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DISSERTATION

Modulation of allergen-specific T-cell responses using
novel antigen-presenting platforms and transgenic
regulatory T cells

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Abstract

T-lymphocytes play a pivotal role in the orchestration of allergic immune responses and thus critically contribute to immediate phase reactions as well as late phase allergic reactions. Consequently, strategies to modulate the pathological T helper 2 (Th2)-biased response might be of therapeutic benefit for allergic individuals. However, until today model systems for allergic T-cell responses based on human-relevant allergens and the corresponding human restriction elements and T-cell receptors (TCR) are still missing. For that reason we cloned and functionally characterized such relevant molecules involved in antigen-presentation and recognition (i.e. the HLA restriction elements and the corresponding TCR alpha and beta chains) from mugwort pollen and birch pollen allergic individuals. By generation of artificial antigen-presenting cells (APC) based on transfection of 293 cells with the HLA molecules, allergenic peptides and costimulatory molecules in concert with retroviral transduction of human T-cells with corresponding TCR we succeeded in rebuilding the 'allergen-specific synapse'. This defined *in vitro* model system allowed us to study signal requirements and plasticity of allergen-specific T-cells. In fact, we could show that TCR transgenic T-cells were strictly dependent on presentation of cognate peptide and costimulatory signals to mount their effector functions. Using this system we tested the capacity of 293 cell-derived virus-like particles (VLP) with distinct compositions of HLA/allergen complexes and accessory molecules to modulate allergen specific T-cell responses. In this context we could demonstrate that allergen-presenting VLP were perfectly suited to assume the role of antigen-presenting cells and to expand, anergize or polarize TCR transgenic T-cells depending on their pre-defined set-up. Similarly, decoration of VLP with the high affinity interleukin-2 receptor allowed for the depletion of IL-2 from T-cell cultures thus mimicking an important effector mechanism of regulatory T-cells (Treg). In a different approach we hypothesized that ectopic expression of transgenes associated with Treg function would enable us to develop also allergen-specific Treg. To that end we applied a multicistronic expression strategy, putting the α and β chains of a Bet v 1 (birch pollen allergen)-specific TCR plus Foxp3, the master regulator of Treg function, along with an IRES-GFP cassette under the control of a retroviral to transduce CD4⁺CD25⁻ T-cells. These TCR/Foxp3 transgenic T-cells displayed the typical phenotypic profile of Treg, showed hyporesponsiveness in response to polyclonal and allergen-specific stimulation and were able to suppress the effector function of Bet v 1-specific T-cells in an activation-dependent manner. The establishment

of such novel T-cell modulatory strategies based on artificial antigen-presenting platforms and regulatory T-cells could possibly lead to novel therapeutic applications in allergic diseases and other immune-mediated disorders.

Kurzbeschreibung

T-Lymphozyten spielen eine wichtige Rolle in der Regulation von allergischen Immunantworten und tragen daher entscheidend sowohl zur Sofort-Typ Reaktion als auch zu den allergischen Spät-Phasen Reaktionen bei. Daher könnten Strategien, die darauf abzielen, die pathologische T Helfer 2 (Th2)- Antwort zu verändern von therapeutischem Wert für allergische Individuen sein. Dennoch fehlen bis heute Modell-Systeme für Allergen-spezifische T-Zell Antworten, die auf human-relevanten Allergenen und den dazugehörigen HLA Restriktionselementen und T-Zell Rezeptoren (TZR) basieren. Daher klonierten und charakterisierten wir solche relevanten Moleküle, die in Antigen-Präsentation und -Erkennung involviert sind (das sind die präsentierenden HLA-Moleküle und die dazu gehörigen TZR α and β Ketten) aus dem Blut von Beifusspollen und Birkenpollen Allergikern. Indem wir 293 Zellen mit den HLA Molekülen, den Allergenen und kostimulatorischen Molekülen transfezierten, und gleichzeitig durch retrovirale Transduktion von humanen T-Zellen mit dem dazugehörigen TZR allergen-spezifische T-Zellen herstellten, gelang es uns, die ‚Allergen-spezifische Synapse‘ auf molekularer Ebene zu rekonstruieren. Dieses definierte *in vitro* Modellsystem erlaubte uns das Verhalten und die Plastizität von allergen-spezifischen T-Zellen nach Stimulation mit unterschiedlichen Konditionen zu untersuchen. Tatsächlich konnten wir zeigen, dass TZR transgene T-Zellen auf die Präsentation des korrekten Peptids und auf kostimulatorische Signale angewiesen waren, um ihre Effektor-Funktion initiieren zu können. In diesem System testeten wir zunächst, ob Virus-ähnlichen Partikeln (‚virus-like particles‘; VLP), die wir in 293 Zellen produzierten und mit definierten Zusammensetzungen an HLA/Allergen Komplexen und accessorischen Molekülen ausstatteten, die Kapazität hatten, allergen-spezifische T-Zellen zu modulieren. In diesen Studien konnten wir beobachten, dass allergen-präsentierende VLP perfekt geeignet waren, die Rolle von antigen-präsentierenden Zellen zu übernehmen, und TZR transgene T-Zellen zu expandieren, zu anergisieren oder zu polarisieren, abhängig von der vordefinierten molekularen Ausstattung. Außerdem konnten VLP, die mit dem hochaffinen Interleukin-2 (IL-2) Rezeptor ausgestattet waren, dieses Zytokin in T-Zell Kulturen neutralisieren, womit sie eine wichtige Effektor-Funktion von regulatorischen T-Zellen (Treg) übernehmen konnten. In einem anderen Ansatz verfolgten wir die Hypothese, ob Transfer von Genen, die mit der Funktion von Treg assoziiert sind, uns erlauben würde, allergen-spezifische Treg zu generieren. Dafür verwendeten wir eine multicistronische Expressions

Strategie, indem wir die α und β Kette eines Bet v 1 (Birkenpollen-Allergen)-spezifischen TZR über 2A Elemente mit Foxp3, dem Hauptregulator-Gen der Treg Funktion, verbanden. Diese Expressionskassette wurde gemeinsam mit einem IRES-GFP Element in einen retroviralen Expressionsvektor kloniert und in $CD4^+CD25^-$ T-Zellen transduziert. Diese Foxp3/TZR transgenen T-Zellen zeigten das charakteristische phänotypische Profil von Treg und stark verminderte Proliferation nach allergen-spezifischer und polyklonaler Stimulation, und konnten Bet v 1-spezifische T-Zellen Aktivierungs-abhängig regulieren. Die Etablierung von solchen neuen T-Zell modulierenden Strategien basierend auf artifiziellen Antigen-präsentierenden Plattformen und regulatorischen T-Zellen könnte zur Entwicklung von neuen Therapien für die Behandlung von Allergien, aber auch von Autoimmunerkrankungen und Episoden von Transplantatabstossung, führen.

Structure of the thesis

The **Introduction** provides a brief overview about the role of T-lymphocytes in allergic reactions, the topic of immunomodulation and the aims of this thesis.

Chapter 1 gives an in depth review about the current knowledge of regulatory T-cells with special emphasis on strategies to propagate, induce or generate regulatory T-cells. This manuscript has been invited to be submitted to the *FASEB Journal*.

Contribution: study of the literature, preparation of the manuscript; first author

Chapter 2 describes the generation of an *in vitro* model system for human allergen-specific T-cell responses. To that end, the relevant HLA restriction element (HLA-DR1), the allergen-derived peptide (Art v 1₂₅₋₃₆) and a corresponding human T-cell receptor (TRAV17/TRBV18) were cloned. T-cell receptor transgenic Jurkat T-cells and peripheral blood T-cells were analyzed for their antigen-specificity and costimulation-dependency. The resulting manuscript was published in *J Allergy Clin Immunol* 2008; 121:64-71

Contribution: establishment of retroviral transduction protocols for peripheral blood T-cells, phenotypic analysis of transgenic peripheral blood T-cells, T-cell proliferation experiments, contribution to the analyses of data and the preparation of the manuscript; co-author

Chapter 3 deals with the potential of virus-like particles as artificial allergen-presenting platforms to modulate human allergen-specific T-cell responses *in vitro*. The capacity of virus-like particles decorated with HLA-DR1 presenting Art v 1₂₅₋₃₄ in concert with different combinations of costimulatory molecules to anergize or polarize Art v 1-specific T-cells was analyzed. The obtained results were published in *J Allergy Clin Immunol* 2009; 124:121-128

Contribution: establishment of T-cell anergization and polarization protocols, provision of allergen-specific transgenic T-cells, T-cell proliferation, restimulation and polarization

assays, contribution to the analyses of data and the preparation of the manuscript; co-author

In the **Chapter 4** we assessed, in a project not directly related to the modulation of allergen-specific T-cells, the molecular requirements for HLA Class II expression using B-cell lines from seven HLA Class II deficient individuals. We especially focused on phenotypic differences regarding invariant chain expression in B-cell lines from the four known different complementation groups. The resulting observations were published in the *Int Arch Allergy Immunol* 2010; 152:390-400

Contribution: establishment of B-cell lines, cloning of CIITA and RFX, retroviral complementation and phenotypic analyses of B-cell lines, preparation of genomic DNAs for sequencing, statistical analyses and contribution to the preparation of the manuscript; first author

Chapter 5 addresses the feasibility to decorate virus-like particles with multimeric receptors such as the interleukin-2 receptor. Binding kinetics of VLP decorated with IL-2R variants of differential affinity were analyzed. Furthermore, the potential of virus-like particles decorated with the high-affinity IL-2R to deplete IL-2 from activated T-cell cultures and the consequently induced T-cell hyporesponsiveness were assessed. The resulting manuscript was published in *FASEB J* 2010; 24:1572-1582

Contribution: provision of allergen-specific transgenic T-cells, T-cell proliferation and restimulation assays, contribution to the analyses of data and preparation of the manuscript; co-author

Chapter 6 describes the generation of transgenic allergen-specific regulatory T-cells by co-introduction of a Bet v 1₁₄₂₋₁₅₃ specific TCR in concert with Foxp3 using a multicistronic expression element. Human TCR/Foxp3 transgenic peripheral blood CD4⁺ T-cells were analyzed for their proliferation, cytokine secretion and suppressive capacity towards allergen-specific effector T-cells. The resulting observations were published in the *J Allergy Clin Immunol* 2009; 127:238-245

Contribution: construction of multicistronic expression elements and retroviral vectors, analyses of transgene expression and phenotype, T-cell proliferation and suppression assays, the analyses of data and the preparation of the manuscript; first author

The chapter **Conclusion and Synopsis** outlines the essential findings and gives a synopsis of the thesis. Furthermore, the ‘open ends’ of this research project are thoroughly discussed.

Introduction

Under physiological conditions a delicate balance between different CD4⁺ T-cell subsets governs adaptive immune responses to foreign antigens. The interplay of T helper subsets (such as Th1, Th2 and Th17) in combination with naturally-occurring and inducible T regulatory cell (Treg) subsets guarantees an appropriate response which serves to eradicate potential pathogens while keeping the integrity of the organism intact. Consequently, imbalances in the CD4⁺ T-cell compartment can promote autoinflammatory, autoimmune and allergic disorders. Indeed, the T-cell response to allergens is dominated by Th2 polarized CD4⁺ T-cells¹ and an apparent lack of Treg numbers and/or function². Following allergen-specific activation, the secretion of Th2 cytokines such as IL-4, IL-5 and IL-13 mediates IgE class switching, activation of eosinophils and basophils and hyperreactivity of epithelial tissues, thereby contributing to the sensitization phase as well as to late-phase reactions and allergic inflammation³ (Fig.1). In this light, strategies to modulate the Th2 CD4⁺ T-cell response to allergens or to induce allergen-specific Treg should present as promising therapeutic options. Evidence for this notion also comes from observations in patients undergoing specific immunotherapy (SIT), in whom amelioration of clinical symptoms was accompanied by an increase in allergen-specific Treg⁴⁻⁷ as well as Th1 polarized CD4⁺ T-cells⁸⁻¹⁰ (and reviewed in *Larche et al*¹¹).

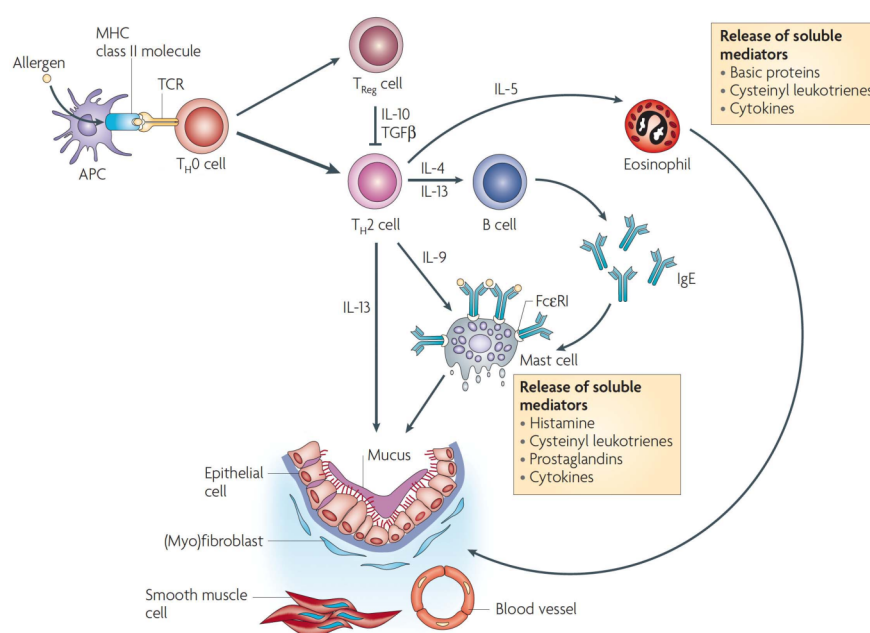


Fig.1 Role of T helper 2 (Th2) cells in allergic immune responses. In allergic individuals, processed allergen is presented to unpolarized CD4⁺ T helper cells by antigen - presenting cells (APC), which induces polarization. Consequently, release of Th2 - specific cytokines promotes IgE class switching in B-cells, activation of eosinophils and mast cells and metaplasia and mucus production in epithelial cells.

(adapted from *Holgate and Polosa, Nat Rev Immunol 2008; 8:218-230*)

Although the central importance of CD4⁺ T-cells in allergic responses is thus indisputable, molecular information about the actual composition of the ‘allergen-specific synapse’ are still scarce. Recent efforts have identified HLA restriction and allergen-derived T-cell epitopes for several clinically relevant allergens¹²⁻¹⁶, yet the T-cell side of the synapse has been largely uncharacterized. Consequently, we set out to clone and ectopically express allergen-specific T-cell receptors derived from allergic patients in order to rebuild the allergen-specific synapse at the molecular level and create an *in vitro* model system to study allergen-specific T-cell activation and modulation.

Transfer of TCR $\alpha\beta$ genes to generate large numbers of antigen-specific T-cells was established in the mid 1980ies¹⁷. Since then, numerous TCR-transgenic murine model systems have been developed and have helped to elucidate mechanisms of T-cell development^{18, 19} and the immune response to autoantigens^{20, 21} and infectious diseases²². More recently, techniques to transfer human TCR $\alpha\beta$ genes have been reported as a novel tool to generate large numbers of tumor-reactive CD8⁺ T-cells²³⁻²⁵. Importantly, those studies could show that TCR-transfer led to the desired transfer of antigen-specificity while leaving key physiological properties of T-cells, such as requirement for costimulation, intact.

As model allergens we chose the major mugwort pollen allergen Art v 1 and the major birch pollen allergen Bet v 1, which both represent clinically relevant entities in the European and American temperate climate zone²⁶. Art v 1 constitutes an ideal model allergen as more than 95% of mugwort pollen allergic patients are sensitized to Art v 1 and the T-cell response is dominated by one immunodominant epitope, Art v 1₂₄₋₃₆. Additionally, several studies have indicated that presentation of this peptide is highly associated with HLA-DRB1*01^{12, 14}. Consequently, we cloned a TCR (TRAV17/TRBV18), which recognizes Art v 1₂₄₋₃₆ in the context of HLA-DR1. In contrast, the T-cell response to birch pollen allergens is more diverse with three frequently recognized epitopes (Bet v 1₄₋₁₅, Bet v 1₁₁₂₋₁₂₃, Bet v 1₁₄₂₋₁₅₃) contained within the major birch pollen allergen Bet v 1^{15, 16}. Similarly, restriction elements are more diverse. For initial studies with focused on a TRAV6/TRBV20 T-cell receptor, which recognizes the C-terminal Bet v 1₁₄₂₋₁₅₃ peptide presented by HLA-DR7.

Once appropriately activated via the TCR, the fate of the T-cell response is dependent on the milieu of costimulatory and accessory signals²⁷. Antigen-presentation in the absence of costimulation induces a state of hyporesponsiveness, called ‘anergy’ in T-cells^{28, 29}, which serves as a major mechanism of T-cell tolerance. Similarly, qualitative differences in costimulation have been described to differentially shape the ensuing T-cell response. In this respect, costimulation solely via the CD58-CD2 axis induces a phenotype characterized by the production of high levels of IL-10 and IFN- γ and low levels of IL-2 and IL-4³⁰. This phenotype is of special interest, as it resembles T regulatory type I cells, which have been implied to play a role in the regulation of immune responses in allergy^{4, 5}.

11.

Apart from APC derived signals, the T-cell response is also influenced by the interplay with different T helper subsets and by regulatory T-cells populations, which can suppress T-cell activation even under optimal stimulation from APC³¹. Consequently, multiple points of modulation exist during a T-cell response, all of them representing potential targets for therapeutic intervention.

In order to assess the modulatory potential of artificial antigen-presenting platforms, we relied on our recently described virus-like particle (VLP) technique^{32, 33} (Fig.2). In these studies we had proven the feasibility to decorate lipid-raft derived VLP with immunostimulatory factors such as HLA molecules, costimulatory ligands, accessory molecules³³ and cytokines³² by anchoring them to GPI-anchor acceptor sequences. Thus equipped VLP constitute modular artificial antigen-presenting platforms that can be decorated with a distinct composition of molecules which allows to shape the VLP induced T-cell response. First formal proof for this capacity was obtained in *in vitro*³³ as well as in *in vivo*³² experiments using antigen-specific activation and lytic function of CD8⁺ T-cells as readout. We hypothesized that HLA Class II decorated VLP should be equally capable of presenting antigen and modulating the effector function of allergen-specific CD4⁺ T-cells. Apart from costimulation via CD80, which promotes robust T-cell activation and thus represents an ideal reference molecule, we focused on allergen-presentation in the absence of costimulation to induce anergy and on CD58 costimulation to induce the above-mentioned Tr1-like T-cell phenotype.

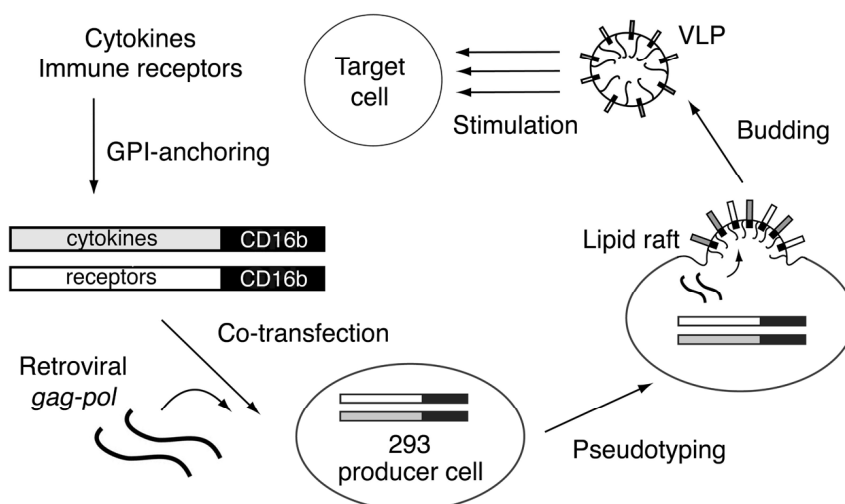


Fig. 2. Scheme for production of cytokine-decorated VLP. Cytokines or immune receptors of interest are modified by GPI anchor acceptor sequences, which, upon transfection into 293 cells, target them to lipid rafts of the plasma membrane. Coexpression of original MoMLV *gag-pol* (OGP) induces the secretion of plasma membrane-derived VLP by the producer cell line. VLP harvested from the supernatant can be used directly or purified by ultracentrifugation in order to stimulate target cells. (adapted from Kueng *et al.*, *J Virol* 2007; 81:8666-76)

Due to their capacity to also accommodate multimeric molecules, VLP are potentially suited to provide, apart from serving as artificial APC, other functions of the immune system. We aimed to use this capacity of VLP to deplete the important survival factor IL-2 from cultures of activated T-cells, thereby mimicking one of the key effector mechanisms of regulatory T-cells³⁴. For that purpose, VLP equipped with the high affinity IL-2 receptor were assessed for their binding capacity and their influence on antigen-specific T-cell activation.

In a second line of strategy we planned to assess the potential of our system to generate allergen-specific regulatory T-cells by genetic engineering. We based our working hypothesis on recent key findings that ectopic overexpression of Foxp3 in CD4⁺ T-cells was sufficient to induce a naturally-occurring Treg (nTreg)-like phenotype and function^{35, 36}. An important feature of nTreg is their property to exert their suppressive function strictly upon appropriate activation via the TCR/CD3 complex^{31, 37, 38}. In this light we hypothesized that co-expression of an allergen-specific TCR in concert with Foxp3 would generate allergen-specific Treg with the TCR serving as a molecular ‘on-switch’ and Foxp3 enforcing the regulatory program. In the light of experimental data that favor the

use of antigen-specific Treg for therapeutic application, strategies to generate antigen-specific human Treg should be of general interest. The current translation of Treg-based therapies from ‘bench to bedside’ with first clinical studies being finished could thus profit from such approaches in the long term. An in-depth review about this topic is given in Chapter II.

In a side project we also studied regulation of MHCII expression, from which we hoped to gain insights into how to further develop and improve artificial antigen-presenting systems. For that purpose, we used EBV transformed B-cell lines from three newly defined Austrian MHCII deficiency patients and four prototype cell lines which had been described before. MHC Class II deficiency is caused by mutations in the genes of four trans-acting factors, the Class II transactivator (CIITA) and three molecules of the restriction factor X complex (RFX), RFXANK, RFX5 and RFXAP, which non-redundantly control expression of the MHCII locus³⁹. Accordingly, four complementation groups can be designated. While expression of HLA-DR, -DP, -DQ and -DM is tightly governed by these factors, expression of the invariant chain (Ii), although located in the MHCII gene cluster, seems to be somewhat independent of the intact CIITA-RFX complex. Indeed, several reports suggest that Ii mRNA can be detected in cell lines from MHC Class II deficient patients^{40, 41}. Consequently, we focused on analysis of invariant chain expression in different complementation groups, which could ultimately also help to improve diagnosis in this rare disease.

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Chapter 1

Naturally-occurring regulatory T-cells: Markers, mechanisms and manipulation

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ABSTRACT

Naturally-occurring $CD4^+CD25^{high}$ forkhead box protein 3 (FOXP3)⁺ regulatory T-cells (nTreg) are key mediators of immunity which orchestrate and maintain tolerance to self and foreign antigens. In the recent 1 1/2 decades, a multitude of studies has aimed to define the phenotype and function of nTreg and to assess their therapeutic potential for modulating immune mediated disorders such as autoimmunity, allergy and episodes of transplant rejection. In this review we summarize the current knowledge on the biology of nTreg. We address the exact definition of nTreg by specific markers and combinations thereof, which is a prerequisite for the state-of-the-art isolation of defined nTreg populations. Furthermore, we discuss the mechanism by which nTreg mediate immunosuppression and how this knowledge might translate into novel therapeutic modalities. With first clinical studies of nTreg-based therapies being finished, questions concerning the reliable sources of nTreg are becoming more and more eminent. Consequently, approaches allowing conversion of $CD4^+$ T-cells into nTreg by co-culture with antigen-presenting cells, cytokines and/or pharmacological agents are discussed. In addition, genetic engineering approaches for the generation of (antigen-specific) nTreg are described.

General Introduction

Regulatory T-cells (Treg) play a fundamental role in the development and maintenance of immunological tolerance. First data about the suppressive capacity of T-cells were already acquired in the late 1960s to early 1980s using mouse thymectomy models^{1, 2}. However, the advent of modern day Treg research can be dated to the year 1995 when *Sakaguchi et al.* first described the anergic and suppressive function of CD4⁺CD25⁺ T-cells in mice and could convincingly show that depletion of these cells resulted in multiorgan autoimmune disorders upon adoptive transfer³. CD4⁺CD25⁺ were defined to express the forkhead box P3 (Foxp3) transcription factor⁴⁻⁷ and form a distinct thymus-derived lineage in both mice⁸ and healthy human individuals⁹ and were consequently termed naturally-occurring Treg (nTreg). Similar to effector T-cell subsets, the function of nTreg (i.e. suppression of immune responses) was shown to be dependent on activation via the TCR/CD3 complex. However, once appropriately activated, this regulatory function was found to be antigen non-specific^{3, 10, 11}. Thus, upon antigen encounter, nTreg create a tolerogenic milieu in order to down-regulate aberrant or harmful immune responses.

In addition to nTreg, the existence of several different inducible Treg subsets has been reported, which are generated in the periphery under tolerogenic conditions. Among them the T regulatory type I (Tr1) subset that produces high levels of IL-10 has been best defined (reviewed by *Roncarolo et al.*¹²).

Treg were demonstrated to restrain the evolution and progression of autoimmune disorders¹³, to play a key role in tolerance against allo-antigens after transplantation¹⁴ and to modulate allergic immune responses¹⁵. Furthermore, several primary immunodeficiencies present with an apparent lack of functional Treg subsets, e.g. Omenn syndrome, Wiskott-Aldrich syndrome, hyper IgE syndrome and the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked)¹⁶. Thus autoimmune disorders, allergies and distinct disorders of the immune system can be attributed to inadequate Treg number and/or function. Consequently, protocols to induce or generate Treg function and/or to expand Treg numbers have gained increased attractiveness as therapeutic options for a broad range of diseases. Such technologies should include the ability to generate large numbers of highly suppressive and antigen-specific Treg that remain stable and viable *in vivo*. In this review we aim to define the two major Treg subsets, i.e. nTreg (naturally-occurring T reg) and Tr1 (T regulatory type I) cells, describe

their effector mechanisms and summarize the so-far used strategies to expand, induce or generate Treg.

Naturally occurring Treg (nTreg)

Naturally occurring CD4⁺CD25⁺ Treg (nTreg) constitute a distinct thymus-derived T-cell lineage that possesses the capacity to suppress activation and function of effector T-cells as well as antigen-presenting cells such as dendritic cells (DC), B-cells and monocytes. In peripheral blood of rodents about 8-10% of lymphocytes express the CD4⁺CD25⁺ phenotype¹⁷⁻²². However, this subset constitutes a heterogeneous population of nTreg and activated T-cells in human blood and thus some authors estimate the actual number of nTreg at 1-2%⁹. Consequently, the more pure nTreg population is confined to the CD4⁺CD25^{high} T-cell subset. Functionally, nTreg show a marked hypoproliferation upon TCR/CD3-mediated stimulation^{3, 9, 17-19, 21}, which can be overcome by the addition of high doses of IL-2^{17, 23} or a strong costimulatory signal^{17, 23}. In contrast to effector T-cells, nTreg also secrete minimal amounts of IL-2 as well as helper subset specific cytokines such as IFN- γ (Th1), IL-4, IL-5 and IL-13 (Th2) and IL-17 (Th17). In co-cultures with CD4⁺CD25⁻ effector T-cells (Teff), nTreg efficiently suppress Teff proliferation^{3, 9, 17} and cytokine production²⁴. This regulatory function is an activation-dependent process that requires cell-cell interactions as shown by experiments in which separation of nTreg and Teff by a semi-permeable membrane and/or transfer of nTreg supernatants do not lead to Teff suppression⁹.

The transcription factor forkhead box protein 3 (Foxp3) is constitutively expressed in nTreg and acts as the master regulator in Treg development and function⁷. Foxp3 contains three discernible functional domains, a single C2H2 zinc-finger motif, a leucine-zipper-like motif and a C-terminal forkhead domain, thereby exerting its positive and negative function as transcription factor. Among those target genes, Foxp3 acts as transcriptional activator for genes that are typically up-regulated in nTreg (e.g. CD25, CTLA-4; see below) while it represses transcription of both Th1 and Th2 cytokines in nTreg⁷. Recent work on the IL-2 promoter in nTreg has shown that Foxp3 physically interacts with NFAT thus displacing AP-1 from the complex and thereby repressing the induction of IL-2 transcription. Similar mechanisms may also contribute to the repression of other cytokines^{25, 26}.

The importance of Foxp3 in Treg development is highlighted by the fact that the scurfy mouse strain that carries a loss-of-function mutation in the Foxp3 gene completely lacks nTreg cells and develops a lymphoproliferative disease characterized by multi-organ infiltration of T-cells, leading to the death of mice at 3-5 weeks of age²⁷. Similarly, human patients suffering from the IPEX syndrome carry mutations in the Foxp3 gene and present with a broad range of autoimmune symptoms that is consistent with the lack of Treg²⁸⁻³⁰.

nTreg express a broad repertoire of α/β TCRs with specificities for both self and non-self antigens. While TCR diversity is similar between Treg and Teff, their repertoire is different with nTreg showing higher TCR affinities for MHC class II/peptide complexes than effector T-cells. However, other studies have indicated that this distinction is not complete and overlaps in the repertoire do exist (reviewed by Pacholczyk and Kern³¹ and Ohkura and Sakaguchi³²). Especially a Foxp3^{low} subpopulation of CD4⁺CD25⁺ nTreg has been reported to show a high degree of TCR similarity with effector T-cells³³.

Markers

While it is now widely accepted that nTreg constitute an independent T-cell lineage, so far no consensus about a distinct phenotype has been reached. The IL-2 receptor α chain (CD25) was the first surface marker on CD4⁺ T-cells, which was proposed as nTreg marker³. CD25 is constitutively expressed on nTreg and most work on nTreg relies on their purification via the CD4⁺CD25⁺ phenotype. However, CD25 is also transiently up-regulated on human effector T-cells upon activation³⁴. Thus, the CD4⁺CD25⁺ population does not represent a pure Treg population but also contains activated T-cells, which can 'contaminate' Treg assays. In peripheral blood CD4⁺ T-cells, the CD25⁺ subset represents no distinct population but contains cells expressing the CD25 molecule at different expression levels from low to high. Among these cells the CD25^{high} subset represents the most reliable Treg population. However, no common directive exists as to which expression level constitutes 'CD25^{high}' cells. Consequently, most researchers use 1-2% of CD4⁺ T-cells, which express the highest CD25 levels.

As stated above, the transcription factor Foxp3 plays an integral role in the development and function of nTreg^{4-6, 35}. Therefore, intracellular Foxp3 expression is now routinely assessed as a marker to define nTreg. However, similar to CD25, Foxp3 is not only constitutively expressed in nTreg but also transiently up-regulated in human effector CD4⁺ T-cells upon activation^{36, 37}. Foxp3, especially in combination with CD4 and CD25 is now

commonly used to monitor Treg numbers, e.g. in comparison between healthy individuals and patients suffering from autoimmune diseases. However, due to its cellular localization, Foxp3 expression can only be detected after permeabilization and thus cannot be used to sort viable nTreg for further functional analyses. In this respect, a useful tool in the basic research of Treg function has been the Foxp3/GFP mouse, which expresses the fluorescent marker GFP under the control of the Foxp3 promoter allowing the purification of nTreg via flow cytometry-based sorting³⁵.

Two further surface markers for the assessment of nTreg are the cytotoxic lymphocyte antigen-4 (CTLA-4/CD152) and the glucocorticoid-induced TNF-receptor related protein (GITR). CTLA-4 is a structural homologue of the costimulatory receptor CD28³⁸ and binds to the two major costimulatory molecules CD80 and CD86 on antigen-presenting cells³⁹⁻⁴¹ and is implied to play a crucial role in Treg function (see below). GITR belongs to the TNF receptor superfamily and has been implied as costimulatory receptor on T-cells⁴². Several reports suggest that GITR may act as a negative regulator of Treg function as ligation of GITR on nTreg has been shown to abrogate their suppressive function in the murine system⁴³⁻⁴⁶, while experiments performed with human nTreg could not confirm these findings⁴⁷. Both, CTLA-4^{48, 49} and GITR⁴⁵ are constitutively expressed on nTreg but are also up-regulated on CD4⁺ effector T-cells following activation, which compromises their suitability as Treg markers.

In contrast to the molecules described above, which show constitutive expression on Treg, expression of the IL-7 receptor α chain (CD127) was found to inversely correlate with the nTreg status⁵⁰. However, this phenotype is not unique for nTreg, as activation of effector T-cells also results in down-regulation of CD127. Yet, the marker combination CD4⁺CD25⁺Foxp3⁺CD127⁻ is currently considered to define the most pure and reliable nTreg population.

While Treg generally show a surface phenotype resembling activated effector T-cells, expression of L-selectin (CD62L) has been described as another marker expressed on Treg. CD62L generally mediates the homing of T-lymphocytes to secondary lymphoid tissues and expression of CD62L is a hallmark of naïve effector T-cells. On CD4⁺CD25⁺ nTreg, the CD62L⁺ phenotype can be used to identify a subpopulation, which has been found to show supreme regulatory capacity *in vitro*⁵¹ as well as in murine models of type-1 diabetes⁵² and GVHD⁵³, when compared to the CD62L⁻ subset. Thus, CD62L is a suitable additional marker in the analysis or purification of murine nTreg. While CD62L is

now routinely assessed on human nTreg as well, no clear-cut correlation to a specific Treg (sub-)set has so far been established.

In recent studies it was observed that surface expression of the ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73) are strongly enhanced on nTreg^{54, 55}. Importantly, expression of CD39 was observed to highly correlate with expression of Foxp3 in CD4⁺ nTreg showing an even higher correlation than CD25 and Foxp3⁵⁵. However, recent research has indicated that CD39 expression does not identify nTreg *per se* but CD39 expression generally defines a memory subset within the CD4⁺ T-cell compartment⁵⁶. Thus, sorting for CD39 expression on CD4⁺ T-cells is not sufficient to purify nTreg but requires additional markers.

Similar to CD39, the homing-associated cell adhesion molecule CD44 (H-CAM) has recently been described as a marker for a special subset among murine CD4⁺CD25⁺ nTreg. CD44 expression was found to strongly correlate with Foxp3, but among CD4⁺CD25⁺ T-cells CD44 levels were found to be varying between low, intermediate and high expression⁵⁷. In functional studies the CD4⁺CD25⁺CD44^{high} subset showed the strongest regulatory capacity, which was mainly mediated by the secretion of IL-10 *in vitro*⁵⁷. However, so far no data have been obtained concerning the origin of this nTreg subset and whether these observations can also be verified in human nTreg.

Another marker that identifies a special nTreg subset is the latency-associated peptide (LAP)⁵⁸. LAP constitutes the amino-terminal domain of the transforming growth factor-beta (TGF-β) precursor peptide⁵⁸. After cleavage LAP remains non-covalently associated with the TGF-β peptide forming the latent TGF-β complex. In studies on murine T-cells *in vitro* and in a murine experimental autoimmune encephalomyelitis (EAE) model it was established that CD4⁺LAP⁺ T-cells, independently of CD25 expression, display regulatory capacity, which critically depends on the secretion of TGF-β. The additional inclusion of CD25 as a further sorting criterion allowed the isolation of nTreg with strongly enhanced suppressive potential compared to CD4⁺CD25⁺ T-cells⁵⁸. This was not due to differential Foxp3 levels, which were found to be similar in LAP⁺ or LAP⁻ CD4⁺CD25⁺ Treg. While these initial studies were performed using murine nTreg, LAP has now also been established as an nTreg marker on human T-cells⁵⁹.

The α_E integrin subunit (CD103) was identified as a putative nTreg marker by comparative gene expression analyses between CD4⁺CD25⁻ and CD4⁺CD25⁺ T-cells⁴⁴. In subsequent studies, CD103 was found to be expressed on about 30% of murine

CD4⁺CD25⁺ nTreg and seems to be involved in the accumulation and retention of Tregs at certain sites within the body⁶⁰. CD103⁺ nTregs have been implied to be beneficial for the amelioration of graft-versus-host disease⁶¹ but also to be highly expressed on tumor-infiltrating nTreg⁶².

Two surface molecules, LAG-3 and GARP have been described to selectively identify activated nTreg. Lymphocyte activation antigen-3 (LAG-3/CD223) is a transmembrane CD4-related protein that contacts MHC class II molecules on antigen-presenting cells⁶³. LAG-3 is expressed on activated effector and regulatory T-cells with activated Tregs showing a higher and more persistent expression⁶⁴. Glycoprotein A repetitions predominant (GARP, LRRC32) is a transmembrane protein that is rapidly up-regulated on nTreg after TCR-mediated stimulation^{65, 66}. GARP provides a highly selective marker, as it strongly correlates with Foxp3 and CD25 expression in nTreg. This specificity of expression is restricted to *bona fide* nTreg underlined by the fact that effector T-cells but also TGF- β induced Foxp3⁺ T-cells and Foxp3-transgenic engineered Treg do not express GARP after activation⁶⁶. Interestingly, retroviral transduction of GARP was sufficient to induce Foxp3 expression and regulatory capacity in CD4⁺CD25⁻ T-cells⁶⁷.

Several molecules of the galectin family have been described as Treg specific markers, among them galectin-1⁶⁸ and galectin-10. The Charcot-Leyden crystal protein galectin-10 was identified as nTreg marker by differential proteomics comparing CD4⁺CD25⁻ and CD4⁺CD25⁺ T-cells⁶⁹. Galectin-10 is specifically expressed by nTreg and, in contrast to other markers described above, is not up-regulated by activated effector T-cells. Interestingly, galectin-10 is differentially expressed in two further nTreg subsets, which can be distinguished by their expression of either $\alpha_4\beta_1$ or $\alpha_4\beta_7$ integrin⁶⁹. However, expression of galectin-10 is restricted to intracellular compartments, which renders galectin-10 unsuitable to enrich viable nTreg populations.

Several DNA microarray studies have identified the transcription factor Helios as nTreg marker^{70, 71}. Indeed, recent work could convincingly show, that Helios is strongly expressed in both murine⁷² and human⁷³ CD4⁺CD25⁺ nTreg but not in activated human Teff or TGF- β induced Foxp3⁺ T-cells. Furthermore, Helios was shown to bind to the Foxp3 promoter and may thus be involved in the maintenance of Foxp3 expression⁷³. However, similar to Foxp3, the absence of surface expression limits its use as an nTreg sorting marker.

In a recent study, the TNF receptor superfamily member 25 (TNFRSF25, DR3) has been identified as a putative marker on murine Treg⁷⁴. In comparison to CD4⁺ effector T-cells, which showed only basal expression levels, murine nTreg strongly expressed DR3, and the relation of DR3 expression between nTreg and effector T-cells was clearly superior to GITR⁷⁴. However, whether DR3 represents a suitable marker for human Treg remains to be determined. A list of all described nTreg markers is given in Table I.

Table I. List of nTreg markers

Molecule	expression	mouse	human	comment	reference
CD25	high; surface	x	x	also expressed on activated T _{eff}	3,34
Foxp3	high; intracellular	x	x	also expressed on activated T _{eff}	4-6,35
CTLA-4	high; surface	x	x	also expressed on activated T _{eff}	48,49
GITR	high; surface	x	x	also expressed on activated T _{eff}	45
CD127	low; surface	x	x		50
CD62L	high; surface	x	n.e.	expressed on naïve T _{eff} , denotes Treg subset with superior suppressive capacity	51-53
CD39	high; surface	x	x		54,55
CD44	high; surface	x	n.d.	denotes IL-10 producing subset	57
LAP	high; surface	x	x	denotes Treg subset with superior suppressive capacity	58,59
CD103	high; surface	x	n.d.	expressed on 30% of CD4 ⁺ CD25 ⁺	44, 60-62
LAG-3	high; surface	x	n.d.	expressed on activated Treg	64
GARP	high; surface	n.d.	x	expressed on activated Treg	65-67
Galectin-1	high; surface	x	x		68
Galectin-10	high; intracellular	n.d.	x		69
Helios	high; intracellular	x	x	expressed exclusively in nTreg, not in iTreg or Foxp3 tg Treg	70-73
TNFRSF25	high; surface	x	n.d.		74

Teff, effector T-cells; n.e., not established; Treg, regulatory T-cells; n.d., not determined

Effector Mechanisms

- surface molecules

One key finding in the early functional definition of nTreg effector mechanisms was that *in vitro* nTreg were not able to suppress effector T-cell function after separation through a semi-permeable membrane. Similarly, transfer of supernatants from nTreg cultures to effector T-cell cultures did not prevent effector T-cell proliferation. Consequently, it was assumed that suppression by nTreg is a ‘contact-dependent’ process and that specific cell-surface molecules must be responsible for the suppressive effects of nTreg.

One of the most attractive targets of interest was the inhibitory B7-superfamily molecule CTLA-4. CTLA-4 contacts the costimulatory molecules CD80 and CD86³⁹⁻⁴¹ and due to its superior affinity out-competes the activating receptor CD28 for binding to these two ligands thereby abrogating the activating signal³⁹⁻⁴¹. The constitutive expression of CTLA-4 on nTreg does not only keep them in their hyporesponsive state but also crucially contributes to their suppressive function. CTLA-4 knockout mice develop fatal multiorgan inflammatory diseases^{75, 76}. Along those lines, blocking of CTLA-4 using specific monoclonal antibodies can elicit autoimmune disorders^{77, 78} and enhance anti-tumour immunity⁷⁹ in murine models. Similarly, conditional knockout of CTLA-4 in murine nTreg severely impairs their suppressive capacity resulting in autoimmune pathology with splenomegaly, lymphadenopathy, autoimmune gastritis and more than 1000-fold increased IgE levels⁸⁰. The suppressive mechanism of CTLA-4 seems to be two-fold: first, CTLA-4 on nTreg ligates CD80 and CD86 on activated T-cells and thereby transmits inhibitory signals directly into effector T-cells⁸¹. A second mechanism is the indirect suppression of T-cell responses via modulation of APC function. Such CTLA-4 mediated mechanisms include the inhibition of DC maturation^{82, 83}, the down-modulation of CD80/CD86 on DC^{80, 84} and the induction of the immune-regulatory enzyme indoleamine desoxygenase (IDO) in DC⁸⁵.

Although deletion of no other surface molecule than CTLA-4 nTreg results in spontaneous development of autoimmune disorders, several other molecules are reported to contribute to the suppressive function of nTreg. The CD4-related protein LAG-3 has been implied to contribute to nTreg suppressive function via direct Treg-Teff interactions and via the modulation of APC function. In this respect, engagement of LAG-3 to MHC class II on dendritic cells has been reported to result in the down-regulation of costimulatory molecules⁸⁶. In a murine model, knockout of LAG-3 severely impaired nTreg suppressive

capacity, while retroviral transduction of LAG-3 into CD4⁺ T-cells was sufficient to induce a hyporesponsive and regulatory function⁶⁴. LAG-3 expression is controlled by the early growth response gene-2 (Egr-2) and over-expression of Egr-2 in murine CD4⁺ T-cells generates CD4⁺CD25⁻LAG-3⁺ regulatory T-cells, which secrete high amounts of IL-10⁸⁷. Studying tumor-infiltrating nTreg in patients suffering from melanoma or colorectal cancer, *Camisaschi et al.* showed that LAG-3⁺ nTreg form a distinct subset that produces significantly increased amounts of IL-10 and TGF- β . However, the *in vitro* suppressive capacity of LAG-3⁺ nTreg was contact-dependent and not mediated by these two cytokines⁸⁸.

The Ig-like type I transmembrane protein CD83 is another molecule that is involved in the regulatory function mediated by nTreg. Several studies have indicated that a soluble form of CD83 (sCD83) is released by B-cells and dendritic cells⁸⁹ and acts as a potent immunosuppressive molecule⁹⁰⁻⁹³. More recent evidence indicates that CD83 is also expressed on the surface of nTreg and directly contributes to contact-dependent suppression⁹⁴. Interestingly, retroviral transduction of CD83 into CD4⁺ T-cells did not only endow them with regulatory function but also led to the up-regulation of CTLA-4 and Foxp3⁹⁴. Consequently, CD83 may also act as an autocrine factor, which regulates the nTreg phenotype. However, so far no receptor has been identified for CD83 and thus the regulatory interaction between CD83 on Treg and a putative receptor on Teff or Treg remains to be investigated.

- cytokines (IL-10, TGF- β , IL-35)

While most *in vitro* assays identified the regulatory capacity of nTreg to be contact-dependent, several soluble factors have been implied in Treg function and are classically associated with nTreg mediated suppression.

One of the most common cytokines mentioned in the context of nTreg is IL-10. IL-10 is a potent immunoregulatory cytokine, which is attributed with anti-inflammatory functions. Along those lines, IL-10 down-modulates the production of Th1 polarizing cytokines such as TNF- α , IL-1, IL-12 and IFN- γ by antigen-presenting cells⁹⁵. IL-10 has also been described to decrease the expression of MHC class II and costimulatory molecules on dendritic cells thereby keeping them in a tolerogenic state⁹⁶⁻⁹⁸. Apart from APC function, IL-10 also regulates the activation and function of mast cells⁹⁹ as well as cytokine production by eosinophils¹⁰⁰ and has been shown to directly suppress T-cell

proliferation¹⁰¹. The immunomodulatory relevance of IL-10 is highlighted by the fact that IL-10 knockout mice spontaneously develop inflammatory bowel disease-like pathology¹⁰².

Various immune cells including monocytes, dendritic cells, B-cells and activated T-cells produce IL-10, but the role of IL-10 in Treg is not fully established as of today. Indeed, several seminal papers, which were the first to functionally assess human nTreg function, could not find any significant secretion of IL-10 upon stimulation^{9, 17}. Along those lines, the regulatory capacity of nTreg was shown to be independent of IL-10^{103, 104}. However, several other studies have proposed important roles for IL-10 for the modulatory function of nTreg both *in vitro*²² and *in vivo*. Taken together, these data suggest that IL-10 plays a role in specialized nTreg subsets or in a distinct tissue-specific milieu but is not essential for nTreg-mediated suppression.

A second soluble factor that is commonly associated with nTreg function is TGF- β . So far, three members of the TGF- β family (TGF- β 1, TGF- β 2, TGF- β 3) have been identified which have pleiotropic physiological effects on a broad range of different cell types. TGF- β is synthesized as a precursor harboring a pre- and a pro-peptide. Upon processing in the Golgi, the N-terminus of pro-TGF- β is removed, forming a novel homodimeric protein called the latency-associated protein (LAP). Via non-covalent association, a homodimer of the mature TGF- β binds to LAP, and can be either secreted or further associates with the latent-TGF- β -binding protein (LTBP). These associations keep TGF- β in an inactive state and in order to bind its receptors, TGF- β has to be dissociated from LAP and/or LTBP via proteolysis or conformational change (reviewed in Li and Flavell¹⁰⁵).

In the immune system, TGF- β 1 is the predominantly expressed isoform and displays mainly regulatory functions. TGF- β knockout mice develop an early fatal autoinflammatory disorder, similar to Foxp3-knockout scurfy mice, which is marked by T-cell hyperactivation and excessive multiorgan inflammation¹⁰⁶. In studies using TGF- β II-receptor knockout mice it was similarly established that nTreg derived TGF-beta is critical for the regulation of auto-reactive effector T-cells¹⁰⁷⁻¹⁰⁹ (and reviewed in Li and Flavell¹⁰⁵). This TGF- β dependency of Treg function could also be shown in murine models of colitis¹¹⁰, anti-tumor immunity¹¹¹ and type I diabetes¹¹² using mice expressing a dominant negative form of the TGF- β II-receptor. However, the situation is not as clear-cut for human CD4⁺CD25⁺ nTreg. Several reports suggest that human nTreg do not

secrete TGF- β and that TGF- β blocking antibodies cannot abrogate regulation^{21, 22, 103, 104, 113} while other groups have gathered exactly contrary data¹¹⁴. The debate is further complicated by the fact that studies with murine Treg suggest that TGF- β may attach to the cell membrane and may thus contribute to the contact-dependent regulatory mechanisms¹¹⁵. In this context, GARP has been described as a receptor for latent human TGF- β on activated nTreg¹¹⁶.

While the role of TGF- β as Treg effector cytokine is still disputed, it has become evident, that TGF- β also plays a role in the induction of Foxp3 expression and regulatory capacity in peripheral T-cells, which are consequently termed induced Treg (iTreg). This effect can be augmented by co-factors such as IL-2 and retinoic acid (RA)(see below). While such induced Treg remain stable and functional in several mouse models, the stability and function of human iTreg has been cast into doubt. Indeed, activation of human CD4⁺CD25⁻ T-cells in the presence of TGF- β does not create functional iTreg and the additional administration of RA seems to be a prerequisite for the induction of a somewhat stable and functional iTreg population.

In two recent works a novel cytokine, termed IL-35, was identified as additional factor important for nTreg function^{117, 118}. IL-35 contains the p35 subunit of IL-12 and the Epstein-Barr-Virus induced gene-3 (Ebi3 which also constitutes the IL-27 beta subunit). IL-35 was found to be constitutively released from murine nTreg but not from effector T-cells. Knockout of either IL12p35 or Ebi-3 severely impaired the regulatory capacity of nTreg *in vitro* and *in vivo* while over-expression conferred regulatory activity to naïve CD4⁺ T-cells¹¹⁷. Interestingly, human nTreg were not found to express Ebi3¹¹⁹ disputing the relevance of IL-35 in human Treg. In contrast to these observations a recent report shows that rhinovirus-treated DC induce IL-35 production in CD4⁺ T-cells¹²⁰ thus indicating that human Treg may produce IL-35 under distinct conditions. Interestingly, in the murine system, IL-35 does not only act as a regulatory cytokine on Teff, but was also found to expand CD4⁺CD25⁺ Treg and to induce CD4⁺Foxp3⁻ Treg with IL-35 dependent regulatory function¹²¹. Thus, IL-35 does not only act as an effector cytokine from Treg but also regulates Treg homeostasis.

- killing

Based on the observation that nTreg express granzyme A and B and perforin^{122, 123}, it was proposed that killing of target cells might be a possible regulatory mechanism exerted by

Treg. Indeed, several studies have convincingly demonstrated that both murine^{124, 125} and human¹²² nTreg at least partially regulate effector T-cell responses by inducing apoptosis. However, the exact contribution of each apoptosis-inducing molecule is still under dispute. While in some mouse models regulation is mainly mediated by granzyme B^{124, 125}, human Treg seem to utilize the complete granzyme/perforin machinery but not FasL (CD95L) in the killing of CD4⁺ effector T-cells¹²². In contrast, more recent reports indicate that nTreg have the capacity to kill CD8⁺ T-cells via FasL-Fas interactions¹²⁶. The induction of apoptosis is not restricted to effector T-cells but also involves antigen-presenting cells. In this respect, human Treg were found to induce apoptosis in B-cells, monocytes and dendritic cells, thereby mediating indirect regulation of T-cell responses¹²².

- *cytokine depletion*

One of the key features of nTreg is their hyporesponsiveness, which is reflected by their low degree of proliferation in response to stimulation but also by their lack of cytokine secretion. Interestingly, nTreg confer this hyporesponsiveness onto effector T-cells as shown in experiments in which effector T-cells both produce and secrete lower amounts of IL-2 when co-cultured with nTreg²³. Apart from this direct effect, nTreg have also been shown to act as ‘cytokine sinks’ by depleting IL-2 through binding to their constitutively highly expressed IL-2 receptor and subsequent internalization. Consequently, effector T-cells, which are deprived of IL-2 by this mechanism undergo apoptosis after 72 hours of co-culture¹²⁷. Along those lines, we have shown that neutralization of IL-2 from T-cell cultures by IL-2 receptor decorated virus-like particles (VLP), which we use as a convenient platform to display immune receptors of choice, blocks T-cell proliferation. However, in contrast to *Pandiyan et al.*, such treated T-cells did not undergo apoptosis but were anergic upon re-stimulation¹²⁸.

- *nucleotides and metabolites*

The finding that nTreg express high levels of CD39 and CD73^{54, 55}, two ectonucleotidases that are involved in the generation of extracellular adenosine, has led to the assumption that Treg released adenosine might play a role in the suppressive function of Treg. Adenosine is known to exert immunosuppressive activity on T-cells via ligation of adenosine receptors, including A_{2A}R¹²⁹, which leads to an increase in intracellular cAMP. Indeed, nTreg express constitutively high levels of A_{2A}R and effector T-cells rapidly up-

regulate A_{2A}R upon activation. Generation of adenosine by Treg is crucial for their suppressive function and knockout of CD39 in murine Treg partially abrogates their regulatory function both *in vitro* and *in vivo*⁵⁵, an effect that can also be reached by using A_{2A}R antagonists¹³⁰.

In a separate study, *Bopp et al.* assessed the role of cAMP for nTreg function. They could show that nTreg contain highly increased levels of cAMP and that cAMP levels also increased in effector T-cells following co-culture with nTreg. Further results demonstrate that effector T-cells and nTreg communicate via the formation of gap-junctions, which allows the transfer of cAMP from Treg to effector T-cells¹³¹. Along those lines, blockade of gap-junction formation could abrogate the regulatory capacity of nTreg thus highlighting the importance of this mechanism in the suppressive repertoire of nTreg¹³¹.

- *induction of IDO in APC*

The regulatory function of Treg is not restricted to the suppression of effector T-cell responses but they also modulate APC function. As described above, several molecular mechanisms contribute to this function, leading to the down-regulation of stimulatory molecules such as MHC and CD80 and thus keeping the APC in a non-stimulatory or even tolerogenic state. In this respect, induction of the enzyme indoleamine-2,3-dioxygenase (IDO) in dendritic cells upon co-culture with Treg seems to form an important bilateral feedback loop involved in the establishment of immune tolerance¹³²⁻¹³⁵. IDO is a key enzyme in the tryptophan metabolism, converting tryptophan to formylkynurenine¹³⁶, which acts as a strong immunosuppressive agent¹³⁷⁻¹³⁹. Apart from its inhibitory effect on effector T-cells, this metabolite has also been shown to induce regulatory T-cells^{140, 141}. While this potent IDO-Treg axis is important for the prolongation of allograft survival, it may also pose a major impediment to anti-tumor immunity and thus remains of interest in several fields of clinical immunology^{82, 140, 141}.

- *Modulation of microenvironment*

Several biochemical factors in the microenvironment of effector T-cells strongly regulate their capacity to fully mount proliferative and functional responses. One limiting factor of crucial importance is the availability of cysteine. T-cells cannot take up cystine, the major extracellular form and consequently critically rely on the secretion of cysteine by co-localized dendritic cells^{142, 143}. In this respect, Treg have been described to suppress this

function in DC, thereby indirectly depleting Teff of cysteine^{144, 145}. Interestingly, this seems to be a general pathway of immunosuppression as similar mechanisms have also been proposed as mode of action of myeloid derived suppressor cells¹⁴⁶.

In another study it was shown that NADPH oxidase derived reactive oxygen species (ROS) produced by nTreg were involved in the suppression of CD4⁺ Teff. Along those lines, murine nTreg have been observed to alter the intracellular distribution of glutathione in Teff upon co-culture thus efficiently inhibiting Teff activation and proliferation¹⁴⁷. Taken together, interference of Treg with both dendritic cell and Teff redox environment seem to emerge as a novel mechanism how Treg create an immunosuppressive milieu.

- *Out-competition of APC stimulation*

In vivo observations on the reconstitution of immune homeostasis in lymphopenic mice have suggested that T-cells are constantly competing for immunological niches and that limitations of such niches may contribute to immune regulation. In this respect, nTreg seem to be especially suited to 'take up space' as their higher avidity for MHC:peptide complexes on APC may allow them to preferentially access T-cell stimulatory niches¹⁴⁸.

Along those lines recent results from *in vitro* experiments suggest that Treg preferentially aggregate with dendritic cells in an LFA-1 dependent manner⁸⁴. Consequently, effector T-cells are out-competed for interaction with stimulatory molecules and thus kept in an inactive state both *in vitro*⁸⁴ and *in vivo*^{149, 150}. While the hypothesis of out-competition seems intriguing in the light of these studies, it cannot explain how Treg regulate effector T-cells in response to cell-free stimuli such as anti-CD3 antibodies. A scheme of the described regulatory mechanisms can be seen in Fig.1.

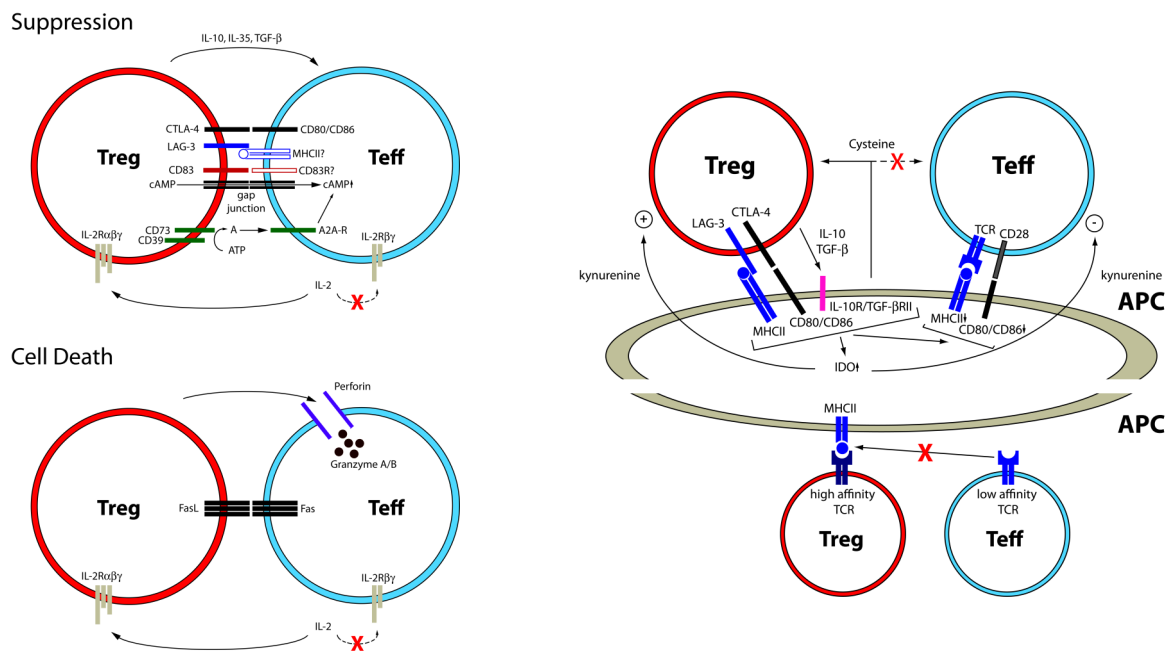


Fig.1 Scheme of suppressive mechanisms of naturally-occurring Treg; left: direct suppression and induction of apoptosis, right: indirect modulation via antigen-presenting cells; A, adenosine; ATP, adenosine triphosphate; A2A-R, adenosine receptor 2A; APC, antigen-presenting cell; cAMP, cyclic adenosine monophosphate; CTLA-4, cytotoxic T-lymphocyte antigen-4; IDO, indoleamine-2,3-dioxygenase; IL-2R, interleukin-2 receptor; IL-10R, interleukin-10 receptor; LAG-3, lymphocyte-activation antigen-3; TCR, T-cell receptor; Teff, effector T-cell; TGF- β , transforming growth factor beta; TGF- β RII, transforming growth factor beta receptor II; Treg, regulatory T-cell

Relevance and evidence for therapeutic benefit

- Autoimmunity

Apart from negative selection in the thymus, which deletes most auto-reactive T-cell clones, peripheral mechanisms, among them regulatory T-cells, are additionally needed to maintain the immunological integrity of the organism. Most notably, breakdown of nTreg mediated immune-regulation, as found in scurfy mice²⁷ and human IPEX patients²⁸⁻³⁰, leads to the development of fatal autoimmune disorders, which affect multiple organs. Similarly, experimental depletion of nTreg in murine models was shown to induce the spontaneous onset of autoimmune diseases. Defects in Treg numbers or function were also noted in patients suffering from established autoimmune diseases such as SLE¹⁵¹⁻¹⁵³, type I diabetes¹⁵⁴ and multiple sclerosis^{155, 156}. In contrast, early post-natal inoculation of mice with indigenous intestinal *Clostridium* bacteria led to an increased number of colonic Foxp3⁺ Treg, which was sufficient to suppress inflammatory bowel disease (IBD). Thus,

physiological Treg homeostasis is also governed by exogenous signals such as microbiota¹⁵⁷. In this light, it can be argued that autoimmunity is a consequence of insufficient immune tolerance and that consequently re-establishment of tolerance mechanisms can prevent or even cure such diseases. Indeed, some of the the first functional observations about the regulatory capacity of CD4⁺CD25⁺ nTreg were observed upon adoptive transfer of Treg in a murine model of autoimmune gastritis³. Since then, nTreg have been assessed for their preventive or curative capacity in a plethora of mouse models of different autoimmune diseases, both spontaneous and induced. Reconstitution of nTreg in murine models of Type I diabetes^{124, 158-160}, multiple sclerosis (EAE)^{161, 162}, rheumatoid arthritis^{163, 164} and systemic lupus erythematoses (SLE)¹⁶⁵ could efficiently prevent disease before onset, however, only in a few studies established disease could actually be reverted by adoptive transfer of nTreg^{159, 166, 167}. In this respect, antigen-specificity of the transferred regulatory T-cell population seems to be of crucial importance, as remission in most models could only be reached by nTreg specific for the pathognomonic antigen (reviewed in Roncarolo and Battaglia¹⁶⁸). Interestingly, in one study using a collagen-induced arthritis model, TCR tg Treg recognizing a non-collagen auto-antigen induced remission of the collagen-induced disease¹⁶³. Similarly, nTreg expanded with dendritic cells pulsed with a single auto-antigen were sufficient to suppress autoimmune diabetes in the non-obese diabetic (NOD) mouse model¹⁶⁰. These data suggest that once appropriately targeted and activated, mono-specific Treg are sufficient to create an immunomodulatory milieu, which abrogates multi-factorial immune responses at the site of Treg activation.

- Allergy

Similar to autoimmune and autoinflammatory disorders, IgE-associated allergies represent disorders of the immune system where crucial mechanisms of immunotolerance are apparently non-functional leading to an aberrant and harmful immune response. Several lines of evidence suggest that allergic disorders might be partially attributed to a dysregulation of regulatory T-cells and that induction and/or propagation of Treg might thus be of therapeutic relevance in allergic patients.

In murine studies, mouse CD4⁺CD25⁺ T-cells were reported to suppress pathognomonic immune parameters such as differentiation and cytokine production of Th2 cells¹⁶⁹ and production of IgE¹⁷⁰. In several inhaled allergen challenge models, it could be shown that

transfer of CD4⁺CD25⁺ T-cells could suppress airway inflammation^{171, 172}. Similarly, repeated exposure to allergen at low doses prior to Th2 sensitization induced tolerance¹⁷³, which was attributed to the induction of Foxp3⁺ regulatory T-cells¹⁷⁴. While this modulatory capacity of nTreg on Th2 responses and airway inflammation is thus well defined, a beneficial effect on airway hyperresponsiveness could not be observed in all studies^{171, 172, 174, 175}. Indeed, the best effects on clinical symptoms could be observed when IL-10 producing regulatory T-cells (mainly Tr1 cells; see below) were used¹⁷⁶⁻¹⁷⁹.

Similar to their murine counterparts, human CD4⁺CD25⁺ Treg are implicated to play a beneficial role in the suppression of allergic immune responses. *In vitro*, CD4⁺CD25⁺ Treg can efficiently suppress proliferation and cytokine production of Th2 cells^{180, 181}. A hallmark study on 12 atopic and 11 patients with seasonal allergic rhinitis to grass pollen demonstrated that nTreg from atopic individuals and allergic patients after allergen-exposure were impaired in their regulatory capacity compared to non-atopic individuals¹⁸². Along those lines, Treg were found to be absent in the skin of atopic dermatitis patients¹⁸³ while outgrowth of cow's milk allergy in initially allergic children is associated with higher numbers of CD4⁺CD25⁺ Treg¹⁸⁴.

Another compelling line of evidence for the importance of Treg in allergy comes from studies on allergic patients undergoing specific immunotherapy (SIT). SIT involves the repetitive, dose-increasing administration of purified allergens to affected individuals thereby leading to tolerance induction and clinical de-sensitization of the patient. Numerous groups have observed the induction/expansion of regulatory T-cells after SIT to a broad range of allergens and using different ways of application¹⁸⁵⁻¹⁹¹. Thus induced Treg seem to mediate, apart from direct suppression of Th2 cells, the suppression of allergen-specific IgE production and the concomitant switch to protective IgG4, the down-modulation of mast cell, eosinophil and basophil function and the amelioration of airway remodeling (reviewed in Akkoc *et al.*¹⁹²). However, whether all these effects are mediated by CD4⁺Foxp3⁺ naturally-occurring Treg is still a matter of debate. Indeed, the immunomodulatory cytokine IL-10 seems to play a crucial role in the efficacy of SIT^{185, 186, 193}. Along those lines, some authors have described the induction CD4⁺Foxp3⁺IL-10⁺ T-cells^{187, 194}. However, given the still disputed relation between nTreg and IL-10 production, Tr1 cells (see below), which are marked by their high level production of IL-10, might be (at least complementarily) required for efficient immunomodulation of allergic responses.

- Transplantation

Although modern day regimen of immunosuppressive medication have significantly prolonged the survival of allografts and decreased the incidence of rejection episodes, the requirement for lifelong medication also bears major disadvantages such as increased susceptibility to infections and increased occurrence of tumors. In this respect, induction of long-lasting tolerance against allografts might offer an attractive therapeutic strategy. Initial observations that regulatory T-cells were enriched in tolerated rat kidney grafts¹⁹⁵ and murine skin grafts¹⁹⁶ have provided first evidence that regulatory T-cells may be major players in transplantation tolerance. Since then a multitude of studies in rodent and primate model systems have addressed this issue and suggested that Treg based therapies could indeed be beneficial for allograft survival. In mouse models of bone marrow transplantation (BMT), concomitant transfer of freshly purified CD4⁺CD25⁺ T-cells together with the bone-marrow allograft was found to facilitate engraftment and ameliorate graft-versus-host disease (GVHD)¹⁹⁷⁻¹⁹⁹. Similarly, nTreg, which were polyclonally expanded *ex vivo* prior to transfer, also showed curative potential in GVHD models²⁰⁰⁻²⁰². Interestingly, Treg populations enriched for alloantigen-specific T-cells showed only slightly improved efficacy over polyclonal Treg populations in this setting²⁰³. This observation may be due to the fact that polyclonal Treg populations contain a sufficiently large proportion of alloreactive Treg to prevent GVHD. Other theories argue that the lymphopenic environment after conditioning for BMT might favor the expansion of Treg resulting in a more beneficial Treg to Teff ratio (reviewed in Roncarolo and Battaglia¹⁶⁸).

In contrast to BMT, antigen-specificity of transferred Treg populations seem to be a prerequisite for the induction of long-lasting tolerance following solid organ transplantation. In several rodent models of pancreatic islet²⁰⁴⁻²⁰⁶, liver²⁰⁷, heart^{208, 209} and skin²¹⁰ transplantation, transfer of alloantigen-specific CD4⁺CD25⁺ Treg proved to be superior to transfer of polyclonal nTreg populations. In this respect, positive effects could be shown for both Treg with direct allo-specificity and indirect allo-specificity, i.e. peptides of donor allo-antigens presented by recipient HLA-molecules. Of note, BALB/c mice, which had been tolerized to B10.BR skin grafts using non-depleting anti-CD4 and anti-CD8 monoclonal antibodies, were shown to subsequently tolerate skin grafts of B10.D2 mice, which share the same minor histocompatibility genes with B10.BR mice²¹¹. Similarly, a TCR-transgenic CD4⁺CD25⁺ Treg line with specificity for one indirect allo-

antigen was sufficient to induce long-lasting tolerance to MHC-mismatched heart allografts²¹². Thus, similar to autoimmune models described above, appropriately located and activated Treg of a singular specificity might be sufficient to create an immunomodulatory milieu, which prevents graft rejection.

Evidence for the importance of nTreg in transplantation tolerance also stems from clinical observations on transplanted patients. In clinically stable kidney transplanted patients, several independent studies have described an increased number of functionally active, alloreactive regulatory T-cells compared to patients suffering from acute or chronic rejection episodes^{213, 214}. In another study on liver transplant recipients, the spontaneous development of tolerance to the graft was strongly associated with increased numbers of graft-infiltrating Treg²¹⁵. Similarly, the outcome of lung transplantation was found to correlate with the frequency of CD4⁺CD25⁺ T-cells in the blood of lung transplant recipients²¹⁶.

These promising experimental results have spurred considerable hope that Treg-based therapies could become a standard regimen for the management of the post-transplant period and several clinical trials are currently underway. A first published study using *ex vivo* expanded Treg for the treatment of GVHD following BMT has been published recently. In this trial, alleviation of symptoms allowing reduction of immunosuppressive medication could be achieved in the two treated patients²¹⁷.

T-regulatory Type 1 cells (Tr1)

In 1997, a novel subset of regulatory T-cells was discovered, which was marked by the high expression of IL-10 and found to prevent colitis after transfer of CD4⁺CD45RB^{high} T-cells into SCID mice²¹⁸. This subset, termed T regulatory type 1 cells (Tr1), was also found in humans and defined by its unique cytokine profile, which includes secretion of high levels of IL-10 and TGF- β , intermediate levels of IL-5 and IFN- γ but no/low secretion of IL-2 and IL-4²¹⁹. In contrast to nTreg, Tr1 cells do not form a unique lineage but are induced in the periphery in the presence of tolerogenic APC and high amounts of IL-10²¹⁹. Similar to nTreg, Tr1 cells are hyporesponsive to stimulation and their regulatory function is dependent on appropriate TCR-mediated activation²¹⁹⁻²²¹. However, once appropriately activated, Tr1 cells mediate bystander suppression irrespective of the antigen-specificity of the co-localized effector T-cells. The main regulatory mechanism of

Tr1 cells is their release of the immunosuppressive cytokines IL-10 and TGF- β as shown by studies using neutralizing antibodies²²²⁻²²⁴. However, additional mechanisms such as the release of granzyme A and B may also contribute to suppression by Tr1 cells²²⁵. So far, no specific cell surface markers have been defined, which would allow for the selective assessment and enrichment of Tr1 cells. Unlike nTreg, Tr1 cells do not express the transcription factor Foxp3, and no Tr1-specific transcription factor has been defined until today (reviewed in Allan et al.²²⁶).

Under physiological conditions Tr1 cells are the main source of IL-10 and thus play an important complementary role to nTreg in the establishment and maintenance of immunological tolerance. Indeed, as described above knockout of IL-10 results in the spontaneous development of IBD in mice¹⁰². In humans, Tr1 cells specific for self MHC molecules²²⁷ and pancreatic-island derived peptides²²⁸ were found in healthy individuals, while patients suffering from rheumatoid arthritis have significantly lower numbers of synovial Tr1 cells²²⁹. In transplantation settings, the induction of Tr1 cells correlates with a lower frequency of GVHD in SCID patients undergoing allogeneic SCT²³⁰. Similarly, Tr1 cells may play a role in the spontaneous development of tolerance to kidney or liver allografts²³¹. In allergic patients, the activity of both nTreg and Tr1 cells is compromised. Following specific immunotherapy (SIT) the number of regulatory T-cells is increased and the concomitant increase of IL-10 and TGF- β correlates with the amelioration of clinical symptoms. In a seminal study by Akdis and co-workers it was demonstrated that desensitization of beekeepers to bee venom following multiple bee-stings correlated with an increase in IL-10¹⁸⁵. In more recent works, Tr1 cells were found to be induced following SIT to house dust mite¹⁸⁸, cat^{232, 233}, birch pollen^{186, 188, 234}, grass pollen²³⁵ and food allergens¹⁸⁸ (reviewed in Jutel and Akdis²³⁶). In summary, Tr1 cells seem to play a crucial role in modulating allergen-specific immune responses and thus represent attractive therapeutic targets.

Inducible and engineered Treg (iTreg, tg Treg)

Expansion of Treg

As described above, one of the hallmarks of CD4⁺CD25⁺ nTreg is their hypoproliferative state upon stimulation *in vitro*. However, several experimental data gathered from *in vivo* mouse models have shown that nTreg can proliferate under conditions of lymphopenia²³⁷ or when stimulated with antigen²³⁸. These observations suggest that standard *in vitro* culture conditions do not support Treg propagation and that additional factors or signals are needed. Indeed, several studies on human Treg have since then shown that expansion of purified CD4⁺CD25⁺ Treg can be achieved using dendritic cells^{239, 240}, B-cells²⁴¹, EBV transformed B-cell blasts²⁴² or anti-CD3/CD28 coated microbeads²⁴³⁻²⁴⁵ and that such protocols allow an up to 1.3 x 10⁴-fold expansion of functional Treg within three to four weeks. In all these studies, expansion of Treg relies on strong TCR stimulation and costimulatory signals and the presence of high concentrations of exogenous IL-2. Strategies to expand antigen-specific Treg include the use of tetramer-sorted Treg as starting population^{239, 240} or allogeneic APC^{241, 242} as stimulator cells. Importantly, regulatory function was found to be maintained or in some cases even enhanced²⁴⁵ in expanded Treg. However, as phenotypic analyses showed that the expression of Treg markers like Foxp3 and CD25 was rather heterogenous in expanded Treg populations, concerns about their stability and about the potential outgrowth of ‘contaminating’ effector T-cells have been raised (reviewed by Allan et al.²²⁶). In order to address these issues and to optimize expansion protocols the additional use of cytokines (IL-7 and IL-15^{246, 247}), costimulatory ligands (4-1BBL^{248, 249}) and pharmacological agents (dexamethasone²⁵⁰) has been proposed.

Induction of Treg using APC

Although nTreg constitute a thymus-derived distinct T-cell lineage, several findings also suggested that conversion of effector T-cells into regulatory Foxp3-expressing T-cells may take place in the periphery. Indeed, several specialized DC subsets were described to induce a CD4⁺CD25⁺Foxp3⁺ phenotype from CD4⁺CD25⁻ effector T-cells in the periphery²⁵¹⁻²⁵⁴. This effect was found to be dependent on cofactors such as TGF- β , the vitamin A metabolite retinoic acid and IL-10. Due to these observations it was assumed that dendritic cells could be used for the *in vitro* induction of Treg from CD4⁺CD25⁻

effector T-cells. A number of studies on murine and human cells has addressed this issue using different approaches to generate ‘tolerogenic’ dendritic cells. Generally, the tolerogenicity of dendritic cells is governed by their costimulatory setup, with low levels of costimulatory molecules and high levels of inhibitory ligands such as PD-L1 and ILT-3/4 favoring Treg induction. Other mechanisms include the up-regulation of IDO and the secretion of cytokines such as IL-10 and TGF- β (reviewed in Maldonado and von Adrian²⁵⁵). Similarly, induction of the tolerogenic state in dendritic cells can be experimentally achieved using pharmacological compounds (such as glucocorticoids^{256, 257}, cyclooxygenase inhibitors^{258, 259} or thiazolidinediones, e.g. rosiglitazone²⁶⁰), hormones (VIP²⁶¹, HGF²⁶²), soluble molecules (HLA-G²⁶³) or cytokines (IL-10^{264, 265}, TGF- β ^{266, 267}, TNF- α ^{268, 269}) and vitamin D3²⁷⁰. Other research has resorted to genetic engineering to generate tolerogenic dendritic cells. Combined knockdown of the costimulatory molecules CD40, CD80 and CD86²⁷¹ or of intracellular factors such as RelB²⁷² or SOCS3²⁷³ has led to considerable success in murine models of EAE²⁷³, CIA²⁷¹ and experimental autoimmune myasthenia gravis²⁷². Similarly, the transduction of IL-10 has led to ‘semi-mature’ dendritic cells, which potently induced CD4⁺CD25⁺Foxp3⁺IL-10⁺ Treg²⁷⁴. Of note, transduction of human mdDC with Foxp3 and co-culture of such DC with Teff led to the induction of Foxp3⁺ iTreg²⁷⁵, which showed potent regulatory capacity *in vitro*. While the exact mechanism of this Treg induction is not yet understood it is intriguing to speculate that the function of Foxp3 may not be solely restricted to the T-cell compartment.

These cumulative observations promise a huge potential for tolerogenic dendritic cells in the induction of Treg and subsequently the establishment of immunological tolerance. However, no comparative analyses on the efficacy of the different strategies exist as of today and no consensus has been reached so far concerning the optimal source of dendritic cells and the exact protocols to induce tolerance. A further point of concern is the type of iTreg generated by tolerogenic dendritic cells. A broad range of different phenotypic entities has been described ranging from CD4⁺CD25⁺ cells (with or without analysis of Foxp3 expression) to CD4⁺IL-10⁺ Treg, which thus resemble a Tr1-phenotype²⁵⁵. In this respect, regulatory capacities as well as stability of iTreg phenotype remain issues, which clearly warrant further assessments.

Induction using Cytokines, Pharmacological Compounds and Vitamins

As described above, the immunoregulatory cytokine TGF- β is a crucial factor involved in the generation of iTreg. Thus it was hypothesized that addition of TGF- β to T-cell cultures may be sufficient to induce Treg and might offer an attractive strategy to create large numbers of iTreg *in vitro*. First formal proof for this hypothesis was provided, when three groups independently showed that activation of murine CD4⁺CD25⁻ T-cells in the presence of recombinant TGF- β led to the up-regulation of Foxp3 and concomitantly the acquisition of a Treg phenotype^{172, 276, 277}. Functional analyses on these nTreg showed that they were anergic to T-cell receptor mediated stimulation and suppressive upon co-culture with responder T-cells in a cell-contact dependent way, thus sharing the typical traits of nTreg. Importantly, the regulatory activity of TGF- β induced iTreg was not restricted to *in vitro* cultures but could be also observed in an ovalbumin peptide TCR transfer model and in a mouse model of house dust mite allergy¹⁷². Since then, successful therapeutic application of iTreg has been assessed in numerous mouse models of autoimmune disease such as type I diabetes²⁷⁸, experimental colitis²⁷⁹, autoimmune gastritis²⁸⁰, EAE²⁸¹ and transplantation settings²⁸².

Mechanistically, several molecules have been assessed for their role in the TGF- β mediated generation of iTreg. A crucial co-factor, which synergizes with TGF- β is IL-2. Neutralization of IL-2 during iTreg induction abolished the development of suppressive function, while iTreg induction using allo-stimulated CD4⁺CD25⁻ T-cells requires IL-2 and TGF- β ²⁸²⁻²⁸⁴. Another factor with an implied role in iTreg generation is CTLA-4 as shown by experiments where CTLA-4 deficient murine T-cells could not be converted into iTreg²⁸⁵. According to this study, TGF- β accelerates the expression of CTLA-4 during T-cell activation, which, when ligated by CD80 or CD86, induces expression of Foxp3. Foxp3 in turn further up-regulates CTLA-4 thus leading to a positive feedback loop between TGF- β /CTLA-4/Foxp3/CTLA-4²⁸⁵.

While these experiments have clearly underlined the importance of TGF- β for the generation of murine iTreg, the situation is not as clear-cut for human Treg. Indeed, one study by the Shevach group indicated that the addition of TGF- β during T-cell stimulation could induce Foxp3 expression in CD4⁺CD25⁻ T-cells, but these T-cells did not show regulatory capacity²⁸⁶. In contrast, other reports have described the induction of fully functional Treg using similar protocols^{172, 287} or repetitive stimulation in the presence of

TGF- β ²⁸⁸. However, observations that additional presence of pro-inflammatory cytokines such as IL-1 β and IL-6 can divert the induction of Treg towards the induction of Th17 cells²⁸⁹ have raised considerable concern whether iTreg would be stable and functional *in vivo*. In this context, vitamin A derivatives such as retinoic acid (RA) seem to play an essential role in generation, stability and expansion of iTreg. First observations that addition of CD103⁺ murine DC from the lamina propria markedly increased TGF- β induced conversion of murine CD4⁺ T-cells into iTreg, could be ascribed to the production of all-trans retinoic acid in these cells^{290, 291}. Several independent studies on murine iTreg suggested that the use of RA independently of accessory cells in combination with TGF- β generated higher numbers of iTreg, which showed enhanced stability upon expansion while at the same time suppressing conversion into Th17 cells²⁹²⁻²⁹⁴. In the human system, RA could be shown to enhance histone acetylation at the Foxp3 promoter and induce Foxp3 expression in human CD4⁺ T-cells²⁹⁵ and to promote *in vitro* expansion of human nTreg²⁹⁶. More recent reports indicate that similar to murine iTreg, RA synergizes with TGF- β in the generation of highly suppressive and functionally stable human iTreg^{297, 298}. While this effect was initially described to be caused by relieving the inhibitory function of CD4⁺CD44^{high} T-cells, which prevent conversion of CD4⁺ T-cells into iTreg²⁹⁸, more recent studies have found direct effects of RA on the converted T-cells²⁹⁷. Importantly, RA plus TGF- β induced human Treg also showed high immunoregulatory capacity upon transfer in a human anti-mouse GVHD model²⁹⁷.

While iTreg generated according to these protocols show so far the most potent and stable suppressive function among iTreg, several points of concern remain. Reports that addition of RA during iTreg generation stabilizes Foxp3 expression but essentially inhibits IL-10 transcription in murine T-cells have pointed out that thus converted iTreg may not be suitable in all therapeutic contexts²⁹⁴. Similarly, comparisons of the gene profile between nTreg and iTreg have shown that the presence of RA slightly reinforced Treg gene-signature but could not overcome certain ‘holes’ in the expression pattern⁷⁰. In this respect it remains to be assessed whether iTreg can fulfill the full regulatory repertoire of nTreg in therapeutic settings.

Apart from vitamin A derivatives such as RA, vitamin 1,2(OH)₂D₃, which constitutes the biologically active form of vitamin D, has been implied in the generation of iTreg. Vitamin D₃ has potent immunomodulatory potency and can induce tolerogenic function in dendritic cells²⁷⁰. Interestingly, similar effects were also observed when purified human

CD4⁺ T-cells were activated in the presence of IL-2 and vitamin D3²⁹⁹. Thus induced Treg showed reduced secretion of IFN- γ and IL-17 and up-regulation of the Treg-specific markers Foxp3 and CTLA-4. Importantly, these cells also showed a highly suppressive capacity in co-cultures stimulated with DC plus anti-CD3 even at a responder:regulator-ratio of 10:1. Similar results could be observed when a synthetic vitamin D analogue, TX527, was used³⁰⁰.

Another pharmacological compound that has been implied to be involved in the induction and/or expansion of regulatory T-cells is rapamycin. Bound in complex with the FK506-binding protein-12, rapamycin inhibits kinase activity of the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, which is essential for cell cycle progression in effector T-cells but not in regulatory T-cells³⁰¹. Due to this difference in sensitivity, addition of rapamycin to CD4⁺ T-cell cultures from rodents²⁰⁵, non-human primates³⁰² and humans³⁰³ has been described to result in the outgrowth of regulatory T-cells from these cultures. While these regulatory T-cells do not represent pure populations, repetitive activation in the presence of rapamycin resulted in sufficiently high percentages to suppress effector T-cell responses *in vitro* and *in vivo*²⁰⁵. In contrast to these observations, other reports describe the *de novo* induction of nTreg in rapamycin-treated cultures of CD4⁺CD25⁻ T-cells^{304, 305}, indicating that Treg induction rather than expansion might be the main mechanism of action of rapamycin in T-cells.

Taking into account that rapamycin-treated T-cell cultures are Treg enriched but not pure populations, this significantly hampers their therapeutic potential. However, given its described functions, the use of rapamycin, possibly in combination with other factors such as RA, may be well warranted in expansion protocols of pre-sorted regulatory T-cell populations.

In this respect direct mTOR-inhibition by application of therapeutic levels of rapamycin or other mTOR-inhibitors such as everolimus *in vivo* has been reported to lead to increased Treg conversion, which can be attributed to direct conversion of effector T-cells to Treg^{306, 307}. Thus it remains to be seen whether rapamycin-mediated *in vitro* or *in vivo* conversion or combinations thereof eventually present highly efficient strategies.

Another immunosuppressive agent that has been implied in the generation of iTreg is anti-thymocyte globulin (ATG). ATG is a polyclonal rabbit, horse or goat IgG preparation derived from animals immunized with peripheral blood T-cells or the Jurkat T-cell line. While originally believed to exert its immunosuppressive activity by depletion of

lymphocytes upon therapeutic administration³⁰⁸⁻³¹⁰, increasing data now also point at a potential role in Treg induction. Several independent studies have reported that *in vitro* culture of human peripheral blood mononuclear cells or purified CD4⁺ T-cells in the presence of ATG results in a dramatic increase in CD4⁺CD25⁺Foxp3⁺ Treg, which display potent regulatory activity *in vitro*^{311, 312}. Along those lines, murine Treg induced by rabbit-anti-murine thymocyte globulin could efficiently inhibit GVHD upon adoptive transfer *in vivo*³¹³.

While this effect of ATG is thus well established, several points remain unresolved. It is still unclear whether ATG induces *de novo* generation of Treg from CD4⁺CD25⁻ T-cells or whether it promotes the expansion of pre-existing nTreg in the treated population. A recent report, that ATG-mediated induction of Foxp3 in T-cells is due to activation and not due to induction of *bona fide* Treg has cast doubt on the concept of ATG-induced Treg³¹⁴. Given that ATG is a very heterogenous product with possibly considerable variability between different preparation methods and batches, it seems possible that not all ATG products may display similar functional activity. This is also supported by reports that rabbit but not horse ATG promotes Treg induction³¹⁵. In this light the exact molecular targets of Treg induction by ATG remain to be determined.

Another disease modifying agent, which has been reported to induce the generation of nTreg is copolymer-I (COP-I). COP-I is a random polypeptide composed of four amino acids and has proven efficacy in the treatment of multiple sclerosis. Mechanistically, the therapeutic value of COP-I is attributed to inducing a shift of the pathological Th1 responses towards a Th2 T-cell response^{316, 317}. Other reports also indicate that treatment with COP-I leads to a decreased production of the pro-inflammatory cytokines IL-6 and IL-17 in murine EAE models^{318, 319}. In 2005 *Hong et al.* described that culture of both murine and human CD4⁺CD25⁻ T-cells in the presence of COP-I led to the up-regulation of Foxp3 in these cells accompanied by the acquisition of regulatory capacity³²⁰. Interestingly, this effect was highly dependent on IFN- γ as shown by the use of IFN- γ deficient murine T-cells or by the addition of recombinant IFN- γ to T-cell cultures. These data have been supported by *in vivo* studies in mouse models of type 1 diabetes³²¹ and EAE³²², where therapeutic efficacy of COP-I administration was associated with induction of CD4⁺CD25⁺Foxp3⁺ Treg. However, further research on the exact functional qualities and the long-term stability of COP-I induced Treg are well warranted.

The peptide hormone vasoactive intestinal peptide (VIP) is another factor that has been implied to play a role in the *in vitro* induction of CD4⁺CD25⁺Foxp3⁺ Treg. First indications about the immunomodulatory capacity of VIP were obtained in murine models of GVHD³²³, EAE³²⁴ and CIA³²⁵ in which application of VIP showed therapeutic efficacy via the *in vivo* induction of CD4⁺CD25⁺ Treg. Along those lines it could be shown that alloantigen-specific stimulation of human CD4⁺ T-cells resulted in the *de novo* induction of CD4⁺CD25⁺ iTreg, which expressed high levels of Foxp3 and CTLA-4 and showed potent suppressive capacity in allogeneic MLR cultures³²⁶. Similar to COP-I induced iTreg, the study of *Pozo et al.* so far represents a singular finding and further assessments on the phenotype, function and stability of VIP-induced iTreg are clearly needed. An overview of all described protocols to induce Treg via addition of cytokines, pharmacological agents and vitamins is provided in Table II.

Table II. *In vitro* Treg induction using cytokines, pharmacological substances and vitamins

Compound	Starting population	Species	Culture Conditions	Ref
TGF-β1	CD4 ⁺ CD25 ⁻ (C57BL/6) splenocytes (DO11.10)	mouse	anti-CD3 (0.5 µg/ml) + APC + TGF-β1 (2 ng/ml) OVA (100 µg/ml) + TGF-β1 (2 ng/ml)	172
TGF-β1	CD4 ⁺ CD25 ⁻	mouse, human	anti-CD3/anti-CD28 + TGF-β1 (1 ng/ml)	276
TGF-β1	CD4 ⁺ CD25 ⁻	mouse	anti-CD3 (plate-bound, 5 µg/ml) + anti-CD28 (soluble 0.5 µg/ml) + TGF-β1 (5 ng/ml)	277
TGF-β1+IL-2	T-cells	mouse	irradiated non-T-cells + TGF-β1 (1-10 ng/ml) + IL-2 (10 U/ml)	282
TGF-β1+IL-2	T-cells	mouse	anti-CD3/CD28 coated microbeads + TGF-β1 (2 ng/ml) + IL-2 (20 U/ml)	284
TGF-β1+IL-2	CD4 ⁺ CD45RA ⁺	human	anti-CD3 and anti-CD28 (plate-bound, 5 µg/ml each) + TGF-β1 (32 U/ml) + IL-2 (65 U/ml) + IL-9 (42 U/ml) + IL-15 (4500 U/ml) + anti-IFN-γ (20 µg/ml) + anti-IL-12 (10 µg/ml)	287
TGF-β1+RA	CD4 ⁺ CD62L ^{high} CD25 ⁻	mouse	anti-CD3 (plate-bound, 2 µg/ml) + anti-CD28 (soluble 2 µg/ml) + TGF-β1 (2 ng/ml) + all- <i>trans</i> RA (10 nM)	292
TGF-β1 +RA+IL-2	CD4 ⁺ Foxp3 ⁻	mouse	anti-CD3 (plate-bound, 10 µg/ml) + anti-CD28 (plate-bound 1 µg/ml) + TGF-β1 (20 ng/ml) + all- <i>trans</i> RA (10 nM) + 100 U/ml IL-2	293
TGF-β1+RA	CD4 ⁺ CD44 ^{lo} GFP ⁻ (10B1T <i>Foxp3</i> ^{gfp} .OT-II mice)	mouse	DC + 1 µg/ml OVA + TGF-β1 + all- <i>trans</i> RA (100 nM)	294
TGF-β1	CD4 ⁺ CD45RO ⁺ CD25 ⁻	human	anti-CD3/CD28 coated microbeads + TGF-β1	297

+RA+IL-2		(5 ng/ml) + all- <i>trans</i> RA (100 nM) + IL-2 (50-100 U/ml)		
Vitamin D	CD4 ⁺ CD25 ⁻	human	anti-CD3/CD28 coated microbeads + 1,25(OH) ₂ D ₃	299
TX527	CD4 ⁺ CD25 ^{high} CD127 ^{low}	human	anti-CD3 and anti-CD28 (plate-bound, 5 µg/ml each) + IL-2 (12.5 ng/ml) + 10 ⁻⁸ M TX527	300
Rapamycin	CD4 ⁺	mouse	anti-CD3 (plate-bound, 10 µg/ml) + anti-CD28 (soluble 1 µg/ml) + IL-2 (50 U/ml) + Rapamycin (100 nM)	205
Rapamycin	CD4 ⁺	baboon	anti-CD3/CD28 coated microbeads + irradiated pig PBMC + IL-2 (500 U/ml) + Rapamycin (0.1-10 nM)	302
Rapamycin	CD4 ⁺	human	anti-CD3 (plate-bound, 10 µg/ml) + anti-CD28 (soluble 1 µg/ml) + IL-2 (100 U/ml) + Rapamycin (100 nM)	303
Rapamycin	CD4 ⁺	human	anti-CD3 (plate-bound, 0.4 µg/ml) + anti-CD28 (soluble 1 µg/ml) + IL-2 (100 U/ml) + Rapamycin (100 nM)	304
Rapamycin	CD4 ⁺ CD25 ⁻	human	anti-CD3 (plate-bound, 5 µg/ml) + anti-CD28 (soluble 1 µg/ml) + IL-2 (200 U/ml) + Rapamycin (100 nM)	305
ATG	PBL	human	Thymoglobulin (10 µg/ml)	311
ATG	PBMC	human	Thymoglobulin (100 µg/ml)	312
ATG	splenocytes	mouse	mATG (100 µg/ml) + IL-2 (200 U/ml)	313
ATG	PBMC	human	rabbit-derived but not horse-derived ATG (10 µg/ml)	315
COP-I	PBMC/T-cells	human	irradiated T-cell depleted PBMC + COP-I (40 µg/ml)	320
VIP	CD4 ⁺ CD25 ⁻	human	HLA-mismatched irradiated PBMC + VIP (100 nM)	326

APC, antigen-presenting cell; RA, retinoic acid; DC, dendritic cells; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; ATG, anti-thymocyte globulin; mATG, anti-murine thymocyte globulin; VIP, vasoactive intestinal peptide

Induction of Treg via Genetic Engineering

The development of modern techniques to genetically modify peripheral blood lymphocyte populations has also opened intriguing possibilities for the development of genetically engineered regulatory T-cells. Observations that forced over-expression of Foxp3 in murine CD4⁺ T-cells could transfer the key features of nTreg phenotype and function, i.e. expression of CD25, CTLA-4 and other markers, hyporesponsiveness to TCR-mediated stimulation and regulatory capacity⁴⁻⁶, spurred hope that similar results could be achieved with human T-cells. In first experiments, retroviral over-expression of either of the two common isoforms of Foxp3, i.e. Foxp3 and Foxp3ΔE2, in CD4⁺ T-cells failed to generate regulatory T-cells³²⁷. However, the use of a lentiviral-vector system, which was designed to allow higher and more stable expression of the Foxp3 transgene, led to the successful generation of engineered regulatory T-cells²⁴². Such artificial

regulatory T-cells showed a typical nTreg phenotype with up-regulation of CD25, GITR and CTLA-4. Following TCR-mediated stimulation, Foxp3-transgenic Treg were hyporesponsive and secreted only low amounts of IL-2, IL-4, IFN- γ and IL-10 compared to control-transduced cells. Upon co-culture with CD4⁺CD25⁻ effector T-cells, Foxp3 transgenic (tg) T-cells were highly suppressive and this suppressive capacity was equal to purified CD4⁺CD25^{high} nTreg. Importantly, the phenotype and function of such artificial Treg were stable even after repetitive expansion over several weeks. At the same time, similar results were obtained independently by *Aarts-Riemens et al.* who showed that retroviral over-expression of either of the two isoforms of Foxp3 was equally sufficient to generate potent engineered Treg³²⁸. These data imply that retroviral expression systems are similarly suited for the generation of engineered Treg as long as stable, high-level expression of Foxp3 can be achieved.

While Foxp3-transduction has been shown to generate T-cells with regulatory function, these cells represent a polyclonal population with the endogenous TCR repertoire of the purified peripheral blood T-cells used as starting population. In this respect, it seems highly probable that such Treg populations will contain a broad range of specificities, some representing important targets for normal immune function. On the other hand, Treg with the desired antigen-specificity (autoreactive, allergen-specific or allo-specific) would most probably only be found at a low frequency. To address this problem, several studies have resorted to the genetic modification of CD4⁺CD25⁺ T-cells in order to introduce antigen-specificity into Treg. In one study, receptor-modified murine CD4⁺CD25⁺ T-cells were transduced with an antigen-MHC class II-TCR- ζ fusion-construct and upon transfer could prevent and cure EAE induced by vaccination with myelin basic protein³²⁹. Similarly, transduction of murine nTreg with a chimeric receptor recognizing trinitrophenol (TNP) generated regulatory T-cells, which could ameliorate TNBS-induced colitis³³⁰.

In the human system, the transduction of CD4⁺CD25⁺ nTreg with a multicistronic expression construct encoding the α - and β -chain of a melanoma antigen-reactive TCR led to the generation of engineered antigen-specific human Treg, which could potentially suppress anti-tumor immune responses in a murine melanoma model³³¹. Along those lines *Hombach et al.* recently described the generation of engineered carcinoembryonic antigen (CEA)-specific human Treg by introduction of a chimeric receptor into purified human CD4⁺CD25⁺ nTreg³³². While these approaches have proven the feasibility to engineer

nTreg with a specific reactivity, they may bear several shortcomings. The purification of nTreg is a tedious process, which is considerably hampered by the low frequency of nTreg in peripheral blood and the need to select for several surface markers in order to exclude ‘contaminating’ activated effector T-cells. Secondly, nTreg from patients suffering from autoimmune or allergic disorders may be compromised in their function, which would significantly impede their usefulness for therapeutic approaches.

In this light, we have recently developed a novel strategy to engineer allergen-specific regulatory T-cells from peripheral blood $CD4^+CD25^-$ T-cells. Using a retroviral vector-based multicistronic expression element, we have introduced the regulatory factor Foxp3 and the α - and β -chain of a TCR specific for the birch pollen allergen derived peptide Bet v 1₁₄₂₋₁₅₃ into human $CD4^+$ T-cells. The accordingly modified engineered Treg potently suppressed the proliferation and cytokine production of human TCR-transgenic effector T-cells in an activation-dependent manner³³³. In our opinion this approach offers the advantage that $CD4^+$ T-cells could be used as starting population, which should in principle be more readily available from any patients’ blood than naturally occurring Treg. A major impediment for the use of human Foxp3-tg Treg may come from studies comparing $CD4^+CD25^+$ nTreg and Foxp3⁺ engineered Treg, which have shown that the gene signature between these two populations differs significantly⁷⁰. Thus it remains to be investigated, whether Foxp3-tg Treg can fulfill the full physiological repertoire of functions of nTreg. Table III gives an overview of the genetic manipulation of human Treg cells.

Apart from Foxp3 over-expression, approaches to exploit other Treg related molecules for genetic engineering have so far been scarce. In murine $CD4^+$ T-cells, the over-expression of LAG-3 has been shown to generate a T-cell population with regulatory properties⁶⁴. Similar observations have been described for T-cells engineered to over-express the LAG-3 regulating factor Egr-2⁶⁴. However, expression of the nTreg-associated marker CD25 was found to be low on these cells, thus raising questions whether such engineered Treg constitute *bona fide* nTreg with similar physiological properties. Additionally, no data about the overexpression of LAG-3 or Egr-2 in human T-cells have so far been published. Another intriguing molecule for the generation of engineered Treg is CD83. In a study on murine $CD4^+CD25^-$ T-cells, ectopic over-expression of CD83 was shown to result in T-cells, which exerted regulatory function towards $CD4^+CD25^-$ effector T-cells *in vitro* and could suppress disease in mouse models of contact-hypersensitivity of the skin and in

EAE⁹⁴. Importantly, over-expression of CD83 also resulted in up-regulation of Foxp3 indicating that these cells may share features with nTreg.

In another study using human T-cells, it could be shown that siRNA-mediated knockdown of the novel Treg marker galectin-10 reversed the anergic and suppressive function of human nTreg. However, in the reverse experiment over-expression of galectin-10 in CD4⁺CD25⁻ effector T-cells led to the induction of apoptosis even when using a range of different viral and non-viral delivery methods⁶⁹. In this light it seems probable that nTreg express a network of factors that are non-redundantly involved in the maintenance of their regulatory function. While the ablation of one factor might be sufficient to significantly disturb this regulatory network, the isolated over-expression of a singular factor may have different consequences in CD4⁺CD25⁻ effector T-cells.

Table III. Transgenic human Treg

Transgene(s)	Starting population	Regulatory function	Antigen-specificity	Suppression model	Ref
Foxp3	CD4 ⁺ CD25 ⁻ CD45RO ⁺	engineered	polyclonal	in vitro	242,328
melanoma-antigen tyrosinase specific TCR	CD4 ⁺ CD25 ⁺ CD127 ^{low/-}	endogenous	engineered	enhanced tumor progression in mice	331
CEA-specific chimeric receptor	CD4 ⁺ CD25 ^{high}	endogenous	engineered	enhanced tumor progression in mice	332
birch-pollen allergen (Bet v 1) specific TCR and Foxp3	CD4 ⁺ CD25 ⁻	engineered	engineered	in vitro	333

CEA, carcinoembryogenic antigen; TCR, T-cell receptor

Conclusion

The definition of regulatory T-cell subsets and the subsequent elucidation of their crucial contribution to peripheral tolerance have spawned hope for the applicability of such regulatory T-cells as therapeutic tools in still badly controllable diseases and medical conditions. Indeed accumulating evidence suggests, that at least at the experimental level, Treg-based therapies can have preventive or even curative effects. In this respect, several strategies have been proposed to achieve sufficiently high Treg numbers, which would be needed for therapeutic application. Isolation and subsequent expansion of nTreg has been so far the method of choice and clinical studies on GVHD patients are currently underway. Other proposed strategies include the induction of nTreg from effector T-cells using antigen-presenting cells, cytokines and pharmacological agents or genetic engineering of T_{eff} or Treg. While all approaches bear their specific advantages and disadvantages, several common problems exist. Under the protocols currently used, no method of Treg induction has been shown to lead to the generation of 100% Foxp3⁺ nTreg. Although suppressive capacity *in vitro* does not require this level of purity, ‘contaminating’ effector T-cells could pose a severe impediment upon adoptive transfer into patients. In this respect, it remains to be determined, which thresholds for nTreg purity must be imposed in order to achieve an acceptable ratio between efficacy and side effects. Given the different entities and subpopulations of Treg cells, it seems conceivable that different therapeutic applications may require different types of Treg. Especially the requirement for the secretion of certain cytokines such as IL-10 and TGF- β , which are necessary for the disease-modifying quality of Treg in some studies, remains a challenge. Consequently, a better definition of subset markers and of signals, which govern site-specific Treg function, is well warranted and requires further efforts in basic research. Another challenge for the therapeutic application of regulatory T-cells is antigen-specificity. A multitude of murine disease models has shown that antigen-specific Treg are superior in their disease-modifying quality as well as their required number when compared to polyclonal Treg populations. While use of TCR-transgenic mouse strains has helped to elucidate these requirements, more elaborate strategies have to be applied for human Treg therapies. Until today, the most promising prospects lie in the field of transplantation tolerance where host Treg can be either induced or expanded using donor APC thus guaranteeing a large percentage of donor/allo-reactive Treg. Other options include the cloning and subsequent expansion of self- or allergen-reactive regulatory T-cells or gene-

transfer of TCR α/β -chains or surrogate T-cell receptors. While all these approaches are technically feasible, it remains to be determined, which strategy offers the best results in regard to yield, applicability for GMP production and safety (reviewed in Riley et al.³³⁴ and Bluestone³³⁵).

Along those lines, more insight into the pathophysiology of human autoimmune diseases, allergies and the processes involved in transplant rejection in respect to which antigens should be the targets of Treg therapies is clearly needed. Currently, such research projects on allergic disorders have defined that some allergies might be rather uniform disorders with a high percentage of allergic individuals reacting to one or only a few immunodominant peptides^{336, 337}.

In conclusion, development of different strategies to isolate, induce, expand or modify regulatory T-cell function and numbers has opened an exciting field of research, which holds great promise for the development of therapies in severe diseases. However, current research has not exceeded the basic level and further assessments as to the detailed definition of specific pathologies and the behavior of differently modified Treg in therapeutic settings is warranted.

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Chapter 2

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

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ABSTRACT

Background: Ninety-five percent of mugwort allergic patients 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 1₂₅₋₃₆ have an HLA-DRB1*01 phenotype.

Objective: Cloning and functional expression of a human $\alpha\beta$ T-cell receptor (TCR) specific for Art v 1₂₅₋₃₆.

Methods: TCR chains were RT-PCR-amplified from an Art v 1₂₅₋₃₆-specific T-cell clone, retrovirally transferred and functionally tested in Jurkat T-cells or alternatively in PB T-lymphocytes of non-allergic individuals.

Results: The α -chain of the TCR is composed of TRAV17 and TRAJ45 segments, 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 V β 18-specific mAb and DRB1*0101/Art v 1 tetramers. Transgenic (tg) Jurkat T-cells specifically recognized syngeneic EBV B-cells pulsed with Art v 1₂₅₋₃₆ peptide and artificial antigen presenting cells (aAPC) expressing invariant chain::Art v 1 fusion proteins. Moreover, transfer of the TCR into PBL generated T-cells that were Art v 1-reactive. Activation of tg T-cells by aAPC was strictly dependent upon co-stimulation.

Conclusion: For the first time, a detailed molecular and functional analysis of a human allergen-specific TCR is presented.

Clinical Implication: Expressible allergen-specific TCRs may contribute to a better definition of the ‘allergen-specific synapse’ and thus the processes leading to allergic diseases and their cure.

INTRODUCTION

T lymphocytes play a central role in the pathogenesis of allergic diseases. During the sensitization, priming of allergen-specific (CD4⁺) T helper 2 (T_H2) cells results in the production of T_H2 cytokines, which are responsible for class switching to the ϵ Ig 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, e.g. in the airways and the skin.² The clonotypic specificity of T-cells is brought about by the TCR, which shows dual specificity for the antigen (allergen) and for self (MHC) determinants.³ Signal transduction by the TCR is conducted by non-covalently associated invariant CD3 subunits.⁴⁻⁶

The antigen specificity of T-cells can be transferred to other T-cells by TCR $\alpha\beta$ gene transfer.⁷⁻⁹ TCR transgenic (tg) model-systems have contributed greatly to our understanding of the immune response to autoantigens and infectious agents¹⁰⁻¹² 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 past¹⁵⁻¹⁹, no molecular or functional analyses of cloned and ectopically expressed human allergen-specific TCR have been reported so far. Consequently, biological model systems based on human allergen-specific TCRs are still missing.

An ideal human allergen-specific model system would be comprised of 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 mugwort allergic patients are sensitized to Art v 1, the major allergen in mugwort pollen. Analyzing the T-cell response to Art v 1 using a panel of specific T-cell lines and clones revealed that the presentation of the sole immunodominant epitope (Art v 1₂₅₋₃₆) 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 genetic TCR transfer. The molecular and functional characterization of human allergen-specific TCRs - as critical tools of defined model systems in which all three components of the 'allergen-specific synapse' are well defined, i.e. restriction element, immunodominant peptide, TCR - shall provide important insights into the pathophysiology of allergic diseases and their possible cure in the future.

RESULTS

*Molecular cloning of the $\alpha\beta$ TCR of an Art v 1₂₅₋₃₆-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 mugwort pollen-allergic individual (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, i.e. Art v 1₂₅₋₃₆, 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 V α gene segment of the TRAV17 gene family to a TRAJ45 segment (Fig 1, nomenclature according to Lefranc²⁹). 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 three non-germline encoded codons are present, while the two C-terminal TRAV17 codons have been deleted. CDR3b is composed of germline sequences except for the addition of a single arginine. Assessment of 23 T-cell clones of ten additional Art v 1 allergic individuals revealed that 12% of the clones expressed TRAV17 and 9% of the clones expressed TRBV18 indicating that Art v 1₂₅₋₃₆-specific T-cell responses are not confined to TCRs consisting of 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 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 V β 18 expression in ~60% of cells (Fig 2). Expression levels of CD3, pan TCR $\alpha\beta$ and CD4 were comparable in wild type (wt) and TCR tg Jurkat cells in bulk transductants as well as in single cell clones, whereas e.g. CD2 expression levels showed considerable variations (Fig 2). A similar variability was observed for V β 8 and V β 18 expression on single cell clones. Clone #3 showed residual co-expression of the endogenous V β 8 chain, whereas clones #5 and #7 showed mutual exclusive expression of the tg V β 18 chain (Fig

2), which might be caused by different levels of over-expression (LTR-driven or via copy-number) of the tg TCR and competition for the

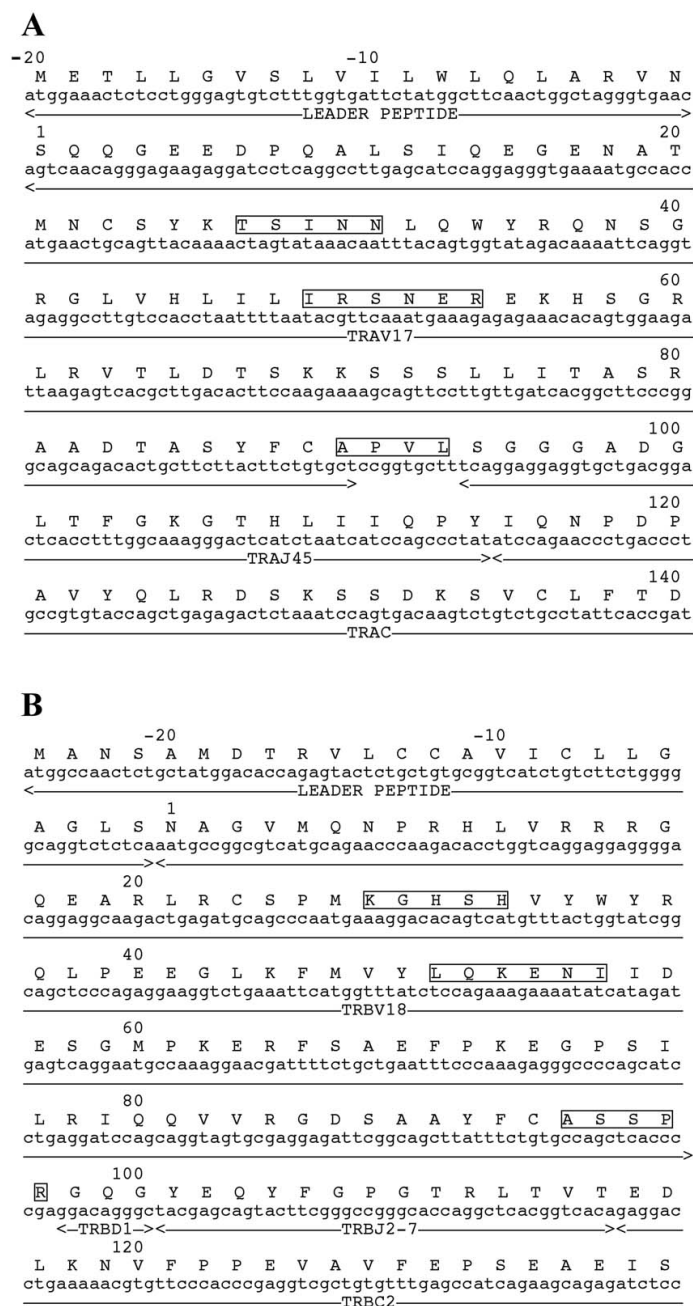


FIG 1. Nucleotide and predicted amino acid sequence of the HLA-DRB1*0101 restricted TCR of the Art v 1-specific T-cell clone SSR20. (A) TCR α -chain sequence; (B) TCR β -chain sequence. Codon numbering and nomenclature according to IMGT-TR²⁹. Only the 5'-ends of the constant region sequences are shown. Complementarity determining regions are boxed.

constitutively expressed CD3 complex. HLA-DRB1*0101/Art v 1₁₉₋₃₆-specific tetramers bound to TCR transductants exclusively (Fig 2) indicating that upon transfer the tg TCR retained its capability to specifically interact with the restriction element/peptide-complex against which it had been selected in vivo.

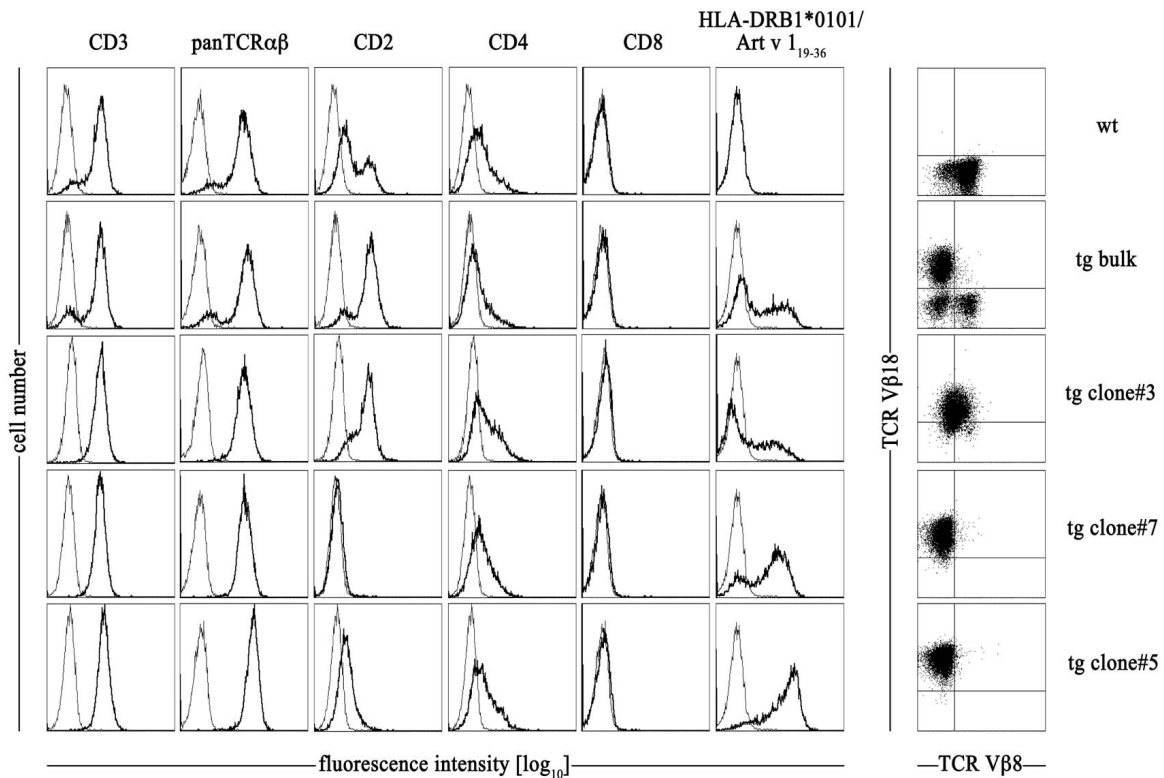


FIG 2. Expression pattern of endogenous TCR, Art v 1-specific tg TCR and accessory molecules on wt, bulk TCR tg and TCR tg single cell clones (#3, #5 and #7) of Jurkat T-cells are shown. Binding of specific mAbs or Art v 1-specific tetramers (HLA-DRB1*0101/Art v 1₁₉₋₃₆) (bold lines) is compared to control antibody binding (thin lines). Two-parameter dot-plot analysis of Jurkat cells stained with TCR Vβ8 and TCR Vβ18 mAb (right panel). Data are representative of two independently performed experiments.

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 1₂₅₋₃₆^{21, 22} also in functional terms, the TCR transductants (subclone #5) were co-incubated with syngeneic EBV B-cells that had been pulsed with saturating concentrations of Art v 1₂₅₋₃₆ or control peptides from Art v 1 (Art v 1₁₉₋₃₀, Art v 1₉₄₋₁₀₅) or influenza hemagglutinin (HA₃₀₆₋₃₁₈). Fig 3, A shows specific recognition of Art v 1₂₅₋₃₆ 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 co-expression 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 1₁₉₋₃₀ or Art v 1₉₄₋₁₀₅), the DRB1*0101/0401-restricted peptide from influenza hemagglutinin (HA₃₀₆₋₃₁₈)³⁰, or medium alone were unable to stimulate TCR tg T-cells. Cellular activation induced by Art v 1₂₅₋₃₆ was strictly dependent on the presence of APC as no T-cell stimulation was observed upon co-incubation of TCR tg Jurkat T-cells with peptides in the absence of EBV B-cells (Fig 3, A). The immunodominant Art v 1₂₅₋₃₆ 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 - 33.4 μ M (12.4-54.8 μ g/ml) peptide for half-maximal stimulation (not shown).

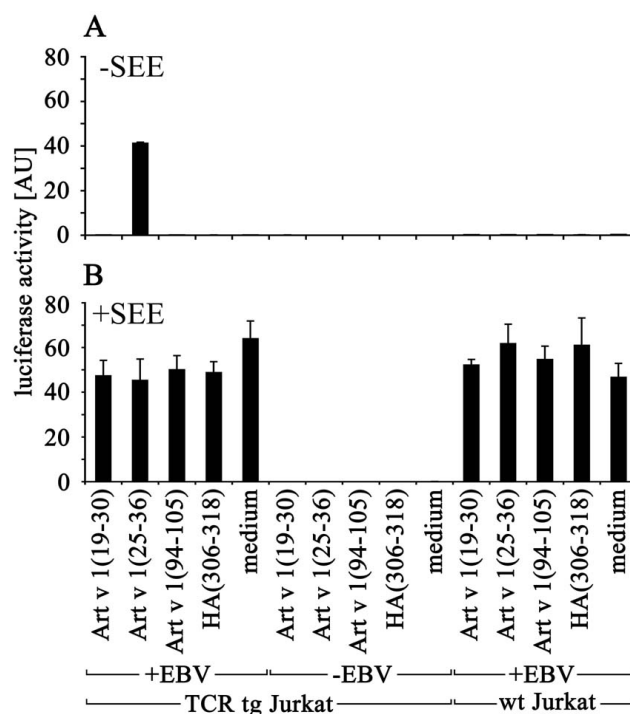


FIG 3. The Art v 1-specific TCR in tg Jurkat T-cells is functionally active. (A) IL-2 promoter activity of Art v 1 TCR tg or wt Jurkat T-cells upon co-culture with or without HLA-DRB1*0101⁺ EBV B-cells and the indicated peptides or with medium alone. (B) Similar to A but supplemented with SEE. Data show mean values + SD of triplicate cultures representative for several independently performed experiments.

TCR tg Jurkat T-cells recognize Art v 1 borne by engineered invariant chains (Ii)

The HLA class II-associated Ii peptide (CLIP) occupies the peptide binding groove during assembly and transport and becomes exchanged to antigenic peptides in endo-/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 aAPC by transfecting human 293 cells with HLA-DRA*0101, HLA-DRB1*0101, CD80, CD54, cathepsin S (CatS) 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 1₂₇₋₃₄ epitope²¹ – were able to stimulate the Art v 1-specific tg Jurkat T-cells similar to the positive control PHA/PMA (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). No SEE dependent response was, however, observed in the absence of aAPC. Fig 4, C shows that HLA Class II expression was similar on all aAPC types whereas Ii expression levels gave a more heterogeneous picture (Fig 4, D). Ii::Art v 1₁₋₁₀₉ fusion protein repeatedly showed the weakest expression, which also resulted in a weaker stimulatory capacity of Ii::Art v 1₁₋₁₀₉ transfected aAPC. Significantly, T-cell activation was only moderate in the absence of co-stimulation (stimulation index (SI): 3.2 ± 0.34), however, became clear-cut when co-stimulatory molecules such as CD80 were co-expressed (SI: 44.7 ± 8.7) (Fig 5). In our system, co-expression of CD80 together with the intercellular adhesion molecule-1 (ICAM-1, CD54) on aAPC revealed only moderate additional synergy (SI: 58.1 ± 15.2 , Fig 5), which may reflect already maximal stimulation by CD80 alone making a further enhancement by CD54 impossible.

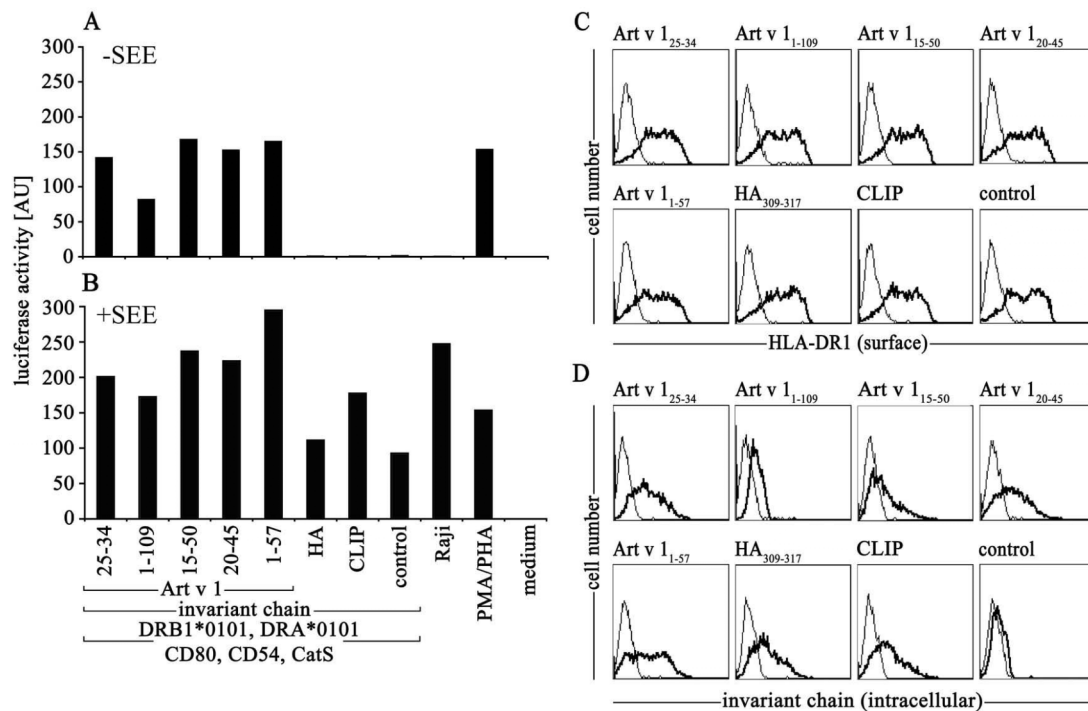


FIG 4. Art v 1-specific TCR tg Jurkat T-cells recognize Ii encoded allergenic peptides presented by aAPC. (A) IL-2 promoter activity of TCR tg Jurkat T-cells (bulk) co-cultured with 293 cells expressing HLA-DRA*0101, HLA-DRB1*0101, CD80::GPI, CD54::GPI, cathepsin S and the indicated forms of Ii or controls.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

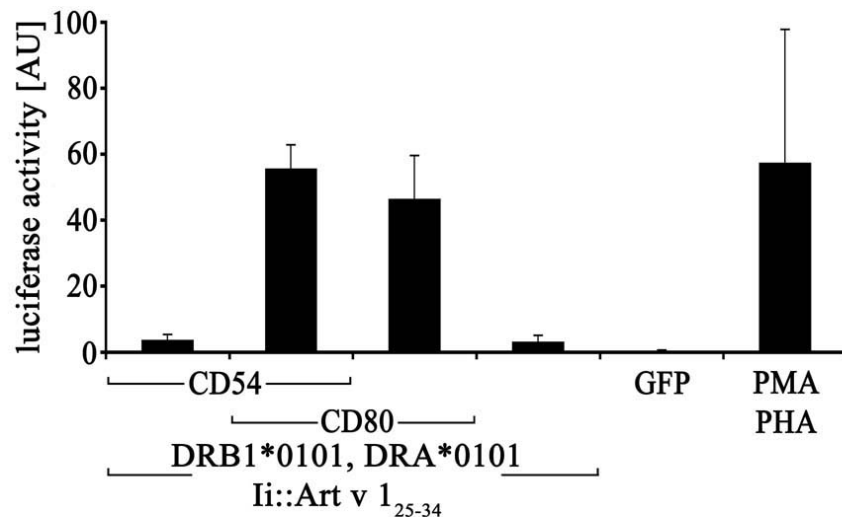


FIG 5. Efficient stimulation of Art v 1-specific Jurkat T-cells requires co-stimulation. IL-2 promoter activity of TCR tg Jurkat T-cells incubated with 293 cells co-transfected with DRA*0101, DRB1*0101, Ii::Art v 1₂₅₋₃₄, CD54, CD80, GFP or control plasmid alone or combined as indicated or PMA plus PHA. Data show mean values + SD of triplicate cultures representative of three independently performed experiments.

Transfer of allergen-specific TCR into human peripheral blood lymphocytes

In a next step it was tested whether it was possible to generate human Art v 1-specific T lymphocytes from non-allergic individuals. For that purpose PBL from six donors (HLA-DRB1*0101 positive or negative) were transduced with the Art v 1-specific TCR. Transduction efficiencies ranged from 10-30 % as determined by flow cytometry of CD3⁺TCR V β 18⁺ T-cells (Fig 6, A). TCR tg T-cells from all donors specifically proliferated upon co-incubation with aAPC expressing Ii::Art v 1₂₅₋₃₆ but not with aAPC expressing Ii::HA₃₀₉₋₃₁₇. Control-transduced T-cells (GFP) of HLA-DRB1*0101 negative donors showed residual reactivity with HLA-DRB1*0101 expressing aAPCs, possibly reflecting alloreactivity, which was not observed with responder T-cells from HLA-DRB1*0101 positive donors. Moreover, stimulation of TCR tg PB T-cells was strongly dependent on CD80 mediated co-stimulation (Fig 6, B). Within ten days, up to 1×10^7 Art v 1-specific T-cells can be generated from 10 ml of peripheral blood of various donors. This cell number can be further increased by a factor of ~ 3 by supplementation of TCR tg T-cells with growth factors, e.g. IL-2. Thus, PB T-cells from non-allergic donors can be genetically modified to react with a well-defined allergen such as Art v 1.

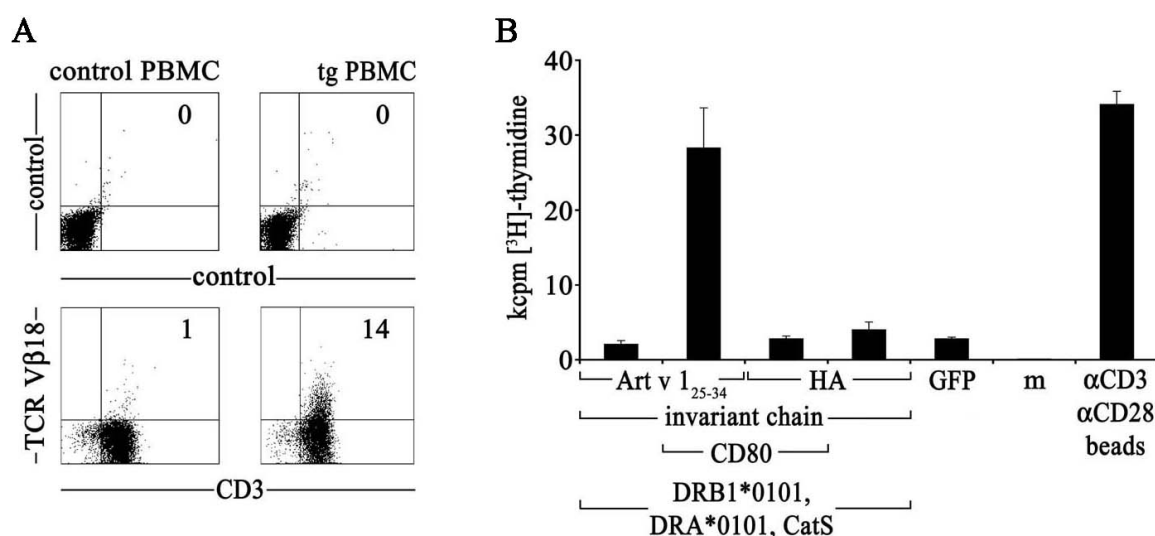


FIG 6. TCR tg PB T-cells proliferate upon challenge with Ii::Art v 1₂₅₋₃₆ expressing aAPC. (A) Expression levels of TCR V β 18 and CD3 on control and tg PB T-cells. Numbers indicate percentage of CD3⁺TCR V β 18⁺ cells. (B) Proliferation of human PBMC transduced with Art v 1-specific TCR co-cultured with aAPC co-expressing the indicated molecules or alternatively with microbeads coated with CD3 plus CD28 mAbs, or with medium alone. Data show mean values + SD of triplicate cultures corrected by mean values (2.6 kcpm, range: 0-8.2 kcpm) of GFP-transduced tg T-cells. Data are representative of several independently performed experiments.

DISCUSSION

We cloned, sequenced and transferred the TCR $\alpha\beta$ -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 non-allergic individuals showed clear-cut expression of the tg TCR and specifically reacted with Art v 1₂₅₋₃₆ peptide-pulsed autologous EBV B-cells as well as with aAPC expressing Ii::Art v 1 fusion proteins. Significantly, stimulation of TCR tg T-cells was dependent on co-expression of co-stimulatory 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 APCs and T-cells. Whereas immunodominant peptides and corresponding restriction elements of major allergens have been characterized in detail during the past two 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 biological model systems in the future, in which all three components of the allergen-specific synapse, i.e. the restriction element, the immunodominant peptide as well as 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 blood of patients with clinically well-defined allergy, a cumbersome task, which 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, independently of the allergic status of the donor and of 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 allergens³³ (allergen (peptide)-fragments,³⁴ -trimers,³⁵ -conjugates,³⁶ shuffled-allergens,³⁷ or hybrid molecules³⁸) demands for test systems able to predict their immunological behaviour. Allergen-specific tg T-cells might represent

such a standardizable tool to e.g. 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 recombinant DNA-technology altered peptide ligands (APL) of immunodominant allergens. Conventionally, APL 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 e.g. error-prone PCR approaches⁴³ might serve as a novel high-throughput screening approach for such purposes.

Moreover, expressible allergen-specific TCRs might also lay the ground 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’ shall represent powerful biological systems to analyze T-cell dependent (but also independent) pathways influencing allergic diseases with the required profoundness in the future.

METHODS

Cell lines and primary cells. 293 cells, 293 OKT3scFv::GPI transfectants,²³ Raji and Jurkat clone 41-19 expressing an IL-2 enhancer/promoter driving luciferase were cultured as described.²³ Art v 1-specific T-cell lines, T-cell clones, and immortalized B-cells were established from PBL of mugwort pollen allergic individuals with typical clinical history and positive skin prick tests to mugwort pollen extract as described.²² The TCR gene usage was determined by RT-PCR with TRAV and TRBV primers (Clontech, Heidelberg, Germany) as described.²²

Cloning and Characterization of $\alpha\beta$ TCRs. mRNA from the Art v 1-specific T_H0-cell clone SSR20, which upon antigen-specific stimulation produced 22 pg/ml IL-4 and 75 pg/ml IFN- γ , was amplified with: TRAV17-for: 5'-CGCGGGAAGCTTGCCACCATGGAACTCTCCTGGGAGTG-3' and TRAC-rev: 5'-CCCGCGGCGGCCGCTTTAGCTGGACCACAGCCGC-3', or TRBV18-for: 5'-CGCGGGAAGCTTGCCACCATGGCCAACTCTGCTATGGAC-3' and TRBC-rev: 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, MA) and DNA-sequenced (VBC Genomics, Vienna, Austria).

Cloning of HLA-DR constructs. SSR (EBV immortalized B-cells) mRNA was amplified with: HLA-DRA-for: 5'-CGCGGGAAGCTTGCCACCATGGCCATAAGTGGAGTCCC-3' and HLA-DRA-rev: 5'-CCCGCGGCGGCCGCTTTACAGAGGCCCCCTGCG-3'; or HLA-DRB1*0101-for: 5'-CGCGGGAAGCTTGCCACCATGGTGTGTCTGAAGCTCCC-3' and HLA-DRB1*0101-rev: 5'-CCCGCGGCGGCCGCTTTAGCTCAGGAATCCTGTTGGCT-3', digested with Hind III (bold) and Not I (underlined) and gel-purified. DNA-fragments were ligated into pEAK12 expression vector (EdgeBio, Gaithersburgh, ME) and DNA-sequenced.

Generation of invariant chain constructs. pcDNA1.1/Amp containing an Ii with a Sfu I – Eco 47III cassette instead of CLIP was kindly provided by J. vanBergen

(Immunohematology and Blood Transfusion, Leiden, The Netherlands).²⁴ Double stranded Art v 1₂₅₋₃₄ oligonucleotides: Art v 1₂₅₋₃₄-upper: 5'-CGAAGTGCATCGAGTGGGAGAAGGCCAGCACCAGGC-3', Art v 1₂₅₋₃₄-lower: 5'-GCCTGGTGCTGGGCCTTCTCCCACTCGATGCACTT-3' were inserted into Sfu I/Eco 47III digested pcDNA1.1/Amp-Ii. CLIP and HA₃₀₉₋₃₁₇ containing Ii were constructed as described.²⁴ Length variants of Art v 1 were constructed by PCR using humArt v 1²⁵ as template amplification with the following primers: humArt v 1₁₋₁₀₉-for: 5'-CGCGGGTTCGAAGATGGCCGGCAGCAAGCTG-3', humArt v 1₁₋₁₀₉-rev: 5'-GGGCGCGGGCAGCGCTTGGTGGGTGCTGGGGGG-3'; humArt v 1₁₅₋₅₀-for: 5'-CGCGGGTTCGAAGAGCGGCAAGTGCGACAACAAG-3', humArt v 1₁₅₋₅₀-rev: 5'-GGGCGCGGGCAGCGCTTGGCAGAAGCAGCTCTCCTTGCC-3'; humArt v 1₂₀₋₄₅-for: 5'-CGCGGGTTCGAAGAACAAGAAGTGCGACAAGAAG-3', humArt v 1₂₀₋₄₅-rev: 5'-GGGCGCGGGCAGCGCTTGCTTGCCGGCCTCCCGCTT-3'; humArt v 1₁₋₅₇-rev: 5'-GGGCGCGGGCAGCGCTTGGCTCTTGCTGCAGTCGAAGTA-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 $\alpha\beta$ TCRs into Jurkat and PB T-cells. Amphotropic TCR $\alpha\beta$ delivering retroviruses were produced by transient transfection using standard procedures.²⁶ Briefly, 293 OKT3scFv were transfected with pMD.gagpol (*Moloney murine leukemia virus* (MoMLV) *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 control. Jurkat clone 41-19 or PB T-cells were transduced with viral supernatant in the presence of 8 μ g/ml polybrene (Sigma, St. Louis, MO). Prior to transduction PB T-cells were pre-stimulated with anti-CD3/CD28 substituted microbeads (Dynabeads, Dynal Biotech) and optionally with 300 U/ml IL-2 (Peprotech, London, UK). Jurkat clones were obtained by limiting dilution.

Immunofluorescence analyses. MAbs CD3-FITC (UCHT1), pan TCR $\alpha\beta$ -PE (BMA031), CD2-FITC (TS2.18), CD4-FITC (VIT4), CD8-PE (VIT8b) and control Ig FITC and PE were obtained from ADG (Kaumberg, Austria). TCR V β 8-FITC (56C5) and TCR V β 18-

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 described.²⁸ For tetramer staining Jurkat cells (4×10^5) were incubated with 4 μ l of PE-labelled DRB1*0101/Art v 1 peptide specific tetramer (Beckman Coulter, Marseille, France) containing Art v 1₁₉₋₃₆ (NKKCDKKCIEWEKAQHGA) at 37°C in the dark for 2 hours, washed once and fixed in 200 μ l 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 pre-incubated with the peptides (3×10^{-5} M) Art v 1₁₉₋₃₀, Art v 1₂₅₋₃₆, Art v 1₉₄₋₁₀₅, influenza HA₃₀₆₋₃₁₈ or medium alone, respectively, for three hours. Subsequently, TCR tg Jurkat cells or wt Jurkat cells (1×10^5) were added and co-cultured with pre-sensitized, syngeneic EBV cells or peptide alone for 6 hours. Alternatively, aAPC (5×10^4) expressing HLA-DR1, CD54, CD80 and Ii constructs served as stimulators. Staphylococcal enterotoxin E (SEE, 0.3×10^{-6} M, Toxin Technologies, Sarasota, FL) or PHA (5 μ g/ml) plus PMA (10^{-7} M) served as positive controls. Luciferase activity was assayed as described.²⁸

Generation of aAPC. 293 cells (3×10^6) were transiently co-transfected with HLA-DRA*0101, HLA-DRB1*0101, CD54, CD80, cathepsin S²⁸ 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 /well) were incubated with aAPC (5×10^4 /well, irradiated with 60 Gy) expressing HLA-DRA*0101, HLA-DRB1*0101, Ii::Art v 1₂₅₋₃₄, Ii::HA₃₀₉₋₃₁₇, CD80, CatS combined as indicated in 96-well flat bottom tissue culture plates for three days. Cells were pulsed with methyl-[³H]-thymidine (1 μ Ci/well) for another 18 h and processed as described.²³ Medium alone or anti-CD3/CD28 microbeads (5×10^4) served as controls.

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ABBREVIATIONS:

APC: antigen presenting cell; aAPC: artificial antigen presenting cell; AU: arbitrary unit; CD: cluster of differentiation; CDR: complementarity determining region; CLIP: Class II associated invariant chain peptide; GFP: green fluorescent protein; HA: Influenza hemagglutinin; HLA: human leukocyte antigen; Ii: invariant chain; IL: interleukin; kcpm: kilo counts per minutes; MHC: major histocompatibility complex; PB: peripheral blood; PBL: peripheral blood lymphocytes; PHA: phytohemagglutinin; PMA: phorbol myristate acetate; RT-PCR: reverse transcriptase polymerase chain reaction; SD: standard deviation; SEE: Staphylococcal enterotoxin E; SI: stimulation index; TCR: T-cell receptor; tg: transgenic; wt: wild type

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Chapter 3

Modulation of allergen-specific T-lymphocyte function by virus-like particles decorated with HLA class II molecules

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ABSTRACT

Background: TH2 lymphocytes play an important role in the induction and maintenance phase of type I allergy. Modulation of the responses of TH2 lymphocytes by novel forms of antigen-presenting platforms may help shape the immune response to allergen and palliate allergic diseases. **Objective:** To present HLA class II/allergen-peptide complexes on virus-like particles (VLPs) and to evaluate their potential to modulate allergen-specific T-cell responses. **Methods:** Virus-like particles that express the immunodominant T-cell epitope Art v 125-34 of the major mugwort pollen allergen in the context of HLA-DR1 and costimulatory molecules were produced by transfection of 293 cells. The effect of VLPs on IL-2 promoter activity, proliferation, and cytokine production of allergen-specific T cells derived from donors with and without mugwort pollen allergy was determined. **Results:** Flow-cytometric analyses showed that HLA class II molecules, invariant chain::Art v 1 fusion proteins, and costimulatory molecules were expressed on 293 cells. Biochemical analyses confirmed that these molecules were efficiently targeted to VLPs. The engineered VLPs activated Art v 1-specific T cells in a costimulation-dependent manner. VLPs lacking costimulators induced T-cell unresponsiveness, which was overcome by addition of exogenous IL-2. Costimulation could be provided by CD80, CD86, or CD58 and induced distinct cytokine profiles in allergen-specific T cells. Unlike the other costimulatory molecules, CD58 induced IL-10/IFN- γ -secreting T cells. **Conclusion:** Virus-like particles represent a novel, modular, acellular antigen-presenting system able to modulate the responses of allergen-specific T cells in a costimulator-dependent fashion. Allergen-specific VLPs show promise as tools for specific immunotherapy of allergic diseases.

Key words: Artificial APC; Art v 1; HLA class II; mugwort pollinosis; T-cell modulation; costimulation; TCR tg T cells; type I allergy; virus-like particles

INTRODUCTION

T lymphocytes play an important role in the pathogenesis of allergic diseases by providing TH2 cytokines that promote class switching to the ϵ immunoglobulin heavy chain in B cells and by contributing to late-phase reactions and allergic inflammation in target organs (1). Among other factors (eg, amount of allergen, cytokine milieu, and genetic background of the patient) T-cell activity is strongly influenced by the functional properties of antigen-presenting cells (APCs) (2). In fact, the delicate balance between the MHC/peptide complex (signal 1) and costimulatory or inhibitory molecules (signal 2) for T-cell activation provided by APCs decides whether a specific T cell responds or remains unresponsive to antigen (3). Thus, APCs or derivatives thereof represent important tools to study activation and/or modulation of allergen-specific T cells to develop efficient strategies for allergen-specific immunotherapy (4, 5).

The numerous activating and inhibitory molecules described on natural APCs (6, 7, 8) make their net effect not easy to predict and direct. Therefore, reductionist antigen-presenting systems gained attractiveness during the last few years. For example, exosome-based vesicular systems able to activate allergen-specific T lymphocytes have been described (9). However, exosome secretion from APCs occurs in a constitutive fashion, and their composition and especially their donor-dependent HLA allotypes are not easily modifiable (10). In contrast, recently we have devised a more flexible system consisting of inducible immunostimulatory virus-like particles (VLPs), which we refer to as *immunosomes* (11, 12). VLPs are released from 293 cells expressing Moloney murine leukemia virus (MoMLV) core proteins group-specific antigen/polymerase (gag/pol) (11) and preferentially incorporate extracellular and intracellular constituents of host cells, a phenomenon central to pseudotyping (13). We have shown that immunosomes can activate naive, antigen-specific CD8⁺ T lymphocytes and induce their differentiation to cytotoxic effector cells (11).

In the current study, we asked whether HLA class II–restricted immunosomes could be generated by a comparable approach and whether omission of costimulatory elements would render VLPs able to induce allergen-specific T-cell unresponsiveness. Furthermore, we studied the impact of different costimulatory molecules expressed on allergen-presenting VLPs on IL-2 promoter induction, proliferative responses, and cytokine responses of allergen-specific T cells. As a clinically relevant, molecularly well defined disease model, we chose mugwort (*Artemisia vulgaris*) pollen allergy. Mugwort is widely

spread in the northern hemisphere and is one of the main causes of hay fever in late summer and autumn. More than 95% of patients with mugwort allergy are sensitized to Art v 1, the major mugwort pollen allergen (14). Using a large panel of Art v 1-specific T-cell lines generated from peripheral blood (PB) of patients with mugwort allergy, we have identified a single immunodominant epitope, Art v 125-36, that is preferentially presented by HLA-DRB1*01 (15, 16, 17). Moreover, we have recently cloned and characterized an Art v 1-specific T-cell receptor (TCR), which allows us to investigate cell fate and phenotype decisions in PB T cells of individuals without allergy instead of using repeatedly stimulated T-cell lines or clones from individuals with allergy (18).

To the best of our knowledge, this is the first description of artificially induced, HLA class II restricted microvesicles (VLPs) able to modulate allergen-specific T-lymphocyte responses. Allergen-specific VLPs shall allow us to gain deeper insights into the pathophysiology of allergic diseases and to devise novel therapeutic approaches for the treatment of type I allergies.

RESULTS

Generation of HLA class II/allergen peptide-expressing 293 cells.

First, producer cells for VLP generation were established and characterized. For the delivery of the antigen-specific signal 1 to CD4⁺ T cells, the HLA-DRA*0101/HLA-DRB1*0101 heterodimer was expressed in 293 cells, which lack constitutive HLA class II and Ii expression (Fig 1). Surface expression of HLA class II molecules was observed when both DRA- and DRB-chains were coexpressed (Fig 1). Peptide-loading of HLA class II molecules was achieved by an Ii-based approach (18, 19). Cytoplasmic expression of control (Ii::class II associated Ii peptide) or allergen-specific (Ii::Art v 125-34) Ii was clear-cut in the absence of HLA class II coexpression (21). Coexpression of Ii did not significantly alter expression levels of HLA class II heterodimers ($n = 5$; $P = .31$, Mann Whitney U test, 2-tailed) (21), whereas HLA class II molecules reduced Ii levels by approximately 50% ($P < .01$). Additional coexpression of molecules providing second signals such as CD80::GPI (Fig 1) or CD86::GPI and CD58::GPI (not shown) did not influence HLA class II ($n = 6$; $P = .70$, Mann Whitney U test, 2-tailed) or Ii expression levels ($n = 4$; $P = .77$).

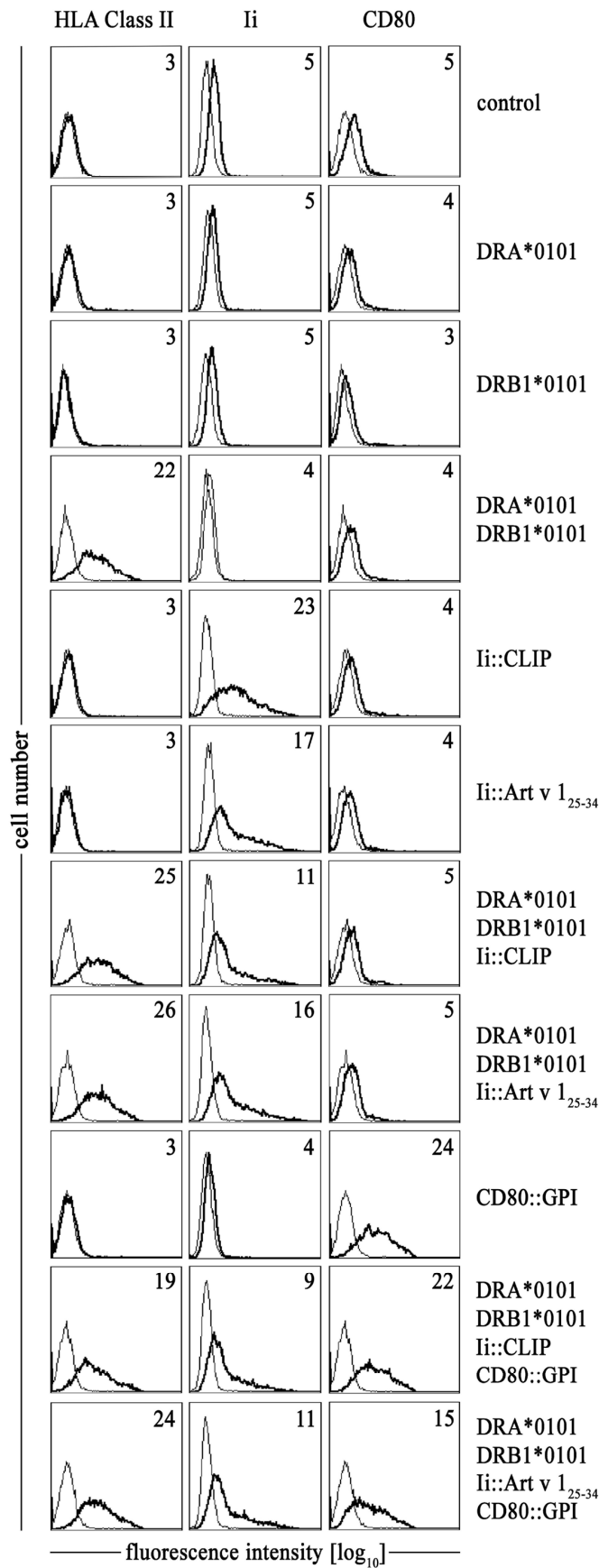


Fig 1. Expression levels of HLA class II molecules, Ii constructs, and CD80 on 293 producer cells. The 293 cells transiently transfected with the indicated constructs were subjected to surface (HLA-DR, CD80) or intracellular (Ii) staining followed by flow-cytometric analysis. Histograms compare non-binding control (thin lines) and specific mAb (thick lines) binding. Data show one representative experiment (n ≥ 4). Numbers indicate geometric mean fluorescence intensity.

VLPs are efficiently decorated with HLA class II and costimulatory molecules.

Next, we assessed targeting of HLA class II and costimulatory molecules to VLPs. Transfected 293 producer cells as well as the MoMLV gag/pol–induced VLPs expressed HLA class II.

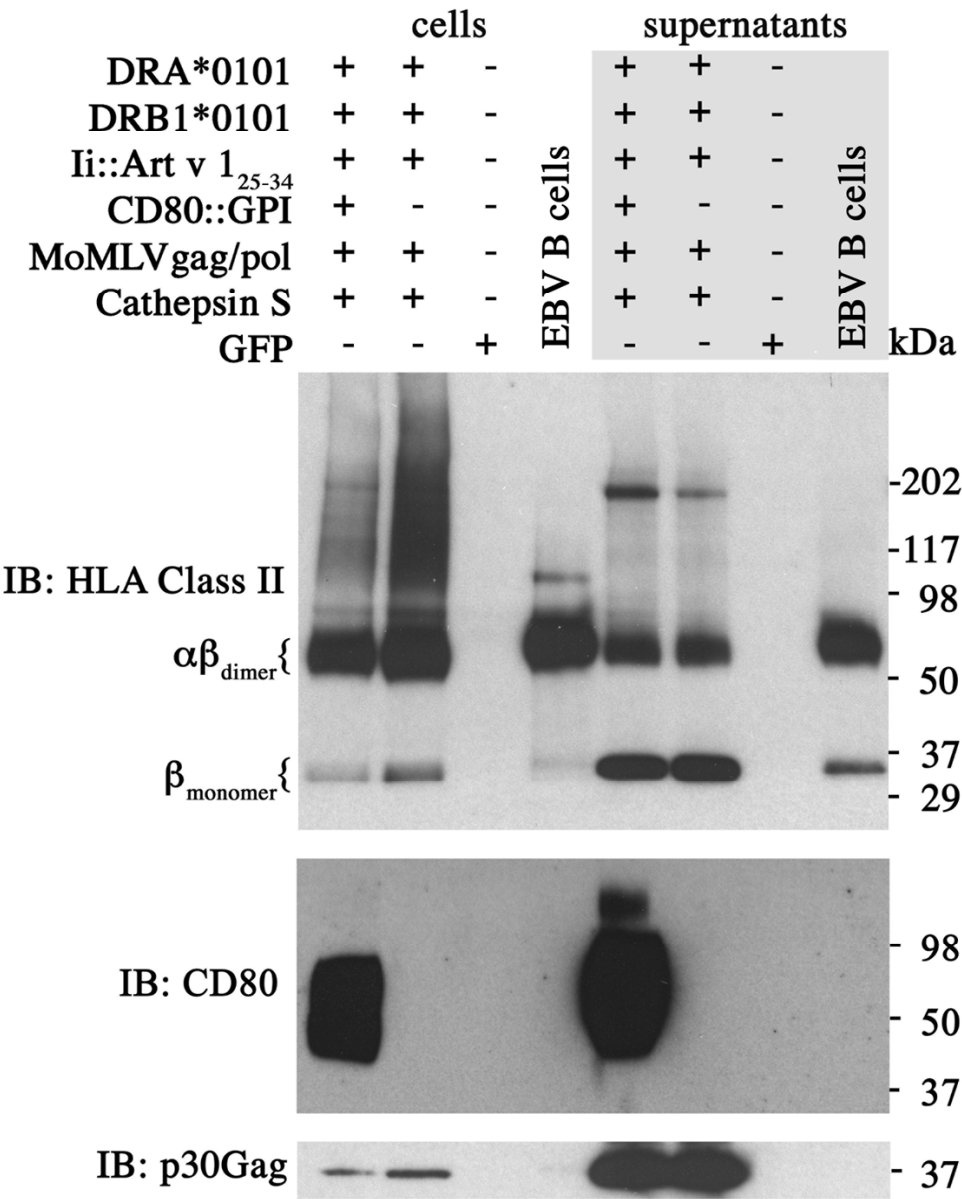


Fig 2. Biochemical characteristics of HLA class II–expressing VLPs. The 293 cells were transfected with the indicated constructs or control plasmid (GFP). Cell lysates and corresponding cell culture supernatants (VLPs) were analyzed by SDS-PAGE followed by immunoblotting (IB) with HLA class II or CD80 mAbs. Vesicle induction and loading were controlled by detection of viral core protein p30Gag. Data show one representative experiment (n = 5).

molecules and CD80::GPI (Fig 2), CD54::GPI, CD86::GPI, and CD58::GPI (not shown), whereas lysates of control transfected 293 cells and their corresponding supernatants were consistently negative for HLA class II and CD80 expression. A considerable fraction of the HLA class II molecules was organized as $\alpha\beta$ -heterodimers on VLPs as demonstrated by binding of the conformation-dependent HLA class II mAb 8-79. In general, HLA class II expression levels on VLPs were comparable to lysates of 293 cell transfectants, lysates of HLA-DR1+ EBV-transformed B cells, and their corresponding exosome preparations. Immunoblotting for p30Gag and HLA class II demonstrated that cotransfection of CD80::GPI changed neither HLA class II expression levels nor VLP amounts produced. HLA class II/Art v 125–34–decorated VLPs activate TCR tg allergen-specific Jurkat T cells in a costimulation-dependent manner

HLA class II/Art v 125–34–decorated VLPs activate TCR tg allergen-specific Jurkat T cells in a costimulation-dependent manner.

To investigate whether HLA class II–decorated VLPs are functional, their ability to activate IL-2 promoter activity in Art v 1–specific TCR tg Jurkat T cells was tested (18). Significantly, the VLP preparations expressing Ii::Art v 125–34 in the context of HLA-DRA*0101/-DRB1*0101 stimulated Jurkat T cells in a costimulation-dependent manner, similar to producer cells (Fig 3, A and B; $P < .001$). PMA plus PHA stimulation served as an independent positive control. Apart from CD80::GPI, IL-2 promoter activity was also strongly induced by CD86::GPI, but only weakly by CD58::GPI (not shown). In contrast, wild-type Jurkat T cells expressing the endogenous TCR remained unstimulated by Art v 1–specific VLPs (not shown). Moreover, VLPs coexpressing Ii::influenza hemagglutinin 309–317, used as an irrelevant HLA-DRA*0101/-DRB1*0101–restricted binding control, or VLPs, in which peptide loading was attempted by Art v 1–minigenes, were unable to stimulate Art v 1–specific TCR tg Jurkat T cells (not shown). Immunoblotting for p30Gag revealed that VLP preparations used for stimulation of Jurkat T cells contained comparable amounts of particles (Fig 3, C).

VLPs decorated with HLA class II/Art v 125-34 complexes activate allergen-specific T-cell clones and TCR tg PB T cells.

Next, VLPs were tested for their potential to induce proliferation of allergen-specific T cells. For that purpose, Art v 1-specific T-cell clones from individuals with mugwort pollen allergy or TCR tg PB T cells of individuals without

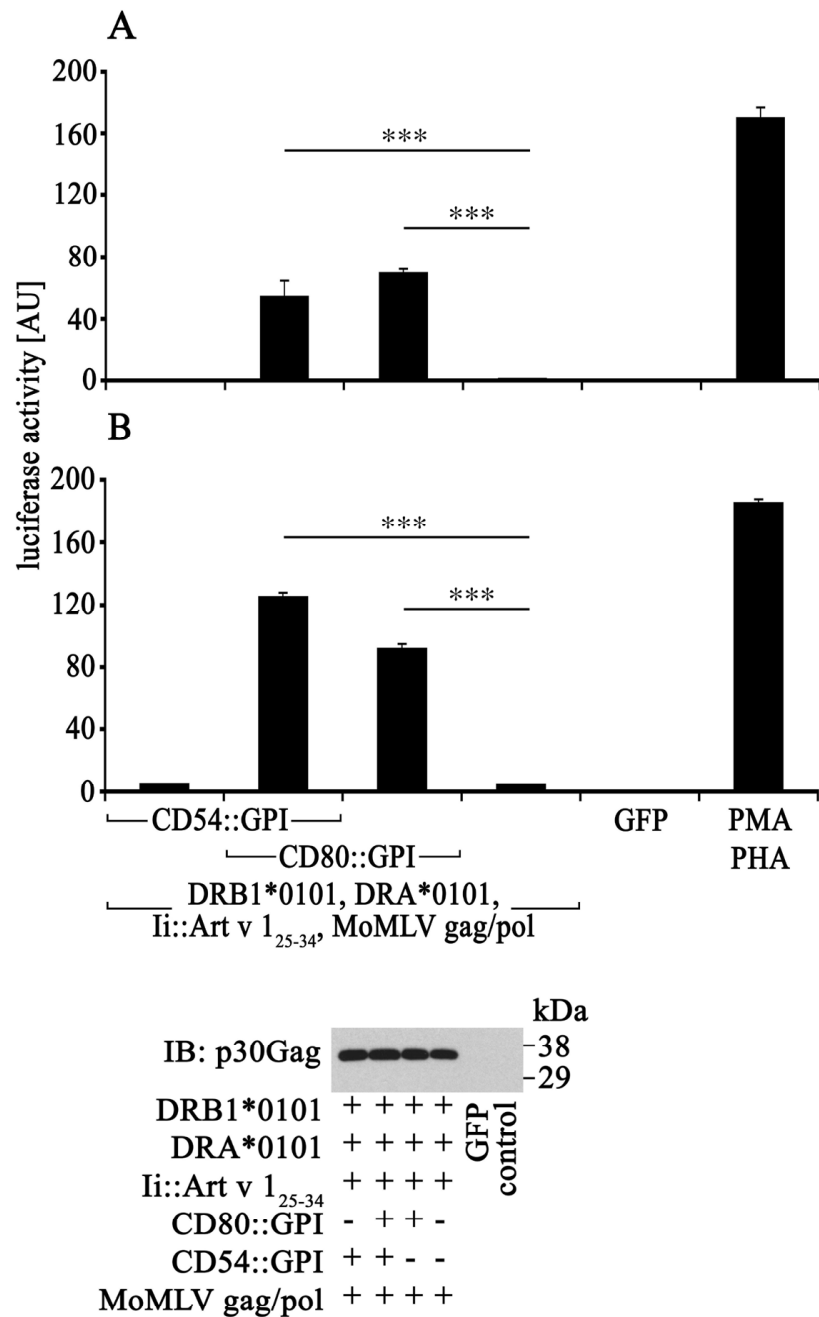


Fig 3. HLA class II/allergen peptide-decorated VLPs activate TCR tg Jurkat T cells. Art v 1-specific TCR tg Jurkat T cells harboring an IL-2 promoter/enhancer driving luciferase expression were coincubated with VLPs (A) or 293 producer cells (B) expressing the indicated molecules. PMA/PHA was used as positive and GFP transfection as negative control. Data show mean + SD of triplicate cultures of 1 representative experiment (n = 3). ****P* < .001, Student t test, paired, 2-tailed. C, Vesicle induction and loading were controlled by detection of viral core protein p30Gag. AU, Arbitrary units.

allergy were incubated with VLPs expressing HL A-DRA*0101, -DRB1*0101, Ii::Art v₁₂₅₋₃₄, CD80::GPI, and CD54::GPI in combinations as indicated. In fact, VLP induced similar levels of T-cell proliferation in T-cell clones or TCR tg PB T cells compared to allergen-presenting 293 cells or microbeads coated with CD3 plus CD28 mAbs as stimulators (Fig 4, A and B). Similar to the IL-2 promoter assays described, induction of T-cell proliferation was significantly dependent on the presence of costimulatory molecules ($P < .01$ and $P < .001$, respectively): VLPs providing the antigen-specific signal 1 alone were unable to induce T-cell proliferation, similar to nondecorated VLPs or medium alone. Apart from CD80::GPI, costimulation could also be provided by CD86::GPI ($P < .001$), the second ligand for CD28, or CD58::GPI ($P < .001$), the CD2 ligand (22), but not by the adhesion molecule CD54::GPI. Furthermore, restimulation of bulk TCR tg PB T cells with Art v₁₂₅₋₃₄-specific VLPs coexpressing CD80::GPI and CD54::GPI and supplemented with IL-2 (100 U/mL) led to a 52.8 ± 11.8 -fold expansion ($n = 6$) of T lymphocytes within 3 weeks (Fig 4, C). During the culture period, the fraction of allergen-specific V β 18-expressing T cells within the bulk transfectants increased from 29% to 84%.

Induction of specific unresponsiveness by HLA class II/Art v₁₂₅₋₃₄-expressing VLPs lacking costimulatory molecules.

In addition to activation/expansion of allergen-specific T cells, VLPs might be useful for anergizing such T cells. In proof of principle experiments, we observed that in primary cultures, allergen-specific T cells from HLA-DR1⁺ individuals with (Fig 5, A) or without (Fig 5, B) mugwort pollen allergy cocultivated with VLPs expressing signal 1 but no costimuli did not proliferate ($P < .01$ and $P < .001$, respectively), similar to cells cultivated with medium alone. Moreover, allergen-specific T cells treated in such a way were unable to proliferate in secondary cultures supplemented with anti-CD3/CD28-coated microbeads ($P < .001$). T-cell unresponsiveness could, however, be overcome by the addition of saturating concentrations of IL-2 (500 U/mL; $P < .001$), indicating that the cells were anergic but still alive. In marked contrast, T cells that received both signal 1 and 2 during the primary stimulation proliferated also after secondary stimulation (Fig 5, A and B).

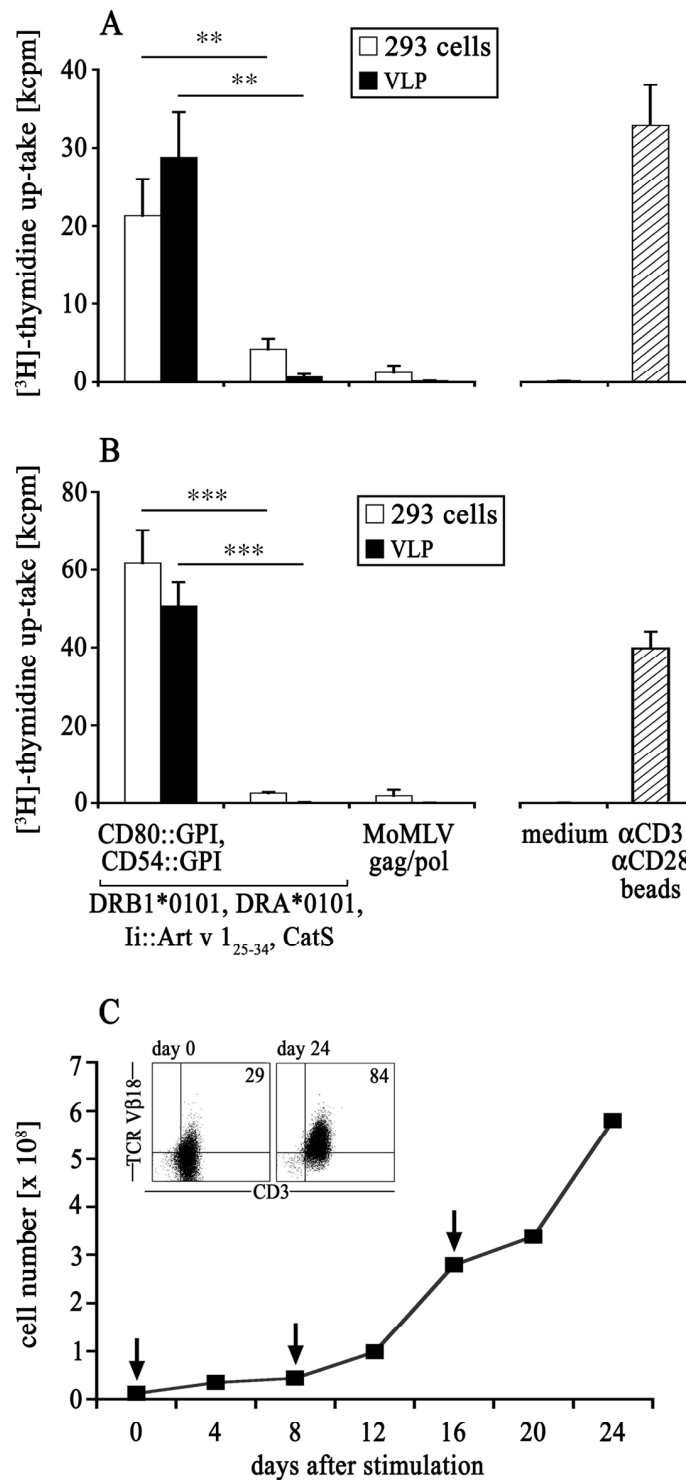


Fig 4. Art v 1-specific VLPs activate allergen-specific T-cell clones and TCR tg PB T cells. VLPs decorated with the indicated molecules were incubated with an Art v 1-specific T-cell clone (A) or TCR tg PB T lymphocytes (B). CD3/CD28 mAb-coated microbeads or culture medium alone served as positive and negative controls, respectively. Cell proliferation is shown (mean + SD of triplicates; $n = 3$). ** $P < .01$, *** $P < .001$, Student t test, paired, 2-tailed. C, Bulk TCR tg PB T cells (12×10^6) stimulated 3 times (arrows) with Art v 1-specific VLPs and supplemented with IL-2 were cultured for 24 days, and total cell numbers were determined at different time points (squares). Insert compares flow-cytometric characteristics of starting culture (day 0) with cells obtained after 24 days. Numbers of CD3+/TCRV β 18+ cells (not, vert, similar 2% in PB T cells) are indicated. Data show one representative experiment ($n = 6$). kcpm, Kilo counts per minute.

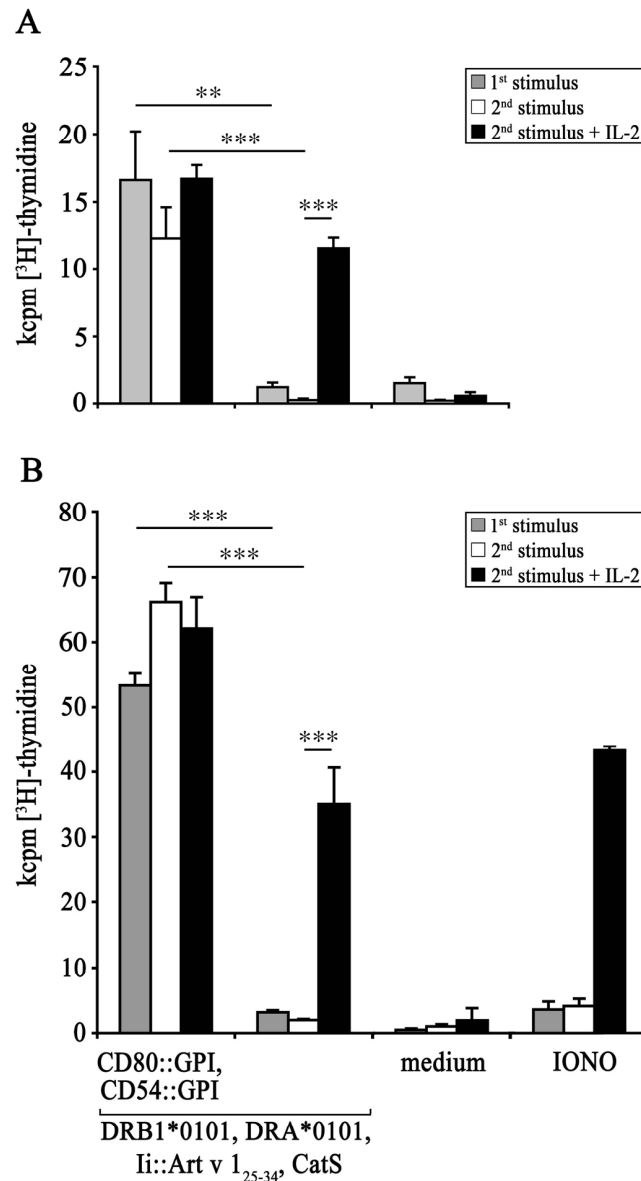


Fig 5. Induction of allergen-specific unresponsiveness by VLPs. Proliferation of TCR tg PB T cells from an individual with mugwort pollen allergy (A) and an individual without allergy (B) cultured with indicated primary stimuli for 4 days is shown (gray bars). Proliferation of T cells obtained on culture with indicated stimuli for 10 days, washed, and recultured with CD3/CD28 mAb-coated microbeads in the absence (white bars) or presence (black bars) of exogenous IL-2 (500 U/mL) is demonstrated. Data show one representative experiment (n = 4). *** $P < .001$, ** $P < .001$, Student t test, paired, 2-tailed. kcpm, Kilo counts per minute.

Allergen-specific VLPs expressing various types of costimulatory molecules modulate the cytokine profiles of allergen-specific T cells differently.

In a final step, we investigated whether the expression of distinct costimulatory molecules on allergen-specific VLPs has a decisive impact on the cytokine profiles of TCR tg allergen-specific T cells from HLA-DR1⁺ individuals with and without mugwort pollen

allergy. Compared with signal 1 alone, allergen-specific VLPs coexpressing CD80::GPI induced significant levels of IFN- γ ($P = .006$), IL-13 ($P = .002$ and $P < .001$), and IL-2 ($P = .001$), whereas no IL-4 and only minute amounts of IL-10 were detectable (Fig 6, A and B). CD86::GPI induced a similar cytokine pattern, although IL-2 levels were lower.

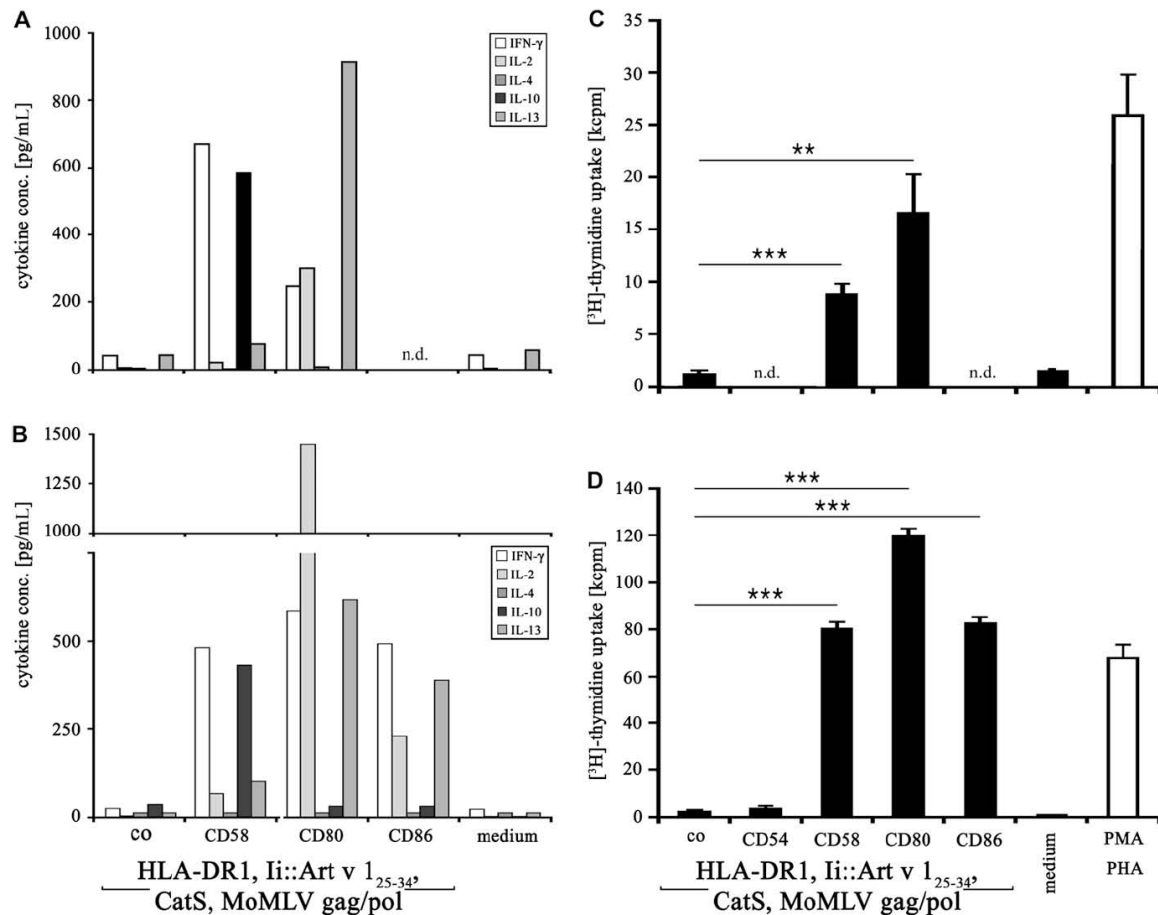


Fig 6. Nature of the costimulatory molecule present on VLPs affects the cytokine profile of allergen-specific T cells. (A and B) Cytokine profiles and (C and D) proliferation of TCR tg allergen-specific PB T cells from an individual with mugwort pollen allergy (A and C) and an individual without allergy (B and D) upon incubation with VLPs expressing the indicated combinations of molecules. One representative experiment is shown ($n = 5$). *** $P < .001$, ** $P < .001$, Student t test, paired, 2-tailed. kcpm, Kilo counts per minute; conc, concentration; n.d., not determined.

In marked contrast, CD58::GPI induced highly significant levels of IFN- γ ($P = .002$ and $P < .001$) and IL-10 ($P = .001$ and $P < .001$), whereas IL-2 and IL-13 levels were low, and IL-4 remained undetectable. Moreover, T-cell proliferation was high in the presence of either of the costimulators ($P < .001$ and $P < .01$, respectively), whereas proliferation

remained undetectable when cells received signal 1 alone (Fig 6, C and D). Thus, VLPs decorated with different flavors of costimulatory molecules might represent novel tools for the modulation of allergen-specific T-cell function.

DISCUSSION

In this study, we have devised a novel, acellular, antigen-presenting platform—VLPs—and examined its potential to modulate allergen-specific T-cell responses. As a model for stimulation/modulation of HLA class II-dependent immune responses, we have used the immunodominant epitope of Art v 1, the major mugwort pollen allergen. To decorate VLP with T-cell ligands of choice, we have built on the recently elucidated mechanisms responsible for viral pseudotyping (13, 23-25), which we have successfully explored for the generation of MHC class I-expressing VLPs (immunosomes) in the past (11). We show that HLA-DR1/Art v₁₂₅₋₃₄-presenting VLPs, which coexpress CD80, induce vigorous T-cell activation even in the absence of natural APCs. In contrast, omission of costimulatory molecules rendered VLPs with anergizing potential for Art v 1-specific T cells. We also demonstrate that depending on the costimulator expressed on VLPs, allergen-specific T cells can be instructed to secrete different cytokine profiles. Similar to MHC class I-decorated VLPs (11), HLA class II-decorated VLPs are robust and can be prepared in a reproducible manner (not shown).

T-cell activation is a delicately balanced process that involves the TCR and HLA/peptide-complex (signal 1) as well as a variety of accessory molecules expressed on T cells and APCs (signal 2) (3). VLPs expressing signal 1 (HLA-DR1/Art v₁₂₅₋₃₄) alone induced unresponsiveness of Art v 1-specific T cells. This unresponsiveness could be overcome by addition of IL-2, which is in line with the functional definition of anergic cells (26, 27).

What could be advantages of VLPs expressing preformed HLA/allergenic peptide-complexes over other systems to influence allergen-specific T-cell function? A special advantage of the VLP platform is its modular nature, allowing accommodation of immunoregulatory molecules of choice adjacent to the HLA/allergenic peptide complex. Coexpression of the CD28 ligands CD80 or CD86 on allergen-specific VLPs induced clear-cut levels of IFN- γ , IL-2, and IL-13, compatible with previous reports (28, 29), which allows strong expansion of specific T cells for further studies. In contrast, CD58 expression on allergen-specific VLPs induced IL-10/IFN- γ -secreting T cells, whereas

IL-2, IL-4, and IL-13 levels remained low. The cytokine profile obtained upon CD58 costimulation is reminiscent of T-regulatory cells described by Groux et al (30). The same group described efficient induction of polyclonal IL-10/IFN- γ -secreting T cells on triggering of the CD58/CD2 axis (31). Of clinical importance in that context are the reports describing induction of IL-10 and/or IFN- γ -secreting T cells on allergen-specific immunotherapy (32-35). These findings have been further substantiated by reports demonstrating the preferential presence of IL-10 and/or IFN- γ -secreting T cells in healthy individuals without allergy (36, 37). Our results obtained with allergen-presenting VLPs coexpressing CD58 suggest an important role of the CD58/CD2 signaling axis for the induction of allergen-specific IL-10/IFN- γ -secreting T cells. On the other hand, the data imply that colocalization of distinct T-cell ligands on allergen-specific VLPs might be a valid strategy to modulate the function of allergen-specific T cells in future immunotherapy protocols. However, future studies will have to assess the detailed function and the reason for the pronounced proliferative behavior of the IL-10/IFN- γ -secreting T cells described. Moreover, because CD58/CD2 signaling also seems to augment T-cell responsiveness for distinct cytokines (38), it will be interesting to examine the impact on allergen-specific T cells of membrane-bound cytokines (12) expressed on VLPs. Of importance, we have recently confirmed the *in vivo* functionality of cytokine-decorated VLPs in an antigen-specific, T-cell-dependent system (12).

A further possible advantage of the VLP platform is the fact that peptide association with HLA class II molecules does not occur randomly. Instead, we have successfully adapted an Ii-based peptide delivery system (18, 20) guiding allergenic peptides into the endo-/lysosomal compartment responsible for HLA class II peptide loading (MIIC) (39). The Ii-based approach turned out to be superior to “minigene” approaches (11, 40), in which strong eukaryotic signal sequences were hooked up to the allergenic peptide sequence with the aim of delivering high concentrations of the peptide into the endoplasmic reticulum of the Ii negative 293 cell (not shown). Possible clinical applications of VLPs will be facilitated by the fact that the producer 293 cells used in this study (41) are suitable for large-scale, good manufacturing practice-adapted production and have been successfully used to prepare retroviruses and adenoviruses for clinical use in the past (42-43).

That VLPs are potent inducers of B-cell-specific immune responses, even when applied via different routes (45), has been described in several studies in the past (46). In fact,

hepatitis B surface antigen (47), human papilloma (48, 49), and Norovirus (50) capsid proteins that assemble particles of defined icosahedral symmetry are currently clinically used as vaccine antigens. In the more recent past, chimeric VLPs used as platforms and chemically conjugated with antigens of microorganisms or allergens have been manufactured and successfully applied *in vivo*, as demonstrated by their induction of protective IgG antibodies (46, 51). Hence, with the exception of the antiviral influenza (52) and HIV approaches (53), VLP platforms studied so far mostly relied on nonenveloped viral cores. Although these particles either had intrinsic antiviral immunogenicity or became chemically coupled with antigens of interest, none of the approaches intended to target T cells with preformed HLA/peptide complexes of choice directly.

In summary, we have shown that, in contrast with exosomes (54, 55), the molecular composition of VLPs can be designed easily at the level of producer cell transfection, which is particularly important for donor-dependent, allergen-presenting HLA allotypes. Furthermore, we present evidence that it is possible to modulate T-cell function directly using VLPs as an integrating platform (11), without the need for cross-presentation by APCs (56). Hence, the VLP platform permits the application or omission of selected costimulation pathways. In contrast with naturally occurring APCs, or exosomes derived from APCs, VLPs offer the possibility to modulate allergen-specific T-lymphocyte responses differentially in a predictable manner and might thus open new avenues for clinical application.

CLINICAL IMPLICATIONS

VLPs decorated with immune receptors of choice may be new tools for specific immunotherapy that offer considerable flexibility to regulate immune responses to antigens provoking allergic diseases.

MATERIALS AND METHODS

Cell lines. Human embryonic kidney 293 cells, 293 OKT3scFv::GPI transfectants (11), Art v 1 TCR transgenic (tg) Jurkat T cells expressing an IL-2 enhancer/promoter driving luciferase (18), and EBV-transformed B cells were cultured as described (11). Art v 1-specific T-cell clones were established from PBMCs of individuals with mugwort pollen allergy with a typical clinical history and positive skin prick test results (≥ 3 mm diameter) to mugwort pollen extract as described (15) (ethics committee approval: EK#497/2005).

Expression constructs. Cloning and characterization of HLA-DR, $\alpha\beta$ TCR, invariant chain (Ii), CD80::GPI, CD86::GPI, CD58::GPI, CD54::GPI, and green fluorescent protein (GFP) constructs has been described previously (18). Art v 1 minigenes were generated by inserting synthetic oligonucleotides coding for Art v 1 peptides with Nhe I and Not I compatible ends into the expression vector pEAK12 downstream of the CD5 signal sequence essentially as described previously (11).

Generation of antigen-presenting 293 cells and VLPs. The 293 cells (3×10^6) were transiently cotransfected with HLA-DRA*0101, HLA-DRB1*0101, CD54::GPI, CD80::GPI, CD86::GPI, CD58::GPI, cathepsin S (19), Ii constructs, minigenes, GFP control vector, or empty control vector (30 μ g DNA) by the calcium phosphate precipitation method in combinations as indicated and used 72 hours after transfection. VLP induction was achieved by cotransfection of MoMLV gag/pol (11, 12). Supernatants were harvested after 72 hours and cleared from cellular debris by filtration (0.45 μ m). VLPs were concentrated by ultracentrifugation at 1×10^5 g for 1 hour, resuspended in PBS, and recentrifuged. VLP concentration was determined by Micro BCA assay (Pierce, Rockford, Ill).

Immunofluorescence analyses. MAbs CD3–fluorescein isothiocyanate (FITC; UCHT1) and control immunoglobulin FITC and phycoerythrin were obtained from ADG (Kaumberg, Austria); MAb CD80 (MEM-233) was obtained from Exbio (Praha, Czech Republic), TCR V β 18–phycoerythrin (BA62) was obtained from Immunotech (Marseille, France); and HLA class II–FITC (1/47), Ii–FITC (5-329), and VIAP–FITC (VIAP) were provided by Dr O. Majdic (Institute of Immunology, Medical University of Vienna, Austria). Oregon-green conjugated goat-anti-mouse IgG was obtained from Invitrogen

(Carlsbad, Calif). Surface and intracellular stainings as well as flow-cytometric analyses were performed on a FACS Calibur cytometer (Becton Dickinson, Palo Alto, Calif) as described (11).

Biochemical analyses of producer cells and VLPs. Lysates from 293 cell transfectants and VLPs were obtained on 1% Nonidet P40 treatment as described previously (11). Cellular and VLP proteins, 1 μ g/slot, as determined by Micro BCA protein assay (Pierce) were resolved by 4% to 20% SDS-PAGE (Anamed, Darmstadt, Germany), transferred to nitrocellulose membranes (Biorad, Hercules, Calif), and probed with HLA class II β -chain specific mAb 2-12 and 8-79 (Dr O. Majdic), CD80 specific mAb MEM-233 (Exbio), and MoMLV p30Gag-specific mAb R187 (ATCC), respectively. Horseradish peroxidase-conjugated secondary reagents were from DAKO (Glostrup, Denmark). Blots were developed with a luminol-based indicator system (Perkin Elmer, Boston, Mass) and exposed to X-ray films (Eastman Kodak, Rochester, NY).

Jurkat luciferase activity assays. TCR tg Jurkat T cells (10^5 /well) were incubated with 293 cells (5×10^4 /well) or VLPs expressing HLA-DRA*0101, HLA-DRB1*0101, Ii::Art v₁₂₅₋₃₄, Ii::influenza hemagglutinin₃₀₉₋₃₁₇, Ii::class II associated Ii peptide, Art v₁₂₅₋₃₄-minigene, Art v₁₁₇₋₃₂-minigene, Art v₁₁₇₋₃₄-minigene, CD80::GPI, CD54::GPI, cathepsin S, or GFP combined as indicated in 96-well flat-bottom tissue culture plates for 6 hours. PHA (5 μ g/mL) plus phorbol 12-myristate 13-acetate (PMA; 10^{-7} mol/L) served as positive control, and GFP-transfected 293 cells or medium alone served as negative control. Luciferase activity was assayed as described (11).

T-cell proliferation assays. Art v 1-specific TCR tg T cells were generated from nonallergic and mugwort pollen allergic HLA-DR1 positive individuals as described previously (18). Transduction efficiencies of CD3⁺ T cells were $29.5 \pm 9.5\%$ ($n = 7$). Art v 1-specific T-cell clones from individuals with allergy or TCR tg PB T cells from individuals without allergy (5×10^4 /well) were incubated with irradiated (60 Gy) 293 cells (5×10^4 /well) or VLPs (2 μ g/mL) expressing HLA-DRA*0101, HLA-DRB1*0101, Ii::Art v₁₂₅₋₃₄, CD80::GPI, CD86::GPI, CD58::GPI, CD54::GPI, cathepsin S, or control plasmid combined as indicated in 96-well flat-bottom tissue culture plates for 3 days. During the last 18 hours, [³H]-thymidine (1 μ Ci/well) uptake was determined as

described (11). Medium alone or anti-CD3/CD28 microbeads (5×10^4) served as controls. Supernatants from replica plates were harvested after 24 and 48 hours and were subjected to cytokine analyses by multiplex analysis using a Luminex 100 apparatus (Luminex, Austin, Tex) according to the manufacturers' recommendations. For expansion, TCR tg T cells (1×10^6 /well) were incubated in 24-well plates with Art v 1-specific VLPs ($2 \mu\text{g/mL}$) coexpressing CD80::GPI and CD54::GPI and supplemented with 100 U/mL IL-2 on day 0 and restimulated on days 8 and 16 of culture. On days 4, 8, 12, 16, and 20, aliquots were counted (Beckman Coulter Z2, Fullerton, Calif) and total numbers of cells calculated.

Induction of T-cell anergy. T-cell receptor tg T cells (1×10^6 cells/well) were cultured in 24-well plates with VLPs ($2 \mu\text{g/mL}$) decorated with the indicated molecules, ionomycin (100 ng/mL), anti-CD3/CD28 microbeads (1×10^6 /well) or medium alone for 10 days. Proliferation of primary cultures was determined in replica plates containing 1×10^5 T cells and corresponding stimuli. After 10 days, cells from 24-well plates were harvested and washed, and 1×10^5 cells/well were restimulated in 96-well flat-bottom plates with anti-CD3/CD28 microbeads (2.5×10^4) alone or in the presence of IL-2 (500 U/mL) for 4 days, and proliferation was determined. Culture supernatants of replica plates were harvested 24 hours after restimulation, and IL-2, IL-4, IL-10, IL-13, and IFN- γ were determined by multiplex analysis. T-cell proliferation was measured as described.

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ABBREVIATIONS

APC: Antigen-presenting cell; FITC: Fluorescein isothiocyanate ; Gag: Group-specific antigen; GFP: Green fluorescent protein; GPI: Glycosyl phosphatidyl inositol; Ii: Invariant chain; MoMLV: Moloney murine leukemia virus; PB: Peripheral blood; PMA: Phorbol 12-myristate 13-acetate; Pol: Polymerase; TCR: T-cell receptor; Tg: Transgenic; VLP: Virus-like particle

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Chapter 4

Two newly diagnosed HLA class II deficient patients identified by rapid vector-based complementation reveal discoordinate invariant chain expression levels

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ABSTRACT

Background: Primary immunodeficiencies (PID) reveal the ‘molecular Achilles’ heels’ of human immunity. Detailed analyses of PID extend our knowledge of pivotal immunological processes, lead to novel diagnostic algorithms and shortens time-to-diagnosis. **Methods:** Clinical/immunological phenotypes of two unrelated combined immunodeficiency (CID) patients from Austria were determined. Leukocyte subpopulations of patients, their parents and healthy controls were analyzed by flow cytometry. Patient-derived EBV transformed B cell lines were established and complemented by candidate cDNAs. Suspected mutations were confirmed by DNA sequencing. **Results:** Phenotyping revealed a lack of constitutive HLA class II expression on antigen presenting cells of both patients, compatible with MHCII deficiency. Rapid vector-based complementation of patients’ B cells identified HLA class II transactivator (CIITA)-deficiency in patient VIP1 and restriction factor X(RFX)AP-deficiency in patient VIP2. CIITA-deficiency was caused by a homozygous p.Glu381X mutation. RFXAP-deficiency resulted from a homozygous p.Ser123ThrfsX15 mutation, not described in the Middle European population so far. Of note, HLA class II associated invariant chain (Ii) expression levels were significantly reduced in VIP1 and three additional EBV transformed B cell lines of CIITA deficient patients but normal in VIP2 EBV transformed B cells. In addition, peripheral blood B cells of VIP1’ parents showed significantly reduced HLA-DR and -DP expression levels compared to healthy controls. **Conclusions:** Analysis of patients’ intracellular Ii and parents surface HLA class II expression levels might help to identify CIITA-deficient patients already during initial phenotyping.

INTRODUCTION

Human leukocyte antigen (HLA) class II molecules play a central role in the induction of adaptive immune responses by displaying extracellular/lysosomal antigens to CD4⁺ T helper lymphocytes [1]. Antigen presenting HLA class II molecules are encoded by a group of closely linked genetic loci (named DR, DQ and DP) on the short arm of chromosome 6. Constitutive expression of HLA class II heterodimers, consisting of polymorphic α - and β -chains, is restricted to professional antigen-presenting cells such as dendritic cells, B lymphocytes and cells of the monocytic lineage [2]. HLA class II expression can, however, also be induced on various other cell types in response to exogenous stimuli such as IFN- γ [3,4].

The HLA class II locus is mainly regulated at the level of transcriptional initiation by four *cis*-acting elements termed W, X1, X2 and Y boxes. They serve as binding sites for several transcription factors including the multi-protein regulatory factors binding to the X box (RFX), the nuclear factor Y and the cyclic-AMP response element binding protein [5]. Additionally, the HLA class II transactivator (CIITA) protein is essential for the initiation of transcription serving as a scaffold by linking the above-mentioned factors to the transcriptional initiation and elongation machinery [6,7].

HLA class II deficiency, formerly also called Bare Lymphocyte Syndrome II (BLS II), is a rare combined immunodeficiency (CID) marked by the complete absence of both constitutive and inducible HLA class II expression. Inability to present antigenic peptides to CD4⁺ T helper lymphocytes results in severely impaired adaptive cellular and humoral immune responses [8]. As a consequence, MHCII deficiency patients are highly susceptible to opportunistic infections with bacterial, viral and fungal pathogens already at an early age. Until today, therapy of MHCII deficiency consists of symptomatic antibiotic therapy and allogeneic stem cell transplantation [9].

MHCII deficiency results from mutations in the genes encoding *trans*-regulatory factors of the HLA class II promoter/enhancer [8]. Genetic complementation analyses of patient-derived cell lines have led to the definition of four complementation groups, i.e. A-D. Group A patients carry mutations in the class II transactivator (CIITA) [10]. Mutations of

RFXANK (group B) [11,12], RFX5 (group C) [13] and RFXAP (group D) [14], constitute the remaining complementation groups.

Here we describe the immunological phenotype and the molecular characteristics of two unrelated, recently identified MHCII deficiency patients from Austria who clinically presented as CID in their early months of life. Moreover, we present evidence that phenotypic analyses of a) intracellular invariant chain (Ii) expression levels of patient-derived EBV transformed B cells as well as b) HLA class II expression levels on B cells and monocytes of the patients' parents might help to assign patients to distinct complementation groups.

MATERIALS AND METHODS

Patients

The female patient VIP1 was born with normal birth weight to unrelated healthy parents of Austrian origin. VIP1 was hospitalized with six months due to acute viral bronchiolitis and pneumonia requiring artificial respiration. A hypoxia-induced cerebral atrophy was diagnosed resulting in a *hydrocephalus e vacuo* accompanied by tetraparesis and visual impairment. Immunologic evaluation revealed hypogammaglobulinaemia with leukocytes and leukocyte-subset numbers within the normal range (table 1). The CD4/CD8-ratio was inverted (0.22) with only 660/ μ l of CD4⁺ T cells indicating a dysregulation within the T cell compartment. PBMC of VIP1 reacted robustly but significantly lower than control PBMC to the HLA class II-independent stimuli PHA and PMA, while no proliferation was observed with superantigens SEA and SEB, indicating a defect in the HLA Class II – T cell receptor interaction (table 2). In addition, soluble CD3 mAb-induced proliferation was strongly reduced (10% of control).

The male patient VIP2 was born after 41 weeks of gestation to unrelated healthy parents of Austrian origin and a healthy sibling of 3 years. VIP2 developed obstructive bronchitis at 2 months of age followed by recurring respiratory infections. A *pneumocystis jirovicii* pneumonia, which was exacerbated by cytomegalo- and adenovirus infections required hospitalization of VIP2. Immunological examination revealed marked hypogammaglobulinaemia and leukocyte, monocyte and lymphocyte numbers below the age-specific normal range (table 1). The CD4/CD8 T lymphocyte ratio was inverted with 0.50 due to severe CD4⁺ T-lymphopenia (80/ μ l). Proliferation of peripheral blood T cells was comparable to the healthy control upon polyclonal stimulation with PHA, but clearly reduced to absent upon stimulation with PMA, soluble CD3 mAb or HLA class II-dependent superantigens (table 2).

Cell lines

The 293 cell line (human embryonic kidney epithelial cells) was maintained in IMDM (Sigma Chemicals, St.Louis, MO) plus 10% fetal calf serum (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-Glutamine, 50 μ M 2-mercaptoethanol and 15 μ g/ml gentamycin sulfate. Peripheral blood mononuclear cells (PBMC) were isolated from

heparinized blood by standard Ficoll density gradient centrifugation using Lymphoprep (Technoclone, Vienna, Austria). EBV transformed lymphoblastoid B cell lines (EBV B cells) were generated by supplementing PBMC with infectious Marmosett P95-8 supernatant plus 200 ng/ml cyclosporine A (Sandimmune, Novartis, Vienna, Austria). After two weeks of incubation cells were washed, re-plated and further cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-Glutamine and 100 µg/ml gentamycin sulfate. EBV B cell lines BLS-1 (RFXANK deficient), BLS-2 and BCH (CIITA deficient) and SJO (RFX5 deficient) were kindly provided by Dr. Walter Reith, Department of Pathology and Immunology, University of Geneva Medical School, Geneva, Switzerland. All cell lines were incubated at 37°C in 5% CO₂.

Expression constructs

pEBO-CIITA, an SV40 promoter-driven eukaryotic expression vector containing the full length *CIITA* cDNA was a kind gift from Dr. Bernhard Mach (Geneva, Switzerland). The human RFXANK, RFX5 and RFXAP specific cDNAs were amplified with the respective primer pairs RFXANKfor (5'-CGCGGGAAGCTTGCCACCATGGAGCTTACCCAGCCTGCAGAA-3') and RFXANKrev (5'-CCCGCGGCGGCCGCTTTACTCAGGGTCAGCGGGCAC-3'), RFX5for (5'-CGCGGGAAGCTTGCCACCATGGCAGAAGATGAGCCTGATGCTAA-3') and RFX5rev (5'-CCCGCGGCGGCCGCTTTATGGGGGTGTTGCTTTTGGGTCTTTA-3') and RFXAPfor (5'-CGCGGGAAGCTTGCCACCATGGAGGCGCAGGGTGTAGCGGAGG-3') and RFXAPrev (5'-GCGCCCGTTAACTTTACATTGATGTTTCCTGGAACTGCTGTTGTC-3') from a human dendritic cell cDNA library [15]. At the 5'-end of the cDNAs optimized translational initiation sites preceded by a Hind III restriction site were generated. 3'-ends were modified by an optimized TAAA STOP codon followed by a Not I restriction site (RFXANK and RFX5) or an Hpa I restriction site (RFXAP). *CIITA* cDNA was amplified and tailed by PCR from pEBO-CIITA with Bsp HI and Hpa I. cDNAs were digested with restriction endonucleases and ligated into the pMMP412 retroviral vector digested by enzymes creating compatible ends. MoMLV gag-pol and vesicular stomatitis virus glycoprotein (VSV-G) were expressed in 293 cells using the expression vector pMD (all kindly provided by Dr. Richard Mulligan, Children's Hospital, Boston, MA) to create infectious virus-particles.

Lymphocyte proliferation assays

PBMC (1×10^5) from patients and healthy control donors were incubated with staphylococcal superantigen A (SEA; 10 ng/ml; Sigma Chemicals), staphylococcal superantigen B (SEB; 20 ng/ml, Sigma Chemicals), phythemagglutinin (PHA; 12,5 µg/ml; Sigma), soluble CD3 mAb (OKT3, 2µg/ml; Ortho, Raritan, NY) or phorbol myristate acetate (PMA; 10^{-7} mol/L; Sigma Chemicals) or plain medium in 96-well round-bottom tissue-culture plates for 72 h. Cells were pulsed with [methyl- ^{3} H] thymidine (1 µCi per well) for another 18 h and processed as described previously [16].

For activation of Jurkat T cells, 5×10^4 complemented VIP1 and VIP2 EBV-transformed B cells or alternatively EBV-transformed B cells from a healthy, HLA-DR1⁺ donor were co-incubated with Jurkat T-cells (10^5) expressing a luciferase gene under the control of the IL-2 promoter in the presence of SEE (0.3×10^{-6} mol/L; Toxin Technologies, Sarasota, FL).

Flow cytometric analyses

The mAbs against CD14 (APC; TUK4) and CD19 (PE; SJ25-C1) were obtained from Caltag/Invitrogen. HLA-DQ (FITC; Leu10) and HLA-DM (PE; MaP.DM1) mAbs were obtained from BD Pharmingen (San Jose, CA), the mAb against HLA-DP (FITC; HI43) was from BioLegend (San Diego, CA). The HLA-DR specific mAb (FITC and PE; L-243) and the invariant chain mAb (FITC; 5-329) were kindly provided by Dr. Otto Majdic (Institute of Immunology, MUV, Vienna, Austria). Isotype matched control antibodies (FITC, PE and APC conjugated) were obtained from ADG (Kaumberg, Austria). Surface and intracellular stainings as well as flow cytometric analyses were performed as described previously [16].

Complementation assays

VIP1 and VIP2 EBV-transformed B cells (10^7) were incubated on ice with 20 µg of CIITA plasmid DNA for 5 min followed by electroporation at 300 V and 300 µF (GenePulser, BioRad, Vienna, Austria). Subsequently, cells were transferred to RPMI supplemented with 20% FCS and cultured for three days before assessment of transgene expression. For retroviral transduction, amphotropic RFXANK-, RFX5- RFXAP- and CIITA-delivering retroviruses were produced as described previously [17].

Genomic sequencing

Genomic DNA was isolated from 2×10^6 EBV B cells (GenElute Mammalian DNA Extraction Miniprep Kit; Sigma Chemicals). Exon sequences of CIITA were determined using primers described previously [18]. Exon sequences of RFXAP were determined with the following primers: exon 1 for: 5'-TGACAGCCCGCGGGCGTACACACGCTGACG-3', exon 1 rev: 5'-AGATGCGGTTTGCGTTAAGATCGGGT-3'; exon 2 for: 5'-CATCTTTGAAATACCTGTAATCATGACTTATTACGTAACTGTT-3'; exon 2 rev: 5'-AATCTCAATTGTTCCATTTATAACAAACGTTTGTTAACACAT-3'; exon 3 for: 5'-CATATGTAGGGACACATCAGAGTAATTAGCATGAAAAC-3', exon 3 rev: 5'-ATTGGCACTGCGGGTGAGACAATCTTTAGTATG-3'.

RESULTS

Lack of HLA class II expression on B lymphocytes and monocytes of two newly identified CID patients

Immunological analysis of two immunodeficiency patients revealed an inverted CD4/CD8-ratio due to CD4⁺ lymphopenia, accompanied by a drastic reduction of superantigen-dependent T cell responses. CD3 mAb and PMA-induced T cell responses were low but deetectable, while PHA-responses were within the normal range (table 2). Flow cytometric analyses revealed a lack of HLA-DR and –DQ expression on nearly all CD19⁺ B cells and CD14⁺ monocytes obtained from VIP1 and VIP2 compared to healthy controls (fig. 1). The absence of HLA class II cell surface expression together with the functional inability to mount a superantigen-driven T cell response was indicative of congenital MHC class II (MHCII) deficiency.

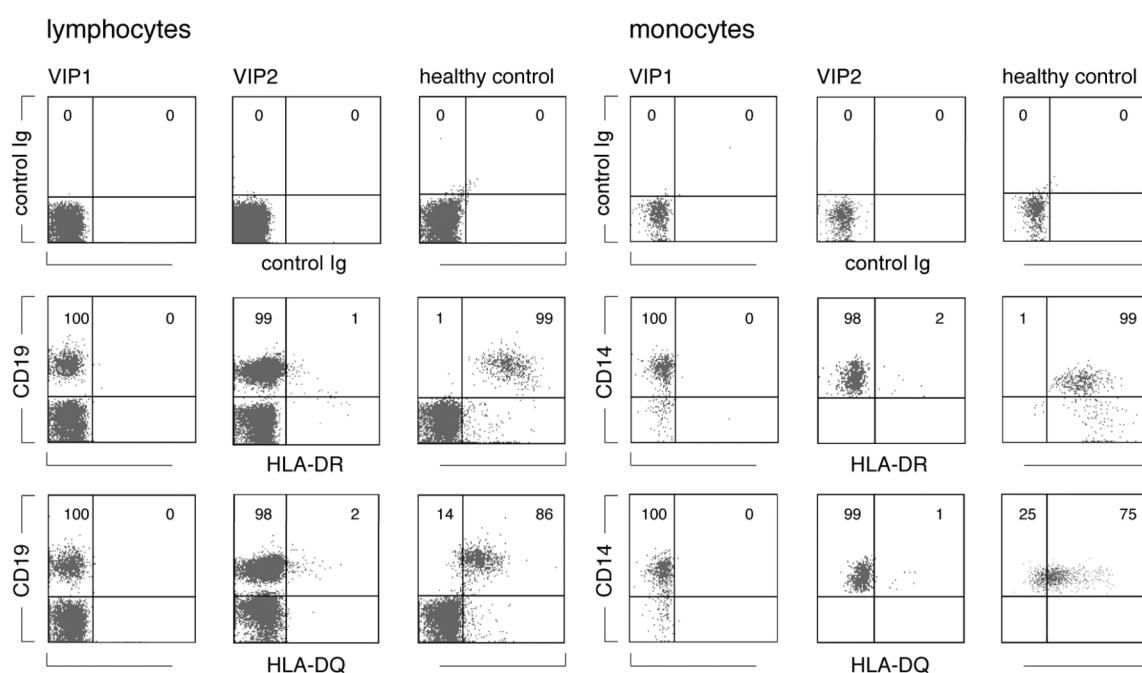


Fig. 1. Defective surface expression of HLA class II molecules on peripheral blood B cells and monocytes of CID patients VIP1 and VIP2. Whole blood cells of patients and a healthy unrelated control individual were stained for major leukocyte subpopulations and analyzed by flow cytometry. Gating on lymphocytes and monocytes was performed according to typical forward-/side-scatter characteristics. HLA-DR and HLA-DQ expression on CD19⁺ B cells or CD14⁺ monocytes is displayed with markers set according to non-binding isotype-matched control antibodies (control Ig). Numbers indicate percentage of HLA class II positive cells within lineage-positive cells.

Table 1. Laboratory findings in MHCII deficiency patients

	VIP1	VIP2	normal range	
leukocytes	9100 ^a	3400	4300-10000	^a cells per microliter
granulocytes	2820	2620	1800-7300	^b mg/dl
monocytes	820	30	200-1000	^c reference values for 2-6 months old children according to [36]
lymphocytes	5460	750	1500-4000	^d not detectable
CD19 ⁺ B cells	1090	410	75-760	
CD3 ⁺ T cells	3440	270	690-3320	
CD3 ⁺ CD4 ⁺ T cells	600	80	480-2480	
CD3 ⁺ CD8 ⁺ T cells	2730	160	195-1800	
CD4 ⁺ /CD8 ⁺ -ratio	0.22	0.50	>1.0	
CD3 ⁻ CD56 ⁺ NK cells	820	50	120-880	
Serum IgG (pre-IVIG)	92 ^b	57	195-794 ^c	
Serum IgM	85	n.d. ^d	9-212	
Serum IgA	n.d.	n.d.	1-59	

Table 2. Lymphocyte proliferative function

Mitogen	VIP1	healthy control	VIP2	healthy control ^b
PHA	109±14 ^a	279±9	225±1	276±7
PMA	40±6	156±8	13±3	76±4
SEA	2±0	205±2	1±0	27±5
SEB	1±0	60±3	25±4	166±12
OKT3 mAb soluble	6±1	50±3	7±7	52±2
none (medium)	1±0	3±1	1±0	3±1

^a kilo cpm^b adult healthy control individual

Rapid restoration of HLA class II expression in patient's cell lines

To analyze the underlying immunodeficiency, EBV lymphoblastoid B cell lines of both patients were either transfected with an HLA class II transactivator (CIITA) encoding expression plasmid or transduced with retroviral vectors encoding RFXANK, RFX5 and RFXAP (efficiency 5-10% and 30-40%, respectively). HLA-DR expression was restored in VIP1 by the CIITA cDNA, and in patient VIP2 by the RFXAP cDNA (fig. 2a). Furthermore, expression of CIITA in VIP1 EBV-blasts and RFXAP in VIP2 EBV-blasts also restored surface expression of HLA-DP and HLA-DQ and intracellular expression of HLA-DM (fig. 2b). Accordingly, VIP1 was assigned to complementation group A and patient VIP2 to complementation group D. Complementation of the respective genes also restored the superantigen response of patients' EBV B cells as tested by IL-2 reporter activity in Jurkat T cells (not shown).

VIP1 is homozygous for a nonsense mutation in amino acid codon 381 of CIITA

The only defect identified in patient VIP1 was a G to T point mutation at nucleotide 1256 of the CIITA cDNA sequence in exon 11 (nomenclature according to GeneBank sequence accession number X74301) resulting in the conversion of codon 381 from a GAA into a TAA encoding a premature stop, i.e. p.Glu381X (fig. 3a). This mutation is identical to the point mutation in allele 1 of patient BCH, described by Bontron et al [19,20]. Homozygosity was confirmed by DNA sequence analyses of parental gDNA, which revealed that both parents carry one p.Glu381X allele (fig. 3a). Thus, VIP1 is the first described MHCII deficiency patient with a homozygous p.Glu381X mutation of CIITA.

In VIP2 a homozygous single-nucleotide deletion in codon 123 induces a premature out-of-frame stop

In patient VIP2 a single base deletion of a guanine in codon 123 of exon 1 of the RFXAP gene was identified, resulting in a frame-shift and premature translational stop signal 15 codons downstream, i.e. p.Ser123ThrfsX15. Analyses of parental gDNA showed a heterozygous configuration from codon 123 onwards, compatible with a heterozygous single nucleotide deletion at position 484 (fig. 3b). This mutation is identical to the mutation described in three independent MHCII deficiency patients (SS, ZM and DA) all three of North African origin [21,22]. Thus, VIP2 is the first patient with a homozygous p.Ser123ThrfsX15 mutation of RFXAP identified within the Austrian population.

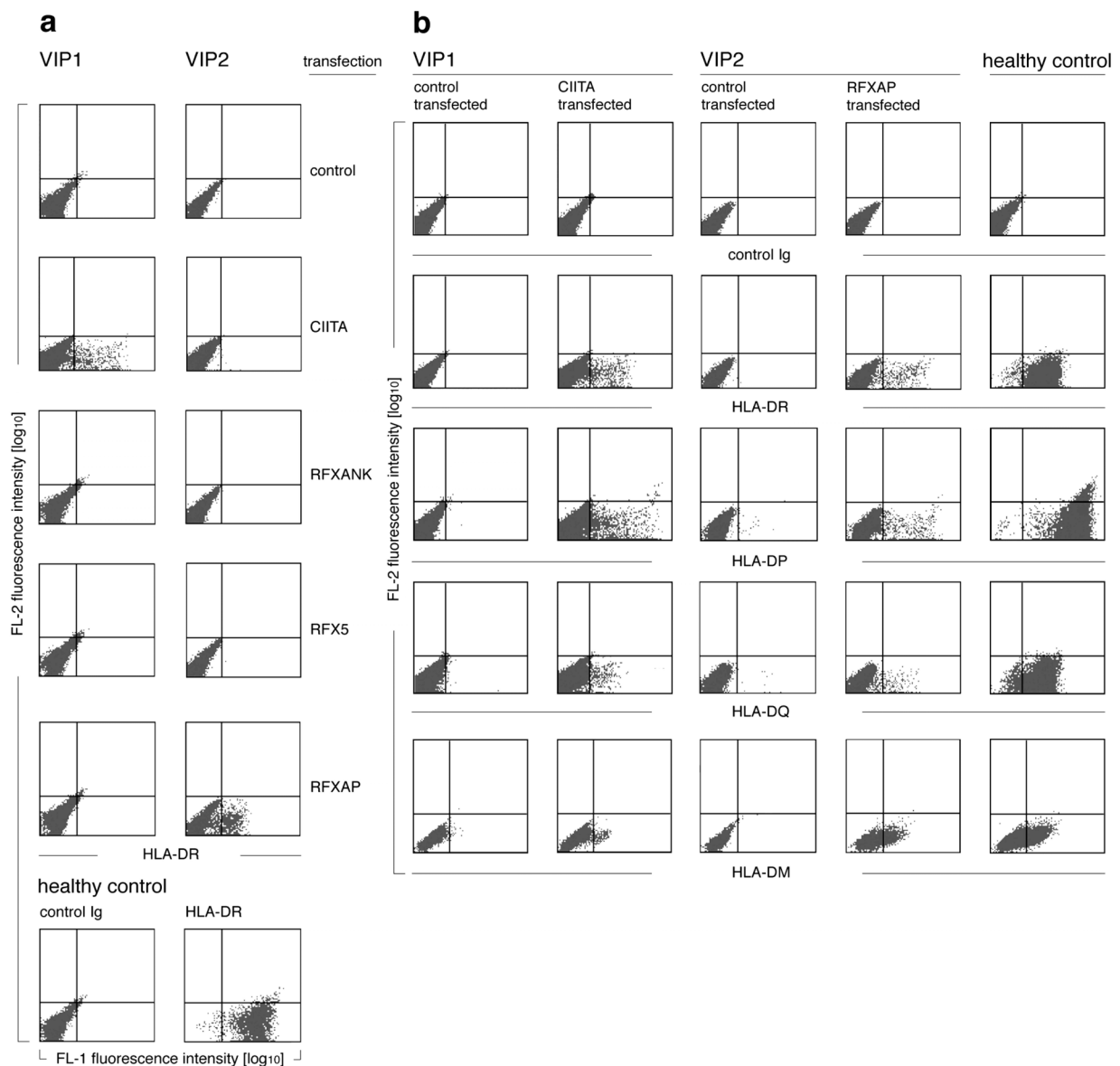


Fig. 2. Restoration of HLA class II expression on patients' EBV B cells. (a) VIP1 and VIP2 EBV B cells transfected with the indicated plasmids and EBV B cells of a healthy unrelated control individual were stained with a HLA-DR specific mAb and analyzed by flow cytometry to assess complementation. (b) HLA isotype expression upon complementation. Complemented or control transfected EBV B cells of VIP1 and VIP2 were stained with the indicated mAbs and analyzed by flow cytometry. EBV B cells of a healthy control individual were used as control. Surface expression of HLA-DR, -DP and -DQ and cytoplasmic expression of HLA-DM is shown. Markers were set according to nonbinding control mAbs. One out of three representative experiments is shown.

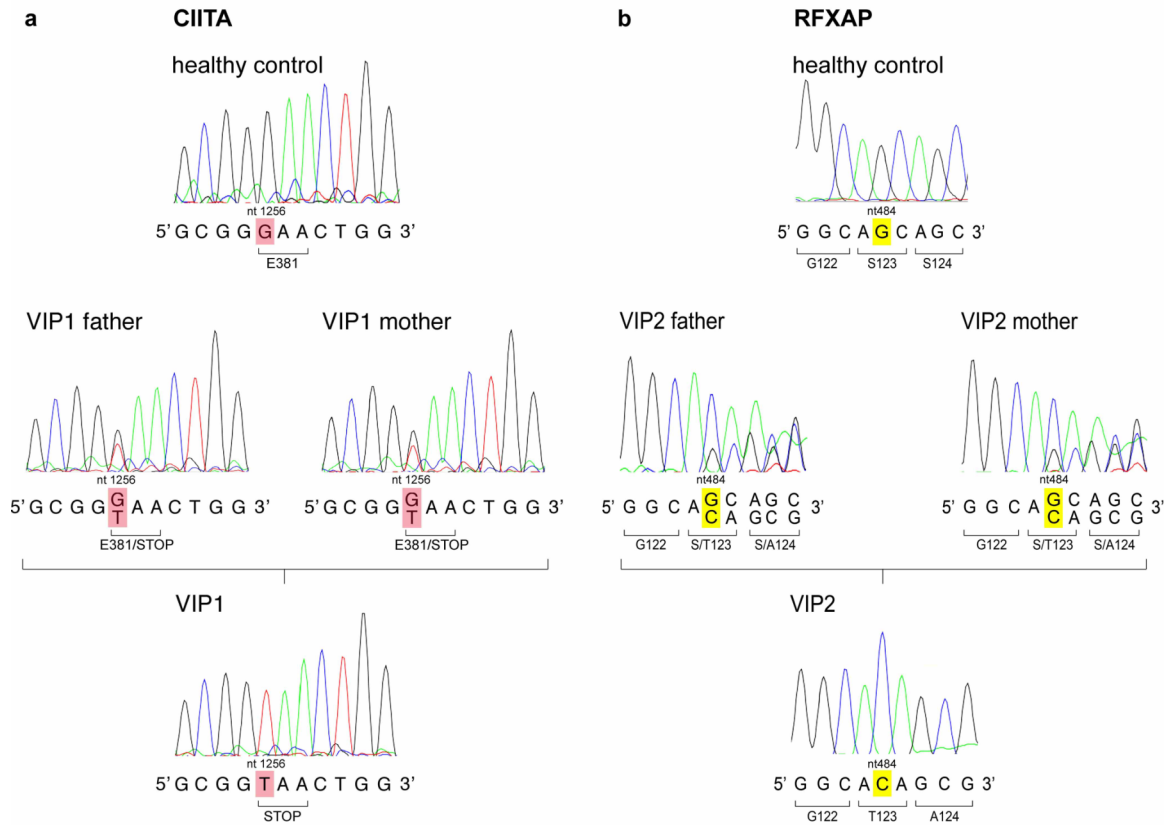


Fig. 3. Sequence analyses of gene mutations in MHCII deficiency patients. (a) VIP1: Sequence of CIITA, exon 11. The wt and mutated nucleotide (nt) 1256 contributing to p.Glu381 in the wt and a premature translational STOP in the patient VIP1 are highlighted (red). Both parents show a heterozygous G/T configuration at this nucleotide position. **(b) VIP2:** Sequence of RFXAP, exon 1. The wt nucleotide 484 contributing to the Serine 123 codon is highlighted (yellow). In the patient VIP2 nt 484 is deleted causing a frame-shift-mutation leading to a pre-mature STOP at codon 137 (not shown). Both parents show the wt and the frame-shifted sequence from nt 484 onwards. Results were confirmed by repetitive bi-directional DNA-sequencing.

Reduced HLA class II expression-levels due to heterozygosity for CIITA mutation

So far, no phenotype has been associated with heterozygosity for a mutated, non-functional *CIITA* allele. However, peripheral blood B cells obtained from the parents of patient VIP1, showed significantly lower cell surface expression of HLA-DR and -DP when compared to a collective of 20 healthy control individuals (fig. 4). A similar, albeit not significant tendency was found for monocytes. Of note, the parents of RFXAP-deficient patient VIP2 had normal HLA class II expression levels on PB B cells and monocytes (not shown). Our findings might point to a gene dosage effect due to the expression of only one functional *CIITA* allele.

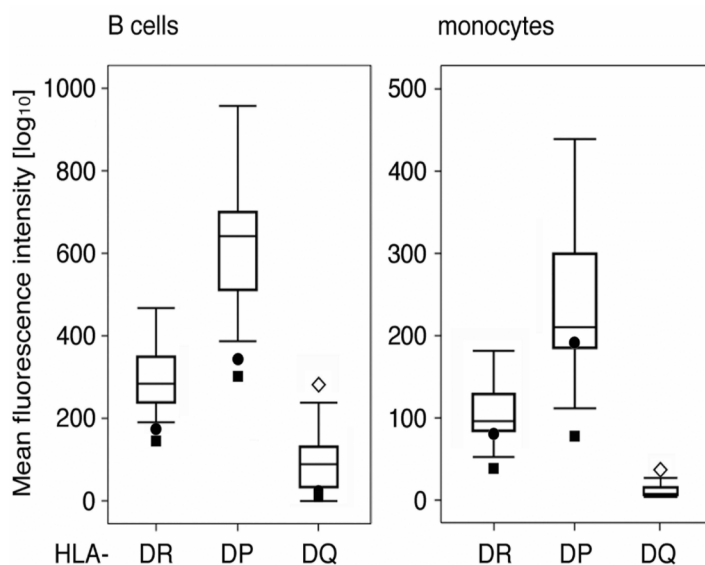


Fig. 4. B cells and monocytes from VIP1 parents show decreased surface expression of HLA class II molecules. Box plots depict the geometric mean fluorescence intensities of HLA-DR, -DP and -DQ molecules on CD19⁺ B-cells and CD14⁺ monocytes of 20 healthy, unrelated control individuals (ten male, ten female; median age 27.7 years; range 20.9 - 45.1). The respective geometric mean expression levels of the indicated HLA isotypes of VIP1 father (black squares; age: 38,2) and VIP1 mother (black circles; age: 35,8) are indicated. Open diamonds represent statistical outliers.

Different complementation groups reveal discoordinate intracellular invariant chain (Ii) expression levels

When analyzing patient-derived EBV B cells differential gene expression was also observed for the invariant chain (Ii, CD74), which regulates peptide loading of HLA class II molecules [23]. Previous studies on the mRNA level have shown that Ii expression is subject to complex gene regulation governed by HLA class II-dependent and -independent transcription pathways [24]. In fact, intracellular Ii expression levels in EBV B cells of patient VIP1 were significantly reduced (fig. 5a, 36.4 ± 4.3 % $p < 0.01$), when compared to healthy controls. To exclude a cell lines effect and to allow generalization of our finding, we tested EBV B cells of three additional *CIITA* deficient patients, i.e. VIP3 (recently identified) and the prototype cell lines BLS-2 and BCH [10,20]. Fig. 5b shows

that VIP3, BLS-2 and BCH also presented with significantly reduced Ii-levels ($p < 0.001$). In marked contrast, Ii expression levels in VIP2 EBV B cells were even slightly higher than that of healthy, unrelated control individuals (fig. 5a and b). The additionally tested cell lines BLS-1 and SJO, from RFXANK and RFX5 patients, showed a clear-cut, but less pronounced, reduction of Ii levels compared to healthy controls. Overall, complementation group A cell lines presented with the lowest Ii levels, complementation groups B and C with intermediate levels, while the group D showed no reduction of Ii expression ($p < 0.01$ for A vs. B or C; and $p < 0.001$ for A vs. D). Complementation studies with retrovirally expressed CIITA led to a significant up-regulation of Ii in the complemented HLA-DR⁺ cell fraction ($p = 0.018$), further confirming the role of CIITA for regulation of Ii expression in B lymphocytes (fig 5c). Interestingly, EBV B cells of VIP1' parents showed normal Ii-expression levels (not shown). Our findings substantiate the observation that the Ii is differentially regulated in hematopoietic cells. High levels of intracellular Ii expression depend on the presence of at least one wild type copy of the CIITA gene, but not on RFXAP gene products. Consequently, intracellular Ii expression levels might help to pre-assign MHCII deficiency patients to distinct complementation groups already during the flow cytometry-based phenotyping step. When combined with the above described analyses of HLA class II expression levels on PB B cells and monocytes of the patients' parents, such assignments might even gain additional weight.

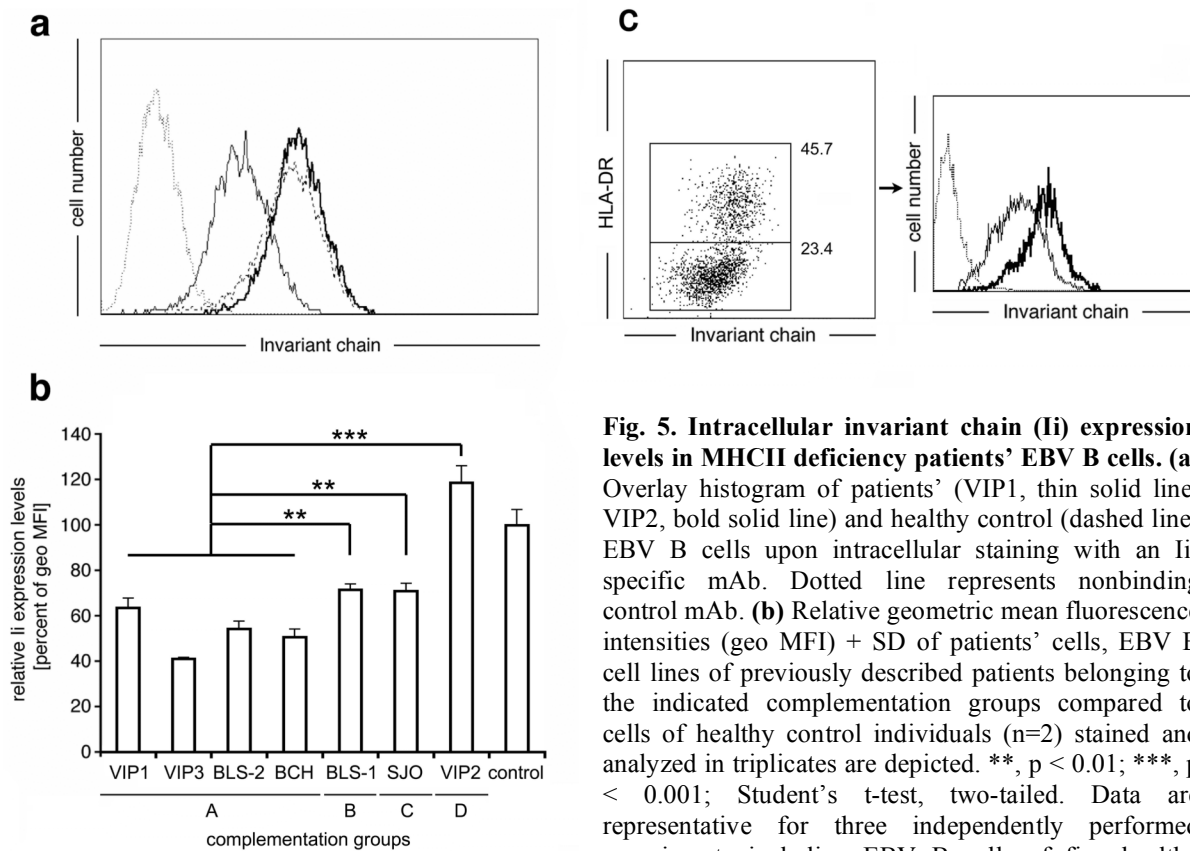


Fig. 5. Intracellular invariant chain (Ii) expression levels in MHCII deficiency patients' EBV B cells. (a) Overlay histogram of patients' (VIP1, thin solid line; VIP2, bold solid line) and healthy control (dashed line) EBV B cells upon intracellular staining with an Ii-specific mAb. Dotted line represents nonbinding control mAb. **(b)** Relative geometric mean fluorescence intensities (geo MFI) + SD of patients' cells, EBV B cell lines of previously described patients belonging to the indicated complementation groups compared to cells of healthy control individuals (n=2) stained and analyzed in triplicates are depicted. **, $p < 0.01$; ***, $p < 0.001$; Student's t-test, two-tailed. Data are representative for three independently performed experiments including EBV B cells of five healthy control individuals. **(c)** Complementation of CIITA in

EBV B cells of patient VIP1 leads to up-regulation of Ii expression. VIP1 EBV B cells transduced with CIITA retrovirus were stained for surface HLA-DR- and cytoplasmic Ii-expression. Complemented versus non-complemented cells were identified by HLA-DR expression and analyzed for Ii expression. Numbers indicate Ii geo MFI of HLA-DR⁺ and -DR⁻ subsets. Right hand panel shows overlay histograms of complemented (bold line) versus non-complemented (thin line) cells, gated as indicated (left panel), and stained with Ii mAb. Dotted line represents staining obtained with nonbinding control mAb.

DISCUSSION

We have identified two non-related patients, designated VIP1 and VIP2, who showed the clinical course of CID owing to a complete lack of HLA class II expression on PB B cells and monocytes compatible with MHCII deficiency. Ectopic overexpression of candidate cDNAs revealed CIITA-deficiency in patient VIP1 (complementation group A) and RFXAP-deficiency in patient VIP2 (complementation group D). Significantly, Ii expression levels were reduced in EBV B cells of the CIITA-deficient but not of the

RFXAP-deficient patient. Moreover, also HLA class II expression levels were affected in the CIITA-deficient patient's parents.

A special advantage of vector-based complementation strategies is the fast assignment of patients to distinct complementation groups providing a cost-effective alternative to first-line DNA-sequencing approaches [25]. Moreover, functional complementation approaches allow immediate conclusions concerning the mono- or oligogenicity of a given deficiency, a test-quality, which can only hardly be provided by gene-sequencing.

The p.Glu381X mutation is identical to the mutation described by Bontron and colleagues in allele 1 of patient BCH [19,20]. Since both parents of VIP1 are heterozygous for the p.Glu381X mutation and no other mutation was found in VIP1, it can be assumed that patient VIP1 is homozygous for the p.Glu381X mutation. So far, only eight MHCII deficiency patients belonging to complementation group A have been described in the literature [26]. Each of them carries a unique set of either homozygous or heterozygous mutations in their CIITA genes. Patient VIP1 described here is the first patient with a homozygous p.Glu381X mutation leading to loss of the C-terminal nuclear localization domain (NLS) of the CIITA which prevents the recruitment of CIITA to the HLAII enhanceosome [7,27]. Since BCH, the first patient described with a (heterozygous) p.Glu381X mutation, is also of Austrian origin, it is well possible that this mutated allele is more widespread in the Austrian population than elsewhere. The assumption, however, that this distinct mutation occurred independently three times, i.e. in one parent of patient BCH and in both parents of patient VIP1, might also point to a region of increased genetic instability within the CIITA gene, which therefore could be a target for fast primary genetic analysis of novel MHCII deficiency patients with a suspected CIITA-deficiency. Given the fact that the two Austrian CIITA deficiency patients VIP1 and VIP3 were born in the years 2005 and 2006, respectively, and given that in those two years a total of 154002 births were reported for Austria (Statistik Austria, http://www.statistik.at/web_de/statistiken/bevoelkerung/geburten/index.html) this would – assuming Hardy-Weinberg equilibrium - correspond to a gene-frequency of 0.0036 and thus imply one heterozygous individual among 139 Austrians. Such estimations, although providing a rough picture on the possible genetic background of a population, will have to

be formally proven by future large-scale investigations. It remains also to be shown whether heterozygous individuals might be endowed with altered immune function.

In patient VIP2 a p.Ser123ThrfsX15 mutation, which leads to a premature translational stop and thus a non-functional RFXAP gene product was identified. So far, three different mutations in eight RFXAP-deficient patients from six unrelated families have been described with three patients carrying the same mutation as patient VIP2 [21,26]. Interestingly, those three patients were all of North African origin. The fact that VIP2 is a homozygous carrier of the p.Ser123ThrfsX15 mutation with both parents showing a heterozygous configuration suggests that also this mutation is more widespread within the Middle European population.

Until today, no phenotype has been associated with heterozygosity for CIITA mutations. We noticed decreased HLA Class II expression-levels on VIP1' parental B lymphocytes compared to cells from healthy control individuals. This points to a gene dosage effect due to the heterozygous loss of CIITA-activity. In contrast, normal HLA class II expression-levels were observed on B lymphocytes of the parents of the RFXAP-deficient patient. Our finding, although based only on two families, could form the basis for initial carrier identification in individuals with suspected CIITA mutations. Moreover, parents' HLA expression levels might give a first hint, which gene might be affected in a given patient.

Moreover, intracellular Ii-expression levels were reduced in EBV B cells of the CIITA-deficient but not of the RFXAP-deficient patient. Previous reports have shown, that CIITA expression is necessary and sufficient for HLA class II expression [28], while Ii expression can also be regulated in a CIITA-independent fashion [21,29]. In the physiological context, the CIITA-independent transcription of Ii might be required in order to exert the other biological functions described for the Ii, e.g. as a ligand for MIF [30] and CD44 [31]. It has been shown in the past that mutations in the CIITA gene lead to a partial block in Ii expression on the mRNA level [18,32], however, information on the protein level, especially in hematopoietic cell types, remained scarce. Similarly, the role of the RFX-complex members for Ii expression is only poorly described. One report described that the RFX-B mutant B cell line BLS-1 had an approx. 50% reduction of Ii mRNA [33], while fibroblasts from two different patients were found to be negative for Ii

mRNA [34]. In RFXAP or RFX5 mutant patients, no Ii mRNA could be detected in IFN- γ treated fibroblasts [21,29,35]. However, no information on intracellular Ii-expression levels of B cells from RFXAP-deficient patients exists. We here show, that Ii expression levels in EBV B cells of the CIITA-deficient but not of the RFXAP-deficient patient are significantly reduced when compared to healthy control cells. Similar results were obtained with three additional EBV B cell lines of unrelated CIITA deficient patients. A less pronounced reduction of Ii expression levels was also found in one RFXANK- and one RFX5-deficient cell line. Thus, strongly reduced Ii expression levels in hematopoietic cells of patients might be indicative of CIITA-deficiency, while they might exclude RFXAP-deficiency. Intermediate Ii-levels might point to RFXANK- or RFX5-deficiency. In fact, reduced Ii expression levels of CIITA-deficient cell lines could be restored to wild type levels by retroviral complementation with CIITA (fig. 5c). Future studies with more patients/cell lines will have to substantiate the findings presented here. However, in combination with the above-described reduction in cell surface HLA class II expression levels on PB B cells of heterozygous parents, our findings might lead to novel algorithms for the quick identification of subsets of MHCII deficiency disorders.

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Chapter 5

Fluorosomes: a convenient new reagent to detect and block multivalent and complex receptor/ligand interactions

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ABSTRACT

We describe for the first time fluorescent virus-like particles (VLP) decorated with biologically active mono- and multi-subunit immune-receptors of choice and the basic application of such *fluorosomes* (FS) to visualize and target immune-receptor/ligand interactions. For that purpose, human embryonic kidney (HEK)-293 cells were stably transfected with Moloney murine leukemia virus (MoMLV) matrix protein (MA) green fluorescence protein (GFP) fusion constructs. To produce FS, interleukins (IL), IL-receptors (IL-R) and co-stimulatory molecules were fused to the glycosyl phosphatidyl inositol (GPI) anchor acceptor sequence of CD16b and co-expressed along with MoMLV group specific antigen-polymerase (gag-pol) in MA::GFP⁺ HEK-293 cells. We show that IL-2 decorated but not control-decorated FS specifically identify normal and malignant IL-2 receptor positive (IL-2R⁺) lymphocytes by flow cytometry. In addition to cytokines and co-stimulatory molecules, FS were also successfully decorated with the heterotrimeric IL-2R, allowing identification of IL-2⁺ target cells. Specificity of binding was proven by complete inhibition with non-labeled, soluble ligands. Moreover, IL-2R FS efficiently neutralized soluble IL-2 and thus induced unresponsiveness of T cells receiving full activation stimuli via T-cell antigen receptor (TCR) and CD28. FS are technically simple, multivalent tools for assessing and blocking mono- and multi-subunit immune-receptor/ligand interactions with natural constituents in a plasma membrane context.

Key words

lipid-rafts, viral pseudotyping, glycosyl phosphatidyl inositol anchored proteins, flow cytometry, fluorescent proteins.

INTRODUCTION

Virus-like particles (VLP) are noninfectious enveloped particles that can be induced in mammalian cells by the expression of viral structural proteins in the absence of viral nucleic acid. They have proven to be powerful tools for the display and transport of diverse biologically active immunomodulatory molecules (1-3). We have shown in the past that VLP can be engineered to modulate immune responses by judicious incorporation of additional membrane-associated proteins that sustain stimulatory or inhibitory immune responses. We have named the resulting engineered microvesicles immunosomes and anergosomes respectively (1-3). Because direct interaction between leukocyte receptors and VLP-resident ligands was found to be a prerequisite for effective receptor triggering, we have in this study explored whether it would be possible to visualize the interaction between VLP decorated with specific ligands and cells expressing the cognate receptors. We hypothesized that sufficient quantities of fluorescently labeled VLP would be detectable once bound to cells, and that such particles might represent both a versatile staining tool and a traceable agent for the efficient modulation of immune responses.

In this study we have focused on VLP labeled *in vivo* by recombinant fluorescent proteins of cnidarian origin (4-6), demonstrating their utility for the visualization of specific immune-receptor/ligand interactions. Translational fusions of viral proteins with GFP have been broadly used to elucidate infectious pathways of viruses (7-10). Since lipid-rafts are the meeting points for GPI-anchored surface molecules and viral core proteins (11) we hypothesized that fluorescent proteins linked to raft-targeted viral core proteins might accumulate in sufficiently high abundance to generate fluorescently labeled VLP that could be used to track the interactions of VLP in different settings. For efficient decoration of VLP, immune-receptors or ligands of choice were fused at their C-termini to the glycosyl phosphatidyl inositol (GPI) anchor attachment sequence of CD16b, a well-defined GPI-anchored molecule of human granulocytes (12). Previous reports have shown that GPI-anchored molecules are efficiently targeted to the lipid-raft regions of producer cells and consequently to VLP (11).

To illustrate the potential of this approach we have chosen cytokine/cytokine-receptor interactions as model system. Cytokines are small to medium sized proteins or glycoproteins, that mediate potent biological programs upon binding to their specific receptors (13). Cytokine receptors are differentially expressed on various cell types and

can be visualized and their density determined by receptor specific mAbs. Flow cytometric detection of cytokine receptors on normal and malignant cells using mAbs has been widely applied in the past (14-16). However, many growth factor receptors consist of multisubunit receptors and frequently similar receptor-chains (subunits) are used by different receptors, e.g. the common γ -chain (CD132) (17), which is part of the IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21 receptors; or the IL-2R β -chain which is part of the IL-2 as well as the IL-15 receptor (13). Thus, the identification of single receptor components does not always allow determination of overall receptor composition.

Consequently, precise enumeration and visualization of growth factor receptors has required radioligand-, affinity- or fluorochrome-based (18, 19) binding assays with in vitro-modified cytokines in the past. Modification of cytokines requires, for the most part, that the molecule of interest be available in sufficient quantity and purity. The most widely used labeling-techniques rely on the existence on the target molecule of a sufficient number of reactive residues susceptible to chemical modification. Alterations to protein sidechains, which in most cases is a random process, can cause changes of the physiological or biophysical properties of the natural protein.

In addition to assessing their staining potential we were interested in determining whether VLP could also be decorated with more complex structures, e.g. multichain cytokine receptors, and whether such particles would bind and neutralize the respective cytokines. The potential utility of this approach has been suggested by recent reports that cytokine deprivation may be one of the mechanisms by which $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) (20) and myeloid dendritic cells (mDC) (21, 22) exert their regulatory function on effector T cells.

MATERIALS AND METHODS

Cell lines and primary cells. The human embryonic kidney epithelial cell line 293 and the murine HT-2 cell line (ATCC, CRL-1841, American Type Culture Collection, Manassas, VA, USA) were maintained in IMDM (Sigma Chemicals, St. Louis, MO, USA) plus 10 % FCS (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 50 μ M 2-mercaptoethanol and 15 μ g/ml gentamycin sulfate. HT-2 cells were re-plated every 48 h at a cell density of 1×10^5 cells/ml in the presence of 100 U/ml human IL-2 (Peprotech, London, UK). BW5147 (23) and BW5147 CTLA-4 cells were described previously (24, 25) and were cultured in RPMI 1640 medium plus 10 % FCS (Invitrogen),

2 mM L-glutamine, 50 μ M 2-mercaptoethanol and 15 μ g/ml gentamycin sulfate. PBMC were isolated from heparinized blood of healthy adult donors upon informed consent by standard density gradient centrifugation with Lymphoprep (Technoclone, Vienna, Austria). Whole blood samples of chronic lymphocytic leukemia (CLL) patients obtained upon informed consent were collected with EDTA anticoagulant and analyzed in standard whole blood stainings using ADG-Lyse (An der Grub, Kaumberg, Austria).

Expression constructs. The modified cytokine expression constructs have been described previously (2). Briefly, the specific cDNAs for human IL-2 and IL-7 were amplified with primers as specified in supplement Table 1 from clone pTCGF-11 (ATCC #39673) and pHumLP-1 (ATCC#67546), respectively. At the 5'-ends, optimized translational initiation sites preceded by a Hind III restriction site were inserted. The 3'stop-codons were replaced with a polyglycine linker sequence followed by a Nhe I restriction site for direct fusion to the minimal CD16 GPI-anchor acceptor sequence (IL-2R constructs) or alternatively to the CD16 GPI-anchor preceded by two Ig-like domains of CD16 (membrane-bound cytokines; D. Haiderer, manuscript in preparation). The DNA-fragments were ligated into the pEAK12-CD80::CD16 plasmid from which the CD80 ectodomain had been released (1). Original MoMLV *gag-pol* (OGP) was expressed from pMD.gagpol (26) kindly provided by Dr. R. Mulligan, Childrens' Hospital, Boston, MA, USA. IL-2R subunits were amplified by RT-PCR using mRNA from PHA plus IL-2 activated (24 h) PB T cells as template and were modified in a similar fashion (primers in supplement **Table S1**). Full length human CD16b served as a control (12). The MA::GFP fusion construct was generated by PCR using pMD.gagpol and pEAK12.GFP as a templates (27). Hind III and Nhe I, or Nhe I and Not I restriction sites, incorporated in the PCR primers allowed the resulting fragments to be inserted into the pEAK12 expression plasmid at the location of the cognate sites in the vector.

Stable 293 transfectants. 293 cells stably expressing MA::GFP, GFP or IL-2::GPI (single cell clones) were generated by transfection with plasmids linearized by digestion with Sfi I (New England Biolabs, Ipswich, MA, USA) (28), selection in puromycin (1 μ g/ml, Sigma Chemicals) and analysis by flow cytometry (FACS Calibur; Becton Dickinson, Palo Alto, CA, USA) using the CellQuest software.

Generation of GFP-labeled VLP decorated with cytokines or co-stimulatory molecules (fluorosomes, FS). For the generation of fluorosomes (FS), 293 cells stably expressing MA::GFP or GFP were transiently transfected using the calcium-phosphate coprecipitation method (28). Briefly, one day prior to transfection, 293 cells were seeded onto 100 mm cell culture dishes (3×10^6 cells/plate) and were transfected the following day with 2 ml transfection mix. For generation of IL-, IL-R- or CD80-decorated FS 293 cells were transfected with a total DNA amount of 30 μg per dish including 7.5 μg of the MoMLV original *gag-pol* (OGP) encoding plasmid pMD.gagpol and 22.5 μg of the indicated IL::GPI or CD80::GPI pEAK12 expression plasmid. For generation of IL-R decorated FS, 293 cells were transfected with 30 μg DNA per dish including 7.5 μg pMD.gagpol and 7.5 μg of each IL-2R subunit expression plasmid. If necessary, DNA amounts were adjusted with control vector (empty pEAK12 vector or, where indicated, full length CD16b in pEAK12). Control particles were generated by transfection of 293 cells with 7.5 μg pMD.gagpol and 22.5 μg of control vector. 18-24 h after transfection, the medium was changed, and transfectants were cultured for another 48 hours before FS-containing supernatants were harvested.

Flow cytometric analysis of 293 producer cells. For membrane staining, 5×10^5 293 cells in 50 μl were incubated at 4°C with fluorochrome conjugated mAbs (20 $\mu\text{g}/\text{ml}$, supplement **Table S2**) for 30 min. After two washes with staining buffer (PBS, 1 % BSA, 0.05 % NaN_3) membrane fluorescence was analyzed by flow cytometry. Treatment of 293 cells with phosphatidylinositol specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (American Radiolabeled Chemicals, St. Louis, MO, USA) was performed according to the manufacturer's recommendations. Briefly, 293 cells were washed in PBS w/o Ca^{2+} and Mg^{2+} and 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 the cells incubated at 37°C for 2 h. Subsequently cells were washed in PBS/ 1 % BSA and subjected to membrane staining.

Isopycnic separation of producer cell lysates. Preparation of lipid raft fractions was performed as described (29). Aliquots of 20 μl of individual fractions collected from top to bottom of 5-40% sucrose gradients were analyzed by SDS-PAGE on 4-20 % gradient gels. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and subjected to Western blotting using mAbs specific for IL-2 (supplement data **Table S2**),

p30gag, GFP, CD59 and CD147. HRP conjugated secondary reagents (supplement data **Table S2**) were used at a dilution of 1:10⁴. Blots were developed with a luminol based indicator system (Western Lightning, Perkin Elmer, Boston, MA, USA) and exposed to x-ray films (Eastman Kodak, Rochester, NY, USA).

Purification of VLP/FS. Supernatants (10 ml/culture plate) were harvested 72 hours after transfection and cleared of cellular debris by filtration through 0.45 µm syringe filters (Millipore), concentrated by ultracentrifugation in a Beckman-Optima LE-80K centrifuge (Beckman Instruments, Palo Alto, CA, USA), using a SW41 Ti rotor, at 100.000 g for 1 h and washed twice in a large volume of PBS. Amounts of VLP/FS were determined by standard protein assay (Micro BCA-Pierce, Thermo Scientific, Rockford, IL, USA) and adjusted to protein concentrations as described in individual experiments. For functional tests, VLP/FS preparations were sterile filtered (0.22 µm syringe filters, Millipore) before use.

Confocal microscopy. 293 cells stably transfected with MA::GFP or GFP were seeded at a density of 4 x 10⁴ cells/cm² in a PeCon POC-R cell cultivation chamber, grown overnight and analyzed on an Axiovert 100 M confocal microscope using a 63x/1.40 oil immersion objective (Carl Zeiss, Göttingen, Germany).

Fluorosome (FS) based staining assays. HT-2 cells (1 x 10⁵ cells) were resuspended in staining buffer (PBS, 0.5% BSA, 0.05% NaN₃) and were incubated at room temperature with) of titrated amounts of either cytokine-decorated or non-decorated FS (starting with 400 µg) in a total volume of 100 µl for > 1 hour until equilibrium binding was reached. Control stainings were performed with IL-2R alpha chain-specific (CD25, supplement data **Table S2**) or nonbinding control mAb all used at 1 µg in a total volume of 100 µl for 30 min. The ED₅₀-value was calculated from mean fluorescence intensity values using nonlinear regression analysis fitted to classical Michaelis-Menten kinetics (Prism 5.0b, GraphPad software, San Diego, CA, USA). Specificity of binding reactions was determined by pre-incubation of HT-2 cells with soluble IL-2 (5 x 10³ U in 100 µl volume). Off-rates were determined by first labeling HT-2 cells (1x 10⁵) with 400 µg IL-2 decorated MA::GFP⁺VLP at 4°C for > 1 hour and by washing them extensively. Following initial binding HT-2 cells were chased in an excess of staining buffer (4 ml) at

4°C, room temperature, 37°C and 37°C in the presence of 4×10^4 U soluble IL-2 (Peprotech, London, UK) for 0, 15, 30, 60 and 120 min and analyzed by flow cytometry. Staining of 293 IL-2R transfectants with VLP ($20 \mu\text{g}/1 \times 10^5$ cells) was performed in a similar fashion, except that control stainings were performed with mAbs against human CD25, CD122, CD132. BW5147 CTLA-4 transfectants were control stained with a CTLA-4 mAb or negative control mAb. Subsequently, cells were washed two times with 4 ml staining buffer (4°C) by centrifugation (300 g for 5 min), resuspended in 150 μl staining buffer and 20 μl propidium iodide (0.5 $\mu\text{g}/\text{ml}$) and analyzed immediately on a FACS Calibur flow cytometer supported by CellQuest software (Becton Dickinson). Activated T cells were generated by incubation of 1×10^6 PBMC/ml with anti-mouse IgG magnetic beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) coated at 150 fg per bead with CD3 and CD28 mAb at a bead to cell ratio of 1:1. After 72 h of stimulation cells were compared to resting cells for their binding of FS ($20 \mu\text{g}/1 \times 10^5$ cells). Activation status of T cells was determined by double staining for CD3 and CD25 or CD3 plus CD69. Peripheral blood of chronic lymphocytic leukemia patients ($n=10$) was analyzed by the whole blood method using ADG-Lyse (An der Grub, Kaumberg, Austria). CD19⁺ B lymphocytes from patient samples were immunophenotyped with a panel of diagnostic mAbs (e.g. CD5, CD20, CD23, CD25, CD43, surface Ig, surface Ig-kappa, surface Ig-lambda), and were reacted with IL-2 decorated GFP⁺ FS or control FS (above). After one wash in PBS, cells were re-suspended in 200 μl sheath fluid, and immediately analyzed (15×10^3 cells) by flow cytometry (FACSCalibur, CellQuest software, Becton Dickinson). Lymphocytes were identified by typical forward and right-angle scatter characteristics.

IL-2 sequestration experiments. PBMC (10^5) of healthy volunteers were co-cultured with $\alpha\text{CD3}/\alpha\text{CD28}$ mAb microbeads at a 1:1 ratio and with titrated amounts of IL-2R $\alpha\beta\gamma::\text{GPI}$, IL-2R $\alpha::\text{GPI}$ or CD16::GPI (control) expressing FS (starting with $2 \mu\text{g}/\text{well}$) or medium alone. Cell proliferation was determined after 4 days by [³H]-thymidine uptake. For antigen-specific experiments Art v 1-specific TCR tg T-cells were generated from non-allergic HLA-DR1 positive individuals as described previously (30). TCR tg T-cells (1×10^6 cells/well) were cultured in 24-well plates with VLP (2 $\mu\text{g}/\text{ml}$) decorated with HLA-DRA*0101, HLA-DRB1*0101, Ii::Art v 1₂₅₋₃₄, CD80::GPI, CD86::GPI, CD58::GPI, CD54::GPI, cathepsin S or control plasmid combined as indicated or medium alone for ten days. Proliferation of primary antigen-specific cultures was determined in

replica plates containing 1×10^5 T-cells and corresponding stimuli. After ten days, cells from 24-well plates were harvested, washed and 1×10^5 cells/well were re-stimulated in 96-well flat bottom plates with anti-CD3/CD28 microbeads (2.5×10^4) alone or in the presence of IL-2 (500 U/ml) for four days and proliferation was determined. Cell proliferation was determined after 4 days by [^{3} H]-thymidine uptake.

The binding capacity of IL-2R $\alpha\beta\gamma$:GPI FS was determined with the help of radioactively labeled ^{125} I IL-2 (Perkin Elmer, Boston, MA, USA). For that purpose, constant amounts of ^{125} I IL-2 corresponding to 25 U IL-2 (i.e. 2,5 ng) were incubated at room temperature with titrated amounts of IL-2R $\alpha\beta\gamma$:GPI FS starting from 30 μ g for 1 hour. FS-bound ^{125} I IL-2 was determined (Packard Cobra II gamma-counter, Packard, Meriden, CT, USA) from the retentate upon size exclusion filtration using Amicon Microcon YM-100 filter units (Millipore) according to the manufacturer's recommendations.

Alternatively, 5 ng non-labeled IL-2 (Peprotech, London, UK) was used to stimulate proliferation of HT-2 cells (5×10^3 /well) in the presence of titrated amounts of IL-2R $\alpha\beta\gamma$:GPI FS. Inhibition of HT-2 cell proliferation was taken as a measure to determine the IL-2 binding capacity of IL-2R $\alpha\beta\gamma$:GPI FS compared to co FS. Proliferation is expressed as [^{3} H]-thymidine uptake after 64 hours of co-cultivation.

RESULTS

Generation of fluorescent VLP. To generate VLP with high membrane fluorescence we amplified and fused the coding sequence of Moloney murine leukemia virus (MoMLV) matrix protein (MA) and GFP (**Fig. 1A**). Expression of MA::GFP in 293 cells resulted in pronounced membrane-associated fluorescence (**Fig. 2A**), whereas expression of untagged GFP led to evenly distributed cytoplasmic fluorescence (**Fig. 2B**). MA::GFP expression in 293 cells was approximately 3-fold lower than expression of soluble GFP (**Fig. 2C, D**). As a model ligand for testing specific interactions we coexpressed human interleukin (IL)-2 fused by a poly-glycine spacer to the GPI-anchor acceptor sequence of human CD16b (2) preceded by two CD16b Ig-like domains (**Fig. 1A**). Membrane fractionation experiments with 293 transfectants showed that IL-2::GPI, MA::GFP, the MoMLV capsid proteins and the GPI-anchored, constitutively expressed CD59 molecule were all targeted to the lipid-raft fractions of 293 cell lysates (**Fig. 2E**) while soluble GFP and the non-lipid-raft marker molecule CD147 (31) remained excluded.

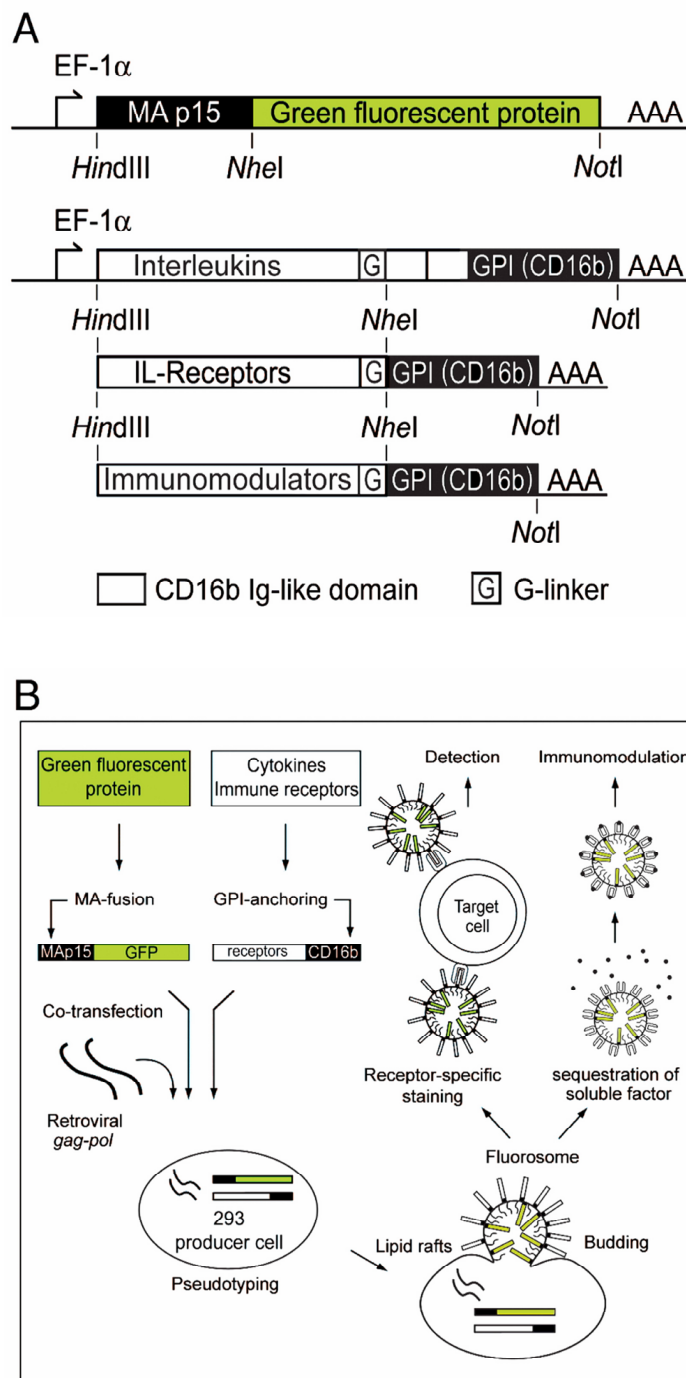


Figure 1. A) *Diagram of expression constructs.* DNA sequences encoding MoMLV matrix protein (MA, p15) were PCR amplified and endowed with flanking Hind III and Nhe I restriction sites. For optimal expression levels, the consensus transcriptional initiation site GCCACC was introduced upstream of the ATG codon. The MA coding region was fused to green fluorescent protein (GFP) sequences previously inserted in the pEAK12 expression vector. Cytokine coding sequences were similarly PCR amplified and prepared for vector insertion. At the 3'-ends of the cytokine cDNAs, the intrinsic stop codons were replaced by a polyglycine linker sequence allowing fusion to a CD16b GPI-anchor acceptor sequence (in the case of interleukins preceded by two CD16b Ig-like domains) previously inserted in the pEAK12 vector. B) *Production scheme for fluorosomes.* FS production is initiated by transfection of MoMLV gag-pol. FS bud from the lipid-raft regions of 293 producer cell membranes. MA::GFP is targeted to the lipid-rafts and consequently to nascent FS by virtue of its fusion to MA. Similarly, the cytokines, cytokine receptors and co-stimulatory molecules fused to the GPI-anchor acceptor sequence of CD16b become enriched in lipid-rafts and FS. After collection and purification from 293 producer cell supernatants, FS can be used to visualize receptor-ligand interactions by standard flow cytometry and microscopy, or, alternatively, for cytokine sequestration.

Binding of IL-2 decorated VLP to IL-2R⁺ target cells. In the next set of experiments we induced secretion of IL-2 decorated fluorescent VLP and asked whether such particles would bind to and stain IL-2R⁺ cells. For that purpose we induced particle formation by transfection (28) of either 293-MA::GFP or 293-GFP stable transfectants with pEAK12_IL-2::GPI and pMD_gag/pol(MoMLV) (production according to **Fig. 1B**).

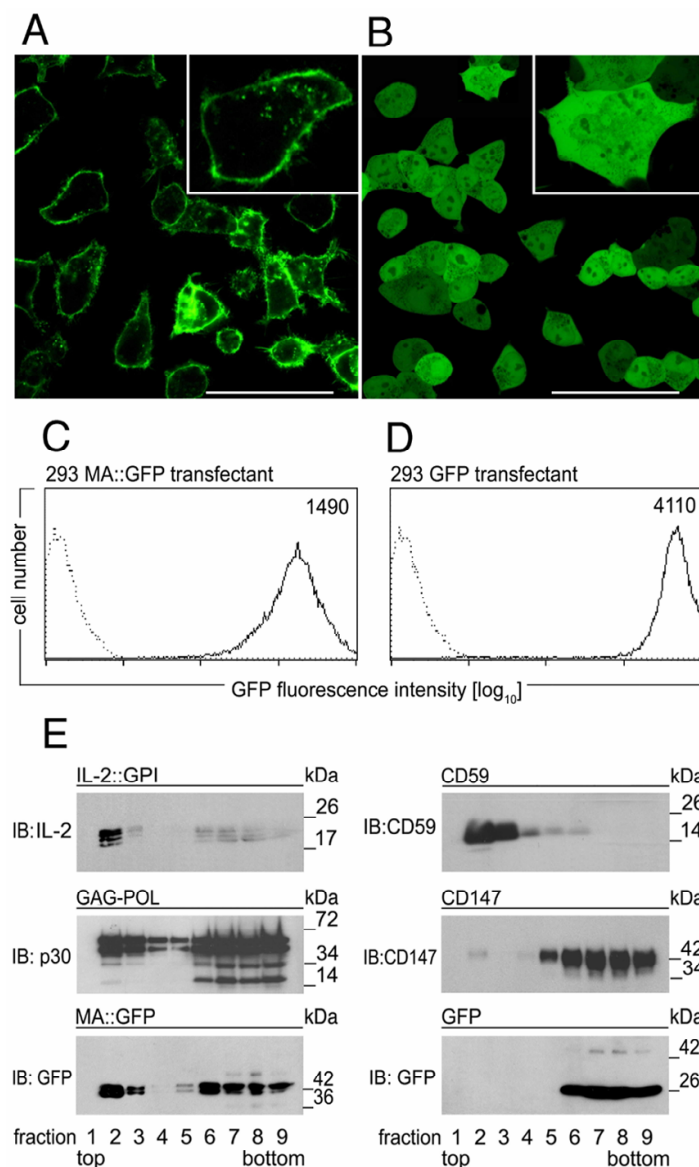


Figure 2. Analysis of 293 producer cells. 293 cells stably transfected with **A**) MA::GFP, or **B**) GFP were seeded in a cell cultivation chamber, grown overnight and analyzed by confocal microscopy (63x/1.40 oil immersion). Pictures (inset: high magnification) show characteristic morphology and fluorescence pattern. Scale bar 50 μ m. Flow cytometric determination of GFP expression levels in 293 producer cells. Overlay histograms show green fluorescence intensity of 293 cells stably expressing **C**) MA::GFP or **D**) GFP (solid lines) compared to native 293 cells (dotted lines) as determined by flow cytometry. Numbers indicate geometric MFI. GPI anchor attachment or fusion to MA targets modified molecules to lipid rafts. **E**) 293 cells transfected with IL-2::GPI, MoMLV gag/pol, MA::GFP or GFP, respectively, were lysed at 4°C in Triton X-100 and fractionated on 5 to 40 % sucrose gradients into nine fractions (top to bottom). Equal amounts of each fraction were resolved by SDS-PAGE, blotted, and probed by immunoblotting (IB) with mAbs specific for IL-2 (all antibodies used are listed in **Table S2**, supplemental material), p30Gag, GFP, CD59 or CD147, respectively. Binding of antibodies was visualized by HRP-conjugated secondary reagents followed by a luminol-based detection reaction. One out of several representative experiments is shown.

Subsequently, the IL-2R⁺ murine T cell line HT-2 (32) was incubated with varying amounts of IL-2::GPI decorated VLP derived from either of the two producer cell lines (1). Binding was analyzed by flow cytometry. Background binding was assessed by comparison to fluorescence resulting from CD16b decorated VLP produced by either of the two cell lines. Binding to > 95 % of HT-2 cells was achieved with 4 μ g (by protein mass) of MA::GFP⁺VLP decorated with IL-2::GPI per 1×10^5 HT-2 cells (**Fig. 3A, D**), with 50 % positive cells obtained with 1 μ g VLP. No binding was observed with control MA::GFP⁺VLP ($p = 0.001$; Mann Whitney (MW) U-test, two-tailed), although similar amounts of VLP were applied (not shown). In marked contrast, IL-2::GPI decorated VLP from 293 cells expressing soluble GFP (GFP⁺VLP) produced significantly weaker signals,

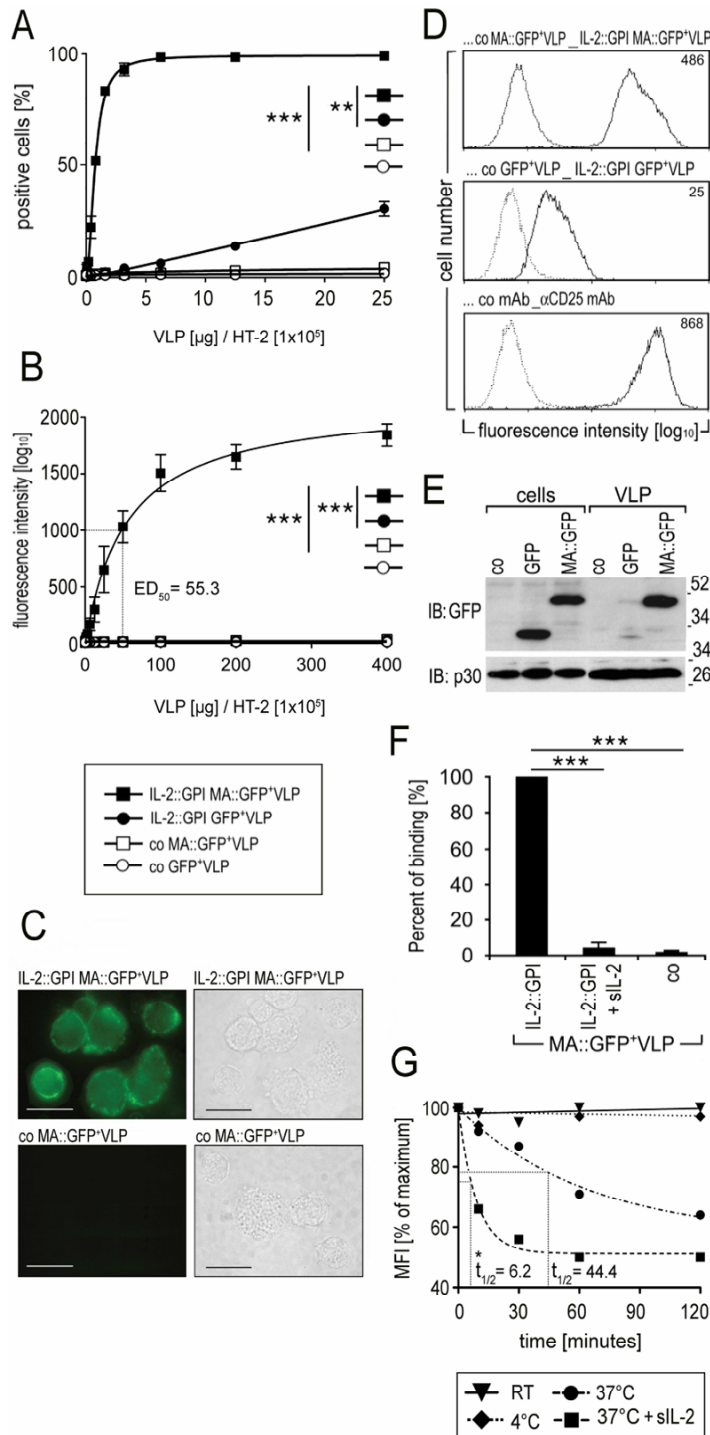


Figure 3. Binding of IL-2 decorated fluorescent VLP to IL-2R⁺ HT-2 cells. Dose-dependent binding. Saturation binding isotherms were generated by reacting two-fold dilutions of the indicated amounts of purified IL-2::GPI MA::GFP⁺VLP (closed squares), control MA::GFP⁺VLP (open squares), IL-2::GPI GFP⁺VLP (closed circles) and control GFP⁺VLP (open circles) with HT-2 cells (starting with 400 μg VLP / 1 × 10⁵ HT-2 cells) followed by flow cytometric analyses. **A)** Percent positive cells and **B)** geometric mean fluorescence intensities (geo MFI) obtained are plotted against the VLP concentrations applied. Native HT-2 cells exhibited a geo MFI of 5 (< 2 % positive cells). ED₅₀ is shown as dotted line. Statistical significance was calculated by the two-tailed Mann Whitney U-test; ** p > 0.01; *** p < 0.001. **C)** VLP-based detection of IL-2R on HT-2 cells by standard microscopy. HT-2 cells were incubated and processed with IL-2::GPI MA::GFP⁺VLP or control MA::GFP⁺VLP as described above. Cell morphology was determined by light microscopy (right), membrane fluorescence by fluorescence microscopy (left). Scale bar 10 μm. **D)** Overlay histograms of HT-2 cells (1 × 10⁵) incubated with 30 μg IL-2::GPI MA::GFP⁺VLP or control MA::GFP⁺VLP (left), IL-2::GPI GFP⁺VLP or control GFP⁺VLP (middle), and anti-CD25 mAb or control mAb (right) are shown. Specific fluorescence (solid lines) is compared to the respective control (dotted lines). Numbers indicate geo MFI values of specific reagents. **E)** Differential targeting of MA::GFP and GFP to VLP. 293 cells were transfected with the indicated expres-

sion plasmids or control plasmid and cell lysates (cells) and corresponding VLP preparations were analyzed by SDS-PAGE under reducing conditions followed by immunoblotting (IB) with a GFP-specific goat antiserum or the p30 Gag specific mAb R187, respectively. Bound antibodies were detected by HRP-conjugated secondary reagents and a luminol-based detection system. Statistical significance was calculated by the paired, two-tailed Student's t-test; *** p < 0.001. **F)** Exogenous IL-2 inhibits binding of IL-2::GPI MA::GFP⁺VLP. Native HT-2 cells (1 × 10⁵) or HT-2 cells pre-incubated with recombinant soluble human IL-2 (5 × 10³ U) were incubated with IL-2::GPI decorated MA::GFP⁺VLP and analyzed by flow cytometry. Results show binding of IL-2::GPI decorated MA::GFP⁺VLP to IL-2 pre-incubated relative to native HT-2 cells (mean + SD). **G)** Temperature dependent dissociation of IL-2::GPI MA::GFP⁺VLP from HT-2 cells. The dissociation of IL-2::GPI MA::GFP⁺VLP from HT-2 cells was monitored after removing excess VLPs and chasing at 4°C, room temperature (RT), 37°C and 37°C in the presence of 4 × 10⁴ U soluble IL-2. MFI was determined and plotted as percentage of maximum, corresponding to the values obtained at start of chase. t_{1/2} denotes dissociation half-life.

with only 30 % positive cells at a concentration of 25 μg ($p = 0.002$; MWU-test, two-tailed). Saturating binding with a geometric mean fluorescence intensity (geo MFI) of 1.842 was reached with 400 μg of IL-2 decorated MA::GFP⁺VLP, compared to a MFI of 38 for IL-2 decorated GFP⁺VLP ($p < 0.001$; MWU-test, two-tailed; **Fig. 3B**), and a MFI 35 for VLP lacking IL-2 ($p < 0.001$; MWU-test, two-tailed). Very similar results showing clear-cut membrane pronounced fluorescence of HT-2 cells preincubated with IL-2::GPI MA::GFP⁺VLP but not control MA::GFP⁺VLP were obtained by fluorescence microscopy (**Fig. 3C**). The reduced fluorescence intensity of VLP bearing soluble GFP compared to MA::GFP was confirmed by GFP-specific immunoblotting (**Fig. 3E**). Specificity of binding reactions was confirmed by pre-incubation of HT-2 cells with 5000 U soluble IL-2, which completely inhibited subsequent binding of IL-2 decorated MA::GFP⁺VLP ($p < 0.001$; Student's t-test, paired, two-tailed; **Fig. 3F**). The interaction of IL-2 decorated MA::GFP⁺VLP with HT-2 cells was remarkably stable at 4°C and room temperature (**Fig. 3G**). At 37°C clear-cut dissociation rates became, however, apparent with a dissociation half-life $t_{1/2} = 44.4$ min in the absence and $t_{1/2} = 6.2$ min in the presence of excess amounts of soluble IL-2 (4×10^4 U) used as competitor (**Fig. 3G**).

For convenience in subsequent exposition we will identify the MA::GFP-expressing VLP as fluorosomes (FS). Importantly, IL-2::GPI FS displayed a high degree of physical stability. Their initial staining activity was reduced by only $8.8 \pm 3.0\%$ and $4.0 \pm 14.4\%$ (geo MFI) upon freezing and re-thawing or storage at 4°C for 3 weeks, respectively (not shown). A rapid decline in staining intensity was evident only upon storage at room temperature.

IL-2::GPI FS discriminate between cells expressing different forms of the IL-2R. To determine whether IL-2::GPI FS are also able to discriminate between different IL-2 receptor subunit combinations (16) we evaluated their binding to respective 293 cell transfectants. **Fig. 4A** and **B** show that similar amounts of IL-2::GPI FS derived from the same production batches bound significantly more strongly to IL-2R $\alpha\beta\gamma$ -transfectants than to IL-2R $\beta\gamma$ -transfectants, and not to control transfected 293 cells ($p < 0.001$; t-test), which mirrors the affinities of IL-2 for the different receptor types (16, 33). In contrast, no binding of control FS to either of the transfectants was evident ($p < 0.001$; t-test).

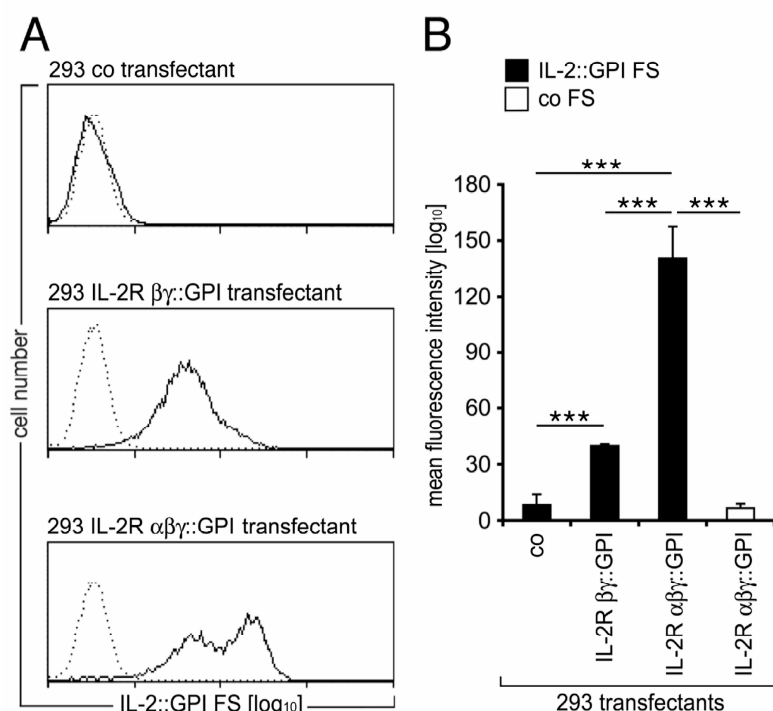


Figure 4. *IL-2::GPI FS discriminate between target cells expressing low- or high-affinity IL-2R.* **A)** Panel shows overlay histograms of 293 cells transfected with control plasmid (upper panel), IL-2R βγ-chain (middle panel) or IL-2R αβγ-chain (lower panel) stained with identical batches of either IL-2::GPI (solid line) or control (dotted line) FS. **B)** Geometric MFI-values of indicated 293 cell transfectants stained with IL-2::GPI (black bars) or control (white bar) FS from three independently performed experiments + SD are shown. Statistical significance was calculated by paired, two-tailed Student's t-test; ns $p > 0.05$; * $p < 0.05$; *** $p < 0.001$.

IL-2::GPI FS identify activated IL-2R⁺ T lymphocytes and B non-Hodgkin lymphoma (B-NHL)-chronic lymphocytic leukemia (CLL) cells. The results above suggest that IL-2::GPI FS could be used to identify distinct leukocyte subpopulations of healthy or diseased individuals. **Fig. 5A** demonstrates that IL-2::GPI FS indeed bound to a small subset of CD3⁺ T lymphocytes in freshly isolated PBMC of healthy individuals, likely representing the CD4⁺CD25⁺ Treg fraction (34). Upon polyclonal activation with CD3 plus CD28 coated microbeads for 48 h, binding of IL-2::GPI FS and CD25 mAb, but not of control FS, to the majority of CD3⁺ T cells was observed (**Fig. 5B**).

In addition to its action on T cells, IL-2 is also an important stimulatory cytokine for B lymphocytes (35, 36). Expression of IL-2R by chronic lymphocytic leukemia (CLL) B cells is one of the distinguishing characteristics of the disease (37-39). IL-2::GPI FS combined with a CD19-APC mAb could clearly identify the IL-2R positive B cell populations in peripheral blood (**Fig. 5C**) and bone marrow (not shown) of CLL patients (11 of 11 patients positive). Compared to the standard staining procedure with CD25 mAbs, somewhat lower overall numbers of positive cells were identified with IL-2::GPI FS ($69,4 \pm 22,6$ % vs. $50,3 \pm 16,4$ %). However, in some patients, the staining intensity obtained with IL-2::GPI FS was identical or even exceeded that obtained with CD25 mAb.

It will be interesting to explore whether the observed differential binding of IL-2::GPI FS to CLL-B cells correlates with disease progression and/or severity (40) and possibly reflects differences in receptor composition.

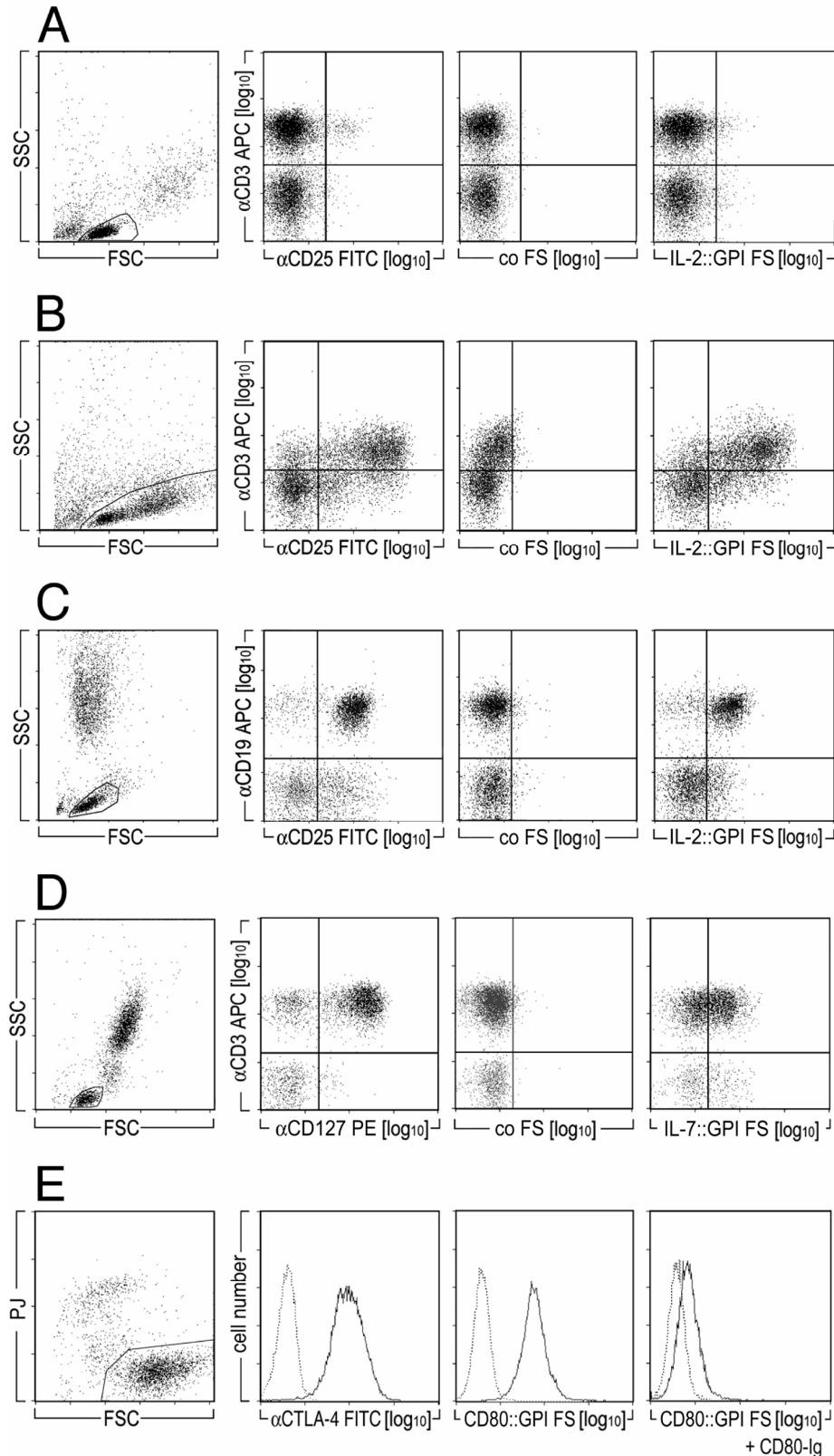


Figure 5. *IL-2::GPI FS identify activated T lymphocytes and CLL B cells.* Two-parameter dot-plot analyses of freshly isolated **A)** or activated (CD3/CD28 coated beads, 48 hours) **B)** PBMC upon incubation with IL-2::GPI FS, control FS, or CD25 FITC-conjugated mAb and counterstained with a CD3 APC-conjugated mAb are shown. Gating for lymphocytes was performed according to typical FSC/SSC-characteristics. Markers were set according to non-binding control mAbs or control FS. Data are representative of three experiments. **C)** Whole blood of a CLL-patient was stained with IL-2::GPI FS, control FS or CD25 FITC-conjugated mAb and counterstained with a CD19 APC-conjugated mAb. Lymphocytes were gated according to typical FSC/SSC characteristics. Markers were set according to staining of non-binding control mAbs or control FS. Data are representative of multiple individuals (n=11). *IL-7::GPI FS or CD80::GPI FS specifically bind to target cells expressing their respective receptors.* **D)** Two-parameter dot-plot analyses of whole blood cells gated on lymphocytes of a representative healthy donor incubated with IL-7::GPI FS, control FS or CD127 PE-conjugated mAb and counterstained with CD3 APC-conjugated mAb are shown. Lymphocytes were gated according to typical FSC/SSC characteristics. Data are representative of four experiments. **E)** Overlay histograms of CTLA-4 transfected BW cells incubated with CD80::GPI FS, control FS or CTLA-4 mAb plus anti-mouse-Ig conjugated to OG are shown (bold lines). Dotted lines show fluorescence obtained with native BW cells. Pre-incubation of CTLA-4 transfected BW cells with soluble CD80-Ig fusion protein (2 µg) was used as specificity control. Data are representative of four experiments.

Generality of the FS approach shown by specific binding of IL-7::GPIFS and CD80::GPI FS to respective target cells. To determine whether the approach can be generalized, we decorated FS with ligands other than IL-2 and investigated their binding characteristics to respective targets. For this we constructed IL-7::GPI (**Fig. 1**) and took advantage of a CD80::GPI construct described previously (1). **Fig. 5D** shows that the IL-7::GPI FS, unlike control FS, specifically bound to resting CD3⁺ T cells ($p < 0.001$; t-test), and binding could be specifically inhibited by addition of exogenous soluble IL-7 (not shown). The IL-7R is expressed as a low ($K_a \sim 1 \times 10^{-6}$ M) and a high ($K_a \sim 5 \times 10^{-9}$ M) affinity receptor on lymphocytes (13). The clearly weaker signal obtained with IL-7::GPI FS as compared to parallel stainings performed with IL-7R α -chain specific mAb might be a reflection of the differential binding of IL-7 to these receptors. Moreover, CD80::GPI FS bound to CTLA-4 transfected (as described in (25)) but not to control transfected BW cells ($p = 0.01$; t-test, **Fig. 5E**). Addition of 2 µg soluble CD80-Ig fusion protein or blocking CD80 mAb 7-480 (not shown) inhibited the binding of CD80::GPI FS to CTLA-4 expressing BW cells to background levels (**Fig. 5E**).

FS probes comprising functional multi-subunit receptors. Conventional labeling approaches typically use monomeric or homomultimeric reagents to identify single epitopes on analyzed cell populations. To establish whether FS probes could be constituted from heteromultimeric reagents we produced FS decorated with various combinations of IL-2R-chains (modified by GPI-anchor acceptor sequences according to

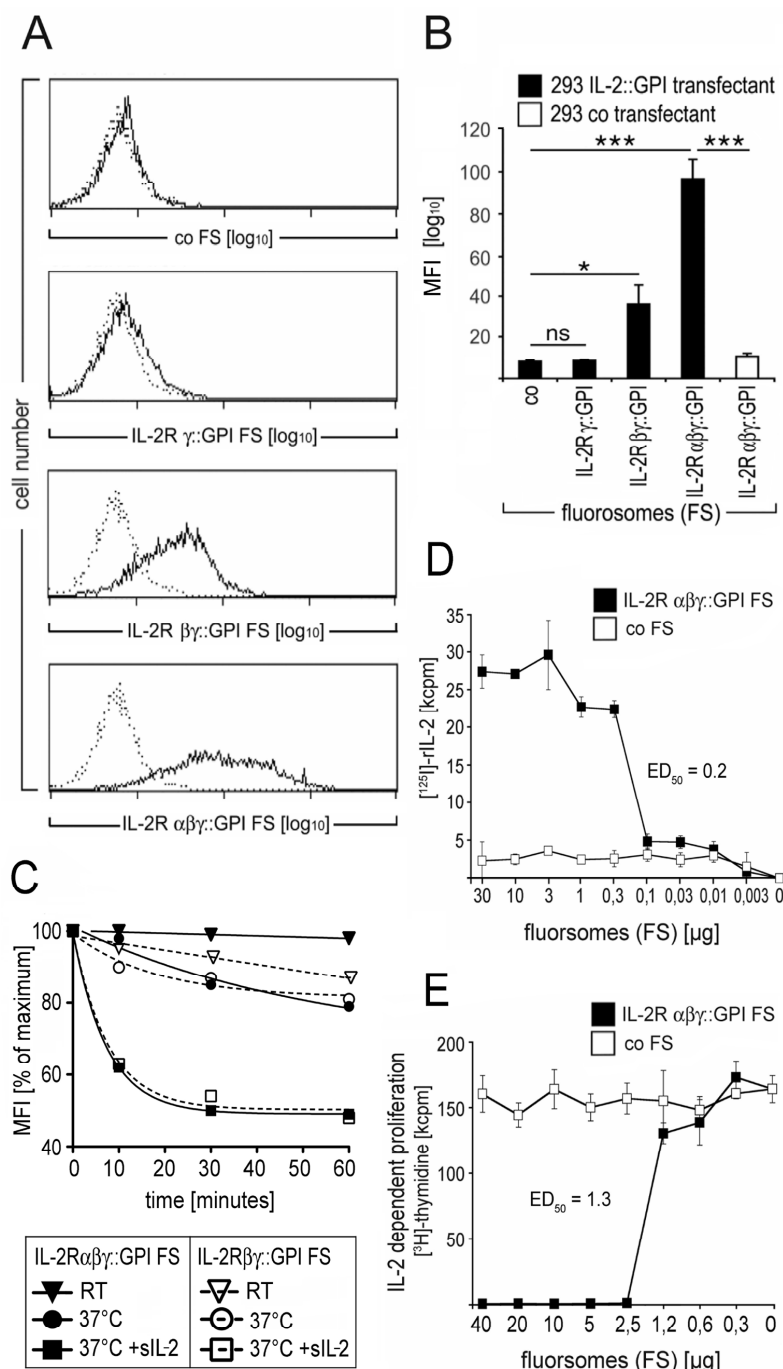


Figure 6. FS decorated with complex combinations of the IL-2R bind to target cells expressing membrane-bound IL-2 and dose-dependently bind soluble IL-2. **A)** Panel shows overlay histograms of 293 cells expressing membrane-bound IL-2::GPI (single cell clone), which were incubated with control FS (upper panel), IL-2R γ-chain::GPI FS (upper-middle panel), IL-2R βγ-chain::GPI FS (lower-middle panel), or IL-2R αβγ-chain::GPI FS (lower panel), respectively (bold lines). Dotted lines represent fluorescence of control transfected 293 cells incubated with the respective FS. **B)** Geometric mean fluorescence values of IL-2::GPI (black bars) or control (white bar) 293 transfectants stained with the indicated FS from three independently performed experiments + SD are shown. Statistical significance was calculated by the paired, two-tailed Student's t-test; ns $p > 0.05$; * $p < 0.05$; *** $p < 0.001$. **C)** Dissociation kinetics of IL-R FS from IL-2⁺ target cells. The dissociation of IL-2R αβγ::GPI FS and IL-2R βγ::GPI from IL-2⁺ 293 cells was monitored after removing excess FS and chasing at room temperature (RT), 37°C and 37°C in the presence of 4×10^4 U soluble IL-2. MFI was determined and plotted as percentage of maximum, corresponding to the values obtained at start of chase. $t_{1/2}$ denotes dissociation half-life. **D)** Binding capacity of IL-2R αβγ::GPI FS. Constant amounts of ¹²⁵I IL-2 (2.5 ng) were incubated with titrated

amounts of IL-2R αβγ::GPI FS or control FS for 1 h at RT. Subsequently, FS were collected by size exclusion filtration and FS-bound ¹²⁵I IL-2 determined by γ-counting. **E)** Constant amounts of soluble IL-2 (5 ng) were incubated with titrated amounts of IL-2R αβγ::GPI FS or control FS and added to 5×10^3 HT-2 cells cultured in 96-well plates. After 48 hours [³H]-thymidine was added, cells were harvested 16 hours later and cell proliferation was determined. Data show mean values ± SD of triplicate cultures ($n = 2$). ED₅₀ denotes effective dose 50 percent.

Fig. 1) and analyzed whether they would specifically bind to target cells expressing membrane-anchored IL-2. **Fig. 6A** and **B** show that this is the case. FS expressing the high-affinity IL-2R (IL-2R αβγ-chain) showed strong and highly significant binding to the

IL-2⁺ target cells when compared to control FS ($p < 0.001$; t-test). FS decorated with the low-affinity IL-2R (IL-2R $\beta\gamma$ -chain) revealed intermediate but still significant binding ($p = 0.03$; t-test), whereas IL-2R γ -chain decorated FS similar to the control FS displayed no significant binding ($p = 0.2$; t-test). In contrast to the staining intensities no significant differences in dissociation rates between IL-2R $\alpha\beta\gamma$::GPI FS and IL-2R $\beta\gamma$::GPI FS in the presence or absence of excess amounts of soluble IL-2 (4×10^4 U) used as competitor were detectable (**Fig. 6C**). To obtain a more accurate estimate also of the valency of FS we have resorted to binding studies using IL-2R FS and ¹²⁵I-labeled IL-2 as the ligand. In parallel experiments the IL-2 binding capacity of IL-2R $\alpha\beta\gamma$::GPI FS was tested in functional assays using HT-2 cell proliferation as the indicator system. The experiments with ¹²⁵I-labeled IL-2 revealed that 1 μ g of IL-2R $\alpha\beta\gamma$::GPI FS bind 6,25 ng IL-2 (**Fig. 6D**). Assuming a mean mass for C-type retrovirus particles of 6.5×10^7 Dalton (41) this would indicate that one IL-2R $\alpha\beta\gamma$::GPI FS has approximately 26 binding sites for ¹²⁵I-IL-2. The functional experiments revealed a 3.25-fold lower IL-2 binding capacity, i.e. approximately 8 sites per FS (**Fig. 6E**). However, these experiments had a much longer duration than the binding assays (64 h versus 1 h) and had to be carried out at 37°C (as opposed to room temperature for binding assays) presumably causing considerably higher off-rates (**Fig. 3G**) making more IL-2 bio-available. In fact, the obtained data are well in line with our previous findings, in which we detected up to 15 IL-2 molecules on one VLP as determined by immuno-gold staining followed by electron microscopy (2).

IL-2 $\alpha\beta\gamma$ -chain::GPI FS neutralize soluble IL-2 and thereby induce novel functional T cell programs. We also examined whether IL-2R decorated FS are capable of neutralizing soluble IL-2. Recently, IL-2 neutralization/depletion has been shown to be one of the effector mechanisms of regulatory T cells (Treg) (20). To determine whether FS decorated with IL-2R components are able to interfere with T cell activation programs we stimulated PBMC of healthy individuals with microbeads coated with CD3 and CD28 mAbs in the presence of IL-2R $\alpha\beta\gamma$ -chain::GPI⁺FS or control FS. In marked contrast to control FS, high concentrations of IL-2R $\alpha\beta\gamma$ -chain::GPI⁺FS almost completely abrogated T cell proliferation. (**Fig. 7A**). Next we asked whether a similar inhibition would take place after antigen-specific T cell stimulation (3) and whether IL-2 deprivation from T

cells receiving a full activation stimulus would render T cells unresponsive during secondary stimulation provided by α CD3 plus α CD28 coated microbeads. **Fig. 7B** shows

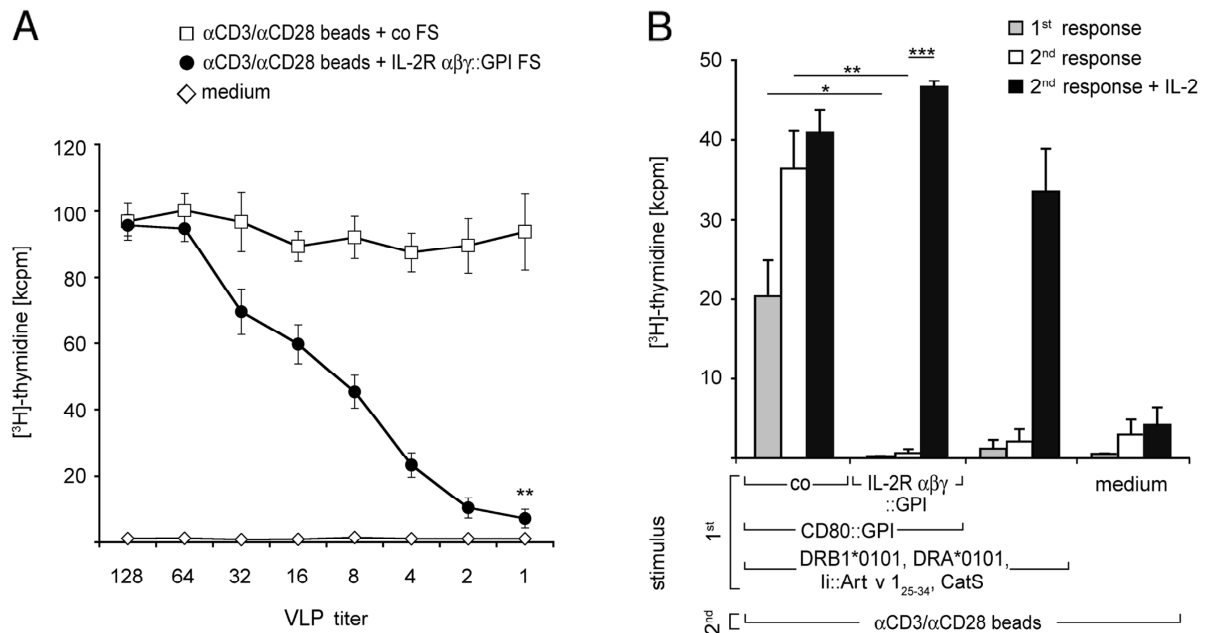


Figure 7. A) *IL-2R $\alpha\beta\gamma$::GPI⁺FS neutralize proliferation of polyclonally activated PB T cells.* PBMC (10^5) were co-cultured with α CD3/ α CD28 mAb substituted microbeads and varying amounts of IL-2R $\alpha\beta\gamma$::GPI or CD16::GPI (control) expressing FS (starting with 2 μ g/well) or medium alone. Cell proliferation was determined after 4 days by [^{3} H]-thymidine uptake. Data show mean values $\pm$ SD of triplicate cultures ($n = 3$); ** $p < 0.01$; Student's t-test, paired, 2-tailed, comparing cultures supplemented with IL-2R $\alpha\beta\gamma$::GPI FS to co FS. **B)** *Induction of antigen-specific unresponsiveness by FS.* Art v 1 allergen-specific V α 17/ $\nu\beta$ 18 TCR tg PB T cells (10^5) were cultured with the indicated primary stimuli and proliferation was measured by [^{3} H]-thymidine incorporation for the last 12 h of a 72 h incubation (gray bars). Proliferation of T cells obtained on culture with indicated stimuli for 10 days, washed, and re-cultured with α CD3/ α CD28 mAb substituted microbeads in the absence (white bars) or presence (black bars) of exogenous IL-2 (500 U/mL) is demonstrated. Data show one representative experiment ($n = 3$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; Student's t-test, paired, 2-tailed. kcpm, Kilo counts per minute.

that allergen-specific T cells react with VLP expressing Art v 1 in the context of HLA-DR1 and CD80 both in primary and secondary T cell stimulation assays. In contrast, the presence of IL-2R $\alpha\beta\gamma$ -chain::GPI FS during primary stimulation not only significantly inhibited the primary, antigen-specific but also completely abrogated secondary polyclonal T cell responses (**Fig. 7B**, $p < 0.05$ and $p < 0.01$, respectively, t-test). T cells did, however, survive the FS-mediated IL-2 deprivation, since they proliferated well upon addition of exogenous IL-2 (500 U/ml, $p < 0.001$, t-test). Similar results were obtained with antigen-specific T cells receiving only antigen stimulation during the primary stimulation, which is in line with our previous results (3), and served as a control. Thus, immune-receptor

decorated FS bind and sequester their respective ligands, shaping the evolution of responses in T cells exposed to these agents.

DISCUSSION

In this work we introduce a technology for the facile preparation of analytical reagents that have high ligand affinity and functional activity. In addition to their specific binding to cell surface receptors, FS are also able to sequester soluble ligands, such as IL-2, with high efficiency, and thereby induce altered cellular programs in T cells that have been activated via their T cell antigen receptor (TCR) and CD28 molecules.

Production of FS relies on the preferential incorporation of the MoMLV MA::GFP fusion protein as fluorochrome into the lipid rafts of 293 producer cells. Co-expressed immune-receptors/ligands of choice can be targeted to FS by attachment of a GPI-anchor. FS can be produced quickly - within four days - and in large amounts - $9.8 \pm 4.5 \mu\text{g}$ per milliliter of producer cell supernatant (not shown). The production procedure relies on transfection of the easy-to-handle 293 embryonic kidney epithelial cell line (42) with an optimized Ca_2PO_4 -precipitation method (28). The strong fluorochrome accumulation in VLP allows direct visualization of fluorescence without the need for secondary reagents, which makes the FS staining procedure quick, typically completed within one hour. In this study, normal and malignant lymphocyte subsets could be identified by IL-2⁺FS. FS containing membrane-targeted GFP were significantly more efficient than FS containing soluble GFP. The generality of the approach was demonstrated by successful application of ligands other than IL-2, i.e. IL-7 and the type I, or single pass, transmembrane T cell surface expressed co-stimulatory molecule CD80.

In contrast to labeled antibodies usually identifying single receptor subunits, FS directly measure receptor/ligand interactions between natural interaction partners, and thereby offer the possibility of a more accurate assessment of activity of the targeted receptor. Interleukin receptor components such as the IL-2R α -chain (CD25) and IL-7R α -chain (CD127) are differentially represented among human T cell subsets (43), and it has been reported that the CD4⁺CD25^{hi}CD127^{lo} (IL-2R α ^{hi}IL-7R α ^{lo}) phenotype identifies natural Treg cells (43). Determination of IL-2R composition in Treg cells based on both IL-2⁺FS and CD25 monoclonal antibodies might lead to new insights into the biology of Treg function, a possibility suggested by a recent report that cell types expressing partial IL-2R with regulatory function can be identified (22). It should also be interesting to explore the possibility of establishing multi-color FS for double or triple staining purposes in the

future. The recently described ‘fruit’-colors developed by Tsien and co-workers and relying on *Discosoma sp.* appear to be interesting candidates in this respect (44).

Another potential application of FS is the characterization of so far unknown receptor-ligand interactions. As shown here, the VLP platform can accommodate multisubunit-ligands or receptors, which are otherwise not easy to express in the appropriate configuration. The membrane environment of VLP should also permit the convenient expression of type III integral membrane proteins, which pass the plasma membrane two to seven times (45) and are associated with lipid rafts (46, 47), for staining purposes and ligand screens. The multivalent nature of FS as such might facilitate the detection of low-affinity interactions between monomeric receptor-ligand pairs.

Reagents that can neutralize biological activity are frequently useful and antibodies can often be found with this capacity, although antibodies rarely achieve the extremely high binding affinity exhibited by natural receptors (48). These studies have shown that IL-2R $\alpha\beta\gamma$ -chain::GPI⁺FS very effectively sequester IL-2 from T cell cultures upon polyclonal and antigen-specific activation. This culminates in a nearly complete inhibition of T cell proliferation. Similar results have been obtained with non-fluorescent IL-2R $\alpha\beta\gamma$ -chain⁺VLP (not shown). T cells exposed to these FS were unresponsive when restimulated in secondary cultures. The state of unresponsiveness could, however, be overcome by the addition of exogenous IL-2. This seems to be of special interest, since Treg cells have been shown to exert their regulatory function on activated effector CD4⁺ T cells - among other mechanisms - by binding and neutralizing of IL-2 and other common γ -chain (γ_c , CD132) associated cytokines (20). Our results indicate that i) IL-2R $\alpha\beta\gamma$ -chain::GPI FS not only bind but also functionally neutralize soluble IL-2; ii) IL-2R $\alpha\beta\gamma$ -chain::GPI FS have functional capabilities usually associated with regulatory cells and iii) the regulatory function of FS is independent of classical transmembrane signaling events, since the intracellular and transmembrane regions of the individual IL-2R chains have been exchanged by GPI anchor acceptor sequences. An advantage of the VLP platform - over other forms of binding reagents - is their ability to accommodate several different functionally active molecules at a time, which potentially allows complex regulatory instructions to be imparted to a given cell by a single type of particle. Because FS are

functionally inert otherwise (compared to intact cells), the effects of their administration to target populations are more easily interpreted.

We here describe for the first time fluorescent VLP decorated with biologically active mono- and multi-subunit immune-receptors of choice and the basic application of such *fluorosomes* to visualize and functionally block immune-receptor/ligand interactions. Due to their simple generation and broad applicability, FS might open new avenues in various fields of basic immunological research as well as for diagnostic purposes.

SUPPLEMENTARY DATA

Table S1. List of primers used in this paper

Primer	Sequence ^a	RE site
GFP for	5'-CCCGCGGCTAGCGTGAGCAAGGGCGAGGAG-3'	Nhe I
GFP rev	5'-GCGCCCGCGGCCGCTTTACTTGTACAGCTCGTCCATG-3'	Not I
IL-2 for	5'-CGCGGGAAGCTTGCCACCATGTACAGGATG-CAACTCCTG-3'	Hind III
IL-2 rev	5'-CCCGCGGCTAGCGCCGCCGCCAGTCAGTGTTGAGATGATGC-3'	Nhe I
IL-2R α for	5'-CGCGGGAAGCTTGCCACCATGGATTCATACCTGCTGATGTGGGGA-3'	Hind III
IL-2R α rev	5'-CCCGCGGCTAGCGCCGCCGCCCTGGTACTCTGTTGTAAATATG-3'	Nhe I
IL-2R β for	5'-CGCGGGAAGCTTGCCACCATGGCGGCCCTGCTCTGTC-3'	Hind III
IL-2R β rev	5'-CCCGCGGCTAGCGCCGCCGCCGGTGTCTTCCCAAG-3'	Nhe I
IL-2R γ for	5'-CGCGGGAAGCTTGCCACCATGTTGAAGCCATCATTACCATTACATCCCTC	Hind III
IL-2R γ rev	5'-CCCGCGGCTAGCGCCGCCGCCATTCTCTTTTGAAGTATTGCTCC-3'	Nhe I
IL-7 for	5'-CGCGGGAAGCTTGCCACCATGTTCCATGTTTCTTTTAGG-3'	Hind III
IL-7 rev	5'-CCCGCGGCTAGCGCCGCCGCCGTGTTCTTTAGTGCCCATC-3'	Nhe I
MLV MA for	5'-CGCGGGAAGCTTGCCACCATGGGCCAGACTGTTACCACT-3'	Hind III
MLV MA rev	5'-GCGCCCGCTAGCGGATCGAGGCGGGGTGCG-3'	Nhe I

^a The underlined regions indicate the restriction enzyme (RE) sites

Table S2. List of mAbs, primary and secondary antisera used.

Specificity	Clone name	Species	conjugated to	Source
mAbs				
CD3	OKT3	mouse	-	eBioscience, San Diego, CA
CD3	UCHT1	mouse	PE ^a	An der Grub, Kaumberg, Austria
CD3	S4.1	mouse	APC	Invitrogen (Caltag), Carlsbad, CA
CD19	SJ25-C1	mouse	APC	Invitrogen (Caltag), Carlsbad, CA
CD25	2A3	mouse	FITC	BD-Pharmingen, San Diego, CA
CD28	28.2	mouse	-	BD-Pharmingen, San Diego, CA
CD59	MEM-43/5	mouse	-	Exbio, Praha, Czech Republic
CD80	MEM-233	mouse	-	Exbio, Praha, Czech Republic
CD80	7-480	mouse	-	Dr. Majdic, Inst. Immunol, Vienna, Austria
CD122	27302	mouse	FITC	RD, Minneapolis, MN
CD127	R34.4	mouse	PE	Beckman-Coulter (Immunotech), Fullerton, CA
CD132	TUGh4	mouse	PE	BD-Pharmingen, San Diego, CA
CD147	MEM-M6/6	mouse	-	Exbio, Praha, Czech Republic
CIAP	VIAP	mouse	FITC	Dr. Majdic, Inst. Immunol, Vienna, Austria
CTLA-4	BN-13	mouse	-	BD-Pharmingen, San Diego, CA
IL-2	MQ1-17H12	rat	PE	BD-Pharmingen, San Diego, CA
IL-2	IL-2.1F10	mouse	-	Dr. Majdic, Inst. Immunol, Vienna, Austria
IL-7	BVD10-40F6	rat	-	BD-Pharmingen, San Diego, CA
p30 Gag MoMLV	R187	rat	-	ATCC, Raritan, NY
control	4H1-A7/VIAP	mouse	FITC/PE	An der Grub, Kaumberg, Austria
primary antisera				
GFP	-	rabbit	-	antibodies-online, Aachen, Germany
secondary antisera				
Mouse Ig	-	goat	Oregon Green	Invitrogen (Molecular Probes), Eugene, OR
Mouse Ig	-	goat	HRP	DAKO, Glostrup, Denmark
Rabbit Ig	-	goat	HRP	DAKO, Glostrup, Denmark
Rat Ig	-	goat	Oregon Green	Invitrogen (Molecular Probes), Eugene, OR
Rat Ig	-	rabbit	HRP	DAKO, Glostrup, Denmark

^a Abbreviations: PE, phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanine; OG, Oregon green; HRP, horse radish peroxidase.

Supplement Figure

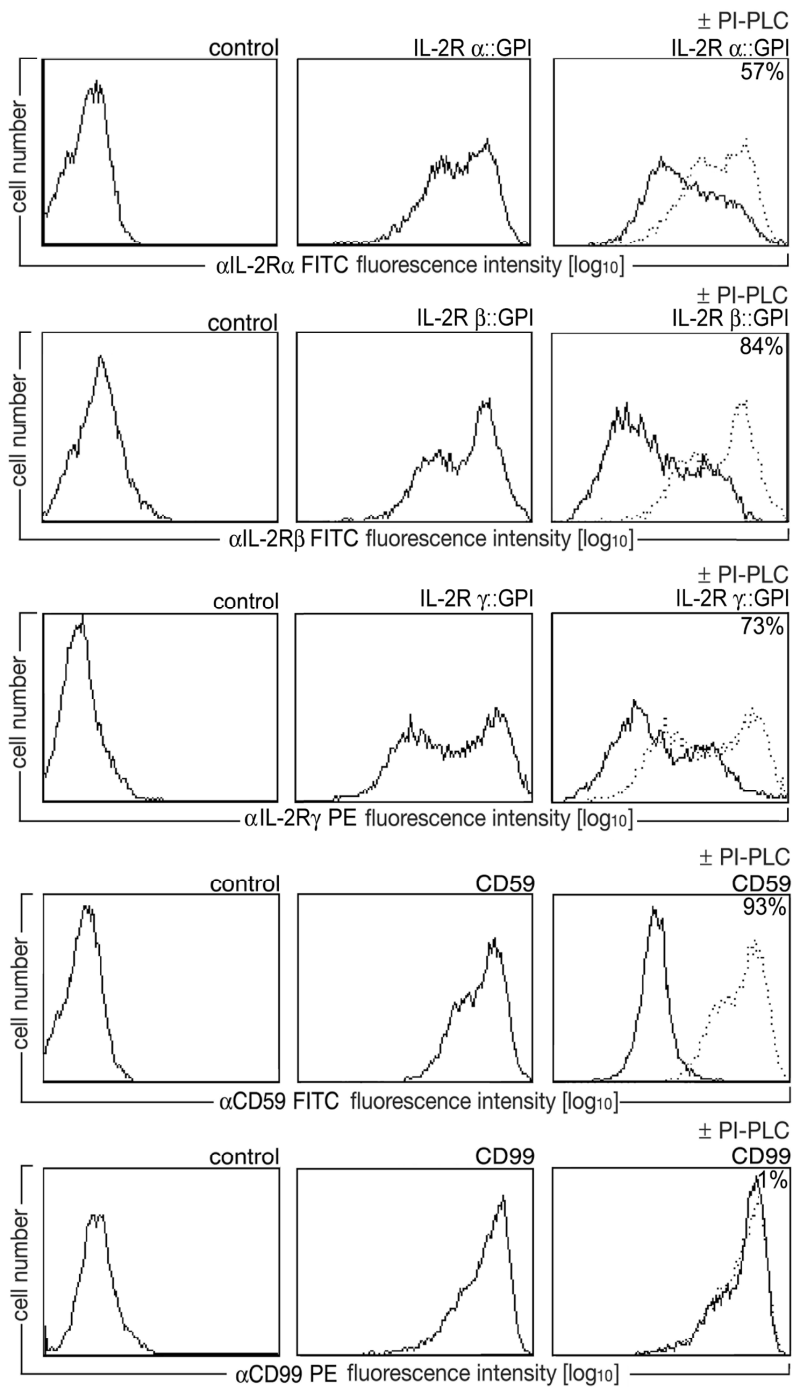


Figure S1. Expression levels of GPI-anchored interleukin-2 receptor α -, β - and γ -chains on 293 producer cells. 293 cells were transfected by the calcium phosphate precipitation method with IL-2R α ::GPI, IL-2R β ::GPI or IL-2R γ ::GPI. Seventy-two hours later, transfectants were harvested, and aliquots (1×10^7) were treated with 100 mU/ml of PI-PLC at 37°C for 2 h. Subsequently, both PI-PLC-treated and non-treated cells (5×10^5) were incubated with the IL-2R α chain-specific mAb 2A3, the IL-2R β chain-specific mAb 27302 and the IL-2R γ chain-specific mAb TUGh4. In addition, the constitutively expressed molecules CD59 (GPI anchored) and CD99 (type I transmembrane molecule) were probed with mAbs MEM-43 and 3B2/TA8, respectively. The resulting cellular fluorescence was analyzed by flow cytometry. Dead cells were excluded from the analyses by means of propidium iodide staining. Overlay histograms show PI-PLC-treated (solid lines) and untreated cells (dotted lines). The percent values indicate the degree of release upon PI-PLC treatment. The data shown are representative of three independently performed experiments.

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Chapter 6

Bet v 1-specific TCR/Foxp3 transgenic T-cells suppress Bet v 1-specific

T-cell effector function in an activation dependent manner

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ABSTRACT

Background: T-regulatory cells (Treg) establish and maintain tolerance to self-antigens and many foreign antigens, e.g. allergens, by suppressing effector T-cell proliferation and function. We have previously shown that human T-cell receptor (TCR) $\alpha\beta$ -chains specific for allergen-derived epitopes confer allergen specificity upon peripheral blood (PB) T-cells of allergic and non-allergic individuals.

Objective: To study the feasibility of generating allergen-specific human Treg by retroviral transduction of a transcription unit encoding Foxp3 and allergen-specific TCR $\alpha\beta$ -chains.

Methods: cDNAs encoding the α - and β -chains of a Bet v 1₁₄₂₋₁₅₃-specific TCR (TRAV6/TRBV20) 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 PB T-cells were tested for expression of transgenes, Treg phenotype and regulatory capacity towards allergen-specific effector T-cells.

Results: Transduced T-cells displayed a Treg phenotype with clear-cut up-regulation of CD25, CD39 and CTLA-4. The transduced cells were hyporesponsive in cytokine production and secretion and, like nTreg, did not proliferate following antigen-specific or antigen-mimetic stimulation. However, proliferation was inducible upon exposure to exogenous IL-2. In co-culture experiments, TRAV6⁺TRBV20⁺Foxp3⁺ transgenic (tg) T-cells, unlike Foxp3⁺ single tg T-cells or nTreg, highly-significantly suppressed T-cell cytokine production and proliferation of corresponding allergen-specific effector T-cells in an allergen-specific, dose-dependent manner.

Conclusions: We demonstrate a transgenic approach to engineer human allergen-specific Treg that exert their regulatory function in an activation-dependent manner. Customized Treg might become useful for tolerance-induction therapies in individuals suffering from allergic and other immune-mediated diseases.

INTRODUCTION

Naturally occurring $CD4^+CD25^{high}Foxp3^+$ regulatory T-cells (Treg) orchestrate and maintain tolerance to self-antigens by suppressing effector T-cell proliferation and function. Depletion of Treg in mice has been shown to lead to overt multiorgan autoimmunity,¹ consistent with widely held views that Treg restrain the evolution of autoimmune disorders,² play a key role in tolerance against allo-antigens after transplantation,³ and modulate allergic immune responses.⁴ Specific immunotherapy (SIT) has been shown to increase regulatory T-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 function and elevate their numbers have gained increased attractiveness as therapeutic options in recent years. Strategies applied include expansion of Treg lines from sorted $CD4^+CD25^+$ populations by stimulation with different forms of antigen-presenting cells and cytokines¹³⁻¹⁶ or the *de novo* generation of ‘inducible’ Treg (iTreg) from $CD4^+CD25^-$ T-cells by TCR mediated stimulation in the presence of TGF- β ^{17, 18} or rapamycin¹⁹.

A recent key finding was that the retro- or lentiviral introduction of Foxp3 was sufficient to generate artificial Treg.^{20, 21} Such tg Treg display the typical features of naturally-occurring Treg – hyporesponsiveness and suppression of effector cells – and can be cultivated and expanded *in vitro* over prolonged periods of time. However, these tg Treg 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 only be found at a low frequency.

The antigen specificity of T-cells is determined by the T-cell antigen receptor (TCR). In fact, transfer of TCR $\alpha\beta$ genes²² has become a convenient tool for transferring designated antigen specificities to PB T-cells. While 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 1₂₅₋₃₆)²⁷ and birch pollen (Bet v 1₁₄₂₋₁₅₃), respectively, in order to study signal requirements

and plasticity of allergen-specific T-cells. These TCRs were chosen because both allergens, their respective HLA restriction elements and their major epitopes are well-characterized,^{28, 29} and allergy to mugwort and birch-pollen are among the main causes of pollinosis in the Northern Hemisphere.³⁰ In fact, more than 95% of birch pollen-allergic individuals elicit an IgE antibody response against Bet v 1, the major birch pollen allergen.³¹

It is well-established that Treg-mediated suppression of effector T-cells (Teff) is based on a functional program that requires the activation of the Treg via the TCR or agonistic ligands to CD3^{1, 32, 33}. We aimed to exploit these principles to generate customized, allergen-specific, human Treg by co-introduction of Foxp3 to provide the regulatory program in concert with TCR $\alpha\beta$ chains to provide allergen-specificity. To ensure reliable and balanced expression of all three transgenes (i.e. the TCR $\alpha\beta$ -chains and Foxp3) a multicistronic vector-based approach was applied³⁴ which allows to express several genes from a single expression unit. The cDNAs encoding TRAV6 and TRBV20 (IMGT nomenclature³⁵) and Foxp3 were linked via picornaviral 2A sequences³⁴ upstream of an IRES-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.

RESULTS

Generation of multicistrons encoding allergen-specific TCR α/β and Foxp3 cDNAs

The cDNA sequences for a TCR (TRAV6/TRBV20) specific for the Bet v 1₁₄₂₋₁₅₃ peptide of the major birch pollen allergen Bet v 1 in the context of HLA-DRB1*07:01 (Neunkirchner et al., manuscript in preparation) were linked via a picornaviral P2A sequence to a bicistronic expression construct and expressed from the retroviral vector pMMP (Fig 1). Additionally, the cDNA encoding Foxp3, was linked to the TCR construct using a picornaviral T2A element. The 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.

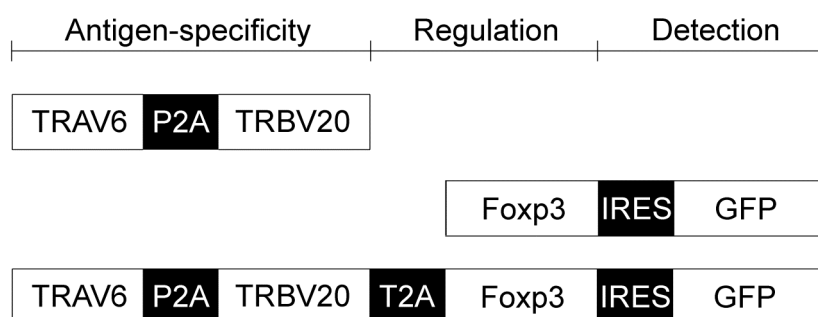


FIG 1. Scheme of multicistronic retroviral expression constructs used in this study. Function of the respective genes is indicated; open boxes, genes; closed boxes, interposed picornaviral 2A or IRES elements

Transduced T-cells express the introduced transgenes

The majority of TRAV6/TRBV20-transduced effector T-cells ($72 \pm 14\%$; Fig 2, A) and of the GFP^{high} fraction of TRAV6/TRBV20/Foxp3-transduced T-cells ($82 \pm 12\%$; Fig 2, B) expressed the tg TCR on their surface as detected by staining for the TRBV20-chain, while the GFP^{high} fraction of control-transduced ($7 \pm 1\%$) or Foxp3-transduced ($8 \pm 2\%$) 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 ($78 \pm 3\%$) and TRAV6/TRBV20/Foxp3-transduced T-cells ($89 \pm 8\%$) (Fig 2, B) whereas control-transduced T-cells exhibited negligible ($1 \pm 1\%$) expression. Importantly, the expression in the non-transduced GFP^{neg} fractions of both TRBV20 and Foxp3 were low and compatible with endogenous levels (Fig 2, B).

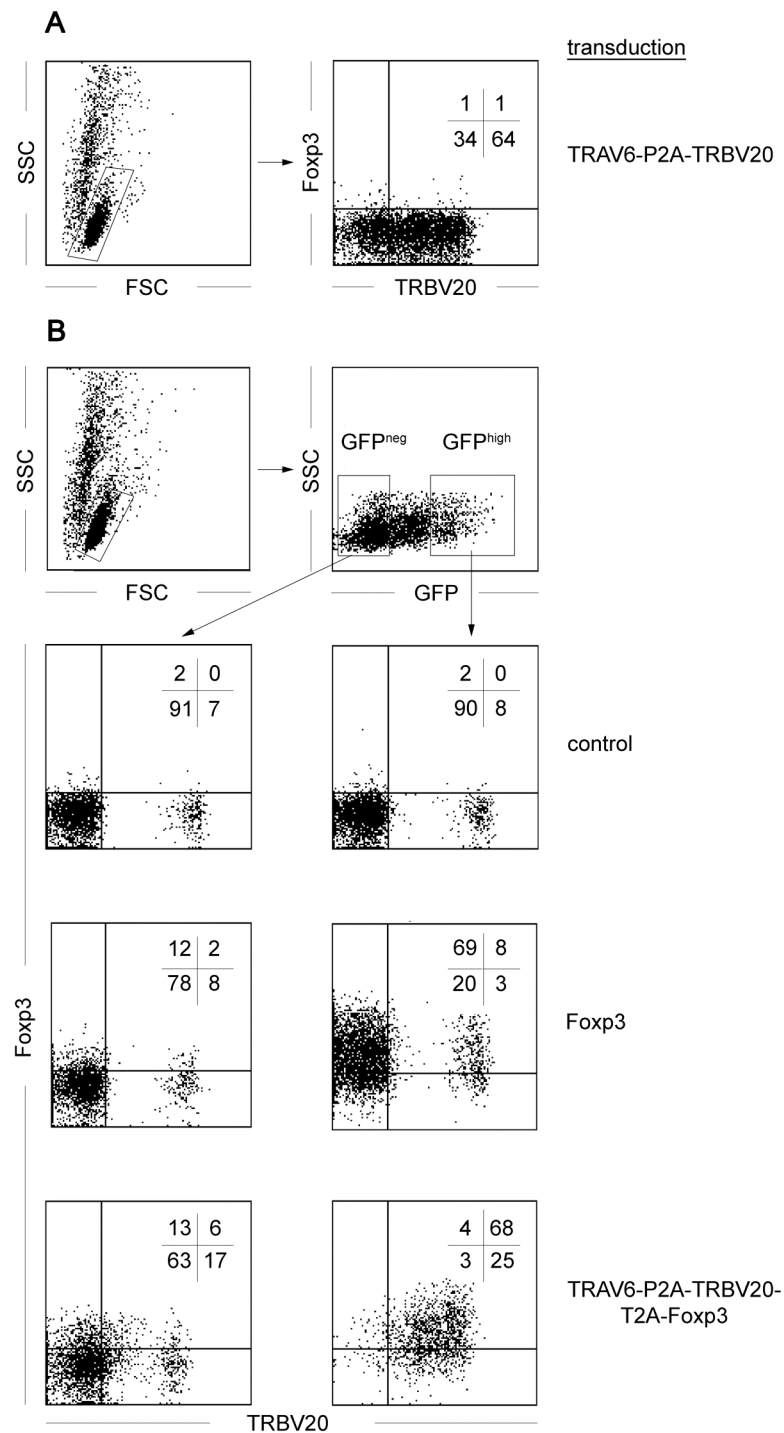


FIG 2. Transgene expression-levels and gating strategy for retrovirally transduced PB T-cells. **(A)** TRAV6⁺TRBV20⁺ T-cells **(B)** TRAV6⁺TRBV20⁺Foxp3⁺, Foxp3⁺ and control T-cells. In B GFP expression served as additional gating marker. Surface TCR expression (TRBV20) and intracellular Foxp3 expression are displayed as two parameter dot-plots. Numbers indicate cell-percentages in respective quadrants

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 $CTLA-4$ ($CD152$)⁴² compared to the $CD3^+CD4^+GFP^{neg}$ non-transduced fraction (Fig 3) or to TRAV6/TRBV20-transduced or mock-transduced PB T-cells (not shown). Furthermore, and in accordance with a regulatory phenotype, we also observed a discrete down-regulation of $CD127$ expression⁴³ on the tg Treg. No changes in $GITR$ expression levels⁴⁴ were observed. In all further functional experiments the flow-cytometrically sorted GFP^{high} fractions of Foxp3-transduced T-cells were used and termed $Foxp3^+$ or $TRAV6^+TRBV20^+Foxp3^+$, respectively.

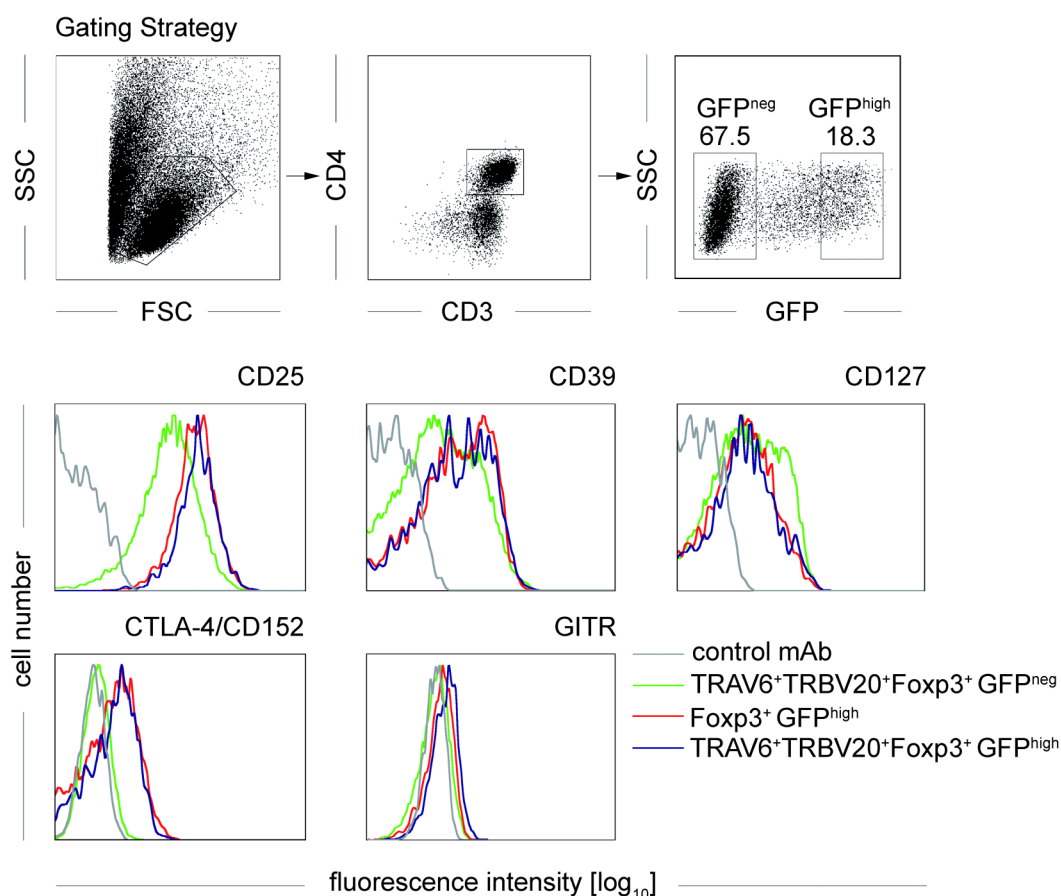


FIG 3. Immunophenotype of transduced T-cells. PB T-cells gated for $CD3^+CD4^+GFP^{neg}$ or $CD3^+CD4^+GFP^{high}$ subsets and analyzed for expression of indicated Treg markers. Blue line, GFP^{high} subset from TRAV6/TRBV20/Foxp3-transduced T-cells, specific mAb; Red line, GFP^{high} subset from Foxp3-transduced T-cells, specific mAb; Green line, GFP^{neg} subset, specific mAb; Grey line, TRAV6/TRBV20/Foxp3-transduced T-cells, control mAb.

TRAV6⁺TRBV20⁺Foxp3⁺ T-cells are hyporesponsive to antigen-specific and polyclonal activation

Treg are hyporesponsive to TCR-dependent stimulation.¹ Upon stimulation with aAPC expressing HLA-DRA*01:01, HLA-DRB1*07:01, CatS, Ii-Bet v 1₁₄₂₋₁₅₃ and CD80, TRAV6⁺TRBV20⁺ T-cells proliferated vigorously while TRAV6⁺TRBV20⁺Foxp3⁺ T-cells were significantly less responsive ($p < 0.001$) (Fig 4, A). Importantly, aAPC-dependent stimulation was highly specific, as control-transduced, Foxp3 single-transgenic T-cells devoid of the Bet v 1-specific TCR expression and nTreg showed only minimal proliferation ($p < 0.001$) (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 while TRAV6⁺TRBV20⁺Foxp3⁺, Foxp3 single-tg T-cells or nTreg did not proliferate ($p < 0.001$) (Fig 4, B.) In eight experiments using cells from four non-related, non-allergic donors, proliferation of TRAV6⁺TRBV20⁺Foxp3⁺ T-cells was reduced by $73.7 \pm 17.3\%$ ($p < 0.001$) and $92.9 \pm 6.6\%$ ($p < 0.001$) respectively compared to 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 ($p < 0.001$) and TRAV6⁺TRBV20⁺Foxp3⁺ T-cells ($p < 0.001$) in both settings,^{16, 20} while freshly isolated nTreg required a polyclonal stimulus plus exogenous IL-2 to proliferate ($p < 0.001$) (Fig 4).

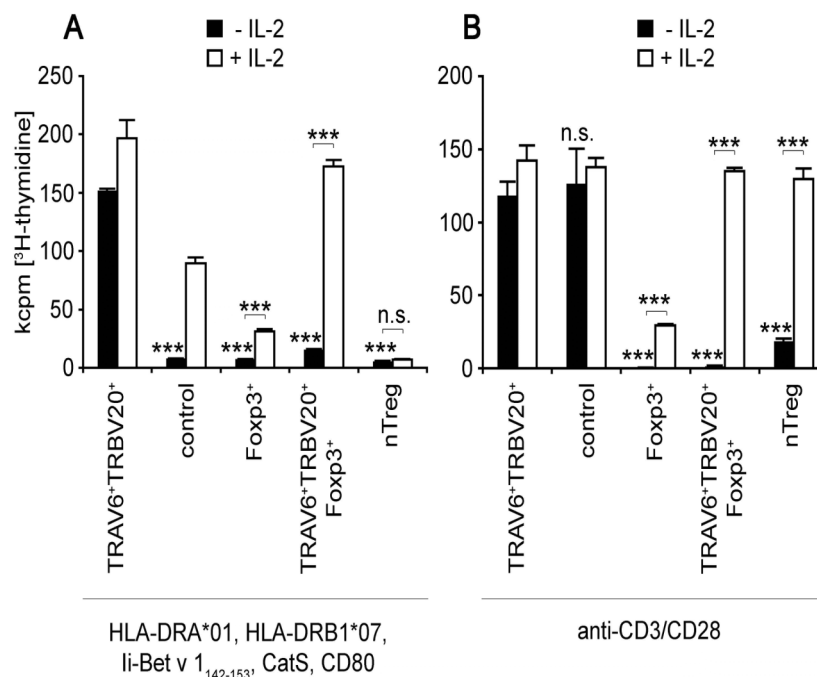


FIG 4. Foxp3-transduced T-cells are hyporesponsive. Proliferation of retrovirally transduced PB T-cells or nTreg stimulated with (A) aAPC expressing indicated molecules or (B) anti-CD3/CD28 coated microbeads in the absence (black bars) or presence (white bars) of recombinant hIL-2 (250 U/ml). Mean + SD of triplicates; control, empty IRES-GFP vector; asterisks indicate significance compared to TRAV6⁺TRBV20⁺ effector T-cells (- IL-2). n.s., not significant; ***, $p < 0.001$.

Similarly, both Foxp3 transgenic T-cell populations secreted only minimal levels of TNF- α , IFN- γ , IL-2 and IL-13, compared to TRAV6⁺TRBV20⁺ or control-transduced T-cells following allergen-specific or polyclonal activation (Table I). In accordance with previous reports on Foxp3-transduced T-cells, both TRAV6⁺TRBV20⁺Foxp3⁺ and Foxp3⁺ T-cells did not secrete significant amounts of IL-10 upon stimulation.^{16, 20} Moreover, no significant differences in TGF- β secretion levels between the different cell populations studied were observed (not shown).

Table I. Cytokine secretion by retrovirally transduced T-cells after 24 h

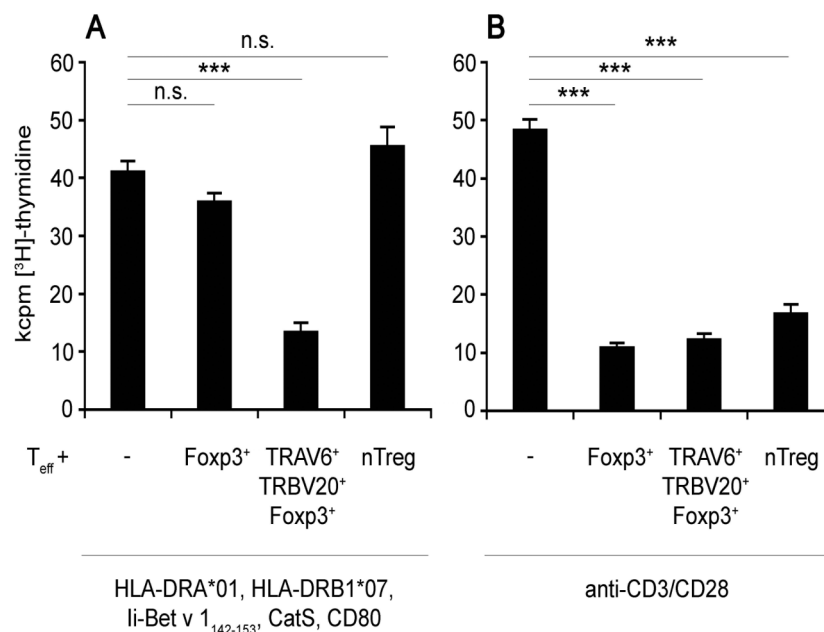
		TRAV6 ⁺ / TRBV20 ⁺	control	Foxp3 ⁺	TRAV6 ⁺ / TRBV20 ⁺ /Foxp3 ⁺
aAPC	TNF- α	94 $\pm$ 29 ^a	<1	<1	1 $\pm$ 1
	IFN- γ	1138 $\pm$ 733	<1	<1	<1
	IL-2	226 $\pm$ 86	5 $\pm$ 2	<1	<1
	IL-4	<13	<13	<13	<13
	IL-10	<1	<1	<1	<1
	IL-13	143 $\pm$ 58	11 $\pm$ 9	7 $\pm$ 6	14 $\pm$ 12
anti-CD3/CD28	TNF- α	73 $\pm$ 16	61 $\pm$ 32	5 $\pm$ 3	3 $\pm$ 2
	IFN- γ	968 $\pm$ 598	537 $\pm$ 428	3 $\pm$ 3	3 $\pm$ 2
	IL-2	35 $\pm$ 12	36 $\pm$ 12	<1	5 $\pm$ 2
	IL-4	<13	<13	<13	<13
	IL-10	<1	<1	<1	<1
	IL-13	67 $\pm$ 29	68 $\pm$ 23	4 $\pm$ 2	2 $\pm$ 1

^a pg/ml; control, IRES-GFP empty vector; data show mean values $\pm$ SD (n=3)

TRAV6⁺TRBV20⁺Foxp3⁺ suppress allergen-specific transgenic effector cell proliferation in response to allergen-specific stimulation

To assess the suppressive capacity of the tg Treg, TRAV6⁺TRBV20⁺ tg effector T-cells were stimulated in the absence or presence of TRAV6⁺TRBV20⁺Foxp3⁺ T-cells, Foxp3⁺ T-cells or CD4⁺CD25⁺ nTreg from the same donor. Upon Bet v 1-specific stimulation using aAPC, TRAV6⁺TRBV20⁺Foxp3⁺ T-cells strongly suppressed allergen-specific effector T-cell proliferation while Foxp3⁺ T-cells and nTreg expressing a non-cognate 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 (Supplementary data Fig E1). Using anti-CD3/CD28 coated microbeads as polyclonal stimulus, TRAV6⁺TRBV20⁺Foxp3⁺, Foxp3⁺ T-cells and nTreg suppressed effector T-cell proliferation to a similar degree (Fig. 5, B). In eight experiments using PB T-cells from four non-allergic donors allergen-specific activation in the presence of TRAV6⁺TRBV20⁺Foxp3⁺ T-cells elicited $67.3 \pm 15.1\%$ suppression of effector T-cell proliferation compared to $19.9 \pm 11.8\%$ suppression by Foxp3⁺ T-cells ($p < 0.001$) and $2.4 \pm 11.2\%$ by nTreg ($p < 0.001$). In response to polyclonal activation TRAV6⁺TRBV20⁺Foxp3⁺ T-cells, Foxp3⁺ T-cells and nTreg showed a similar degree of suppression of effector cell proliferation ($71.6 \pm 15.5\%$, $p < 0.001$, $74 \pm 15.8\%$, $p < 0.001$ and $65.0 \pm 2.9\%$, $p < 0.001$). The obtained results were similar irrespective whether magnetic beads-purified CD4⁺CD25⁻ T-cells (purity >95%) or bulk PB T-cells were used as the starting population (not shown).

**FIG 5.**

TRAV6/TRBV20/Foxp3-transduced T-cells unlike Foxp3-transduced T-cells suppress Bet v 1-specific effector T-cells upon allergen-specific activation. Proliferation of TRAV6⁺TRBV20⁺ tg effector T-cells (T_{eff}) cultured in the absence or presence of Foxp3⁺, TRAV6⁺TRBV20⁺Foxp3⁺ T-cells or nTreg at a 1:1 ratio upon stimulation with (A) Bet v 1-presenting aAPC or (B) anti-CD3/CD28 coated microbeads. Data show means + SD from triplicates.

Similarly, proliferation of eFluor670 CPD-labeled effector T-cells was suppressed by TRAV6⁺TRBV20⁺Foxp3⁺ T-cells but not by Foxp3⁺ T-cells or nTreg upon Bet v 1-specific activation (Fig 5, C). Moreover, upon 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).

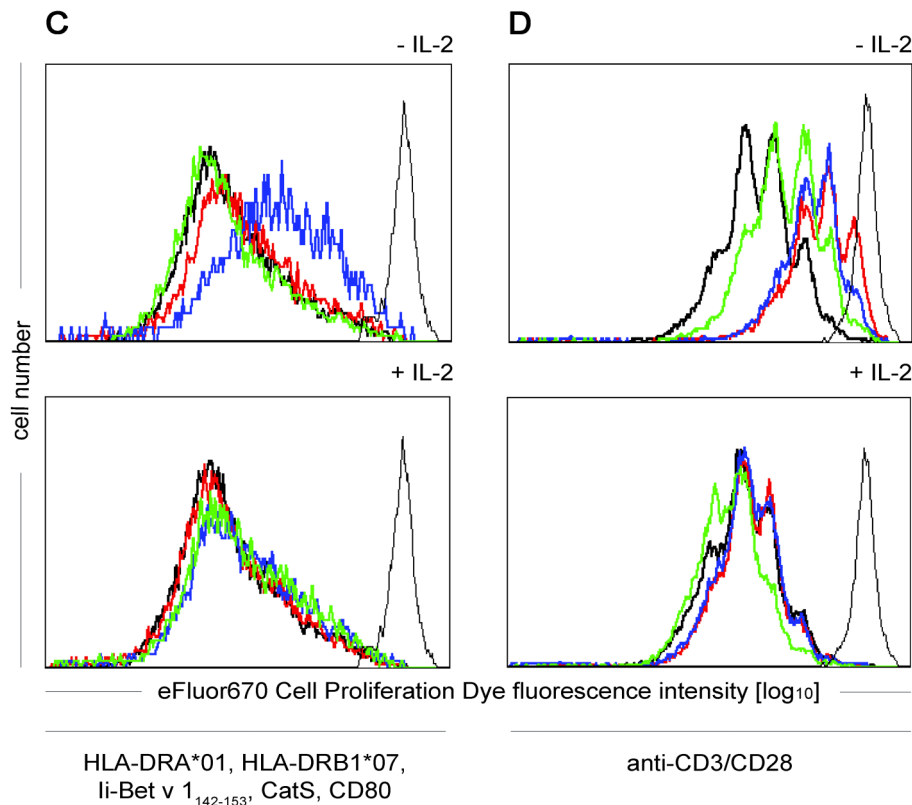


FIG 5. Flow cytometric analyses of eFluor 670 CPD-labeled T_{eff} in the absence (black line) or presence of $Foxp3^+$ (red line), $TRAV6^+TRBV20^+$ $Foxp3^+$ (blue line) T-cells or nTreg (green line) at a 1:1 ratio upon stimulation with (C) Bet v 1-presenting aAPC or (D) anti-CD3/CD28 coated microbeads in the absence (upper panel) or presence (lower panel) of exogenous IL-2 (250 U/ml). n.s., not significant; ***, $p < 0.001$.

TRAV6⁺TRBV20⁺Foxp3⁺ T-cells efficiently suppress cytokine production of tg Bet v 1-specific effector T-cells

Upon allergen-specific activation, significantly decreased levels of the cytokines $TNF-\alpha$, $IFN-\gamma$, IL-2 and IL-13 (range of inhibition: 72-95%) were found in supernatants of co-cultures of $TRAV6^+TRBV20^+$ effector T-cells with $TRAV6^+TRBV20^+Foxp3^+$ regulatory cells (Fig 6, A). Co-culture with $Foxp3^+$ Treg showed a much less pronounced inhibition of cytokine secretion (range 32-55%) while nTreg 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 non-fluorescent effector T-cells and GFP^+ tg Treg cells or CFSE-labeled nTreg allowed a strong and significant reduction of $TNF-\alpha$, $IFN-\gamma$, IL-2 and IL-13 production to be detected in the effector T-cell population (GFP^- , non-fluorescent). Thus, activated tg Treg have the capacity to directly and highly significantly inhibit cytokine production by effector cells.

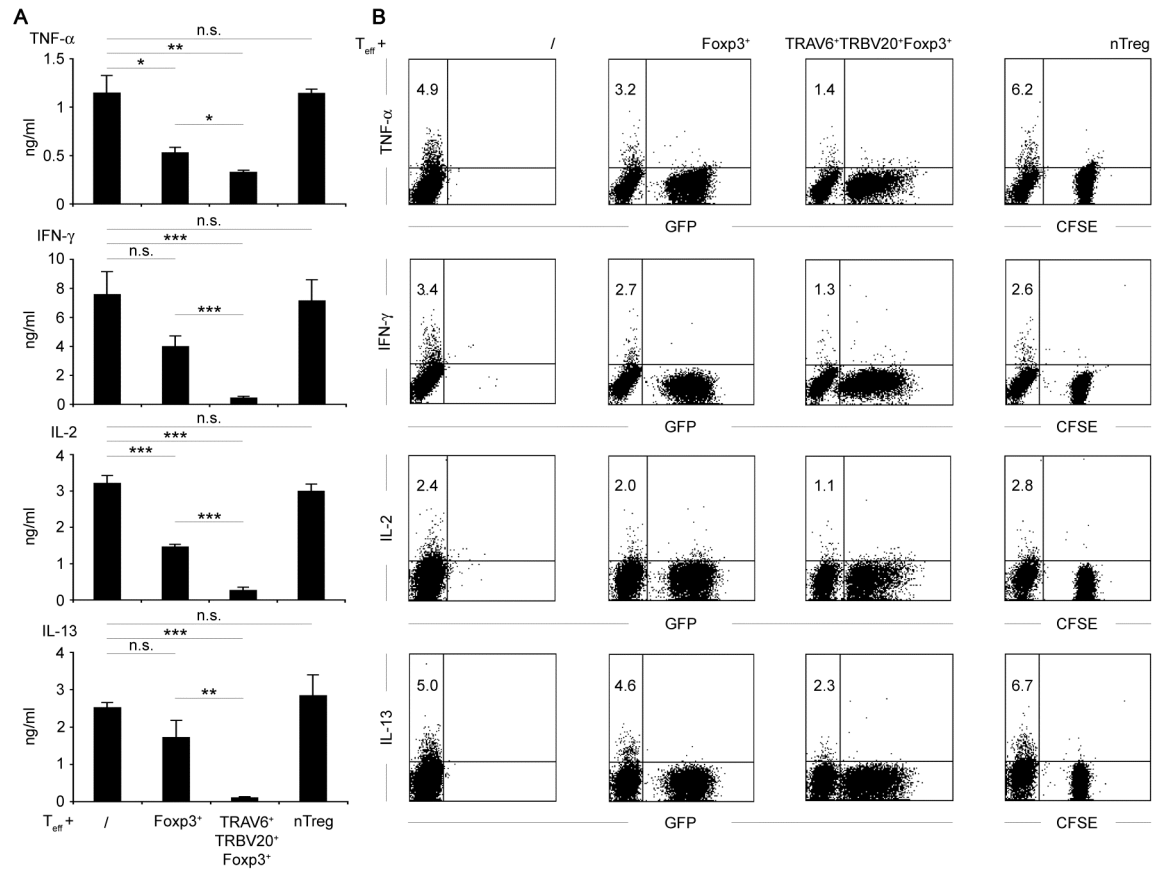


FIG 6. Suppression of cytokine secretion of effector T-cells by engineered Treg. **(A)** Cytokine levels from cell-culture supernatants collected after 24 h (TNF- α , IL-2) or 48 h (IFN- γ , IL-13). **(B)** Intracellular staining of indicated cytokines. Numbers indicate percentage of cytokine positive cells among GFP^{neg} (effector) cells. One representative experiment out of several is shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

DISCUSSION

We show that co-introduction of human TCR $\alpha\beta$ -chains of predefined allergen specificity in concert with human Foxp3, the master regulator of Treg, into human PB T-cells generates tg allergen-specific Treg. These cells show the characteristic phenotype and functional properties of regulatory T-cells, i.e. 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. While 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 has not, to date, been explored. This may be due to the fact that, although TCR transfer technologies have been 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., manuscript in preparation) 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 upon allergen-specific and polyclonal stimulation. Importantly, Foxp3 single-tg T-cells, which express their endogenous, non-cognate TCR, as well as CD4⁺CD25⁺ nTreg from the respective donors showed only weak regulatory capacity in the allergen-specific test-system but were able to suppress tg effector T-cell proliferation to a similar degree as TRAV6⁺TRBV20⁺Foxp3⁺ T-cells upon polyclonal stimulation. This observation indicates that suppression by allergen-specific Foxp3 tg T-cells is - for the most part - an activation-dependent process and that signaling via the tg 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 in perfect agreement with Treg studies in the murine system, showing that Treg exert their suppressive function only upon TCR/CD3 engagement. ¹ However, once appropriately activated, both antigen-specific and non-specific Treg show equal suppressive capacity.

Allergen-specific tg Treg could become potent, highly specific immunotherapeutic tools. One could envision to re-infuse Treg engineered from autologous T-cells of severely affected individuals by TCR/Foxp3 transduction. Upon seasonal exposure to birch pollen,

transferred Treg would become specifically triggered and start to exert their regulatory program leading to tolerance against the immunodominant epitope of Bet v 1, i.e. Bet v 1₁₄₂₋₁₅₃.

Will such an epitope/mono-specific therapeutic tool benefit poly-sensitized individuals suffering from multiple allergies?

Larché and co-workers have demonstrated that tolerization against one epitope of an allergen induces also tolerance to adjacent epitopes, a phenomenon termed ‘linked suppression’.⁴⁶ Whether a similar mechanism might also be induced by epitope/mono-specific tg Treg remains to be determined. In favor of this proposal, in a collagen-induced arthritis model, a B47 TCR tg Treg recognizing a non-collagen autoantigen induced remission of the collagen-induced disease.⁴⁷

Although allergen-specific for the most part, the regulatory potential of Foxp3 tg T-cells was not entirely dependent on TCR-mediated activation. This might be due to ‘bystander’ effects of activated effector T-cells secreting IL-2 and/or providing cellular contact and thus supporting initial activation of co-localized Treg.⁴⁸ Both possible mechanisms will be the focus of future studies. Transgenic Treg directly modulated cytokine production which may be the main mechanism of suppression rather than cytokine consumption.⁴⁵ Like nTreg, Foxp3 tg T-cells are responsive to IL-2 (Fig 4), which should facilitate expansion and longevity *in vivo*. The observation that the tg Treg 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.⁴⁹

In conclusion, given the well-documented relation between impaired Treg function and allergy¹¹ and the fact that Treg play an important role in the efficacy of SIT⁵⁻¹⁰, our approach could add a novel tool to the still poorly endowed therapeutic armamentarium for treatment of allergies. While our studies suggest that nTreg and Foxp3 tg Treg 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 upon 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.

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.

SUPPORTING INFORMATION

Table E1. List of primers

Primer	Sequence ^a	RE site
Foxp3 for	5'- CGCGGGA <u>AAGCTT</u> GCCACCATGCCCACCCCAGGCC -3'	<i>Hind</i> III
Foxp3 rev	5'- CGCGGGG <u>CGGCCGCTT</u> AGGGGCCAGGTGTAGGG -3'	<i>Not</i> I
Foxp3 E1 rev	5'- CGTGGGCATCCACCGTTGACAGCTGCAGCTGCGATGG -3'	
Foxp3 E3 for	5'- CCATCGCAGCTGCAGCTGTCAACGGTGGATGCCACG -3'	
TRAV6 for	5'- CGCGGGA <u>AAGCTT</u> GCCACCATGGAGTCATTCCTGGGAGGTGT -3'	<i>Hind</i> III
TRBV rev	5'- CCCGCGG <u>CGGCCGCTT</u> AGAAATCCTTTCTCTTGACCATGGC -3'	<i>Not</i> I
P2A-TRAV6 rev	5'- GTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGATC CGCTGGACCACAGCCGACG -3'	
P2A-TRBV20 for	5'- GCCACGAACCTTCTCTGTAAAGCAAGCAGGAGACGTGGAAG AAAACCCCGGTCCTATGCTGCTGCTTCTGCTGCTTCTG -3'	
T2A-TCRBV rev	5'- GTCACGCATGTTAGCAGACTTCCTCTGCCCTCTCCGGATCCGAA ATCCTTTCTCTTGACCATGGCCAT -3'	
T2A-Foxp3 for	5'- GAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAA TCCTGGCCCAATGCCCAACCCCAGGCCTG -3'	

^a The underlined regions indicate the restriction enzyme (RE) sites

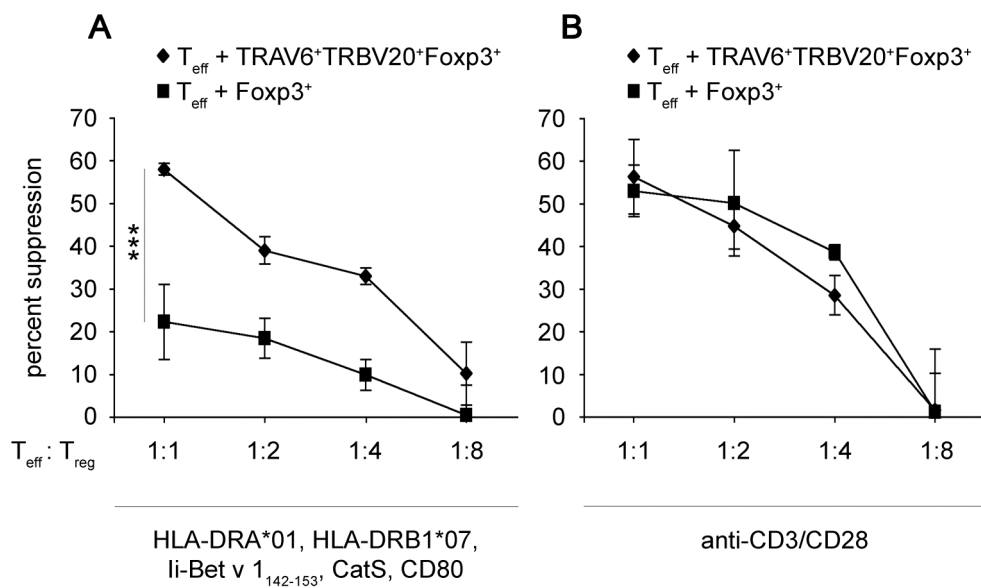


FIG E1. Percent suppression of titrated amounts of indicated transgenic Treg co-cultured with T_{eff} upon stimulation with (A) Bet v 1-presenting aAPC or (B) anti-CD3/CD28 coated microbeads. n.s., not significant; *, $p < 0.05$; ***, $p < 0.001$.

MATERIALS AND METHODS

Molecular cloning and generation of multicistronic vectors

The Foxp3 cDNA was amplified from a human T-cell cDNA library (primer sequences Supplemental Table I). Multicistronic constructs were generated via 2-step overlapping PCR using P2A and T2A sequences.³⁴ and the Bet v 1₁₄₂₋₁₅₃-specific DRB1*07:01-restricted TCR constructs (TRAV6/TRBV20) (Neunkirchner et al., manuscript in preparation). All constructs were digested with *Hind* III and *Not* I and ligated into either the retroviral pMMP³⁶ vector or the pMMP-IRES-GFP vector.

Cell lines and primary cells

The HEK-293 cell line (human embryonic kidney cells) were cultured as described.^{36, 37} PBMC were isolated from healthy HLA-DR7⁺ volunteers in compliance with the ethics-committee of the Medical University of Vienna. CD4⁺CD25⁺ nTreg 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 aAPC, 293 cells were transfected with 5µg each of pEAK12.HLA-DRA*01:01, pEAK12.HLA-DRB1*07:01, pcDNA.Ii::Bet v 1₁₄₂₋₁₅₃, pcDNA.CatS 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 peripheral blood T-cells

CD4⁺CD25⁻ or PB-T cells (5 x 10⁶/well) were stimulated in 6-well flat bottom plates with 5 x 10⁶ anti-CD3/CD28 coated microbeads (Dynabeads, Invitrogen, Camarillo, CA) 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 described⁴⁰ using the mAbs indicated in Supplementary Table II (20 µg/ml). Foxp3 was detected by the Foxp3 Staining Set (eBioscience, San Jose, CA). Intracellular cytokine production was analyzed after pre-treatment for 6 h with Golgi-Stop (1:1500; Becton Dickinson, Palo Alto, CA) using 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 (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, London, UK) for 72 h, pulsed with [methyl-³H] thymidine (1 µCi per well, Perkin Elmer, Boston, MA) and processed as described.³⁶ To test the suppressive capability of tg Treg, 5×10^4 TRAV6⁺TRBV20⁺ effector T-cells were co-cultured with flow cytometrically-sorted TRAV6⁺TRBV20⁺Foxp3⁺, Foxp3⁺ T-cells or CD4⁺CD25⁺ nTreg and stimulated with aAPC or anti-CD3/CD28 coated microbeads and processed as above. Similarly, TRAV6⁺TRBV20⁺ effector T-cells were labeled with the far-red cell proliferation dye eFluor 670 (eBioscience; 1,5µM), co-cultured with tg Treg or CFSE-labeled nTreg (Invitrogen; 1 µM) and analyzed by flow cytometry after 96 h.

Statistical analysis

For multiple group comparisons ANOVA followed by Bonferroni correction was performed (SPSS, IBM, Chicago, IL). Two-group comparisons were performed using the Student's t-test or the Mann-Whitney U-test. Data represent mean values $\pm$ SD. Statistically significant values are denoted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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ABBREVIATIONS

APC: antigen presenting cell; aAPC: artificial antigen presenting cell; CatS: Cathepsin S; CD: cluster of differentiation; CFSE: carboxyfluorescein succinimidyl ester; CPD: eFluor 670 cell proliferation dye; CTLA-4: cytotoxic T-lymphocyte antigen-4; Foxp3: forkhead box P3; GFP: green fluorescent protein; HLA: human leukocyte antigen; Ii: invariant chain; IL: interleukin; IRES: internal ribosomal entry site; kcpm: kilo counts per minute; PB: peripheral blood; SD: standard deviation; TCR: T-cell receptor; tg: transgenic; Treg: T regulatory cell; nTreg: naturally occurring T regulatory cells

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Conclusion and Synopsis

Although a broad range of symptomatic and therapeutic strategies for the treatment of allergic disorders are currently in use, severely affected patients still pose a considerable problem in medical care. In this respect the exact understanding of the pathophysiological mechanisms operative in allergy is clearly needed and thus remains a field of interest in basic research. To the day *in vivo* studies of respiratory allergy mostly rely on mouse models using the hen egg derived ovalbumin as model allergens¹⁻⁵. While these studies have significantly helped to elucidate mechanisms of allergy and potential treatment strategies, they do not entirely reflect the actual situation in respiratory allergy. Ovalbumin, which belongs to the serpin protein family⁶ and represents a major food allergen⁷, considerably differs in its source, glycosylation pattern and structure from most major respiratory allergens⁸⁻¹⁰. Thus, experimental models based on the use of human-relevant allergens and immune receptors are well warranted. Consequently, we have initially set out to develop an *in vitro* system of the human ‘allergen-specific’ synapse by cloning the relevant HLA-molecules, immunodominant peptides and correlating T-cell receptor α and β chains for the ragweed allergen Art v 1 (TRAV17/TRBV18)¹¹ and the major birch pollen allergen Bet v 1 (TRAV6/TRBV20) (Neunkirchner *et al*; in press). Respective cDNAs were derived from T-cell clones and EBV transformed B-cell lines obtained from allergic individuals. Using human 293 cells transfected with the cloned HLA-molecules, allergenic peptides and costimulatory signals as artificial antigen-presenting cells, we could demonstrate the antigen-specific and costimulation-dependent reaction pattern of TCR-transduced T-cells and T-cell lines¹¹. In contrast to generation of allergen-specific T-cell clones/lines, which is a time-consuming and sometimes tedious process, TCR-transgenic T-cells can be generated in large quantities independently of the allergen season. Furthermore, retroviral transduction of TCR α/β chains should allow to target distinct T-cell populations and thus to study the influence of diverse modulatory strategies on different T-cell subsets (e.g. Th1, Th2, Th17 and Treg).

In a first study we have relied on the recently established technique of targeting HLA-molecules, costimulatory molecules and cytokines via GPI-anchoring to virus-like particles (VLP)^{12, 13} in order to target and modulate T-cell responses of Art v 1-specific transgenic T-cells. In these experiments we could show that VLP expressing HLA-DR1 plus invariant-chain borne Art v 1₂₅₋₃₄ peptide were able to activate Art v 1-specific tg T-

cells in a costimulatory dependent manner, thus validating the ability of HLA-decorated VLP to specifically target T-cells. Importantly, omission of costimulatory molecules on such stimulatory VLP led to the induction of anergy, which concurs with the classical observations summarized in the ‘two-signal hypothesis’. Additionally, we made use of the recently published observation that costimulation of CD4⁺ T-cells solely with CD58 induces a Tr1-like phenotype¹⁴. Indeed, we could show that VLP decorated with CD58 induced an IL-10^{high}IFN- γ ^{int}IL-2^{low}IL-13^{low} phenotype in Art v 1-specific TCR tg T-cells¹⁵. However, whether such CD58-induced T-cells constitute a fully functional regulatory population has not been assessed in our laboratory so far. Given recently aired doubts that CD58 costimulation might not induce *bona fide* Tr1 cells¹⁶ it seems possible that we have, similar to *Groux et al.*, observed a phenotype which resembles but does not constitute regulatory T-cells. Still, it would be tempting to speculate that such CD58-induced T-cells, although *per se* not regulatory to other effector T-cells, might be able to modulate Th2-mediated diseases *in vivo* due to their production of the anti-inflammatory cytokine IL-10 and the Th1 polarizing cytokine IFN- γ .

In a second VLP-based approach we made use of the ability of VLP to accommodate multiple GPI-anchored proteins on the same particles. This observation allowed us to decorate VLP with multimeric receptors such as the high affinity IL-2 receptor consisting of the IL-2 receptor α , β and γ chain (CD25, CD122, CD132)¹⁷. In order to visualize the impact of different receptor compositions on binding and affinity, we used an influenza matrix protein:GFP fusion protein (MA:GFP) as fluorescence marker. With this approach we could assess that VLP expressing the high-affinity IL-2 receptor showed a 2.5-fold higher staining intensity of IL-2 transfected 293 cells when compared to VLP expressing only the IL-2R β and γ chain.

The IL-2R is of special importance in T-cell homeostasis and activation and regulatory T-cells can be distinguished from effector T-cells by their constitutive expression of the CD25 molecule¹⁸. This seems to be of mechanistical importance, as recent reports indicated that regulatory T-cells use this higher affinity to IL-2 to deplete effector T-cells from this important cytokine, which consequently undergo apoptosis¹⁹. We hypothesized that this function could be mimicked by VLP decorated with the high affinity IL-2R. Indeed, addition of VLP decorated with CD25:GPI, CD122:GPI and CD132:GPI to Artv1-tg T-cells stimulated with artificial antigen-presenting cells strongly abrogated proliferation. However, in contrast to the report by *Pandiyan et al.*, we could show in

restimulation experiments that IL-2 depleted CD4⁺ T-cells were not apoptotic but anergic¹⁷.

While VLP-based IL-2 depletion thus seems to be a promising strategy to modulate T-cell responses, several practical questions have to be further assessed. First, systemic application of IL-2R decorated VLP could probably result in a general immunosuppression, a state that would certainly not be tolerable for the treatment of allergic diseases. Recent reports indicate that IL-2/anti-IL-2 immune-complexes favor the induction and expansion of regulatory T-cells *in vivo*^{20, 21}. In this light it seems conceivable that IL-2 bound to VLP may have similar functions, thus even potentiating the level of immunosuppression. Consequently, targeting of such VLP to specific T-cells poses a strict requirement. Whether additional decoration with MHC:peptide molecules is sufficient to meet this requirement will be the focus of further studies. Secondly, it remains to be shown whether the induction of anergy/hyporesponsiveness in allergen-specific T-cells can fully abrogate pathological T-cell responses. As an ongoing allergic inflammation may provide a highly stimulatory environment with potent antigen-presentation, this could potentially override anergy-inducing signals. Thus strategies to induce allergen-specific Treg may be more promising in the long run.

While VLP-based approaches have potent immunomodulatory capacity *in vitro*, so far no data about kinetics and dynamics *in vivo* especially regarding tissue distribution and stability of VLP have been obtained. Given that decoration with MA:GFP allows to follow the fate of VLP, further studies will elucidate the *in vivo* behaviour of VLP and will aim to optimize these parameters.

VLP-based strategies represent artificial antigen-presenting platforms of well-defined and thus predictable stimulatory/modulatory potential. However, during T-cell activation, APC derived signals are complemented by the crosstalk of different T-cell subsets, which have the ability to potentiate, polarize or modulate each other. As outlined in the chapter Introduction, regulatory T-cells offer huge promise in experimental models of a multitude of immune-mediated diseases and disorders. A broad range of different approaches to induce, generate or propagate regulatory T-cells with or without pre-defined antigen-specificity has been described. The cloning and characterization of allergen-specific TCRs endowed us with the unique opportunity to generate allergen-specific regulatory T-cells by genetic engineering of purified human CD4⁺ T-cells. We hypothesized that co-introduction of Foxp3 in concert with an allergen-specific TCR would result in transgenic

allergen-specific regulatory T-cells. In order to achieve a reliable expression of the three transgenes we fused the cDNAs encoding the Bet v 1₁₄₂₋₁₅₃-specific TCR α and β chains (TRAV6/TRBV20) to the cDNA encoding Foxp3 using the recently described picornaviral 2A elements.

Since its implementation as biotechnological tool in 2004²², the use of 2A elements in multicistronic expression elements has gained increased attractiveness and has been used in studies to express transgenic TCRs. This may be due to the fact that transgenes linked via 2A elements were found to show a higher correlation of expression than transgenes linked via IRES elements²². However, in contrast to IRES elements, 2A elements are translatable sequences, which add a short amino-acid sequence to the C-terminus of the 5' expressed protein after translation. This additional sequence has not been reported to interfere with the function of the linked transgenes (in our case the transgenic TCR α and β chains). However, the introduction of an additional foreign sequence may create novel antigenic peptides, which could limit the viability of transgenic cells *in vivo*, a notion that so far has not been thoroughly investigated.

In order to achieve a facile detection and sorting possibility for positively transduced cells, the TCR/Foxp3 expression element was cloned upstream of an IRES-GFP element into a retroviral vector. Indeed, positively-transduced (GFP⁺) CD4⁺ T-cells showed strong co-expression of the introduced transgenes and displayed the typical phenotype of nTreg (CD25^{high}CTLA-4^{high}CD39^{high}CD127^{low}). In functional experiments using Bet v 1₁₄₂₋₁₅₃ presenting artificial APC or anti-CD3/CD28 coated microbeads as polyclonal stimulus, these cells were found to be hyporesponsive in both proliferation and cytokine secretion. Importantly, TCR/Foxp3 tg T-cells were highly suppressive in co-cultures with TRAV6/TRBV20 tg effector T-cells both upon allergen-specific (aAPC-mediated) and polyclonal (microbeads-mediated) stimulation. In control experiments, Foxp3 tg or CD4⁺CD25⁺ nTreg, expressing their ectopic, polyclonal TCR repertoire, showed only minimal suppression upon antigen-specific activation but equal suppression when activated polyclonally. These findings validate that Treg suppressive activity is an activation-dependent process and that over-expression of a tg TCR can indeed serve as a 'molecular switch' for Treg function²³. While direct transduction of pre-sorted nTreg represents another strategy that has already proven feasible, our 'fully transgenic approach' offers several advantages in our eyes: Firstly, the use of nTreg sorted for the CD4⁺CD25⁺ phenotype denotes an ill-defined population, which potentially contains

‘contaminating’ activated effector T-cells. The additional use of markers such as CD127 might result in a more pure nTreg population but considerably decreases the already low yield. In this respect CD4⁺CD25⁻ T-cells, as used in our study, represent a more well-defined population, which is readily available at high yields. Secondly, as backed by several studies, nTreg from allergic individuals or individuals suffering from autoimmune diseases might be compromised in their suppressive function and might thus be not suitable for therapeutic application²⁴⁻²⁶. Consequently, the high level overexpression of Foxp3, as practiced in our approach, could prove to be superior.

Yet, several points of concern remain. Firstly, a TCR tg approach demands the exact knowledge about all factors involved in the ‘pathology-specific synapse’, i.e. the restriction element, the auto-antigenic/allogeneic/allergenic peptides and the corresponding TCRs. Although some allergies might present as rather uniform disorders in this respect²⁷⁻³⁰ other allergies and especially autoimmune diseases could be more heterogenous and would thus demand for a large collection of allergen/autoantigen-specific TCRs. Secondly, Foxp3 overexpression does not reconstitute the complete gene expression profile of nTreg³¹, thus raising questions whether Foxp3 tg T-cells can be used as therapeutic tools for all mentioned applications. Whether additional introduction of genes involved in T-cell tolerance (for example ubiquitin ligases like Cbl-b³² or signaling molecules like STAT3³³) can overcome this deficiency will be the focus of further studies. Thirdly, the retro- or lenti-viral introduction of transgenes harbors the risk of insertional mutagenesis, which could at least theoretically lead to potentially severe complications such as malignant transformation. However, clinical trials in cancer patients receiving TCR transduced CD8⁺ T-cells have so far proven to be safe in this respect³⁴.

In conclusion, by building an *in vitro* system of the allergen-specific synapse we have succeeded in developing a system to study human allergen-specific T-cell responses. Consequently, we have developed and evaluated aAPC/VLP- and Treg-based strategies for the immunomodulation of Art v 1- and Bet v 1-specific T-cell responses. First studies show that these approaches have the capacity to modify or suppress allergen-specific T-cell responses *in vitro*. However, questions about the *in vivo* applicability of VLP- or Treg based strategies concerning stability and disease-modifying potential remain to be experimentally addressed. To be able to study these issues in detail, we have recently developed a novel mouse model based on the introduction of the human TRAV17/TRBV18 TCR (Art v 1-specific) into HLA-DR1 tg mice, thereby rebuilding the

human allergen-specific synapse *in vivo*. First evaluations of TCR/HLA-DR1 tg mice have shown that T-cells show high expression levels of the respective transgenes and react specifically to Art v 1 protein and the Art v 1₂₄₋₃₆ peptide. This may enable us to take the next step in the “bench-to-bedside” process and ultimately develop novel therapies for immune-mediated disorders.

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Zusammenfassung

T-Lymphozyten spielen eine entscheidende Rolle im Ablauf einer allergischen Immunantwort. In Folge der Präsentation von Allergenen an spezifische T-Helfer Zellen, kommt es in Allergikern zur Polarisierung in Richtung einer T-Helfer 2 (Th2) Antwort. Die daraus resultierenden Effektormechanismen, vor allem die Sekretion von Th2-Zytokinen wie IL-4, IL-5 und IL-13, tragen entscheidend zu sowohl der Akut-Phase Reaktion als auch zu Spät-Phasen Reaktionen der allergischen Immunantwort bei. Daher könnten Strategien, die diese pathologische Th2-Antwort modulieren, für die Behandlung von allergischen Patienten von therapeutischem Wert sein. Um solche Ansätze zu untersuchen, besteht dringender Bedarf nach Modellsystemen, die auf human-relevanten Allergenen, Präsentationsmolekülen und T-Zell-Rezeptoren (TZR) basieren. Daher wurde von uns die „allergen-spezifische Synapse“ von zwei klinisch äußerst relevanten Allergenen, dem immun-dominanten Beifuss-pollen Allergen (Art v 1) und dem Haupt-Allergen des Birkenpollens (Bet v 1), auf molekularer Ebene nachgestellt. Dafür wurden die relevanten antigen-präsentierenden HLA-Moleküle und die korrespondierenden TZR aus Zell-Linien von Beifuss- bzw. Birkenpollen-Allergikern kloniert. Artifizielle antigen-präsentierende Zellen (APZ) wurden durch Transfektion von 293 Zellen mit den jeweiligen HLA-Restriktionselementen, Allergenen und kostimulatorischen Molekülen hergestellt, während die dazugehörigen TZR in CD4⁺ T-Zellen retroviral transduziert wurden. Dieses Modell-System erlaubte uns, die Vorgänge der allergen-spezifischen T-Zell Aktivierung unter definierten Bedingungen *in vitro* zu untersuchen. Zunächst konnten wir zeigen, dass TZR transgene T-Zellen in ihrer Aktivierung von der Erkennung des passenden Peptids und dem Vorhandensein von kostimulatorischen Signalen abhängig waren. Aufgrund dieser Beobachtungen konnten wir schließen, dass TZR transgene T-Zellen in ihrem Verhalten dem physiologischen Verhalten von T-Zellen entsprachen.

In einem ersten Ansatz zielten wir darauf ab, die Funktion von transgenen T-Zellen durch das Anbieten von unterschiedlichen kostimulatorischen Signalen zu modulieren. Dafür verwendeten wir ein in unserem Labor etabliertes System, bei dem Virus-ähnliche Partikel (‘virus-like particles’; VLP) als artifizielle antigen-präsentierende Plattform verwendet werden. In diesen Experimenten konnten wir zeigen, dass VLP optimal geeignet sind, die Rolle von APZ zu übernehmen und je nach ihrer spezifischen Ausstattung allergen-

spezifische T-Zellen modulieren können. Während die Ausstattung von VLP mit antigen-präsentierenden HLA/Peptid-Komplexen und CD80 als kostimulatorischem Molekül zu einer massiven Expansion der TZR transgenen T-Zellen führte, konnten antigen-präsentierende VLP ohne Ko-Signale spezifisch einen Zustand der Anergie, induzieren. Ebenso führte VLP-medierte Aktivierung mit CD58 als kostimulatorischem Molekül zu einer Polarisierung der antigen-spezifischen T-Zell Antwort in Richtung einer T regulatorischen Typ I (Tr1) Antwort. Dies ist von besonderem Interesse, da Tr1 Zellen wichtige Stellglieder in der Unterdrückung von allergischen Reaktionen darstellen.

Weiters konnten wir zeigen, dass die Ausstattung von VLP mit dem hochaffinen IL-2 Rezeptor uns erlaubte, dieses wichtige Zytokin in Kulturen von aktivierten allergen-spezifischen T-Zellen zu neutralisieren. Das hatte zum Effekt, dass die T-Zell Aktivierung unterbunden wurde, und diese T-Zellen in den Zustand der Anergie übergangen. Damit konnten wir zeigen, dass VLP auch die Kapazität haben, wichtige Effektor-Mechanismen von T regulatorischen Zellen (Treg) zu übernehmen.

In einem weiteren Unterprojekt untersuchten wir die Regulation des HLA Klasse II Komplexes, wovon wir uns Rückschlüsse für eine effektive Erstellung von artifiziellen antigen-präsentierenden Systemen erhofften. Zu diesem Zweck untersuchten wir B-Zell-Linien von HLA Klasse II defizienten Individuen. Dabei konnten wir beobachten, dass die Invariante Kette im Gegensatz zu den anderen Molekülen des HLA Klasse II Locus in diesen Zellen nicht vollständig fehlte, sondern, abhängig vom auslösenden Gendefekt, verschieden stark exprimiert war. Dieses Resultat erlaubt nicht nur ein besseres Verständnis der Biologie des antigen-präsentierenden HLA Klasse II Komplexes, sondern könnte auch von diagnostischem Wert sein.

Zusätzlich zu den Signalen, die von APZ an T-Zellen präsentiert werden, wird die T-Zell-Antwort auch durch das Zusammenspiel von verschiedenen T-Zell-Untergruppen beeinflusst. Eine besonders wichtige Rolle in diesem Zusammenhang spielen Treg, die Aktivierung von T Zellen auch unter optimalen stimulatorischen Bedingungen unterbinden können. Aus diesem Grund bieten Treg ein attraktives Ziel zur Entwicklung von Therapien für Erkrankungen, die durch das Immunsystem vermittelt werden, wie Autoimmunerkrankungen, Allergien und Transplantatabstossung.

Vor kurzem konnte von zwei Gruppen unabhängig gezeigt werden, dass sich durch die Einbringung des Forkhead Box Protein 3 (Foxp3) Gens in T-Zellen, diese in Treg konvertieren lassen. Diesem Prinzip folgend erstellten wir allergen-spezifische Treg durch die Einbringung von einem Bet v 1-spezifischen TZR gemeinsam mit Foxp3 in CD4⁺ T-Zellen. Eine verlässliche Einbringung und Expression aller drei Transgene (TZR α/β Ketten und Foxp3) erreichten wir durch die Konstruktion eines multicitronischen Expressionselements bei dem die entsprechenden cDNAs über 2A-Elemente fusioniert wurden. Um Identifizierung und Isolierung von transgenen Zellen zu erleichtern, wurde diese Expressionskassette 5' vor ein IRES-GFP Element als Fluoreszenzmarker inseriert. Durch Transduktion mit diesem Konstrukt erstellte und isolierte TZR/Foxp3 transgene T-Zellen zeigten die typischen Eigenschaften von Treg. Dazu gehört sowohl das spezifische Profil von hoher Expression von CD25, CD39 und CTLA-4 und die verminderte Expression von CD127, als auch das verminderte Ansprechen auf antigen-spezifische und polyklonale Stimulation. Weiters zeigten diese allergen-spezifischen Treg hohe suppressive Kapazität in Ko-Kulturen mit allergen-spezifischen T-Zellen. Von besonderer Signifikanz erwiesen sich Beobachtungen, dass nur TZR/Foxp3 transgene T-Zellen nach allergen-spezifischer Aktivierung suppressiv wirkten, während Foxp3 transgene T-Zellen, die einen nicht-spezifischen TZR exprimierten, keinen Effekt in solchen Ansätzen zeigten. Das validiert nicht nur die bekannte Beobachtung, dass Treg-medierte Suppression Aktivierungs-abhängig ist, sondern beweist auch, dass ein künstlich eingebrachter TZR in Treg als molekularer Schalter für deren Funktion wirken kann.

Aus diesen Experimenten gewonnene Schlüsse könnten entscheidend dazu beitragen, neue therapeutische Ansätze für Allergien, aber auch für andere Erkrankungen, in denen T-Zellen eine wichtige pathophysiologische Rolle spielen, zu finden. Um diese Hypothese weiter zu überprüfen, sind wir zur Zeit damit beschäftigt, ein Mausmodell zu entwickeln, das auf dem *in vitro* System der „allergen-spezifischen Synapse“ basiert.

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