

Generation of Antigen-Specific, Foxp3-Expressing CD4⁺ Regulatory T Cells by Inhibition of APC Proteasome Function¹

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We tested the hypothesis that immature APC, whose NF- κ B-signaling pathway and thus maturation was blocked by the proteasome inhibitor benzyloxycarbonyl-isoleucyl-glutamyl(*O*-tert-butyl)-alanyl-leucinal (PSI), could be a source of Ag-specific regulatory T (Treg) cells. DO11.10 CD4⁺ T cells that were incubated with Ag- and PSI-pulsed APC proliferated poorly, produced less IL-2, IFN- γ , and IL-10 in secondary cultures, and inhibited the response of both naive and memory CD4⁺ T cells stimulated by Ag-pulsed APC. The generation of PSI-APC Treg cells required IL-10 production by APC. PSI-APC Treg cell inhibition required cell-cell contact but not IL-10 or TGF- β . Addition of IL-2 did not reverse, but Ab to CTLA-4 did reverse partially the inhibitory effect. Depletion of CD25⁺ T cells before initial culture with PSI-APC did not affect Treg generation. PSI-APC Treg cells expressed high levels of Foxp3, inhibited proliferation of naive DO11.10 T cells *in vivo*, and abrogated colitis driven by a memory Th1 response to bacterial-associated Ag. We conclude that NF- κ B-blocked, immature APC are able to induce the differentiation of Treg cells that can function *in vitro* and *in vivo* in an Ag-specific manner. *The Journal of Immunology*, 2005, 174: 2787–2795.

The inhibitory activity of regulatory T (Treg)³ cells is believed to be central to the prevention of autoimmune and chronic inflammatory diseases. Multiple types of Treg cells appear to exist, including CD4⁺CD25⁺ T cells, T regulatory-1 (Tr1) cells, Th3 cells, and vitamin D₃/dexamethasone-induced, IL-10-producing T cells. Treg cells mediate their effects via immunosuppressive cytokines or by contact-dependent mechanisms (1–5). CD4⁺CD25⁺ T cells are one of the best-characterized populations of Treg subsets in both humans and rodents. After TCR triggering, CD4⁺CD25⁺ Treg cells inhibit immune responses *in vivo* and *in vitro* in an Ag-nonspecific and MHC-nonrestricted manner (4, 6–9). CD4⁺CD25⁺ Treg cells are anergic and express intracellular CTLA-4, a costimulatory receptor that delivers a negative or off signal to T cells (6, 10). They can express also the glucocorticoid-induced TNFR (11, 12). Recently, the forkhead/winged helix transcription factor gene *Foxp3* has been shown to program CD4⁺CD25⁺ T cell development and function (13–16).

The ability of Treg cells, particularly the CD4⁺CD25⁺ subset, to control responses to autoantigens is well established. The role of these cells in regulating the adaptive response to environmental Ags such as those of the enteric microbiota is unclear. High doses of CD4⁺CD25⁺ T cells were able to prevent (17) and to treat (18)

disease in a model of experimental colitis in immunodeficient RAG^{-/-} mice, a pathology known to involve CD4 Th1 cell reactivity to the enteric bacterial flora. Tr1 cells can prevent experimental colitis as well (1). These studies did not address the mechanism of immune regulation of the adaptive T cell response to the bacterial flora in normal hosts. We previously reported the presence of CD4⁺ cells in the normal murine intestine that had the properties of Tr1 cells, producing high amounts of IL-10 when stimulated with bacterial Ag-pulsed APC (5). These CD4⁺ Tr1 cells were generated *in vitro* using high amounts of exogenous IL-10, conditions not likely to be met *in vivo*. In this study, we asked whether immature dendritic cells (DC) could generate Ag-specific Treg cells because the intestine is known to have a high turnover of DC, and thus, immature DC-T cell interactions are likely to occur in this microenvironment. We used NF- κ B blockade of DC to ensure their immaturity, based on reports that such blockade prevents the transition from immature to mature DC (19–21). We report here that benzyloxycarbonyl-isoleucyl-glutamyl(*O*-tert-butyl)-alanyl-leucinal (PSI) treatment down-regulated DC MHC class II, CD86, and CD40 expression and inhibited IL-12 but not IL-10 production. These DC were impaired in their ability to stimulate naive CD4⁺ T cell responses to Ag and induced CD4⁺ T cells to develop into regulatory cells. These Treg cells, termed PSI-APC Treg cells, expressed Foxp3 and inhibited naive and memory T (Tmem) cell responses by cell-cell contact interactions rather than by soluble factors. Such inhibition was abrogated by anti-CTLA-4 mAb. More importantly, PSI-APC Treg cells inhibited T cell activation *in vivo* and prevented colitis in an Ag-driven animal model of inflammatory bowel disease.

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Received for publication August 13, 2004. Accepted for publication December 21, 2004.

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¹ This work supported by a research grant from Sankyo Company Ltd. and National Institutes of Health Grants DK60132 and DK064400.

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³ Abbreviations used in this paper: Treg, regulatory T; Tr1, T regulatory-1; DC, dendritic cell; PSI, benzyloxycarbonyl-isoleucyl-glutamyl(*O*-tert-butyl)-alanyl-leucinal; Tmem, memory T; BMDC, bone marrow-derived DC; SN, supernatant; KLH, keyhole limpet hemocyanin.

Materials and Methods

Mice

BALB/c were obtained from The Jackson Laboratory. DO11.10 and DO11.10.RAG-2^{-/-} OVA TCR-transgenic mice were bred in the Animal Facility at the University of Alabama. Female mice of 6- to 12-wk-old were used in these experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama.

Reagents and materials

Reagents and materials were purchased from the following sources. PSI was purchased from Calbiochem. GM-CSF was a gift kindly provided by DNAX (Palo Alto, CA). Anti-CD4, CD25, and CTLA-4 mAb were purchased from BD Biosciences. Anti-glucocorticoid-induced TNFR and anti-TGF- β were purchased from R&D Systems. Anti-IL-10R1 was a gift from Dr. K. Moore (DNAX).

CD4⁺ T cell purification

CD4⁺ T cells were isolated by using anti-mouse CD4-magnetic beads (BD Pharmingen). Briefly, spleen or mesenteric lymph node cells were washed twice and incubated with anti-CD4-beads at 4°C for 30 min and then separated by magnetic field. When checked by flow cytometry, >95% of the cells were CD4⁺ T cells.

Generation of bone marrow-derived DC (BMDC)

Bone marrow cells were isolated by flushing the marrow cavities of mouse femurs with ice-cold RPMI 1640 medium and gently refluxing the expelled cell plug through a 25-gauge needle to form a single-cell suspension (22). Bone marrow cells from at least 10 femurs isolated from five mice were pooled for each experiment and suspended at 1×10^6 cells/ml in complete RPMI 1640 medium containing 10% heat-inactivated FCS (Atlanta Biologicals), 25 mM HEPES, 2 mM sodium pyruvate (BioWhittaker), 50 mM 2-ME, 2 mM L-glutamine (Mediatech), 100 U/ml penicillin, and 100 mg/ml streptomycin (Mediatech). The cells were cultured in the presence of 20 ng/ml GM-CSF in T-75 flasks (Corning Glass) in 5% CO₂ in humid air. The nonadherent cells were collected at day 6 of culture and put back into culture in media containing 20 ng/ml GM-CSF. After 3 additional days of culture, nonadherent cells were collected and washed three times with fresh media. More than 95% of the adherent cells were CD11c⁺, CD3⁻, and B220⁻ when checked by flow cytometry. The cells were plated at $1 \times 10^6/0.5$ ml per well in 48-well plates (Costar) in the presence of various agents as indicated. After incubation at 37°C in 5% CO₂ and humid air for 24 h, supernatants (SN) were collected, centrifuged to remove contaminating cells, and stored at -70°C until analysis of cytokines. The cells were stained for flow cytometric analysis.

Generation of PSI-APC Treg cells

BMDC generated from BALB/c mice were treated with 1 μ M PSI dissolved in DMSO for 4 h and then 5 μ g/ml OVA peptide for an additional 20 h at 1×10^6 /ml. After washing twice, the pretreated BMDC were added at 1×10^5 /ml into cultures of 1×10^6 freshly isolated CD4⁺ T cells from DO11.10.RAG2^{-/-} mice. Seven days later, the CD4⁺ T cells were reisolated with CD4-magnet beads (BD Pharmingen) and used to test for regulatory activity (PSI-APC Treg). Freshly isolated CD4⁺ T cells from DO11.10.RAG2^{-/-} mice were also cultured with 5 μ g/ml OVA peptide-pulsed BMDC as a control and used as Tmem cells.

Generation of a keyhole limpet hemocyanin (KLH)-specific CD4⁺ T cell line

BALB/c mice were immunized with 100 μ g of KLH in CFA i.p. twice at day 1 and day 14. The mice were sacrificed 7 days later, and splenic CD4⁺ T cells were isolated and stimulated with KLH-pulsed APC for a week, then restimulated every 2 wk for several cycles.

T cell proliferation assay

CD4⁺ T cells at 1×10^5 cells/well were incubated in triplicate in the presence of 4×10^5 Ag-pulsed spleen cells as APC in the wells of a 96-well plate at 37°C in 5% CO₂-humidified air. After 4 days of incubation, 0.5 μ Ci of [³H]thymidine was added to each culture for the last 18 h of the incubation period. The cells were harvested on glass fiber filters on a PHD cell harvester (Cambridge Technology), and proliferation was assessed as the amount of incorporation of [³H]thymidine into cell DNA, as measured by beta scintillation counting of the harvested samples. Data are expressed as cpm \pm SD.

Cytokine assays

CD4⁺ T cells were stimulated in the presence of OVA peptide plus irradiated spleen cells as APC. The culture SN were collected at different times and pooled together for assay. SN collected after 24 h of culture were used for IL-2 assay, and SN collected at 72 h of culture were used for IL-10, IL-4, and IFN- γ assays. The cytokine content in SN was determined by ELISA as described previously (23).

Measurement of nuclear NF- κ B

BMDC were treated with media alone, 1 μ g/ml LPS, or LPS plus 1 μ M PSI for 60 min. The nuclear extracts were made by using the TransFactor Extraction kit (BD Clontech) based on the protocol recommended by the manufacturer. NF- κ B p65 was measured by using Mercury TransFactor kits (BD Clontech) according to manufacturer's protocol.

Flow cytometric analysis

After washing in PBS with 0.1% sodium azide plus 2% heat-inactivated newborn calf serum, the cells were incubated with various FITC-, Red 670-, or PE-conjugated mAbs, washed, and fixed in 1% buffered paraformaldehyde. A total of 1.5×10^5 stained cells was quantitated using a FACStar flow cytometer (BD Biosciences). A FITC- or PE-labeled mAb of the same isotype but irrelevant specificity was used as a negative control in all experiments.

Analysis of Foxp3 mRNA expression

Expression of FoxP3 on T cells was detected using real-time PCR with the primers 5'-GGCCCTTCTCCAGGACAGA-3' and 5'-GCTGATCATGGCTGGGTGT-3' at a final concentration of 800 nM and a FAM-labeled internal probe, 5'-ACTTCATGCATCAGCTCTCCACTGTGGAT-3', at a final concentration of 150 nM. As an endogenous reference, β_2 -microglobulin was simultaneously measured using primers 5'-CCTGCAGAGTAAAGCATGCCAG-3' and 5'-TGCTTGATCACATGTCTCGATCC3-3' (final concentration of 30 nM) and a Texas Red-labeled internal probe 5'-TGGCCGAGCCCAAGACCGTCTAC-3' (final concentration of 50 nM). All primers and probes were obtained from Integrated DNA Technologies. Multiplex reactions were performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) and amplified with the cycling parameters of 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min on a Bio-Rad iCycler (Bio-Rad).

CFSE labeling and adoptive transfer of DO11.10-transgenic T cells and Treg cells

CFSE in the form of a 5 mM stock solution in DMSO was added to 2×10^7 DO11.10 CD4 T cells/ml in a final concentration of 2 μ M. The cells were incubated at 37°C for 10 min and then washed twice with the same volume of FCS and twice with culture media.

Adoptive transfer of OVA-specific Th1 cells and induction of colitis

To generate OVA-specific pushed Th1 cells, CD4⁺ T cells from DO11.10 mice were cultured with 2 μ g/ml OVA peptide and 5 μ g/ml anti-IL-4 mAb (11B11) and 10 ng/ml IL-12 in the presence of irradiated APC for 7 days. OVA-expressing *Escherichia coli* and Tet-expressing *E. coli* were prepared as described previously (24). Briefly, 5×10^6 in vitro pushed Th1 cells alone, 5×10^6 Th1 cells plus 5×10^6 PSI-APC Treg cells, or 5×10^6 PSI-APC Treg cells alone were each injected into groups of five BALB.B.RAG2^{-/-} mice i.v., and the recipients were then given OVA-expressing *E. coli* into the colon. Control BALB.B.RAG2^{-/-} mice were reconstituted with 5×10^6 in vitro pushed Th1 cells and then given Tet-expressing *E. coli* into the colon. Two months later, the mice were sacrificed, and histopathology was examined.

Statistical analysis

The results were expressed as the mean \pm SD. The significance of the difference between means was determined by the Mann-Whitney *U* test, and differences were considered statistically significant at $p < 0.05$.

Results

PSI-treated APC inhibited T cell proliferation in vitro

PSI-pulsed BMDC stimulated with LPS did not up-regulate expression of costimulatory molecules, such as CD40, CD86, and MHC class II, or IL-12p70 cytokine production, whereas IL-10 production was not affected (data not shown) as reported previously (19). PSI-pulsed BMDC stimulated by LPS had reduced levels of NF- κ B p65 in nuclear extracts compared with control (Fig. 1A). These data confirmed the observation that PSI blocks the maturation of immature to mature DC (19).

To investigate the ability of PSI-treated DC to present Ag to CD4⁺ T cells, BMDC of BALB/c mice were pulsed with PSI for

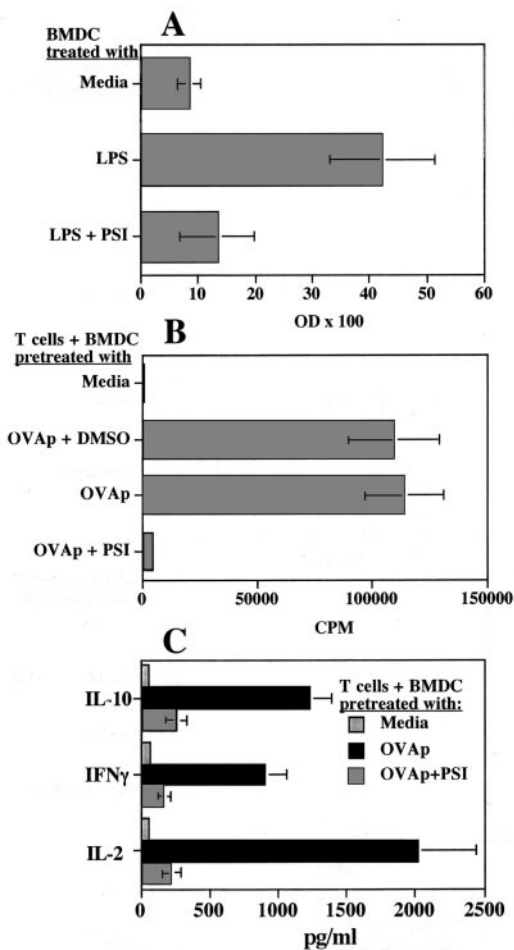


FIGURE 1. PSI treatment inhibited BMDC NF- κ B activation, and PSI-treated BMDC inhibited T cell proliferation and cytokine production. *A*, BMDC derived from BALB/c mice were cultured with media alone, with 1 μ g/ml LPS, or with LPS and 1 μ M PSI for 60 min. NF- κ B p65 in nuclear extracts was measured. *B*, BMDC derived from BALB/c mice were pulsed with or without 1 μ M PSI for 4 h and then with 5 μ g/ml OVA peptide for an additional 20 h, and then 2×10^4 pulsed BMDC were cultured with 1×10^5 CD4⁺ T cells from DO11.10 RAG2^{-/-} mice. [³H]TdR was added in the final 18 h of a 3-day culture. The results are expressed in mean cpm of triplicates \pm SD. *C*, Culture SN were collected at day 3 for measurement of IL-4, IL-10, and IFN- γ production and day 1 for IL-2 production. Cytokines were measured by ELISA. One representative of three experiments is shown.

4 h and then 5 μ g of OVA peptide were pulsed for an additional 20 h. Then the pretreated BMDC were irradiated and put into culture with naive DO11.10.RAG2^{-/-} TCR-transgenic CD4⁺ T cells. Naive DO11.10 CD4⁺ T cells proliferated strongly to stimulation of OVA peptide-pulsed BMDC. However, this T cell response was greatly decreased in cultures with PSI-pretreated, OVA peptide-pulsed BMDC ($p < 0.05$; Fig. 1*B*). BMDC pretreated with solvent DMSO plus OVA peptide had no effect on T cell proliferation (Fig. 1*B*).

Naive DO11.10 CD4⁺ T cells produced high amounts of IL-2 and moderate amounts of IFN- γ and IL-10 but no IL-4 upon stimulation of OVA-peptide-pulsed BMDC. Culture with PSI-pretreated, OVA peptide-pulsed BMDC greatly reduced T cell IL-2 (89%), IFN- γ (81%), and IL-10 production (79%). There was still no IL-4 production (Fig. 1*C* and data not shown).

CD4⁺ T cells induced with PSI-treated BMDC inhibited naive and memory CD4⁺ T cell responses

To test whether T cells induced with PSI-treated BMDC have regulatory function, DO11.10.RAG2^{-/-} CD4⁺ T cells were cultured with BMDC that were pretreated with PSI for 4 h and then OVA peptide for an additional 20 h. CD4⁺ T cells were harvested 7 days later and reisolated with anti-CD4-magnetic beads. The cell yield was ~15–20% of the original T cell input. After washing twice, the T cells were added into a second culture with freshly isolated naive DO11.10 CD4⁺ T cells at various ratios. As shown in Fig. 2*A*, CD4⁺ T cells that had been incubated with PSI- and OVA-pulsed BMDC inhibited naive T cell proliferation at 2:1, 1:1, and 1:2 ($p < 0.05$) ratios of PSI-APC Treg:naive T cells. At a ratio of 1:4, these T cells did not inhibit naive T cell response ($p > 0.05$). Tmem cells (cell yield ~130% of the original T cells) and T cells that had been incubated with PSI-treated BMDC that were not pulsed with OVA (T (PSI-APC) cell, yield ~10% of the original T cells) had little effect on naive T cell proliferation ($p > 0.1$).

To determine the effects of such Treg cells on Tmem cells, Tmem cells were generated by culture of DO11.10 CD4⁺ T cells with OVA peptide in the presence of BMDC for 7 days. These Ag-primed memory CD4⁺ T cells (Tmem) were then restimulated

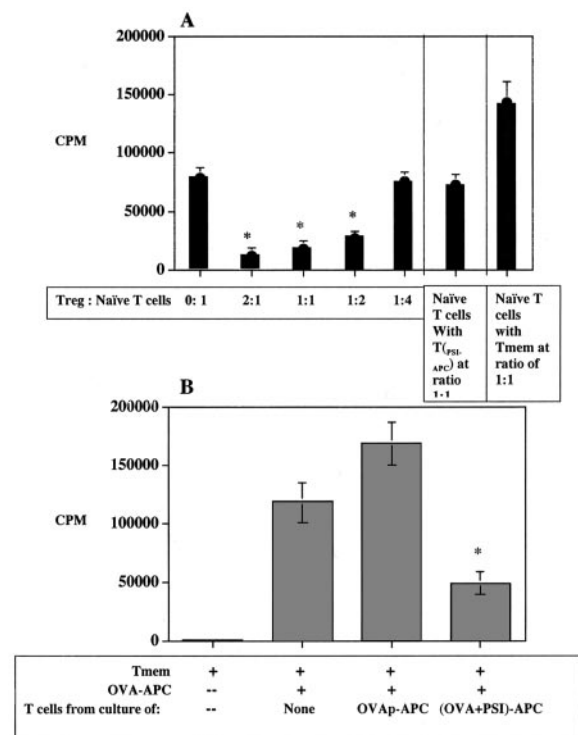


FIGURE 2. PSI-BMDC-generated T cells inhibited naive and Tmem cell responses to Ag stimulation. *A*, BMDC of BALB/c mice were pulsed with or without 1 μ M PSI for 4 h, then with 5 μ g/ml OVA peptide for an additional 20 h, and then 2×10^4 pulsed APC were cultured with 1×10^5 CD4⁺ T cells from DO11.10.RAG2^{-/-} mice. One week later, the T cells were harvested and cultured with naive CD4⁺ T cells of DO11.10 mice at various ratio in the presence of 4×10^5 fresh OVA-pulsed APC. Naive T cells were also cultured at a 1:1 ratio with T cells that had been incubated with PSI-treated BMDC that were not pulsed with OVA (T(PSI-APC)). [³H]TdR was added in the final 18 h of a 3-day culture. *B*, PSI-APC-cultured T cells were cocultured with memory DO11.10 CD4⁺ T cells in the presence of OVA peptide-APC. [³H]TdR was added in the final 16 h of a 3-day culture. The results were expressed in average of cpm of triplicates \pm SD. One representative of three experiments is shown. *, A value of $p < 0.05$ compared with cultures with only naive T cells.

with fresh, OVA peptide-pulsed APC in the absence or presence of the putative Treg cells. T cell proliferation was measured at day 3 of culture. As shown in Fig. 2B, Tmem cells proliferated well to OVA peptide stimulation, but T cells generated with PSI-treated BMDC inhibited Tmem cell proliferation significantly ($p < 0.05$) at a 1:1 ratio of Treg:Tmem cells.

To test whether PSI-treated, normal spleen APC could also generate putative Treg cells as well as PSI-treated BMDC did, spleen cells of BALB/c mice were treated with 1 μ M PSI and OVA peptide as the same fashion. DO11.10.RAG2^{-/-} CD4⁺ T cells were then cultured with these pretreated-splenic APC for 7 days. DO11.10.RAG2^{-/-} CD4⁺ T cells that were cultured with PSI and OVA-pulsed splenic APC inhibited both naive and Tmem cell proliferation significantly (data not shown). These data demonstrated that Ag-pulsed, PSI-treated BMDC and normal spleen APC induced T cells with regulatory function. These regulatory cells are thus denoted as PSI-APC Treg cells below.

Induction of PSI-APC Treg cells required IL-10 production by APC but not the presence of CD25⁺ T cells

IL-10 has been implicated as an important differentiating factor for some subtypes of Treg cells (17, 25, 26). To investigate the role of APC IL-10 in the induction of PSI-APC Treg cells, adherent splenic APC from wild-type or IL-10-deficient BALB/c mice were treated with OVA plus PSI for 24 h and used to generate Treg cells. Treg cells generated by culture with wild-type APC with pulsed PSI-OVA peptide inhibited naive T cell responses to OVA peptide stimulation ($p < 0.05$). In contrast, T cells cultured with IL-10^{-/-} APC had no effect on naive T cell responses to OVA peptide stimulation ($p > 0.05$), indicating that generation of PSI-APC Treg cells required IL-10 production by APCs (Fig. 3A).

CD4⁺CD25⁺ T cells have been studied intensively as Treg cells; however, the relationship between CD4⁺CD25⁺ T cell and other subsets of Treg cells is unclear. To determine whether CD4⁺CD25⁺ T cells were the precursor of PSI-APC Treg cells, CD25⁻ T cells were depleted from naive DO11.10 CD4⁺ spleen cells by FACS sorting. The remaining CD4⁺CD25⁻ T cells (> 99% purity) were cultured with PSI- and OVA-peptide-pulsed APC for a week, and then their inhibitory function was measured. Unfractionated naive CD4⁺ T cells were cultured in a similar manner as a positive control. As shown in Fig. 3B, CD4⁺CD25⁻ T cells that were cultured with APC pretreated with OVA and PSI strongly inhibited naive CD4⁺ T cell proliferation similar to control unfractionated CD4⁺ T cells, indicating that PSI-APC Treg could be generated from CD4⁺CD25⁻ T cell fraction and that CD25⁺ cells are not required for the generation of PSI-APC Treg cells.

PSI-APC Treg cells express Foxp3

Foxp3 has been shown as a transcription factor specific for CD25⁺ Treg cell development and function (13–15). To determine whether PSI-APC Treg cells express Foxp3, RNA was isolated from PSI-APC Treg cells and memory CD4⁺ T cells, and Foxp3 expression was measured by real-time PCR. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells served as controls. As shown in Fig. 3C, Foxp3 expression in PSI-APC Treg cells was ~60-fold higher than that in memory CD4⁺ T cells, similar to the expression found in CD25⁺ Treg cells.

Activation of PSI-APC Treg cell function was Ag specific

To investigate the Ag specificity of PSI-APC Treg cell activation, a CD4⁺ T cell line reactive to KLH was generated and used as target cells to investigate the Ag specificity of PSI-APC Treg cells. OVA-specific PSI-APC Treg cells were generated as described

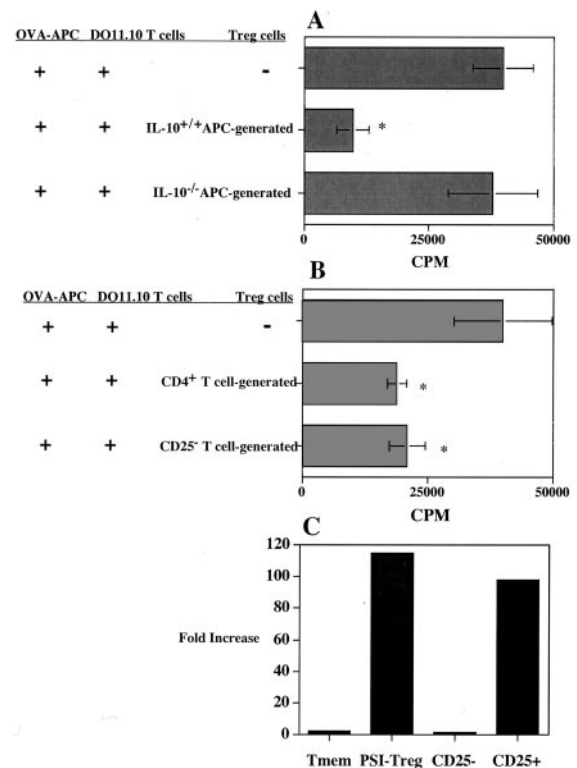


FIGURE 3. PSI-APC Treg cells required IL-10 for their generation and are not derived from CD25⁺ T cells but expressed Foxp3. Spleen cells of wild-type or IL-10^{-/-} BALB/c mice were pulsed with 2 μ g/ml OVA peptide and 1 μ M PSI for 24 h, and then 4×10^5 pulsed APC were cultured with 1×10^5 CD4⁺ T cells from DO11.10 mice. One week later, the T cells were harvested and cocultured with naive DO11.10 CD4⁺ T cells in the presence of fresh OVA-pulsed APC. [³H]TdR was added in the final 16 h of a 3-day culture. **B**, CD25⁻ CD4⁺ T cells and whole CD4⁺ T cells from DO11.10 mice were cultured with APC that had been pulsed with 2 μ g/ml OVA peptide and 1 μ M PSI for 24 h. Seven days later, these CD4⁺ T cells were reisolated and cocultured with naive DO11.10 CD4⁺ T cells in the presence of fresh OVA-pulsed APC. [³H]TdR was added in the final 16 h of a 3-day culture. The results are expressed as mean cpm of triplicates \pm SD. *, A value of $p < 0.05$ compared with cultures with only naive T cells. **C**, PSI-APC Treg cell expression of Foxp3. Seven days after culture with OVA-pulsed APC or OVA- plus PSI-pulsed APC, DO11.10 CD4⁺ T cells were lysed, and Foxp3 expression was measured by real-time PCR. One representative of three experiments is shown.

above and cocultured with KLH-specific T cells and APC in the presence of 100 μ g/ml KLH, 2 μ g/ml OVA peptide, or both KLH and OVA peptide. As shown in Table I, KLH-specific T cells proliferated well in the presence of KLH (cpm, 13,701 \pm 2,755). This proliferation was inhibited by OVA-specific PSI-APC Treg

Table I. PSI-AOC Treg cells required TCR activation by specific Ag^a

KLH-T cells	Treg cells	APC	KLH	OVAp	CPM \pm SD
+	-	+	-	-	738 \pm 50
+	-	+	+	-	13,701 \pm 2,755
+	+	+	+	-	13,215 \pm 2,184
+	+	+	+	+	9,585 \pm 897
+	-	+	+	+	13,963 \pm 2,137
-	+	+	-	+	4,333 \pm 566

^a KLH-specific memory CD4⁺ T cells were cultured with PSI-APC Treg cells and APC in the presence of KLH, OVA peptide, or both KLH and OVA peptide. [³H]TdR was added in the final 16 h of a 5-day culture. The results were expressed as mean cpm of triplicates \pm SD.

cells only in the presence of OVA peptide (cpm, $9,585 \pm 897$, $p < 0.05$) but not in its absence (cpm $13,963 \pm 2,137$, $p > 0.05$), indicating that these Treg cells require specific Ag signaling through their TCR to activate their regulatory program.

Addition of IL-2 did not reverse Treg cell inhibition

Exogenous IL-2 can block the regulatory activity of CD4⁺CD25⁺ T cells and of anergic T cells (27). To investigate the effects of IL-2 on the inhibitory activity of PSI-APC-induced Treg cells, CFSE-labeled naive DO11.10 CD4⁺ T cells were cultured with Treg cells in the presence of OVA-APC with or without a high dose of IL-2 (100 U/ml). Three days later, the cells were stained with PE-labeled KJ1-26 Ab for flow cytometric analysis. The KJ1-26⁺ CFSE-labeled naive CD4⁺ T cells proliferated well, with a large fraction of the T cells having four to six cell divisions (Fig. 4A). Addition of Treg cells to these cultures greatly inhibited proliferation of OVA-specific CD4⁺ T cells (89% of cells did not divide compared with 20% in the absence of Treg cells; Fig. 4B). Addition of IL-2 did not reverse the Treg cell inhibition of cell-cycle progression of naive CFSE-labeled CD4⁺ T cells (87% of cells did not divide compared with 89% in the presence of IL-2; Fig. 4C).

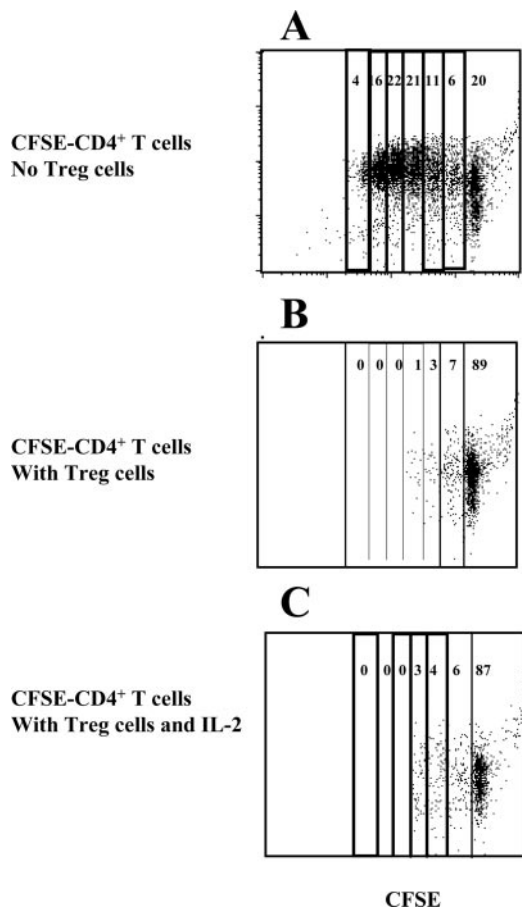


FIGURE 4. PSI-APC Treg inhibition is not reversed by exogenous IL-2. PSI-APC Treg cells were generated as in the legend to Fig. 3. Seven days later, the T cells were harvested and cocultured with CFSE-labeled DO11.10 CD4⁺ T cells in the presence of OVA-pulsed APC with or without 100 U/ml IL-2 for 3 days. The cells were harvested and stained with PE-KJ1-26 mAb and analyzed by flow cytometry. One representative of three experiments is shown. The percentage of cells present in each cell division are shown at the top of each profile.

Inhibitory activity of Treg cells in vitro did not require IL-10 or TGF-β

To determine the potential role of TGF-β and IL-10 in the inhibitory function of PSI-APC Treg cells, anti-IL-10R1 and anti-TGF-β mAb were added into the cultures of Treg cells and naive T cells in the presence of OVA-pulsed APC. Addition of 5 μg/ml anti-IL-10R1 and anti-TGFβ mAb, which partially reversed Tr1 inhibitory function in another of our systems (5), separately or together did not abrogate the inhibitory function of PSI-APC Treg cells, indicating that IL-10 and TGF-β were not involved in the inhibitory function of PSI-APC Treg cells (Fig. 5A). Additionally, to investigate the role of IL-10, PSI-APC Treg cells were generated from CD4⁺ T cells from wild-type (IL-10^{+/+}) or IL-10 deficient (IL-10^{-/-}) DO11.10 mice. As shown in Fig. 5B, both IL-10^{+/+} and IL-10^{-/-} Treg cells inhibited naive T cell responses to OVA peptide stimulation, confirming that IL-10 is not required for PSI-APC Treg cell inhibitory function.

Cell-cell contact is required for PSI-APC Treg cell inhibitory function

To investigate whether other soluble factors or cell-cell contact were involved in the PSI-APC Treg cell inhibitory function, Transwell experiments were performed using CFSE-labeled naive

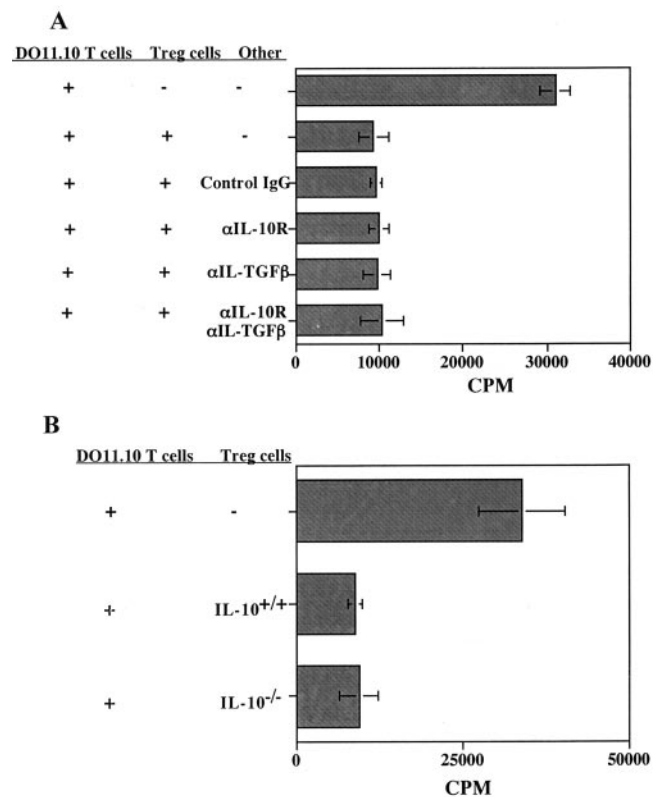


FIGURE 5. IL-10 and TGF-β were not required for inhibitory function. A, PSI-APC Treg cells were generated as in the legend to Fig. 3. Seven days later, the T cells were harvested and cocultured with DO11.10 CD4⁺ T cells with OVA-APC in the presence of 5 μg/ml of different mAbs or control IgG as shown. [³H]TdR was added in the final 16 h of a 3-day culture. The results are expressed as mean cpm of triplicates ± SD. B, CD4⁺ T cells from wild-type (IL-10^{+/+}) or IL-10^{-/-} DO11.10 mice were cultured with OVA and PSI-pulsed APC for 7 days and then cocultured with naive DO11.10 CD4⁺ T cells in the presence of fresh OVA-pulsed APC. [³H]TdR was added in the final 16 h of a 3-day culture. The results were expressed as mean cpm of triplicates ± SD. One representative of three experiments is shown.

DO11.10 CD4⁺ T cells. When CFSE-labeled naive DO11.10 CD4⁺ T cells were cultured alone in the lower wells with OVA-pulsed APC, the naive T cells proliferated vigorously with a large fraction of the T cells completing five to six cell divisions (Fig. 6A). When naive CD4⁺ T cells and Treg cells were cultured in the same wells, T cell cycle progression was inhibited with 71% of cells not dividing compared with 16% in the absence of Treg cells (Fig. 6B). However, when naive CD4⁺ T cells were cultured in the lower wells and Treg cells in the upper wells, Treg cells no longer inhibited naive T cell proliferation, in that most T cells completed five to seven cell divisions (Fig. 6C). Similar data were obtained when [³H]TdR was used to measure T cell proliferation (Fig. 6D).

PSI-APC Treg cell required B7-CTLA-4 interactions for their inhibitory function

Because B7-CTLA-4 interactions are required for the regulatory activity of certain Treg subsets and CD25 is a marker for CD4⁺CD25⁺ T regulatory cells (6, 10), CTLA-4 and CD25 expression on PSI-APC Treg cells was determined. Naive DO11.10 CD4⁺ T cells stimulated with OVA-APC expressed a low level of CD25, and T cells cultured with OVA peptide-pulsed and PSI-treated APC had a similar level of CD25 (Fig. 7A). CTLA-4 ex-

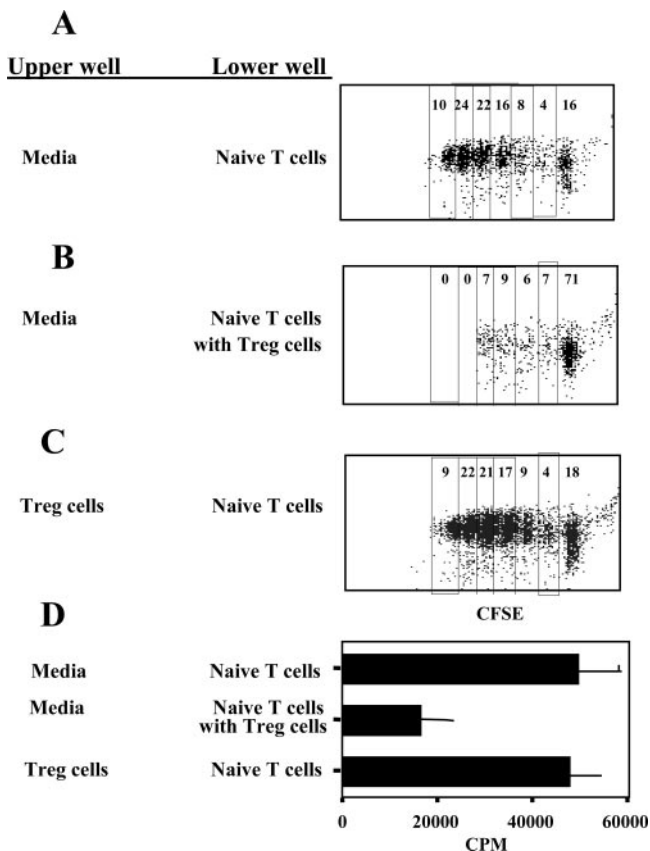


FIGURE 6. Cell-cell contact interactions were required for inhibitory function. A–C, PSI-APC Treg cells were generated as in the legend to Fig. 3. CFSE-labeled naive DO11.10 CD4⁺ T cells were cultured with Treg cells in the same or different wells of the Transwell plate with OVA-APCs for 3 days. The cells were harvested and stained with PE-KJ1-26 mAb and analyzed by flow cytometry. The percentage of cells in each cell division is shown at the top of each profile. D, Treg cells were cultured with DO11.10 CD4⁺ T cells in the same or different wells of the Transwell plate with OVA-APC. [³H]TdR was added in the final 16 h of a 3-day culture. The results are expressed as mean cpm of triplicates \pm SD. One representative of three experiments is shown.

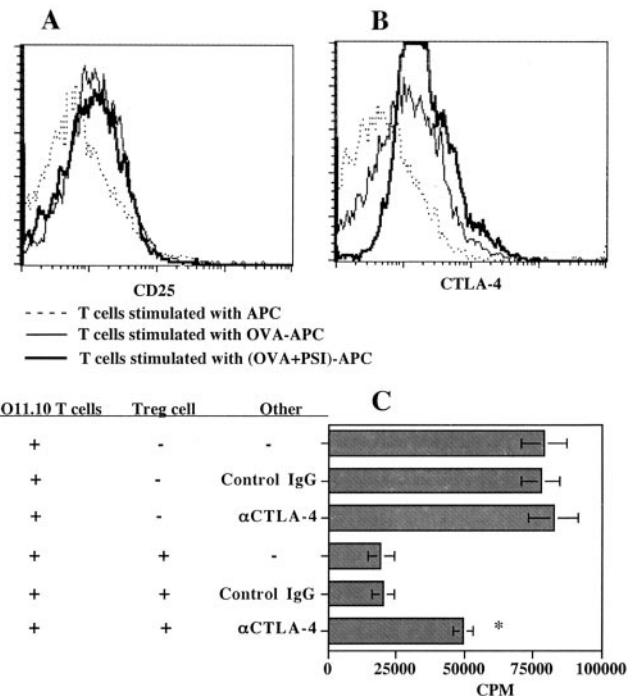


FIGURE 7. CTLA-4 partially mediated PSI-APC Treg cell inhibitory function. PSI-APC Treg cells were generated as in the legend to Fig. 3 using CD4⁺ T cells from DO11.10.RAG2^{-/-} mice. Seven days later, the T cells were stained with PE-anti-CD25 mAb (A) or stained intracellularly with PE-anti-CTLA-4 mAb (B). C, PSI-APC Treg cells were cocultured with DO11.10 CD4⁺ T cells with OVA-APC in the presence of 5 μ g/ml anti-CTLA-4 mAb or control Ab. [³H]TdR was added in the final 16 h of 3-day culture. The results are expressed as mean cpm of triplicates \pm SD. One representative of three experiments is shown. *, A value of $p < 0.05$ compared with the culture without addition of Ab.

pression on T cells cultured with OVA peptides and PSI-pulsed APC was modestly increased compared with that on T cells stimulated with OVA-APC (Fig. 7B).

To investigate the potential functional role of increased CTLA-4 expression on PSI-APC Treg cells, anti-CTLA-4 mAb or control Ab was added into the cultures of naive DO11.10 CD4⁺ T cells with Treg cells in the presence of OVA-APC. Addition of anti-CTLA-4, but not control Ab, significantly ($p < 0.05$) reversed Treg cell-mediated inhibition (Fig. 7C).

PSI-APC Treg cells expanded and inhibited CD4⁺ T cell proliferation in vivo and prevented colitis development in a OVA-specific mouse model

To investigate whether PSI-APC Treg cells induced in vitro could function in vivo, 5×10^6 CFSE-labeled naive DO11.10 RAG2^{-/-} CD4⁺ T cells were transferred together with same number of unlabeled DO11.10 RAG2^{-/-} CD4⁺ cells or of unlabelled PSI-APC Treg cells into BALB/c mice i.v. One day after cell transfer, the recipients were immunized with 100 μ g of OVA in CFA i.p. Five days later, the mice were sacrificed, and CD4⁺ T cells were isolated from the spleen, stained with PE-KJ1-26 mAb, and analyzed by flow cytometry. CFSE-labeled naive DO11.10 CD4⁺ T cells proliferated strongly in vivo after immunization with OVA, with 90% of CFSE-labeled naive T cells dividing and 69% completing four or more cell divisions (Fig. 8A). However, the proliferation of naive DO11.10 CD4⁺ T cells was significantly inhibited when PSI-APC Treg cells were cotransferred. In these recipients, 36% of the naive T cells failed to divide and only 32% completed four or

The NF- κ B-signaling pathway appears to be a key molecular switch in DC activation and maturation (19, 20). NF- κ B can be activated by a large number of signals, including TLR-ligand interactions and various cytokines. Mature DC up-regulate costimulatory and MHC class II molecules and produce large amounts of cytokines, such as IL-12, IL-1, and TNF- α . In studies on human monocyte-derived DC, blockade of NF- κ B activation with NF- κ B inhibitors interfered with maturation of DC manifested by impaired up-regulation of MHC class II and CD86 and inhibition of IL-12 and TNF- α but not of IL-10 production (19, 20, 28). PSI inhibits I κ B degradation and hence inhibits NF- κ B activation without affecting other transcription factors (29–32). In the current study, we found that treatment of mouse DC with PSI had similar effects, inhibiting NF- κ B activation, preventing up-regulation of MHC class II and costimulatory molecule CD40 and CD86 expression on mouse DC, and down-regulating their IL-12 but not IL-10 production. When cultured with naive T cells, PSI-treated DC had a poor capacity to stimulate T cell proliferation and cytokine production, i.e., were functionally immature. These data indicate that blockade of NF- κ B signaling in DC results in a prolonged immature state.

The mechanisms that drive Treg cell differentiation are still largely unknown. CD25⁺ Treg cells are generated mainly in the thymus due to high-affinity TCR interactions with self ligands (33, 34). However, Treg cells can also be generated in the periphery. For example, transgenic expression of an agonist peptide by non-activated hemopoietic cells produced mostly CD4⁺CD25⁺ Treg cells in the periphery, even in the absence of a functioning thymus (35). Another mechanism of Treg generation is Ag stimulation by immature DC (3), and a role for DC in the induction of peripheral tolerance and of Treg cells is supported by multiple studies (36–38) in mouse and humans. Indeed, human Treg cells can be induced by stimulation with immature DC in an allogeneic system (39, 40). In addition, IL-10-treated human monocyte-derived DC, which have an immature or tolerant phenotype, induce anergic CD4⁺ and CD8⁺ T cells with suppressive capability (41). In the current study, Treg cells were induced in vitro by PSI-treated APC that displayed an immature or nonactivated phenotype. These data strongly support the notion that immature DC can drive Treg cell development and may be involved in the generation of Treg cells reactive to enteric bacterial Ags in the normal intestine (5).

The properties of the PSI-APC Treg cells overlapped with some of those of other Treg subsets, but PSI-APC Treg cells did not fall into any previously defined subset. PSI-APC Treg cell inhibition requires cell-cell contact interactions, or at least close apposition, in common with the CD4⁺CD25⁺ subset and with Tr1 cells. PSI-APC Treg cells up-regulated the transcription factor Foxp3, which is characteristic of CD4⁺CD25⁺ Treg cells, but has not been described in Tr1 cells (42). The high expression of Foxp3 transcription factor in PSI-APC Treg argues that Foxp3 may be involved in multiple lineages of Treg cells, can be induced depending on microenvironmental signals such as TGF- β , and is not restricted to CD25⁺ Treg cells generated in the thymus (43). The requirement for IL-10 for induction of PSI-APC Treg cells is shared with Tr1 cells, but not with CD4⁺CD25⁺ Treg cells. From the data presented here and the data of others, Treg cells appear to represent a continuum of activities and properties that can adapt to the microenvironment, and this plasticity may account for the different types of Treg cells that have been described previously (8, 42, 44). We have found that alteration of culture conditions used to induce PSI-APC Treg cells can alter their properties, i.e., addition of a compound that induces IL-10 results in the production of high amounts of Treg IL-10 production in secondary cultures (data not shown). Treg cells exhibit at least three distinct inhibitory pro-

grams, namely cell-cell contact inhibition, production of IL-10, and production of TGF- β . Differences among putative subsets may be due to which one of these mechanisms predominate under the particular microenvironment in which the Treg cells are generated.

PSI-APC Treg cell inhibition in vitro was of moderate potency based on ratios of Treg to naive T cells, with loss of inhibition at ratios less than 1:4 (Fig. 2). Yet, these Treg cells had impressive in vivo effects after adoptive transfer, inhibiting a splenic T cell response to a very strong stimulus of Ag in complete Freund's Ag. PSI-APC Treg cells expand in vivo after adoptive transfer to RAG2^{-/-} mice, although these T cells could not be expanded in vitro or even carried in culture beyond 10–14 days. This expansion in vivo may explain the ability of PSI-APC Treg cells to completely prevent a colitis induced by adoptive transfer of memory effector Th1 cells. Another element that might be important to the in vivo effects and that may not be evident in short-term assays is time. The colitis model evolves over 8 wk, and the sustained activity of Treg cells of even moderate activity over time may be greater than would be predicted from short-term assays in vitro.

In summary, we demonstrate in this report that PSI-treated APC can induce Ag-specific Treg cell differentiation. These Treg cells had in vitro and in vivo inhibitory activity. When cultured in vitro, they inhibited both naive and memory OVA-specific T cell responses to Ag stimulation. When adoptively transferred into recipient mice, these Treg cells inhibited naive T cells proliferation in vivo. More importantly, such Treg cells prevented colitis induction in vivo in an Ag-specific model of inflammatory bowel disease. This is a novel and simple approach to generate Treg cells that could be useful in the clinical treatment of autoimmune and chronic inflammatory diseases. A protocol of in vitro pulsing of NF- κ B-blocked APC with autoantigens and subsequent injection in vivo might generate a Treg cell population able to down-regulate autoreactivity. Alternatively, such PSI-APC Treg cells could be induced in vitro and reinfused. Although complex, the reinfusion of DC or T cells after ex vivo manipulation has already been used in humans for the treatment of cancer. Because the activation of Treg cells requires specific Ag, but their effector phase is non-specific, in theory, an Ag expressed in the relevant organ would be sufficient for this approach to work, and identification of the Ags actually driving disease would not be necessary.

Disclosures

The authors have no financial conflict of interest.

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