenges. One group comprised mice with functional Tregs, while the other had its Tregs deleted and either anti-TIGIT or isotype control Ig Ab was administered to each group. By comparing the amelioration of allergic asthma upon TIGIT blockade in the presence or absence of Tregs, this thesis provided insight in the understanding of the role that TIGIT exerts in augmenting allergic airway inflammation.

2. MATERIALS & METHODS

2.1 Animals

All the *in vivo* experiments were conducted using C57BL/6-Tg Foxp3-DTR/EGFP DEREG mice, which were provided by Tim Sparwasser (Centre for Experimental and Clinical Infection Research, Hannover, Germany). Mice that were used in the experiments were 8-12 weeks old age-matched males and were accommodated at the Animal Facility of the Biomedical Research Foundation, Academy of Athens (BRFAA). All procedures undertaken were in agreement with US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736-01) and with the European Union Directive 86/609/EEC for animal research.

2.1.1 Genotyping

In order to decide which animals should be used in experimental and which in control groups, all litters of every mating were genotyped. In brief, the last 2 mm of each mouse's tail were obtained and incubated overnight (O/N) at 55^{o}C with 2% Proteinase K (PK) (5mg/ml) in lysis buffer (50mM Tris pH 8, 100mM NaCl, 1% SDS, 100mM ethylendiaminetetraacetic acid [EDTA]). The next day, samples were vortexed and centrifuged at 13000 rpm for 10 min. Supernatant was transferred in new tubes where equal volume of isopropanol was added and tubes were mixed so that the DNA could precipitate. After a centrifugation at 13000 rpm for 5 min, disposal of supernatant and addition of 100 μl 75% ethanol, samples were again centrifuged as previously. Following, supernatant was discarded and the tubes were left for 30 min at room temperature (RT) before the DNA pellets were re-dissolved in distilled water (dH_2O). The DNA was either stored at -20^{o}C or directly used in the rest of the process.

The next step was to enhance the DNA sequence of interest via polymerase chain reaction (PCR). The reagents used in the procedure are summarized in Table 2. A master mix containing the aforementioned components, except for the DNA, that covered for the number of the samples together with positive, negative and distilled water controls was prepared. The master mix was, then, divided into PCR tubes (24 μl/tube) and 1 μl of each DNA sample or the controls was added. The tubes were inserted into the PCR machine (SimpliAmp™ Thermal Cycler, Applied Biosystems,

CA, USA.), which was set at the program that Table 3 presents. Both the primers and the cycling program were the ones suggested by The Jackson Laboratory.

Table 2: PCR master mix for DEREG genotyping. DreamTaq™ Hot Start DNA Polymerase was purchased from Thermo Fisher Scientific (MA, USA). dNTP, deoxyribonucleotide triphosphate

Reaction Component	µl per reaction
DNA	1
10x DreamTaq™ Buffer	2.5
25 mM dNTPs mixture	0.25
20 µM Transgene Forward Primer 10012	0.625
20 µM Transgene Reverse Primer oIMR9402	0.625
20 µM Wild type Forward Primer oIMR8744	0.625
20 µM Wild type Reverse Primer oIMR8745	0.625
DreamTaq™ Hot Start DNA Polymerase	0.125
dH_2O	18.625
Total	25

Table 3: PCR thermal profile of DEREG genotyping.

Stages	Temperature (°C)	Time
1	94	2 min
2 (10x cycles)	94	20 sec
	65	15 sec (-0.5°C/cycle)
	68	10 sec
3 (28x cycles)	94	15 sec
	60	15 sec
	72	10 sec
4	72	2 min
	10	∞

Subsequently, an agarose gel was prepared for electrophoresis of the PCR products. Since the expected DNA bands for the transgene and the internal positive control were at 370 bp and 200 bp, respectively, a 1,5% agarose gel was considered appropriate. Agarose was dissolved in Tris Acetate-EDTA (TAE) running buffer 1x (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) via a 3 min heating in microwave oven and, then, 5 µl of SafeView nucleic acid stain (NBS biologicals, Huntingdon, UK) were added per 100 ml gel. TAE 1x that was used in electrophoresis was also enriched with 5 µl

SafeView/100 ml for better band clarity. Electrophoresis was held at 90 Volt for 1 h and, eventually, the gel was exposed to ultraviolet (UV) light in order for the DNA bands to be visualized.

2.2 In vivo experimental protocol

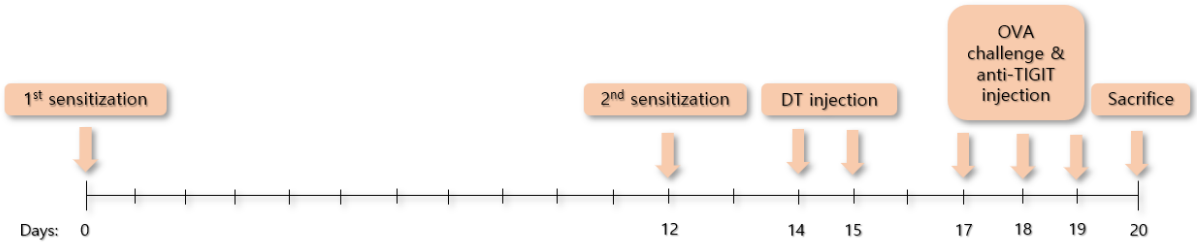
Days 0 and 12: Intraperitoneal (i.p.) injections of 0.01 mg chicken OVA (Sigma-Aldrich, MO, USA) in 0.2 ml aluminum hydroxide (alum; Serva Electrophoresis GmbH, Heidelberg, Germany) were carried out on mice so that allergen sensitization could occur. OVA was at first added in sterile Dulbecco's phosphate buffer saline (PBS) and, after vortexing and alum addition, the mixture was left rotating for 1 h at 4^{o}C.

Days 14 and 15: 1 μg/mouse of Diphtheria Toxin (DT; Sigma-Aldrich) dissolved in sterile PBS was administered i.p. to the groups of mice that Treg population deletion was desirable. According to Katharina Lahl and Tim Sparwasser[52], treating mice with 1 μg DT for two consecutive days results in efficient Foxp3^{+} cell depletion by day 3 (day of first allergen challenge in our setting). It is not until day 5 (day of last allergen challenge in our setting) that Tregs start to reappear in peripheral blood and immunological organs, reaching normal levels at day 14.

Days 17,18 and 19: Three consecutive OVA challenges were performed through administration of aerosolized OVA (5%) in sterile PBS for 30 min for allergic airway disease induction. Mice were treated with 250 μg of the mouse purified blocking monoclonal (clone 10A7) antibody against TIGIT, that was kindly provided by Genentech (or Ig isotype control Ab) 30 min before each challenge. A small blood sample was, also, obtained after the first and third OVA challenge through tail bleeding to assess the extent of Treg depletion.

Day 20: Mice were sacrificed via isoflurane inhalation (IsoFLo, Abbott, IL, USA) and, mediastinal lymph nodes (MLNs), as well as lung tissue, were obtained. LN-derived cells were, then, cultured and lung sections were conducted in order for the cytokine production of T cells and the histopathological image of the lungs to be estimated, respectively. A fraction of the isolated cells was stored at -80^{o}C as cell pellets for RNA extraction and qPCR performance.

Picture 4 visualizes the steps followed during the allergic airway disease induction, according to the aforementioned experimental protocol.



Picture 4: Illustration of the experimental protocol for induction of allergic asthma *in vivo*.

2.3 Histopathology

The harvested lungs were inoculated with 10% formalin (Sigma-Aldrich) and remained at 4°C O/N. After that, they were submersed in 70% ethanol and sent to the Histology Department of the BRFAA for tissue sectioning and staining. Inflammation was evaluated on transversal sections stained with hematoxylin and eosin (H&E), whereas periodic acid schiff (PAS) staining was informative on mucus secretion. H&E section scoring of inflammation was estimated as follows: 0 for sections that displayed up to three foci of peribronchial and perivascular infiltration, 1, 2 or 3 for sections with four, five or six inflammatory foci, respectively, while score 4 was used for sections that carried more than six foci of peribronchial and perivascular inflammation.

2.4 Evaluation of immunological parameters in MLNs of allergic mice

2.4.1 Cell isolation from Mediastinal Lymph Nodes

MLNs of each mouse were isolated in RPMI, smashed on 70 μm cell strainers (BD Falcon™) and cells were suspended in fresh RPMI. A centrifugation at 1200 rpm for 10 min at 4°C and resuspension of cell pellet in complete RPMI followed. A cell sample was mixed with Trypan Blue 0,4% (Sigma®) in 1:1 dilution and cells were counted using a haemocytometer. In a 96-well microplate, 10^6 cells per well were added and primed with further reagents to conduct subsequent assays.

2.4.2 Flow Cytometry

Flow cytometry is a technique that mediates the analysis of various individual cell characteristics out of heterogeneous populations, giving also the potentiality to sort different cell subpopulations through fluorescence-activated cell sorting (FACS). Among other applications of this tool, like multi-parametric DNA analysis, fluorescent protein, proliferation and cell counting, a quite common one is immuno-phenotyping, which involves antibodies directed against specific antigens that some cells express, resulting in clustering of cells into populations, according to the expression or not of several proteins[53].

The blood acquired from tail bleeding (~400 μl) was centrifuged at 1800 rpm, 15^{o}C for 30 min without stop, after adding histopaque (Sigma-Aldrich) up to 1 ml below the blood. That way, stratification of blood cell populations was achieved and peripheral blood mononuclear cells (PBMCs), forming a white ring between the plasma and histopaque, were collected. To wash away histopaque, PBS 1x was added and a centrifugation at 5000 rpm, 4^{o}C for 10 min followed. Pellets were resuspended in 1% fetal bovine serum (FBS) in PBS 1x and incubated with Phycoerythrin (PE)-fluorescing anti-CD3 Ab for 20 min at 4^{o}C in the dark. Single stain, as well as unstained controls were also created. After that, cells were washed with PBS to remove the excess Ab and pellets were resuspended in 1% FBS and transferred to FACS tubes. Detection of Tregs was carried out exploiting the fluorochrome of anti-CD3 Ab as well as the green fluorescence of eGFP expressed in these cells.

To detect the presence of the cytokines IL-4, IL-5, IL-13, IFN-γ, IL-17, IL-10, as well as the transcription factor Foxp3 expressed by the lymphocytes isolated from MLNs, intracellular cytokine staining needed to be done, since they are secreted proteins, using the Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit by BD Biosciences (NJ, USA). For this purpose, the isolated cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin for 4 hours or 10 ng/ml and 250 ng/ml, respectively, for 12 hours. Protein transportation was blocked using 1 μg GolgiPlug™ (BD Biosciences) per ml culture. Following, cells were harvested, washed with RPMI (1200 rpm/10 min) and resuspended in 1% FBS, containing Fc Block™ (1 μg/10^6 cells), which binds FcγII/III receptors and prevents nonspecific staining. After an incubation for 10 min at 4^{o}C, cells were centrifuged at 1200 rpm for 10 min to remove unbound Fc blocker and were resuspended in 1% FBS,

before they were plated so as to be stained for the surface antigens CD3, CD4 and CD44.

In order to finally detect as many cytokines as possible according to the lasers available, two master mixes containing differently fluorescent Abs that bind the aforementioned markers were prepared and applied to each sample for a 20-min incubation at 4ºC in the dark. A small number of cells was intended for single staining and unstained controls. The plate was, thereafter, centrifuged as previously, supernatants were discarded and pellets were re-dissolved in 100 μl PBS/FBS 1% adding 100 μl Fixation/Permeabilization solution. After an incubation for 20 minutes at 4°C, cells were washed two times with Perm/Wash™ buffer 1x. Two master mixes of Abs against T cell surface markers and the cytokines of interest diluted in Perm/Wash™ buffer were prepared and each mix was added to each sample. Table 4 summarizes the different mixes used in two independent experiments. Incubation for 20 minutes at 4°C, then, followed and, after two washes with Perm/Wash™ buffer, stained cells were resuspended in 1% FBS and transferred into FACS tubes for Flow Cytometry analysis. Running of samples was performed either at Attune™ Acoustic Focusing Cytometer (Applied Biosystems) or at BD FACSAria™ cell sorter (BD Biosciences) and data were analyzed with FlowJo v10 software.

Table 4: Ab composition of each of the master mixes added to the samples in two experimental repetitions, A and B. APC, Allophycocyanin; BV, Brilliant Violet; Cy, Cyanine; FITC, fluorescein isothiocyanate; PE, phycoerythrin

A	FITC	PE	PE-Cy5	PE-Cy7	BV421	BV510
Mix 1	CD44	IL-17	CD4	IL-13	IL-5	CD3
Mix 2	CD44	CD4	Foxp3	CD3	IL-4	IFN-γ

B	FITC	PE	PE-Cy5	PE-Cy7	Pacific Blue	APC-Cy7
Mix 1	CD4	IL-17	CD44	IL-13	IL-5	CD3
Mix 2	IL-10	IL-4	CD44	IFN-γ	CD4	CD3

2.4.3 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a sensitive quantitative method that measures the concentration of specific molecules, such as peptides/proteins, vitamins, hormones or drugs in biological fluids. This is achieved by using enzyme-conjugated Abs that bind to the molecule of interest and react with the enzyme substrate, eliciting a color change. This method was first described by Engvall and Perlmann in 1971 via modifying the radioimmunoassay (RIA), in which the radioactive isotope iodine 125 was used instead, and is very broadly utilized as a diagnostic tool[54].

The cells isolated from the MLNs were stimulated *ex vivo* with 125 μg/ml OVA in 96-well plates. After a 67/72-and a 96-hour incubation at 37^{o}C, plates were centrifuged at 1200 rpm for 2 min and supernatants were collected in new plates and stored at -80^{o}C for use in ELISA. The above-mentioned timepoints were selected based on the peak production of the cytokines evaluated, that is 67-72 h for IL-4, IL-5, IL-13 and IFN-γ, whereas IL-10 production peaks at 96 h of incubation.

All the ELISA kits were purchased from R&D systems® (MN, USA). The procedure included an initial priming of high binding ELISA plates with 100 μl of capture Ab/well diluted in sterile PBS. After an O/N incubation at RT, the plates were washed 3 times with PBS/Tween 0,05% (Wash Buffer) and left for at least 1 hour for blocking with 300μl PBS/BSA (bovine serum albumin) 1% (Block Buffer) added to each well. Then the plates were again washed 3x with Wash Buffer and sample plating was done by adding 100 μl sample per well. The samples were already diluted 3 times in Reagent Diluent, which for IFN-γ was BSA 0,1% Tween 0,05% in PBS, while for the rest of the cytokines was the same as the Block Buffer. Concomitantly, seven 2-fold serial dilutions of recombinant cytokine in Reagent Diluent were prepared and added to the plates (100 μl/well) to generate a standard curve. Blank controls of Reagent Diluent alone were also applied. The plates were left at RT for 2 h and then washed with Wash Buffer 3 times.

Following, addition of 100 μl/well biotinylated detection Ab, diluted in Reagent Diluent, took place and plates were incubated for 2 h at RT before they were washed 3x with Wash Buffer. Streptavidin conjugated to horseradish peroxidase (HRP) was then added (100 μl/well) and the plates were incubated in the dark for 20 min, RT. After that, 3,3',5,5'-tetramethylbenzidine (TMB) was mixed with hydrogen peroxide (H_2O_2) in 1:1 ratio to produce the peroxidase substrate solution, which was applied to the plates

(100 μl/well). A 20-min incubation in the dark at RT followed, producing a blue color of density proportional to the amount of protein, before the reaction was stopped with 50μl 2N sulfuric acid (H_2SO_4) to each well. As the addition of H_2SO_4 turns the blue color into yellow, which absorbs at 450 nm, optical density (O.D.) was measured at such wavelength with correction at 630 nm in ELX-800 reader (BioTek Instruments, Inc., VT, USA).

2.4.4 Gene expression analysis

2.4.4.1 RNA extraction

RNA extraction was accomplished via NucleoSpin® RNA Plus kit (Macherey-Nagel GmbH & Co KG, Dueren, Germany) using the cell pellets stored at -80ºC. The first step was cell lysis, for which the pellets were suspended in 350 μl lysis buffer LBP and, after vortexing, the lysate was transferred to the NucleoSpin® gDNA removal columns placed in collection tubes. Centrifugation took place at 11000 x g for 30 sec and, to fine-tune RNA binding conditions, 100 μl binding solution BS was added to the flowthroughs. The samples were, then, mixed gently, transported to the NucleoSpin® RNA Plus columns and centrifuged at 11000 x g for 30 sec. Flowthrough was discarded and the silica membrane was washed via adding 200 μl WB1 buffer to the columns and centrifuging at 11000 x g for 30 sec. After transferring the columns to new collection tubes, two more washes followed, adding 600 and 250 μl WB2 buffer, respectively, to the columns and centrifuging as previously, except for the last centrifugation, which was at 11000 x g for 2min to completely dry the silica membrane. Finally, after transferring the columns into new collection tubes, 30 μl RNase-free water were added to the centre of each membrane to elute the RNA and samples were centrifuged at 11000 x g for 1 min. The last step was repeated one more time and the eluted RNA was stored at -80ºC or used directly for cDNA synthesis. RNA was quantified with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) by measuring 1 μl of each RNA sample.

2.4.4.2 Complementary DNA synthesis

The RNA extracted from the cell pellets was utilized for complementary DNA (cDNA) synthesis. The tubes containing 1-5 µg of total RNA were put to centrifugation at 30ºC until the volume of every sample reached 10 µl. Master mix I, shown on Table 5, was, then, prepared and 2 µl of mix were added to each sample, since RNA was already dissolved in the desired water volume.

Table 5: RT-PCR master mix I for cDNA synthesis. Total volume of RNA yield (X), together with RNase-free water (Y), reaches 10 µl.

Reaction Component	µl per reaction
RNA (1-5 µg)	X
10mM dNTPs mixture	1
Oligo-dT_{12-18} (500 µg/ml)	1
RNase-free water	Y
Total	12

Samples were then placed into the SimpliAmp™ Thermal Cycler for a 5 min incubation at 65ºC and, then, immediately transferred to ice for 2-3 min, before 8 µl of the second master mix (II) (Table 6) were added to each sample. The reducing agent dithiothreitol (DTT) is used to disrupt the disulfide bonds of RNases, thereby constraining their activity[55]. Following, the samples were incubated in the PCR machine at 42ºC for 50 min and, finally, at 70ºC for 15 min. The yielded cDNA was stored at -20ºC for subsequent use.

Table 6: RT-PCR master mix II for reverse transcription of the RNA samples. SuperScript™ II Reverse Transcriptase was purchased from Invitrogen™ (CA, USA).

Reaction Component	µl per reaction
5x First-Strand buffer	4
0.1 M DTT	2
RNase OUT™ (40 units/µl)	1
SuperScript™ II Reverse Transcriptase	1
Total	8

2.4.4.3 Quantitative PCR

Real-Time, or else, quantitative PCR (qPCR) is a molecular technique based on conventional PCR, but, in contrast with the latter, it incorporates fluorescent dyes in the

mix that enables monitoring the increases in amplification of the desirable DNA sequence during the reaction. This results in assessment of parameters throughout the exponential phase, before the reaction reaches a plateau, as in the case of standard PCR. Its high sensitivity and enhanced throughput of small tissue samples rendered qPCR quite the most popular tool for nucleic acid quantification[56].

The cDNA acquired from the reverse transcription reaction was utilized in qPCR experiments. Beside the enhancement of the target genes, *Foxp3* and the housekeeping (reference) gene, hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), was also amplified to allow normalization later on the procedure. For that reason, a master mix for *Hprt1* along with the one intended for the target gene was prepared, the synthesis of which is shown on Table 7. All the primers used were previously designed and ordered and their sequences are indicated on Table 8.

Table 7: Master mix for qPCR performance. Sybr® Green I Nucleic Acid Gel Stain was acquired from Molecular Probes Inc, whereas Platinum® Taq DNA Polymerase from Invitrogen™.

Reaction Component	μl per reaction
10x PCR buffer, minus Mg^{2+}	2.5
50 mM $MgCl_2$	1.25
10 mM dNTPs mixture	0.2
10 μM Forward primer	0.75
10 μM Reverse primer	0.75
10000x Sybr® Green I Nucleic Acid Gel Stain	0.05
Platinum® Taq DNA Polymerase	0.25
dH_2O	9.25
Total	15

Table 8: qPCR primers used for enhancement of *Foxp3* or *Hprt1* genes.

Primer	Sequence (5'→3')
Hprt1 Forward	GTGAAACTGGAAAAGCCAAA
Hprt1 Reverse	GGACGCAGCAACTGACAT
Foxp3 Forward	CCTCCACTCCACCTAAAG
Foxp3 Reverse	TGAAACCAGACAACTAACAG

A 96-well qPCR MicroAmp® Fast Optical plate (Applied Biosystems) was used to add triplicates of the samples as well as of the standards and distilled water controls (25 µl/well). Concentration of sample cDNA was 5 ng/µl, while for the standards, five four-fold serial dilutions were prepared. Once the plate was loaded, it was sealed with optical adhesive film to prevent evaporation and inserted into StepOnePlus Real-Time PCR system (Applied Biosystems). Table 9 describes the program that was used to achieve the enhancement. Acquirement of data took place at the end of each of the 40 cycles and data analysis was carried out using StepOne™ Software v2.1.

Table 9: Cycling program for qPCR performance.

Stages	Temperature (°C)	Time
1	95	4 min
2 (40x cycles)	95	1 min
	60	1 min
3 (Melting Curve)	95	15 sec
	70	1 min (+0.3°C until 95 °C)
	95	15 sec
4	95	10 min

2.5 Image and statistical analysis

Both H&E and PAS stained sections were utilized to obtain images through Leica DM LS2 (Leica Microsystems GmbH, Wetzlar, Germany) optical microscope and the Leica Application Suite V3.6.0 software. Data were analyzed using GraphPad Prism 7.0 with the implementation of two-way analysis of variance (ANOVA) for all the figures. P-value under 0.05 indicated statistical significance.

3. RESULTS

3.1 Treg cells are efficiently depleted upon diphtheria toxin administration during allergen challenges

To investigate the role of Treg cells in the ameliorated allergic responses observed following anti-TIGIT antibody administration in OVA-induced asthma model[51], 8-12-week-old male DEREG mice were sensitized and challenged with the allergen ovalbumin and also injected with DT before challenges to delete the $Foxp3^+$ Treg cell compartment. Since the majority of $TIGIT^+$ Tregs consist of natural Tregs[39], which are $Foxp3^+$, utilization of the DEREG murine model is believed to efficiently eliminate $TIGIT^+$ Treg population. A critical step was to confirm that during the three allergen challenges Treg cell levels remained adequately low. For this reason, blood samples were obtained the first and third day of challenges from DEREG mice that had received DT. Mice either wild type (WT) or DEREG to which DT was not administered were used as controls. The number of $Foxp3^+$ T cells was estimated through FACS analysis after staining for the T cell marker, CD3 (Fig. 1). The fact that $Foxp3^+$ Tregs of the DEREG mouse model express DTR conjugated with eGFP, allows for direct detection of this particular subset by the green fluorochrome it carries[52]. Thus, $Foxp3^+$ Tregs of WT mice, albeit present, would not be detected, because they do not express eGFP. On the contrary, in DEREG mice where $Foxp3^+$ Tregs were not deleted, a positive eGFP signal was expected by these cells. Depletion of Treg cells in DT-treated DEREG mice was confirmed on the first challenge day, as their $CD3^+Foxp3^+$ T cell numbers were comparable to those of WT mice, which represented background fluorescence, while remarkably lower than those of DEREG mice that had not received DT (Fig. 1A). This pattern was also evident on the third day of OVA challenges, indicating a long-lasting depletion of $Foxp3^+$ Tregs (Fig. 1A). Representative pseudocolor flow cytometric plots gated on $CD3^+$ T cells reflect the above observations, with DT-treated DEREG mice having significantly lower percentages of $CD3^+Foxp3^+$ Tregs than DEREG mice without DT, while these percentages remained close to those of WT mice (Fig. 1B).

Figure 1

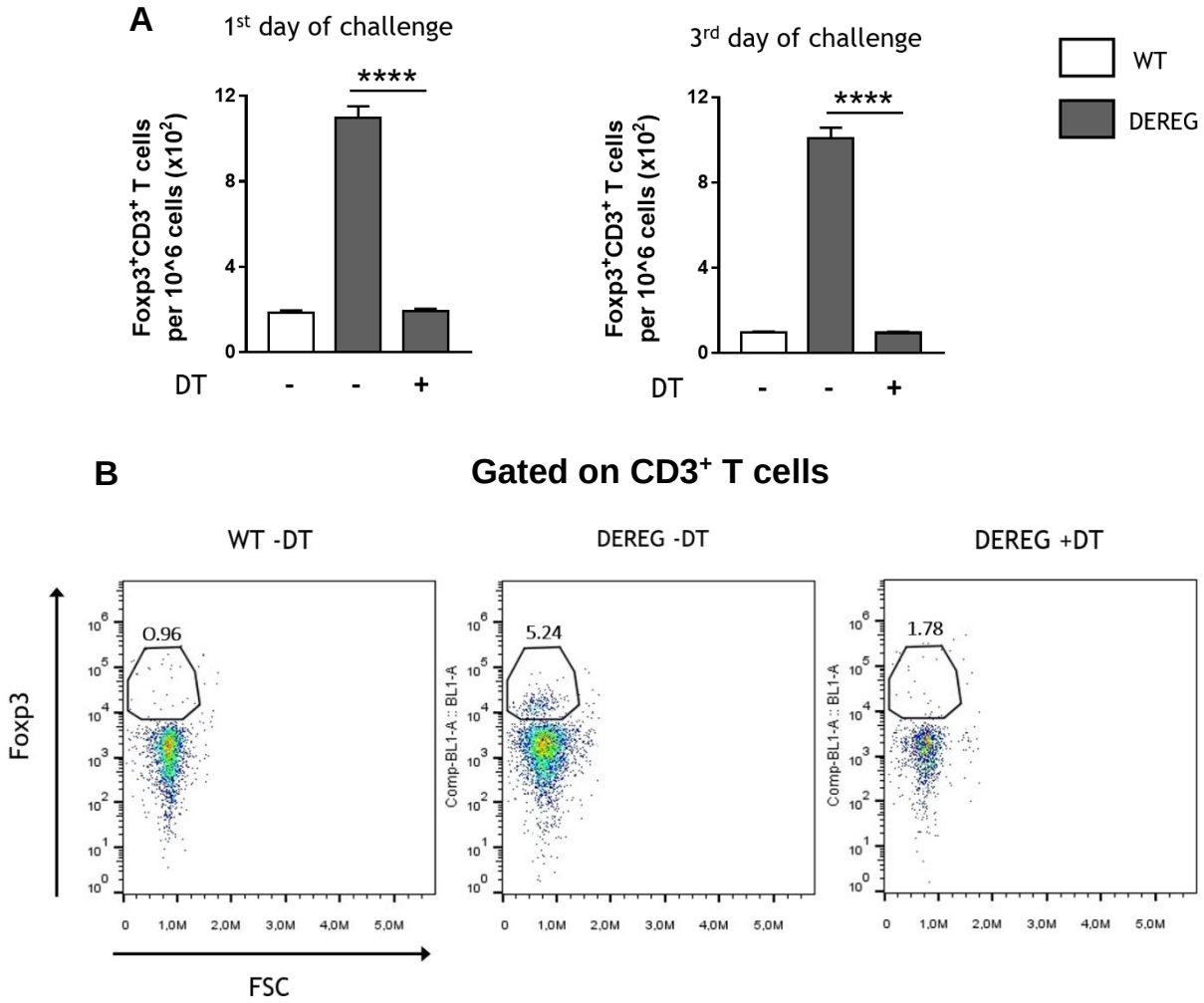


Figure 1: FACS analysis of lymphocytes from blood samples obtained from either WT and DEREG mice without DT treatment or DT-administered DEREG mice to certify sufficient depletion of $Foxp3^+$ Tregs of the latter. **(A)** Numbers of $CD3^+Foxp3^+$ Tregs during the first and third day of OVA challenges. **(B)** Percentages of $CD3^+Foxp3^+$ Treg cells as illustrated in representative pseudocolor plots of gated $CD3^+$ cells. Data are expressed as mean ± standard error of the mean (SEM). **** $p<0.0001$

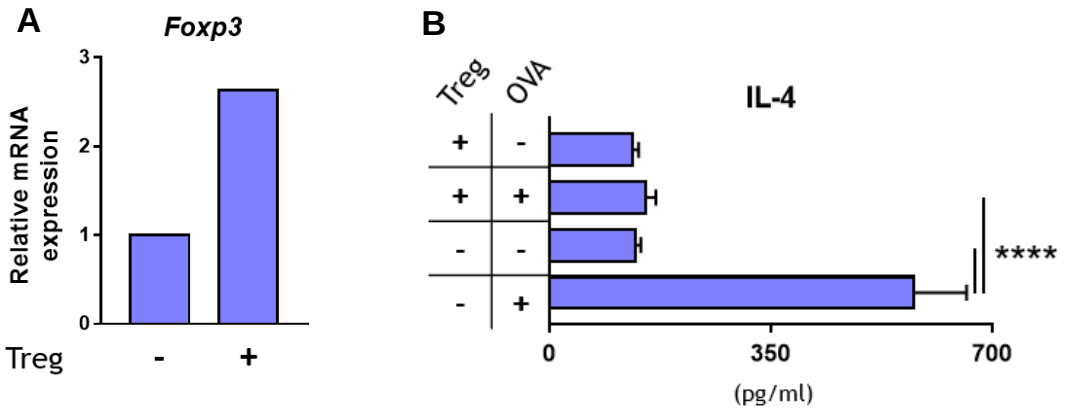
3.2 Treg cell depletion upon allergen challenges exacerbates allergic airway inflammation

Preliminary experiments to set up the protocol included two groups, each containing three mice, subjected to OVA-mediated allergic asthma induction. Both groups received anti-TIGIT Ab, while the first group was Treg cell-depleted after administration of DT prior to OVA challenges. Anti-TIGIT Ab was used in both groups to mitigate immune responses so as to exclude the possibility of impaired comparability between the groups due to extensive inflammation. Cells derived from MLNs of these mice were either cultured in the presence of the allergen OVA to measure antigen (Ag)-

specific responses, or used for RNA extraction and FACS analysis. qPCR for evaluation of *Foxp3* expression harnessing the cDNA obtained from extracted RNA revealed that the qPCR product for mice that received DT was approximately 2-fold down compared to the product for untreated mice (Fig. 2A). These data further confirmed the previous observations regarding Foxp3$^+$ Treg depletion upon DT administration (Fig. 1).

To estimate the severity of allergic inflammation elicited by allergen inhalation during the challenges, Ag-specific responses were measured (Fig. 2). In more detail, the production of several Th2 cytokines (IL-4, IL-5 and IL-13), as well as of the anti-inflammatory cytokine IL-10 was measured. IFN-γ and IL-17, which are the signature cytokines for Th1 and Th17 responses, respectively, were also assessed, as they could play a role in asthmatic responses[20]. After a 67h-incubation with OVA, Ag-specific responses in MLN cell cultures were evaluated through ELISA. As IL-10 production peaks at 96 hours of incubation, this cytokine was measured at that timepoint[57]. Supernatants of OVA-untreated cells were also collected, although minimum T cell activation should be anticipated in the absence of the antigen *ex vivo*. Results indicated a significant increment in the levels of all type 2 cytokines, IL-4, IL-5 and IL-13, expressed from OVA-treated cells of mice lacking Foxp3$^+$ Tregs compared to those of Treg-sufficient mice (Fig. 2B-D). IL-10 and IFN-γ production in response to OVA was also significantly increased, when Foxp3$^+$ Tregs were absent (Fig. 2E-F). Finally, as expected, no significant difference was observed in the production of the cytokines measured between the two groups, when the allergen was not added in the culture (Fig. 2).

Figure 2



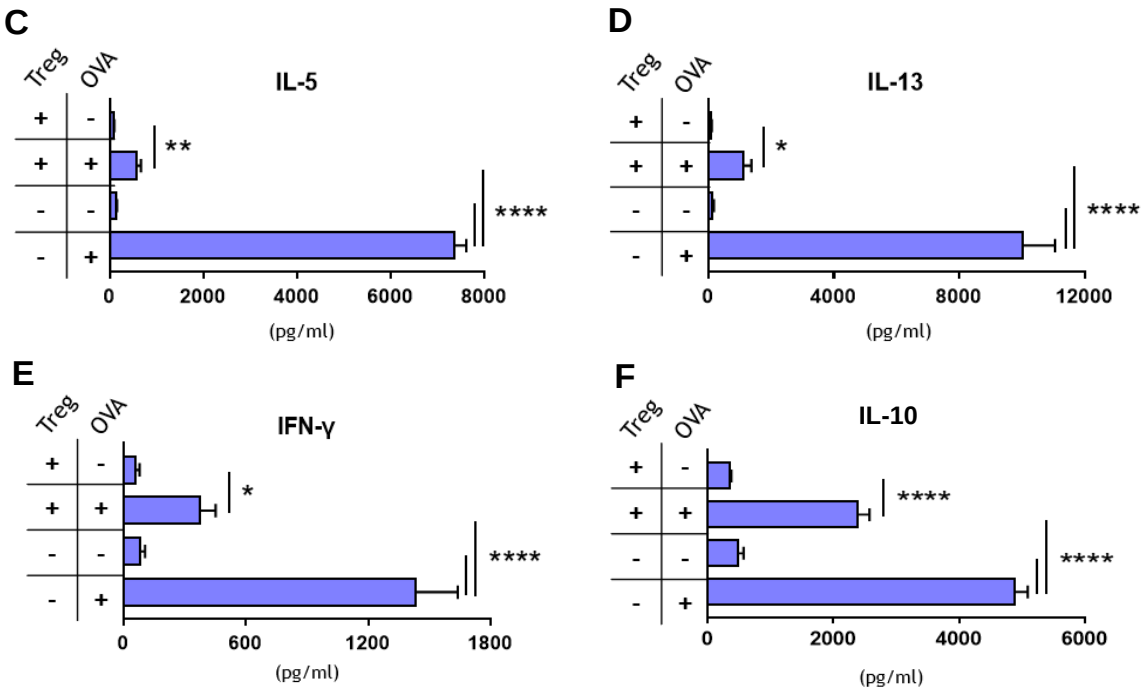


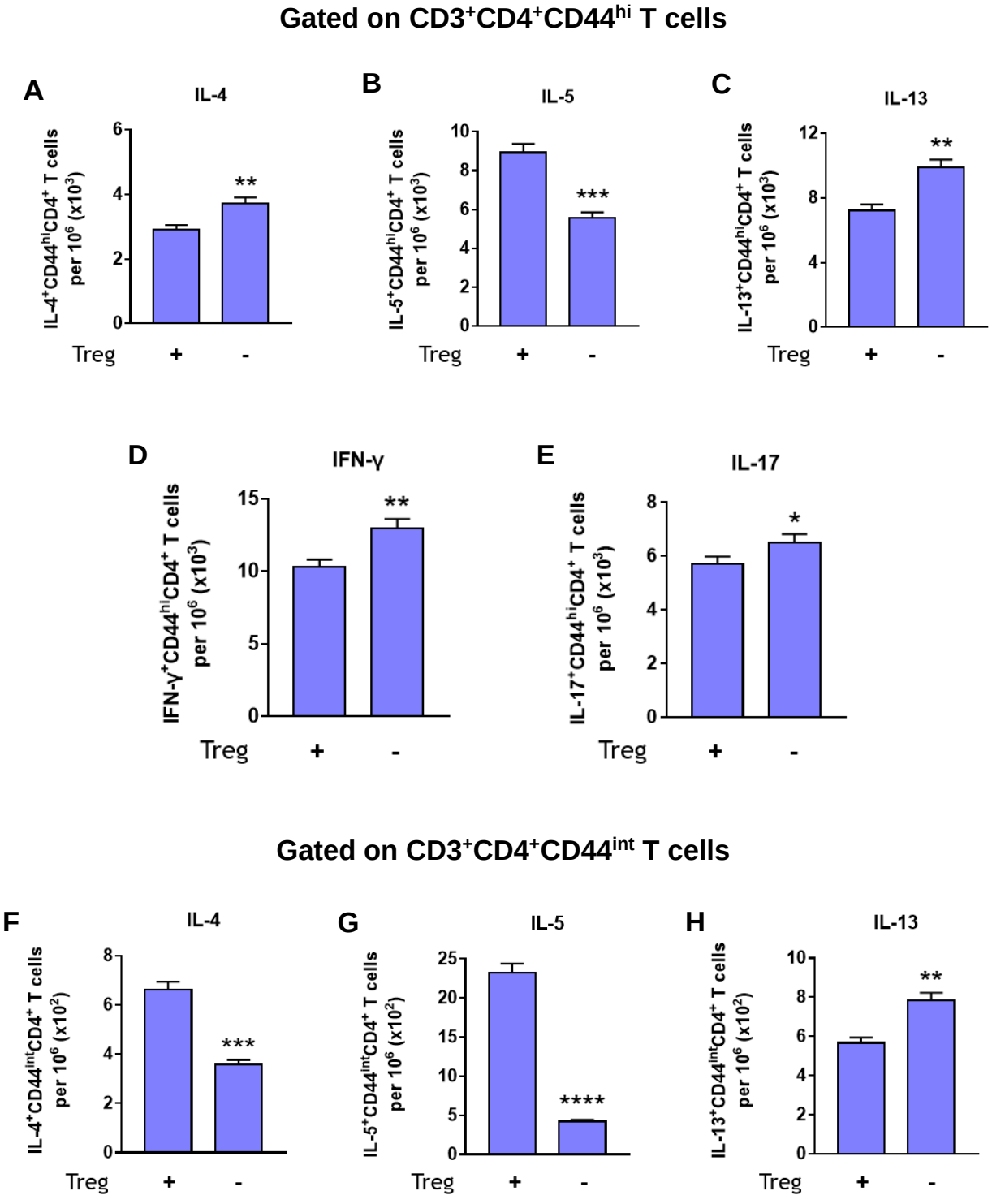
Figure 2: **(A)** Relative expression of *Foxp3* mRNA in MLN cells of Treg-depleted vs non Treg-depleted mice. **(B-F)** Levels of (B) IL-4, (C) IL-5, (D) IL-13, (E) IFN-γ and (F) IL-10 secretion after a 67- or 96-hour incubation of cells derived from MLNs of mice with either deleted Foxp3$^+$ Tregs or not, ± OVA. Data are expressed as mean ± SEM. **** $p<0.0001$

Similar results were obtained from FACS analysis, where PMA/ionomycin-stimulated cells isolated from MLNs were stained for the cytokines IL-4, IL-5, IL-13, IL-17 and IFN-γ as well as for T cell surface markers (Fig. 3). Treg-depleted mice appeared to have significantly elevated numbers of highly activated CD4$^+$CD44^{hi} T cells expressing either IL-4, IL-13, IL-17 or IFN-γ compared to Treg-bearing mice (Fig. 3A,C-E). IL-5-producing CD4$^+$CD44^{hi} T cells, though, were remarkably fewer in Treg-depleted group (Fig. 3B). However, combined with the previous results it seems that, albeit lower in numbers, these cells secrete more IL-5 than the corresponding cells obtained from Treg efficient mice.

Interestingly, the CD4$^+$ T cell subpopulation with intermediate CD44 expression, which comprises less activated cells compared to CD44^{hi} T cells, consisted of fewer cells that were positive for each of the cytokines studied in mice lacking Tregs in comparison with Treg-bearing mice except for IL-13, for which CD4$^+$CD44^{int} T cells followed the pattern of CD4$^+$CD44^{hi} T cells (Fig. 3F-J). However, CD4$^+$CD44^{hi}IL-13$^+$ T cells of mice with depleted Tregs still outnumbered CD4$^+$CD44^{int}IL-13$^+$ cells, which was also evident in the rest of the measured cytokines, indicating that highly activated CD4$^+$ T cells are mainly responsible for secretion of the above cytokines in this setting. Lastly, beside MLNs, lungs were also harvested for histological evaluation of

inflammation. In line with the previous results, mice lacking Foxp3^{+} Tregs seemed to have a more extensive inflammation and mucus secretion than mice with Tregs in H&E and PAS stained lung sections (Fig. 4).

Figure 3



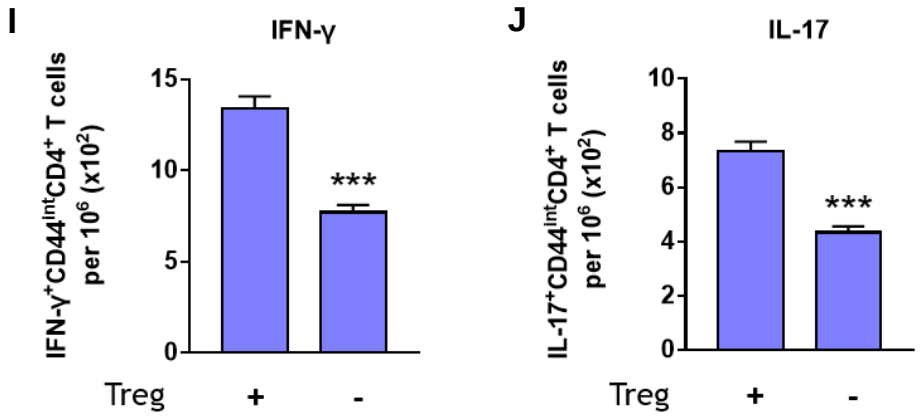


Figure 3: Flow cytometry results for CD4$^+$ T cells isolated from MLNs of Treg-depleted vs non Treg-depleted mice. **(A-E)** Numbers of positive cells for (A) IL-4, (B) IL-5, (C) IL-13, (D) IFN-γ and (E) IL-17 within the high-CD44 subpopulation of activated CD4$^+$ T cells. **(F-J)** Numbers of positive cells for (F) IL-4, (G) IL-5, (H) IL-13, (I) IFN-γ and (J) IL-17 within the intermediate-CD44 subpopulation of moderately activated CD4$^+$ T cells. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

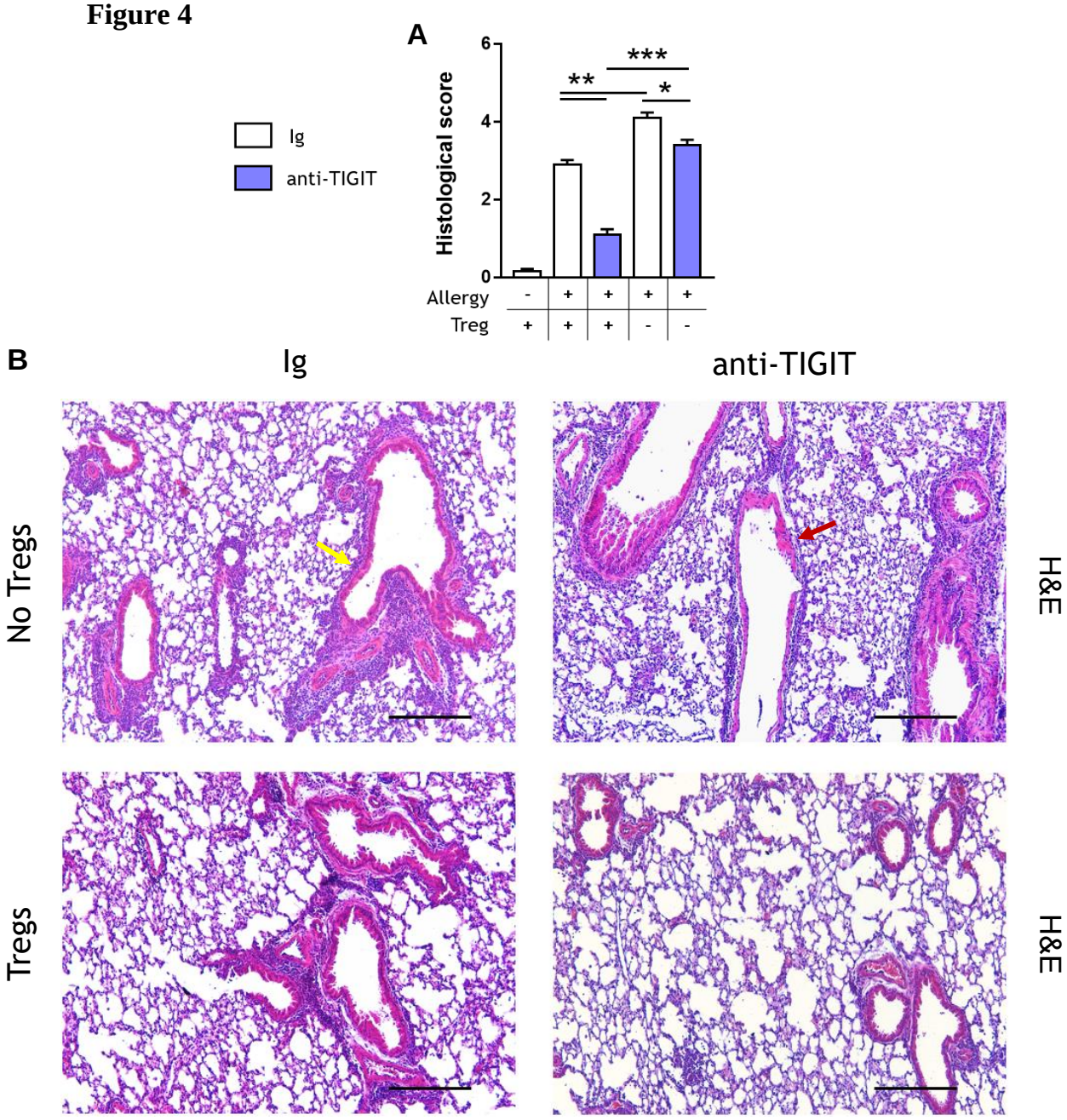
3.3 TIGIT-expressing Treg cells are dispensable for anti-TIGIT-mediated dampening of allergic recall responses

According to Joller *et al.*, TIGIT-expressing Treg cells do not inhibit Th2 responses[39]. In addition, previous findings of our lab support a promotive role of TIGIT in the context of allergic airway inflammation and specifically of Th2 responses in this context, as *in vivo* blockade of this molecule confined the hallmarks of allergic airway disease in an OVA-induced allergic asthma mouse model[51]. However, whether the mechanism leading to this alleviation involves interruption of Th2- and/or Treg-derived TIGIT activity is not clear yet. To investigate the presumable implication of TIGIT$^+$ Treg cells in the attenuation of allergic inflammation through TIGIT blockade, we performed deletion of these cells during allergic asthma induction combined with anti-TIGIT Ab administration. In two of the four 8-12-week old male DEREG mice groups, each composed of 2-3 mice, Tregs were depleted, while the other two had this population intact, and within each condition one group received anti-TIGIT, while the other received control Ig Ab. Depletion of Foxp3$^+$ Tregs through DT administration was conducted right before the stage of OVA challenges. 24 hours post last challenge, mice were sacrificed and MLNs, as well as their lungs were harvested.

Histopathological analysis in lung sections stained with H&E revealed an increased histological score in the absence of Tregs, as estimated by the degree of peribronchial and perivascular infiltration (Fig. 4A). In particular, lung inflammation was

significantly more acute in the control group than in the anti-TIGIT-administered one. The same pattern was observed between the score of control (Ig-treated) and experimental (anti-TIGIT-treated) group in the presence of functional Tregs. Sections of healthy lung tissue from mice without allergic asthma were used as a control and received a zero score. In figure 4B, H&E-stained sections illustrate the severity of inflammation of each group in terms of cell infiltration around the bronchi and vessels. PAS staining, revealing the mucus-producing goblet cells, which exhibit hyper-proliferation upon inflammatory conditions, shows a similar inflammation level, proportionate to that of the H&E-stained sections (Fig. 4C).

Figure 4



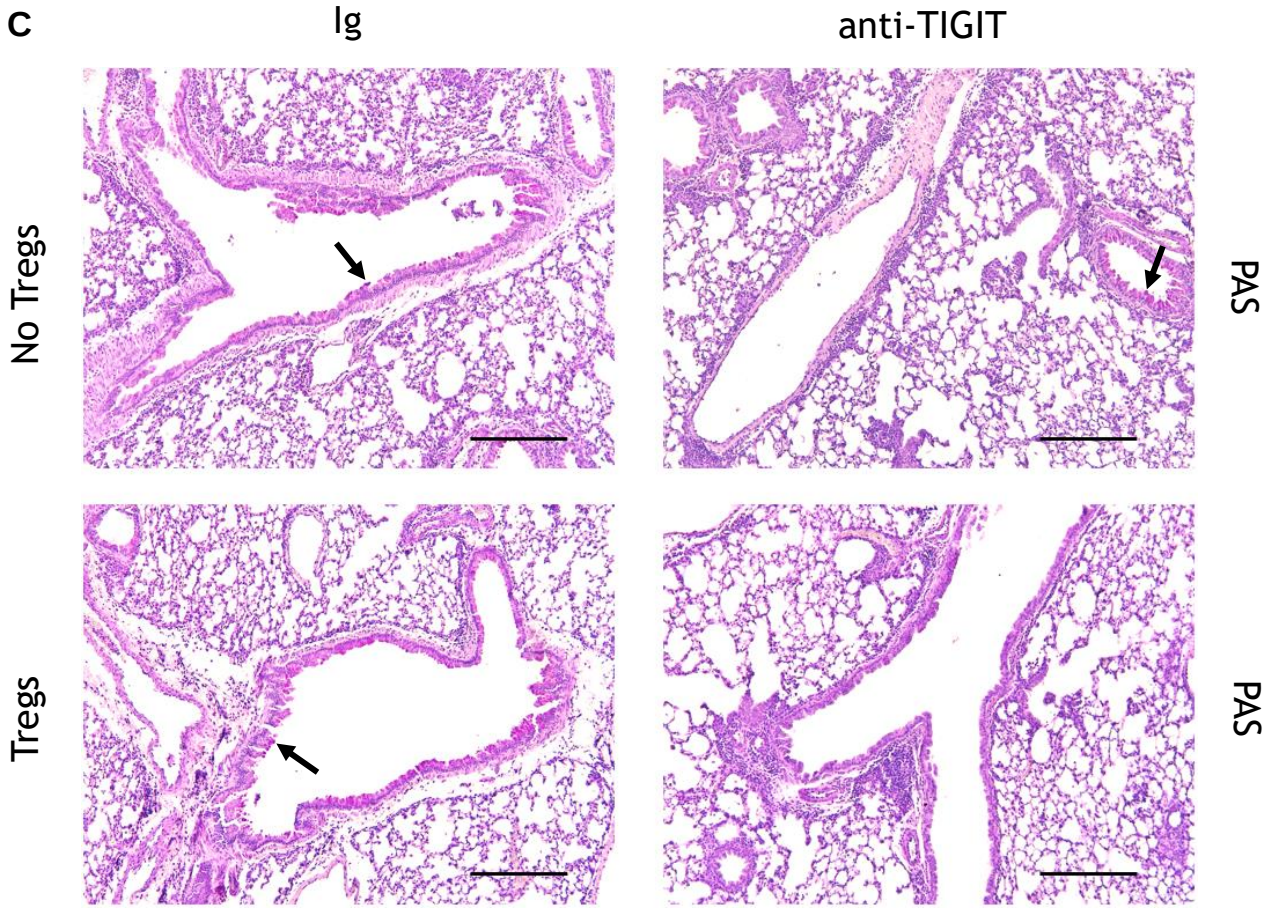
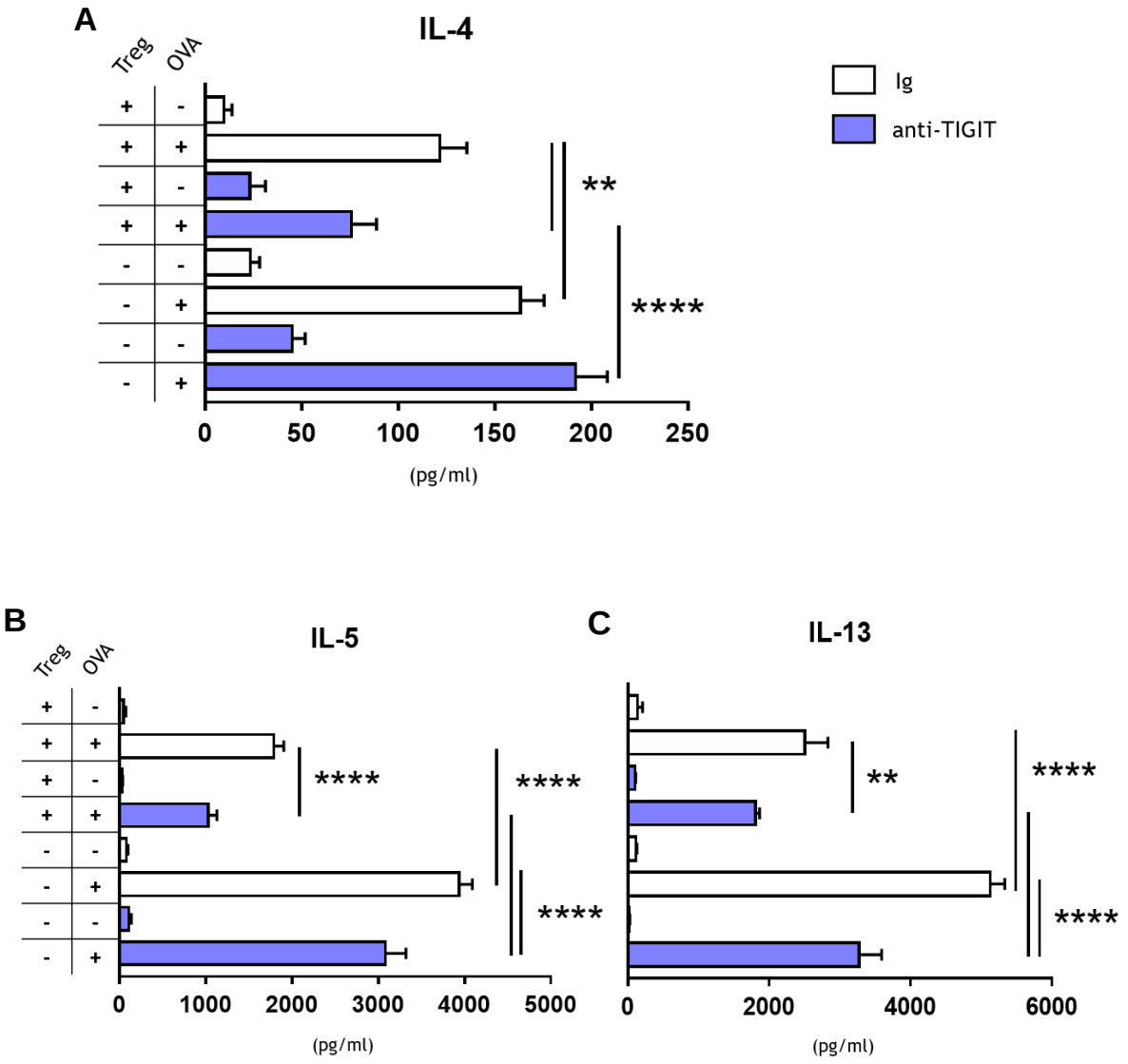


Figure 4: Microtome sections of lungs harvested from mice with allergic asthma that either had their Treg cells depleted or not, together with anti-TIGIT or Ig control Ab administration. **(A)** Histological score for each group as estimated by the number of peribronchial and perivascular infiltration foci in H&E sections. **(B)** Representative images of H&E-stained lung sections depicting the degree of cell infiltration for each group. The yellow arrow points to a bronchus, while the red one to a vessel. **(C)** Representative images of sections stained with PAS, indicating the level of mucus production by the goblet cells. The black arrows show the mucus-secreting goblet cells. Scale bars, 300 μm. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

Cells isolated from MLNs were subjected to the same analyses described in the previous experiment in order to assess the magnitude of inflammation. Figures 5 and 6 include representative data of two independent experiments. Following a 72-hour incubation with OVA, culture supernatants were collected for detection of IL-4, IL-5, IL-13 and IFN-γ production via ELISA (Fig. 5). IL-10 was again measured at 94 hours of incubation. Results of this assay indicated a significantly augmented production of all these cytokines under $Foxp3^+$ Treg deletion compared to when Tregs are present in anti-TIGIT-treated groups (Fig. 5A-E), which further confirmed the results of the previous experiment (Fig. 2B-F). The same applied for the Ig-treated groups for most cytokines (Fig. 5A-C), with the exception of IL-10 and IFN-γ, where the increase was not significant (Fig. 5D-E). In addition, anti-TIGIT Ab administration led to significant

decrease in production of all the cytokines measured compared to Ig-treated mice in groups with functional Treg compartment. Interestingly, Th2 cytokines IL-5 and IL-13 displayed a notable reduction when anti-TIGIT was administered comparing to Ig control in groups where Tregs were depleted (Fig. 5B-C). On the contrary, an increase was observed in IL-4 production upon anti-TIGIT treatment, albeit with no statistically significant difference (Fig. 5A). Moreover, IFN-γ secretion was robustly enhanced when anti-TIGIT was administered compared to the control group (Fig. 5D). Finally, IL-10 production exhibited a minor reduction with no statistical significance (Fig 5E).

Figure 5



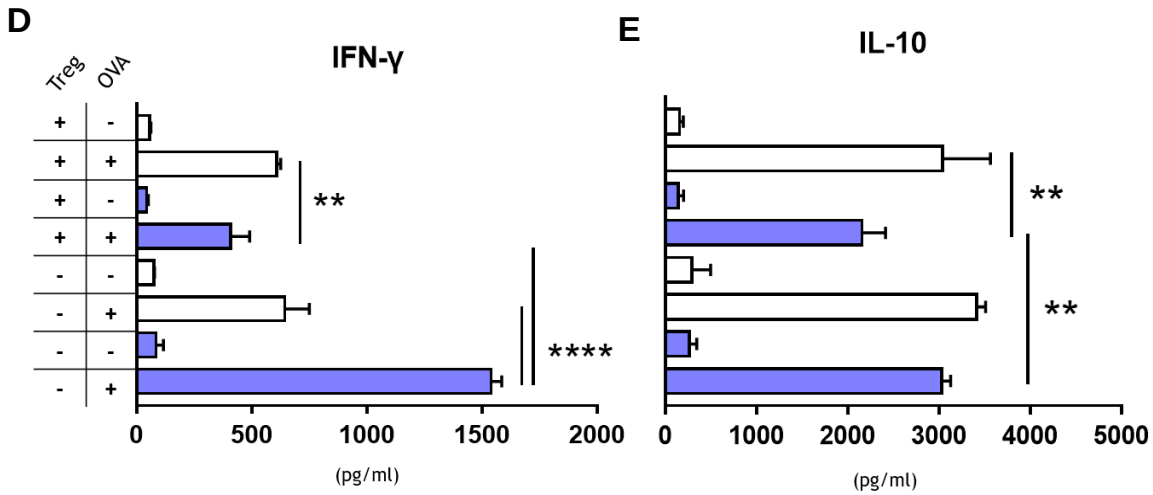


Figure 5: Levels of **(A)** IL-4, **(B)** IL-5, **(C)** IL-13, **(D)** IFN-γ and **(E)** IL-10 secretion after a 72- or 96-hour incubation with OVA of cells derived from MLNs of mice that were treated either with anti-TIGIT Ab or Ig isotype control in the presence or absence of $Foxp3^+$ Treg cells. Data are expressed as mean ± SEM. **p<0.01, **** p<0.0001

The numbers of cytokine-secreting MLN-derived cells were also estimated through flow cytometry (Fig. 6). After PMA/ionomycin stimulation, cells were intracellularly stained for the above cytokines, as well as IL-17 and the transcription factor Foxp3. Extracellular staining for T cell surface markers was also performed. Regarding Foxp3, cells expressing this transcription factor, thus exerting regulatory functions, are not exclusively $CD44^+$, therefore such cells were detected after gating on $CD3^+CD4^+$ T cells. Levels of $CD4^+Foxp3^+$ T cells remained the same among mice administered anti-TIGIT and control mice both in the presence and absence of Treg cells (Fig. 6A). A minor increase was observed in $CD4^+Foxp3^+$ T cells in both Ig- and anti-TIGIT-treated mice when Tregs were deleted compared to when Tregs were not impaired. However, this increase was not statistically significant (Fig. 6A). Cells positive for the cytokines were searched in the $CD44^{hi}$ compartment of $CD4^+$ T cells. This fraction of highly activated T cells seemed to be enlarged in the absence of $Foxp3^+$ Tregs (Fig 6B). Under these conditions, mice injected with anti-TIGIT Ab demonstrated a significantly lower number of $CD4^+CD44^{hi}$ T cells than the Ig-treated mice. Such differences were also observed between the experimental and control groups that had intact $Foxp3^+$ Treg cells (Fig. 6B).

Figure 6

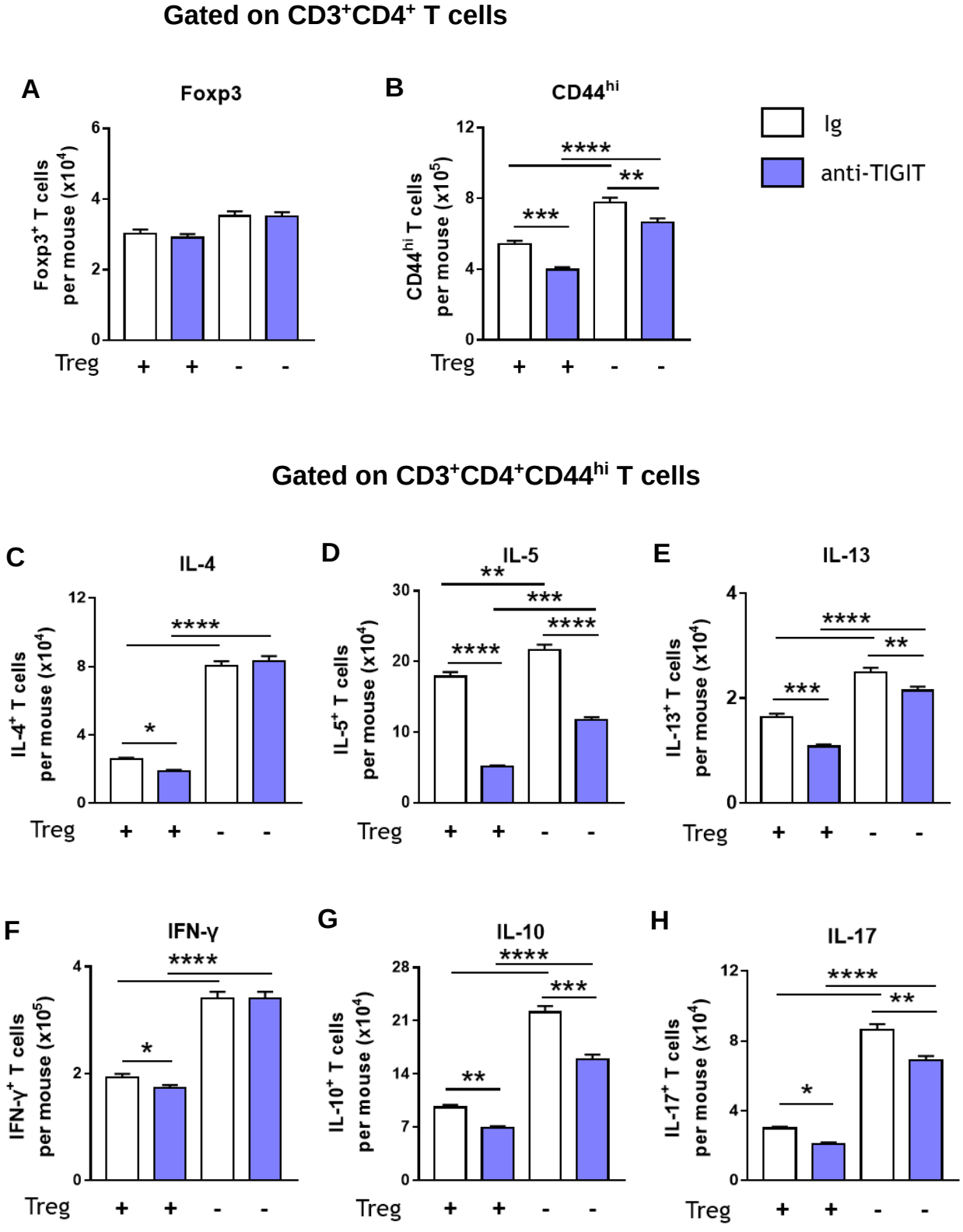


Figure 6: Flow cytometry results for cells isolated from MLNs of mice that were treated either with anti-TIGIT Ab or Ig isotype control in the presence or absence of Foxp3$^+$ Treg cells **(A)** Foxp3$^+$ cells within gated CD4$^+$ T cells. **(B)** Numbers of CD4$^+$CD44^{hi} T cells within each group. **(C-H)** Numbers of positive cells for (C) IL-4, (D) IL-5, (E) IL-13, (F) IFN-γ, (G) IL-10 and (H) IL-17 within the high-CD44 subpopulation of activated CD4$^+$ T cells. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

$CD4^+CD44^{hi}$ T cells positive for each of the cytokines stained were significantly more abundant in mice lacking $Foxp3^+$ Tregs compared to Treg-bearing mice, upon not only anti-TIGIT but also Ig Ab administration (Fig. 6C-H). This further supports our previous results of FACS analysis regarding the anti-TIGIT-treated groups (Fig. 3A-E). Moreover, significantly greater numbers of $CD4^+CD44^{hi}$ T cells that produce each of the studied cytokines, were measured in mice received anti-TIGIT Ab compared to controls, within the Treg-sufficient group (Fig. 6C-H). Upon $Foxp3^+$ Treg cell depletion, the experimental group exhibited lower numbers of $CD4^+CD44^{hi}$ T cells producing either IL-5 or IL-13 compared to the control group, with IL-5-secreting cell numbers displaying a more significant difference (Fig. 6D-E). IL-4, on the other hand, was produced by comparable numbers of $CD4^+CD44^{hi}$ T cells among the two groups (Fig. 6C). Similar results were also obtained for IFN-γ, as numbers of $CD4^+CD44^{hi}$IFN-γ^+ T cells were comparable between the experimental and control group (Fig. 6F). Furthermore, IL-10- and IL-17-producing $CD4^+CD44^{hi}$ T cells appeared significantly reduced in the anti-TIGIT-treated mice compared with controls, upon $Foxp3^+$ Treg depletion (Fig 6G-H).

Taking everything into account, both the secretion levels and cell numbers for the IL-5 and IL-13 cytokine-producing $CD4^+CD44^{hi}$ T cells, as well as the numbers of $CD4^+CD44^{hi}$IL-17^+ T cells demonstrate similar changes between the experimental and control group independently of whether Tregs are impaired or not. These data suggest that, TIGIT expressed on Treg cells is not overall involved in asthma amelioration that TIGIT blockage mediates. However, IL-4, IL-10 and IFN-γ seem to be somehow affected by the absence of Tregs upon anti-TIGIT Ab administration, as they display different patterns of alterations between the control and experimental group when Tregs are missing compared to when they are present.

4. DISCUSSION

Asthma is a disease with alarmingly increased predominance in industrialized countries and high socio-economic impact[3,4]. Recent findings highlighted the complex and multiparametric nature of this disease, resulting in differing phenotypes, thus obscuring the development of effective therapies[3,5]. Therefore, unravelling the mechanisms underlying the pathogenesis of asthma is mandatory for designing remedies specific for the distinct phenotypes. In this context, the main focus of this dissertation was to identify the role of the co-inhibitory receptor TIGIT expressed on Treg cells in the pathogenesis of allergic asthma, which is the most usual type encountered[5].

Although Tregs are generally thought as critical for immune tolerance induction to allergens and prevention of extensive Th2-driven inflammation that leads to asthma[4,33], this $TIGIT^+$ Treg cell subset was found to selectively spare Th2 responses, while retaining the capacity to inhibit Th1 and Th17 responses[39]. More precisely, transfer of $TIGIT^+$ OVA-specific OT-II Treg cells in WT recipients before OVA-mediated asthma induction, led to enhanced Th2 responses and lung eosinophilia compared to when $TIGIT^-$ Tregs were transferred[39]. Therefore, this study showed that $TIGIT^+$ Treg compartment does not suppress Th2-mediated inflammation. Additional evidence provided by previous research of our lab supports a proinflammatory role for TIGIT expressed on T cells in an OVA-induced allergic asthma model, as its blockade significantly impaired allergic responses[51]. The blocking anti-TIGIT Ab, though, could act on both Th2 effector and Treg cells, therefore this study aimed to investigate whether $TIGIT^+$ Tregs are implicated in anti-TIGIT-mediated diminution of allergic airway inflammation.

In order to investigate the aim of this study there was a need to delineate the role of $Foxp3^+$ Tregs in our setting. Results of preliminary experiments demonstrated that $Foxp3^+$ Treg ablation during allergen challenges leads to enhanced numbers in MLNs of highly activated $CD4^+$ T cells, which produce substantial amounts of Th2 cytokines, as well as IFN-γ and potentially IL-17, suggesting a more aggravated inflammation under these conditions (Fig. 2,3). In addition, the elevated IL-10 production measured in MLNs may reflect the great extent of undergoing inflammation. Of note, one study came to opposing conclusions[58]. In particular, Treg depletion via DT administration upon allergen provocation did not exacerbate airway inflammation and lung pathology,

as indicated by the unaltered Th2 cytokine production in MLNs and lung infiltration of immune cells[58].

The observed contradictions between the above-mentioned and the present study may be due to differences in the experimental procedures that were followed. For instance, the protocol for OVA-mediated asthma induction that Baru *et al*. utilized differed and led to vigorous inflammatory response, as indicated by the remarkably high levels of Th2 cytokines in MLNs[58]. This could have resulted in diminution of the differences between the groups due to large-scale inflammation. We, on the other hand, used a protocol provoking a more moderate asthma and also administered an anti-TIGIT Ab, which has been reported to limit Th2 inflammatory responses[51]. Moreover, our experiments confirmed the elevated cytokine production upon Treg cell depletion not only through Ag-specific responses in MLN cells, but also via FACS analysis, revealing the numbers of activated T cells secreting each cytokine.

The same scientific group had previously found that when Treg cells were deleted during sensitization phase, dramatic augmentation of allergic airway inflammation was observed, thus suggesting a time-dependent regulation by Tregs[59]. In light of new evidence provided by our study, further investigation is needed in order to reassess the possibility of a temporal role for Tregs in blunting inflammatory responses in allergy.

Regarding subsequent experiments of this dissertation, the patterns of cytokine production levels were generally quite similar to those of cytokine-secreting cell numbers for the Th2 cytokines investigated, but not for IFN-γ and IL-10 upon Treg-depleting conditions (Fig. 5,6). The results obtained from mice with functional Treg compartment were in agreement with corresponding findings of the previous work our lab did[51], as anti-TIGIT Ab administration led to significant decrease in the production of IL-4, IL-5, IL-13 and IL-10 compared to Ig-treated mice. In addition, the reduced numbers of cells producing the above-mentioned cytokines in the experimental group when compared to controls were also consistent with this study[51].

The fundamental Th2 cytokines, IL-4, IL-5 and IL-13, contribute to the development of allergic inflammation and AHR, each through distinct functions. Results of the present study showed that both in presence and absence of Tregs, IL-5 and IL-13 levels, as well as numbers of cells positive for each of these cytokines were reduced upon anti-TIGIT administration compared to Ig controls (Fig. 5B-C&6D-E). This indicates lower inflammation in anti-TIGIT-treated mice, as IL-5 promotes mast cell hyperplasia[10] and lung eosinophilia[3,15], while IL-13 mediates goblet cell hyperplasia, mucus production

and IgE secretion by activated B cells[3,10]. Since Treg depletion does not reverse the anti-inflammatory effects conferred by the anti-TIGIT Ab, one can speculate that TIGIT expressed on Tregs is not implicated in IL-5 and IL-13 expression induction during allergen challenges.

Nevertheless, TIGIT$^+$ Tregs seem to be involved in IL-4 production, since its reduction both in Ag-specific responses and in the number of positive cells upon TIGIT blockade in Treg-bearing mice is not observed when Tregs are deleted (Fig. 5A&6C). This suggests a non-redundant role for Treg-derived TIGIT in promoting IL-4 expression. Indeed, it has been found that TIGIT engagement in TIGIT$^+$ Tregs upregulates the expression of Fgl2, which is, in turn, pivotal for sustaining IL-4 production by Th2 cells[39]. However, the most predominant Th cell compartment that secretes IL-4 inside the lymph nodes upon allergic responses comprises Tfh cells[60] and IL-4 produced by these cells is responsible for isotype class switching and production of IgE Abs by B lymphocytes[14,15]. Therefore, it would be possible that TIGIT-expressing Treg cells interact with Tfh cells in a way resembling interactions with Th2 cells. Measuring the IgE production in MLNs in this setting could provide a clearer view with regard to this hypothesis.

Moreover, reduced numbers of Tfh cells have been reported in LNs of mice administered anti-TIGIT Ab[51]. In the present study, a decrease in IL-4-producing CD4$^+$CD44^{hi} T cells was evident after TIGIT blockade in Treg presence, however this reduction was inverted in anti-TIGIT-treated mice upon Treg depletion (Fig. 6C). Considering these, Tregs could also be promoting Tfh cell proliferation by acting through TIGIT. The fact that Tfh cells do not co-express IL-13[60] and this cytokine presented a different pattern of production than that of IL-4, further endorses the notion of Treg-expressed TIGIT interacting with Tfh rather than Th2 cells, which express both cytokines[1]. However, a regulation by TIGIT on Tregs towards Th2 cells that have not exited LNs yet cannot be ruled out.

IL-10 is an immunomodulatory cytokine considered as a global suppressor of Th responses[61,62]. Its role, however, in regulation of asthma remains unclear[61], with different studies detecting either elevated or decreased levels of IL-10 in BAL fluid of asthmatic patients[62]. Increased production of IL-10 could be interpreted as an attempt to limit the excess inflammation caused by allergic responses. However, it has been reported that high IL-10, albeit increasing Treg cell numbers, seems to impair their immunosuppressive capacity in mouse models of parasitic infection with *Schistosoma*

japonicum and asthma[63]. This is consistent with findings of the present dissertation suggesting elevated IL-10 levels, along with insufficient control of inflammation. In line with that, previous work from our lab has shown a positive correlation between IL-10 production and Th2 inflammation, too[57]. Furthermore, IL-10 can also be produced by Th2 cells to contribute to allergic inflammatory responses[51].

Interestingly, the remarkably higher numbers of IL-10-producing cells upon Treg ablation in both the experimental and control group compared to Treg presence could be attributed to a mechanism compensating for the lack of $Foxp3^+$ Treg population (Fig. 6G). This could include the proliferation of other IL-10-producing $CD4^+CD44^{hi}$ T cells, such as Tr1 cells, which are known to secrete considerable amounts of this cytokine[1]. Production of IL-10 by Th2 cells upon Treg depletion is also possible, associated with the augmented Th2 responses observed under these conditions.

Elevated numbers of IL-10-secreting $CD4^+CD44^{hi}$ T cells during aggravated inflammation were observed in Ig-treated compared to anti-TIGIT-treated mice regardless of whether Tregs were depleted or not (Fig. 6G). However, in the absence of $Foxp3^+$ Tregs, the levels of IL-10 production after anti-TIGIT administration were not significantly reduced (Fig. 5E), suggesting that these cells might produce adequate amounts of IL-10.

Alternatively, the discrepancies between the changes in levels of production and cell numbers in the absence of Tregs when TIGIT was blocked could reflect an additional source producing IL-10 in the MLNs aside from $CD4^+CD44^{hi}$ T cells, since ELISA's measurements were performed on whole MLN-derived cell culture supernatants, while FACS results concern only $CD4^+CD44^{hi}$ T cells. Such IL-10-producing populations could comprise cells of the innate compartment. In fact, TIGIT is known to interact with CD155 on DCs and increase IL-10 expression by these cells[42]. A similar crosstalk has been reported between TIGIT and macrophages, where CD155 engagement on these cells leads to enhanced IL-10 secretion[64]. Since the main cell type constitutively expressing TIGIT is Tregs, it is possible that a significant portion of these effects on APCs could be mediated by TIGIT-expressing Tregs[39]. In that sense, blockage of interactions among $TIGIT^+$ Tregs and APCs might, at least in part, explain the reduction in IL-10 levels when anti-TIGIT is administered in mice with intact Tregs, and why this reduction is no longer apparent when Tregs are depleted (Fig. 5E). Importantly, a previous research supports a critical role for IL-10-secreting DCs in facilitating the development of Th2 responses in a mouse model of allergic dermatitis[61]. All of the

above could propose a mechanism through which TIGIT exerts its Th2-promoting functions, albeit further investigation is needed.

The role of IFN-γ asthma pathogenesis is more obscure. Many studies have identified through a plethora of approaches either a promotive or a protective function of IFN-γ in this disease[65]. Therefore, it seems that, despite its well documented role in counterbalancing Th2 responses, IFN-γ has been also reported to act in concert with Th2 cytokines to sustain airway inflammation[66]. The results for IFN-γ in this study showed a decrease in Ag-specific responses and IFN-γ-producing cells in mice with Tregs, when TIGIT was blocked (Fig. 5D&6F). This was contradictory to previous results during allergy, where no significant change in neither parameter was observed[51]. A possible explanation to this would be that those experiments were conducted in BALB/c mice, a more Th2-prone strain producing less IFN-γ. In addition, our results were also opposed to other studies presenting TIGIT as inhibitory for Th1 cell function[47,67]. Nevertheless, in our context it has not been identified whether IFN-γ is produced by Th1 or Th2 cells.

Notably, when Tregs were depleted, a remarked increment in IFN-γ production was evident upon anti-TIGIT treatment, while the numbers of IFN-γ-secreting cells were unaltered (Fig. 5D&6F). This could imply that either Th1/Th2 cells highly produce IFN-γ or a population other than $CD4^+CD44^{hi}$ T cells might be producing such high levels of this cytokine. In any case, the elevated IFN-γ production in Ag-specific responses when TIGIT was blocked in the absence of Treg cells, cannot explain whether $TIGIT^+$ Tregs are implicated in anti-TIGIT-mediated regulation of IFN-γ production. Perhaps cell intrinsic signaling through TIGIT ligation could take place in $CD4^-$ cells, such as $CD8^+$ T cells, NK cells and a subset of NKT cells, which are all known to express IFN-γ upon activation[68]. Indeed, the presence of these cells is correlated mostly with augmented allergic inflammation and AHR, often attributed to IFN-γ production[69-71]. Interestingly, it was found that IgE antibodies can mediate FcγRIII-dependent activation of NK cells, which, among other induced molecules, express increased amounts of IFN-γ[72]. A similar process might also drive NKT cell activation, as FcγRIII has been implicated in activation of these cells[73].

In this respect, TIGIT could transduce an inhibitory cell intrinsic signal to these cell types, thus limiting IFN-γ release, which could be reversed after anti-TIGIT antibody administration, as observed upon Treg deletion. However, when $Foxp3^+$ Tregs are intact, this otherwise exaggerated IFN-γ production upon TIGIT blockade would be

masked due to additional Treg-mediated suppressive mechanisms, not including TIGIT, such as immunosuppressive cytokine production. In any event, although asthmatic responses in anti-TIGIT-treated mice seem to be ameliorated compared to the controls both in presence and absence of Treg cells, IFN-γ levels when TIGIT is blocked appear to be elevated upon Treg depletion, while they are diminished when Tregs are intact. Thus, the contribution of this cytokine during the development of type 2 responses in the OVA-induced model of asthma requires further study.

IL-17 is another cytokine with ambiguous role in allergic asthma. Mainly produced by the Th17 cell subset, this cytokine is considered as a potent mediator of neutrophil activation and recruitment into the lungs, leading to a severe asthma phenotype[74]. However, an involvement of IL-17 has also been reported in Th2-driven eosinophilic asthma having either a protective or favoring effect. This likely depends on when this cytokine is manipulated during the experimental procedures, with various studies supporting an inhibitory role upon the effector phase, while others a promotive function during initiation of Th2 responses[74,75].

The present study demonstrated elevated $CD4^+CD44^{hi}IL\text{-}17^+$ T cell numbers in Ig controls compared to anti-TIGIT-treated mice regardless of the presence or absence of $Foxp3^+$ Tregs (Fig. 6H). According to this observation, a specific role for $TIGIT^+$ Tregs in promoting Th17 responses upon asthma development seems implausible. The functional relevance of these numbers might concern a positive correlation between Th17 cell activation and allergic inflammation severity, as control groups, exhibiting more extended inflammation than the experimental ones, had elevated IL-17-producing cells both in presence and absence of Tregs. In fact, Th17 differentiation in mice requires the cytokine IL-6 in the presence of TGF-β[74]. IL-6 is produced, among others, by APCs and B cells and increased levels of this cytokine have been observed in serum and BAL fluid of asthmatic patients compared to control subjects[76]. In addition, IL-6 is known to promote Th2 cell differentiation, thereby contributing to asthma[77]. This raises the possibility that elevated IL-6 during Th2 responses could potentiate the differentiation of IL-17-secreting Th17 cells in Ig controls compared to anti-TIGIT-treated mice, thus explaining the higher numbers in the former and the lower in the latter group. It would be, therefore, interesting to evaluate the production of IL-6 in our context. Additionally, data for IL-17 production upon Ag-specific responses could further enlighten the TIGIT-mediated regulatory mechanisms regarding this cytokine during allergic airway inflammation.

According to the above, it appears that the amelioration of allergic airway inflammation observed upon anti-TIGIT administration during allergen challenges in the OVA-induced mouse model of asthma is not mediated by $TIGIT^+$ Treg cells. The assumed association of these cells with some of the studied cytokines does not seem to play a significant role in the overall severity of inflammation. This could be attributed to the complex interplay among a plethora of cells and molecules that contribute to the development of immune responses.

5. FUTURE PERSPECTIVES

The present dissertation aimed to shed light on the mechanisms underlying the promotive role of TIGIT during asthmatic responses. Based on estimations regarding cytokines related to allergic inflammation on cells isolated from MLNs, as well as histological scores in lung tissue sections, it was found that Tregs are unlikely to augment allergic inflammation via TIGIT expression during allergen challenges. However, although MLNs constitute organs critically involved in the development of immune responses, a clearer vision would be provided by estimating the impact of such responses also at the site of inflammation, namely the lungs. Thus, along with lung sections, in which peribronchial and perivascular infiltration, as well as goblet cell hyperplasia were assessed, additional techniques can further determine the hallmarks of asthma.

In this context, following experiments could include the evaluation of eosinophil and neutrophil recruitment into the lungs, together with cytokine secretion in BAL fluid and OVA-specific IgE production in serum. Cytokines, such as IL-6[77], IL-9[29] and IL-22[26] could also be measured, since they are reported to play a role during Th2 responses. Moreover, staining for Th lineage-specific markers can provide a better understanding on the relative contribution to cytokine release by each Th cell subset. Lung homogenate supernatants can be obtained to evaluate the production of the airway epithelium-derived cytokines IL-25 and IL-33, which are known to variously promote Th2 responses[11,12], as well as macrophage-derived chemokine (MDC)[78] and thymus- and activation-regulated chemokine (TARC)[79] that both facilitate Th2 cell migration. Finally, qPCR analysis can be performed for genes like *Gata3*, *T-bet*, *RORγt* and *Fgl2* to quantify their expression both in T cells isolated from lung tissue and in MLNs.

Given that a potential involvement of Treg-expressed TIGIT in IL-4 secretion may exist, it would be interesting to investigate this relationship during the sensitization phase, when production of IgE antibodies by B lymphocytes and differentiation of allergen-specific Th2 cells first takes place. However, it is possible that in our experiments Treg cell ablation could cause such a robust inflammatory response in Ig-treated mice that differences between the experimental and control group would be indistinct in the case of IL-4 due to overstimulation of Th cells leading to enhanced cell death or even exhaustion. Therefore, staining for specific markers indicative of

proliferation, apoptosis and exhaustion could provide valuable information for the activation status of Th cells.

Moreover, having suggested a putative mechanism through which IL-10 produced by APCs after engagement with TIGIT could actually facilitate Th2 responses, more research is needed in order to clarify these interactions. For this purpose, the IL-10 knockout (IL-$10^{-/-}$) mouse strain can be exploited so as to be perceived whether the enhancing effect of TIGIT on allergic inflammatory responses is still apparent.

In this thesis we utilized the OVA-induced asthma model because it facilitates a Th2-mediated allergic airway disease where Th1, Th17 and Th9 populations are not largely implicated[57], as anti-TIGIT Ab may exert differential effects on them. However, previous and proposed experiments can be also performed on a more clinically relevant model of asthma, like house dust mite (HDM)-induced allergic airway inflammation model, as HDM has been denoted as a pronounced allergen responsible for allergic asthma induction in humans[80].

Overall, understanding the mechanisms of TIGIT function in allergic responses is of great importance, as this molecule appears to be a compelling target for development of therapeutic approaches against asthma. Since current therapeutic field concerning this disease attempts to harness Treg tolerogenic and immunosuppressive capacity, elucidating the mechanisms of TIGIT function on this cell population during allergic airway inflammation is mandatory. Such data in the Th2 context would also be enlightening for cancer immunotherapy focused on TIGIT blockade to enhance immune responses against tumors.

6. REFERENCES

1 Palomares, O., Akdis, M., Martin-Fontecha, M. & Akdis, C. A. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunological reviews* **278**, 219-236 (2017).

2 Palomares, O. *et al.* Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. *Genes and immunity* **15**, 511-520 (2014).

3 Martin, H. & Taube, C. Regulatory T cells and regulation of allergic airway disease. *American journal of clinical and experimental immunology* **1**, 166-178 (2012).

4 Venuprasad, K., Kong, Y. C. & Farrar, M. A. Control of Th2-mediated inflammation by regulatory T cells. *The American journal of pathology* **177**, 525-531 (2010).

5 Kim, H. Y., DeKruyff, R. H. & Umetsu, D. T. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nature immunology* **11**, 577-584 (2010).

6 Bellanti, J. A. Genetics/epigenetics/allergy: The gun is loaded ... but what pulls the trigger? *Allergy and asthma proceedings* **40**, 76-83 (2019).

7 Potaczek, D. P. *et al.* Epigenetics and allergy: from basic mechanisms to clinical applications. *Epigenomics* **9**, 539-571 (2017).

8 Noval Rivas, M. & Chatila, T. A. Regulatory T cells in allergic diseases. *The Journal of allergy and clinical immunology* **138**, 639-652 (2016).

9 Xystrakis, E., Boswell, S. E. & Hawrylowicz, C. M. T regulatory cells and the control of allergic disease. *Expert opinion on biological therapy* **6**, 121-133 (2006).

10 Kubo, M. Innate and adaptive type 2 immunity in lung allergic inflammation. *Immunological reviews* **278**, 162-172 (2017).

11 Caminati, M., Pham, D. L., Bagnasco, D. & Canonica, G. W. Type 2 immunity in asthma. *The World Allergy Organization journal* **11**, 1-10 (2018).

12 Hirose, K., Iwata, A., Tamachi, T. & Nakajima, H. Allergic airway inflammation: key players beyond the Th2 cell pathway. *Immunological reviews* **278**, 145-161 (2017).

13 Khare, A. *et al.* Cutting edge: inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103+ but not plasmacytoid dendritic cells to induce Foxp3 de novo in CD4+ T cells and promote airway tolerance. *Journal of immunology* **191**, 25-29 (2013).

14 Locksley, R. M. Asthma and allergic inflammation. *Cell* **140**, 777-783 (2010).

15 Licona-Limon, P., Kim, L. K., Palm, N. W. & Flavell, R. A. TH2, allergy and group 2 innate lymphoid cells. *Nature immunology* **14**, 536-542 (2013).

16 Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J. F. & Bonnefoy, J. Y. IgE versus IgG4 production can be differentially regulated by IL-10. *Journal of immunology* **160**, 3555-3561 (1998).

17 von Moltke, J. & Pepper, M. Sentinels of the Type 2 Immune Response. *Trends in immunology* **39**, 99-111 (2018).

18 Klein Wolterink, R. G. *et al.* Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *European journal of immunology* **42**, 1106-1116 (2012).

19 Halim, T. Y., Krauss, R. H., Sun, A. C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* **36**, 451-463 (2012).

20 Durrant, D. M. & Metzger, D. W. Emerging roles of T helper subsets in the pathogenesis of asthma. *Immunological investigations* **39**, 526-549 (2010).

21 Schnyder-Candrian, S. *et al.* Interleukin-17 is a negative regulator of established allergic asthma. *The Journal of experimental medicine* **203**, 2715-2725 (2006).

22 Hellings, P. W. *et al.* Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *American journal of respiratory cell and molecular biology* **28**, 42-50 (2003).

23 Busse, W. W. *et al.* Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *American journal of respiratory and critical care medicine* **188**, 1294-1302 (2013).

24 McKinley, L. *et al.* TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Journal of immunology* **181**, 4089-4097 (2008).

25 Pichavant, M. *et al.* Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *The Journal of experimental medicine* **205**, 385-393 (2008).

26 Pennino, D. *et al.* IL-22 suppresses IFN-gamma-mediated lung inflammation in asthmatic patients. *The Journal of allergy and clinical immunology* **131**, 562-570 (2013).

27 Sonnenberg, G. F. *et al.* Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *The Journal of experimental medicine* **207**, 1293-1305 (2010).

28 Besnard, A. G. *et al.* Dual Role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A. *American journal of respiratory and critical care medicine* **183**, 1153-1163 (2011).

29 Temann, U. A., Geba, G. P., Rankin, J. A. & Flavell, R. A. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *The Journal of experimental medicine* **188**, 1307-1320 (1998).

30 McMillan, S. J., Bishop, B., Townsend, M. J., McKenzie, A. N. & Lloyd, C. M. The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. *The Journal of experimental medicine* **195**, 51-57 (2002).

31 Ray, A., Khare, A., Krishnamoorthy, N., Qi, Z. & Ray, P. Regulatory T cells in many flavors control asthma. *Mucosal immunology* **3**, 216-229 (2010).

32 Yamaguchi, T., Wing, J. B. & Sakaguchi, S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Seminars in immunology* **23**, 424-430 (2011).

33 Zhao, S. T. & Wang, C. Z. Regulatory T cells and asthma. *Journal of Zhejiang University. Science. B* **19**, 663-673 (2018).

34 Mucida, D. *et al.* Oral tolerance in the absence of naturally occurring Tregs. *The Journal of clinical investigation* **115**, 1923-1933 (2005).

35 Smith, P. M. *et al.* The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569-573 (2013).
36 Round, J. L. *et al.* The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* **332**, 974-977 (2011).
37 Gelfand, E. W., Joetham, A., Wang, M., Takeda, K. & Schedel, M. Spectrum of T-lymphocyte activities regulating allergic lung inflammation. *Immunological reviews* **278**, 63-86 (2017).
38 Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. & DeKruyff, R. H. Asthma: an epidemic of dysregulated immunity. *Nature immunology* **3**, 715-720 (2002).
39 Joller, N. *et al.* Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* **40**, 569-581 (2014).
40 Boles, K. S. *et al.* A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. *European journal of immunology* **39**, 695-703 (2009).
41 Levin, S. D. *et al.* Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *European journal of immunology* **41**, 902-915 (2011).
42 Yu, X. *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nature immunology* **10**, 48-57 (2009).
43 Anderson, A. C., Joller, N. & Kuchroo, V. K. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* **44**, 989-1004 (2016).
44 Dougall, W. C., Kurtulus, S., Smyth, M. J. & Anderson, A. C. TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy. *Immunological reviews* **276**, 112-120 (2017).
45 Manieri, N. A., Chiang, E. Y. & Grogan, J. L. TIGIT: A Key Inhibitor of the Cancer Immunity Cycle. *Trends in immunology* **38**, 20-28 (2017).
46 Kurtulus, S. *et al.* TIGIT predominantly regulates the immune response via regulatory T cells. *The Journal of clinical investigation* **125**, 4053-4062 (2015).
47 Joller, N. *et al.* Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *Journal of immunology* **186**, 1338-1342 (2011).
48 Lozano, E., Joller, N., Cao, Y., Kuchroo, V. K. & Hafler, D. A. The CD226/CD155 interaction regulates the proinflammatory (Th1/Th17)/anti-inflammatory (Th2) balance in humans. *Journal of immunology* **191**, 3673-3680 (2013).
49 Lozano, E., Dominguez-Villar, M., Kuchroo, V. & Hafler, D. A. The TIGIT/CD226 axis regulates human T cell function. *Journal of immunology* **188**, 3869-3875 (2012).
50 Negishi, N. *et al.* CD155-Transducing Signaling through TIGIT Plays an Important Role in Transmission of Tolerant State and Suppression Capacity. *ImmunoHorizons* **2**, 338-348 (2018).
51 Kourepini, E. *et al.* TIGIT Enhances Antigen-Specific Th2 Recall Responses and Allergic Disease. *Journal of immunology* **196**, 3570-3580 (2016).
52 Lahl, K. & Sparwasser, T. In vivo depletion of FoxP3+ Tregs using the DEREG mouse model. *Methods in molecular biology* **707**, 157-172 (2011).

53 Picot, J., Guerin, C. L., Le Van Kim, C. & Boulanger, C. M. Flow cytometry: retrospective, fundamentals and recent instrumentation. *Cytotechnology* **64**, 109-130 (2012).
54 Aydin, S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides* **72**, 4-15 (2015).
55 Chen, Z., Ling, J. & Gallie, D. R. RNase activity requires formation of disulfide bonds and is regulated by the redox state. *Plant molecular biology* **55**, 83-96 (2004).
56 Peirson, S. N. & Butler, J. N. Quantitative polymerase chain reaction. *Methods in molecular biology* **362**, 349-362 (2007).
57 Xanthou, G. *et al.* Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nature medicine* **13**, 570-578 (2007).
58 Baru, A. M. *et al.* Absence of Foxp3+ regulatory T cells during allergen provocation does not exacerbate murine allergic airway inflammation. *PloS one* **7**, e47102 (2012).
59 Baru, A. M. *et al.* Selective depletion of Foxp3+ Treg during sensitization phase aggravates experimental allergic airway inflammation. *European journal of immunology* **40**, 2259-2266 (2010).
60 Liang, H. E. *et al.* Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nature immunology* **13**, 58-66 (2011).
61 Laouini, D. *et al.* IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. *The Journal of Clinical Investigation* **112**, 1058-1066 (2003).
62 Kips, J. C. Cytokines in asthma. *European Respiratory Journal* **18**, 24-33 (2001).
63 He, L. *et al.* The regulation of regulation: interleukin-10 increases $CD4^+CD25^+$ regulatory T cells but impairs their immunosuppressive activity in murine models with schistosomiasis japonica or asthma. *Immunology* **153**, 84-96 (2017).
64 Chen, X. *et al.* TIGIT negatively regulates inflammation by altering macrophage phenotype. *Immunobiology* **221**, 48-55 (2016).
65 van Oosterhout, A. Th1/Th2 paradigm: not seeing the forest for the trees?, *European Respiratory Journal* **25**, 591-593 (2005).
66 Ngoc, L., Gold, D., Tzianabos, A., Weiss, S. & Celedón, J. Cytokines, allergy, and asthma. *Current Opinion in Allergy and Clinical Immunology* **5**, 161-166 (2005).
67 Levin, S. *et al.* Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *European Journal of Immunology* **41**, 902-915 (2011).
68 Schoenborn, J. & Wilson, C. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. *Advances in Immunology* **96**, 41-101 (2007).
69 Stock, P. & Akbari, O. Recent advances in the role of NKT cells in allergic diseases and asthma. *Current Allergy and Asthma Reports* **8**, 165-170 (2008).
70 Karimi, K. & Forsythe, P. Natural Killer Cells in Asthma. *Frontiers in Immunology* **4**, 1-8 (2013).
71 O'Sullivan, S. *et al.* Activated, cytotoxic $CD8^+$ T lymphocytes contribute to the pathology of asthma death. *American Journal of Respiratory and Critical Care Medicine* **164**, 560-564 (2001).
72 Arase, N. *et al.* IgE-Mediated Activation of NK Cells Through FcγRIII. *The Journal of Immunology* **170**, 3054-3058 (2003).

73 Kim, H. Y., Kim S. & Chung D. H. FcgRIII engagement provides activating signals to NKT cells in antibody-induced joint inflammation. *Journal of Clinical Investigation* **116**, 2484-2492 (2006).

74 Nembrini, C., Marsland, B. & Kopf, M. IL-17-producing T cells in lung immunity and inflammation. *Journal of Allergy and Clinical Immunology* **123**, 986-994 (2009).

75 Lambrecht, B. & Hammad, H. The immunology of asthma. *Nature Immunology* **16**, 45-56 (2014).

76 Rincon, M. & Irvin, C. G. Role of IL-6 in asthma and other inflammatory pulmonary diseases. *International Journal of Biological Sciences* **8**, 1281-1290 (2012).

77 Diehl, S. & Rincón, M. The two faces of IL-6 on Th1/Th2 differentiation. *Molecular Immunology* **39**, 531-536 (2002).

78 Mantovani, A., Gray, P. A., Damme, J. V. & Sozzani, S. Macrophage-derived chemokine (MDC). *Journal of Leukocyte Biology* **68**, 400-404 (2000).

79 Sekiya, T. *et al.* Inducible Expression of a Th2-Type CC Chemokine Thymus- and Activation-Regulated Chemokine by Human Bronchial Epithelial Cells. *The Journal of Immunology* **165**, 2205-2213 (2000).

80 Woo, L. *et al.* A 4-Week Model of House Dust Mite (HDM) Induced Allergic Airways Inflammation with Airway Remodeling. *Scientific Reports* **8**, 1-11 (2018).