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DISSERTATION

Titel der Dissertation

„Functional characterization of human allergen-specific T-cell receptors *in vitro* and *in vivo*“

verfasst von

Mag.rer.nat. Alina NEUNKIRCHNER

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Doktorin der Naturwissenschaften (Dr.rer.nat.)

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Abstract

T-lymphocytes play a central role in the pathogenesis of allergic diseases. During allergen-sensitization the exposure of the respiratory epithelium to allergens leads to the differentiation of antigen-presenting cells that induce the differentiation/activation of allergy-orchestrating T-lymphocytes. To investigate the specific contribution of T-lymphocytes in allergy we rely on the observation that the antigen specificity of T-cells can be conveniently transferred to other T-cells by means of TCR gene transfer. We have rebuilt the 'allergen-specific synapse' *in vitro* by cloning and expression of the relevant molecules involved in antigen-presentation (i.e. HLA-molecules) and antigen-recognition (i.e. TCR alpha and beta chains) specific for the major mugwort pollen allergen (*Artemisia vulgaris*), Art v 1, and the major birch pollen allergen (*Betula verrucosa*), Bet v 1. Transient transfection of 293 cells with allergenic peptides, the corresponding HLA-molecules and co-stimulatory molecules proved to be a reliable method for the generation of antigen-presenting cells (APC). Transfer of either of the two TCR into the human Jurkat T-cell line or PB-lymphocytes of allergic or non-allergic individuals restored the fine-specificity of the original TCR, i.e. allergen-specificity as well as cross-reactivity to homologous allergens. Moreover, similar to the original allergen-specific T-cell clone the activation of transgenic (tg) T-cells was also found to be strictly dependent on co-stimulation. These valuable tools for rebuilding the allergen-specific synapse enabled us to develop several immunomodulatory strategies with the focus on the inhibition/attenuation of disease-eliciting allergen-specific T-cell responses. In fact, allergy is thought to result from an imbalance in Th1/Th2 homeostasis leading to a predominance of Th2 cell responses. Along those lines we demonstrated that co-expression of the immunodominant T-cell epitope of Bet v 1 and membrane-bound human IL-12 polarizes Bet v 1-specific TCR tg T-cells towards Th1-like T helper cells with predominant IFN-γ-secretion. Moreover, in co-culture experiments tg Th1-polarized cells had the potential to inhibit the effector function of Bet v 1-specific Th2 cells after allergen-specific restimulation. In addition, another subset of $CD4^+$ T-lymphocytes, i.e. naturally occurring regulatory T-cells (nTreg), was shown to play important roles during immune regulation including the induction and maintenance of immune tolerance. Apart from Th2 cells, Treg are a possible explanation why not all experimental and clinical observations can be easily explained by the Th1/Th2 balance hypothesis. Therefore, we tested the possibility to generate allergen-specific TCR tg regulatory T-cells by genetic engineering. In a multicistronic expression vector containing an IRES-GFP reporter element we combined the TCR alpha- and beta-chains of the Bet v 1-specific TCR plus the master regulator of Treg function, FOXP3. Retroviral transduction of $CD4^+CD25^-$ PB T-cells with

this mulitcistron generated TCR^+FOXP3^+ tg T-cells that displayed a regulatory phenotype and function. Bet v 1-specific TCR^+FOXP3^+ tg T-cells remained hyporesponsive after allergen-specific or polyclonal activation and suppressed the effector function and proliferation of allergen-specific T-cells when activated by allergen. To study the contribution of T-helper cells in detail and to investigate protocols to modulate the immune response against a human-relevant aeroallergen not only *in vitro* but also *in vivo* we generated double tg mice co-expressing an Art v 1-specific TCR (TRAV17/TRBV18) and the corresponding restriction element HLA-DR1. In a first step, a detailed immunological work-up of the various mouse lines was performed. Among other results, these investigations revealed that splenocytes of double tg mice specifically proliferated upon incubation with the human-relevant immuno-dominant Art v 1_{25-36} peptide or whole Art v 1 protein. In contrast, neither wild type mice nor single HLA-DR1 or TCR tg mice showed signs of proliferation. Similar data were obtained for secreted cytokines. *In vivo* evaluations of the different groups of mice showed that after allergen-specific sensitization, only double tg mice showed enhanced airway hyperreactivity (AHR) as assessed by methacholine or allergen challenge. Three consecutive exposures to nebulized allergen were found to be sufficient for induction of altered airway function and were accompanied by airway and lung inflammation due to massive accumulation of inflammatory cells in bronchoalveolar lavage fluids as well as peribronchiolar and perivascular infiltration with inflammatory cells. Of importance, the observed airway responses were entirely IgE-independent, since no allergen-specific serum IgE (or IgG) was detectable. In conclusion, allergen-specific tg T-cells are a promising tool for the further development of immunomodulatory strategies to alter the course of allergic diseases. Moreover, humanized allergy models are amenable to the evaluation of human-relevant allergen formulations or cellular intervention protocols and will contribute to the further understanding of allergic diseases and their cure.

Kurzbeschreibung

T-Lymphozyten spielen in der Pathogenese von allergischen Erkrankungen eine zentrale Rolle. Während der Sensibilisierungsphase führt die Allergen-Exposition des respiratorischen Epithels zur Differenzierung von Antigen-präsentierenden Zellen (APZ), welche die Aktivierung von allergen-spezifischen T-Lymphozyten begünstigen.

Um die Beteiligung von T-Zellen an allergischen Reaktionen zu analysieren, wurde die "Allergen-spezifische Synapse" auf molekularer Ebene rekonstruiert. Zu diesem Zweck wurden die notwendigen Moleküle, welche für die Antigen-Präsentation (*i.e.* HLA-Moleküle) sowie für die Antigen-Erkennung (*i.e.* α- und β-Kette des T-Zellrezeptor (TZR)) für das Hauptallergen des Beifusspollens (Art v 1, *Artemisia vulgaris*) und für das Hauptallergen des Birkenpollens (Bet v 1, *Betula verrucosa*), verantwortlich sind, kloniert. In diesem Zusammenhang hat sich die transiente Transfektion von 293 Zellen mit den allergenen Peptiden, den dazugehörigen HLA-Molekülen sowie unterschiedlichen kostimulatorischen Molekülen als eine zuverlässige Methode zur Erzeugung von APZ erwiesen. Des Weiteren haben wir die Tatsache genutzt, dass durch die genetische Übertragung der spezifischen TZR α- und β-Ketten einer allergen-spezifischen T-Zelle die Antigen-Spezifität von dieser T-Zelle auf eine andere übertragen werden kann. Tatsächlich konnten wir beobachten, dass die Übertragung der beiden allergen-spezifischen TZR auf eine humane T-Zelllinie bzw. auf T-Zellen aus dem peripheren Blut von allergischen und nicht allergischen Individuen dazu führte, dass die solcherart hergestellten transgenen T-Zellen die Spezifität des ursprünglichen T-Zellklons annahmen (*i.e.* Allergen-Spezifität, Kreuzreaktivität mit homologen Allergenen). Die Aktivierung der transgenen T-Zellen war dabei, vergleichbar mit dem ursprünglichen T-Zellklon, strikt abhängig vom Vorhandensein eines kostimulatorischen Zweitsignals. Die solcherart erwirkte Rekonstruktion der Allergen-spezifischen Synapse ermöglichte uns, unterschiedlichste Strategien zu entwickeln und zu testen, um pathogene Allergen-spezifische T-Zell Antworten zu verändern oder aber ganz zu inhibieren. Eine Hypothese besagt, dass allergische Erkrankungen aus einem Ungleichgewicht zwischen den beiden T-Helferzelltypen Th1 und Th2 hervorgehen und daher allergische Reaktionen auf ein Überwiegen von Th2-Antworten zurückzuführen wären. Aus diesem Grund haben wir untersucht, ob APZ, welche das immundominante T-Zell-Epitop von Bet v 1 präsentieren und gleichzeitig Membran-gebundenes IL-12 auf der Zelloberfläche exprimieren, Bet v 1-spezifische TZR tg T-Zellen zu IFN-γ-produzierenden Th1-Zellen polarisieren können. Wir haben in Kokultur Experimenten

gefunden, dass diese Th1-polarisierten T-Zellen tatsächlich die Effektorfunktionen von Allergen-spezifischen Th2-Zellen zu inhibieren vermögen.

Eine weitere Untergruppe der $CD4^+$ T-Zellen stellen natürlich vorkommende regulatorische T-Zellen (Treg) dar, welche eine wichtige Rolle im Rahmen der Induktion und Aufrechterhaltung von immunologischer Toleranz spielen. Das Vorhandensein von Treg wäre eine mögliche Erklärung dafür, warum in der Vergangenheit nicht alle experimentellen und klinischen Beobachtungen in der Allergieforschung restlos durch ein Ungleichgewicht zwischen Th1- und Th2-Zellen erklärt werden konnten. Daher haben wir uns hier auch die Frage gestellt, ob sich $CD4^+$ T-Zellen, welche mit Genen transduziert werden, die mit der Treg Funktion assoziiert sind und die für allergen-spezifische TZR kodieren, zu Allergen-spezifischen Treg entwickeln würden. Mit Hilfe eines multicistronischen Vektors, der ein IRES-GFP Reporter Element trägt wurde der Bet v 1-spezifische TZR mit FOXP3, dem Hauptregulatorgen für die Treg Funktion kombiniert. Tatsächlich führte die retrovirale Transduktion von $CD4^+CD25^-$ T-Zellen aus dem peripheren Blut mit dem mulitcistronischen Expressionskonstrukt zur Ausbildung von TZR^+FOXP3^+ T-Zellen, welche einen regulatorischen Phänotyp und regulatorische Funktion aufwiesen. Die solcherart hergestellten Bet v 1-spezifischen TZR^+FOXP3^+ T-Zellen ließen sich sowohl durch Allergen-spezifische als auch durch polyklonale Stimulation nur schwer zum Wachstum anregen (hyporesponsiv), waren aber dazu im Stande, die Effektorfunktion und die Proliferation von Allergen-spezifischen T-Zellen in aktivierungsabhängiger Form zu hemmen.

Für die detaillierte Untersuchung von Allergen-spezifischen T-Zellen und um neue Therapiemodelle für humanrelevante Aero-Allergene *in vivo* zu etablieren, haben wir doppelt transgene Mäuse erzeugt, welche neben dem Art v 1-spezifischen TZR auch das dazugehörige humane Restriktionselement HLA-DR1 exprimieren. Im ersten Schritt wurde eine umfangreiche immunologische Charakterisierung der verschiedenen Maus-Linien vorgenommen (Wildtyp, Einzeltransgen, Doppelttransgen). Die Untersuchungen haben klar gezeigt, dass Splenozyten von doppelt transgenen Mäusen (TZR/DR1) spezifisch sowohl mit dem immun-dominanten Art v $1_{25\text{-}36}$-Peptid als auch mit dem Art v 1-Gesamtprotein reagierten. Im Gegensatz dazu zeigten weder die Splenozyten von Wildtyp-Mäusen noch jene von einfach transgenen Mäusen (TZR oder DR1) Wachstum nach Inkubation mit Art v 1 Protein oder Peptid. Ähnliche Ergebnisse wurden für die Zytokin-Produktion beobachtet. Die *in vivo*-Evaluierung der verschiedenen Maus-Gruppen hat nun gezeigt, dass nach aerogener Sensibilisierung mit dem Allergen nur doppelt transgene Mäuse eine erhöhte Atemwegs-Reaktivität nach neuerlicher Exposition mit Allergen aufwiesen. Tatsächlich waren drei

aufeinander folgende Expositionen mit dem vernebelten Allergen ausreichend, um eine stark veränderte Atemwegsfunktion in den TZR/DR1 Mäusen zu erzielen, welche von starken Entzündungszeichen der Atemwege und der Lunge begleitet wurden. Die solcherart behandelten Tiere wiesen eine massive Akkumulation von inflammatorischen Immunzellen in den bronchoalveolären Lavageflüssigkeiten auf, welche von starken peribronchiolären und perivaskulären entzündlichen Infiltraten der Lungen begleitet wurden. Trotz der starken pathologischen Veränderungen, war in diesen Tieren keine Allergen-spezifischen Immunglobuline (weder IgE noch IgG) nachweisbar.

Humane Allergen-spezifische TZR stellen vielversprechende Werkzeuge zur Entwicklung von neuartigen immunmodulatorischen Strategien in der Allergieforschung dar. In diesem Sinne, werden die in dieser Arbeit erstmals hergestellten human-relevanten Allergiemodelle einen entscheidenden Beitrag zum besseren Verständnis von allergischen Erkrankungen und zur Entwicklung effizienterer Therapien leisten.

Structure of the thesis

The section **Introduction** gives a short overview on the cascade of events leading to allergen-sensitization and the role of T-lymphocytes and their subsets in allergic disease. It includes present knowledge of the immunomodulatory capacity of T-cell responses, *in vivo*-models frequently used in allergy research and formulates the aim of the thesis.

Chapter I comprises a detailed review of "*Lymphocyte-based model systems in allergy research*". This review article describes common model systems, which allow to study distinct lymphocytes (B- and T-lymphocytes, Natural Killer cells and Natural Killer T-cells) for their contribution to allergic diseases and asthma. This review aritcle has been submitted to the *International Archives of Allergy and Immunology*.
Contribution: study of the literature, preparation of the manuscript; first authorship

Chapter II gives insights into the cloning and functional characterization of a human T-cell receptor specific for the major mugwort pollen allergen, Art v 1. The allergen-specific synapse was fully rebuilt *in vitro* by cloning the corresponding HLA-molecules, allergenic peptides, costimulatory molecules and the TCR α and β chains. The transgenic TCR was successfully transferred into the human T-cell line Jurkat and peripheral blood T-cells of non-allergic individuals and retained the allergen-specific and co-stimulation-dependent reaction pattern of the original T-cell clone when stimulated with artificial APC. This work was published in the *J Allergy Clin Immunol 2008;121:64-71*
Contribution: retroviral transduction of Jurkat T-cells and measurement of Jurkat T-cell activation by luciferase assays, contribution to the analysis of data and preparation of the manuscript; co-authorship

Chapter III: assesses the feasibility of host-encoded reporters for general and quantitative detection of enveloped viruses. Host cells expressing GPI-anchored alkaline phosphatase were analyzed for the detection, isolation and purification of different enveloped viruses including RSV, HSV1, HIV and human coronavirus 229E.
Contribution: biochemical and functional evaluation of GPI-anchored alkaline phospatase on human producer cells and MoMLV-induced virus-like particles, contribution to the analysis of data and preparation of the manuscript; co-authorship

Chapter IV describes the cloning and application of a birch pollen (Bet v 1)-specific T-cell receptor in cross-reactivity studies and immunomodulation of allergen-specific T-cell responses. TCR tg Jurkat T-cells were analyzed for their potential to cross-react with Bet v 1-related food and pollen allergens presented by monocyte-derived DC. aAPC co-expressing membrane-bound human IL-12 polarized allergen-specific TCR tg T-cells towards the Th1-phenotype with predominant IFN-γ secretion. In co-culture experiments Th1-polarized TCR tg T-cells were potent enough to inhibit the effector function of Th2-differentiated Bet v 1-specific T-cells. The resulting manuscript was published in the *J Immunol 2011;187:4077-87*
Contribution: TCR cloning, retroviral transduction of Jurkat T-cells, establishment and identification of TCR-tg Jurkat T-cell clones, measurement of Jurkat T-cell activation by luciferase assays, generation of monocyte-derived DC, isolation of peripheral blood T-cells from allergic and non-allergic individuals, retroviral transduction of peripheral blood T-cells, T-cell proliferation, polarization and co-culture assays, analyses of data and preparation of the manuscript; first authorship

Chapter V addresses the feasibility to genetically engineer allergen-specific regulatory T-cells upon co-introduction of the Bet v 1-specific TCR together with the transcription factor FOXP3 using a multicistronic expression strategy. Resulting human TCR/FOXP3 transgenic T-cells were characterized for their phenotype, proliferation potency, cytokine profile and their potential to suppress allergen-specific effector T-cells after polyclonal and allergen-specific activation. The results were published in the *J Allergy Clin Immunol 2011;127:238-45.*
Contribution: Bet v 1-specific TCR cloning, contribution to generation of the multicistronic expression cassette, isolation of peripheral blood T-cells, retroviral transduction of T-cells, contribution to analysis of data and preparation of the manuscript; co-authorship

Chapter VI: describes the generation of allergy mice, i.e. of double transgenic mice expressing a human Art v 1-specific TCR and the corresponding human restriction element HLA-DR1. In this model the human-relevant allergen is presented by the primary human restriction element for Art v 1, i.e. HLA-DR1, and is recognized by a human TCR. Double transgenic mice were characterized for transgene expression and leukocyte distribution by immunophenotyping of spleen, thymus and peripheral blood and analyzed for antigen-specific T-cell reactivity *in vitro* and *in vivo*. *In vivo*, the effects of the transgenes on the T-cell phenotype, specific antibody production and lung function upon mild aerosol provocation

with specific allergen are described. The manuscript is currently in preparation for publication.
Contribution: creation and evaluation of transgene expression for pronucleus injection, genotyping and breeding of trangenic mouse lines, supervision of immunophenotyping, performance of proliferation assays, *in vitro*-polarization studies, immunization and challenge of mice, assessment of antibody production, lung function and lung histology, analysis of data and preparation of the manuscript; first authorship

The final chapter **Conclusion and Synopsis** summarizes and discusses the most important findings and gives a synopsis of the research topics of this thesis.

Introduction

Allergic reactions are characterized by an excessive immune response to normally harmless environmental antigens (1), so called allergens, and can lead to several symptomatic manifestations including rhinoconjunctivitis, sinusitis, certain forms of asthma, food allergy, atopic dermatitis, angioedema and urticaria, as well as life-threatening anaphylaxis either occurring alone or in various combinations (2). Over the last 50 years asthma and related allergic diseases have continued to increase in association with the Western lifestyle (3). Worldwide asthma affects about 300 million people. Moreover, up to 250 million people suffer from food allergies and 400 millions from rhinitis, whereas one-tenth of the worldwide population is estimated to suffer from drug-allergies (4). Consequently, allergic diseases represent complex disorders varying from patient to patient (2) and putting enormous burden on the economics of the affected countries. Accordingly, new model systems which allow to study the human disease in great detail are urgently warranted to help to better understand the human disease and to develop novel and efficient therapeutic strategies for their prevention and cure.

Allergic sensitization

Development of allergic diseases like bronchial asthma is most likely a multifactorial process, which is governed on the one hand by the interactions of allergens with the host immune system, on the other by infections and other environmental stimuli as well as genetically determined parameters (e.g. barrier function). Therefore, the onset of allergic asthma is probably dependent on the coincidence of several (immunological) events that result eventually in a pathological outcome. The first step in the sensitization process takes place when the allergen reaches the first barrier represented by the epithelial layer of the respiratory tract. These interactions between allergens and epithelial cells have an important influence on the way the immune system responds to the allergens, either by leading to the induction of tolerance or alternatively by inducing allergic sensitization. The airway epithelium and the submucosa are in close contact with macrophages and professional antigen-presenting cells (APC) known as dendritic cells (DC) (5). DC are characterized by their expression of innate immune receptors and the ability to process and present antigens via the major histocompatibility complexes (MHC) class I and II to T-cells. After allergen-encounter resident tissue cells in concert with hematopoietic cells usually induce a tolerogenic immune response in which migratory DC instruct allergen-specific naïve T-cells to differentiate into

inducible Foxp3$^+$ Treg (iTreg) (6, 7). These regulatory T-cells then counter-act the subsequent processes of allergic sensitization. In contrast, in the pathological situation the augmented exposure of the respiratory epithelium to allergens and other environmental factors leads to the differentiation of inflammatory DC (8) that induce the differentiation of allergy-orchestrating T helper 2 (Th2)-cells and the secretion of Th2 cytokines. These factors are responsible for the induction of IgE production by B-cells through class switching to the immunoglobulin ε heavy chain (interleukin (IL)-4 and 13), T-cell survival (regulated by IL-4), mast cell differentiation (IL-3, IL-9 and IL-13), eosinophil maturation and survival (IL-3, IL-5 and GM-CSF) and basophil recruitment (IL-3 and GM-CSF) (9).

However, the exact mechanisms that drive the differentiation of naïve T-cells into allergen-specific Th2-cells are not completely understood as of today. It is already well accepted that the secretion of IL-4 induces the activation of the transcription factors STAT6 and GATA3, which are necessary for the induction of the Th2-phenotype. The initial/original source of this Th2-skewing cytokine remains however still enigmatic. One possible explanation would be that the Th2-progam is automatically switched on when a strong Th1- or Th17 response is missing while another hypothesis postulates that weak MHC class II T-cell receptor (TCR) interactions favor IL-4 production from naïve CD4$^+$ T-cells (10). The physiological importance of the TCR signal strength-concept as a possible explanation for the generation of Th2-prone allergy perpetuating T-cells is, however still controversial (11). It has been shown that a weak TCR signal for the induction of a Th2 response can be elicited indirectly by manipulation of allergen-presenting DC. Of note, the T2-ribonuclease (Omega-1) from *Schistosoma manosoni* eggs, which suppresses IL-12 production of DC, reduces, in fact, the overall TCR-mediated allergen-specific signals for naive CD4$^+$ T-cells. This results in Th2 differentiation although sufficient antigen would be available (12, 13).

Paradoxically, allergic sensitization does not mandatorily lead to the clear-cut burst of allergic diseases, indicating that not all sensitized individuals become necessarily affected by allergic diseases. In addition, not all allergic patients develop allergic asthma (14), which is mediated by the interplay between the structural cells of the airways, the lung and inflammatory cells.

Transcription factors and T-cell plasticity

The induction of specific functional T-cell subsets is characterized by the predominant activation of key transcription factors regulating the expression of the distinct cytokine genes and regulatory genes. The transcription factor T-bet (T-box-containing protein) directs the induction of the differentiation program for the T helper 1-cell subtype (Th1) and initiates the

production of interferon-γ (IFN-γ) responsible for the defense against intracellular pathogens (15). Whereas the phosphorylation of the transcription factor GATA-3 (GATA binding protein 3) leads to the differentiation of the Th2 cell linage and the secretion of their signature cytokines IL-3, IL-4, IL-5, IL-9 and IL-13 required for efficient immune responses against helminths and extracellular pathogens (16).

Although these cell types were found to be irreversibly committed, plasticity of T cell subsets has been described (reviewed in (17)). *Hegazy et al.* addressed the plasticity of the Th2 cell subset. It was demonstrated that $GATA3^{+}$ Th2 cells could be reprogrammed upon TCR triggering in the context of a LCMV-infection *in vivo* as well as by the combination of type I and type II interferons in the presence of IL-12 *in vitro*. Th2 cell re-programming was dependent on T-bet and resulted in the co-expression of GATA-3 and T-bet as well as Th2 and Th1 cytokines (Th2+1 phenotype) (18).

Th1/Th2 dichotomy

Since the 1980s allergic diseases were explained according to the Th1/Th2 dichotomy concept, which postulates that allergy is a Th2-mediated disease due to an imbalance of the immune system towards Th2 responses, which might be amenable to regulation by counteracting IFN-γ secreting Th1 cells (19). Although numerous studies support this concept, especially results from mouse models weakened the general validity of this dogma. In fact, adoptive transfer experiments of *in vitro*-polarized T-cells showed that Th1 cells and their key cytokine IFN-γ can in fact worsen lung inflammation, do not have therapeutic effects on airway hyperreactivity and fail to counter-regulate Th2 cell responses (20-23). In contrast, others demonstrated that the transfer of Th1-polarized T-cell clones is able to attenuate the asthmatic phenotype in mice (24). Co-transfer of both T cell subsets demonstrated that allergen-specific Th1 cells are more capable of down-regulating asthma-related pathology like mucus production when compared to exclusive Th2 cell transfer, and this inhibition is mediated by IFN-γ (25). Multiple parameters possibly influencing the outcome of such transfer models including among other explanations the administration of differentially polarized T-cells as well as timing and routing of the transfer.

Th1-skewing conditions

Another approach to promote an 'anti-allergic' Th1-response is the use of Th1-skewing factors such as cytokines or bacteria *in vivo*. AHR as well as eosinophilic lung inflammation were inhibited by the administration of IL-12, IL-18 or bacterially-derived CpG-DNA in several

reports (26-30). The Th1-associated cytokine IL-12 is a heterodimeric protein composed of the covalently linked 35 kDa L-chain (p35) and the 40 kDa H-chain (p40) secreted by activated monocytes, macrophages, neutrophilic granulocytes and DC (31). This cytokine has been described to be a major factor capable of inducing a Th1-phenotype (32). Furthermore, IL-12 has been demonstrated to be able to repolarize Th2 cells towards the Th0 or Th1 phenotype (33). Intraperitoneal injection of IL-12 during allergic sensitization with the major house dust mite allergen Der p 1 significantly reduced allergen-specific IgE and IgG1 production whereas the IgG2a production was elevated in a murine model of asthma (34). Moreover, treatment with IL-12 inhibited airway inflammation by preventing allergen-induced infiltration of eosionophils into bronchoalveolar tissues (34). IL-12 might act as a potent immunomodulatory cytokine in allergic inflammation, however its mode of action strongly depends on the timing of administration. *In vivo* ragweed-induced airway inflammation was reduced by IL-12 administration during allergen-sensitization (early treatment) or a combination of early and late dosage (during allergen challenge). In contrast, late administration of IL-12 had no significant effects in this experimental model on the development of asthma symptoms (35). Blocking of endogenous IL-12 with neutralizing antibodies in the sensitization phase enforced airway hyperreactivity (AHR), bronchial eosinophilia, Th2 cytokine and allergen-specific IgE production. In contrast, IL-12 neutralization in the effector phase, after repeated airway challenges, alleviated the observed asthma symptoms indicating that endogenous IL-12 plays a protective role in allergen sensitization but has an aggravating effect during ongoing allergen exposure (36). To avoid the risk of Th1-driven side effects, a combination of adenovirus-mediated IL-12 and IL-10 gene therapy was tested in a murine model of allergic inflammation (37). Low dosage of this cytokine combination exhibited a synergistic therapeutic effect and was able to inhibit the development of AHR with reduced pulmonary infiltration by eosinophils and neutrophils (37). Of note, the fold of an allergenic protein has the potential to shape the outcome of an immune response. Along those lines, BM4 a folding derivative of the Bet v 1 protein induced an altered immune response compared to the wild type form of the major birch pollen allergen. It was demonstrated that BM4 induced increased levels of T-cell proliferation of human PBMC. Moreover, immunization of mice with Bet v 1 adsorbed to aluminum hydroxide elicited a strong Th2 polarization, whereas BM4 immunization additionally recruited Th1 cells (38). In contrast to conventional Th1 cells, which can contribute to the exacerbation of allergic airway inflammation in sensitized mice, Th1-like regulatory cells (Th1-like TR) producing IL-10 and IFN-γ have been described to suppress AHR and allergic

inflammation. These cells arise from naive $CD4^+CD25^-$ T cells within a Th1-biased environment and are induced by IL-10 and IL-12 producing $CD8a^+$ DC. Th1-like TR are characterized by expression of Foxp3 and the master regulator of Th1-cells, T-bet, indicating their dual commitment (39).

Allergen-specific regulatory T-cells

In addition, another subset of $CD4^+$ T-cells, naturally occurring regulatory T-cells (nTreg) plays an important role in immune regulation and in the induction and maintenance of immune tolerance. Regulatory T-cells helped to explain several observations, which did not fit into the Th1/Th2 balance concept (40). Indeed, allergic disorders might be partially attributed to a dysfunction of regulatory T-cells. Thus induction and/or propagation of Treg might be a promising therapeutic approach in allergic patients.

In murine studies, $CD4^+CD25^+$ T-cells were reported to suppress differentiation and cytokine production of Th2 cells (41) and IgE synthesis (42). Moreover, with the help of inhaled allergen challenge models it was demonstrated that transfer of $CD4^+CD25^+$ T-cells is able to suppress airway inflammation (43, 44). Similarly, repeated allergen exposures at low doses prior to Th2 sensitization induced tolerance (45), which was attributed to the induction of $Foxp3^+$ regulatory T-cells (46). While the immunomodulatory effect of nTreg on Th2 responses and airway inflammation is well defined, a beneficial effect on AHR was not observed in all studies (43, 44, 46, 47).

Of note, pulmonary DC from mice exposed to respiratory antigen produced IL-10, which in turn stimulated the development of IL-10 producing $CD4^+$ T regulatory 1-like cells (Tr1) thereby mediating tolerance to the respiratory allergen (48). The importance of Treg in allergy was also confirmed by studies of patients undergoing specific immunotherapy (SIT). SIT comprises the repetitive and dose-increasing administration of purified allergens to allergic individuals, thereby leading to the induction of tolerance and clinical de-sensitization of the patients and induction of blocking antibodies. Induction/expansion of Treg after SIT to a variety of allergens has been reported (49-54). Treg seem to play a multifunctional role in the regulation of allergy including the suppression of Th2 cells and allergen-specific IgE production, induction of production of protective IgG4 antibodies, down-modulation of mast cell, eosinophil and basophil function (reviewed in (55)). However, whether all these effects are mediated by $CD4^+Foxp3^+$ naturally-occurring Treg is still a controversial topic (49, 50, 56).

T-cell receptor transfer studies

During the late 1980ies the first transfer experiments with αβ-TCR were performed in order to generate large numbers of antigen-specific T-cells (57) to facilitate studies on tolerance induction and selection. Subsequently, different TCR-transgenic models have been developed to study aspects of T-cell development (58, 59) and the role of T-cells in autoimmune (60, 61) as well as infectious diseases (62).

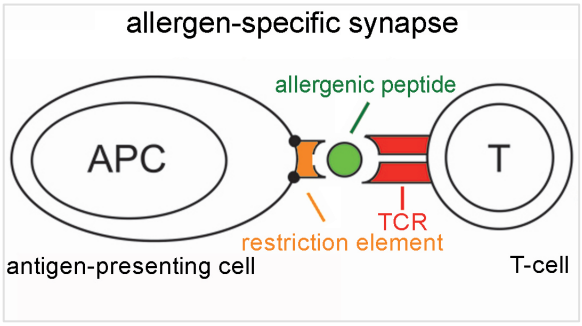

Figure 1. Illustration of the allergen-specific synapse formed between an antigen-presenting cell and a T-cell.

To contribute to the detailed analysis of the T-cell side of the allergen-specific synapse, formed between an antigen-presenting cell, harboring the restriction elements presenting the allergenic peptides and T-cells expressing the corresponding TCR (Figure 1), I here cloned and functionally characterized TCR recognizing human-relevant allergens *in vitro* and *in vivo*. TCR transfer allows the generation of high numbers of allergen-specific T-cells and to develop novel strategies for immunomodulation of allergen-specific T-cell responses *in vitro*.

Model allergens

To study the precise role of allergen-specific T-cells in allergic diseases we have choosen two human-relevant aeroallergens.

The major mugwort pollen allergen from *Artemisia vulgaris,* Art v 1, harbors key features of an ideal respiratory model allergen. Mugwort is not confined to the European temperate climate zone, but is also spread over North America and parts of Asia (63) and is one of the main causes of hay fever in late summer and autumn (64). Moreover, sensitization to mugwort mainly occurs to Art v 1, the major mugwort pollen allergen and the presentation of its immunodominant T-cell epitope, Art v $1_{25\text{-}36}$, is highly restricted to HLA-DRB1*01 (65, 66). Therefore, a TCR, which recognizes Art v $1_{25\text{-}36}$ in the context of HLA-DR1, was cloned and further characterized. The Art v 1-specific TCR is composed of a TRAV17 and a TRBV18 chain. In addition, birch pollen are one of the main causes of pollinosis in the Northern Hemisphere from spring to early summer (67). In fact, more than 95% of birch

pollen-allergic individuals mount an IgE antibody response to Bet v 1, the major birch pollen allergen from *Betula verrucosa*. T-cells from these patients preferentially recognize the immunodominant epitope of Bet v $1_{142-156}$ located at the C-terminus of the allergen as well as the other frequently recognized major epitopes Bet v 1_{4-15} (N-terminal) and Bet v $1_{112-123}$ (intermediate). In the following studies a focus was put on the TRAV6/TRBV20 T-cell receptor, which recognizes the immunodominant epitope at the C-terminus (Bet v $1_{142-156}$) of the Bet v 1 molecule.

Detection and quantification of enveloped viruses with host encoded reporter proteins

In previous projects we used the modular nature of virus-like particles (VLP) to generate artificial antigen-presenting platforms for T-cell activation or anergization (68-70). VLP can be decorated with HLA/peptide complexes, corresponding HLA-molecules, and co-stimulatory molecules by modification of the respective molecules with a GPI-anchor acceptor sequence. The modular decoration of VLP allows shaping of the VLP-induced immune response. Allergen-presenting VLP expressing the co-stimulatory molecule CD80 induced massive expansion of TCR tg T-cells, whereas allergen-presenting VLP lacking expression of co-stimulatory molecules led to anergic T-cell responses (70). Moreover, we demonstrated the usefulness of fluorescent VLP, which can be decorated with biologically active multi-subunit immune-receptors. With the help of fluorescent VLP we were able to target and visualize immune-receptor/ligand interactions (71).

Using our VLP technology, we investigated in a side project the possibility to use host encoded reporter proteins for the detection and purification of enveloped viruses. Enveloped viruses constitute a large group of viruses including *e.g.* human immunodeficiency virus (HIV), hepatitis C virus (HCV) and influenza virus. Genome-wide high-throughput screening methods facilitated the identification of host factors that regulate virus replication and/or virus budding (72-77). Virus genomes have been engineered by the introduction of reporter sequences such as green fluorescent protein or luciferase to measure virus activities such as replication (73, 78). Furthermore, detection of virus particles was facilitated by fusion of virus components to reporter genes (e.g. chloramphenicol transferase (79, 80), luciferase (81, 82) or alkaline phosphatase (83)). However, several reports indicated that the incorporation of reporter enzymes possibly impairs virus infectivity and budding (84, 85). Of note, the membrane of enveloped viruses is derived from infected host cells, and thus contains the lipid bilayer and membrane proteins of host cells on the virus particle surface. Virus budding takes place in lipid rafts and as a consequence lipid-raft resident host cell molecules are highly

susceptible for incorporation into viral membranes thereby contributing to the phenomenon known as pseudotyping (86). Pseudotyping is observed after mixed infection of a host cell with two different enveloped viruses and results in the production of virus particles that contain the genome of one virus that is packaged with its own envelope protein together with the envelope protein of the second virus (86-90). Therefore, enzymatic reporter activities can be conveniently targeted to the virus membrane upon modification of the reporter protein with a lipid raft anchor. In this study it was shown that host cells expressing glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase as reporter molecule is suitable to detect the formation of diverse enveloped viruses such as RSV, HSV1, HIV and human coronavirus 229E. Therefore, this strategy can be reliably applied for the isolation and characterization of known and unknown enveloped viruses and might thus become a valuable tool for quick virus detection.

Allergen-specific TCR tg T-cells

Transfer of the Bet v 1-specific TCR into peripheral blood (PB) T-lymphocytes revealed that the TCR retained its original fine specificity as well as its ability to cross-react with Bet v 1-related food and pollen allergens. Thus the TCR tg T-cells resembled the essential features of the original T-cell clone. Next to T-cell clones and T-cell lines (91) TCR tg T-cells are useful tools for the preclinical evaluation of hypoallergens (92), allergenic variants and allergen vaccines.

Two strategies of how to alter the imbalance in favor a Th2 response using the Bet v 1-specific TCR *in vitro* were followed. It was tested if it is possible to promote an allergen-specific Th1-response with the use of Th1-skewing factors like cytokines or to generate allergen-specific T-cells with regulatory function to counteract disease-mediating Th2 cell responses.

First, the capacity of aAPC co-expressing membrane-bound human IL-12 to strictly polarize TCR tg T cells towards the Th1 phenotype, identified by production of high levels of their signature cytokine IFN-γ has been analyzed and whether such Th1-polarized T-cells would have the potential to suppress the effector function of Bet v 1-specific Th2-differentiated T-cells in co-culture experiments (Figure 2).

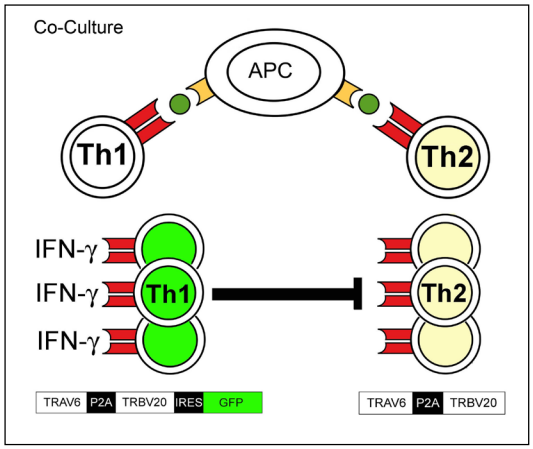

Figure 2. In co-culture experiments TCR tg Th1-like T-cells are able to inhibit the effector function of Bet v 1-specific Th2-cells after antigen-specific re-stimulation.

In the second approach the Bet v 1-specific TCR was used to generate allergen-specific regulatory T-cells by genetic engineering. In the past it has been shown that overexpression of the transcription factor FOXP3 in $CD4^+$ T-cells is sufficient to induce naturally-occurring Treg (nTreg) phenotype and corresponding function (93, 94). To fully exert their suppressive potential nTreg have to become activated via their CD3/TCR complex (95-97). Therefore, it was tested whether co-expression of the Bet v 1-specific TCR along with the transcription factor FOXP3 would result in the generation of Bet v 1-specific Treg. The TCR being important for ensuring T-cell specificity and the FOXP3 transcription factor being responsible for initiating and perpetuating the regulatory program.

Murine models of allergic disease

Mouse models have proven to be valuable tools in allergy and asthma research and have helped to understand the principal mechanisms and mediators that contribute to the establishment of allergic asthma. However, not all results obtained in mouse models resemble the human pathology and therefore cannot be directly translated into concepts for human therapies. A lot of efforts are undertaken to improve current mouse models, in order to establish novel model systems that match the human situation as close as possible. Since allergic asthma represents a chronic disease, the frequently used acute ovalbumin models (reviewed in (98)) get more and more replaced by newly emerging chronic models and models utilizing human relevant allergens. The main focus is on the induction of the hallmarks of chronic allergic asthma such as persisting eosinophilic inflammation and airway remodeling. Along those lines, several non-OVA-based mouse models have been established, which apart from differences in the applied model allergens, also take advantage of alternative

adjuvants, variations in the duration of provocations and the genetic backgrounds of the strains under investigation.

In fact, several mouse models for the investigation of allergic diseases relying on recombinant allergens (99, 100) or model antigens (101, 102) have been established in the past. Since T-cells play a key role in the development of allergic diseases, several of these models are based on transgenic TCR recognizing model allergens. Among them are the frequently used DO11.10 TCR transgenic mice (BALB/c background) (101-103), which express a murine alpha/beta TCR (104) specific for chicken ovalbumin ($OVA_{323-339}$) in the context of murine MHC class II ($I\text{-}A^d$) (103). Later a comparable model has been established on the C57BL/6 background and termed OT-II mice (105). Although OVA is often used as model allergen for the induction of airway inflammation in mice (106), this antigen is only rarely implicated to be the cause for human asthma (107). In addition, other allergens with more clinical relevance in allergy such as house dust mite (HDM) (108), cockroach extracts (109) or birch and grass pollen allergens are in experimental use.

Humanized mouse models for allergy research

Humanized mice comprise immunodeficient mice engrafted with functional human immune cells or immunocompetent mice expressing human transgenes.

Along those lines, SCID mice are a valuable tool to study human-relevant aspects of immunopathology. Since these mice are not able to reject allogeneic or xenogeneic transplants, transferred human peripheral blood mononuclear cells (PBMC) can survive in these mice for prolonged periods of time (110, 111). In one model, human PBMC from Der p 1 sensitive allergic individuals were *i.p.* injected into SCID mice and challenged two weeks after cell transfer exclusively via the airways in the absence of adjuvant. Inhalation of the allergen resulted in the detection of allergen-specific IgE antibodies and an inflammatory response (112). Co-injection of human PBMC from atopic donors and the allergen into SCID mice induced comparable allergen-specific IgE-levels as in the donor-sera at the time of transfer. Activation of the transferred T-cells was dependent on the presence of human APC in the PBMC SCID model, as the absence of human APC caused a state of T cell unresponsiveness to the allergen (113). In another work human PBMC from non-allergic individuals were intratracheally transferred into SCID mice (114). These mice developed AHR following aerosol exposure after systemic sensitization with Der p 1 caused by lung-infiltrating human IL-4- and IL-5-producing Th2-cells (114). To study how T-cells get

attracted to inflamed airways, SCID mice were reconstituted with PBMC from patients with HDM allergy before allergen challenge. Pretreatment with a CCR4-blocking antibody abolished airway inflammation and AHR, indicating that the DC-derived chemokines CCL17 and CCL22 are responsible for the recruitment of allergen-specific Th2 cells (115). Moreover, systemic administration of human-induced pluripotent stem cells derived mesenchymal stem cells (iPSC-MSC) prior to allergen-challenge prevented airway inflammation in BALB/c mice. Similar therapeutic effects were observed when iPSC-MSC were transferred before allergen sensitization (116).

So far, only a restricted number of humanized TCR tg mouse models have been established to study immune-mediated diseases in more detail, e.g those for the analysis of the T cells' role in autoimmune diseases such as multiple sclerosis (117) and rheumatoid arthritis (118). However, humanized allergy mouse models, based on alpha/beta TCRs specific for human relevant respiratory allergens to investigate the contribution of allergen-specific T-lymphocytes to the pathogenesis of allergic diseases, were still missing. *Jarman et al.* described the establishment of transgenic mice expressing a murine αβ-TCR recognizing the immunodominant epitope of Der p 1, a prominent inhalant indoor allergen affecting a large number of allergic individuals. Two intratracheal instillations of the allergen were sufficient to induce asthmatic features in these mice including accumulation of eosinophiles and lymphocytes in the airways and the lung as well as goblet cell hyperplasia and mucus production (119). Since also this model entirely relies on murine receptors, it remains to be shown whether the involved affinities of the murine receptors in these mice as well as the recognized allergenic peptides are comparable to the human situation.

To circumvent this problem and to form the basis for more human-relevant *in vivo* studies we first cloned and functionally evaluated an Art v 1-specific TCR after transfer into human T-lymphocytes in *vitro* (120). I here demonstrate that allergen-specific TCR tg T cells represent an excellent tool to study the immunomodulatory capacity of allergen-specific T cells (70, 121, 122). Moreover, I have proven the feasibility of creating double tg mice expressing a human TCR specific for the immunodominant epitope of the major mugwort (*Artemisia vulgaris)* pollen allergen Art v 1 in the context of the human restriction element HLA-DR1 (Figure 3). This novel humanized allergy model, in which all components of the allergen-specific synapse are well defined, enables us now to analyze in more detail the relevant T-cell dependent pathways by which allergic diseases are influenced *in vivo* and will provide important insights into the pathophysiology of allergic diseases and their possible cure in the future.

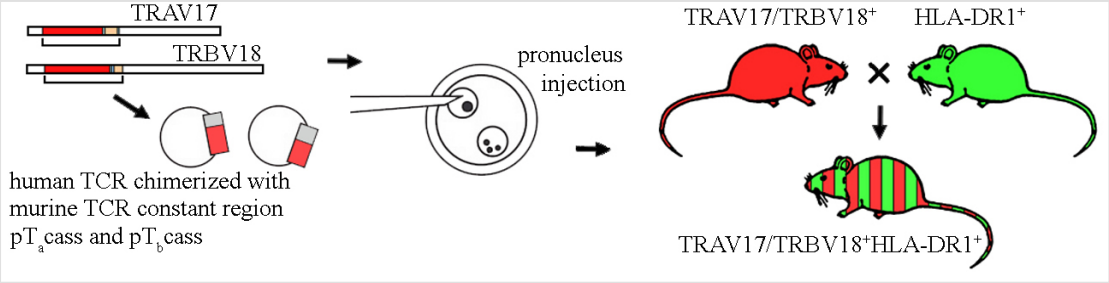

Figure 3. Establishment of humanized mice expressing a human Art v 1-specific TCR and HLA-DR1*01:01

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Chapter I

Lymphocyte-based model-systems for allergy research

Running head: Allergy model-systems

Alina Neunkirchner,[1,2] Klaus G. Schmetterer[2,3] and Winfried F. Pickl[1,2,*]

[1]Christian Doppler Laboratory for Immunomodulation, Vienna, Austria. [2]Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria; [3]Department of Laboratory Medicine, Medical University of Vienna, Austria;

* Corresponding author. Mailing address: Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Borschkegasse 8A, 1090 Vienna, Austria. Phone: (+431) 40160 33245. Fax: (+431) 40160 933201. E-mail: winfried.pickl@meduniwien.ac.at.

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Abstract

During the last decades a multitude of studies applying distinct *in vitro* and *in vivo* model systems contributed largely to the better understanding of the initiation and regulation of inflammatory processes leading to allergic diseases and/or asthma. Over the years it has become more and more evident that among lymphocytes not only IgE-producing B-cells and allergy-orchestrating $CD4^+$ Th2-cells but also cytotoxic $CD8^+$ T-cells, γδ-T-cells as well as natural killer (NK) cells and NK T-cells (NKT) and various subtypes thereof, as well as regulatory T-cells might be critically involved in the development of allergic diseases. In this review we provide an overview on human and mouse lymphocyte-based model systems used in allergy research. We address established model systems for the analysis of specific contributions of particular lymphocytes and their subsets *in vitro* and *in vivo*. Approaches for the investigation of effector cell activation/function, polarization conditions as well as depletion methods and adoptive transfer models along with gene knockout and transgenes utilizing different model allergens are explained and discussed. In addition novel attempts for the establishment of humanized mouse models for allergy research are described.

List of abbreviations

AHR	airway hyperreactivity
APC	antigen presenting cell
BAL	bronchoalveolar lavage
CD	cluster of differentiation
CRL	complement receptor positive cells
CTL	cytotoxic T-lymphocytes
DC	dendritic cells
GPI	glycosyl phosphatidyl inositol (anchor)
HEK-293	human embryonic kidney cells
HDM	house dust mite
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LDC	lung dendritic cell
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MSC	mesenchymal stem cells
NK	Natural killer cell
NKT	Natural killer T-cell
OVA	ovalbumin
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
Penh	enhanced pause
RAG	recombination activation gene
SCID	severe combined immunodeficiency
TCC	T-cell clone
TCR	T-cell receptor
T_{eff}	effector T-cell
tg	transgenic
TLR	Toll-like receptor
Th	T-helper cell

T_{MEM}	memory T-cell
Treg	regulatory T-cell
FOXP3	forkhead box protein 3
WBP	whole body plethysmograph

Introduction

IgE-associated immune reactions (allergies) affect more than 25% of individuals within our population. The manifestations of IgE-associated allergies include allergic rhinitis, conjunctivitis and dermatitis but also severe organ-specific forms such as asthma, food allergy and life-threatening anaphylaxis. Today, we speak of an allergy-epidemic affecting not only the highly-developed countries of the world but also putting an ever-growing toll on developing countries. The prevalence of allergic diseases has constantly increased over the last decades posing an enormous economic burden onto health care systems especially also due to the chronicity of the disease. It is well established that allergic diseases develop in early childhood and if not diagnosed and treated properly progress to severe life-threatening manifestations. Thus, there is a strong need for a holistic approach towards allergy research and treatment. While clinically approved treatments can be readily applied in human individuals, our ethical codex is not compatible with applying experimental therapies or even tests in populations of affected (and for control purposes non-affected) individuals.

Nevertheless, in order to ensure progress in the understanding of IgE-associated diseases and to formulate novel hypotheses for therapies but also to develop new therapeutics - and this is true and valid for the entire biomedical field - alternative, ethically appropriate test systems and evaluation methods must be constantly further developed. Such systems might still rely on human immune cells or entire parts of target tissues or single cell suspensions thereof, however, they are not being evaluated and tested in the bodily context but *in vitro*. The remaining ethical issues, related to the sampling of tissues and blood cells, can be settled much more straightforward and are covered by societal and institutional regulations.

In vitro test systems can however only represent surrogates when compared to the putative options experimental procedures in the affected whole organism could offer.

Nevertheless, reductionist *in vitro* test systems also harbor considerable advantages over complex *in vivo* systems: they are well accessible, simplified and amenable to easy manipulation. For instance, *in vitro* test systems allow to analyze specific aspects (functions) of a pre-specified (selected) cell type under standardized conditions, and provide the opportunity to address questions regarding, e.g. signaling programs or effector functions in great detail and with the highest accuracy. Accordingly, several important *in vitro* test systems for allergy research including cells of the priming and/or the effector phase of allergic diseases such as T- and B-lymphocytes, antigen-presenting cells (DC, monocytes and macrophages), eosinophils, basophils and mast cells, as well as epithelial tissue-models (*i.e.*

mouth, nasal, and respiratory tract epithelial cell lines grown as monolayers) have been established. Moreover, antibody quantification and binding assays to determine overall IgE- as well as allergen-specific IgE-levels represent gold standards nowadays. Of note, also these assays have experienced dramatic innovations over the last decades, allowing for instance on one small glass-slide (allergy chip) the determination of dozens of molecular species against which a patient has mounted a specific IgE response.

To better understand the biological complexity of single molecular events and even of elaborate networks and to be able to integrate individual helper and effector mechanisms as well as tissue and organ functions within the mammalian body, the detailed study of entire model organisms is, however, a *conditio sine qua non*. Model organisms help to uncover pathomechanisms at the molecular, cellular and target organ level, which only become apparent in the diseased body but cannot be appreciated and followed in simplified *in vitro* systems. Pathophysiological manifestations like eczematous skin lesions, rhinoconjunctivitis or airway hyperresponsiveness along with increased bronchoconstriction and accelerated heart frequencies can only be explored in entire model organisms. This also concerns the initiation and maintenance of immune reactions but also therapeutic interventions intended to prevent or cure allergic diseases.

Rodents certainly belong to the preferred mammal species in experimental immunology research nowadays. Accordingly, model antigens and corresponding receptors, which have been established by addressing basic research questions, have been adapted to and extensively used in allergy research over the last decades. While these model systems represent valuable tools to address certain aspects of the allergic disease, they are at the same time limited. This is owed to the use of murine receptor-ligand pairs and to the sometimes very artificial and/or artificially-applied antigenic sources. As a result, as of today, only a limited set of human-relevant test systems, be they *in vitro* or *in vivo* systems, especially for testing the contribution of adaptive immune responses are available.

In this review article we give a comprehensive overview on *in vitro* and *in vivo* model systems (summarized in **Table I**) in allergy research with a special focus on the cells belonging to the adaptive immune system, i.e. B- and T-lymphocytes, and discuss new developments in this area.

B-cell model-systems for allergy research

B-cells belong to the lymphoid lineage of immune cells and are derived from precursor cells, which are resident in the bone marrow. Upon exposure to specific antigen, i.e. substances for which BCR have sufficiently high affinity to become cross-linked on the B-cell surface, individual B-cells become activated, start to proliferate and differentiate into antibody secreting cells. Only during secondary B-cellular responses (boosting), and depending on the surrounding milieu in which B-cell activation occurs, class-switch recombination takes place endowing the antibodies, with additional biological properties.

To evaluate the role of B-cells, either the relevance of allergen-specific IgE antibodies or their otherwise immunoregulatory features, several strategies have been followed ranging from the creation of simple *in vitro* systems to sophisticated *in vivo* models by the use of gene transfer technologies. The following section is intended to give an overview on classical and modern models to study B-cell function, with a special focus on allergy research.

In vitro B-cell model-systems

Spontaneous production of IgE *in vitro*

Studies on the spontaneous *in vitro* production of IgE have been spearheaded, among others, by *Sergio Romagnani* and colleagues, who showed in the early 1980ies that, in fact, peripheral blood mononuclear cells obtained from atopic individuals and when kept in culture for a period of seven days spontaneously release significant amounts of IgE into the culture supernatant [1,2] (**Table I**). IgE release was not due to passive release of cell-bound IgE from $Fc\varepsilon^{+}$ cell types but recognized as an active process, since addition of mitomycin B, a cytostatic agent with cross-linking activity for DNA, significantly reduced IgE secretion [2]. Moreover, the IgE-secreting cell population was inhibited by high numbers of autologous T-cells, which was probably due to the release of inhibitory factors.

Induced production of IgE *in vitro*

While low levels of IgE are produced spontaneously upon culture of PBMC derived from donors suffering from allergic diseases, more robust IgE secretion protocols allowing to study regulation of IgE production also in B-cells derived from healthy donors were established soon thereafter. Protocols for the induced production of IgE by human B-cells *in vitro* were, in fact, pioneered independently by the groups of *Vercelli* and *Banchereau* [3,4]. *Vercelli* and co-workers demonstrated that anti-CD40 monoclonal antibodies in the presence of IL-4 leads to vigorous IgE production in a T-cell independent manner [3]. IgE production was not only

observed with negatively purified B-cells, but also with positively, anti-CD20, enriched B-cells. IL-6 was demonstrated to play an important role within the system of IL-4-induced IgE production, since addition of neutralizing anti-IL-6 antibodies abrogated IgE production. That in fact class-switching takes place *in vitro* and is not the consequence of culturing minute amounts of B-cells which have already IgE-switched *in vivo*, was shown by sorting of B-cell for IgE negativity [3]. Along a similar path, *Banchereau* and co-workers further developed the anti-CD40 stimulation system by developing an improved culture system, relying on murine fibroblast L cells expressing FcγRII (CD32), which can be loaded with mAb binding to FcγRII via their Fc-portions [4], thus providing a strong cross-linking environment for the applied monoclonal antibodies. Decoration of CD32 positive L cells with anti-CD40 mAb and co-culturing them with purified human tonsillar B-cells induced vigorous B-cell proliferation. CD40-dependent B-cell proliferation could be co-stimulated up to 5-fold by the addition of exogenous IL-4, and additional synergistic effects were observed upon addition of IL-1a or IFN-γ. Co-stimulation of B-cell proliferation could be induced with IL-4 concentrations as low as 2.5 Units/ml, with an IC_{50} of approximately 10 Units/ml, and a maximum at around 100 Units/ml of IL-4. Most impressively, the combination of anti-CD40 with IL-4 led to an 30- to 40-fold increase in B-cell numbers after a culture period of three weeks, which could be further augmented to reach an increase of up to 80-fold of input B-cells numbers upon supplementation with IFN-γ. While anti-CD40 stimulated B-cells produced only minute amounts of immunoglobulins, the addition of IL-4 induced clear-cut levels of IgG and IgM, and most interestingly also of IgE. While the further addition of IL-2 at concentrations of around 50 Units/ml had little impact of IL-4/CD40 triggered IgE secretion it maximally induced IgM and IgA production. Importantly, IgA production was otherwise found to be very low in these cultures. In this system, both anti-CD40 and IL-4 represent a factor for long lasting B-cell growth [5], which is not the case when B-cells were stimulated via their BCR (via anti-IgM) instead of the CD40 molecule. Due to this unique feature, the CD32 L cell model was, in fact, compared to the *in vitro* equivalent of germinal centers within lymphnodes [6]. In summary, the model developed by the group of *Banchereau* represents a unique model to investigate the regulation of isotype switching of purified human B-cells *in vitro*. Both anti-CD40 systems allowed to define factors positively [3,4] or negatively [7] regulating IgE-production and were also the basis for generating human monoclonal antibodies against allergens for subsequent functional studies [8]. Moreover, *Fasler et al.* have shown that co-culture of allergen-specific T-cell clones, in contrast to bulk peripheral blood T-cells [1,2] with tonsillar B-cells induces the secretion of significant levels of IgE [9].

Cytokines as direct or indirect repressors of B-cell produced IgE

As demonstrated above, simple or more sophisticated co-culture systems were applied to evaluate the biological effects of cytokines on B-cell function and IgE production. Along those lines it was shown that supplementation of peripheral blood mononuclear cells with IL-4 and culturing them for 12 days induces substantial IgE synthesis, while the low-level spontaneous IFN-γ secretion was reduced to detection limit. Addition of e.g. IL-12 to PBMC induces a massive surge of IFN-γ production, and, most interestingly, dramatically inhibited the IL-4 induced IgE production. Of note, the inhibitory effect of IL-12 was already seen at the picogram-level and reached maximum action at concentrations around 0.5 ng/ml.

B-cell-specific depletion methods

Levy and Scott have shown in their landmark papers that complement receptor positive lymphocytes (CRL) can be efficiently and specifically depleted by *in vitro* treatment of splenocytes with cobra venom factor and fresh serum [10]. The principle used in this method relies on the activation of the terminal complement pathway from complement component 3 (C3) to C9 onwards. Since B-lymphocytes constitutively express C3-receptors they were referred to as 'complement receptor lymphocytes', CRL in those times. When incubated in cobra venom factor plus fresh serum, B-cells become opsonized by the abundantly generated proteolytic C3 fragments and subsequently lysed. Depletion of B-lymphocytes (CRL) was validated by introduction of treated spleen cell populations into irradiated hosts followed by immunization with sheep red blood cells. The (decreased) numbers of hemolytic plaque-forming cells obtained after seven days (Jerne plaque assay) was used as a measure for the degree of B-cell depletion. In fact, cobra venom treatment induced a three-fold reduction in plaque-forming cells while leaving the numbers of GvHD-inducing cells (mostly T-cells) unchanged. This rather crude way of removing B-cells from complex mixtures of leukocytes was further refined by using erythrocytes sensitized with antibodies and complement via the classical complement activation pathway. In this setting binding of erythrocytes to B-cells depends on the amount of deposited C3 on anti-erythrocyte antibodies [11]. Since those B-cells, which form rosettes with antibody and complement sensitized erythrocytes have a higher density, it was soon recognized that they can be conveniently removed from the whole 'gemisch' of leukocytes by simple density gradient centrifugation over albumin. This methodology, in addition to the then new observation that certain lymphocytes express

immunoglobulins on their surface helped to characterize the bone-marrow derived lymphocyte subset (B-cells) and to distinguish it from thymus-derived (T-cells) lymphocytes. Other rather unspecific ways to remove B-cells from complex mixtures of leukocytes included protocols relying on basic adhesive properties of B-lymphocytes to charged surfaces such as tissue culture plastic or nylon wool. Moreover, several groundbreaking methods were developed at that time, including magnetic particle based sorting of hematopoietic cell types and the fluorescence-based flow cytometry technology. With the advent of monoclonal antibody technology [12], novel unprecedented opportunities for the specific enrichment of B-cells, via positively selecting surface structures or by negative depletion of non-B-cells from complex mixtures of cells became feasible [13]. Today, either flow cytometry-based or magnetic particle-based sorting/depletion strategies are the state-of the art for B-cell enrichment/depletion [14-16].

In vivo B-cell model-systems

Systemic B- lymphocyte depletion with antisera and monoclonal antibodies

Until the advent of recombinant DNA technologies, antibody-based depletion strategies represented a useful tool to investigate the role and importance of distinct cell types for defined pathophysiological processes (**Table I**). Along those lines, B-cell depletion studies were also performed in allergy research [17-19]. The advantage of depletion strategies is their speed of action once appropriate reagents are available. Their obvious disadvantage is the possibility that either incomplete removal of the targeted cell types or even unintended removal of additional cell types might compromise the experiments. Since B-cells are professional antigen presenting cells they constitutively express high levels of MHC class II molecules on their surface. Accordingly, anti-MHC (anti-Ia) mAb were among the first to be used for the systemic depletion of B-lymphocytes [20]. In fact, after a single injection of 4 mg of anti-Ia antibody into SJL mice more than half of splenic B-cells were deleted. B-cell depletion reaches a maximum with almost complete removal of all B-cells after 5 days and complete recovery takes approximately three weeks. Even one tenth of that dose, i.e. 0.4 mg, depleted roughly 50 percent of B-cells. Although antibody mediated depletion was found to be efficient in some mouse strains, others were more resistant to this treatment, indicating genetic differences in the susceptibility to B-cell depletion. Interestingly, additional injections did not further enhance B-cell depletion. In fact, already before the era of monoclonal antibodies, similar depletion strategies relying on the injection of anti-μ antisera have been established and used with success [21].

Apart from defining basic biological functions of B-lymphocytes these studies opened important new avenues towards the development of today's therapies with biologicals. These early studies provided the proof of principle that monoclonal antibodies, when applied systemically, have the ability to deplete distinct cellular populations with high specificity, while sparing others entirely. Of note, genetic differences especially gender differences, became also apparent in the response of human individuals to antibody therapy, with significantly higher complete remission rates among female patients treated for follicular lymphoma with anti-CD20 therapy [22].

B-cell specific knock-out model - µMT mice

A more elegant way to eliminate B-cells was realized by the targeted disruption of the µ-chain locus by one of the pioneers of gene knock-out technology, *Klaus Rajewski* and co-workers. The resultant µMT mice are devoid of B-cells expressing IgM or IgD on their surface, and do not harbor B-cells in their peritoneal cavity [23]. Furthermore, they completely lack serum immunoglobulin M. When µMT mice were sensitized by one i.p. injection of OVA plus alum followed by seven daily exposures to aerosolized OVA (1%) 14 days later, they presented with swollen, discolored lungs and exhibited marked eosinophilia in large airway subepithelial tissues and perivascularly and peribronchially in the lungs [24]. These findings were accompanied by eosinophilia in local lymph nodes and an increase in mucus producing cells in the large airway epithelia. Along with the marked eosinophilia, $CD4^+$ T-cells and macrophages were found to be increased in the lungs. Comparable findings were obtained in wild type mice who underwent the same treatment, although, most interestingly the observed changes were much less dramatic. This demonstrated that B-cells are not a *conditio sine qua non* for antigen-presentation in the lungs and for the induction of lung pathology induced by OVA used as a model aeroallergen. Consequently, these data suggested a major role of $CD4^+$ T-cells in the induction of airway eosinophilia, which is also in-line with later findings of *Kips et al*. who have demonstrated that airway eosinophilia is dependent on the capability of experimental animals to produce IL-4 and on the presence of sufficient amounts of $CD4^+$ T-cells [25,26]. µMT mice do not produce detectable levels of IgE and thus the observed lung pathology can be regarded as being IgE and B-cell independent. Consequently, the authors question treatment protocols for allergic asthma bronchiale, which solely aim at the reduction of IgE and/or the targeting of B-cell function. *Epstein* and co-workers convincingly demonstrated that priming of virgin T-cells by a whole set of antigens (non-allergens) is independent of B-cells as antigen-presenting cells [27]. While a further study performed by

Hamelmann et al. [28] essentially confirmed the results obtained by *Korsgren et al*. [24] (see above), additional information regarding lung pathology has been provided. They showed that neither IgE nor B-cells were essential to induce increased Th2 cell responses, as determined by IL-4 and IL-5 production of peribronchial lymph node T-cells and eosinophilic infiltration of the lungs of OVA sensitized mice. Furthermore, sensitization did not cause *bona fide* airway hyperresponsiveness (AHR) as determined by tracheal smooth muscle contractile responses as primary read-out. The mode of sensitization consisted of 10 daily exposures to nebulized 1% OVA followed by analysis two days after the last exposure, i.e. on day 12. In fact, measurable AHR was only inducible upon passive transfer of anti-OVA Ig into µMT mice, indicating that AHR, at least in this model of airway sensitization, was strictly dependent on the presence of allergen-specific IgE.

While the µMT-model has provided important insights into B-cellular developmental processes and basic aspects of allergic diseases, several recent reports have challenged the 'purity' of the µMT mouse model, by demonstrating that distinct worm and fungal antigens, ranging from *Heiligmosomioides polygyrus*, *Trichuris muris*, *Schistosoma mansoni* [29] and *Aspergillus fumigatus* [30] are actually able to drive B1-type B-cell growth and even antibody production in µMT mice. To achieve B-cellular activation, mice had to undergo quite harsh sensitization protocols including simultaneous i.p. and s.c. injections of *A. fumigatus* antigen, followed after two weeks by three weekly i.n. doses of Ag and finally two weekly challenges with live *A.fumigatus* conidia. Although clear-cut IgG2a and IgE levels could be induced in µMT mice with these protocols no Ag-specificity for the inducing agent, i.e. *A. fumigatus* could be detected. This leaves the reader with the important question by which antigen-independent pathway such B-cells actually become activated and develop into Ig-secretors.

Ig-Cε (IgE) ko mice

A similar, although, by far more specific approach to test the role of allergen-specific immunoglobulins has been undertaken by the group of *Phil Leder* and colleagues who disrupted the Cε exons in the germ-line of mice [31]. While IL-4 plus LPS triggered IgE production of such mice is not detectable at all, the induced levels of the other immunoglobulin classes were comparable to wt mice. In further experiments $IgE^{-/-}$ mice along with control mice were i.p. immunized with ovalbumin, alum and pertussis toxin, which represents a classical way to induce IgE and an anaphylactic state in rodents. To induce the disease, animals were challenged by i.v. injection of OVA 18-21 days later. The wt mice presented with significantly increased heart rates, decreased pulmonary conductance and

increased dynamic compliance, with a maximal amplitude three minutes after challenge. Death usually occurred 20 minutes later. Importantly, IgE-deficient mice had the same response as wt mice. A similar clinical picture was obtained upon i.v. injection of cross-linking anti-IgE in wt mice, while this maneuver was without effect in IgE ko mice. Cobra venom experiments, neutralizing the anaphylatoxins C3a and C5a, did not abrogate the induced anaphylactic reactions. Since in Ig-deficient SCID/SCID or RAG-2/RAG-2 immunodeficient mice no anaphylactic phenotype could be induced it was assumed that immunoglobulins other than IgE and binding to FcγR on mast cells or other innate immune cells are responsible for the observed anaphylactic reactions. Today we know, in fact that macrophages are responsible for IgG-mediated anaphylaxis [32]. Of note, anaphylactic reactions were not restricted to OVA used as the model antigen but could also be induced by other sensitizing agents such as bovine immunoglobulins.

CD23 Fcε (low affinity IgE receptor) ko mice

Among other cell types, such as Langerhans cells, distinct T-cell subsets, monocytes, dendritic cells, macrophages platelets and neutrophils, also B-lymphocytes express two forms of the low affinity Fc epsilon receptor (CD23) commonly referred to as FcεRIIa and FcεRIIb [33]. The type II transmembrane proteins are responsible for the internalization of receptor bound antigens (allergens) and are also believed to exert negative signaling functions, possibly involved in negative feedback regulation of IgE synthesis [34-36]. Importantly, a serum soluble form of CD23 is generated by proteolytic cleavage. In allergic individuals, CD23 becomes strongly up-regulated on B-cells, monocytes and alveolar macrophages [37,38]. To study the relationship between CD23 expression and the development of allergic diseases CD23 deficient animals were created [39], sensitized and challenged with ovalbumin applied as model aeroallergen exclusively via the airways [40]. This was achieved by three different protocols, either by exposure of mice to 1% ovalbumin, by nebulization on ten consecutive days, or by i.p. injection of OVA followed by nebulization of OVA on days 24, 25, and 26 or by passive sensitization with OVA-specific IgE. Monitoring of the ensuing IgE levels in sera of mice revealed that CD23 ko mice produced, in fact, higher rather than lower specific IgE titers than wt mice. Significantly, passive sensitization of mice with IgE antibodies from a hybridoma cell line induced significantly higher IgG and IgE levels in wt mice but not in CD23 ko mice. Similar increases of IgG and IgE levels were obtained in related studies and using haptenated antigens as the immunogens [41]. Moreover, lung digests of CD23 ko mice contained higher eosinophil numbers after nebulization of OVA for ten days

when compared to wt control mice. After three aerosol challenges, no eosinophilia was detectable. In fact, eosinophilia became only apparent when two i.p. sensitizations were followed by three aerosol exposures. The eosinophilia induced in this way involved exclusively the peribronchial and perivascular tissue but not the rest of lung parenchyma, which remained almost eosinophil free. Surprisingly, CD23 ko mice were more responsive than wt mice to the action of methacholine used as a non-specific bronchoconstricting agent. Taken together, the lack of CD23 neither inhibited the production of IgE nor the development of airway inflammation and the development of AHR. Thus, CD23 seems to be dispensable for the development of allergen induced lung pathology. Of note, in situations in which CD23 becomes overexpressed, i.e. in CD23 transgenic mice, reduced levels of IgE (and IgG) production have been observed upon challenge of mice with anti-IgD antibodies, *Nipostrongylus brasiliensis* infestation or keyhole limpet hemocyanin-conjugated benzylpenicilloyl absorbed on aluminium hydroxide [36]. Similar inhibition in IgE production can be induced by a monoclonal antibody directed against the CD23 molecule, lumiliximab, *in vitro* [34] and *in vivo* [35].

What might be the mechanism for this inhibitory effect of CD23 antibodies?

It cannot be excluded that blockade of CD23 on T-cells prevents its interaction with complement receptor 2 (CR2), CD21 on B-cells and thus inhibits IL-4 facilitated, T-cell dependent IgE production [42]. Of interest in line with these observations, *in vitro* IgE production by splenocytes of CD23 transgenic (tg) mice induced by anti-CD3 stimulation in the presence of IL-2, IL-4, IL-5, IL-6 and anti-IFN-γ was not impaired when compared to splenocytes of wt mice.

Knock-out of innate immune receptors with importance for B-cell function – example of TLR9 ko mice

TLR9 is a well-known innate immune receptor, which is expressed intracellularily among other cell types also in B-cells, i.e. in endosomes, and senses cytosine phosphate guanosine (CpG) motifs within DNA of bacterial or viral origin. Since TLR9 has a strong modulatory capacity on the mucosal immune system, which represents the first contact site with potential food allergens, TLR9 was genetically deleted in mice and its effect on peanut allergy was studied [43]. Peanut allergy can be induced in mice by feeding them with peanuts in the presence of a mucosal adjuvant, e.g. cholera toxin [44,45]. Such treated mice develop high titers of peanut-specific IgE and respond to i.p. challenge with systemic anaphylaxis. Significantly, TLR9 ko mice had fewer signs of anaphylaxis when compared to wt mice upon

challenge, suggesting an important role of TLR9 for the induction of anaphylaxis. Lower anaphylactic susceptibility of TLR 9 ko mice was associated with much lower IgE titers. Passive transfer of specific IgE to TLR9 ko mice restored the anaphylactic phenotype, strongly indicating that mast cells of TLR9 ko mice are intact and thus suggesting that the defect is at the level of IgE production. Significantly, TLR9 ko mice presented with a reduction in the numbers and size of Payer's patches in the small intestine, which was highly indicative of a B-cell differentiation problem. However, whether mice are affected by a primary or secondary B-cell differentiation problem remained unclear. Moreover, a direct involvement of B-cells becoming triggered by TLR9-ligands and leading to overt IgE production was rather unlikely since it has been described in the past that engagement of TLR9 reduces class switching of naïve B-cells towards IgE [46] and systemic challenge of mice with TLR9 ligands, i.e. CpG oligonucleotides, was generally associated with the induction of reduced IgE titers [47]. Therefore, other routes of immunization e.g. via the peritoneal cavity or the skin were investigated in TLR9 ko mice and found to be similarly reduced, indicating that the reduction of the anti-peanut response was systemic rather than restricted to the intestinal route/environment. In addition, reconstitution experiments using RAG ko mice and B-cells derived from wt or TLR9 ko mice were performed. RAG ko mice in addition received $CD4^+$ T-cells from wt mice, which had been sensitized by allergen. Such reconstituted mice were challenged with peanut allergen and specific IgE titers were determined. Interestingly, in the absence of transferred T-cells no IgE was detectable in both types of mice, while in the presence of transferred $CD4^+$ T-cells, which had been sensitized previously, the levels of induced, peanut-specific IgE were similar in both wt and TLR9 ko mice. This argues for a non B-cell-dependent defect, since TLR9 deficient B-cells, when brought into a 'wild type milieu' are able to produce pathogenic IgE titers. This study indicated that TLR9 expressed on a non-B-cell might be important for modulating the IgE response to ingested allergens, most probably within a (narrow) developmental period early during life. Candidate cell types conferring the TLR9 ko phenotype might be intestinal epithelial cells or dendritic cells but this is currently a matter of intensive investigations.

B regulatory cells

B-cells fulfill several biological tasks. Apart from being the precursors of antibody producing plasma cells, they act as antigen-presenting cells and via secretion of cytokines and other factors they execute important immunomodulatory functions. Only very recently it has been appreciated that a distinct subset of B-cells secretes IL-10 and has, in fact, regulatory

properties [48]. These IL-10 producing B-cells are commonly referred to as Breg or B10 cells and have a $CD5^+IgM^+IgD^+CD1d^+$ phenotype, which resembles B1 cells in rodents. Most interestingly, Breg numbers are highly up-regulated upon infestation with parasites such as *Schistosoma mansoni* worms and can thus be isolated in high numbers from the spleens of experimental animals suffering from *S. mansoni* infection [49]. Of note, *S. mansoni* infestation reduces acute experimental anaphylaxis as well as airway hyperresponsiveness after challenge with model allergens such as ovalbumin. This effect has been shown to depend on IL-10 by the use of IL-10 specific blocking antibodies. To formally prove that Breg are actually the sole factor responsible for the modified acute and chronic allergic responses observed upon *S. mansoni* infestation, Breg transfer experiments were performed recently [50]. These experiments revealed a clear-cut protective effect of adoptively transferred Breg in ovalbumin-sensitized mice, in which they suppressed airway inflammation upon allergen challenge. Breg might, however, not be the suppressive principle *per se*, since they induced considerable influx of $CD4^+CD25^+FOXP3^+$ regulatory T-cells to the site of challenge. In fact, Treg might eventually be responsible for executing the regulatory program under these circumstances.

T-cell model-systems for allergy research

It is already well established that T-lymphocytes play a crucial role during the sensitization phase as well as in late phase reactions of allergic reactions. Consequently, several *in vitro* and *in vivo* models have been established over the last decades to study the precise role of $CD4^+$ T-cells during the development and maintenance of allergic reactions.

In vitro T-cell systems

Allergen-specific T-cell lines

Early studies have shown that peripheral blood mononuclear cells derived from allergic individuals proliferate when incubated with allergen [51-54]. In those times it was still disputed whether the observed proliferation is the result of a specific stimulus or the consequence of polyclonal lymphocyte activation. It took, however, more than ten years to establish the first allergen-specific long term cultures of T-cell lines with the help of allergen-extracts (*Lolium perenne*) or house dust mite allergens (*Dermatophagoides pteronyssinus*) and interleukin-2, which was the first T-cell derived growth factor available [55]. As possible restriction elements HLA-DR molecules were brought into focus since anti-DR antibodies were able to specifically block the allergen-induced T-cell responses. Limiting dilution cultures led to the establishment of T-cell clones that had the same specificities as the founder lines. While most of the obtained clones belonged the CD4 helper lineage some exhibited CD8 expression [55].

Allergen-specific T-cell clones

Already back then it was claimed that the experimental availability of allergen-specific T-cell clones might help to understand the regulation of IgE production. Since the degree of T-cell help was rather unknown in those times, large efforts were undertaken to characterize the molecular regions within major allergens responsible for T-cellular activation. Along those lines *Baskar et al.* [56] delineated the T-cell stimulatory capacity of three *Lol p* allergens. Interestingly, some degree of cross-reactivity for these proteins was observed, which allowed defining sequence similarities between the molecules, and finally led to the characterization of the first immunodominant 20-mer peptides able to induce allergen-specific T-cellular responses similar to the native allergenic proteins. Similar work was published by *Hans Yssel* and colleagues at about the same time, who cloned HDM-specific T-cell clones and determined their peptide-specificity along with their detailed HLA-restriction [57]. In fact, two T-cell epitopes of *Der p I* were identified and shown to be restricted by HLA-DR7, while

a third displayed a rather promiscuous restriction-pattern comprising HLA-DR2, -DR11 and -DR8. Of note, all three T-cell epitopes induced Th2-type cytokine responses indicated by high levels of IL-4 and IL-5 production. Interestingly, the Th2 cytokine production pattern depended on the T-cell stimulus applied. While ConA induced similarly low IFN-γ and IL-2 levels as allergen, the combination of phorbolesters with ionomycin was able to also induce high-levels of IFN-γ and IL-2 along with IL-4 and IL-5-production, giving testimony of the plasticity of *in vitro*-generated T-cell clones. Significantly, when such T-cells are co-cultivated with B-cells they induced the secretion of IgE [9]. As a major outcome of these studies, T-cell activating peptides, which lack IgE binding capability could be defined, paving the way for novel therapeutic concepts, e.g. those relying on tolerogenic peptides [9].

Subsequently, *Ebner* and colleagues established 35 TCC specific for the major birch pollen allergen Bet v 1 [58]. These clones not only reacted with the purified allergen, but also with recombinant Bet v 1 protein. All clones belonged to the αβ TCR subset and expressed the CD4 co-receptor. Most interestingly, 9 of 11 clones secreted significantly more IL-4 than IFN-γ and thus qualified as Th2 clones. Both an N-terminal and a C-terminal epitope were characterized by means of overlapping 12-mer peptides used to stimulate the TCC. In addition, several peptides representing sequences scattered over the middle portion of Bet v 1 were shown to activate distinct TCC. Most significantly, none of the T-cell stimulatory peptides was able to inhibit binding of allergen-specific IgE to Bet v 1 [59]. Collectively, it was hoped that the identification of T-cell stimulatory epitopes might eventually lead to the definition of ‘disease-related epitopes’ in patients. Subsequent studies from the same group disproved this hypothesis quite clearly by showing that TCC derived from either non-allergic or allergic individuals react with the same set of immunodominant Bet v 1 T-cell epitopes. TCC from non-allergic individuals differed from TCC of allergic individuals by their low IL-4/IFN-γ ratio [60,61]. While the initial cloning procedures generally relied on the bulk activation of whole peripheral blood mononuclear cell followed by limiting dilution preparations, more selective technologies have been developed recently. They either rely on the recognition and enrichment by flow cytometry-based sorting of allergen-specific T-cells with the help of fluorochrome conjugated tetramers [62-64], or the identification in bulk cultures of proliferating antigen-specific cells with the help of carboxyfluorescein succinimidyl ester (CFSE)-based dilution [65,66].

TCR-transgenic models

T-cell clones derived by the specific selection and expansion of peripheral blood T-lymphocytes from allergic and non-allergic donors have certainly revolutionized the field of allergy research and allowed to obtain deep insights into the adaptive, helper-dependent aspects of immune reactions. This technology led to the definition of immunodominant T-cell epitopes, provided a clinically relevant model for the dichotomy of Th1/Th2 cell development and in turn helped to define allergen-specific influences on antigen-presenting cells by using TCC as a valuable functional read-out tool. However, once established TCC cannot be expanded *ad infinitum* since natural restrictions such as telomere shortening after n-numbers of cell divisions [67-69] along with approaching of the Hayflick limit [70] often lead to a sudden stop of proliferation of TCC. Thus, strategies for the 'immortalization' of the TCC itself or at least of its specific principle, i.e. the individual T-cell antigen receptor, were warranted. Therefore the authors of this review article have cloned and expressed relevant human T-cell antigen receptors (TCR) with the aim of transferring them on the one hand into immortalized human T-cell lines to or, alternatively, for into primary T-cells by gene transfer technology [71]. This approach has led to the detailed characterization of two TCR recognizing human-relevant major pollen allergens derived from birch and mugwort (Bet v 1 an Art v 1, respectively) along with novel concepts of how to use TCR tg cells for specific immunomodulation [71,72]. In one approach *Neunkirchner et al.* proved that TCR tg T-cells polarized by virtue of engineered APC decorated with allergen-specific pHLA complexes and membrane-bound IL-12 are potent inhibitors of the effector phase of Th2 cells [73]. Therefore TCR tg T-cells are a valuable functional tool to test the growing number of newly developed recombinant allergens and hypoallergens [74]. In another approach *Schmetterer et al.* provided evidence that allergen-specific Treg can be engineered by co-transfer into T-cells of allergen-specific TCR chains plus the master regulator of natural regulatory T-cells, FOXP3 [75]. This study also proved that the regulatory program becomes only activated when the allergen-specific TCR becomes ligated by specific antigen. Since recombinant material is sheer unlimited and can be easily stored, this approach represents a major breakthrough in T-cell based allergy research. Moreover, it offers the possibility to generate entire model organisms, e.g. TCR transgenic mice, which offer the possibility to study the effects of the respective allergen in an *in vivo* model.

***In vivo* T-cell systems**

CD4-specific depletion approaches

Depletion of distinct T-cells supposed to promote a given disease can also be achieved by the systemic administration of mAb targeting the respective cellular population of interest (**Table I**). Anti-CD4 treatment of experimental animals is an important tool to study the specific contribution of $CD4^+$ helper T-cells during the establishment of airway inflammation, AHR and airway remodeling. However, $CD4^+$ T-cell depletion studies led to somewhat conflicting results. Studies on murine models of acute allergen challenge demonstrated that depletion of $CD4^+$ T-cells by administration of a monoclonal anti-CD4 antibody inhibits AHR [76,77] and also eosinophilic lung inflammation [76]. Similar findings were obtained in a chronic mouse model of asthma by *Foster et al.* who showed that functional depletion of $CD4^+$ T-cells before repeated allergen challenges not only clearly reduced AHR but also diminished pathological airway remodeling processes such as subepithelial fibrosis, epithelial thickening and mucus cell hyperplasia/metaplasia [78]. In a further study administration of anti-$CD4^+$ mAb but not of an anti-$CD8^+$ mAb shortly before starting allergen-challenges reduced AHR in response to acetylcholine, decreased the number of eosinophils in BAL fluids and affected airway remodeling demonstrated by attenuated allergen-induced goblet cell hyperplasia in the epithelium and reduced subepithelial fibrosis [79].

Although several reports confirmed the crucial contribution of $CD4^+$ T-cells to the development of airway inflammation and AHR in mouse models of allergic asthma, the precise role of T-cells in airway remodeling is still controversial. In fact, others have reported that the T-cell-mediated inflammation occurring after a brief allergen exposure is not necessary to maintain airway dysfunction in mice [80]. Depletion of $CD4^+$ and $CD8^+$ T-cells by administration of co-receptor specific mAb three weeks after termination of chronic allergen challenge did not reverse several features of airway remodeling, such as mucus hyperproduction, collagen deposition and the amount of airway wall contractile tissue staining [80]. In contrast to previous studies *Doherty et al.* showed, however, that $CD4^+$ T-cells are required for the establishment of chronic eosinophilic lung inflammation but again are not necessary for airway remodeling during ongoing allergen challenges [81]. In contrast to previous studies, the CD4-depleting antibody was not administered before allergen challenge but after establishment of acute airway inflammation during chronic exposure to allergen. Although these mice showed reduced peribronchiolar inflammation and reduced eosinophil numbers in BAL fluids, airway remodeling was not affected. Depletion of $CD4^+$ T-cells also failed to reduce mucus metaplasia, peribronchiolar supepithelial fibrosis and smooth muscle

mass. The authors postulated that $CD4^+$ T-cells next to DC, iNKT cells (invariant natural killer T-cells) and LTi cells (lymphoid tissue inducer cells) also expressing the CD4 coreceptor might be involved in the induction of airway inflammation but are not essential for the further progression and maintenance of airway remodeling during prolonged periods of chronic allergen challenge [81]. Thus, monoclonal antibody-based depletion of $CD4^+$ T-cells approved to be a valid tool in several mouse models to study the precise contribution of these cells in allergic asthma, however, conflicting results point to the importance of proper timing of specific interventions within different protocols, which will have an important impact on the outcome of the studies. Apart from monoclonal antibody-mediated depletion of T-cell populations, genetic manipulations leading to cellular deficiencies of choice became state-of-the-art with the advent of gene-targeting technologies pioneered by *Capecci* [82,83].

Köntgen et al. established mice lacking mature $CD4^+$ T-cells due to a targeted disruption of the MHC II Aa gene in C57BL/6 mice ($B6.Aa^{-/-}$) to elucidate the role of MHC class II expression in lymphocyte development and immune responses [84]. It is well known that lack of MHC class II expression in one of the reasons for CD4-lymphopenia commonly referred to as bare lymphocyte syndrome [85]. Using these mice to study allergic asthma, it was shown, that they failed to develop BAL eosinophilia and airway inflammation even after repeated exposure to allergen [86].

Another approach to investigate the involvement of $CD4^+$ helper T-cells in allergic asthma is to use $CD4^{-/-}$ mice [87]. In this model the CD4 gene was disrupted in embryonic stem cells by homologous recombination, resulting in mutant mice. Using CD4 knock out mice *Koya et al*. demonstrated that intratracheally administered allergen-pulsed bone-marrow derived DC were unable to induce airway inflammation and AHR, indicating that the interaction of allergen-presenting DC and $CD4^+$ T-cells is a prerequisite for the induction of AHR [88]. Along those lines *Corry and co-workers* have shown that in $RAG^{-/-}$ mice adoptively transferred $CD4^+$ T-cells are sufficient to restore many of the pathognomonic alterations that are typically observed in human asthma [89]. This study was performed by applying *Aspergillus fumigatus* as allergen intranasally at 4-day intervals five times or by 3 s.c. injections with 4 day intervals followed by three 4-day i.t. applications of antigen. Moreover, neutralization of IL-4 but not of IL-5 was found to be sufficient to abrogate allergic asthma in mice [89]. That IL-4 is of critical importance for the induction of asthma pathology has been demonstrated by *Renz* and colleagues by applying soluble IL-4 receptor to BALB/c mice during sensitization, which in fact decreases IgE production and inhibits AHR development [90]. These models suggest that

immediate interventions censoring T-cellular responses might help to abrogate subsequent tissue modifying alterations [89].

Adoptive transfer models

Adoptive transfer models have proved to be a valuable tool in allergy research to delineate the critical role of a specific cell type in the initiation and maintenance of airway inflammation and bronchial hyperreactivity (**Table II**). *Kaminuma et al.* showed that adoptive transfer of $5x10^6$ cells of an allergen-specific, IL-5-producing Th2-clone into wild type mice orchestrates eosinophil inflammation and tissue destruction of the bronchial mucosa following allergen challenge [91]. Subsequent reports confirmed these results by demonstrating that transfer of $CD4^+$ Th2-cells isolated from the spleen of OVA-sensitized C57BL/6 mice is sufficient to induce eosinophilia in blood and airways as well as airway hyperreactivity in IL-$5^{-/-}$ mice after aeroallergen-challenge [92]. In fact, data from this mouse model imply that $CD4^+$ Th2 cells set the initial steps for the recruitment of eosinophils and orchestrate allergic inflammation of the airways through secretion of the Th2-associated cytokine IL-5 [93]. *Cohn et al*. investigated the role of adoptively transferred Th1- or Th2-polarized cells after aerosol allergen challenge. In this model only Th2 cells were able to induce eosinophilic inflammation of the airways and AHR, whereas mice receiving Th1-polarized cells rather developed neutrophilic inflammation without concomitant AHR [94].

Since the 1980s it is well appreciated that allergic diseases among many other confounding factors can be regarded as a Th2-mediated entity. Such an imbalance in favor of Th2 responses, was demonstrated to be ameliorated by counteracting Th1 cells [95]. In mice, co-transfer of both T-cell subsets demonstrated that allergen-specific Th1 cells are potent enough to down-regulate asthma-related pathology induced by transfer of Th2 cells alone, and this inhibition is primarily mediated by IFN-γ [96]. Adoptive transfer of allergen-specific Th1-clones into OVA-sensitized mice just before aerosol challenge leads to reduction of Th2-mediated allergic asthma exemplified by clearly decreased lung eosinophila, diminished Th2-associated cytokine levels, alleviated AHR and increased levels of the Th1-associated cytokine IFN-γ [97]. In contrast to the Th2-mediated lung eosionophila, transfer of Th1 cells causes non-eosinophilic inflammation mainly comprised of macrophages, lymphocytes [97,98] and neutrophils [94]. Although allergen-specific Th1 cells were originally shown to counter-regulate Th2-mediated allergic diseases, it has been demonstrated that this intervention might not be beneficial in all instances since Th1 cells might, indeed, worsen the situation by contributing to increased airway inflammation and airway hyperreactivity

[98,99]. Transfer models where the OVA-specific TCR tg Th1 or Th2 polarized effector cells were rested for 24 days – thereby allowing the cells to convert from their active effector phenotype to a more quiescent memory state - were investigated and gave a slightly different picture. Indeed, allergen-challenge demonstrated that only those mice, which received Th2 cells developed airway eosinophilia and AHR. In contrast, co-transfer of Th1 cells along with Th2 cells had neither a beneficial effect on the reduction of asthma symptoms nor did this maneuver increase inflammation [100]. It remains to be addressed whether adoptive transfer models applying *in vitro*-polarized T-cells are able to sufficiently recapitulate the pathophysiological situation of allergic diseases. Adoptive transfer models commonly rely on the administration of large numbers of polarized T-cells (up to 1 x 10^7 cells). In contrast, less than 1.2 x 10^3 of *in vivo* generated allergen-specific $CD4^+$ T-cells seem to be sufficient to induce eosinophilic lung inflammation [101].

TCR tg $CD4^+$ T-cells mouse models

A frequently used *in vivo*-tool to study different aspects of immune diseases, in which T-lymphocytes seem to play an important role, was established by *Murphy et al.* in 1990 [102] (**Table I**). They generated MHC class II-restricted (I-A^d) αβ-TCR tg BALB/c mice expressing a TCR specific for chicken ovalbumin (cOVA) recognizing the immunodominant peptide $OVA_{323-339}$ [103], termed DO11.10 tg mice [102]. This I-A^d restricted TCR was isolated from the cOVA-specific T-cell clone DO-11.10 generated by the group of *Kappler and Marrack* [104,105]. Apart from DO11.10 mice a second MHC class II-restricted (I-A^b, C57BL/6-background) $cOVA_{323-339}$-specific tg model system was generated by the group of *Carbone* and co-workers, and termed OT-II.2 [106]. For the establishment of this model they used the T-cell clone 1.1 as a source for the tg TCR [106]. Although ovalbumin does not represent a *bona fide* human-relevant respiratory allergen, this model peptide/protein along with the TCR tg mice is increasingly used for studies aiming at the better understanding of immune regulatory mechanisms operative in allergic diseases with a special focus on asthma research (reviewed in [107]).

In fact, naive $CD4^+$ T-cells from TCR tg mice have been used to study the role of cytokines and antigen-presenting cells (APC) in respect to the differentiation of the T helper-subsets Th1 and Th2. *Hsieh et al*. has shown that *in vitro* stimulation of naive $CD4^+$ T-cells by the cognate antigen in the presence of IL-4 drives Th2 cell development [108]. This early results on the influence of Th-subset differentiation were subsequently confirmed by using TCR tg mice specific for pigeon cytochrome C peptide 88-104 (PCC_{88-104}) in the context of I-E^k

[109]. In contrast, presence of IL-12 produced by accessory cells like *Listeria monocytogenes* induced macrophages [110] or dendritic cells [111] was demonstrated to be critical for the development of IFN-γ producing Th1 cells. In the mouse lung, two major subpopulations of DC can be defined, $CD103^{+}CD11b^{low/neg}$ LDC and $CD103^{-}CD11b^{high}$ LDC [112]. Naive $CD4^{+}CD62L^{high}$ T-cells isolated from DO11.10 tg mice that had been antigen-specifically primed by $CD103^{+}CD11b^{low/neg}$ LDC displayed a Th1 or Th17 phenotype after re-stimulation shown by their predominant IFN-γ and IL-17A secretion. In contrast, priming of naive T-cells with $CD103^{-}CD11b^{high}$ LDC led to the evocation of a Th2 cytokine profile marked by the production of elevated levels of IL-4, IL-6 and IL-10 *in vitro* [113]. Using the same mouse model for the investigation of allergic airway inflammation *in vivo Raymond et al*. showed that upon allergen contact $CD103^{-}CD11b^{high}$ LDC migrate from the lung to mediastinal lymph nodes where they induce a Th2-biased T-cellular immune response [114].

Knott et al. showed that a single OVA-aerosol challenge of non-sensitized TCR tg DO11.10 BALB/c mice is sufficient to activate resident lung $CD4^{+}$ T-cells to drive allergic airway inflammation [115]. One aerosol challenge caused characteristic Th1-mediated lung inflammation. Typically, rapid and dominant recruitment of neutrophils in concert with predominant IFN-γ secretion into BAL-fluids as well as Th2-associated lung inflammation exemplified by peribronchiolar eosinophila and mucus hyperproduction of the respiratory epithelium was observed. Despite these obvious immunological changes even four consecutive aerosol exposures did not lead to the development of airway hyperreactivity in response to methacholine used as a pharmacological bronchoconstricting agent [115]. In contrast, *Wilder et al.* demonstrated by using hemizygous DO11.10 TCR tg mice that three aerosol challenges, one every week, caused pulmonary recruitment of OVA-specific T-cells along with the induction of AHR. The infiltrating T-cells displayed an activated phenotype as shown by the neo-expression of the activation marker CD69 [116]. Interestingly, this exposure regimen did neither induce eosinophilia of BAL fluids nor eosinophilic infiltration of lung parenchyma, which is in accordance with previous findings [115,116]. Accordingly, the authors claim that the resulting AHR was directly facilitated by the recruited and activated antigen-specific T-cells [116]. A chronic challenge model based on enhanced exposure (6 h per day, 5 days per week for up to 6 weeks) of homozygous DO11.10 mice to OVA aerosol resulted in the detection of elevated levels of total and OVA-specific serum IgE, increased numbers of lymphocytes and neutrophils in BAL fluids and AHR [117].

Interplay between T-cells and eosinophilic granulocytes

Although eosinophilic granulocytes were initially thought to act solely as terminal effector cells in allergic airway diseases new studies indicate that they might be also critically involved in the induction phase of allergic lung diseases. In particular the interrelationship between eosinophils and Th2 cells in allergic disease has gained a lot of interest. Recent advances suggest that eosinophils play a multifunctional role during the development of allergic airway disease by (i) acting as antigen-presenting cells for T-cells, (ii) inducing Th2-cell development, and by (iii) actively producing cytokines leading to the recruitment of T-cells to the lungs with subsequent airway inflammation (reviewed in [118]). Of note, the genetic background of the chosen mouse strain has, however, an important impact on the outcome of those investigations. These discrepancies surfaced due to reports claiming that eosinophils are not essential for the development of allergic asthma in BALB/c mice [119] while it was demonstrated that within the C57BL/6 background eosinophils are indispensable for disease induction [120,121].

What might be the reason for these opposing outcomes? Studies by the group of *Lee* and co-workers clearly demonstrated that pulmonary eosinophils are critically responsible for the recruitment and/or accumulation of effector T-cells into the lungs [120,122]. These studies were performed in PHIL mice. PHIL mice (C57BL/6 background) are eosinophil deficient mice due to the lineage-specific expression of the cytotoxic protein diphtheria toxin A under the control of eosinophil peroxidase promoter [123]. These mice have a normal distribution of all other hematopoietic cell types, however, they are almost completely devoid of eosinophils. Adoptive transfer of Th2 polarized OT-II cells followed by OVA challenge revealed reduced T-cell recruitment to the lungs along with diminished Th2 cytokine production. Only adoptive transfer of OVA-specific Th2-polarized T-cells from OT-II mice along with eosinophils was able to restore endogenous Th2-cell migration to the lungs and concomitant cytokine production in the lungs of recipient *PHIL* mice. In this report the suggested mechanism for the recruitment to the lungs of Th2-cells was explained by the release of the chemokines [120,122] CCL22 [124] and CCL17 [125] by eosinophils after allergen-challenge, which are well-known to attract $CCR4^+$ T-lymphocytes [124,125]. Moreover, eosinophils seem to be required for the proper recruitment of myeloid dendritic cells to pulmonary draining lymph nodes [122]. Using DO11.10 TCR tg BALB/c mice *Wang et al.* demonstrated that eosinophils might also function as APC. One day after adoptive transfer of OVA-specific naive $CD4^+$ T-cells, BALB/c recipient mice received intratracheally administered OVA-pulsed eosinophils. Similar to conventional APC, antigen-loaded eosinophils induced activation and polarization

of OVA-specific T-cells in paratracheal lymph nodes (LN) as mirrored by their up-regulation of CD69 expression, the induction of proliferation and the secretion of the Th2-associated cytokine IL-4 [126].

Models addressing the regulatory capacity of (allergen-specific) - lymphocytes *in vitro*

Numerous studies both in murine model systems as well as on patients suffering from diverse allergies suggest that, apart from a disequilibrium in the Th1-Th2 balance, crucial mechanisms of immune tolerance are apparently dysfunctional in IgE-mediated hypersensitivity. In the T-cell compartment, the $CD4^+CD25^+$ naturally-occurring regulatory T-cells (nTreg) are the main mediators of tolerance [127] by actively suppressing immune responses of T- and B-cells, mast-cells and eosinophilic and basophilic granulocytes (reviewed in [128]). Indeed, in a hallmark study on 12 atopic and 11 patients with seasonal allergic rhinitis to grass pollen, it could be demonstrated that nTreg from atopic individuals and allergic patients after allergen-exposure were impaired in their regulatory capacity compared to non-atopic individuals [129]. Along those lines, Treg were found to be absent in the skin of atopic dermatitis patients [130] while outgrowth of cow's milk allergy in initially allergic children known to be associated with increasing numbers of $CD4^+CD25^+$ Treg [131]. Although the status of nTreg number and functional capacity may thus be an important factor in the development of allergic diseases, research on this topic seems rather under-represented in the field of allergy research. Especially standardized model systems using human nTreg are clearly lacking. Most studies rely on the isolation of nTreg from allergic patients and subsequent functional analyses using polyclonal as well as allergen-specific stimulation. In those studies it could be shown that $CD4^+CD25^+$ Treg can efficiently suppress proliferation and cytokine production of Th2 cells [132,133] although other publications suggest that Th2 clones are less susceptible to nTreg-mediated suppression when compared to Th1 clones [134]. Our recent observation that cloning of allergen-specific TCR and subsequent transduction into peripheral blood T-cells transfers allergen-reactivity and fine-specificity of the original clone [71,73], has also led us to the development of an nTreg-based *in vitro* approach. For that purpose we made use of the fact that gene transfer of *FOXP3* is sufficient to generate T-cells with nTreg phenotypic and functional properties [135,136]. Consequently, we combined overexpression of a Bet v 1-specific TCR and human FOXP3 in human $CD4^+$ T-cells by use of a multicistronic expression construct. These TCR/FOXP3 T-cells displayed strong suppressive capacity in response to both allergen-specific as well as polyclonal stimulation [75]. Importantly, the suppressive capacity of these cells was strictly dependent on

appropriate TCR/CD3-based activation. This was shown by control experiments where FOXP3 single-transgenic T-cells, which express an endogenous TCR and do not recognize Bet v 1, could not suppress allergen-specific T-cell responses following allergen-specific stimulation. In conclusion, our approach offers the possibility to generate large numbers of allergen-specific Treg independently of the allergen-season and donor availability. These cells thus represent a convenient model system to assess allergen-specific T-cell regulation.

Models addressing the regulatory capacity of (allergen-specific) T-lymphocytes *in vivo*

In an acute allergen challenge model it was demonstrated that adoptive transfer of $5x10^5$ of OVA-specific $CD4^+CD25^+$Treg from DO11.10 mice was sufficient to clearly attenuate AHR, recruitment of eosinophils and Th2-associated cytokine expression in the lungs after allergen challenge of OVA-sensitized wt mice. The observed inhibition was dependent on IL-10 produced by recipient $CD4^+$ T-cells but not on IL-10 production of $CD4^+CD25^+$ Treg themselves since transfer experiments with Treg obtained from IL-10 gene-deficient mice [137] were similarly suppressive [138]. In further studies using OVA-specific Treg from DO11.10 mice in a chronic challenge model of allergic asthma the same group observed that therapeutic transfer of Treg lead to the reduction of allergen-induced inflammation [139]. Interestingly, the therapeutic effect of Treg was dependent on the proper timing of the adoptive transfer. In situations of chronic allergen challenge the transfer of Treg immediately after establishment of acute airway inflammation was able to block the process of airway remodeling by reducing airway mucus hypersecretion, smooth muscle hypertrophy and collagen synthesis. In contrast, transfer of the Treg at later time-points, i.e. when airway remodeling had already taken place, was not able to significantly change lung pathology [139].

In some models it was shown, that apart from Th2 cells also Th1 cells migrate into the lungs of sensitized and challenged mice and that Th1 cells induce inflammatory responses after allergen exposure [98,99]. Along those lines, *Dehzad et al.* used a strictly T-cell-dependent approach to compare the suppressive capacity of nTreg on allergen-specific Th1 and/or Th2 cells. Transfer of OVA-specific $CD4^+CD25^+$Treg into B-cell- and T-cell-deficient $Rag2^{-/-}$mice prior to transfer of Th1- or Th2-polarized T-cells from DO11.10 tg BALB/c mice demonstrated that nTreg are more efficient in inhibiting the Th1-associated neutrophil inflammation and AHR than the Th2-associated eosinophilic inflammation after allergen-exposure [140]. Although the suppressive mechanism of nTreg, *e.g.* those mediated by cAMP

transfer, is similar for both T-cell subsets, it seems that Th1-associated AHR and inflammation is more sensitive to nTreg-mediated inhibition [140].

Animal models of allergic airway disease have clearly contributed to the further understanding of the role of regulatory T-cells *in vivo*. Increased AHR and inflammation was measured after depletion of $CD4^+CD25^+$ Treg in C3H mice, which are known to be largely resistant to the induction of airway hyperresponsiveness [141]. Most importantly, the depletion of Treg from C3H mice was associated with increased AHR and eosinophilia, IgE and Th2-cytokine production and accompanied by increased numbers of pulmonary myeloid DC with increased stimulatory potential (*i.e.* high expression of MHCII, CD80, CD86) in treated animals [141]. Moreover, other reports clearly demonstrated that intratracheal administration of $CD4^+CD25^+$ nTreg isolated from the lungs of naive mice one day before allergen-challenge diminished AHR, airway inflammation, and Th2 cytokine levels and at the same time increased the levels of IL-10 and TGF-β [142]. This protective effect was clearly dependent on IL-10, since transfer of nTreg from $IL\text{-}10^{-/-}$ mice did not change airway responsiveness, however, prior incubation of the $IL\text{-}10^{-/-}$ Treg with rIL-10 again resolved AHR. The authors further demonstrated that the regulatory cells required IL-10 to produce TGF-β in order to exhibit their full suppressive activity [142].

In vivo studies support the hypothesis that IL-10-induced tolerogenic DC (DC10) can partly induce the differentiation of OVA-specific T effector cells (T_{eff}) from asthmatic DO11.10 mice into $CD4^+CD25^{hi}Foxp3^+$ Treg when co-transferred into sensitized recipient mice [143].

Xu et al. showed that *i.v.* injection of iTreg before but also after allergen-challenge is sufficiently potent to mitigate airway inflammation as demonstrated by reduced AHR, diminished recruitment of eosinophils to the lungs, decreased mucus production and decreased overall airway remodeling as well as reduced serum IgE levels [144]. Of note, in this therapeutic setting the potency of iTreg is comparable to that of expanded nTreg [144].

Models addressing the role of $CD8^+$ T-cells in allergic responses

Despite the prominent role of $CD4^+$ T-cells in allergic responses, $CD8^+$ T-cells seem to be critically involved in the development of allergic diseases in animal models as well as in humans [145]. The precise function of MHC class I–restricted $CD8^+$ T-cells in allergic airway inflammation is not completely understood. Allergens, among other exogenous antigens, can also be processed and presented in the context of MHC class I [146,147]. It remains open how the respiratory tract facilitates antigen-presentation of inhaled antigen through MHC class I thereby inducing $CD8^+$ T-cell mediated responses. $CD8^+$ T-cells were, in fact, demonstrated

to execute an inhibitory function on IgE production [148,149] and to counteract Th2-mediated responses in the airways [150-152]. Moreover, it has been shown, that $CD8^+$ T-cells contribute to the regulatory function of Treg in allergic lung diseases [153].

However, the role of $CD8^+$ T-cells is controversial [154] since several reports demonstrated that these cells exert a protective role in allergy while other findings support the contribution of $CD8^+$ T-cells in allergic responses [155-157]. $CD8^+$ T-cells were originally believed to predominantly secrete IFN-γ [158], until *Seder et al*. showed that cultures containing murine IL-2 and IL-4 can induce murine $CD8^+$ T-cells to produce IL-4 [159]. It became apparent, that different subsets of $CD8^+$ T-cells co-exist. These subsets were termed Tc1 and Tc2 in accordance with $CD4^+$ T helper subsets Th1 and Th2 [160-162]. Several reports indicate that Tc1 are able to suppress Th2 mediated allergic inflammation [150,152]. After OVA-sensitization transgenic mice overexpressing IL-15 developed a Tc1 response caused by $CD8^+$ T-cells and following OVA-challenge they demonstrated reduced airway inflammation with attenuated eosinophilia and Th2-cytokine production in the airways.

In contrast, *in vivo* depletion of $CD8^+$ T-cells by administration of an anti-CD8 mAb enhanced airway inflammation in IL-15 tg mice. The beneficial effect of the $CD8^+$ T-cell-mediated Tc1 response has an important role in the induction phase of allergic diseases, prior to sensitization, as adoptive transfer of $CD8^+$ T-cells from OVA-sensitized IL-15 tg mice into naive animals prevented OVA induced Th2 responses [150,152]. In another study adoptive transfer of $CD8^+$ T-cells from OT-I tg mice into naive mice revealed characteristic features of Tc1 cells after OVA-specific challenge [152]. The hypothesis that Tc1 cells might counteract allergen-specific Th2 cells is supported by the observation that transfer of OT-I $CD8^+$ T-cells into OVA-sensitized mice resulted in reduced IL-13 and IL-5 levels but enhanced production of IL-12 into BAL fluids with concomitantly decreased eosinophil recruitment after allergen-challenge. The authors state that the allergen-specific $CD8^+$ T-cells suppress allergic airway inflammation by induction of IL-12 in the lung through interaction with respiratory dendritic cells [152]. *Takeda et al.* showed that adoptive transfer of lung $CD8^+$ T-cells that had been immunized with a vaccine comprised of OVA and cationic liposome DNA complexes (CLDC) inhibited the development of AHR, eosinophilia, goblet cell metaplasia and Th2 cytokine production in sensitized mice after allergen-challenge. This suppression was dependent on IFN-γ production of the transferred T-cells [163].

In addition to their counteracting role in the establishment of allergic inflammation of $CD8^+$ T-cells cells are also implicated in the pathogenesis of allergic airway diseases in some studies. Dependent on the timing, $CD8^+$ T-cells have the potential to protect the organism

from systemic sensitization at an early stage, most likely by an increased production of IFN-γ. In contrast, after established systemic sensitization, $CD8^+$ T-cells may contribute as bystanders or take over a pro-inflammatory role upon allergen challenge [164].

Interestingly, after establishment of AHR, $CD8^+$ T-cells displayed a different cytokine profile depending on their location in the different compartments of the same animals. Lung infiltrating $CD8^+$ T-cells produced high levels of IL-4, IL-5 and IL-10 but only minute amounts of IFN-g, whereas $CD8^+$ T-cells isolated from the peribronchial lymph nodes or the spleen secreted high levels of IFN-γ and no or only low levels of Th2-associated cytokines. These observations can be explained by different homing properties of the different subpopulations. Tc2 cells might preferentially be recruited to the site of allergic inflammation. This hypothesis is supported by results from *Weninger et al.* showing that $CD8^+$ T-cells with a functionally different phenotype can exhibit distinct migratory behavior [165]. Moreover, the different cytokine profiles of lung and spleen $CD8^+$ T-cells might be caused by local polarization of these cells in the tissues following homing [165]. It seems that Tc2 polarization is favored in the lung during allergic sensitization.

Models addressing the role of $CD8^+$ T-cells using CD8 ko mice

In 1991 *Fung-Leung et al*. described the generation of $CD8^{-/-}$ mice on the C57BL/6 background by targeting the CD8a gene. Disruption of the Lyt-2 gene with the help of embryonic stem cell technology resulted in mice lacking CD8 (Lyt-2 and Lyt-3) cell surface expression. This mutant mouse strain displays no $CD8^+$ T-cells in the peripheral lymphoid organs, whereas $CD4^+$ T-cells remain unaffected [166]. These mice have been used to study the role of antigen-primed $CD8^+$ T-cells in allergic responses [157]. OVA-sensitized and aerosol-challenged $CD8^{-/-}$ mice showed reduced airway reactivity in response to inhaled metacholine concomitant with decreased levels of eosinophils in the lung. In addition, reduced IL-13 production was detected in BAL-fluids and in supernatants from *ex vivo* cultured PBLN cells [157]. Adoptive transfer of OVA-primed $CD8^+$ T-cells into $CD8^{-/-}$ mice restored the development of AHR, lung eosinophila as well as the production of IL-13 in BAL and cultures of PBLN cells. Transfer experiments using naive $CD8^+$ T-cells or OVA-primed $CD8^+$ T-cells from IL-13 deficient mice failed to establish the observed characteristics of allergic airway inflammation. The authors stated that the development of AHR and inflammation is dependent on the IL-13 production of Ag-primed $CD8^+$ T-cells [157]. In further studies the same authors analyzed the specific contribution of the two subpopulations of Ag-experienced $CD8^+$ T-cells [165,167]. They demonstrated that adoptive transfer of *in*

vitro-generated $CD8^+$ effector T-cells (T_{eff}) but not of $CD8^+$ memory T-cells (T_{MEM}) is responsible for the establishment of AHR, eosinophilia and IL-13 production [168]. $CD8^+$ T_{eff} might facilitate AHR and airway inflammation through their Tc2-type cytokine profile and their potential to migrate into the lung [168]. Further studies attributed Notch signaling an essential role in the $CD8^+$ T_{eff} mediated allergic airway inflammation [169]. The potential of $CD8^+$ T_{eff} to induce AHR and airway inflammation in sensitized and challenged $CD8^{-/-}$ mice was abolished following treatment of the cells with a g-secretase inhibitor suppressing Notch signaling [169].

TCR tg $CD8^+$ T-cells mouse models

Generation of OT-I tg mice (originally termed OVA-tcr-I) [170,171], were generated using the TCR derived from the K^b-restricted CTL-clone 149.42 recognizing $OVA_{257-264}$. The alpha chain of the rearranged TCR is composed of Vα2-Jα26 and the beta chain consists of Vb5-Dβ2-Jβ2.6 [172]. TCR tg mice proved to be an excellent tool to study the role of $CD8^+$ Tc1 or Tc2 cells in more detail. Transfer of *in vitro*-polarized OVA-specific Tc2 cells producing high levels of IL-4 and only minute amounts of IFN-γ into naive C57BL/6 mice before allergen-challenge led to recruitment of eosinophils into the airways and bronchial hyperreactivity comparable to *in vitro*-primed OVA-specific Th2 cells [173]. Interestingly, transfer of IFN-γ-producing Tc1 cells resulted in the influx of neutrophils into the airways without the development of bronchial hyperreactivity [173]. In 1994 perforin-deficient mice (PKO) were generated by homologous recombination [174]. These mice exhibited normal numbers of $CD8^+$ T-cells and natural killer (NK) cells but were not able to lyse virus-infected or allogeneic fibroblasts as well as natural killer target cells *in vitro*. Moreover, the *in vivo* characterization demonstrated that perforin-deficient mice fail to clear lymphocytic choriomeningitis virus (LCMV) infection and their potential to kill fibrosarcoma tumor cells was clearly reduced. Using OT-I/PKO mice it has been demonstrated that the protective effect of allergen-specific $CD8^+$ cytotoxic T-cells in allergic inflammation is dependent on the expression of the granule component perforin and not on the secretion of IFN-γ [175]. Most interestingly, PKO/OVA-specific $CD8^+$ cytotoxic T-cells lost their ability to inhibit eosinophil infiltration and mucus production in the airways after allergen-challenge when compared to OVA-specific $CD8^+$ T-cells. The authors hypothesize that the allergen-specific $CD8^+$ cytotoxic T-cells might exert their inhibitory function by elimination of allergen-presenting DC in the airway, thereby stopping allergen-presentation and activation of $CD4^+$ Th2 cells. This suggestion is supported by the observation that mice treated with allergen-

specific $CD8^+$ cytotoxic T-cells showed reduced numbers of DC in the mediastinal lymph node, whereas treatment with perforin-deficient $CD8^+$ cytotoxic T-cells did not affect the numbers of DC [175]. These results are in contrast to recent observations from *Tang et al*. who demonstrated that the inhibitory role of $CD8^+$ T-cells in allergic airway is mainly facilitated by IFN-g secretion and regulation of DC function [176]. In this study, OT-I tg T-cells were transferred into OVA-sensitized mice three days prior to allergen-challenge. Only allergen-specific $CD8^+$ T-cells from OT-I tg mice but not from OT-I/IFN-$\gamma^{-/-}$ [177] mice revealed a Tc1-like profile and reduced levels of eosinophila and mucus production in the lung. The influence of allergen-specific $CD8^+$ T-cells on DC function was studied *ex vivo* with sorted lung parenchymal DC from treated animals co-cultured with $CD4^+$ T-cells. $CD11b^+CD103^-$DC isolated from mice that had been reconstituted with OT-I $CD8^+$ T-cells had the potential to polarize naive $CD4^+$ T-cells and lung parenchymal $CD4^+$ T-cells isolated from recipient mice towards the Th1 subset. In contrast, IFN-$\gamma^{-/-}$OT-I mice developed a Tc2/Tc17-like profile and promoted eosinophil as well as neutrophil inflammation in treated mice. The authors state that only IFN-g -competent $CD8^+$ T-cells have beneficial effects during allergic disease shown by reduced inflammation and their influence on lung DC to polarize $CD4^+$ T-cells towards the Th1-phenotype. IFN-γ deficiency promotes the development of Tc2/Tc17-like $CD8^+$ T-cells can contribute to the exacerbation of airway inflammation [176].

Contribution of γδ-T cells

During the last years γδ-T-cells attracted increasing interest in the evaluation of allergic airway responses. γδ-T-cells become usually activated by the presentation of small organic molecules, i.e. alkyl amines, lipids and phosphate-containing antigens presented by the HLA-class I-like CD1 transmembrane surface molecules on DC and are able to elicit pro-inflammatory as well as regulatory functions [178]. Using a γδ-T-cell deficient mouse model based on a genetically deficient TCR δ-chain [179] on the BALB/c background it has been shown that gd-T-cells might be required for Th2-type eosinophilic lung inflammation, T-cell infiltration and AHR upon allergen-challenge [180]. In fact, γδ-T-cell deficient mice do not produce detectable levels of allergen-specific IgE and also specific IgG titers were found to be extremely low [180]. Supplementation of OVA with IL-4 complexed to anti-IL-4 mAbs used for i.p. sensitization induced clearly detectable OVA-specific serum IgE levels and restored eosinophil accumulation and IL-5 production in BAL fluids of γδ-T-cell deficient mice. This is suggestive of an important role of γδ-T-cell-produced IL-4 early on during sensitization

[180]. *Wen et al*. have in fact directly demonstrated that γδ-T-cell support IgE and IgG1 production in αβ-T-cell deficient mice [181,182]. These findings are, however, in sharp contrast to other reports [183] claiming that γδ-T-cells negatively regulate airway hyperresponsiveness. These authors suggest, that, apart from regulatory effects on eosinophilic granulocytes, γδ-T-cells might exert their regulatory functions by modulating lung-resident cell types, such as alveolar macrophages, airway epithelial cells or airway smooth muscle cells [183]. A possible explanation for these differences might be the existence of different subsets of γδ-T-cells. Of note, it was shown, that various subtypes of γδ-T-cells exert different functions, depending on their predominant TCR V-gene segment usage [184]. Along those lines, it has been reported that $V\gamma4^+$ γδ-T-cells are, involved in the suppression of allergic diseases, whereas $V\gamma1^+$ γδ-T-cells contribute to the development of AHR in mouse models of allergic disease [184].

How precisely γδ-T-cells exert their regulatory function is still a matter of debate. In a recent report *Huang et al*. adoptively transferred $V\gamma4^+$ γδ-T-cells isolated from the spleens of TCR $\alpha\beta^{-/-}$ mice, which had been tolerized either by aerosolized hen egg lysozyme or OVA antigen into γδ-T-cell deficient mice [185]. Before cell transfer, γδ-T-cell deficient mice had been immunized via a single injection with antigen (HEL or OVA) plus alum. Most interestingly, cell transfer prevented antigen-specific IgE production in the γδ-T-cell deficient recipient mice. During the tolerization process via the airways, small amounts of inhaled allergens reach the blood stream by crossing the barrier posed by the bronchial epithelial cells [186,187]. Blood-borne allergens become filtered during their passage through the spleen [188] where they get taken-up by antigen presenting cells that present the processed allergen to the γδ-T-cells and thereby possibly inducing their regulatory program. Moreover, the transfer of allergen onto γδ-T-cells could convert them into APC. Thus γδ-T-cells might fulfill both, allergen-presenting and at the same time IgE-regulatory functions [185].

Contribution of natural killer cells to allergic disease

In humans $CD3^-CD56^+$ natural killer (NK) cells represent the third population of lymphocytes distinct from T-and B-lymphocytes and increasing knowledge suggests that different NK cell subsets play a role in allergic diseases [189]. NK cells have been extensively studied for their important contribution to innate immunity, mostly by their ability to recognize and kill virus-infected cells as well as tumor cells. NK cells might however also modulate adaptive immune responses, e.g. by the stimulation of DC maturation, the elimination of immature DC [190,191] and induction of T-cell activation and proliferation [192,193]. The regulatory

function of NK cells is mainly mediated by cytotoxicity, production of cytokines and modulation of other cell types [194-196].

Peripheral blood NK cells can be further subdivided according to their expression of CD16 and CD56 [197]. The majority of NK cells in blood are $CD16^{+}CD56^{dim}$ whereas the rest is $CD16^{+}CD56^{bright}$ [198-200]. $CD16^{+}CD56^{dim}$ cells have the potential to home to inflamed peripheral sites and contain perforin to execute their cytotoxic effects. The other subtype ($CD16^{+}CD56^{bright}$) is present in the secondary lymphoid organs, produces IFN-γ and TGF-β upon activation but lack perforin expression [198]. Mouse NK cell subsets can be identified by differential CD27-expression levels [201].

NK cells can be further subdivided according to their secreted cytokine profiles (**Table I**). The most important effector cytokine of NK cells is IFN-γ [200,202]. In addition, these cells are able to produce Th2 cytokines. According to their cytokine profile, NK cells are classified as NK1, NK2 or regulatory NK cells similar to $CD4^{+}$ T-helper cell subsets and regulatory T-cells. The two NK cell subsets can be differentiated *in vitro*. NK1 cells can be induced by IL-12 [203] or the combination of IL-12 and IL-18 [204]. NK cells stimulated with IL-4 differentiate into NK2 cells characterized by the production of IL-5 and IL-13 [203,205]. It has been shown that NK cells are involved in human inflammatory diseases [206] like atopy and asthma [207] Moreover, IL-10 producing NK cells are able to suppress allergen-stimulated T-cells [208] indicating the existence of regulatory NK cells with an immune function similar to those described for Treg. NK cell function in allergic disease is not fully clarified with possible disease-promoting or counter regulatory functions of NK1 or NK2 cells in allergic immune responses, respectively. NK cells were shown to induce DC cells to initiate Th1-dominated responses [209-211]. Using co-cultures of *in vitro*-differentiated NK1 and NK2 cells from patients with atopic dermatitis with peripheral blood mononuclear cells or B-cells it was demonstrated that NK1 cells might play an IgE counter-regulatory role in allergy. In these studies NK1, cells inhibited IL-4 dependent and soluble CD40-ligand stimulated IgE production. In contrast, NK2 cells did not exert any effect and did not induce IgE. The inhibitory effect of NK1 cells was mediated by IFN-γ since the inhibitory effect of NK1 cells on IgE production was abolished by neutralization of IFN-γ [212].

Contribution of Natural killer T-cells in allergic responses

Natural killer T (NKT) cells belong to the T-cell lineage but express typical NK cell receptors including CD56 [196]. NKT cells encompass distinct subpopulations, with the most important subpopulation comprising invariant NKT-cells (iNKT). iNKT cells are CD1d restricted

lymphocytes arising from double positive thymocytes and expressing a restricted TCR repertoire. In mice only Vα14 and Jα18 TCR chains are used by iNKT cells and become mainly combined with Vβ8.2 TCR chains, whereas human iNKT cells express Vα24 and Jα18 in concert with Vβ11 TCR chains. iNKT cells recognize glycolipid antigens, and can be specifically identified by a-galactosylceramide (α–GalCer), which can be extracted from a marine sponge [213], bacterial glycosylceramide [214] and mammalian isoglobotrihexosylceramide [215]. Besides their cytotoxic properties iNKT cells are characterized as innate immune cells by the very rapid production, upon TCR stimulation, of high amounts of both IFN-g (Th1) and Th2 (IL4 and IL-13) [216], cytokines which enables these cells to regulate bronchial asthma responses. Studies with NKT-deficient mice ($J\alpha18^{-/-}$, $J\alpha281^{-/-}$ [217] and $CD1^{-/-}$[218]) revealed a role of iNKT cells for the development of AHR and other asthma-related parameters [219-221].

The role of iNKT cells in human allergic disease is controversially discussed with suppressive functions as well as disease-promoting effects described [222,223]. Several studies demonstrate that activation of iNKT cells in the sensitization phase has the potential to counteract allergic inflammation and Th2 responses. This protective effect of iNKT-cells was mediated via the secretion of IFN-γ [224-226]. Both OVA-induced airway inflammation and AHR were not inducible in iNKT cell deficient mice indicating an important role for these cells [220]. These observations were supported by experiments applying anti-CD1d antibodies to animals and by treatment with CD1d-dependent antagonist both suppressing OVA-induced AHR and inflammation [219,227]. Of special note, administration of α-GalCer induced lung inflammation and AHR in naïve MHC II-deficient mice lacking $CD4^{+}$T-cells entirely [228].

Humanized mouse models of allergy research

Humanized mice are either immunodeficient mice engrafted with functional human immune cells or immunocompetent mice expressing human transgenes.

SCID mice are a valuable tool to study various aspects of human pathology since these mice are not able to reject allogeneic or xenogeneic transplants. In fact, transferred human peripheral blood mononuclear cells (PBMC) or bone marrow cells can survive in these mice for prolonged periods of time [229,230] (Table III). In one model, human PBMC from HDM-sensitive allergic individuals were *i.p*. injected into SCID mice which were challenged two weeks later exclusively via the airways in the absence of adjuvant. Inhalation of the allergen resulted in the detection of allergen-specific IgE antibodies in serum and caused inflammatory

responses in the lungs [231]. Co-injection of human PBMC from atopic donors plus specific allergen into SCID mice induced comparable allergen-specific IgE-levels as in the donor-sera at the time of transfer. In these models activation of the transferred T-cells is dependent on the presence of human APC, in fact the absence of human APC causes a state of T-cell unresponsiveness to the allergen [232]. In another study, human PBMC from non-allergic individuals were intratracheally transferred into SCID mice [233]. These mice developed AHR following aerosol exposure after systemic sensitization with HDM. This pathology was caused by lung-infiltrating human IL-4- and IL-5-producing Th2 cells [233]. To study how T-cells get attracted to inflamed airways, SCID mice were reconstituted with PBMC from patients suffering from HDM allergy before allergen challenge. Pretreatment with a CCR4-blocking antibody abolished airway inflammation and AHR, indicating that the DC-derived chemokines CCL17 and CCL22 are responsible for the recruitment of allergen-specific Th2 cells [234]. Moreover, systemic administration of human induced pluripotent stem cells (iPSC) derived from mesenchymal stem cells (MSC) prior to allergen-challenge prevented airway inflammation in BALB/c mice. Similar therapeutic effects were observed when iPSC-MSC were transferred before allergen sensitization [235].

So far, few humanized TCR tg mouse models have been established to study important immune-mediated diseases, e.g. for the analysis of the T-cell role in autoimmune diseases such as multiple sclerosis [236] and arthritis [237]. In contrast, humanized allergy mouse models based on alpha/beta TCR specific for human relevant respiratory allergens have been sorely missing. *Jarman et al.* described the establishment of transgenic mice expressing a murine αβ-TCR recognizing the immunodominant epitope of Der p 1, a prominent inhalant indoor allergen affecting a multitude of allergic individuals. Two intratracheal instillations of the allergen were sufficient to induce asthmatic features in these mice including accumulation of eosinophils and lymphocytes in the airways and the lung as well as goblet cell hyperplasia and mucus production [238].

We recently established the basis for humanized studies by cloning and functional evaluation of an Art v 1-specific TCR after transfer into human T-lymphocytes in *vitro* [71]. We showed that Art v 1-specific TCR tg T-cells represent an excellent tool to study immunomodulation of allergen-specific T-cells such as anergization of pathological Th2 cell responses [72,239]. Accordingly, we generated double transgenic mice co-expressing a human Art v 1-specific TCR (TRAV17/TRBV18) and the corresponding human restriction element HLA-DR1 on the C57BL/6 background. The variable domains of the TCR α and β chain were chimerized with the murine TCR constant regions thereby putting them under the influence of murine

regulatory elements that guarantee efficient expression in mice. Established single tg mice expressing the Art v 1-specific TCR were crossed with HLA-DR1 tg mice [237]. Immunophenotyping of double tg TCR/DR1 mice revealed a normal distribution of leukocyte subsets in spleen, thymus and peripheral blood when compared to control wild type, single tg HLA-DR1 or single tg TCR mice. Moreover, the allergen-specific TCR tg T-cells are properly selected on the HLA-DR1 background. Clear-cut expression of the tg TCR on $CD3^+$ T-cells was found (60 % of the $CD3^+$ T-cells) in the spleen, whereas about 80 % of the peripheral blood $CD4^+$ T-cells in are TCR tg. Moreover, T-cells isolated from the spleens of double tg mice mounted a strong allergen-specific proliferative response against the full allergenic protein, Art v 1, or the Art v $1_{25\text{-}36}$-peptide, containing the immunodominat epitope, when presented by HLA-DR1. Of note, this cellular proliferation was accompanied by a balanced cytokine milieu without a significant bias towards a certain T helper cell subset.

Mouse models resembling the human pathology by using exclusive allergen-aerosol exposures without systemic sensitization as sensitization/provocation protocols are scarce. Using humanized TCR/DR1 double tg mice with a large precursor frequency of allergen-specific T-cells eliminates the need of prior systemic sensitization with adjuvant before allergen-specific aerosol challenge. In our model three consecutive aerosol exposures are sufficient to elicit massive airway reactivity in unprimed double tg mice in response to the specific allergen as measured by whole body plethysmography. Lung histology revealed airway and lung inflammation with massive peribronchiolar and perivascular infiltration with immune cells. Interestingly, lung function of double tg mice started to show altered airway reactivity already with the second allergen exposure, whereas all groups of control mice did not show impaired lung function at any point of intervention. These results are in accordance with observations form *Wilder et al.*, who demonstrated that 3 weekly OVA aerosol challenges each lasting for an hour are sufficient to induce AHR in naive heterozygous $DO11.10^{+/-}$ mice [116]. Of importance, *Knott et al.* showed by using homozygous DO11.10 mice that a single OVA aerosol exposure or four OVA aerosol exposures delivered on four consecutive days failed to develop AHR, as measured by WBP and expressed in Penh (enhanced pause) in response to methacholine [115]. The authors show that a single allergen-exposure of mice was accompanied exclusively with an increased influx of neutrophils and minimal recruitment of eosinophils into the lungs and the detection of mucus in the bronchial epithelium. In our model, double tg mice show a diverse cellular composition of BAL fluids with high levels of granulocytes, composed of mainly eosinophils and neutrophils as well as T-lymphocytes, monocytes and alveolar macrophages following a short-term provocation

protocol (three exposures on three consecutive days). In contrast, control groups of mice showed significantly different BAL compositions lacking the pronounced eosionophilic and neutrophilic components. Lung histology revealed only minor mucus production in allergen-exposed double transgenic mice, most probably caused by the fact that mucus production and structural remodeling of the lung is a chronic feature of allergic asthma elicited only upon long-term exposure. Such protocols resembling clinical pathological effects are currently evaluated. However, in control experiments using systemic Art v 1-specific sensitization in the presence of alum as adjuvant showed the induction of comparable allergen-specific IgE-levels in BALB/c mice and the double tg TCR/DR1 (C57BL/6 background) mice after three intraperitoneal injections. In contrast, four exclusive intranasal challenges in the absence of adjuvant only induced significant allergen-specific IgE titers in double transgenic mice but not in control mice.

In the light of these cumulative observations it is evident that numerous unresolved issues about the contribution of T-cells in allergic diseases exist. Humanized TCR tg mice should prove as model systems to understand the fundamental basics of the pathophysiology as well as to improve the development and evaluation of such T-cell based therapeutic approaches.

Table I. Summary of lymphocyte-based *in vitro* and *in vivo* model systems in allergy research

Read-out	Species (strain, ref.)	Outcome	References*)
B-cell function (in vitro)			
IgE production (spontaneous)	human	*in vitro* production of IgE by PBMC from atopic patients	[5, 6]
IgE production (induced)	human	co-culture of B-cells with α-CD40 mAb decorated $CD32^+$ L-cells plus exogenous IL-4 induced significant levels of IgG, IgM and IgE	**[3, 4]**
B-cell depletion (cobra venom factor)	rat	*in vitro* depletion of CRL in splenocyte preparations by addition of cobra venom factor and fresh serum	[10]
B-cell depletion (complement R)	human	B-cells rosette with erythrocytes sensitized with Ab and C3, CRL separation from non-CRL on albumin gradients, no CRL in thymus	[11]
B-cell function (in vivo)			
B-cell depletion (anti-μ)	mouse	depletion of Ig^+ B-cells by continuous treatment of with rabbit α-μ-chain antiserum	[21]
B-cell depletion (anti I-A)	SJL, CKB	injection of α-I-A mAb depletes nearly all B-cells in SJL mice and 50% of B-cells in CKB mice; reduced primary, secondary and tertiary immune response	[20]
B-cell knock-out mice (μMT-mice)	C57BL/6 [23]	no $B220^+$, IgM^+ or IgD^+ cells in periphery or peritoneal cavity; no IgM in serum; after allergen sensitization and challenge marked eosinophilia and mucus production, no induction of AHR	[**24,** 27, 28]
IgE knock-out mice (Ig-Cε ko)	*129/sv*	no IgE-levels but wt-levels of other Ig-classes; allergen-sensitization/challenge induced strong, non-complement-dependent anaphylaxis in wt and ko mice (*IgG-dependent mast cell activation)*	[31]
CD23 Fcε $^{-/-}$ mice	C57BL/6 [39]	allergen-sensitization/challenge leads to higher IgE-titers in $CD23^{-/-}$ mice and more eosinophilia in lung compared to wt mice	[40]
TLR9$^{-/-}$ mice	C57BL/6 [241	allergen-sensitization/challenge induced less peanut-specific IgE in $TLR9^{-/-}$ than wt mice, smaller Peyer's patches evident, similar effects upon sensitization via skin, i.p. sensitization, i.e. systemic IgE production affected	[43]
Breg transfer model	BALB/c, C57BL/6	*S. mansoni* infection induces high numbers of $IL\text{-}10^+CD1d^+$ Bregs, Bregs transfer into sensitized mice leads to reduction of airway inflammation, reduced overall numbers in BAL, BAL-resident eosinophils and lung resistance, Breg induce Treg influx into lungs	[49, 50]
T- and NK-cell function (in vitro)			
Allergen-specific T-cell lines/clones	human	establishment of allergen-specific T-cell clones from PBMC of atopic individuals	[**55**, 57-60]
polarization of NK-cells	human	stimulation of PBL with γ-irradiated RPMI 8866 cells plus polarizing culture conditions lead to NK-cell polarization, NK1: production of IFN-γ & IL-10; NK2: production of IL-5 & IL-13	**[203,** 204, 205**]**
T- and NK-cell function (in vivo))***			
$CD4^+$T-cell depletion (anti-CD4)	BALB/c	administration of α-CD4 mAb in chronic model of allergic disease reduced AHR, eosinophilia in BAL and airway remodeling	[76, 78, **79**]
	C57BL/6	reduced eosiniphilia in BAL, no effect on airway remodeling	[81]
$CD8^+$ T-cell depletion (anti-CD8)	IL-15tg mice [240], C57BL/6	allergen-sensitization/challenge reduced eosinophilia & Th2-cytokine production, pronounced Tc1 response depletion of CD8 in IL-15 tg mice induces aggravation of allergic airway inflammation	[150]
$CD4^+CD25^+$ Treg depletion (anti-CD25)	C3H/HeJ	reduced AHR, eosinophilia & IgE, Th2 cytokine production, increased pulmonary myeloid DC (high MHCII, CD80 & CD86)	[141]
TCR depletion (anti-TCR ß and δ)	BALB/c, C57BL/6	antibody-depleted mice resembled gene-deficient mice in BALB/c and C57BL/6; mice lacking γδ-T-cells developed increased AHR	[183]
iNKT-cell depletion (anti-CD1d)	C57BL/6	blocking of iVα14 T-cells to promote eosinophil recruitment to airways & allergen-induced Th2 cytokine & IgE production	[219]
CD4 knock out mice ($CD4^{-/-}$)	C57BL/6(B6.129S2-d4^{tm1Mak}/J) [87]	no induction of airway inflammation and airway remodeling	[88]
CD8 knock out mice ($CD8^{-/-}$)	CD8 ko mice [166]	reduced lung eosinophila, IL-13 production in BAL and AHR, (adoptive transfer of OVA-primed $CD8^+$ T-cells restorated of symptoms)	[157]
TCR knock out mice (TCR $\gamma\delta^{-/-}$)	TCR $\gamma\delta^{-/-}$ [179] (BALB/c)	reduced specific IgE & IgG1, reduced eosinophil and T-cell infiltration in lung, pulmonary IL-5 and reduced AHR;	[180]
iNKT-cell deficient mice	$Cd1d^{-/-}$ [218], $Ja281^{-/-}$ [217] BALB/c	no induction of AHR in $Ja281^{-/-}$mice or $Cd1d^{-/-}$	[219, **220**, 227]
TCR tg $CD4^+$ mice	DO11.10 (BALB/c) [102]	one OVA-aerosol challenge induces dominant neutrophilic lung inflammation, eosinophilia, IFN-γ-secretion in BAL fluids, mucus hyperproduction	[**115**, 116, 117]
TCR tg /eosinophil$^{-/-}$ mice	OT-II.2 [106], OT-II/*PHIL* [123]	eosinophils indispensable for disease induction, Th2 migration & cytokine production in lungs, goblet cell metaplasia/airway epithelial cell mucus production	[120]
TCR tg $CD8^+$ mice	C57BL/6 OT-I [170]	reduced IL-5 & IL-13 in BAL and airway eosinphilia, but enhanced levels of IL-12 in BAL	[**152**, 175,176]

E-RFC, erythrocyte rosette forming cell; SRBC, sheep red blood cell; AHR, airway hyperreactivity; AR, airway remodeling; AI, airway inflammation; $^{+/-}$ hemizygous; BAL, bronchoalveolar lavage; i.t., intratracheal; LN, lymph node; mAb, monoclonal antibody; CFSE, carboxyfluorescein diacetate succinimidyl ester; PKO; perforin knockout mice

*) Main references for each topic: bold;
**) For a detailed overview of T cell adoptive transfer models please refer to Table II.

Table II. T-cell transfer model systems for allergy research

Strain	Allergen	Sensitization	Challenge/Exposure	Timing of Transfer	T-cell type & Amounts	Outcome	Reference
BALB/c	OVA	-	day 1, aerosol 100 mg/ml OVA/20 min	day 0	5x 10^6 OVA-specific T-cell **clones** (IL-5^+) (i.v.)	eosinophilic inflammation & AHR, increased IL-5-concentration in BALF	[91]
C57BL/6 IL-$5^{-/-}$ [92]	OVA	day 0 & 12, i.t. 50µg OVA/alum	day 25 plus onwards every second day for 8 d aerosol 3x 1h 10 mg/ml OVA /30 min	day 23	2x 10^6 OVA-primed $CD4^+$**Th2-cells** (IL-4^+IL-5^+) from sensitized wt mice	blood and airway eosinophilia, lung damage, AHR in IL-$5^{-/-}$ mice	[93]
BALB/c, DO11.10	OVA	-	day 1, aerosol challenge 1% OVA/20 min for 7d	day 0	5x 10^6 of ***in vitro***-generated TCR tg **Th1** or **Th2** cells (i.v.)	Th2 cells > eosinophilia & AHR; Th1 cells > neutrophilia & no AHR	[94]
BALB/c	OVA	day 0, 7 & 14, i.p. 10 µg OVA/alum	day 15-19, daily aerosol challenge 5% OVA/20 min	day 15	5x 10^6 OVA-specific **Th1-*clones*** (i.v.)	reduced eosinophila in BALF & peribronchial area; suppression of goblet cell hyperplasia & peribronchial fibrosis & decreased AR, increased IFN-γ & reduction of IL-5 & IL-13 in BALF	[97]
BALB/c, DO11.10, IFN- $\gamma^{-/-}$	OVA	-	day 1-9, aerosol challenge 1% OVA/20 min (4d challenge, 2d resting, 3d challenge)	day 0	co-transfer of 5x 10^6 or of ***in vitro***-generated TCR tg **Th1** and **Th2** cells (i.v.)	counter-regulation by Th1 cells, reduced airway eosinophilia and mucus production, IFN-γ dependent	[96]
BALB/c, DO11.10, C.B-17Icr$^{scid/scid}$ [242]	OVA	BALB/c: day 0 & 14, i.p. 50 µg OVA/alum	BALB/c: day 14,25-27 i.n 50 µg OVA SCID: day 0, 2, 3 i.n 50 µg OVA	day 14 and 25 day 1	2.5-7.5x 10^6 *in vitro*-generated DO11.10 TCR tg **Th1, Th2** or **Th0** cells (i.v.)	Th1-cell transfer induces airway inflammation, no counter-regulation of Th2-induced airway inflammation, no induction of AHR but no counter-regulation of Th2-induced AHR	[99]
BALB/c, DO11.10	OVA	-	day 24-32, aerosol challenge 1% OVA/40 min	day 0	1-5x 10^6 ***in vitro***-generated TCR tg **Th1 and**/or **Th2** cells (i.v.)	Th2 cells induce airway eosinophilia & AHR, Th1 cell co-transfer neither enhanced nor reduced inflammation	[100]

BALF, bronchoalveolar lavage fluid; tg, transgenic, AHR, airway hyperresponsiveness; OVA, ovalbumin; i.t., intratracheal; i.v. intravenous; i.n., intranasal; d, day;

Table III. Humanized mouse models for allergy research

Model	Allergen	Starting population	Experimental protocol and Outcome	Reference
Hu-PBMC-SCID	Dpt	PBMC of sensitized and non-sensitized individuals CB.17 SCID mice	day-14, i.p. transfer of $1x10^7$ PBMC into SCID mice, day 0-4, aerosol challenge with 200 µg Dpt *2 months after challenge, detection of $CD45^+$, $CD45RO^+$, $CD20^+$ & $HLA\text{-}DR^+$ human cells in perivascular, peribronchial areas & lung parenchyma, mRNA-$IL\text{-}5^+$ cells & eosinophils in peribronchial infiltrates*	[231]
Hu-PBMC-SCID	Dpt	PBMC of atopic donors C.B.-17 scid/scid mice	day 0, i.p. transfer of $5\text{-}10x10^7$ PBMC day 0 & 7, i.p. immunization with 20 µg Dpt *specific IgE levels in hu-PBL-SCID sera comparable to donor sera, location of human T-cells in spleen, with hIL-4, hIL-5, and hIFN-γ mRNA expression after stimulation with PHA & PMA*	[232]
Hu-PBMC-SCID	Der p 1	PBMC of non-sensitized individuals C.B17-SCID	day -1, i.p. injection of 900 µg α-mouse CD122; day 0, i.t. transfer of $1x10^7$ PBMC; day 1 & 9 i.p. immunization with 10 µg Der p 1/alum, day 2-20, daily aerosol challenge with HDM extract (300 µg/ml/ 30 min) *hIL-4 &h IL-5-dependent AHR, infiltration of airways & lungs with hT-cells, no eosinophils, no increases in human IgE*	[233]
Hu-PBMC-SCID	Dpt	PBMC of HDM allergic asthmatic patients (NOD/SCID mice)	day 0, i.p. injection of $1x10^7$ PBMC, days 1, 3 & 7, i.p. injection of 100 µg of α-hCCR4 mAb & after 30 min i.t. injection of Dpt- extract *allergic inflammation & BHR, treatment with CCR4 blocking antibody, abolished airway eosinophilia, goblet cell hyperplasia, IgE synthesis & BHR*	[234]
iPSC-MSC	OVA	BALB/c mice	every other day from day 1-13, i.p. injection of 40 µg OVA/alum day 21 -27, aerosol challenge with 5% OVA/30 min & subsequent i.n. administration of 800 µg OVA, day 0 or 20, transfer of $5x10^6$ human iPSC-MSC *reduced inflammatory cell infiltration & mucus production reduced nasal eosinophil infiltration, and reduced in inflammatory cell infiltration in BAL & nasal lavage fluids. reduced serum IgE and decreased IL-4, IL-5, IL-13 BAL and/or nasal lavage fluids,*	[235]
human TCR/HLA-tg	Art v 1	C57BL/6	day 0, 1, 2 aerosol exposure (1% mugwort-extract/15 min), *AHR in response to allergen, massive eosinophilia, peribronchial and perivascular lung infiltration*	*Neunkirchner et. al* (in preparation)

Dpt, Dermatophagoides pteronyssinus; PHA, Phytohaemagglutinin; PMA, Phorbol-12-myristat-13-acetat; BHR, bronchial hyperreactivity; iPSC-MSC, induced pluripotent stem cell derived mesenchymal stem cells

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Chapter II

Molecular and functional analysis of the antigen receptor of Art v 1–specific helper T lymphocytes

Victoria M. Leb, MSc,[a] Beatrice Jahn-Schmid, PhD,[b] Klaus G. Schmetterer, MSc,[a] Hans J. Kueng, MSc,[a] Daniela Haiderer, MSc,[a] Alina Neunkirchner, MSc,[a] Gottfried F. Fischer, MD,[c] Karl Nissler, PhD,[d] Arnulf Hartl, PhD,[e] Josef Thalhamer, PhD,[f] Barbara Bohle, PhD,[b] Brian Seed, PhD,[g] and Winfried F. Pickl, MD[a] *Vienna and Salzburg, Austria, Jena, Germany, and Boston, Mass*

Background: Ninety-five percent of patients with mugwort allergy are sensitized to Art v 1, the sole major allergen in mugwort *(Artemisia vulgaris)* pollen. Sixty-nine percent of patients recognizing the single immunodominant T-cell epitope Art v 1_{25-36} have an HLA-DRB1*01 phenotype.
Objective: We studied cloning and functional expression of a human αβ T-cell receptor (TCR) specific for Art v 1_{25-36}.
Methods: TCR chains were RT-PCR amplified from an Art v 1_{25-36}–specific T-cell clone, retrovirally transferred, and functionally tested in Jurkat T cells or alternatively in peripheral blood T lymphocytes of nonallergic individuals.
Results: The α-chain of the TCR is composed of TRAV17 and TRAJ45 segments, and the β-chain uses TRBV18, TRBD1, and TRBJ2-7. Analyses of 23 other Art v 1–specific T-cell clones did not reveal preferential usage of the TRAV17, TRBV18, or other TCR gene families. Efficient TCR transfer into Jurkat T cells was shown by binding of TCR Vβ18–specific mAb and DRB1*0101/Art v 1 tetramers. Transgenic Jurkat T cells specifically recognized syngeneic EBV B cells pulsed with Art v 1_{25-36} peptide and artificial antigen-presenting cells expressing invariant chain::Art v 1 fusion proteins. Moreover, transfer of the TCR into peripheral blood lymphocytes generated T cells that were Art v 1 reactive. Activation of transgenic T cells by artificial antigen-presenting cells was strictly dependent on costimulation.
Conclusion: For the first time, a detailed molecular and functional analysis of a human allergen-specific TCR is presented. (J Allergy Clin Immunol 2008;121:64-71.)

Key words: *Type I allergy, mugwort pollinosis, Art v 1, human T-cell receptor, T-cell receptor transgenic T cells, artificial antigen-presenting cell*

T lymphocytes play a central role in the pathogenesis of allergic diseases. During sensitization, priming of allergen-specific ($CD4^+$) T_H2 cells results in the production of T_H2 cytokines, which are responsible for class switching to the ε immunoglobulin heavy chain, allowing IgE production by B cells.[1] Once primed, allergen-specific T cells critically contribute to late-phase reactions and allergic inflammation in target organs (eg, airways and skin).[2] The clonotypic specificity of T cells is brought about by the T-cell receptor (TCR), which shows dual specificity for the antigen (allergen) and for self (MHC) determinants.[3] Signal transduction by the TCR is conducted by means of noncovalently associated invariant CD3 subunits.[4-6]

The antigen specificity of T cells can be transferred to other T cells by means of TCRαβ gene transfer.[7-9] TCR-transgenic (tg) model systems have contributed greatly to our understanding of the immune response to autoantigens and infectious agents[10-12] and have shed light on fundamental processes in T-cell development.[5,13,14] Although TCR gene usage by allergen-specific T lymphocytes has been studied to a considerable degree in the past,[15-19] no molecular or functional analyses of cloned and ectopically expressed human allergen-specific TCRs have been reported thus far. Consequently, biologic model systems based on human allergen-specific TCRs are still missing.

An ideal human allergen-specific model system would comprise an immunodominant epitope, which is presented by a well-characterized restriction element expressed by the majority of the individuals affected. These features apply to mugwort pollen allergy: mugwort *(Artemisia vulgaris)* is widely spread throughout the European temperate climate zone, North America, and parts of Asia and is one of the main causes of hay fever in late summer and autumn.[20] More than 95% of patients with mugwort allergy are sensitized to Art v 1, the major allergen in mugwort pollen. Analyzing the T-cell response to Art v 1 by using a panel of specific T-cell lines and clones revealed that the presentation of the sole immunodominant epitope (Art v 1_{25-36}) is highly associated with HLA-DRB1*01.[21,22] Thus allergy to Art v 1 is characterized by a uniform T-cell response.

In the present study we addressed the feasibility of engineering allergen-specific T lymphocytes by means of genetic TCR transfer. The molecular and functional characterization of human allergen-specific TCRs as critical tools of defined model systems

From [a]the Institute of Immunology and [b]the Department of Pathophysiology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna; [c]Department of Blood Group Serology, Medical University of Vienna; [d]the Institute of Pathology, Friedrich Schiller University, Jena; [e]the Institute of Physiology and Pathophysiology, Paracelsus Medical University, Salzburg; [f]the Department of Molecular Biology, Division of Allergy and Immunology, University of Salzburg; and [g]the Center for Computational and Integrative Biology, Harvard Medical School, Boston.

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The nucleotide sequences for the Art v 1–specific T-cell receptor α and β chains have been deposited in the GenBank database under accession numbers EU030677 and EU030678, respectively.

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Reprint requests: Winfried F. Pickl, MD, Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, A-1090 Borschkegasse 8A, Vienna, Austria. E-mail: winfried.pickl@meduniwien.ac.at.

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Abbreviations used
aAPC: Artificial antigen-presenting cell
CLIP: Class II–associated invariant chain peptide
FITC: Fluorescein isothiocyanate
HA: Influenza hemagglutinin
Ii: Invariant chain
PB: Peripheral blood
PBL: Peripheral blood lymphocytes
PE: Phycoerythrin
SEE: Staphylococcal enterotoxin E
SI: Stimulation index
TCR: T-cell receptor
tg: Transgenic
WT: Wild-type

in which all 3 components of the "allergen-specific synapse" are well defined (ie, restriction element, immunodominant peptide, and TCR) will provide important insights into the pathophysiology of allergic diseases and their possible future cure.

METHODS

Cell lines and primary cells

For this investigation, 293 cells, 293 OKT3scFv::GPI transfectants, 23 Raji cells, and Jurkat clone 41-19 expressing an IL-2 enhancer/promoter-driving luciferase were cultured as previously described.[23] Art v 1–specific T-cell lines, T-cell clones, and immortalized B cells were established from peripheral blood lymphocytes (PBLs) of individuals with mugwort pollen allergy with a typical clinical history and positive skin prick test responses to mugwort pollen extract, as described.[22] The TCR gene usage was determined by means of RT-PCR with TRAV and TRBV primers (Clonetech, Heidelberg, Germany), as previously described.[22]

Cloning and characterization of αβ TCRs

mRNA from the Art v 1–specific T_H0 cell clone SSR20, which on antigen-specific stimulation produced 22 pg/mL IL-4 and 75 pg/mL IFN-γ, was amplified with TRAV17 forward 5′-CGCGGGAAGCTTGCCA**CCATGG**AAA CTCTCCTGGGAGTG-3′ and TRAC reverse 5′-CCCGCGGCGGCCGCT TTAGCTGGACCACAGCCGC-3′ or TRBV18 forward 5′-CGCGGG AAGCTTGCCA**CCATGG**CCAACTCTGCTATGGAC-3′ and TRBC reverse 5′-CCCGCGGCGGCCGCTTTAGAAATCCTTTCTCTTGACCATG-3′, digested with *Nco*I (bold) and *Not*I (underlined), and gel purified. DNA fragments were ligated into the retroviral expression vector pMMP412 (R.C. Mulligan, Childrens Hospital, Boston, Mass) and DNA sequenced (VBC Genomics, Vienna, Austria).

Cloning of HLA-DR constructs

SSR (EBV-immortalized B cells) mRNA was amplified with HLA-DRA forward 5′-CGCGGG**AAGCTT**GCCACCATGGCCATAAGTGGAGTCCC-3′ and HLA-DRA reverse 5′-CCCGCGGCGGCCGCTTTACAGAGGCCC CCTGCG-3′ or HLA-DRB1*0101 forward 5′-CGCGGG**AAGCTT**GCCAC CATGGTGTGTCTGAAGCTCCC-3′quo; and HLA-DRB1*0101 reverse 5′-CCCGCGGCGGCCGCTTTAGCTCAGGAATCCTGTTGGCT-3′, digested with *Hind*III (bold) and *Not*I (underlined), and gel purified. DNA fragments were ligated into pEAK12 expression vector (EdgeBio, Gaithersburg, Md) and DNA sequenced.

Generation of invariant chain constructs

pcDNA1.1/Amp containing an invariant chain (Ii) with a *Sfu*I–*Eco*47III cassette instead of class II–associated Ii peptide (CLIP) was kindly provided by J. vanBergen (Immunohematology and Blood Transfusion, Leiden, The Netherlands).[24] The double-stranded Art v $1_{25\text{-}34}$ oligonucleotides Art v $1_{25\text{-}34}$ upper 5′-CGAAGTGCATCGAGTGGGAGAAGGCCCAGCACCAGGC-3′ and Art v $1_{25\text{-}34}$ lower 5′-GCCTGGTGCTGGGCCTTCTCCCACTCG ATGCACTT-3′ were inserted into *Sfu*I/*Eco*47III-digested pcDNA1.1/Amp-Ii. CLIP and $HA_{309\text{-}317}$ containing Iis were constructed as previously described.[24] Length variants of Art v 1 were constructed by means of PCR with codon optimized Art v 1[25] as template amplification with the primers codon optimized Art v $1_{1\text{-}109}$ forward 5′-CGCGGGTTCGAAGATGGCCGG CAGCAAGCTG-3′quo; and codon optimized Art v $1_{1\text{-}109}$ reverse 5′-GGGCGCGGGCAGCGCTTGGTGGGTGCTGGGGGG-3′; codon optimized Art v $1_{15\text{-}50}$ forward 5′-CGCGGGTTCGAAGAGCGGCAAGTGCGA CAACAAG-3′ and codon optimized Art v $1_{15\text{-}50}$ reverse 5′-GGGCGCG GGCAGCGCTTGGCAGAAGCAGCTCTCCTTGCC-3′; codon optimized Art v $1_{20\text{-}45}$ forward 5′-CGCGGGTTCGAAGAACAAGAAGTGCGACAA GAAG-3′ and codon optimized Art v $1_{20\text{-}45}$ reverse 5′-GGGCGCGGGC AGCGCTTGCTTGCCGGCCTCCCGCTT-3′; and codon optimized Art v $1_{1\text{-}57}$ reverse 5′-GGGCGCGGGCAGCGCTTGGCTCTTGCTGCAGTC GAAGTA-3′. After PCR amplification, digestion, and gel purification, the DNA fragments were ligated into the pcDNA1.1/Amp-Ii expression vector, followed by DNA sequencing (VBC Genomics).

Transduction of αβ TCRs into Jurkat and peripheral blood T cells

Amphotropic TCRαβ-delivering retroviruses were produced by means of transient transfection with standard procedures.[26] Briefly, 293 OKT3scFv::GPI cells were transfected with pMD.gagpol (Moloney murine leukemia virus *gag-pol*), pMD-VSV-G (vesicular stomatitis virus protein G; both provided by Dr R. C. Mulligan),[27] pMMP412.TRAV17, and pMMP412.TRBV18. Alternatively, pMMP412.GFP was used as a control. Jurkat clone 41-19 or peripheral blood (PB) T cells were transduced with viral supernatant in the presence of 8 μg/mL polybrene (Sigma, St Louis, Mo). Before transduction, PB T cells were prestimulated with anti-CD3/CD28 substituted microbeads (Dynabeads, Dynal Biotech, Oslo, Norway) and optionally with 300 U/mL IL-2 (Peprotech, London, United Kingdom). Jurkat clones were obtained by limiting dilution.

Immunofluorescence analyses

The mAbs CD3–fluorescein isothiocyanate (FITC; UCHT1), pan-TCRαβ–phycoerythrin (PE; BMA031), CD2-FITC (TS2.18), CD4-FITC (VIT4), CD8-PE (VIT8b), and control immunoglobulin FITC and PE were obtained from ADG (Kaumberg, Austria). TCR Vβ8-FITC (56C5) and TCR Vβ18-PE (BA62) were from Immunotech (Marseille, France). HLA class II–FITC (1/47) and Ii-FITC (5-329) were provided by O. Majdic (Institute of Immunology, MUV, Vienna, Austria). Surface and intracellular staining, as well as flow cytometric analyses, were performed as previously described.[28] For tetramer staining, Jurkat cells (4×10^5) were incubated with 4 μL of PE-labeled DRB1*0101/Art v 1 peptide–specific tetramer (Beckman Coulter, Marseille, France) containing Art v $1_{19\text{-}36}$ (NKKCDKKCIEWEKAQHGA) at 37°C in the dark for 2 hours, washed once, and fixed in 200 μL of 0.5% paraformaldehyde-PBS at room temperature for 1 hour, followed by immediate flow cytometric analysis.

Jurkat luciferase activity assays

SSR EBV-immortalized B cells (5×10^4, HLA-DRB1*0101$^+$) were preincubated with the peptides (3×10^{-5} mol/L) Art v $1_{19\text{-}30}$, Art v $1_{25\text{-}36}$, Art v $1_{94\text{-}105}$, influenza HA306-318 or medium alone, respectively, for 3 hours. Subsequently, TCR tg Jurkat cells or wild-type (WT) Jurkat cells (1×10^5) were added and cocultured with presensitized syngeneic EBV cells or peptide alone for 6 hours. Alternatively, artificial antigen-presenting cells (aAPCs; 5×10^4) expressing HLA-DR1, CD54, CD80, and Ii constructs served as stimulators. Staphylococcal enterotoxin E (SEE; 0.3×10^{-6} mol/L; Toxin Technologies, Sarasota, Fla) or PHA (5 μg/mL) plus phorbol 12-myristate 13-acetate (10^{-7} mol/L) served as positive controls. Luciferase activity was assayed as previously described.[28]

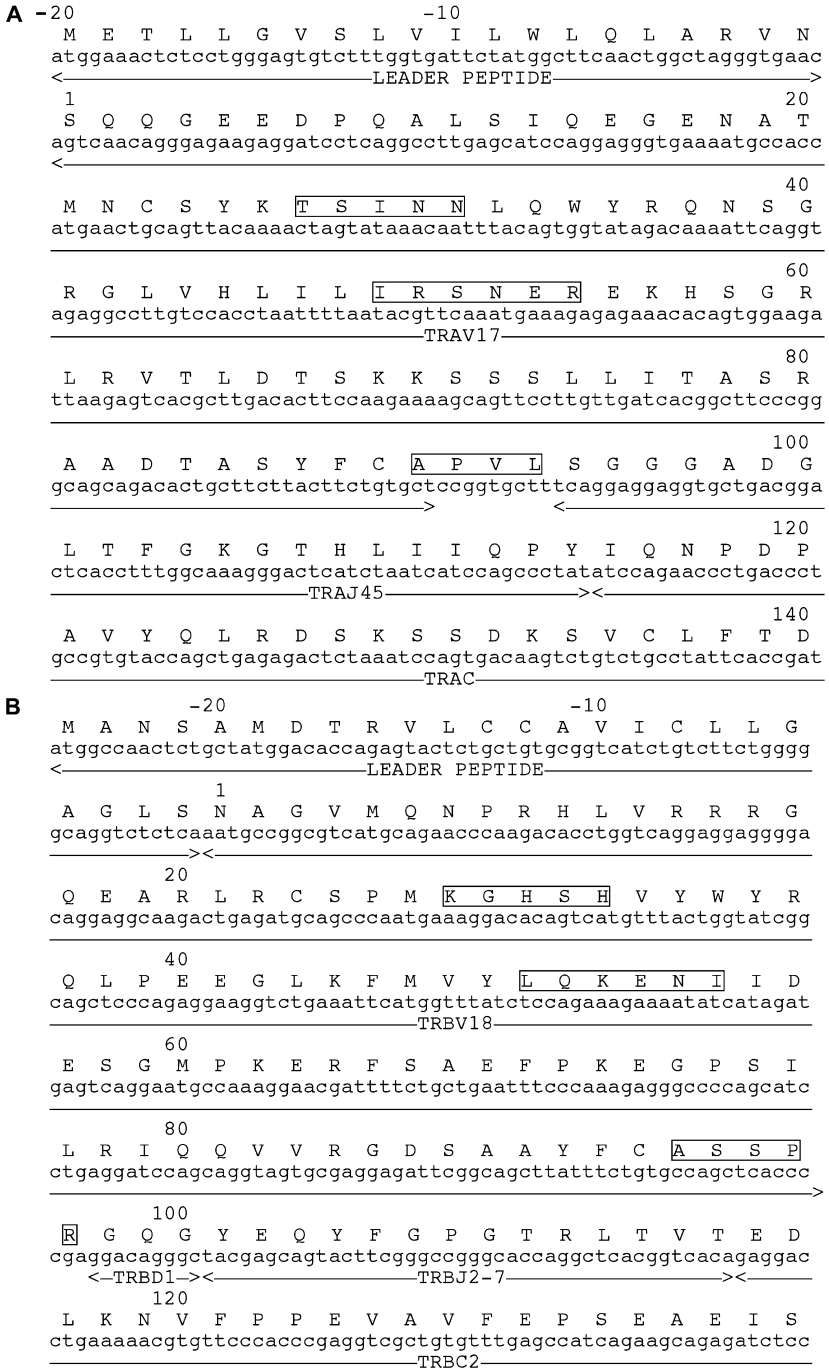

FIG 1. Nucleotide and predicted amino acid sequence of the HLA-DRB1*0101–restricted TCR of the Art v 1–specific T-cell clone SSR20. **A,** TCR α-chain sequence. **B,** TCR β-chain sequence. Codon numbering and nomenclature are according to the international immunogenetics information system.[29] Only the 5′-ends of the constant-region sequences are shown. Complementarity-determining regions are *boxed*.

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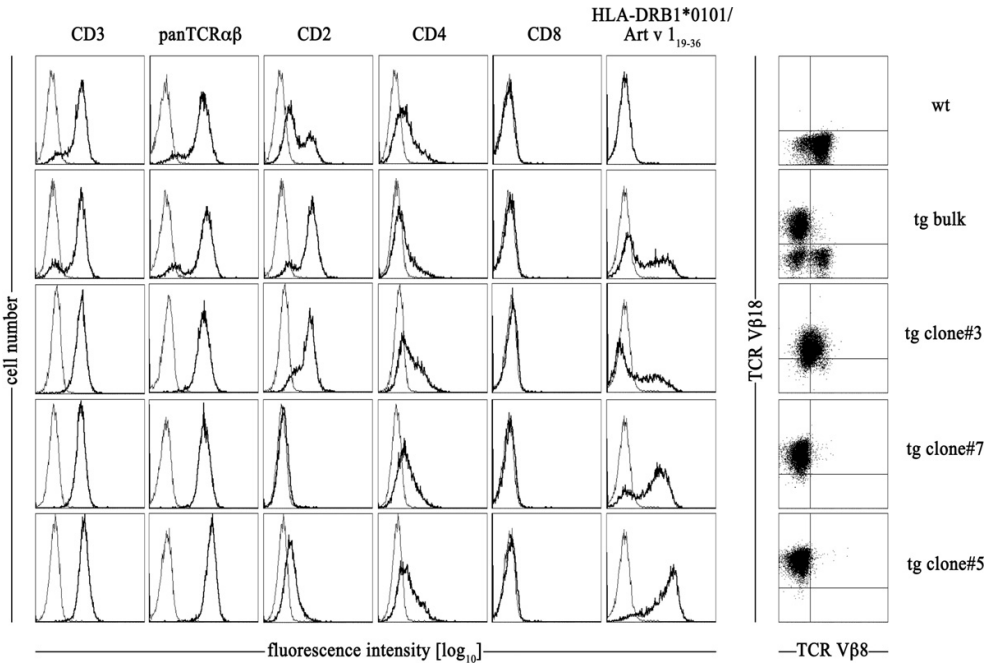

FIG 2. Expression pattern of endogenous TCR, Art v 1–specific tg TCR, and accessory molecules on WT, bulk TCR tg, and TCR tg single-cell clones (nos. 3, 5, and 7) of Jurkat T cells are shown. Binding of specific mAbs or Art v 1–specific tetramers (HLA-DRB1*0101/Art v 1_{19-36}; *bold lines*) is compared with control antibody binding *(thin lines)*. Two-parameter dot-plot analysis of Jurkat cells stained with TCR Vβ8 and TCR Vβ18 mAbs *(right panel)*. Data are representative of 2 independently performed experiments.

Generation of aAPCs

In this study, 293 cells (3×10^6) were transiently cotransfected with HLA-DRA*0101, HLA-DRB1*0101, CD54, CD80, cathepsin S,[28] and Ii constructs or empty control vector in combinations as indicated and used 72 hours after transfection.

T-cell proliferation assays

TCR tg T cells (5×10^4 per well) were incubated with aAPCs (5×10^4 per well irradiated with 60 Gy) expressing HLA-DRA*0101, HLA-DRB1*0101, Ii::Art v 1_{25-34}, Ii::$HA_{309-317}$, CD80, and cathepsin S combined, as indicated in 96-well flat-bottom tissue-culture plates for 3 days. Cells were pulsed with [methyl-^{3}H] thymidine (1 μCi per well) for another 18 hours and processed as previously described.[23] Medium alone or anti-CD3/CD28 microbeads (5×10^4) served as controls.

RESULTS

Molecular cloning of the αβ TCR of an Art v 1_{25-36}–specific, HLA-DRB1*0101–restricted T-cell clone

The Art v 1–specific $CD4^+$ T-cell clone (SSR20) was isolated from the blood of a subject with mugwort pollen allergy (HLA-DRB1*0101, *0301; DRB3*0101; DQB1*0201, *0501) shortly after the mugwort pollen season. The clone specifically reacted to stimulation with the immunodominant peptide of Art v 1, Art v 1_{25-36}, in an HLA-DRB1*0101–restricted manner.[21] The TCR α- and β-chains of SSR20 were RT-PCR amplified, ligated into the retroviral expression vector pMMP412, and DNA sequenced. The functional α-chain gene of SSR20 was formed by the rearrangement of a Vα gene segment of the TRAV17 gene family to a TRAJ45 segment (Fig 1, nomenclature according to Lefranc et al[29]). The β-chain of SSR20 is derived from a TRBV18-TRBD1-TRBJ2-7-TRBC2 rearrangement (Fig 1). The junctional or N-diversification in CDR3s is low for both chains. In the α-chain 3 nongermline-encoded codons are present, whereas the 2 C-terminal TRAV17 codons have been deleted. CDR3β is composed of germline sequences, except for the addition of a single arginine. Assessment of 23 T-cell clones of 10 additional subjects with Art v 1 allergy revealed that 12% of the clones expressed TRAV17 and 9% of the clones expressed TRBV18, indicating that Art v 1_{25-36}–specific T-cell responses are not confined to TCRs consisting of the TRAV17 and TRBV18 gene families.

Retroviral transfer and expression of the Art v 1–specific TCR

In a first instance expression of the cloned TCR was tested by means of retroviral transduction of Jurkat T cells harboring a human IL-2 promoter/enhancer controlling luciferase expression.[23] Immunofluorescence staining of bulk transductants revealed stable TCR Vβ18 expression in approximately 60% of cells (Fig 2). Expression levels of CD3, pan-TCRαβ, and CD4 were comparable in WT and TCR tg Jurkat cells in bulk transductants, as well as in single-cell clones, whereas, for example, CD2 expression levels showed considerable variation (Fig 2). A similar variability was observed for Vβ8 and Vβ18 expression on single-cell clones. Clone 3 showed residual coexpression of the

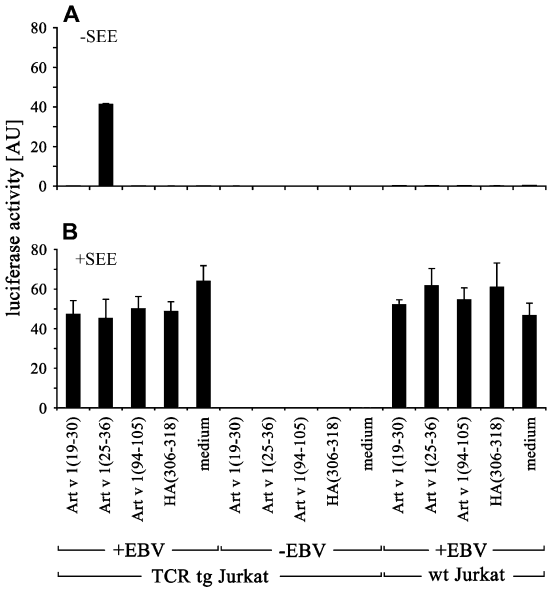

FIG 3. The Art v 1–specific TCR in tg Jurkat T cells is functionally active. **A**, IL-2 promoter activity of Art v 1 TCR tg or WT Jurkat T cells on coculture with or without HLA-DRB1*0101$^+$ EBV B cells and the indicated peptides or with medium alone. **B**, Similar to Fig 3, *A*, but supplemented with SEE. Data show mean values ± SDs of triplicate cultures representative of several independently performed experiments.

endogenous Vβ8 chain, whereas clones 5 and 7 showed mutual exclusive expression of the tg Vβ18 chain (Fig 2), which might be caused by different levels of overexpression (long terminal repeat–driven or through copy number) of the tg TCR and competition for the constitutively expressed CD3 complex. HLA-DRB1*0101/Art v $1_{19\text{-}36}$–specific tetramers bound to TCR transductants exclusively (Fig 2), indicating that on transfer the tg TCR retained its capability to specifically interact with the restriction element/peptide complex against which it had been selected *in vivo*.

The allergen-specific TCR is functionally intact in TCR tg Jurkat T cells

To determine whether Jurkat T cells transduced with the allergen-specific TCR recognize the HLA-DRB1*0101–restricted immunodominant T-cell epitope Art v $1_{25\text{-}36}$[21,22] also in functional terms, the TCR transductants (subclone 5) were coincubated with syngeneic EBV B cells that had been pulsed with saturating concentrations of Art v $1_{25\text{-}36}$ or control peptides from Art v 1 (Art v $1_{19\text{-}30}$ or Art v $1_{94\text{-}105}$) or influenza hemagglutinin ($HA_{306\text{-}318}$). Fig 3, *A*, shows specific recognition of Art v $1_{25\text{-}36}$ by the TCR tg Jurkat T cells, as revealed by IL-2 promoter–driven luciferase activity. Similar results were obtained with other subclones or the bulk transductants (not shown). Of note, residual coexpression of endogenous TCR chains, as observed for clone 3, did not influence subsequent antigen-specific activation (not shown). EBV B cells pulsed with control peptides from Art v 1 (Art v $1_{19\text{-}30}$ or Art v $1_{94\text{-}105}$), the DRB1*0101/0401–restricted peptide from influenza hemagglutinin ($HA_{306\text{-}318}$),[30] or medium alone were unable to stimulate TCR tg T cells. Cellular activation induced by Art v $1_{25\text{-}36}$ was strictly dependent on the presence of antigen-presenting cells because no T-cell stimulation was observed on coincubation of TCR tg Jurkat T cells with peptides in the absence of EBV B cells (Fig 3, *A*). The immunodominant Art v $1_{25\text{-}36}$ peptide did not activate WT Jurkat T cells, which were perfectly able to mount HLA class II–dependent responses in the presence of SEE (Fig 3, *B*). Dose-response determinations revealed that TCR tg Jurkat T cells required between 7.6 and 33.4 μmol/L (12.4-54.8 μg/mL) peptide for half-maximal stimulation (not shown).

TCR tg Jurkat T cells recognize Art v 1 borne by engineered invariant chain

The HLA CLIP occupies the peptide-binding groove during assembly and transport and becomes exchanged to antigenic peptides in endosomes/lysosomes.[24] Does the allergen-specific TCR also recognize Art v 1, which was genetically exchanged against CLIP in Ii expression constructs?

To answer this question, we generated aAPCs by transfecting human 293 cells with HLA-DRA*0101, HLA-DRB1*0101, CD80, CD54, and cathepsin S plus Ii constructs harboring different length variants of a codon-optimized version of the Art v 1 gene.[25] Alternatively, 293 cells were transfected with the control Ii constructs Ii::HA309-317 or Ii::CLIP. 293 Cells transfected with the Ii::Art v 1 versions, all harboring the minimal Art v $1_{27\text{-}34}$ epitope,[21] were able to stimulate the Art v 1–specific tg Jurkat T cells similar to the positive control PHA/phorbol 12-myristate 13-acetate (Fig 4, *A*). No stimulation was induced by 293 cells transfected with Ii::HA309-317, Ii::CLIP, or control vector, although these cell types were perfectly able to functionally bind SEE (Fig 4, *B*). However, no SEE-dependent response was observed in the absence of aAPCs. Fig 4, *C*, shows that HLA Class II expression was similar on all aAPC types, whereas Ii expression levels provided a more heterogeneous picture (Fig 4, *D*). Ii::Art v $1_{1\text{-}109}$ fusion protein repeatedly showed the weakest expression, which also resulted in a weaker stimulatory capacity of Ii::Art v $1_{1\text{-}109}$–transfected aAPCs.

Significantly, T-cell activation was only moderate in the absence of costimulation (stimulation index [SI], 3.2 ± 0.34); however, it became clear-cut when costimulatory molecules, such as CD80, were coexpressed (SI, 44.7 ± 8.7; Fig 5). In our system coexpression of CD80, together with the intercellular adhesion molecule 1 (CD54), on aAPCs revealed only moderate additional synergy (SI, 58.1 ± 15.2; Fig 5), which might reflect already maximal stimulation by CD80 alone, making a further enhancement by CD54 impossible.

Transfer of allergen-specific TCR into human PBLs

In a next step we tested whether it was possible to generate human Art v 1–specific T lymphocytes from nonallergic individuals. For that purpose, PBLs from 6 donors (HLA-DRB1*0101 positive or negative) were transduced with the Art v 1–specific TCR. Transduction efficiencies ranged from 10% to 30%, as determined by flow cytometry of CD3$^+$ TCR Vβ18$^+$ T cells (Fig 6, *A*). TCR tg T cells from all donors specifically proliferated on coincubation with aAPCs expressing Ii::Art v $1_{25\text{-}36}$ but not with aAPCs expressing Ii::$HA_{309\text{-}317}$. Control-transduced T cells (green fluorescent protein) of HLA-DRB1*0101–negative donors showed residual reactivity with HLA-DRB1*0101–expressing aAPCs, possibly reflecting alloreactivity, which was not observed

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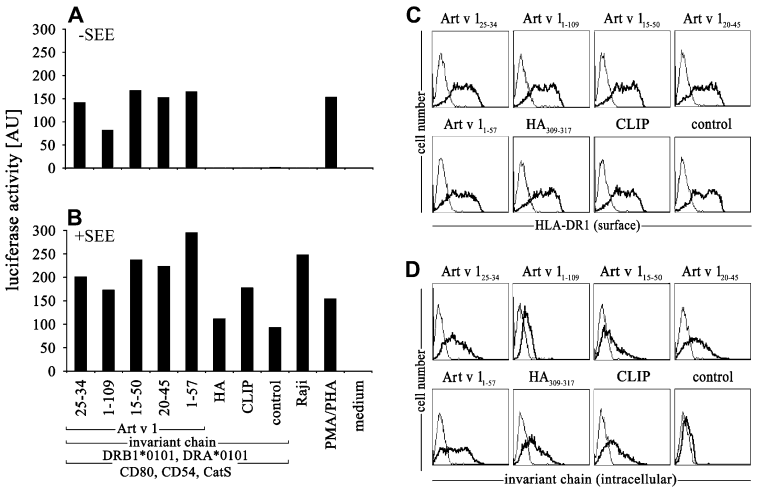

FIG 4. Art v 1–specific TCR tg Jurkat T cells recognize Ii-encoded allergenic peptides presented by aAPCs. **A**, IL-2 promoter activity of TCR tg Jurkat T cells (bulk) cocultured with 293 cells expressing HLA-DRA*0101, HLA-DRB1*0101, CD80::GPI, CD54::GPI, cathepsin S, and the indicated forms of Ii or controls. Coculture with Raji B cells, PHA plus phorbol 12-myristate 13-acetate *(PMA)*, or medium served as a control. **B**, Similar to Fig 4, *A*, but supplemented with SEE. Expression levels of HLA-DR1 **(C)** or Ii **(D)** in 293 aAPC transfectants *(bold lines)* superimposed by negative control staining *(thin lines)* is also shown.

with responder T cells from HLA-DRB1*0101–positive donors. Moreover, stimulation of TCR tg PB T cells was strongly dependent on CD80-mediated costimulation. Within 10 days, up to 1×10^7 Art v 1–specific T cells can be generated from 10 mL of PB of various donors. This cell number can be further increased by a factor of approximately 3 by supplementation of TCR tg T cells with growth factors, such as IL-2. Thus PB T cells from nonallergic donors can be genetically modified to react with a well-defined allergen, such as Art v 1.

DISCUSSION

We cloned, sequenced, and transferred the TCR α- and β-chain genes of a $CD4^+$ T-cell clone (SSR20) specific for the immunodominant T-cell epitope of the major mugwort pollen allergen Art v 1 to human T lymphocytes. The human Jurkat T-cell line, as well as PB T cells of nonallergic individuals, showed clear-cut expression of the tg TCR and specifically reacted with Art v $1_{25\text{-}36}$ peptide-pulsed autologous EBV B cells, as well as with aAPCs expressing Ii::Art v 1 fusion proteins. Significantly, stimulation of TCR tg T cells was dependent on coexpression of costimulatory molecules, such as CD80 or CD86 (not shown) and, to a much lesser degree, on CD58 (not shown). To our knowledge, this is the first report describing the successful genetic transfer of a human allergen-specific TCR into human T lymphocytes.

We here attempted to contribute to a better definition of the critical molecular players within the "allergen-specific synapse" formed between antigen-presenting cells and T cells. Whereas immunodominant peptides and corresponding restriction elements of major allergens have been characterized in detail during the past 2 decades,[31,32] the information on the molecular and functional definition of human allergen-specific TCRs remained scarce. The availability of recombinant, allergen-specific TCRs will allow us to set up biologic model systems in the future in which all 3 components of the allergen-specific synapse (ie, the

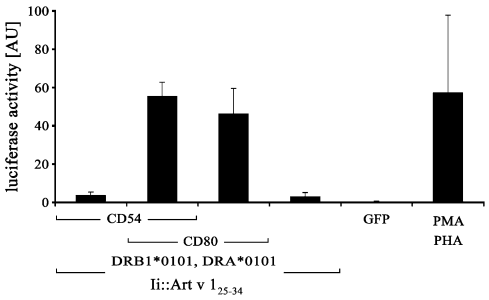

FIG 5. Efficient stimulation of Art v 1–specific Jurkat T cells requires costimulation. IL-2 promoter activity of TCR tg Jurkat T cells incubated with 293 cells cotransfected with DRA*0101, DRB1*0101, Ii::Art v $1_{25\text{-}34}$, CD54, CD80, green fluorescent protein *(GFP)*, or control plasmid alone or combined as indicated or phorbol 12-myristate 13-acetate *(PMA)* plus PHA. Data show mean values ± SDs of triplicate cultures representative of 3 independently performed experiments.

restriction element, the immunodominant peptide, and the corresponding TCR) are defined at a molecular level and are based on a relevant allergic disease. Currently, allergen-specific T cells have to be expanded from the blood of patients with clinically well-defined allergy, a cumbersome task that is only successful shortly after the pollen season. In addition, the overall cell yields are often negligible. The transgenic approach allows the generation of high numbers of allergen-specific T cells in a short period of time, independent of the allergic status of the donor and the allergy season. Future studies will have to show whether and how allergen-specific TCR tg T cells can be polarized to exert defined effector functions. The steadily growing number of modified/recombinant allergens[33] (allergen [peptide] fragments,[34] trimers,[35] conjugates,[36] shuffled allergens,[37] or hybrid molecules[38]) demands test systems able to predict their immunologic

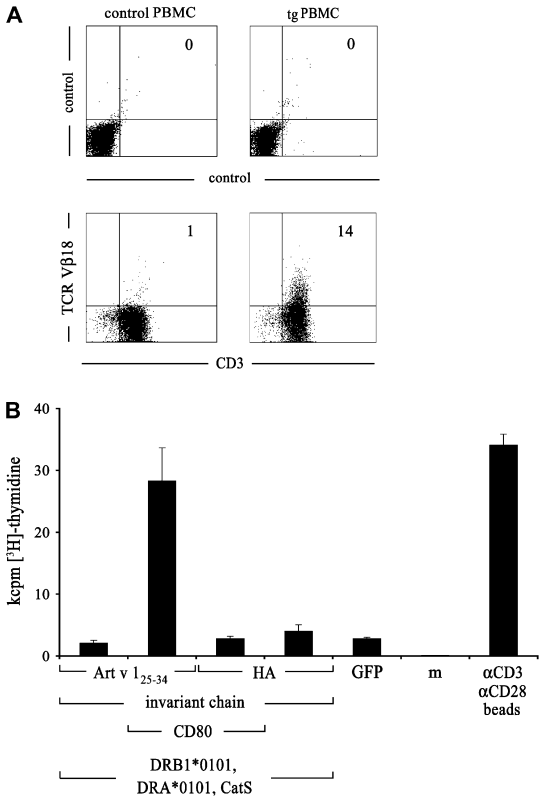

FIG 6. TCR tg PB T cells proliferate on challenge with Ii::Art v $1_{25\text{-}36}$–expressing aAPCs. **A**, Expression levels of TCR Vβ18 and CD3 on control and tg PB T cells. Numbers indicate percentage of $CD3^+$ TCR $V\beta18^+$ cells. **B**, Proliferation of human PBMCs transduced with Art v 1–specific TCR cocultured with aAPCs coexpressing the indicated molecules or alternatively with microbeads coated with CD3 plus CD28 mAbs or with medium alone. Data show mean values ± SDs of triplicate cultures corrected by mean values (2.6 kcpm; range, 0-8.2 kcpm) of green fluorescent protein *(GFP)*–transduced tg T cells. Data are representative of several independently performed experiments.

behavior. Allergen-specific tg T cells might represent such a standardizable tool to, for example, test for retained T-cell reactivity of modified/recombinant allergens.

In addition, the Ii-based sensitization strategy,[24] which we adapted for allergenic peptides in this report, might prove to be useful to define by means of recombinant DNA technology–altered peptide ligands of immunodominant allergens. Conventionally, altered peptide ligands are created by introducing single amino acid changes into peptide ligands of MHC, generating peptides able to influence autoimmune diseases but also allergies.[39-42] The Ii-based allergen (peptide) expression system in combination with, for example, error-prone PCR approaches,[43] might serve as a novel high-throughput screening approach for such purposes.

Moreover, expressible allergen-specific TCRs might also lay the groundwork for the generation of valuable *in vivo* systems in the future, such as TCR tg "allergy mice" based on a human disease and based on human immune receptors. Allergy mice will represent powerful biologic systems to analyze T cell–dependent (but also T cell–independent) pathways influencing allergic diseases with the required profoundness in the future.

We thank Dr van Bergen for providing the Ii construct and Dr Majdic for providing human HLA class II (1/47) and Ii (5-329) mAbs.

Clinical implications: Expressible allergen-specific TCRs might contribute to a better definition of the "allergen-specific synapse" and thus the processes leading to allergic diseases and their cure.

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Chapter III

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Host-encoded reporters for the detection and purification of multiple enveloped viruses

Robin Ketteler[a,b,1], Vesselin Tomov[a,b,2], Alina Neunkirchner[c], Qiang Xie[a,b], Winfried F. Pickl[c], Brian Seed[a,b,*]

[a] Center for Computational and Integrative Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114, USA
[b] Department of Genetics, Harvard Medical School, 185 Cambridge Street, Boston, MA 02114, USA
[c] Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Vienna, Austria

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The identification of host cell factors for virus replication holds great promise for the development of new antiviral therapies. Recently, high-throughput screening methods have emerged as powerful tools to identify candidate host factors for therapeutic intervention. The development of assay systems suitable for large-scale automated screening is of particular importance for novel viruses with high pathogenic potential for which limited biological information can be developed in a short period of time. This report presents a general enzymatic reporter system for the detection and characterization of multiple enveloped viruses that does not rely on engineering of the virus. Instead, reporter enzymes are incorporated into virus particles by targeting to lipid microdomains in producer cells. The approach allows a variety of human pathogenic enveloped viruses to be detected by sensitive, inexpensive and automatable enzymatic assays. Tagged viruses can be purified quickly and efficiently by a magnetic bead-based capture method. The method allows general detection of enveloped viruses without prior reference to their sequence.

1. Introduction

Enveloped viruses constitute a significant global health problem and potential bioterror hazard. This group of viruses includes human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza virus, members of the coronavirus family, and all of the high risk pathogenic viruses, including the arboviruses, filoviruses and poxviruses. All viruses require host cell factors for completion of their life cycle and the identification of these factors presents an opportunity to discover therapeutic targets that in principle should be less susceptible to virus evasion by mutation (Lama and Planelles, 2007). Genome-wide high-throughput screening has emerged as a powerful technique for identifying host factors that regulate virus replication and budding (Brass et al., 2008; Konig et al., 2008; Krishnan et al., 2008; Randall et al., 2007; Tai et al., 2009; Zhou et al., 2008). This is of particular interest with respect to developing effective methods to characterize novel or emerging viruses (Snowden, 2008). Although identification of specific viruses requires at least partial knowledge of the protein or DNA composition of the virus, improvements in sequencing technology have made it possible to discover novel viruses using microarray chips (Wang et al., 2002), cDNA amplification (van der Hoek et al., 2004) or next-generation sequencing methods (Briese et al., 2009; Zhang et al., 2006). However, few of these methods are compatible with high-throughput screening approaches.

Many known viruses have been engineered by the introduction of reporter sequences such as GFP or Luciferase in their genomes to provide activities that can be measured to provide a surrogate for viral replication (Hao et al., 2008; Konig et al., 2008). Alternatively, reporter virus particles can be produced by translational fusion of a specific virus component to a reporter gene such as chloramphenicol transferase (Sato et al., 1995; Wu et al., 1995; Yao et al., 1999), luciferase (Asokan et al., 2008; Kolokoltsov and Davey, 2004; McCarthy et al., 2006) or alkaline phosphatase (Iro et al., 2009). A similar concept is the incorporation of translational fusions with fluorescent proteins to observe trafficking of individual virions (Fritz et al., 2008; McDonald et al., 2002; Muller et al., 2004). However, in many cases the reporter gene interferes with infectivity of the virus (Furnes et al., 2008; Shih et al., 2004) and careful optimization is required for each individual virus construct. To develop more general labeled-virus assays, the reporter gene

* Corresponding author at: Center for Computational and Integrative Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114, USA. Tel.: +1 617 726 5975; fax: +1 617 643 3328.
E-mail address: bseed@ccib.mgh.harvard.edu (B. Seed).
[1] Present address: MRC Laboratory for Molecular Cell Biology, London, United Kingdom.
[2] Present address: University of Pennsylvania, Department of Medicine, Division of Gastroenterology, Philadelphia, USA.

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should be incorporated by a means common to a large group of viruses.

The membrane of enveloped viruses is derived entirely from the host cell, and contains both the lipid bilayer and some host cell membrane proteins (Brugger et al., 2006; Hammarstedt et al., 2000). It has been proposed that virus budding originates in lipid rafts, and as a consequence lipid raft-localized host cell proteins are highly enriched in viral membranes (Hammarstedt and Garoff, 2004; Hammarstedt et al., 2000; Manes et al., 2000; Ono and Freed, 2001). This property can be used to target enzymatic reporter activities to the virus membrane by modification of the reporter with a lipid raft anchor, a strategy that has previously been used for pseudotyping of virus particles (Pickl et al., 2001). The present report shows that host cells expressing GPI-anchored alkaline phosphatase can be used to detect diverse enveloped viruses as RSV, HSV1, HIV and human coronavirus 229E. The method thus allows the detection, isolation and characterization of a wide variety of known enveloped viruses and in principle could be applied to unknown or poorly characterized emerging viruses.

2. Materials and methods

2.1. Plasmids

For construction of AP::CD16, SEAP::APP pEAK12 (Schobel et al., 2006) was digested with *Xba*I and *Not*I and the PCR amplified, *Nhe*I and *Not*I tailed, GPI-anchor acceptor sequence of CD16b was inserted to create AP::CD16 pEAK12. A PCR fragment of placental alkaline phosphatase (PLAP, IMAGE clone, Open Biosystems) was subcloned into pEAK14 to generate pEAK14-PLAP. The leader peptide was replaced with the signal peptide of Gaussia *princeps* luciferase and the flag peptide sequence DDDDDYDK was inserted upstream of the signal peptide to generate a Flag-PLAP. Flag-PLAP was inserted in pMOWS (Ketteler et al., 2002), pEAK14, and pEAK14-PDG, a vector containing a fusion cassette of puromycin, dihydrofolate reductase (DHFR), and green fluorescent protein (GFP) linked by F2A and T2A self-cleaving peptide sequences to generate single peptides of each marker gene.

2.2. Cell lines

Cell lines used were human embryonic kidney cells 293ET, Vero monkey kidney cells (ATCC, #CCL-81), lung fibroblast MRC5 cells (ATCC, #CCL-171) and the T cell line CEMx174-T2 (MT2; ATCC, #CRL-1992). All cell lines were maintained in DMEM supplemented with 10% calf serum (supplemented with iron), 0.25 μg/mL gentamycin and 50 μM β-mercaptoethanol. MT2 cells were maintained in IMEM with the same supplements. 293ET cells were transfected using calcium-phosphate precipitation as described (Ketteler et al., 2002). DHFR vectors were transfected in Vero cells using Nucleofection (Amaxa, Cologne, Germany) using Solution V and program V-01. Cells were selected in 6.0 μg/mL Puromycin. MRC5 cells were transduced with retroviral supernatants generated upon co-transfection of MoMLV Gag-Pol and VSV-G with pMOWS-FlagPLAP in 293ET cells. Stable AP::CD16 transfectants were established by electroporation (1.5×10^7 cells/400 medium, 950 μF, 400 V, 4 mm cuvettes) of 293 cells with 50 μg of *Sfi*I linearized plasmid DNA and selection with puromycin (1 μg/mL, Sigma-Chemicals, St. Louis, USA).

2.3. Immunofluorescence analysis

For membrane staining, 50 μL of 293 or 293 AP::CD16 cells (1×10^7/mL) were incubated for 30 min at 4 °C with mouse–anti-human alkaline phosphatase-specific mAb (20 μg/mIgG2a, clone 8B6, Sigma-Chemicals), control mAb VIAP (negative control, IgG1, kindly provided by Dr. Otto Majdic, Vienna) or CD99 mAb 3B2/TA8 (positive control, IgG1, Caltag Laboratories, Burlingame, CA). Subsequently, cells were washed twice (1% BSA and 0.05% NaN_3) followed by incubation with Oregon-green conjugated goat-anti-mouse IgG (Molecular Probes, Eugene, OR) at 4 °C for 30 min. After another wash cells were resuspended (200 μL PBS, 50 μL propidium iodide (0.5 μg/mL, Sigma-Chemicals) and subjected to flow cytometry (FACS Calibur, CellQuest software, Becton Dickinson, San Jose, CA). GPI-anchoring of AP::CD16 was determined by PI-PLC treatment with phosphatidylinositol specific phospholipase C from *Bacillus thuringiensis* (American Radiolabeled Chemicals, St. Louis, MO) according to the manufacturer's recommendations. Briefly, 293 AP::CD16 cells were washed in PBS w/o Ca^{2+} and Mg^{2+}, resuspended at a concentration of 1×10^7 cells/mL in PBS. 100 mU of PI-PLC were added to 1×10^7 cells and incubated at 37 °C for 2 h. Subsequently cells were washed in PBS/1% BSA and subjected to surface staining for alkaline phosphatase (mAb 8B6), CD59 (mAb MEM-43/5), or CD99 (mAb 3B2/TA8). Percent release was calculated by relating the geometric mean expression of untreated cells to PI-PLC treated cells corrected by the geo-mean of a non-binding control mAb (VIAP).

2.4. Generation of MoMLV particles

293 or 293 AP::CD16 cells were transiently transfected by the calcium-phosphate precipitation method using 25 μg of plasmid DNA (either vector without insert, or bearing Gag-Pol). Supernatants were harvested after 48 h, cleared of cellular debris by filtration through 0.45 μm syringe filters (Millipore, Billerica, MA) and subjected to direct analyses (crude supernatant). Alternatively, membrane vesicles were concentrated by ultracentrifugation in a Beckman-Optima LE-80K centrifuge (Beckman Instruments, Palo Alto, CA), using a SW41 Ti rotor, at 100,000 × *g* for 1 h and were washed once in PBS. For determination of phosphatase activity, pellets were resuspended in culture medium at the original volume. This particulate fraction was analyzed and compared to cleared and crude supernatants. To destroy endogenous AP activity of 293 cells 1 mL aliquots of the various preparations were incubated at 56 °C for 15 min. Afterwards, 50 μL of the various preparations was added to 50 μL of p-NNP substrate buffer, incubated at 37 °C for 1 h and analyzed for substrate conversion at 405 nm (Thermomax Microplate Reader, Molecular Devices, Sunnyvale, CA, supported by the Softmax Pro software).

2.5. Animal viruses

Human respiratory syncytial virus (RSV; strain B WV/14617/'85; ATCC, #VR-1400), Aguacate virus (ATCC, #VR900), and human herpesvirus HSV1, strain F (ATCC, #VR-733) were propagated in Vero cells maintained in 2% calf serum (supplemented with iron) at 35 °C. Human coronavirus 229E (ATCC, #VR-740) was propagated in MRC5 cells (ATCC, #CCL-171) maintained with 2% calf serum (supplemented with iron) at 35 °C. Titer of viruses provided by ATCC was $10^{4.5}$ TCID[50]/0.2 mL for HCoV 229E, $10^{7.75}$ TCID[50]/0.2 mL for HSV1 and $10^{4.25}$ TCID[50]/0.2 mL for RSV1. For all viruses, 100 μL of virus stocks were used for infection in 12-well plates. HSV1 and Aguacate virus infection was confirmed by rounding of cells and cytopathic effects. RSV was detected by the Light Diagnostics™ respiratory virus detection kit (Millipore, Billerica, MA) according to the manufacturer's instructions. HSV1 was detected by anti-HSV1 antibody (Sigma, St. Louis), HCoV 229E by anti-coronavirus 229E antiserum (Millipore, clone #401-4A).

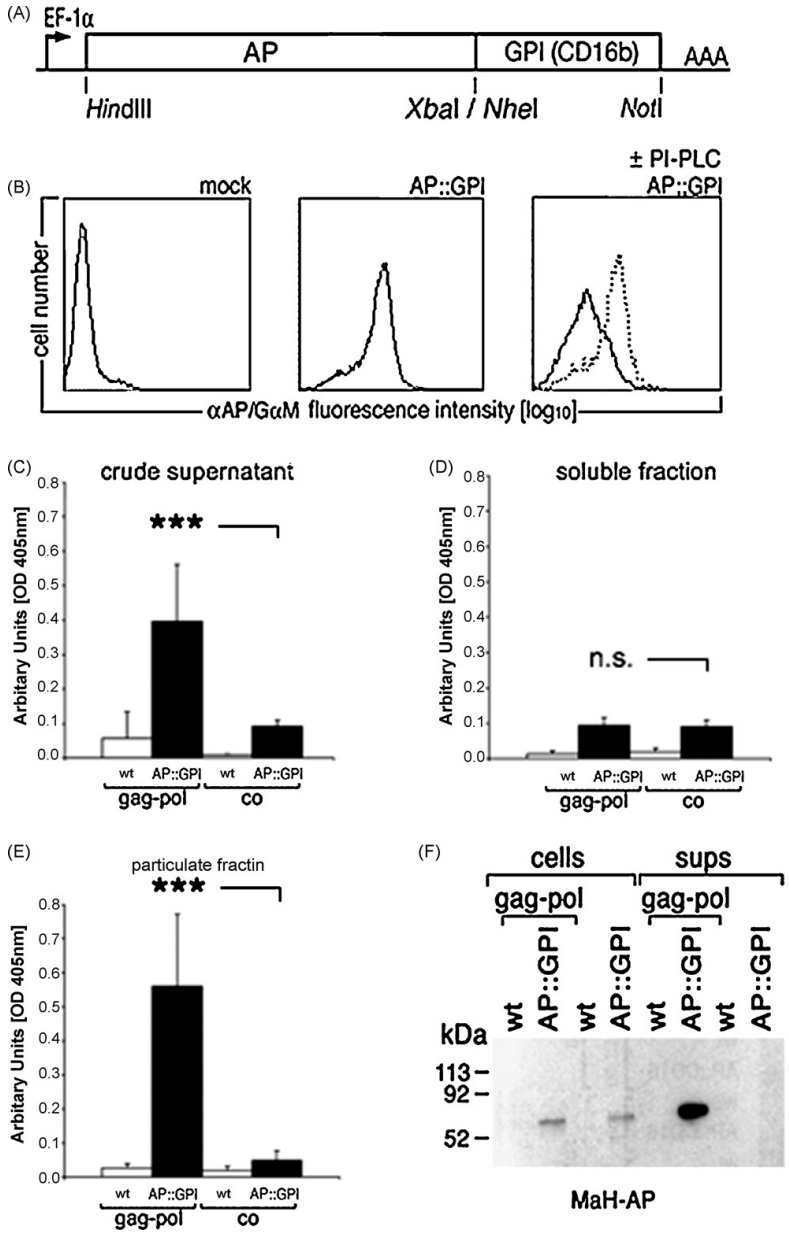

Fig. 1. Incorporation of alkaline phosphatase activity in Gag-Pol derived virus-like particles. (A) Schematic representation of AP::CD16b. (B) Incubation of 293 AP::CD16 cells with phosphatidylinositol specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* removes AP::CD16b from the cell surface. (C) Gag-Pol derived particles release alkaline phosphatase activity in SN. 293 wild type (wt) and AP::CD16 cells were transfected with MLV Gag-Pol, supernatants were harvested 72 h later, cleared of cellular debris by filtration and analyzed for alkaline phosphatase activity. (D) AP activity is not associated with the soluble fraction after ultracentrifugation of Gag-Pol derived virus-like particles. (E) AP activity is associated with the particulate fraction after ultracentrifugation of Gag-Pol derived virus-like particles. (F) AP-CD16b is detected in the supernatants (sups) of Gag-Pol expressing cells. Lysates of cells and vesicles concentrated from cell supernatants by ultracentrifugation were resolved by SDS-PAGE and probed with mouse–anti-human (MaH)AP-specific mAb. n.s., not significant; ***$p < 0.001$; Student's *t*-test, paired, two-tailed.

2.6. Alkaline phosphatase

Secreted alkaline phosphatase was determined using the Phospha-Light™ secreted alkaline phosphatase reporter assay system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. In all experiments, the supernatants were centrifuged at 10,700 × *g* prior to testing. Briefly, 50 μL of supernatant was mixed with 150 μL 1× Dilution buffer/1% Triton X-100

and incubated at 70 °C for 20 min. 50 μL of diluted supernatant was mixed with 50 μL of assay buffer and 50 μL of substrate solution before assaying in the TopCount luminescence plate reader.

2.7. Polyacrylamide gel electrophoresis and Western blotting

Samples were resolved onto 10% polyacrylamide gels (BioRad) and transferred to PVDF membranes (Millipore, Billerica, MA), stained with Ponceau Red (Sigma, St. Louis, MO), blocked with 2% BSA and incubated with AP specific mAb 8B6 (Sigma), MoMLV Gag specific mAb R187 (ATCC), anti-Flag M2 antibody (Sigma, St. Louis, MO), anti-HSV glycoprotein D (Sigma, St. Louis, MO) or anti-coronavirus (Millipore, Billerica, MA). After incubation with appropriate secondary antibodies (Jackson Immunoresearch, West Grove, PA), the bands were visualized with enhanced chemoluminescence (ECL) reagent.

2.8. Immunoprecipitation of virus particles

Viral supernatants were collected, anti-Flag M2 antibody (Sigma, St. Louis) and magnetic anti-mouse IgG coated Dynabeads® (Invitrogen) were added for 2 h at 4 °C, and 1/4 of the beads were used for re-infection of MT2 cells.

2.9. ELISA

ELISA for p24 (PerkinElmer Alliance Kit) was performed according to the manufacturer's instructions.

3. Results

3.1. Virus-like MLV particles incorporate membrane-anchored placental alkaline phosphatase

To evaluate the feasibility of establishing a general and quantitative detection method for enveloped viruses based on host-encoded reporter proteins targeted to lipid rafts (Hammarstedt and Garoff, 2004; Pickl et al., 2001), a number of candidate reporter systems were evaluated. One of these, placental alkaline phosphatase (AP) fused to CD16b GPI-anchor (AP::CD16b; Fig. 1A) proved suitable for the purpose. AP::CD16b was expressed on the cell surface of cells as shown by flow cytometry analysis using specific antibody against AP (Fig. 1B). Treatment with PI-PLC resulted in a reduction (67.7 ± 11.2%) of the amount of AP::CD16b on the cell surface, confirming that AP::CD16b is anchored by a glycosylphosphatidyl lipid. The release of AP activity into supernatant (SN) upon co-expression of MoMLV Gag-Pol was measured as a surrogate for virus infection. The MoMLV Gag protein has been shown to be sufficient to form virus-like particles in the absence of retroviral RNA or other structural proteins and thus serves as a good model system for membrane incorporation of host proteins (Mergener et al., 1992). In the absence of Gag-Pol, very little AP activity was detected in supernatant (Fig. 1C). In the presence of Gag-Pol, a 4-fold increase in AP activity in supernatant of cells expressing AP::CD16b was detected, indicating specific release of AP activity as a consequence of Gag-Pol expression (Fig. 1C). To determine, whether AP activity was associated with virus-like particles (VLP), AP activity in the soluble (Fig. 1D) and particulate fraction (Fig. 1E) after ultracentrifugation was measured. Very little AP activity was associated with the soluble fraction, while the majority of the activity was recovered in the particulate fraction (Fig. 1D and E). In the presence of Gag-Pol, a 10-fold increase of AP activity in the particulate fraction was detected (Fig. 1E). AP::CD16b was present at similar levels in cell lysates in the presence or absence of Gag-Pol. In contrast, full-length AP::CD16b was detected only in supernatants from cells expressing Gag-Pol, but not control cells (Fig. 1F). We conclude that

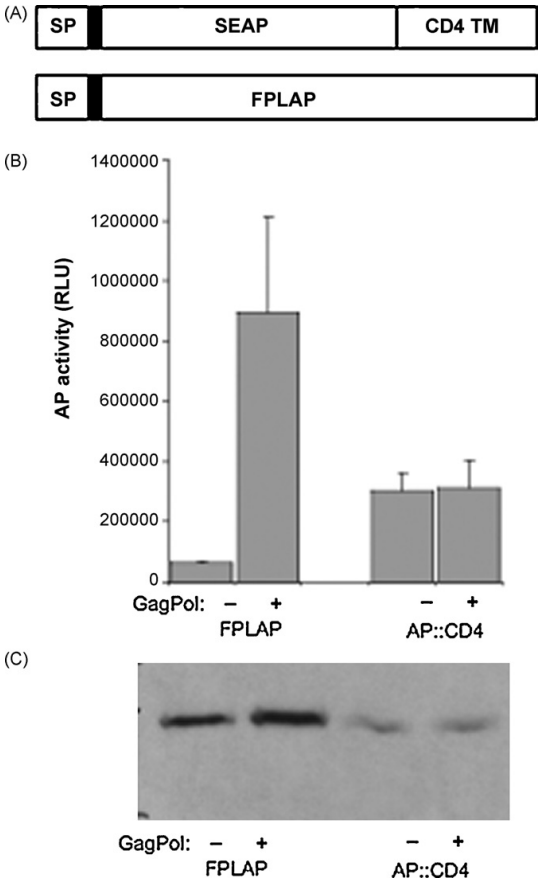

Fig. 2. Incorporation of alkaline phosphatase activity in Gag-derived virus-like particles. (A) Schematic representation of AP::CD4 and FPLAP. The Flag-tag is indicated by a black box following the signal peptide (SP). TM, transmembrane domain. (B) 293ET cells were transiently co-transfected with MLV Gag-Pol and FPLAP or AP::CD4. Supernatants were harvested after 3 days and analyzed for alkaline phosphatase activity and compared to cells that were transfected with FPLAP constructs only. Results from three independent infections + standard deviation (SD) are shown. RLU, relative light units. (C) Cell lysates from A were analyzed by immunoblotting of FPLAP and AP::CD4 in the presence or absence of Gag-Pol using anti-Flag M2 antibody.

AP::CD16b is expressed on the surface of virus-like particles and allows monitoring Gag-Pol induced particle formation.

As an alternative to the CD16b anchor, placental alkaline phosphatase with the natural GPI-anchor and an N-terminal Flag-tag (FPLAP) was tested for uptake by virus-like particles of MLV Gag-Pol. In addition, the GPI-anchor sequence was replaced with the transmembrane sequence of CD4 (AP::CD4) and compared to wild-type placental alkaline phosphatase with the natural GPI-anchor (Fig. 2A). In the presence of Gag-Pol, a 13.8-fold increase in alkaline phosphatase activity in the supernatant of 293ETN cells expressing FPLAP was observed, while AP::CD4 did not result in enhanced release of AP activity in the presence of Gag-Pol (Fig. 2B). Expression levels in cell lysates for FPLAP and AP::CD4 were not changed by expression of Gag-Pol (Fig. 2C). We therefore conclude that GPI-anchored alkaline phosphatase can be used to detect virus-like particles in the supernatant of transduced cells releasing viral particles.

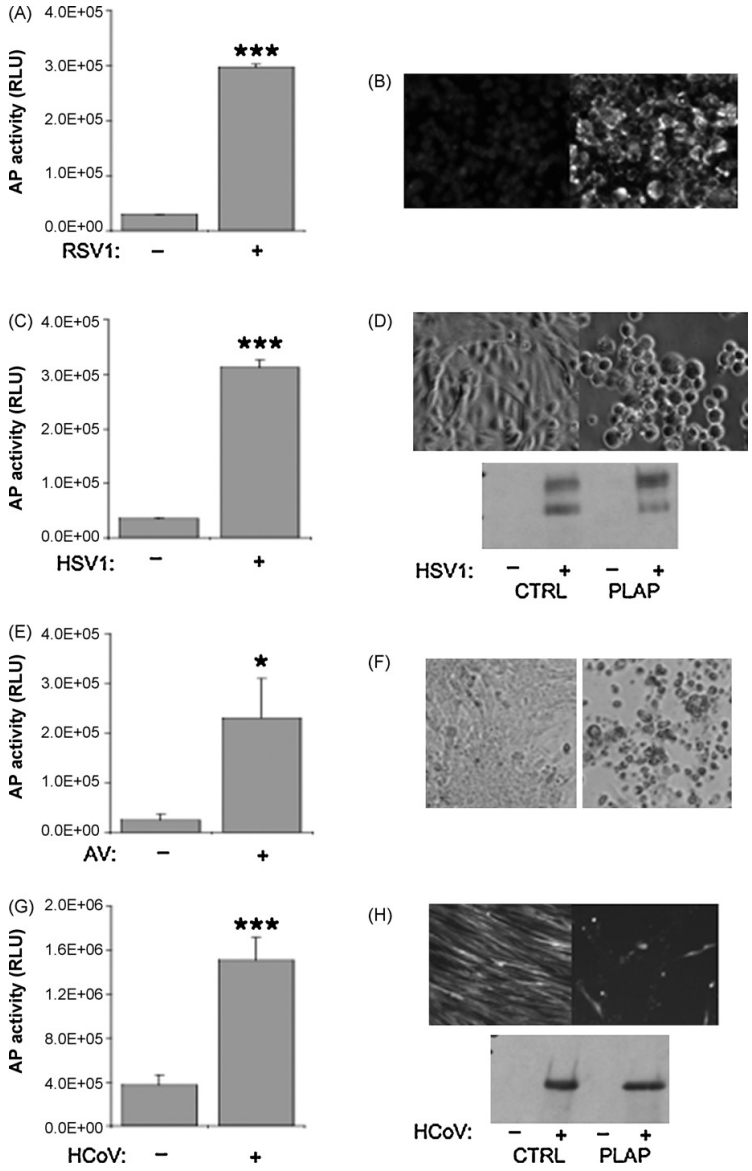

Fig. 3. Alkaline phosphatase can be incorporated into multiple enveloped viruses. (A) Vero reporter cell lines were infected with Respiratory Syncytia Virus (RSV) and assayed for alkaline phosphatase (AP) activity in supernatants after 7 days. (B) The presence of RSV was confirmed with the respiratory detection kit (Chemicon) by orange-red fluorescence specific for RSV (lower panel). (C) Vero reporter cell lines were infected with Herpes Simplex Virus 1 (HSV1) and cultured for up to 3 days. Supernatants were collected, centrifuged and analyzed for alkaline phosphatase activity. (D) Vero cells were infected with HSV1 as in (C). After 24 h, cell rounding was observed as a hallmark of efficient infection with HSV1 by light microscopy in infected cells (lower panel) compared to uninfected cells (upper panel). The presence of HSV1 was confirmed by immunoblotting with antibodies raised against HSV1 (lower panel). (E) MRC5 cells transduced with GFP or FPLAP were infected with human coronavirus 229E in 2% serum at 35 °C. After 3 days, supernatants were analyzed for alkaline phosphatase activity. (F) Cytophathic effects were visible after 3 days in cells infected with HCoV 229E (lower panel) compared to the confluent layer of GFP-positive cells in uninfected MRC5 cells (upper panel). The cells were lysed and subjected to immunoblotting with anti-HCoV antibody (Chemicon, as recommended by ATCC) that is highly specific for the 229E sub-type of HCoV, but not for OC43. A single band was detected only in cells that were infected with HCoV 229E. Results from three independent infections + standard deviation (SD) are shown. RLU, relative light units. $^{*}p < 0.05$; $^{***}p < 0.001$; Student's *t*-test, paired, two-tailed.

3.2. Generation of reporter cell lines to detect enveloped viruses

To investigate the potential use of the reporter system for detection of other enveloped viruses, stable reporter cell lines expressing FPLAP in combination with a Puromycin$_{F2A}$DHFR$_{T2A}$GFP cassette driven by the HSV1 TK promoter were generated. Vero cells are a good host for a variety of enveloped viruses such as Respiratory Syncytial Virus (RSV) and Herpes Simplex Virus (HSV). Vero cells have very little endogenous alkaline phosphatase activity and thus serve as a good reporter cell line. Vero cells were trans-

Table 1
Detection of alkaline phosphatase and cytopathic effects in serial dilutions of HSV1.

Dilution	$1/10^4$	$1/10^5$	$1/10^7$
Alkaline phosphatase (fold)	5.52 ± 1.06	2.78 ± 0.46	0.97 ± 0.22
Cytopathic effects	Yes	No	No

Serial dilutions of HSV1 virus stock ($10^{7.75}$ TCID[50]/0.2 mL) were incubated with Vero cells expressing FPLAP. After 7 days, cells were analyzed for cytophathic effects and supernatants were tested for alkaline phosphatase activity.

fected by nucleofection (Amaxa) and selected with puromycin at 6.0 μg/mL. AP activity was measured in supernatants upon infection with respiratory syncytial virus (RSV1), Herpes Simplex Virus (HSV1) and Aguacate Virus (AV). Upon infection with RSV1, HSV1 and AV, 10.4 ± 0.2-fold, 9.0 ± 0.4-fold and 9.7 ± 3.3-fold increases in AP activity, respectively, were observed in supernatants compared to uninfected cells (Fig. 3A, C and E). Successful virus infection was confirmed with virus-specific assays. Infection with RSV was confirmed by immunofluorescence (Fig. 3B). An orange-red fluorescence specific for RSV was detected in infected Vero cells, but not uninfected cells. Immunoblotting with an anti-HSV1 antiserum recognizing glycoprotein D precursor showed a double band in infected cells but not in uninfected cells. Successful HSV1 infection was confirmed by cell rounding as early as 24 h and by cytopathic effects thereafter (Fig. 3D). Infection with AV resulted in cytopathic effects after 3 days in culture (Fig. 3F).

As an alternative host cell, MRC5 cells were transduced with FPLAP or GFP and a 4.1 ± 0.5-fold increase of AP activity in supernatant upon infection with human coronavirus 229E (HCoV) compared to uninfected cells (Fig. 3G) was detected. After 24 h, the majority of MRC5 cells was GFP-positive, indicating high transduction efficiencies. The cells were subsequently infected with HCoV 229E in 2% serum at 35 °C. After 3 days, cytopathic effects became visible (Fig. 3H), supernatants were harvested and AP activity was measured in SN. A 4.1 ± 0.5-fold increase of AP activity in supernatant upon infection with HCoV was observed compared to uninfected cells (Fig. 3G). No difference in AP activity in supernatant was detected in cells expressing GFP. To determine whether cells had been infected with HCoV 229E, the cells were lysed and subjected to immunoblotting with an anti-HCoV antibody that is highly specific for the 229E sub-type of HCoV, but not for OC43. A single band was detected in infected cells but not in uninfected cells (Fig. 3H). Thus, this system can be used to detect viruses of the coronavirus family in MRC5 cells.

To determine the sensitivity of this assay, serial dilutions of HSV1 (TCID50: $10^{7.75}$ TCID[50]/0.2 mL) were used for infection of Vero cells. At 10^4-fold and 10^5-fold dilutions, a 5.52-fold and 2.78-fold increase in alkaline phosphatase activity compared to uninfected cells was detectable in supernatants, respectively (Table 1). Cytopathic effects were detectable at up to 10^4-fold dilution, but not at higher dilutions.

3.3. Flag-tagged placental alkaline phosphatase can be incorporated in replicating HIV particles

To determine whether Flag-tagged PLAP can be incorporated in infectious HIV particles, FPLAP and HIV-GFP were co-expressed in 293ET cells. High levels of HIV expression were confirmed by fluorescence microscopy for expression of GFP (data not shown), which has been cloned in the nef open reading frame. Upon transfection of HIV-GFP and FPLAP in 293ET cells, a 4.2 ± 0.3-fold increase in AP activity in supernatant was detected compared to cells transfected with HIV-GFP only (Fig. 4A).

To explore whether this strategy could be used to purify unknown virus particles, supernatants of cells transfected with HIV and FPLAP were exposed to anti-Flag M2 antibody coupled

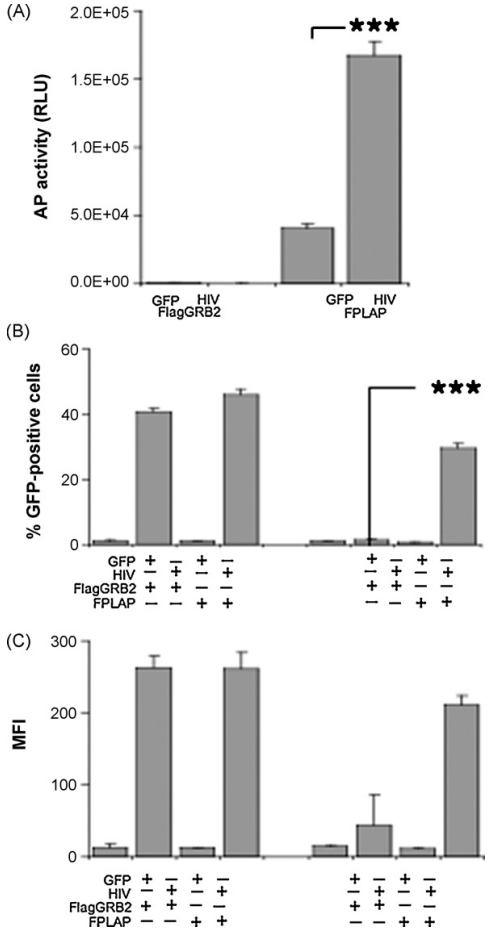

Fig. 4. Incorporation of Flag-tagged alkaline phosphatase in replicating HIV. (A) 293ET cells were transfected with Flag-GRB2 (FGRB2) as control and FPLAP in combination with GFP or HIV. AP activity was determined after 2 days in SN. (B and C) Supernatants from 293ET cells expressing HIV and FPLAP or GFP were collected, pre-incubated with Flag M2 antibody and magnetic beads for 2 h at 4 °C and subsequently used for infection of CEMx174-T2 (MT2) cells. After 5 days, MT2 cells infected with plain supernatant or with captured virus were analyzed by flow cytometry for percentage of MT2 cells displaying green fluorescence (B) or mean fluorescence intensity (MFI) (C). Results from three independent infections + standard deviation (SD) are shown. RLU, relative light units. ***$p < 0.001$; Student's t-test, paired, two-tailed.

to magnetic beads. CEMx174-T2 cells (MT2) were incubated with the magnetic beads or supernatants and analyzed by flow cytometry for GFP expression indicative of infection by HIV. Supernatants from cells transfected with HIV resulted in successful infection with 40–46% of cells displaying green fluorescence with high mean fluorescence intensities after 5 days (Fig. 4B and C). Coincubation of immunoadsorbed beads from cells expressing FPLAP and HIV resulted in successful infection of 30% MT2 cells displaying green fluorescence and a mean fluorescence intensity of 210.4 ± 13.4 (Fig. 4B and C), whereas similar coincubation with beads exposed to supernatant from cells expressing HIV and FGRB2 did not result in infection of MT2 cells. We conclude that FPLAP is incorporated into infectious virus particles and the Flag-tag is exposed on the virus surface in a way that permits the virus to be purified and delivered to recipient cells.

4. Discussion

High-throughput screening of RNAi or shRNA libraries enable the identification of host cell factors required for virus replication. Successful screening of several viruses for cellular host factors has been reported including HIV (Brass et al., 2008; Konig et al., 2008; Yeung et al., 2009; Zhou et al., 2008), HCV (Lupberger et al., 2008; Randall et al., 2007; Tai et al., 2009), West Nile virus (Krishnan et al., 2008) and Influenza virus (Hao et al., 2008). Targeting host cell factors for antiviral strategies may overcome virus resistance to conventional virus-targeted therapies that arises due to the high mutation rate of the viral genome. It would be interesting to identify host cell factors that are common to different pathogenic viruses, thus enabling "pan"-viral therapeutic targets. However, screening of multiple different viruses is difficult to achieve, largely because individual assays for each virus have to be established and optimized. The development of general virus detection methods would allow cross-virus screening. Prior to our study, a small molecule screen for SARS based on the observation of cytopathic effects was reported (Ivens et al., 2005). In principle, the detection of cytopathic effects is applicable to multiple viruses, but effects on general toxicity need to be controlled for. In addition, quantitation of cytopathic effects is much harder to achieve and less sensitive than enzymatic reporter systems. As such, cytopathic effects are largely a qualitative measure of general cytotoxicity rather than virus infectivity.

This study presents a host cell-based assay system for general detection of enveloped viruses based on lipid raft-mediated uptake of the reporter gene by the virus. The natural GPI-anchor of placental alkaline phosphatase incorporates effectively into the membrane of diverse enveloped virus particles. Other anchor sequences such as the extracellular domain of the low-affinity nerve growth factor receptor that has been used to funtionalize lentiviral particles (Nesbeth et al., 2006) may be used as alternative targeting motif. Several studies have demonstrated that the incorporation of reporter enzymes in the virus genome impacts budding and infectivity of the virus. The system described here is based on expression of the reporter in *trans*, thus enabling the detection of multiple viruses. The impact on budding, viral yields, potency and the immune profile when expressed in *cis* need to be addressed separately in future studies as these would be important aspects to address the suitability of this system for gene delivery and gene therapy approaches. Since the system described here does not modify integral virus components, but rather decorates the virus particle with the reporter enzyme, it has potentially a minimal effect on the virus structure itself. This is underscored by the demonstration that infectious HIV particles can be obtained by immuno-capture using anti-Flag antibodies.

The general virus detection system described here offers several advantages. First, it allows the detection of multiple enveloped viruses that bud from the plasma membrane. Second, the assay is amenable to high-throughput screening and can be used for multiple enveloped viruses. Third, a magnetic purification scheme is described that enables the recovery of infectious virus for further analysis. Thus, this system enables detection and subsequent analysis of enveloped viruses without prior reference to their composition. One limitation of this system is cell culture adaptation as a pre-requisite for virus screening. Vero cells have proven as suitable host for multiple cell culture adapted viruses, but may not be suitable for all enveloped viruses. In such cases, the use of alternate hosts such as MRC5 or MDCK cells can be explored. We propose that this tool will be highly useful as a research tool for characterization of known and novel enveloped viruses.

Conflict of interest

The authors declare no conflict of interest.

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Chapter IV

The Journal of Immunology

Human TCR Transgenic Bet v 1-Specific Th1 Cells Suppress the Effector Function of Bet v 1-Specific Th2 Cells

Alina Neunkirchner,[*,†,1] Victoria M. Leb-Reichl,[*,†,1] Klaus G. Schmetterer,[†] Sonja Mutschlechner,[*,‡] Hans J. Kueng,[†] Daniela Haiderer,[†] Karina Schuch,[*,†] Michael Wallner,[§,¶] Beatrice Jahn-Schmid,[‡] Barbara Bohle,[*,‡] and Winfried F. Pickl[*,†]

Pollinosis to birch pollen is a common type I allergy in the Northern Hemisphere. Moreover, birch pollen-allergic individuals sensitized to the major birch pollen allergen Bet v 1 frequently develop allergic reactions to stone fruits, hazelnuts, and certain vegetables due to immunological cross-reactivity. The major T cell epitope Bet v $1_{142–153}$ plays an important role in cross-reactivity between the respiratory allergen Bet v 1 and its homologous food allergens. In this study, we cloned and functionally analyzed a human αβ TCR specific for the immunodominant epitope Bet v $1_{142–153}$. cDNAs encoding TCR α- and β-chains were amplified from a Bet v $1_{142–153}$-specific T cell clone, introduced into Jurkat T cells and peripheral blood T lymphocytes of allergic and nonallergic individuals, and evaluated functionally. The resulting TCR transgenic (TCRtg) T cells responded in an allergen-specific and costimulation-dependent manner to APCs either pulsed with Bet v $1_{142–153}$ peptide or coexpressing invariant chain:: Bet v $1_{142–153}$ fusion proteins. TCRtg T cells responded to Bet v 1-related food and tree pollen allergens that were processed and presented by monocyte-derived dendritic cells. Bet v $1_{142–153}$-presenting but not Bet v $1_{4–15}$-presenting artificial APCs coexpressing membrane-bound IL-12 polarized allergen-specific TCRtg T cells toward a Th1 phenotype, producing high levels of IFN-γ. Coculture of such Th1-polarized T cells with allergen-specific Th2-differentiated T cells significantly suppressed Th2 effector cytokine production. These data suggest that human allergen-specific TCR can transfer the fine specificity of the original T cell clone to heterologous T cells, which in turn can be instructed to modulate the effector function of the disease initiating/perpetuating allergen-specific Th2-differentiated T cells. ***The Journal of Immunology*****, 2011, 187: 4077–4087.**

Birch pollen is one of the main causes of pollinosis in the Northern Hemisphere from spring to early summer (1). More than 95% of birch pollen-allergic individuals mount an IgE Ab response against Bet v 1, the major birch pollen allergen (2). T cells from these patients preferentially recognize the immunodominant epitope Bet v $1_{142–153}$. This region is located at the highly conserved C terminus of Bet v 1 and shares considerable homology with related food and pollen allergens (3). Recent studies have demonstrated that various Bet v 1 peptides bind with high affinity to different HLA-DR molecules (4). The structures targeted by both the humoral and cellular immune responses to Bet v 1 are sufficiently characterized at the molecular level to allow the development of knowledge-based therapies for treatment of birch pollen atopic disorders (3, 5).

Individuals allergic to birch pollen frequently develop type I hypersensitivity reactions against certain foods; for example, apple, celery, carrot, or hazelnut (6, 7). Ingestion of these foods causes the oral allergy syndrome, such as an itching, burning, or tingling sensation of the lips, mouth, or pharynx (8, 9). The molecular basis for these symptoms is Bet v 1-specific IgE Abs, which cross-react with tertiary structural determinants on homologous proteins within these foods (10, 11). Moreover, Bet v 1-specific T cells are also efficiently activated by Bet v 1-homologous food allergens (3). But, in marked contrast to IgE-mediated effects, T cell cross-reactivity is not abolished by cooking or by digestion of birch pollen-related food allergens (12) because these processes do not destroy T cell epitopes (13). Thus, ingestion of pollen-related food allergens can lead to aggravation of T cell-mediated immunopathology, such as exacerbation of eczema in patients with atopic dermatitis (14).

In the past, experimental tools have been devised that allow exploration of T cell responses in more detail. For instance, the Ag specificity of T cells can be conveniently transferred to other T cells by αβ TCR gene transfer (15–17). How might allergy research benefit from recombinant TCRs recognizing major allergens? The answers are severalfold: first, recombinant allergen-specific TCRs should allow the generation of large numbers of specific T cells in a short period of time independent of the allergic status of the donor and the allergy season; second, the availability of well-defined allergen-specific T cells favors the creation of biological model systems in which all three components of the allergen-

*Christian Doppler Laboratory for Immunomodulation, Vienna A-1090, Austria; †Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna A-1090, Austria; ‡Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna A-1090, Austria; §Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Salzburg A-5020, Austria; and ¶Department of Molecular Biology, University of Salzburg, Salzburg A-5020, Austria

[1]A.N. and V.M.L.-R. contributed equally to this work.

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Address correspondence and reprint requests to Prof. Winfried F. Pickl, Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, A-1090 Borschkegasse 8A, Vienna, Austria. E-mail address: winfried.pickl@meduniwien.ac.at

The online version of this article contains supplemental material.

Abbreviations used in this article: aAPC, artificial Ag-presenting cell; Ii, invariant chain; MDDC, monocyte-derived dendritic cell; PB, peripheral blood; TCC, T cell clone; TCRtg, TCR transgenic.

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specific synapse (HLA molecule, allergen peptide, and TCR) are well defined; and third, the recombinant TCR technology should allow engineering of allergen-specific T cells with immunomodulatory capacity (e.g., for adoptive transfer to modulate an existing Th2-dominated allergen-specific response).

Initial steps toward the realization of these objectives have been made possible by the transfer of a human allergen (Art v 1)-specific TCR into human T lymphocytes (18). The resulting TCR transgenic (TCRtg) T cells strictly responded according to the two-signal model described by Bretscher and Cohn (18, 19). In the absence of costimuli, TCRtg T cells receiving solely allergen-specific signal 1 developed a state of unresponsiveness (20). These studies have afforded a rational strategy for anergizing allergen-specific $CD4^+$ T lymphocytes.

In the current study, we characterized a Bet v 1-specific TCR that recognizes the immunodominant epitope Bet v $1_{142–153}$ (3). For this purpose, we isolated cDNA encoding the α- and β-chains of the TCR of a Bet v 1-specific T cell clone (TCC), transferred the TCR chains into Jurkat T cells and peripheral blood (PB) T cells of nonallergic individuals, and studied T cell function upon coculture with APCs presenting allergen plus various combinations of costimulators. Furthermore, we analyzed whether the transferred TCR retained its original fine specificity (i.e., the capacity to cross-react with Bet v 1-related food and pollen allergens). Finally, by combining recently developed APC technologies, we asked whether strictly Th1-polarized TCRtg Bet v 1-specific PB T cells could be conveniently generated and whether such T cells would be able to modulate the effector function of Th2-differentiated cells.

Materials and Methods

Cell lines and primary cells

293 cells and Jurkat TCC 41-19 expressing an IL-2 enhancer/promoter driving luciferase were cultured as described (21). Bet v 1-specific TCCs and immortalized B cells were established from PBMCs of birch pollen-allergic individuals with typical clinical history and positive skin-prick tests to birch pollen extract as described (22). TCR gene usage was determined by RT-PCR with TRAV and TRBV primers (Clontech, Heidelberg, Germany) as described (23). PBMCs from allergic and nonallergic individuals were obtained upon informed consent in accordance with institutional ethics guidelines.

Recombinant allergens

Recombinant allergens (Api g 1.0101; Bet v 1.0101; Dau c 1.0103; Cor a 1.0103; Mal d 1.0108) were purchased from Biomay AG (Vienna, Austria) or produced and purified to homogeneity [Car b 1.0109 (EU283857); Cas s 1.0101 (FJ390843.1); Fag s 1.0101 (FJ390846.1); Que a 1.0301 (EU283863); accession numbers in parentheses were submitted to GenBank] as described elsewhere (24).

Cloning and characterization of αβ TCRs

mRNA from the Bet v $1_{142–153}$-specific HLA-DRB1*07:01–restricted, Th2-like TCC SD334 (3) was amplified with TRAV6.01-forward 5′-CGCGGG*AAGCTT*GCCA**CCATGG**AGTCATTCCTGGGAGGTGT-3′ and TRAC-reverse 5′-CCCGCGGCGGCCGCTTTAGCTGGACCACAGCCGC-3′ or TRBV20.01-forward 5′-CGCGGG*AAGCTT*GCCA**CCATGCTGCTG-CTTCTGCTGCTT**-3′ and TRBC-reverse 5′-CCCGCGGCGGCCGCTTT-AGAAATCCTTTCTCTTGACCATG-3′, digested with NcoI (bold) or HindIII (italics) and NotI (underlined), and gel purified. DNA fragments were ligated into the retroviral expression vector pMMP412 (R.C. Mulligan, Children's Hospital, Boston, MA) and sequenced (VBC Genomics, Vienna, Austria). [Note: HLA nomenclature in this article is given according to the 2010 update and using colons (:) in the allele names to act as delimiters of the separate fields according to Marsh et al. (25).]

Cloning of HLA-DR constructs

mRNA from autologous EBV-immortalized B cells was amplified with HLA-DRB1*07:01-forward 5′-CGCGGG**AAGCTT**GCCACCATGGTGT-GTCTGAAGCTCCC-3′ and HLA-DRB1*07:01-reverse 5′-CCCGCG-GCGGCCGCTTTAGCTCAGGAATCCTGTTGGCT-3′, digested with HindIII (bold) and NotI (underlined). Gel-purified DNA fragments were ligated into pEAK12 expression vector (EdgeBio, Gaithersburg, MD) and sequenced.

Generation of Bet v 1-containing invariant chain constructs

pcDNA1.1/Amp containing an invariant chain (Ii) with an SfuI–Eco47III cassette instead of CLIP was kindly provided by J. van Bergen (Department of Immunohematology and Blood Transfusion, Leiden, The Netherlands) (26). The following double-stranded oligonucleotides were inserted into SfuI/Eco47III digested pcDNA1.1/Amp-Ii: Bet v $1_{4–15}$-upper 5′-CGAACTACGAGACCGAGACCACCAGCGTGATCCCCGC-3′, Bet v $1_{4–15}$-lower 5′-GCGGGGATCACGCTGGTGGTCTCGGTCTCGTAGTT-3′; Bet v $1_{112–123}$-upper 5′-CGTCCATCCTGAAGATCTCCAACAAGT-ACCACACCAA-3′, Bet v $1_{112–123}$-lower 5′-TTGGTGTGGTACTTGT-TGGAGATCTTCAGGATGGA-3′; Bet v $1_{142–153}$-upper 5′-CGACCCT-GCTGCGCGCCGTGGAGTCCTACCTGCTGGC-3′, Bet v $1_{142–153}$-lower 5′-GCCAGCAGGTAGGACTCCACGGCGCGCAGCAGGGT-3′. Ii containing CLIP or $HA_{309–317}$ was constructed as described (26). Proper DNA insertion was confirmed by sequence analysis (VBC Genomics).

Transduction of αβ TCRs into Jurkat and PB T cells

Bet v $1_{142–153}$-specific TCR constructs in pMMP412.TRAV6 and pMMP412.TRBV20 or Art v $1_{25–36}$-specific TCR constructs pMMP412.TRAV17 and pMMP412.TRBV18 were transduced into Jurkat T cells, bulk or sorted naive $CD4^+CD45RA^+$ PB T cells (Miltenyi, Bergisch Gladbach, Germany; purity of sorted T cells >98%) as described previously (18). In some experiments, a multicistronic strategy was used to express the Bet v 1-specific TCR, linking TRAV6 and TRBV20 via a P2A sequence in an IRES-GFP–containing version of pMMP. TCRtg Jurkat clones were obtained by limiting dilution. (The nucleotide sequences for the Bet v $1_{142–153}$-specific TCR α and β chains have been deposited in the GenBank database under accession numbers GQ179994 and GQ179995, respectively; http://www.ncbi.nlm.nih.gov/genbank/.)

Immunofluorescence analyses and intracellular cytokine determination

mAbs CD3–FITC (UCHT1), pan TCRαβ–PE (BMA031), CD4–FITC (VIT4), CD8–PE (VIT8b), and control Ig FITC and PE were obtained from An der Grub (Kaumberg, Austria). TCRβ-chain–specific mAbs TRBV12–FITC (56C5) and TRBV20–PE (MPB2D5) were from Immunotech (Marseille, France), and CD45RA (MEM-56) was from Invitrogen (Carlsbad, CA). HLA class II–FITC (1/47) and Ii–FITC (5-329) were kindly provided by O. Majdic (Institute of Immunology, Medical University of Vienna, Vienna, Austria). Staining and flow cytometry was performed as described (18). Intracellular cytokine production was analyzed after pretreatment for 6 h with GolgiStop (1:1500; Becton Dickinson, Palo Alto, CA) using Fix and Perm (An der Grub) and using Abs specific for IFN-γ (25723-PerCP; R&D Systems, Minneapolis, MN), IL-2 (MQ1-17H12-allophycocyanin; eBioscience, San Diego, CA), IL-4 (MP4–25D2-PE; Invitrogen, Camarillo, CA), IL-5 (JES1-39D10-PE; BioLegend, San Diego, CA), IL-10 (JES3-9D7; eBioscience), and IL-13 (JES10-5A2-allophycocyanin; BioLegend).

Generation of artificial APCs

293 cells (3×10^6) were transiently cotransfected with CD80::GPI, CD54::GPI, cathepsin S, HLA-DRA*01:01, and either HLA-DRB1*07:01 or HLA-DRB1*01:01 in combinations as indicated. Alternatively, artificial Ag-presenting cells (aAPCs) were transfected in addition with Ii–allergen fusion constructs Ii::Bet v $1_{4–15}$, Ii::Bet v $1_{112–123}$, Ii::Bet v $1_{142–153}$, or Ii::Art v $1_{25–34}$ and used 72 h after transfection. For polarization experiments, aAPCs were variably transfected with single-chain IL-12::GPI. IL-12::GPI had been constructed from pORF–hIL-12 (Invivogen, San Diego, CA) with primers IL-12 forward 5′-GCGAAGGAGGGCCA**CCATGG**GTC-3′ and IL-12 reverse 5′-CCCGCGGCTAGCGGAGCCGCCGCCGCCGCTGCCGCC-GCCGCCGCTGCCGCCGCTGGAGGAAGCATTCAGATAGCTCATC-3′ followed by digestion with NcoI (bold) and NheI (underlined) and ligation together with a HindIII–NcoI linker into pEAK12_CD80::GPI (21).

Generation of monocyte-derived dendritic cells

Monocyte-derived dendritic cells (MDDCs) were differentiated as described previously (27) and incubated at 2×10^5/well in 96-well flat-bottom culture plates with allergens (range, 0.01×10^{-6} to 9.4×10^{-6} M) in a total volume of 100 μl at 37°C overnight. Subsequently, TCRtg Jurkat T cells (1×10^5/well) were added and cocultured (total volume 200 μl) for 6 h.

Jurkat IL-2 promoter activity assays

aAPCs expressing the earlier described Ii constructs or aAPCs preincubated with peptides (3×10^{-5} M) Bet v $1_{4–15}$, Bet v $1_{112–123}$, Bet v $1_{142–153}$, Art v $1_{25–36}$, or medium alone (control) for 3 h were generated. Subsequently, Bet v $1_{142–153}$-specific or Art v $1_{25–36}$-specific TCRtg Jurkat cells (1×10^5) were added to 5×10^4 of the above-described aAPCs and cocultured for 6 h. PMA (10^{-7} M) plus PHA (5 μg/ml) served as positive and medium alone as negative control. Luciferase activity was assayed as described (21). Stimulation of Jurkat T cells with MDDCs followed an analogous protocol.

T cell proliferation and Th1 polarization

TCRtg T cells (5×10^4; starting population bulk PB T cells or naive $CD4^+$ $CD45RA^+$ T cells) were incubated with 5×10^4 irradiated (60 Gy) aAPCs expressing recombinant immunomodulatory molecules in 96-well flat-bottom plates for 3 d. After 24 to 72 h, cell culture supernatants were harvested and concentrations of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ determined by multiplex analysis (20). Duplicate plates were pulsed after 72 h with methyl-[^{3}H]thymidine (1 μCi/well) for 18 h and processed as described (20). Medium alone or anti-CD3/CD28 microbeads (5×10^4) served as controls. Proliferation assays with allergen-specific TCC and Bet

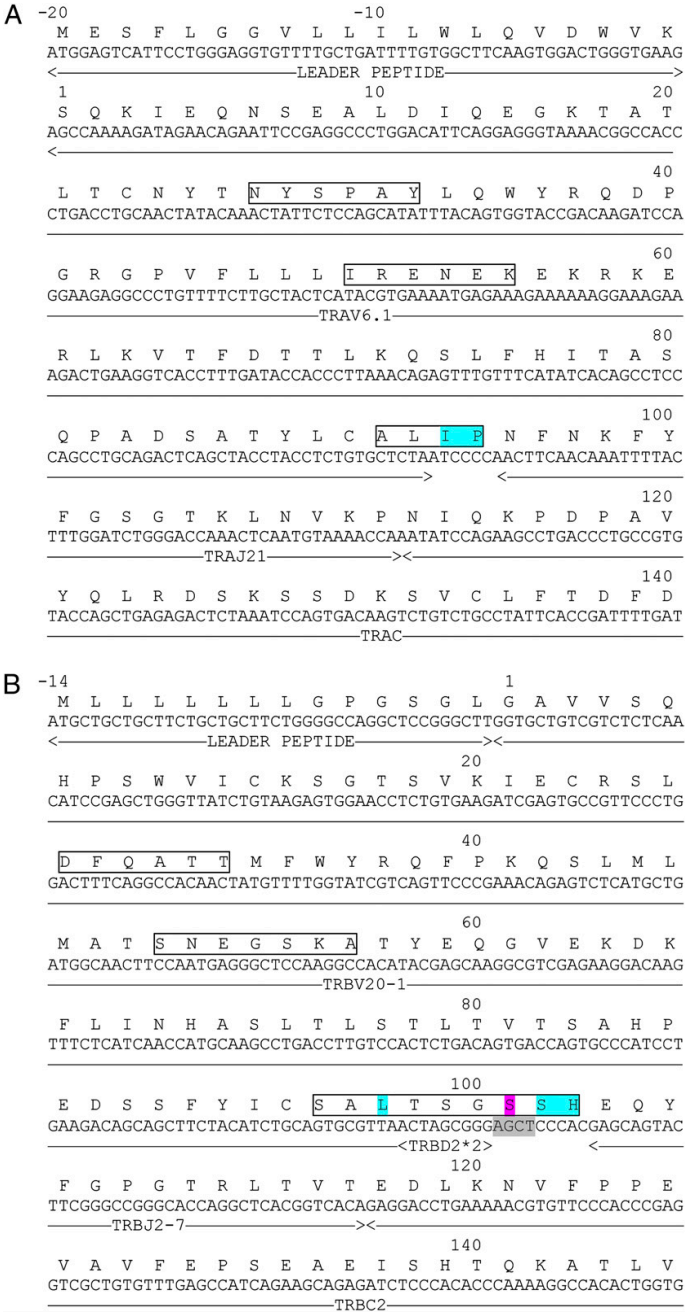

FIGURE 1. Nucleotide and amino acid sequence of the HLA-DRB1*07:01–restricted TCR of the Bet v $1_{142–153}$-specific TCC SD334. *A*, TCR α-chain sequence. *B*, TCR β-chain sequence. Codon numbering and nomenclature according to the ImMunoGeneTics web resource for T cell receptors (44). The 5′-ends of C-region sequences are shown. CDRs (boxed), P-nucleotides (gray), and amino acids from P-diversification (magenta) and from N-diversification (turquoise) are indicated.

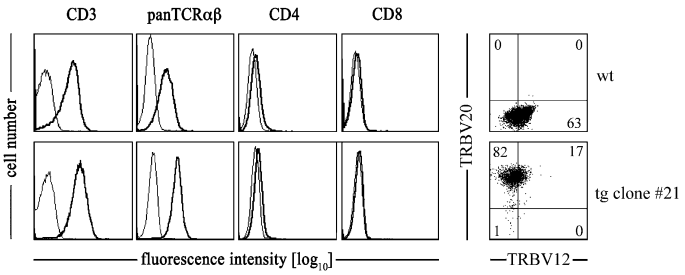

FIGURE 2. Bet v $1_{142–153}$-specific TCRtg Jurkat T cells. Expression pattern of TCR/CD3, Bet v $1_{142–153}$-specific transgenic TCR, and coreceptor molecules on wild-type and TCRtg single-cell clone no. 21 are shown. Expression of endogenous TRBV12 and transgenic TRBV20 is shown in the *right panel*. Numbers indicate percentage values within quadrants. Data are representative of two independently performed experiments.

v 1-related food and pollen allergens were performed as described (3). Th1-polarized cells were generated by coculture with irradiated (60 Gy) allergen-specific aAPCs coexpressing IL-12::GPI for 24 h, harvested, washed extensively, and cytometrically sorted for expression of GFP cotransduced with the recombinant TCR.

Inhibition of effector function of Th2-differentiated T cells

For the generation of TCRtg T cells, $CD4^+CD45RA^+$ PB T cells of allergic and nonallergic individuals were isolated, preactivated for 2 d with CD3/CD28-coated microbeads (ratio 1:2 of beads/cells), followed by transduction with the allergen-specific TCR as described. For Th1 differentiation, T cells were supplemented with IL-2 (100 U/ml; Peprotech) throughout the 9-d culture period. Final Th1 polarization was achieved by coculture of these T cells with irradiated (60 Gy) allergen-specific aAPCs coexpressing IL-12::GPI for 24 h. Th2 differentiation was achieved by supplementing T cells with IL-2 (10 ng/ml; Peprotech), IL-4 (50 ng/ml; Peprotech), anti–IFN-γ mAb (10 μg/ml; R&D Systems, Minneapolis, MN), and anti–IL-12 mAb (3 μg/ml; Ustekinumab; Jansen-Cilag, Titusville, NJ) for the 10-d culture period. After harvesting and extensive washing, Th2-differentiated cells (0.5×10^5/well) and Th1-polarized cells (0.5×10^5 to 1.5×10^5/well) were either cultured alone or in combination with allergen-specific or allergen-nonspecific aAPCs coexpressing CD80 (5×10^4/well, irradiated) in 96-well flat-bottom culture dishes in triplicate for up to 72 h. At various times, supernatants were harvested and subjected to multiplex cytokine analyses. After 4 d, cellular proliferation was monitored by pulsing with methyl-[^{3}H]thymidine (1 μCi/well) for the last 18 h of cultivation.

Statistical analyses

For multiple group comparisons, a linear mixed-model ANOVA was performed using SPSS software (IBM SPSS, Chicago, IL). For comparison of more than three groups, a Bonferroni correction was applied. Comparison between two groups was performed using the Student *t* test. Statistically significant values are denoted as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

*Molecular cloning of the αβ TCR of a Bet v $1_{142–153}$-specific, HLA-DRB1*07:01–restricted TCC*

A Bet v $1_{142-153}$-specific $CD4^+$ TCC (SD334) was isolated from PB of a birch pollen-allergic individual (HLA-DRB1*07, *15; DRB4*01; DRB5*01; DQB1*02, *06). This clone specifically reacted with the epitope RAVESY located within the immunodominant peptide Bet v $1_{142–153}$ in an HLA-DRB1*07:01–restricted manner (3). cDNA cloning and sequencing revealed that the functional α-chain gene of SD334 is formed by the rearrangement of TRAV6*01 to a TRAJ21*01 segment. The β-chain resulted from a TRBV20-1*01–TRBD2*02–TRBJ2-7*01–TRBC2 rearrangement (Fig. 1). In the α-chain, two non-templated codons resulting from N-diversification are present, whereas the C-terminal TRAV6*01 codon is deleted. In the β-chain CDR3 region, the C-terminal codon of TRBV20-1 has been deleted with P- and N-diversification giving rise to three non-germline-encoded codons at the 3′-end of TRBD2*02.

Retroviral transfer and expression of the Bet v $1_{142–153}$-specific TCR

cDNAs encoding the Bet v $1_{142–153}$-specific TCR α- and β-chains were introduced by retroviral transduction into Jurkat T cells harboring a human IL-2 promoter/enhancer controlling luciferase expression (21). The $tgTRBV20^+$ TCR was expressed on >99.8% of the cells of Bet v 1 TCRtg Jurkat clone no. 21 obtained by limiting dilution from bulk cultures of transduced cells (Fig. 2). Both the parental line and clone no. 21 expressed CD3 and TCR αβ, low levels of CD4, and no CD8. Similar results were obtained with other clones (data not shown).

The Bet v $1_{142–153}$-specific TCR is functionally intact in Jurkat T cells

To assess whether Bet v 1-specific TCRtg Jurkat T cells retain the antigenic specificity of the original TCC, Jurkat clone no. 21 was

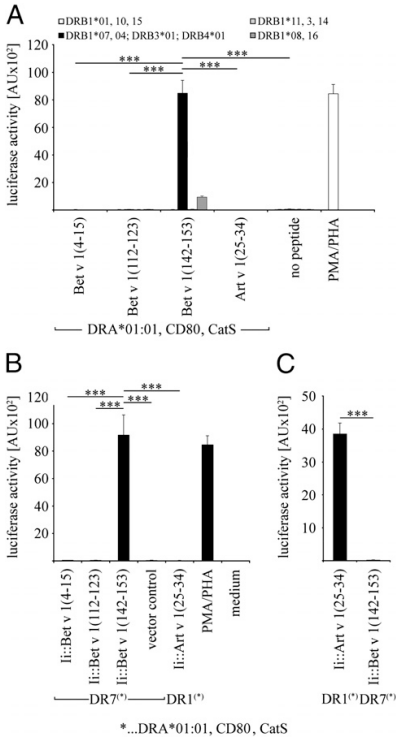

FIGURE 3. Functional activity of the transgenic Bet v $1_{142–153}$-specific TCR. *A*, IL-2 promoter activity of Bet v 1 TCRtg Jurkat T cells upon coculture with aAPCs transfected with the indicated pools of HLA class II cDNAs and pulsed with the indicated peptides or medium (no peptide). PMA/PHA (white bar) served as positive control ($n = 3$). Recognition of aAPCs expressing Ii-borne allergenic peptides. *B* and *C*, aAPCs expressing the indicated molecules and Ii::fusion proteins or vector control were coincubated with Bet v $1_{142–153}$-specific ($n = 6$) (*B*) or Art v 1-specific ($n = 6$) (*C*) TCRtg Jurkat T cells. Medium or PMA/PHA in the absence of APCs served as controls. Mean values + SEM of triplicates are shown. $***p < 0.001$. AU, arbitrary units; CatS, cathepsin S.

coincubated with aAPCs expressing pools of defined HLA class II molecules in medium alone or after pulsing with saturating concentrations of Bet v $1_{4–15}$, Bet v $1_{112–123}$, Bet v $1_{142–153}$ peptides or with Art v $1_{25–36}$ peptide, the immunodominant epitope of the major mugwort pollen allergen Art v 1, the latter serving as a negative control. Fig. 3*A* illustrates the specific recognition by Jurkat clone no. 21 of Bet v $1_{142–153}$-peptide presenting by HLA-DRB1*07:01$^+$ aAPCs, resulting in a significant increase in IL-2 promoter activity ($p < 0.001$). Coculture of Jurkat clone no. 21 with Bet v 1- or Art v 1-pulsed aAPCs expressing a collection of non-DR7 HLA class II molecules consistently revealed negative results. Experiments with aAPCs expressing single HLA-DRB1 specificities confirmed the HLA-DRB1*07:01 restriction of Jurkat clone no. 21 (data not shown). Half-maximal stimulation (ED_{50}) was achieved with 0.30 ± 0.07 μM Bet v $1_{142–153}$ peptide (data not shown). Wild-type Jurkat T cells did not react with Bet v $1_{142–153}$ but otherwise responded well to the superantigen staphylococcal enterotoxin E (data not shown).

aAPCs harboring an Ii::Bet v $1_{142–153}$ fusion construct along with expression of HLA-DRA*01:01, HLA-DRB1*07:01, CD80::GPI, CD54::GPI, and cathepsin S also significantly stimulated ($p < 0.001$) Bet v $1_{142–153}$-specific TCRtg Jurkat T cells comparable with the positive control PMA/PHA (Fig. 3*B*). In contrast, clone no. 21 was not activated by aAPCs transfected with Ii::Bet v $1_{4–15}$, Ii::Bet v $1_{112–123}$, or control vector. Furthermore, Art v $1_{25–36}$ had no stimulatory effect on Bet v $1_{142–153}$-specific TCRtg T cells in the context of HLA-DR1, which otherwise significantly ($p < 0.001$) stimulated Art v 1-specific TCRtg Jurkat T cells (Fig. 3*C*). Generally, T cell activation was moderate in the absence (stimulation index: 3.2 ± 0.6 fold background) but became pronounced in the presence (stimulation index: 24.2 ± 3.4 fold background) of costimulation, for example, by CD80 (Supplemental Fig. 1).

Bet v $1_{142–153}$-specific TCRtg T cells cross-react with Bet v 1-related food and pollen allergens

In a further step aimed at evaluating the fine specificity of the recombinant TCR, TCRtg Jurkat T cells were incubated with HLA-DR7$^+$ MDDCs pulsed with Bet v 1, Bet v 1-related food (Dau c 1, carrot; Api g 1, celery; Mal d 1, apple) or pollen (Cor a 1, hazelnut; Cab a 1, horn beam; Cas s 1, chestnut; Fag s 1, beech; and Que a 1, oak) allergens. Fig. 4*A* and 4*B* show that, apart from Bet v 1 (ED_{50} 0.22 ± 0.01 μM), the food-derived Api g 1 (ED_{50} 0.78 ± 0.09 μM) and the pollen-derived Cor a 1 (ED_{50} 0.16 ± 0.05 μM) and Car b 1 (ED_{50} 0.38 ± 0.08 μM) were recognized by TCRtg T cells. In contrast, Dau c 1 was only poorly stimulatory, and Mal d 1 remained unrecognized. Among the pollen allergens, Cas s 1 was poorly recognized, with responses seen only at the highest concentrations, whereas Que a 1 and Fag s 1 remained unrecognized by the TCRtg T cells. Art v 1 from mugwort used as negative control did not activate TCRtg Jurkat T cells (data not shown). The original TCC SD334 from which the TCR was derived displayed an almost identical reaction pattern to the tested food and pollen allergens when incubated with 0.3 μM allergens (Fig. 4*C*, 4*D*). Neither reactivity with Bet v 1 nor cross-reactivity with related allergens was observed when HLA-DR7$^-$ MDDCs were used as aAPCs (data not shown).

The Bet v $1_{142–153}$-specific TCR is functionally active in human PB T cells

Next, we tested whether it was possible to generate human Bet v 1-specific T lymphocytes from nonallergic individuals. PBLs from five donors (HLA-DR7$^+$ or HLA-DR7$^-$) were transduced with the Bet v $1_{142–153}$-specific TCR, resulting in 63 ± 2% compared with 7 ± 2% CD3$^+$TRBV20$^+$ T cells in TCR versus control transduced T cells, respectively (Fig. 5*A*). TCRtg T cells from all four donors significantly proliferated in an HLA-DR7–restricted manner upon coincubation with aAPCs coexpressing Ii::Bet v $1_{142–153}$ but not with aAPCs coexpressing Ii::Bet v $1_{4–15}$ or Ii::Bet v $1_{112–123}$ (Fig. 5*B*). Moreover, Ii::Bet v $1_{142–153}$-induced proliferation was strictly costimulation dependent (Fig. 5*C*).

Th1 polarization of TCRtg PB T lymphocytes by aAPCs coexpressing HLA/allergen plus membrane-bound IL-12

We next asked whether aAPCs could polarize de novo-generated, TCRtg allergen-specific T cells from nonallergic and allergic

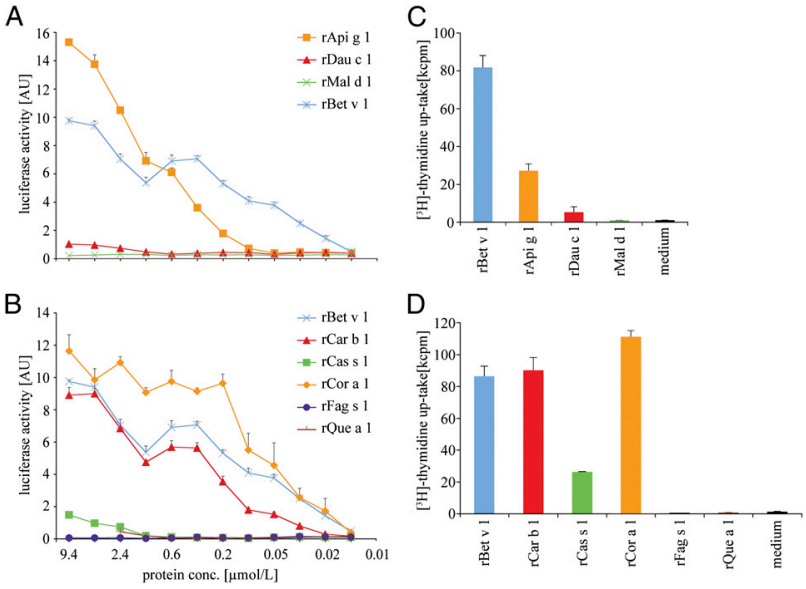

FIGURE 4. The Bet v $1_{142–153}$-specific TCR cross-reacts with Bet v 1-related food and pollen allergens. *A* and *B*, HLA-DR7$^+$ MDDCs preincubated overnight with Bet v 1 and Bet v 1-related (*A*) food or (*B*) pollen allergens were cocultured the next day with TCRtg Jurkat cells for 6 h before luciferase activity was determined. Allergens: Bet v 1 (birch), Api g 1 (celery), Dau c 1 (carrot), Mal d 1 (apple), Cor a 1 (hazelnut), Car b 1 (hornbeam), Cas s 1 (chestnut), Fag s 1 (beech), Que a 1 (oak). *C* and *D*, Proliferation of the original TCC from which the TCR was derived in response to (*C*) food and (*D*) pollen allergens. Data show mean values + SD of triplicate cultures representative of two independently performed experiments. AU, arbitrary units; conc., concentration.

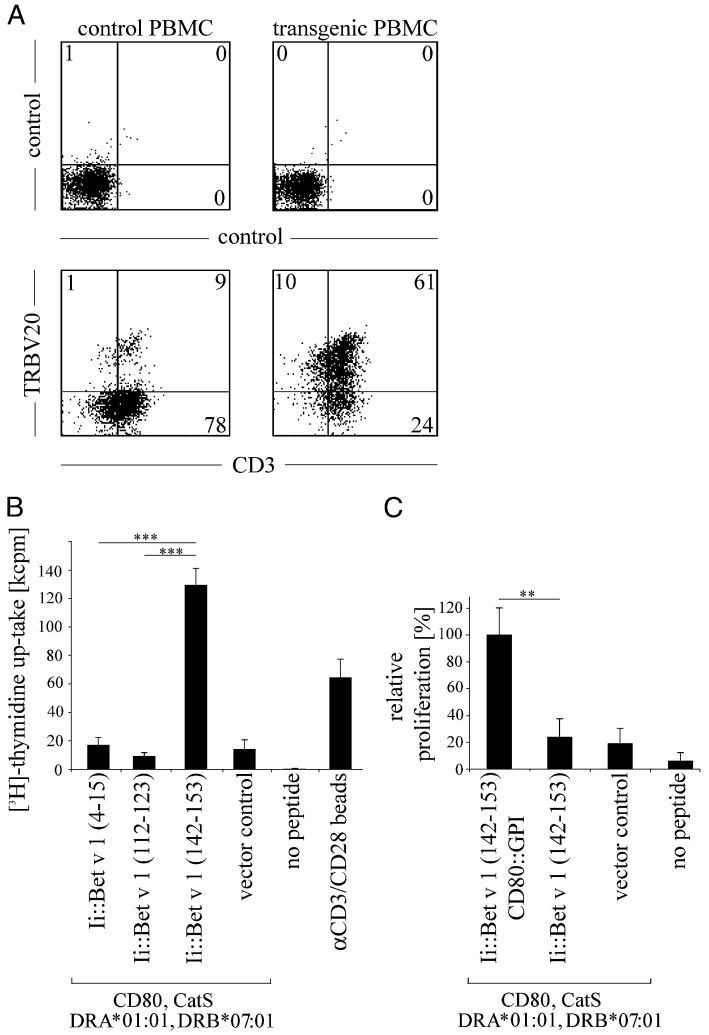

FIGURE 5. TCRtg PB T cells are specific for Bet v $1_{142–153}$. *A*, Expression levels of TRBV20 and CD3 on control and TCRtg PB T cells. Data are representative for five independent experiments using five different donors. Numbers indicate percentage values within indicated quadrants. *B*, Proliferation of TCRtg PB T cells cocultured with aAPCs coexpressing the indicated molecules. Control, control vector. Anti-CD3/CD28 microbeads and medium (no peptide) were used as controls. Data show mean proliferation + SD of triplicate cultures corrected for proliferation of GFPtg T cells (9.8 kcpm; range, 8.1–12.5 kcpm). *C*, Costimulation requirement of TCRtg PB T cells. Data show relative proliferation rates of TCRtg PB T cells cocultured with aAPCs expressing Ii::Bet v $1_{142–153}$ in the context of HLA-DR7 in the presence or absence of CD80::GPI. Data are representative for four different donors and seven experiments performed. $^{**}p < 0.01$, $^{***}p < 0.001$. CatS, cathepsin S.

donors toward the Th1 phenotype. For that purpose, we created aAPCs expressing HLA/allergen in the absence or presence of the costimulatory molecule CD80 and/or membrane-bound IL-12 (28). Allergen-specific aAPCs coexpressing CD80 induced significant levels (>40 pg/ml) of IFN-γ, IL-2, and IL-13, whereas no IL-4 (data not shown) and only minute amounts of IL-10 were detectable (Fig. 6 and Supplemental Table I). Significantly, coexpression of membrane-bound IL-12 (p40::p35::GPI fusion protein) (28) in the absence of CD80 strongly increased IFN-γ secretion levels during primary allergen-specific stimulation ($p < 0.05$), which was even more pronounced in the additional presence of CD80 ($p < 0.05$). When compared with CD80 costimulated T cells, IL-12–costimulated allergen-specific T cells (in the absence of CD80) produced significantly less IL-2 and IL-13 (<15 pg/ml; $p < 0.05$ and $p < 0.001$, respectively). In the absence of specific Ag, the levels of CD80-induced or IL-12–induced cytokines were consistently low similar to cytokine levels obtained upon stimulation of T cells with signal 1 alone (Fig. 6 and Supplemental Table I). In contrast, the combination of CD80 and IL-12 led to Ag-independent IFN-γ production. However, the additional presence of an Ag-specific signal 1 increased IFN-γ levels by 23.0 ± 10.4 fold ($p < 0.01$).

Similar results were obtained with an Art v 1-specific TCR introduced into T cells of an HLA-DR1$^+$ individual (Supplemental Table II). Comparable Th1-biased polarization was observed, irrespective of whether bulk PB or naive CD4$^+$CD45RA$^+$ PB T cells were used as the starting population (data not shown).

Next, we assessed cytokine expression levels in allergic and nonallergic individuals ($n = 5$) also on the cellular level by flow cytometry. Fig. 7 shows a clear-cut increase in the percentage of IFN-γ–positive cells in allergic and nonallergic donors upon costimulation with IL-12 compared with that of T cells receiving signal 1 alone (mean ± SD: 5.3 ± 1.6 fold and 4.6 ± 1.6 fold, respectively). Of note, the IL-12–induced percentage of IFN-γ–producing cells was even higher than upon CD80 costimulation (mean ± SD: 1.9 ± 1.3 fold and 2.4 ± 0.3 fold, respectively). In contrast, the percentages of IL-2– and IL-13–positive cells remained low compared with CD80-costimulated cells (mean ± SD of IL-2: 0.4 ± 0.1% and 0.9 ± 0.6% versus 5.7 ± 2.1% and 6.5 ± 3.1%, respectively; mean ± SD of IL-13: 0.9 ± 0.6% and 1.3 ± 0.5% versus 3.6 ± 0.9% and 5.2 ± 2.4%, respectively). Similar intracellular cytokine pattern was obtained in an HLA-DR1–positive individual and applying the Art v 1-specific TCR (Supplemental Fig. 2).

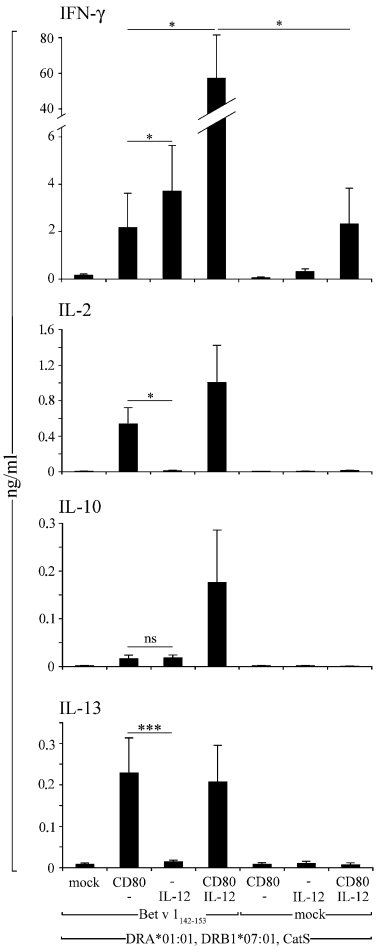

FIGURE 6. Predominant IFN-γ secretion by Bet v $1_{142–153}$–TCRtg PB T cells stimulated with aAPCs expressing Bet v $1_{142–153}$ in the context of HLA-DR7 plus membrane-bound IL-12. TCRtg allergen-specific T cells were generated from allergic and nonallergic donors ($n = 6$) and stimulated with irradiated (60 Gy) aAPCs equipped with or without (mock) the indicated costimulatory molecules in the presence (Ii::Bet v $1_{142–153}$) or absence (mock) of allergen. Data show mean cytokine levels (ng/ml) of supernatants (24 h) of triplicates + SEM. $*p < 0.05$, $***p < 0.001$. CatS, cathepsin S; ns, not significant.

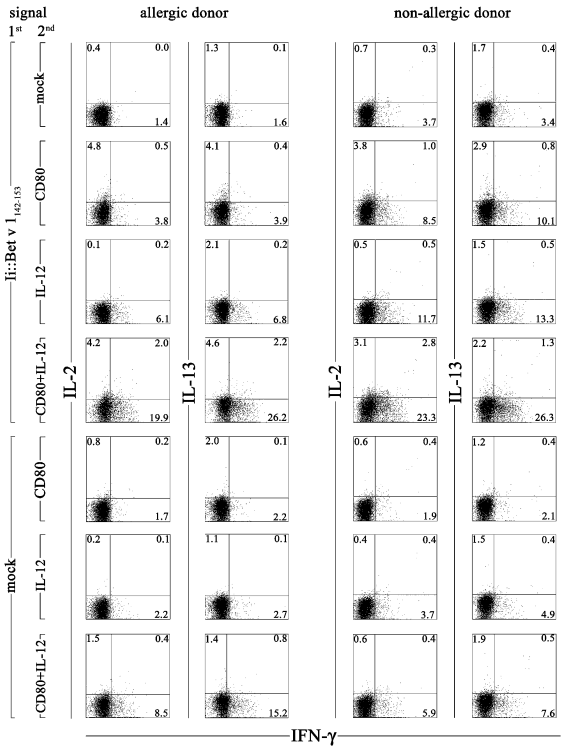

FIGURE 7. Intracellular cytokine levels of allergen-specific TCRtg T cells upon stimulation with different forms of allergen-specific or nonspecific APCs. TCRtg allergen-specific T cells were generated from allergic ($n = 2$) and nonallergic ($n = 3$) donors and stimulated with the indicated irradiated (60 Gy) aAPCs in the presence or absence of allergen (1st signal: Ii::Bet v $1_{142–153}$) and equipped or nonequipped with costimulatory molecules (2nd signal). After 24 h, cocultures were incubated with GolgiStop (Becton Dickinson) for 6 h, followed by intracellular cytokine staining using Fix and Perm (An der Grub) and analysis by flow cytometry. Data show dual parameter dot plots of one representative allergic and nonallergic individual. Markers were set according to the results obtained with nonbinding control Abs. Numbers indicate percentage positive cells in respective quadrants.

Allergen-specific Th1-polarized TCRtg T cells inhibit the effector function of allergen-specific Th2-differentiated T cells

To investigate whether IL-12–induced Th1 cells, differentiated in the absence of CD80 costimulation, could inhibit allergen-specific Th2 cells, we introduced the TCR into naive $CD4^+CD45RA^+$ T cells of allergic and nonallergic donors undergoing Th1 (as described earlier) or Th2 polarization. Fig. 8 shows that both cell types when cultured individually secreted typical signature cytokines upon restimulation with Bet v $1_{142–153}$-specific aAPCs coexpressing CD80. However, upon coculture of Th2-polarized cells of allergic (Fig. 8*A*) or nonallergic (Fig. 8*B*) individuals with syngeneic Th1-polarized cells at a 1:2 ratio (i.e., 0.5×10^5 Th2 cells to 1×10^5 Th1 cells), Th2-differentiated T cells significantly reduced production of their signature cytokines IL-5 and IL-13 ($p < 0.01$, respectively) and also significantly downregulated IL-2 and IL-10 production ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 8*A*, 8*B*). Subset-specific cytokine secretion was strongly dependent on allergen-specific activation by aAPCs coexpressing CD80, as only minute quantities of cytokines (e.g., IFN-γ <10 pg/ml) were secreted in the absence of allergen-specific aAPCs (data not shown) or in the presence of aAPCs expressing the noncognate Bet v $1_{4–15}$ peptide and CD80 (Fig. 8, white bars). Furthermore, allergen specificity was also confirmed by determination of intracellular cytokine levels (Fig. 9 and Supplemental Fig. 3).

The observed downregulation of Th2-signature cytokine levels was also apparent when looking at the single-cell level of T cells from allergic individuals using intracellular cytokine staining. In fact, the percentage of IL-4–, IL-5–, and IL-13–positive cells was reduced from 3.3 to 0.7%, 1.0 to 0.3%, and 1.9 to 1.0%, respectively (Fig. 9*A*). These experiments also revealed that IL-2 consumption does not seem to be the major factor accounting for the observed decrease of IL-2 levels in cocultures, as also the percentage of IL-2–positive cells within the Th2 population was reduced upon coculture from 12.3% in Th2 cultures to 3.1% in Th1/Th2 cultures. Similar results were obtained with allergic and nonallergic donors (data not shown). Data concerning intracellular cytokine levels were confirmed by using an Art v $1_{25–36}$-specific TCR and T cells from

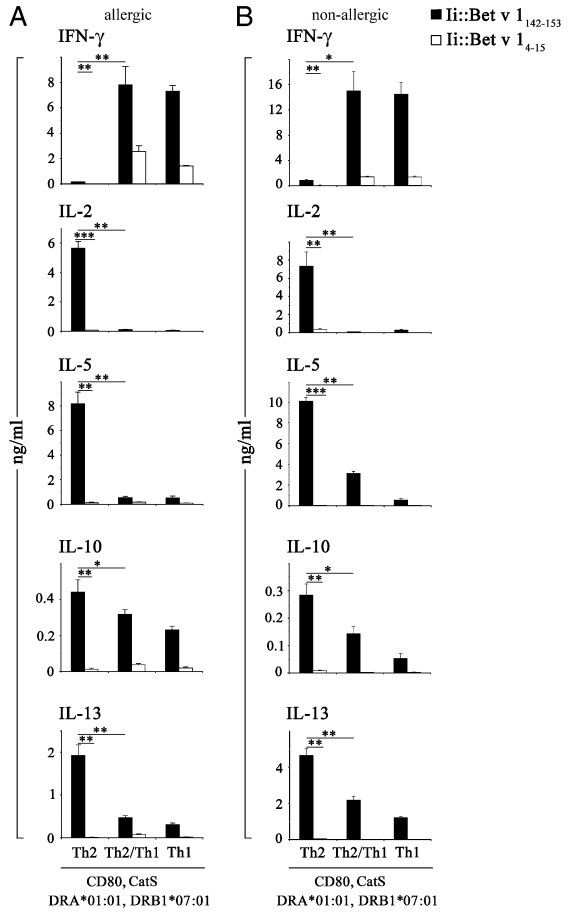

FIGURE 8. Allergen-specific TCRtg Th1-polarized T cells suppress the effector function of allergen-specific Th2-differentiated T cells. *A* and *B*, Naive $CD4^+CD45RA^+$ T cells from (*A*) allergic and (*B*) nonallergic individuals were transduced with the Bet v $1_{142–153}$-specific TCR and cultured under Th2-differentiating conditions in the presence of IL-2, IL-4, anti–IFN-γ, and anti–IL-12. Th1-polarized T cells were generated by coculture of Bet v $1_{142–153}$-specific TCRtg T cells with APC Bet v $1_{142–153}$ peptide in the context of HLA-DR7 and membrane-bound IL-12. Data show mean + SD of secreted cytokine levels of individual Th cell types or Th1/Th2 cocultures upon incubation with allergen-specific (Ii::Bet v $1_{142–153}$, black bars) or allergen-nonspecific (Ii::Bet v $1_{4–15}$, white bars) aAPCs coexpressing CD80 for 48 h (IFN-γ, IL-2) or 72 h (IL-5, IL-10, IL-13). Data are representative of four individuals and seven experiments performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. CatS, cathepsin S.

an HLA-DR1–positive individual (Supplemental Fig. 3). Cytokine expression was strictly Ag dependent, as no significant numbers of cytokine-positive cells were detectable when aAPCs used for coculture experiments presented the noncognate Bet v $1_{4–15}$ peptide in the presence of CD80 (Fig. 9*B*). In summary, these results demonstrate the concept that the effector function of human Th2-differentiated T cells can be efficiently inhibited by allergen-specific TCRtg Th1-polarized T cells generated by priming with allergen-specific aAPCs coexpressing membrane-bound IL-12.

Discussion

In the current study, we have cloned, sequenced, and functionally characterized the TCR α- and β-chains of a $CD4^+$ TCC (SD334) specific for the immunodominant T cell epitope of the major birch pollen allergen Bet v 1. Jurkat T cells as well as PB T cells of nonallergic individuals stably expressing this transgenic TCR after retroviral transduction specifically reacted with Bet v $1_{142–153}$ peptide-pulsed aAPCs, with aAPCs expressing Ii::Bet v $1_{142–153}$ fusion proteins, and with MDDC-processed Bet v 1 protein. Reactivity of TCRtg T cells depended on coexpression of costimulatory molecules such as CD80. The fine specificity of the TCC for Bet v 1-related food and pollen allergens was preserved in the TCRtg T cells. Coexpression of membrane-bound IL-12 on allergen-presenting aAPCs induced TCRtg T cells derived from allergic and nonallergic individuals to secrete high levels of IFN-γ, whereas IL-2 and IL-13 levels remained low, compatible with the induction of a Th1-polarized phenotype. Of note, analysis of cytokines at the single-cell level revealed similar results. Such Th1-polarized T cells, upon allergen-specific but not -nonspecific activation, significantly inhibited the effector function of Th2-differentiated allergen-specific T cells derived from naive $CD4^+$ $CD45^+$ T cells. Similar results were obtained with an HLA-DR1–restricted TCR recognizing the mugwort major pollen allergen, Art v $1_{25–34}$, confirming the general validity of our findings.

Birch pollen allergy and its associated oral allergy syndrome significantly reduce the quality of life of affected individuals throughout the year (8). Individuals with accompanying atopic dermatitis may additionally suffer cutaneous exacerbations (e.g., aggravation of atopic eczema) upon ingestion of Bet v 1-related food allergens (13). Significantly, Bet v 1-related food allergy cannot be palliated by conventional specific immunotherapy, leaving this aspect of birch pollen allergy currently intractable (29). To contribute to a better understanding of the Bet v 1-specific T cell response, in this study we devised a novel tool, a molecularly defined and transferable Bet v $1_{142–153}$-specific food and pollen cross-reactive TCR, which allows the creation of biological model systems in which the Bet v 1-specific T cell response can be studied in greater detail and which can be subjected to hypothesis-based modulation. In the Bet v 1-specific model system, all three components of the allergen-specific synapse (restriction element, immunodominant peptide, TCR) are well defined at the molecular level allowing the reproducible study and evaluation of new allergen variants/formulations for specific immunotherapy. The system also provides a solid basis for the establishment of "birch pollen-specific TCRtg mice" to study cross-reactivity and late-phase reactions in vivo in the near future. In principle, the recombinant TCR could also be used to evaluate the contribution of allergen-specific IgE in T cell-dominated in vivo model systems, for example, by adoptive transfer of recently developed mAbs with allergen specificity (30).

Upon transfer into heterologous T cells the Bet v $1_{142–153}$-specific TCR retained its typical cross-reactivity with the Bet v 1-related food allergen Api g 1 from celery and its weak but significant cross-reactivity with Dau c 1 from carrot. Similarly, cross-reactivity remained strong with the tree pollen allergens Cor a 1 from hazelnut and Car b 1 from hornbeam and weak with Cas s 1 from chestnut (3). Notably, the presence of the immunodominant epitope RAVESY, representing the amino acid residues 145–150 of Bet v 1, was not an absolute requirement for cross-reactivity with related food and pollen allergen. Only one of the cross-reactive allergens (i.e., Car b 1) has complete sequence identity at positions 145–150 with Bet v 1. Although Cor a 1 (hazelnut) and Api g 1 (celery) have one or three amino acid exchanges in the core binding region, respectively, they still functioned as highly cross-reactive allergens. These results together with the reliable identification of anchor residues and the availability of a high-throughput cellular test system should be useful for the design

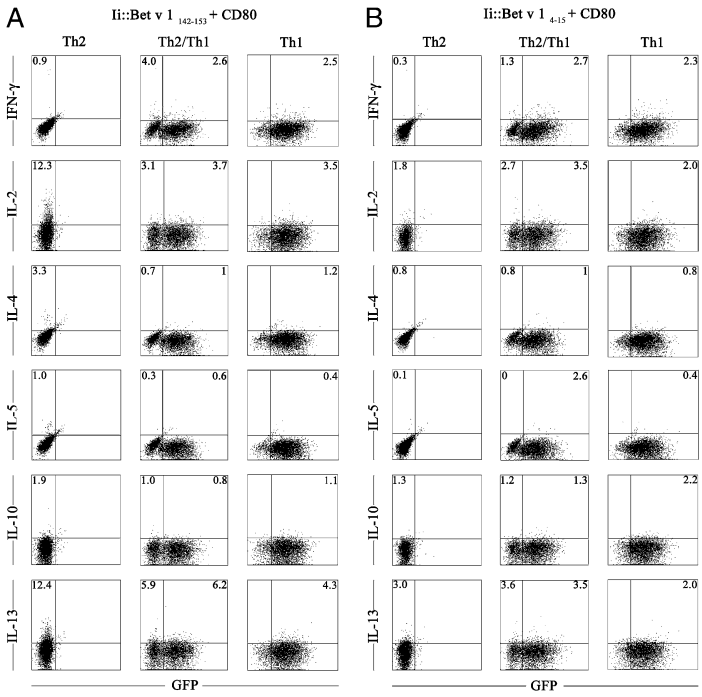

FIGURE 9. Intracellular cytokine levels of polarized allergen-specific T cells upon individual culture and coculture. *A* and *B*, Th1 (GFP^+) and Th2 (GFP^-) differentiated T cells from an allergic individual cocultured with (*A*) allergen-specific (Ii::Bet v $1_{142–153}$) or (*B*) allergen-nonspecific (Ii::Bet v $1_{4–15}$) aAPCs coexpressing CD80 were analyzed for intracellular cytokine levels individually or upon Th1/Th2 coculture. After 48 h, cultures were incubated with GolgiStop (Becton Dickinson) for 6 h, followed by intracellular cytokine staining using Fix and Perm (An der Grub) and the indicated directly conjugated cytokine-specific mAbs and analyzed by flow cytometry. Data show dual parameter dot plots of one representative experiment of several performed ($n = 5$). Markers were set according to negative controls; numbers indicate percentage positive cells within the GFP^- and GFP^+ cell fractions.

of therapeutically valuable immunomodulatory peptide ligands covering the immunodominant C-terminal epitope of Bet v 1 in the future.

In allergic individuals, the net output of the cognate allergen/APC–T cell interaction is an imbalanced, Th2-dominated immune response favoring IgE production along with recruitment and arming of potent effector cells (31) such as eosinophils and mast cells. It is generally believed that, among other mechanisms, Th1 cells inhibit the de novo generation of Th2 cells by virtue of their secreted signature cytokines. Soluble IL-12 is a potent inducer of Th1 cells (32, 33); however, systemic administration of the cytokine has been shown to induce a plethora of adverse reactions (34, 35). To provide a more general solution for this problem and to restrict the action of cytokines to the site of Ag presentation, we have resorted to membrane attachment of cytokines to aAPCs (28). In this report, we sought to assess whether allergen-specific aAPCs expressing membrane-bound IL-12, in the absence of CD80 coexpression, would induce Th1-polarized Bet v 1-specific T cells. Our results clearly demonstrate that membrane-tethered IL-12p35::p40 fusion proteins in concert with HLA/allergen complexes elicited allergen-specific TCRtg T cells with a strict Th1 phenotype, exemplified by the sole secretion of large amounts of IFN-γ (Fig. 6 and Supplemental Table I). The increased IFN-γ levels were mirrored by elevated numbers of IFN-γ–producing cells as determined by intracellular cytokine measurements at the single-cell level (Fig. 7). Importantly, similar results were obtained irrespective of whether T cells were derived from allergic or nonallergic individuals. Of note, Th1 polarization was not a salient feature of the Bet v 1-specific TCR but was similarly observed with a mugwort, Art v $1_{25–34}$-specific, TCR (Supplemental Table II and Supplemental Fig. 2). Moreover, we have demonstrated that such Th1-polarized T cells are capable of dominantly inhibiting the effector function of Th2-differentiated T cells with the same allergen specificity. Besides the significantly reduced Th2-signature cytokine levels in coculture supernatants, these experiments also revealed distinct inhibition of cytokine production at the single-cell level (Fig. 9). This indicates that cytokine consumption/deprivation (36) might not play a major role for the observed downregulation of cytokine levels in cocultures. Our findings with TCRtg T cells (i.e., inhibition of cytokine synthesis) are consistent with previous results obtained with Th2-differentiated human and murine TCCs, which were stimulated Ag-specifically in the presence of exogenously added IFN-γ (37, 38). In principle, redirection of Th cell polarization represents a potentially important strategy for treating Th2 cell-dominated diseases, such as type I allergies. However, future studies, which certainly are beyond the scope of this investigation, will have to address the question whether the Th2 cells under investigation have retained their flexibility and were thus diverted from their previous phenotype. Alternatively, suppression with subsequent deletion from cultures could explain the effects observed. The availability of well-characterized Bet v 1-specific TCRs along with adjustable aAPCs and noncellular Ag-presenting platforms (20) offers the possibility to generate allergen-specific, Th1-polarized T lymphocytes ex vivo on a large scale (e.g., for subsequent adoptive transfer into severely allergic individuals in the future).

Before initiation of such studies, however, it remains to be explored whether and how polarized T cells could be applied in vivo to restore the Th1/Th2 balance without harming the host. In fact, certain allergic diseases have a biphasic T cell response in which a systemic allergen-specific Th2 response is followed by a more Th1-dominated organ pathology, as for example in atopic dermatitis (39). Along those lines, also IL-17 has been described to have a dual nature in the pathogenesis of allergic diseases (40). While IL-17 is required for the establishment of asthma in sensitized mice, it suppresses asthma in the effector phase of the disease (41). Consequently, as of today our current study cannot exclude that adoptive transfer of allergen-specific Th1 cells might enhance disease-promoting mechanisms in the recipient. To clarify these important issues experimentally in the near future,

humanized "allergy mice," expressing the relevant Bet v 1-specific TCR and the human restriction elements described in this article, would offer an attractive model.

As an alternative to the above strategy, the engineering and application of allergen-specific regulatory T cells modified by direct gene transfer appears to represent an attractive option. In a pilot study, we have transduced PB T cells with multicistronic expression cassettes (42) harboring Bet v 1-specific TCR α- and β-chains along with regulatory genes, such as the *FOXP3* gene, as a single translation product. This maneuver endows T cells with Ag specificity, a regulatory cell surface phenotype, and regulatory capabilities. The multicistronic approach reliably prevents inappropriate expression of single-gene products, avoiding the potential generation of undesired T cell specificities. Recently, we have demonstrated that Bet v 1-specific TCR/Foxp3 double-transgenic T cells suppress the effector function of Bet v 1-specific T cells in an activation-dependent manner (43).

In conclusion, the availability of well-characterized allergen-specific TCRs offers a broad range of possibilities for creating hypothesis-based, human-relevant in vitro and in vivo model systems to study the multifaceted aspects of allergic diseases. Biologically relevant pathways amenable to immunomodulation have been experimentally addressed in this report. The findings are of potential clinical importance and should also provide novel insights into the pathophysiology of allergic diseases and their possible cure in the future.

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Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTAL DATA FIGURE LEGENDS

FIGURE S1. Activation of Bet v $1_{142-153}$-specific TCRtg T-cells is co-stimulation dependent. IL-2 promoter activity of **A**,Bet v $1_{142-153}$-specific TCRtg Jurkat T-cells (n=4) or **B,** Art v 1_{25-34}-specific TCRtg Jurkat T-cells (n=3)upon co-culture with aAPC transfected with the indicated HLA class II cDNAs and Ii in the absence (grey bars) or presence (black bars) of the co-stimulatory molecule CD80. PMA/PHA (white bars) served as positive medium (no peptide) as negative control. Data show mean values + SD of triplicates and are representative for the number of experiments indicated.AU, arbitrary units. ***, $p < 0.001$.

FIGURE S2. Intracellular cytokine levels of allergen-specific T-cells upon stimulation with different forms of allergen-specific APC. TCRtg allergen-specific T-cells were generated from non-allergic (n=3) and allergic donors (n=2) and stimulated with the indicated irradiated (60 Gy) aAPC equipped with co-stimulatory molecules (2^{nd} signal) in the presence or absence of allergen (1^{st} signal: Bet v $1_{142-153}$). After 24 hours, co-cultures were incubated with Golgi-Stop (Becton Dickinson) for 6 hours, followed by intracellular staining using Fix and Perm (An der Grub) and the indicated directly conjugated cytokine-specific mAbs and analyzed by flow cytometry. Data show dual parameter dot plots of representative examples of several experiments performed (n=5). Numbers indicate percent positive cells in respective quadrant.

FIGURE S3. Intracellular cytokine levels of polarized allergen-specific T-cells upon co-culture. Th1 ($CFSE^+$) and Th2($CFSE^-$) differentiated T-cells from a non-allergic individual, co-cultured with **A,** allergen-specific (Art v 1_{25-34}) or **B,** allergen-nonspecific (Bet v $1_{142-153}$) aAPC were analyzed for intracellular cytokine levels individually or upon co-culture for 48 hours. Subsequently, co-cultures were incubated with Golgi-Stop (Becton Dickinson) for 6 hours, followed by intracellular staining using Fix and Perm (An der Grub) and the indicated

directly conjugated cytokine-specific mAbs and analyzed by flow cytometry. Data show dual parameter dot plots of representative examples of several experiments performed (n=5). Numbers indicate the percent positive cell fractions within the $CFSE^-$ and $CFSE^+$ cell fractions.CFSE, Carboxy-fluorescein succinimidyl ester.

Figure S1

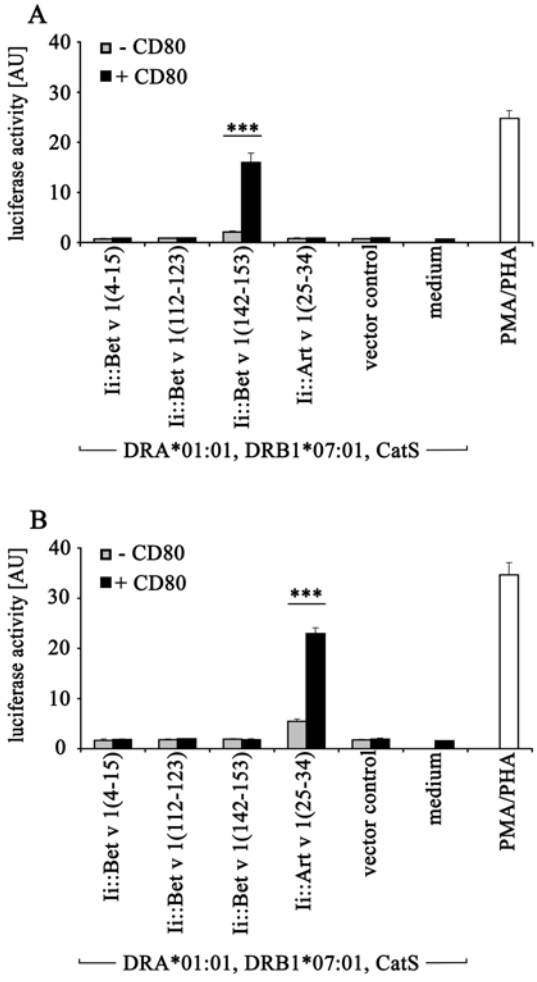

Figure S2

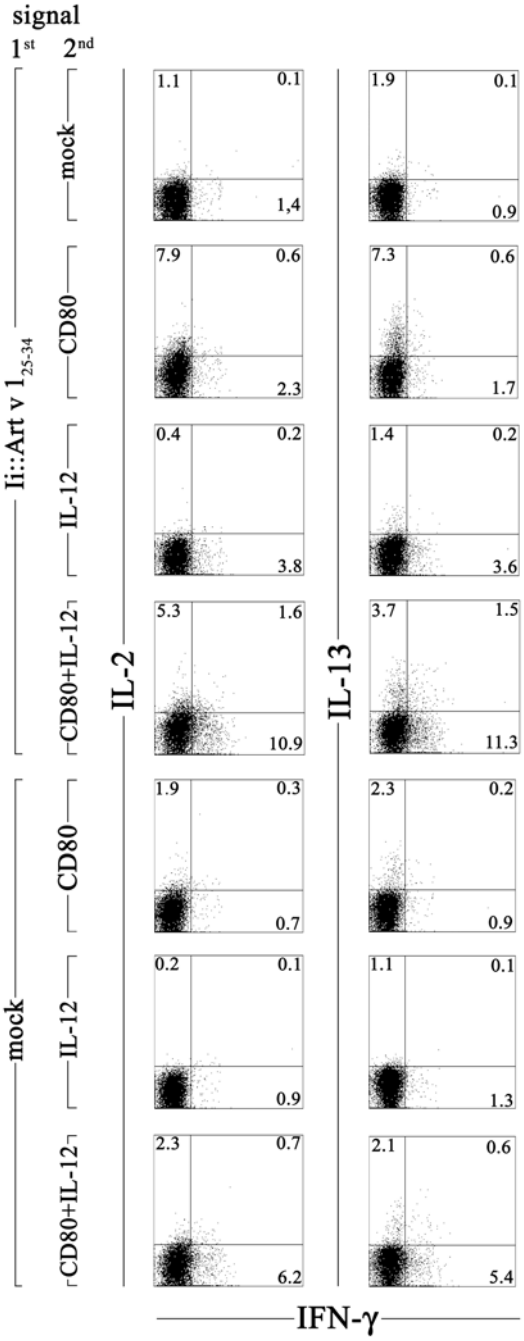
signal
1st
2nd
Ii::Art v 1 25-34
mock
CD80
IL-12
CD80+IL-12
mock
CD80
IL-12
CD80+IL-12
IL-2
IL-13
IFN-γ
1.1
0.1
1,4
1.9
0.1
0.9
7.9
0.6
2.3
7.3
0.6
1.7
0.4
0.2
3.8
1.4
0.2
3.6
5.3
1.6
10.9
3.7
1.5
11.3
1.9
0.3
0.7
2.3
0.2
0.9
0.2
0.1
0.9
1.1
0.1
1.3
2.3
0.7
6.2
2.1
0.6
5.4

Figure S3

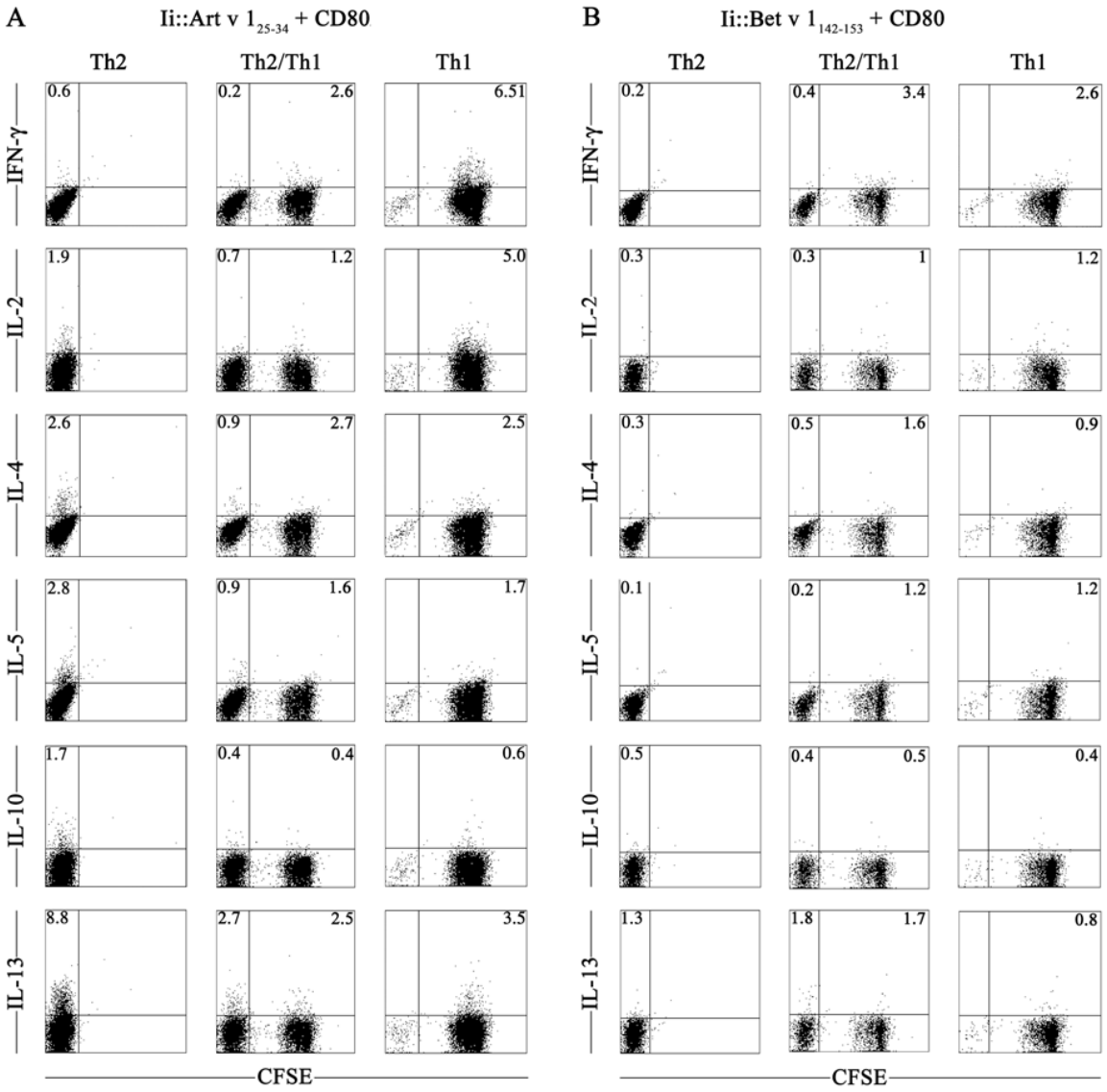
A
Ii::Art v 1 25-34 + CD80
B
Ii::Bet v 1 142-153 + CD80
Th2
Th2/Th1
Th1
IFN-γ
IL-2
IL-4
IL-5
IL-10
IL-13
CFSE
0.6
0.2
2.6
6.51
1.9
0.7
1.2
5.0
2.6
0.9
2.7
2.5
2.8
0.9
1.6
1.7
1.7
0.4
0.4
0.6
8.8
2.7
2.5
3.5
0.2
0.4
3.4
2.6
0.3
0.3
1
1.2
0.3
0.5
1.6
0.9
0.1
0.2
1.2
1.2
0.5
0.4
0.5
0.4
1.3
1.8
1.7
0.8

TABLE SI. Predominant IFN-γ secretion by TCRtg PB T-cells stimulated with aAPC expressing Bet v $1_{142-153}$ in the context of HLA-DR7 plus membrane-bound IL-12

		1st signal												
		Ii::Bet v $1_{142-153}$							mock					
		2nd signal												
		mock	CD80		IL-12		CD80 + IL-12		CD80		IL-12		CD80 + IL-12	
	Cytokines	pg/ml	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%
donor #1	IFN-γ	36±10[a]	149±10	100[b]	1165±384	782	32750±12070	21980	10±5	7	115±34	77	154±46	103
	IL-2	11±4	659±65	100	22±6	3	1463±291	222	10±9	2	4±4	1	18±10	3
	IL-4	0	0	100	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
	IL-10	3±1	13±2	100	20±4	154	80±12	615	0	0	1±1	8	2±0	15
	IL-13	0	47±2	100	4 ±3	9	33±1	70	0	0	0	0	0	0
donor #2	IFN-γ	17±0	133±18	100[b]	3367±1420	2531	77210±4190	58053	4±4	3	35±6	26	73±21	55
	IL-2	12±5	1324±17	100	37±6	3	2871±163	217	7±0	1	2±4	0	19±4	1
	IL-4	0	0	100	0	n.d.	1±1	n.d.	0	n.d.	0	n.d.	0	n.d.
	IL-10	2±1	15±1	100	41±4	273	78±3	520	0	0	0	0	1±1	7
	IL-13	4±3	73±7	100	23±5	32	61±8	84	0	0	0	0	0	0
donor #3	IFN-γ	104±8	601±92	100[b]	974±101	162	4045±198	673	38±17	6	139±15	23	626±197	104
	IL-2	9±1	509±78	100	9±1	2	415±80	82	8±2	2	17±4	3	1±1	0
	IL-4	0	0	100	0	n.d.	1±1	n.d.	0	n.d.	0	n.d.	0	n.d.
	IL-10	0	1±2	100	5±4	500	38±11	3800	0	0	0	0	1±1	100
	IL-13	17±11	391±44	100	31±4	8	391±66	100	11±3	0	35±7	9	3±5	1
donor #4	IFN-γ	187±28	975±122	100[b]	1328±151	136	3354±683	344	129±28	13	344±50	35	1002±63	103
	IL-2	3±1	76±5	100	4±1	5	76±14	100	5±1	7	10±3	13	1±1	1
	IL-4	0	0	100	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
	IL-10	0	2±2	100	5±4	250	49±9	2450	0	0	0	0	0	0
	IL-13	5±6	172±13	100	13±2	8	124±24	72	8±7	5	9±8	5	0	0
allergic donor #1	IFN-γ	460±54	9287±2640	100[b]	13155±142	136	145991±21662	1572	1±2	0	574±236	6	9657±1565	104
	IL-2	4±0	535±46	100	2±1	0	779±126	146	1±1	0	0	0	32±10	6
	IL-4	0	0	100	0	n.d.	0	n.d.	0	n.d.	0	n.d.	2±0	n.d.
	IL-10	1±1	12±2	100	8±1	67	85±2	708	0	0	1±1	8	0	0
	IL-13	0	119±34	100	1±1	1	72±8	61	0	0	0	0	9±1	8
allergic donor #2	IFN-γ	96±16	1874±125	100[b]	2232±242	119	8014±619	428	140±20	7	695±178	37	2441±643	1302
	IL-2	3±1	120±23	100	3±1	3	420±46	350	8±3	7	2±0	2	11±4	9
	IL-4	0	0	100	0	n.d.	0	n.d.	0	n.d.	0	n.d.	33±5	n.d.
	IL-10	3±6	53±13	100	30±4	57	725±38	1368	8±7	15	6±6	11	0	n.d.
	IL-13	20±5	570±100	100	12±10	2	561±38	98	28±3	5	14±12	2	30±11	5

[a] Mean cytokine levels of triplicates ± SD.

[b] Percent cytokine levels compared to allergen/HLA plus CD80 stimulated T-cells.

n.d., not determined.

TABLE SII. Predominant IFN-γ secretion by TCRtg PB T-cells stimulated with aAPC expressing Art v 1_{25-34} in the context of HLA-DR1 plus membrane-bound IL-12

		1st signal												
		Ii:Art v 1_{25-34}							mock					
		2nd signal												
		mock	CD80		IL-12		CD80 + IL-12		CD80		IL-12		CD80 + IL-12	
	Cytokines	pg/ml	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%
donor #5	IFN-γ	534±71[a]	2008±177	100[b]	3399±640	169	7276±1138	362	170±17	8	966±150	48	3966±757	198
	IL-2	5±1	76±9	100	4±1	5	27±2	36	10±3	13	2±0	3	24±6	32
	IL-4	0	0	100	0	n.d.	0	n.d.	0	n.d.	0	n.d.	11±3	n.d.
	IL-10	7±4	19±0	100	10±10	53	51±7	268	1±2	5	6±6	32	0	n.d.
	IL-13	293±46	1732±98	100	144±19	8	1190±59	69	100±46	6	45±6	3	292±76	17

[a] Mean cytokine levels of triplicates ± SD.

[b] Percent cytokine levels compared to allergen/HLA plus CD80 stimulated T-cells.

n.d., not determined.

Chapter V

Bet v 1–specific T-cell receptor/forkhead box protein 3 transgenic T cells suppress Bet v 1–specific T-cell effector function in an activation-dependent manner

Klaus G. Schmetterer, MD,[a] Daniela Haiderer, MSc,[a] Victoria M. Leb-Reichl, PhD,[a,c] Alina Neunkirchner, MSc,[a,c] Beatrice Jahn-Schmid, PhD,[b] Hans J. Küng, PhD,[a] Karina Schuch, BSc,[a] Peter Steinberger, PhD,[a] Barbara Bohle, PhD,[b,c] and Winfried F. Pickl, MD[a,c] *Vienna, Austria*

Background: Regulatory T (Treg) cells establish and maintain tolerance to self-antigens and many foreign antigens, such as allergens, by suppressing effector T-cell proliferation and function. We have previously shown that human T-cell receptor (TCR) αβ-chains specific for allergen-derived epitopes confer allergen specificity on peripheral blood T cells of individuals with and without allergy.

Objective: To study the feasibility of generating allergen-specific human Treg cells by retroviral transduction of a transcription unit encoding forkhead box protein 3 *(FOXP3)* and allergen-specific *TCR αβ*-chains.

Methods: cDNAs encoding the *α* and *β*−chains of a Bet v $1_{142\text{-}153}$−specific *TCR* (TCR alpha variable region 6/TCR beta variable region 20) and human *FOXP3* were linked via picornaviral *2A* sequences and expressed as single translational unit from an internal ribosomal entry site—green fluorescence protein−containing retroviral vector. Retrovirally transduced peripheral blood T cells were tested for expression of transgenes, Treg phenotype, and regulatory capacity toward allergen-specific effector T cells.

Results: Transduced T cells displayed a Treg phenotype with clear-cut upregulation of CD25, CD39, and cytotoxic T-lymphocyte antigen 4. The transduced cells were hyporesponsive in cytokine production and secretion and, like naturally occurring Treg cells, did not proliferate after antigen-specific or antigen-mimetic stimulation. However, proliferation was inducible upon exposure to exogenous IL-2. In coculture experiments, $TRAV6^+TRBV20^+FOXP3^+$ transgenic T cells, unlike $FOXP3^+$ single transgenic T cells or naturally occurring Treg cells, highly significantly suppressed T cell cytokine production and proliferation of corresponding allergen-specific effector T cells in an allergen-specific, dose-dependent manner.

Conclusion: We demonstrate a transgenic approach to engineer human allergen-specific Treg cells that exert their regulatory function in an activation-dependent manner. Customized Treg cells might become useful for tolerance induction therapies in individuals with allergic and other immune-mediated diseases. (J Allergy Clin Immunol 2011;127:238-45.)

Key words: *Immune regulation, regulatory T cells, allergen-specific T-cell receptor, FOXP3, type I allergy, birch pollen, Bet v 1*

Naturally occurring $CD4^+CD25^{high}$ forkhead box protein 3 $(FOXP3)^+$ regulatory T (Treg) cells orchestrate and maintain tolerance to self-antigens by suppressing effector T cell proliferation and function. Depletion of Treg cells in mice has been shown to lead to overt multiorgan autoimmunity,[1] consistent with widely held views that Treg cells restrain the evolution of autoimmune disorders,[2] play a key role in tolerance against alloantigens after transplantation,[3] and modulate allergic immune responses.[4] Specific immunotherapy has been shown to increase Treg cell prevalence, which correlates with amelioration of clinical symptoms.[5-11] In addition, several primary immunodeficiencies are characterized by an apparent lack of functional Treg subsets.[12] Consequently, protocols to induce Treg cell function and elevate their numbers have gained increased attractiveness as therapeutic options in recent years. Strategies applied include expansion of Treg cell lines from sorted $CD4^+CD25^+$ populations by stimulation with different forms of antigen-presenting cells and cytokines[13-16] or the *de novo* generation of inducible Treg cells from $CD4^+CD25^-$ T cells by T-cell receptor (TCR)–mediated stimulation in the presence of TGF-β[17,18] or rapamycin.[19]

A recent key finding was that the retroviral or lentiviral introduction of *FOXP3* was sufficient to generate artificial Treg cells.[20,21] Such transgenic Treg cells display the typical features of naturally occurring Treg (nTreg) cells—hyporesponsiveness and suppression of effector cells—and can be cultivated and expanded *in vitro* over prolonged periods. However, these transgenic Treg cells represent a polyclonal population with a broad range of antigenic specificities, some representing important targets for normal immune function. On the other hand, Treg cells with the desired antigen specificity would most probably be found only at a low frequency.

The antigen specificity of T cells is determined by the TCR. In fact, transfer of *TCRαβ* genes[22] has become a convenient tool for transferring designated antigen specificities to peripheral blood (PB) T cells. Although first developed to generate large numbers of tumor-reactive T cells for therapy,[23-26] TCR transfer approaches can be used to generate T cells reactive against peptides derived from the major allergens in mugwort (Art v $1_{25\text{-}36}$)[27] and birch pollen (Bet v $1_{142\text{-}153}$), respectively, to study signal requirements and plasticity of allergen-specific T cells. These TCRs were

From [a]the Institute of Immunology and [b]the Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna; and [c]the Christian Doppler Laboratory for Immunomodulation.

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Reprint requests: Winfried F. Pickl, MD, Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, A-1090 Borschkegasse 8A, Vienna, Austria. E-mail: winfried.pickl@meduniwien.ac.at.

0091-6749/$36.00

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Abbreviations used
aAPC: Artificial antigen-presenting cell
FOXP3: Forkhead box protein 3
GFP: Green fluorescent protein
IRES: Internal ribosomal entry site
nTreg: Naturally occurring regulatory T cells
PB: Peripheral blood
TCR: T-cell receptor
Treg: Regulatory T

chosen because both allergens, their respective HLA restriction elements, and their major epitopes are well characterized,[28,29] and allergies to mugwort and birch pollen are among the main causes of pollinosis in the Northern Hemisphere.[30] In fact, more than 95% of individuals with birch pollen allergy elicit an IgE antibody response against Bet v 1, the major birch pollen allergen.[31]

It is well established that Treg cell–mediated suppression of effector T cells is based on a functional program that requires the activation of the Treg cells via the TCR or agonistic ligands to CD3.[1,32,33] We aimed to exploit these principles to generate customized, allergen-specific, human Treg cells by co-introduction of *FOXP3* to provide the regulatory program in concert with TCRαβ chains to provide allergen specificity. To ensure reliable and balanced expression of all 3 transgenes (ie, the *TCR αβ*-chains and *FOXP3*), a multicistronic vector-based approach was applied,[34] which allows expression of several genes from a single expression unit. The cDNAs encoding *TRAV6* and *TRBV20* (international immunogenetics information system nomenclature[35]) and FOXP3 were linked via picornaviral *2A* sequences[34] upstream of an internal ribosomal entry site *(IRES)*–green fluorescent protein (*GFP*) element into a retroviral expression vector. PB T cells were transduced with these constructs, monitored for transgene expression and immunophenotype, tested for hyporesponsiveness, and evaluated for their ability to suppress proliferation and cytokine production of allergen-specific effector T cells.

METHODS

Molecular cloning and generation of multicistronic vectors

The *FOXP3* cDNA was amplified from a human T-cell cDNA library (for primer sequences, see this article's Table E1 in the Online Repository at www.jacionline.org). Multicistronic constructs were generated via 2-step overlapping PCR by using porcine *Teschovirus-1* 2A peptide and *Thosea asigna* virus 2A peptide sequences[34] and the Bet v $1_{142-153}$–specific DRB1*07:01-restricted TCR constructs (*TRAV6/TRBV20;* Neunkirchner et al, July 2010, unpublished data). All constructs were digested with *Hind* III and *Not* I and ligated into either the retroviral pMMP[36] vector or the pMMP-IRES-GFP vector.

Cell lines and primary cells

The HEK-293 cell line (human embryonic kidney cells) was cultured as described.[36,37] PBMCs were isolated from healthy HLA-DR7$^+$ volunteers in compliance with the ethics committee of the Medical University of Vienna. CD4$^+$CD25$^+$ nTreg cells and CD4$^+$CD25$^-$ T cells were isolated by using the CD4$^+$CD25$^+$Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Transfection of 293 cells

293 cells were transfected as described.[27,36,37] For the generation of artificial antigen-presenting cells (aAPCs), 293 cells were transfected with 5 μg each of pEAK12.HLA-DRA*01:01, pEAK12.HLA-DRB1*07:01, pcDNA.invariant chain::Bet v $1_{142-153}$, pcDNA.cathepsin S, and pEAK12.CD80.[38] Amphotropic T cell transducing retroviruses using multicistronic expression cassettes were produced, purified, and applied as described.[27,36,37,39]

Retroviral transduction of PB T cells

CD4$^+$CD25$^-$ or PB T cells (5×10^6/well) were stimulated in 6-well flat-bottom plates with 5×10^6 anti-CD3/CD28–coated microbeads (Dynabeads; Invitrogen, Camarillo, Calif) and 300 U/mL IL-2 (Peprotech, London, United Kingdom) for 48 hours. Transduction leading to stable integration of transgenes into the genome of PB T cells was performed as described.[27]

Flow-cytometric analyses

Cells were stained as described[40] by using the mAbs indicated in this article's Table E2 in the Online Repository at www.jacionline.org (20 μg/mL). FOXP3 was detected by the FOXP3 Staining Set (eBioscience, San Jose, Calif). Intracellular cytokine production was analyzed after pretreatment for 6 hours with Golgi-Stop (1:1500; Becton Dickinson, Palo Alto, Calif) using the Fix and Perm Kit (An der Grub, Kaumberg, Austria).

Determination of cytokine secretion

Transduced T cells (5×10^4) were stimulated in 96-well plates with either allergen-specific aAPC (5×10^4 irradiated with 60 Gy) or anti-CD3/CD28 coated microbeads (5×10^4). After 24, 48, or 72 hours, supernatants were harvested and cytokine concentrations determined by multiplex analysis (Luminex 100IS; Biomedica, Vienna, Austria).

Proliferation and suppression assays

Transduced T cells or nTreg cells (5×10^4) were stimulated in 96-well plates with allergen-specific aAPC (5×10^4 irradiated with 60 Gy) or anti-CD3/CD28–coated microbeads (5×10^4) in the absence or presence of IL-2 (250 U/mL; Peprotech) for 72 hours, pulsed with [methyl-^{3}H] thymidine (1 μCi per well; Perkin Elmer, Boston, Mass), and processed as described.[36] To test the suppressive capability of transgenic Treg cells, 5×10^4 TRAV6$^+$TRBV20$^+$ effector T cells were cocultured with flow-cytometrically sorted TRAV6$^+$TRBV20$^+$FOXP3$^+$ T cells, FOXP3$^+$ T cells, or CD4$^+$CD25$^+$ nTreg cells and stimulated with aAPC or anti-CD3/CD28–coated microbeads and processed as described. Similarly, TRAV6$^+$TRBV20$^+$ effector T cells were labeled with the far-red cell proliferation dye eFluor 670 (eBioscience; 1.5 μmol/L), co-cultured with transgenic Treg cells or carboxyfluorescein succinimidyl ester–labeled nTreg cells (Invitrogen; 1 μmol/L) and analyzed by flow cytometry after 96 hours.

Statistical analysis

For multiple group comparisons, ANOVA was performed, followed by Bonferroni correction (SPSS; IBM, Chicago, Ill). Two-group comparisons were performed by using the Student *t* test or the Mann-Whitney *U* test. Data represent means ± SDs. Statistically significant values are denoted ($^*P < .05$; $^{**}P < .01$; $^{***}P < .001$).

RESULTS

Generation of multicistrons encoding allergen-specific *TCRα/β* and *FOXP3* cDNAs

The cDNA sequences for a TCR (*TRAV6/TRBV20*) specific for the Bet v $1_{142-153}$ peptide of the major birch pollen allergen Bet v 1 in the context of HLA-DRB1*07:01 (Neunkirchner et al, July 2010, unpublished data) were linked via a picornaviral porcine *Teschovirus-1* 2A peptide sequence to a bicistronic expression construct and expressed from the retroviral vector pMMP (Fig 1). In addition, the cDNA encoding *FOXP3* was linked to the TCR construct by using a picornaviral *T2A* element. The

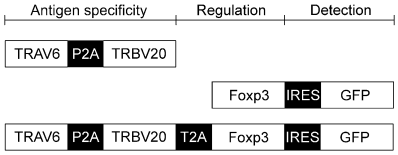

FIG 1. Scheme of multicistronic retroviral expression constructs used in this study. Function of the respective genes is indicated. *Open boxes,* genes; *closed boxes,* interposed picornaviral *2A* or *IRES* elements.

tricistronic construct and the respective FOXP3 single construct were transferred into the pMMP-IRES-GFP vector to allow transduction and to facilitate detection and sorting.

Transduced T cells express the introduced transgenes

The majority of *TRAV6/TRBV20*–transduced effector T cells (72 ± 14%; Fig 2, *A*) and of the GFP^{high} fraction of *TRAV6/TRBV20/FOXP3*–transduced T cells (82 ± 12%; Fig 2, *B*) expressed the transgenic TCR on their surfaces as detected by staining for the TRBV20-chain, whereas the GFP^{high} fraction of control-transduced (7 ± 1%) or *FOXP3*-transduced (8 ± 2%) T cells contained only low numbers of $TRBV20^+$ T cells, corresponding to the endogenous levels of polyclonal $TRBV20^+$ T cells (Fig 2, *B*). Similarly, intracellular staining for FOXP3 revealed high levels of FOXP3 in the GFP^{high} fractions of *FOXP3*-transduced (78 ± 3%) and *TRAV6/TRBV20/FOXP3*–transduced T cells (89 ± 8%; Fig 2, B) whereas control-transduced T cells exhibited negligible (1 ± 1%) expression. Importantly, the expression in the nontransduced GFP^{neg} fractions of both TRBV20 and FOXP3 were low and compatible with endogenous levels (Fig 2, *B*).

FOXP3-transduced T cells display a Treg cell surface phenotype

Flow-cytometric analyses performed on resting transduced PB T cells revealed that the $CD3^+CD4^+GFP^{high}$ fraction of both *FOXP3*-transduced and *TRAV6/TRBV20/FOXP3*–transduced T cells exhibited elevated expression of the Treg markers CD25,[1] CD39,[41] and cytotoxic T-lymphocyte antigen 4 (CD152)[42] compared with the $CD3^+CD4^+GFP^{neg}$ nontransduced fraction (Fig 3) or with *TRAV6/TRBV20*–transduced or mock-transduced PB T cells (not shown). Furthermore, and in accordance with a regulatory phenotype, we also observed a discrete downregulation of CD127 expression[43] on the transgenic Treg cells. No changes in glucocorticoid-induced tumor necrosis factor receptor-related protein expression levels[44] were observed. In all further functional experiments, the flow-cytometrically sorted GFP^{high} fractions of *FOXP3*-transducted T cells were used and termed $FOXP3^+$ or $TRAV6^+TRBV20^+FOXP3^+$ T cells, respectively.

$TRAV6^+TRBV20^+FOXP3^+$ T cells are hyporesponsive to antigen-specific and polyclonal activation

Treg cells are hyporesponsive to TCR-dependent stimulation.[1] On stimulation with aAPC expressing HLA-DRA*01:01, HLA-DRB1*07:01, cathepsin S, invariant chain–Bet v $1_{142\text{-}153}$, and CD80, $TRAV6^+TRBV20^+$ T cells proliferated vigorously whereas $TRAV6^+TRBV20^+FOXP3^+$ T cells were significantly less

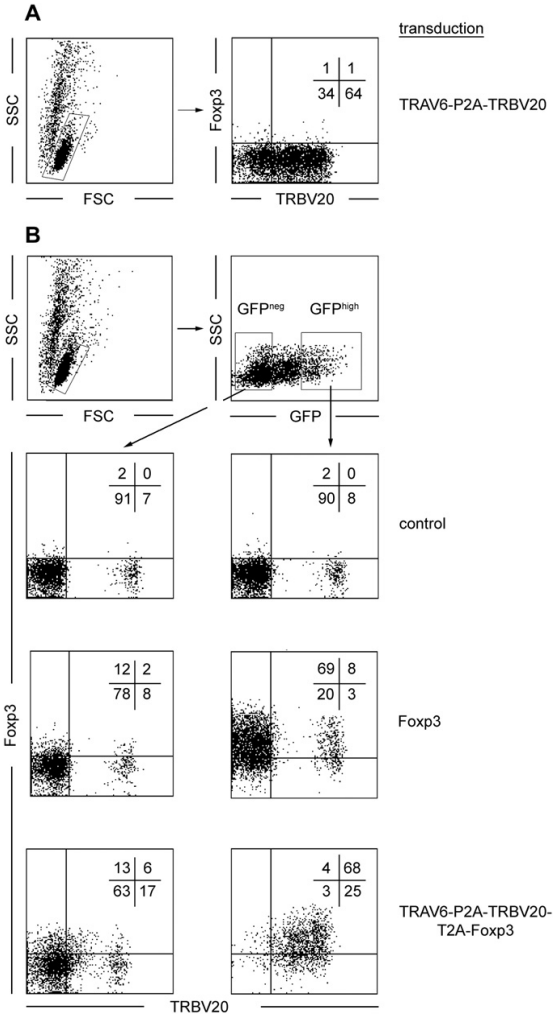

FIG 2. Transgene expression levels and gating strategy for retrovirally transduced PB T cells. **A,** $TRAV6^+TRBV20^+$ T cells. **B,** $TRAV6^+TRBV20^+FOXP3^+$, $FOXP3^+$, and control T cells. In *B,* GFP expression served as additional gating marker. Surface TCR expression (TRBV20) and intracellular FOXP3 expression are displayed as 2 parameter dot plots. Numbers indicate cell percentages in respective quadrants. *FSC,* Forward scatter; *SSC,* side scatter.

responsive ($P < .001$; Fig 4, *A*). Importantly, aAPC-dependent stimulation was highly specific, because control-transduced, *FOXP3* single-transgenic T cells devoid of the Bet v 1–specific TCR expression and nTreg cells showed only minimal proliferation ($P < .001$; Fig 4, *A*). Moreover, polyclonal stimulation using anti-CD3/CD28–coated microbeads led to a strong proliferative response of both $TRAV6^+TRBV20^+$ and control-transduced T cells whereas $TRAV6^+TRBV20^+FOXP3^+$ T cells, *FOXP3* single-transgenic T cells, and nTreg cells did not proliferate ($P < .001$; Fig 4, *B*.) In 8 experiments using cells from 4 nonrelated donors without allergy, proliferation of $TRAV6^+TRBV20^+FOXP3^+$ T cells was reduced by 73.7 ± 17.3% ($P < .001$) and 92.9 ± 6.6%

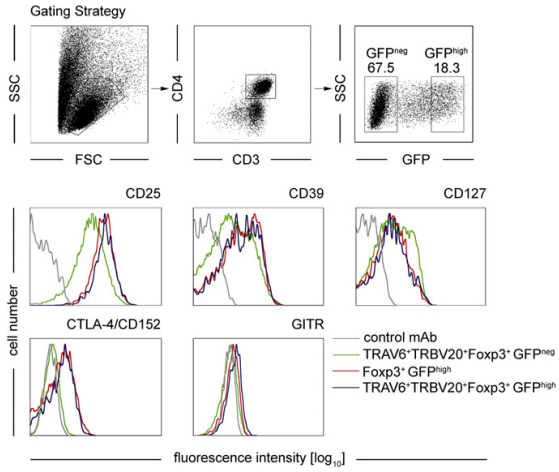

FIG 3. Immunophenotype of transduced T cells. PB T cells gated for $CD3^+CD4^+GFP^{neg}$ or $CD3^+CD4^+GFP^{high}$ subsets and analyzed for expression of indicated Treg markers. *Blue line,* GFP^{high} subset from *TRAV6/TRBV20/FOXP3*–transduced T cells, specific mAb; *red line,* GFP^{high} subset from *FOXP3*-transduced T cells, specific mAb; *green line,* GFP^{neg} subset, specific mAb; *gray line, TRAV6/TRBV20/FOXP3*–transduced T cells, control mAb. *FSC,* Forward scatter; *SSC,* side scatter.

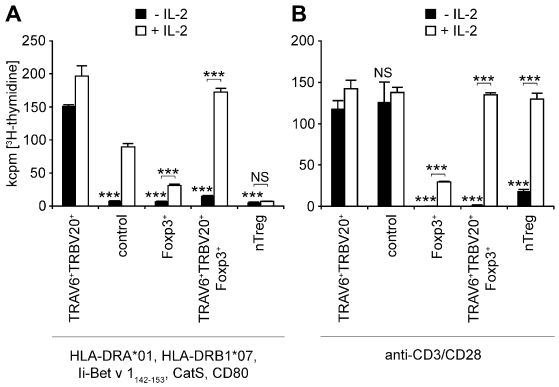

FIG 4. *FOXP3*-transduced T cells are hyporesponsive. Proliferation of retrovirally transduced PB T cells or nTreg cells stimulated with aAPCs expressing indicated molecules **(A)** or anti–CD3/CD28 coated microbeads in the absence *(black bars)* or presence *(white bars)* of recombinant human IL-2 (250 U/mL; **B**). Means + SDs of triplicates; control, empty *IRES-GFP* vector; asterisks indicate significance compared with $TRAV6^+TRBV20^+$ effector T cells *(- IL-2). NS,* Not significant. ***$P < .001$. *kcpm,* Kilo counts per minute.

($P < .001$), respectively compared with $TRAV6^+TRBV20^+$ effector T cells in response to allergen-specific and polyclonal stimulation. In agreement with previous reports, the addition of exogenous IL-2 led to a significant induction of proliferation of $FOXP3^+$ T cells ($P < .001$) and $TRAV6^+TRBV20^+FOXP3^+$ T cells ($P < .001$) in both settings,[16,20] whereas freshly isolated nTreg cells required a polyclonal stimulus plus exogenous IL-2 to proliferate ($P < .001$; Fig 4). Similarly, both *FOXP3*-transgenic T cell populations secreted only minimal levels of TNF-α, IFN-γ, IL-2, and IL-13 compared with $TRAV6^+TRBV20^+$ or control-transduced T cells after allergen-specific or polyclonal activation (Table I). In accordance with previous reports on *FOXP3*-transduced T cells, $TRAV6^+TRBV20^+FOXP3^+$ and $FOXP3^+$ T cells did not secrete significant amounts of IL-10 on stimulation.[16,20] Moreover, no significant differences in TGF-β secretion levels between the different cell populations studied were observed (not shown).

$TRAV6^+TRBV20^+FOXP3^+$ T cells suppress allergen-specific transgenic effector cell proliferation in response to allergen-specific stimulation

To assess the suppressive capacity of the transgenic Treg cells, $TRAV6^+TRBV20^+$ transgenic effector T cells were stimulated in the absence or presence of $TRAV6^+TRBV20^+FOXP3^+$ T cells, $FOXP3^+$ T cells, or $CD4^+CD25^+$ nTreg cells from the same donor. Upon Bet v 1–specific stimulation using aAPCs, *$TRAV6^+$ $TRBV20^+FOXP3^+$* T cells strongly suppressed allergen-specific effector T-cell proliferation, whereas $FOXP3^+$ T cells and nTreg cells expressing a noncognate T-cell receptor showed only weak suppressive capacity at a 1:1 ratio (Fig 5, *A*). Significant suppression by $TRAV6^+TRBV20^+FOXP3^+$ T cells was evident up to an effector-to-regulator ratio of 4:1 (see this article's Fig E1 in the Online Repository at www.jacionline.org). Using anti-CD3/CD28–coated microbeads as polyclonal stimulus, $TRAV6^+TRBV20^+FOXP3^+$ T cells, $FOXP3^+$ T cells, and nTreg cells suppressed effector T-cell proliferation to a similar degree (Fig, 5, *B*). In 8 experiments using PB T cells from 4 donors without allergy, allergen-specific activation in the presence of $TRAV6^+TRBV20^+FOXP3^+$ T cells elicited 67.3 ± 15.1% suppression of effector T-cell proliferation compared with 19.9 ± 11.8% suppression by $FOXP3^+$ T cells ($P < .001$) and 2.4 ± 11.2% by nTreg cells ($P < .001$). In response to polyclonal activation, $TRAV6^+TRBV20^+FOXP3^+$ T cells, $FOXP3^+$ T cells, and nTreg cells showed a similar degree of suppression of effector cell proliferation (71.6 ± 15.5%, $P < .001$; 74.0 ± 15.8%, $P < .001$; and 65.0 ± 2.9%, $P < .001$, respectively). The obtained results were similar irrespective of whether magnetic beads–purified $CD4^+CD25^-$ T cells (purity > 95%) or bulk PB T cells were used as the starting population (not shown). Similarly, proliferation of eFluor 670 cell proliferation dye–labeled effector T cells was suppressed by $TRAV6^+TRBV20^+FOXP3^+$ T cells but not by $FOXP3^+$ T cells or nTreg cells on Bet v 1–specific activation (Fig 5, *C*). Moreover, on polyclonal stimulation, all assessed Treg populations suppressed effector T cell proliferation (Fig 5, *D*). This suppressive capacity could, however, be overcome by exogenously added IL-2 (Fig 5, *C and D, lower panel*).

$TRAV6^+TRBV20^+FOXP3^+$ T cells efficiently suppress cytokine production of transgenic Bet v 1–specific effector T cells

On allergen-specific activation, significantly decreased levels of the cytokines TNF-α, IFN-γ, IL-2, and IL-13 (range of inhibition, 72% to 95%) were found in supernatants of cocultures of $TRAV6^+TRBV20^+$ effector T cells with $TRAV6^+TRBV20^+FOXP3^+$ regulatory cells (Fig 6, *A*). Coculture with $FOXP3^+$ Treg cells showed a much less pronounced inhibition of cytokine secretion (range, 32% to 55%), whereas nTreg cells did not affect cytokine secretion at all. To assess whether the observed results were caused by decreased effector cell cytokine production or by cytokine depletion,[45] intracellular cytokine expression levels were monitored by flow cytometry. Discrimination between nonfluorescent effector

TABLE I. Cytokine secretion by retrovirally transduced T cells after 24 hours

	TRAV6$^+$/ TRBV20$^+$	Control	FOXP3$^+$	TRAV6$^+$/ TRBV20$^+$/FOXP3$^+$
aAPC				
TNF-α	94 ± 29*	<1	<1	1 ± 1
IFN-γ	1138 ± 733	<1	<1	<1
IL-2	226 ± 86	5 ± 2	<1	<1
IL-4	<13	<13	<13	<13
IL-10	<1	<1	<1	<1
IL-13	143 ± 58	11 ± 9	7 ± 6	14 ± 12
anti-CD3/CD28				
TNF-α	73 ± 16	61 ± 32	5 ± 3	3 ± 2
IFN-γ	968 ± 598	537 ± 428	3 ± 3	3 ± 2
IL-2	35 ± 12	36 ± 12	<1	5 ± 2
IL-4	<13	<13	<13	<13
IL-10	<1	<1	<1	<1
IL-13	67 ± 29	68 ± 23	4 ± 2	2 ± 1

*pg/mL; control, *IRES-GFP* empty vector; data show mean values ± SDs (n = 3).

T cells and GFP$^+$ transgenic Treg cells or carboxyfluorescein succinimidyl ester–labeled nTreg cells allowed a strong and significant reduction of TNF-α, IFN-γ, IL-2, and IL-13 production to be detected in the effector T-cell population (GFP$^-$, nonfluorescent). Thus, activated transgenic Treg cells have the capacity to inhibit cytokine production by effector cells directly and highly significantly.

DISCUSSION

We show that co-introduction of human TCR αβ-chains of predefined allergen specificity in concert with human FOXP3, the master regulator of Treg cells, into human PB T cells generates transgenic allergen-specific Treg cells. These cells show the characteristic phenotype and functional properties of Treg cells: Treg marker expression, hyporesponsiveness to both allergen-specific and polyclonal stimulation, low cytokine secretion levels, and suppression of effector T cell proliferation and cytokine production. Although it has been well documented in several transgenic mouse models[1,32,33] that Treg-mediated suppression is an activation-dependent process, the general applicability of this understanding to human allergen-specific Treg cells has not been explored to date. This may be because, although TCR transfer technologies were described some 25 years ago,[22] molecular information about human allergen-specific TCR has been slow to develop.

We here used the Bet v 1–reactive TRAV6/TRBV20 TCR (Neunkirchner et al, unpublished data) to establish a proof-of-principle system. TRAV6$^+$TRBV20$^+$FOXP3$^+$ PB T cells showed a strong regulatory capacity for Bet v 1–specific effector T cells on allergen-specific and polyclonal stimulation. Importantly, *FOXP3* single-transgenic T cells, which express their endogenous, noncognate TCR, as well as CD4$^+$CD25$^+$ nTreg cells from the respective donors showed only weak regulatory capacity in the allergen-specific test system but were able to suppress transgenic effector T-cell proliferation to a similar degree as TRAV6$^+$TRBV20$^+$FOXP3$^+$ T cells on polyclonal stimulation. This observation indicates that suppression by allergen-specific *FOXP3*-transgenic T cells is—for the most part—an activation-dependent process and that signaling via the transgenic human TCR can be indeed used as a specific "molecular switch" to turn on allergen-specific T-regulatory programs in response to the defined allergen. The data obtained herein are thus

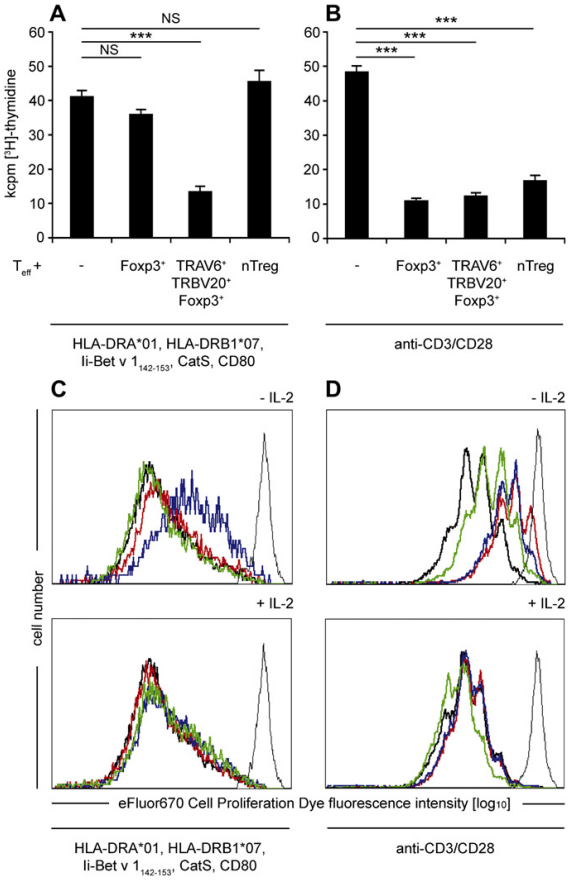

FIG 5. *TRAV6/TRBV20/FOXP3*-transduced T cells, unlike *FOXP3*-transduced T cells, suppress Bet v 1–specific effector T cells on allergen-specific activation. Proliferation of TRAV6$^+$TRBV20$^+$ transgenic effector T cells *(T_{eff})* cultured in the absence or presence of FOXP3$^+$, TRAV6$^+$TRBV20$^+$FOXP3$^+$ T cells, or nTreg cells at a 1:1 ratio on stimulation with Bet v 1–presenting aAPCs **(A)** or anti-CD3/CD28 coated microbeads **(B)**. Data show means ± SDs from triplicates. Flow-cytometric analyses of eFluor 670 cell proliferation dye–labeled T_{eff} in the absence *(black line)* or presence of FOXP3$^+$ *(red line)*, TRAV6$^+$TRBV20$^+$FOXP3$^+$ T cells *(blue line)*, or nTreg cells *(green line)* at a 1:1 ratio on stimulation with Bet v 1–presenting aAPCs **(C)** or anti-CD3/CD28 coated microbeads **(D)** in the absence *(upper panel)* or presence *(lower panel)* of exogenous IL-2 (250 U/mL). *NS,* Not significant. ***P < .001. *kcpm,* Kilo counts per minute.

in perfect agreement with Treg studies in the murine system, showing that Treg cells exert their suppressive function only on TCR/CD3 engagement.[1] However, once appropriately activated, both antigen-specific and nonspecific Treg cells show equal suppressive capacity.

Allergen-specific transgenic Treg cells could become potent, highly specific immunotherapeutic tools. One could envision reinfusing Treg cells engineered from autologous T cells of severely affected individuals by TCR/FOXP3 transduction. On seasonal exposure to birch pollen, transferred Treg cells would become specifically triggered and start to exert their regulatory program, leading to tolerance against the immunodominant epitope of Bet v 1—that is, Bet v $1_{142\text{-}153}$.

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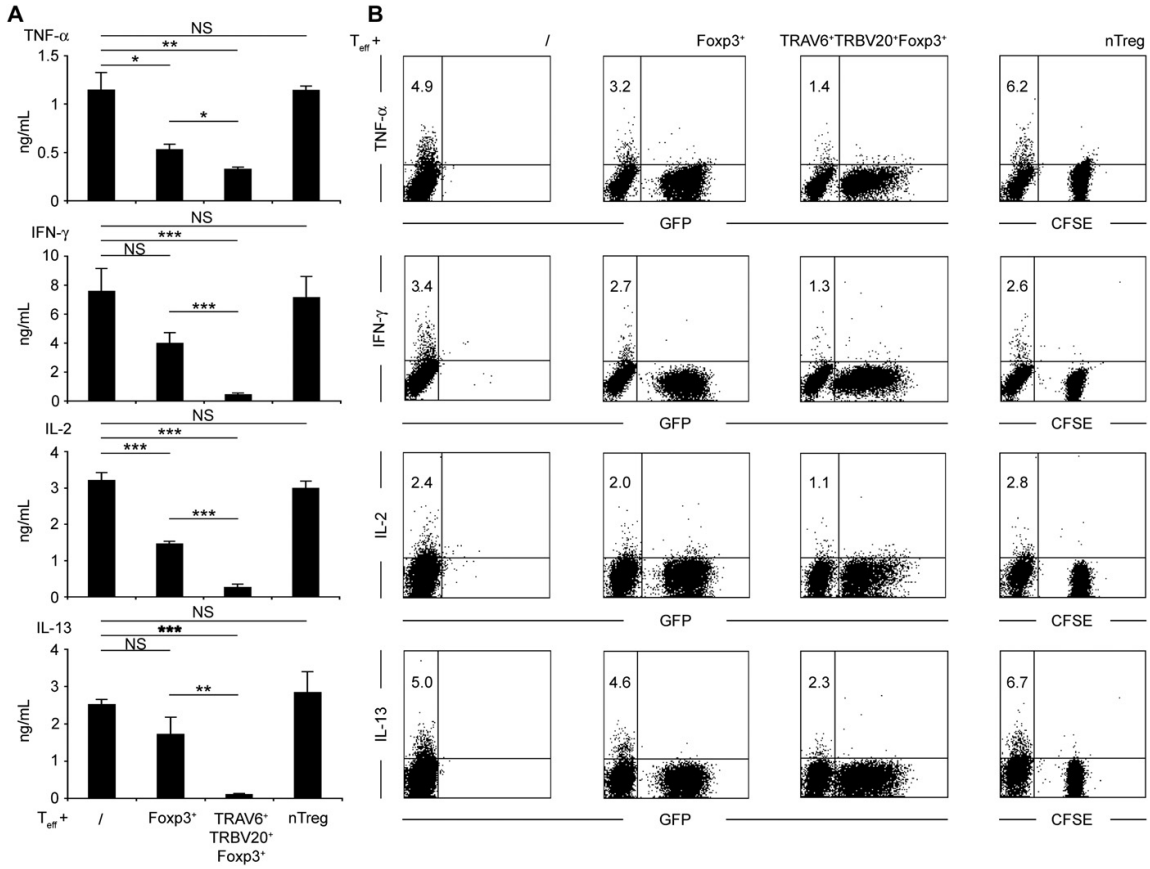

FIG 6. Suppression of cytokine secretion of effector T cells by engineered Treg cells. **A,** Cytokine levels from cell culture supernatants collected after 24 hours (TNF-α, IL-2) or 48 hours (IFN-γ, IL-13). **B,** Intracellular staining of indicated cytokines. *Numbers* indicate percentages of cytokine positive cells among GFP^{neg} (effector) cells. One representative experiment of several is shown. **P* < .05; ***P* < .01; ****P* < .001. *CFSE,* Carboxyfluorescein succinimidyl ester.

Will such an epitope/monospecific therapeutic tool benefit polysensitized individuals with multiple allergies?

Larché and colleagues[46] have demonstrated that tolerization against one epitope of an allergen also induces tolerance to adjacent epitopes, a phenomenon termed "linked suppression." Whether a similar mechanism might also be induced by epitope/monospecific transgenic Treg cells remains to be determined. In favor of this proposal, in a collagen-induced arthritis model, a B47 TCR transgenic Treg cell recognizing a noncollagen autoantigen induced remission of the collagen-induced disease.[47]

Although allergen-specific for the most part, the regulatory potential of *FOXP3*-transgenic T cells was not entirely dependent on TCR-mediated activation. This might be a result of bystander effects of activated effector T cells secreting IL-2 and/or providing cellular contact and thus supporting initial activation of colocalized Treg cells.[48] Both possible mechanisms will be the focus of future studies. Transgenic Treg cells directly modulated cytokine production, which may be the main mechanism of suppression rather than cytokine consumption.[45] Like nTreg cells, *FOXP3*-transgenic T cells are responsive to IL-2 (Fig 4), which should facilitate expansion and longevity *in vivo*. The observation that the transgenic Treg cells described here are suppressive in the absence of changes in significant secretion of IL-10 or TGF-β (not shown) supports the view that secretion of "regulatory" cytokines represents a tissue-specific auxiliary mechanism rather than a critically required core feature of Treg cells.[49]

In conclusion, given the well documented relation between impaired Treg cell function and allergy[11] and the fact that Treg cells play an important role in the efficacy of specific immunotherapy,[5-10] our approach could add a novel tool to the still poorly endowed therapeutic armamentarium for treatment of allergies. Although our studies suggest that nTreg cells and *FOXP3*-transgenic Treg cells are functionally equal, we cannot exclude certain differences in fine specificity and/or transcriptional signature as has been suggested.[50] This aspect will be the focus of detailed future studies. The human origin of the introduced functional transgenes should minimize their immunogenicity and thus in addition to their IL-2 responsiveness allow longevity of customized Treg cells on transfer *in vivo*. The approach presented here could help pave the way for novel therapeutic strategies to abrogate

undesired immune responses in the setting of allergy, autoimmunity, and transplant rejection.

We are in debt to Ms Margarethe Merio for perfect technical assistance with cytokine determinations, to Mr Dieter Printz for invaluable support with fluorescence-activated cell sorting and multicolor fluorescence-activated cell sorting analysis, and to Ms Sophie Frantal for excellent supervision of statistical analyses. We express our gratitude to Dr Brian Seed for critically reading and commenting on the article.

Clinical implications: Transgenic expression of allergen-specific TCRs and FOXP3 generates customized allergen-specific Treg cells, which may help to develop adoptive immunotherapy protocols for allergic diseases.

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Supplement

TABLE E1. List of primers

Primer	Sequence*	RE site
FOXP3 for	5'- CGCGGGAAGCTTGCCACCATGCCCACCCCCAGGCC -3'	*Hind* III
FOXP3 rev	5'- CGCGGGGCGGCCGCTTTAGGGGCCAGGTGTAGGG -3'	*Not* I
FOXP3 E1 rev	5'- CGTGGGCATCCACCGTTGACAGCTGCAGCTGCGATGG -3'	
FOXP3 E3 for	5'- CCATCGCAGCTGCAGCTGTCAACGGTGGATGCCCACG -3'	
TRAV6 for	5'- CGCGGGAAGCTTGCCACCATGGAGTCATTCCTGGGAGGTGT -3'	*Hind* III
TRBV rev	5'- CCCGCGGCGGCCGCTTTAGAAATCCTTTCTCTTGACCATGGC -3'	*Not* I
P2A-TRAV6 rev	5'- GTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGATC CGCTGGACCACAGCCGCAGC -3'	
P2A-TRBV20 for	5'- GCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAG AAAACCCCGGTCCTATGCTGCTGCTTCTGCTGCTTCTG -3'	
T2A-TCRBV rev	5'- GTCACCGCATGTTAGCAGACTTCCTCTGCCCTCTCCGGATCCGAA ATCCTTTCTCTTGACCATGGCCAT -3'	
T2A-FOXP3 for	5'- GAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAA TCCTGGCCCAATGCCCAACCCCAGGCCTG -3'	

For, Forward; *Rev,* reverse.
*The underlined regions indicate the restriction enzyme *(RE)* sites.

TABLE E2. List of mAbs

Specificity	Clone name	Species	Conjugated to	Source
CD3	OKT3	Mouse	eFluor 450	eBioscience, San Diego, Calif
CD4	S3.5	Mouse	Pacific Orange	Invitrogen, Camarillo, Calif
CD25	BC96	Mouse	APC-eFluor 780	eBioscience, San Diego, Calif
CD39	eBioA1	Mouse	PE-Cy7	eBioscience, San Diego, Calif
CD127	eBioRDR5	Mouse	PE-Cy5	eBioscience, San Diego, Calif
CD152	14D3	Mouse	PE	eBioscience, San Diego, Calif
GITR	eBioAITR	Mouse	APC	eBioscience, San Diego, Calif
TRBV2	MBP2D5	Mouse	PE	Immunotech, Marseille, France
TNF-α	6401.111	Mouse	PE	BD Biosciences, San Jose, Calif
IFN-γ	B27	Mouse	PerCP	Invitrogen, Camarillo, Calif
IL-2	MQ1-17H12	Mouse	APC	eBioscience, San Diego, Calif
IL-13	JES10-5A2	Mouse	APC	BioLegend, San Diego, Calif
FOXP3	PCH101	Mouse	APC	eBioscience, San Diego, Calif
Control	4H1-A7/VIAP	Mouse	FITC/PE	An der Grub, Kaumberg, Austria
Control	MOPC-21	Mouse	APC	BioLegend, San Diego, Calif

APC, Allophycocyanin; *CD*, cluster of differentiation; *Cy,* cyanine; *FITC,* fluorescein isothiocyanate; *IFN*, interferon; *IL*, interleukin; *PE,* phycoerythrin; *PerCP,* peridinin chlorophyll protein; *TNF*, tumor necrosis factor.

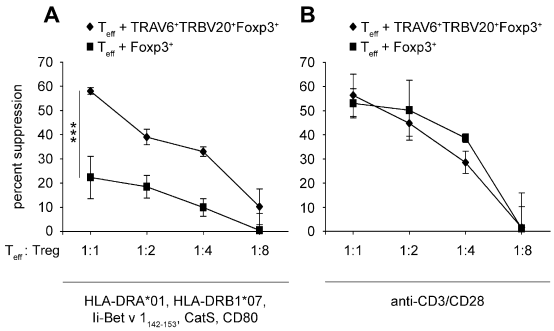

FIG E1. Percent suppression of titrated amounts of indicated transgenic Treg cells cocultured with effector T cells *(T_{eff})* on stimulation with Bet v 1–presenting aAPCs **(A)** or anti–CD3/CD28 coated microbeads **(B)**. *NS,* Not significant. *$P < .05$; ***$P < .001$.

Chapter VI

Creation of a humanized model for respiratory allergy using a human mugwort-specific T-cell receptor and HLA-DR1

Modulation and control of allergen-specific T-cell responses in humanized allergy-mice

Alina Neunkirchner[1,2], Daniela Wojta-Stremayr[1,2], Klaus G. Schmetterer[2,3], Lukas F. Mager[2] Victoria M. Leb-Reichl[1,2], Edward Rosloniec[4], Ronald Naumann[5], Gerhard Dekan[6], Beatrice Jahn-Schmid[7], Barbara Bohle[1,7] and Winfried F. Pickl[1,2*]

[1]Christian Doppler Laboratory for Immunomodulation, Vienna, Austria
[2]Institute of Immunology, Medical University of Vienna, Vienna, Austria
[3]Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria
[4]Veterans Affairs Medical Center, Memphis, TN, USA
[5]Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany
[6]Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria
[7]Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

* Corresponding author. Mailing address: Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, 1090 Borschkegasse 8A, Vienna, Austria. Phone: (+431) 40160 33245. Fax: (+431) 40160 933201. E-mail: winfried.pickl@meduniwien.ac.at.

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Abstract

T-cells play a key role in the development of allergic diseases thus representing promising targets for therapeutic interventions. Mice expressing an I-A^d restricted murine T-cell receptor (TCR) specific for chicken ovalbumin (OVA) are frequently used to mimic allergic diseases induced by aeroallergens *in vivo*. However, implications for human allergic diseases remain questionable, since OVA does not represent a major human-relevant aeroallergen. To circumvent this problem we here created double tg mice expressing a human TCR specific for the major mugwort (*Artemisia vulgaris*) pollen allergen Art v 1 and HLA-DR1. In tg mice ninety percent of peripheral blood $CD4^+$ T-lymphocytes expressed the Art v 1-specific TCR, while $CD14^+$ monocytes and $B220^+$ B-lymphocytes revealed HLA-DR1 expression. Splenocytes of tg mice specifically proliferated upon incubation with the human-relevant immuno-dominant Art v $1_{25\text{-}36}$ peptide or whole Art v 1 protein when compared to control mice. *In vivo*, following allergen-specific sensitization, only tg mice showed enhanced airway hyperreactivity (AHR) as assessed by methacholine or allergen-specific challenge. Three consecutive exposures to nebulized allergen were found to be sufficient for induction of altered airway function, as mirrored by airway and lung inflammation, due to massive peribronchiolar and perivascular infiltration with immune cells. Rapid induction of airway reactivity after a short period of aerosol exposure was not mediated by allergen-specific Ig-levels, since neither Art v 1-specific IgG or IgE were detectable in mouse sera. Analysis of bronchoalveolar lavage (BAL) fluids, whole lung cell preparations and lung histology revealed a drastic increase of eosinophils and neutrophils. Our study provides firm evidence that a human Art v 1-specific TCR in concert with HLA-DRB1*01:01 confers susceptibility to induced disease resembling allergic asthma by presenting the immunodominat Art v $1_{25\text{-}36}$ peptide to T-cells. Humanized allergy models are amenable to the evaluation of human-relevant allergen formulations for vaccination purposes as well as cellular intervention protocols and will contribute to the further understanding of allergic diseases and their cure.

Abbreviations

AHR airway hyperreactivity

BAL broncheoalveolar lavage

HDM house dust mite

HLA human leukocyte antigen

Ig immunoglobulin

mAb monoclonal antibody

Penh enhanced Pause

TCR T-cell receptor

Th T helper cell

WBP whole body plethysmograph

Introduction

Allergic diseases are characterized by an excessive and aberrant immune response to usually innocuous environmental antigens (1). While major effector mechanisms of the disease are triggered by the allergen binding to specific IgE antibodies attached to FcR expressed on tissue resident effector cells, such as mast cells, there is no doubt that T-lymphocytes play a pivotal role in the pathogenesis of allergic diseases (2). In fact, $CD4^+$ T-cells have been shown to provide the initial surge of the so-called switch-factors interleukin (IL)-4 and IL-13, which are a prerequisite for immunoglobulin class switching towards IgE. Moreover, T-cells also seem to be critically involved in late-phase reactions contributing to organ-pathology in the airways, skin and gastrointestinal tract. Although critically involved in the initiation and maintenance phase of allergic diseases, allergen-specific T-cell precursor frequencies in peripheral blood are generally low, precluding them from direct and detailed investigation in diseased individuals. Consequently, and in order to be able to ask detailed experimental questions regarding their primary function and fate in the disease, allergen-specific T-lymphocytes have to become enriched from peripheral blood by repetitive allergen-specific stimulation *in vitro*, leading to the establishment of T-cell lines and clones (3-5). While having been invaluable for *e*.g. the elucidation of major allergen-specific T-cell epitopes, antigenically-enriched T-cell populations might, however, represent a skewed cellular phenotype due to prolonged periods of stimulation within *ex vivo* cell culture milieus. Thus, allergen-specific T-cell lines and clones might not necessarily represent the 'true' phenotype when it comes to studies interrogating allergen-specific Th cell polarization, plasticity and regulation.

Because of these limitations a number of mouse models have been established and used in order to investigate distinct aspects of allergic diseases in more detail *in vivo*. Along those lines recombinant allergens (6, 7), model antigens (8, 9) and murine T-cell antigen receptors (TCR) directed against *bona fide* allergens or model allergens and expressed as transgenes in murine T-cells have been applied in the past. Among these frequently used tools DO11.10 TCR transgenic, ovalbumin (OVA)-specific mice stand out (8-10). DO11.10 TCR tg mice express a murine alpha/beta TCR (11), which is specific for chicken ovalbumin ($OVA_{323-339}$) in the context of murine MHC class II ($I\text{-}A^d$) (10). Although frequently used to study airway inflammation in mice (12) OVA represents a negligible cause for asthma induction in humans (13). Moreover, harsh prime-boost regimen are required to induce an allergic phenotype in these mice. Furthermore, a murine TCR specific for the

immunodominant T-cell epitope of the indoor inhalant allergen of house dust mite (*Dermatophagoides pteronyssinus*, Der p 1) has been established recently (14). Two intratracheal instillations with Der p 1 at a 5-day interval without prior systemic sensitization were sufficient to induce symptoms of allergic asthma in these TCR tg mice on the C57BL/6 background (14). Moreover, in another attempt to humanize currently available mouse models for allergic diseases *Tournoy et al*. reconstituted SCID mice with PBMC from non-allergic individuals. Systemic sensitization with Der p 1 plus alum followed by aerosol exposure to house dust mite (HDM) allergen induced airway hyperresponsiveness of these mice, which was dependent on the Th2-associated cytokines IL-4 and IL-5 (15).

The clear advantage of a fully humanized model for allergy research would be that it could offer the unique chance of moving directly to investigations of the equivalent human reagents and to analyze the relevant T-cell dependent (and independent) pathways, which drive allergic diseases. Humanized disease models, in which the immunological synapse between TCR and MHC molecules has been rebuilt with the human-relevant receptors, have been developed for a small number of T-cell-driven pathologies in the past, among them are tg mice for multiple sclerosis (MS) using HLA-DR2 and a human TCR (16). In these murine models both human relevant TCR and the corresponding human MHC molecules are overexpressed in the form of transgenes on T-cells and antigen-presenting cells, respectively (16-18). However, a humanized allergy mouse model based on specific alpha/beta TCR specific for a human-relevant respiratory allergen presented in the context of a human restriction element was still missing. Consequently, we have selected mugwort allergy driven by the major mugwort pollen allergen from *Artemisia vulgaris,* Art v 1, as an important model model disease since it offers all key features of an ideal respiratory allergen. Mugwort is found in the European temperate climate zone, as well as throughout North America and parts of Asia (19) and is one of the main causes of hay fever in late summer and autumn (20). Moreover, sensitization to mugwort nearly exclusively occurs to Art v 1, the major mugwort pollen allergen (95% of affected allergic patients) and the presentation of its immunodominant T-cell epitope, Art v $1_{25\text{-}36,}$ is highly restricted to HLA-DRB1*01:01 (21, 22).

In the present study we describe the generation of allergen-specific TCR/HLA-DRB1*01:01 double transgenic mice. We provide phenotypic data describing tissue specific expression of the transgenes and provide functional evidence for the allergen-specificity of the transgenes by applying cellular activation and proliferation assays along with cytokine production/secretion experiments. Moreover, the mice have been used to investigate specific

responsiveness towards the airborne allergen, including lung function, organ pathology as well as local and systemic immune reactions.

Materials and Methods

Recombinant allergens and peptides

Purified recombinant Art v 1.0101 allergen was purchased from Biomay AG (Vienna, Austria). Allergenic peptides from mugwort pollen (Art v 1_{25-36}) and birch pollen (Bet v $1_{142-156}$) or antigenic peptides from Influenza Hemagglutinin ($HA_{306-318}$) were obtained from thinkpeptides (ProImmune, Oxford, UK).

Animals

C57BL/6-J and transgenic mice were kept in a conventional animal facility at the Institute of Immunology (Medical University of Vienna, Vienna) and were used for experiments at 6-10 weeks of age according to animal ethics regulations and approved by the Ethic committee of the Medical University of Vienna. Homozygous B10.M-$DR1^{d1AB1-Ea}$ mice (23) were obtained from the Veterans Affairs Hospital, Memphis, TN. Sentinel mice were screened for mouse pathogenic viruses, bacteria and parasites according to FELASA 2002 recommendations.

Generation of TCR transgenic mice

To generate TCR tg mice, rearranged V(D)J regions of the TCR from the human Art v 1-specific and HLA-DRB1*01:01 restricted TH0 cell clone SSR20, as described previously (22, 24), were cloned into the TCR cassette vectors pTαcass and pTβcass (25) (kindly provided by Dr. Diane Mathis, Division of Immunology, Department of Microbiology & Immunology, Harvard Medical School, Boston, MA), which contain mouse TCR α and β constant sequences and mouse promotor/enhancer elements. Variable TCR regions were amplified by PCR from genomic DNA of the original T-cell clone SSR20 using the oligonucleotide primers 5'-CGC GGG CCC GGG AGG TCT TCT GTG ATT TCA ATA AGG A-3' (sense) and 5'-CCC GCG GCG GCC GCC CCC ATG AGG ACT GCA TTT TG-3' (antisense) for the alpha-chain and 5'-CGC GGG CTC GAG GTG CCT TTG CCC TGC CTG T-3' (sense) 5'-CCC GCG CCG CGG ACA CCC AGC TCC TCC AGC-3' (antisense) for the beta-chain. Both PCR fragments (size: 653 bp, 809 bp, respectively) were digested with appropriate restriction enzymes (α-chain: *Xma I*/*Not I*; β-chain: *Xho I*/*Sac II*) and cloned into the puc 19 derived vector pBluescript SK^+ (Stratagene, Heidelberg, Germany). After sequence verification, the fragments were subcloned into the correspondingly digested cassette vectors. The constructs were tested for expression *in vitro* upon Ca_2PO_4-transfection of HEK-293 cells along with the murine CD3 complex. Subsequently pTα cass (*Sal* I) and pTβ cass (*Kpn* I) vectors were linearized to remove prokaryotic vector sequences followed by microinjection

into pronuclei of fertilized eggs of C57BL/6-J mice. Five founder mice were obtained, two of which revealed germ-line transmission of transgenes. Offsprings were analyzed by PCR of tail biopsy DNAs using TCR α or TCR β clonotype-specific PCR and applying the above described primer devoid of restriction sites and 5'-clamp sequences. Mice from the positive founder line A003, showing the highest TCR expression levels on $CD4^+$ T-cells, were crossed with B10.M-$DR1^{d1AB1\text{-}Ea}$ and backcrossed onto the C57BL/6-J background for ≥ 10 generations before experimental use and were bred towards homozygosity. Corresponding mouse lines were termed *TCR/DR1* for double tg mice, *TCR* for TCR single tg mice, *DR1* for HLA-DRB1*0101 single tg mice and *wt* for wild type C57BL/6-J mice.

Whole blood, tissue collection and treatment

Whole blood samples were collected by incision of the tail vein and anticoagulation with 10 U/ml heparin. Spleens and thymi of animals were obtained upon anatomical dissection of sacrificed animals according to standard procedures. Briefly, tissues were homogenized in medium by mincing organs cut into 5 mm pieces with the pestle of a 10 ml syringe through a sterile metal mesh followed by sieving through a 70 µm cell strainer to obtain single cell suspensions, which were washed subsequently in IMDM plus 10 % FCS, 2 mM L-glutamine, essential amino acids, 0.1 mM 2-mercaptoethanol, 1mM sodium pyruvate and 25 mM HEPES (pH7.3) at 200 g for 10 minutes. Erythrocytes were removed by incubation of cell suspension in standard ammonium chloride lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM EDTA at pH 7.40) at room temperature for 5 minutes. Subsequently, cells were washed twice in IMDM at 200 g for 10 minutes and adjusted to a concentration of 1 x 10^7/ml in medium. Lung homogenates were prepared according to standard procedures. Briefly, lungs were excised, chopped and incubated with digest-solution containing 1.8 µg/ml collagenase and 40 µg/ml DNase (Sigma Aldrich) at 37°C for 60 min, then cells were disaggregated and filtered through a 70 µm nylon cell strainer (BD). The filtrate was then centrifuged at 500 x g for 5 min at 4°C. The cell pellet was resuspended in amonium-chloride lysis buffer, incubated at room temperature for 5 minutes, washed once in 1 x IMDM before subjection to flow cytometric analyses.

Flow cytometric analyses of leukocyte populations in peripheral blood, thymus and lymphnodes

20 µl aliquots of whole blood were incubated with the indicated mAb-combinations (20 µg/ml) at room temperature for 20 minutes. After addition of 100 µl lysis solution (An der

Grub, Kaumberg, Austria) and incubation for 10 minutes at room temperature, 5 ml of aqua bidest were added, incubated for 5 minutes followed by centrifugation at 500 g for 5 minutes. Subsequently, supernatants were discarded and the samples were analyzed by flow cytometry. Splenocytes and thymocytes were harvested as described above, re-suspended in 1 x 10^6 cells/ 50 µl of phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and incubated with 20 µl of the indicated mAb-combinations on ice for 30 minutes. Afterwards, cells were washed once with PBS plus 0.5% BSA and submitted to flow cytometric analyses. Data were obtained exclusively from viable cells by using appropriate forward and side scatter gating. Analysis was performed on a LSRII flow cytometer (Becton-Dickinson, Palo Alto, CA) followed by data analysis using the FlowJo v 9.3.3 software (Tree Star Inc, Ashland, OR). For phenotypic analysis of leukocytes, the following monoclonal antibodies listed in **Supplementary Table S1** were used.

Proliferation assays

Single cell suspensions of splenocytes (2 x 10^5/well) were incubated in 96-well flat bottom plates with Art v 1_{25-36} peptide, Art v 1 protein or Bet v $1_{142-156}$ peptide used as an independent specificity control. Concentrations of Art v 1 protein and peptide stimulation ranged from 0.125 µmol/L to 0.001 µmol/L and 1.5µmol/L to 0.01 µmol/L, respectively. Cells were pulsed after 72 hours with methyl-[^{3}H]thymidine (1 µCi/well), for 18 hours and T-cell proliferation was quantified on a Betaplate Counter (Packard, Meriden, CT). Similarly, splenocytes of mice were labeled with carboxyfluorescein succinimidyl ester (Molecular Probes, 1.5 µmol/L) or cell proliferation dye-V450 (eBioscience, 5 µmol/L) stimulated with Art v 1_{25-36} peptide, Bet v $1_{142-156}$ peptide, PMA/Ionomycin or medium alone and analyzed by flow cytometry after 72-96 hours using CD3, CD4, CD8 and TRBV18 mAB. Division index of $CD3^+CD4^+$ T-cells was calculated according to (26) using the formula

n= [log ((MFI of unstimulated population)/ (MFI of stimulated population))] / log [2], which estimates the mean number of cell divisions between the unstimulated and the simulated cultures.

Cytokine measurements

Supernatants of splenocytes incubated with the indicated stimuli were harvested at various time points after initiation of culture (24 h, 48 h, 72 h and 96 h) and subjected to multiplex cytokine analyses. Determination of secreted cytokines was performed using FlowCytomix Multiple Analyte Detection System (eBioscience) a bead based multiplex immune assay for

flow cytometry according to the manufacturer's recommendations. Beads were analyzed on a FACScalibur cytometer (Becton Dickinson). Alternatively, cytokine measurements were performed by multiplex analysis using the Luminex system (Luminex 100IS, Biomedica, Vienna, Austria).

Pollen extract preparation

Artemisia vulgaris pollen (Allergon AB, Engelholm, Sweden) was used for the preparation of mugwort-pollen extract. Briefly, ten grams of the mugwort-pollen were extracted in 100 ml of 1 x PBS by stirring at 4°C overnight. After centrifugation at 52000 g for 60 minutes at 4°C, the supernatant was filtered and subsequently dialyzed (Spectra/Por Dialysis Membrane, MWCO: 6-8000, Spectrum Laboratories, Rancho Dominues, CA) against 1 x PBS for 48 hours. The total protein concentration of the dialysate was determined by using standard procedures (BCA (bicinchoninic acid) protein Kit, Pierce, Rockford, IL). LPS content of the mugwort-pollen extract was determined according to the LAL reaction (Lonza, Walkersville, MD). The extract was lyophilized and aliquots were stored at –80°C.

Allergen-sensitization and challenge for the induction of Art v 1-specfic Ig-levels

For the induction of Art v 1-specific IgG1, IgG2a and IgE levels three different protocols were compared. *Protocol A*: Four-five groups (in some experiments sera were compared to sera of immunized BALB/c mice) of 6-10 weeks old mice (n=4) were sensitized i.p with 50 µg of mugwort-pollen extract (LPS contents ≤ 0,024 U/mg) (Allergon, Sweden) adsorbed to aluminium hydroxide ($Al(OH)_3$, Alu-Gel-S, SERVA Electrophoresis, Heidelberg, Germany) and i.p. challenged two times with the same amounts within 14 day intervals up to 8 weeks. *Protocol B:* Mice were sensitized by i.n. administration of 30 µl of a 1.5 % mugwort-pollen extract solution once every two weeks for up to 10 weeks. Blood samples were collected prior to the first immunization and 10 days after each immunization by tail vein incision. *Protocol C:* Mice were challenged via the airways with nebulized mugwort-pollen extract (1 %) for 16 min on three consecutive days. Blood samples were taken one day after the last exposure and serum stored for each mouse individually at -20 °C.

Serum collection and ELISA for immunoglobulin determinations

ELISA was performed to determine Art v 1-specific Ig in the sera of immunized mice. ELISA plates (Costar) were coated over night with either 50 µg/ml mugwort-pollen extract or alternatively with 2 µg/ml of recombinant Art v 1 protein in carbonate buffer (pH 9.6). Plates

were washed with PBS plus 0.05% Tween (PT) two times and blocked with PT plus 1 % BSA at room temperature for 2 hours. Sera of immunized mice were diluted in PT plus 0.5 % BSA 1:10 for IgE, 1:500 for IgG2a and 1:2000 for IgG1. Plates were incubated with 50 µl of diluted sera at 4°C over night and washed five times with PT. Bound mouse Ig were detected by addition of 100 µl of monoclonal rat-anti-mouse Ig-subclass specific antibodies (BD Pharmingen) diluted 1:500 in PT/0.5 % BSA at 37°C for 3 hours. After 5 washes with PT 100 µl of 1:2000 diluted goat-anti-rat horseradish peroxidase coupled antibody (GE Healthcare) was added and incubated at 37°C for 30 minutes and at 4°C for 30 minutes. Again plates were washed 5 times with PT and incubated with 100 µl ABTS substrate (Sigma-Aldrich, St. Louis, Mo). Plates were developed at room temperature in the dark and extinction (optical density 405 nm) was measured with an ELISA reader (Softmax Pro) after one hour.

Mononuclear cell culture for Th1- and Th2-polarization experiments

Sorted naive $CD4^+CD62L^+$ T-cells (Macs beads, Miltenyi, Bergisch Gladbach, Germany, purity of T-cells > 98%) from splenocytes of TCR/DR1 mice were polarized towards the Th1 or Th2 subset according to (27) using 1.5 x 10^6 irradiated (60 Gy) splenocytes from DR1 mice, that had been pulsed with Art v $1_{25\text{-}36}$ peptide (Proimmune) or $1x10^5$ anti-CD3/CD28 coated microbeads (Dynabeads Mouse T-Activator CD3/CD28, Invitrogen). For Th1-differentiation, cells (5 x 10^5 cells/ 0.5 ml) were stimulated in the presence of 10 ng/ml recombinant mouse IL-12 (Peprotech) and 10 µg/ml purified anti-IL-4 antibody (11B11, eBiosience). For Th2-differentiation, cells (5 x 10^5 cells/ 0.5 ml) were stimulated in the presence of recombinant mouse IL-4 (1000 U/ml, Peprotech) and 10 µg/ml purified anti-IFN-γ antibody (RA-6A2, eBiosience) and 6 µg/ml anti-IL-12/IL-23 p40 (C17.8, eBiosience). After 24 hours both cultures were supplemented with 20 U/ml mouse rIL-2 (Peprotech). On the fourth day after antigen-specific or polyclonal stimulation both T-cell subsets were expanded by the addition of fresh culture medium containing the above described cytokine concentrations in the absence of TCR stimulation. After 7 days of culture T-cells were restimulated as described before by omitting anti-IL-12 for the Th2-cell culture conditions. 48-96 hours after restimulation, intracellular cytokine production of cells was assessed after pretreatment for 6 hours with Golgi-Stop (1:1500 BD) in the presence of polyclonal activation with PMA/Ionomycin using the Fix and Perm Kit (An der Grub, Kaumberg, Austria). Alternatively, splenocytes from TCR/DR1 mice were polarized towards the Th1 subset using aAPC co-expressing Ii::Art v $1_{25\text{-}36}$ and membrane-bound version of murine IL-12 (28).

Challenge protocols for the induction of airway hyperreactivity (AHR)

Four groups of mice (wt, DR1 or TCR and TCR/DR1 mice) were immunized via different routes and analyzed for symptoms of allergic asthma.

Protocol #1: Mice were immunized intraperitoneally with 50 µg of allergen extract plus alum on days 0 and 7. Isofluran-anesthetized mice were challenged on day 15 intranasally with 30 µl of a 1.5% solution of mugwort-pollen extract. Two days after the allergen-specific challenge airway hyperreactivity responses were assessed. *Protocol #2:* Mice were exposed exclusively to aersolized allergen (3-8x) by nebulizing 1 % mugwort-pollen extract solution for 16 minutes using whole body plethysmography chambers and the aerosol delivery system (Buxco, Winchester, UK).

Pulmonary physiology – In vivo determination of allergen-induced changes in lung function

Airway hyperresponsiveness (AHR) was assessed by measurement of Penh by unrestrained whole-body plethysmography (Buxco) after induction of allergic asthma. Animals were challenged with increasing doses of methacholine (in 1xPBS) according to (29) or 1% mugwort-pollen extract solution.

Bronchoalveolar lavage (BAL) fluid

Two days after the last i.n. challenge or one day after the last aerosol challenge BAL was performed with 2 x 1 mL of Ca^{2+} and Mg^{2+} free, ice-cold PBS and samples were centrifuged for 5 minutes at 500 g. Cell-free supernatants were stored for future cytokine analyses at -80°C. Total leukocyte cell numbers were determined and cytospin preparations of BAL cells were stained using a modified Wright`s stain. Leukocytes subsets were determined by morphological criteria and routinely 200 cells were counted per cytospin slide using a Nikon Eclipse E600 light microscope with a x60 objective. In parallel, BAL cells were stained with mAb panel as described for "whole blood staining" (**Supplementary Fig. S1**). To prevent non-specific binding to Fc receptors 2.4G2 blocking reagent (6 µg/ml, BD) was added to the monoclonal antibody mix. Cellular composition of BAL fluids was determined with a BD Fortessa flow cytometer using FACS DIVA (BD) and FlowJo Software (Treestar, Costa, Mesa, CA).

Footpad swelling assays and determination of delayed type hypersensitivity

Animals were anesthetized by 1 mg Ketanest® (Ketamin hydrochloride, Bayer) und 0.2 mg Rompun® (Xylazine hydrochloride, Bayer). Subsequently, 25 µl of allergen extract preparation (containing 375 µg whole protein content) were injected by using a 1 ml syringe equipped with a hypodermic needle (26G, 0.45 x 10 mm, BD) intradermally into the hinder right footpad, while the contralateral hinder food pad was challenged with 1x PBS alone or left untreated. After 24, 48, 72 and 96 hours the appearances of the injection sites were inspected and documented. Thickness of the footpads before and after immunization was measured using a digital thickness gauge and Δ-levels of footpad swelling were determined as: Footpad swelling (mm) = Footpad thickness after allergen-provocation - Footpad thickness before allergen-provocation.

Upon scarification of animals, reactive footpads and skin injection sites were removed, fixed in 10 % formaldehyde, decalcified in 5 % formic acid, and embedded in paraffin. Serial sections (10 µm) of the footpads and skin explants were stained with hematoxylin and eosin.

Lung Histology

Lungs were fixed initially with 10 % formaldehyde (Rotifix, Lactan) for 6 hours and kept in 4 % formaldehyde (Rotifix) for 1-2 days before paraffin-embedding. Tissue sections were stained with hematoxylin and eosin (H&E) dye or periodic acid–Schiff (PAS) for airway mucus production by using standard protocols.

Statistical data analyses

For multiple group comparisons, ANOVA was performed, followed by Bonferroni correction (SPSS; IBM, Chicago, Ill). Two-group comparisons were performed by using the Students *t*-test or the Mann-Whitney U test. Data represent means ± SD/SEM. Statistically significant values were denoted (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$).

Results

Generation of human Art v 1-specific TCR/HLA-DRB1*01:01-restricted double transgenic mice

In previous experiments we have cloned and functionally characterized a human allergen-specific T-cell antigen receptor (TCR), which is specific for the major mugwort pollen allergen, Art v 1, in the context of HLA-DRB1*01:01 (24). The rearranged TCR chains, which were RT-PCR-amplified from the Art v 1_{25-36}-specific T-cell clone SSR20, were formed by the rearrangement of TRAV17-TRAJ45 and TRBV18-TRBD1-TRBJ2-7 segments, respectively. Functional investigations upon retroviral transfer of the Art v 1-specific TCR into Jurkat and PB T-cells revealed that the TCR retained both its original fine specificity for the Art v 1_{25-36} peptide as well as its typical HLA-DRB1*01:01 restriction (24).

We here generated Art v 1-specific TCR/HLA-DRB1*01:01 double transgenic mice by expressing the genes *TRAV17-TRAJ45* and *TRBV18-TRBD1-TRBJ2-7* under the control of mouse T-cell antigen receptor promoter and enhancer sequences guaranteeing tissue-specific expression (25). To ensure proper interaction with the mouse CD3 complex and to facilitate interactions with mouse CD4 co-receptors, the variable domains of the Art v 1-specific TCR were fused to murine TCR constant regions within the vectors pTαcass and pTβcass, respectively (**Supplementary Fig. S1**). Proper expression of the chimeric human::mouse TCR constructs in comparison to the non-modified full-length human TCR constructs was confirmed *in vitro* by transient transfection of HEK-293 cells along with expression constructs encoding murine CD3 γ, δ, ε and ζ followed by staining with the αβ TCR-specific mAb BA62.6. Thereafter, Art v 1-specific TCR transgenic mice were generated by injecting a mixture of both α- and β-chain-encoding plasmids into the pronuclei of fertilized eggs of C57BL/6-J x C57BL/6-J matings. Of the five founder mice established, two showed clear-cut germ-line transmission of the transgene, of which line A003 showed the highest transgene expression levels on $CD4^+$ T-cells. Accordingly, Art v 1-specific TCR tg founder mice of the A003 line were crossbred with HLA-DR1/I-E tg mice, which have been established previously (23).

Characterization of human Art v 1-specific TCR/HLA-DRB1*01:01-restricted double transgenic mice

Detailed immunophenotyping of peripheral blood leukocytes, thymocytes and splenocytes of mouse strains was performed in wt, DR1, TCR and TCR/DR1 mice. Absolute peripheral blood $CD3^+$ T-lymphocyte numbers (**Table 1**) were comparable in wt (3.0±0.0 x 10^3/µl), DR1 (3.1±0.0 x 10^3/µl) and TCR/DR1 mice (3.1±0.0 x 10^3/µl), while TCR mice presented with significantly diminished T-cell numbers (1.7±0.1 x 10^3/µl). The fraction of TRBV18 tg T-cells among all $CD3^+$ T-cells in TCR/DR1 mice amounted 66.6%, while wt and DR1 mice had negligible amounts of $TRBV18^+$ T-cells (<1 %) (**Fig. 1**). Interestingly, the allergen-specific TCR was also selected in TCR mice, however, with much lower efficiency causing overall T-lymphopenia. Moreover, TCR/DR1 mice had a markedly reduced $CD8^+$ T-cell fraction (0.9±0.5%), reaching only about 1/10 of the level usually found in wt animals (9.7±1.4%). The obvious problem in proper T-cell selection was also evident by looking at overall thymocyte numbers, which were significantly decreased in TCR mice (57.0±0.9 x 10^6/organ) when compared to wt mice (120.2±1.6 x 10^6 cells/organ). Of note, also the TCR/DR1 mice presented with reduced thymic cellularity (77.4±1.1 x 10^6 cells/organ) indicating some degree of selection disadvantage.

The size of the splenic B-lymphocyte compartments of TCR/DR1 mice (34.6±3.0 x 10^6cells/organ) was almost identical when compared to that of wt mice (37.4±2.0 x 10^6cells/organ), while DR1 mice displayed reduced B-cell numbers (25.0±0.9 x 10^6cells/organ). The fraction of $CD45RB^+$ B-lymphocytes expressing the tg DR1 molecule ranged between 75.2% in DR1 mice and 80.3% in TCR/DR1 mice as detected with the HLA-DR-specific mAb L243 (**Fig. 1**). Similarly, $CD14^+$ monocytes in DR1 and TCR/DR1 mice co-expressed to a large extent HLA-DR1, while no human HLA class II molecules were detected on wt or TCR tg monocytes.

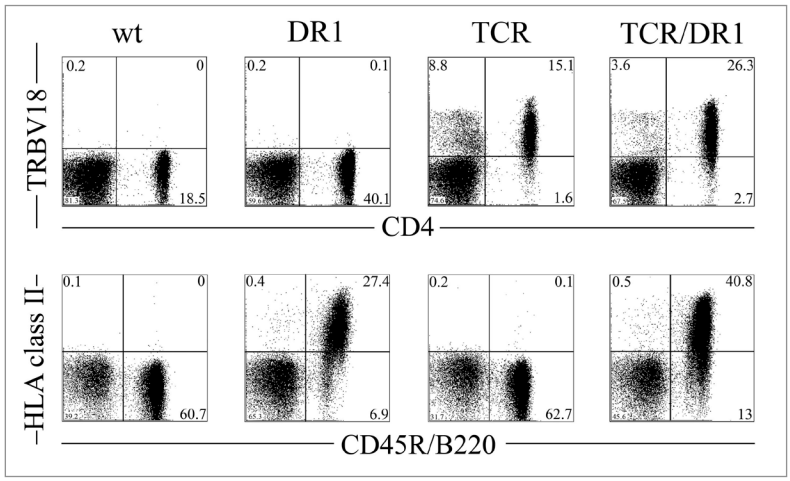

Figure 1. Expression of chimeric Art v 1-specific TCR and HLA-DR1 by PB lymphocytes from mice. Multi-color flow cytometry was performed to analyze the expression of the tg TCR β-chain TRBV18 using mAb Ba62.6 (PE-labeled) and of HLA-DR1 using mAb L243 (PerCP-labeled). Whole PB leukocytes were stained and analyzed by flow cytometry. Lymphocytes were identified by typical FSC and SSC characteristics and CD3- or CD45/B220-staining. T- and B-lymphocyte lineages from wt, DR1, TCR and TCR/DR1 mice are shown in two parameter dot plots. Markers were set according to the staining intensity of isotype matched negative control mAb, numbers indicate percent of cells within respective quadrant. Dot plots are based on 5 x 10^4 cells analyzed. Data are representative of four independently analyzed mice within each group.

Transgenic T-lymphocytes and HLA-DR1 transgenic antigen-presenting cells are functionally active *in vitro*

The existence of Art v 1-specific HLA-DR1-restricted T-cells was tested *in vitro* by incubating splenocytes obtained from wt, DR1, TCR and TCR/DR1 mice with the immunodominant Art v 1_{25-36} peptide, full length recombinant Art v 1 protein as well as immunodominant Bet v $1_{142-156}$ peptide and medium alone used for negative control purposes. Cellular proliferation and cytokine secretion assays were applied as the read-out systems. Significantly, only splenocytes from TCR/DR1 mice proliferated massively in response to Art v 1_{25-36} peptide and Art v 1 protein but not to Bet v $1_{142-156}$ peptide used as negative control (**Fig. 2A**). In contrast, splenocytes from all groups of mice proliferated upon activation with the TCR independent, polyclonal stimulus delivered by PMA/Ionomycin (**Fig. 2B**). When Art v 1 protein was titrated into splenocyte cultures of TCR/DR1 mice, T-cells, dose-dependently responded with proliferation, reaching a plateau of maximal proliferation (kcpm: 61±7.5) at a peptide dose of 125 nM, with half-maximal responses at 57.5 nM of protein (**Fig. 2C**). When immunodominant Art v 1_{25-36} peptide was titrated into splenocyte cultures proliferation reached a plateau of maximal proliferation (kcpm: 51±8.2) at a peptide dose of 1.5 µM, with a half-maximal response at 0.28 µM of peptide (**Fig. 2D**). In contrast, splenocytes from TCR or DR1 mice or wt mice did neither proliferate in response to Art v 1_{25-36} peptide nor to Art v 1

protein (**Fig. 2A**). In addition, they did not respond to peptide. Thus, while splenocytes from single tg mice remained unstimulated by Art v 1-specific antigens, co-cultured splenocytes of TCR and DR1 mice clearly responded by vigorous proliferation to incubation with Art v $1_{25\text{-}36}$ peptide and recombinant full-length Art v 1 protein, while again Bet v $1_{142\text{-}156}$ peptide failed to do so (data not shown). The antigen-specificity of T-cells derived from TCR mice is indicative for successful selection of the Art v 1-specific TCR on the murine MHC (H-2^b) background.

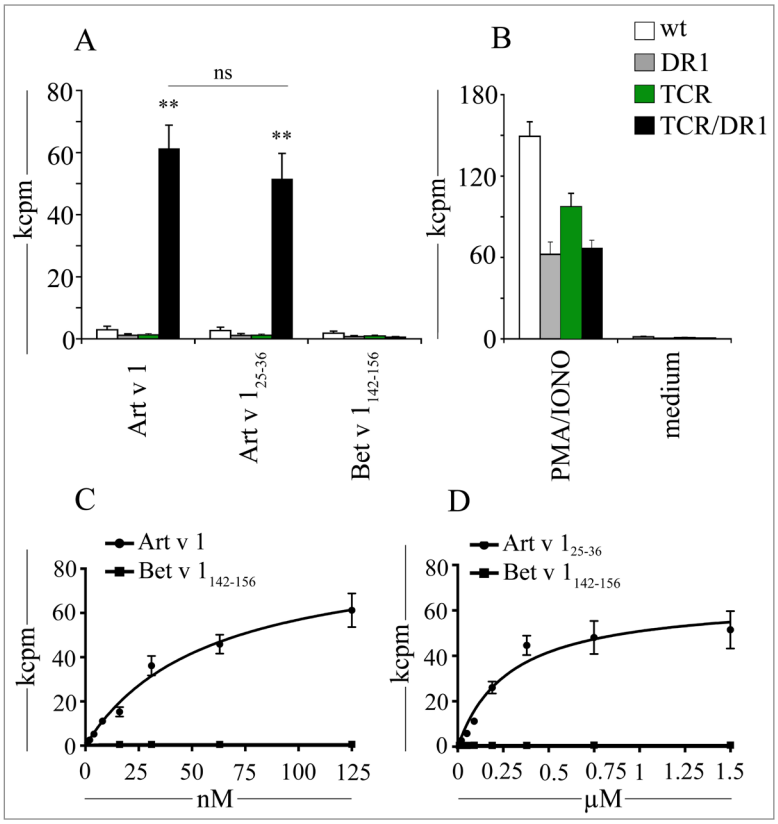

Figure 2. *In vitro*-proliferation of splenocytes stimulated with allergen-specific immunodominant peptide or full-length Art v 1 protein. (A) Splenocytes (2 x 10^5/well) from wt, DR1, TCR and TCR/DR1 mice were co-cultured with Art v 1 protein (3 µM), the Art v $1_{25\text{-}36}$ peptide (10 µM) and for control purposes with equimolar concentrations of the immunodominant peptide of birch pollen (Bet v $1_{142\text{-}156}$) *in vitro*. (B) PMA/Ionomycin stimulation or medium alone served as additional controls, respectively. (C) Dose dependent proliferation of splenocytes from TCR/DR1 mice upon incubation with the indicated concentrations of Art v 1 protein (EC_{50}: 57.5 nM) or (D) Art v $1_{25\text{-}36}$ peptide (EC_{50}: 0.28 µM) in comparison to Bet v $1_{142\text{-}156}$. Cellular proliferation was determined after 4 days by [^{3}H]-thymidine incorporation and is expressed as kilo counts per minute (kcpm). Data show mean values + SEM of triplicate cultures which are representative of four independently performed experiments. ** $p < 0.01$ Art v 1 protein/Art v $1_{25\text{-}36}$ peptide stimulation compared to Bet v $1_{142\text{-}156}$ peptide stimulation of TCR/DR1 splenocytes, ns (not significant, Art v 1 protein compared to Art v $1_{25\text{-}36}$ peptide.

Results obtained in proliferation assays were confirmed by determination of secreted cytokine levels. In fact, Art v $1_{25\text{-}36}$ peptide-induced cellular proliferation of TCR/DR1 splenocytes was

regularly accompanied by the production of high levels (> 500 pg/ml per $2x10^5$ cells /well) of IL-2, intermediate levels (499-200 pg/ml) of IFN-γ, IL-13 and IL-17 and low levels (199-10 pg/ml) of IL-4 and IL-6 (**Fig. 3**) while no indication for TNF-α, IL-5 and IL-10 secretion was observed. In addition, no cytokine secretion was evident upon incubation of splenocytes of TCR/DR1 mice with a control peptide or medium alone (**Fig. 3**). In contrast, the positive control provided by incubation with PMA/Ionomycin induced clear-cut secretion (>10 pg/ml) of IL-2, IFN- γ, IL-10, IL-6, TNF-α, IL-4 and IL-13 (**Fig. 3**).

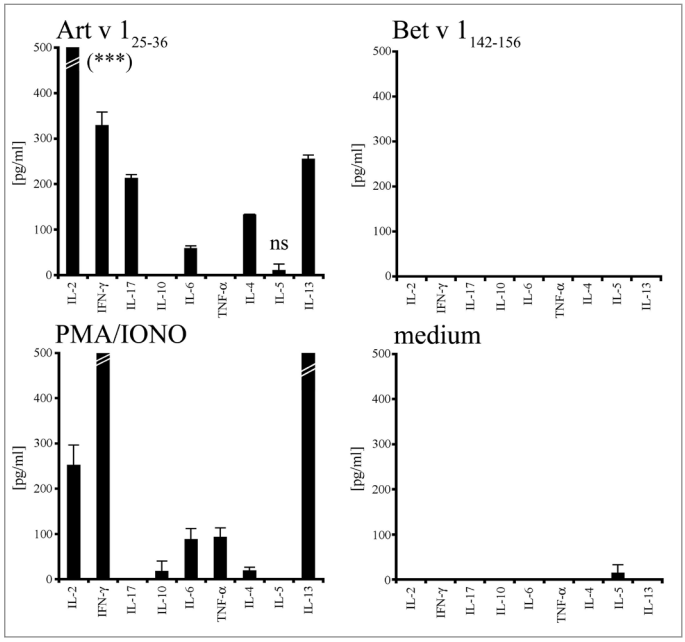

Figure 3. Cytokine secretion pattern of splenocytes derived from TCR/DR1 mice. Splenocytes (2 x 10^5/well) were co-incubated with the immunodominant Art v 1_{25-36} peptide (10 μM), the immunodominant Bet v $1_{142-156}$ peptide (10 μM), medium alone or PMA/Ionomycin applied as polyclonal stimulus. Culture supernatants were harvested after 48 h and analyzed for secreted cytokine levels by using the FlowCytomix system. Data show mean values of triplicate cultures and are representative for one out of three independently performed experiments. *t* test *** $p < 0.001$ Art v 1_{25-36} stimulation compared to Bet v $1_{142-156}$ stimulation for all cytokines except for IL-5 ns (not significant) and IL-10 not determined.

Sole Art v 1-specific activation apparently did not induce a bias towards either of the Th1 or Th2 helper subset. However, Art v 1-specific stimulation of naive $CD4^+CD62L^+$ T-cells in the presence of either Th1- or Th2-priming culture conditions evoked clear-cut polarization towards the different T helper cell subsets. Intracellular cytokine staining showed the induction of Th1-like cells with predominant secretion of IFN-γ (87.6% positive cells) with no production of the Th2-associated cytokines IL-4 and IL-13. In contrast, Th2-polarized cells were identified by their high percentage of $IL\text{-}4^+$ (29.4%) and $IL\text{-}13^+$ (31.9%) T-cells accompanied by low IFN-γ production (8.4%) (**Supplementary Fig. S2**).

Determination of the proliferative potential of T-cells from TCR/DR1 mice on the cellular level

Splenocytes from wt, DR1, TCR and TCR/DR1 mice were isolated, labeled with cell proliferation dye eFluor450 and stimulated with Art v 1_{25-36} peptide, Bet v $1_{142-156}$ peptide, medium alone or PMA/Ionomycin (**Fig. 4**). Art v 1-specific T-cell proliferation (black line) was exclusively detected in the $CD3^+CD4^+$ T-cell fraction of TCR/DR1 mice as shown by the dilution of the cell proliferation dye (**Fig. 4A**). These cells demonstrated an average number of divisions (expressed by the division index (DI)) of three. (**Fig. 4B**). In contrast, no significant proliferation in response to the non-cognate peptide Bet v $1_{142-156}$ (blue line, DI = 0) or in response to medium (grey line) was observed. Moreover, $CD3^+CD4^+$ T-cells from wt, DR1 or TCR mice showed only minimal proliferation upon incubation with Art v 1_{25-36} peptide, Bet v $1_{142-156}$ peptide or medium alone. Importantly, $CD3^+CD4^+$ T-cells from all mouse groups proliferated to a similar degree upon polyclonal activation with PMA/Ionomycin (**Fig.4B**. DI 2.7, 3.4, 3.5, 3.1, respectively) excluding that the differences in Art v 1-induced T-cell proliferation result from unequal distribution of cell viability.

Since it cannot be entirely excluded that the tg TRBV18 chain also pairs with endogenous TRAV-chains, and due to the fact that representative panels of anti-human TRAV-specific binding reagents for exact phenotyping are missing, experiments using cell proliferation dye-labeled splenocytes were also used to enumerate the percentage/fraction of allergen-specific T-cells in TCR/DR1 mice. These experiments revealed that within the splenic pool of $CD3^+$ T-cells of TCR/DR1 mice 67.6% belong to the $CD4^+TRBV18^+$ phenotype and 93.1% of these cells proliferated upon stimulation with the Art v 1_{25-36} peptide, whereas these cells showed only marginal proliferation in response to Bet v $1_{142-156}$ peptide or medium alone (5.2% and 3.4%, respectively). Polyclonal activation of these cells induced 96.4 % of these cells to undergo proliferation within the $CD3^+CD4^+TRBV18^+$ fraction (**Supplementary Fig. S3**). Thus we conclude that T-cells from TCR/DR1 mice show a high degree of antigen specificity since they regularly responded with dilution of the incorporated cell proliferation dye (cellular proliferation) upon incubation with specific but not irrelevant peptide. (**Fig. 4)**.

Moreover, we performed delayed type hypersensitivity experiments as an *in vivo* assay for cell mediated immune responses (**Supplementary Fig. S7)**. Unprimed mice were injected intradermally with mugwort-pollen extract in the right hind foot and footpad swelling was monitored after 72-96 h and measured with a thickness gauge. TCR/DR1 mice showed a clear-cut swelling of the footpad, whereas the control mice did not develop significant swelling.

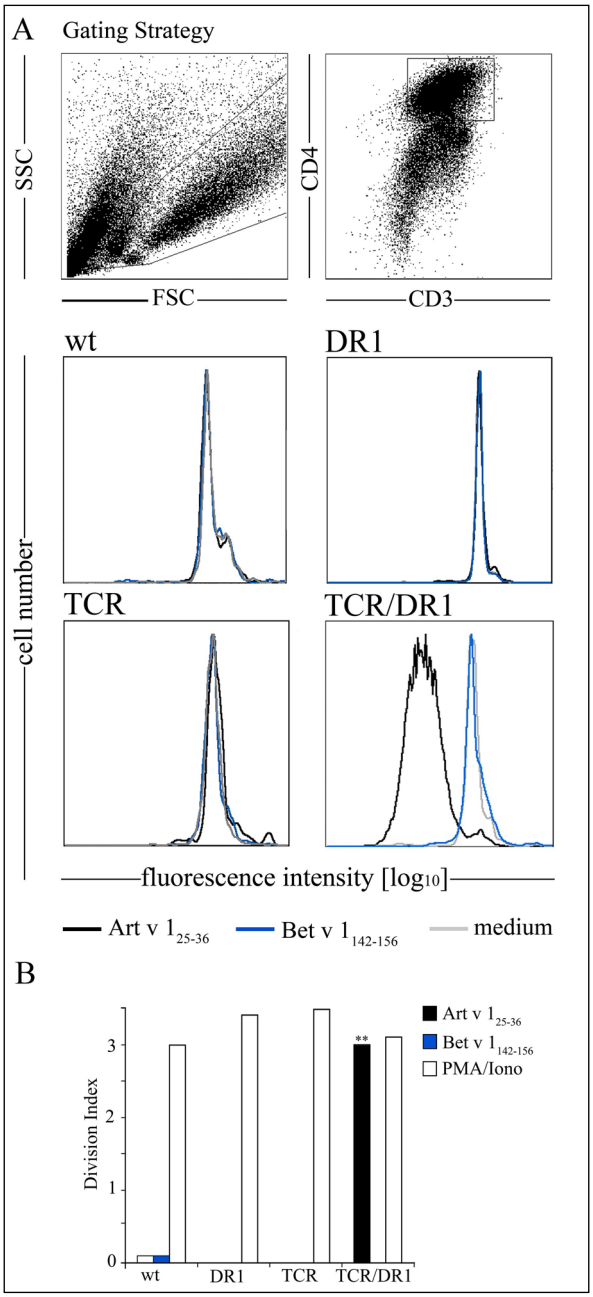

Figure 4. Art v 1-specific proliferation as determined by CPD eFluor® 450 dilution. (A) Splenocytes were labeled with 5 µM CPD eFluor® 450 (cell proliferation dye) and incubated with either Art v 1_{25-36} (black line) or Bet v $1_{142-156}$ peptide (blue line, 10 µM), or medium (grey line) alone for 96 hours. Subsequently, cells were harvested and counter-stained with CD3 and CD4 mAb. (B) The division index of life CPD-labeled $CD3^+CD4^+$ T-cells, which underwent antigen-specific proliferation was calculated according to established methods (26) as described in Material and Methods. Data are representative for one out of three independently performed experiments, *t* test ** $p < 0.01$ Art v 1_{25-36} stimulation compared to Bet v $1_{142-156}$ stimulation.

TCR/DR1 mice reveal specific airway reactivity upon allergen-specific sensitization and challenge

To test if TCR/DR1 mice are a suitable model to study the role of T-cells in the development and treatment of allergic diseases we used a conventional immunization and challenge protocol for the induction of allergic asthma. TCR/DR1 mice and the indicated control mice were sensitized to mugwort-pollen extract (50 µg) in the presence of the adjuvant aluminium hydroxide (alum) by two intraperitoneal injections (day 0 and 7). One week after the booster injection, mice were challenged by one i.n. administration of mugwort-pollen extract. Two

days after allergen-specific challenge airway reactivity of mice was assessed by methacholine induced airflow obstruction in conscious mice placed in a whole body plethysmograph (**Fig. 5A**). Airway reactivity was expressed as the enhanced Pause (Penh) in response to inhalation of increasing methacholine concentrations, which acts as a pharmacological bronchoconstrictor. Interestingly, the baseline level of TCR/DR1 mice (blue line, PBS response, no methacholine) was clearly increased (Penh 3.2±2.1) compared to control wt, DR1 and TCR mice showing Penh levels of 0.6±0, 1.3±0.7, 0.7±0.3 respectively (**Fig. 5B**). Airway reactivity of TCR/DR1 mice increased with increasing concentrations of methacholine peaking at Penh levels of 7.4±4.1 when applying the maximum methacholine concentration (32 mg/ml). In control mice airway reactivity stayed relatively low and slightly increased over baseline levels until a methacholine concentration of 8 mg/ml was reached. High doses of methacholine (16-32 mg/ml) induced enhanced airway reactivity in control mice but never reached Penh levels similar to TCR/DR1 mice (wt Penh 4.6±3.5; DR1 Penh 5.1±1.4; TCR Penh 3.8±1.4; TCR/DR1 Penh 7.4±4.1) (**Fig. 5B**).

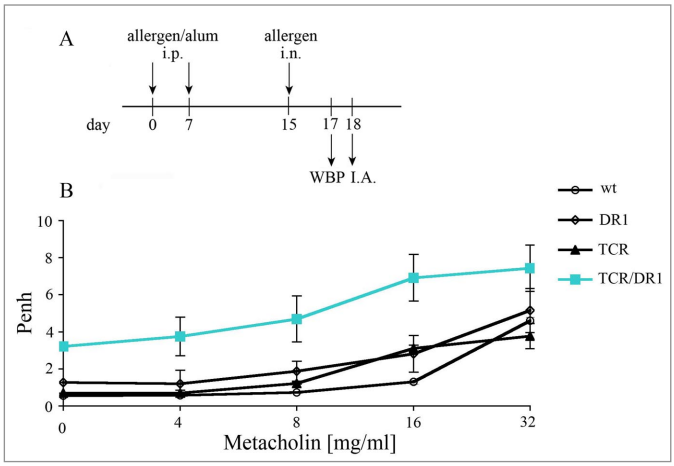

Figure 5. TCR/DR1 mice show enhanced airway reactivity after Art v 1-specific systemic sensitization and challenge compared to control mice. (A) Mice were immunized by two i.p. injections of 50 µg mugwort-pollen extract plus alum on days 0 and 7 and i.n. challenged with 30 µl of a 1.5% solution of mugwort-pollen extract on day 15. (B) Two days after challenge airway reactivity of different mice (wt, DR1, TCR and TCR/DR1; n=4) was assessed by methacholine-induced airflow obstruction in conscious mice placed in a whole body plethysmograph (Buxco Electronics). Results are expressed as the mean enhanced Pause (Penh) after inhalation of the indicated methacholine concentrations.

Sole aerosol exposure of TCR/DR1 mice to mugwort-pollen extract induces significant airway reactivity

In order to closely mimic the human pathology of allergic asthma and to avoid non-physiological prime/boost protocols in the presence of adjuvant we tested mice for enhanced

airway reactivity in response to sole aerosol exposure with mugwort-pollen extract. All groups of mice were exposed to nebulized mugwort-pollen extract on three consecutive days and the specific airway reactivity was monitored in a whole body plethysmograph and expressed as Penh. Single aerosol exposure to mugwort-pollen extract did not induce significant differences in Penh levels of control groups (**Fig. 6**). Further exposure to nebulized allergen significantly increased the airway reactivity of TCR/DR1 mice (mean Penh levels 3.4±3.4) whereas control mice showed no signs of airway reactivity demonstrated by low Penh levels (wt 0.4±0.1, DR1 0.5±0.1, TCR 0.5±0.1). Third exposure to nebulized mugwort-pollen extract did not lead to the induction of airway reactivity in control mice as Penh levels remained low after allergen treatment in all tested control groups. In contrast, TCR/DR1 mice again displayed clear airway reactivity, demonstrated by significantly increased Penh values when compared to control mice (mean Penh levels 2.5±1.6). Rapid induction of airway reactivity was not due to elevated levels of Art v 1-specific IgE levels (**Supplementary Fig. S4**). No detectable Art v 1-specific IgG1, IgG2a or IgE levels were found by ELISA in sera of mice that had been exposed to nebulized mugwort-pollen extract on three consecutive days.

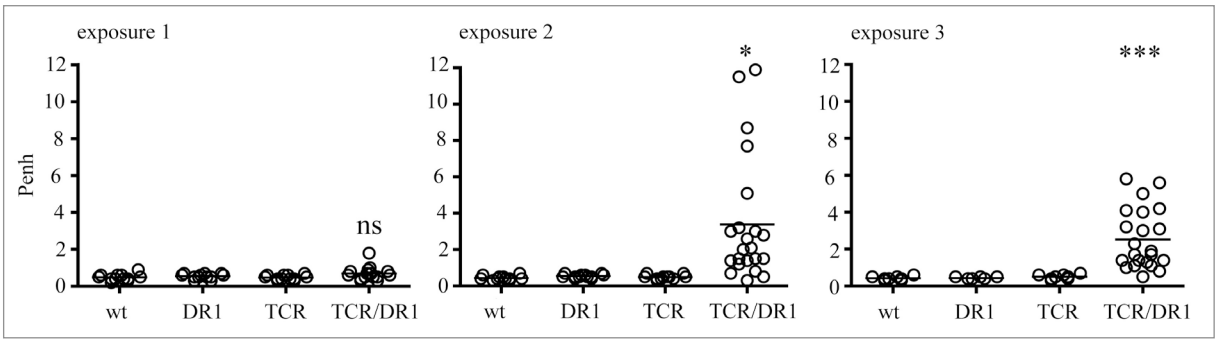

Figure 6. Rapid changes in airway reactivity of TCR/DR1 mice after exposure to nebulized mugwort-pollen extract. All groups of mice (wt, DR1, TCR (n=12) and TCR/DR1 (n=20)) were exposed to 1% nebulized mugwort-pollen extract for 16 minutes using the Buxco-WBP apparatus on three consecutive days. Specific airway reactivity was measured at the end of the exposure and is expressed as the mean enhanced pause (Penh); each circle represents an individual mouse. *t* test * $p < 0.05$; *** $p < 0.001$ DR1/TCR

Allergen-specific aerosol exposure induces recruitment of inflammatory cells detected in the BAL fluids of TCR/DR1 mice

In order to correlate the observed airway hyperreactivity (AHR) to immunological parameters, animals were exposed three times at 24 h intervals with 10 µg/mouse of mugwort-pollen extract in single whole body plethysmography chambers for 16 minutes. During exposure allergen-induced airway reactivity was monitored as Penh for each animal

and exposure. After the last of three exposures, animals were sacrificed and BAL was performed followed by processing of lungs for subsequent histological analyses. Analyses of BAL fluids revealed that the total numbers of leukocytes recovered from TCR/DR1 mice were significantly higher than in the single tg or wt mice (wt: 4.3±2.8 x 10^6, DR1: 3.2±1.6 x 10^6, TCR: 4.4±1.0 x 10^6, TCR/DR1: 12.5±6.7 x 10^6). Moreover, while almost no neutrophilic and eosinophilic granulocytes were present in the three groups of control mice (wt, DR1, TCR), TCR/DR1 mice presented with significant levels of both eosinophils and neutrophils amounting to 19.8±4.6 10^5 cells/BAL and 10.8±2.5 x 10^5 cells/BAL (**Fig. 7**). Accordingly, the proportion of eosinophils and neutrophils reached 17.4±2.7 % and 9.7±1.2 % in the TCR/DR1 mice, while it was negligible in the three groups of control mice. Moreover, clear signs of lymphocytic infiltration were evident in double tg mice and control groups. The degree of mast cells in BAL fluids was generally low, however, mast cell counts were comparable in BAL of TCR/DR1 mice (0.5±0.2 x 10^5) and DR1 mice (0.5±0.1 x 10^5) the numbers in TCR mice (0.4±0.2 x 10^5) and wt mice (0.2±0.1 x 10^5) were marginally lower (data not shown). In close agreement with the outcome of BAL analyses, flow cytometric analysis of lung digests showed pronounced recruitment of eosinophilic and neutrophilic granulocytes in the lungs obtained from double tg mice. As above, they were found to be significantly elevated when compared to the single tg or wt control mice (preliminary results). Moreover, cellular composition of BAL fluids showed elevated polymorphic infiltration of inflammatory immune cells after 8 aerosol challenges in BAL fluids of TCR/DR1 mice (**Supplementary Fig. S4**).

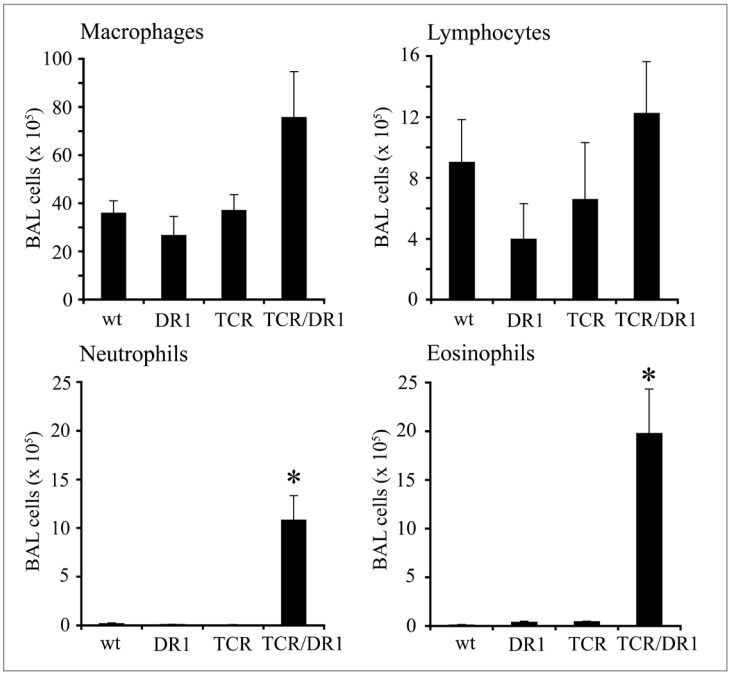

Figure 7. Morphological determination of cellular composition of BAL fluids of mugwort sensitized mice. Groups of animals (wt, DR1, TCR or TCR/DR1) were sensitized with 1% mugwort-pollen extract on three consecutive days as described in Materials and Methods. Cytospin preparations of isolated BAL cells were stained with a modified Wright's stain, counted by using a light microscope at magnification x60/1.40 oil immersion) and characterized morphologically as macrophages, lymphocytes, neutrophilic and eosinophilic granulocytes. Data show mean values + SEM and are representative of 3 experiments (wt, DR1, TCR (n=12) and TCR/DR1 (n=20), *t* test, * $p < 0.05$

Pathological findings in lung histology upon exposure of TCR/DR1 mice to nebulized mugwort-pollen extract

A minimal lymphocytic cell infiltrate is typical for the periarterial space lining a normal bronchiole, while no such infiltrates are usually seen in the alveolar walls of healthy individuals. Upon challenge with nebulized mugwort-pollen extract for three days, wt animals were not different from non-challenged mice, displaying only sparse periarteriolar lymphocytic infiltration not affecting the alveolar walls. This was in marked contrast to TCR/DR1 mice, which presented with a massive cellular infiltrate including the whole lung tissue and including central but also peripheral alveolar tissues (**Fig. 8**). Lung-tissue infiltrating cells were dominated by eosinophilic granulocytes, as demonstrated by high-power magnification, as well as immunophenotypic evaluation of whole lung digests (preliminary results). Of note, no such changes were observed in single tg or wt mice. In addition lung sections from each mouse group were stained with Alcian Blue and Periodic Acid Schiff for the detection of mucus glycoproteins and positive-staining cells in all airways were scored. There were only few PAS-positive cells observed in all lungs of TCR/DR1 mice, with most lungs of control mice being completely devoid of mucus-producing cells. No significant difference between groups was observed (data not shown).

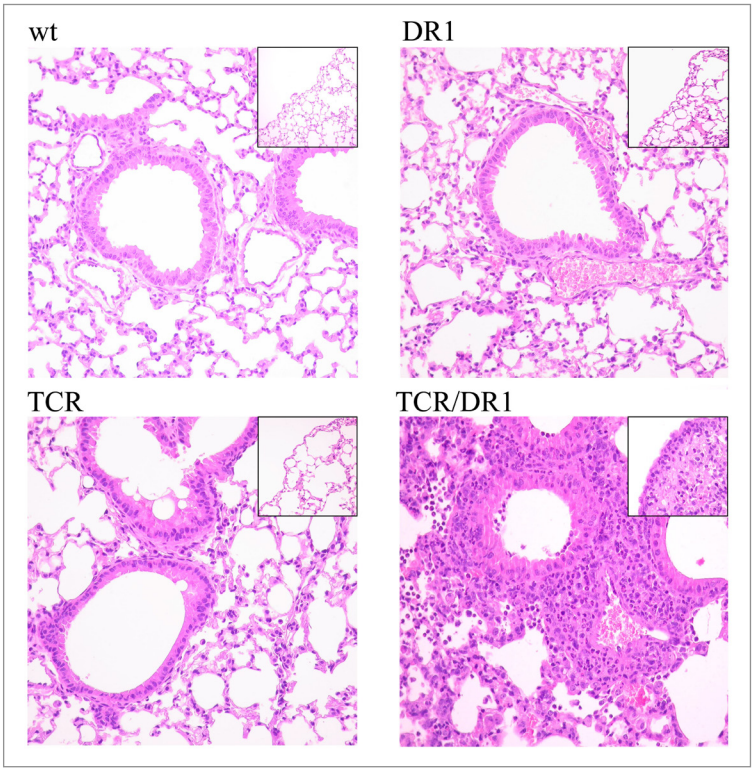

Figure 8. Lung histology of TCR/DR1 mice demonstrates severe peribronchiolar and perivascular inflammation after exposure to nebulized mugwort-pollen extract. Representative light micrographs of lung tissue sections (10 µm) from mice that had been exposed to three aerosol-exposures with 1% mugwort-pollen extract on three consecutive days were stained with hematoxylin and eosin. Central lung tissues of wt, DR1, TCR and TCR/DR1 mice were analyzed using a light microscope (magnification x60/1.40 oil immersion), insets show pheripheral areas of the lung tissues including the pleura (n=12, wt, DR1, TCR; n=20, TCR/DR1).

Table I. Immunophenotyping of leukocyte subsets in spleen, thymus and peripheral blood.

		Spleen		Thymus		Peripheral Blood	
		Percentage of Leukocytes	Absolute numbers per 10^6	Percentage of Leukocytes	Absolute numbers per 10^6	Percentage of Leukocytes	Absolute numbers per 10^6
T cells							
CD3	*wt*	28.5± 5.8	18.5±1.3	82.6±4.0	119.0±1.4	31.8± 5.5	3.0±0
(TRBV18+)		0.1± 0	0.1±0	0.5±0.1	0.5±0.0	0.1± 0	0±0
CD3	*DR1 tg*	30.3± 8.0	17.6±1.5	79.7±5.8	103.4±2.9	44.4± 3.7	3.1±0
(TRBV18+)		0.1± 0	0.1±0	0.5±0.3	0.6±0.1	0.1± 0.1	0±0
CD3	*TCR tg*	19.0± 4.0	16.1±0.5	78.2±2.5	55.5±0.7	23.0± 4.4	1.7±0.1
(TRBV18+)		5.8± 3	4.9±0.4	21.8±9.9	12.1±2.3	16.4± 4.3	1.2±0.1
CD3	*TCR/DR1 tg*	25.2± 6.0	16.2±1.6	79.6±3.7	76.9±1.1	32.5± 3.4	3.3±0
(TRBV18+)		16.0± 7.0	10.3±1.7	42.0±12.9	33.4±3.0	21.0± 3.5	2.2±0
CD4	*wt*	15.4± 5.0	10±1	11.4±0.6	13.6±0.2	17.2± 3.6	1.6±0
(TRBV18+)		0.1± 0	0.1±0	0.1±0.1	0.1±0	0,1±0	0±0
CD4	*DR1 tg*	23.6± 7.0	13.7±1.3	21.9±3.9	22.7±1.6	29.9±3.0	2.1±0
(TRBV18+)		0.1± 0	0.1±0	0.1±0.1	0.1±0.0	0.3±0.2	0±0
CD4	*TCR tg*	12.8± 2.0	10.8±0.2	12.8±1.4	7.1±0.3	13.2.±2.6	1.0±0
(TRBV18+)		6.6±3.0	5.6±0.4	3.7±2.0	2.1±0.5	11.5±2,4	0.9±0
CD4	*TCR/DR1 tg*	22.8±6.0	14.7±1.4	23.6±5.7	18.8±1.3	22.5± 3.4	2.3±0
(TRBV18+)		18.2±6.4	11.7±1.6	15.3±7.1	12.1±1.6	20.2±3.1	2.1±0
CD8	*wt*	10.0± 3.0	6.5±0.7	4.2±0.1	5.0±0.0	9.7± 1.4	0.9±0
(TRBV18+)		0.1± 0	0.1±0	0±0	0±0	0.1± 0	0±0
CD8	*DR1 tg*	4.7± 2.0	2.7±0.3	3.5±0.8	3.6±0.3	7.0± 0.4	0.5±0
(TRBV18+)		0.1± 0	0.1±0	0±0	0.0±0.0	0.1± 0.1	0±0
CD8	*TCR tg*	4.5± 2.3	3.8±0.3	4.1±0.8	2.3±0.2	5.4± 1.6	0.4±0.4
(TRBV18+)		1.9± 1.2	1.6±0.2	1.9±0.7	1.1±0.2	4.7± 1.5	0.4±0.4
CD8	*TCR/DR1 tg*	1.0± .7.0	0.7±0.2	3.2±1.1	2.6±0.3	0.9± 0.5	0.1±0.1
(TRBV18+)		0.6± 0.4	0.4±0.1	2.5±1.2	2.0±0.3	0.9± 0.4	0.1±0.1
B cells							
CD45R	*wt*	61.3±9.6	37.4±2.0	1.1±0.9	1.2±0.2	51.3±5.3	5.0±0
*HLA-DRB1*01:01*$^+$		0.1±0.1	0.1±0.1	0±0	0±0	0.1±0.1	0±0
CD45R	*DR1 tg*	46.4±4.8	25.0±0.9	0.2±0.1	0.2±0	30.±2.6	2.2±0
*HLA-DRB1*01:01*$^+$		34.9±3.6	18.8±0.7	0.2±0.1	0.1±0	26.5±1.8	1.9±0
CD45R	*TCR tg*	67.8±5.9	53.8±0.7	3.0±1.1	1.5±0.2	55.2±3.7	4.2±0.1
*HLA-DRB1*01:01*$^+$		0.2±0.1	0.2±0	0±0	0±0	0.5±0.1	0±0
CD45R	*TCR/DR1 tg*	55.8±12.5	34.6±3	0.7±0.2	0.5±0	47.3±5.8	4.9±0.1
*HLA-DRB1*01:01*$^+$		44.9±12.8	27.8±3	0.6±0.1	0.4±0	41.1±4.5	4.3±0
Lymphocytes							
	wt	89.8 ±15.4	55.9±2.3	83.7±4.9	120.2±1.6	83.1±10.8	8.0±0
	DR1 tg	76.7 ±12.8	42.6±2.4	79.9±5.9	103.6±2.9	99.6±6.3	5.3±0
	TCR tg	86.8 ±9.9	69.9± 1.3	81.2±3.6	57.0±0.9	78.2±8.1	5.9±0.1
	TCR/DR1 tg	81.0±18.5	50.8±4.6	80.3±3.9	77.4±1.1	79.8±8.7	8.2±0.1
Monocytes							
CD14	*wt*	0.6±0.2	0.4±0	0.0±0	0.0±0	0.3±0	0.0±0
*HLA-DRB1*01:01*$^+$		0.3±0.1	0.2±0	0.0±0	0.0±0	0.0±0	0.0±0
CD14	*DR1 tg*	0.6±0.1	0.3±0	0.0±0	0.0±0	0.3±0.1	0.0±0
*HLA-DRB1*01:01*$^+$		0.5±0.1	0.3±0	0.0±0	0.0±0	0.2±0	0.0±0
CD14	*TCR tg*	0.6±0.2	0.5±0	0.0±0	0.0±0	0.4±0	0.0±0
*HLA-DRB1*01:01*$^+$		0.4±0.2	0.3±0	0.0±0	0.0±0	0.0±0	0.0±0
CD14	*TCR/DR1 tg*	0.6±0.1	0.4±0	0.0±0	0.0±0	0.4±0.1	0.0±0
*HLA-DRB1*01:01*$^+$		0.5±0.1	0.3±0	0.0±0	0.0±0	0.3±0	0.0±0
Granulocytes							
Ly6C$^+$Ly6G$^+$	*wt*	1.0±0.6	0.6±0.1	0.2±0.1	0.0±0	10.4±3.0	1.0±0
	DR1 tg	1.5±0.5	0.8±0.1	0.1±0.1	0.0±0	17.0±5.0	1.7±0
	TCR tg	0.6±0.3	0.4±0.	0.2±0.1	0.0±0	9.6±1.1	0.9±0
	TCR/DR1 tg	0.9±0.2	0.5±0	0.0±0	0.0±0	12.0±7.3	1.2±0.1
Leukocytes	*wt*		84.2±29.4		144.0±34.0		11.0±0.8
	DR1 tg		75.0±25.0		129.8±50.4		8.2±1.6
	TCR tg		105.7±16.0		71.0±30.0		8.9±1.6
	TCR/DR1 tg		89.0±34.0		100.0±29.1		12.0±1.2

Discussion

We here established a novel humanized mouse model that will contribute to a better understanding of the precise mechanisms by which allergen-specific T-cells contribute to allergic diseases elicited by human-relevant aeroallergens *in vivo*. To build a strong basis for the detailed investigation of the contribution of allergen-specific T-cells we have recently cloned an allergen-specific T-cell receptor specific for the immunodominant T-cell epitope of the major mugwort-pollen allergen, Art v 1_{25-36} (24). Chimerization of the variable domains of these human TCR αβ chains with the constant regions of the murine TCR-chains was facilitated by using the pTcass expression system described by *Kouskoff et. al.* to direct the expression of T-cell receptor genes in transgenic mice (25). Subsequent pairing of the TCR transgenic mice with mice expressing the human restriction element HLA-DR1 (23) resulted in the generation of double transgenic mice. Using a short-term protocol relying on the exlusive exposure to allergen via the airways these mice develop rapid and impressive airway reactivity almost after three nebulizations with allergen. Airway reactivity is accompanied by massive airway inflammation as demonstrated by peribronchiolar and perivascular infiltration with inflammatory immune cells into the lungs. While systemic sensitization via the i.p. route in the presence of alum led to the induction of allergen-specific IgE and IgG1 and IgG2a in TCR/DR1 mice but also in all of the control mice studied, preliminary results suggest that sole intranasal exposure of mice to the allergen extracts almost exclusively induced allergen-specific IgE production in the absence of IgG2a in TCR/DR1 tg mice but not in control mice.

In the recent decades, several mouse models have been established and used to study the various disease-eliciting factors involved in allergy and asthma (30-32). Among them, the frequently used models based on chicken ovalbumin, which use murine alpha/beta TCR specific for chicken ovalbumin ($OVA_{323-339}$) in the context of murine MHC class II ($I\text{-}A^d$) termed DO11.10 (10) on the BALB/c background. In addition, a C57BL/6-based TCR transgenic model termed OT-II has been established recognizing the same OVA peptide ($OVA_{323-339}$) in the context of $I\text{-}A^b$ (33). While OVA has largely contributed to the better understanding of many immune-mediated mechanisms operative in a variety of diseases, ranging from cancer to autoimmune diseases and transplantation immunology, OVA certainly does not represent a classical human-relevant aeroallergen. Therefore, conclusions drawn from respiratory OVA-models have to be interpreted with caution. Others have addressed this potential conflict and have introduced models recognizing clinically relevant allergens such as those derived from house dust mite. In fact, *Jarman et al.* established transgenic mice

expressing a murine αβ-TCR recognizing a dominant T-cell epitope of Der p 1, a pervasive inhalant indoor allergen (34). Two intratracheal instillations of the allergen at a 5-day interval were sufficient to induce asthmatic features in these mice including accumulation of eosinophiles and lymphocytes in the airways and the lungs as well as goblet cell hyperplasia and mucus production (14). Although this model focuses on a human-relevant aeroallergen, the downside of this model is the fact, that the involved receptors presenting and recognizing the allergen are entirely of murine origin and thus might display other interaction affinities as well as differences in immunodominance when compared to the human situation.

One way to circumvent these flaws is to mimic the human situation more closely and to create humanized mouse models. Humanized mouse models comprise both immunodeficient mice engrafted with functional human immune cells or immunocompetent mice expressing human transgenes. Severe combined immunodeficiency (SCID) mice are, for instance, not able to reject allogeneic or xenogeneic transplants. Therefore, transferred human peripheral blood mononuclear cells (PBMC) can survive in these mice for prolonged periods of time (35, 36). Along those lines, intraperitoneal injections of human PBMC from Der p 1 allergic individuals into SCID mice followed by exclusive airway challenge in the absence of adjuvant resulted in the detection of allergen-specific IgE antibodies and the development of an allergic inflammatory response (37). Moreover, human PBMC from non-allergic individuals were intratracheally transferred into SCID mice (15). These mice developed AHR following aerosol exposure after systemic sensitization with Der p 1 caused by lung-infiltrating human IL-4- and IL-5-producing Th2 cells (15). To study how T-cells get attracted to inflamed airways, SCID mice were reconstituted with PBMC from patients with HDM allergy before allergen challenge in one study (38). Pretreatment with CCR4-blocking antibodies, indeed, abolished airway inflammation and AHR, indicating that the DC-derived chemokines CCL17 and CCL22 are responsible for the recruitment of allergen-specific Th2 cells to the airways (38). Although these models resemble the human situation much more closely, they are dependent on human cell sources and, in general, are only transient in nature, i.e. every single mouse has to be reconstituted with the respective human immune cells individually, making large scale studies very cumbersome and hard to standardize. Transgenic models relying on the use of human immune receptors introduced into mice represent an attractive exit strategy from this dilemma. Indeed, several humanized tg mouse models have been established to study, e.g. autoimmune diseases such as multiple sclerosis (17, 23) rheumatoid arthritis (23) or diabetes (39). However, humanized allergy mouse models based

on an alpha/beta TCR specific for a human relevant respiratory allergen and the corresponding human restriction elements were still missing. Therefore, the here described humanized allergy mice expressing an allergen-specific T-cell receptor for a human-relevant allergen in the context of its corresponding human restriction element represent a considerable advancement of current allergy disease models.

Previous reports have shown that the genetic background of the used mouse strain has an important impact on the outcome of different disease models, which has to be considered when comparing results obtained in different mouse models (40, 41). One of the hallmarks of allergic airway diseases is the development of airway hyperreactivity (AHR). To test the susceptibility of TCR/DR1 mice, which have been established on the C57BL/6 background, to develop bronchial hyperreactivity after allergen-specific sensitization, we systemically immunized mice by two intraperitoneal injections of mugwort-pollen extract in the presence of alum followed by one intranasal allergen-challenge (42). The i.p. route was chosen for initial experiments, since this route is part of many classical sensitization protocols. In fact, upon sensitization TCR/DR1 mice presented with airway hyperreactivity in response to increasing doses of the bronchoconstrictor methacholine as assessed by WBP. Interestingly, TCR/DR1 mice revealed elevated Penh levels already at steady-state, which were further increased by increasing methacholine doses when compared to control mice. These results are in accordance with several reports demonstrating that systemic sensitization with allergens in the presence of a Th2-skewing adjuvant followed by antigen-challenge induces allergic lung responses in mice (42-45). BALB/c mice are well-known to be high IgE-responders when challenged with a broad range of allergens, which clearly makes them more allergy-prone than the C57BL/6 mouse strain (40). While several reports have shown that OVA applied as model allergen induced AHR in BALB/c and AKR mice no such response was observed C57BL/6 and 129/Sv mouse strains, which remained hyporesponsive after equal allergen treatment (46, 47), our results clearly confirm that a robust lung phenotype can be induced also on the C57BL/6 background. The differences in AHR observed in different mouse strains have been attributed to differences in the production of allergen-specific IgE or eosinophil recruitment into the lungs of mice (46-48). Yet these observations failed to completely explain the underlying pathophysiology (41) and do not seem to play a major role in our short-term sensitization model.

A clear advantage of TCR tg mouse models is the preexistence of a large number of naive antigen-specific T-cells recognizing the respective model antigen. This makes also our novel human allergen-specific TCR/DR1 model particularly useful. The high allergen-specific precursor frequencies enable the scientist to take a close look on even very small subsets of allergen-specific T-cells and facilitates the observation of minimal changes within these compartments during therapeutic and prophylactic interventions. In addition, imitation of the natural route of sensitization resembling the human-relevant situation, e.g. low concentrations of nebulized allergen allows to omit harsh prime-boost regimen in combination with the administration of adjuvant. Previous studies have shown, that non-sensitized BALB/c mice do not develop an allergic inflammatory response after a single exposure to OVA aerosol (49) and only few studies use exclusive exposure (even when multiple exposures are taken into account) via the airways to induce the allergic disease (50-57). In our model, three consecutive aerosol exposures were sufficient to elicit massive IgE independent airway reactivity in unprimed TCR/DR1 mice in response to the specific allergen as measured by WBP. Lung histology revealed airway and lung inflammation with massive peribronchiolar and perivascular infiltration of immune cells. Next to non-sensitized wt mice, TCR and DR1 mice served as appropriate controls for mugwort pollen-specific aerosol exposure. Control mice belonging to either of the three groups did not show impaired lung function at any point of intervention. In marked contrast to short term aerosol exposure of mice, intranasal exposure to allergen-extracts elicited high levels of allergen-specific IgE in TCR/DR1 mice, but not in control mice, demonstrating that various aspects of the allergic disease, i.e. those highly IgE-dependent and those non-IgE-dependent can be conveniently studied in in the new model.

To induce a significant eosinophilia in the lungs of previously naïve BALB/c mice it is necessary that mice become exposed to nebulized OVA up to ten times (50). Therefore, it can be excluded that the assessed airway reactivity observed in TCR/DR1 mice is the result of a non-specific protein-induced reaction. Moreover, prolonged allergen-exposure of control mice (up to eight during a time period of two weeks) did not induce significant airway reactivity as shown for double tg mice (Supplementary Fig. 5). Interestingly, first changes regarding altered airway reactivity became apparent already during the second allergen exposure, which followed the first exposure with a 24 hour interval. The third allergen-exposure via the airways did not further enhance the measured Penh levels but rather increased the number of mice presenting with strong airway responses. These results are to some degree in accordance

with previous observations from *Wilder et al.*, who demonstrated that 3 OVA aerosol exposures (in contrast to our daily exposure these mice were exposed once a week) each lasting for an hour were, indeed, sufficient to induce AHR in naive heterozygous DO11.10$^{+/-}$ mice (9). In contrast to our model and of importance, *Knott et al.* showed by using homozygous DO11.10 mice that a single OVA aerosol exposure or four OVA aerosol exposures delivered on four consecutive days failed to develop AHR, as measured by WBP and expressed in enhanced pause (Penh) in response to methacholine (58). The authors showed that a single allergen-exposure of mice was accompanied exclusively with an increased influx of neutrophils and minimal recruitment of eosinophils into the lungs and the detection of mucus in the bronchial epithelium. In our model, double tg mice show a polymorphic cellular composition of BAL fluids with high levels of granulocytes, composed mainly of eosinophils and neutrophils, as well as T-lymphocytes, monocytes and alveolar macrophages following a short-term provocation protocol (three exposures on three consecutive days). In contrast, BAL fluids of control mice presented mostly with macrophages. Lung histology revealed only minor mucus production in allergen-exposed double transgenic mice, most probably caused by the facts that both mucus production and structural remodeling of the lung represent rather chronic features of allergic lung inflammation and are usually not induced upon short-term pulmonary exposure. Of note, more escalated exposure protocols, i.e. eight aerosol exposures within two weeks, revealed even more polymorphic cellular infiltrates into BAL fluids of TCR/DR1 mice (Supplementary Fig. 5), caused by the additional presence of mast cells. Interestingly, after eight repetitive exposures also DR1 control mice showed, next to alveolar macrophages, considerable recruitment of eosinophils to BAL fluids. This result was rather unexpected since DR1 control mice did not demonstrate elevated levels of airway reactivity (preliminary results).

The rapid induction of airway reactivity of TCR/DR1 mice after the short-term aerosol exposure protocol cannot be explained by production of Art v 1-specific IgE levels since we did not detect Art v 1-specific IgE nor IgG1 in sera of treated mice (Supplementary Fig. 4). These results are not entirely unexpected since as the short-term protocol, using only three consecutive aerosol challenges, does not provide enough time for activated B-cells in allergen-naïve mice to undergo class switch recombination and IgE production.

Do allergen-specific TCR tg T-cell *a priori* respond with a polarized immune response? In fact, *ex vivo* Art v 1-specific stimulation of splenocytes from aerosol-challenged TCR/DR1 tg mice showed a mixed cytokine profile with detectable amounts of IFN-γ, IL-2, IL-17, IL-4,

IL-13 rather than an exclusive Th2-skewed profile. Nevertheless, *in vitro*-polarization studies, i.e. by applying appropriate cytokine cocktails, revealed that naive T-cells from TCR/DR1 mice have the principle potential to become polarized towards either the Th1 or Th2 subset. Similar results were obtained by exposing DO11.10 tg mice (BALB/c-background) with OVA *in vivo*, which did not result in a clear-cut Th2-response in lung-resident allergen-specific T-cells (58). Moreover, previous findings report that *ex vivo*-stimulation of lung-resident T-cells isolated from OVA-aerosol challenged DO11.10 mice did not lead to proliferation and IL-2 production upon OVA-specific or polyclonal activation (59). Although these cells did not proliferate in response to the allergen, their effector function were found to be intact because they were able to produce IFN-γ, IL-4 or IL-5. The authors claimed that the anti-proliferative response of lung parenchymal T-cells was tissue-specific and facilitated by $F4/80^+$ interstitial macrophages. Removal of these macrophages restored proliferation and IL-2 production to some degree. Macrophage-mediated inhibition of proliferation was suggested to keep clonal expansion of T-cells in the lung compartment at the site of inflammation in check to prevent chronic pulmonary reactions.

In summary, we here established a novel humanized mouse model for allergy research expressing a TCR for the human-relevant mugwort-pollen allergen, Art v 1, and the corresponding human restriction element HLA-DR1. TCR/DR1 tg mice specific for a clinically-relevant human allergen and presented by the human-relevant restriction element will provide new insights into the pathophysiological mechanisms underlying allergic diseases and promote the development and evaluation of novel therapeutic strategies for the regulation of allergic immune responses.

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Disclosure of potential conflict of interest: The authors have no conflict of interest.

SUPPLEMENT

Supplementary Tables

Table SI. List of mAbs used for flow cytometric analyses

Specificity	Clone name	Species	conjugated to	Source
CD3	500A2	hamster	FITC	eBioscience, San Diego, CA, USA
CD3	17A2	rat	eFluor450	eBioscience, San Diego, CA, USA
CD4	RM4-5	rat	eV450	eBioscience, San Diego, CA, USA
CD4	RM4-5	mouse	APC	eBioscience, San Diego, CA, USA
CD8	5H10	rat	APC	Invitrogen, Camarillo, CA, USA
CD14	rmC5-3	rat	PE	Becton Dickinson, Palo, Alto, CA
CD45R/B220	RA3-6B2	rat	FITC	eBioscience, San Diego, CA, USA
CD45R/B220	RA3-6B2	rat	PB	eBioscience, San Diego, CA, USA
CD90.2 (Thy1.2)	53-2.1	rat	APC	eBioscience, San Diego, CA, USA
Ly6C+6G	RB6-8C5	rat	FITC	Becton Dickinson, Palo, Alto, CA
HLA-CII	L243	mouse	FITC	BioLegend, San Diego, CA, USA
HLA-CII	L243	mouse	PerCP	BioLegend, San Diego, CA, USA
TRBV18	BA62.6	mouse	PE	Immunotech, Marseille, France
TCRγ/δ	GL3	hamster	FITC	BioLegend, San Diego, CA, USA
control	eBio299Arm	hamster	FITC	eBioscience, San Diego, CA, USA
control	P3.6.2.8.1	mouse	PE	eBioscience, San Diego, CA, USA
control	MOPC-21	mouse	PerCp	BioLegend, San Diego, CA, USA
control	MOPC-21	rat	APC	BioLegend, San Diego, CA, USA
control	RTK2758	rat	PO/PB	BioLegend, San Diego, CA, USA

Abbreviations: APC, allophycocyanine; PE, phycoerythrin; Cy, cyanine; PerCP, peridinin chlorophyll protein; FITC, fluorescein isothiocyanate

Table SII. List of mAbs used for flow cytometric analysis BAL fluids

Specificity	Clone name	Species	conjugated to	Source
CD16/CD32	2.4G2	rat	-	Becton Dickinson, Palo, Alto, CA
CD45	30-F11	rat	Alexa Fluor 700	Becton Dickinson, Palo, Alto, CA
CD3	500A2	hamster	PE	eBioscience, San Diego, CA, USA
CD4	L3T4	rat	Pe-Cy7	Becton Dickinson, Palo, Alto, CA
CD11c	HL3	hamster	APC-Cy7	Becton Dickinson, Palo, Alto, CA
CD45R/B220	RA3-6B2	rat	PB	BioLegend, San Diego, CA, USA
CD19	1D3	rat	V450	Becton Dickinson, Palo, Alto, CA
CCR3	83103	rat	Alexa Fluor 647	Becton Dickinson, Palo, Alto, CA
Siglec-F	E50-2440	rat	Alexa Fluor 647	Becton Dickinson, Palo, Alto, CA
Ly6C+6G	RB6-8C5	rat	FITC	Becton Dickinson, Palo, Alto, CA
Ly6C+6G	RB6-8C5	rat	Pacific Orange	Molecular Probes
CD117	2B8	rat	PE-CF594	Becton Dickinson, Palo, Alto, CA
IgE	R35-72	rat	FITC	Becton Dickinson, Palo, Alto, CA
NK-1.1	PK136	rat	PerCP-Cy5.5	Becton Dickinson, Palo, Alto, CA
HLA-DR	L243	mouse	PerCP	BioLegend, San Diego, CA, USA
MHC I-A/I-E	M5/114.15.2	rat	PerCP	BioLegend, San Diego, CA, USA
control	eBio299Arm	hamster	FITC	eBioscience, San Diego, CA, USA
control	P3.6.2.8.1	mouse	PE	eBioscience, San Diego, CA, USA
control	MOPC-21	mouse	PerCp	BioLegend, San Diego, CA, USA
control	MOPC-21	rat	APC	BioLegend, San Diego, CA, USA
control	RTK2758	rat	PO/PB	BioLegend, San Diego, CA, USA

Abbreviations: APC, allophycocyanine; PE, phycoerythrin; Cy, cyanine; PerCP, peridinin chlorophyll protein; FITC, fluorescein isothiocyanate, PO/PB, pacific orange/pacific blue

Table SIII. List of mAbs used for Luminex

Specificity	Clone name	Species	Source
IFN-γ purified	AN-18	rat	eBioscience, San Diego, CA, USA
IL-2 purified	JES6-1A12	rat	eBioscience, San Diego, CA, USA
IL-4 purified	11B11	rat	eBioscience, San Diego, CA, USA
IL-5 purified	TRFK5	rat	eBioscience, San Diego, CA, USA
IL-10 purified	JES5-16E3	rat	Invitrogen, Camarillo, CA, USA
IL-13 purified	eBio13A	rat	Becton Dickinson, Palo, Alto, CA
IL-17 purified	eBio17CK15A5	rat	eBioscience, San Diego, CA, USA
IFN-γ Biotin	R4-6A2	rat	eBioscience, San Diego, CA, USA
IL-2 Biotin	JES6-5H4	rat	eBioscience, San Diego, CA, USA
IL-4 Biotin	BVD6-24G2	rat	eBioscience, San Diego, CA, USA
IL-5 Biotin	TRFK4	rat	eBioscience, San Diego, CA, USA
IL-10 Biotin	JES5-2A5	rat	Invitrogen, Camarillo, CA, USA
IL-13 Biotin	eBio1316H	rat	Becton Dickinson, Palo, Alto, CA
IL-17 Biotin	eBio17B7	rat	eBioscience, San Diego, CA, USA

Supplementary Figures

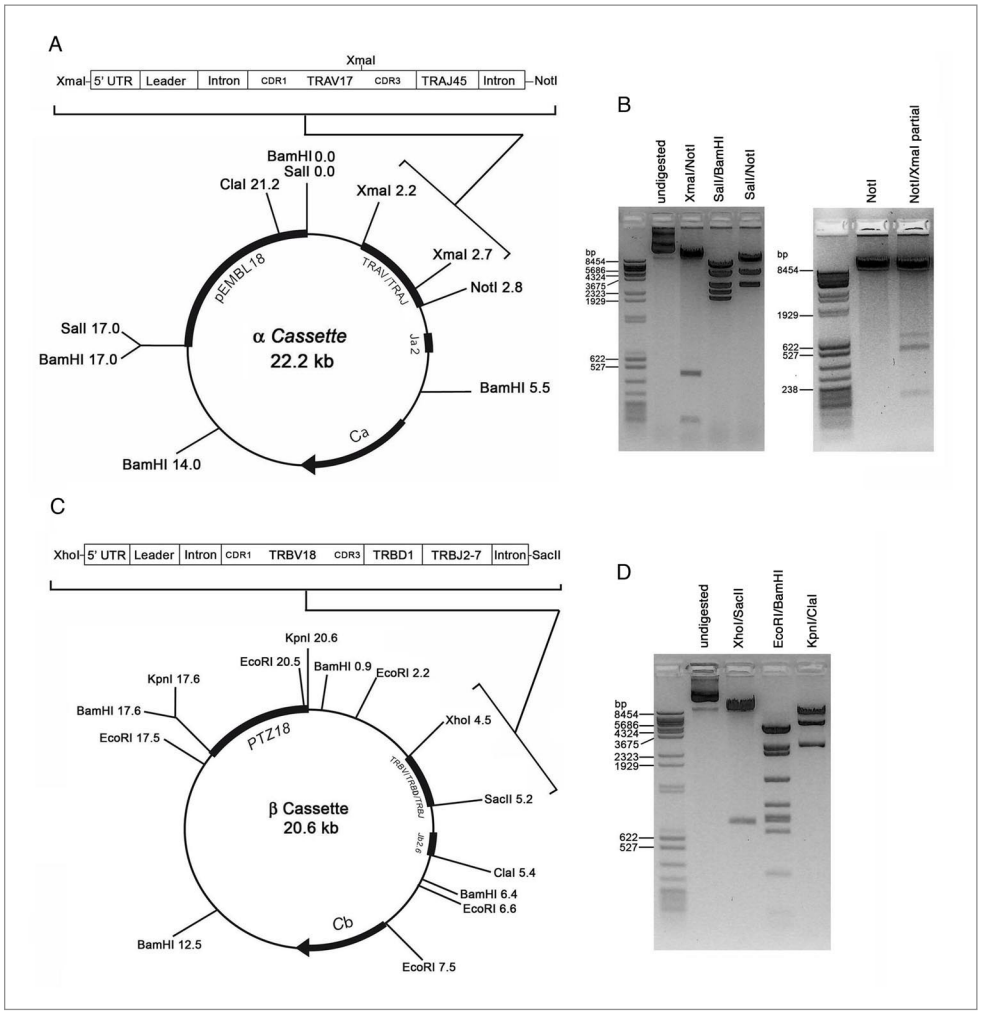

Figure S1. Generation of TCR transgenic mice by using genomic TCR cassette vectors. (A) Schematic depiction of the TCR alpha transgenic expression vector. The vector (22.3 kb) consists of 2.2 kb of genomic DNA upstream of the murine TCR alpha leader, variable and joining sequence (0.7 kb) and 14.2 kb of downstream homologous murine TCR sequences including the Jα2, the Cα exon and the 3' enhancer elements according to (25) and based on (60). The position of the 5.2 kb prokaryotic vector sequences (pEMBL18) are indicated. Unique restriction sites, *Xma I* and *Not I*, were used for inserting the respective human TCR alpha leader, variable and joining genomic sequences, while *Sal I* sites were used to remove the prokaryotic plasmid sequences to linearize the entire TCR gene (17.1 kb). (B) *Xma I/Not I*-digested vector DNA was used to identify properly inserted TCR variable sequences and comparisons between the undigested (lane 1) *Xma I/Not I*-digested (lane 2), *SalI/BamHI*-digested (lane 3) and *SalI/NotI*- vector (lane 4) and partially digested vector DNA *NotI* and *XMA/Not I* are shown. (C) Schematic depiction of the TCR beta transgenic expression vector. The vector (20.58 kb) consists of 4.47 kb of genomic DNA upstream of the murine TCR beta leader, variable and joining sequence (0.76 kb) and 12.42 kb of downstream homologous murine TCR beta sequences including the Jb2.6, Cb exon and 3' enhancer elements according to (25) and based on (61). The position of the 2.93 kb prokaryotic vector sequences (PTZ18) are indicated. Unique restriction sites are indicated, *Xho I* and *Sac II* restriction sites used for inserting the respective human TCR beta leader, variable, diversity and joining genomic sequences are indicated in bold letters, while *Kpn I* sites used to remove the prokaryotic plasmid sequences to linearize the entire TCR beta gene (17.65 kb). (D) *Xho I/Sac II*-digested vector DNA was used to identify properly inserted TCR variable sequences (lane 2) and was compared to undigested vector DNA (lane1) digest profile of EcoRI/BamHI (lane 3) or KpnI/ClaI (lane 4) digestion. Positions of DNA size markers (mixture of λ *Bst EII* digest and pBR322 *Msp I* digest) are indicated.

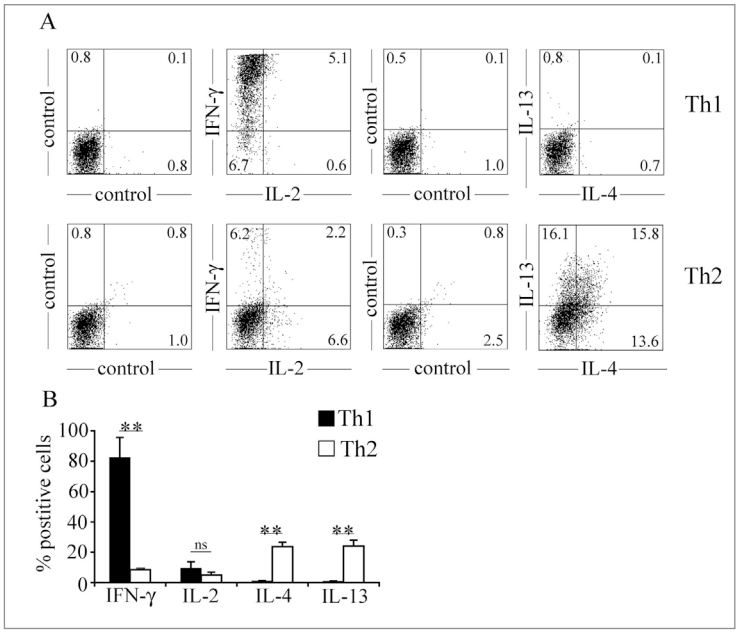

Figure S2. T-cells from TCR/DR1 mice can be polarized towards Th1 and Th2 phenotypes *in vitro*. Polarization of naive T-cells isolated from splenocytes of TCR/DR1 mice was performed as described in Material and Methods. (A) Intracellular cytokine levels of polarized T-cells were assessed after pretreatment with Golgi-Stop in the presence of polyclonal activation with PMA/Ionomycin for 6 hours, followed by intracellular cytokine staining using Fix and Perm (An der Grub) and analysis by flow cytometry. (B) Data show percent positive cells of dual parameter dot plots of one out of three independently performed experiments. Markers were set according to the results obtained with non-binding control mAb. Numbers indicate percentage of positive cells in respective quadrants. *t* test, ** $p < 0.01$, ns not significant.

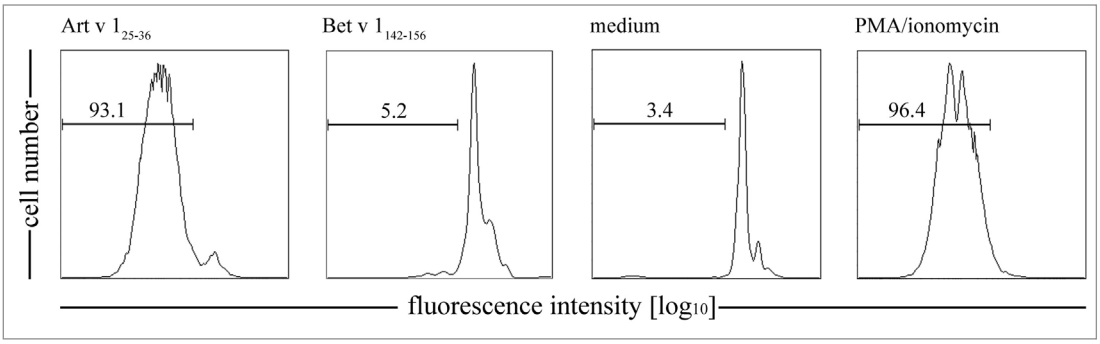

Figure S3. Art v 1-specific proliferation of CPD-labeled $CD3^+CD4^+TRBV18^+$ splenocytes from double TCR tg mice. Splenocytes were labeled with 5 µM CPD eFluor® 450 (cell proliferation dye) and incubated with either Art v 1_{25-36} peptide (10 µM), Bet v $1_{142-156}$ (10 µM), medium alone or PMA/Ionomycin for 96 hours. Subsequently, cells were harvested and counter-stained with CD3, CD4 and TRBV18 mAb. Data are representative for one out of three independently performed experiments. Numbers indicate percentage of cells which underwent proliferation.

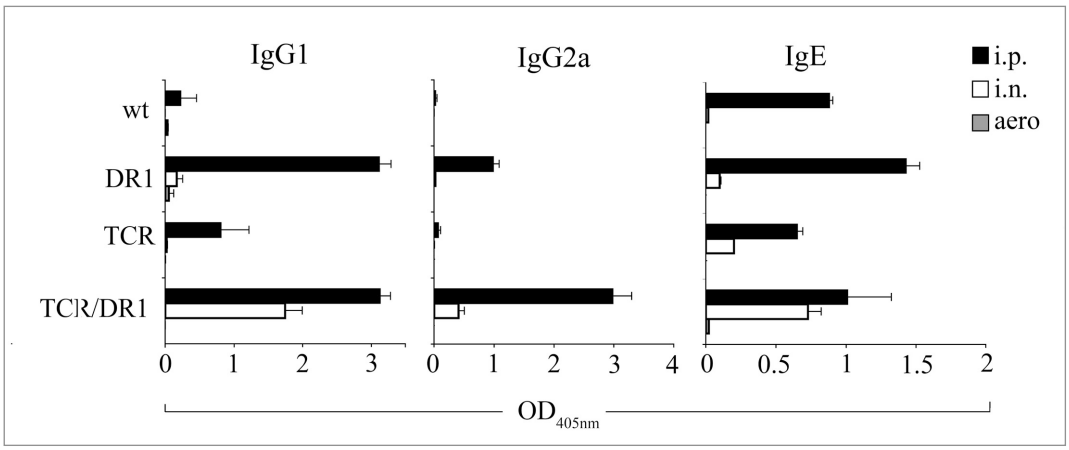

Figure S4. Rapid induction of airway reactivity of TCR/DR1 mice after exposure to nebulized mugwort-pollen extract is IgE independent. Data show Art v 1-specific IgG1, IgG2a and IgE-levels in sera derived from mice that had been immunized three times i.p. with 50 µg mugwort-pollen extract plus alum (black bars indicated as *i.p.*), mice that had been exposed to four i.n. challenges with 30 µl of 1.5% mugwort-pollen extract (white bars indicated as *i.n.*) or mice that had been exposed to three aerosol exposures of 1 % mugwort-pollen extract on three consecutive days (grey bars indicated as *aero*). Serum Ig-levels were determined using a standard ELISA reader (OD_{405nm}, Softmax Pro) Data show mean values of triplicate measurements of each sample + SEM (numbers of mice for individual groups ≤ 4).

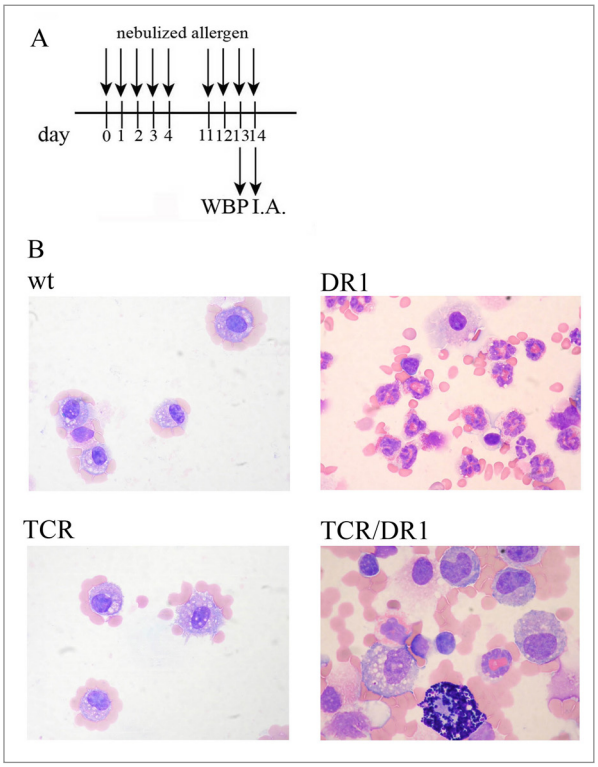

Figure S5. Cytospin preparations of BAL fluids of mice who underwent repetitive sensitization with mugwort-pollen extract. (A) Groups of animals (wt, DR1, TCR or TCR/DR1; n=4) were sensitized with 1% mugwort-pollen extract for 8 times (B). Cytospin preparations of isolated BAL cells were stained with a modified Wright's stain, counted by using a light microscope (at x60/1.40 oil immersion magnification) and characterized morphologically as macrophages, lymphocytes, neutrophilic and eosinophilic granulocytes. Data show a representative picture of one mouse each (n=4); I.A. immunological analyses.

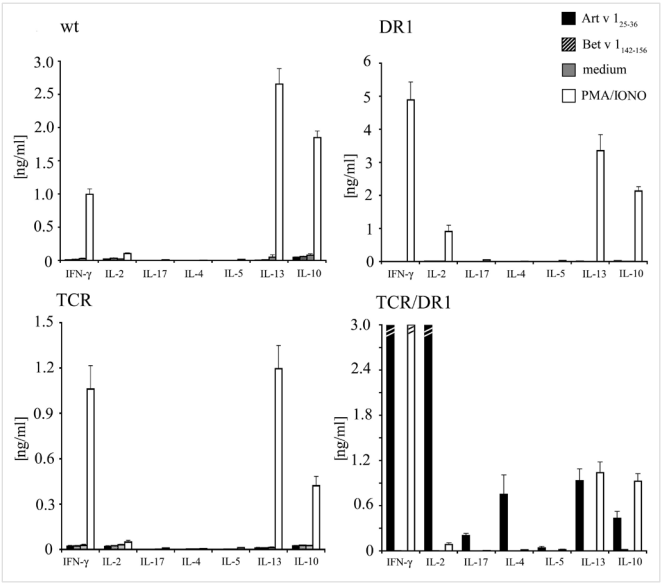

Figure S6. Three consecutive exposures to nebulized mugwort-pollen allergen do not induce systemic polarization of splenocytes. Splenocytes (2 x 10^5/well) of wt, DR1, TCR and TCR/DR1 mice that had been exposed to three aerosol challenges with nebulized mugwort-pollen extract were co-cultured with Art v 1_{25-36} peptide (10 µM), Bet v $1_{142-156}$ (10 µM), medium alone or PMA/Ionomycin. Culture supernatants were harvested after 72 h and analyzed for cytokine secretion levels (Luminex) system. Data show mean values + SD of triplicate cultures and are representative for one out of three independently performed experiments.

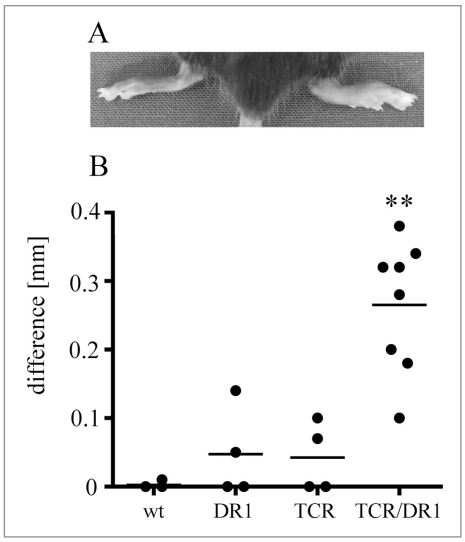

Figure S7. TCR/DR1 mice develop massive delayed-type hypersensitivity reactions upon injection of antigen. Anesthesized mice were immunized by subcutaneous injection of 20 µl mugwort-pollen extract into the food pad of the right hindfoot. After 96 h delayed type hypersensitivity reactions (A) were determined by measuring the thickness of the footpad using a digital thickness gauge. (B) Footpad swelling was calculated as described in Material and Methods. Data show individual values for each mouse, horizontal bar indicates mean and are representative for one out of two experiments; wt, DR1, TCR (n= 4), *t* test, ** $p < 0.01$ compared to TCR/DR1 mice (n=8).

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Conclusion and Synopsis

The aim of this thesis was to investigate the precise mechanisms by which allergen-specific T-cells contribute to allergies elicited by human-relevant allergens *in vitro* and *in vivo*. The antigen specificity of a distinct T-cell is determined by its T-cell receptor (TCR) and can be transferred to other T cells by means of TCR αβ gene transfer (1-3). For this reason we cloned TCR specific for the immunodominant epitope of two highly human-relevant aeroallergens, Art v 1, (*Artemisia vulgaris,* TRAV17/TRBV18) the major mugwort pollen allergen and Bet v 1, the major birch pollen allergen (*Betula verrucosa,* TRAV6/TRBV20). Respective cDNAs for TCR and HLA genes were derived from T-cell clones and corresponding EBV transformed B-cell lines obtained from allergic individuals. Within the scope of this thesis I generated allergen-specific T-lymphocytes by transfer of allergen-specific TCR into T-cell lines and peripheral blood T-cells (PBL) from allergic and non-allergic individuals. To imitate the allergen-specific synapse formed between antigen-presenting cells and T-cells I used human embryonic 293 cells transiently transfected with the corresponding allergen-presenting HLA-molecules, allergenic peptides and co-stimulatory molecules as artificial antigen-presenting cells (aAPC) (4). In addition I employed and further developed technology for generation of virus-like particles (VLP) as modular, non-cellular antigen-presenting platforms. Along those lines we have recently shown that decoration of VLP with molecules of choice can be accomplished by introduction of GPI-anchors into the respective molecules (5, 6). Of note, in a side project together with colleagues from the Harvard Medical University I have developed technology to measure and quantify virus-particle formation (7). Importantly, activation of TCR-transduced T-cells and T-cell lines was strictly dependent on antigen-specific and co-stimulatory signals provided either by monocytes-derived DC, artificial APC or VLP (4, 8, 9). Thus TCR transgenic (tg) T-cells represent a promising tool, which can be used to study targeting fo different T-cell populations and to develop strategies for immunomodulation of allergen-specific T-cell responses to exert defined effector functions (e.g Th1, Th2, Th17 and Treg) *in vitro* and *in vivo*.

In the first study, using the Art v 1-specific TCR I was able to show together with my colleagues that allergen-specific TCR-transgenic T-cells can be generated in large quantities independently of the allergic status of the donor and the allergy season. This is in contrast to generation of conventional allergen-specific T-cell clones/lines, which usually represents a time consuming and sometimes tedious process.

Transfer of the TCR α and β-chains into the human Jurkat T-cell line or human PB-T cells of non-allergic individuals resulted in clear-cut expression of the tg TCR on the surface of the T-cells. Transgenic human T-cells functionally resembled the original Art v 1-specific T-cell clone (SSR20) as they specifically reacted with Art v 1_{25-36} pulsed autologous EBV B-cells or aAPC expressing invariant chain (Ii)::Art v 1 fusion proteins. Moreover, the activation of tg T-cells was strictly dependent on co-stimulation via CD80 or CD86.

In a second approach, I demonstrated that the transfer of a Bet v $1_{142-156}$ -specific TCR retains the fine specificity of the original T-cell clone (SD334). Birch pollen allergic individuals frequently suffer from the oral allergy syndrome (OAS) triggered by cross-reactivity with Bet v 1-related food and pollen allergens affecting patients throughout the year. The TCR tg T-cells showed typical cross-reactivity with Bet v 1-related food allergens from celery (Api g 1) and carrot (Dau c 1) as well as the cross-reactivity with the tree pollen allergens Cor a 1 and Car b 1, derived from hazelnut and hornbeam. The growing number of recombinant/modified allergens designed for allergy therapy demands reliable test systems to predict their immunological behavior. Bet v 1-specific tg T-cells might represent a standardizable tool to assay for retained T-cell reactivity of modified allergens covering the C-terminal part of Bet v 1. Since the 1980s allergic diseases have been explained by the Th1/Th2 hypothesis, stating that allergy is a Th2-mediated disease with an imbalance in favor of Th2 responses. One strategy to regulate overwhelming Th2 responses would be the promotion of counteracting IFN-γ secreting Th1 cells (10). Consequently, I tested whether I could use the Bet v 1-specific TCR to induce these two different counteracting allergen-specific T-helper cell subsets, Th1 and Th2 cells with a typical defined cytokine profile. For the induction of the Th1-phenotype I have chosen IL-12 as the major Th1-inducing cytokine (11). The feasibility of Th1-skewing strategies was recently shown by attempts to define novel therapeutic mechanisms for the treatment of allergic diseases. In fact, farm-derived dust samples have been screened for bacteria that exert an allergo-protective potential. *In vitro*, stimulation of human monocyte-derived dendritic cells (moDC) with the gram-negative bacteria *A. lwoffii-F78* induced the production of IL-12, which polarized naive T-cells towards the Th1 subset shown by predominant IFN-γ secretion (12, 13). Several strategies have utilized IL-12 for the induction of a Th1-phenotype, but the systemic administration of this cytokine in human therapy bears a lot of adverse reactions (14, 15). To restrict the action of this cytokine to the site of antigen-presentation I generated a membrane-anchored version of human IL-12 (9). Allergen-specific aAPC expressing membrane-bound IL-12 in the absence of the classical co-stimulatory

molecule CD80 induced strictly Th1-polarized T-cells shown by their predominant IFN-γ secretion. Furthermore, such Th1-polarized TCR tg T-cells were able to suppress the effector function of allergen-specific Th2-differentiated T-cells in co-culture experiments.

To be able to distinguish between Th1- and Th2-polarized T-cells in co-culture experiments I used a multicistronic expression strategy to express the Bet v 1-specific TCR linking the TRAV6 and TRVB20 chains via a picornaviral 2A sequence upstream of an IRES-GFP element. Picornaviral 2A elements have been described as a convenient biotechnological tool by *Szymczak et al.* (16). 2A elements are translatable sequences, which add a short amino-acid sequence to the C-terminus of the 5' expressed protein after translation. These sequences mediate ribosomal skipping, thereby allowing translation of two or more proteins from a single transcript. I did not observe any interference of the additional sequence with the function of the linked TCR alpha and beta chains. Importantly, these experiments were reproducible with similar results obtained by a second unrelated TCR, *i.e*. Art v 1-specific TCR tg T-cells, indicating that the obtained results are not a sole feature of the Bet v 1-specific TCR. The characterization of the Bet v 1-specific TCR along with modular aAPC offers the possibility to generate large amounts of Bet v 1-specific Th1-polarized T-cells *ex vivo* for adoptive transfer experiments to counter-regulate allergen-specific Th2 cell responses. Although allergen-specific Th1-polarized T-cells have proven to have the potential to suppress allergen-specific Th2 cells, some questions still remain open. Among them questions concerning the stability of the IL-12-induced Th1 cell phenotype, the precise mechanism(s) of suppression, is it the sole function of the key cytokine IFN-γ or mediated via a synergistic effect between different soluble factors?

The use of transgenic TCR for therapeutic approaches is a very promising strategy but concerns exist that the transfer of TCR α and β chains might be technically demanding and bears certain risks. Studies performed with cytotoxic T-lymphocytes expressing tumor-specific TCR for improved anti-tumor immune responses in cancer research show that the transgenic TCR chains can combine with endogenous, native TCR chains and thus form hybrid receptors with unknown specificities. Such cross-pairing can lead to loss of antigen-specificity, thereby reducing the beneficial effect of the modified cells and might even result in the gain of new functions, in the worst case the development of autoreactivity (17). Several strategies have been applied to prevent the potential risk of cross-pairing events between endogenous and transgenic TCR chains, such as silencing of the endogenous TCR with small interfering RNAs (18), transfer of TCR αβ-chains into γδ T-cells (19) or introduction of minimally "murinized" TCR constant domains (20). I tested a strategy described by *Kuball et*

al. who described the avoidance of cross-pairing through introduction of an additional disulfide bond into the transgenic TCR chains as well as increased functional avidity of a TCR upon removal of a defined N-glycosylation site in the constant region of the TCR alpha chain. Although I observed a slightly enhanced expression of the TCR beta chain of the Bet v 1-specific TCR upon introduction of such additional cysteine residues, we did not observe an increased functional activity of the tg T-cells compared to T-cells transduced with the unmodified TCR chains (unpublished data). These results were not entirely unexpected as it was described that different TCR might display strongly different expression levels on the cell surface (21, 22). Only TCR with high-level cell surface expression are able to transfer and integrate the antigen-specific signal into the cell (20). The high expression levels achieved with the unmodified Bet v 1-specific TRBV20 chain (up to 70% of $CD3^+$ T-cells) probably already represent optimal transgenic expression levels which can not be further increased by this strategy. Consequently, this strategy although a convenient method to enhance the cell surface expression of TCR with intrinsically weak surface expression, did not enhance the expression of the Bet v 1-specific TCR significantly.

In vivo mouse models utilizing the administration of recombinant IL-12, other Th1-skewing factors or Th1-polarized T-cells to shut down allergic reactions orchestrated by Th2 cells have revealed controversial results and question their potential for therapy. Adoptive transfer of Th1-polarized T-cells or systemic administration of IL-12 has in some reports shown to exacerbate ongoing allergic reactions (23-26) while others demonstrated down-regulation of ongoing allergic reactions (27). Comparisons of experimental models are complicated by the fact that several factors might influence the outcome of the studies including the genetic background of the mouse strains used, the allergens applied, the presence or absence of adjuvant administered, as well as timing of therapeutic intervention etc.

On the other hand the existence of regulatory T-cells as an important, additional T-cell subset orchestrating effector functions, could be a possible explanation why not all observed results fit to the Th1/Th2 balance hypothesis (28).

Indeed, allergic disorders might be partially attributed to a dysfunction of naturally occurring regulatory T-cells (nTreg). Thus induction and/or propagation of nTreg might be a promising therapeutic approach in allergic patients. Since the regulatory program of Treg is dependent on the engagement of the TCR/CD3 complex, in a third approach, we tested whether co-introduction of the Bet v 1-specific TCR together with FOXP3 into PB-T cells generates T-cells with regulatory properties. Reliable expression of the three transgenes was facilitated

upon introduction of two picornaviral 2A elements. As described before, the linkeage of the TCR α and β cDNAs was mediated by a porcine *Tescovirus-l* 2A peptide (P2A) and the cDNA expressing the human FOXP3 gene was linked to TCR via a *Thosea asigna* virus 2A (T2A) peptide sequence. To be able to detect and sort for positively transduced T-cells this multicistronic TCR/FOXP3 expression cassette was cloned upstream of an IRES-GFP element. After polyclonal activation using anti-CD3/CD28 coated microbeads or Bet v 1-specific aAPC these tg Treg remained hyporesponsive, which is one of the hallmarks of regulatory T-cells. Importantly, these engineered Treg displayed regulatory function in co-cultures where they suppressed TRAV6/TRBV20 tg responder T-cells after polyclonal or allergen-specific activation. In contrast, control transduced T-cells only expressing FOXP3 or naturally Treg isolated from peripheral blood, expressing their polyclonal TCR repertoire, only exerted regulatory function upon polyclonal activation but not upon Bet v 1-specific activation. These results show that the initiation of the suppressive capacity of Treg is an activation-dependent process and that the TCR integrates the antigen-specific signal into Treg function. The fully transgenic Treg approach represents several advantages: Sorting of nTreg for the $CD4^+CD25^+$ phenotype bears the risk of isolating a heterogenous T-cell population potentially also containing activated effector T-cells. This problem can be overcome by the use of additional surface markers like CD127, resulting in the isolation of a more pure Treg population, but on the other hand further decreases the already low cell yield obtainable. Therefore, the $CD4^+CD25^-$ T-cells used in the transgenic approach represents a well-defined population, which is available at much higher yields. In addition, nTreg from allergic individuals or from individuals affected by autoimmune diseases might in fact be impaired in their regulatory potential and might therefore not be the best choice for therapeutic application (29-31). On the other hand, the over-expression of human FOXP3 does not constitute the complete gene expression profile of nTreg, leaving the question open, whether the transgenic counterparts are reliable alternatives for therapeutic applications.

At the moment a frequently used mouse model in allergy research is the DO11.10 TCR tg mouse that is based on a murine TCR (32) specific for ovalbumin ($OVA_{323-339}$) in the context of a murine restriction element ($I\text{-}A^d$, *i.e.* on the BALB/c background) (33). A related model, termed OT-II, was established on the $I\text{-}A^b$ background in C57BL/6 mice (42). Although this model has been extensively used and broadened our knowledge in the understanding of the role of T-cells in allergic diseases (reviewed in (34), chicken ovalbumin does not represent a typical human-relevant aeroallergen and is rarely implicated in human asthma. Thus,

development of *in vivo* models more close to the actual pathophysiological situation was clearly warranted.

Cloning and transfer of allergen-specific TCR enabled us to rebuild the allergen-specific synapse *in vitro* in several human-relevant allergen-specific systems. In a fourth approach and to provide the basis for the evaluation of immunomodulatory strategies in a human-relevant background *in vivo* I took advantage of a human allergen-specific TCR to establish a humanized mouse model. Along those lines I generated double transgenic mice co-expressing the human Art v 1-specific TCR (TRAV17/TRBV18) and the corresponding human restriction element HLA-DR1. The variable domains of the TCR α and β chain were chimerized with the murine TCR constant regions thereby putting them under the influence of murine regulatory elements that guarantee efficient expression in mice. In addition, chimerization of the human TCR enabled proper interaction of the transgenic TCR with the murine CD4 co-receptor.

One advantage of the humanized TCR/DR1 mice is the existence of a large number of naive allergen-specific T-cells recognizing the human-relevant allergen. This enables to take a look on even very small subsets of allergen-specific T-cells and facilitates the observation of changes within these compartments during therapeutic and prophylactic interventions. In addition, imitation of the natural route of sensitization resembling the human-relevant situation by mild aerosol application protocols allows the avoidance of harsh prime-boost regimen in combination with the administration of adjuvant.

Immunophenotyping of double tg TCR/DR1 mice revealed a relatively normal distribution of leukocyte subsets in spleen, thymus and peripheral blood when compared to control wild type (wt), single tg DR1 or single tg TCR mice. Moreover, the allergen-specific TCR tg T-cells are properly selected on the HLA-DR1 background. Clear-cut expression of the tg TCR was observed on $CD3^+$ T-cells (60 % of the $CD3^+$ T-cells) in the spleen, whereas about 80 % of the peripheral blood $CD4^+$ T-cells in were TCR tg. Due to the restriction of the transgene to $CD4^+$ T-cells we observed reduced numbers of $CD8^+$ T-cells of double tg mice in spleen, thymus and peripheral blood. Moreover, T-cells isolated from the spleens of double tg mice mounted a strong allergen-specific proliferative response against the full allergenic protein, Art v 1, or the Art v $1_{25\text{-}36}$-peptide, containing the immunodominat T-cell epitope, when presented by HLA-DR1. Of note, this cellular proliferation was accompanied by a balanced cytokine milieu without a significant bias towards a certain T helper cell subset.

Most mouse models of allergic disease require systemic priming to expand the precursor pool of allergen-specific T-cells. These protocols, which most often rely on the use of adjuvant,

might bias the subsequent outcome of functional studies. For that very reason comparable mouse models using exclusive allergen-aerosol exposures without systemic sensitization as sensitization/provocation protocols are scarce. Using humanized TCR/DR1 mice with a large precursor frequency of allergen-specific T-cells eliminates the need of prior systemic sensitization with adjuvant before allergen-specific aerosol challenge, thereby closely mimicking the human pathological situation. In our model three consecutive aerosol exposures are sufficient to elicit massive airway reactivity in unprimed double tg mice in response to the specific allergen as measured by whole body plethysmography (WBP). Lung histology revealed airway and lung inflammation with massive peribronchiolar and perivascular infiltration with immune cells.

Interestingly, lung function of double tg mice started to show altered airway reactivity as soon as with the second allergen exposure, whereas all groups of control mice did not show impaired lung function at any point of intervention. These results are in accordance with previous observations form *Wilder et al.*, who demonstrated that 3 weekly OVA aerosol challenges each lasting for an hour are sufficient to induce AHR in naive heterozygous DO11.10$^{+/-}$ mice (35). Of importance, *Knott et al.* showed by using homozygous DO11.10 mice that a single OVA aerosol exposure or four OVA aerosol exposures delivered on four consecutive days failed to develop AHR, as measured by WBP and expressed in Penh (enhanced pause) in response to methacholine (36). The authors show that a single allergen-exposure of mice was accompanied exclusively with an increased influx of neutrophils and minimal recruitment of eosinophils into the lungs and the detection of mucus in the bronchial epithelium. In our model TCR/DR1 double tg mice show a diverse cellular composition of BAL fluids with high levels of granulocytes, composed of mainly eosinophils and neutrophils as well as T-lymphocytes, monocytes and alveolar macrophages following a short-term provocation protocol (three exposures on three consecutive days). In contrast, control groups of mice showed significantly different BAL compositions lacking the pronounced eosionophilic and neutrophilic components. Lung histology revealed only minor mucus production in allergen-exposed double transgenic mice, most probably caused by the fact that mucus production and structural remodeling of the lung is a chronic feature of allergic asthma, that is elicited only upon long-term exposure. Such protocols resembling clinical pathological effects are currently evaluated. Many *in vivo* results are obtained using the DO11.10 model on the BALB/c background. BALB/c are known to be high IgE-responder mice to many allergens, which makes them more allergy-prone than the C57BL/6 mouse strain. However, control experiments using systemic Art v 1-specific sensitization in the

presence of alum as adjuvant showed the induction of comparable allergen-specific IgE-levels in BALB/c mice and the double tg TCR/DR1 (C57BL/6 background) mice after three intraperitoneal injections. In contrast, four exclusive intranasal challenges in the absence of adjuvant only induced significant allergen-specific IgE titers in double transgenic mice but not in control mice. In addition, the level of lung inflammation and altered lung function can differ between distinct strains (37). Therefore, the usefulness of mouse models of allergic airway disease are often controversially debated. In fact, diverse results obtained with different mouse strains are often attributed as a general weakness of mouse models. On the other hand the dissimilar results obtained with different mouse strains reflect genetic diversity that might mirror the influence of human polymorphic genes on asthma (38). In fact, animal models have greatly expanded our knowledge regarding the pathophysiological mechanisms underlying human respiratory diseases and contributed to the development of new therapeutics. Recent technological progress allows accurate measurement of pathophysiological changes responsible for airflow obstruction and hyperresponsiveness *in vivo*. However, the complexity of the human airways is unique and differs without doubts from rodent lungs. This is underscored by the fact that only humans and a small number of other mammalian species like horses or cats develop asthma and asthma-like disorders (39-41), thus complicating the establishment of models resembling the characteristics of human disease. Therefore, there is an urgent need for the improvement of murine models in the field of allergic diseases to provide *in vivo*-model systems that reflect the human pathology as close as possible. Especially, novel settings furnished to fit well to mice as the model organism turned out to represent very helpful models to study pathophysiological processes associated with respiratory disorders *in vivo*.

In the light of these cumulative observations it is evident that numerous unresolved issues about the contribution of T-cells in allergic diseases exist. Humanized TCR tg mice should prove as model systems to understand the fundamental basics of the pathophysiology as well as to improve the development and evaluation of such T-cell based therapeutic approaches.

In conclusion, by characterization of human-relevant allergen-specific TCR we have established valuable *in vitro* and *in vivo* models to study the contribution allergen-specific T-cell responses in the development of allergic diseases. Rebuilding of the allergen-specific synapse on a molecular basis enabled us to study various aspects of allergen-specific T-cell activation regarding signal requirement, T-cell plasticity and genetic engineering of certain T-cell subsets. First immunomodulation experiments with Bet v 1-specific Th1-like T-cells

show that these polarized T-cells are potent inhibitors of allergen-specific Th2 cells. These studies were further advanced by the generation of specific T-cell subsets upon genetic engineering. Introduction of FOXP3 and the Bet v 1-specific TCR into human T-cells induced the regulatory phenotype and function comparable to nTreg. However questions about the *in vivo* applicability of polarized TCR tg T-cells or engineered Treg remain to be investigated. Therefore humanized allergy mice are a promising new model to study the allergen-specific synapse *in vivo*, which allow detailed analysis of disease-eliciting T-cell responses as well as the evaluation of the immunomodulatory potential and longevity of drug-induced or engineered T-cell subsets in prophylactic or therapeutic settings.

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Zusammenfassung

Das Ziel dieser Arbeit war es die genauen Vorgänge, durch welche allergen-spezifische T-Zellen zur Entstehung von allergischen Erkrankungen beitragen, *in vitro* und *in vivo* zu untersuchen.

Neben den IgE-produzierenden B-Lymphozyten spielen vor allem T-Lymphozyten im Krankheitsverlauf von allergischen Erkrankungen eine bedeutende Rolle. Es wurde gezeigt, dass spezielle $CD4^+$ T-Zell-Untergruppen, sogenannte Th2-Zellen, die Zytokine IL-4 und IL-13 produzieren, welche für den Antikörper-Klassenwechsel in Richtung IgE notwendig sind. T-Zellen sind aber nicht nur an der Sensibilisierungsphase beteiligt, sondern auch an den sogenannten Spät-Phase-Reaktionen, welche durch allergische Entzündungsreaktionen in den betroffenen Endorganen bedingt werden. Zusätzlich spielen regulatorische $CD4^+$ T-Lymphozyten (Treg) im Gesunden eine wichtige Rolle bei der Etablierung und Aufrechterhaltung der immunologischen Toleranz.

Die Antigen-Spezifität einer T-Zelle wird durch den T-Zell-Rezeptor (TZR) auf ihrer Oberfläche bestimmt und kann durch Gentransfer der spezifischen TZR-αβ Ketten von einer T-Zelle auf eine andere übertragen werden. Aus diesem Grund haben wir humane TZR, welche spezifisch für die beiden klinisch-relevanten Aeroallergene, Art v 1, von *Artemisia vulgaris*, das Hauptallergen des Beifusspollens und Bet v 1, *Betula verrucosa*, das Hauptallergen des Birkenpollens sind, kloniert und exprimiert. Die korrespondierenden cDNAs für die TZR und HLA-Moleküle wurden aus T-Zell-Klonen und EBV-transformierten B-Zellen aus allergischen Individuen gewonnen. Durch Gentransfer von Allergen-spezifischen TZR in humane T-Zell-Linien und T-Zellen aus dem peripheren Blut (PB) von allergischen und nicht allergischen Individuen konnten transgene Allergen-spezifische T-Zellen erzeugt werden. Um die "Allergen-spezifische Synapse", welche zwischen einer Antigen-präsentierenden Zelle (APZ) und einer T-Zelle gebildet wird, *in vitro* nachstellen zu können, wurden artifizielle APZ (aAPZ) verwendet, welche durch transiente Transfektion von Zellen der humanen Zelllinie 293 mit den entsprechenden HLA-Molekülen, den allergenen Peptiden und den kostimulatorischen Signalen hergestellt wurden.

Mit Hilfe des Art v 1-spezifischen TZR konnten wir zeigen, dass wir in kurzer Zeit große Mengen an Allergen-spezifischen T-Zellen herstellen können, unabhängig vom Allergie-Status des Spenders oder der Allergie-Saison. Der retrovirale Transfer der TZR αβ-Ketten in die humane T-Zelllinie Jurkat oder humane PB T-Zellen von nicht allergischen Individuen

führte zu einer deutlichen Expression des transgenen TZR auf der Oberfläche. Die transgenen Allergen-spezifischen T-Zellen ähnelten dem urspünglichen T-Zellklon (SSR20) in ihrer Spezifität und konnten nur durch Aktivierung mit autologen, Art v 1_{25-36} Peptid-präsentierenden EBV-Zellen oder aAPZ, aktiviert werden. Darüber hinaus war die Aktivierung von transgenen (tg) T-Zellen vom Vorhandensein eines kostimulatorischen Signals abhängig.

Neben aAPZ haben wir auch Erfahrung mit der Erzeugung und Verwendung von künstlichen Antigen-präsentierenden Plattformen ('virus-like particles', VLP) gesammelt. VLP können mit HLA/Peptid-Komplexen und kostimulatorischen Molekülen ausgestattet werden und daher die Rolle von aAPZ übernehmen. Unter anderem konnten wir zeigen, dass VLP, welche das allergene Peptid und das kostimulatorische Molekül CD80 exprimieren zu einer massiven Expansion von Antigen-spezifischen T-Zellen führen.
In einem Nebenprojekt, das gemeinsam mit Kollegen der Harvard Medical School in Boston durchgeführt wurde, haben wir gezeigt, dass es möglich ist zelleigene Proteine für die Detektion und Aufreinigung von Hüllenviren zu verwenden. Die Membran eines umhüllten Virus stammt von der infizierten Zelle ab und enthält daher nicht nur deren Lipid-Doppelschicht sondern auch zelleigene Oberflächenproteine. Der Zusammenbau der Virusbestandteile und die nachfolgende Sprossung des fertigen Viruspartikels von der Zelle findet in den sogenannten "Lipid Raft-Regionen" der Wirtszellmembran statt. Lipid Rafts sind Cholesterin-reiche Mikrodomänen der Zellmembran. Modifizierung von Reporterproteinen mit einem Lipid Raft-Anker, z.B.: Glykosylphosphatidylinositol (GPI) sorgt dafür, dass das Reporterprotein in die Lipid Rafts geschleust wird und sich nach Virussprossung an der Oberfläche des Viruspartikels befindet. In dieser Studie konnten wir zeigen, dass mit Hilfe einer GPI-verankerten alkalinen Phosphatase als Reporterenzym die Bildung von verschiedenen Hüllenviren (RSV, HSV1, HIV und humaner Coronavirus 229E) nachgewiesen werden kann. Diese Strategie stellt daher nicht nur eine verlässliche Methode dar, um bekannte und unbekannte Hüllenviren zu isolieren, sondern ermöglicht auch eine rasche Virusdetektion.

In einem weiteren Teil dieser Arbeit konnten wir zeigen, dass durch den Transfer des Bet v 1-spezifischen TZR die Feinspezifität des ursprünglichen T-Zellklons (SD334) beibehalten wird. Um eine gemeinsame und vergleichbare Expression der beiden T-Zell-Rezeptor Ketten zu gewährleisten wurden die spezifischen cDNAs durch ein retrovirales 2A-Element

miteinander verbunden, welches die Translation von einem gemeinsamen Transkript erlaubt. Die Birkenpollen-Allergie ist von höchster klinischer Relevanz, häufig leiden Birkenpollen-Allergiker am sogenannten "oralen Allergie Syndrome" (OAS), welches durch Kreuzreaktivität mit Birkenpollen verwandten Baumpollen- und Nahrungsmittelallergenen entsteht. Die transgenen T-Zellen zeigten vergleichbare Kreuzreaktivität mit den Bet v 1-verwandten Nahrungsmittelallergenen von Sellerie (Ap g 1) und Karrotte (Dau c 1) sowie mit den Bet v 1-verwandten Baumpollenallergenen von Haselnuss (Cor a 1) und Hainbuche (Car b 1). Diese Experimente haben eindeutig bewiesen, dass transgene T-Zellen ein verlässliches Werkzeug darstellen, um die stetig steigende Anzahl an rekombinanten und/oder modifizierten Allergenen auf ihre immunologische Wirkung zu testen.
Ein möglicher Erklärungsansatz für die Entstehung von allergischen Erkrankungen liegt in dem Ungleichgewicht der beiden T-Helfer (Th) Zell-Subgruppen Th1 und Th2. In dieser Hypothese wird die Allergie durch überschießende Th 2-Antworten erklärt. Eine Strategie, um die pathologische Reaktion von allergen-spezifischen Th2-Zellen zu unterbinden, ist die Immunantwort von allergen-spezifischen Th1-Zellen zu fördern, welche den allergen-spezifischen Th2-Zellen entgegenwirken. Ein wichtiger Faktor, der zur Entstehung von Th1-Zellen führt, ist IL-12. Zur Polarisierung von allergen-spezifischen transgenen T-Zellen haben wir IL-12 mit einem GPI-Membrananker ausgestattet, sodass es auf der Oberfläche von aAPZ exprimiert wird. Allergen-spezifische Stimulation mit IL-12 ko-exprimierenden APZ führte zur Polarisierung von T-Zellen zu Th1-Zellen, die durch ausschließliche Sekretion des Th1-assoziierten Zytokins IFN-γ identifiziert wurden. Weiters haben Experimente mit Kokulturen der beiden T-Helfer Subsets gezeigt, dass tg Bet v 1-spezifische Th1-polarisierte T-Zellen die Effektorfunktion von allergen-spezifischen Th2-Zellen inhibieren können.

Eine weitere Erklärungsmöglichkeit für die Entstehung von Allergien kann im Fehlen von natürlich-vorkommenden regulatorischen T-Zellen (nTreg) begründet sein. Treg haben unter anderem die Aufgabe T-Zell-Antworten zu unterbinden, daher scheint die Förderung und Expansion von allergen-spezifischen Treg in allergischen Patienten ein viel versprechender Therapieansatz zu sein. Für die Induktion des regulatorischen Programms ist die Aktivierung der Treg über ihren TZR/CD3 Komplex notwendig. Daher haben wir getestet, ob die gemeinsame Einführung des Bet v 1-spezifischen TZR gemeinsam mit dem Transkriptionsfaktor Forkhead Box Protein 3 (FOXP3), dem Masterregulator für Treg-Ausbildung zur Entstehung von transgenen allergen-spezifischen Treg führt. Verlässliche Expression der drei Transgene wurde durch Verbindung mit 2A-Elementen von Picornaviren

erreicht. Die so hergestellte entstandene multicistronische Expressionskassette wurde vor das 5'-Ende eines IRES-GFP Elements kloniert, welches als Fluoreszenzmarker die schnelle und zuverlässige Isolierung von transgenen Zellen ermöglicht. Retrovirale Transduktion von $CD4^+$ T-Zellen mit dem Multicistron führte zur Ausbildung von T-Zellen mit regulatorischem Phänotyp und regulatorischer Funktion, vergleichbar mit den Eigenschaften von nTreg. Transgene Treg wiesen dabei eine hohe Oberflächenexpression von CD25, CD39 und CTL-4 auf sowie eine niedrige Oberflächenexpression von CD127, ein Erscheinungsbild das typisch für nTreg ist. Neben der vergleichbaren Oberflächenexpression zeichneten sich TCR tg Treg durch vermindertes Ansprechen auf antigen-spezifische und polyklonale Aktivierungssignale aus. Darüber hinaus zeigten TZR tg Treg starkes regulatorisches Potential in Kokulturen mit allergen-spezifischen Effektor-T-Zellen. Nach Allergen-spezifischer Aktivierung konnten tg Treg das Wachstum und auch die Effektorfunktion von allergen-spezifischen T-Zellen signifikant unterdrücken. Mit Hilfe dieser Experimente konnten die bekannten Beobachtungen, wie etwa dass regulatorische T-Zellen aktivierungsabhängig ihre regulatorischen Eigenschaften entfalten, bestätigt werden. Nur TZR^+FOXP3^+ tg T-Zellen konnten nach einer Allergen-spezifischen Aktivierung ko-kultivierte Allergen-spezifische T-Zellen hemmen. Im Gegensatz dazu zeigten transgene $FOXP3^+$ T-Zellen, welche einen nicht-spezifischen TZR exprimierten, keine suppressive Wirkung.

Um die unterschiedlichen Aspekte der Allergieentstehung und deren Beeinflussung *in vivo* testen zu können, wurden in der Vergangenheit mehrere Mausmodelle entwickelt. Eines der am häufigsten verwendeten Tiermodelle um z.B. Asthmainduktion und -therapie zu erforschen, ist die transgene DO11.10 Maus, deren T-Zellen einen T-Zellrezeptor (TZR) exprimieren, welcher Hühnerei-Albumin (Ovalbumin) im Kontext von Maus MHC Klasse II ($I\text{-}A^d$) erkennt. Ovalbumin ist jedoch nur sehr selten an der Entstehung von Asthma bronchiale im Menschen beteiligt, da es sich um kein human-relevantes respiratorisches Allergen handelt. Daher erscheinen humanisierte Tiermodelle, welche spezifisch für klinisch relevante Allergene sind, für die Erforschung, der durch Allergien verursachten Erkrankungen im Menschen, wesentlich sinnvoller zu sein.

Um die Aktivierung von allergen-spezifischen T-Zellen *in vivo* besser analysieren zu können, haben wir ein doppelt-transgenes Mausmodell entwickelt, welches sowohl den humanen TZR, welcher spezifisch für das immundominante T-Zellepitop von Art v 1 (Art v $1_{25\text{-}36}$) ist, als auch das dazugehörige Restriktionselement (HLA-DRA*01:01/DRB*01:01) exprimiert. *In*

vitro-Versuche mit Milzzellen von doppelt-transgenen TZR/DR1 Mäusen haben gezeigt, dass der transgene TZR deutlich auf der Oberfläche von CD3 positiven T-Zellen nachweisbar ist. In Gegenwart von syngenen APZ und Inkubation mit dem Gesamtallergen (Art v 1-Protein) oder Allergenpeptid (Art v 1_{25-36}), welche jeweils das immun-dominante T-Zellepitop beinhalten, weisen T-Zellen der tg TZR/DR1 Maus ein deutliches Zellwachstum auf. Ein bedeutender Vorteil von humanisierten "Allergiemäusen" ist die hohe Vorläuferfrequenz von naiven allergen-spezifischen T-Zellen. Dadurch können einerseits auch sehr feine Änderungen in T-Zellkompartimenten während einer prophylaktischen und/oder therapeutischen Behandlung nachgewiesen werden. Andererseits können durch die bereits existierenden naiven allergen-spezifischen T-Zellen harsche "prime-boost" Protokolle durch milde Sensibilisierungs- und Provokationsmethoden z.B.: Inhalation, welche der physiologischen Situation im Menschen ähneln, ersetzt werden.
In vivo-Evaluierung verschiedener Maus-Gruppen hat gezeigt, dass nach Sensibilisierung mit dem Allergen nur doppelt transgene TZR/DR1 Mäuse eine erhöhte Atemwegs-Reaktivität nach Exposition mit dem Allergen oder dem Bronchokonstriktor Methacholin aufwiesen. Drei aufeinander folgende Expositionen mit dem vernebelten Allergen waren ausreichend, um eine veränderte Atemwegsfunktion zu erzielen, welche von einer Entzündung der Atemwege und der Lunge begleitet wurden. Eine massive Akkumulation von inflammatorischen Immunzellen in der bronchoalveolaren Lavage sowie peribroncholäre und perivaskuläre Infiltration der Lunge konnten beobachtet werden.
In künftigen Experimenten soll geklärt werden, wie sich die Menge an vernebeltem (Antigen) Allergen, das Vorhandensein von bestimmten Mengen an Allergen-spezifischen T-Zellen sowie die Exposition gegenüber Allergen-Varianten (Hypoallergene, Peptide, Allergoide, etc.) sowie unterschiedlichen Immunisierungsrouten auf die Lungenfunktion auswirken.

Allergen-spezifische T-Zellen sind ein viel versprechendes Werkzeug um neuartige immunmodulatorische Strategien zur Behandlung oder Verminderung von Allergien zu entwickeln. Sie stellen ein verlässliches Werkzeug dar mit dem nicht nur neue rekombinante/modifizierte Allergene schnell und effizient getestet werden können, sondern ermöglichen auch das bestehende Wissen über wichtige Vorgänge in der T-Zell Biologie (Aktivierung, Polarisierung, Plastizität) zu erweitern. In diesem Sinn stellen humanisierte Maus-Allergie-Modelle für die Evaluierung von neuen human-relevanten Allergen-Formulierungen und Interventionsprotokollen essentielle Innovationen dar und werden einen

entscheidenden Beitrag zum besseren Verständis der allergischen Erkrankung und deren Therapien leisten.

Curriculum Vitae

NAME

Alina NEUNKIRCHNER

PERSONAL DATA

Date of birth: 13th June 1982
Place of birth: Linz, Austria
Nationality: Austria

CURRENT WORK ADDRESS

Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology,
Medical University of Vienna
Borschkegasse 8A,
A-1090 Vienna, Austria
alina.neunkirchner@meduniwien.ac.at

SCIENTIFIC EDUCATION AND CAREER HISTORY

1988 - 1992	Primary School, Schulstraße 5, Neuhofen/Krems, Austria
1992 - 2000	High School, Bundesrealgymnasium Traun, Schulstraße 59, Traun, Austria
2000	A-Level, Bundesrealgymnasium Traun, Austria
2000 - 2006	Studies in Genetics and Microbiology, University of Vienna, Austria
2002	Practical training for 2 months, Department of Hematology at the Erasmus MC Rotterdam (group of Prof. Dr. Ivo Touw and Dr. Marieke von Lindern), Rotterdam, The Netherlands

2005 - 2006	Diploma Thesis at the Institute of Immunology, Medical University Vienna, Austria
2006	M.Sc. Degree with distinction in Genetics and Microbiology, University of Vienna, Austria
2006 - present	PhD thesis, Institute of Immunology, Medical University of Vienna, Austria

SCIENTIFIC PUBLICATIONS

1. Schmetterer K., **Neunkirchner, A**. and W. F. Pickl. 2012. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *Faseb J 26:2253-76.*
2. **Neunkirchner, A**., V. Leb-Reichl, K. Schmetterer, S. Mutschlechner, H-J. Kueng, D. Haiderer, K. Schuch, M. Wallner, B. Jahn-Schmid, B. Bohle, and W. F. Pickl. 2011. Human TCR transgenic, Bet v 1-specific T helper 1 cells suppress the effector function of Bet v 1-specific T helper 2 cells. *J Immunol 187:4077-87.*
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PARTICIPATION IN SCIENTIFIC MEETINGS

December 1th-3rd, 2005	Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Graz, Austria (*poster presentation)*
June 10th - 14th, 2006	XXV Congress of the European Academy of Allergology and Clinical Immunology, Vienna, Austria (*poster presentation)*
September 7th-9th, 2006	1st Joint Meeting of European National Societies of Immunology, Paris, France *(oral presentation)*
December 7th-9th, 2006	Clemens von Pirquet Symposium, Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Vienna, Austria (*poster presentation)*
December 13th-15th, 2007	Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Alpbach, Tyrol, Austria *(poster presentation)*
September 3rd-6th, 2008	Joint annual meeting of Immunology of the Austrian and German Societies (ÖGAI and DGfI), Vienna, Austria *(oral presentation)*
September 13th-16th, 2009	2nd European Congress of Immunology ECI 2009, Berlin, Germany *(poster presentation)*
November 6th-7th, 2009	Karl Landsteiner Meeting, Salzburg, Austria *(poster presentation)*

June 5th-9th, 2010	29th Congress of the European Academy of Allergy and Clinical Immunology (EAACI), London, United Kingdom (*poster presentation)*
December 3rd-5th, 2010	Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Vienna, Austria (*oral presentation)*
February 3rd-6th, 2011	9th ECCCI GA^{2}LEn Immunolgy Winter School, Basic Immunology Research in Allergy, February 3-6, 2011, Davos, Switzerland (*poster presentation)*
September 15th-17th, 2011	Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Graz, Austria *(poster presentation)*
December 4th-8th, 2011	XXII World Allergy Congress, Cancun, Mexico (*oral presentation)*
September 5th-8th, 2012	European Congress of Immunology ECI 2012, Glasgow, Scotland *(poster presentation)*
November 30th, 2012	Annual meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Klosterneuburg, Austria *(poster presentation)*
May 3rd-7th, 2013	Immunology 2013, AAI Annual meeting, Honolulu, Hawaii, USA (*oral* and *poster presentation*)

SOCIETY MEMBERSHIPS

Since 2005	Austrian Society for Allergology and Immunology (ÖGAI)
Since 2010	European Academy of Allergology and Clinical Immunology (EAACI)
Since 2012	The American Association of Immunologists (AAI)

TRAVEL AWARDS

2006	ÖGAI travel grant for 1st Joint Meeting of European National Societies of Immunology in Paris, France
	EFIS travel grant 1st Joint Meeting of European National Societies of Immunology in Paris, France
2009	ÖGAI travel grant for the 2nd European Congress of Immunology in Berlin, Germany

2010	ÖGAI travel grant for the 29th Congress of the European Academy of Allergy and Clinical Immunology (EAACI), London, United Kingdom
2011	EAACI travel grant for the 9th EAACI-GA²LEN Immunology Winter School in Davos, Switzerland
	World Allergy Organization travel grant for the XXII World Allergy Congress, Cancun, Mexico
2012	ÖGAI travel grant for the XXII World Allergy Congress in Cancun, Mexico
2013	AAI travel grant for the AAI Annual Meeting, Immunology 2013, Honolulu, Hawaii, USA

AWARDS

2006	Achievement Scholarship, University of Vienna
2009	Poster Prize of the Austrian Society of Allergology and Immunology (ÖGAI)
2010	Poster Prize of the JMA of the European Academy Allergy and Clinical Immunology (EAACI)
2012	Poster Prize of the Center for Pathophysiology, Infectiologie and Immunology at the 3rd center retreat
2012	Clemens of Pirquet Prize of the Austrian Society of Allergology and Immunology (ÖGAI)