Interleukin 10-modulated Dendritic Cells Induce Tolerance in Patients with Birch Pollen and Associated Hazelnut Allergies

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„All we have to decide is what to do with the time given to us.”

Gandalf in
The Fellowship of the Ring by J.R.R. Tolkien

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ABSTRACT

Birch pollen allergy is one of the most prevalent type 1 allergic diseases in Northern Europe. Among birch pollen allergic individuals approximately 70% experience secondary food allergies, which are then classified as pollen-associated food allergies. These patients are first sensitized towards a birch pollen allergen (Bet v 1, [Bet]) and subsequently develop an allergy towards a homologous food allergen such as hazelnut (Cor a 1, [Cor]). However, the only disease-modifying therapy for type 1 allergies called allergen immunotherapy (AIT) has little to no effect on secondary food allergies.

In this study, allergen-loaded IL-10-modulated dendritic cells (IL-10 DC) were investigated in regard to their potential to induce allergen-specific and cross-reactive tolerance in the context of pollen-associated food allergies. Therefore $CD4^+$ T cells from birch pollen (Bet)-allergic donors with associated hazelnut (Cor) allergies were primed by Bet-loaded IL-10 DC to obtain Bet-specific iTreg. The T cell response was evaluated during priming and after restimulation by analysis of cytokine concentration, phenotype and proliferation. The suppressive capacity of iTreg was analysed *in vitro* and *in vivo*.

This study revealed that Bet-specific iTreg were anergic during priming, but proliferated vigorously after Bet- and Cor-induced restimulation. They displayed a high capacity to suppress allergen-specific and cross-reactive immune responses: *in vitro* they reduced the proliferation of Bet- and Cor-specifically stimulated responder T cells which was accompanied by a reduction of the allergy-related T_H2 cytokine IL-13 and a profound increase in IL-10 secretion. Flow cytometric analysis also revealed a highly activated and suppressive phenotype of Bet-specific iTreg. In a humanised mouse model of allergic intestinal and airway inflammation, Bet-specific iTreg were able to ameliorate birch pollen- and hazelnut-induced symptoms and to reduce Bet-specific IgE. Compared to non-specific iTreg, Bet-specific iTregBet showed a significantly increased capacity to suppress allergen-specific and cross-reactive T cell responses *in vitro*, indicating that allergen-specific priming does have a beneficial effect and that IL-10 DC-induced Treg do not only facilitate a general immunosuppression.

These pieces of evidence combined strongly suggest that iTreg which were primed by allergen-loaded IL-10 DC are able to facilitate highly allergen-specific as well as cross-reactive tolerance to both pollen and associated food allergens. IL-10 DC should

therefore be considered as top candidates for cellular tolerance-inducing therapies in pollen-associated food allergies.

ZUSAMMENFASSUNG

Die Birkenpollenallergie ist eine der häufigsten Typ 1 Allergien in Nordeuropa. 70 % aller Birkenpollenallergiker erkranken zusätzlich an sekundären Nahrungsmittelallergien, welche dann als Pollen-assoziierte Nahrungsmittelallergien eingestuft werden. Diese Patienten erleiden zuerst eine Sensibilisierung gegen ein Birkenpollenallergen (Bet v 1, [Bet]) und entwickeln daraufhin eine Allergie auf ein homologes Nahrungsmittelallergen, wie zum Beispiel Cor a 1 [Cor] aus der Haselnuss. Jedoch hat die einzig verfügbare krankheitsverändernde Therapie für Typ 1 Allergien, die sogenannte allergen-spezifische Immuntherapie (AIT), keine oder nur geringe Auswirkungen auf die sekundäre Nahrungsmittelallergie.

In dieser Studie wurden Allergen-beladene IL-10-modulierte dendritische Zellen (IL-10 DC) untersucht, um ihr Potential zur Induktion von Allergen-spezifischer und kreuzreaktiver Toleranz bezüglich Pollen-assoziierter Nahrungsmittelallergien zu bewerten. Dafür wurden $CD4^+$ T Zellen von Birkenpollenallergikern mit assoziierter Haselnussallergie mit Bet-beladenen IL-10 DC induziert, um Bet-spezifische iTreg zu erhalten. Die T-Zellantwort wurde während der Induktion und nach der Restimulation durch Analyse von Zytokinkonzentrationen, Phänotyp und Proliferation sowie der suppressiven Kapazität *in vitro* und *in vivo* untersucht.

Es zeigte sich, dass Bet-spezifische iTreg während der Induktion anerg sind, sich jedoch nach Bet- und Cor-spezifischer Restimulation sehr proliferativ verhalten. Sie wiesen trotzdem eine starke suppressive Kapazität bezüglich allergenspezifischer- und kreuzreaktiver Immunantworten auf: *in vitro* reduzierten sie die Proliferation von Bet- und Cor-stimulierten Responder- T Zellen und verringerten dabei die Konzentration des Allergie-assoziierten T_H2 Zytokins IL-13 und erhöhten die Konzentration des regulatorischen Zytokins IL-10. Dabei zeigten Bet-spezifische iTreg einen aktivierten und suppressiven Phänotyp. In einem humanisierten Mausmodel zu der allergischen Darm- und Atemwegsentzündung konnten Bet-spezifische iTreg Birkenpollen- und Haselnuss-induzierte Symptome lindern und Bet-spezifisches IgE reduzieren. Verglichen mit unspezifischen iTreg zeigten Bet-spezifische iTreg ein größeres Potential um Allergen-spezifische und kreuzreaktive T-Zellantworten *in vitro* zu hemmen. Dies zeigt die Bedeutung der Allergen-spezifischen Aktivierung und dass IL-10 induzierte Treg nicht nur eine generelle Immunsuppression vermitteln.

All diese Hinweise zusammen deuten darauf hin, dass iTreg, welche durch Allergen-beladene IL-10 DC induziert wurden, eine Allergen-spezifische und kreuzreaktive Toleranz gegenüber Pollen- und assoziierten Nahrungsmittelallergenen vermitteln. Deshalb können IL-10 DC als Kandidaten für zelluläre Toleranz-induzierende Therapien bei Pollen-assoziierte Nahrungsmittelallergien betrachtet werden.

ABBREVIATIONS

AIT	allergen-specific immunotherapy
CD	cluster of differentiation
CFDA	carboxyfluorescein diacetate
CFSE	5(6)-carboxyfluorescein diacetate N-succinimidyl ester
CTLA-4	cytotoxic T-lymphocyte-associated Protein 4
DAMP	danger-associated molecular pattern
DC	dendritic cells
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
eFluor670	cell Proliferation Dye eFluor™ 670
FACS	Fluorescence Activated Cell Sorting
Foxp3	forkhead box protein P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSA	human serum albumin
ICOS	inducible T cell costimulator
iDC	immature DC
Ig	immunoglobulin
IL	interleukin
ILT	immunoglobulin-like transcript
IL-10 DC0	unloaded IL-10 DC
IL-10 DCBet	Bet v 1-loaded IL-10 DC
ILC	innate lymphoid cells
iTreg	induced Treg
iTreg0	IL-10 DC0-primed iTreg
iTregBet	IL-10 DCBet-primed iTreg
KCl	potassium chloride
KH_2PO_4	potassium dihydrogenphosphate
LAG3	lymphocyte activation gene 3
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
mCH	methacholine
mDC	mature DC
mDC0	unloaded mDC
mDCBet	Bet v 1-loaded mDC

mDCCor	Cor a 1-loaded mDC
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
Na_2HPO_4	sodium dihydrogenphosphate
NaCl	sodium chloride
nTreg	natural Treg
OAS	oral allergy syndrome
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PC	primary culture
PD-1	programmed cell death protein 1
PFA	pollen-associated food allergy
PGE_2	prostaglandin E2
PR	pathogenesis-related
PRR	pathogen recognition receptor
rpm	rounds per minute
RS	restimulation
SCIT	subcutaneous allergen-specific immunotherapy
SLIT	sublingual allergen-specific immunotherapy
TCR	T cell receptor
Teff	effector T cells
Teff0	mDC0-primed Teff
TeffBet	mDCBet-primed Teff
TGF-β	transforming growth factor β
T_H1	T helper cells type 1
T_H2	T helper cells type 2
TNFR2	tumour necrosis factor receptor 2
TNF-α	tumour necrosis factor α
tolDC	tolerogenic dendritic cells
Tr1	T regulatory type 1
Treg	regulatory T cells
Tresp	responder T cells
TSLP	thymic stromal lymphopoietin

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1 INTRODUCTION

1.1 Allergies

An allergy is an exaggerated immune reaction towards a usually harmless environmental substance, the allergen.[1] They are often titled as diseases of the modern or industrialised culture. However, allergy-like symptoms were already described in ancient Egypt and ancient Rome.[2] This indicates that allergies have existed for thousands of years, but through their increasing prevalence and impact on everyday life they have gained great importance since then. The number of people suffering from allergic asthma or rhinitis has gone up from 75 million in 2002 to 400 million in 2011, as estimated by the *World Health Organisation* and 20 % of German adults suffer from at least one allergy.[1,3,4] In a worldwide online survey, birch pollen was reported as the third most-diagnosed allergen for respiratory allergies.[5] In addition, around 70 % of patients allergic to birch also develop a secondary food allergy, a condition called pollen-associated food allergy (PFA).[6,7] These numbers elucidate the comprehensive impact of allergies on personal quality of life, work or school performance and on the socio-economic burden.[8,9]

1.1.1 Classification of Allergies

Strictly speaking, there are four different types of allergies or hypersensitivity reactions. Immunoglobulin (Ig) E mediates immediate reactions (type I), which are usually triggered by airborne (e.g. pollen) or food allergens.[10] IgG- and IgM-mediated reactions can either be cytotoxic reactions direct against cell surface antigens (type II, e.g. autoimmune haemolytic anaemia),[11] or are immune complex-mediated (type III, e.g. serum sickness).[11,12] T cell-dependent delayed type hypersensitivity (type IV) usually manifests in contact dermatitis.[13] Gell and Coombs first categorised these types dependent on the allergen properties and the involved immune components such as cell types, antibody classes and the complement system in 1963.[14] Yet colloquially spoken the term “allergy” refers to the most prominent type I allergy to which PFA belong. The mechanism behind type I allergies will be explained in detail in the next paragraph.

1.2 Type 1 Allergies

Type 1 allergies are also called immediate-type allergies, because allergic symptoms are displayed only seconds or minutes after allergen contact.[11] Airborne allergens induce allergic rhinitis and sinusitis, the main symptoms of which are nasal and ocular

pruritus, angioedema and watery discharge.[15,16] Patients can also develop allergic asthma with more severe symptoms like shortness of breath, chest tightness and cough.[17] Food allergens trigger mostly labial and oropharyngeal pruritus and angioedema (oral allergy syndrome, OAS) and more rarely urticaria, nausea and anaphylaxis.[6]

The prevalence of type 1 allergies is increasing worldwide and a great variety of causing factors are discussed. The spread is associated with the progression of industrialisation, urbanisation and western life styles.[18] Air pollution and cigarette smoke directly trigger pulmonary inflammation and create a microenvironment that promotes IgE sensitisation and asthma, and air pollutants indirectly enhance the allergenic potency of pollen.[19–21] Observations of the correlation between increased hygiene and increasing incidence of allergic diseases led to the formulation of the hygiene hypothesis.[22,23] Originally this was attributed to higher incidences of viral, bacterial and parasitic infections under less hygienic conditions,[24,25] but many research groups have contributed to the theory with the consideration of different aspects including bacterial and allergen exposure during pregnancy and early life and the constitution of the microbiome.[20,21,23,26,27] In addition to a hereditary predisposition, some factors that favour allergic development are obesity, lack of physical exercise, a diet rich in industrially processed food as well as growing up in an urban environment and with little contact to other infants.[20,28,29]

1.2.1 The Development of Type 1 Allergies

The progression of type 1 allergies is divided into two phases: sensitisation and effector phase. The first allergen contact happens during the sensitisation, but allergic symptoms are only displayed in the effector phase after the second exposure to the allergen. The allergen usually enters the body through a mucosal interphase, where it is taken up by dendritic cells (DC) (Figure 1 A).[30] DC can migrate towards a draining lymph node where the adaptive immune response is initiated.[31]

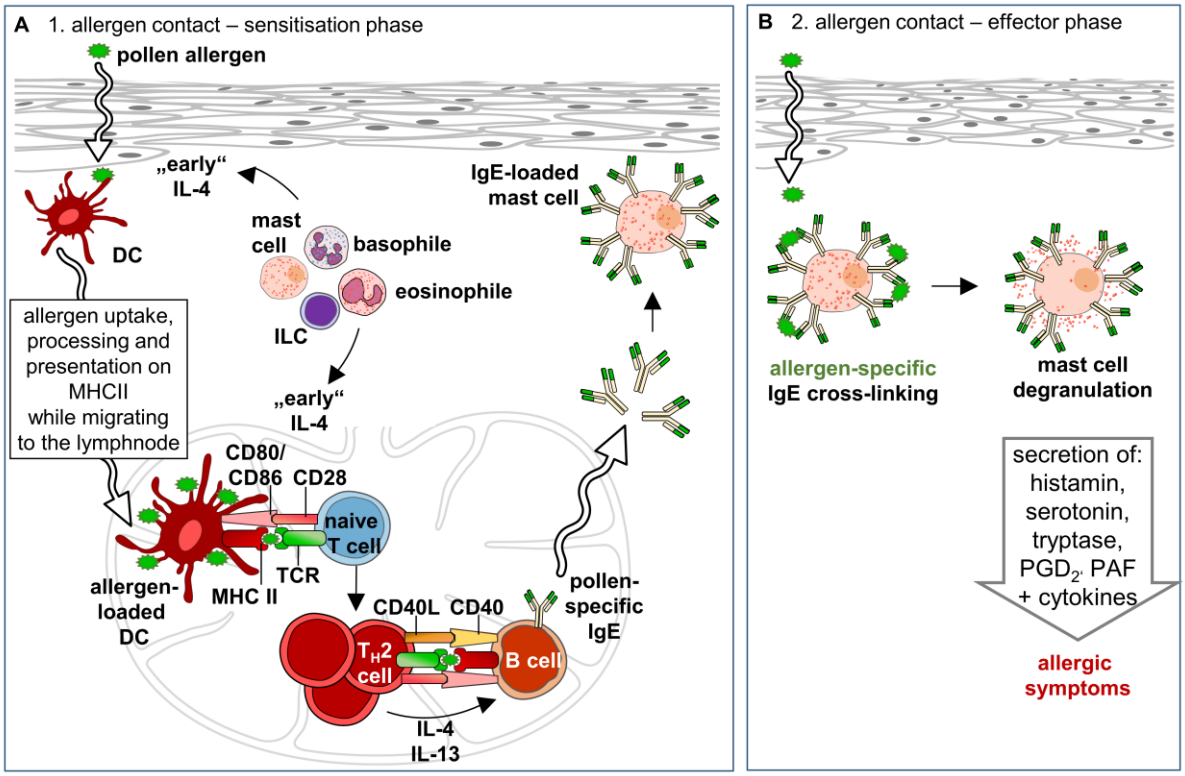

Figure 1: Mechanism of type 1 pollen allergies. A The sensitisation phase begins with pollen allergens crossing through a mucosal interphase into the tissue, where they are taken up and processed for presentation on major histocompatibility complex (MHC) II by dendritic cells (DC). The microenvironment, which is shaped by pathogens and immune or non-immune cells, determines the further differentiation of the DC. Mast cells, basophiles, eosinophils and innate lymphoid cells (ILC) can be sources of „early“ IL-4 which drives a T helper cell 2 (T_H2)-promoting DC phenotype. DC displaying the antigen migrate to a draining lymph node where they stimulate naive T cells with matching T cell receptors (TCR) and provide costimulation through CD80/86-CD28 interaction. The DC phenotype and soluble factors drive T cell differentiation towards T_H2 and T cell proliferation. T_H2 cells provide IL-4 and IL-13 as well as CD40L which stimulate B cells with matching B cell receptor to produce pollen-specific IgE which circles the periphery. The sensitisation phase is concluded by binding of pollen-specific IgE to Fcε-receptors on mast cells in the mucosa and skin. **B** Upon second contact with the pollen allergen the effector phase of the primary allergy is initiated. The pollen allergens cross-link the pollen-specific IgE on the mast cells which leads to mast cell degranulation and secretion of soluble factors which trigger allergic symptoms.

In brief, DC present the processed allergen to naïve $CD4^+$ T cells. T cells with matching T cell receptor (TCR) recognise the allergen, proliferate and differentiate into T helper type 2 (T_H2) effector cells, which can stimulate matching B cells to produce allergen-specific IgE antibodies.[32,33] These bind with high affinity to FcεRI receptors on mast cells residing in the skin and mucosa.[34,35] This event concludes the sensitisation phase and the effector phase starts upon second allergen contact (Figure 1 B). When allergens cross-link the mast cell-bound IgE, the mast cells degranulate, which results in the release of stored immune-mediators such as histamine, serotonin and several proteases (tryptase, chymase), as well as to the formation of lipid-mediators (prostaglandin E_2, platelet activating factor) and a variety of cytokines (inflammatory,

T_H1, T_H2).[36] Within seconds to minutes these trigger allergic symptoms by increased vascular permeability, contraction of the smooth muscles, recruitment of inflammatory cells and enhanced secretion of mucus in the respiratory organs for air-borne allergens or OAS for food allergens.[12,34,37]

1.2.2 The Function of DC in Type I Allergies

DC are the link between the antigen-independent innate and antigen-specific adaptive immune system.[38] Although their numbers are relatively small compared to other immune cell populations, they are at the centre of immunity and their role in immune regulation is very powerful.[39] Under steady-state conditions, DC reside in an immature state and display a barely immunogenic phenotype: reduced secretion of inflammatory cytokines and intermediate to low expression of major histocompatibility complex (MHC) and costimulatory molecules such as CD80 and CD86. [40–43] Immature DC (iDC) constantly take up antigens which are processed into small epitopes and are displayed on MHC molecules on the surface of DC to be recognised by T cells.[44,45] But iDC are incapable of inducing an appropriate T cell response.[42] Pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP) as signs of imminent thread are needed for DC maturation. [39,40] PAMPS are recognised by receptors of the innate immune system called pattern recognition receptors (PRR).[44] Activation of PRR on DC and inflammatory cytokines secreted by innate immune cells or epithelial cells initiate a signalling cascade that completely changes the DC phenotype into a highly migratory and T cell-stimulating mature DC (mDC).[41,46] mDC display high expression of costimulatory molecules (CD80/CD86) and the maturation marker CD83.[47] They migrate towards the draining lymph nodes through chemotaxis and the expression of adhesion molecules and chemokine receptors like CCR7. [40,48–50] Also, the mDC morphology changes: to increase the cell surface for cell-to-cell contacts, long stellate or dendrite-like branches are formed which gave the DC its name.[40,51] In the lymph node, mDC meet naïve T cells and initiate antigen-specific immune responses.

1.2.3 Tolerogenic Dendritic Cells

DC play a critical role in adaptive immunity as they provide the main T cell triggers that decide between allergen-specific immune activation or tolerance induction.[52] Tolerogenic DC (tolDC) prevent unnecessary immune responses towards harmless antigens, mainly by induction of regulatory T cells (Treg). Thereby, they ensure the tolerance of self-antigens, commensal bacteria or environmental substances and

prevent autoimmune diseases or allergies. Compared to mDC, tolDC are characterised by high expression of immunoglobulin-like transcript (ILT)-3, ILT4 and HLA-G as well as low expression of costimulatory molecules and MHC.[47,53] Secretion of transforming growth factor β (TGF-β) and interleukin (IL)-10 as well as tumour necrosis factor α (TNF-α) promotes Treg induction by tolDC. [53–55]

Many different protocols for *in vitro* tolDC generation have been established, leading to a vast variety of tolDC with different characteristics.[56] The protocols have in common that DC precursor cells are incubated with a tolerogenic substance, which can be a drug (rapamycin, dexamethasone, acetylsalicylic acid) or an endogenous biomolecule (IL-10, TGF-β, vitamin D3), but only some protocols involve the addition of a maturation stimulus (lipopolysaccharide (LPS), IL-1β, IL-6, TNF-α, prostaglandin E2 (PGE_2).[47] For IL-10-modulated tolDC there are two main protocols: DC-10 are created by incubation of peripheral blood mononuclear cells (PBMC) with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4 and IL-10 for 7-9 days, whereas for IL-10 DC induction PBMC are cultured with GM-CSF and IL-4 for 5 days, and subsequently with IL-10 and a maturation cocktail (consisting of IL-1β, IL-6, PGE_2 and TNF-α for stable and migratory phenotype) for 2 days. Both tolDC are capable of inducing antigen-specific Treg with strong suppressive capacities, although the DC show very different characteristics.[49,57–60] DC-10 show a rather mature phenotype with MHC, CD80, CD83 and CD86 expression comparable to mDC, but they also secrete high amounts of IL-10.[53] IL-10 DC display intermediate expression of MHC, CD80, CD83 and CD86 and an increase in suppression-associated molecules (ILT3, ILT4).[49] Their tolerogenic phenotype is extremely stable under inflammatory conditions, which is due to the presence of the maturation cocktail during IL-10 DC differentiation.[49,61]

1.2.4 The Role of T_H2 Cells in Type 1 Allergies

T cells are able to recognise and directly or indirectly attack a vast variety of pathogens, which highly depends on the diversity of their TCR.[62] Thereby, they provide wide-ranging protection on the one hand, but on the other hand they pose the risk of wrongfully attacking a harmless allergen and initiating allergic development. Naïve T cells are antigen- or allergen inexperienced cells that scan the secondary lymphoid organs for their cognate antigen.[63] mDC which carry the cognate allergen on MHC molecules, are the only cells that are able to prime naïve T cells and subsequently initiate an allergen-specific immune response (Figure 1 A).[39,41,64] Apart from signalling through a matching TCR, two more signals are needed to prime naïve T cells:

costimulation provided by mDC expression of CD80 and CD86 and proinflammatory cytokine signals which can also be derived from other cells such as basophiles, eosinophils, mast cells or innate lymphoid cells (ILC).[41] The engagement of TCR with antigen:MHC leads to the formation of the immunological synapse between the T cell and the DC: more TCR and costimulatory or adhesion molecules are recruited and concentrated to the site of interaction.[63] In the context of type 1 hypersensitivity, the T cells are driven towards $CD4^+$ T_H2 effector differentiation by the mDC.[46,65,66]

In host defence, T_H2 cells are believed to be primarily responsible for battling helminth parasites.[32] But also bacterial triggers like LPS or allergens derived from for example house dust mite can drive DC towards a T_H2-promoting phenotype.[67,68] The complete mechanism of T_H2-induction is much less understood than for T_H1 or T_H17 polarisation.[68,69] Activation of both transcription factors interferon regulatory factor 4 and Krüppel-like factor 4 is necessary to induce T_H2-promoting DC.[70,71] The expression pattern of DC surface molecules might also play a role. For instance, CD40, OX40 ligand, inducible T cell costimulator (ICOS) ligand and CD86 seem to favour T_H2 differentiation.[72] Induction of transcription factor GATA3 in T cells is essential for T_H2 polarisation and can be achieved by Stat5 or Stat6 phosphorylation induced by IL-2 or IL-4 stimulation, respectively.[69] Simple absence of IL-12 as signal for T_H2 differentiation is also discussed.[68,72] Thymic stromal lymphopoietin (TSLP) is expressed at mucosal interphases and in the tonsils where it is believed to suppress T_H1 responses in favour of T_H2 responses, and overexpression leads to allergic hyperreactivity or atopic dermatitis.[73,74] Interestingly, IL-10 which is a very prominent inducer of tolerance, has been linked with the promotion of T_H2 differentiation.[71]

T_H2 cells secrete IL4, IL5, IL-9 and IL-13, which have effector functions on different cell types.[68,71] IL-5 promotes tissue eosinophilia and IL-9 mast cell hyperplasia, whereas IL-13 stimulates goblet cells to produce mucus.[37,67] These cytokines are mainly produced by effector T_H2 cells in the tissue, whereas in the thymic B cell zone follicular T_H2 cells release IL-4 and IL-13 which, together with CD40/CD40L interaction, promotes the class switch towards IgE in B cells (Figure 1 A, bottom).[75,76] IL-4 also induces IgE secretion in the respiratory- or gut-associated mucosa, where IgE binds to Fcε receptors on basophils and mast cells.[35]

T_H2 cytokines can also be provided by a variety of other cells such as epithelial cells, innate immune cells and the more recently discovered ILC. ILC show characteristics

of lymphocytes and the ILC2 subtype is similar to T_H2 cells in regard to transcription factors and cytokine secretion.[37] However, they do not express a TCR or PRR and rely on other immune or epithelial cells to activate them with IL-25, IL-33 or TSLP upon pathogen encounter.[67,75] ILC2 together with T_H2 cells were found to be the main contributors of IL-5 and IL-13 in allergic airway inflammation in mice.[77] ILC2 express MHCII and costimulatory molecules and potentiate T cell responses and IL-2 production directly by antigen-specific TCR-MHCII interactions.[78] ILC have been discovered in 2001 and since then they have been extensively studied, but their true position within immunity has yet to be revealed.

1.2.5 Regulatory T Cells

The practically endless variety of the TCR inevitable creates auto-reactive T cells and T cells recognising harmless environmental antigens.[79,80] To suppress unnecessary excessive T cell responses and thereby protect from autoimmune and allergic diseases, antigen-specific Treg are induced in two ways: in the thymus directly during T cell development ((natural) nTreg, central tolerance) or later in the periphery ((induced) iTreg, peripheral tolerance).[54] During thymic T cell development, T cells with strong affinity to self-antigens are deleted, inactivated or driven towards nTreg differentiation.[54,81] But some auto-reactive or allergen-specific T cells escape this negative selection and patrol the periphery.[82] To prevent unwanted immune responses, these T cells can still become regulatory or anergic T cells, or they can be deleted by apoptosis.[48] Anergy is a state of long-term hyporesponsiveness to antigens, facilitated by active repression of TCR and IL-2 signalling.[83] In mice, Treg can be easily identified by forkhead box protein P3 (Foxp3) expression, which is necessary and sufficient for murine Treg development and function,[82] but in humans Foxp3 can also be upregulated by activated effector T cells (Teff) and not all Treg subsets express Foxp3.[84,85] CD25, a subunit of the high affinity IL-2 receptor, is constitutively expressed on Treg and was the original Treg marker, but has since been found to be also upregulated in activated Teff.[54] In conclusion, a reliable marker for human Treg has not been identified at present. In addition, both nTreg and iTreg are present in the periphery but cannot be distinguished due to the lack of defining surfaces markers.[86,87] Helios and Neuropilin-1 have been proposed as specific markers for nTreg in mice, but some evidence suggests their unreliability.[88–90] Since iTreg cannot be clearly distinguished from nTreg in the peripheral blood, the mechanism of *in vivo* iTreg induction is less clear than that of nTreg induction.[87] But *in vitro* studies revealed

several ways of iTreg differentiation from naïve $CD4^+$ T cells: TGF-β stimulation, repeating low doses of antigen, microbiotic metabolites (propionate, butyrate), and by priming with tolDC.[87] The diversity of induction protocols and the lack of *in vivo* population markers complicate the attribution of distinct characteristics and functional mechanisms to nTreg and iTreg, especially in humans. However, nTreg seem to prevent autoimmunity while iTreg suppress unwanted immune responses to environmental airborne and food allergens,[91] which is also reflected in their non-overlapping T cell receptor repertoire.[92] Allergen-specific iTreg are also enriched in the gastrointestinal tract and in the lung during chronic inflammation.[86]

T regulatory type 1 (Tr1) cells are a subset of iTreg, which do not express Foxp3 and are characterised by lymphocyte activation gene 3 (LAG3) and CD49b expression.[93] Tr1 cells were first generated *in vitro* by chronic activation of T cell clones in the presence of IL-10 but have since been identified in human peripheral blood.[94] Decreased numbers or defective function of Tr1 cells is often associated with autoimmunity or allergic diseases.[95–99] Tr1 cells produce large amounts of IL-10 as well as TGF-β and IL-5, low levels of IL-2 and IFN-γ, but no IL-4.[100] Tr1 cells need to be activated by their antigen via TCR signalling, but then exert their suppressive function antigen independently, leading to bystander suppression.[100]

Treg can exhibit their suppressive functions on many different immune cells via cytokine secretion or cell contact-dependently, but understanding of the mechanisms is still limited.[54] Treg control T cell responses via IL-10, which in mice was shown to activate a feed-forward loop of IL-10 production through IL-10 receptor α signalling.[101] IL-10 suppresses effector T cells by inhibition of IL-2 production and proliferation.[102] Also, Treg derived IL-10 was shown to drive DC towards a regulatory phenotype in mice.[103] TGF-β secreted by Treg suppresses Teff differentiation and proliferation while promoting Treg differentiation.[104] IL-10 and TGF-β are the main soluble suppressive molecules and Tr1 cells secret both to suppress T_H1 and T_H2 responses, but cell contact-dependent mechanisms might be involved.[105] CD25-dependent deprivation of the essential T cell survival stimulus IL-2 is another means by which Treg can suppress Teff.[106]

Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) is highly expressed on Treg, where it inhibits intrinsic activation and depletes costimulatory molecules from DC via trans-endocytosis, thereby inducing a tolDC phenotype.[107]

Another molecule implicated in DC inhibition by Treg is LAG3, which has a high binding affinity to MHCII and might be required for maximal suppressive capacity.[108] Programmed cell death protein 1 (PD-1) is highly expressed on allergen-specific $CD4^+$ T cells and inhibits T cell responses towards aeroallergens.[109]

The mechanism behind allergen-specific immunotherapy (AIT) is dependent on the induction of allergen-specific IL-10 producing Treg.[110] The therapy is especially associated with an increase of Tr1 cells.[111]

1.2.6 Therapy

The simplest – but by far not the easiest – way to manage an allergy is allergen avoidance, which can hugely restrict personal choices and the quality of life. The only disease-modifying treatment available for type 1 allergies is AIT, which can be applied subcutaneously (SCIT) or sublingually (SLIT).[9,112] Independent of the application route, the therapy requires numerous allergen exposures over a long period of time.[113–115]

For seasonal and all-season air-borne allergens, both AIT options seem to be similar in efficiency.[116,117] A 30-60% of symptom reduction can be achieved by SCIT or SLIT with grass pollen allergens.[118–120] However, AIT studies are difficult to compare due to a vast heterogeneity in therapeutic measures and assessment of clinical parameters and direct comparisons are scarce.[116] There are only a few studies about AIT in the context of primary and secondary food allergies. For peanut-allergic patients SCIT was effective but unsafe, whereas SLIT showed efficacy and safety in peanut, hazelnut or peach allergic patients.[117,121] The effect of pollen-specific AIT on related food allergy remains unsolved.[115,117,122–126]

The adverse effects of SCIT are few but rather severe, whereas for SLIT there are more frequent but very moderate side effects.[127] For SCIT frequent injections at a medical practice or hospital are needed, whereas SLIT can be self-administered at home but requires daily intake and both therapies have to be undertaken for at least 3 years.[112,127] Therefore patient adherence for these therapies are a considerable problem and drop-out rates are high.[127] Not all patients respond optimally, but biomarkers for outcome prediction are not available.

The mechanisms by which AIT induces allergen-specific tolerance are not yet fully understood, but several possibilities have been observed.[113] Induction of allergen-specific blocking antibodies IgG and IgA can inhibit IgE-facilitated antigen

presentation.[125,128] IL-10 secreting regulatory B cells that particularly express the blocking antibody IgG4 increase during AIT.[129] On T cell level, the effects include a shift from T_H2 towards T_H1 responses and induction of Treg.[113,116]

1.3 Pollen-associated Food Allergies and Allergen Cross-Reactivity

Food allergies can develop either by direct sensitisation to the food allergen in the gastrointestinal tract or through secondary reaction after primary sensitisation to a different allergen, which is the cause of pollen-associated food allergies (PFA).[130] This is possible because many allergens from different plants are highly conserved and therefore they have similar amino acid sequences and tertiary protein structures which may trigger cross-reactions.[66,131] In PFA allergic individuals are sensitised towards a pollen allergen (primary allergy, Figure 1 A). In addition to developing a pollen allergy, the pollen-specific IgE and T cells cross-react with a food allergen (Figure 2) initiating the cascade which manifests in a food allergy (secondary allergy).[66]

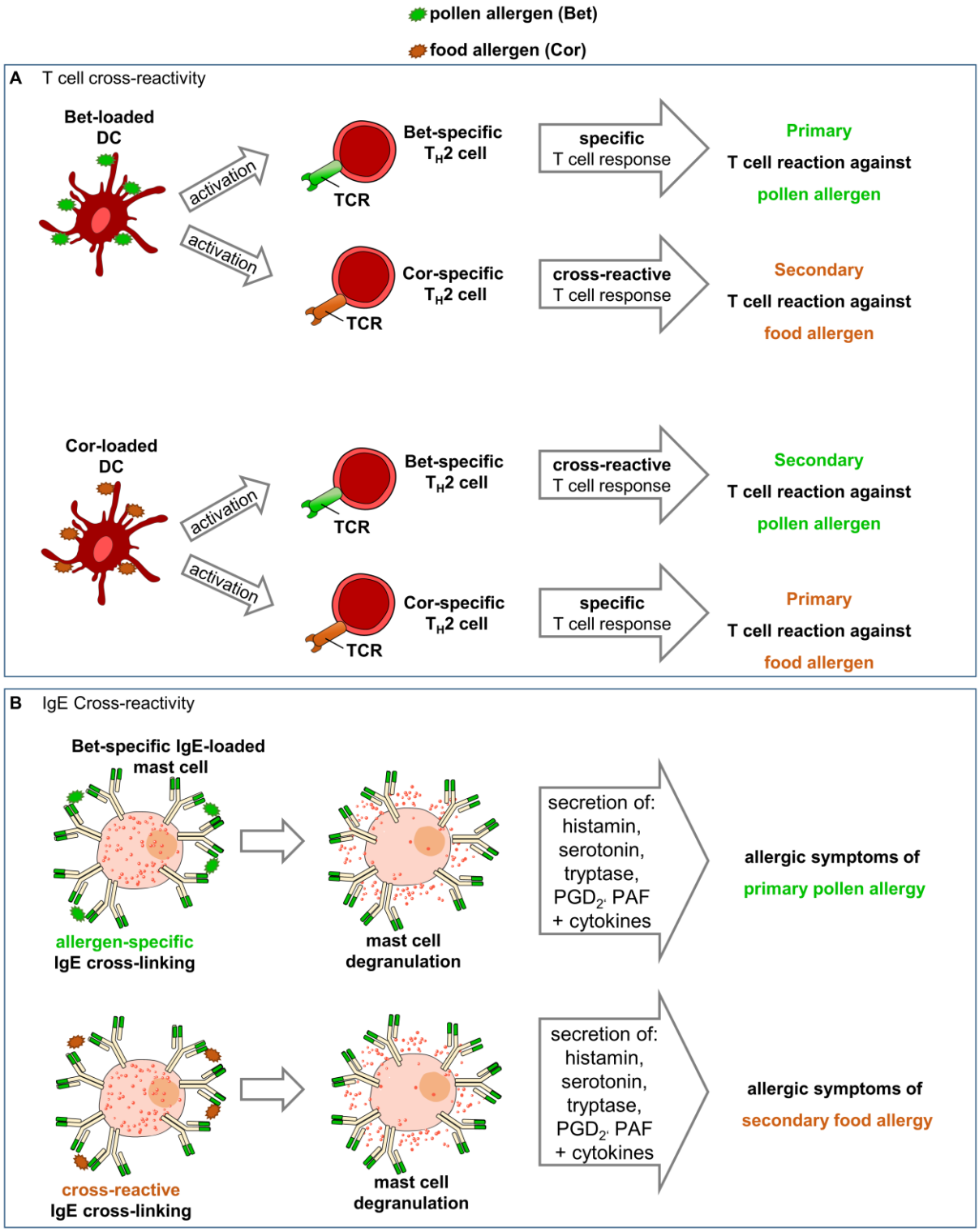

Figure 2: T cell and IgE cross-reactiveness between pollen and associated food allergens. A T cell cross-reactivity: Pollen allergen (Bet)-loaded DC activate Bet-specific T_H2 cells to proliferate. Activated T_H2 cells stimulate B cells to produce Bet-specific IgE, which drives the progression of the primary pollen allergy. Due to sequential allergen homology, Cor-loaded DC are also able to stimulate Bet-specific T_H2 cells, resulting in cross-reactive T cell activation and Bet-specific IgE secretion. **B** IgE cross-reactivity: Mast cells loaded with pollen allergen (Bet)-specific IgE are cross-linked by Bet and degranulate. Subsequent secretion of multiple soluble factors initiates allergic symptoms of the primary pollen allergy. Due to structural allergen homology, Cor is also able to cross-link Bet-specific IgE on mast cells, which drives the development of allergic symptoms of the secondary food allergy.

The 3D structure and protein surface are essential for the recognition of antigens by the respective antibody. The homologue allergen structure therefore causes allergen-specific IgE to also bind to related allergens.[7,29] On the other hand, only small amino acid sequences suffice to be recognised by the T cell receptor. Cross-reactivity between homologous allergens and allergen-specific T cells have first been confirmed in T cell clones, but could also be identified in primary human cells.[66,132]

Birch pollen-associated food allergies are the most abundant and important PFA.[29] The major birch pollen allergen Bet v 1 belongs to the pathogenesis-related protein (PR)-10 group.[133] PR proteins are plant proteins involved in innate host defence mechanisms and PR proteins, which cross-react with pollen, are therefore found in most plants including fruits, vegetables and nuts.[6,7,29,134] Allergens from the PR-10 group are present in apple, carrot, celery, hazelnut, peanut, soybean and strawberries.[135] Cor a 1 is a PR-10 hazelnut allergen that shares 67 % of sequence homology and a similar tertiary structure with Bet v 1.[66] Due to their severe and life-threatening symptoms, hazelnut allergies are listed among the top five serious food allergies, but hazelnuts are often dangerously “hidden” food ingredients.[136]

1.4 Tolerance-inducing Cellular Therapies

Transplant rejections, autoimmune disorders and severe allergic diseases have in common that their treatment requires immunosuppression which except for AIT can only be applied non-specifically. The generalised and mostly systemic immunosuppression has severe side effects and leaves patients vulnerable for infections and tumours.[137] These immunosuppressive therapies are also not disease-modifying, but focus on symptom relieve which leads to a livelong necessity of immunosuppressive drugs. Therefore, new ways to establish tolerance specific for autoantigens, allergens or transplants are being explored and cellular therapies using regulatory T cells, tolerogenic macrophages or DC are promising candidates.[137]

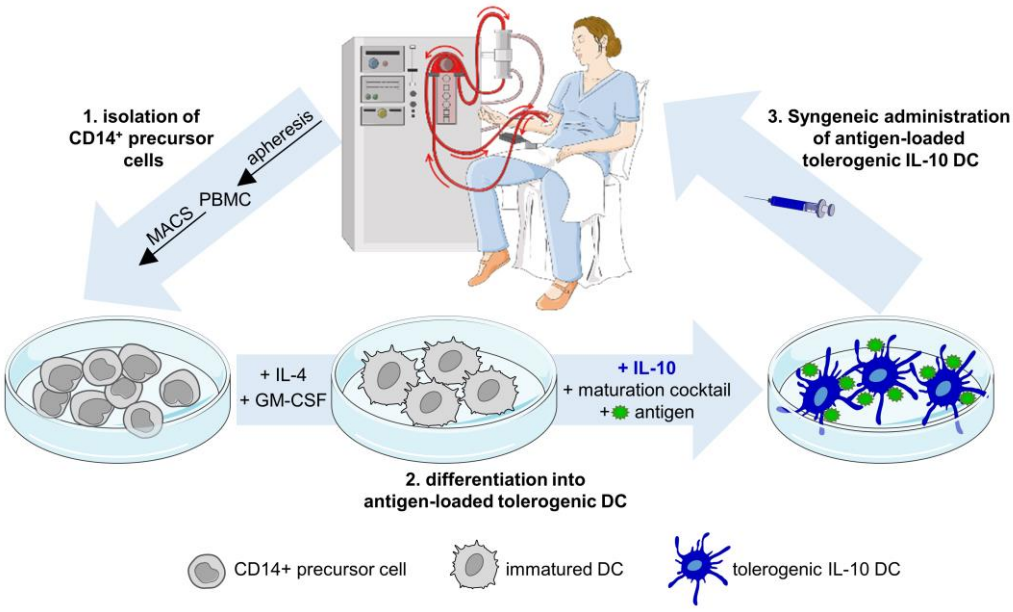

Figure 3: IL-10-modulated dendritic cell therapy. The patient's immune cells are separated from the peripheral blood by apheresis and $CD14^+$ precursor cells are isolated by magnetic cell separation (MACS). During an *ex vivo* cultivation phase the precursor cells are differentiated into immature DC with IL-4 and GM-CSF. Then a maturation cocktail (consisting of IL-1β, IL-6, PGE_2 and TNF-α for stable and migratory phenotype) and IL-10 (for tolerogenic phenotype) are added to the culture and the cells are incubated with the antigen to obtain antigen-loaded IL-10 DC. The syngeneic antigen-loaded tolerogenic DC are administered into the patient by for example intravenous injection.

tolDC seem specifically qualified, as they stand at the centre of adaptive immunity, but also regulate its induction at a very early stage. Their progenitors are easily isolated from peripheral blood and can be differentiated *ex vivo* in an antigen-dependent and tolerogenic manner.[47,58,138] One possible way to prepare a DC therapy is explained in 3 steps (Figure 3): (1) isolation of monocytic precursor cells from peripheral blood by apheresis, (2) *ex vivo* differentiation of antigen-specific tolDC, (3) readministration of syngeneic tolDC into patients.[47]

tolDC can be differentiated *ex vivo* using numerous tolerogenic agents and a great variation of protocols are in use, but IL-10 DC are the top candidates for cellular tolerance-inducing therapies, as they have a stable tolerogenic phenotype and induce iTreg with strong suppressive capacities.[47,56,61] None of the tolDC applied in type 1/2A clinical trials have induced any severe side effects or diseases worsening.[139–142] So far, only very few trials have investigated disease-modifying effects, but the preliminary results are very promising.[141,143,144]

2 MATERIALS

2.1 Laboratory Equipment and Disposables

Laboratory equipment:

autoclave	Varioklav Steam Sterilizer, H+P Laborechnik GmbH, Oberschleißheim, Germany
flow cytometer	BD LSR II Flow Cytometer, BD Biosciences, Heidelberg, Germany
incubator	Hera Cell 240, Thermo Fisher Scientific, Karlsruhe, Germany
heating cabinet	Ehret Labor- und Pharmatechnik, Freiburg Germany
photometer	BioPhotometer® 6131, Eppendorf AG, Hamburg, Germany
microscope	LH50A, Olympus Deutschland GmbH, Hamburg, Germany
flow bench	HeraSafe, Thermo Fisher Scientific, Langenselbod, Germany
β-scintillation counter	1205 Betaplate®, LKB Wallac, Victoria, Australia
water bath	TW 12, Julabo, Seelbach, Germany
centrifuge	Heraeus Megafuge 1.0R, Thermo Fisher Scientific, Karlsruhe, Germany
hemocytometer	Neubauer Improved, Marienfeld, Lauda-Königshofen, Germany
cell harvester	Semiautomatic Cell Harvester, Skatron AS, Lier, Norway
pipettes	1000/100/20/10 µL: HTL Lab Solutions, Warszawa, Poland 200 µL: Gilson Incorporated, Middleton (WI), USA
MACS separator	MidiMACS™ Separator on MACS MultiStand, Miltenyi Biotech, Bergisch Gladbach, Germany
mini-endoscope	Coloview System, 1,9mm, Karl Storz, Tuttlingen(D)
plethysmograph	flexiVent, SCIREQ, Montreal, Canada
Immuno-CAP analyser	Phadia™ 250: ImmunoCAP™, Thermo Fisher Scientific, Karlsruhe, Germany
plastic film sealer	Polystar® 601M, Rische + Herfurth, Hamburg Germany

Disposables:

adapter	Membrane Adapter, Sarsted, Nümbrecht, Germany
syringes	20 mL/2 mL: Omnifix, B. Braun AG, Melsungen, Germany
glass fibre filter map	Printed Filtermap A, PerkinElmer, Waltham (MA), USA
glass pipette	Graduated Pipette, BLAUBAND®, Brand, Wertheim Germany Measuring Pipette, Hirschmann Laborgeräte GmbH, Eberstadt, Germany
hypodermic needle	Sterican® 0.5 x 25 mm BL/LB, B. Braun AG, Melsungen, Germany
cell depletion column	LD Column, Miltenyi Biotech, Bergisch Gladbach, Germany
cell separation column	LS Column, Miltenyi Biotech, Bergisch Gladbach, Germany
sample bag	Sample Bag for Betaplate™ 102 x 258 mm, PerkinElmer, Waltham (MA), USA
pipette tips	1000/200/10 µL, Greiner Bio-One GmbH, Kremsmünster, Austria
sterile filter	Filtropur S 0.2 µm, Sarsted, Nümbrecht, Germany
cell culture plates	6 well/ 12 well/ 96 well, Costar® Cell Culture Plate, Corning B.V. Life Sciences, Amsterdam, The Netherlands
centrifugation tubes	15 mL/ 50 mL, Cellstar®, Greiner Bio-One GmbH, Kremsmünster, Austria
butterfly needle	Venofix® safety, B. Braun AG, Melsungen, Germany
pasteur pipette	Glas Pasteuer Pipettes 225 mm, Brand, Wertheim Germany
flow cytometry tube	Falcon® 5 mL polysterene round-bottom tube, Corning B.V. Life Sciences, Amsterdam, The Netherlands

2.2 Common Reagents, Buffers and Solutions

water (sterile)	Ampuwa® Sterile Water for Irrigation, Fresenius Kabi GmbH, Bad Homburg, Germany

EDTA	EDTA sodium salt dehydrate p.a., Applichem GmbH, Darmstadt, Germany
ethanol	Ethanol 70% denatured, Carl Roth GmbH, Karlsruhe, Deutschland
HSA	Human-Albumin 20 % Behring salzarm, CSL Behring, King of Prussia (PA), USA
KCl	potassium chloride p.a., Merck KGaA, Darmstadt, Germany
KH_2PO_4	potassium dihydrogen phosphate p.a. ISO, Merck KGaA, Darmstadt, Germany
NaCl	sodium chloride p.a., Carl Roth GmbH, Karlsruhe, Deutschland
Na_2HPO_4	di-sodium hydrogen phosphate p.a., Carl Roth GmbH, Karlsruhe, Deutschland
scintillation cocktail	Rotiszin® eco plus, Carl Roth GmbH, Karlsruhe, Deutschland
trypan blue stock	Trypan Blue Solution 0.4 %, Merck KGaA, Darmstadt, Germany
[^{3}H]-thymidine	[methyl-^{3}H]-thymidine, PerkinElmer, Waltham (MA), USA
DMSO	Dimethyl sulfoxide Hybrimax^{TM}, Merck KGaA, Darmstadt, Germany
xylazine	2 %, Rompun , Bayer Healthcare, Leverkusen, Germany
ketamine	500mg/10ml Injektionslösung Ratiopharm GmbH, Ulm, Germany
methacholine	methacholine chloride, Merck KGaA, Darmstadt, Germany
pentobarbital	Narcoren® 160mg/ml, Merial, Halbermoos, Germany
antibody dilution	0,5% HSA in PBS
PBS buffer	NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10.0 mM, KH_2PO_4 1.8 mM in water
FACS buffer	0,5% HSA, EDTA 3 mM in PBS
Tris/HCl buffer	Tris-HCL 50 mM pH 7.6 in PBS
trypan blue solution	1:10 trypan blue stock in PBS

0.9 % NaCl solution	NaCl 0.9 %, B. Braun AG, Melsungen, Germany

2.3 Kits

BCA Protein Assays Kit	Pierce® BCA Protein Assay Kit, Thermo Fisher Scientific, Karlsruhe, Germany
intracellular staining kit	Fixation and Permeabilization Solution Kit with BD GolgiStop™, BD Biosciences, Heidelberg, Germany
cytokine analysis Kit	eBioscience™ ProcartaPlex Human Th1/Th2/Th9/Th17/Th22/Treg Zytokin-Panel (18-plex), Thermo Fisher Scientific, Karlsruhe, Germany

2.4 Allergens

Bet v 1	rBet v 1.0101, Paul Ehrlich Institut, Langen, Germany Bet v 1a (Bet v 1.0101), biomay, Wien, Austrua
Cor a 1	Cor a 1.0401 Paul Ehrlich Institut, Langen, Germany
birch pollen extract	Allergovit® 108 Birke 100 %, Allergopharma, Reinbek, Germany
hazelnut extract	Allergovit® 129 Hasel 100 %, Allergopharma, Reinbek, Germany

2.5 Antibodies and Proliferation Dyes

Antibodies:

CD14 Pacific Blue	Pacific Blue™ anti-human CD14, Clone M5E2, Biolegend, San Diego (CA), USA
CD80 APC	APC anti-human CD80, Clone 2D10, Biolegend, San Diego (CA), USA
CD83 PE	Anti-human CD83 PE, Clone HB15e, Thermo Fisher Scientific, Karlsruhe, Germany
HLA-DR Fitc	Anti-HLA-DR Fitc, Clone L243,BD Biosciences, Heidelberg, Germany
CD4 Fitc	CD4-Fitc, Clone 12B8.2, Beckman Coulter GmbH, Krefeld, Germany

CD25 PE	CD25-PE, Clone 4E3, Miltenyi Biotech, Bergisch Gladbach, Germany
CD25 BV510	Brilliant Violet™ 510 anti-human CD25, Clone M-A251, Biolegend, San Diego (CA), USA
CD45RA APC-H7	APC-H7 mouse anti-human CD45RA, Clone HI100, BD Biosciences, Heidelberg, Germany
CD45RO BV650	Brilliant Violet™ 650 anti-human CD45RO, Clone UCHL1, Biolegend, San Diego (CA), USA
CTLA-4 PE	PE mouse anti-human CD152, Clone BNI3, BD Biosciences, Heidelberg, Germany
PD-1 BV711	Brilliant Violet™ 711 anti-human CD279 (PD-1), Clone H12.2H7, Biolegend, San Diego (CA), USA
TNFR2 Pacific Blue	Pacific Blue™ anti-human CD14, Clone 3G7A02, Biolegend, San Diego (CA), USA
HLA-DR PerCp/Cy5	PerCP/Cyanine 5.5 anti-human HLA-DR, Clone L243, Biolegend, San Diego (CA), USA
LAG3 PE	Anti-human LAG-3 Phycoerythrin conjugated, Clone 874501, R&D Systems Inc, Minneapolis (MN), USA
CD49b BV711	BV711 BD OptiBuilt™ Mouse Anti-Human CD49b, Clone AK-7, BD Biosciences, Heidelberg, Germany
ICOS PE/Cy7	PE/Cyanine7 anti-human/mouse/rat CD278 (ICOS), Clone C398.4A, Biolegend, San Diego (CA), USA
IL-10 BB700	Horizon™ BB700 rat anti-human IL-10, Clone JES3-19F1, BD Biosciences, Heidelberg, Germany

Proliferation dyes:

cell trace violet	CellTrace™ Violet Cell Proliferation Kit, Thermo Fisher Scientific, Karlsruhe, Germany
CFSE	5(6)-CFDA, SE; CFSE (5-(und-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester), Thermo Fisher Scientific, Karlsruhe, Germany
eFluor 670	eBioscience™ Cell Proliferation Dye eFluor® 670, Thermo Fisher Scientific, Karlsruhe, Germany

2.6 Reagents for Cell Isolation and Cell Culture

sodium-heparin	Heparin-Natrium 25000, ratiopharm GmbH, Ulm, Germany
cell separating solution	Biocoll Separating Solution, Biochrom GmbH, Berlin, Germany
IMDM	Iscove's Modified Dulbecco's Medium, Lonza, Basel, Switzerland
RPMI	RPMI-1640, Lonza, Basel, Switzerland
GM-CSF	Leukine® Sargramostim, Partner Therapeutics, Lexington (MA) USA
IL-1β	Human IL-1β premium grade, Miltenyi Biotech, Bergisch Gladbach, Germany
IL-4	Recombinant Human Interleukin-4, Immunotools, Friesoythe, Germany
IL-6	Human IL-6 premium grade, Miltenyi Biotech, Bergisch Gladbach, Germany
IL-10	Recombinant Human Interleukin-10, Cellgenix GmbH, Freiburg, Germany
TNF-α	Human TNF-α premium grade, Miltenyi Biotech, Bergisch Gladbach, Germany
PGE_2	Prostaglandine E_2, Cayman Chemical, Michigan (MI), USA
CD4 MicroBeads	CD4 MicroBeads, Miltenyi Biotech, Bergisch Gladbach, Germany
CD25 MicroBeads	CD25 Microbeads, Miltenyi Biotech, Bergisch Gladbach, Germany

2.7 Mouse Strain

The *NOD-scid-γc*$^{-/-}$ mice (*CB17-PRkdc*^{scid}*/Jγc*$^{-/-}$ purchased from Jackson Laboratory) were kept under pathogen free conditions in the Translational Animal Research Center of the University Medical Center of the Johannes Gutenberg-University in Mainz.

3 METHODS

All experiments were carried out under sterile conditions on a flow bench, except for antibody staining for flow cytometry and preparing cell culture supernatants for cytokine analysis by ProcartaPlex. If not stated otherwise, primary human cells were isolated from peripheral blood directly before culture. Cells were cultured under standard conditions: at 37 °C in an incubator with 5 % CO_2 and 95 % humidity. All cell co-cultures and the plasma used for cell culture media and stainings were autologous.

Cells were counted with a hemocytometer with exclusion of dead cells by trypan blue staining. Trypan blue is a dye that can pass through the damaged membrane of dead cells.

Flow cytometry samples were analysed on an LSR II and with FACSDiva™ 8.0 software (both BD Biosciences). Cytokines in cell culture supernatants were analysed on a MAGPIX® system (LUMINEX®) with ProcartaPlex Analyst 1.0 software (ThermoFisher Scientific). Statistical analysis and graph design were performed with Prism6.0 (GraphPad Software).

3.1 Study Cohort

All 38 participants reported a history of allergic symptoms after being exposed to air-borne birch pollen and after ingesting hazelnuts. The sensitisation against birch pollen and hazelnut extracts as well as against recombinant Bet v 1 and Cor a 1 was confirmed by Immuno-CAP Specific IgE test. Patients with CAP classes ≥ 3 for birch pollen (Bet v 1 and birch pollen extract) and CAP class ≥ 2 for hazelnut (Cor a 1 and hazelnut extract) were included in the study, except for one participant, who was diagnosed with CAP class 2 for birch, hazelnut and Cor a 1, but CAP class 3 for Bet v 1. On average the participants had CAP class 4.1 ± 0.97 and 4.2 ± 0.92 for birch pollen and Bet v 1 and 3.3 ± 0.90 and 3.6 ± 1.02 for hazelnut and Cor a 1, respectively. Individual data for each participant is presented in Table 1. It was further assured that none of the participants suffered from autoimmune syndromes or underwent permanent immunomodulatory treatment. Seasonal corticosteroid treatment due to pollen allergies was terminated at least 4 weeks before the first blood donation for the study. If the participants had undergone AIT against birch pollen, the treatment was finished at least 7 years before the study and the participants reported persistent or recurrent allergic symptoms.

On average, the participants were 31.7 ± 11.09 years old ranging from 19 to 62 years. 27 participants were female and 11 were male.

Table 1 Characterisation of the study cohort

			ImmunoCAP							
			Birch extract		Bet v1		Hazelnut extract		Cor a1	
ID	age	sex	kUA/l	class	kUA/l	class	kUA/l	class	kUA/l	class
VA 7	62	f	3.31	2	4.62	3	2	2	3.03	2
VA 8	47	m	10	3	9.74	3	2.15	2	2.77	2
VA 9	31	f	8.05	3	10.02	3	3.69	3	6.4	3
VA 10	39	f	5.11	3	5.9	3	3.02	2	4.21	3
VA 11	25	m	4.66	3	4.87	3	2.24	2	3.4	2
VA 13	30	m	47.6	4	31.9	4	13.4	3	19.4	4
VA 14	42	m	49.4	4	59.6	5	15.9	3	31.4	4
VA 15	30	f	77.6	5	95	5	25.5	4	51.6	5
VA 16	29	m	100	6	100	6	100	6	100	6
VA 17	22	f	37.8	4	34.9	4	19.5	4	26.9	4
VA 18	26	f	58	5	60.6	5	35.4	4	49.4	4
VA 19	24	f	80.6	5	78.9	5	48.7	4	65.8	5
VA 20	30	m	15.1	3	19	4	10.8	3	14.9	3
VA 21	27	f	100	6	100	6	100	6	100	6
VA 22	20	f	30.9	4	26.2	4	12.5	3	15.5	3
VA 23	29	f	71.4	5	79.9	5	37.7	4	58.9	5
VA 25	51	f	43.8	4	40.4	4	17	3	21.7	4
VA 26	28	m	19.6	4	21.3	4	4.46	3	9.93	3
VA 27	37	m	55.6	5	57.3	5	29.6	4	38.5	4
VA 28	24	f	80.6	5	78.9	5	48.7	4	65.8	5
VA 29	49	f	6.8	3	4.24	3	3.62	3	5.08	3
VA 30	23	f	100	6	100	6	43.3	4	62.3	5
VA 33	19	f	55.5	5	50.3	5	14.6	3	23.2	4
VA 34	53	f	13.6	3	8.98	3	6.94	3	10.5	3
VA 35	19	f	8.9	3	7.57	3	3.62	3	5.71	3
VA 36	23	f	31.7	4	21.3	4	13.3	3	23	4
VA 37	25	m	66.3	5	50.1	5	28.7	4	43.7	4
VA 38	27	f	22.7	4	20.6	4	9.75	3	14.3	3
VA 39	27	f	32.1	4	36.2	4	20.6	4	27.5	4
VA 40	26	m	22.8	4	28.8	4	11.2	3	15.6	3
VA 41	24	f	27.9	4	29.9	4	4.29	3	7.31	3
VA 43	28	m	9.5	3	10.3	3	2.99	2	4.32	3
VA 44	43	f	24.4	4	28	4	6.14	3	11.6	3
VA 45	29	f	18.3	4	17.5	4	5.61	3	8.88	3
VA 46	20	f	70	5	78.7	5	30.8	4	53.3	5
VA 47	20	f	14.3	3	13.7	3	6.46	3	8.78	3
VA 48	51	f	21.1	4	18.5	4	8.84	3	13.6	3
VA 49	44	f	21.5	4	21.4	4	7.98	3	12.5	3
mean:	31.7	(27 f /11 m)	37.7	4.1	37.5	4.2	19.4	3.3	26.5	3.7
SD	11.09		29.63	0.97	30.76	0.92	23.35	0.90	26.09	1.02

3.2 Overview of Experimental Procedure *in vitro*

On day 0, PBMC were isolated from the peripheral blood of confirmed birch pollen and hazelnut allergic patients (Methods 3.3.1) and monocytic precursors were separated to generate four populations of DC (Figure 4 A and Methods 3.4): unloaded mDC (mDC0), Bet-loaded mDC (mDCBet), unloaded IL-10 DC (IL-10 DC0) and Bet-loaded IL-10 DC (IL-10 DCBet).

Then, $CD4^+$ T cells (Methods 3.3.2) were isolated from a fresh blood sample of the same patient. The $CD4^+$T cells were primed with the four populations of DC mentioned above, resulting in the generation of effector T cells 0 (Teff0) (mDC0), TeffBet (mDCBet), iTreg0 (IL-10 DC0) and iTregBet (IL-10 DCBet), respectively. On day 4 of priming, T cell proliferation was assessed by [^{3}H]-thymidine incorporation (Methods 3.7). After five days of priming, cell culture supernatants were harvested and stored for cytokine analysis and the T cells were rested for 3 days.

Subsequently, the Teff0, TeffBet, iTreg0 and iTregBet populations were restimulated with Bet- or Cor-loaded mDC (mDCBet or mDCCor), respectively, which were generated from monoctyic precursors of the same patient (Methods 3.4). The T cell proliferation was determined by [^{3}H]-thymidine incorporation (Methods 3.7).

The two iTreg populations (iTreg0 and iTregBet) were applied in suppressor assays (Figure 4 B and Methods 3.8) to assess their capacity to suppress Bet- and Cor-stimulated T cell proliferation. After 5 days, half of the cells from the suppressor assay sample were directly used to assess T cell proliferation by flow cytometry, whereas the other part was stained with fluorescently-labelled antibodies against intra- and extracellular T cell markers prior to flow cytometry-based phenotype analysis.

The cell culture supernatants of these assays were collected and stored for cytokine analysis (Methods 3.9).

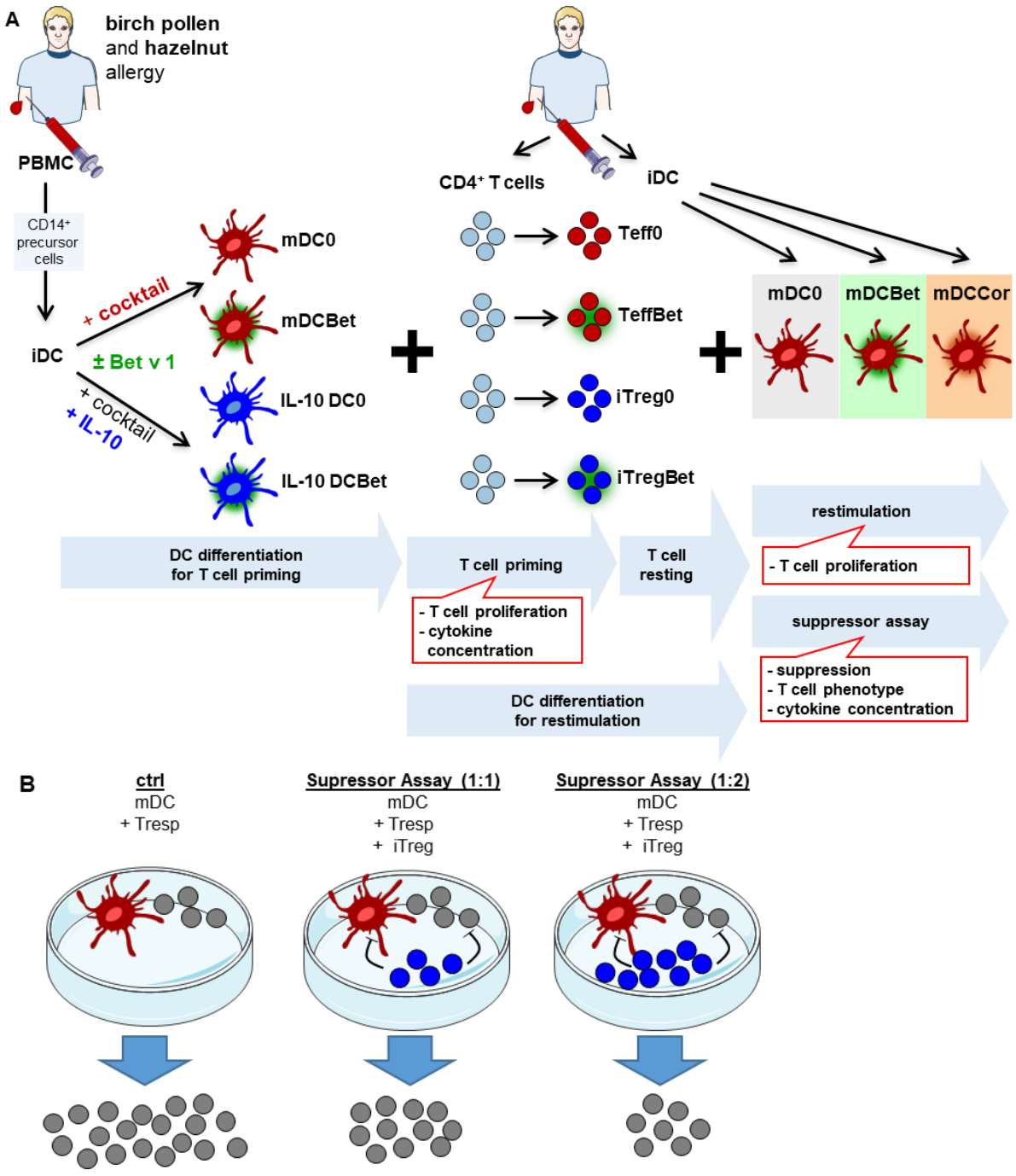

Figure 4: Experimental protocol *in vitro*. A PBMC are isolated from peripheral blood of donors with birch pollen and hazelnut allergies. Immature dendritic cells (iDC) are generated from CD14$^+$ precursor cells and are differentiated into mature DC (mDC;+ maturation cocktail) or IL-10 DC (+ cocktail and IL-10) which are unloaded or loaded with Bet v 1 (Bet), respectively. Syngeneic CD4$^+$ T cells are isolated from the same donor and are cocultured with the four different DC populations to prime effector T cells (Teff0/TeffBet) and induced regulatory T cells (iTreg0/Bet). During T cell priming, the T cell proliferation and cytokine profile in cell culture supernatants are assessed. Meanwhile from the same blood donation, unloaded and Bet-loaded or Cor a 1 (Cor)-loaded mDC, respectively, are differentiated for restimulation, which starts after a T cell resting phase. The investigated parameters are T cell proliferation after restimulation and suppression, T cell phenotype and cytokine secretion after suppressor assay. **B** In suppressor assays (SA) responder T cells (Tresp) from allergic donors were stimulated with allergen-loaded mDC (positive control; ctrl) and syngeneic iTreg were added in 1:1 or 1:2 Tresp:iTreg ratio (SA 1:1 or SA 1:2). As a parameter of suppression, the responder T cell proliferation after 5 days of coculture was compared to the ctrl (= 100%).

3.3 Cell Isolation

3.3.1 PBMC Isolation

PBMC can be isolated from peripheral human blood by density gradient centrifugation. The blood sample is layered over a cell separating solution, which consists of a highly branched, hydrophilic polysaccharide. Due to the solution's density, the components of the blood are separated according to their sedimentation velocity. The different phases of the separated blood sample are shown in Figure 5. The immune cells are located in the thin separation layer called buffy coat.

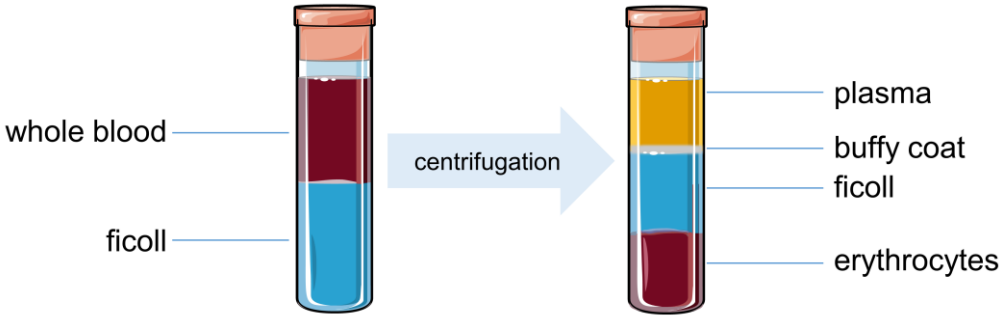

Figure 5: Isolation of PBMC by density gradient centrifugation. The whole blood of allergic donors is layered over ficoll, an aqueous polysaccharide solution with a strictly defined density. By centrifugation the components of the blood are separated according to their size and weight. Small and heavy cells like erythrocytes precipitate at the bottom under the ficoll layer, which still contains some immune cells like granulocytes. The plasma with the soluble factors remains at the top and is separated from the ficoll by a thin diffuse layer called buffy coat, where the PBMC accumulate.

120 mL (DC generation and $CD4^+$ T cell isolation) or 60 mL ($CD4^+CD25^{low}$ T cell isolation) of peripheral blood were drawn from allergic patients using 20 mL-syringes filled with 200 µL heparin. In a 50 mL tube, 30 mL of blood were layered over 15 mL of cell separating solution. Tubes were centrifuged (25 min, 1500 rpm, 25 °C, breaks off). If needed, about 7 mL/ tube plasma were taken off and were inactivated for further use: the plasma was centrifuged (5 min, 4000 rpm, 4 °C) and the supernatant was incubated in a water bath (30 min, 56 °C). After two further centrifugation steps (5 min, 4000 rpm, 4 °C), the inactivated plasma was stored at 4 °C. The buffy coat of the separated blood sample was carefully taken off with a glass pipette and washed in phosphate buffered saline (PBS) (centrifugation: 10 min, 1500 rpm, 4 °C) until the supernatant was clear. The cells were resuspended in 20 mL of PBS and counted. PBMC were either used for the generation of monocyte-derived DC (see 3.4) or for T cell isolation.

3.3.2 **T cell** Isolation

Magnetic-activated cell sorting (MACS) is often used for the selection of cell populations that have a collective and fairly specific surface antigen, such as CD4 for T_H cells. This method uses nano-sized superparamagnetic beads loaded with specific antibodies against the surface marker. After incubation with a mixed cell solution, cells bound to the beads can be isolated on a column in a strong magnetic field. The unlabelled cells in the effluent can be collected directly (negative selection) and the labelled cells can be eluted from the column after removing the magnetic field (positive selection). Different bead concentrations during incubation also allow for the separation of cell types according to differing amounts of surface antigen expression, such as $CD4^{low}$ monocytes from $CD4^{high}$ T cells or differentiation between $CD25^{high}$ and $CD25^{low}$ T cells.

Here, $CD4^+$ T cells were positively selected while $CD4^+CD25^{low}$ T cell were first negatively selected for CD25 and then positively selected for CD4. Prior to use, cell separation columns were placed in the magnet of a MACS Separator and were equilibrated with 3 mL of FACS buffer.

$CD25^{high}$ cell depletion: For every 1 $x10^7$ PBMC 10 µL FACS buffer and 1 µL CD25 microbeads were added to the cell pellet. The cell suspension was incubated on a tumbler (20 min, on ice). Then 20 mL of FACS buffer were added and the cells were centrifuged (10 min, 1200 rpm, 4 °C). The cell pellet was resuspended in 0.5 mL of FACS buffer and loaded onto an equilibrated cell depletion column. To remove unbound cells, the column was washed three times with 1 mL of FACS buffer while the effluent containing $CD25^{low}$ PBMC was collected in a 50 mL tube. The effluent was diluted in 20 mL FACS buffer. The cells were counted and then washed in FACS buffer once.

$CD4^+$ cell isolation: PBMC or $CD25^{low}$ PBMC were resuspended in FACS buffer (16 µL for every 1 $x10^7$ PBMC or 4 µL for every 1 $x10^7$ $CD25^{low}$ PBMC) and CD4 microbeads were added (12 µL for every 1 $x10^7$ PBMC or 3 µL for every 1 $x10^7$ $CD25^{low}$ PBMC). For the following procedure PBMC and $CD25^{low}$ PBMC were treated the same. The cell suspension was incubated on a tumbler (20 min, on ice). After adding 20 mL of FACS buffer, cells were centrifuged (10 min, 1200 rpm, 4 °C) and the cell pellet was resuspended in 1 mL of FACS buffer. The cell suspension was loaded onto an equilibrated cell selection column and the column was washed three times with 5 mL

of FACS buffer to remove unbound cells. The effluent was collected in a 50 mL tube. After washing, the column was removed from the magnet and placed on a 15 mL tube. T cells were flushed from the column by twice firmly pushing 5 mL of FACS buffer through the column with a plunger. The cell suspension was centrifuged (10 min, 1500 rpm, 4 °C), the cell pellet was resuspended in 2 mL of culture medium and the cells were counted. Samples of PBMC and isolated T cells were saved for the flow cytometric analysis of the T cell purity (Methods 3.6.1).

The effluent of the positive selection contains the remaining PBMC. After $CD4^+$ T cell isolation, they were used to generate monocyte-derived DC for the restimulation (Methods 3.4). If needed, $CD4^+$ T cells were cryopreserved for 8 days at -80 °C in 450 µL of IMDM, 450 µL of autologous plasma and 100 µL of DMSO.

3.4 Generation of Monocyte-derived DC

3.4.1 Isolation and Culture of Monocytes for iDC Generation

Monocytes can be isolated from PBMC by positive selection for CD14 or through their adherence to plastic. If incubated in a plate, monocytes adhere on the bottom, whereas T and B cells act as suspension cells in the culture media. The specificity of this isolation method can be adjusted by differing protein levels through choice of incubation medium or addition of human plasma. High protein levels are selective for highly adherent cells, therefore favouring monocytes over for example B cells, but might lead to loss of less adherent monocytes. Thus isolated monocytes can be differentiated into iDC by culture with IL-4 and GM-CSF for several days. Addition of a maturation cocktail consisting of IL-1β, IL-6, TNF-α and PGE_2 drives DC polarisation towards mature DC (mDC) whereas adding the cocktail and IL-10 generates tolerogenic IL-10 DC.

1 $x10^7$ PBMC in 3 mL of RPMI + 0.75 % plasma were incubated in 6-well culture plates under standard cell culture conditions. Then the adherent cells were rinsed carefully with a glass pipette and the incubation medium. After removing the medium, the rinsing was repeated twice with PBS (37 °C). The remaining cells were cultured in 2 mL of IMDM + 2.5 % plasma overnight. On day 1, the medium was replaced by 3 mL of IMDM + 2.5 % plasma supplemented with IL-4 (150 IU/mL) and GM-CSF (400 IU/mL). Thus, the monocytes were differentiated into immature DC (iDC) for six days. Meanwhile on day 3, 1 mL of the culture medium was exchanged with 1 mL of IMDM + 2.5 % plasma supplemented with IL-4 (150 IU/mL) and GM-CSF (800 IU/mL).

3.4.2 Differentiation of iDC into mDC and IL-10 DC

On day 6, iDC were harvested by cooling the culture plates (15 min, on ice) and then rinsing the cells with a glass pipette. The cells were centrifuged (10 min, 1500 rpm, 4 °C) and counted. 1 x10^6 iDC were taken up in 3 mL of IMDM + 2.5 % plasma supplemented with IL-4 (150 IU/mL) and GM-CSF (400 IU/mL) and were plated in 6-well plates (1-2 mL/well) or 12-well plates (2-3 mL/well). For allergen-loaded DC, 20 µg/mL of either Bet v 1 or Cor a 1 were added, whereas unloaded control DC received the respective volume of allergen buffer. After one hour, DC differentiation towards mDC was initiated by adding the maturation cocktail (IL-1β: 10 ng/ml, IL-6: 100 IU/ml, PGE_2: 1 µg/ml and TNF-α: 10 ng/ml) or tolerogenic IL-10 DC were generated by adding the cocktail as well as IL-10 (20 ng/mL).

After culture under these condition for two days, unloaded and allergen-loaded mDC or IL-10 DC were harvested and counted as described for iDC on day 6. Before further use, a cell sample of the cells was saved for the flow cytometric analysis of the DC phenotype.

3.5 Priming and Resting of T Cell Populations

T cells are primed by DC which present the T cells' specific antigen on a MHCII molecule. The fate of the T cells is determined by the microenvironment and above all the DC phenotype: mDC can drive T cell differentiation towards an effector or memory phenotype whereas tolDC such as IL-10 DC promote the generation of iTreg.

Freshly isolated $CD4^+$ T cells were primed with the four different DC populations mentioned above: unloaded and allergen-loaded mDC or IL-10 DC. The cells were diluted in IMDM + 2.5 % of plasma at different concentrations (T cells 2.5 x10^6 /mL; DC 2.5 x10^5 /mL). Then the same volume of the T cell and respective DC suspension was plated in 6-well plates (final volume: 2-3 mL/well) or 12-well plates (final volume: 1-2 mL/well), resulting in a 1:10 DC/T cell ratio. In addition, a 96-well culture plate was prepared to analyse the proliferative response of the $CD4^+$ T after 4 days by [^{3}H]-thymidine incorporation (Methods 3.7).

The T cells were primed for 5 days, after which they were harvested by gently rinsing the plates with the medium. After centrifugation (10 min, 1500 rpm, 4 °C), the supernatant was taken off and stored at -20 °C for cytokine analysis (Methods 3.9). The cells were resuspended in IMDM + 2.5 % plasma at a concentration of 1 x10^6 /3 mL and were plated in a culture dish to rest for 3 days.

3.6 Flow Cytometric Analysis

Flow cytometry is a method used to analyse intracellular or surface expression of cellular proteins. Single molecules such as receptors on the surface or cytokines inside the cell can be tagged with a specific antibody coupled to a fluorophore. Within the flow cytometer, the cells run past several lasers. They split the beam of the first laser into forward scattered (FSC) and sideward scattered (SSC) light. These parameters are detected and indicate the size (FSC) and granularity (SSC) of the cell. The other lasers excite the different fluorophores and multiple detectors then catch the emitted light. The emission spectra are specific for each fluorophore and the intensity is directly proportional to the amount of expressed surface or intracellular marker.

Flow cytometry was used to analyse the DC phenotype after differentiation and T cell purity after isolation. Furthermore iTreg and responder T cell (Tresp) phenotypes were characterised in suppressor assays. Due to limited cell numbers, the minimum amount of necessary cells was determined: 2.5 x10^4 DC, 5 x10^4 $CD4^+$ or $CD4^+CD25^{low}$ T cells.

3.6.1 Extracellular Antibody Staining

The cells were transferred to a 96-well round bottom plate and were washed once in 150 µL FACS buffer. The antibody mix was prepared according to Table 2 and 20 µL thereof was added to each well. The cells were incubated on ice for 20 min in the dark. Then the staining process was stopped by adding 150 µL of FACS buffer. After washing the cells, they were resuspended in 100 µL FACS buffer and transferred to flow cytometry tubes.

Table 2: Antibody mixtures for flow cytometry staining

DC	T cells CD4+ or $CD4+CD25^{low}$	iTreg and Tresp (suppressor assay)	
		panel 1	panel 2
CD14 (1:10)	CD4 (1:30)	CD25** (1:60)	CD25** (1:60)
CD80 (1:30)	CD25* (1:10)	CD45RA (1:30)	CD45RA (1:30)
CD83 (1:2)		CD45RO (1:100)	CD45RO (1:100)
HLA-DR (1:10)		CTLA-4 (1:4)	LAG3 (1:5)
		PD-1 (1:10)	CD49b (1:2)
		TNFαRII (1:5)	ICOS (1:60)
		HLA-DR (1:100)	[IL-10*** (1:10)]

dilution indicated in round brackets.

* CD25-PE, **CD25-BV 510, *** for intracellular staining, not included in antibody mix

3.6.2 Intracellular Antibody Staining

The *Fixation/Permeabilization Solution Kit with BD GolgiStop™* (BD, Franklin Lakes, United States) was used for intracellular staining of IL-10. *BD GolgiStop™* was diluted 1:100 in IMDM + 2.5 % plasma and 4 µL were added to 60 µL of cell culture for 4 hours. Then the surface markers of panel 2 were stained (see 4.6.1), but the samples were not yet transferred to flow cytometry tubes. Stained cells were resuspended in 100 µL *Fixation/Permeabilisation solution* and were incubated on ice for 20 min in the dark. Then, cells were centrifuged (3 min, 2000 rpm, 4 °C) and washed in 100 µL of BD *Perm/Wash™ Buffer*. 20 µL of diluted IL-10 antibody was added and cells were incubated for 30 min on ice in the dark. After one washing step with 100 µL of *BD Perm/Wash™ Buffer*, cells were taken up in 100 µL of FACS buffer and were transferred to flow cytometry tube for flow cytometric analysis.

3.6.3 Staining with Proliferation Dyes

All proliferation dye stainings were performed in 15 mL tubes. Prior to staining, cells were washed in PBS once and incubations were carried out under standard cell culture conditions, if not stated differently. Warm reagents were heated in a 37 °C water bath.

For carboxyfluorescein diacetate (CFSE) staining, a maximum of 2 x10^7 cells were resuspended in 1 mL of warm PBS and 10 µL of CFSE staining solution (1:10 CFSE in warm PBS) were added. Cells were incubated for 20 min and then washed in warm IMDM + 2.5 % plasma.

A cell trace violet staining solution was prepared by adding 20 µL of DMSO to one vial of cell trace violet dye and diluting 1 µL thereof in 10 mL of warm PBS. Each 1 x10^6 cells were resuspended in 1 mL of cell trace violet staining solution and were incubated for 20 min. The staining was stopped by filling up the tube with warm IMDM + 2.5 % plasma. After resting the cells for 5 min at room temperature in the dark, cells were centrifuged and the medium was taken off.

2 µL of cell Proliferation Dye eFluor™ 670 (eFluor670) were diluted in 10 mL of warm PBS and 1 mL thereof were used to resuspend each 1 x10^7 cells. The cell suspension was incubated for 10-15 min, then the staining was stopped by adding cold IMDM + 2.5 % plasma (4 °C) and resting for 5 min on ice in the dark. Cells were centrifuged and the supernatant was taken off.

3.7 [^{3}H]-thymidine Assay

[^{3}H]-thymidine is a radioactively labelled nucleoside with weak β-radiation. If added to a cell culture for several hours, it will be taken up and incorporated into the DNA of replicating cells. After harvesting, the cells are treated with a scintillator, which converts the ionizing radiation into visible or ultraviolet light. This process called scintillation is proportional to the number of proliferated cells. However, the method is a relative one, meaning absolute numbers of dividing cells cannot be calculated, but different samples can be compared.

The [^{3}H]-thymidine assay was used to assess the proliferation of $CD4^+$ T cells during priming or of Teff and iTreg during restimulation.

T cells and DC were suspended in IMDM + 2.5 % of plasma (T cells 5 x10^5 /mL; DC 2.5 x10^4 /mL). 100 µL of T cells and 100 µL of DC were cocultured in a 96-well culture plate for four days (DC/T cell ratio = 1:20). All experiments were carried out in triplicates. In the afternoon of the third day, one drop of [^{3}H]-thymidine dispersed in RPMI medium was added to each well using a syringe and needle. After 16-18 hours, a semi-automatic cell harvester was used to place the cells on a glass fibre filter map, which was dried for 20 min at 250 °C in a heating cabinet. Then the filter map was inserted into a plastic sample bag, where it was soaked evenly in 10 mL of scintillation solution. The radiation of the samples was then analysed in a β-scintillation counter.

3.8 Suppressor Assay

The capability of Treg to suppress the proliferation of responder T cells (Tresp) can be investigated by suppressor assays. In this method, the proliferation of stimulated Tresp cocultured with Treg is compared to the proliferation of stimulated Tresp alone (positive control).

Fluorescent proliferation dyes are used in flow cytometry based proliferation assays. Cells take up the dye into the cytosol and pass it on to the daughter cells during proliferation. Naturally, the dye is diluted during every division cycle. After flow cytometric analysis, bright undivided cells can be distinguished from dimmer cells that have proliferated. The actual percentage of proliferating cells can be determined and compared between different samples.

In this study, suppressor assays were conducted with syngeneic mDC, iTreg and $CD4^+CD25^{low}$ Tresp from birch pollen and hazelnut allergic patients. mDC were stained with CFSE, iTreg with cell trace violet and Tresp with eFluor670 (Methods 3.6.3). Cells

were cultured in 200 µL of IMDM + 2.5 % plasma in 96-well culture plates. 1 x10^5 freshly isolated Tresp were stimulated with 1 x10^4 mDC loaded with Bet or Cor. Either 1 x10^5 or 2 x10^5 of iTreg were added, so the positive control could be compared to samples with a Tresp/ iTreg ratio of 1:1 or 1:2. On day 5, cells were harvested and culture supernatants were stored at -20 °C until cytokine analysis. Cell samples were divided into three groups: one sample was analysed on a flow cytometer directly, whereas the other two samples were further stained with two different antibody combinations prior to analysis (Methods 3.6.1 and 3.6.2).

3.9 Cytokine Analysis

After primary culture and after suppressor assay, the cell cultures were harvested, centrifuged and the supernatants were stored at -20 °C. For analysis of cytokine concentrations the ProcartaPlex Human Th1/Th2/Th9/Th17/Th22/Treg Cytokine-Panel (18-plex) (Invitrogen™) was applied according to the manufacturer's protocol.

3.10 Humanised Mouse Model of Allergen-induced Intestinal and Airway Inflammation

The human and murine immune system are closely related, but not identical. Therefore, not all findings from murine experiments can be directly transferred to human immunology. Humanised mice are a remarkable investigative tool, which close the gap between exclusively murine and human studies with regard to transferability and restriction of possibilities. The model consists of immunodeficient mice which are engrafted with human PBMC. The immune system of *NOD-scid-γc*$^{-/-}$ mice is compromised in multiple aspects: reduced natural killer cell numbers and activity, no development of T- and B cells, reduced macrophage function, and complement-dependent haemolytic activity.[145,146] They therefore tolerate the xenogeneic engraftment with human PBMC and are used in a well-established model of allergen-induced intestinal and airway inflammation.[147,148]

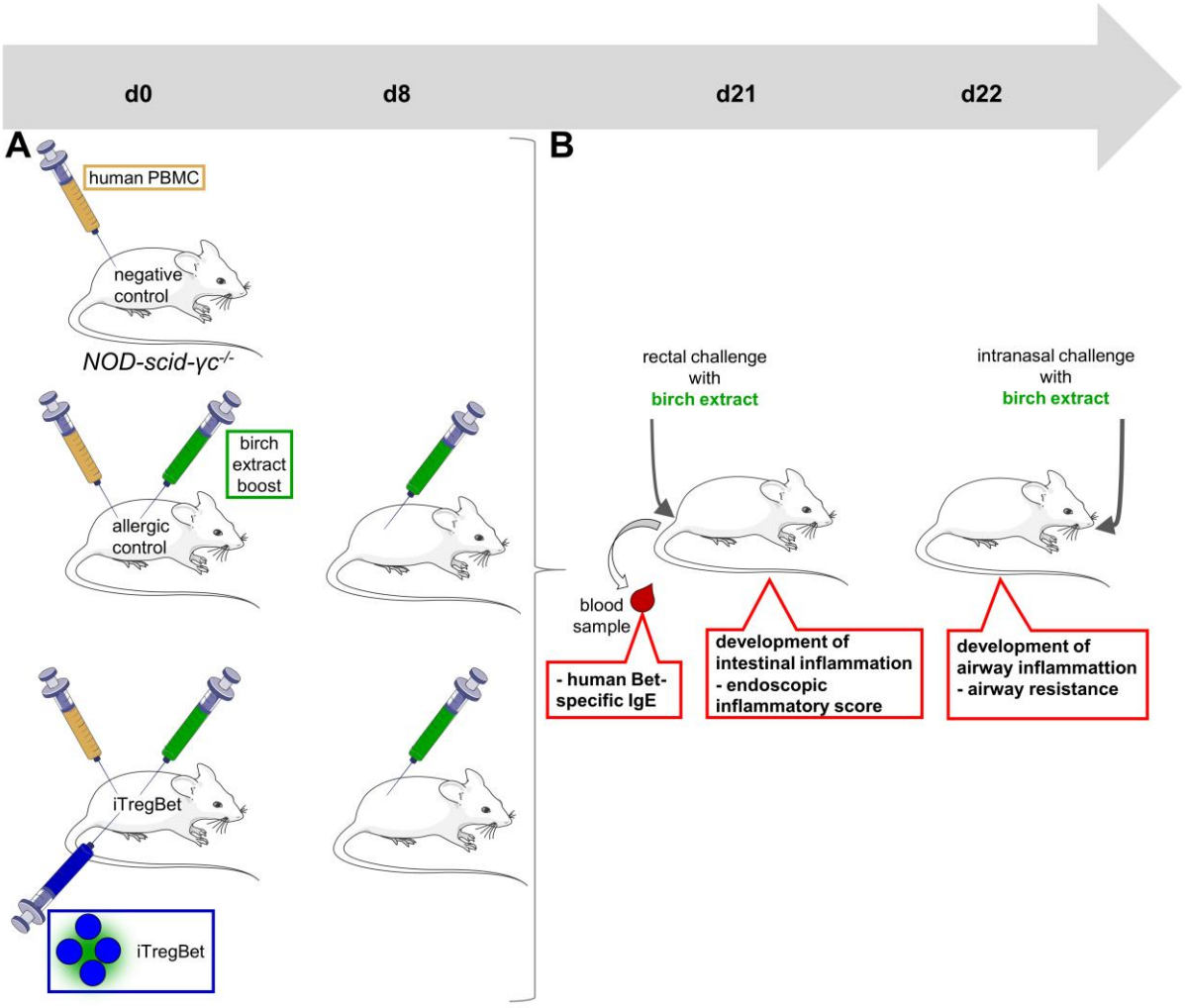

Figure 6: Humanised mouse model of allergen-induced intestinal and airway inflammation. A Immunodeficient *NOD-scid-γc*$^{-/-}$ mice were engrafted with human peripheral blood mononuclear cells (PBMC) from donors allergic to birch pollen and hazelnut and were boosted twice (d0 an d8) with birch extract to establish an allergic immune reaction in the animals (allergic control). iTreg were coinjected at the day of engraftment to investigate their impact on allergic symptoms. Negative controls only received human PBMC. **B** After 3 weeks, blood samples for Bet-specific IgE analysis were taken and the mice were challenged rectally with birch extract. Allergic animals develop an intestinal inflammation, which was scored by mini-endoscopy (colitis). The next day, mice were challenged intranasally with birch extract and the airway inflammation developed by allergic animals was evaluated by assessment of airway resistance.

3.10.1 Overview of the Humanised Mouse Model

iTregBet were generated according to Methods 3.3.1, 3.4 and 3.5, but with the use of cryopreserved CD4$^+$ T cells (Methods 3.3.2). At day 0, *NOD-scid-cγ*$^{-/-}$ mice were reconstituted with human PBMC (Figure 6 and Methods 3.10.2). As negative control, mice were reconstituted with PBMC only, whereas the allergic control received PBMC and a boost with birch pollen extract. iTregBet syngeneic to the PBMC were additionally injected into PBMC reconstituted and allergen boosted mice to analyse the effect on the allergic immune response *in vivo*. The allergen boosts were repeated after

7 days. After three weeks, blood samples for analysis of human IgE were taken. Then the animals were challenged rectally with birch pollen or hazelnut extract to score the allergen-induced intestinal inflammation (colitis) by mini-endoscopy (Methods 3.10.3). In some experiments, the mice were also challenged intranasally with birch pollen extract and changes in the airway resistance provoked by methacholine (mCH) were measured to evaluate the allergen-induced airway inflammation (asthma; Methods 3.10.4).

3.10.2 Reconstitution of *NOD-scid-γc*$^{-/-}$ Mice

PBMC were isolated from patients with confirmed allergies against birch pollen and hazelnut (ImmunoCAP class ≥3; average CAP class for birch pollen extract = 4.7 ± 0.9 and for hazelnut extract = 4.0 ± 0.6). Male and female NOD-scid-*γc*$^{-/-}$ mice aged 4-8 weeks were injected intraperitoneally with 1 x10^7 PBMC in the absence or presence of 20 µg birch pollen extract (negative and allergic control) and 1 x10^6 iTregBet were coinjected with the extract (Figure 6). For the injections, all cells and allergens were resuspended and combined in 200 µL of 0.9 % NaCl. On day 7, all mice except for the negative control group were boosted with another injection of 20 µg birch pollen extract in 50 µL 0.9 % NaCl.

3.10.3 Analysis of Human total and Bet-specific IgE in Humanised Mice

3 weeks after reconstitution, blood samples of the mice were collected for the analysis of human total and Bet-specific IgE. The sera of two mice from the same experimental group were pooled to reach the volume necessary for both analysations by ImmunoCAP specific/total IgE blood test (Phadia GmbH).

3.10.4 Rectal Allergen Challenge and Mini-endoscopic Analysis of Intestinal Inflammation

After 3 weeks, the mice were anaesthetised with ketamine/xylazine and challenged rectally with 20 µg of birch pollen or hazelnut extract through a small plastic tube inserted into their anus. 2 hours later the intestinal inflammation was scored by mini-endoscopy under isoflurane anaesthesia. Therefore a mini-endoscope was introduced into the anus and the colon was carefully inflated with an air pump to obtain high quality pictures of up to 4 cm into the colon. The intestinal inflammation was evaluated according to the murine endoscopic index of colitis severity: 5 parameters (translucent structure, granularity, fibrin, vascularity, and stool consistency) are graded from 0 to 3 adding up to an overall score between 0 (no inflammation) and 15 (severe inflammation).

3.10.5 Intranasal Allergen Challenge and Airway Hyperreactivity

1 day after the rectal challenge, the mice were challenged intranasally with 20 µg of birch pollen or hazelnut extract. 24 hours later the mice were anesthetised with pentoarbital, then they were intubated and placed in an invasive whole-body plethysmograph. Increasing concentrations (3.125 – 50 mg/mL) of methacholine (mCH) were administered, the airway resistance was measured and was related to a baseline value taken before mCH challenge.

4 RESULTS

In this study, allergen-specific and cross-reactive tolerance induction by IL-10 DC from birch pollen and hazelnut allergic individuals was explored. Using T cell lines and T cell clones, T cell cross-reactivity had already been identified as an underlying mechanism of pollen-associated food allergies.[149–151] Our group previously used sera and primary T cells from allergic donors showing that Bet-specific IgE and Bet-specific Teff cross-react with Cor.[66] It is also known that tolerogenic human IL-10 DC induce iTreg with high antigen-specific suppressive capacities.[57,60] Here, it was investigated whether IL-10 DC-induced allergen-specific iTreg are able to exert allergen-specific (birch) and cross-reactive tolerance (hazelnut) and are thereby able to facilitate cross-tolerance between cross-reactive allergens.

4.1 Induction of Bet-specific and Non-specific iTreg by Human IL-10 DC

4.1.1 DC Phenotype

After DC differentiation, the DC phenotype was analysed by flow cytometric analysis of HLA-DR, CD14, CD80 and CD83 to control the maturation status and to exclude an additional maturation effect of the allergen (Figure 7). HLA-DR, a human MHC II receptor, is expressed on both mDC and IL-10 DC, but is usually found in higher amounts on mDC compared to IL-10 DC.[61] CD14 is a differentiation marker of monocyte subpopulations and its expression level is also characteristic for different monocyte-derived DC types (CD14^{-} mDC, CD14^{low} tolDC).[49] CD80 is a co-stimulatory molecule and CD83 a DC maturation marker, which are both highly expressed on mDC and intermediate on IL-10 DC.[47]

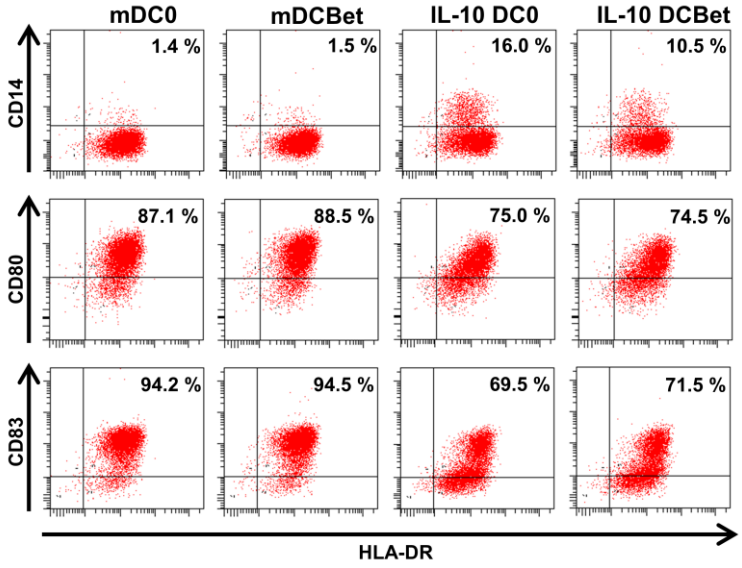

Figure 7: mDC and IL-10 DC phenotype. mDC and IL-10 DC were generated, and loaded with Bet v 1 (Bet) or left unloaded (0) as described in Methods 3.4. The cells were stained with anti-CD14, -CD80, -CD86 and -HLA-DR antibodies and were analysed by flow cytometry. HLA-DR and CD14/80/86 double positive cells of one representative experiment out of 38 are shown.

The DC surface markers of one representative experiment are shown in Figure 7. mDC displayed negligible expression of CD14, but high expression of CD80 and CD83. IL-10 DC showed slight expression of CD14 and intermediate expression of CD80. The overall expression of CD83 by IL-10 DC was also intermediate, but divided the cells into $CD83^{high}$ and $CD83^{low}$ subpopulations. All DC were close to 100 % HLA-DR positive, but the mean fluorescence intensity (MFI) of IL-10 DC was lower compared to mDC, indicating that the amount of HLA-DR molecules on the surface of IL-10 DC was lower compared to mDC. There were no notable differences between allergen-loaded (mDCBet, IL-10 DCBet) and unloaded (mDC0, IL-10 DC0) DC, neither in mDC nor in IL- 10 DC, confirming that the allergens were free of maturation stimuli such as endotoxins.

4.1.2 T Cell Proliferation during Primary Culture

$CD4^+$ T cells were primed with syngeneic unloaded or Bet-loaded mDC (mDC0/ mDCBet) and unloaded or Bet-loaded IL-10 DC (IL-10 DC0/ IL-10 DCBet), resulting in four different T cell populations (Teff0, TeffBet, iTreg0, iTregBet). After four days of primary culture, the T cell proliferation was analysed by [^{3}H]-thymidine incorporation. Due to great individual differences in the number of the counts in each experiment, the results were normalised to the non-specific background proliferation of T cells stimulated with mDC0 and are represented as stimulation index (SI) of 38 pooled experiments in Figure 8. Thereby the SI for mDC0-stimulated T cells is defined as SI=1.

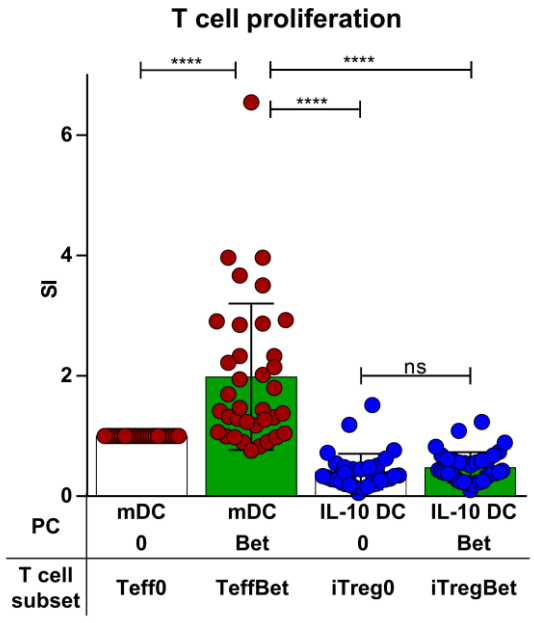

Figure 8: T cell proliferation after priming. mDC0/Bet and IL-10 DC0/Bet from allergic donors were generated as described in Methods 3.4 and were cocultured with syngeneic $CD4^+$ T cells. After 3 days T cells were pulsed with [^{3}H]-thymidine for 16-18 h and were analysed on a β-scintillation counter. The proliferation was normalised to the background proliferation of T cells stimulated with mDC0 (SI=1) and is pooled from 38 independent experiments. SI = stimulation index, ns = not significant, p values: **** $p < 0.0001$, ns = not significant ($p > 0.05$).

Compared to the control of mDC0 and to IL-10 DC0 or IL-10 DCBet stimulated T cells, T cells stimulated with mDCBet showed significantly increased proliferation demonstrating a Bet v 1-specific T cell response due to the donor's allergic sensitisation. In contrast, T cells stimulated with both unloaded or Bet-loaded IL-10 DC exhibited low proliferative potential as was previously shown for iTreg induced by IL-10 DC.[49]

4.1.3 Cytokine Secretion after Primary Culture

After 5 days of primary culture the cell culture supernatants were harvested and their cytokine content was analysed. The cytokine concentrations were normalised to T cells stimulated with mDC0 (control =1) and are represented in Figure 9.

Supporting the data from T cell proliferation, supernatants of cocultures with mDCBet contained elevated levels of IL-5, IL-9 and IL-13 (T_H2 cytokines) as well as IL-2 (T cell activation) compared to the mDC0 control, demonstrating the activation of a T_H2 response (Figure 9). In contrast, in primary T cell cultures stimulated with both IL-10 DC0 and IL-10 DCBet reduced amounts of T_H2 cytokines and IL-2 were released, confirming the anergic phenotype of IL-10 DC induced iTreg with reduced T_H2 activation. IL-4, another important T_H2 cytokine, was only detected in negligible amounts in all samples (data not shown). Interestingly, elevated levels of the immunosuppressive cytokine IL-10 were found in cocultures with mDCBet and IL-10 DCBet, although when comparing the two, IL-10 DCBet cocultures contained significantly higher amounts of IL-10, suggesting that the cytokine acts as suppressive mechanism of IL-10 DC induced iTreg. However, T cells stimulated with IL-10 DCBet exhibited significantly higher amounts of the T_H2 cytokines IL-5 and IL-13 as well as IL-10, compared to T cells primed in the presence of IL-10 DC0, indicating an activated iTreg phenotype only after allergen-specific stimulation.

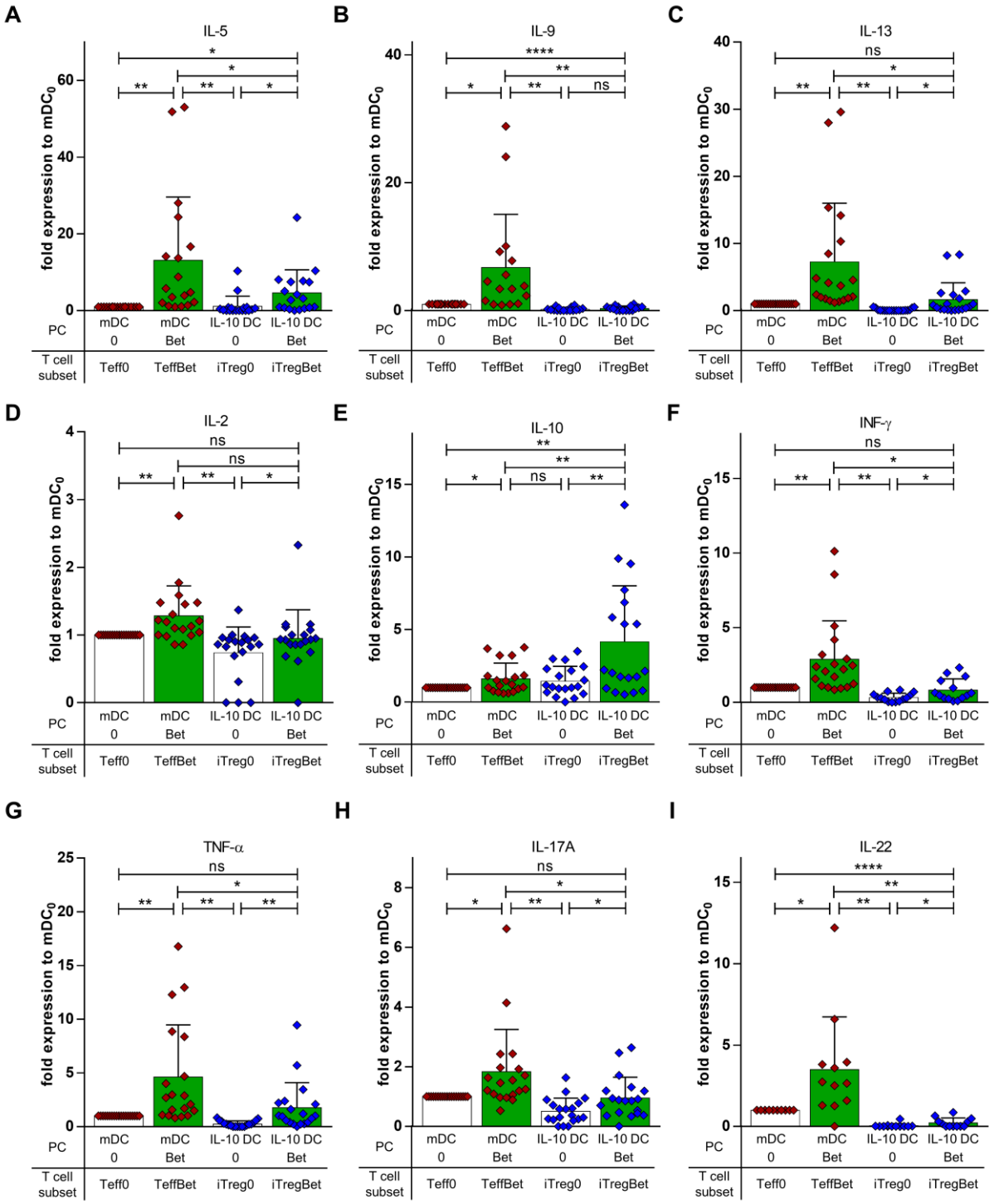

Figure 9: Cytokine concentrations in cell culture supernatants after T cell priming. mDC0/mDCBet and IL-10 DC0/IL-10 DCBet from allergic donors were generated as described in Methods 3.4 and were cocultured with syngeneic $CD4^+$ T cells. After 5 days the culture supernatants were harvested and stored at -20 °C until analysis with eBioscience™ ProcartaPlex. The cytokine concentrations for IL-5, IL-9, IL-13, IL-2, IL-10, INF-γ, TNF-α, IL-17A and IL-22 were normalised to the control (mDC0 stimulation (=1)). p values: **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

The immune response of the primary cultures was further analysed regarding cytokines related to other T helper cell types (T_H1: IFN-γ and TNF-α, T_H17: IL-17A, T_H22: IL-22). Compared to the mDC0 control, all of them were found to be significantly

increased in mDCBet cultures but were similar or decreased in IL-10 DC cultures. Supernatants of T cells primed with IL-10 DCBet did contain moderate amounts of INF-γ, TNF-α, IL-22 and IL-17A, which were significantly lower compared to T cells stimulated with mDCBet (Figure 9). This cytokine diversity indicates a more complex immune response than strict T_H2 polarisation, especially in mDCBet primed T cell cultures. Since $CD4^+$ T cells were isolated and cocultured with mDC or IL-10 DC, the T cells contained a mix of different $CD4^+$ T cell subsets with different T cell differentiations, including naïve and memory phenotypes. Thereby memory T_H1, T_H9, T_H17 and T_H22 cells could be activated to produce cell type specific cytokines and it is possible that not all proliferating Teff are Bet-specific T_H2 cells. Furthermore, it is known that the polarisation status of T cell subtypes may allow the secretion of different cytokines.

4.2 Restimulation of Bet-specific and Non-specific iTreg Compared to Teff

Treg are usually described as being anergic and do not proliferate when restimulated with their specific antigen, as was shown for IL-10 DC induced iTreg.[60,152] We also wanted to prove the induction of anergic iTreg by allergen-loaded IL-10 DC. Therefore the T cell populations induced by unloaded mDC0, mDCBet, IL-10 DC0 and IL-10 DCBet were restimulated with syngeneic mDC0, mDCBet and Cor a 1 (Cor)-loaded mDC (mDCCor) and the T cell proliferation after restimulation was analysed. Again, the level of proliferation between individuals differed and the results presented in Figure 10 were normalised to the background proliferation of Teff0 stimulated with mDC0 (control, SI=1). Compared to this control, TeffBet showed a significantly increased proliferation after stimulation with mDCBet, demonstrating an allergen (Bet)-specific Teff response. The proliferative response after restimulation with mDCCor was more moderate in most donors, resulting in an overall not significant increase in T cell proliferation, although the absolute values are comparable to the homologous restimulation (Bet-stimulation: 2.07 ±1.27 vs Cor-stimulation: 2.09 ±1.60). This suggests the cross-reactive activation of allergen-specific T cell responses in T cell/mDC cocultures from donors with birch pollen (bet)-associated hazelnut (Cor) allergies. These results also confirm that in PFA the primary allergy against pollen allergens is usually more profoundly established than the associated food allergy, which is also represented by the difference in CAP classes in our study cohort (Table 1). Restimulation of iTregBet with mDCBet and mDCCor prompted highly increased proliferation compared to the control and also compared to TeffBet with respective

stimulation. These results reveal that iTregBet do not display an anergic phenotype after restimulation with their cognate or an associated allergen. In some individuals, Bet-specific stimulation triggered vigorous proliferation which was up to 20-times higher than the background and almost 10-times higher than the respectively stimulated TeffBet (Figure 10).

Contrary to these findings, mDCBet and mDCCor stimulation of iTreg0 did not induce T cell proliferation above background level. In contrast to iTregBet, iTreg0 remained anergic with significantly decreased proliferation, suggesting that the vigorous proliferation of stimulated iTregBet is antigen-dependent. This data indicated an allergen-specific activation of iTregBet, thereby confirming the results of activated cytokine production including IL-10 as demonstrated in Figure 9.

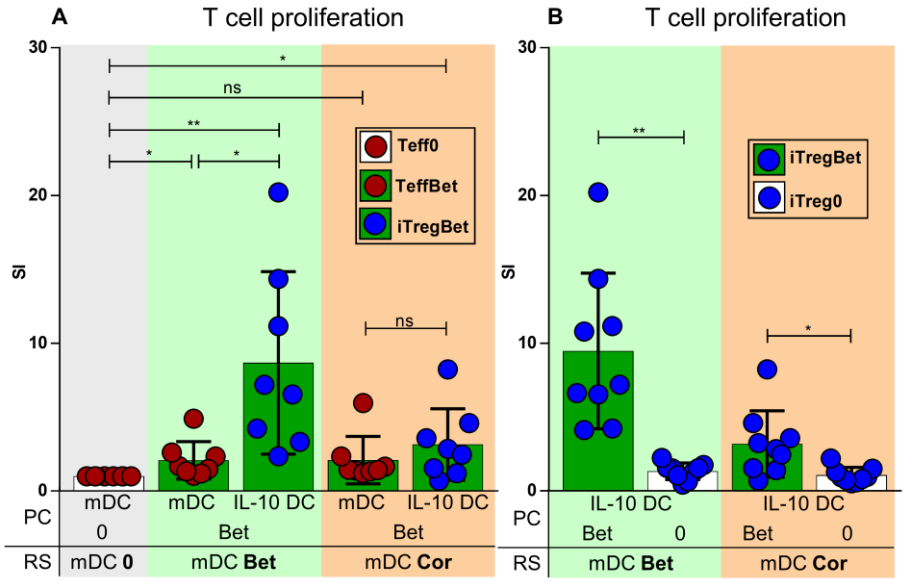

Figure 10: T cell proliferation during restimulation. Teff0, TeffBet, iTreg0 and iTregBet were primed by coculture of $CD4^+$ T cells with syngeneic mDC0, mDCBet, IL-10 DC0 and IL-10 DCBet from allergic donors. After 5 days of priming, the T cells were rested for 3 days. Then they were restimulated with syngeneic mDC0, mDCBet or mDCCor respectively, as indicated. After 3 days of restimulation, the cell cultures were pulsed with [^{3}H]-thymidine for 16-18 h and were analysed on a β-scintillation counter. The results were normalised to the proliferation of Teff0 restimulated with mDC0 (SI=1). **A** Restimulation of Teff0, TeffBet and iTregBet. **B** Restimulation of iTregBet and iTreg0, as indicated. PC = primary culture, RS = restimulation, SI = stimulation index, ns = not significant, p values: ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

4.3 Suppressive Capacity and Phenotype of iTreg *in vitro*

In order to analyse the allergen-specific and cross-reactive suppressive capacity of iTreg induced by allergen-loaded IL-10 DC, we have performed so-called suppressor assays. In suppressor assays, responder T cells are stimulated in the absence (=positive control) or presence of suppressor T cells. The suppressive capacity of the latter can then be evaluated as the percentage of proliferating responder T cells relative to the positive control. In this assay, $CD4^+CD25^{low}$ responder T cells (Tresp) were stimulated with syngeneic mDCBet or mDCCor to induce allergen-specific (Bet) or cross-reactive (Cor) T cell proliferation. Syngeneic iTregBet or iTreg0 were added as suppressor cells (see samples of suppressor assay in Figure 4 B). All cell populations (DC, iTreg, Tresp) were stained with different proliferation dyes (Methods 3.6.3), so they could be distinguished in the coculture and their proliferation could be determined by flow cytometric analysis (gating strategy in Figure 11). Additionally, the phenotype of the T cells was analysed by intra- and extra-cellular antibody staining and the cytokine content in the cell culture supernatant was determined.

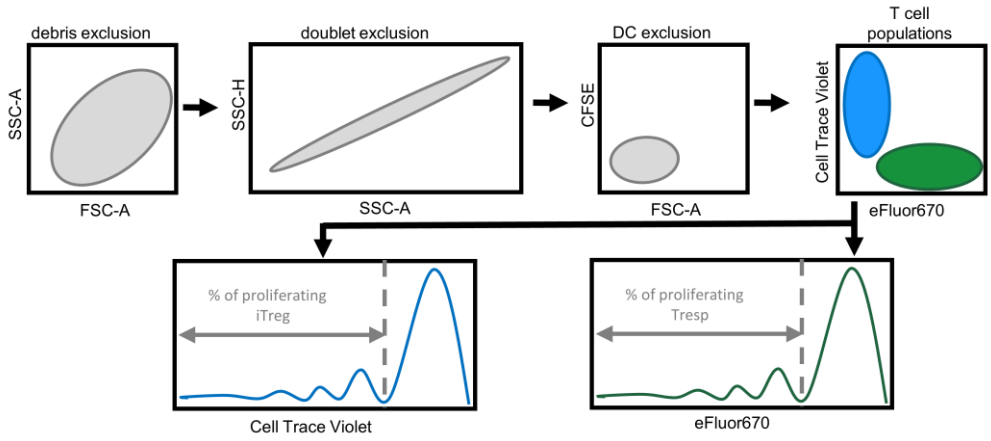

Figure 11: Gating strategy for suppressor assays. Debris, doublets and CFSE-stained DC were excluded. iTreg and Tresp were differentiated by cell trace violet (iTreg) and eFluor670 (Tresp) staining. Proliferating T cells were identified by dye dilution in histograms.

4.3.1 Analysis of Bet-specific and Cross-reactive Suppression

To evaluate the allergen-specific and cross-reactive suppressive capacity of iTregBet and iTreg0, the iTreg were applied to suppress Tresp proliferation induced by mDCBet (allergen-specific) or mDCCor (cross-reactive). The proliferation of Tresp cocultured with iTreg is depicted as flow cytometric blot from one representative experiment and as pooled data relative to the Tresp control (= 100 %) (Figure 12 A, B). Addition of iTregBet to Tresp in 1:1 ratio led to a decrease in the Bet and Cor-stimulated responder T cell proliferation. This proves that Bet-specific iTreg are able to suppress allergen-specific and also cross-reactive responder T cell proliferation. A further significant reduction of Tresp proliferation was achieved by doubling the number of iTregBet (Tresp:iTreg ratio = 1:2), indicating that the suppression is dependent on iTreg numbers, although the difference between the 1:1 and 1:2 ratio seems rather insubstantial. To underline the evidence for dose dependency the results from each individual donor are listed in Figure 12 C. Here it is evident that in most experiments doubling the amount of iTreg substantially increased the suppressive activity (Bet-stimulation: 12 out of 18 (66.6 %) experiments, Cor-stimulation: 10 out of 13 (76.9 %) experiments).

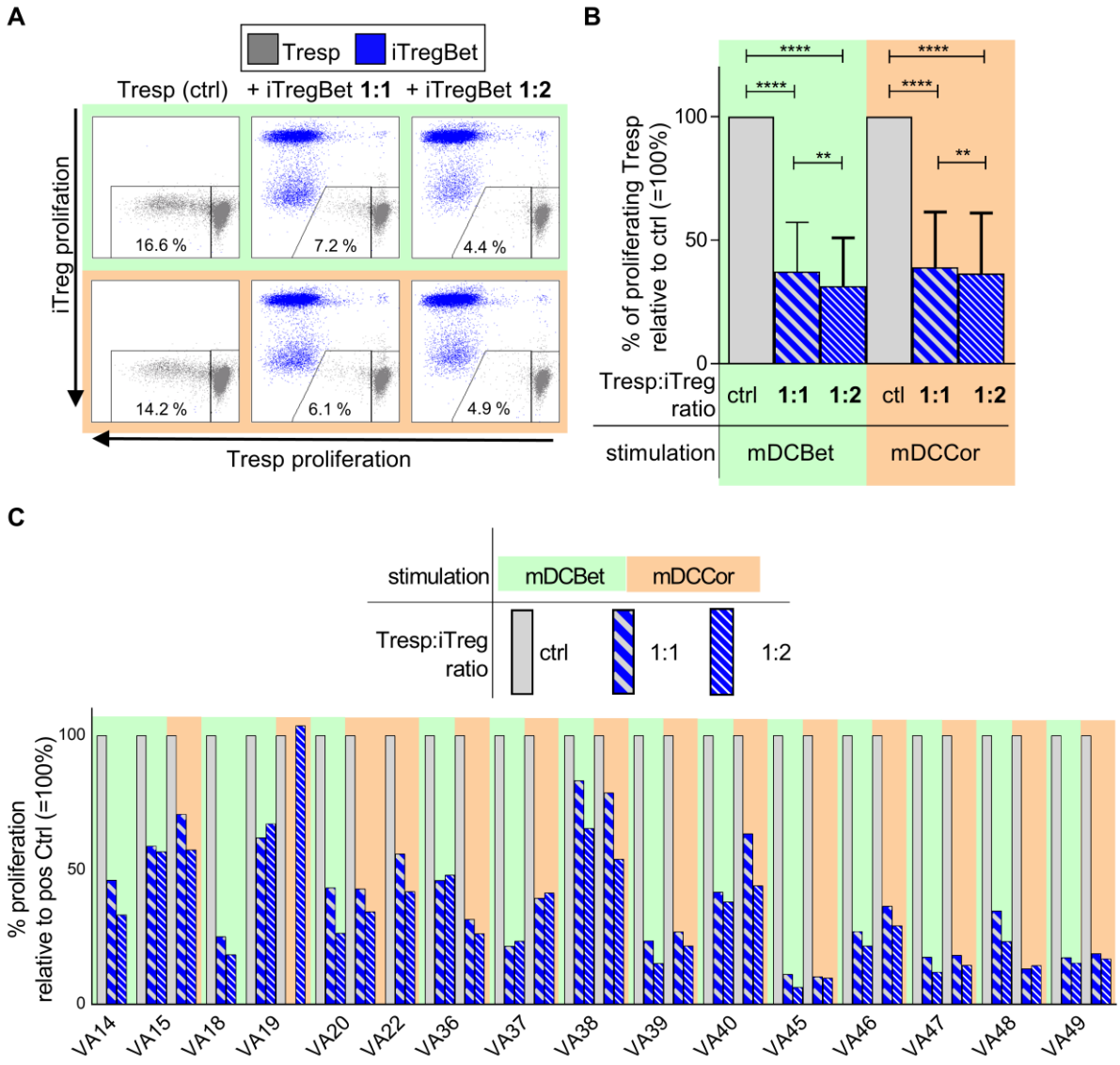

Figure 12: iTregBet suppressed allergen-specific and cross-reactive Tresp proliferation. Tresp were stimulated with syngeneic mDCBet or mDCCor (= ctrl) and iTregBet were added in 1:1 or 1:2 Tresp:iTreg ratio (see assay samples Figure 4 B). To differentiate between T cells and to assess T cell proliferation, the T cells were stained with different proliferation dyes (see gating strategy in Figure 11). The percentage of proliferating Tresp are shown of one representative experiment (**A**), as pooled data normalised to the ctrl (=100 %, **B**) and for each individual experiment (**C**). ctrl = control, p values: **** $p < 0.0001$, ** $p < 0.01$.

To further investigate the specificity of iTreg primed in the presence of Bet v 1, the suppressive capacity of iTregBet and iTreg0 was compared (Figure 13 A, B). Addition of iTreg0 reduced the Bet- and Cor- stimulated responder T cell proliferation moderately. However, the effect was significantly pronounced in experiments with iTregBet, demonstrating a superior capacity of iTregBet compared to iTreg0 with regard to suppression of allergen-specific and cross-reactive T cell responses. In 8 out of 10 experiments (80 %) with Bet-stimulation and in 6 out of 8 (75 %) experiments with Cor-stimulation, iTregBet exhibited stronger suppressive functions compared to

iTreg0, highlighting the advantage of allergen-specific iTreg priming for both – allergen-specific and cross-reactive – tolerance induction (Figure 13 C). iTreg were previously shown to facilitate tolerance induction antigen-independently,[49,106] as is verified here by the suppressive capacity of iTreg0. However, the superior capacity of iTregBet indicates that iTreg can also direct immune tolerance specifically towards an allergen (e.g. birch) and can induce cross-tolerance to cross-reactive allergens (e.g. hazelnut).

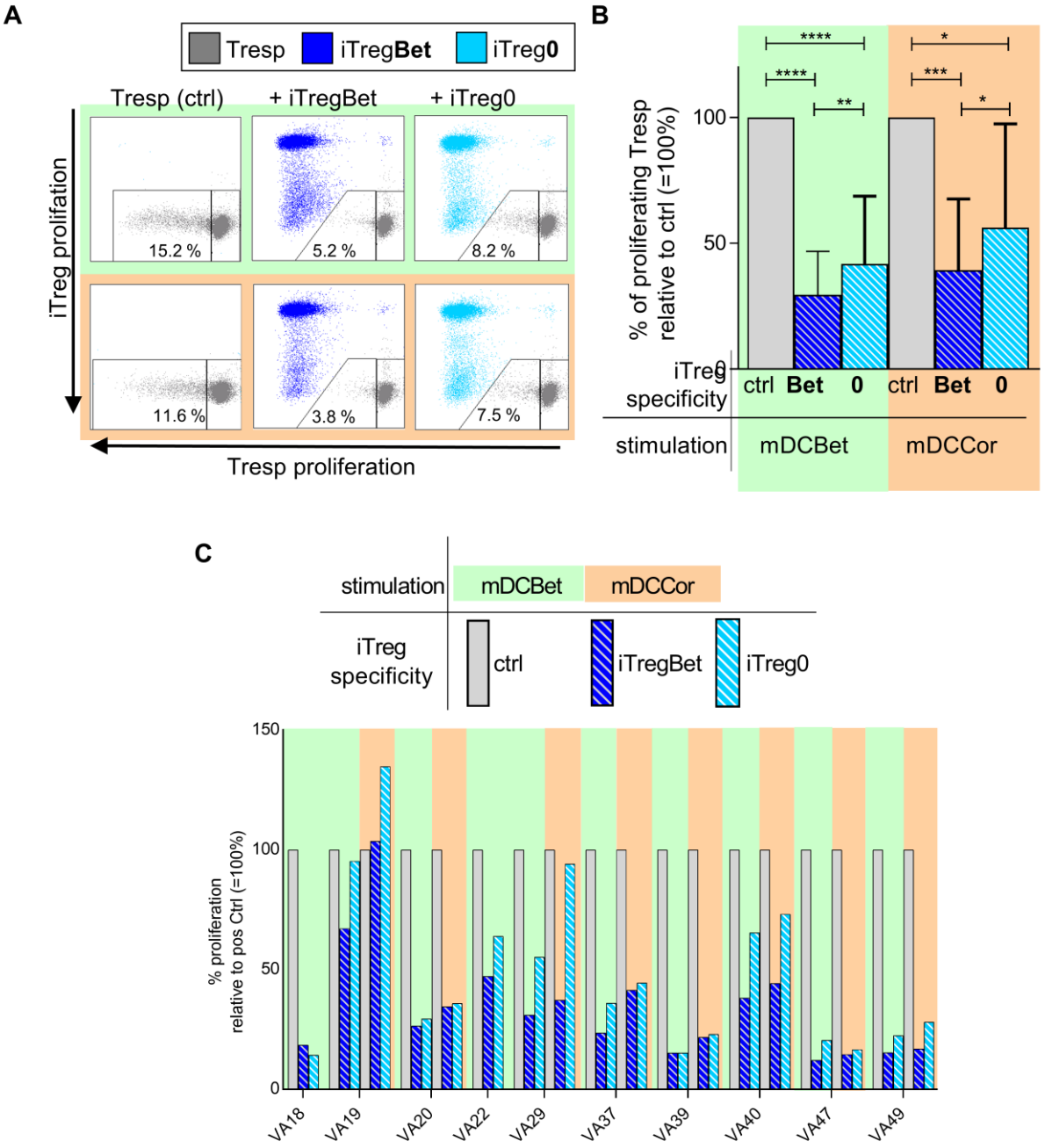

Figure 13: iTregBet exhibited stronger allergen-specific and cross-reactive suppressive capacities than iTreg0. Tresp were stimulated with syngeneic mDCBet or mDCCor (= ctrl) and iTregBet or iTreg0 were added in 1:2 Tresp:iTreg ratio (see Figure 4 B). To differentiate between T cells and to assess T cell proliferation, the T cells were stained with different proliferation dyes (see gating strategy in Figure 11). The percentage of proliferating Tresp are shown of one representative experiment (**A**), as pooled data normalised to the ctrl (=100 %, **B**) and for each individual experiment (**C**). ctrl = control, p values: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.3.2 Cytokine Secretion in Suppressor Assays

In order to further characterise the suppressive mechanisms of iTregBet, different T cell cytokines were analysed in the cell culture supernatants of suppressor assays. Figure 14 shows the cytokine concentrations normalised to the positive controls (Tresp

stimulated with mDCBet/mDCCor). Supernatants of suppressor assays with iTregBet showed a decrease in the T_H2 cytokine IL-13 after mDCBet as well as mDCCor stimulation, indicating a shift away from T_H2 immune responses. Differences in the T_H2 cytokines IL-5 and IL-9 could not be detected. The regulatory cytokine IL-10 was increased significantly in mDCBet-stimulated samples with iTregBet in 1:1 and 1:2 ratio, but under Cor-stimulation the increase was only significant in 1:1 ratio. This identifies IL-10 as a possible mechanism for tolerance induction by iTreg. Notably, there was no increase in IL-10 secretion in samples with iTreg0, supporting proliferation data of the suppressor assays and demonstrating that iTreg0 exhibited inferior suppressive capacity compared to iTregBet. This further indicates that iTreg might have to be activated allergen-specifically in order to secrete IL-10 and exert their full suppressive potential, providing a potential IL-10-dependent mechanism of allergen-specific tolerance induction. In the context of pollen and related food allergens, the allergen-specificity is extended to the cross-reactive allergen, enableing allergen-specific iTreg to suppress cross-reactive immune responses.

To investigate the shift of the immune response towards T_H1 immunity, the T_H1 cytokines IFN-γ and TNF-α were analysed. The only differences detected were a decrease in IFN-γ in samples with iTregBet (1:2 ratio) - but only under Cor-stimulation - and a decrease of TNF-α in samples with iTregBet (1:1 ratio) stimulated with mDCBet. Therefore there is no clear evidence for T_H1 polarisation as a possible mechanism of tolerance induction by iTreg. Since restimulation assays revealed that iTregBet proliferate profoundly when stimulated with mDCBet, IL-2 consumption may play a role in iTreg suppressive acivity. However compared to the control, IL-2 amounts were not reduced in samples with iTregBet, suggesting that IL-2 depletion did not occur as regulatory mechanism.

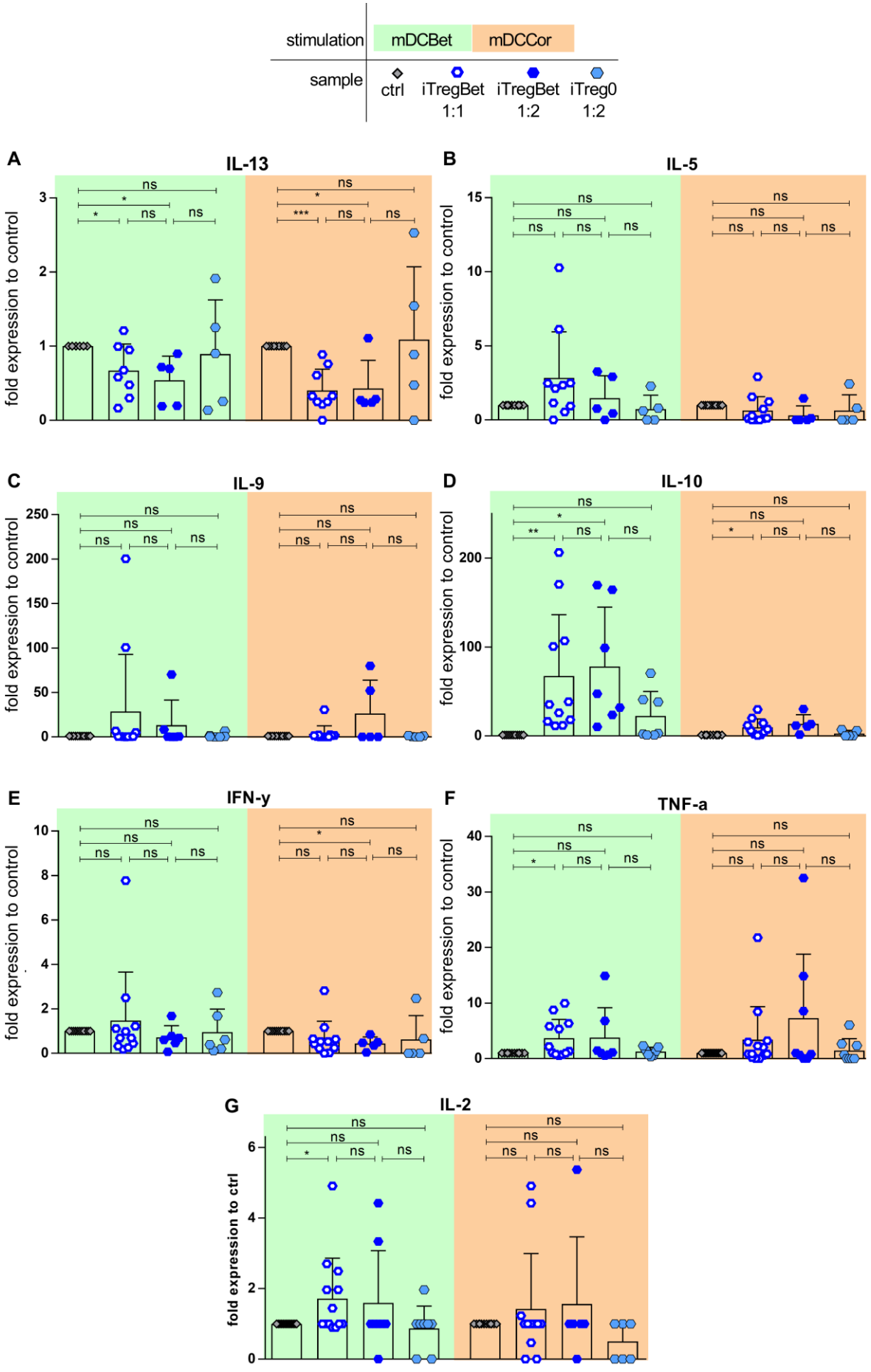
stimulation
mDCBet
mDCCor
sample
ctrl
iTregBet 1:1
iTregBet 1:2
iTreg0 1:2
A
IL-13
B
IL-5
C
IL-9
D
IL-10
E
IFN-y
F
TNF-a
G
IL-2
fold expression to control
fold expression to ctrl
ns

Figure 14 (previous page): Cytokine concentrations in cell culture supernatants after suppressor assay. After the suppressor assay the cell culture supernatants were harvested and stored at -20 °C until analysis with eBioscience™ ProcartaPlex. The concentrations of IL-13, IL-5, IL-9, INF-γ, TNF-α and IL-2 from suppressor assays with iTregBet in 1:1 and 1:2 Tresp:iTreg ratio and with iTreg0 in 1:2 ratio were compared to the control with allergen-stimulated Tresp. Therefore the concentrations were normalised to the control (=1) and were pooled. ns = not significant, p values: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

4.3.3 Tresp and iTreg Phenotypes in Suppressor Assays

As we have found excellent capacities of Bet-specific iTreg to suppress allergen-specific and cross-reactive T cell response, the T cell phenotypes were analysed by flow cytometry to further support those findings. Therefore, the T cells were additionally stained for intracellular and surface markers after the suppressor assay, so that the labelling with different proliferation dyes allows the distinction of iTreg and Tresp as well as the differentiation between proliferating and non-proliferating T cells (gating strategy in Figure 15). To differentiate between naïve and memory T cells CD45RA, CD45RO and CD49b were used. CD45RA/RO are classical markers to differentiate between naïve and memory T cells[153] and CD49b is a marker for functionally mature Teff as well as regulatory and effector memory T cells.[154,155] CD25 and HLA-DR were used to identify activated T cells.[156] CTLA-4, PD-1, tumour necrosis factor receptor 2 (TNFR2), LAG3, IL-10 and ICOS are molecules associated with immunosuppressive and regulatory activities.[157–159] CD49b and LAG3 are also regularly used to identify Tr1 cells, a subpopulation of Treg that was found to be predominantly induced by IL-10 DC.[93,160]

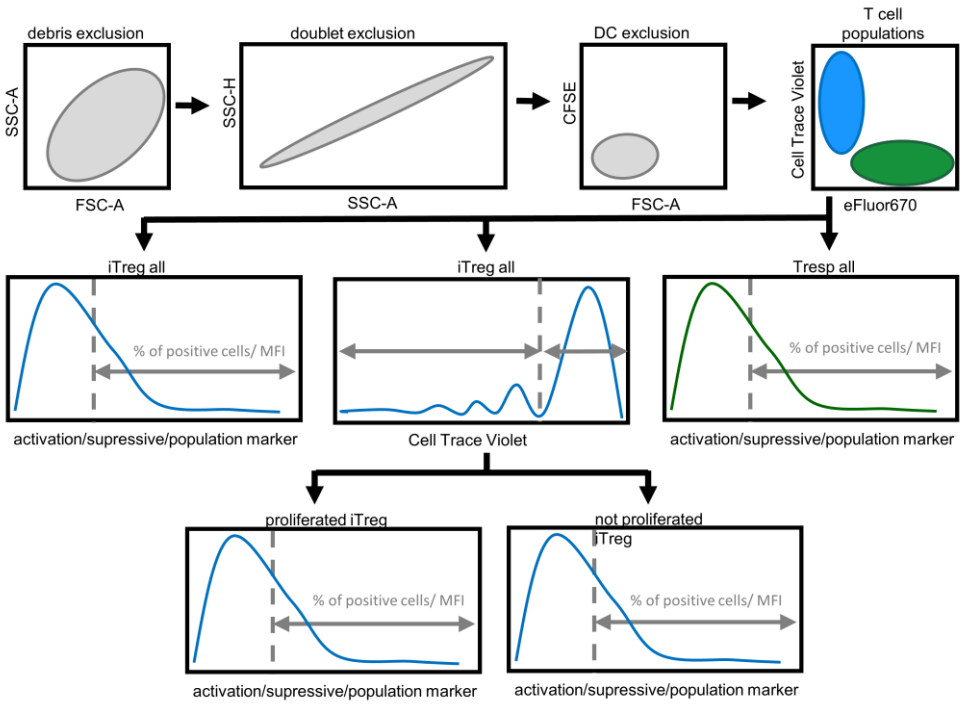

Figure 15: Gating strategy for T cell phenotype during suppressor assays. Debris, doublets and CFSE-stained DC were excluded. iTreg and Tresp were differentiated by cell trace violet (iTreg) and eFluor670 (Tresp) staining, and proliferating and non-proliferating T cells were distinguished by dye dilution. Percentages of positive cells or MFI for activation, suppression or population markers were obtained from histograms. MFI = mean fluorescent intensity.

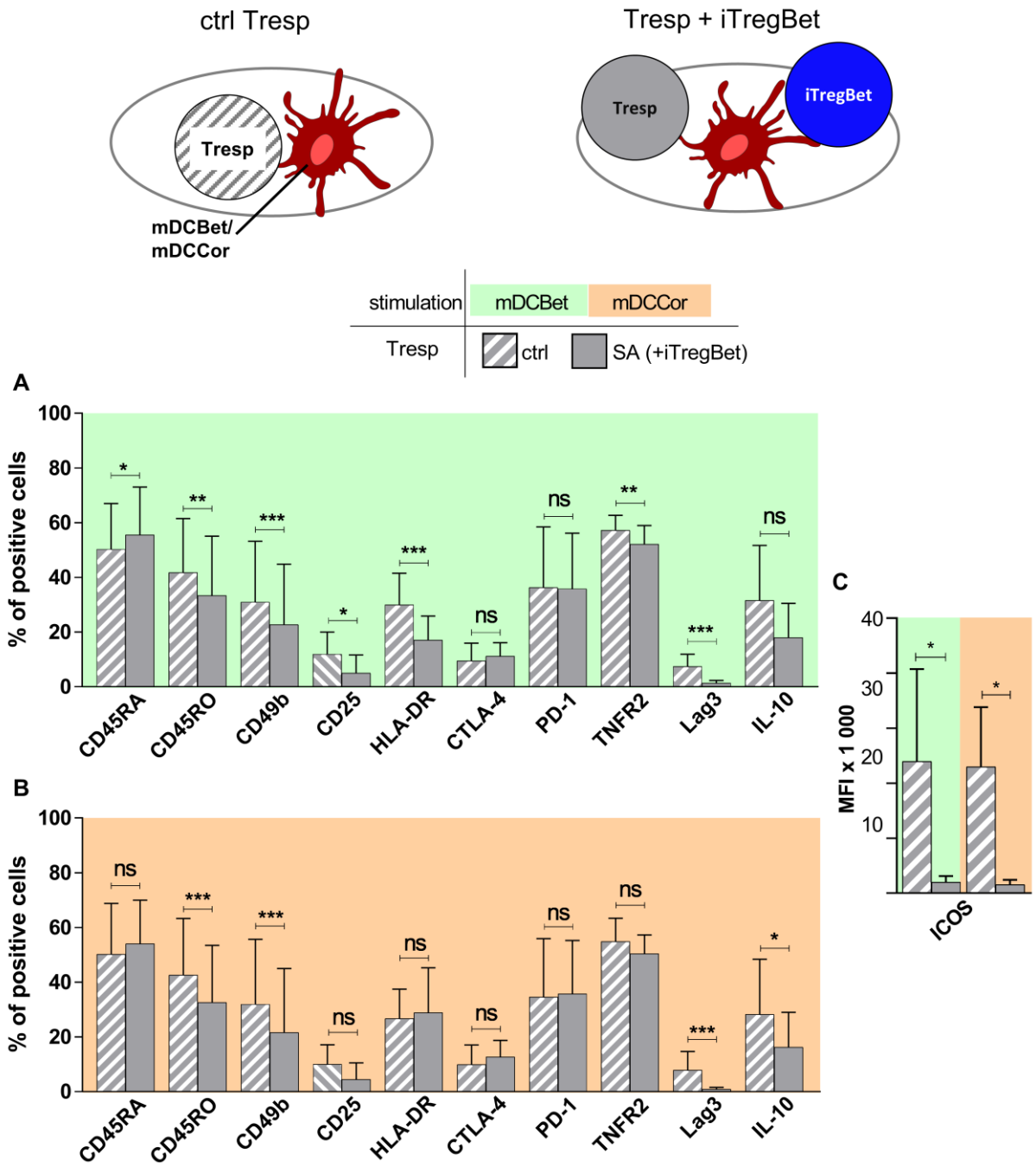

Figure 16: T cell phenotype of control Tresp vs. Tresp cocultured with iTreg in suppressor assays. iTregBet were primed and rested as described (Methods 3.5) and were cocultured with syngeneic mDCBet- or mDCCor-stimulated Tresp (Tresp:iTreg = 1:1). Positive control (ctrl) Tresp were cocultured with mDCBet or mDCCor alone for Bet- or Cor-specific activation. Before coculture, iTregBet and Tresp were stained with different proliferation dyes for identification. Prior to flow cytometric analysis of suppressor assays (SA), T cell populations were stained for expression of extra- and intracellular markers, which are compared between control Tresp and Tresp cocultured with iTreg. Percentage of positive cells for different phenotypic markers are shown for mDCBet- (**A**) and mDCCor- (**B**) stimulated cell cultures. The MFI of ICOS expression is shown in C. Ctrl =control, SA =suppressor assay, MFI = mean fluorescent intensity, p values: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

In Figure 16 the phenotype of allergen-stimulated control Tresp and Tresp stimulated in the presence of iTregBet (Tresp:iTreg ratio = 1:1) are compared. In comparison to

Bet-stimulated control Tresp, presence of iTreg resulted in enhanced CD45RA (polarisation towards naïve phenotype) and reduced CD45RO and CD49b (decrease in memory phenotype) expression on Tresp and simultaneously the activation associated molecules CD25 and HLA-DR were decreased (Figure 16 A). A similar trend was found for Cor-stimulated Tresp (Figure 16 B). This suggests downregulation of the Tresp activation status by iTreg as a mechanism of the iTreg-mediated suppression. Compared to Bet-stimulated control Tresp, Tresp cocultured with iTreg expressed lower amounts of the immunosuppressive markers TNFR2 and LAG3, but no changes were seen for CTLA-4, PD-1 and IL-10. Under Cor-stimulation Tresp cocultured with iTreg expressed significantly less IL-10. A similar, but not significant, trend is seen under Bet-stimulation. In all samples almost 100 % of cells expressed ICOS (data not shown) but there were differences in the mean fluorescence intensity (MFI), which was lower in Tresp in the presence of iTreg, meaning that the amount of ICOS molecules expressed on each cell surface was reduced compared to control Tresp (Figure 16 C).. The downregulation of immunosuppression-associated molecules in Tresp which are cocultured with iTreg suggests a polarisation towards activation of T cell effector functions. However, the downregulation of these molecules could also result from decreased T cell activation as is supported by the decrease in CD25 and HLA-DR and the shift to a naïve rather than memory phenotype (CD45RA/RO).

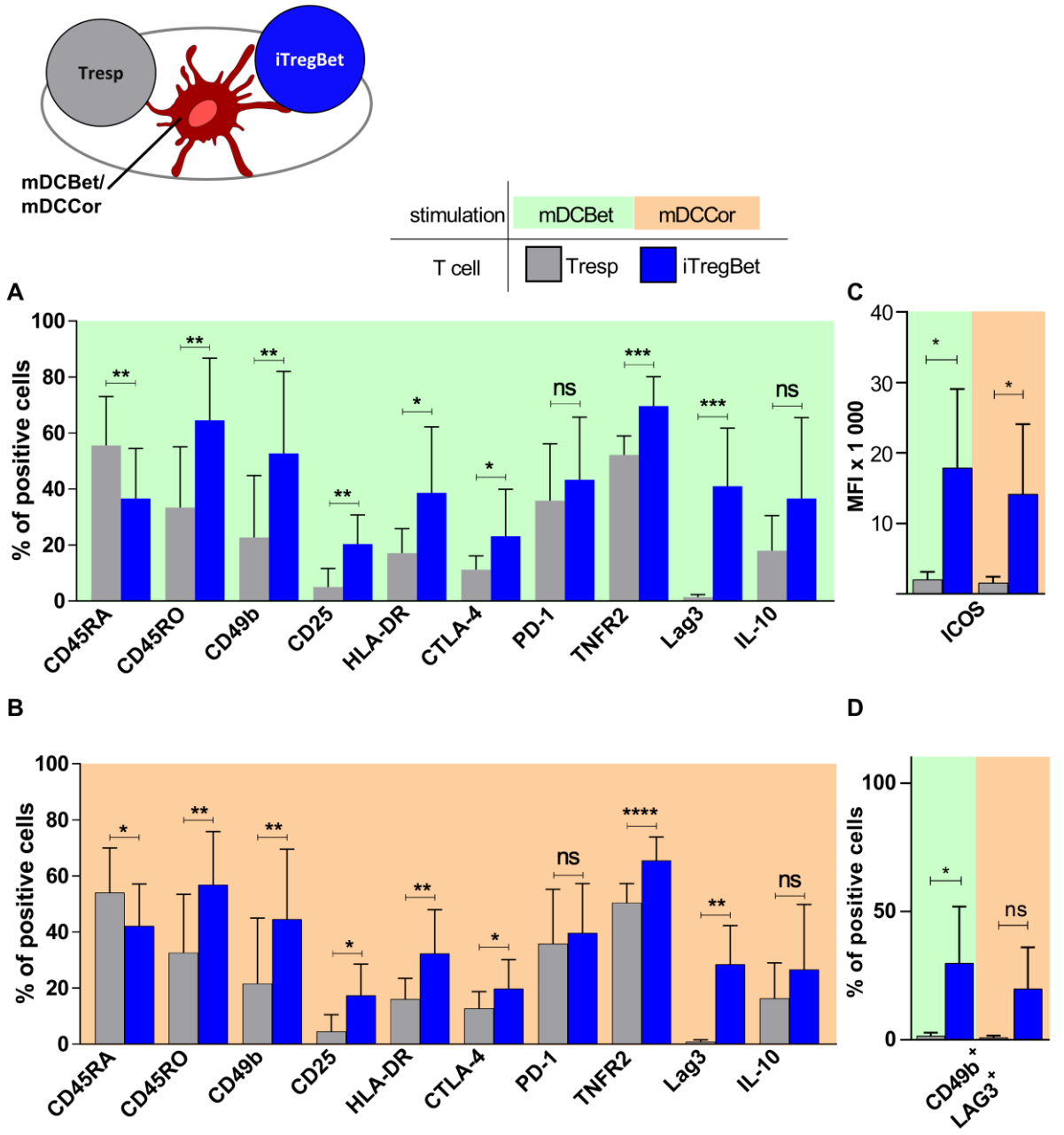

Figure 17: T cell phenotype of iTreg vs Tresp in suppressor assays. Tresp, iTregBet and mDCBet or mDCCor were prepared, cocultured and stained as described for Figure 16. The percentages of phenotypic markers of Tresp and iTregBet are compared under mDCBet- (**A**) and mDCCor- (**B**) stimulation. The MFI of ICOS expression and percentages of $CD49b^{+}LAG3^{+}$ are shown in **C** and **D**, respectively. MFI = mean fluorescent intensity, p values: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

In order to characterise the phenotype of Bet-specific iTreg, they were cocultured with and compared to Tresp in Bet- and Cor-stimulated suppressor assays (Figure 17). Bet-specific iTreg showed a decrease in CD45RA (naïve phenotype) expression and an increase in activation (CD25 and HLA-DR) and memory markers (CD45RO and CD49b) (Figure 17 A, B). In addition, Bet-specific iTreg were characterised by elevated expression of the suppression-associated molecules CTLA-4, TNFR2 and LAG3

(Figure 17 A, B). No significant increase was found in the expression of PD-1 and IL-10. ICOS expression (MFI) was significantly higher in Bet-specific iTreg (Figure 17 C). iTreg showed increased percentages of $CD49b^{+}LAG^{+}$ cells compared to Tresp, although this was not significant under Cor-stimulation (Figure 17 D). The activated and suppressive iTreg phenotype is in line with the finding of highly activated, proliferating iTreg with strong suppressive capacities in the restimulation and suppressor assays. This surface expression pattern highlights that in Bet- and Cor-stimulated cocultures of iTreg and Tresp, the iTreg are more activated and polarised towards immunosuppressive functions compared to the Tresp.

Further, we wanted to distinguish between proliferating and non-proliferating iTreg, which can be identified by the use of proliferation dyes (Figure 18). Thus, it was revealed that Bet- or Cor-stimulated proliferated iTreg show an even more profoundly activated and suppressive phenotype: decreased expression of CD45RA and increased expression of CD45RO, CD49b, CD25 and HLA-DR (memory type and activation) as well as CTLA-4, TNFR2, PD-1, ICOS and IL-10 (suppression) compared to total iTreg as well as to non-proliferated iTreg (Figure 18 A,B). ICOS expression (MFI) was significantly increased on proliferated iTreg compared to total iTreg and non-proliferated iTreg (Figure 18 C). Proliferated iTreg also showed the highest percentage of $CD49b^{+}LAG3^{+}$ Tr1 cells (Figure 18 D). In summary, the subpopulation of proliferated iTreg displays a highly activated phenotype with explicit expression of immunosuppressive markers. These results suggest activation and consequent proliferation as a prerequisite for the iTreg's suppressive function. As suppressor assays revealed a higher suppressive capacity of iTregBet compared to iTreg0, it is likely that allergen-specific iTreg stimulation is necessary for the activation of their full suppressive potential.

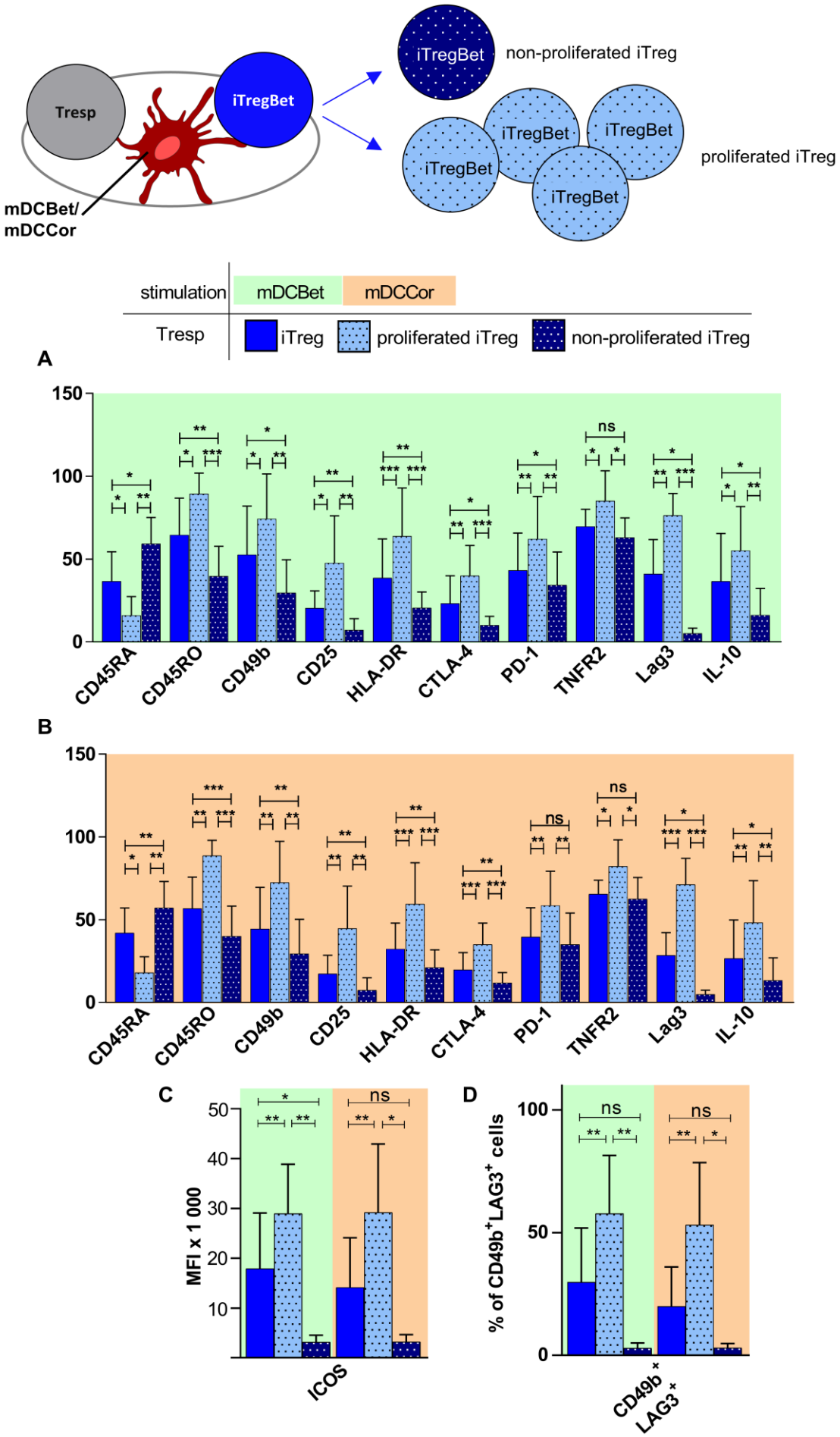
Tresp
iTregBet
mDCBet/
mDCCor
iTregBet
non-proliferated iTreg
iTregBet
iTregBet
iTregBet
iTregBet
proliferated iTreg
stimulation
mDCBet
mDCCor
Tresp
iTreg
proliferated iTreg
non-proliferated iTreg
A
B
150
100
50
0
CD45RA
CD45RO
CD49b
CD25
HLA-DR
CTLA-4
PD-1
TNFR2
Lag3
IL-10
ns
C
D
MFI x 1 000
ICOS
% of CD49b+LAG3+ cells
CD49b+
LAG3+

Figure 18 (previous page): T cell phenotype of proliferated and non-proliferated iTreg. Tresp, iTregBet and mDCBet or mDCCor were prepared, cocultured and stained as described for Figure 15. A cell proliferation dye was used to identify iTregBet and to further distinguish between proliferated and non-proliferated iTregBet by dilution of the proliferation dye. Percentages of iTreg positive for different phenotypic markers are shown for mDCBet- (A) and mDCCor- (B) stimulation and the MFI of ICOS expression and percentages of $CD49b^{+}LAG3^{+}$ are shown in C and D, respectively. p values: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant ($p > 0.05$).

4.4 Suppressive Capacity of iTregBet *in vivo*

The suppressive capacity of Bet-specific iTreg was further investigated in a humanised mouse model of allergen-induced intestinal and airway inflammation (see protocol in Figure 6). Immunodeficient NOD-scid-$\gamma^{-/-}$ mice were engrafted with PBMC from birch pollen and hazelnut allergic donors and boosted with birch pollen extract (allergen boost) to simulate a birch pollen and cross-reactive hazelnut allergic immune response in the animals (PBMC +birch, allergic control group). Human syngeneic, Bet-specific iTreg were coinjected at the day of engraftment to analyse their impact on the development of the allergic T cell reaction (PBMC +birch +iTregBet). Mice which received PBMC but not the allergen boost served as negative control group (PBMC).

After three weeks, blood samples for IgE analysis were collected and the mice were challenged rectally with birch pollen or hazelnut extract, which provoked the development of intestinal inflammation (colitis) in allergic animals.[161] 24 h later, the mice were challenged intranasally with birch pollen extract to initiate the development of allergic airway inflammation (asthma).[147]

4.4.1 Impact of iTregBet on Allergic Intestinal Inflammation

After rectal challenge, the allergic intestinal inflammation was evaluated blindedly by mini-endoscopy using an endoscopic score to assess the capacity of iTregBet to suppress allergen-induced intestinal immune responses (Methods 3.10.3).

Figure 19 shows the intestinal inflammation as endoscopic pictures of one representative experiment (A) and pooled data from independent experiments (B, C). Mice in the allergic control (PBMC + birch) developed severe symptoms of allergen-induced intestinal inflammation, which was significantly increased compared to the negative control (PBMC). This confirms the development of an allergen-induced intestinal inflammatory response in this model. Coinjection of iTregBet led to a significant decrease of the allergen-induced colitis, indicating the alleviation of allergic symptoms and the inhibition of allergy development by iTregBet. These results are in line with the high suppressive capacity of Bet-specific iTreg found in the *in vitro*

suppressor assay, highlighting that Bet-specific iTreg can suppress allergen-induced immune responses *in vivo* as well.

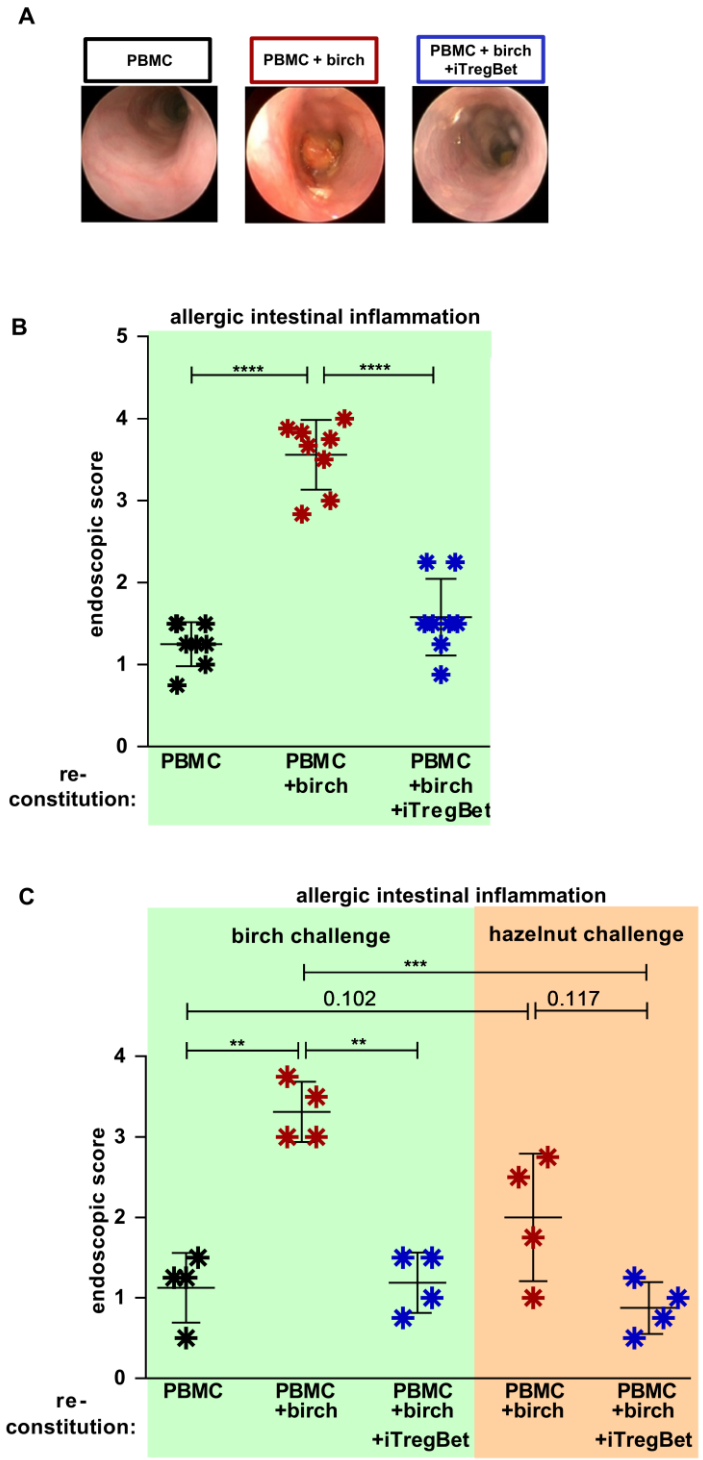

Figure 19: Effect of iTregBet on allergic intestinal inflammation. Immunodeficient mice were engrafted with human PBMC from birch pollen and hazelnut allergic donors (PBMC, negative control). For stimulation of an allergen-specific immune reaction in the animals, they were boosted with birch pollen extract (PBMC + birch, allergic positive control). iTregBet were coinjected at the day of engraftment to analyse their impact on intestinal inflammation. The protocol of the humanised mouse model and the animal groups are visualised in Figure 6. After 3 weeks, the mice were challenged rectally with birch pollen extract (**A, B**) or with birch pollen or hazelnut extract (**C**) (Methods 3.10.3). Endoscopic

pictures of intestinal inflammation from one representative experiment (**A**) and pooled data (**B, C**) are shown. p values: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, ns = not significant ($p > 0.05$).

In some experiments the mice treated with iTregBet were divided into two groups which were challenged rectally with either birch pollen or hazelnut extract (Figure 19 C). The allergic control group with hazelnut challenge showed less severe signs of intestinal inflammation compared to the allergic control group challenged with birch. Still, compared to the negative control, the allergic control group challenged with hazelnut showed an increased inflammatory response although it was not significantly enhanced. This could result from the lack of boost with hazelnut extract during and after the engraftment with PBMC and/or the donors usually less sever sensitisation towards hazelnut compared to birch pollen. However, iTregBet injection resulted in a profound but not significant reduction of allergen-induced intestinal inflammation, suggesting that Bet-specific iTreg are able to induce cross-reactive tolerance *in vivo*.

4.4.2 Effect of iTregBet on Allergic Airway Inflammation

To further evaluate the effect of iTregBet *in vivo*, iTregBet were applied in a model of allergic airway inflammation, which resembles human asthma (Figure 20).

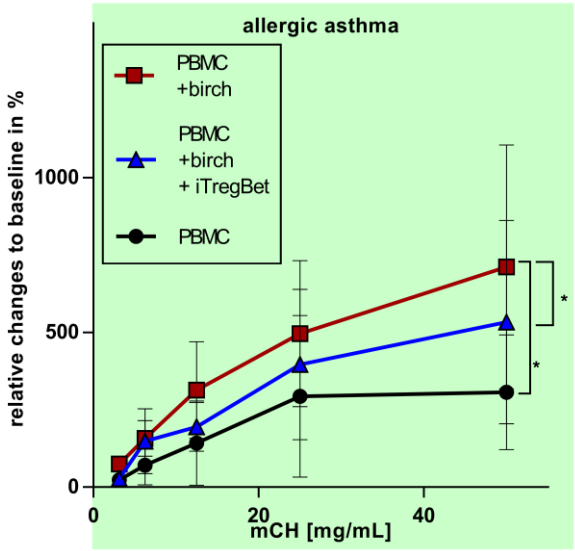

Figure 20: Influence of iTregBet on allergen-induced airway inflammation *in vivo*. Immunodeficient mice were engrafted with human PBMC as described for Figure 19 and after three weeks were challenged intranasally with birch pollen extract. 24 h later their airway resistance was evaluated (Methods 3.10.4). The airway resistance was pooled from 4 independent experiments and is presented as relative changes to a baseline value, which was taken before mCH challenge. p value: * $p < 0.05$.

Before the lung reaction was provoked with the first dose of methacholine (mCH), a baseline value for the airway resistance was determined for each mouse. The airway resistance in Figure 20 is presented as relative changes to the baseline. Escalating doses of mCH resulted in an increase in the airway resistance in all groups, although the extent varied: the negative control group (PBMC) shows the mildest symptoms

provoked by mCH. Compared to that, the airway resistance in the allergic control group (PBMC +birch) increased considerably with escalating doses of mCH, confirming the development of an allergic airway response in the mouse model. Mice treated with iTregBet showed a significant decrease in the airway resistance compared to the allergic control, indicating the ability of Bet-specific iTreg to ameliorate birch pollen-induced allergic airway inflammation.

4.4.3 Analysis of Human IgE in Humanised Mice

Analysis of allergen-specific and total IgE is used in the diagnosis of allergic diseases. Human IgE can also be detected in the humanised mouse model and represents a parameter for the severity of the established allergic immune response. The experiments did not reveal any differences in total IgE concentrations of all experimental groups (PBMC, PBMC+ birch and PBMC +birch +iTregBet) (Figure 21 A). Human birch-specific IgE was increased in the allergic control group (PBMC +birch) compared to the negative control group (PBMC), confirming the development of a birch pollen-specific immune reaction in the positive control animals (Figure 21 B). By coinjection of iTregBet at the day of PBMC engraftment a significant reduction in the production of birch-specific IgE was achieved, confirming the abrogation of clinical symptoms (allergic inflammation) by iTregBet. In conclusion, these results demonstrate that the *in vivo* application of Bet-specific iTreg can improve clinical parameters of allergic diseases, in addition to the alleviation of intestinal and airway symptoms.

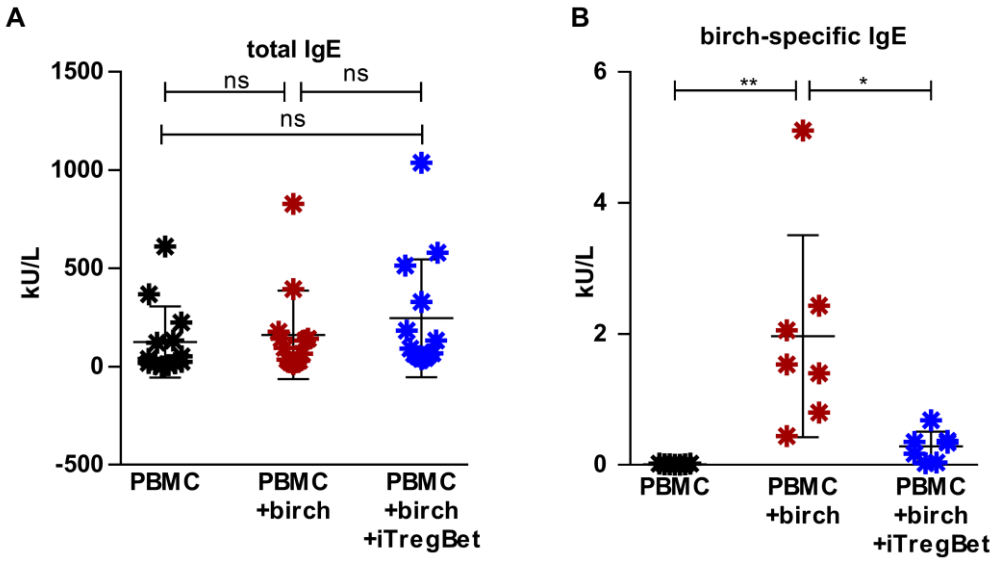

Figure 21: Total and birch-specific IgE levels of humanised mice. Immunodeficient mice were engrafted with human PBMC as described in Figure 19, and blood samples for IgE analysis were taken three weeks after engraftment. Total (**A**) und birch-specific (**B**) IgE concentrations in the plasma are presented in kU/L. p values: ** $p < 0.01$, ns = not significant ($p > 0.05$).

5 DISCUSSION

Due to cross-reactive pollen and food allergens, many individuals allergic to pollen (e.g. birch) develop a secondary food allergy (e.g. hazelnut), also called pollen-associated food allergy (PFA). Most allergic patients are polysensitised to different primary allergens and PFA additionally promotes the sensitisation towards multiple food allergens, but allergen-specific immunotherapy (AIT) can only target few allergens simultaneously and several years of therapy are needed to achieve a long-lasting effect.[162,163] To date the therapeutic effect of pollen AIT on the associated food allergy is unclear, as convincing studies are scarce.[126] Many studies which originally reported excellent effects of pollen AIT on associated food allergies did not include a placebo control,[164,165] and the few available placebo-controlled studies report little statistically proven clinical improvement of the associated food allergies.[166,167] These results imply that AIT might be insufficient in treating PFA and therefore novel therapeutic approaches need to be explored.

Efforts to improve AIT include co-application of adjuvants with the allergens as fusion proteins or packed in nanocapsules and the development of hypoallergic allergen variants.[168–170] In a different approach a food allergen (Mal d 1 from apple) was used for AIT, which decreased Bet v 1- and Mal d 1-specific T cell reactivity and IgE levels.[171,172] But official guidelines do not recommend food AIT for routine clinical use due to the risk of severe side effects and lack of evidence for sustained efficacy.[162] This study aims to investigate the possibility of a completely different approach: cellular tolerance-inducing therapies. For this purpose, we used allergen-loaded IL-10 DC generated from the peripheral blood of birch pollen allergic patients with associated hazelnut allergies. In this study, we explored the potential of these allergen-loaded IL-10 DC to induce iTreg with strong capacity to suppress allergen-specific (birch pollen, Bet) as well as cross-reactive (hazelnut, Cor) allergic immune responses of birch pollen and hazelnut allergic patients.

Human tolDC can be generated *ex vivo* by several protocols. Genetical modifications of DC directly allow the recombinant expression of FasL, PD-L1, TRAIL, IL-10 or TGF-β, all of which result in a tolerogenic DC phenotype and the ability to facilitate immunosuppression.[47,173,174] Several protocols have been established for the *ex vivo* differentiation of tolDC from iDC, which are generated by culture of $CD14^+$ precursor cells with IL-4 and GM-CSF.[47–49,53,175] iDC themselves show some tolerogenic

properties and are able to regulate immune responses, but under inflammatory conditions they mature and can drive the activation of T cell responses.[61] However their tolerogenicity can be enhanced and stabilised by culture with several immunosuppressive drugs (dexamethasone, rapamycin and acetylsalicylic acid) or biomolecules (vitamin D3, TGF-β, IL-10).[47,48] In most protocols, a maturation stimuli is added to further improve phenotypic stability.[175] All these culture conditions result in the downregulation of costimulatory molecules (CD80, CD86, CD40), DC maturation markers (CD83) and MHCII, as well as the upregulation of immunosuppressive markers (ILT3, ILT4, PD-L1, TRAIL) and secretion of immunosuppressive mediators (IL-10, TGF-β, IDO).[47] Consequently tolDC are able to regulate immune responses by induction of T cell apoptosis, anergy or iTreg differentiation. In our protocol, IL-10 DC were generated by culture of iDC with IL-10 and a maturation mix containing IL-1β, TNF-α, IL-6 and PGE_2, resulting in a semi-mature DC phenotype.[47,49] In other protocols IL-10 is added in the absence of maturation stimuli at the beginning of the $CD14^+$ precursor culture, which induces IL-10-modulated DC (DC10), that are $CD14^+CD16^+$ but are more mature than IL-10 DC as their expression levels of HLA-DR, CD40, CD80, CD83 and CD86 are similar to mDC.[53]

A comparative study of tolDC-induced by IL-10, vitamin D_3, dexamethasone, TGF-β and rapamycin identified IL-10 DC as the tolDC subset with the most prominent and stable tolerogenic characteristics and as the only potent inducer of Treg with strong suppressive capacities.[61] With the protocol established in our lab we further increased the suppressive and migratory capacity of IL-10 DC by sorting for a $CD83^{high}CCR7^{high}$ subset.[49] This undoubtedly puts IL-10 DC among the top candidates for antigen-specific cellular tolerance-inducing therapies. This study was conducted to further investigate the potential of allergen-loaded IL-10 DC to prime allergen-specific and cross-reactive iTreg *in vitro* to reveal their potential utility for cellular tolerance inducing therapies for PFA.

In this study, mDCBet-stimulation of syngenic $CD4^+$ T cells from allergic donors induced a Bet-specific T_H2 response. Allergen-specific *in vitro* proliferation of T cells derived from allergic donors has been previously proven for different allergens and is an established method to investigate allergic T_H2 responses.[176–178] Here, we could reproduce results from a previous study in our group, which additionally demonstrated that $CD4^+$ T cells of healthy donors do not react to stimulation with Bet-loaded mDC in contrast to T cells from allergic donors. Thereby, it was proven that allergen-induced

Teff proliferation is restricted to allergen-sensitised patients.[66] In the present study, T cell priming with IL-10 DCBet did not stimulate T cell proliferation but induced secretion of IL-5, IL-9 and IL-10. Induction of non-proliferating iTreg by IL-10 DC was also previously shown by us and others in an antigen-independent experimental setting,[49,179] and antigen-dependently.[58,60,178]

The cross-reactive proliferation of T cells induced by pollen and related food allergens was first shown in T cell lines and T cell clones.[132,150,151] The T cell cross-reactivity between Bet and Cor using primary T cells from allergic donors was confirmed in our previous study[66] and this *in vitro* model was used in this study. In the present study, Bet-specific Teff proliferated significantly when restimulated with Bet-loaded mDC, but only showed slightly increased proliferation after Cor-specific restimulation. The study cohort was much smaller and therefore Cor-induced proliferation of Bet-specific Teff did not reach significance, although the absolute values and SD were in the same range as restimulation with Bet-loaded mDC. This indicates that in this study T cell cross-reactivity between the homologous allergens would probably have been statistically significant, if the study cohort was larger.

We also found in this study that Bet-specific iTreg showed high proliferative activity after restimulation with Bet v 1 or Cor a 1. In a study by another group, it was revealed that Der p 1 (major house dust mite allergen)-specific Treg induced by DC-10 were hyporesponsive after restimulation with Der p 1.[58] The different proliferative behaviour after homologous restimulation could be due to the different DC phenotypes induced with IL-10 DC and DC-10 generation protocols, which also create different Treg inducing capabilities.

When Treg were discovered and studied *in vitro*, it was appreciated that Treg are anergic after restimulation. However, after testing TCR-transgenic Treg *in vivo* it was concluded that Treg proliferate in an antigen-specific manner while exhibiting suppressive functions.[180–182] In fact, Treg that have been stimulated to proliferate can even display an enhanced suppressive capacity.[182–184] Therefore, although extensive Treg stimulation with IL-2 or anti-CD28 antibodies impairs their suppression,[59,180] proliferation itself is not an imperative characteristic for impairment of Treg function. In our *in vitro* system, Bet- and Cor-stimulated Bet-specific iTreg proliferated vigorously, whereas restimulated non-specific iTreg remained anergic, indicating allergen-specific and cross-reactive proliferation as prerequisite for Bet-specific iTreg activation.

Despite their proliferative activity, Bet-specific iTreg exhibited strong capacities to inhibit allergen-specific (Bet, birch) and cross-reactive (Cor, hazelnut) T cell response, indicating allergen-specific and cross-reactive suppression facilitated by Bet-specific iTreg. In contrast to our results, another study of peanut allergen-specific iTreg induced by IL-10-modulated DC from allergic patients found that these T cells (=peaT_{10}) were functionally impaired.[185] However, they induced iTreg with DC-10, which compared to IL-10 DC have different immunoregulatory properties and induce iTreg with different phenotypes and functions. Also, they studied iTreg in the context of peanut allergy, which in most cases is not a secondary food allergy associated with a pollen allergy.[186] Therefore, the immunological pathogenesis and possible Treg defect could differ from PFA. However, there are two reasons why it is still possible that iTreg cells in peanut allergy are functional: (1) peaT_{10} were expanded in the presence of IL-10 and IL-2. But high amounts of exogenous IL-2 can break the suppressive capacity of Treg, [59,180] indicating the possibility that peaT_{10} are not impaired *in vivo* but through *ex vivo* expansion. (2) The authors suggested that peaT_{10} are functionally impaired because peaT_{10} were not anergic and they found increased amounts of IL-4 and IL-5 in supernatants of restimulated peaT_{10} from allergic compared to healthy donors, but they did not specifically test the suppressive functions. In our study, primary Bet-specific iTreg were investigated without the need for further expansion with IL-2 and IL-10. These cells were highly proliferative and secreted T_H2 cytokines, but were not functionally impaired. Combined with the data from T cell phenotype analysis and cytokine secretion, we suggest that activation and subsequent proliferation is a prerequisite for suppressive capacity, rather than a sign of defective suppressive function.

The induction of primary iTreg with strong suppressive capacities by IL-10-modulated DC has been shown before in an antigen-independent context,[49] and also in T cell clones and T cell lines with allergen-specificity.[58,60] Here, we further showed that it is possible to facilitate strong allergen-dependent suppression as indicated by superior suppressive functions of primary Bet-specific compared to non-specific iTreg. Additionally, we showed that this allergen specificity is extended to tolerance induction towards cross-reactive food allergies.

One possible mechanism behind allergic development is a shift of the allergen-induced immune response from T_H1 towards T_H2, which might be a consequence of the reduced microbial exposure in western countries (hygiene hypothesis).[187] It is therefore

desirable to restore the immune balance during therapies, and a shift back from T_H2 towards T_H1 is seen as a mechanism of tolerance induction by AIT and successful (pre-)clinical outcome.[188,189] In our *in vitro* testing system for suppressive capacity, a decrease in IL-13 and increase in TNF-α indicate such a shift, but it is not supported by the other T_H1 and T_H2 cytokines. IL-13 has been shown to be substantially involved in the development of type-1 allergies, especially in the induction of food allergen-specific IgE in the intestinal mucosa.[190–192] A reduction of IL-13 might therefore be most beneficial for tolerance induction towards food allergens. Although IL-5 and IL-9 are proinflammatory T_H2 cytokines, they are also known to be produced by Treg: type 1 regulatory T cells (Tr1) are known to secrete IL-5[100] and nTreg can secrete large amounts of IL-9.[193] It is therefore possible that we did not find a decrease of IL-5 and IL-9 secretion by Tresp, because Bet-specific iTreg might produce the cytokines by themselves.

Flow cytometric analysis of Tresp and iTreg in suppressor assays revealed that IL-10 was produced by Tresp as well as iTreg in comparable amounts and only the subset of proliferating iTreg showed an increase in the tolerogenic cytokine. However, analysis of the cell culture supernatants revealed that only very little IL-10 was actually secreted by Tresp, whereas in suppressor assays with Bet-specific iTreg very large amounts of IL-10 were released. Teff are known to secrete IL-10 in case of excessive T cell activation due to chronic antigen exposure in order to protect from tissue damage.[194] Here, we show that Bet-specifically stimulated Tresp produce and store IL-10 rather than secreting it. On the other hand, IL-10 secretion is a major mechanism of iTreg-mediated suppression.[100,195,196] IL-10 inhibits T cell activity and proliferation indirectly via its tolerogenic effects on DC and directly by binding to the IL-10 receptor on T cells, which results in downregulation of cytokine production and a change in surface molecule expression.[197] Analysis of the expression of activation-, memory- or suppression-associated surface molecules on Tresp revealed a shift from activated, memory-like Tresp in the control towards resting/naïve Tresp in suppressor assays with iTregBet, which could be the individual impact of IL-10. This strongly suggests IL-10 as one mechanism of the suppressive capacity of IL-10 DC-induced iTreg. In another study, allergen-specific iTreg differentiated in the presence of TGF-β were shown to facilitate immunosuppression by IL-10-dependent downregulation of pro-inflammatory molecules on mDC,[103] which therefore could also be a mechanism of iTreg in our system.

Tr1 cells are characterised by high expression of the population markers CD49b and LAG3[93] and are known to produce large amounts of IL-10 and IL-5 as well as low amounts of INF-γ and IL-2.[100] Further characterisation of Bet-specific iTreg revealed that especially the proliferating subset expresses high amounts of CD49b and LAG3. In addition, IL-10, IL-5, INF-γ and IL-2 were found in suppressor assay supernatants containing Bet-specific iTreg. This indicates that at least a proportion of the suppressive iTreg induced by IL-10 DC exhibit characteristics of Tr1-like cells. Human Tr1 cells have been identified *in vivo* and abnormal Tr1 differentiation or numbers are implied in several allergic and autoimmune diseases.[95,97] In healthy subjects the majority of allergen-specific T cells are Tr1 cells, whereas in allergic patients, the balance between allergen-specific Tr1 and T_H2 cells is inverted.[198] Therefore IL-10 DC as potent inducers of Tr1-like cells could restore the immune balance in allergic diseases.

Large amounts of the T cell activating cytokine IL-2 were found in suppressor assays with Bet-specific iTreg, but drawing conclusions from IL-2 in the supernatants of suppressor assays is difficult, as it is unknown which T cell population (iTreg or Tresp) produced the cytokine and how much of the IL-2 has already been consumed. Contrary to the earlier idea that Treg cannot produce IL-2, Tr1 cells are known to be able to secrete IL-2, although in low amounts.[100] The high concentration of IL-2 in suppressor assays with iTregBet implies that it was rather produced by mDC-stimulated Tresp. A study of iTreg activity in murine lymph nodes revealed that high IL-2 concentrations function as a negative feedback loop increasing Treg activity in order to prevent excessive T cell responses.[199] Considering these findings, it is possible that Tresp were activated by mDCBet to produce IL-2 which in turn enhanced the suppressive capacity of iTreg. Subsequently, iTreg suppressed Tresp activity, as also indicated by the Tresp phenotype. The inactivated status of Tresp as revealed by flow cytometric analysis implies that iTreg downregulate Tresp activity, which might also decrease IL-2 responsiveness. IL-2 deprivation and consequent starving of T cells is a possible antigen-independent mechanism of Treg function[200], but due to the large amount of IL-2 found in suppressor assays with iTregBet this seems unlikely in our setting.

In a previous study, we used IL-10 DC which were not loaded with specific antigens and cocultured them with allogenic $CD4^+$ T cells. iTreg did not proliferate after allogenic restimulation while displaying a more naïve ($CD45RO^{low}$, $CD45RA^{high}$) but also suppressive ($CTLA\text{-}4^+$, $ICOS^+$, $PD\text{-}1^+$) phenotype.[49] In the present study, Bet-specific

iTreg displayed Bet-stimulated proliferation and an activated and suppressive phenotype. We therefore suggest that allergen-specific stimulation further activates cognate iTreg and enhances the suppressive capacity, as is also demonstrated by the higher suppressive capacity of Bet-specific iTreg compared to non-specific iTreg. These findings indicate that allergen-specific iTreg induced by IL-10 DC are especially equipped for tolerance induction in allergic diseases.

We confirmed the strong capacity of iTregBet to suppress allergen-induced inflammation in a humanised mouse model, which was established in our institution.[150, 163] In an inducible mouse model of type 1 allergy murine IL-10 DC-induced allergen-specific iTreg were also found to have strong tolerance inducing capabilities.[201] These findings are supported by the fact that mice deficient in iTreg generation develop spontaneous intestinal and airway T_H2 reactions.[202] In our mouse model, the injection of allergen-specific iTreg might have re-established a healthy immune balance and thereby alleviated the allergic symptoms.

Although total IgE levels are sometimes still used in the diagnosis of food and inhalant allergies, it is not a very reliable predictor, as a study revealed that in 44 % percent of patients allergies could not be excluded although they had normal total IgE levels.[203] It is therefore not necessarily expected to be increased in the allergic control of the humanised mouse model, as was seen in our study and was shown before.[204] However, another study of the humanised mouse model showed that although total IgE levels were not increased in the allergic control, application of $CD4^+CD25^+Foxp3^+$ nTreg resulted in the reduction of total IgE,[161] which we did not achieve by injection of Bet-specific iTreg. But more importantly, we could prove the iTreg's positive effect on clinical level by the reduction of human Bet-specific IgE, which is a much more reliable clinical parameter for birch pollen allergies.[4]

Application of Bet-specific iTreg in allergen-sensitised mice proves that iTreg-mediated suppression is functional *in vivo*, but it is unclear whether these are long-lasting tolerogenic effects. In fact, adoptive transfer of Tr1-like cells did not facilitate long-term tolerogenic memory in a mouse model of house dust mite allergy.[205] It is therefore possible that the effect in our model is also only transient, unfortunately long-term effects cannot be studied in the humanised mouse model due to the onset of graft versus host disease.[206] But immunological tolerance can be mediated by so-called "infectious" mechanisms, meaning that it sustains itself through self-renewal.[207,208]

Therefore it should be possible to establish immune tolerance with long-lasting effects, which could be achieved by the injection of allergen-loaded IL-10 DC instead of allergen-specific iTreg.

In addition to the induction of iTreg, tolDC have several mechanisms to maintain central and peripheral tolerance, such as the induction of anergy in antigen-specific memory T cells[209] or the downregulation of pro-inflammatory phenotype and T cell stimulation capacity in mDC.[47] Application of tolDC instead of iTreg might therefore have mechanisms to prolong the persistence of allergen-specific immunosuppression. This is supported by a study with a murine ovalbumin-induced model of asthma, where reduced allergen-specific IgE and a decrease in airway eosinophilic inflammation were detected 8 months after injection of a single dose of ovalbumin-loaded IL-10 DC.[210] These findings also imply that for tolerance-inducing DC therapies a single or few repeated injections might suffice. Contrary to that, AIT is a tedious procedure that requires repeated allergen application for several years. Clinical trials of AIT report a 30-40% reduction in symptom or medication scores, but the persistence of the therapeutic effect is only followed up for 3 years or even less.[112,113,118] Only one study directly investigated longer lasting effects of SLIT against house dust mite allergy and revealed that 4 or 5 years of therapy were needed to achieve a lasting therapeutic success for 8 years.[163] High drop-out rates due to this time-consuming therapy further diminishes the therapeutic success.[127] Cellular tolerance-inducing therapies with allergen-loaded IL-10 DC could therefore solve the problem of patient adherence.

Several phase 1 clinical studies applying *ex vivo* generated tolDC in different disease context have been conducted. None reported any severe side effects and the DC were generally well tolerated, highlighting the explicit safety.[139–142] Only few clinical outcomes were investigated so far but they are very promising. In rheumatoid arthritis a decrease in effector T cells and increased ratio of regulatory to effector T cells as well as reduction of proinflammatory serum cytokines were found.[141] Decreased disease activity scores were achieved in Crohn's disease trials.[144] In a recent phase 1/2A clinical study, kidney transplant recipients were treated with different regulatory cells (Treg, tolDC, regulatory macrophages) while their immunosuppressive treatment was reduced compared to conventional therapies.[143] This did not increase the transplant rejection rate but resulted in decreased incidences of post-surgical infectious complications, providing proof for the efficacy and utility of cellular tolerance-inducing cellular therapies.

6 CONCLUSION

Cellular tolerance-inducing therapies are promising therapeutic strategies for the induction of antigen-specific immune tolerance in autoimmune diseases, transplant rejection and allergic diseases. IL-10 DC are optimal candidates as they are phenotypically stable and have strong migratory and suppressive capacities. They are able to induce allergen-specific iTreg and thereby they are especially suitable to restore the imbalance of allergen-specific iTreg and T_H2 effector cells in type 1 allergic diseases.

In this study, IL-10 DC were investigated in regard to their potential to induce immune tolerance specific to multiple allergens in patients with pollen-associated food allergies. It was revealed that they induce iTreg which in addition to allergen-specific suppressive function also have the ability to suppress cross-reactive immune responses *in vitro* and *in vivo*. IL-10 DC are therefore suitable to fill the gap in conventional allergen-specific immunotherapies, which have unsatisfying effects on associated food allergies.

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8 CURRICULUM VITAE

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