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

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

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

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

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

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

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

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

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

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

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Supported by grants from the Austrian Science foundation (SFB F1816-B13, SFB F1807-B13, and FWF 20011-B13), the Österreichische Forschungsförderungsgesellschaft #812079 and Biomay AG, and the Christian Doppler Research Association, Austria.

Disclosure of potential conflict of interest: B. Jahn-Schmid receives research support from the Austrian Science Fund. B. Bohle receives research support from the Austrian Science Fund and the Christian Doppler Laboratory. The rest of the authors have declared that they have no conflict of interest.

Received for publication July 23, 2010; revised October 19, 2010; accepted for publication October 20, 2010.

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0091-6749/$36.00

doi:10.1016/j.jaci.2010.10.023

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

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

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

METHODS

Molecular cloning and generation of multicistronic vectors

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

Cell lines and primary cells

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

Transfection of 293 cells

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

Retroviral transduction of PB T cells

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

Flow-cytometric analyses

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

Determination of cytokine secretion

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

Proliferation and suppression assays

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

Statistical analysis

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

RESULTS

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

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

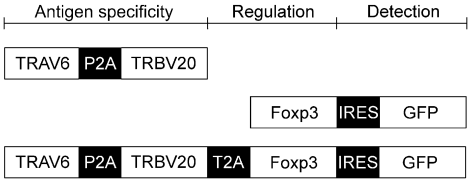

FIG 1. Scheme of multicistronic retroviral expression constructs used in this study. Function of the respective genes is indicated. *Open boxes,* genes; *closed boxes,* interposed picornaviral *2A* or *IRES* elements.

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

Transduced T cells express the introduced transgenes

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

FOXP3-transduced T cells display a Treg cell surface phenotype

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

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

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

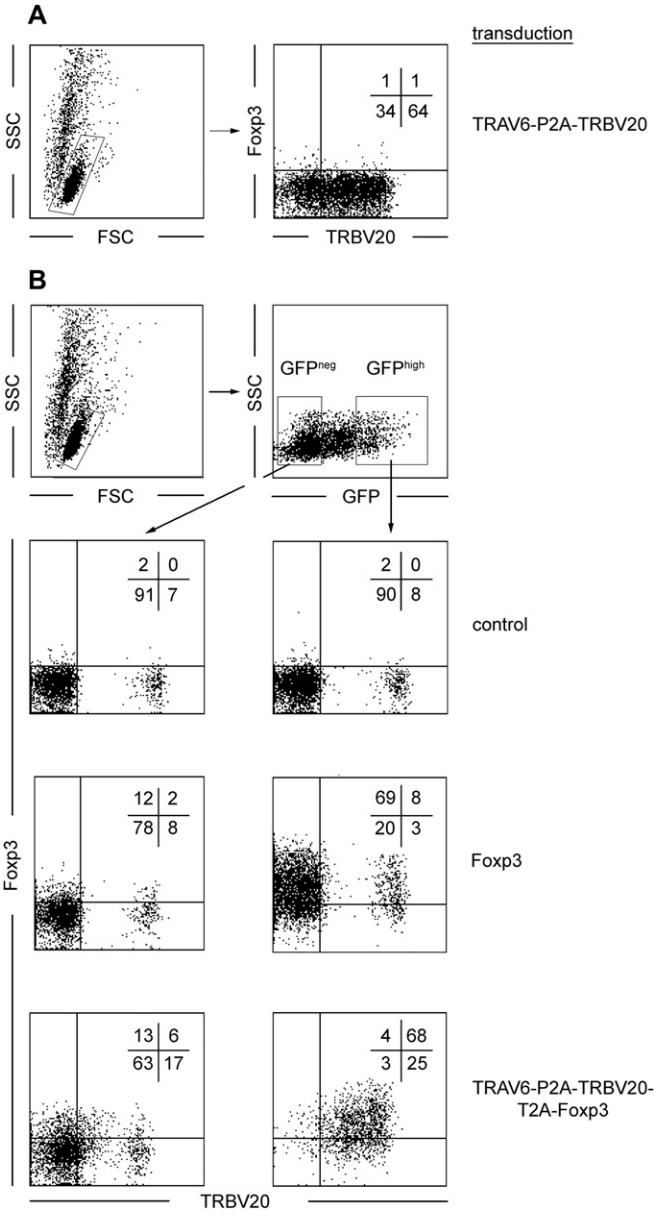

FIG 2. Transgene expression levels and gating strategy for retrovirally transduced PB T cells. **A,** TRAV6$^+$TRBV20$^+$ T cells. **B,** TRAV6$^+$TRBV20$^+$FOXP3$^+$, FOXP3$^+$, and control T cells. In *B,* GFP expression served as additional gating marker. Surface TCR expression (TRBV20) and intracellular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

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

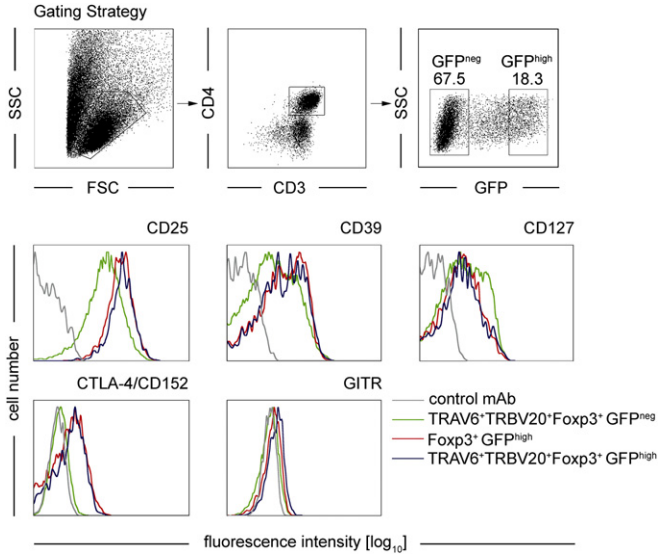

FIG 3. Immunophenotype of transduced T cells. PB T cells gated for $CD3^+CD4^+GFP^{neg}$ or $CD3^+CD4^+GFP^{high}$ subsets and analyzed for expression of indicated Treg markers. *Blue line,* GFP^{high} subset from *TRAV6/TRBV20/FOXP3*–transduced T cells, specific mAb; *red line,* GFP^{high} subset from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

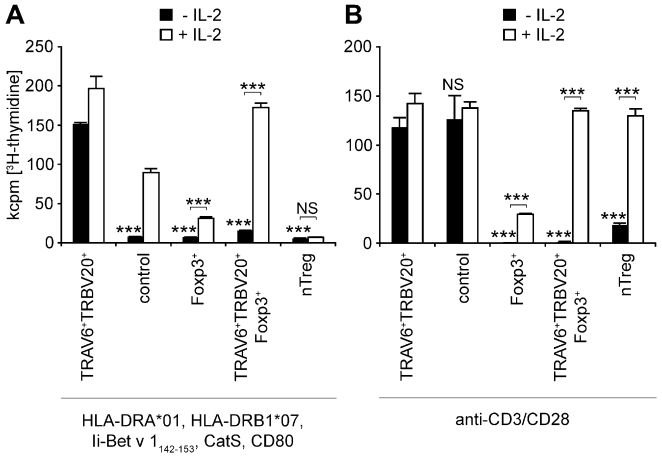

FIG 4. *FOXP3*-transduced T cells are hyporesponsive. Proliferation of retrovirally transduced PB T cells or nTreg cells stimulated with aAPCs expressing indicated molecules **(A)** or anti–CD3/CD28 coated microbeads in the absence *(black bars)* or presence *(white bars)* of recombinant human IL-2 (250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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

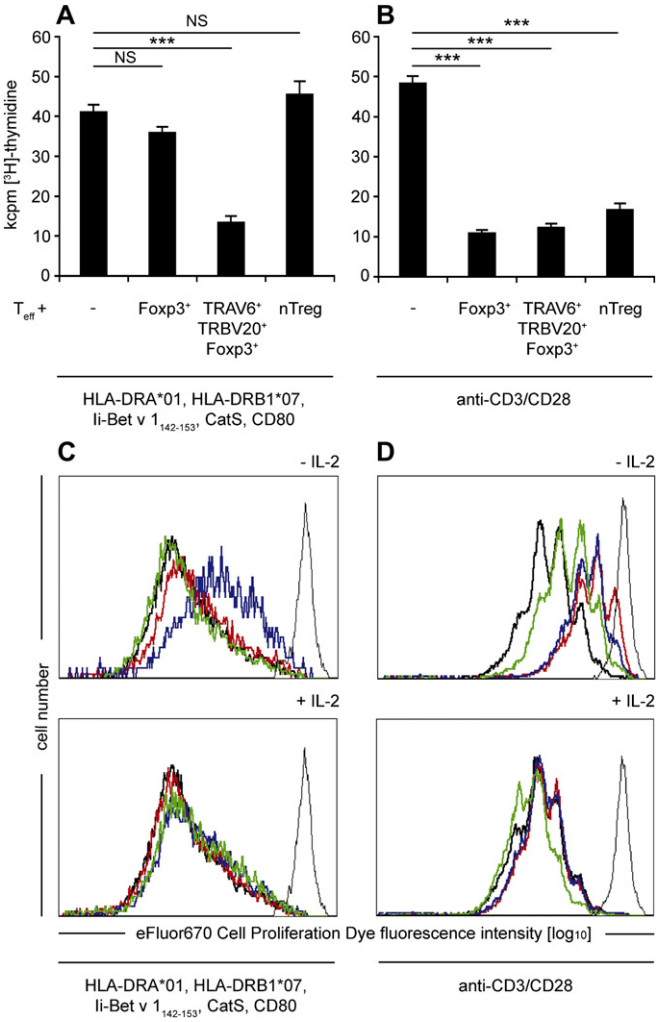

FIG 5. *TRAV6/TRBV20/FOXP3*–transduced T cells, unlike *FOXP3*-transduced T cells, suppress Bet v 1–specific effector T cells on allergen-specific activation. Proliferation of TRAV6$^+$TRBV20$^+$ transgenic effector T cells *(T_{eff})* cultured in the absence or presence of FOXP3$^+$, TRAV6$^+$TRBV20$^+$FOXP3$^+$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

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

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

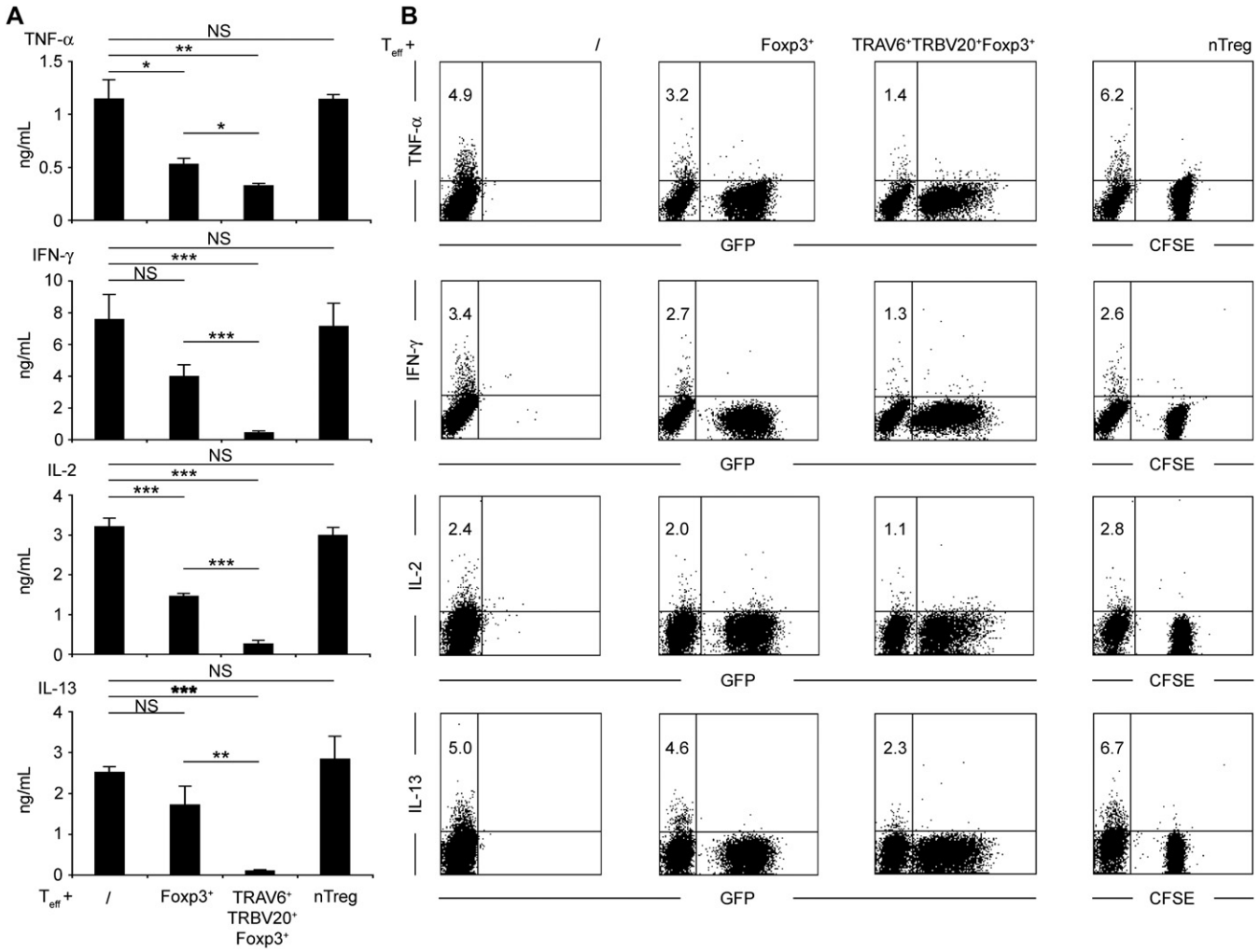

FIG 6. Suppression of cytokine secretion of effector T cells by engineered Treg cells. **A,** Cytokine levels from cell culture supernatants collected after 24 hours (TNF-α, IL-2) or 48 hours (IFN-γ, IL-13). **B,** Intracellular staining of indicated cytokines. *Numbers* indicate percentages of cytokine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

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

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

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

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

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

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

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

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TABLE E1. List of primers

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

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

TABLE E2. List of mAbs

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

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

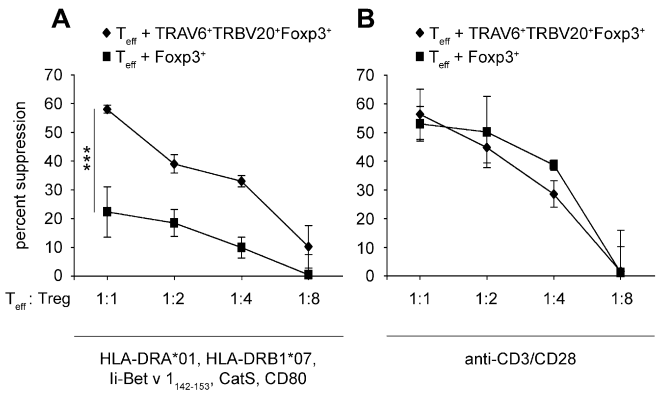

FIG E1. Percent suppression of titrated amounts of indicated transgenic Treg cells cocultured with effector T cells *(T_{eff})* on stimulation with Bet v 1–presenting aAPCs **(A)** or anti–CD3/CD28 coated microbeads **(B)**. *NS,* Not significant. *$P < .05$; ***$P < .001$.