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ORIGINAL ARTICLE

Basic Study

Regulatory T cells suppress autoreactive CD4$^+$ T cell response to bladder epithelial antigen

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Abstract

AIM: To investigate the role of regulatory T (Treg) cells in CD4$^+$ T cell-mediated bladder autoimmune inflammation.

METHODS: Urothelium-ovalbumin (URO-OVA)/OT-II mice, a double transgenic line that expresses the membrane form of the model antigen (Ag) OVA as a self-Ag on the urothelium and the OVA-specific CD4$^+$ T cell receptor specific for the I-A^b/OVA$_{323-339}$ epitope in the periphery, were developed to provide an autoimmune environment for investigation of the role of Treg cells in bladder autoimmune inflammation. To facilitate Treg cell analysis, we further developed URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice, a derived line of URO-OVA/OT-II mice that express the green fluorescent protein (GFP)-forkhead box protein P3 (Foxp3) fusion protein.

RESULTS: URO-OVA/OT-II mice failed to develop bladder inflammation despite the presence of autoreactive CD4$^+$ T cells. By monitoring GFP-positive cells, bladder infiltration of CD4$^+$ Treg cells was observed in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice. The infiltrating Treg cells were functionally active and expressed Treg cell effector molecule as well as marker mRNAs including transforming growth factor-β, interleukin (IL)-10, fibrinogen-like protein 2, and glucocorticoid-induced tumor necrosis factor receptor (GITR). Studies further revealed that Treg cells from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice were suppressive and inhibited autoreactive CD4$^+$ T cell proliferation

and interferon (IFN)-γ production in response to OVA Ag stimulation. Depletion of GITR-positive cells led to spontaneous development of bladder inflammation and expression of inflammatory factor mRNAs for IFN-γ, IL-6, tumor necrosis factor-α and nerve growth factor in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice.

CONCLUSION: Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4$^+$ T cell-mediated bladder autoimmune inflammation.

Key words: Bladder; Autoimmunity; Regulatory T cell; CD4$^+$ T cells; Antigen

Core tip: Evidence suggests that autoimmune inflammation may cause interstitial cystitis/bladder pain syndrome (IC/BPS) in subgroups of patients. However, the role of regulatory T (Treg) cells in the control of bladder autoimmunity has not yet been identified. In this study we developed novel transgenic autoimmune cystitis models and demonstrated that Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4$^+$ T cell-mediated bladder autoimmune inflammation. Our results suggest that loss of functional Treg cells may contribute to IC/BPS pathology in subgroups of patients.

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INTRODUCTION

The mechanisms of autoimmune responses in the urinary bladder have not been well studied. Regulatory T (Treg) cells, a special subset of CD4$^+$ T cells, are crucial for immunological homeostasis and play an important role in preventing autoimmune pathogenesis. Predisposition to immunopathology due to loss of functional Treg cells has been observed in numerous autoimmune diseases and animal models[1]. Studies have shown the involvement of Treg cells in the pathogenesis of bladder carcinoma[2-4], suggesting the importance of Treg cells in bladder immunosurveillance. Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory condition of the bladder characterized by pelvic pain, irritative voiding symptoms, and sterile and cytologically normal urine. The etiology of IC/BPS is currently unknown and may involve multiple causes. Although autoimmunity is debated as a potential cause of IC/BPS, clinical evidence suggests that it may play an important role in the pathophysiology of the disease. It has been reported that IC/BPS patients develop antinuclear and anti-urothelium autoantibodies[5-11], overexpress urothelial HLA-DR molecules[12-14], and co-present with other autoimmune diseases such as bronchial asthma, systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis and ulcerative colitis[15-21]. Considerable data have been published on the histopathology of bladder specimens, demonstrating a role of cell-mediated immune mechanisms in IC/BPS[14,22]. Hence, autoimmune inflammation may be a component in the pathophysiology of IC/BPS in subgroups of patients. However, despite these observations, the role of Treg cells in bladder autoimmunity has not been identified.

Prior studies on bladder autoimmunity have been based on the use of rodent models of experimental autoimmune cystitis (EAC) in which animals developed bladder inflammation after immunization with urothelial components[23-28]. These EAC models demonstrated many clinical correlates seen in IC/BPS, offering a unique property for controlled examination of specific aspects of the disease. Using genetic engineering technology, we previously developed a novel transgenic model of EAC (URO-OVA mice) that expresses the membrane form of the model antigen (Ag) ovalbumin (OVA) as a self-Ag on the urothelium and develops bladder inflammation upon introduction of OVA-specific T cells[29-32]. In addition to the many features of conventional EAC models, the transgenic EAC model demonstrates T cell tolerance, activation and autoimmune responses[29,32], facilitating the investigation of the mechanisms underlying bladder autoimmune pathogenesis.

To investigate the role of Treg cells in bladder autoimmunity, we established an autoimmune environment through crossbreeding of URO-OVA mice with OT-II mice that expressed the CD4$^+$ T cell receptor (TCR) specific for the I-A^b/OVA$_{323\text{-}339}$ epitope[33,34]. To further facilitate the analysis of Treg cells, we generated URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice that expressed green fluorescent protein (GFP)-fused forkhead box protein P3 (Foxp3), a Treg cell lineage specification factor[35,36], enabling direct identification of Treg cells based on GFP fluorescence[37]. By using these transgenic EAC models, we have found that CD4$^+$ Treg cells play an important role in immunological homeostasis and the control of bladder autoimmune inflammation.

MATERIALS AND METHODS

Mice

URO-OVA mice [C57BL/6 (B6) genetic background] were previously developed in our laboratory[29]. B6 mice were obtained from the National Cancer Institute/Frederick Cancer Research Animal Facility (Frederick, MD). OT-II mice (B6 genetic background), a line originally developed by Barnden *et al*[33,34], were

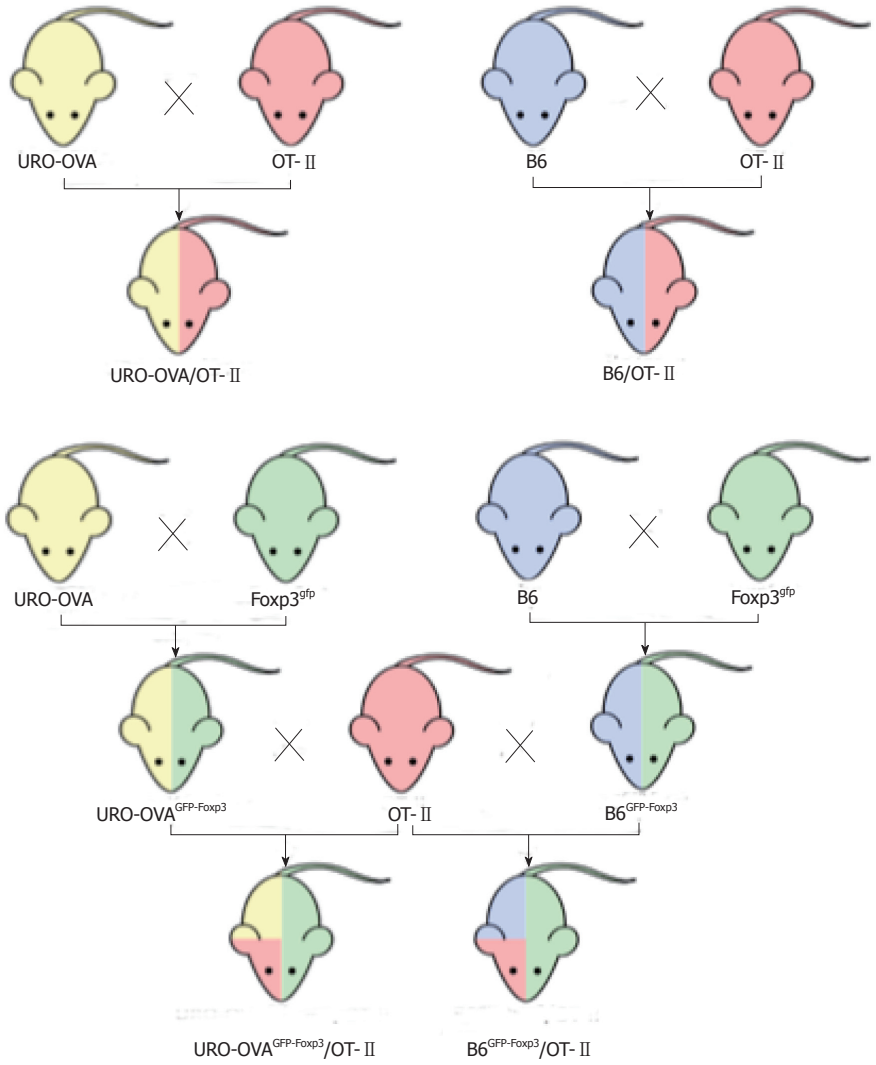


Figure 1 Animal crossbreeding. URO-OVA/OT-Ⅱ mice were generated through crossbreeding of URO-OVA mice with OT-Ⅱ mice. B6/OT-Ⅱ mice were generated through crossbreeding of B6 mice with OT-Ⅱ mice. URO-OVA$^{GFP\text{-}Foxp3}$ mice were generated through crossbreeding of URO-OVA mice with Foxp3^{gfp} mice. B6$^{GFP\text{-}Foxp3}$ mice were generated through crossbreeding of B6 mice with Foxp3^{gfp} mice. Both URO-OVA$^{GFP\text{-}Foxp3}$ and B6$^{GFP\text{-}Foxp3}$ mice were further crossed with OT-Ⅱ mice to generate URO-OVA$^{GFP\text{-}Foxp3}$/OT-Ⅱ and B6$^{GFP\text{-}Foxp3}$/OT-Ⅱ mice, respectively. URO: Urothelium; OVA: Ovalbumin.

obtained from Dr. Ratliff (Purdue Cancer Center, West Lafayette, IN). As shown in Figure 1, URO-OVA/OT-Ⅱ mice were generated through crossbreeding of URO-OVA mice with OT-Ⅱ mice and B6/OT-Ⅱ mice were generated through crossbreeding of B6 mice with OT-Ⅱ mice, respectively. Foxp3^{gfp} mice, a line developed by Fontenot *et al*[37], were obtained from Dr. Rudensky (University of Washington, Seattle, WA). URO-OVA$^{GFP\text{-}Foxp3}$ mice were generated through crossbreeding of URO-OVA mice with Foxp3^{gfp} mice (Figure 1), while B6$^{GFP\text{-}Foxp3}$ mice were generated through crossbreeding of B6 mice with Foxp3^{gfp} mice (Figure 1). All progeny mice were selected for transgenic OVA by tail genotyping and for GFP-positive CD4$^+$ T cells by flow cytometry. Both URO-OVA$^{GFP\text{-}Foxp3}$ and B6$^{GFP\text{-}Foxp3}$ mice were further crossed with OT-Ⅱ mice to generate URO-OVA$^{GFP\text{-}Foxp3}$/OT-Ⅱ and B6$^{GFP\text{-}Foxp3}$/OT-Ⅱ mice, respectively (Figure 1). Male OT-Ⅱ mice and their derived mice were used because only the Y chromosome carries the transgenic CD4$^+$ TCR specific for the I-A^b/OVA$_{323\text{-}339}$ epitope. Mice were housed in a pathogen-free facility at the University of Iowa Animal Care Facility. All procedures involving animals were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee.

In vitro CD4$^+$ T cell response to OVA

Splenocytes were prepared from OT-Ⅱ, B6/OT-Ⅱ and URO-OVA/OT-Ⅱ mice as described previously[32], resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin, and seeded in 96-well plates at a density of 4 × 10^5 cells/200 μL per well. Cells were cultured in the absence or presence of OVA$_{257\text{-}264}$ peptide (10 μg/mL) or OVA$_{323\text{-}339}$ peptide (10 μg/mL) for 3 d at 37 ℃ in a humidified incubator with 5% CO_2. Culture supernatants were then collected and

analyzed for IFN-γ by enzyme-linked immunosorbent assay (ELISA) with paired antibodies (Endogen; clones: R4.6A2 and XMG1.2; Woburn, MA).

In vitro Treg cell suppression assay

OT-Ⅱ splenocytes were prepared as described previously[32], resuspended in the above-mentioned culture medium, seeded in 96-well plates at a density of 3 × 10^5 cells/200 μL per well, and cultured in the absence or presence of $OVA_{323-339}$ peptide (10 μg/mL) for 3 d at 37 ℃ in a humidified incubator with 5% CO_2. To evaluate the effect of Treg cells, OT-Ⅱ splenocytes were also incubated at a 1:1 ratio with GFP-positive (Foxp3$^+$) CD4$^+$ T cells sorted from the spleens of URO-OVA$^{GFP\text{-}Foxp3}$/OT-Ⅱ mice using FACSAria (BD Biosciences; San Jose, CA). As control, GFP-negative CD4$^+$ T cells were collected and incubated with OT-Ⅱ splenocytes at a 1:1 ratio. Proliferation was assessed by pulsing the cells with 1 μCi of [methyl-^{3}H]thymidine (Amersham; Piscataway, NJ) per well for the last 18 h and then assayed for thymidine incorporation by liquid scintillation counting. Culture supernatants from a parallel plate were collected after a 3-d incubation period and analyzed for IFN-γ by ELISA as described above.

In vivo Treg cell depletion assay

Monoclonal antibodies (mAb) specific for CD25 (clone: PC61) and *glucocorticoid-induced tumor* necrosis factor receptor (GITR; clone: DTA-1) were prepared from hybridomas provided by Dr. Ratliff through ammonium sulfate precipitation and protein-A/G affinity chromatography as described previously[38]. URO-OVA$^{GFP\text{-}Foxp3}$/OT-Ⅱ mice were injected intraperitoneally (i.p.) with 500 μg of PC61 or 250 μg of DTA-1 every other day beginning at 6 wk of age and sacrificed for analysis at 10 wk. The bladders were then collected and processed for histological hematoxylin and eosin (H and E) staining and analysis of inflammatory factor mRNAs by reverse transcriptase-polymerase chain reaction (RT-PCR).

Bladder histological analysis

The standard paraffin-embedded histological sections of the bladder were prepared and stained with H and E solution as described previously[29-32]. Bladder inflammation was scored in a blinded manner based on cellular infiltration in the lamina propria and interstitial edema as follows: 1+ (mild infiltration with no or mild edema); 2+ (moderate infiltration with moderate edema); 3+ (moderate to severe infiltration with severe edema). Statistical analysis was performed using Student's *t* test with SPSS11.0 software.

Flow cytometric analysis

In various experiments single-cell suspensions of the thymus, spleen, bladder draining lymph nodes (BLNs) and bladder were prepared by mechanical disruption as described previously[29,32]. Briefly, cells were washed with staining buffer [1% FBS, 0.09% (w/v) NaN_3 in Mg^{2+} and Ca^{2+} free PBS], stained with a FITC-, PE- or PE-Cy5-labeled antibody (eBioscience, San Diego, CA) to various surface markers including CD4 (clone: RM4-5), CD44 (clone: IM7), CD45RB (clone: C363.16A), CD62L (clone: MEL-14), CD69 (clone: H1.2F3), and OT-Ⅱ CD4$^+$ TCR clonal phenotype Vα2 (clone: B20.1) and Vβ5 (clone: MR9-4) at 4 ℃ for 15 min, fixed in 2% formalin, and analyzed using a FACScan equipped with CellQuest (BD Biosciences). For GFP analysis, the FITC channel was used. Post-acquisition analysis was carried out using FlowJo software (Tree Star, Ashland, OR).

RT-PCR analysis

RT-PCR was used to analyze mRNAs expressed by bladder infiltrating Treg cells and the inflamed bladders of URO-OVA$^{GFP\text{-}Foxp3}$/OT-Ⅱ mice. Total RNAs were extracted using Qiagen RNAeasy Kit (Valencia, CA) from FACS-sorted bladder infiltrating CD4$^+$ T cells (both GFP positive and negative cells) and the bladders of mice untreated or treated with depleting mAbs. Three microgram of total RNAs were used for cDNA synthesis using Invitrogen Superscript Ⅲ RNase H Reverse Transcriptase (Carlsbad, CA) and oligo dT according to the manufacturer's instructions. Two microlitre of the cDNA products were further processed for PCR amplification using sequence-specific primer pairs and Invitrogen Taq DNA polymerase. The following primer pairs were used: 5'-agcccgaagcggactactat-3' and 5'-agccctgtattccgtctcct-3' for transforming growth factor (TGF)-β (357 bp); 5'-tgcctgctcttactgactgg-3' and 5'-gctccactgccttgctctta-3' for interleukin (IL)-10 (397 bp); 5'-tcaacagtttggatggcaag-3' and 5'-ctgccgtgccattgtagtta-3' for FGL2 (468 bp); 5'-tggagtctcgatgctctgtg-3' and 5'-atcctcagctgacaactgcac-3' for GITR (583 bp); 5'-cgctacacactgcatcttgg-3' and 5'-aaattcaaatag-tgctggcaga-3' for interferon (IFN)-γ (522 bp); 5'-ctgatg-ctggtgacaaccac-3' and 5'-gccactccttctgtgactcc-3' for IL-6 (505 bp); 5'-gtccccaaagggatgagaag-3' and 5'-aagtagacctgcccggactc-3' for tumor necrosis factor (TNF)-α (520 bp); 5'-agtgtcagtgtgtgggttgg-3' and 5'-gccttgacgaaggtgtgagt-3' for nerve growth factor (NGF; 218 bp); and 5'-agcttgtcatcaacgggaag-3' and 5'-gtcttctgggtggcagtgat-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 364 bp). PCR cycle numbers were initially optimized to achieve desirable discrepancies between the experimental groups. PCR was then performed for GAPDH with 30 cycles, IFN-γ, TNF-α and NGF with 36 cycles, and other molecules with 40 cycles. The cycling condition consisted of denaturing at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and elongation at 72 ℃ for 1 min. DNA fragments were run on a 1% agarose gel, stained with ethidium bromide, and imaged by EpiChemi digital image system (Upland, CA).

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test with SPSS11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Constitutive expression of urothelial OVA causes clonal deletion of OVA-specific CD4$^+$ T cells in URO-OVA/OT-II mice

URO-OVA/OT-II mice (F1 generation), a crossed line of URO-OVA mice with OT-II mice, expressed self-Ag OVA on the urothelium and the TCR (Vα2Vβ5) specific for I-A^b/OVA$_{323-339}$ epitope on CD4$^+$ T cells. URO-OVA/OT-II mice showed T cell tolerance in potentially autoreactive OVA-specific CD4$^+$ T cells. Compared to control B6/OT-II mice (F1 generation) that expressed the same OVA-specific CD4$^+$ TCR but no urothelial OVA, URO-OVA/OT-II mice showed severe reduction in CD4$^+$Vα2$^+$ cells, CD4$^+$Vβ5$^+$ cells, and Vα2$^+$Vβ5$^+$cells in the thymus (Figure 2, top panel; 1% *vs* 25% for all 3 populations). The severe population reduction was also observed in the spleen (Figure 2, middle panel; 4% *vs* 19% for CD4$^+$Vα2$^+$ cells, 1% *vs* 19% for CD4$^+$Vβ5$^+$ cells, and 2% *vs* 18% for Vα^{2+}Vβ^{5+}cells) and the BLNs (Figure 2, bottom panel; 7% *vs* 55% for CD4$^+$Vα2$^+$ cells, 3% *vs* 50% for CD4$^+$Vβ5$^+$ cells, and 9% *vs* 43% for Vα2$^+$Vβ5$^+$cells). However, this population reduction was incomplete, suggesting the presence of additional regulatory mechanism(s) in the control of autoreactive CD4$^+$ T cells in URO-OVA/OT-II mice.

Deletion-escaped OVA-specific CD4$^+$ T cells are responsive to OVA and gain activation in URO-OVA/OT-II mice

We next investigated whether OVA-specific CD4$^+$ T cells that had escaped from clonal deletion retained OT-II CD4$^+$ T cell responsiveness to OVA. Splenocytes were prepared from URO-OVA/OT-II mice and incubated with OVA$_{323-339}$ peptide specific for the OT-II CD4$^+$ TCR for 3 d *in vitro*. Cells were also incubated with OVA$_{257-264}$ peptide as control. Splenocytes from age-matched OT-II and B6/OT-II mice were included for comparison. As expected, cells from both OT-II and B6/OT-II mice produced similar levels of IFN-γ in response to OVA$_{323-339}$ peptide stimulation (Figure 3). Interestingly, cells from URO-OVA/OT-II mice also produced IFN-γ in response to OVA$_{323-339}$ peptide stimulation ($P < 0.001$), although the level was 2-3 fold less than those of OT-II and B6/OT-II cells. This reduced IFN-γ production suggested that the autoreactivity of OVA-specific CD4$^+$ T cells was compromised in URO-OVA/OT-II mice. However, despite the reduction of autoreactivity, OVA-specific CD4$^+$ T cells gained activation *in vivo*. Compared to B6/OT-II mice, CD4$^+$ T cells from the BLNs of URO-OVA/OT-II mice showed up-regulated expressions of CD44 and CD69 and down-regulated expressions of CD45RB and CD62L (Figure 4). In addition, the bladders of URO-OVA/OT-II mice contained 6-15 fold more infiltrating CD4$^+$, Vα2$^+$ and Vβ5$^+$ cells than those of B6/OT-II mice (Figure 5A). Further analysis revealed that the majority of bladder infiltrating CD4$^+$ T cells were Vα2$^+$ and Vβ5$^+$ cells (Figure 5B), suggesting that they were OT-II CD4$^+$ T cells. These observations indicated that endogenous OVA-specific CD4$^+$ T cells retained the ability to respond to self-Ag OVA, gained activation in the BLNs, and infiltrated into the bladders in URO-OVA/OT-II mice. Interestingly, despite T cell activation and bladder infiltration, URO-OVA/OT-II mice developed no bladder histopathology, further suggesting the presence of additional regulatory mechanism(s) in these mice.

Bladder infiltrating CD4$^+$ T cells consist of Treg cells in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice

Since URO-OVA/OT-II mice contained activated OVA-specific CD4$^+$ T cells but failed to develop bladder inflammation, we hypothesized that Treg cells might play an important role in the control of autoreactive CD4$^+$ T cells in these mice. To facilitate the analysis of Treg cells, we crossed URO-OVA mice with Foxp3^{gfp} mice, a Foxp3^{gfp} allele knock-in line that expresses GFP-fused Foxp3[36], to generate URO-OVA$^{GFP\text{-}Foxp3}$ mice. As control, B6$^{GFP\text{-}Foxp3}$ mice were generated in parallel. To investigate the role of Treg cells in bladder autoimmunity, we further crossed URO-OVA$^{GFP\text{-}Foxp3}$ mice with OT-II mice to generate URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice. As control, B6$^{GFP\text{-}Foxp3}$/OT-II mice were generated through crossbreeding of B6$^{GFP\text{-}Foxp3}$ mice with OT-II mice. Similar to URO-OVA/OT-II mice, URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice showed severe but incomplete reduction in OVA-specific CD4$^+$ T cell population in the thymus, spleen and BLNs compared to B6$^{GFP\text{-}Foxp3}$/OT-II mice (data not shown). Also, similar to the bladders of URO-OVA/OT-II mice, the bladders of URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice showed increased infiltrating CD4$^+$ T cells compared to B6$^{GFP\text{-}Foxp3}$/OT-II mice (Figure 6A). However, like URO-OVA/OT-II mice, URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice developed no bladder histopathology.

Analysis of bladder infiltrating CD4$^+$ T cells revealed an increased number of GFP-positive (Foxp3$^+$) cells in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice compared to B6$^{GFP\text{-}Foxp3}$/OT-II mice (Figure 6B). Further analysis of bladder infiltrating CD4$^+$ T cells in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice indicated that the majority of the cells were GFP positive (Foxp3$^+$) cells (Figure 7A; 64% *vs* 36%). These GFP-positive (Foxp3$^+$) CD4$^+$ T cells were functionally active, as they expressed increased CD44 and CD69 and decreased CD45RB and CD62L compared to GFP-negative (Foxp3$^-$) CD4$^+$ T cells (Figure 7B). Consistently, these GFP-positive (Foxp3$^+$) CD4$^+$ T cells expressed increased levels of Treg cell effector molecule TGF-β, IL-10 and FGL2 mRNAs and Treg cell marker GITR mRNA compared to GFP-negative (Foxp3$^-$) CD4$^+$ T cells (Figure 7C). These observations suggested that Treg cells were actively involved in bladder autoimmune responses in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice.

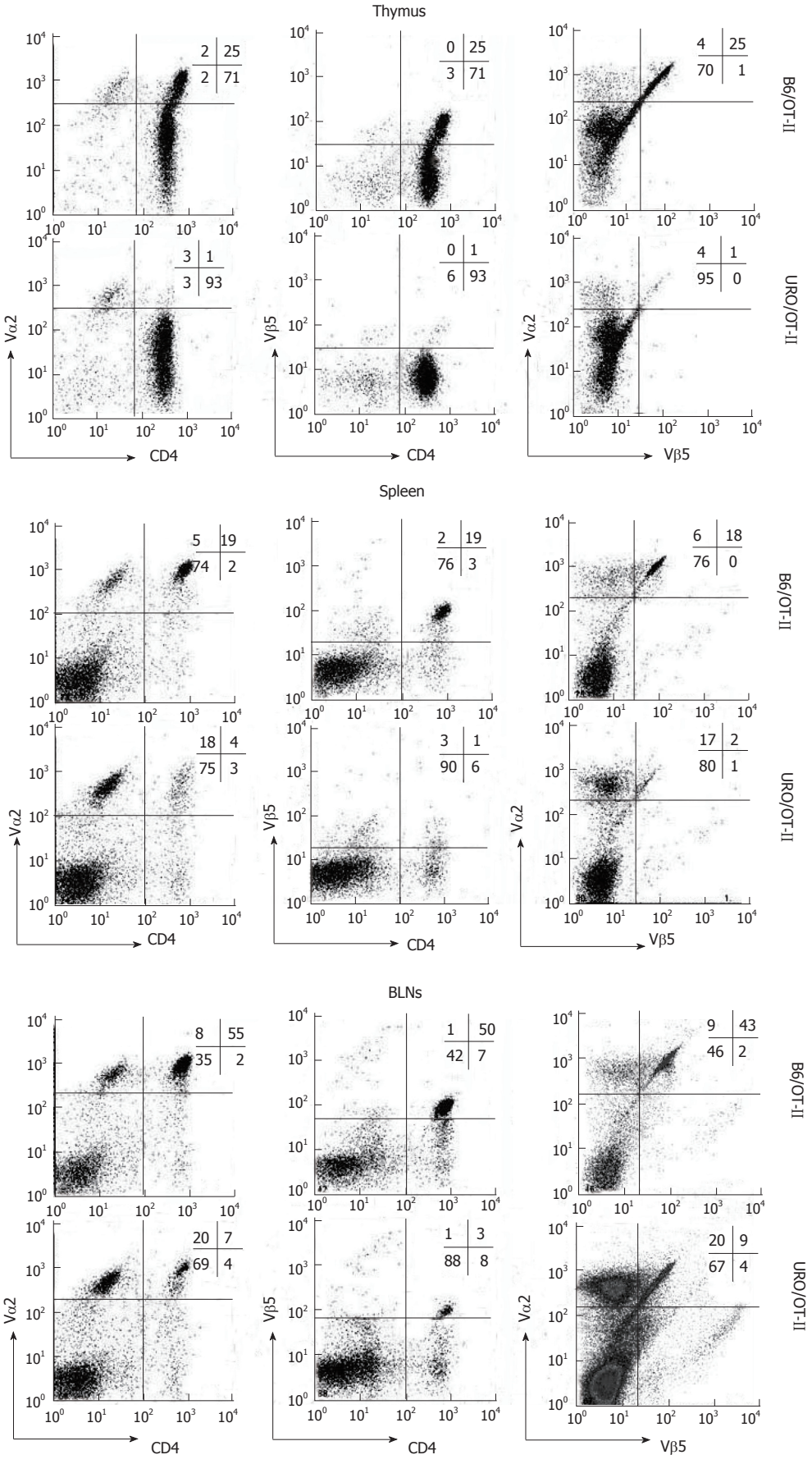


Figure 2 Clonal deletion of OT-II CD4$^+$ T cells in urothelium-ovalbumin/OT-II mice. Cells from the thymus (top panel), spleen (middle panel), and BLNs (bottom panel) of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on lymphocytes according to scatter criteria. Percentages of single- and double-positive cells are indicated. Results are representative of 3 separate experiments consisting of 4-6 mice per group. URO/OT-II: Urothelium-ovalbumin/OT-II mice.

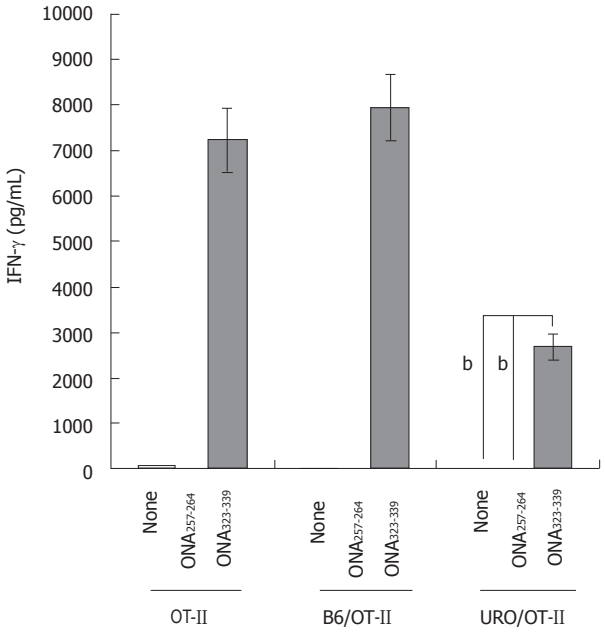


Figure 3 Deletion-escaped OT-II CD4$^+$ T cells retain responsiveness to ovalbumin. Splenocytes from URO-OVA/OT-II mice (8 wk) were cultured alone or in the presence of $OVA_{257-264}$ peptide (10 μg/mL) or $OVA_{323-339}$ peptide (10 μg/mL) for 3 d, followed by ELISA analysis of IFN-γ production in culture supernatants. Splenocytes from age-matched OT-II and B6/OT-II mice were included for comparison. Data are presented as the mean ± SD from duplicate determinations. $^bP < 0.001$ compared with non-stimulated or $OVA_{257-264}$-stimulated splenocytes (two-tailed Student's *t* test). URO/OT-II: Urothelium-ovalbumin/OT-II mice.

Treg cells from URO-OVA$^{GFP-Foxp3}$/OT-II mice are suppressive to OVA-specific CD4$^+$ T cells

To determine whether Treg cells found in URO-OVA$^{GFP-Foxp3}$/OT-II mice were suppressive, we prepared GFP-positive (Foxp3$^+$) CD4$^+$ T cells from the spleens of URO-OVA$^{GFP-Foxp3}$/OT-II mice. GFP-negative (Foxp3$^-$) CD4$^+$ T cells were prepared for comparison. The purity of both cell types was > 95%. Responder OT-II splenocytes were incubated with or without $OVA_{323-339}$ peptide in the presence or absence of GFP-positive (Foxp3$^+$) or GFP-negative (Foxp3$^-$) CD4$^+$ T cells at a 1:1 ratio for 3 d *in vitro*, followed by analysis of cell proliferation and IFN-γ production (Figure 8). Compared to OT-II cells incubated with $OVA_{323-339}$ peptide alone, OT-II cells incubated with $OVA_{323-339}$ peptide in the presence of CD4$^+$Foxp3$^-$ cells showed similar high levels of proliferation and IFN-γ production. However, when incubated with $OVA_{323-339}$ peptide in the presence of CD4$^+$Foxp3$^+$ cells, OT-II cells showed significantly reduced levels of proliferation ($P < 0.001$) and IFN-γ production ($P < 0.05$). These observations indicated that CD4$^+$ Treg cells were suppressive, suggesting their importance in the control of bladder autoimmunity in URO-OVA$^{GFP-Foxp3}$/OT-II mice.

Depletion of CD4$^+$ Treg cells results in spontaneous development of bladder autoimmune inflammation in URO-OVA$^{GFP-Foxp3}$/OT-II mice

To determine whether CD4$^+$ Treg cells played an inhibitory role in bladder autoimmune inflammation, we depleted CD25$^+$ cells or GITR$^+$ cells in URO-OVA$^{GFP-Foxp3}$/OT-II mice. Mice were injected i.p. with anti-CD25 mAb (PC61) or anti-GITR mAb (DTA-1) every other day beginning at 6 wk and sacrificed for analysis at 10 wk. Depletion of CD4$^+$ Treg cells was verified by flow cytometric analysis of splenocytes showing the lack of GFP-positive (Foxp3$^+$) CD4$^+$ T cells. Interestingly, depletion of CD25$^+$ cells led to the development of bladder histopathology in only 2 of 12 mice (score: +), whereas depletion of GITR$^+$ cells led to the development of bladder histopathology in 11 of 12 mice (score: + for 3 bladders, ++ for 6 bladders, and +++ for 2 bladders) (Table 1 and Figure 9A, $P < 0.001$). Consistently, the bladders of mice treated with anti-GITR mAb expressed increased levels of IFN-γ, IL-6, TNF-α and NGF mRNAs compared to the bladders of mice treated with anti-CD25 mAb (Figure 9B). Indeed, the latter bladders showed no clear increase in the mRNA expressions compared to the bladders of non-treated mice. These observations indicated that depletion of GITR$^+$ cells but not CD25$^+$ cells resulted in spontaneous development of bladder inflammation in URO-OVA$^{GFP-Foxp3}$/OT-II mice.

Table 1 Summary of bladder histological inflammation

	Bladder histologic score^b			
	-	+	++	+++
Anti-CD25 (n = 12)	10	2	0	0
Anti-GITR (n = 12)	1	3	6	2

$^bP < 0.001$ compared between two groups (two-tailed Student's *t* test). GITR: Glucocorticoid-induced tumor necrosis factor receptor.

DISCUSSION

The role of Treg cells in bladder autoimmunity has not been identified due to the lack of a proper animal model. In this study we used transgenic EAC models to investigate the role of Treg cells and found that CD4$^+$ Treg cells played an important role in the control of bladder autoimmune inflammation. Acquirement of autoreactive CD4$^+$ T cells was not sufficient to cause bladder inflammation; however, depletion of CD4$^+$ Treg cells led to spontaneous development of bladder inflammation in the transgenic EAC models.

We generated URO-OVA/OT-II mice to investigate bladder autoimmunity, because CD4$^+$ T cells are preferentially induced in IC/BPS compared to CD8$^+$ T cells[14,22,39-41]. The ability of OT-II CD4$^+$ T cells to induce bladder inflammation was previously demonstrated in URO-OVA mice[32]. To facilitate the analysis of Treg cells, we generated URO-OVA$^{GFP-Foxp3}$ mice that expressed the GFP-Foxp3 fusion protein. We further crossed URO-OVA and URO-OVA$^{GFP-Foxp3}$ mice with OT-II mice to establish an autoimmune environment in mice. Constitutive expression of urothelial OVA resulted in clonal deletion

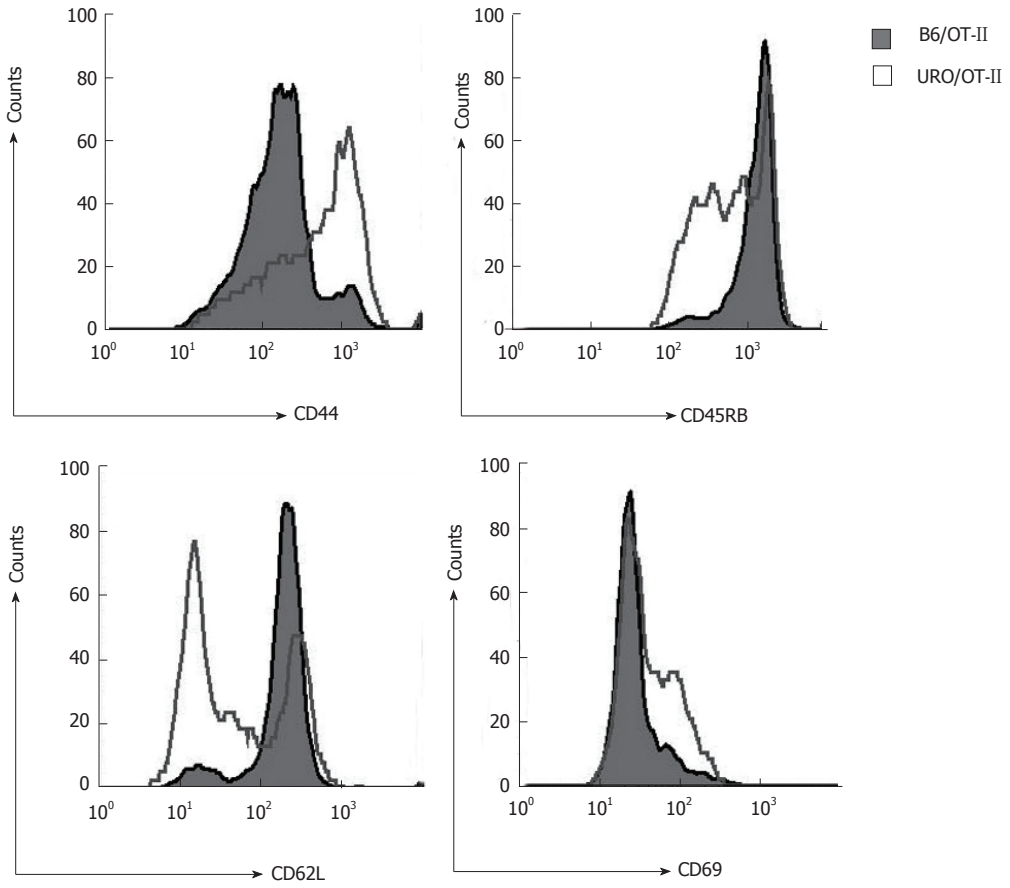
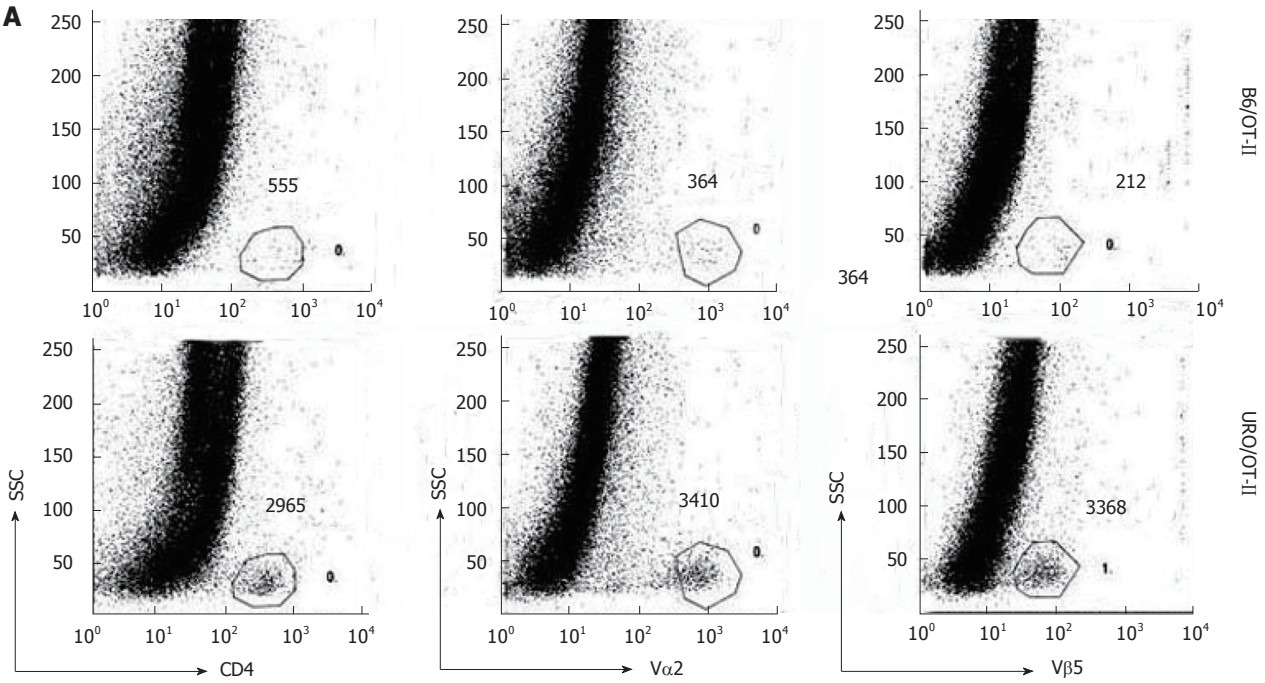


Figure 4 OT-II CD4$^+$ T cells gain activation in the bladder draining lymph nodes of urothelium-ovalbumin/OT-II mice. BLN cells of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD44, CD45RB, CD62L, and CD69 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on CD4$^+$ T cells. Results are representative of 3 separate experiments consisting of 5 mice per group. Filled histograms: B6/OT-II mice; Gray line histograms: URO-OVA/OT-II mice. URO/OT-II: Urothelium-ovalbumin/OT-II mice; BLN: Bladder draining lymph nodes.



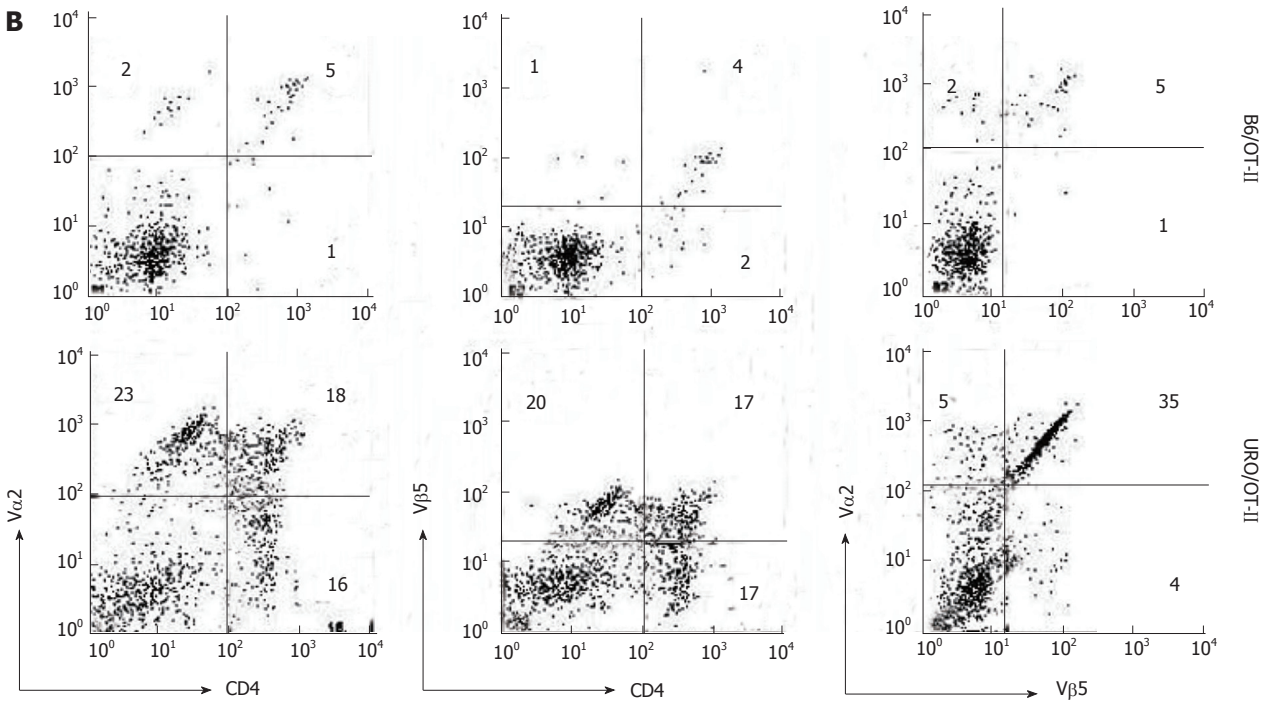


Figure 5 OT-II CD4$^+$ T cells infiltrate into the bladders of urothelium-ovalbumin/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA/OT-II mice (8 wk) and analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set on lymphocytes according to scatter criteria. Total numbers of CD4$^+$, Vα2$^+$ and Vβ5$^+$ cells per bladder are indicated in (A) and percentages of single- and double-positive cells per bladder indicated in (B). Results are representative of 3 separate experiments consisting of 5-8 mice per group. URO/OT-II: Urothelium-ovalbumin/OT-II mice.

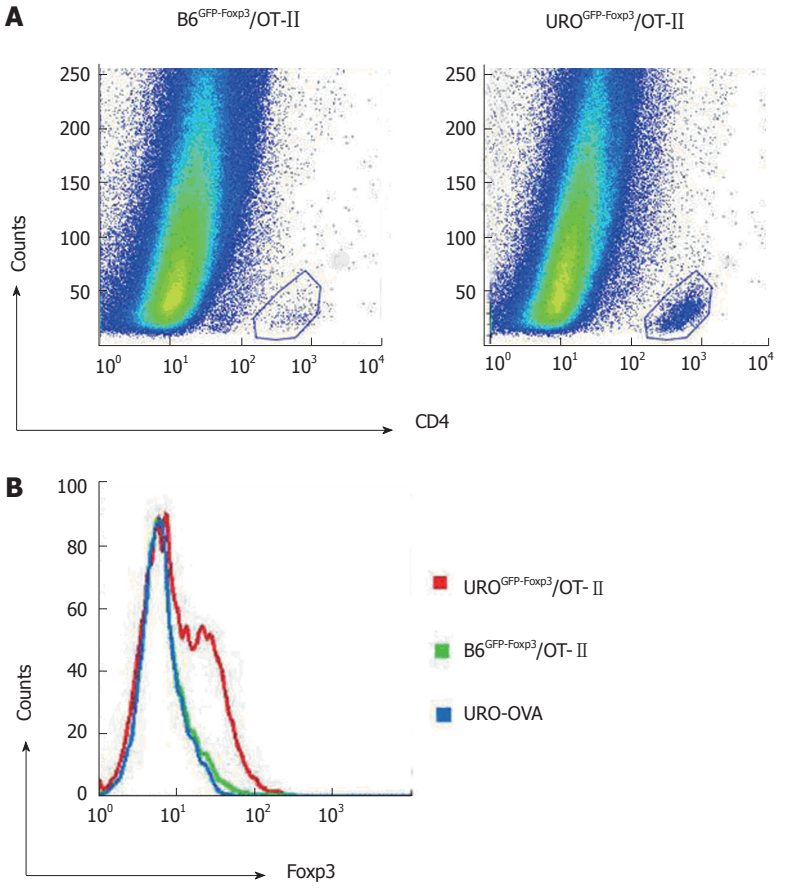


Figure 6 Bladder infiltrating CD4$^+$ T cells consist of Treg cells in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice (8 wk) and analyzed by flow cytometry. Age-matched B6$^{GFP\text{-}Foxp3}$/OT-II mice were included for comparison. A: Flow cytometric analysis of bladder infiltrating CD4$^+$ T cells. Gate was set on lymphocytes according to scatter criteria; B: Flow cytometric analysis of bladder infiltrating GFP-positive CD4$^+$ T cells (*i.e.*, Foxp3$^+$CD4$^+$ T cells). Gate was set on CD4$^+$ T cells. Results are representative of 3 separate experiments consisting of 6 mice per group. URO$^{GFP\text{-}Foxp3}$/OT-II: Urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice.

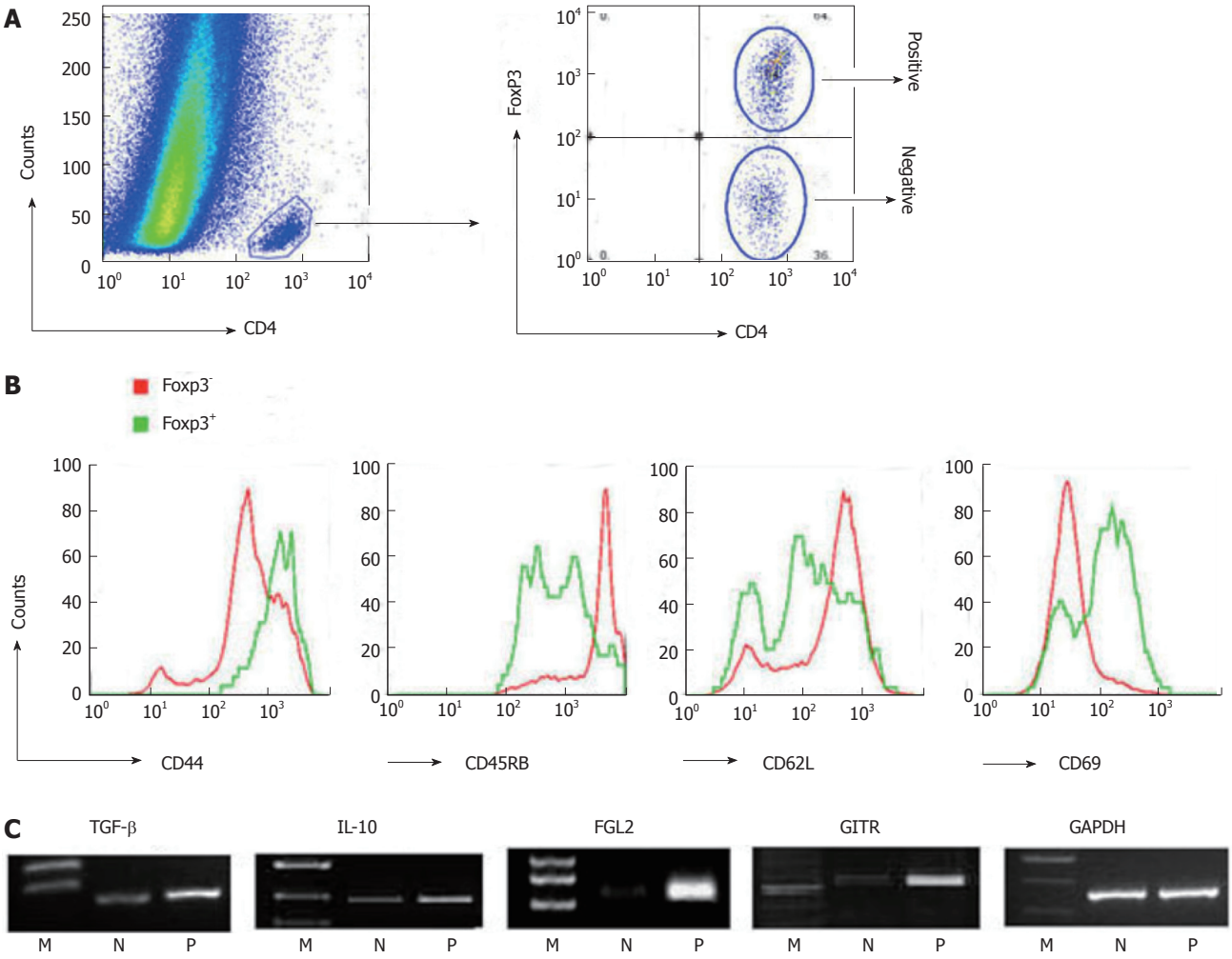


Figure 7 Bladder infiltrating CD4$^+$ Treg cells are functionally active and express inhibitory effector molecules in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice (8 wk) and analyzed by flow cytometry or sorted for GFP-positive (Foxp3$^+$) and GFP-negative (Foxp3$^-$) CD4$^+$ T cells. A: Bladder infiltrating CD4$^+$ T cells consist of both GFP-positive (Foxp3$^+$) and GFP-negative (Foxp3$^-$) populations by flow cytometry. Gate was set on lymphocytes according to scatter criteria; B: Flow cytometric analysis of surface CD44, CD45RB, CD62L and CD69 on bladder infiltrating GFP-positive (Foxp3$^+$) and GFP-negative (Foxp3$^-$) CD4$^+$ T cells. Gate was set on CD4$^+$ T cells; C: RT-PCR analysis of TGF-β, IL-10, FGL2 and GITR mRNAs in bladder infiltrating GFP-positive (Foxp3$^+$) and GFP-negative (Foxp3$^-$) CD4$^+$ T cells. GAPDH was used as an internal control. M: Marker; N: GFP-negative (Foxp3$^-$) CD4$^+$ T cells; P: GFP-positive (Foxp3$^+$) CD4$^+$ T cells; URO$^{GFP\text{-}Foxp3}$/OT-II: Urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice; RT-PCR: Reverse transcriptase-polymerase chain reaction; TGF: Transforming growth factor; GITR: Glucocorticoid-induced tumor necrosis factor receptor.

of autoreactive CD4$^+$ T cells in both URO-OVA/OT-II and URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice. However, this clonal deletion was incomplete, as a tiny population of autoreactive CD4$^+$ T cells was observed in both central and peripheral compartments. Such incomplete clonal deletion of autoreactive T cells has been observed in our previously reported autoimmune cystitis model (URO-OVA/OT-I mice)[29] and others' organ-specific transgenic inflammation models[42-44].

Due to urothelial OVA expression, deletion-escaped OVA-specific CD4$^+$ T cells gained activation in the BLNs and infiltrated into the bladders in URO-OVA/OT-II mice. These observations suggested that bladder urothelial OVA was antigenic and could access the immune system for CD4$^+$ T cell activation. However, despite the CD4$^+$ T cell activation and bladder infiltration, URO-OVA/OT-II mice developed no bladder inflammation. This observation differed from our previous observation in URO-OVA/OT-I mice as these mice spontaneously developed bladder inflammation at 10 wk of age[29,31]. This discrepancy might be attributed to differential expression levels of I-A^b *vs* H2-K^b on the bladder urothelium, which directly influences Ag recognition by autoreactive CD4$^+$ and CD8$^+$ T cells, respectively. Alternatively, the presence of different numbers of deletion-escaped autoreactive T cell subsets might lead to different autoimmune responses in these mice. However, despite this discrepancy, Treg cells appeared to play a predominant role in the control of bladder autoimmune responses. To support this, splenocytes from URO-OVA/OT-II mice showed a substantially reduced ability to produce IFN-γ in response to OVA$_{323\text{-}339}$ peptide stimulation *in vitro*, suggesting that the autoreactivity of OVA-specific CD4$^+$ T cells was greatly compromised in these mice. Also, depletion of Treg cells *in vivo* by anti-GITR mAb has been observed

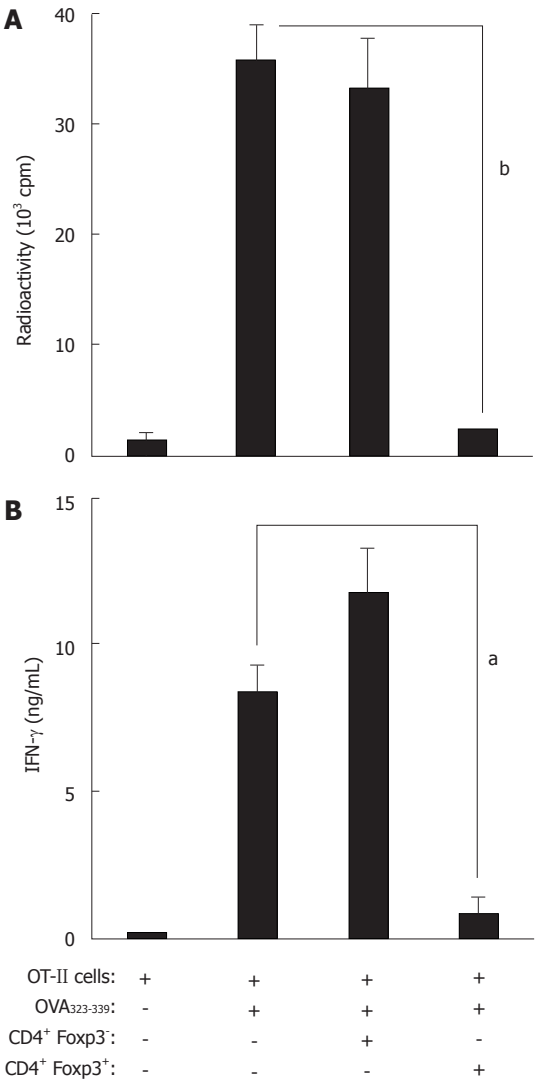


Figure 8 Treg cells from urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice are suppressive to ovalbumin-specific CD4$^+$ T cells. A: OT-II splenocytes were incubated alone or in the presence of OVA323-339 peptide (10 μg/mL), GFP-positive (Foxp3$^+$) CD4$^+$ T cells (at a 1:1 ratio), and/or GFP-negative (Foxp3$^-$) CD4$^+$ T cells (at a 1:1 ratio) sorted from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice for 3 d. Proliferation was assessed by labeling the cultures with 3H-thymidine for the final 18 h. Data are presented as the mean ± SD from triplicate cultures. $^bP < 0.001$ compared with OT-II cells stimulated with OVA$_{323\text{-}339}$ peptide alone (two-tailed Student's *t* test); B: Culture supernatants from a parallel plate were collected after 3-d incubation and analyzed for IFN-γ by ELISA. Data are presented as the mean ± SD from duplicate cultures. $^aP < 0.05$ compared with OT-II cells stimulated with OVA$_{323\text{-}339}$ peptide alone (two-tailed Student's *t* test). URO$^{GFP\text{-}Foxp3}$/OT-II: Urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice.

to result in spontaneous development of bladder inflammation in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice. Therefore, Treg cells appeared to counteract autoreactive CD4$^+$ T cells for the induction of bladder inflammation in these mice. However, our observations cannot exclude the possibility that other cell types with regulatory activities may contribute to the control of bladder autoimmune responses, since the bladders of mice depleted of GITR$^+$ cells showed varying degrees of inflammation.

By monitoring GFP for Foxp3$^+$ cells we observed a considerable number of CD4$^+$Foxp3$^+$ T cells, along with CD4$^+$Foxp3$^-$ T cells, in the bladders of URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice. Compared to CD4$^+$Foxp3$^-$ T cells, CD4$^+$Foxp3$^+$ T cells expressed increased levels of Treg cell effector molecule TGF-β, IL-10 and FGL2 mRNAs as well as Treg cell marker GITR mRNA. In addition, CD4$^+$Foxp3$^+$ T cells exhibited an activated phenotype with up-regulated expressions of CD44 and CD69 and down-regulated expressions of CD45RB and CD62L. Such Treg cell activation *in vivo* has been observed in other animal models[45,46]. Moreover, we have observed the inhibitory effect of CD4$^+$Foxp3$^+$ T cells on OVA-specific CD4$^+$ T cells in co-culture assays and the spontaneous development of bladder inflammation in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice after depletion of GITR$^+$ cells. All these observations support the important role of CD4$^+$ Treg cells in the control of bladder autoimmune responses in the transgenic EAC models.

As direct evidence for the role of Treg cells in the control of bladder autoimmunity, URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice spontaneously developed bladder inflammation after depletion of GITR$^+$ cells. Interestingly, mice depleted of CD25$^+$ cells failed to develop clear bladder inflammation. This phenomenon might result from the elimination of CD25-expressing autoreactive CD4$^+$ T cells, together with CD4$^+$CD25$^+$ Treg cells, by anti-CD25 mAb (PC61). Our observation was consistent with previous studies demonstrating that anti-GITR mAb (DTA-1) but not PC61 was effective in the control of cancer in diverse animal models[47-49]. These studies revealed the differential activities of DTA-1 and PC61, *i.e.,* DTA-1 specifically depleted Treg cells whereas PC61 depleted both CD25$^+$ effector T cells and Treg cells. In addition, studies have also shown that DTA-1 co-stimulates conventional effector T cells while disabling Treg cells[50,51].

The origin of CD4$^+$ Treg cells in URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice is unknown. It is generally accepted that naturally-occurring Treg cells specific for self-Ag presented by the thymic epithelium are positively selected in the thymus and then colonize in secondary lymphoid organs[52-55]. It has also been shown that peripheral CD4$^+$CD25$^-$ naïve T cells can be converted into CD4$^+$CD25$^+$ Treg cells under certain circumstances[56-58]. In the presence of a physiologically low level of cognate self-Ag, resting autoreactive Treg cells can gain activation in the draining lymph nodes and then enter circulation[45,59]. Therefore, it is possible that in the transgenic EAC models the urothelial self-Ag OVA is transported to the BLNs and presented to OVA-specific CD4$^+$ Treg cells as well as effector CD4$^+$ T cells by Ag-presenting cells. This Ag presentation activates both autoreactive CD4$^+$ T cell types, leading to proliferation in the BLNs and infiltration into the bladders. However, because of the co-presence of Treg cells *in situ*, effector CD4$^+$ T cells are suppressed and cause no bladder inflammation. This assumption is supported by our observations that URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice spontaneously develop bladder inflammation after depletion of GITR$^+$ cells. The origin of Treg cells

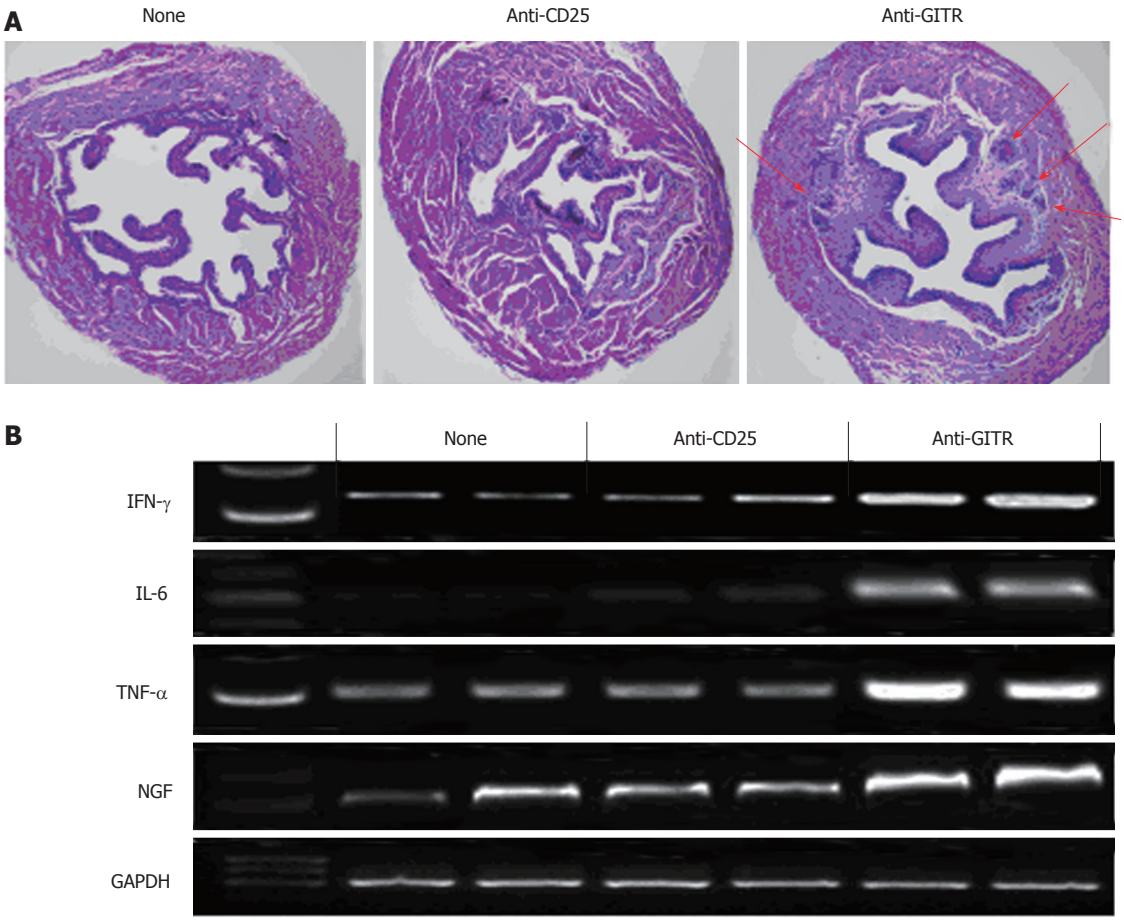


Figure 9 Depletion of CD4$^+$ Treg cells results in bladder autoimmune inflammation in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice were treated with anti-CD25 or anti-GITR mAb every other day beginning at 6 wk of age and sacrificed for analysis at 10 wk. A: Bladder histological H and E staining. The slides are representative of 12 bladders for each of anti-CD25 and anti-GITR mAb treated groups. Cellular infiltration is indicated by red arrows. The bladder of an untreated mouse is included for comparison. The summary of bladder histological inflammation is shown in Table 1; B: RT-PCR analysis of IFN-γ, IL-6, TNF-α and NGF mRNA expressions in the bladders of mice treated with anti-CD25 or anti-GITR mAb. GAPDH was used as an internal control. The bladders from untreated mice are included for comparison. URO$^{GFP\text{-}Foxp3}$/OT-II: Urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice; RT-PCR: Reverse transcriptase-polymerase chain reaction; IFN: Interferon; TNF: Tumor necrosis factor; NGF: Nerve growth factor.

and the mechanisms underlying Treg cell action are interesting topics in bladder autoimmunity research and warrant further investigation.

In summary, we have demonstrated that CD4$^+$ Treg cells play an important role in immunological homeostasis and the control of bladder autoimmune inflammation in the transgenic EAC models. This study, together with our previous studies[29,32], sheds light on the cellular mechanisms of bladder autoimmunity. Clear understanding of bladder autoimmune responses will add to future development of novel therapies for bladder inflammatory diseases that contain an autoimmune component in the pathophysiology such as IC/BPS in subgroups of patients.

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COMMENTS

Background

Interstitial cystitis/bladder pain syndrome (IC/BPS) is one of the most refractory diseases in urology today. Since the etiology of IC/BPS remains elusive, current treatments are largely empirical, often dissatisfactory, and vary in efficacy. Therefore, effort to identify the mechanisms of the disease for therapeutic development is greatly needed. Evidence suggests that autoimmune inflammation may cause IC/BPS in subgroups of patients. However, the role of Treg cells in immunological homeostasis and the control of bladder autoimmune inflammation has not yet been identified.

Research frontiers

Rodent models of experimental autoimmune cystitis (EAC) have been actively used in IC/BPS research for identifying the importance of bladder autoimmunity in the disease pathology.

Innovations and breakthroughs

This is the first study demonstrating that Treg cells specific for bladder epithelial Ag play an important role in immunological homeostasis and the control of CD4$^+$ T cell-mediated bladder autoimmune inflammation.

Applications

The authors have demonstrated the presence of Treg cells in the developed

transgenic EAC models. The authors have also demonstrated that depletion of Treg cells causes bladder autoimmune inflammation in the transgenic EAC models. The results suggest that loss of functional Treg cells may contribute to IC/BPS pathology in subgroups of patients.

Terminology

IC/BPS is a chronic and debilitating inflammatory condition of the urinary bladder characterized by the hallmark symptom of pelvic pain in the absence of other identified etiologies for the symptom. IC/BPS patients also frequently have voiding dysfunction such as increased urinary frequency and urgency. This urologic condition is significant and severely affects quality of life. The etiology of IC/BPS is currently unknown and may involve multiple causes. Increasing evidence suggests that autoimmune inflammation may be causative in subgroups of IC/BPS patients.

Peer-review

The manuscript describes the role of regulatory T cells in IC/BPS model mice. The experiments are well designed and the results are clearly presented.

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