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REVIEW

New insights on chitinases immunologic activities

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Abstract

Mammalian chitinases and the related chilectins (ChiLs) belong to the GH18 family, which hydrolyse the glycosidic bond of chitin by a substrate-assisted mechanism. Chitin the fundamental component in the coating of numerous living species is the most abundant natural biopolymer. Mounting evidence suggest that the function of the majority of the mammalian chitinases is not exclusive to catalyze the hydrolysis of chitin producing pathogens, but include crucial role specific in the immunologic activities. The chitinases and chitinase-like proteins are expressed in response to different proinflammatory cues in various tissues by activated macrophages, neutrophils and in different monocyte-derived cell lines. The mechanism and molecular interaction of chitinases in relation to immune regulation embrace bacterial infection, inflammation, dismetabolic and degenerative disease. The aim of this review is to update the reader with regard to the role of chitinases proposed in the recent innate and adaptive immunity literature. The deep scrutiny of this family of enzymes could be a useful base for further studies addressed to the development of potential procedure directing these molecules as diagnostic and prognostic markers for numerous immune and inflammatory diseases.

Key words: Chitinases; Chitinase like proteins; Chronic inflammation; Immune regulation; Autoimmunity

Core tip: The chitinases and chitinase-like proteins are expressed in response to different pro-inflammatory signals by activated macrophages and in different monocyte-derived cell lines. The mechanism and molecular interaction of chitinases in the immune regulation embrace bacterial infection, inflammation, dismetabolic and degenerative disease. The concept of the chitinases involvement in human diseases discussed herein may stimulate the development of new studies leading to a deeper understanding on the biochemical mechanisms inducing chitinases regulation and on the consequences that the increases in chitinases levels impact with immunity and autoimmunity in different conditions. The future understanding on chitinase functions will lead to the opportunity to develop selective and specific chitinase inhibitors.

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INTRODUCTION

Mammalian chitinases and the related chilectins (ChiL) belong to the GH18 family[1]. Chitinases embraces members both with and without glycohydrolase enzymatic activity against chitin. Chitotriosidase (CHIT1) and acidic mammalian chitinase (CHIA) are recognized as true chitinase because are the only two chitinases demonstrating chitinolytic (glycohydrolase) activity[2]. In contrast none of the other mammalian chitinases, encompassing chitinase 3-like-1 (CHI3L1), chitinase 3-like-2 (CHI3L2), chitinase domain–containing 1 (CHID1), display enzymatic activity in the face of the retention and conservation of the substrate-binding cleft of the chitinases[3] and for this reason they are called chitinase-like-lectins (Chi-lectins) or chitinase-like proteins (C/CLPs). Mammalian chitinases with enzymatic activity have a chitin binding domain containing six cysteine residues predisposed for the binding of chitin[4]. Instead, CLPs do not contain such typical chitin-binding domains, but still can bind to chitin with high affinity[5]. A number of evidence reports that the expression of the majority of the mammalian chitinases is differentially regulated during specific immunologic activities and has important biological roles in chronic inflammatory diseases[6-8]. Additionally chitinases have been widely shown to have an antipathogen function, through their capability to degrade both colloidal chitin and chitin in the cell wall of the fungal pathogen. Similarly, mammalian ChiLs may play a role in immunomodulation. The majority of chitinase families are produced by monocyte/macrophages lineage. In addition, macrophages induce inflammatory responses by producing cytokines, chemokines, and lipid mediators. Interestingly, chitinase play a role in modulating the local and/or circulating concentration of chitins in the body and, therefore, in regulating the immune response to this polysaccharide. Hypothetically, when exogenous chitin from sources such as fungi or dust mites are present in the tissues, chitinases act by cleaving chitin which consequently prevent chitin from stimulating immune responses. Hence, it is possible that without active chitinases, chitin accumulate in tissues triggering an excessive inflammatory response. Therefore is clear that induction of chitinase and CLPs is associated with inflammatory disease, including allergy, asthma, dismetabolic and degenerative diseases and several types of cancer[9].

In the last decade various investigations have brought new insights on the immune properties of chitinases and their functions in inflammatory pathologies. Both chitinases and CLPs can activate specific receptors and signaling pathways stimulating immune mediators' generation and amplification of inflammation. New studies are helping to understand the beneficial as well the detrimental properties of chitinases. Characterizing the role of induced chitinases activity promises interesting perspectives. As well, understanding the molecular signalling pathways involved in the immune function influenced by chitinases might be a valuable approach to investigate new therapeutic alternatives for pathological conditions in which the increased immune response and inflammation are involved.

CHITOTRIOSIDASE AND IMMUNITY

CHIT1 was the first mammalian chitinase measured in disease states[4]. CHIT1 has been encompassed as one of the secreted biomarkers for Gaucher's disease[10]. The elevation of CHIT1 in these patients may reflect a particular state of activation of macrophages[11]. CHIT1 is a very critical enzyme to regulate the susceptibility to infection of organisms containing chitin as structural components[2].

The *CHIT1* gene is localized in chromosome 1q31-q32[12] and consists of 12 exons and spans approximately 20 kb of genomic DNA[12]. Recombinant CHIT1 inhibits hyphal growth of fungi, suggesting a physiological role in the host defense mechanism against the invasion/attack of chitin-containing pathogens[13] which to act as adjuvants by stimulating the production of cytokines and chemokines[6]. Further evidence indicates that the enzymatic role of CHIT1 extends to bacteria[4,13]. Usually, CHIT1 activity is very low and originates in the circulating polymorphonuclear cells[12]. CHIT1 rises significantly in response to various pro-inflammatory signals in a complementary fashion in neutrophils and macrophages[4]. The evidence that TLR signaling is a potent inducer in neutrophils, while NOD-2 signaling induces *CHIT1* in macrophages[14], strongly confirms the importance of this enzyme in the immune response. A defect in *CHIT1* gene consisting of 24-bp duplication in exon 10 that activates a cryptic 39 splice site in the same exon generates an abnormally spliced mRNA with an in-frame deletion of 87 nucleotides. This spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids[12]. CHIT1 deficiency appears as an autosomal incompletely dominant disorder, with no activity in homozygous subjects for the defective allele and approximately half-normal activities in heterozygous subjects. *CHIT1* gene mutation has been encountered with high incidence in different Caucasian populations[12], instead, in African peoples living in malaria parasite endemic areas CHIT1 mutation shows a low prevalence. The absence of homozygosis for CHIT1 deficiency in malaria endemics area suggests the hypothesis that susceptibility to parasitic disease influences the CHIT1 allele composition. In sub-Saharan regions the maintenance of the wild-type *CHIT1* gene confirms that CHIT1 provides innate protection from malaria infection[15]. As well the studies reporting that

individuals bearing the mutant allele exhibit an increased susceptibility to chitin-containing pathogens including *Wuchereria bancrofti* filarial, *Plasmodium falciparum* malaria, *Cryptococcus neoformans* and *Candida albicans*[16] confirmed the CHIT1 allele arrangement hypothesis. Nevertheless, in others studies have been reported that a functional polymorphism produces protective effect in human longevity[17] and protects from nonalcoholic fatty liver disease progression[18]. CHIT1 may have organ- as well as cell-specific effects in the setting of infectious diseases and inflammatory disorders. In fact, CHIT1 overexpression in Kupffer cells is involved in the modulation of the tissue remodeling processes in fibroblastic hepatic tissue[18]. Furthermore, the CHIT1 produced by macrophages enhances atherosclerotic plaques formation and subsequent thrombosis[19]. Therefore this enzyme produced by differentiated macrophages can also be damaging to host tissues and are implicated in the progression of a number of chronic inflammatory diseases[20]. In this context, it is important to note that CHIT1 displays different role in the specialized macrophages. CHIT1 modulation changed during the diverse stages of macrophages maturation and in polarized M1 and M2 macrophages[6]. This data could explain why the expression of CHIT-1 is particularly elevated in the later inflamed stages of infection-induced diseases such as tuberculosis and leprosy[21,22]. Remarkably, was also reported that in monocytes interleukin-4 (IL-4) treatment induced a significant increase on CHIT-1 expression[6]. Since IL-4 promotes immune responses to parasites, this finding set straight why CHIT-1 increased secretion is closely associated with pathophysiological conditions dominated by T-helper type 2 (Th2) cells including infections with fungal pathogens and malaria parasites, fibrosis, allergy, and asthma[23-25]. Macrophages are involved in both generation of fibrosis and its resolution. Conversely M2 polarization generates a positive feedback loop during resolution of inflammation, therefore it is unclear what are the events influencing M2 differentiation and interrupting tissue repair/remodeling as well fibrotic outcomes. The finding reporting that CHIT1 increases in M2 subset suggest that CHIT1 could be involved in the modulation of the extracellular matrix affecting cell adhesion and migration during the tissue remodeling processes that take place in fibrogenesis[26,27]. CHIT1 is also involved in human airway hyper-responsiveness and asthma[28], as well as being active to IL-13-driven alveolar fibrosis by augmenting transforming growth factor beta (TGFβ) and mitogen-activated protein kinase signaling in mice[29]. Therefore, it is conceivable that chitinase inhibition might have beneficial effects on the expression of genes associated with tissues remodeling. Additionally, the recent findings demonstrating that CHIT1 is not exclusively produced by macrophages but is expressed in other cells involved in the immune response such as osteoclasts[30,31] and monocyte-derived DCs[32] confirm the active role of CHIT-1 in the immune response and in disease states where inflammatory responses prevail[21,22,28,33-35].

ACIDIC MAMMALIAN CHITINASE AND IMMUNITY

The second true chitinase called AMCase or CHIA has a 30-kDa N-terminal catalytic domain that hydrolyze chitin, and it expressed mainly in the gastrointestinal tract and lung of both mouse and human[36]. Similarly to CHIT1, is located on chromosome 1q13.1e 21.3, and in addition to the N-terminal catalytic domain acidic mammalian chitinase (AMCase) contain a C-terminal chitinase binding domain[5]. The presence of AMCase in the gastrointestinal tract and lung indicates that it plays a crucial role as a food processor in stomach and its involvement in lung inflammation[5,37]. As well, the expression of AMCase in the lung suggests that the enzyme may have a dual function in digestion of chitinous substrates and host defense[38]. This enzyme plays protective role against parasites. AMCase acts as chemotactic agents and synergistically with other chemokines attracting eosinophils and T cells to sites of parasitic infection, appears to modulate tissue inflammation, immunity, and therefore plays active roles in anti-infective defense and repair responses[8]. Recently it has been demonstrated that AMCase and CHIT1 play different rule in the immune response[8]. Comparing the modulation of both AMCase and CHIT1 expression during monocyte/macrophages differentiation and polarization was found that AMCase was not selectively expressed and highly regulated in activated macrophages. The slight increases of AMCase in M1 stage following treatment with pro-inflammatory stimuli indicated AMCase is ineffective against infections and therefore may be involved only in innate immunity[8]. It has been reported that AMCase is specifically upregulated in response to Th2 inflammation in the lung, and is strictly related to pathophysiological conditions dominated by Th2 type cells such as allergy and asthma[39-41]. The early up regulation of AMCase expression in undifferentiated monocytes treated with IL-4 suggested that an inhibition of AMCase prevents this immune response[8]. In addition, genetic studies of the AMCase gene have indicated that certain polymorphisms and haplotypes of AMCase are associated with bronchial asthma in humans[40]. In contrast, other studies revealed that a haplotype encoding an AMCase isoform displaying a significant enzymatic activity was associated with protection from asthma in several United States ethnic populations[41]. These data indicated that an increased AMCase enzymatic activity could be protective against the development of human asthma, possibly through cleavage of inflammatory chitin polymers[41]. This protective isoform of AMCase may reproduce an improved activity in the stomach, where the degradation of ingested polymeric environmental chitin or chitin-containing microorganisms could induce changes of the bowel commensal flora or to alterations in immune

responses to ingested allergens[42,43]. Ingested polymeric chitin has been observed to disrupt interactions with host proteins involved in regulating bacterial adherence to the gastrointestinal epithelium, such as RegⅢ, and to be used as the preferred energy source by certain gut bacteria[44,45]. Alterations in intestinal microflora alter the subsequent immune response to allergens in the lung in experimental models[46]. It has been reported that inhibition with the transition-state analog allosamidin, an inhibitor of chitinase, enhanced the Th2 driven, IL-13-dependent inflammation, endorsing that its chitinase activity play a role in asthma, even in the absence of chitin[47]. The enzymatic activity of AMCase was found critical in the regulation of pulmonary Th2 inflammation in both murine models exposed and unexposed to polymeric chitin. Since AMCase expression is regulated by active Th2 inflammation it is possible that the active isoform predominates in severe asthmatics and/or during asthma exacerbations. Furthermore, expression of the active isoform could be up-regulated by environmental chitin exposures. Chitin microparticles induce alternative macrophage activation through CCL2 signaling in response to binding of chitin by airway epithelial cells[45]. Moreover, chitin induces the release of IL-25, IL-33 and thymic stromal lymphopoietin that are able to activate the production of the type 2 cytokines such as IL-5 and IL-13 in innate lymphoid type 2 cells. This induction also led to both eosinophilia and alternative activation of macrophages[48]. It has been reported that chitin itself is a pattern recognition molecule stimulating the tissue accumulation of innate immune cells associated with asthma, such as eosinophils and basophils[43]. In addition, AMCase preserves airway epithelial cells from undergoing apoptosis by stimulating phosphoinositide 3-kinase (PI3K) and AKT signaling, through a mechanism associated to its chitin-binding site[45].

CHITINASE-3-LIKE-1 AND IMMUNITY

Chitinase-3-like-1 (CHI3L1) protein or YLK-40 binds chitin polymers in the absence of the active site residues necessary for cleavage. CHI3L1 is produced by neutrophils, monocytes/macrophages, monocyte derived dendritic cells and osteoclasts[32,49,50]. CHI3L1 is a pro-inflammatory biomarke[51] and is capable of inducing inflammatory mediators including chemokines (CCL2, CXCL2) and metalloproteases (MMP-9)[51]. Local inflamed tissues including intestinal mucosa in inflammatory bowel disease (IBD)[52] and adipose tissues in type 2 diabetes produce CHI3L1[53]. Induction of CHI3L1 has been reported in autoimmune disorders, in pulmonary sarcoidosis, systemic sclerosis, liver fibrosis, rheumatoid arthritis, bronchial asthma, coronary artery disease, Alzheimer's disease and inflammatory-related illnesses in humans[54-62]. CHI3L1 secretion is induced by interferon (IFN)-γ[5] and IL-6[61] and is an acute phase reactant associated with disease severity and mortality in numerous infectious diseases. The expression of CHI3L1 has been reported to be significantly associated with migration of human macrophages[52] bronchial smooth muscle cells[62] and glioma cells[63]. In inflammation activated macrophages are the major CHI3L1 producers[50]. Substantial evidence supports a role of CHI3L1 in endothelial dysfunction and atherosclerosis[52,60]. CHI3L1 expression was found variably modulated during macrophages activation and polarization supporting that CHI3L1 plays a crucial role during the initial innate immune responses at the site of pathogen invasion[64]. The modulation of CHI3L1 following treatment with pro-inflammatory stimuli in monocytes and its strong increases in M1 polarized macrophages indicates that the antimicrobial pathway in human macrophages involves also a vigorous activation of CHI3L1. Additionally the higher expression in M2 polarized macrophages highlight that CHI3L1 is a mediator of innate and acquired immunity[7]. Remarkably, some evidence indicated that CHI3L1 may play a role in type 2 helper cell-mediated inflammation[65]. Additionally, CHI3L1 is involved in intestinal inflammation and diverse pathologies concerning the mucosal barriers of the stomach and gastrointestinal tract integrity such as inflammatory bowel disorders. Specifically, *CHI3L1* is upregulated in inflammatory conditions of the gut. Moreover, infection studies have suggested a function in both development and resolution of intestinal inflammation as well as bacterial removal[66]. The infection stimulating effects have been found to arise from enhanced adhesion of bacteria to intestinal epithelial cells (IECs)[66], precisely through bacterial interaction with N-glycosylation patterns on *CHI3L1* expressed by IECs[66]. *CHI3L1* also stimulates clearance and resolution of bacterial infections and inflammation in colitis *via* Stat3 signaling[66]. Moreover, elevated serum levels of *CHI3L1* promote a marked protection against *Streptococcus pneumoniae* infection, improving the aptitude of macrophages to kill bacteria and simultaneously protecting the immune cells from pyroptosis by inhibiting IL-1β-driven inflammasome activation[66]. Serum levels of *CHI3L1* are elevated in patients with pathogen-induced inflammation, including purulent meningitis, and endotoxaemia caused by endotoxin of *Escherichia coli*[66]. In both meningitis and pneumonia, CHI3L1 is secreted by locally activated macrophages[66] and neutrophils[67], and thus, has been proposed as a specific supplementary serological marker for the activation of granulocytes and macrophages in inflamed tissues[68]. These evidences confirm that CHI3L1 may have a particular affinity with some pathogenic bacteria. Though chitin is not expressed in bacteria, the majority of chitinase-producing pathogenic microorganisms enclose a gene encoding for the chitin binding protein, which possibly interacts with the binding ability between chitinase producing bacteria and chitin[69]. In a knock-out model of the murine CHI3L1 analogue, CHI3L1 is important in establishing Th2 polarized immune responses and enhance the

recruitment of macrophages, dendritic cells and T-cells by inhibiting apoptosis[70].

Genetic variants of CHI3L1 are associated with reduced lung function in asthmatics[71]. The increase of this protein in the lung has been found also in patients with COPD and pulmonary sarcoidosis[72]. Both macrophages and giant cells in pulmonary sarcoid granuloma express CHI3L1, and serum levels of CHI3L1 are indicative for sarcoid disease activity and ongoing fibrosis[73]. In addition, CHI3L1 promotes the proliferation and antagonizes catabolic or degradative processes during the inflammatory response of connective tissues[74]. Increased concentrations of CHI3L1 have been detected also in serum of patients with rheumatoid arthritis (RA). The ability of CHI3L1 to regulate cell proliferation, adhesion, migration, and activation, as well as to regulate extracellular matrix assembly, correlates well with elevated level of CHI3L1 in the sites of chronic inflammation and active connective tissue turnover. Local release of CHI3L1 in the arthritic joint is followed by a secondary increase of CHI3L1 concentration in serum. Neutrophil-released CHI3L1 acts as an autoantigen in RA. In contrast to healthy individuals, who show strong bias to regulatory response to CHI3L1, patients with RA exhibit polarization towards Th1 phenotype[73]. At the same time CHI3L1 is able to suppress the TNFα and IL-1-induced secretion of matrix metalloproteases and IL-8 in both human skin fibroblasts and articular chondrocytes[74]. In contrast, in RA the serum levels of CHI3L1 positively correlated with serum levels of IL-6 and CRP[75]. Increased levels of CHI3L1 in serum reflect the degree of the synovial inflammation and joint destruction in patients with RA and OA[76]. Moreover, elevated level of CHI3L1 is a marker for joint involvement in IBD[77] and for the activity of the disease[59]. Rheumatic symptoms are also common for extra-intestinal manifestations of IBD, which is an autoimmune inflammatory disorder of the colon and small intestine. CHI3L1 also colocalises with lactoferrin, but not with gelatinase in both stimulated and non-stimulated neutrophils. Moreover, release of CHI3L1 from specific neutrophil granules was suggested to lead to the post-transfusional complications, which were avoided depleting leukocytes by filtration of whole blood in order to inhibit extracellular CHI3L1 accumulation during storage of erythrocyte components[78]. CHI3L1 promotes proliferation of human synovial cells, skin and fetal lung fibroblasts, an effect that occurs in synergy with the insulin-like growth factor[79]. CHI3L1 is upregulated in distinct subsets of macrophages, particularly, in early atherosclerotic lesions and in macrophages which infiltrated deep in the lesion[80]. Later proteomics study identified elevated levels of CHI3L1 in supernatants of macrophage cell line THP-1 treated with oxidized LDL[81], proving that CHI3L1 expression is indicative for the differentiation of macrophages during formation of atherosclerotic plaque[79].

CHI3L2 AND IMMUNITY

CHI3L2 was originally isolated from the cultured medium of primary human articular cartilage chondrocytes[82]. CHI3L2 is homologous to the family 18 chitinases in the human genome, it lacks of chitinase activity but possesses a chitinase-like fold and putative lectin properties[83]. CHI3L2 is recognized as a biochemical marker for the activation of chondrocytes and the progress of the osteoarthritis in human. CHI3L2 mRNA is significantly up-regulated in cartilage of patients with osteoarthritis (OA) *vs* normal subjects, while no significant up-regulation was detected for CHI3L2 mRNA in OA cartilage[82]. Particularly CHI3L2 expression is upregulated both in early degenerative and late stage of osteoarthritis. Proteomic analysis established that CHI3L2 is secreted by human osteoarthritic cartilage in culture[5]. The contribution of CHI3L2 to the OA progression is suggested by the induction of autoimmune response[84] and by its involvement in tissue remodeling. However these finding suggested that synovial fibroblasts do not represent the exclusive producers of CHI3L2 in OA. Recently, CHI3L2 has been found slightly expressed in macrophages differentiated in the presence of IFN-γ or IL-4[85]. Only classically activated or M1 macrophages are able to produce CHI3L2, whereas in response to IFN-γ and LPS stimulation undifferentiated monocytes were unable to produce CHI3L2[85]. Thus, IFN-γ which is one of the main cytokines in OA tissues that is able to induce the production of CHI3L2 by monocyte-derived macrophages. In patients with OA, the amount of autoantibodies to CHI3L2 and other autoantigens on early phases of disease indicates that the autoimmune response occurs during the initial phase of cartilage degeneration[86]. It has been demonstrated that Th1 cells prevail in the synovium of patients with OA[87]. In addition the co-treatment of IL-4 and TGF-β promotes stimulatory effect on the expression of CHI3L2 in macrophage cultures[88]. So far, the studies on biological activity of CHI3L2 are limited, therefore further studies are necessary to elucidate the role of CHI3L2 in immunopathology and inflammatory diseases.

CONCLUSION

Chitinases synthesis occurs in most innate immune responses against fungi, bacteria and other non-viral pathogens. In the context of infectious diseases, it is likely that chitinases activity can be both detrimental and beneficial for the host organism. In addition, it cannot be excluded that chitinases augmentations have negative consequences in those conditions in which they are regarded as biochemical markers of macrophage activation. Although we do not yet fully understand the implications of chitinases production in response to chitinous pathogens, the concept of their function as "more than just antipathogens and antifungicidals" seems reasonable. In support to this opinion, the

aforesaid investigations confirming that CHIT-1, CHI3L1 and CHI3L2 can be regarded as mediators of the immune and inflammatory responses and are involved in the progression of degenerative and dismetabolic disorders. The general concept of the chitinases involvement in human diseases discussed in this review may stimulate the development of new planning and experiments leading to a deeper understanding, not only on the biochemical mechanisms inducing chitinases regulation, but also on the consequences that the increases in chitinases levels impact with immunity and autoimmunity in different conditions. The future understanding will lead to the opportunity to develop selective and specific chitinase inhibitors.

REFERENCES

1 **Bussink AP**, Speijer D, Aerts JM, Boot RG. Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. *Genetics* 2007; **177**: 959-970 [PMID: 17720922 DOI: 10.1534/genetics.107.075846]

2 **Boot RG**, Blommaart EF, Swart E, Ghauharali-van der Vlugt K, Bijl N, Moe C, Place A, Aerts JM. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. *J Biol Chem* 2001; **276**: 6770-6778 [PMID: 11085997 DOI: 10.1074/jbc.M009886200]

3 **van Aalten DM**, Komander D, Synstad B, Gåseidnes S, Peter MG, Eijsink VG. Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proc Natl Acad Sci USA* 2001; **98**: 8979-8984 [PMID: 11481469 DOI: 10.1073/pnas.151103798]

4 **Malaguarnera L**. Chitotriosidase: the yin and yang. *Cell Mol Life Sci* 2006; **63**: 3018-3029 [PMID: 17075695 DOI: 10.1007/s00018-006-6269-2]

5 **Kzhyshkowska J**, Gratchev A, Goerdt S. Human chitinases and chitinase-like proteins as indicators for inflammation and cancer. *Biomark Insights* 2007; **2**: 128-146 [PMID: 19662198]

6 **Di Rosa M**, Malaguarnera G, De Gregorio C, D'Amico F, Mazzarino MC, Malaguarnera L. Modulation of chitotriosidase during macrophage differentiation. *Cell Biochem Biophys* 2013; **66**: 239-247 [PMID: 23152091 DOI: 10.1007/s12013-012-9471-x]

7 **Di Rosa M**, Malaguarnera G, De Gregorio C, Drago F, Malaguarnera L. Evaluation of CHI3L-1 and CHIT-1 expression in differentiated and polarized macrophages. *Inflammation* 2013; **36**: 482-492 [PMID: 23149946 DOI: 10.1007/s10753-012-9569-8]

8 **Di Rosa M**, De Gregorio C, Malaguarnera G, Tuttobene M, Biazzo F, Malaguarnera L. Evaluation of AMCase and CHIT-1 expression in monocyte macrophages lineage. *Mol Cell Biochem* 2013; **374**: 73-80 [PMID: 23129258 DOI: 10.1007/s11010-012-1506-5]

9 **Qureshi AM**, Hannigan A, Campbell D, Nixon C, Wilson JB. Chitinase-like proteins are autoantigens in a model of inflammation-promoted incipient neoplasia. *Genes Cancer* 2011; **2**: 74-87 [PMID: 21779482 DOI: 10.1177/1947601911402681]

10 **Hollak CE**, van Weely S, van Oers MH, Aerts JM. Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. *J Clin Invest* 1994; **93**: 1288-1292 [PMID: 8132768 DOI: 10.1172/JCI117084]

11 **Boven LA**, van Meurs M, Boot RG, Mehta A, Boon L, Aerts JM, Laman JD. Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages. *Am J Clin Pathol* 2004; **122**: 359-369 [PMID: 15362365 DOI: 10.1309/BG5VA8JRDQH1M7HN]

12 **Boot RG**, Renkema GH, Verhoek M, Strijland A, Bliek J, de Meulemeester TM, Mannens MM, Aerts JM. The human chitotriosidase gene. Nature of inherited enzyme deficiency. *J Biol Chem* 1998; **273**: 25680-25685 [PMID: 9748235 DOI: 10.1074/jbc.273.40.25680]

13 **Wiesner DL**, Specht CA, Lee CK, Smith KD, Mukaremera L, Lee ST, Lee CG, Elias JA, Nielsen JN, Boulware DR, Bohjanen PR, Jenkins MK, Levitz SM, Nielsen K. Chitin recognition via chitotriosidase promotes pathologic type-2 helper T cell responses to cryptococcal infection. *PLoS Pathog* 2015; **11**: e1004701 [PMID: 25764512 DOI: 10.1371/journal.ppat.1004701]

14 **van Eijk M**, Scheij SS, van Roomen CP, Speijer D, Boot RG, Aerts JM. TLR- and NOD2-dependent regulation of human phagocyte-specific chitotriosidase. *FEBS Lett* 2007; **581**: 5389-5395 [PMID: 17976376 DOI: 10.1016/j.febslet.2007.10.039]

15 **Malaguarnera L**, Simporè J, Prodi DA, Angius A, Sassu A, Persico I, Barone R, Musumeci S. A 24-bp duplication in exon 10 of human chitotriosidase gene from the sub-Saharan to the Mediterranean area: role of parasitic diseases and environmental conditions. *Genes Immun* 2003; **4**: 570-574 [PMID: 14647197 DOI: 10.1038/sj.gene.6364025]

16 **van Eijk M**, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, Sugar A, Verhoeven AJ, Boot RG, Aerts JM. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. *Int Immunol* 2005; **17**: 1505-1512 [PMID: 16214810 DOI: 10.1093/intimm/dxh328]

17 **Malaguarnera L**, Ohazuruike LN, Tsianaka C, Antic T, Di Rosa M, Malaguarnera M. Human chitotriosidase polymorphism is associated with human longevity in Mediterranean nonagenarians and centenarians. *J Hum Genet* 2010; **55**: 8-12 [PMID: 19881466 DOI: 10.1038/jhg.2009.111]

18 **Di Rosa M**, Mangano K, De Gregorio C, Nicoletti F, Malaguarnera L. Association of chitotriosidase genotype with the development of non-alcoholic fatty liver disease. *Hepatol Res* 2013; **43**: 267-275 [PMID: 22971072 DOI: 10.1111/j.1872-034X.2012.01063.x]

19 **Artieda M**, Cenarro A, Gañán A, Lukic A, Moreno E, Puzo J, Pocoví M, Civeira F. Serum chitotriosidase activity, a marker of activated macrophages, predicts new cardiovascular events independently of C-reactive protein. *Cardiology* 2007; **108**: 297-306 [PMID: 17290100 DOI: 10.1159/000099099]

20 **Kanneganti M**, Kamba A, Mizoguchi E. Role of chitotriosidase (chitinase 1) under normal and disease conditions. *J Epithel Biol Pharmacol* 2012; **5**: 1-9 [PMID: 23439988]

21 **Bargagli E**, Margollicci M, Nikiforakis N, Luddi A, Perrone A, Grosso S, Rottoli P. Chitotriosidase activity in the serum of patients with sarcoidosis and pulmonary tuberculosis. *Respiration* 2007; **74**: 548-552 [PMID: 17347558 DOI: 10.1159/000100555]

22 **Iyer A**, van Eijk M, Silva E, Hatta M, Faber W, Aerts JM, Das PK. Increased chitotriosidase activity in serum of leprosy patients: association with bacillary leprosy. *Clin Immunol* 2009; **131**: 501-509 [PMID: 19307157 DOI: 10.1016/j.clim.2009.02.003]

23 **Cho SJ**, Weiden MD, Lee CG. Chitotriosidase in the Pathogenesis of Inflammation, Interstitial Lung Diseases and COPD. *Allergy Asthma Immunol Res* 2015; **7**: 14-21 [PMID: 25553258 DOI: 10.4168/aair.2015.7.1.14]

24 **Malaguarnera L**, Musumeci M, Di Rosa M, Scuto A, Musumeci S. Interferon-gamma, tumor necrosis factor-alpha, and lipopolysaccharide promote chitotriosidase gene expression in human macrophages. *J Clin Lab Anal* 2005; **19**: 128-132 [PMID: 15900564]

25 **Stein M**, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992; **176**: 287-292 [PMID: 1613462]

26 **Malaguarnera L**, Di Rosa M, Zambito AM, dell'Ombra N, Di Marco R, Malaguarnera M. Potential role of chitotriosidase gene in nonalcoholic fatty liver disease evolution. *Am J Gastroenterol* 2006; **101**: 2060-2069 [PMID: 16848812 DOI: 10.1111/j.1572-0241.2006.00680.x]

27 **Malaguarnera L**, Di Rosa M, Zambito AM, dell'Ombra N, Nicoletti F, Malaguarnera M. Chitotriosidase gene expression in Kupffer cells from patients with non-alcoholic fatty liver disease. *Gut* 2006; **55**: 1313-1320 [PMID: 16825325 DOI: 10.1136/gut.2005.075697]

28 **Gavala ML**, Kelly EA, Esnault S, Kukreja S, Evans MD, Bertics PJ, Chupp GL, Jarjour NN. Segmental allergen challenge enhances

chitinase activity and levels of CCL18 in mild atopic asthma. *Clin Exp Allergy* 2013; **43**: 187-197 [PMID: 23331560 DOI: 10.1111/cea.12032]
29 **Lee CG**, Herzog EL, Ahangari F, Zhou Y, Gulati M, Lee CM, Peng X, Feghali-Bostwick C, Jimenez SA, Varga J, Elias JA. Chitinase 1 is a biomarker for and therapeutic target in scleroderma-associated interstitial lung disease that augments TGF-β1 signaling. *J Immunol* 2012; **189**: 2635-2644 [PMID: 22826322 DOI: 10.4049/jimmunol.1201115]
30 **Di Rosa M**, Tibullo D, Vecchio M, Nunnari G, Saccone S, Di Raimondo F, Malaguarnera L. Determination of chitinases family during osteoclastogenesis. *Bone* 2014; **61**: 55-63 [PMID: 24440516 DOI: 10.1016/j.bone.2014.01.005]
31 **Di Rosa M**, Szychlinska MA, Tibullo D, Malaguarnera L, Musumeci G. Expression of CHI3L1 and CHIT1 in osteoarthritic rat cartilage model. A morphological study. *Eur J Histochem* 2014; **58**: 2423 [PMID: 25308850 DOI: 10.4081/ejh.2014.2423]
32 **Di Rosa M**, Tibullo D, Cambria D, Distefano G, Saccone S, Di Raimondo F, Malaguarnera L. Chitotriosidase Expression during Monocyte-Derived Dendritic Cells Differentiation and Maturation. *Inflammation* 2015; **38**: 2082-2091 [PMID: 26026464 DOI: 10.1007/s10753-015-0190-5]
33 **Di Rosa M**, Dell'Ombra N, Zambito AM, Malaguarnera M, Nicoletti F, Malaguarnera L. Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. *Eur J Neurosci* 2006; **23**: 2648-2656 [PMID: 16817867 DOI: 10.1111/j.1460-9568.2006.04780.x]
34 **Di Rosa M**, Zambito AM, Marsullo AR, Li Volti G, Malaguarnera L. Prolactin induces chitotriosidase expression in human macrophages through PTK, PI3-K, and MAPK pathways. *J Cell Biochem* 2009; **107**: 881-889 [PMID: 19415692 DOI: 10.1002/jcb.22186]
35 **Di Rosa M**, Musumeci M, Scuto A, Musumeci S, Malaguarnera L. Effect of interferon-gamma, interleukin-10, lipopolysaccharide and tumor necrosis factor-alpha on chitotriosidase synthesis in human macrophages. *Clin Chem Lab Med* 2005; **43**: 499-502 [PMID: 15899671 DOI: 10.1515/CCLM.2005.088]
36 **Zhu Z**, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elias JA. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004; **304**: 1678-1682 [PMID: 15192232 DOI: 10.1126/science.1095336]
37 **Chou YT**, Yao S, Czerwinski R, Fleming M, Krykbaev R, Xuan D, Zhou H, Brooks J, Fitz L, Strand J, Presman E, Lin L, Aulabaugh A, Huang X. Kinetic characterization of recombinant human acidic mammalian chitinase. *Biochemistry* 2006; **45**: 4444-4454 [PMID: 16584180 DOI: 10.1021/bi0525977]
38 **Adrangi S**, Faramarzi MA. From bacteria to human: a journey into the world of chitinases. *Biotechnol Adv* 2013; **31**: 1786-1795 [PMID: 24095741 DOI: 10.1016/j.biotechadv.2013.09.012]
39 **Zimmermann N**, Mishra A, King NE, Fulkerson PC, Doepker MP, Nikolaidis NM, Kindinger LE, Moulton EA, Aronow BJ, Rothenberg ME. Transcript signatures in experimental asthma: identification of STAT6-dependent and -independent pathways. *J Immunol* 2004; **172**: 1815-1824 [PMID: 14734765 DOI: 10.4049/jimmunol.172.3.1815]
40 **Bierbaum S**, Nickel R, Koch A, Lau S, Deichmann KA, Wahn U, Superti-Furga A, Heinzmann A. Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. *Am J Respir Crit Care Med* 2005; **172**: 1505-1509 [PMID: 16179638 DOI: 10.1164/rccm.200506-890OC]
41 **Seibold MA**, Reese TA, Choudhry S, Salam MT, Beckman K, Eng C, Atakilit A, Meade K, Lenoir M, Watson HG, Thyne S, Kumar R, Weiss KB, Grammer LC, Avila P, Schleimer RP, Fahy JV, Rodriguez-Santana J, Rodriguez-Cintron W, Boot RG, Sheppard D, Gilliland FD, Locksley RM, Burchard EG. Differential enzymatic activity of common haplotypic versions of the human acidic Mammalian chitinase protein. *J Biol Chem* 2009; **284**: 19650-19658 [PMID: 19435888 DOI: 10.1074/jbc.M109.012443]
42 **Cash HL**, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 2006; **313**: 1126-1130 [PMID: 16931762 DOI: 10.1126/science.1127119]
43 **Chen HC**, Chang CC, Mau WJ, Yen LS. Evaluation of N-acetylchitooligosaccharides as the main carbon sources for the growth of intestinal bacteria. *FEMS Microbiol Lett* 2002; **209**: 53-56 [PMID: 12007653]
44 **Noverr MC**, Falkowski NR, McDonald RA, McKenzie AN, Huffnagle GB. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. *Infect Immun* 2005; **73**: 30-38 [PMID: 15618138 DOI: 10.1128/IAI.73.1.30-38.2005]
45 **Hartl D**, He CH, Koller B, Da Silva CA, Kobayashi Y, Lee CG, Flavell RA, Elias JA. Acidic mammalian chitinase regulates epithelial cell apoptosis via a chitinolytic-independent mechanism. *J Immunol* 2009; **182**: 5098-5106 [PMID: 19342690 DOI: 10.4049/jimmunol.0803446]
46 **Roy RM**, Wüthrich M, Klein BS. Chitin elicits CCL2 from airway epithelial cells and induces CCR2-dependent innate allergic inflammation in the lung. *J Immunol* 2012; **189**: 2545-2552 [PMID: 22851704 DOI: 10.4049/jimmunol.1200689]
47 **Van Dyken SJ**, Mohapatra A, Nussbaum JC, Molofsky AB, Thornton EE, Ziegler SF, McKenzie AN, Krummel MF, Liang HE, Locksley RM. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and γδ T cells. *Immunity* 2014; **40**: 414-424 [PMID: 24631157 DOI: 10.1016/j.immuni.2014.02.003]
48 **Rehli M**, Niller HH, Ammon C, Langmann S, Schwarzfischer L, Andreesen R, Krause SW. Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J Biol Chem* 2003; **278**: 44058-44067 [PMID: 12933821 DOI: 10.1074/jbc.M306792200]
49 **Lee JH**, Kim SS, Kim IJ, Song SH, Kim YK, In Kim J, Jeon YK, Kim BH, Kwak IS. Clinical implication of plasma and urine YKL-40, as a proinflammatory biomarker, on early stage of nephropathy in type 2 diabetic patients. *J Diabetes Complications* 2012; **26**: 308-312 [PMID: 22705282 DOI: 10.1016/j.jdiacomp.2012.04.012]
50 **Di Rosa M**, Tibullo D, Saccone S, Distefano G, Basile MS, Di Raimondo F, Malaguarnera L. CHI3L1 nuclear localization in monocyte derived dendritic cells. *Immunobiology* 2016; **221**: 347-356 [PMID: 26466985 DOI: 10.1016/j.imbio.2015.09.023]
51 **Libreros S**, Garcia-Areas R, Shibata Y, Carrio R, Torroella-Kouri M, Iragavarapu-Charyulu V. Induction of proinflammatory mediators by CHI3L1 is reduced by chitin treatment: decreased tumor metastasis in a breast cancer model. *Int J Cancer* 2012; **131**: 377-386 [PMID: 21866546 DOI: 10.1002/ijc.26379]
52 **Chen CC**, Pekow J, Llado V, Kanneganti M, Lau CW, Mizoguchi A, Mino-Kenudson M, Bissonnette M, Mizoguchi E. Chitinase 3-like-1 expression in colonic epithelial cells as a potentially novel marker for colitis-associated neoplasia. *Am J Pathol* 2011; **179**: 1494-1503 [PMID: 21763261 DOI: 10.1016/j.ajpath.2011.05.038]
53 **Nielsen AR**, Erikstrup C, Johansen JS, Fischer CP, Plomgaard P, Krogh-Madsen R, Taudorf S, Lindegaard B, Pedersen BK. Plasma YKL-40: a BMI-independent marker of type 2 diabetes. *Diabetes* 2008; **57**: 3078-3082 [PMID: 18650368 DOI: 10.2337/db08-0182]
54 **Coffman FD**. Chitinase 3-Like-1 (CHI3L1): a putative disease marker at the interface of proteomics and glycomics. *Crit Rev Clin Lab Sci* 2008; **45**: 531-562 [PMID: 19003601 DOI: 10.1080/10408360802334743]
55 **Johansen JS**, Milman N, Hansen M, Garbarsch C, Price PA, Graudal N. Increased serum YKL-40 in patients with pulmonary sarcoidosis--a potential marker of disease activity? *Respir Med* 2005; **99**: 396-402 [PMID: 15763444 DOI: 10.1016/j.rmed.2004.09.016]
56 **Nordenbaek C**, Johansen JS, Halberg P, Wiik A, Garbarsch C, Ullman S, Price PA, Jacobsen S. High serum levels of YKL-40 in patients with systemic sclerosis are associated with pulmonary involvement. *Scand J Rheumatol* 2005; **34**: 293-297 [PMID: 16195162 DOI: 10.1080/03009740510018598]
57 **Lebensztejn DM**, Skiba E, Werpachowska I, Sobaniec-Łotowska ME, Kaczmarski M. Serum level of YKL-40 does not predict

advanced liver fibrosis in children with chronic hepatitis B. *Adv Med Sci* 2007; **52**: 120-124 [PMID: 18217402]

58 **Choi J**, Lee HW, Suk K. Plasma level of chitinase 3-like 1 protein increases in patients with early Alzheimer's disease. *J Neurol* 2011; **258**: 2181-2185 [PMID: 21562723 DOI: 10.1007/s00415-011-6087-9]

59 **Vind I**, Johansen JS, Price PA, Munkholm P. Serum YKL-40, a potential new marker of disease activity in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2003; **38**: 599-605 [PMID: 12825867 DOI: 10.1080/00365520310000537]

60 **Wang Y**, Ripa RS, Johansen JS, Gabrielsen A, Steinbruchel DA, Friis T, Bindslev L, Haack-Sørensen M, Jørgensen E, Kastrup J. YKL-40 a new biomarker in patients with acute coronary syndrome or stable coronary artery disease. *Scand Cardiovasc J* 2008; **42**: 295-302 [PMID: 18615353 DOI: 10.1080/14017430802220567]

61 **Nielsen AR**, Plomgaard P, Krabbe KS, Johansen JS, Pedersen BK. IL-6, but not TNF-α, increases plasma YKL-40 in human subjects. *Cytokine* 2011; **55**: 152-155 [PMID: 21478032 DOI: 10.1016/j.cyto.2011.03.014]

62 **Bara I**, Ozier A, Girodet PO, Carvalho G, Cattiaux J, Begueret H, Thumerel M, Ousova O, Kolbeck R, Coyle AJ, Woods J, Tunon de Lara JM, Marthan R, Berger P. Role of YKL-40 in bronchial smooth muscle remodeling in asthma. *Am J Respir Crit Care Med* 2012; **185**: 715-722 [PMID: 22281830 DOI: 10.1164/rccm.201105-0915OC]

63 **Di Rosa M**, Sanfilippo C, Libra M, Musumeci G, Malaguarnera L. Different pediatric brain tumors are associated with different gene expression profiling. *Acta Histochem* 2015; **117**: 477-485 [PMID: 25792036 DOI: 10.1016/j.acthis.2015.02.010]

64 **Mizoguchi E**. Chitinase 3-like-1 exacerbates intestinal inflammation by enhancing bacterial adhesion and invasion in colonic epithelial cells. *Gastroenterology* 2006; **130**: 398-411 [PMID: 16472595 DOI: 10.1053/j.gastro.2005.12.007]

65 **Ahangari F**, Sood A, Ma B, Takyar S, Schuyler M, Qualls C, Dela Cruz CS, Chupp GL, Lee CG, Elias JA. Chitinase 3-like-1 regulates both visceral fat accumulation and asthma-like Th2 inflammation. *Am J Respir Crit Care Med* 2015; **191**: 746-757 [PMID: 25629580 DOI: 10.1164/rccm.201405-0796OC]

66 **Tran HT**, Lee IA, Low D, Kamba A, Mizoguchi A, Shi HN, Lee CG, Elias JA, Mizoguchi E. Chitinase 3-like 1 synergistically activates IL6-mediated STAT3 phosphorylation in intestinal epithelial cells in murine models of infectious colitis. *Inflamm Bowel Dis* 2014; **20**: 835-846 [PMID: 24694795 DOI: 10.1097/MIB.0000000000000033]

67 **Dela Cruz CS**, Liu W, He CH, Jacoby A, Gornitzky A, Ma B, Flavell R, Lee CG, Elias JA. Chitinase 3-like-1 promotes Streptococcus pneumoniae killing and augments host tolerance to lung antibacterial responses. *Cell Host Microbe* 2012; **12**: 34-46 [PMID: 22817986 DOI: 10.1016/j.chom.2012.05.017]

68 **Rathcke CN**, Vestergaard H. YKL-40--an emerging biomarker in cardiovascular disease and diabetes. *Cardiovasc Diabetol* 2009; **8**: 61 [PMID: 19930630 DOI: 10.1186/1475-2840-8-61]

69 **Kawada M**, Chen CC, Arihiro A, Nagatani K, Watanabe T, Mizoguchi E. Chitinase 3-like-1 enhances bacterial adhesion to colonic epithelial cells through the interaction with bacterial chitin-binding protein. *Lab Invest* 2008; **88**: 883-895 [PMID: 18490894 DOI: 10.1038/labinvest.2008.47]

70 **Lee CG**, Hartl D, Lee GR, Koller B, Matsuura H, Da Silva CA, Sohn MH, Cohn L, Homer RJ, Kozhich AA, Humbles A, Kearley J, Coyle A, Chupp G, Reed J, Flavell RA, Elias JA. Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *J Exp Med* 2009; **206**: 1149-1166 [PMID: 19414556 DOI: 10.1084/jem.20081271]

71 **Ober C**, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R, Radford S, Parry RR, Heinzmann A, Deichmann KA, Lester LA, Gern JE, Lemanske RF, Nicolae DL, Elias JA, Chupp GL. Effect of variation in CHI3L1 on serum YKL-40 level, risk of asthma, and lung function. *N Engl J Med* 2008; **358**: 1682-1691 [PMID: 18403759 DOI: 10.1056/NEJMoa0708801]

72 **Létuvé S**, Kozhich A, Arouche N, Grandsaigne M, Reed J, Dombret MC, Kiener PA, Aubier M, Coyle AJ, Pretolani M. YKL-40 is elevated in patients with chronic obstructive pulmonary disease and activates alveolar macrophages. *J Immunol* 2008; **181**: 5167-5173 [PMID: 18802121 DOI: 10.4049/jimmunol.181.7.5167]

73 **Kim SH**, Choi H, Yoon MG, Ye YM, Park HS. Dipeptidyl-peptidase 10 as a genetic biomarker for the aspirin-exacerbated respiratory disease phenotype. *Ann Allergy Asthma Immunol* 2015; **114**: 208-213 [PMID: 25592153 DOI: 10.1016/j.anai.2014.12.003]

74 **Ling H**, Recklies AD. The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha. *Biochem J* 2004; **380**: 651-659 [PMID: 15015934 DOI: 10.1042/BJ20040099]

75 **van Bilsen JH**, van Dongen H, Lard LR, van der Voort EI, Elferink DG, Bakker AM, Miltenburg AM, Huizinga TW, de Vries RR, Toes RE. Functional regulatory immune responses against human cartilage glycoprotein-39 in health vs. proinflammatory responses in rheumatoid arthritis. *Proc Natl Acad Sci USA* 2004; **101**: 17180-17185 [PMID: 15569925 DOI: 10.1073/pnas.0407704101]

76 **Matsumoto T**, Tsurumoto T. Serum YKL-40 levels in rheumatoid arthritis: correlations between clinical and laborarory parameters. *Clin Exp Rheumatol* 2001; **19**: 655-660 [PMID: 11791636]

77 **Bernardi D**, Podswiadek M, Zaninotto M, Punzi L, Plebani M. YKL-40 as a marker of joint involvement in inflammatory bowel disease. *Clin Chem* 2003; **49**: 1685-1688 [PMID: 14500601 DOI: 10.1373/49.10.1685]

78 **Cintin C**, Johansen JS, Skov F, Price PA, Nielsen HJ. Accumulation of the neutrophil-derived protein YKL-40 during storage of various blood components. *Inflamm Res* 2001; **50**: 107-111 [PMID: 11289654 DOI: 10.1007/s000110050732]

79 **Recklies AD**, White C, Ling H. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. *Biochem J* 2002; **365**: 119-126 [PMID: 12071845 DOI: 10.1042/BJ20020075]

80 **Boot RG**, van Achterberg TA, van Aken BE, Renkema GH, Jacobs MJ, Aerts JM, de Vries CJ. Strong induction of members of the chitinase family of proteins in atherosclerosis: chitotriosidase and human cartilage gp-39 expressed in lesion macrophages. *Arterioscler Thromb Vasc Biol* 1999; **19**: 687-694 [PMID: 10073974 DOI: 10.1161/01.ATV.19.3.687]

81 **Nagy ZS**, Czimmerer Z, Szanto A, Nagy L. Pro-inflammatory cytokines negatively regulate PPARγ mediated gene expression in both human and murine macrophages via multiple mechanisms. *Immunobiology* 2013; **218**: 1336-1344 [PMID: 23870825 DOI: 10.1016/j.imbio.2013.06.011]

82 **Steck E**, Breit S, Breusch SJ, Axt M, Richter W. Enhanced expression of the human chitinase 3-like 2 gene (YKL-39) but not chitinase 3-like 1 gene (YKL-40) in osteoarthritic cartilage. *Biochem Biophys Res Commun* 2002; **299**: 109-115 [PMID: 12435396 DOI: 10.1016/S0006-291X(02)02585-8]

83 **De Ceuninck F**, Marcheteau E, Berger S, Caliez A, Dumont V, Raes M, Anract P, Leclerc G, Boutin JA, Ferry G. Assessment of some tools for the characterization of the human osteoarthritic cartilage proteome. *J Biomol Tech* 2005; **16**: 256-265 [PMID: 16461950]

84 **Du H**, Masuko-Hongo K, Nakamura H, Xiang Y, Bao CD, Wang XD, Chen SL, Nishioka K, Kato T. The prevalence of autoantibodies against cartilage intermediate layer protein, YKL-39, osteopontin, and cyclic citrullinated peptide in patients with early-stage knee osteoarthritis: evidence of a variety of autoimmune processes. *Rheumatol Int* 2005; **26**: 35-41 [PMID: 15378262 DOI: 10.1007/S00296-004-04972]

85 **Ishii H**, Tanaka H, Katoh K, Nakamura H, Nagashima M, Yoshino S. Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis. *Osteoarthritis Cartilage* 2002; **10**: 277-281 [PMID: 11950250 DOI: 10.1053/joca.2001.0509]

86 **Di Rosa M**, Tibullo D, Malaguarnera M, Tuttobene M, Malaguarnera L. Comparison of CHI3L2 and CHIT-1 expression during macrophages differentiation and polarization. *Modern Res Inflamm*

2013; **2**: 82-89 [DOI: 10.4236/mri.2013.24011]

87 **Tsuruha J**, Masuko-Hongo K, Kato T, Sakata M, Nakamura H, Sekine T, Takigawa M, Nishioka K. Autoimmunity against YKL-39, a human cartilage derived protein, in patients with osteoarthritis. *J Rheumatol* 2002; **29**: 1459-1466 [PMID: 12136906]

88 **Gratchev A**, Schmuttermaier C, Mamidi S, Gooi L, Goerdt S, Kzhyshkowska J. Expression of Osteoarthritis Marker YKL-39 is Stimulated by Transforming Growth Factor Beta (TGF-beta) and IL-4 in Differentiating Macrophages. *Biomark Insights* 2008; **3**: 39-44 [PMID: 19578492]

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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

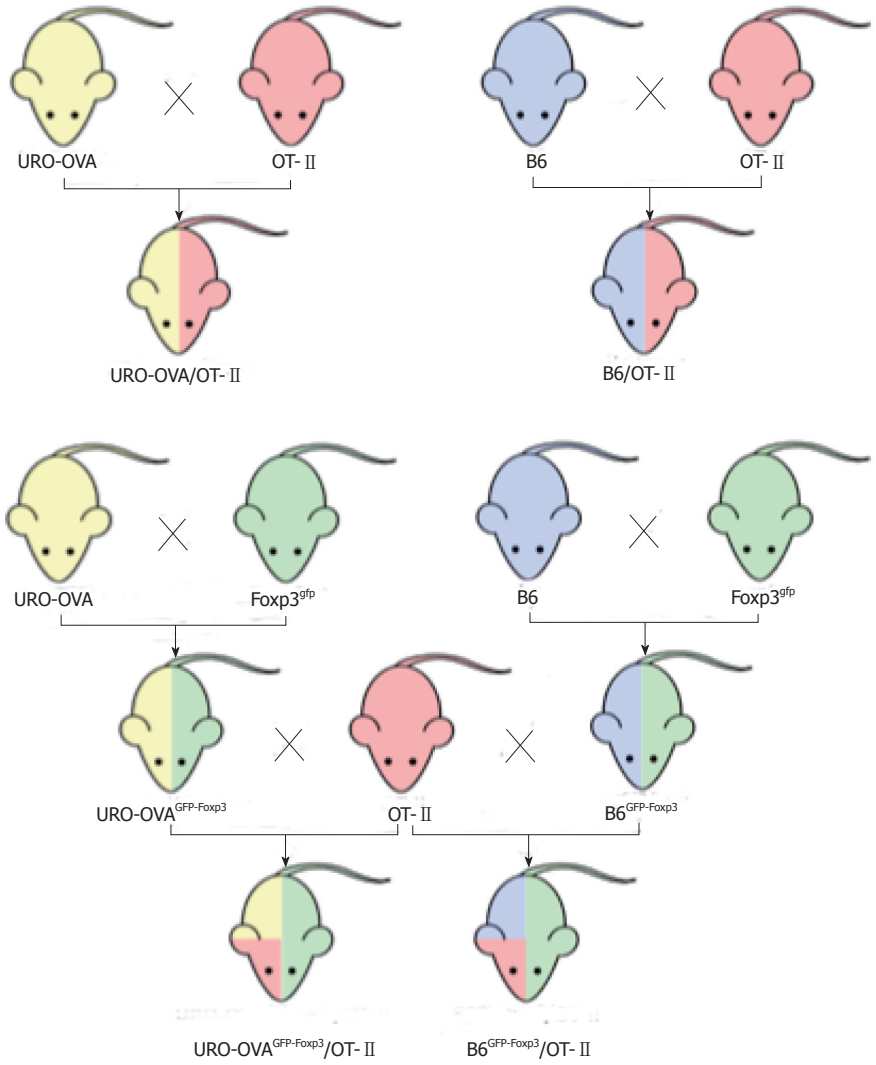


Figure 1 Animal crossbreeding. URO-OVA/OT-Ⅱ mice were generated through crossbreeding of URO-OVA mice with OT-Ⅱ mice. B6/OT-Ⅱ mice were generated through crossbreeding of B6 mice with OT-Ⅱ mice. URO-OVA$^{GFP\text{-}Foxp3}$ mice were generated through crossbreeding of URO-OVA mice with Foxp3^{gfp} mice. 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.

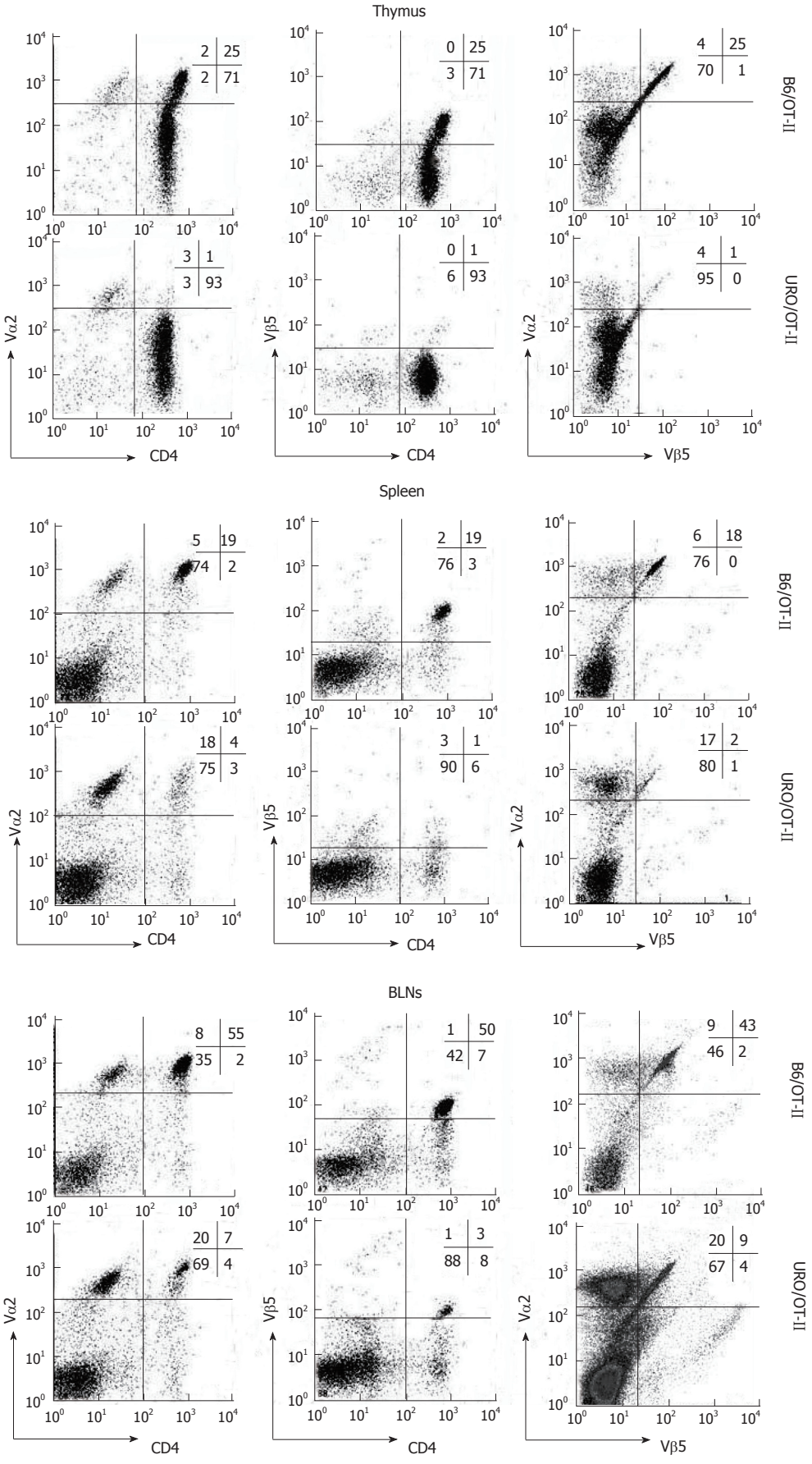


Figure 2 Clonal deletion of OT-II CD4$^+$ T cells in urothelium-ovalbumin/OT-II mice. Cells from the thymus (top panel), spleen (middle panel), and BLNs (bottom panel) of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for 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.

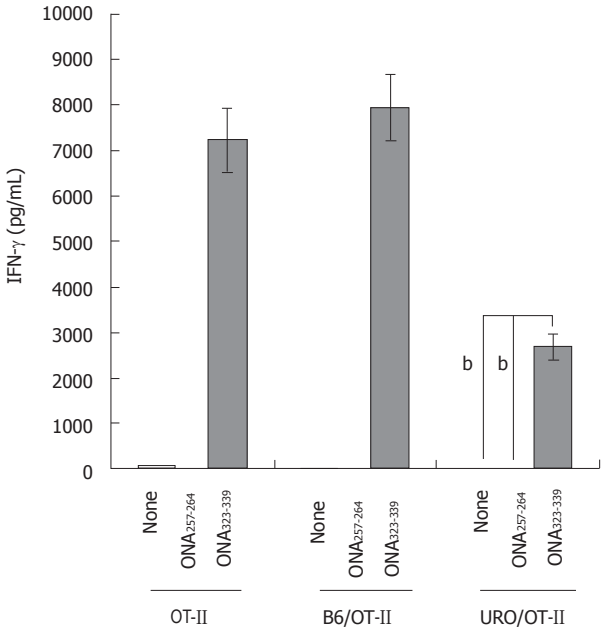


Figure 3 Deletion-escaped OT-II CD4$^+$ T cells retain responsiveness to ovalbumin. Splenocytes from URO-OVA/OT-II mice (8 wk) were cultured alone or in the presence of $OVA_{257-264}$ peptide (10 μg/mL) or $OVA_{323-339}$ peptide (10 μg/mL) for 3 d, followed by ELISA analysis of IFN-γ production in 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

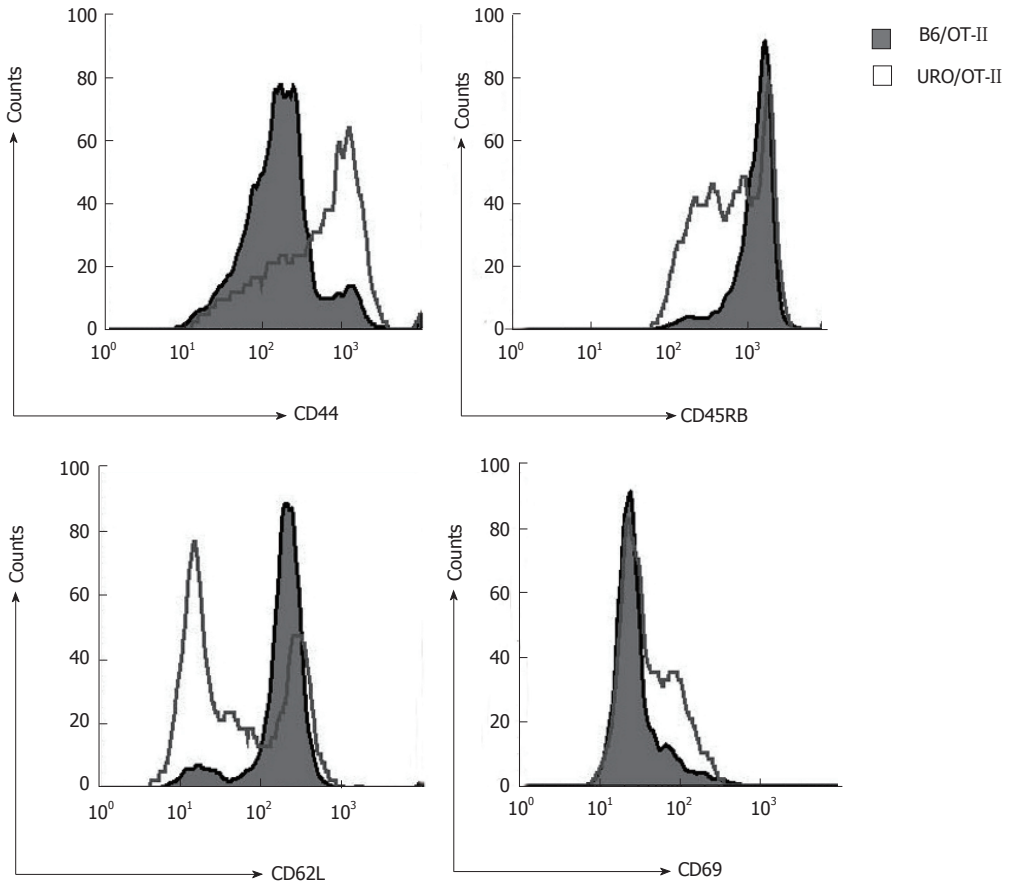
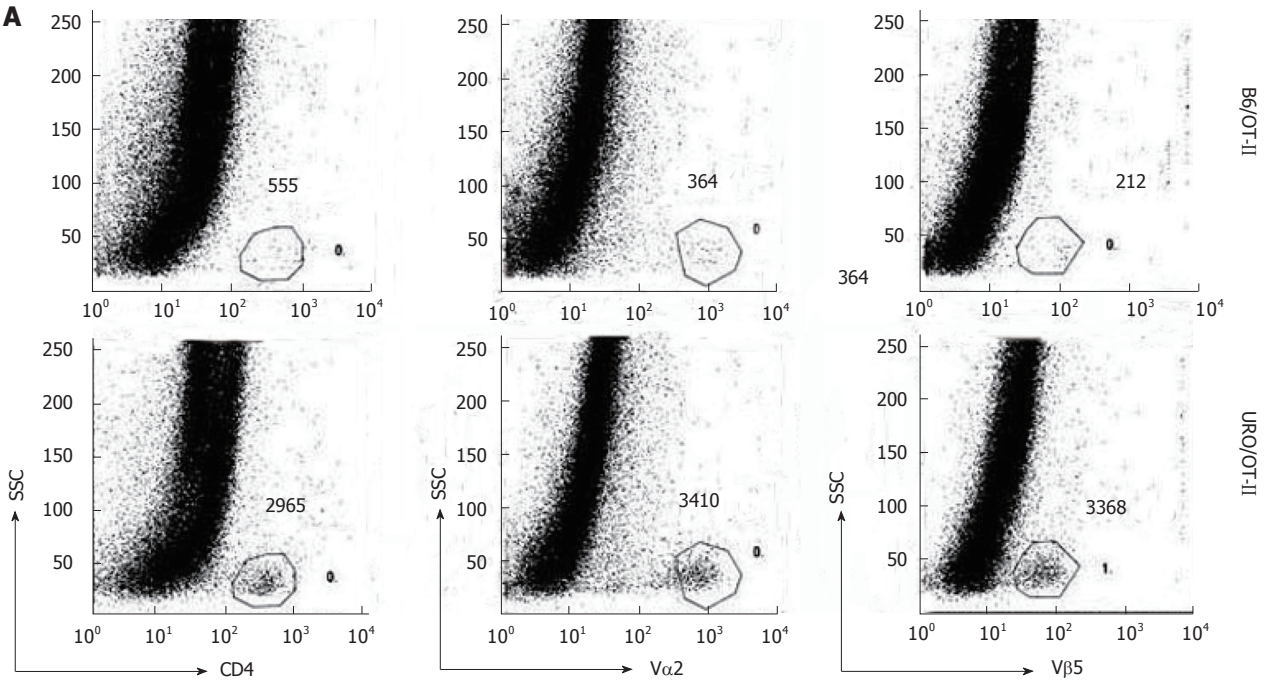


Figure 4 OT-II CD4$^+$ T cells gain activation in the bladder draining lymph nodes of urothelium-ovalbumin/OT-II mice. BLN cells of URO-OVA/OT-II mice (8 wk) were analyzed for surface CD44, CD45RB, CD62L, and CD69 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set 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.



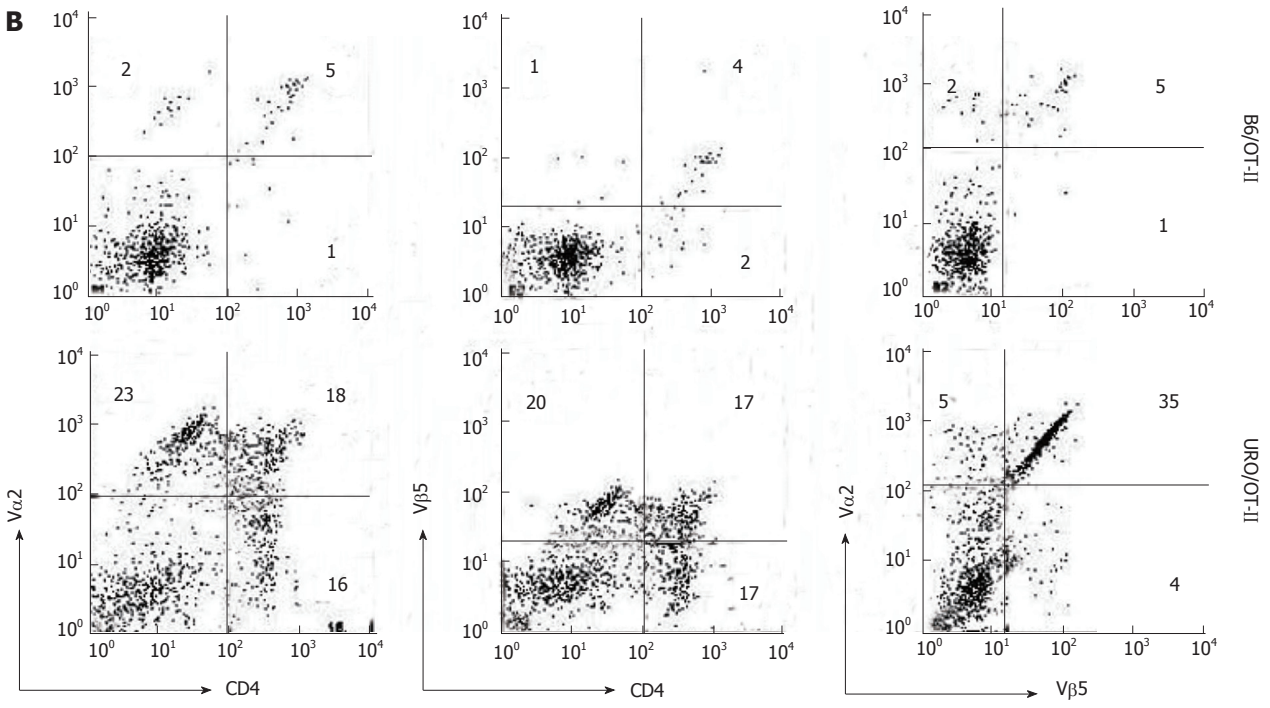


Figure 5 OT-II CD4$^+$ T cells infiltrate into the bladders of urothelium-ovalbumin/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA/OT-II mice (8 wk) and analyzed for surface CD4, Vα2 and Vβ5 by flow cytometry. Age-matched B6/OT-II mice were included for comparison. Gate was set 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.

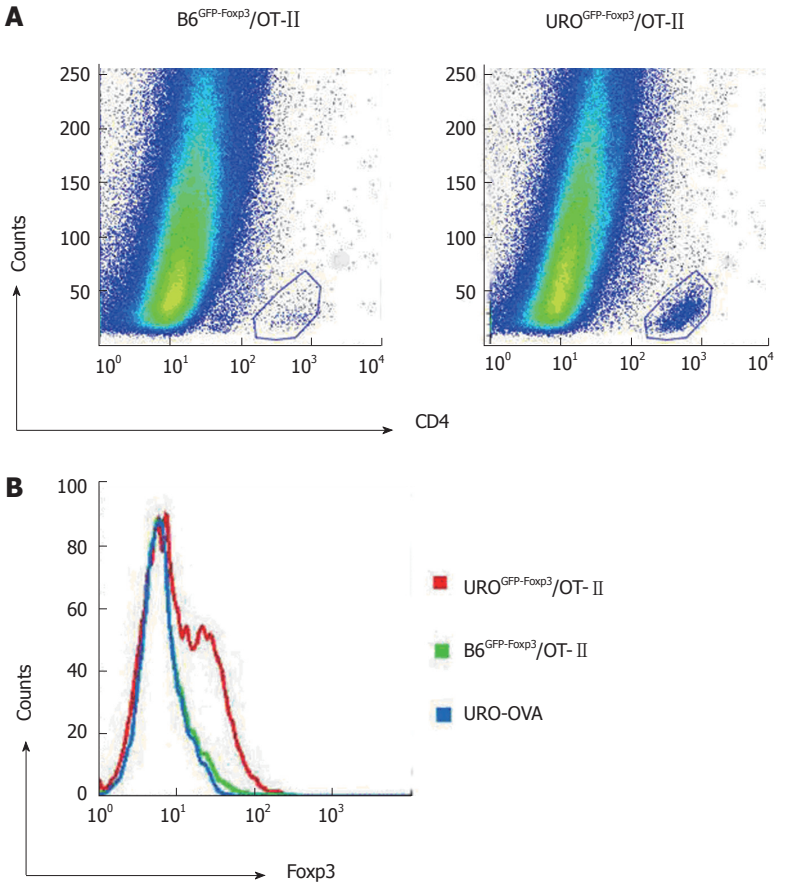


Figure 6 Bladder infiltrating CD4$^+$ T cells consist of Treg cells in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice (8 wk) and analyzed by flow cytometry. Age-matched B6$^{GFP\text{-}Foxp3}$/OT-II mice 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.

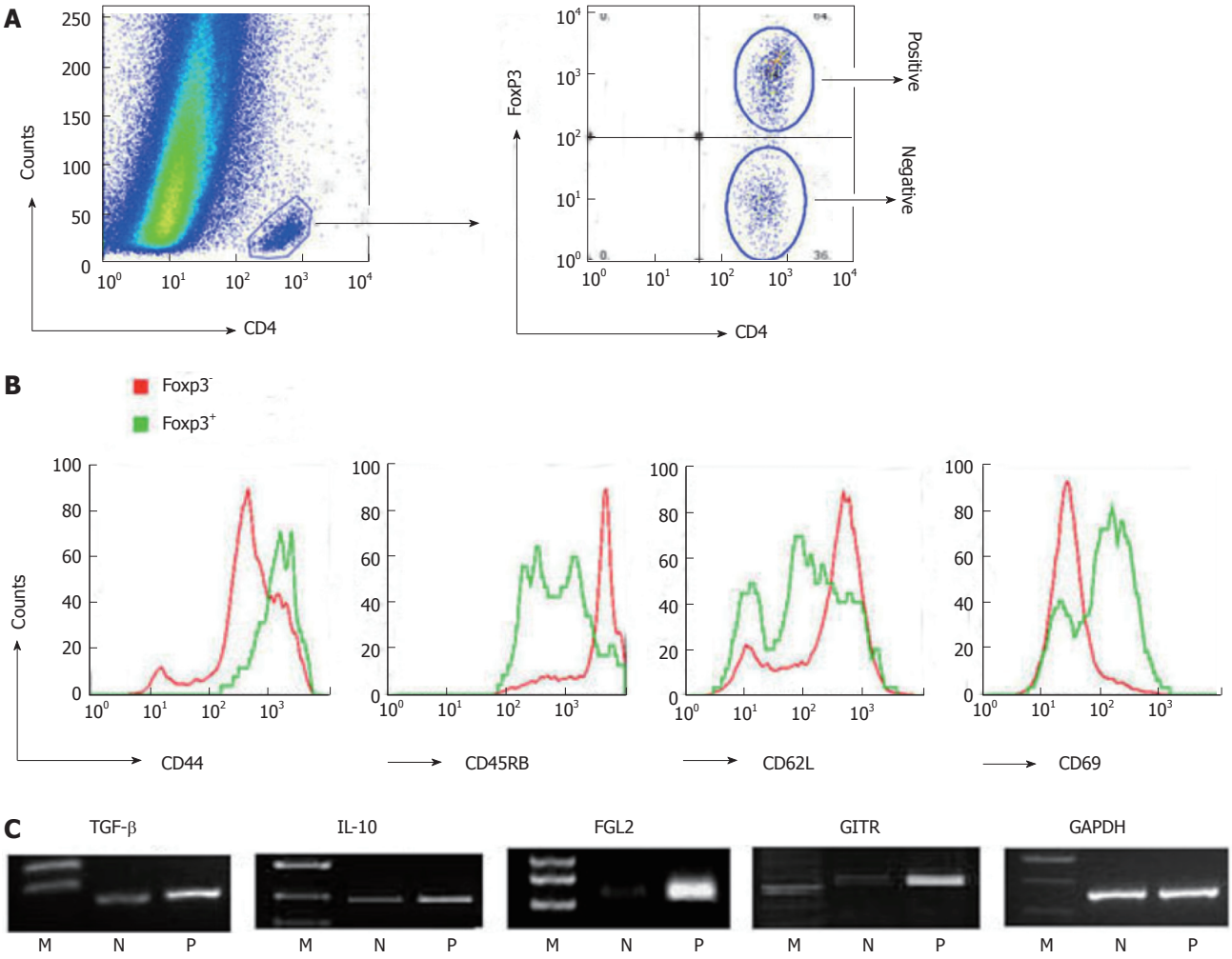


Figure 7 Bladder infiltrating CD4$^+$ Treg cells are functionally active and express inhibitory effector molecules in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. Bladder single-cell suspensions were prepared from URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice (8 wk) and analyzed by flow cytometry 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

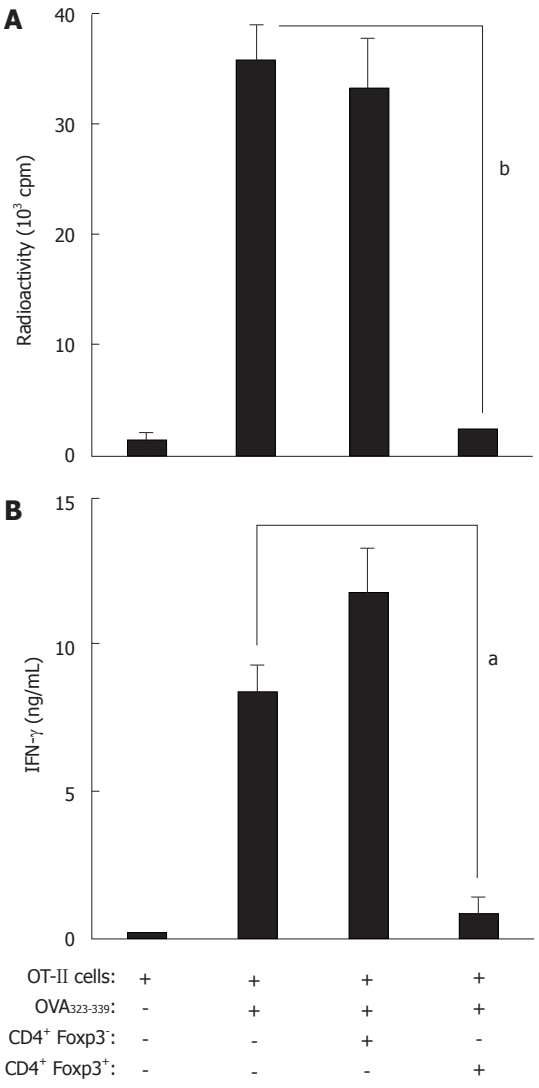


Figure 8 Treg cells from urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice are suppressive to ovalbumin-specific CD4$^+$ T cells. A: OT-II splenocytes were incubated alone or in the presence of OVA323-339 peptide (10 μg/mL), GFP-positive (Foxp3$^+$) CD4$^+$ T cells (at a 1:1 ratio), and/or GFP-negative (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

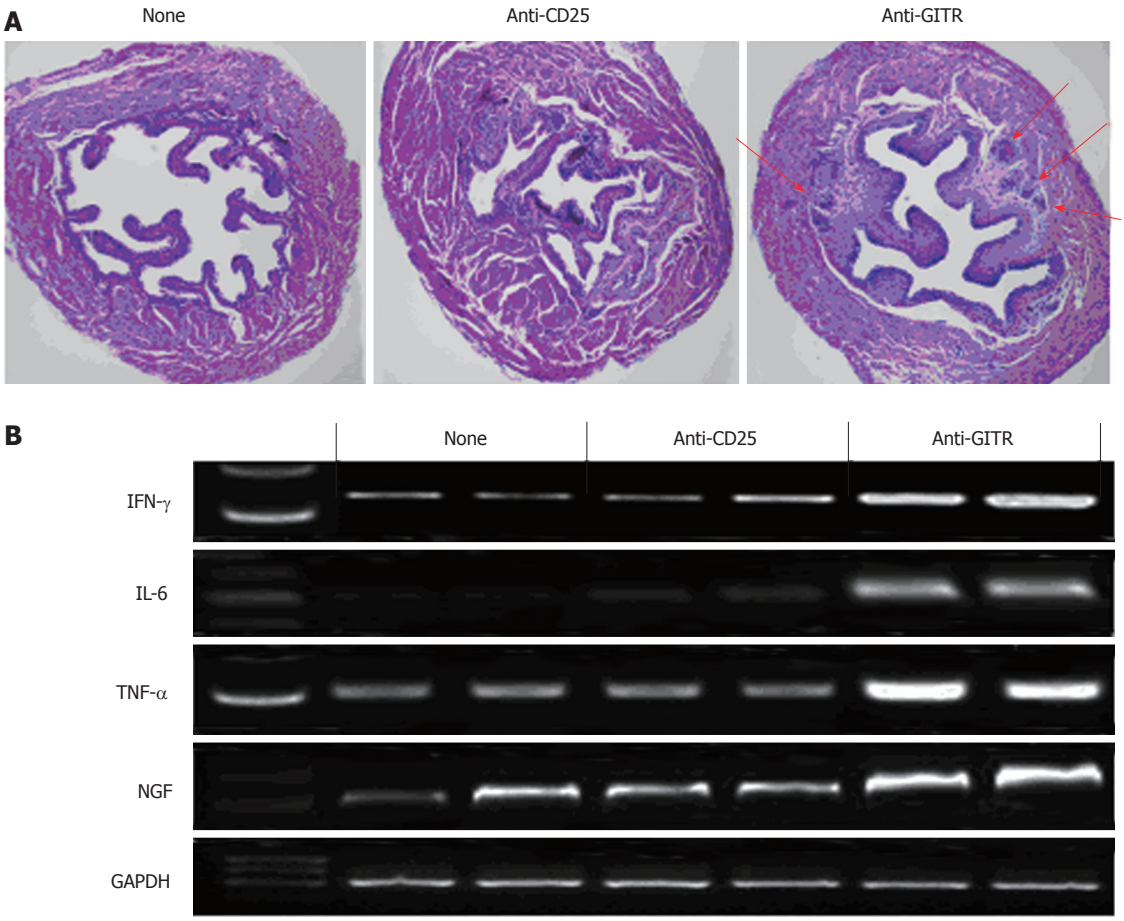


Figure 9 Depletion of CD4$^+$ Treg cells results in bladder autoimmune inflammation in urothelium-ovalbumin$^{GFP\text{-}Foxp3}$/OT-II mice. URO-OVA$^{GFP\text{-}Foxp3}$/OT-II mice were treated with anti-CD25 or anti-GITR mAb every other day beginning at 6 wk of age and sacrificed for analysis at 10 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.

REFERENCES

1 **Sakaguchi S**, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008; **133**: 775-787 [PMID: 18510923 DOI: 10.1016/j.cell.2008.05.009]

2 **Winerdal ME**, Marits P, Winerdal M, Hasan M, Rosenblatt R, Tolf A, Selling K, Sherif A, Winqvist O. FOXP3 and survival in urinary bladder cancer. *BJU Int* 2011; **108**: 1672-1678 [PMID: 21244603 DOI: 10.1111/j.1464-410X.2010.10020.x]

3 **Loskog A**, Ninalga C, Paul-Wetterberg G, de la Torre M, Malmström PU, Tötterman TH. Human bladder carcinoma is dominated by T-regulatory cells and Th1 inhibitory cytokines. *J Urol* 2007; **177**: 353-358 [PMID: 17162090 DOI: 10.1016/j.juro.2006.08.078]

4 **Chi LJ**, Lu HT, Li GL, Wang XM, Su Y, Xu WH, Shen BZ. Involvement of T helper type 17 and regulatory T cell activity in tumour immunology of bladder carcinoma. *Clin Exp Immunol* 2010; **161**: 480-489 [PMID: 20646003 DOI: 10.1111/j.1365-2249.2010.04215.x]

5 **Mattila J**, Linder E. Immunoglobulin deposits in bladder epithelium and vessels in interstitial cystitis: possible relationship to circulating anti-intermediate filament autoantibodies. *Clin Immunol Immunopathol* 1984; **32**: 81-89 [PMID: 6733983 DOI: 10.1016/0090-1229(84)90045-X]

6 **Neal DE**, Dilworth JP, Kaack MB. Tamm-Horsfall autoantibodies in interstitial cystitis. *J Urol* 1991; **145**: 37-39 [PMID: 1984095]

7 **Anderson JB**, Parivar F, Lee G, Wallington TB, MacIver AG, Bradbrook RA, Gingell JC. The enigma of interstitial cystitis--an autoimmune disease? *Br J Urol* 1989; **63**: 58-63 [PMID: 2920261 DOI: 10.1111/j.1464-410X.1989.tb05124.x]

8 **Ochs RL**, Stein TW, Peebles CL, Gittes RF, Tan EM. Autoantibodies in interstitial cystitis. *J Urol* 1994; **151**: 587-592 [PMID: 8308964]

9 **Ochs RL**. Autoantibodies and interstitial cystitis. *Clin Lab Med* 1997; **17**: 571-579 [PMID: 9316774]

10 **Keay S**, Zhang CO, Trifillis AL, Hebel JR, Jacobs SC, Warren JW. Urine autoantibodies in interstitial cystitis. *J Urol* 1997; **157**: 1083-1087 [PMID: 9072548 DOI: 10.1016/S0022-5347(01)65146-2]

11 **Ochs RL**, Muro Y, Si Y, Ge H, Chan EK, Tan EM. Autoantibodies to DFS 70 kd/transcription coactivator p75 in atopic dermatitis and other conditions. *J Allergy Clin Immunol* 2000; **105**: 1211-1220 [PMID: 10856157 DOI: 10.1067/mai.2000.107039]

12 **Liebert M**, Wedemeyer G, Stein JA, Washington R, Faerber G, Flint A, Grossman HB. Evidence for urothelial cell activation in interstitial cystitis. *J Urol* 1993; **149**: 470-475 [PMID: 8094760]

13 **Christmas TJ**, Bottazzo GF. Abnormal urothelial HLA-DR expression in interstitial cystitis. *Clin Exp Immunol* 1992; **87**: 450-454 [PMID: 1544229 DOI: 10.1111/j.1365-2249.1992.tb03018.x]

14 **Erickson DR**, Belchis DA, Dabbs DJ. Inflammatory cell types and clinical features of interstitial cystitis. *J Urol* 1997; **158**: 790-793 [PMID: 9258082 DOI: 10.1016/S0022-5347(01)64317-9]

15 **Yamada T**, Murayama T, Mita H, Akiyama K. Bladder hypersensitivity of interstitial cystitis complicated by allergic diseases. *Urology* 2001; **57**: 125 [PMID: 11378117 DOI: 10.1016/S0090-4295(01)01091-3]

16 **Kim HJ**, Park MH. Obstructive uropathy due to interstitial cystitis in a patient with systemic lupus erythematosus. *Clin Nephrol* 1996; **45**: 205-208 [PMID: 8706364]

17 **Vicencio GP**, Chung-Park M, Ricanati E, Lee KN, DeBaz BP. SLE with interstitial cystitis, reversible hydronephrosis and intestinal manifestations. *J Rheumatol* 1989; **16**: 250-251 [PMID: 2746573]

18 **Sugai S**. Interstitial cystitis and Sjögren's syndrome. *Intern Med* 2004; **43**: 174-176 [PMID: 15098594 DOI: 10.2169/internalmedicine.43.174]

19 **Shibata S**, Ubara Y, Sawa N, Tagami T, Hosino J, Yokota M, Katori H, Takemoto F, Hara S, Takaichi K, Fujii A, Murata H, Nishi T. Severe interstitial cystitis associated with Sjögren's syndrome. *Intern Med* 2004; **43**: 248-252 [PMID: 15098610 DOI: 10.2169/internalmedicine.43.248]

20 **Van de Merwe J**, Kamerling R, Arendsen E, Mulder D, Hooijkaas H. Sjögren's syndrome in patients with interstitial cystitis. *J Rheumatol* 1993; **20**: 962-966 [PMID: 8350331]

21 **Lorenzo Gómez MF**, Gómez Castro S. Physiopathologic relationship between interstitial cystitis and rheumatic, autoimmune, and chronic inflammatory diseases. *Arch Esp Urol* 2004; **57**: 25-34 [PMID: 15119315]

22 **MacDermott JP**, Miller CH, Levy N, Stone AR. Cellular immunity in interstitial cystitis. *J Urol* 1991; **145**: 274-278 [PMID: 1671106]

23 **Bullock AD**, Becich MJ, Klutke CG, Ratliff TL. Experimental autoimmune cystitis: a potential murine model for ulcerative interstitial cystitis. *J Urol* 1992; **148**: 1951-1956 [PMID: 1433651]

24 **Luber-Narod J**, Austin-Ritchie T, Banner B, Hollins C, Maramag C, Price H, Menon M. Experimental autoimmune cystitis in the Lewis rat: a potential animal model for interstitial cystitis. *Urol Res* 1996; **24**: 367-373 [PMID: 9008331 DOI: 10.1007/BF00389795]

25 **Mitra S**, Dagher A, Kage R, Dagher RK, Luber-Narod J. Experimental autoimmune cystitis: further characterization and serum autoantibodies. *Urol Res* 1999; **27**: 351-356 [PMID: 10550523 DOI: 10.1007/s002400050162]

26 **Phull H**, Salkini M, Purves T, Funk J, Copeland D, Comiter CV. Angiotensin II plays a role in acute murine experimental autoimmune cystitis. *BJU Int* 2007; **100**: 664-667 [PMID: 17550411 DOI: 10.1111/j.1464-410X.2007.07035.x]

27 **Lin YH**, Liu G, Kavran M, Altuntas CZ, Gasbarro G, Tuohy VK, Daneshgari F. Lower urinary tract phenotype of experimental autoimmune cystitis in mouse: a potential animal model for interstitial cystitis. *BJU Int* 2008; **102**: 1724-1730 [PMID: 18710451 DOI: 10.1111/j.1464-410X.2008.07891.x]

28 **Altuntas CZ**, Daneshgari F, Sakalar C, Goksoy E, Gulen MF, Kavran M, Qin J, Li X, Tuohy VK. Autoimmunity to uroplakin II causes cystitis in mice: a novel model of interstitial cystitis. *Eur Urol* 2012; **61**: 193-200 [PMID: 21719190 DOI: 10.1016/j.eururo.2011.06.028]

29 **Liu W**, Evanoff DP, Chen X, Luo Y. Urinary bladder epithelium antigen induces CD8+ T cell tolerance, activation, and autoimmune response. *J Immunol* 2007; **178**: 539-546 [PMID: 17182594 DOI: 10.4049/jimmunol.178.1.539]

30 **Liu W**, Deyoung BR, Chen X, Evanoff DP, Luo Y. RDP58 inhibits T cell-mediated bladder inflammation in an autoimmune cystitis model. *J Autoimmun* 2008; **30**: 257-265 [PMID: 18162370 DOI: 10.1016/j.jaut.2007.10.005]

31 **Kim R**, Liu W, Chen X, Kreder KJ, Luo Y. Intravesical dimethyl sulfoxide inhibits acute and chronic bladder inflammation in transgenic experimental autoimmune cystitis models. *J Biomed Biotechnol* 2011; **2011**: 937061 [PMID: 21113298 DOI: 10.1155/2011/937061]

32 **Liu W**, Chen X, Evanoff DP, Luo Y. Urothelial antigen-specific CD4+ T cells function as direct effector cells and induce bladder autoimmune inflammation independent of CD8+ T cells. *Mucosal Immunol* 2011; **4**: 428-437 [PMID: 21270773 DOI: 10.1038/mi.2010.90]

33 **Barnden MJ**, Allison J, Heath WR, Carbone FR. Defective TCR

expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 1998; **76**: 34-40 [PMID: 9553774 DOI: 10.1046/j.1440-1711.1998.00709.x]

34 **Robertson JM**, Jensen PE, Evavold BD. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J Immunol* 2000; **164**: 4706-4712 [PMID: 10779776 DOI: 10.4049/jimmunol.164.9.4706]

35 **Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061 [PMID: 12522256 DOI: 10.1126/science.1079490]

36 **Fontenot JD**, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330-336 [PMID: 12612578 DOI: 10.1038/ni904]

37 **Fontenot JD**, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005; **22**: 329-341 [PMID: 15780990 DOI: 10.1016/j.immuni.2005.01.016]

38 **Page M**, Thorpe R. Purification of monoclonal antibodies. *Methods Mol Biol* 1998; **80**: 113-119 [PMID: 9664368 DOI: 10.1007/978-1-59259-257-9_11]

39 **Ratliff TL**, Klutke CG, Hofmeister M, He F, Russell JH, Becich MJ. Role of the immune response in interstitial cystitis. *Clin Immunol Immunopathol* 1995; **74**: 209-216 [PMID: 7859410 DOI: 10.1006/clin.1995.1031]

40 **Christmas TJ**. Lymphocyte sub-populations in the bladder wall in normal bladder, bacterial cystitis and interstitial cystitis. *Br J Urol* 1994; **73**: 508-515 [PMID: 8012772 DOI: 10.1111/j.1464-410X.1994.tb07635.x]

41 **Harrington DS**, Fall M, Johansson SL. Interstitial cystitis: bladder mucosa lymphocyte immunophenotyping and peripheral blood flow cytometry analysis. *J Urol* 1990; **144**: 868-871 [PMID: 2204728]

42 **Westendorf AM**, Templin M, Geffers R, Deppenmeier S, Gruber AD, Probst-Kepper M, Hansen W, Liblau RS, Gunzer F, Bruder D, Buer J. CD4+ T cell mediated intestinal immunity: chronic inflammation versus immune regulation. *Gut* 2005; **54**: 60-69 [PMID: 15591505 DOI: 10.1136/gut.2003.037663]

43 **Mangialaio S**, Ji H, Korganow AS, Kouskoff V, Benoist C, Mathis D. The arthritogenic T cell receptor and its ligand in a model of spontaneous arthritis. *Arthritis Rheum* 1999; **42**: 2517-2523 [PMID: 10615996 DOI: 10.1002/1529-0131(199912)42:12<2517::AID-ANR3>3.0.CO;2-W]

44 **Mamalaki C**, Murdjeva M, Tolaini M, Norton T, Chandler P, Townsend A, Simpson E, Kioussis D. Tolerance in TCR/cognate antigen double-transgenic mice mediated by incomplete thymic deletion and peripheral receptor downregulation. *Dev Immunol* 1996; **4**: 299-315 [PMID: 8924765 DOI: 10.1155/1995/54219]

45 **Fisson S**, Darrasse-Jèze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL. Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. *J Exp Med* 2003; **198**: 737-746 [PMID: 12939344 DOI: 10.1084/jem.20030686]

46 **Ring S**, Enk AH, Mahnke K. ATP activates regulatory T Cells in vivo during contact hypersensitivity reactions. *J Immunol* 2010; **184**: 3408-3416 [PMID: 20208014 DOI: 10.4049/jimmunol.0901751]

47 **Coe D**, Begom S, Addey C, White M, Dyson J, Chai JG. Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy. *Cancer Immunol Immunother* 2010; **59**: 1367-1377 [PMID: 20480365 DOI: 10.1007/s00262-010-0866-5]

48 **Ko K**, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, Shimizu J, Nomura T, Chiba T, Sakaguchi S. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. *J Exp Med* 2005; **202**: 885-891 [PMID: 16186187 DOI: 10.1084/jem.20050940]

49 **Ramirez-Montagut T**, Chow A, Hirschhorn-Cymerman D, Terwey TH, Kochman AA, Lu S, Miles RC, Sakaguchi S, Houghton AN, van den Brink MR. Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. *J Immunol* 2006; **176**: 6434-6442 [PMID: 16709800 DOI: 10.4049/jimmunol.176.11.6434]

50 **Shevach EM**, Stephens GL. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? *Nat Rev Immunol* 2006; **6**: 613-618 [PMID: 16868552 DOI: 10.1038/nri1867]

51 **Nocentini G**, Riccardi C. GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. *Eur J Immunol* 2005; **35**: 1016-1022 [PMID: 15770698 DOI: 10.1002/eji.200425818]

52 **Apostolou I**, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 2002; **3**: 756-763 [PMID: 12089509 DOI: 10.1038/ni816]

53 **Jordan MS**, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2001; **2**: 301-306 [PMID: 11276200 DOI: 10.1038/86302]

54 **Kawahata K**, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, Miyazaki J, Yamamoto K. Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J Immunol* 2002; **168**: 4399-4405 [PMID: 11970982 DOI: 10.4049/jimmunol.168.9.4399]

55 **Romagnoli P**, Hudrisier D, van Meerwijk JP. Preferential recognition of self antigens despite normal thymic deletion of CD4(+)CD25(+) regulatory T cells. *J Immunol* 2002; **168**: 1644-1648 [PMID: 11823492 DOI: 10.4049/jimmunol.168.4.1644]

56 **Chen W**, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886 [PMID: 14676299 DOI: 10.1084/jem.20030152]

57 **Zheng SG**, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 2004; **172**: 5213-5221 [PMID: 15100259 DOI: 10.4049/jimmunol.172.9.5213]

58 **Rao PE**, Petrone AL, Ponath PD. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-{beta}. *J Immunol* 2005; **174**: 1446-1455 [PMID: 15661903 DOI: 10.4049/jimmunol.174.3.1446]

59 **Green EA**, Choi Y, Flavell RA. Pancreatic lymph node-derived CD4(+)CD25(+) Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. *Immunity* 2002; **16**: 183-191 [PMID: 11869680 DOI: 10.1016/S1074-7613(02)00279-0]

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