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Amelioration of Colitis by Genetically Engineered Murine Regulatory T Cells Redirected by Antigen-Specific Chimeric Receptor

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Background & Aims: **The therapeutic application of regulatory T cells (Tregs) for the treatment of inflammatory diseases is limited by the scarcity of antigen-specific Tregs. A preferred approach to endow effector T cells (Teff) with a desired specificity uses chimeric immune receptors with antibody-type specificity. Accordingly, employing such chimeric immune receptors to redirect Tregs to sites of inflammation may be a useful therapeutic approach to alleviate a broad scope of diseases in which an uncontrolled inflammatory response plays a major role.** ***Methods:*** **To enable application of the approach in clinical setting, which requires the genetic modification of the patient's own Tregs, we describe here a novel protocol that allows the efficient retroviral transduction and 2,4,6-trinitrophenol-specific expansion of murine naturally occurring regulatory T cells (nTregs), with a 2,4,6-trinitrophenol-specific tripartite chimeric receptor.** ***Results:*** **Transduced Tregs maintained their Foxp3 level, could undergo repeated expansion upon ex vivo encounter with their cognate antigen in a major histocompatibility complex-independent, costimulation-independent, and contact-dependent manner and specifically suppressed Teff cells. Adoptive transfer of small numbers of the transduced nTregs was associated with antigen-specific, dose-dependent amelioration of trinitrobenzenesulphonic acid colitis.** ***Conclusions:*** **This study demonstrates that nTregs can be efficiently transduced to express functional, antigen-specific chimeric receptors that enable the specific suppression of effector T cells both in vitro and in vivo. This approach may enable future cell-based therapeutic application in inflammatory bowel disease, as well as other inflammatory disorders.**

CD4$^+$CD25$^+$ regulatory T cells (Tregs) are potent inducers of peripheral tolerance that are characterized by constitutive expression of the transcription factor Foxp3.[1] Treg activation is believed to be antigen-specific,[2,3] although their suppressor function is considered to be nonantigen-specific and is mediated by contact-dependent mechanisms as well as by secretion of suppressive cytokines such as transforming growth factor (TGF)-β, interleukin (IL)-10, and IL-35.[4,5] Impaired Treg accumulation and function in inflammatory sites have been suggested to be central in the pathogenesis of multiple autoimmune disorders, including autoimmune thyroiditis, gastritis, insulitis, arthritis, encephalomyelitis, and colitis, whereas adoptive transfer of Tregs was demonstrated in animal models to be beneficial in both preventing and ameliorating these disorders.[6–9] Because antigen-specific Tregs are extremely rare, studies utilizing this cell type have generally employed adoptive transfer of large numbers of nonspecific Tregs or isolated Tregs from transgenic mice expressing T-cell receptor (TCR) specific to a particular major histocompatibility complex (MHC)-peptide complex.[10,11]

We have recently described the development of Tregs that transgenically express a chimeric receptor (CR) composed of an antibody variable region specific for 2,4,6-Trinitrophenol (TNP) that was fused to the extracellular and transmembrane domain of the CD28 costimulatory molecule and intracellular domain of the stimulatory Fc-γ receptor chain. A transgenic mouse expressing this receptor on all T cells was found to be nearly resistant to 2,4,6-trinitrobenzenesulphonic acid (TNBS) colitis.[12] Moreover, adoptive transfer of small numbers of Tregs expressing this tripartite chimeric receptor (TPCR) resulted in antigen-specific, MHC-nonrestricted, and costimulation-independent Treg accumulation and activation at inflamed colonic sites, leading to cure of acute experimental colitis. To employ this approach in the nontransgenic setting (such as in human disease), Tregs must be transduced with vectors encoding a CR, then selected on the basis of TPCR expression, and finally expanded ex vivo to enable their clinical use. The partial acquisition of regulatory functions by retroviral or lentiviral transduction of Teff cells with vectors encoding Foxp3 has been reported.[13] However, nTregs are considered extremely resistant to transduction with retroviral or lentiviral vectors, which may stem from nTreg anergy that impedes preactivation of Tregs, a prerequisite step

Abbreviations used in this paper: CR, chimeric receptor; Teff, effector T cells; Tg, transgenic; nTregs, naturally occurring regulatory T cells; TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNP, 2,4,6-trinitrophenol; TPCR, tripartite chimeric receptor; Tregs, regulatory T cells.

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for successful and efficient retroviral transduction.[14] In this report, we describe, for the first time, the development of a protocol that enables efficient and reproducible retroviral transduction of murine nTregs as well as their selection and expansion, leading to a highly enriched population of antigen-specific nTregs that retain Foxp3 expression and suppressive capabilities both in vitro and in vivo.

Materials and Methods

Mice

Transgenic mice expressing the TNP-specific CR (TNP-TPCR) were generated as previously described.[15] In all experiments, 8- to 10-week-old female Balb/C or C57bl mice were used. All manipulations were carried out in accordance with guidelines of the Institutional Animal Care and Use Committee.

Antibodies, Cell Lines, and Reagents

Anti-CD3-APC and anti CD3-PERCP antibodies were purchased from Biolegend (San Diego, CA). Foxp3 expression in lymphocytes was determined using either FITC or APC-labeled anti-mouse Foxp3 staining kit (Ebioscience, San Diego, CA) and FACS sorting. Anti-idiotypic monoclonal antibodies (mAb) GK 20.5 (anti-Sp6) was a gift from the late Prof G. Köhler[16]; 2C11, a hamster anti-mouse CD3ε mAb was kindly provided by Prof J. Bluestone (UCSF, San Francisco, CA). Anti-mouse CD28 (37.51) was purchased from Southern Biotechnology Associates, Inc (Birmingham, AL).

Flow Cytometry

Lymphocytes (1×10^6) were incubated with the appropriate antibodies in staining buffer (5% fetal calf serum [FCS], 0.05% sodium azide in phosphate-buffered saline) for 30 minutes on ice. Cells were analyzed by flow cytometry using a FACSort (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson).

Induction of Hapten-Mediated Colitis

TNBS colitis was induced as previously described.[12]

Adoptive Transfer Experiments

In adoptive transfer experiments, mice were preconditioned with intraperitoneal cyclophosphamide (200 mg/kg) 10 days prior to induction of colitis. Tregs (1×10^5) were intraperitoneally injected into mice 16 hours following induction of colitis.

Murine Colonoscopy

For monitoring colitis, we employed a high-resolution murine video endoscopic system, consisting of a miniature endoscope (scope, 1.9-mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Coloview; Karl Storz, Tuttlingen, Germany) to achieve regulated inflation of the mouse colon.[17] The endoscopic procedure was viewed on a color monitor and digitally recorded in anesthetized mice. Video files were processed with the Windows Movie Maker software (Microsoft Corp, Ra'anana, Israel). Endoscopic quantification of colitis was graded for bowel thickening (0–3), changes in bowel vascularity (0–3), presence of fibrin deposits (0–3), granularity of the mucosal surface (0–3), and bowel consistency (0–3).

Preparation of GP+e Packaging Cells

The DNA for the anti-TNP scFv-CD28-FcRγ TPCR was previously described[16] and was cloned into the retroviral vector p-Bullet, which contains a GFP encoding segment cotranslated with the TPCR through an internal ribosomal entry site. This plasmid along with Gag/Pol/Env (Gp+e) constructs were transfected using the Ping-Pong method by a Ca_2PO_4 kit (Mammalian Transfection Kit; Stratagene, La Jolla, CA) in accordance with the manufacturer's directions. Briefly, the "Ping-Pong" method involves transfection of an equal mix of HEK293 and Phoenix packaging cells at 80% confluence and incubation for 48 hours. After transfection, the viral supernatant was collected and used to stably transduce GP+e packaging cells. Cultures of infected GP+e were then sorted. Packaging cells with the highest levels of green fluorescent protein expression were collected and regrown. The sorting procedure was repeated several times to achieve a stable monoclonal GP+e cell line, which produces high titers of retrovirus encoding the plasmid for the TNP-TPCR.

Retroviral Transduction of $CD4^+CD25^+$Tregs

To produce viral supernatant, selected virus-producing GP+e packaging cells were cultured overnight in 100×20-mm Petri dishes at 80% confluence with 5 mL complete Dulbecco's modified Eagle medium (DMEM) (DMEM supplemented with 10% FCS, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, nonessential amino acids, and streptavidin/penicillin). $CD4^+CD25^+$Tregs were isolated from wild-type Balb/c splenocytes using a $CD4^+CD25^+$Tregs isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) to achieve a purity of over 92%. The isolated cells were preactivated for 4 to 18 hours with 2 μg/mL Concanavalin A or with plate bounded anti-CD3 and anti-CD28 antibodies (plated at 1 ng/mL and 5 ng/mL, respectively). Activation and following transduction steps were performed either in Biotarget-1 serum-free medium (Biological Industries, Beit Haemek, Israel) or in complete medium (RPMI supplemented with 10% FCS); both media were supplemented with 750 U/mL IL-2, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.01 mol/L HEPES, 50 μmol/L 2-ME, nonessential amino acids, and streptavidin/penicillin. For ret-

roviral transduction, viral supernatant was filtered through a 0.45-μm filter and supplemented with 750 U/mL recombinant mouse IL-2. Next, 750-μL cell-free viral supernatant was administered to each well of 24-well plates precoated with 5 μg/mL retronectin (Takara Bio Inc, Shiga, Japan). Plates were centrifuged at 1000*g* for 30 minutes at room temperature. The activated Tregs, resuspended in 750-μL cell-free viral supernatant, were distributed to the same retronectin-coated, virus-containing wells (1.5×10^6 activated Tregs/well). Plates were then centrifuged again at 1000*g* for 1 hour at room temperature and incubated for 5 hours. After incubation, viral supernatant was replaced with either Biotarget-1 or RPMI 1640 complete medium. Plates were incubated in 5% CO_2 for an additional 2 days before flow cytometry analysis or further experimentation.

Statistical Analysis

Statistical evaluation was performed using the unpaired Student *t* test or analysis of variance parametric tests. The log-rank test was used for all experiments in which survival was assessed as an end point. $P < .05$ was considered significant.

Results

Development of a Protocol for Efficient Retroviral Transduction of Murine nTregs With Vectors Encoding TNP-Specific TPCR

Therapeutic application of the Treg-T body approach necessitates that nTregs be efficiently transduced ex vivo with vectors encoding a CR to enable their timely antigen-specific suppression of the inflammatory response. To develop such a protocol, we transduced mouse nTregs with retroviruses encoding anti-TNP-TPCR (under the control of the cytomegalovirus promoter), produced in high titers by a mouse-specific packaging cell line (GP+e).

Our conventional retroviral transduction protocols,[18] consisting of a 36- to 48-hour preactivation period with plate-coated anti-CD3 and anti-CD28 antibodies, and use of RPMI medium supplemented with 10% FCS yielded disappointingly low levels of nTreg transduction (less than 5%, on average), in comparison with 30%–50% transduction levels in Teff cells (data not shown). Such levels were insufficient to provide usable numbers of cells. To improve these low transgene expression levels, multiple modifications of the transduction protocols were attempted; the main parameters modified included mode of Treg activation (soluble Concanavalin A vs plastic bound anti-CD3 and anti-CD28 antibodies), transduction medium (serum containing medium vs serum-free Biotarget medium, number of retroviral transductions (single vs double incubation with viral supernatant), and duration of Treg preactivation (4–72 hours). In all combinations, preactivation of Tregs with Concanavalin A was clearly superior to anti-CD3 and anti-CD28 antibodies, with best transduction efficacy obtained with a shortened preactivation period of 14–18 hours (Figure 1*A*, *panel IV*, and Figure 2). Using these optimal conditions, comparable Tregs transduction was obtained with use of full-medium, serum-free medium, and single or repeated retroviral transduction. Nevertheless, because use of serum-free medium and single transduction yielded only the lowest transduction rate (13%) of Teff cells, we favored this protocol for Tregs transduction for the following experiments. Under these conditions, over 20%–40% of the Foxp3-positive cells could be efficiently transduced (Figure 1*B*).

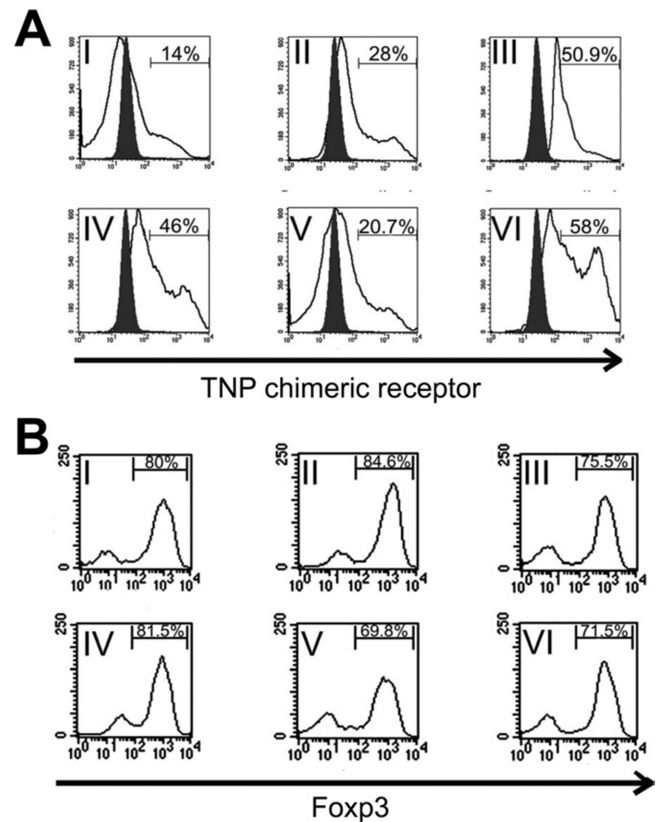

Figure 1. Retroviral transduction of Tregs with a vector encoding anti-TNP TPCR. (*A*) Assessment of optimal retroviral transduction of isolated $CD4^+CD25^+$ Tregs by comparison of the following incubation and preactivation protocols: (*I*) Complete medium (containing 10% fetal calf serum) and plastic bound anti-CD3/anti-CD28; (*II*) serum-free medium and anti-CD3/anti-CD28; (*III*) full medium and Concanavalin A; (*IV*) serum-free medium and Concanavalin A; (*V*) full medium and Concanavalin A, repeated retroviral induction after 24 hours; (*VI*) serum-free medium and Concanavalin A, with repeated retroviral induction after 24 hours. (*B*) Intracellular Foxp3 expression of the $CD4^+CD25^+$ Treg population 48 hours following transduction using the different protocols described above. Results show a representative experiment out of 5 performed.

Enrichment and Expansion of Retrovirally Transduced Tregs

Whereas a Tregs transduction level of 20%–40% could be reproducibly achieved using the above protocol,

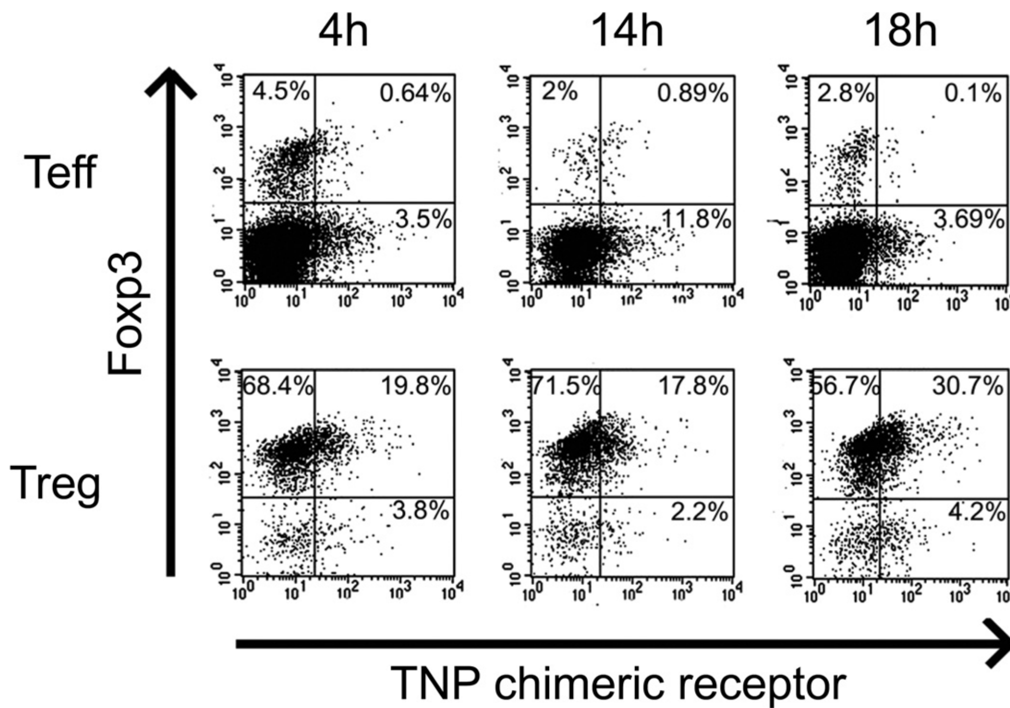

Figure 2. Optimal duration of preactivation of Teff and Treg cells during retroviral transduction. Following isolation, 2×10^6 Teff cells or Tregs were preactivated with 2 mg/mL soluble Concanavalin A for the indicated time periods. Activated cells were then retrovirally transduced with a vector encoding anti-TNP chimeric receptor. Following transduction, cells were incubated for 48 hours and then double stained for intracellular Foxp3 and surface expression of the anti-TNP TPCR. Data shown are from 1 representative experiment out of 3.

to obtain efficient suppressive activity of transduced Tregs in in vivo models of inflammation, further improvement of transduction efficiency was required. To enrich the fraction of TPCR-expressing nTreg, we incubated the retrovirally transduced Tregs on plastic-bound bovine serum albumin (BSA)-TNP, assuming that antigen-specific activation of posttransduction Tregs would induce specific proliferation of the desired transduced nTreg population. Indeed, as demonstrated in Figure 3*A*, a 6-day incubation period of transduced nTregs in the presence of BSA-TNP resulted in enrichment of the GFP-positive nTreg population to 80% on average, all transformed cells being uniformly positive for both Foxp3+ and the anti-TNP CR (Figure 3*B*). As early as 3 days after initiation of the incubation (Figure 3*C*), proliferation centers were observed throughout the BSA-TNP-coated plates, and, after 6 days, nearly all non-GFP-positive cells disappeared, whereas GFP-positive cells actively proliferated. Furthermore, this enrichment technique enabled the long-term expansion of transduced nTregs, reaching an average weekly expansion rate of more than 7-fold, while maintaining their Foxp3 and CR expression. TNP-TPCR-transduced and enriched nTregs could be repeatedly frozen at −80°C and thawed and readily regrown on TNP-bound plates, allowing for cell storage and retrieval (data not shown).

Retrovirally Transduced nTregs Demonstrate Antigen-Specific Suppressor Function, Both In Vitro and In Vivo

To demonstrate the in vitro suppressive activity of retrovirally transduced TNP-specific nTregs, 5×10^4 TNP-TPCR transgenic (TNP-Tg) Teff cells were cocultured with 2×10^4-irradiated, TNP-loaded CD11c+ mouse dendritic cells, in the presence of wild-type, TNP-Tg, or TNP-TPCR-transduced nTregs. As depicted in Figure 4*A*, wild-type Tregs exhibited no suppressive effect of TNP-Tg Teff cell proliferation. In contrast, both TNP-Tg and TNP-TPCR-transduced Tregs, upon activation by dendritic cells preloaded with TNP, induced comparable levels of significant and dose-dependent suppression of Teff cell proliferation. Similar results were obtained when Teff IFN-γ secretion was evaluated by enzyme-linked immunosorbent assay (ELISA) (Figure 4*B*). Suppression of the transduced Tregs was as effective as that of the native nTreg, derived from the TNP-Tg mouse, and could be achieved at up to 8:1 Teff/Treg ratios (Figure 4*C*).

To demonstrate the in vivo suppressive capabilities of TNP-TPCR-transduced nTregs, 1.5×10^5 wild-type, TNP-Tg, or TNP-TPCR-transduced nTregs were adoptively transferred into wild-type mice that were induced with acute TNBS colitis (Figure 5). Both TNP-Tg and TNP-TPCR-transduced nTregs induced comparable improvement (60%) in survival (Figure 5*A*), colonoscopy colitis score (Figure 5*B* and *C*), and macroscopic (Figure 5*D*) and microscopic (data not shown) colitis scores. TNP-TPCR-transduced nTreg protective effect was dose dependent, with mild survival advantage noted with as little as 5×10^4 TNP-TPCR-transduced nTregs, and with survival reaching 70% when 1.5×10^5 TNP-TPCR-transduced nTregs were transferred (Figure 5*E*). In contrast, transfer of as many as 2.5 ×

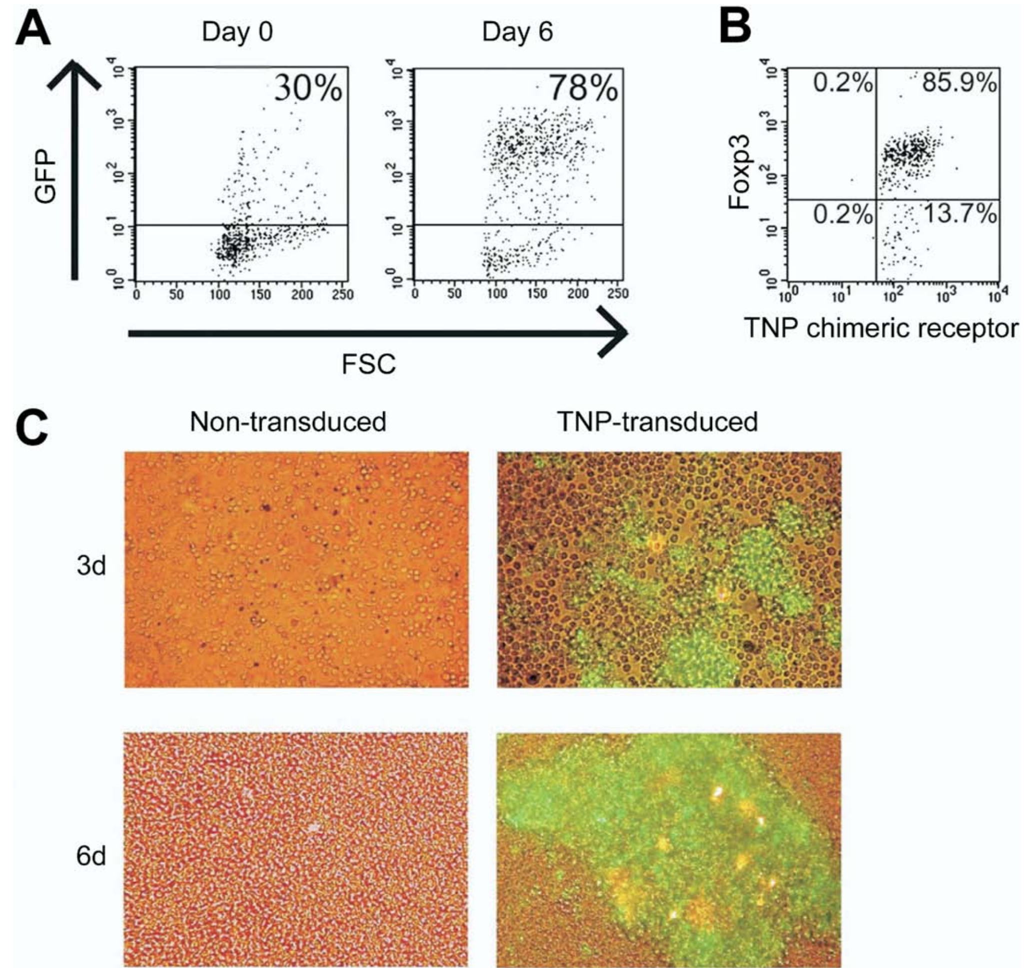

Figure 3. Enrichment and expansion of the TNP-TPCR-transduced Treg population. Following retroviral transduction with a vector encoding anti-TNP TPCR, Tregs were cultured on plastic-bound 50 μg/mL TNP-BSA. After 6 days of culture, (*A*) GFP expression and (*B*) intracellular Foxp3 and anti-TNP-TPCR expression were determined. (*C*) Appearance and GFP fluorescence of the enriched cell population after 3 and 6 days of culture.

10^5 wild-type nTregs was not associated with significant amelioration of disease severity or improved survival. Interestingly, reinduction of TNBS colitis in TNP-TPCR Treg-administered mice, 3 weeks following recovery from the first TNBS challenge, was associated with 75% survival, as compared with only 33% survival in WT-Treg-administered mice. These results demonstrate that TNP-TPCR-transduced nTregs are fully functional as suppressors of Teff cells and TNBS-induced inflammation both in vitro and in vivo, in an antigen-specific, dose-dependent, and persistent manner.

In Vitro Suppression Induced by TNP-Tg and TNP-TPCR-Transduced nTregs Is Mediated by IL-10-Independent, TGF-β-Independent, and Cell-Contact-Dependent Mechanisms

To understand the mode of suppression of TPCR-transduced nTregs, we cocultured TNP-Tg Teff cells, TNP-preloaded dendritic cells, and wild-type TNP-Tg or TNP-TPCR-transduced nTregs without exogenous IL-2 and measured the levels of cytokine secretion (Figure 6). Interestingly, IL-10 was found to be secreted by TNP-Tg Teff cells even in the absence of nTregs (Figure 6*A*), suggesting the type 2 helper T cells polarization of some or all of these Teff cells (Friedmann–Morvinski et al, submitted for publication). The fact that IL-10 secretion was inhibited by either TNP-Tg or TNP-TPCR-transduced Tregs, in concordance with Teff suppression, suggested that IL-10 is dispensable as a mediator of Teff suppression in our system. TGF-β levels seemed to positively correlate with the degree of antigen-specific Treg suppression (Figure 6*B*), yet the addition of neutralizing soluble TGF-β receptor reduced TGF-β to background levels but did not affect the suppression profile (thymidine uptake or the same experiment, Figure 6*C*). Coculture of TNP-TPCR-transduced TGF-β receptor knockout Teff cells with either TNP-TPCR-transduced wild-type or TNP-TPCR-transduced IL-10 knockout nTregs resulted in comparable levels of suppression, demonstrating that IL-10, TGF-β, or their combination were dispensable as mediators of Teff suppression in our system (Figure 6*D*). To test the possibility of Treg-mediated IL-2 deprivation (by binding to CD25) as a contributory suppressive mechanism (the "sink" hypothesis),[19] excess IL-2 (2000 U/mL) was added into

the coculture plates but resulted in no significant attenuation of antigen-specific suppression (Figure 6*E*).

In an attempt to determine whether cell-cell contact is required for suppression, similar coculture experiments were performed in transwells. Under these conditions, TNP-Tg Treg-mediated suppression was abolished, and TNP-TPCR-transduced Treg-mediated suppression of TNP-specific Teff was significantly attenuated (less than 40% suppression in average, Figure 6*E*) as compared with 75%–95% suppression when cell contact was allowed (Figure 4*A*). Similar results were obtained when Teff IFN-γ secretion was evaluated by ELISA (data not shown). Thus, these results suggest that IL-10 and TGF-β do not seem to play a major role in TNP-specific Teff suppression in vitro, whereas this suppression is partially cell-contact dependent.

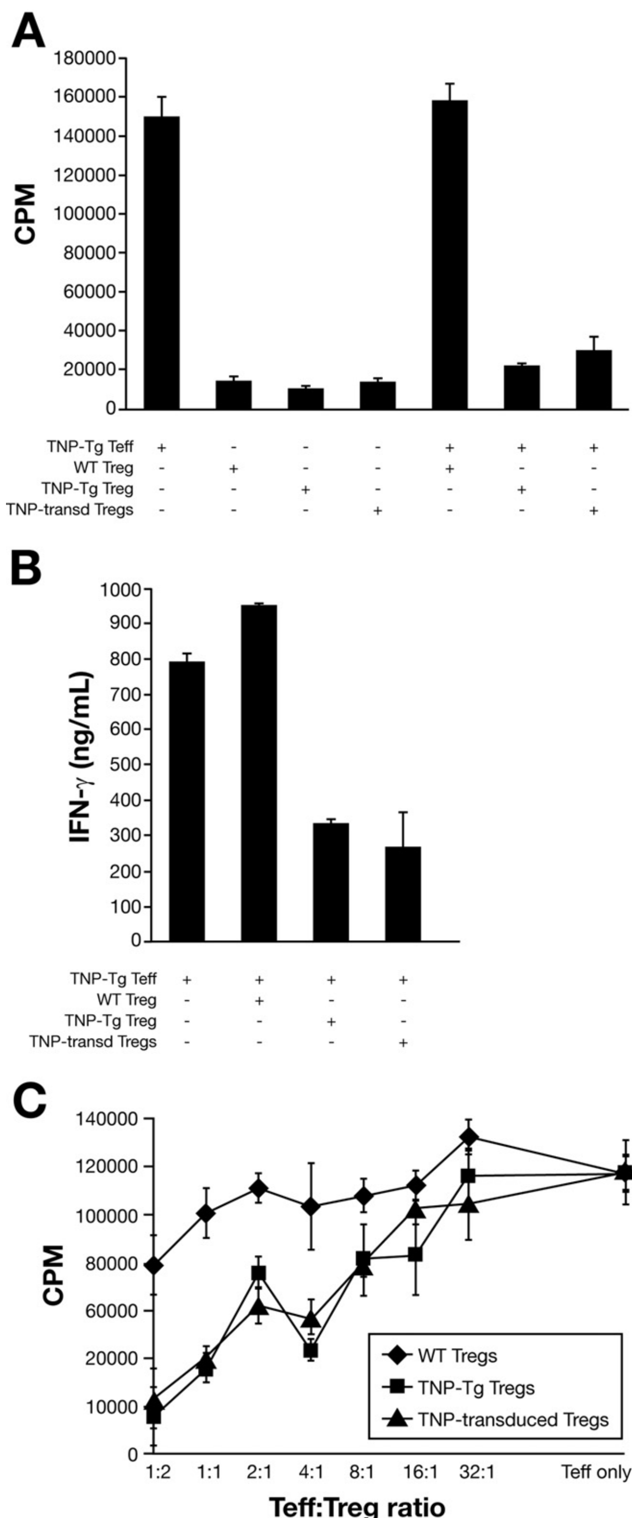

Figure 4. TNP-TPCR-transduced Treg antigen-specific suppression of Teff proliferation. (*A*) TNP-Tg Teff cells (5×10^4) were cocultured with wild-type (WT), TNP-Tg, or TNP-TPCR-transduced Tregs in the presence of 2×10^4 TNP-preloaded irradiated CD11c+ dendritic cells. Teff proliferation was measured after 72 hours by ^{3}H-Thymidine incorporation. Each group was cultured in quadruplicate, and the experiment was repeated 3 times. The data shown represent mean (±SD) of quadruplicate cultures of a single experiment representative of 3. TNP-Tg Teff cell proliferation was significantly lower when cocultured with TNP-Tg and TNP-TPCR transduced Tregs ($P < .05$). (*B*) IFN-γ levels from the experiment depicted in *A* were determined by ELISA. IFN-γ levels were significantly lower in TNP-Tg and TNP-TPCR transduced Treg coculture wells ($P < .05$). (*C*) TNP-Tg Teff cells (5×10^4) were cocultured with WT, TNP-Tg, or TNP-TPCR transduced Tregs (1×10^5 in the 2:1 ratio, and progressively reduced doses thereafter) in the presence of 2×10^4 TNP-preloaded irradiated CD11c+ dendritic cells. Teff proliferation was measured after 72 hours by ^{3}H-Thymidine incorporation. Each group was cultured in quadruplicate, and the experiment was repeated 3 times. The data shown represent mean (±SD) of quadruplicate cultures from 1 experiment representative of 3.

Discussion

In this study, we developed an efficient protocol for retroviral transduction, enrichment, and expansion of murine nTregs with a vector encoding an anti-TNP antibody-based CR to enable antigen-specific alleviation of acute experimental colitis. The TNP-TPCR-transduced Tregs maintain high Foxp3 expression and demonstrate antigen-specific suppressive activity in vitro that is TGF-β and IL-10 independent. In vivo, the redirected Tregs significantly alleviate acute TNBS colitis. Collectively, these data describe the preparation of highly specific and reactive Tregs that are suitable for research purposes and provide guidelines and protocols for future therapeutic applications not only for colitis but for other types of autoimmune inflammatory diseases.

Practically, efficient Tregs transduction requires different conditions from those used for Teff cells. Briefly, these conditions include a short (14–18 hours) preactivation of Tregs with Concanavalin A, incubation with serum-free medium, and supplementation with high doses of IL-2. Notably, prolonged preactivation, considered optimal for Teff transduction, resulted in massive Tregs cell death and consequently in poor transduction efficacy. Avoiding these "Teff favor-

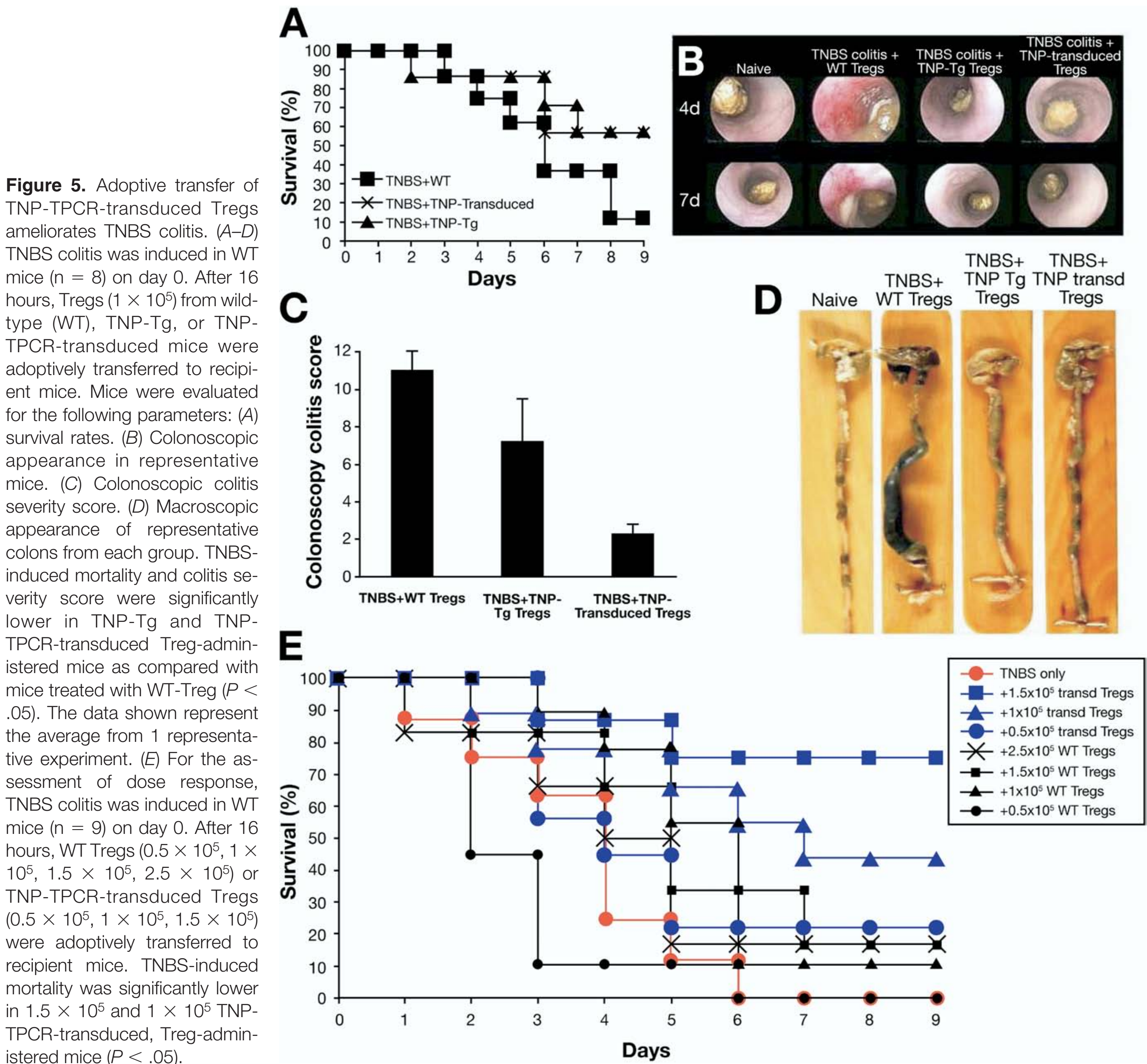

Figure 5. Adoptive transfer of TNP-TPCR-transduced Tregs ameliorates TNBS colitis. (*A–D*) TNBS colitis was induced in WT mice ($n = 8$) on day 0. After 16 hours, Tregs (1×10^5) from wild-type (WT), TNP-Tg, or TNP-TPCR-transduced mice were adoptively transferred to recipient mice. Mice were evaluated for the following parameters: (*A*) survival rates. (*B*) Colonoscopic appearance in representative mice. (*C*) Colonoscopic colitis severity score. (*D*) Macroscopic appearance of representative colons from each group. TNBS-induced mortality and colitis severity score were significantly lower in TNP-Tg and TNP-TPCR-transduced Treg-administered mice as compared with mice treated with WT-Treg ($P < .05$). The data shown represent the average from 1 representative experiment. (*E*) For the assessment of dose response, TNBS colitis was induced in WT mice ($n = 9$) on day 0. After 16 hours, WT Tregs (0.5×10^5, 1×10^5, 1.5×10^5, 2.5×10^5) or TNP-TPCR-transduced Tregs (0.5×10^5, 1×10^5, 1.5×10^5) were adoptively transferred to recipient mice. TNBS-induced mortality was significantly lower in 1.5×10^5 and 1×10^5 TNP-TPCR-transduced, Treg-administered mice ($P < .05$).

able" conditions decreases the risk of contaminating effector T cells that may have a proliferation and survival advantage and could have a deleterious effect, if introduced in vivo, by enhancing the autoimmune disorder.

Our finding that preactivation of murine Tregs with Concanavalin A is superior to that using anti-CD3/anti-CD28 antibodies may be due in part to the reduced secretion of IL-2 following Concanavalin A activation, which has been shown to bear tolerogenic effects on Teff cells.[20] In contrast, anti-CD3/anti-CD28 antibodies were suggested to effectively promote Tregs proliferation only in the presence of high levels of anti-CD28, while promoting enhanced activation-induced apoptosis, which is only partially preventable by high levels of IL-2.[21]

The use of a defined serum-free medium (Biotarget 1) obviates the need for serum and provides a clinically certified reagent for ex vivo manufacture of Tregs. The use of serum-free conditions has the added advantage of a reduction in the amounts of nonspecific growth factors and inhibitors of T-cell activation, elimination of lot to lot variation in serum preparations, and reduction in background levels of cytokines that may adversely influence Tregs activation and transduction efficacy.[22] The addition of high IL-2 doses to the retroviral transduction medium was required for successful nTreg transduction. IL-2 is the key cytokine responsible for survival signaling of nTregs[23] and has been suggested to activate STAT5 via the JAK1/3 kinase and to promote Treg survival and proliferation by transcriptional activation of D cyclins 2 and 3.[23,24]

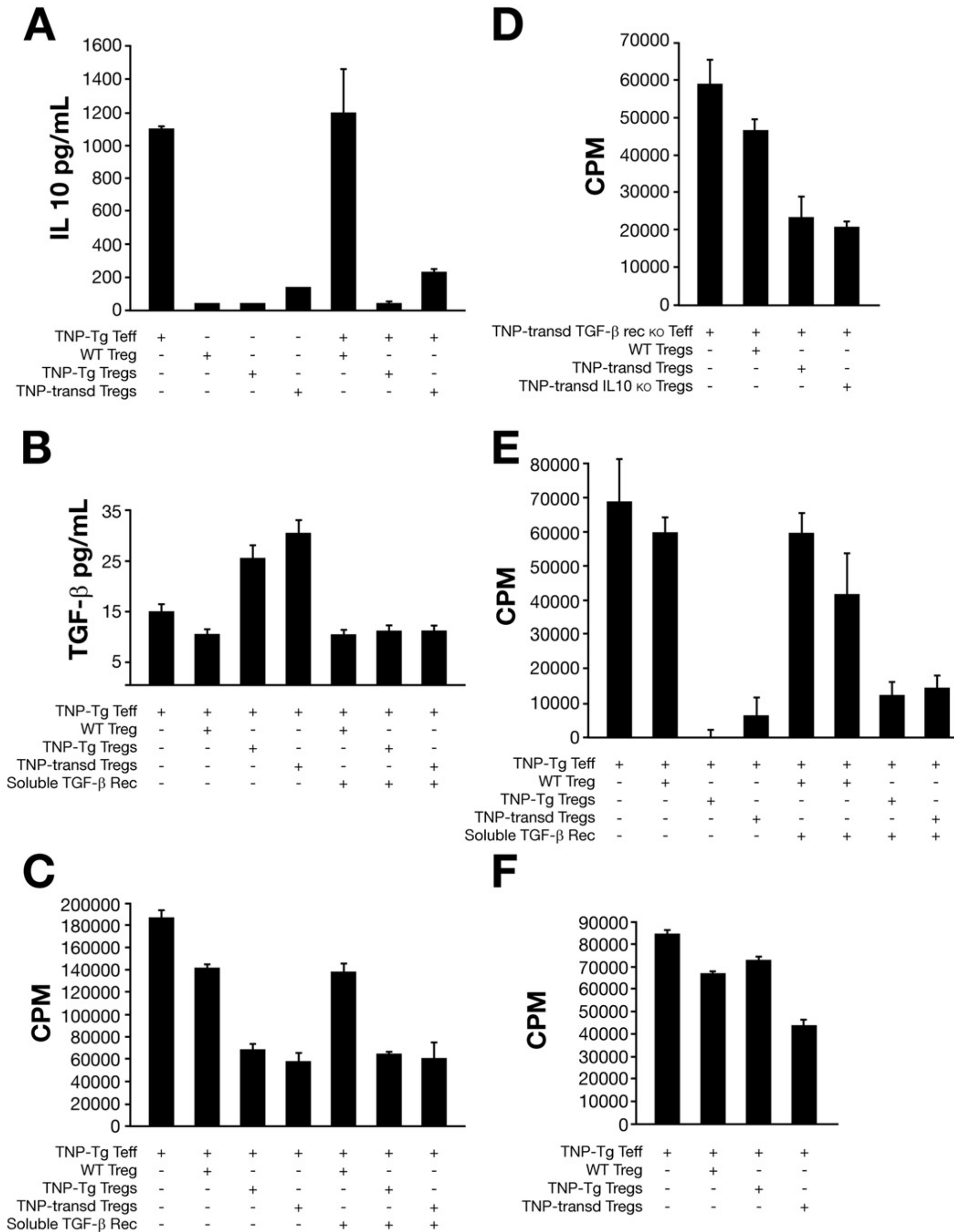

Figure 6. Mechanisms of suppression of TNP TPCR-transduced and TNP-Tg cells. (*A*) TNP-Tg Teff cells (5×10^4) were cocultured with wild-type (*WT*), TNP-Tg, or TNP-TPCR-transduced Tregs (5×10^4) in the presence of 2×10^4 TNP-preloaded irradiated CD11c+ dendritic cells. The IL-10 content in the supernatants was measured after 72 hours by ELISA and was significantly lower when Teff cells were cocultured with TNP-Tg and TNP-TPCR-transduced Tregs ($P < .05$). (*B* and *C*) Coculture experiments (as in *A*) with the addition of 10 μg/mL soluble TGF-β receptor to neutralize TGF-β. TGF-β secretion (ELISA; *B*) and Teff proliferation (^{3}H-Thymidine incorporation; *C*) were measured after 72 hours. TNP-Tg Teff cell proliferation, even after elimination of TGF-β, was significantly lower when cocultured with TNP-Tg and TNP-TPCR-transduced Tregs ($P < .05$). (*D*) Coculture experiments (as in *A*) using TNP-TPCR-transduced TGF-β receptor knockout (KO) CD4+ cells as effector cells that were cocultured with either wild-type, TNP-TPCR-transduced, or IL-10-knockout TNP-TPCR-transduced Tregs. Teff proliferation (^{3}H-Thymidine incorporation) was measured after 72 hours and was significantly lower when cocultured with either TNP-TPCR or IL-10-knockout TNP-TPCR-transduced Tregs ($P < .05$). (*E*) Coculture experiments (as in *A*) with and without addition of excess exogenous mouse IL-2 (2000 units/mL). Teff proliferation (^{3}H-Thymidine incorporation) was measured after 72 hours and was significantly lower when cocultured with TNP-Tg and TNP-TPCR-transduced Tregs, with or without IL-2 ($P < .05$). (*F*) To study the dependence of Tregs on intercellular contacts, coculture experiments were performed in transwells. Teff cells were incubated in the lower chamber and Tregs in the upper chamber, whereas 2×10^4 irradiated CD11c+ dendritic cells were added to both chambers to enable antigen-specific Teff and Tregs activation. Teff proliferation was measured after 72 hours by ^{3}H-Thymidine incorporation. The data shown represent mean (±SD) of quadruplicate cultures of a representative experiment of 3 performed.

In vitro, we demonstrated that suppression induced by TNP-TPCR-transduced Tregs, similar to native TNP-Tg Tregs, is mediated via pathways that are IL-10- and TGF-β independent. In contrast to the absolute need of IL-2 in the transduction phase, excess exogenous IL-2 had no effect on Treg suppression in the coculture experiments (Figure 6*E*), thereby ruling out the possibility of Treg IL-2 deprivation as a contributory suppressive mechanism (the "sink" hypothesis).[19] In contrast, both TNP-Tg and TNP-TPCR-transduced-Treg mediated suppression was partially contact dependent (Figure 6*F*). Indeed, the importance of contact dependency for nTregs suppression has been previously studied. Proximity-dependent mechanisms that have been suggested include Perforin–Granzyme-dependent Tregs lysis of Teff cells,[25,26] LAG-3-induced Teff suppression through interaction with MHC class II proteins,[27] and nTreg induction of Teff cell apoptosis via activation of the Fas-Fas ligand pathway[28–30] as well as contact-dependent repression of Teff cytolytic function.[31,32] Additional, less defined, mechanisms of suppression include Treg's repression of Teff migration by induction of reduced interferon-α-dependent CXCR3 expression[33] and induction of Teff anergy by inhibition of early IL-2 secretion.[34–36] Another potentially important Treg-related inhibitory cytokine is the recently discovered IL-35, which has been shown to potently inhibit Teff cell proliferation and activity.[37] Indeed, any combination of these Treg suppressive functions may play a role in TNP-TPCR-transduced Tregs antigen-specific suppression.[38] These diverse contact-dependent and -independent mechanisms, as well intracellular-signaling events following TPCR-antigen binding, merit further studies.

Our results have both research and therapeutic implications. For research use, the protocol we described herein enables the efficient retroviral transduction of murine nTregs, allowing the use of animal models to study nTreg generation, activation, homeostatic proliferation, and function by stable expression of proteins and RNAi. Whereas transduction of Teff cells with vectors encoding ectopic Foxp3 has been previously shown to confer a Treg phenotype,[13,39,40] the nTreg gene expression profile only partially overlaps that of cells ectopically expressing Foxp3+.[41] Thus, observations made on the basis of study of these Foxp3-modified Teff cells may now be confirmed in studies utilizing nTregs, using the transduction protocol we describe here. In addition, our approach allows for rapid and efficient selection of transduced antigen-specific Tregs, whose rarity and anergy preclude the use of conventional purification methods such as cell sorting and antibiotic selection.[14] We suggest that nTregs may be transduced with vectors coencoding the TNP-specific TPCR (or any other one) and additional genes of interest (through an internal ribosomal entry site, as we used here for the GFP marker). Successfully transduced cells can then be isolated on the basis of growth on plastic-coated antigen, allowing for expansion, purification, and subsequent experimentation with sufficient numbers of nTregs that strongly express the gene of interest.

Therapeutically, our approach allows for redirection of nTregs with specificity against any predetermined antigen in a clinical setting. This, along with the ensuing expansion and purification protocol, paves the road for future therapeutic application of Tregs therapy in inflammatory bowel disease, as well as other organ-specific and nonorgan-specific autoimmune disorders.[42,43] Compared with polyclonal Tregs, our approach allows for the use of smaller and easily obtainable Treg numbers for significant therapeutic effect (Figure 5*E*). The preferential survival of TNP-TPCR-transduced, Treg-administered mice even after a second TNBS challenge may point to the development of persistent tolerance. This phenomenon may suggest the potential of maintenance therapy, yet its nature and mechanisms need to be further investigated in future studies. As we previously showed, lack of known disease-causing antigens in most autoimmune disorders can be overcome by redirection of Tregs against a disease-associated or tissue-specific antigen, resulting in a "bystander" tissue-specific Treg activation and suppressive function.[12] In contrast to nonspecific approaches that necessitate massive nonantigen-specific nTreg expansion and subsequent transfer to diseased animals,[44,45] our approach is expected to require transfer of only small numbers of redirected nTregs to achieve a meaningful clinical effect. As such, it may be also used in the transplantation setting, in which redirected Tregs can be used to prevent and cure both rejection episodes and graft-vs-host disease.[46]

In summary, this report provides a proof of concept for retroviral transduction of nTregs that allows redirection of the specificity of Tregs by a TPCR targeting a predetermined model antigen, TNP. Such retrovirally transduced, TNP-redirected Tregs are specifically activated in a non-MHC, non-TCR, and non-costimulation-dependent manner; their mechanism of action is partially contact dependent and suppresses acute colitis in an antigen-specific manner. This approach may be valuable in directing Tregs to combat autoimmune disease even in cases when the inciting, pathogenic antigen(s) is, as yet, unknown.

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Conflicts of Interest

The authors disclose no conflicts.

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