Redirection of Regulatory T Cells With Predetermined Specificity for the Treatment of Experimental Colitis in Mice

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Background & Aims: **Treatment with ex vivo expanded regulatory T cells (Tregs) is regarded as a promising therapeutic approach in inflammatory bowel disease but is hampered by impaired Treg accumulation and function at inflammatory sites. We aim to study whether antigen-specific redirected Tregs can overcome these limitations.** ***Methods:*** **We developed transgenic mice whose T cells, including Tregs, express chimeric receptor (CR) made of antibody variable region as recognition unit and T-cell stimulatory and costimulatory domains to activate specifically in response to the predetermined model antigen 2,4,6-trinitrophenol (TNP).** ***Results:*** **TNP-specific CR-bearing Tregs were potently and specifically activated by exogenous TNP and suppressed effector T cells in the absence of costimulatory B7-CD28 interaction. TNP-specific transgenic (Tg) mice were resistant to 2,4,6-trinitrobenzene sulphonic acid (TNBS) colitis but not to other hapten-mediated colitis. Adoptive transfer of CR-bearing Tregs to wild-type mice with TNBS colitis was associated with significant amelioration of colitis and improved survival. Although TNP-specific CR-bearing Tregs did not suppress oxazolone colitis, they cured it after addition of traces of TNBS to oxazolone-inflamed colons, demonstrating a "bystander" effect. In vivo imaging of adoptively transferred CR-bearing Tregs demonstrated that they preferentially migrate to TNBS-induced colonic mucosal lesions within hours of induction of colitis.** ***Conclusions:*** **Tregs can be redirected with specificity distinct from that of pathogenic lymphocytes, accumulate at colonic inflammatory lesions, and suppress effector T cells in a specific, nonmajor histocompatibility complex-restricted, and noncostimulatory-dependent manner, resulting in significant amelioration of colitis. Hopefully, this approach will lead to a novel therapy for inflammatory bowel disease, as well as other inflammatory diseases.**

$CD4^+CD25^+$ (regulatory T cells [Tregs]) are a unique subpopulation of suppressive peripheral regulatory T cells that comprise 5%–10% of peripheral $CD4^+$ cells in mice and 3%–6% of $CD4^+$ cells in humans.[1] They are characterized by constitutive expression of the transcription factor foxp3 and of the inhibitory costimulatory molecule CTLA-4.[2] $CD4^+CD25^+$ cells are potent inducers of self-tolerance in animals and in humans. Aberration in the suppressive function of Tregs and in their accumulation within inflammatory colonic lesions has been suggested to be central in the pathogenesis of autoimmune colitis, and adoptive transfer of large numbers of nonantigen specific Tregs has been demonstrated to prevent or ameliorate experimental colitis in animal models.[3–5] Similarly, in experimental allergic encephalitis (EAE), adoptive transfer of myelin basic protein (MBP)-specific (but not of nonspecific) Tregs to MBP TCR-Tg $Rag^{-/-}$ mice prevents the onset of disease.[6] Indeed, adoptively transferred Tregs have been demonstrated to prevent other organ-specific autoimmune diseases including autoimmune thyroiditis, autoimmune gastritis, insulitis, and arthritis.[7] Because of the extreme scarcity of antigen-specific Tregs, many of these studies have employed adoptive transfer of nonspecific Tregs; alternatively, Tregs have been derived from transgenic mice expressing either a T-cell receptor (TCR) specific to a particular major histocompatibility complex (MHC)-peptide complex[8] or a chimeric immune receptor containing the same MHC:peptide autoantigen complex that is recognized by the autoimmune $CD4^+$ T cells.[9]

The repertoire and mode of action of Tregs has been extensively investigated. Most studies suggest that Treg activation is antigen specific and that their interaction with autoantigens is required for Treg maintenance in vivo.[10,11] On the other hand, the suppressor effector function of Tregs is not antigen specific and is mediated by suppressive cytokines such as transforming growth factor β (TGF-β) and interleukin (IL)-10,[12,13] which are released following antigen recognition by the Treg's TCR.

In this study, we demonstrate the efficacy of Tregs derived from transgenic mice expressing a genetically

Abbreviations used in this paper: CR, chimeric receptor; EAE, experimental allergic encephalitis; MBP, myelin basic protein; TNBS, 2,4,6-trinitrobenzene sulphonic acid; TCR, T-cell receptor; TPCR, tripartite chimeric receptor; Tregs, regulatory T cells.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.02.060

engineered anti-TNP scFv-CD28-FcRγ tripartite chimeric receptor (TPCR),[14,15] with specificity toward a predetermined colitis-associated antigen. Such a TPCR promoted antigen-specific, MHC-nonrestricted, and costimulation-independent Treg accumulation and activation at inflammatory sites, leading to cure of acute experimental colitis. When adoptively transferred into mice in which 2,4,6-trinitrobenzene sulphonic acid (TNBS) colitis was induced, these Tregs could indeed alleviate the disease. Moreover, our results support a "bystander" effect by demonstrating that Tregs specific to an antigen (TNP) distinct from the pathogenic/eliciting autoimmune antigen (oxazolone) can ameliorate acute oxazolone colitis, providing that the Treg-specific antigen is present at the inflamed site.

Materials and Methods

Antibodies, Cell Lines, and Reagents

Anti-mouse CD28 (37.51) was purchased from Southern Biotech (Birmingham, AL). Anti-CD25-PE, anti-Thy 1.2-APC (CD90.2), anti-CD3-APC, anti-CD4-PERCP, anti-CD4 FITC, and anti-CD8-FITC were purchased from BioLegend (San Diego, CA). Antiidiotypic monoclonal antibody (mAb) GK 20.5 (specific to the Sp6 anti-TNP) was a gift from the late Prof G. Kohler; 2C11, a hamster anti-mouse CD3 mAb, was kindly provided by Prof J. Bluestone (UCSF, San Francisco, CA). Foxp3 expression in lymphocytes was determined using FITC anti-mouse Foxp3 staining kit (Ebioscience, San Diego, CA) and FACS sorting.

P815, an H-2^d mastocytoma line that does not express the CD28 ligands B7.1 (CD80) and B7.2 (CD86), and a subclone that was transfected with B7 complementary DNA (cDNA) (kindly provided by Prof L. Lanier; UCSF, San Francisco, CA) were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS).

Animals

Experiments used transgenic mouse lines that specifically express a tripartite chimeric receptor (TPCR) with anti-TNP or anti-Erb B2 specificity under the control of a CD2 promoter, as well as a transgenic mouse line expressing anti-TNP CRs lacking the cytoplasmatic signaling CD28 domain (see Supplementary Figure 1 online at www.gastrojournal.org), were generated as described before and backcrossed to a Balb/c background (for at least 9 generations[14]). All invasive procedures were conducted under ketamine and xylazine general anesthesia (127.5 and 4.5 mg/kg, respectively). Subcutaneous injections were conducted under local anesthesia with 10% Xylocaine spray (Astra, Södertalje, Sweden).

Isolation of Tregs

Regulatory $CD4^+CD25^+$ lymphocytes were purified from bulk splenocytes by magnetic cell sorting ((Miltenyi Biotec, Inc, Auburn, CA). Purity of the enriched T-cell population was routinely more than 92%. In experiments in which highly purified (>99%) Treg and effector T-lymphocyte subpopulation were required, high-speed cell sorting was applied, using BD FACSaria cell-sorting system (Becton Dickinson Bioscience, Mountain View, CA).

Flow Cytometry

Lymphocytes (1×10^6) were incubated with the appropriate antibodies in staining buffer (5% FCS, 0.05% sodium azide in phosphate-buffered saline [PBS]) for 30 minutes on ice. Cells were spun down and washed with staining medium, resuspended in PBS, and analyzed by flow cytometry (FACSort, Becton Dickinson) and CellQuest software (Becton Dickinson). For Treg isolation using cell sorting, splenic lymphocytes were labeled with anti-CD4 and anti-CD25 and sorted by flow cytometry using the FACSaria fluorescence-activated cell sorter (Becton Dickinson). Treg purity using this method was routinely greater than 98%.

Effector and Treg T-Cell Proliferation

Isolated $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teff cells were cocultured in various ratios in stimulation medium (RPMI 1640 medium supplemented with 2 mmol/L glutamine, 10 mmol/L 2-mercaptoethanol, 100 μ/mL penicillin, 100 μg/mL streptomycin, and 10% FCS). Irradiated, T cell-depleted splenocytes were used as antigen-presenting cells (APC) in all coculture experiments. In nonantigen-specific activation experiments, 1 μg/mL Concanavalin A or anti-CD3 was used for Treg activation. In antigen-specific experiments, splenic-derived APCs were modified by TNBS. For proliferation assays, cells were cultured in a humidified atmosphere at 5% CO_2 at 37°C. Cells were harvested at various time points as indicated in the Results section. Proliferation was assessed by ^{3}H-Thymidine incorporation.

Induction of Hapten-Mediated Colitis

To induce hapten-mediated colitis, mice were sensitized with 150 mL of the haptenizing agent TNBS (Sigma–Aldrich, St Louis, MO) at a concentration of 2.5% in 50% ethanol by skin painting on day 1. On day 8, 150 μL of 1% TNBS in 50% ethanol was administered intrarectally via a 3.5-French catheter under general anesthesia. Oxazolone colitis was induced by sensitizing mice with oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma–Aldrich) at a concentration of 3% in 100% ethanol by skin painting on day 1, followed by intrarectal administration of 1% oxazolone in 50% ethanol on day 8.

Colitis was macroscopically assessed in all mice in each experiment using the following parameters: degree of colonic ulcerations, intestinal and peritoneal adhesions, wall thickness, and degree of mucosal edema. Each pa-

rameter was graded on a scale from 0 (completely normal) to 4 (most severe) by experienced examiners, blinded to the treatment protocol. For histologic evaluation of inflammation, distal colonic tissue (last 10 cm) was removed and fixed in 10% formaldehyde, and 5 paraffin sections from each mouse were stained with H&E. The degree of inflammation observed in microscopic cross sections of the colon was graded semiquantitatively from 0 to 4 as follows: grade 0, normal with no signs of inflammation; grade 1, very low level of leukocyte infiltration; grade 2, low level of leukocyte infiltration; grade 3, high level of infiltration with high vascular density, and bowel wall thickening; grade 4, transmural infiltrates with loss of goblet cells, high vascular density, wall thickening, and disruption of normal bowel architecture.

In the combined TNBS oxazolone experiments, wild-type (WT) mice were presensitized to oxazolone only and then were intrarectally administered with a mixture of 0.7%–1% oxazolone in 50% ethanol together with lower doses (0.3%) of TNBS.

Isolation of Colonic Lamina Propria Lymphocytes

To isolate colonic lamina propria lymphocytes, colonic mucosa were dissected, incubated with Ca^{2+} Mg^{2+}-free Hank's balanced salt solution containing 1 mmol/L dithiothreitol (Sigma–Aldrich) for 30 minutes to remove mucus, and then serially incubated twice in medium containing 0.75 mmol/L EDTA for 60 minutes. The supernatants containing the epithelium and intraepithelial lymphocyte population were removed and residual fragments pooled and treated with 2 mg/mL collagenase A (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington Biomedical) at 37°C for 2 hours. The cells were then pelleted twice through a 40% isotonic Percoll solution, followed by Ficoll-Hypaque density gradient (40%/75%) centrifugation.

Adoptive Transfer Experiments

In adoptive transfer experiments, mice were preconditioned with intraperitoneal cyclophophamide (200 mg/kg) 10 days prior to induction of colitis. Tregs (1×10^5) were intraperitonealy injected into mice 24 hours following induction of colitis. In imaging experiments, each mouse received 1×10^6 Tregs.

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.[16] The endoscopic procedure was viewed on a color monitor and digitally recorded. Video files were processed with the Windows moviemaker software (Microsoft, Israel). Endoscopic quantification of colitis was performed as previously described,[16] namely, 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).

In Vivo Imaging

To follow trafficking of redirected effector T cells and Tregs, the whole body cooled CCD camera system was used (IVIS 100 Series Imaging System; Xenogen, Alameda, CA). Redirected Tregs were labeled using the near-infrared lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR; Invitrogen). This dye, having absorption and fluorescence maxima at 750 and 782 nm, respectively, enabled the safe direct labeling of membranes of lymphoid cells with very low light absorption and autofluorescence levels in living tissues.[17] Quantification of average radiance was performed using the Living Image software.

Additional visualization of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Tregs within the colonic mucosa was performed by intrarectal insertion of a 650-μm diameter fluorescent microendoscope (Cell Vizio; MKT, Paris, France). This modality allowed the repeated in vivo assessment of CFSE-labeled redirected Treg homing to the most inner colonic layer following induction of colonic inflammation. To allow for better background visualization of colonic mucosa, 100 μL Fluorescin (diluted 1:5000) was intravenously injected to mice 2 minutes prior to their examination.

Statistical Analysis

Statistical evaluation was performed using the unpaired Student *t* test or analysis of variance (ANOVA) 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

Mice Expressing TNP-Specific CR Transgene Are Protected From Colitis Induced by TNBS but Not by Oxazolone

To determine whether antigen-specific, genetically redirected Tregs can treat colitis, we first studied whether the naturally occurring Tregs in transgenic (Tg) mice that express TNP-specific scFv-CD28-FcRγ TPCR could prevent the development of acute TNBS-mediated colitis. The construct used to generate the transgenic mice was described before[14] and is schematically depicted in online Supplementary Figure 1 (see Supplementary Figure 1 online at www.gastrojournal.org). The scFv was derived from the Sp6 anti-TNP antibody linked to the extracellular, transmembrane, and cytoplasmic domains of the human CD28 (excluding the B7 binding site) linked to the FcR γ signaling domain. Expression of the TPCR

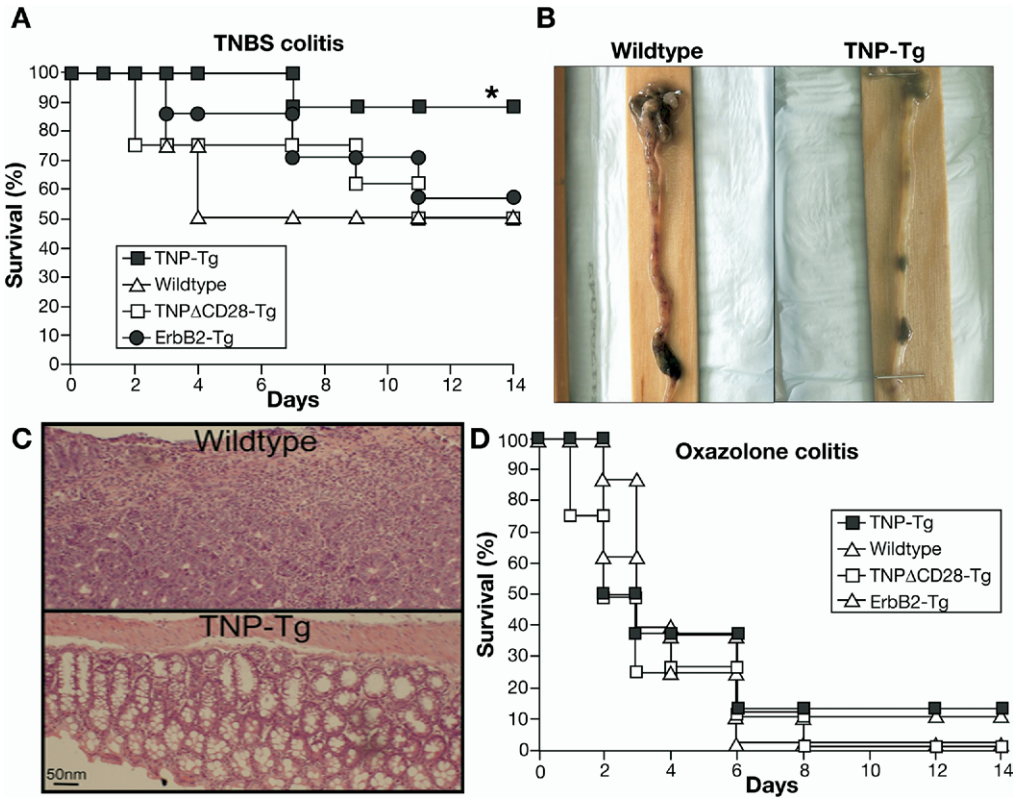


Figure 1. TNP-Tg mice are resistant to TNBS colitis. (*A–C*) TNBS colitis was induced in wild-type (WT), TNP-Tg, TNP-CD28Δ-Tg, and erbB2 Tg mice (n = 8) by intrarectal instillation of TNBS at day 0. (*D*) As a control for Treg specificity, colitis was induced using unrelated hapten, oxazolone, which was intrarectally instilled in similar strains of mice (n = 10). (*A* and *D*) Survival curves following disease induction. (*B*) Macroscopic appearance of a representative colon from a WT mouse (*left*) vs a TNP-Tg mouse (*right*) following induction of TNBS colitis. (*C*) Microscopic appearance of the colons from *B*. Results represent 1 experiment out of 3. Differences in TNBS-induced mortality between TNP-Tg and WT and TNP-CD28Δ-Tg and erbB2-Tg mice were significant (*$P < .05$).

construct in Tg mice was driven by the CD2 promoter/enhancer, which directed its expression only in T and natural killer cells. As shown in Figure 1, induction of TNBS colitis in the TNP-Tg mice resulted in a significantly reduced mortality rate (10%) in comparison with a mortality rate of approximately 50% among WT mice with TNBS-induced colitis, Tg mice expressing an erbB2-specific CR, or TNP-CD28Δ-Tg mice lacking the CD28 activation domain ($P = .03$, Figure 1*A*). Induction of more severe TNBS colitis (150 μl of 1.5% TNBS in 50% ethanol) resulted in 90% mortality in WT, TNP-CD28Δ-Tg, and erbB2-Tg mice, as opposed to only 20% mortality among TNP-Tg mice ($P < .01$, data not shown). Macroscopically, colons of WT, TNP-CD28Δ-Tg, and erbB2-Tg mice were graded 12.4 ± 2.4, 13.0 ± 2.3, 12.9 ± 1.2, respectively, whereas those of TNP-Tg mice were graded as 3.2 ± 1.2 ($P < .01$, Figure 1*B*). Microscopically (Figure 1C), colons of WT, TNP-CD28Δ-Tg, and erbB2-Tg mice exhibited dense transmural lymphocytic inflammatory infiltrate, loss of normal colonic architecture, mucosal erosions, and submucosal necrosis (microscopic colitis score of 3.2 ± 0.8, 3.4 ± 0.8, and 3.2 ± 1, respectively), in contrast to colons from TNP-Tg mice, which exhibited normal morphology or only mild inflammation (0.8 ± 0.4, $P < .01$).

Further evidence for the specificity of the protection from hapten-mediated colitis was obtained by comparing oxazolone-induced colitis in TNP-Tg mice with that in WT, TNP-CD28Δ-Tg, and erbB2-Tg mice. As demonstrated in Figure 1*D*, a comparable rate of mortality was obtained for all groups ($P = .89$). The same was true to macroscopic and microscopic colitis scores (data not shown).

The percentage of TNP-specific Tregs in the spleens of the TNP-Tg mice is shown in Figure 2. In TNP-Tg mice, over 99% of Foxp3$^+$ Tregs express the TNP-specific CR (Figure 2*A*). All Foxp3$^+$ TNP-specific Tregs express CD3 (Figure 2*B*) as well as the endogenous TCR. In naïve TNP-Tg mice, the percentage of peripheral Foxp3$^+$ T cells is significantly increased in comparison with WT mice. Induction of TNP colitis results in further increase in Foxp3$^+$ TNP-specific Tregs (Figure 2*C*).

Regulatory T Cells Bearing CR Specifically Suppress the Activity of Effector T Cells in an MHC and CD28 Independent Manner

Because the CR gene in our transgenic mice is expressed under the CD2 promoter, most of the mature T cells coexpress both the TNP-specific CR and their endogenous TCR. Accordingly, as shown in Figure 2, virtually all Foxp3 cells from these mice expressed the TPCR. To assess whether T cells from the TNP-Tg mice retain their "classic" functions, both effector (CD4$^+$CD25$^-$) and regulatory (CD4$^+$CD25$^+$) T cells from WT and TNP-Tg mice were isolated, and the ability of the Treg to suppress effector activity was tested in vitro. Tregs from both WT and TNP-Tg mice completely suppressed the Concanavalin A-mediated polyclonal proliferation (Figure 3*A*) and anti-

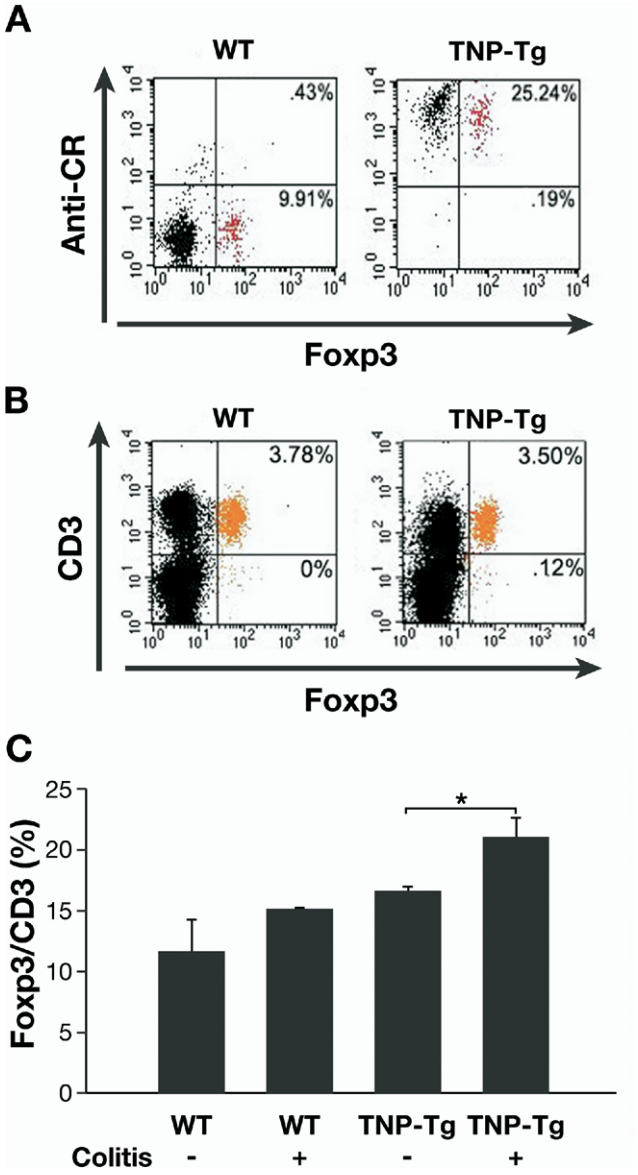
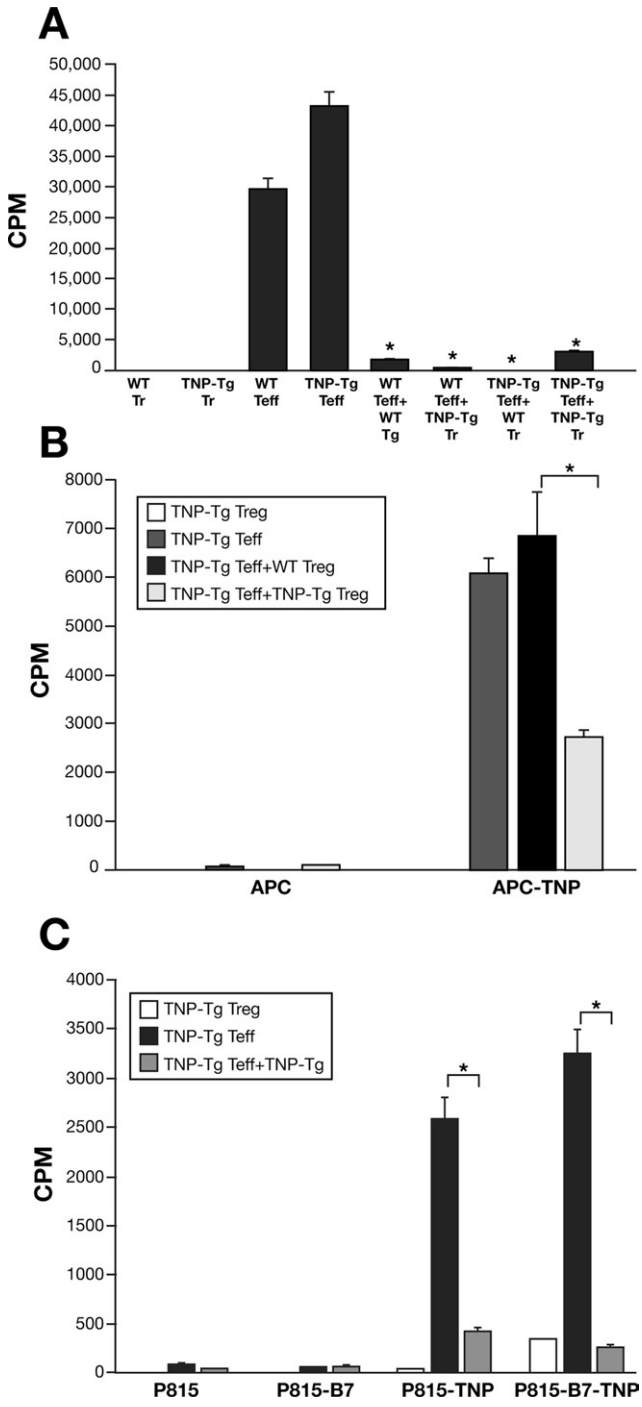


Figure 2. Expression profile of Tregs in TNP-Tg mice. Data shown are from representative analysis for an individual mouse out of 5 tested mice. (*A*) Splenocytes isolated from WT and TNP-Tg mice were stained for CD3, TNP-specific chimeric receptor using anti-idiotypic antibody to the Sp6 scFv, and for intracellular Foxp3. Analysis of gated $CD3^+$ T cells is shown. Percentages indicate $CD3^+Foxp3^+CR^-$ cells (*lower right quadrant*) and $CD3^+Foxp3^+CR^+$ (*upper right quadrant*). (*B*) FACS analysis of splenocytes double stained for CD3 and Foxp3. *Percentages* indicate $CD3^+Foxp3^-$ cells (*lower right quadrant*) and $CD3^+Foxp3^+$ (*upper right quadrant*). (*C*) Percentage of $Foxp3^+$ splenocytes in the total $CD3^+$ T-cell population. Splenic lymphocytes were isolated from WT and TNP-Tg mice prior to or 48 hours following induction of TNP colitis and double-stained with anti-Foxp3 and anti-CD3 antibodies. The percentage of $Foxp3^+$ lymphocytes in the $CD3^+$ population is presented as the average Foxp3/CD3 ratio ± SD of each 5-mouse group. Data shown are averages of 2 independent experiments performed. Differences in ratios between naïve and colitis-induced TNP-Tg mice were significant (*$P < .05$).



CD3-mediated polyclonal proliferation (data not shown) by both WT and TNP-Tg effector T (Teff) cells. In contrast, only TNP-Tg and not WT Tregs suppressed the specific proliferation of the TNP-Tg Teff in a TNP-specific manner (Figure 3*B*).

To assess the role of costimulatory signaling in our TPCR-Tg model, coculture experiments were performed using, as APCs, TNP-modified P815 stimulator cells that do not express B7 or P815 cells into which the B7 gene was stably transfected (Figure 3*C*). Stimulation of

Figure 3. Specific activation of TNP-Tg Tregs and their suppression of effector T cells requires TNP and CD28 costimulation. (*A*) Nonspecific (Concanavalin A) activation of Tregs. WT or TNP-Tg Tregs (1×10^5) were cocultured with WT or TNP-Tg Teff (1×10^5) in the presence of irradiated, T-cell depleted, splenic APC (3×10^5) and 1 μg/mL Concanavalin A. Teff proliferation was measured after 48 hours by ^{3}H-Thymidine incorporation. (*B*) Specific (TNP) activation of Tregs. WT or TNP-Tg Tregs (5×10^4) were cocultured with WT or TNP-Tg Teff (5×10^4) in the presence of irradiated, T-cell depleted, TNPylated splenic APC (1.5×10^5). Teff proliferation was measured after 48 hours by ^{3}H-Thymidine incorporation. (*C*) Coculture experiments were performed as in *A* using APC irradiated P815 mastocytoma cells (1.5×10^5) that were either stably transfected (or not) with B7 cDNA. Teff cell proliferation was measured after 48 hours by ^{3}H-Thymidine incorporation. Each group was cultured in triplicate, and the experiment was repeated 3 times. The data shown represent mean (±SD) of triplicates of a representative experiment. Differences in CPM between Teff+WT Tregs and Teff+TNP-Tg Tregs were significant (*$P < .01$).

TNP-Tg Teff cells with both TNP-modified APC resulted in strong proliferation regardless of B7 expression, whereas the addition of TNP-Tg Tregs resulting in potent inhibition of Teff-cell proliferation, regardless of B7 expression. Notably, such in vitro B7-independent Treg-induced suppression did not occur when TNP-CD28Δ-Tg Tregs were used (data not shown). These results suggest that stimulation through the CD28 cytoplasmatic signaling domain of the TPCR enables full Treg activation leading to suppression of Teff proliferation, regardless of the presence of B7-CD28 costimulation.

Adoptively Transferred TNP-Tg Tregs Protect Wild-Type Mice From TNBS Colitis

One of the most attractive clinical applications of antigen-specific, redirected Tregs is their administration to patients with autoimmune inflammatory diseases. To demonstrate the feasibility of this approach as therapeutic modality, we adoptively transferred small numbers (1×10^5) of WT or TNP-Tg Tregs to WT mice 24 hours after induction of TNBS colitis. Only the TNP-Tg Tregs, but not the WT Tregs, conferred protection from colitis, manifested as reduction in mortality rate (25% mortality in TNP-Tg administered mice vs 60% in WT-Treg administered mice and 60% in non-Treg administered mice, $P = .02$, Figure 4*A*). Similarly, a significant improvement was noted in macroscopic colitis severity score (3.5 ± 0.7 vs 12.6 ± 1.2 and 12 ± 1.5, respectively, Figure 4*B* and *C*), as well as the microscopic colitis score (1.4 ± 0.9 vs 3.0 ± 1.5 and 3.1 ± 1, respectively, Figure 4*D*). In similar experiments, adoptive transfer of 1×10^5 TNP-CD28Δ-Tg Tregs to WT mice did not have any protective effect against acute TNP colitis (data not shown). Collectively, these data indicate that antigen-specific triggering of Tregs results in their potent in vivo activation and suppression of inflammation.

Administration of TNP-Tg Tregs Specific to a Bystander Antigen, TNBS, Cures Colitis Mediated by a Pathogenic Antigen, Oxazolone

In contrast to hapten-mediated colitis, in which the eliciting antigen is predefined, the disease-causing antigen in inflammatory bowel disease is unknown. To enable application of the "T body" approach in inflammatory bowel disease, we tested whether naïve TPCR-bearing Tregs can potentially be activated to induce their antigen-nonspecific suppressive function by a predetermined "bystander" colon or colitis-associated antigen. To this end, we presensitized WT and TNP-Tg mice to oxazolone only and then luminally introduced a mixture of oxazolone and very low doses of TNBS (0.3%, in the absence of TNBS presensitization). Using this approach, we expected a predominant oxazolone-induced T helper cell 2 activity, while the coapplication with minute TNBS quantities served as means of Treg attraction to the inflamed site. TNBS administration at a low dose and in the absence of presensitization was associated with no colitis development and no mortality (data not shown). As is shown in Figure 5*A*, concomitant challenge of WT mice with TNBS and oxazolone was associated with a 100% 12-day mortality rate, as compared with only a 37.5% 12-day mortality of TNP-Tg mice ($P < .01$). Endoscopically, a significant mucosal inflammation was evident in both WT and TNP-Tg mice with oxazolone colitis (data not shown) and was most severe in WT mice with TNBS+oxazolone (13.9 ± 0.6; Figure 5*B*, *box I*; and see only Supplementary Video 1 online at www.gastrojournal.org) resulting in severe bleeding, fibrin deposition, and sloughing off of colonic mucosa. In utter contrast, TNP-Tg mice administered TNBS+oxazolone featured normal-appearing colonic mucosa with scattered areas of mild colitis (6.5 ± 1.5, $P < .01$; Figure 5*B*, *box II*; and see Supplementary Video 1 online at www.gastrojournal.org). Both macroscopically and microscopically, colons of concomitantly TNBS and oxazolone-treated WT mice also featured severe colitis, as opposed to near-normal colons in TNP-Tg mice (macroscopic colitis score of 4.6 ± 1.2 vs 14 ± 1, $P < .01$, and microscopic score of 1.7 ± 0.8 vs 3.5 ± 0.5, $P < .01$, for TNP-Tg vs WT mice, respectively; Figure 5*C* and *D*).

Notably, this "bystander" protective effect also occurred when TNP-Tg Tregs were adoptively transferred to oxazolone-presensitized WT mice that were intrarectally boosted with a mixture of oxazolone and low doses of TNBS (43% survival vs 12% survival in oxazolone and TNP colitis mice, Figure 5E, $P < .01$). In contrast, adoptively transferred WT Tregs did not have curative effect (12% survival, $P = .8$), and, again, the very low TNBS doses in the absence of presensitization were insufficient by themselves to induce TNBS colitis (100% survival). These results demonstrate that Treg activation by a bystander antigen (TNBS) may improve colitis induced by a different noncross-reactive antigen (oxazolone).

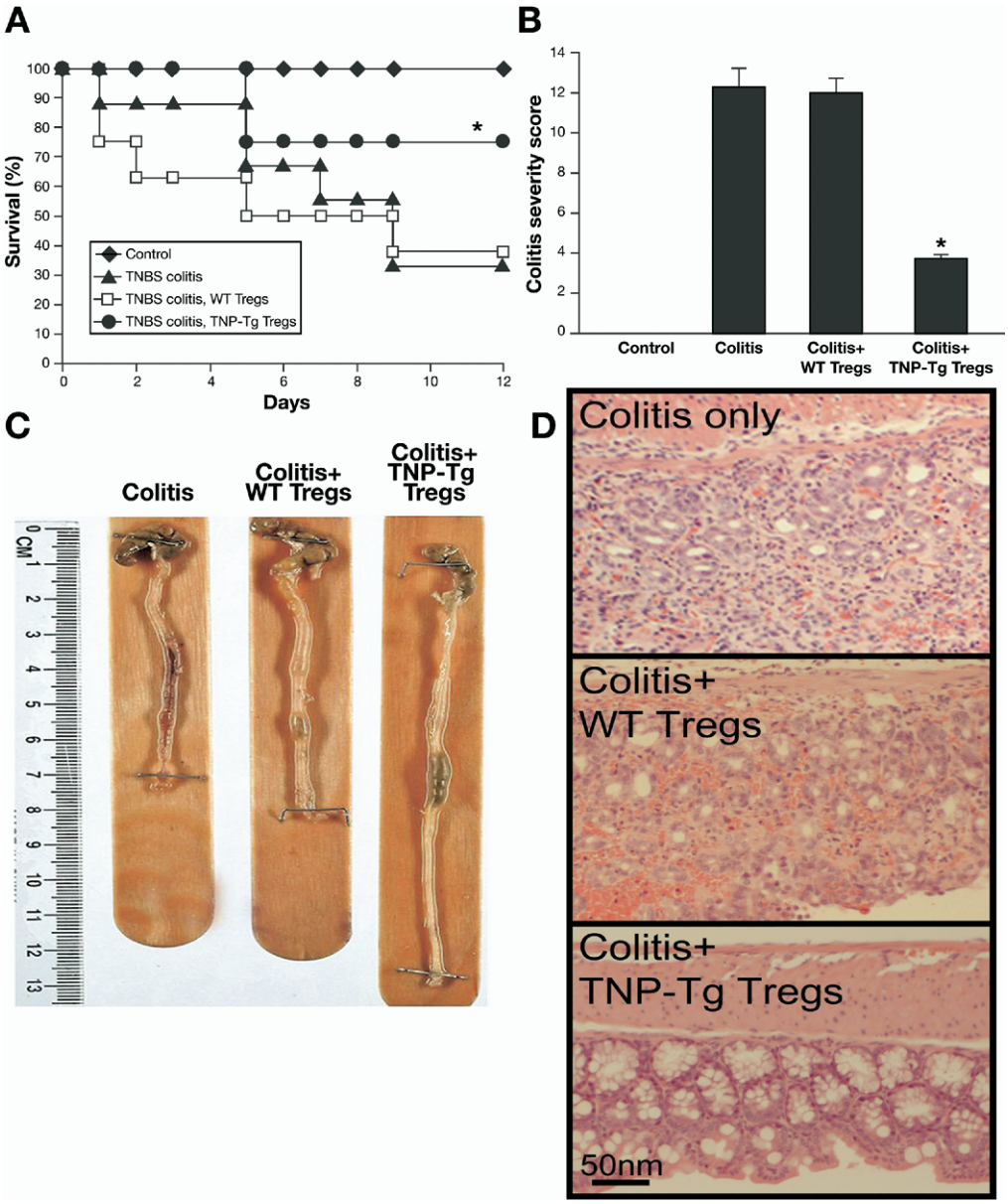


Figure 4. Adoptive transfer of TNP-Tg Tregs ameliorates TNBS colitis. TNBS colitis was induced in WT mice (n = 8) on day 0. After 16 hours, Tregs (1×10^5) from TNP-Tg or WT mice were adoptively transferred to the recipient mice. Each experiment was repeated 3 times. The data shown represent the average of a representative experiment. (*A*) Survival rates. (*B*) Disease severity score of the various mouse groups. (*C*) Macroscopic appearance of representative colons from various mouse groups. (*D*) Microscopic appearance of colons shown in *C*. TNBS-induced mortality and colitis severity score were significantly lower in TNP-Tg Treg-administered mice as compared with noncell and WT-Treg-administered mice (*$P < .05$).

Adoptively Transferred TNP-Tg Tregs Accumulate in TNP-Sensitized Colon

A prerequisite for Treg therapy is their localization and accumulation in diseased organs, in which they exert their suppressive effects. To study whether TNP-Tg Tregs localize to colons afflicted with TNBS-induced colitis, we labeled Treg cells with the DiR lipophilic carbocyanine dye, adoptively transferred the labeled cells, and monitored their accumulation daily, employing the IVIS camera (Figure 6*A*). An anterior-abdominal fluorescent signal, reflecting the bulk of injected cells in Treg-administered mice and colonic autoflurescence in colitis-induced mice, was noted in all mice 0–48 hours following intraperitoneal Treg administration. Thereafter, this signal disappeared in the control groups. In mice with colitis that received labeled WT Tregs, a weak lower abdominal fluorescent signal could be recognized following Treg transfer. In contrast, a distinctive and persistent signal was seen only in TNP-Tg Treg recipients with TNBS colitis, featuring a sustained abdominal fluorescent signal for up to 1 week following cell transfer, with an average radiance significantly higher than that of mice receiving labelled WT Tregs at all time points (10.33 ± 1.53 vs 4.8 ± 0.72 at 72 hours, respectively, $P = .01$; 6.23 ± 1.25 vs 1.83 ± 0.76 at 96 hours, respectively, $P = .01$; 4.5 ± 0.26 vs 0 ± 0 at 120 hours, respectively, $P < .001$; Figure 6*B*).

To obtain the Treg accumulation pattern at earlier time points (when background staining prevents accurate quantification using the DiR method), WT and TNP-Tg Tregs were labeled with CFSE and transferred into WT mice 24 hours following induction of TNBS colitis. At

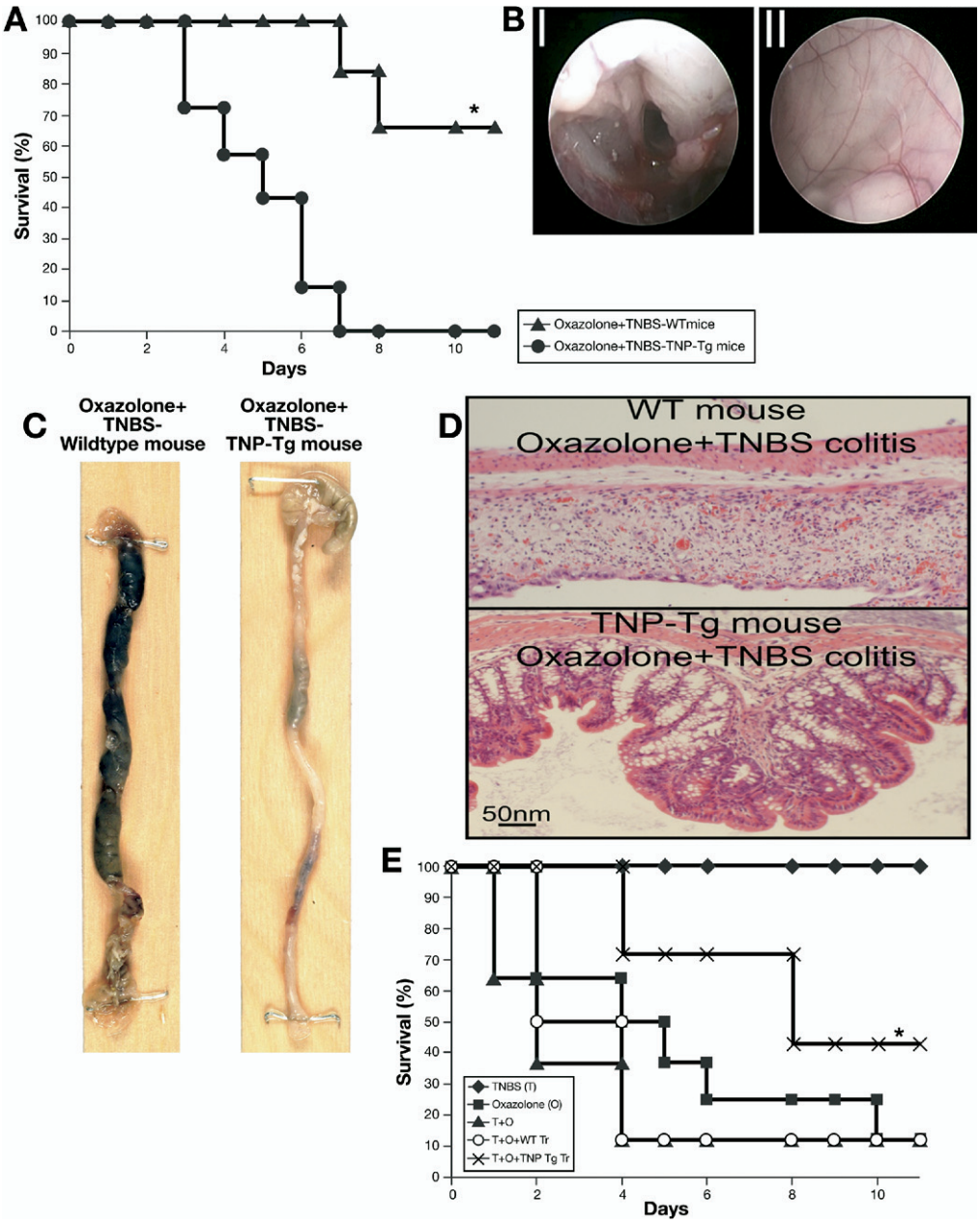


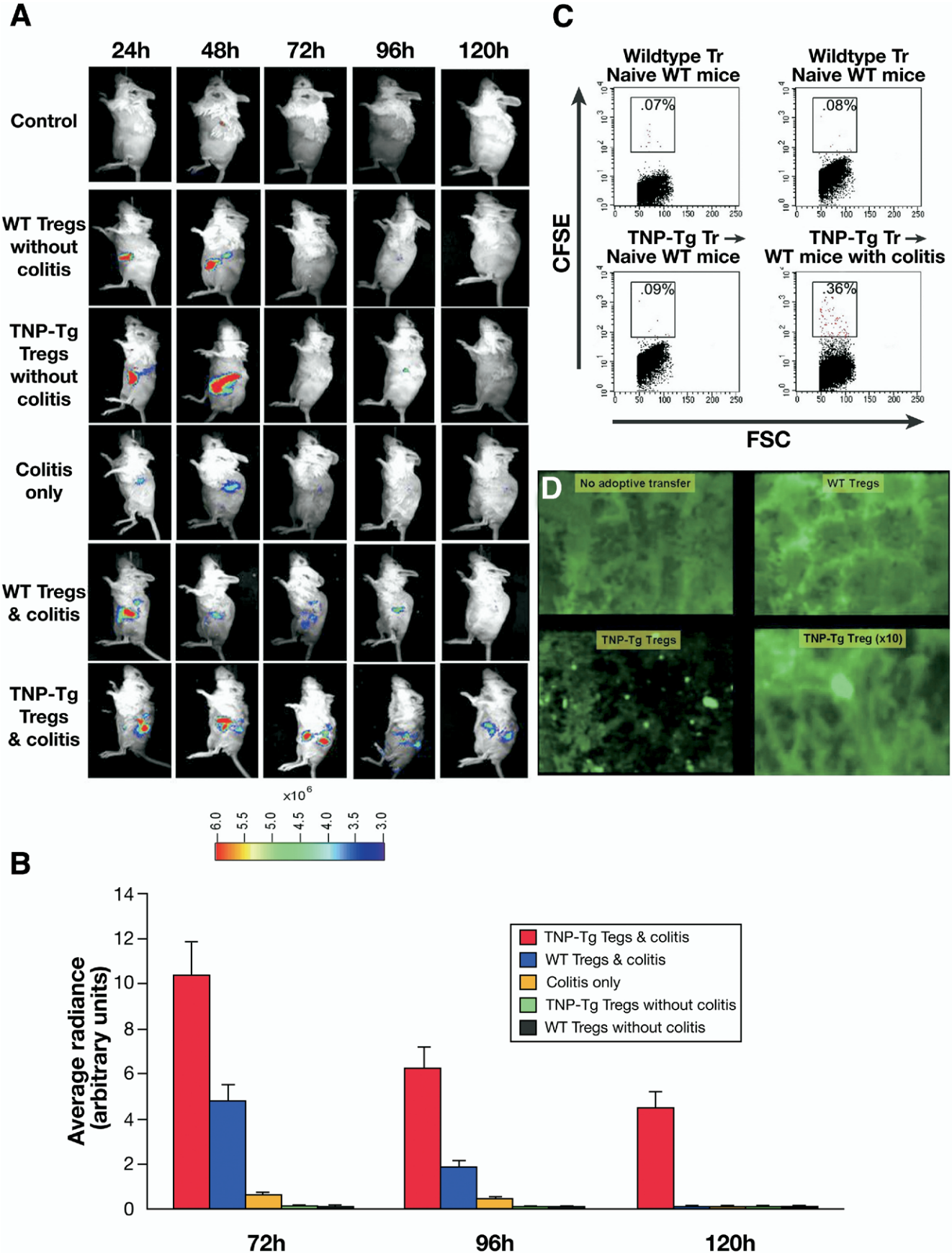
Figure 5. Intrarectal administration of TNBS results in TNP-Tg Treg-mediated protective effect from oxazolone colitis. (*A*) Mortality rates of wild-type and TNP-Tg mice administered oxazolone ± low doses of TNBS, 1 week following presensitization with oxazolone only. (*B*) Murine colonoscopy images of representative WT and TNP-Tg mice. (*C*) Macroscopic appearance of representative colons from various mouse groups. (*D*) Microscopic appearance of colons shown in *C*. (*E*) Adoptive transfer of Tregs (Tr) to oxazolone (O) presensitized mice induced 1 week later with oxazolone (O) colitis in the presence of low dose of TNBS (T). WT or TNP-Tg Tregs were administered to mice (n = 8) 16 hours after the induction of colitis. Oxazolone-induced mortality was significantly reduced in TNP-Tg mice (*A*) and TNP-Tg Treg-administered mice (*E*) as compared with WT mice and WT Treg-administered mice (*$P < .05$).

various time points, the colons were removed, and cell suspensions were prepared from the excised tissue. Labeled cells were analyzed by FACS. Very small numbers of CFSE-labeled WT Tregs were noted in colonic lamina propria lymphocytes of naïve or TNBS colitis-induced mice. In contrast, as early as 16 hours following induction of TNBS colitis (Figure 6*C*), a 4-fold increase in TNP-Tg Tregs was noted among the lymphocytes extracted from the lamina propria, and this elevation in cell number was maintained for 96 hours (data not shown).

To determine whether TNP-Tg Tregs reach the inner colonic mucosal layer, where most of TNBS-induced mucosal damage takes place, we employed the Cell Vizio microendoscopy system (Figure 6*D* and see Supplementary Video 2 online at www.gastrojournal.org). Numerous adoptively transferred CFSE-labeled TNP-Tg, but not WT Tregs, could be visualized in the inner mucosal layer of WT mice with TNBS colitis as early as 12 hours following Treg transfer. The highest number of intramucosal Tregs was visualized 48 hours following adoptive transfer. Taken together, these results indicate that antigen-specific, redirected Tregs localize to colonic antigens within hours of their administration and that the cells reach the deepest colonic mucosal layers, where they exert their potent suppressor functions.

Discussion

In this study, we employed Tregs expressing an antibody-based CR to specifically alleviate acute experimental


A
24h
48h
72h
96h
120h
Control
WT Tregs without colitis
TNP-Tg Tregs without colitis
Colitis only
WT Tregs & colitis
TNP-Tg Tregs & colitis
x10^6
6.0
5.5
5.0
4.5
4.0
3.5
3.0
B
Average radiance (arbitrary units)
14
12
10
8
6
4
2
0
72h
96h
120h
TNP-Tg Tegs & colitis
WT Tregs & colitis
Colitis only
TNP-Tg Tregs without colitis
WT Tregs without colitis
C
Wildtype Tr Naive WT mice
.07%
Wildtype Tr Naive WT mice
.08%
TNP-Tg Tr Naive WT mice
.09%
TNP-Tg Tr WT mice with colitis
.36%
CFSE
FSC
D
No adoptive transfer
WT Tregs
TNP-Tg Tregs
TNP-Tg Treg (x10)

colitis. We demonstrate that (1) Tregs expressing an anti-TNP TPCR are specifically and functionally activated through their engineered receptor; (2) this activation leads to potent suppression of Teff-cell proliferation in a B7-independent manner; (3) naïve, TNP-specific Tregs can protect mice from TNBS-induced colitis; (4) when activated by their predetermined antigen, TNP, redirected Tregs can, through a "bystander effect," cure colitis induced with a different antigen, oxazolone; (5) adoptive transfer of naïve TNP-Tg Tregs leads to their accumulation in colonic mucosa and suppression of colitis. Thus, this study lays the foundation for a novel approach to the treatment not only of colitis but of other types of autoimmune inflammatory diseases. The accumulation of the adoptively transferred TNP-Tg Tregs in the colonic mucosa of the recipient WT mice with TNP colitis proves that these cells enter and are retained at the site of inflammation, a prerequisite step to the execution of their suppressive activity.

It has been demonstrated that adoptive transfer of antigen-specific Tregs prevents spontaneous EAE.[6] However, once EAE develops, antigen-specific Tregs that reach the inflamed site fail to undergo activation and thus do not prevent the expansion and function of pathogenic effector lymphocytes. This effect in EAE was suggested to be mediated by enhanced IL-6 and TNF production by effector T cells.[18] In our model, adoptively transferred redirected antigen-specific Tregs preferentially accumulate within the site of inflammation and are capable of both preventing and ameliorating the inflammatory process when administered after its induction. This profound advantage of redirected Tregs over TCR-specific Tregs can be attributed to the costimulatory activity of the CD28 domain that is an integral part of the antigen-specific CR independently of extracellular B7-CD28 interaction.

CD28-CD80/86 (B7.1/7.2) costimulatory signaling has been demonstrated to play an important role in Treg homeostasis, proliferation, and survival.[19,20] Administration of superagonistic anti-CD28 antibodies was shown to support Treg expansion in vivo, whereas the absence of CD80/86 expression on dendritic cells (in double knockout mice or in mice treated with anti-CD80/CD86 antibody), results in impaired proliferative capacity of adoptively transferred Tregs.[21,22] In EAE, CD80 expression on B cells is required for late Treg activation and suppression of inflammation.[23] However, although high CD80 levels are detected on APCs at early stages of the disease (when pathogenic Teff cells are at their peak of activity), later in disease when Treg activation is expected, a marked reduction in MHC-II and CD80 expression occurs.[24] These observations suggest that, in addition to the inhibitory effect of the local inflammatory milieu on Tregs, lack of sufficient costimulatory signaling may contribute to impaired Treg function. In our system, Treg activation at the inflamed site is facilitated by the CD28 costimulatory domain included in the CR construct. This enables a full Treg activation upon TPCR binding to antigen in a manner independent of the native CD80/86-CD28 interaction.

One of the major advantages of an antibody-based CR approach over approaches such as those employing a preformed MHC-II:peptide complex recognizable by the TCR of the effector cells[12] is that the antibody-based CR enables Treg activation in an MHC-independent manner. In addition, this approach enables specific activation of Treg by predefined bystander antigens expressed within the target tissue itself, even in the absence of known pathogenic antigens. Inflammatory bowel disease is an excellent example of an autoimmune process that affects individuals with broad HLA diversity and in which disease-associated antigens have not yet been identified. Thus, in diseases such as inflammatory bowel disease, a therapeutic approach that is specific for antigen and MHC-II cannot be readily implemented. The antibody-based approach, on the other hand, allows for full Treg activation regardless of MHC compatibility. Moreover, as was demonstrated in our study, activation of redirected Tregs toward tissue-specific bystander antigens (trace levels of TNBS) rather than toward the disease-associated antigen (oxazolone) results in a "bystander" local Treg activation and suppressive function regardless of the disease-eliciting antigen.

Mechanisms of Treg suppressive function involved in colitis are diverse and nonmutually exclusive including IL-10, TGF-β, cell contact, CTLA-4, LAG-3, IDO, Perforin/Granzyme, GITR agonists, and IL-35 and most likely a combination of several of these mechanisms.[25] Indeed, the diversity of mechanisms of TNP-Tg Treg

Figure 6. Adoptively transferred TNP-Tg Tregs localize in inflamed colonic mucosa. (*A*) In vivo imaging of WT mice receiving DiR-labeled wild-type and TNP-Tg Tregs (1×10^6) 16 hours following induction of TNP colitis (n = 3). Mice were subjected to a whole body imaging (IVIS 100 Series Imaging System) at 12-hour intervals. A single representative mouse out of 3 in each group is shown at all time points. Two independent experiments were performed, with similar results. (*B*) Quantification of abdominal fluorescent emission of a representative IVIS experiment at 3 time points following absorbance of IP-administered Tregs (72–120 hours). Results are given as average radiance. At all time points, average radiance of TNP-Tg Treg-administered mice was significantly higher than all other mouse groups (*$P < .01$) (*C*) Levels of adoptively transferred, CFSE-labeled WT and TNP-Tg Tregs extracted from colons of recipient WT mice. Labeled Tregs were intraperitonealy injected 24 hours following induction of TNBS colitis. The Figure depicts Treg accumulation 16 hours following adoptive transfer. Data shown represent the percentages of CFSE-positive cells in the corresponding gates of 1 representative mouse of each 4-mouse group. Each experiment was repeated twice. (*D*) In situ fluorescent microendoscopic (Cell Vizio) evaluation of CFSE-labeled Tregs accumulating at the colonic preluminal mucosal layer. The experimental design is identical to the one described in *B*. The Figure shows representative frames taken 48 hours following adoptive transfer. Each group consisted of 4 mice, and each experiment was repeated twice.

CR-mediated suppression, although not fully investigated in this study, is most likely mediated through the same mechanisms as mediated by the native TCR and warrants further investigation.

In summary, this report provides a proof of concept for redirection of the specificity of regulatory T cells by a tripartite CR targeting a predetermined model antigen, TNP. Such TNP-redirected Tregs are specifically activated in a non-MHC- and non-TCR-dependent manner. They localize to the TNP hapten presented within the inflamed colon and suppress colitis even when disease is induced through a different antigen. 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.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2008.02.060.

References

1. Jonuleit H, Schmitt E, Stassen M, et al. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001;193:1285–1294.
2. Chen W, Jin W, Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor β (TGF-β) production by murine CD4(+) T cells. J Exp Med 1998; 188:1849–1857.
3. Maul J, Loddenkemper C, Mundt P, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005;128:1868–1878.
4. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med 2000;192:295–302.
5. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 2003;170:3939–3943.
6. Hori S, Haury M, Coutinho A, et al. Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T-cell receptor transgenic mice. Proc Acad Sci U S A 2002;99:8213–8218.
7. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155:1151–1164.
8. Skapenko A, Kalden JR, Lipsky PE, et al. The IL-4 receptor α-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. J Immunol. 2005;175:6107–6116.
9. Mekala DJ, Alli RS, Geiger TL. IL-10-dependent infectious tolerance after the treatment of experimental allergic encephalomyelitis with redirected CD4+CD25+ T lymphocytes. Proc Natl Acad Sci U S A 2005;102:11817–11822.
10. Hsieh CS, Zheng Y, Liang Y, et al. An intersection between the self-reactive regulatory and nonregulatory T-cell receptor repertoires. Nat Immunol 2006;7:401–410.
11. Tachnot C, Vasseur F, Pontoux C, et al. Immune regulation by self-reactive T cells is antigen specific. J Immunol 2004;172: 4285–4291.
12. Asano M, Toda M, Sakaguchi N, et al. Autoimmune disease as a consequence of developmental abnormality of a T-cell subpopulation. J Exp Med 1996;184:387–396.
13. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2:389–400.
14. Friedmann-Morvinski D, Bendavid A, Waks T, et al. Redirected primary T cells harboring a chimeric receptor require costimulation for their antigen-specific activation. Blood 2005;105:3087–3093.
15. Eshhar Z, Waks T, Bendavid A, et al. Functional expression of chimeric receptor genes in human T cells. J Immunol Methods 2001;248:67–76.
16. Becker C, Fantini MC, Wirtz S, et al. In vivo imaging of colitis and colon cancer development in mice using high-resolution chromoendoscopy. Gut 2005;54:950–954.
17. Kalchenko V, Shivtiel S, Malina V, et al. Use of lipophilic near infrared dye in whole body optical imaging of hematopoietic cell homing. J Biomed Opt 2006;11:0505071–3.
18. Korn T, Reddy J, Gao W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 2007;13:423–431.
19. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12:431–440.
20. Tang Q, Henriksen KJ, Boden EK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J Immunol 2003;171:3348–3352.
21. Beyersdorf N, Gaupp S, Balbach K, et al. Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. J Exp Med 2005;202:445–455.
22. Yamazaki S, Iyoda T, Tarbell K, et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. J Exp Med 2003;198:235–247.
23. Mann MK, Maresz K, Shriver LP, et al. B-cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. J Immunol 2007;178:3447–3456.
24. Juedes AE, Ruddle NH. Resident and infiltrating central nervous system APCs regulate the emergence and resolution of experimental autoimmune encephalomyelitis. J Immunol 2001;166: 5168–5175.
25. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. Trends Mol Med 2007;13:108–116.

Received October 18, 2007. Accepted February 14, 2008.

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Supported in part by the Moross Cancer Center at the Weizmann Institute of Science and the joint Weizmann Institute-Tel Aviv Medical Center Research Fund.

The authors thank Dr Alon Harmelin for histologic analyses; Dr Vyacheslav Kalchenko for his excellent help with the in vivo imaging experiments; Professor Daniel Gazit and Dr Yoram Zilberman for their help with experiments using the Cell Vizio system; Dr Shelley Schwarzbaum for editorial work; Professors Markus Neurath and Zamir Halpern for their support with the murine colonoscopy system; Keren Francis Koifmann for statistical processing of the data; and Einat Finkelstein, Meirav Katz, and Nitzan Adam for their technical assistance.

Conflicts of interest: The authors declare no competing financial interests.