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Development and Characterization of Experimental Autoimmune Cystitis (EAC)

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DEVELOPMENT AND CHARACTERIZATION OF EXPERIMENTAL AUTOIMMUNE CYSTITIS (EAC)

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DEDICATION

To My Mother, Ayşe izgi, My Father, Abidin izgi, my Brothers and my first Teacher

Tulay Dolgun

DEVELOPMENT AND CHARACTERIZATION OF EXPERIMENTAL AUTOIMMUNE CYSTITIS (EAC)

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ABSTRACT

Interstitial Cystitis/Painful Bladder Syndrome(IC/PBS) is a chronic inflammation of the urinary bladder, consisting of irritative voiding symptoms; frequent and urgent urination and pain referred to the pelvic region or the bladder upon filling, lack of other pathology. The pathophysiology of interstitial cystitis (IC) is enigmatic. The impaired urothelium of bladder and autoimmunity might lead the underlying pathology. One of the shortcomings in IC/PBS research has been the lack of an appropriate animal model.

In the current study, we show that the bladder specific Uroplakin 3A (UPK3A)-derived immunogenic UPK3A 65-84 peptide is capable of targeting experimental autoimmune cystitis (EAC) in BALB/c mice. We determined an immunogenic peptide from the known sequence of UPK3A based on having the binding motif for IA^d MHC class II molecules expressed in BALB/c mice. The highly antigen-specific proliferative response to UPK3A 65-84 was determined by proliferation assays. Active immunization with the UPK3A 65-84 peptide resulted in increased micturition frequency and decreased urine output per micturition by FVC along with the increased pelvic pain response to von Frey Filaments, and decrease intercontractile intervals, bladder compliance and bladder capacity in CMG after 5 weeks of immunization compared to control group.

The recall responses of LNC to UPK3A 65-84 showed selectively activated CD4+ T-cells with a proinflammatory Th1-like phenotype. Immunocytochemical analysis

showed that immunization with UPK3A 65-84 peptide resulted in T-cell infiltration of the bladder. The ratio of bladder weight to body weight was increased in EAC mice simply showing bladder inflammation. The elevated gene expression levels of TNF-α, IFN-γ, IL-17A, and IL-1β were confined to the bladder but not in other organs. T-cell induction of EAC was identified by showing significantly increased micturition frequency and decreased output per micturition by FVC along with the increased pelvic pain response to von Frey monofilament stimulus after adoptive transfer of peptide-activated CD4+ T-cells into naïve BALB/c hosts.

Our study provides the creation of an advanced and more specific EAC model manifesting all three major phenotypes of IC/PBS by inoculation of mice with, and eliciting an immune response to, tissue specific peptide derived from the Uroplakin of the bladder urothelium. These findings qualify this animal model as a contemporary model for future exploration of pathogenesis and therapeutic intervention of IC/PBS.

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LIST OF ABBREVIATIONS

ANA	Antinuclear Antibodies
ALPS	Autoimmune Lymphoproliferative Syndrome
APC	Antigen Presenting Cell
APECED	Autoimmune Poliendocrinopathy Candidiasis Ectodermal Distrophy
APF	Antiproliferative Factor
AUM	Asymetric Unit Membrane
BSA	Bovine Serum Albumin
CAMHC	Cardiac Alpha Myosin Heavy Chain
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CMG	Cystometrogram
cpm	counts per minute
CYP	Cyclophosphamide
DM	Diabetes Mellitus
dsDNA	Double Stranded DNA
EAC	Experimental Autoimmune Cystitis
EAE	Experimental Autoimmune Encephalomyelitis
EAMC	Experimental Autoimmune Myocarditis
EAO	Experimental Autoimmune Oophoritis
ELISA	Enzyme Linked Immunosorbent Assay
EGF	Epidermal Growth Factor
FVC	Frequency Voiding Charts
GATA 3	GATA Binding Protein 3
GAG	Glycosaminoglycan
GP-51	Glycoprotein 51
GVHD	Graft-Versus-Host-Disease
HB-EGF	Heparin-Binding EGF-like Growth Factor
H2	Histocompatibility Complex 2
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
IA	Mouse MHC Class II Molecule
ICAM	Intercellular Adhesion Molecule
IC	Interstitial Cystitis
IC/PBS	Interstitial Cystitis/Painful Bladder Syndrome
ICS	International Continence Society
IFN-γ	Interferon-Gamma
IGFBP3	Insulin-like Growth Factor Binding Protein 3
IL	Interleukin
i.v.	Intravenous
i.p.	Intraperitoneal

LFA	Lymphocyte Function Associated Antigen
LNC	Lymph Node Cells
LPS	Lipopolysaccharide
M3R	M3 Muscarinic receptor
MHC	Major Histocompatibility Complex
MOBP	Myelin Oligodendrocyte Glycoprotein
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NGF	Nerve Growth Factor
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NOD	Non-Obese Diabetic
OVA	Ovalbumin
p-ANCA	Perinuclear Antineutrophil Cytoplasmic Antibodies
PLP	Myelin Proteolipid Protein
RA	Rheumatoid Arthritis
SS	Sjögren Syndrome
Stat 5	Signal Transducer and Activator of Transcription 5
Stat 6	Signal Transducer and Activator of Transcription 6
SFB	Segmental Filamentous Bacteria
Th0	T helper Cell Type 0
Th1	T helper Cel l Type 1
Th2	T helper Cell Type 2
Th3	T helper Cell Type 3
TH9	T helper Cell Type 9
Th17	T helper Cell Type 17
TGF-β	Tumor Growth Factor Beta
Foxp3	Forkhead Box P3
Treg	Regulatory T cells
T-bet	T box expressed in T cells
THP	Tam Horsfall Protein
TNF	Tumor Necrosis Factor
TCR	T Cell Receptor
TLR	Toll-Like Receptor
UPK	Uroplakin
UPK3A	Uroplakin 3A
UP	Uroplakin

CHAPTER I

BACKGROUND AND INTRODUCTION

1.1. AUTOIMMUNITY

1.1.1. Immune System

The immune system is competent to recognize and horse effectively specific immune response to the vast variety of foreign antigens, pathogens or cancer cells without any damage to host itself. This amazing diversity of immune system is achieved by somatic recombination and rearrangement of gene segments coding for B-cells, antibody molecules, and T-cell receptors (TCR) (French, Laskov et al. 1989; Livak, Burtrum et al. 2000; Li, Woo et al. 2004). The CD4+ CD8+ double positive (DP) cells go into apoptosis if they do not recognize any antigens, and their TCRs bind high affinity to self-peptide/MHC complex (Kappler, Roehm et al. 1987; McDuffie, Roehm et al. 1987). On the other hand, if the DP cells are acquainted with self-antigens or MHC with low affinity, they enter into positive selection and alter to cytotoxic-T cells and T-helper cells (Kappler, Roehm et al. 1987; McDuffie, Roehm et al. 1987). The immature T cells

that recognize the self-antigens binding the Major Histocompatibility Complex(MHC) molecules develop maturation (Robey and Fowlkes 1994).

These generated T-cells express very diverse set of TCRs. The positive selection based on the MHCs narrows the T cells population in the periphery (Sospedra, Ferrer-Francesch et al. 1998). The 3% of the differentiated T cells continue to exist after positive and negative selection (Shortman, Egerton et al. 1990). The mature T cells travel to spleen and lymphoid nodes where they are ready to act in response to foreign antigens. One TCR cross-react with various peptides and this process is required to sustain immune system with adequate flexibility to get used to an incessantly varying antigenic milieu (Garcia and Teyton 1998; Mason 1998). A few T cells with the intermediate affinity to self-antigen flee from the thymic negative selection and are freed in to the periphery(Jiang and Chess 2006).These escaped self-reactive T cells are competent for self-peptide originated proliferative response and can transform into pathogenic effector cells inducing autoimmunity(Mason and Powrie 1998; Kuchroo, Anderson et al. 2002; Jiang and Chess 2006).

1.1.2. General Features of Autoimmunity

Autoimmunity is the outcome of impairment and breakdown of self –tolerance mechanisms due to cause of genetic factors, environmental factors, bystander effect and cross reactivity of infectious agents, Toll-Like Receptor (TLR) cross-linking (Romagnani 2006). The autoimmune disease leads broad changes of immune system along with changes in cytokine profile and autoantibodies causing tissue injure and impaired immune response(Winer and Winer 2012). Autoimmune response attacks the own self-

antigens of body and leads continuous auto-reactivity to targeted tissues or organ causing chronic inflammatory damage(Brower 2004). It is eminent that T-cells play a major role in autoimmune diseases development and initiation via antigen-specific immune response (O'Garra, Steinman et al. 1997) as seen in multiple sclerosis(Swanborg 1995), type 1 diabetes mellitus (Delovitch and Singh 1997), rheumatoid arthritis((Anthony and Haqqi 1999; Fitzpatrick, Green et al. 2011).

Genetic mutations can cause autoimmunity. The autoimmune poliendocrinopathy, candidiasis, ectodermal dystrophy (APECED) is the result of genetic mutation of AIRE gene in central tolerance mechanism (Peterson and Peltonen 2005), and autoimmune lymphoproliferative syndrome (ALPS) is the outcome of mutations in Fas/FasL-mediated apoptosis (Worth, Thrasher et al. 2006).

It was shown that ischemic injury and trauma could elicit autoimmune condition as seen in post-traumatic uveitis (Rahi, Morgan et al. 1978) and orchitis after vasectomy (Goldacre, Abisgold et al. 2006). Moreover, the infectious agent can cause autoimmunity through eliciting sequestered auto-antigen (Miller, Vanderlugt et al. 1997) inducing the inflammatory cytokines and co-stimulatory molecules (Klinman, Yi et al. 1996; Infante-Duarte, Horton et al. 2000) and also elicit T-cells by super antigens(Kearney, Pape et al. 1994).

1.1.3. Organ Specific Autoimmunity

While the systemic autoimmune diseases affect multiple organs, the organ-specific autoimmune diseases mainly target one specific organ or tissue. The immunological reactivity to self-antigen mediated tissue or organ specific autoimmune

disorders have shown in several diseases. Autoimmune hepatitis(T-cell-mediated attack on liver antigens), Hashimoto's thyroiditis (Autoantibodies to thyroglobulin of thyroid gland), Graves' disease (Thyrotropin receptor autoantibodies, antibody to the thyroid-stimulating hormone receptor), type 1 diabetes(autoimmune response to pancreatic islets beta cells) , Addison's disease (autoimmunity against adrenal glands),rheumatoid arthritis(autoimmune attack to synovial tissues of joints), multiple sclerosis(autoimmune attack to central nervous system) and myasthenia gravis(antibody to the acetylcholine receptor) are some of the more common organ-specific autoimmune conditions(Golub 1991; Gossard and Lindor 2012) (Chazenbalk, Portolano et al. 1993; Swanborg 1995; King and Sarvetnick 1997; Anthony and Haqqi 1999) (Martin Martorell, Roep et al. 2002; Wilson 2011) (Ratliff, Klutke et al. 1995). Sjögren's syndrome (autoimmune attacks the exocrine glands) and systemic lupus erythematosus (SLE) (autoantibodies Double-stranded DNA (dsDNA) are well-known examples of systemic autoimmune diseases.

1.1.4. Experimental Organ-Specific Autoimmunity

The conception of the employing of experimental autoimmunity through the stimulation of T-cell mediated targeted self-antigens has utilized to the establishment of practical models of autoimmune conditions such as autoimmune encephalomyelitis (EAE)(Tuohy, Lu et al. 1989; Yu, Johnson et al. 1996), autoimmune myocarditis (Jane-wit, Yu et al. 2002), autoimmune oophoritis(Altuntas, Johnson et al. 2006),autoimmune hearing loss(Solares, Edling et al. 2004) and experimental autoimmune cystitis (EAC) that imitates the phenotype of human interstitial cystitis(IC)(Lin, Liu et al. 2008;

Altuntas, Daneshgari et al. 2011) . For development of organ specific autoimmunity, the lyophilized animal whole organ tissue homogenate, proteins or peptides of targeted organs were used. The animals are immunized with immunogenic antigen in a emulsion of complete Freund's adjuvant (CFA) which augments the inflammatory response to the selected immunogenic protein or peptide. Myelin proteolipid protein (PLP), myelin-associated oligodendrocytic basic protein (MOBP), myelin oligodendrocyte glycoprotein (MOG) are used for induction of EAE in animal that mimics human clinical aspects of multiple sclerosis (Tuohy, Lu et al. 1989; Yu, Johnson et al. 1996; Kaushansky, Zilkha-Falb et al. 2007). The cardiac alpha-myosin heavy chain (CAMHC) is used for induction of experimental autoimmune myocarditis (Jane-wit, Yu et al. 2002), inner ear specific proteins cochlin (Coch) and β-tectorin are used for targeting experimental autoimmune hearing loss (EAHL) in mice(Solares, Edling et al. 2004), and bladder specific uroplakin 2 protein is used for induction of experimental autoimmune cystitis(EAC)(Altuntas, Daneshgari et al. 2011).

1.1.5. T Cells and Autoimmunity

The self-tolerance is preserved through the elimination of immature T cells that recognize self -antigens in the thymus by negative selection (Kappler, Roehm et al. 1987), and T regulatory cells(Treg)(Sakaguchi, Sakaguchi et al. 1995), deletion by apoptosis, T cell anergy (Jenkins and Schwartz 2009) in the periphery (Walker and Abbas 2002; Saeki and Iwasa 2011). Self-reactive T cells recognize and attack to own tissues or organs that lead autoimmunity in the periphery (Seddon and Mason 1999; Jordan, Boesteanu et al. 2001). Genetic mutations or deletions can also breakdown the tolerance

and contribute the development of autoimmunity (Abbas, Lohr et al. 2004). Some auto-reactive T cells flee from the thymic negative selection due to absence of self-antigen manifestation in the thymus (Ohashi 2003). The splice variants of self-proteins which do not have the proper T-cell epitope avoid the thymic deletion (Klein, Klugmann et al. 2000). Moreover, it was suggested that autoimmunity could be induced from mutations in self-proteins making them immunogenic (Houghton and Guevara-Patino 2004) (Engelhorn, Guevara-Patino et al. 2006) as seen in mutated pigment-related proteins associated to melanoma showed autoimmune T-cell response to this self-antigen(Engelhorn, Guevara-Patino et al. 2006; Guevara-Patino, Engelhorn et al. 2006).

1.1.6. Major Histocompatibility Complex (MHC)

Whereas nearly all nucleated cells comprise MHC Class I molecules, MHC class II molecules are located outer surface of antigen presenting cells (APCs); dendritic cells, macrophages, B cells and a few other types. T cell receptors (TCR) are antigen-specific receptors that define the specificity T cells. T-cells recognize the peptides presented through the MHC molecule on the surface of APCs (Babbitt, Allen et al. 1985; Townsend, Rothbard et al. 1986). Major histocompatibility complex (MHC) class I molecules present the peptides from the proteolytic degradation of inside antigens including viruses and intracellular bacteria, introduce antigens to antigen-specific CD8+ cytotoxic T lymphocytes, whereas MHC class II molecules that are presented on the surface of antigen presenting cells inrtoduce the antigenic peptides from extracellular and soluble antigens to the CD4+ T cells, leading activation, proliferation and differentiation of T cells subsets (McHeyzer-Williams, Altman et al. 1996; Zhu, Cote-Sierra et al. 2003).

The strong association of MHC class II molecule alleles with diseases lead them to become the most important genetic risk factors for autoimmune diseases (Jones, Fugger et al. 2006) such as human leukocyte antigen (HLA) DR4 in insulin depended diabetes and HLA DR2 haplotype linkage in Multiple Sclerosis(Kent, Chen et al. 2005; Sospedra and Martin 2005). The link of HLA-B27 with ankylosing spondylitis is another example of genetically mediated autoimmune disease (Thomas and Brown 2010).

1.1.7. T Cells

T cells are white blood cells well-known as lymphocytes, they mediate cellular immunity. T cells are differentiated from other cells by T cell receptor (TCR) on their cell surface. CD4 T cells have two different subtypes; Th1 and Th2. The differentiation of Th1 and Th2 response is based on cytokine expressions. While Th1 response generates IL-2 and Interferon gamma (IFN-γ), Th2 response secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13(Romagnani 1995). CD8 T cells also have different subsets corresponding to different cytokine expressions; Tc1 subsets produce IFN-γ but not IL-4 or IL-5, Tc0 produce IL-4, IFN-γ and other cytokines, Tc2 subsets produce IL-4 but not IFN-γ (Croft 1994; Sad, Marcotte et al. 1995; MacAry, Holmes et al. 1998; Vukmanovic-Stejic, Vyas et al. 2000). IL-12 stimulate Tc1 subsets CD8 T cell proliferation, while IL-4 inhibits proliferation of clones (Vukmanovic-Stejic, Vyas et al. 2000). IL-4 producing CD8 T cell clones were shown in some diseases such as HIV(Maggi, Giudizi et al. 1994),cutaneous leishmaniasis (Uyemura, Pirmez et al. 1993), lepromatous leprosy(Salgame, Abrams et al. 1991), periodontitis(Wassenaar, Reinhardus et al. 1996), and in asthma(Till, Li et al. 1995).

1.1.8. T helper cells

Naive T-cells is called Th0 cells which facilitate moderate effect, relying on the ratio of Th1/Th2 cytokines. Th3 is another T cell subset that produces TGF-β(Chen, Kuchroo et al. 1994) named regulatory T cells(Treg) having Foxp3(Fontenot, Gavin et al. 2003; Carrier, Yuan et al. 2007).TGF-β mediates the differentiation of Foxp3 negative naive CD4 T cells into Foxp3 positive CD4 T cells called Treg cells(Chen, Jin et al. 2003).

Th1 cells play role in the pathogenesis of Hashimoto's disease, type 1 DM, autoimmune gastritis and MS (Romagnani 1997; Romagnani, Parronchi et al. 1997). IFN-γ secreted by Th1 cells is vital to macrophage activation in terms of elevating the microbicidial response (Suzuki and Remington 1988). IL-2 production by Th1 cells is important for CD4 T cell memory (Darrah, Patel et al. 2007). IFN-γ induces Th1 subset differentiation (Lighvani, Frucht et al. 2001) through the stimulation of Stat-1 and T-bet (Szabo, Kim et al. 2000). After the T cell activation through the TCR interaction with APCs, bulk amount of IL-12 is expressed by APCs. Stat4 augments the Th1 response through IL-12 mediated phosphorylation transduction leading T-bet activation of IFN-γ (Kaplan, Sun et al. 1996; Thierfelder, van Deursen et al. 1996).

Th2 subset differentiation is promoted by IL-4. Stat 6 is necessary for inducing response to IL-4 and Th2 differentiation (Kaplan, Sun et al. 1996). Additionally, Stat 6 activation leads the expression of GATA-3 which is Th2 chief regulatory gene (Jankovic, Kullberg et al. 2000; Zhu, Guo et al. 2001). GATA-3 is important for the production of IL-5 and IL-13(Zhu, Min et al. 2004; Zhu, Yamane et al. 2006).The IL-2- induced stat5 activation and IL-4 mediated GATA-3 stimulation through stat6 leading complete Th2

differentiation(Yamane, Zhu et al. 2005; Zhu, Yamane et al. 2006). Th1 cells are pivotal for immune defense to pathogens inside the cells, whereas Th2 cells are for immune defense to extracellular pathogens such as helmints (Mosmann and Coffman 1989; Paul and Seder 1994).The balance between Th1 and Th2 determines the severity of autoimmune disease; While Th1 cells are considered as developer pathogenic effect, Th2 cells considered as having inhibitory effects (Nicholson and Kuchroo 1996).

The Th17 cells have been associated with different diseases. Th17 cells are promoted from naive T cells through inducing TGF-β and IL-6 (Bettelli, Carrier et al. 2006; Veldhoen, Hocking et al. 2006). The function of IL-17 was documented in human autoimmune diseases such as multiple sclerosis (Matusevicius, Kivisakk et al. 1999), rheumatoid arthritis(Aarvak, Chabaud et al. 1999), psoriasis (Teunissen, Koomen et al. 1998), tissue inflammation (Steinman 2007). Th17 cells generate IL-17A, IL-17F, IL-21, IL-22, and have been shown to transfer EAE to naïve recipient (Langrish, Chen et al. 2005) and also Th17 cells induce EAE (O'Connor, Prendergast et al. 2008). It was shown that IL-23 is the major contributor in induction of EAE along with IL-17(Harrington, Hatton et al. 2005; Park, Li et al. 2005) and promotes naïve CD4+ T cells into Th17 lineage by inhibiting T-bet and Foxp3 (Mus, Cornelissen et al. 2010).

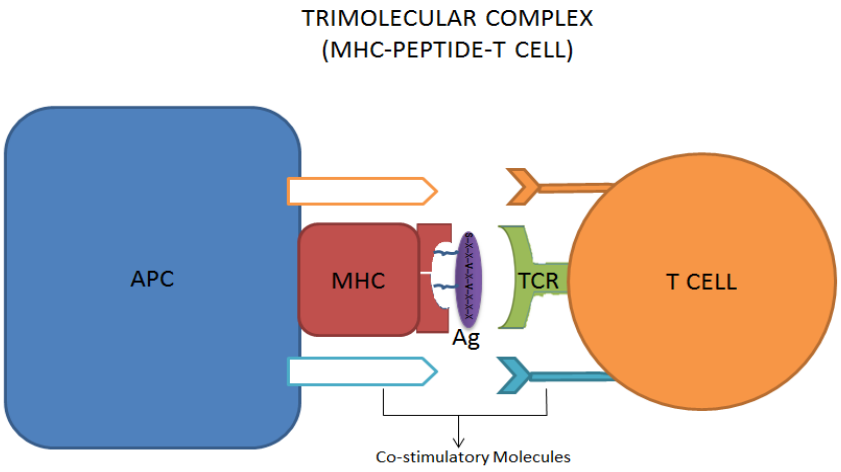
CD4 Th1 and Th17 T cells are primary mediators of autoimmune diabetes (Haskins 2005) (Wagner, Newell et al. 1999; Emamaullee, Davis et al. 2009). It was shown that the levels of Th17-associated proinflammatory cytokines such as IL-1β, IL-6 and IL-23, were increased in NOD mice, insulin-dependent diabetes (Type 1DM) and IFN-γ induced by IL-12 application stop diabetes development via blocking the IL-17 expression(Zhang, Huang et al. 2012).Th17 cells have been shown to be more effective

than Th1 cells in disease progress (Langrish, Chen et al. 2005). Moreover, the changed balance between Th1 and Th17 cells in circulation was considered as a sign showing the impairment in murine acute Graft-Versus-Host Disease (GVHD) (Pan, Zeng et al. 2012).

Additionally another subset of T cells has been defined called Th9 cell which are formed after induction with TGF-β and IL-4 and produce IL-9 and IL-10(Dardalhon, Awasthi et al. 2008; Veldhoen, Uyttenhove et al. 2008; Lin, Chen et al. 2012). It was shown that Th9 cells can mediate tissue inflammation in a colitis model (Dardalhon, Awasthi et al. 2008) and in EAE upon adoptive transfer with Th9 cells from myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic T cells (Jager, Dardalhon et al. 2009).

1.1.9. Trimolecular Complex

For the full T cell Activation APC s are required to present peptides through MHC molecules to TCR of T cells, however this interaction is weak and some co-stimulatory signals such as CD28/B7-1(CD80) and B7-2(CD86), 4-1BB/4-1BBL and adhesion structures (Sharpe and Abbas 2006) CD4/MHC-class II or CD8/MHC class I, LFA-1/ICAM-1, CD2/LFA-3 , CD40/CD40L are contributed this trimolecular complex and these interactions generate signal and fully activation of T cells along with cytokines production(Smith, Graham et al. 1994; Wingren, Parra et al. 1995; Perrin, Scott et al. 1996; Shuford, Klussman et al. 1997). Moreover, co-stimulatory signals through OX40/OX40L and CD27/CD70 enrich T cell activation in along with CD28/B7 (Croft 2003).



1.2. INTERSTITAIL CYSTITIS/PAINFUL BLADDER SYNDROME(IC/PBS)

According to The International Continence Society (ICS), the definition for IC/PBS is

> "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and night-time frequency, in the absence of proven urinary infection or other obvious pathology"(Abrams, Cardozo et al. 2002).

IC is a chronic germ-free inflammation of the urinary bladder with the nonappearance of verified infection (Forrest and Vo 2001; Bogart, Berry et al. 2007), and is presented by the symptoms of frequent and urgent urination a long with pain or irritation in the bladder and lower urinary tract (LUT), necessitating the adding of the new terminology painful bladder syndrome (PBS)(Toft and Nordling 2006; Hanno 2008) (Bogart, Berry et al. 2007). The prevalence of IC/PBS had been estimated as 55 per 100,000 or 45 per 100,000 by using the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

criteria(Curhan, Speizer et al. 1999; Clemens, Meenan et al. 2005). IC occurs mainly in female group with a ratio of 5:1 to male(Bogart, Berry et al. 2007). Based on the high sensitivity and specificity criteria it was anticipated that nearly 3.5 to 8 million women at age 18 or older suffer from bladder pain syndrome/interstitial cystitis symptoms in the United States (Berry, Elliott et al. 2011). According to the NIDDK diagnostic criteria IC/PBS occurs 17 times more in the relatives of IC/PBS patients than general population (Warren, Jackson et al. 2004).

The medical costs are predicted to surpass the $100 million per year(Nickel 2004). The diagnostic standard criteria for IC/PBS is compose of presence Hunner's ulcer on cystoscopy with urinary symptoms of pelvic pain, urgent to urinate, frequent urination and ease of dyspareunia after voiding(Hanno, Levin et al. 1990). While the only 20% of patients present Hunner's ulcer, 80% of patients do not have ulcer in their bladder (Messing and Stamey 1978; Koziol 1994). Patients suffer considerable morbidity over the course of their lives, especially during the most productive years. Advancement in addressing this disease has been painfully slow due to a lack of understanding of the underlying pathophysiology.

1.2.1. Etiology of IC/PBS

The etiopathogenesis of IC/PBS has not been explicated yet. Several different theories have been proposed on the underlying pathology of IC/PBS(Oberpenning, van Ophoven et al. 2002); defects of bladder barrier glycosaminoglycan(GAG) layer, infection induced, mast cell activation(Hofmeister, He et al. 1997; Parsons 2003; Theoharides 2004) autoimmunity, antiproliferative factor(Keay, Zhang et al. 2001),

decreased sialylation and glycosylation of Tam Horsfall Protein(THP)(Argade, Vanichsarn et al. 2009), TNF-related apoptosis-inducing ligand(TRAIL) and its receptor(Kutlu, Akkaya et al. 2010), elevated transient receptor potential subtype 1 vaniloid receptor in nerve fibers(Mukerji, Yiangou et al. 2006), neuroendocrine and neuroimmune mechanisms(Butrick 2003).

1.2.2. Pathological Changes in IC/PBS

IC/PBS is chronic urinary bladder inflammation with the manifested symptoms of pelvic pain, frequency, urgency, nocturia, irritative micturition, dyspareunia and sterile urine (Parsons 1996; Warren, Langenberg et al. 2008). The csytoscopic findings of IC patients compose of glomerulations which are caused by mechanical and congestive ruptures of capillaries during hydrodistention (Johansson and Fall 1990; Tamaki, Saito et al. 2004)(MASAHIRO TAMAKI, 2004; Johansson SL, 1990) and Hunner's ulcers.

1.3. IC/PBS AND AUTOIMMUNITY

IC/PBS exhibits some characteristic features that can be suggested as the signs for autoimmune chronic disorder (van de Merwe 2007). These features are; no existence microorganism in the bladder of IC patients, the higher incidence of disease in the people with certain HLA type(Christmas and Bottazzo 1992), more frequent in women than in men and existence of autoantibodies in serum of IC patients(Alarcon-Segovia, Abud-Mendoza et al. 1984; Ochs 1997; Peeker, Atanasiu et al. 2003),the occurrence of another autoimmune disease in patients and other family members(Warren, Jackson et al. 2004), improvement with immunologically affective drugs (Sairanen, Tammela et al. 2005;

Evans, Moldwin et al. 2011) and immune suppressive agents(Liu, Deyoung et al. 2008). Anti-NGF monoclonal antibody (Tanezumab) is used for treatment of IC/PBS symptoms showing improvement in pain, urgency and frequency (Evans, Moldwin et al. 2011).Moreover the induction of disease in naïve hosts after adoptive transfer of lymphocytes or antibodies(Bullock, Becich et al. 1992), and also observing satisfactory effects after the treatment of a drug that is effecting the immune response(Kim, Liu et al. 2011).

1.3.1. HLA-Linkage in IC/PBS

The upregulation of HLA-DR and antinuclear antibodies (ANA) were observed in IC patients samples as the sign of immune response (Jokinen, Oravisto et al. 1973; Christmas and Bottazzo 1992; Liebert, Wedemeyer et al. 1993). In another study the 36% of patients with IC showed increased antinuclear antibody (Ochs, Stein et al. 1994). IC also has been connected with HLA DR6 allele of MHC II as a relative risk factor (Christmas and Bottazzo 1992; Christmas 1994).

1.3.2. Immune Disorders Associated with IC/PBS

The occurrences of IC/PBS with different diseases give some ideas about the cause of etiology and potential treatment strategy. The high incidence of IC association with the nonspecific autoimmune disorders including systemic lupus erythematosis (SLE), rheumatoid arthritis (RA), thyroiditis, and Sjögren syndrome (SS) has been shown(Oravisto 1980; Fall 1985; Golstein, Manto et al. 1994; Van De Merwe and

Arendsen 2000; Peeker, Atanasiu et al. 2003; van de Merwe, Yamada et al. 2003). Moreover, In a case report the chronic IC was revealed through the superimposing course from rheumatoid arthritis to lupus (Golstein, Manto et al. 1994). Autoantibodies to SS-A/Ro and SS-B/La were revealed in approximately 13% of IC patients (van de Merwe, Yamada et al. 2003).The antibodies to M3R of bladder in IC patients with Sjögren Syndrome were suggested leading initial symptoms and later local inflammation (Van De Merwe and Arendsen 2000). IC patients show different diseases concomitantly such that allergic diseases, sensitive skin diseases irritable, bowel disease, migraine headaches, fibromyalgia, vulvodynia and pelvic floor dysfunction were higher in IC/PBS patients than in normal population (Alagiri, Chottiner et al. 1997; Moldwin 2002; Yamada 2003; Shorter, Kushner et al. 2006).

1.4. BLADDER AUTOIMMUNITY

1.4.1. Autoantibodies in IC/PBS

The accumulation immunoglobulin (IgG, IgA, and IgM) and complement to elastic structures of bladder of IC were revealed (Helin, Mattila et al. 1987; Gillespie, Said et al. 1990). It was reported that nearly 50% of the IC patient exhibit autoantibodies (Ochs 1997). The high incidence of IC in women with waxing and waning symptoms along occurrence of infiltrating lymphocytes, antinuclear antibodies, anti-tissue antibodies, and anti-Tamm-Horsfall antibodies are considered as autoimmune sign in IC pathogenesis (Mattila and Linder 1984; Said, Van de Velde et al. 1989; Gillespie, Said et al. 1990; Neal, Dilworth et al. 1991).

1.4.2. Cellular Immune Abnormalities in IC/PBS

In most of the IC patients' bladder biopsies the main pathological alterations were the increased vascularity, lymphocytes infiltration, and mast cells accumulation (Elbadawi 1997). The elevation of T helper cells, B cell nodules, and plasma cells deposition in bladder biopsies of IC patients was showed indicating a cell mediated immune reaction (Harrington, Fall et al. 1990; MacDermott, Miller et al. 1991; Elbadawi 1997). In Interstitial cystitis patients' bladder biopsies the IgG accumulation, lymphocytes, mast cells and plasma cells infiltration were observed (Boye, Morse et al. 1979; Rosin, Griffiths et al. 1979). In IC patients, the accumulation of CD8+ T cells in urothelium and CD4+ T cells in lamina propria were demonstrated in bladder biopsies (Harrington, Fall et al. 1990; Christmas 1994). In some IC patients the massive accumulation of plasma cells in bladder mucosa and increased perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) was observed (Thaxton, Eggener et al. 2004; Pacella, Varca et al. 2010).

The role of autoreactive CD4+ T cells in induction of bladder autoimmune inflammation was shown in transgenic a URO-OVA mouse which is a model self-antigen producing ovalbumin in the bladder surface epithelium, derived by UPK2 gene promoter(Liu, Deyoung et al. 2008; Liu, Chen et al. 2011). The preactivated OVA-specific CD4+ T cells induce autoimmune inflammation in the bladder of this mice and CD8+ T cell-lacking URO-OVA mice (Liu, Chen et al. 2011).

1.5. ANIMAL MODELS FOR IC/PBS

Numerous cystitis animal models have been generated in different ways such as through intravesical administration of immune stimulants, systemic and environmentally impelled inflammation, immunization with bladder homogenate, transgenic URO-OVA mice, and naturally occurring models.

1.5.1. EAC Induced by Immunization with Bladder Homogenate

In different mice strains such as BALB/cAN, C3H/HEN, and C57BL/6, experimental autoimmune cystitis(EAC) animal model were generated with bladder homogenate and showed the some physiopathological features of cystitis (Bullock, Becich et al. 1992; Phull, Salkini et al. 2007). The initial experimental autoimmune cystitis was developed in BALB/cAN mice immunized with a syngeneic bladder homogenate that induced cell mediated autoimmunity after adoptive transfer of spleen cells from immunized mice into naïve host(Bullock, Becich et al. 1992). The edema, fibrosis, perivascular lymphocytic infiltrations and also the deposition of mast cell in detrusor muscle of bladder were demonstrated in this bladder homogenate induced cystitis model (Bullock, Becich et al. 1992).

Bladder homogenate obtained from syngeneic animal in Complete Freund's adjuvant was used to induce experimental autoimmune cystitis in Lewis rats, demonstrating urinary frequency, mast cell accumulation and vascular congestion (Luber-Narod, Austin-Ritchie et al. 1996). In another study SWXJ mice that are prone to the generation of autoimmune diseases were immunized with bladder homogenate and

developed experimental autoimmune cystitis showing the symptoms of increase urination frequency and decrease micturition volume along with histopatlogical alterations such as increased leukocytes infiltration, mast cell infiltration and thickened lamina propria (Lin, Liu et al. 2008).

Moreover, in guinea pig models, which were sensitized to Ovalbumin (OVA), developed experimental cystitis demonstrating mast cell infiltration after administration of Ovalbumin antigen into bladder (Saban, Christensen et al. 1991). In another parallel study in mice showed that Ovalbumin instillation transurethrally developed experimental cystitis, on the other hand mast cell deficient mice did not developed bladder inflammation (Saban, Saban et al. 2001). It was suggested that T helper cells could play a role in immune response in the bladder of antigen induced cystitis (Ratliff, Klutke et al. 1994; Ratliff, Klutke et al. 1995).

1.5.2. Naturally Occurring Cat Model for IC/PBS

Feline model of IC showed the similar symptoms of IC/PBS such as increase urinary frequency and pain (Buffington, Chew et al. 1997). The lesser urinary excretion of GP-51 glycosaminoglycan, increased bladder wall permeability, elevated substance P immunoreactivity and neurokinin-1 receptors in this model were observed (Gao, Buffington et al. 1994; Caito and Masty 1995; Press SM 1995; Buffington and Wolfe 1998; Moldwin 2002). The major advantage of this cat model is generation with the absence of external stimulants. The restriction of this model is dissimilar gender distribution in cats compared to human; in human more than 90% of IC is women, in cats

about half to half distribution (Pontari MA 1995). And also the etiology of cystitis development on this cat model is unknown. Other issues are such as handling problems that require veterinarian involvement, and cost highly expensive handling this animal in animal facilities (Westropp and Buffington 2002).

1.5.3. Intravesical Irritant Instillation Models

Urine of IC patients instillated into bladder of rabbits to test the histological and functional changes (Kohn, Filer-Maerten et al. 1998), intravesical acetone applied to induce IC in rats, monkeys, and rabbits (Kato, Kitada et al. 1990; Ghoniem, Shaaban et al. 1995; Shimizu, Kawashima et al. 1999). Moreover, intravesical acid administration showed increase neutrophil accumulation and edema(Elgebaly, Allam et al. 1992). An antineoplastic drug -Cyclophosphamide is mostly used for induction of cystitis in animals. It damages the urothelium, mucosa, and causes edema, leukocytes accumulation in bladder tissue and hemorrhage (Locher and Cooper 1970; Cox and Abel 1979; Lanteri-Minet, Bon et al. 1995). Mustard oil administration intravesically and electrical stimulation of pelvic ganglia were studied to test to bladder distension in regard to afferent fibers in visceral pain (Koltzenburg and McMahon 1986). Instillation of Xylene, lipopolysaccharide (LPS), and polyinosinic-polycytidilic acid incited inflammation in rat bladder (Luber-Narod, Austin-Ritchie et al. 1996).

1.5.4. EAC Induced by Immunization with Recombinant mUPK2 Protein

The immune response to self-antigen mediated organ specific autoimmune diseases EAC model was generated by targeting uroplakins with recombinant rmUPK2

protein(Altuntas, Daneshgari et al. 2011). Bladder specific protein UPK2 was used to immunize the mice and assessed bladder urodynamic, showing the symptoms of increase urination frequency and decrease micturition volume along with increased T cell infiltration in the bladder.

1.5.5. URO-OVA Mice

EAC model with URO-OVA mice (produce a membrane structure of the Ag Ovalbumin(OVA) like a self-antigen on the urothelium deriven by Uroplakin II gene promoter) formed autoimmune cystitis through OVA-specific OT-I $CD8^+$ T-cells; however OVA is not endogenous antigen of bladder and also the bladder urodynamic changes and pain correlated to IC/PBS phenotype have not been characterized (Liu, Evanoff et al. 2007; Liu, Deyoung et al. 2008).

1.5.6. Immune Stimulant Induced Cystitis Models

The bacterial product of LPS administration intravesically resulted into cystitis showing neutrophil invasion, hemorrhage and edema (Jerde, Bjorling et al. 2000). Neurokinin-1 knockout mice did not show inflammation and edema after instillation of dinitropheny14-ovalbumin even the existing of high mast cells (Saban, Saban et al. 2000). In mast cell knockout mice, the intravenous administration of substance P or LPS incited cystitis in these mice (Bjorling, Jerde et al. 1999). In another study, the tail-side muscle injection of pseudorabies virus revealed bladder inflammation in rats (Jasmin, Janni et al. 1998).In mice model of cystitis with pseudorabies virus (PRV) injected, the

mast cell infiltration in lamina propria led by necrosis factor alpha (TNF) along with pelvic pain mediated by histamine have been demonstrated(Rudick, Schaeffer et al. 2008; Rudick, Schaeffer et al. 2009). In addition, the Cathelicidin (LL-37) an antimicrobial peptide were used to generate bladder inflammation in mice (Oottamasathien, Jia et al. 2011).

1.5.7. Noxious Environmental Stimulus Induced Cystitis Animal Models

Environmental stimulus induced cystitis animal model has been reported by administration of physical and physchological stress factors in such studies that restraint stress induced mast activation in bladder(Spanos, Pang et al. 1997), applied acute cold stress induced edema, leukocyte invasion, and mast cell degranulation in rat bladder(Ercan, San et al. 1999). In another study, it was shown the temperature mediated bladder pathological changes such as distraction of tight junctions, desquamation and damages of subapical vesicles that are alike observed in IC patients(Jezernik, Medalia et al. 1995; Elbadawi 1997; Lavelle, Meyers et al. 2000).

1.6. UROEPITHELIUM

It was suggested that painful bladder syndrome occurs due to some modification on uroepithelium in IC. Urothelium, which have three cell layer containing basal cells, intermediate and umbrella cells, forms the inner surface of urinary bladder providing a barrier against urine and other metabolites (Khandelwal, Abraham et al. 2009).The tight junctions in balder urothelium support the resistance of cells (Lewis and Kleine 2000).The defect of urothelial barrier such as damaging of tight junctions, umbrellas

cells, cell adhesions which are mostly observed in IC/PBS patients' bladder, could allow urine, noxious elements and chemical metabolites to invade into underneath layers of tissue and causing the symptoms of frequent o urinate, urgent to urinate and pelvic pain (Birder 2011; Birder, Hanna-Mitchell et al. 2011). Urothelium is in a web of interaction with immune and inflammatory cells along with bladder nerves, smooth muscle, urothelial cells and afferent nerves exist closer to the uroepithelium that could be stimulated by substances secreted by urothelium cells and bladder nerves(Birder and de Groat 2007; Birder 2011).

1.7. UROPLAKINS

The mammalian Uroplakins comprising human, bovine and mouse, are highly conserved (Wu, Lin et al. 1994). Where UPII and UPIII contain a single transmembrane domain(Sun 2006) , UPIa and UPIb have four trans membrane domain (Levy and Shoham 2005). It is suggesting that Uroplakins take a part as a protective role for the Asymmetric Unit Membrane (AUM) from breaching during bladder expansion (Born, Pahner et al. 2003; Kong, Deng et al. 2004) . AUM particles consist of five Uroplakins (UPs), which are tetraspan structured UPIa and UPIb and single spanned UPII, UPIIIa, and UPIIIb proteins, are generated specifically in the urothelium except UPIb that is also expressed in the eye and reside in the umbrella cell layer (Wu, Medina et al. 1995; Deng, Liang et al. 2002). UPIa (27 kDa), UPIb (28 kDa), UPII (15kDa) and UPIIIa (47 kDa) are integral membrane proteins of urothelial plaques (Yu, Lin et al. 1994). The heterodimerization of UPII with UPIa and heterodimerization of UPIIIa or UPIIIb with UPIb are pivotal for transferring from endoplasmic reticulum to the cell surface (Wu,

Medina et al. 1995; Deng, Liang et al. 2002; Tu, Sun et al. 2002). The existences of the uroplakins in bladder are well chosen target for the establishment of EAC.

1.7.1. Function of Uroplakins

Uroplakins possess different function in bladder such as contributing the barrier formation of apical membrane (Hu, Meyers et al. 2002), mediating bladder function alteration (Aboushwareb, Zhou et al. 2009) and cell signaling (Hasan, Ou et al. 2007; Thumbikat, Berry et al. 2009). Uroplakins (Ia, Ib) have substantial roles in immunologic signaling, infection, membrane structure, cell migration (Hemler 2003; Levy and Shoham 2005). The urodynamic with cystometry (CMG) studies on Uroplakin II and IIIa knockout mice showed significant bladder function alteration including increased nonvoiding contractions, the pressure between micturitions, remaining volume after voiding(Aboushwareb, Zho et al. 2007; Aboushwareb, Zhou et al. 2009). UPs are expressed mostly in urothelial tissues and are detected in the urothelial originated carcinomas more than 50% (Wu, Osman et al. 1998; Huang, Shariat et al. 2007).

1.7.2. UPIIIa and Plaques

The apical surface of umbrella cells comprise hinges and plaques which form the scalloped appearance and plaques containing nearly 3000 AUM particles cover the more than 80% of umbrella cell surface(Kachar, Liang et al. 1999; Apodaca 2004). It was suggested that urothelial plaques could provide the sustainability of urothelium permeability barrier along with the apical membrane of umbrella cells and UPIIIa

knockout mice revealed a small number of plaques (Hu, Deng et al. 2000). And also the UPII deficiency caused totally absence of urothelial plaques (Kong, Deng et al. 2004).

The role of UPIIIa on permeability of apical membranes of umbrella cells was tested on UPIIIa knockout mice showing the increased permeability to water and urea, increase urothelial leakage through the umbrella cell layer (Hu, Deng et al. 2000; Hu, Meyers et al. 2002). IC/PBS commonly demonstrates the increase of bladder urothelium permeability (Parsons 2007). Moreover the diminished UPIIIa manifestation led hyperplastic uroepithelium, expanded ureters and vesicoureteral reflux (Hu, Deng et al. 2000). It was suggested that UPIIIa containing large cytoplasmic domain interrelates with cytoskeleton and these cooperation might stabilize membrane domains in umbrella cells (Hu, Deng et al. 2000; Apodaca 2004).

1.8. BIOMARKER FOR IC/PBS

The possible biomarkers that are suggested for IC are the urine Antiproliferative factor (APF),Heparin-Binding EGF-like Growth Factor (HB-EGF), Epidermal Growth Factor (EGF), IL-6 and Insulin Like Growth Factor Binding Protein-3(IGFBP3) (Erickson, Xie et al. 2002). Antiproliferative factor(APF), which is produced by urothelium of IC/PBS patients, showed the inhibitory effect on bladder cell proliferation and causing failure of damaged bladder tissues(Keay, Szekely et al. 2004; Hanno 2007).

CHAPTER II

METHODS

2.1. Peptide

We employed an online database (http://www.syfpeithi.de/) (Hans-Georg Rammensee 1999) research of MHC and their recognized peptide motifs to predict potentially immunogenic peptides of known sequences for bladder-specific Uroplakins. The amino acid sequences of all three Uroplakins (Ia, Ib II, IIIa-b) were placed in online T- cell epitope, and MHC motif prediction program to figure out possible immunogenic peptide MHC motif sequence for each different mice strains(C57BL/6-**H2** ^{b}, BALB/c-**H2** ^{d}, C3H-**H2** ^{k}).

For the SWXJ mice, the peptides derived from the known sequences of Uroplakins were chosen based on having the -KXXS- tetrapeptide-binding motif for IA^{s} and IA^{q} MHC class II molecules expressed in this mouse (Table 1). We identified that UPK3A 65-84, 20-mer peptide derived from the mouse bladder specific Uroplakin 3A protein, contain -SXXVXV- binding motif for IA^{d} MHC class II molecules expressed in

BALB/c mice was predicted the uppermost immunogenic according to online database. UPK3A 65-84 peptide, derived from the sequences of mouse bladder specific Uroplakin 3A protein and other peptides for SWXJ mice were synthesized and purified by the Molecular Biotechnology Core Facility of the Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OHIO. The standard solid phase approach and FMOC side chain-protected amino acids were used to synthesize the peptides. The peptides were purified >97% with Reverse Phase HPLC followed by mass spectrometry to check the amino acid sequences of peptides.

PEPTIDES (UROPLAKINS)	MOUSE STRAIN	SEQUENCES & MHCII BINDING MOTIF
UPK1A 167-186	SWXJ	WVNYTSAFRAATPEVVFPWP
UPK2 115-134	SWXJ	YYISYRVQKGTSTESSPETP
UPK3A 36-55	SWXJ	TLTTVALEKPLCMFDSSEPL
UPK3A 65-84	SWXJ	AMVDSAMSRNVSVQDSAGVP
UPK3A 65-84	BALB/c	AMVDSAMSRNVSVQDSAGVP
UPK3B 159-178	SWXJ	VMDAAGPPKAETKWSNPIYL
UPK3B 132-151	SWXJ	GNDFGCYQRPYCNAPLPSQG
UPK3B 238-257	SWXJ	RIGSFMGKRYMTHHIPPSEA

Table 1. Uroplakin derived peptide sequences and MHC II binding motifs for SWXJ mice strain and BALB/c mice strain

2.2. Mice and immunization

BALB/c female mice and SWXJ female mice were purchased from Jackson Laboratory, and at 6-8 weeks of aged mice were injected subcutaneously (s.c.) in the abdominal flank with or without 200μg of the peptides (Table 1) in 200 μl of an emulsion of equal volumes of water and Complete Freund's Adjuvant (CFA) containing 400 μg of Mycobacteria tuberculosis H37RA (Difco Laboratories, Detroit, Michigan, USA). All protocols were pre-approved by the institutional animal care and use committee of the Case Western Reserve University in compliance with the Public Health Service policy on humane care and use of laboratory animals.

2.3. Weighting Bladder Tissues

At 5 weeks after immunization the mice and bladder weight were measured. The ratio of bladder weight/body weight was calculated and compared with control-CFA immunized group.

2.4. Cell culture and Proliferation Assays

As it described previously (Jane-wit, Yu et al. 2002; Altuntas, Johnson et al. 2006; Altuntas, Daneshgari et al. 2011), to find out the immunogenicity of peptides, inguinal and axillary lymph node cells (LNCs) were removed from mice at 10 days after immunization with UPK3A 65-84 or the other peptides. The removed LNCs were placed on a tissue culture dish and teased them apart into single cell suspension by squashing

with 3ml syringe plunger through with a cell strainer(nylon mesh) removing the clumps and debris. The cell suspensions were washed with Hank's Buffered Salt Solution (HBBS) (Life Technologies, Grand Island, NY) and centrifuged for 10 minutes at 300xg at 4^0C. The cell pellets were suspended and the cell count was made to check viability. The cells were cultured in a way of single-cell suspension in 96-flat-bottom-well microtiter Falcon plates (BD Biosciences, San Jose, CA, USA) at $3x10^5$ cells/well in Dulbecco modified Eagle medium (DMEM) (Mediatech CellGro, Herndon, VA, USA) with 10% fetal bovine serum (HyClone), 5% HEPES buffer, 2% L-glutamine, and 1% penicillin/streptomycin (Invitrogen Life Technologies) included. And then the peptides and Ovalbumin (as a control) were added in serial 10-fold dilutions to triplicate wells with positive control wells containing 2 µg/ml antimouse CD3 (BD Biosciences).

T cell isolation-The CD4+ and CD8+ T cells proliferative response to the UPK3A 65-84 peptide was determined. CD4+ and CD8+T cells were purified and isolated from 10-day primed LNC by using anti-CD4- and anti-CD8-coated magnetic bead sand through a MACS LS column using a Midi MACS cell separator (Miltenyi Biotec, Auburn, CA, USA). The purified $3x10^5$ cells/well of CD4+ or CD8+ T cells were cultured with 5×10^5 cells/well gamma-irradiated (2000 rads) syngeneic splenocyte feeders.50 µg/ml of UPK3A 65-84 peptide or Ovalbumin (control) were inserted into triplicate cultured wells with positive control wells containing 2 µg/ml antimouse CD3 (BD Biosciences).

All the cultured cells were incubated at 37 °C in humidified air containing 5% CO_2. After 96 hours of culture, wells were pulsed with [methyl-3H] thymidine (l µCi per

well; specific activity 6.7 Ci/mmol; New England Nuclear, Boston, MA, USA) and harvested 16 h after pulsing by aspiration onto glass fiber filters. The level of incorporated radioactivity was determined by scintillation spectrometry. The results are showed as mean counts per minute (cpm) of triplicate experimental cultures and/or cpm of cultures with antigen divided by mean cpm of cultures without antigen (stimulation index; (Jane-wit, Yu et al. 2002).

2.5. Cytokine Analysis

As described in previous studies (Altuntas, Johnson et al. 2006; Altuntas, Daneshgari et al. 2011); IFN-γ, IL-2, IL-4, IL-5 and IL-10 cytokine concentrations were determined by ELISA. The measurement was performed on the 48 hour supernatants of 10-day-primed LNC cultured in supplemented DMEM at 5x106 cells/well in 24-well flat-bottom Falcon plates (BD Biosciences) in the presence of 25 µg/ml UPK3A 65-84 and Ovalbumin (as a control) in a final volume of 2.0 ml/well. Purified capture/detection Ab pairs and recombinant cytokines were obtained commercially (BD Biosciences) and included anti-mouse IFN-γ (R4-6A2 and biotin XMG1.2), anti-mouse IL-2 (JES6-1A12 and biotin JES6-5H4), anti-mouse IL-4(11B11 and biotin BVD6-24G2), anti-mouse IL-5 (TRFK5 and biotin TRFK4), and anti-mouse IL-10 (JES5-2A5 and biotin SXC-1). Microtiter plates were coated with anti-cytokine capture mAb (0.5-2µg/ml) in 50 µl PBS for 6 hours at room temperature (RT). Wells were blocked with 200µl of 3% BSA in PBS for 2 hours at RT. 100 µl/well from each samples were added and incubated at RT for 4 hours. From the biotinylated anti-cytokine detecting mAb(1-2µg/ml) 100µl were added after washing steps and incubated at RT for 45 minutes. Following the washing steps

100µl/well Avidin-peroxidase were incubated 30 minutes at RT. Working substrate solution, ABTS, was added to all wells for 20-30 minutes and followed by the addition of stop solution. Absorbance was measured at 405 nm using a model Versamax ELISA microplate reader (Molecular Devices, CA). Standard values were plotted as absorbance vs cytokine concentration, and sample cytokine concentrations were determined as values within the linear part of the standard curve established using known concentrations of each cytokine.

2.6. Total Antibody Titer

The total antibody titer was determined by enzyme-linked immunosorbent assay (ELISA) in sera. 5 week after immunization the serum samples were obtained from the each immunized mice with UPK3A 65-84 and from Ovalbumin injected mice. After the coating the microwell plate with peptide antigens, the different serial dilutions (1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600) of serum samples of each mice (n=5) were used. The goat-anti-mouse antibody IgG (H+L)-HRP (Horseradish Peroxidase) conjugated (1/8000) (Southern Biotech) was used. The plates were read by ELISA reader at 405 nm.

2.7. Isotype-Specific Antibody Titers

As described in (Altuntas, Johnson et al. 2006; Altuntas, Daneshgari et al. 2011), Isotype-specific antibody titers to UPK3A 65-84 was determined in the serum samples of immunized mice 5 week after immunization, according to the manufacturer's instructions

using the mouse Mono AB ID/SP ELISA kit (Zymed, Invitrogen, Carlsbad, CA, USA). In brief, UPK3A 65-84-antigen (10µg/ml) in 50µl PBS coated the microtiter plates and incubated at 4^0C overnight. Wells were blocked with 1% of BSA in PBS for 1 hour at 37^0C after washing. The serum samples (n=4) were serially diluted and 50µl of sera was added to each well and incubated for 30 minutes at 37^0C. The biotinylated antibodies specific to each mouse antibody isotypes (IgG1, IgG2a, IgG2b, IgG3) in 50µl PBS were added and incubated for 30 minutes at 37^0C, and followed by addition of horse radish peroxidase (HRP)-streptavidin (50µl/well, 30 minutes at 37^0C) and substrate solution ABTS ((2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) administration and then the absorbance was measured at 405 nm by ELISA reader.

2.8. Real Time-PCR

The inflammatory genes(IFN-γ, TNF-α, IL-1β, IL-17A) expression levels was performed 5 weeks after immunization in the bladders of immunized and control mice(CFA injected group and Naïve mice) comparing with the kidney, ovary, uterus, and liver. Total RNA was extracted from bladder of EAC mice and controls and then converted to cDNA for gene expressions analysis. RNAs were extracted from bladders following Invitrogen TRIzol protocol (Invitrogen). cDNAs were synthesized with Invitrogen Super Script III cDNA Synthesis Kit. The primers were designed with online Universal Probe Library Assay Design Center of Roche. qRT- PCR using the Sybr Green PCR Master kit (AB, USA) with ABI Prism 7500 Sequence Detection System was conducted using the following primer pairs: interferon (IFN) γ, sense, TGATGGCCTGATTGTCTTTCAA, antisense, GGATATCTGGAGGAACTGGCAA;

tumor necrosis factor (TNF) α, sense, CAAAGGGAGAGTGGTCAGGT, antisense, ATTGCACCTCAGGGAAGAGT; interleukin (IL) 1β, sense, GAGTGTGGATCCCAAGCAAT, antisense, AGACAGGCTTGTGCTCTGCT; IL-17A, sense, TCCACCGCAATGAAGAC, antisense, CTTTCCCTCCGCATTGAC; β-actin, sense, GGTCATCACTATTGGCAACG, antisense, ACGGATGTCAACGTCACACT. The quantitation of gene expression levels were calculated with comparative Ct method. The Ct values of the samples from immunized mice and the control mice are normalized to endogenous housekeeping gene, β-Actin.

2.9. Histologic Analysis

Mice were sacrificed and bladders weighed. The bladder was sectioned at the equatorial midline and fixed in 10% neutral formalin. After fixation, the tissues were dehydrated and embedded in paraffin. Serial 5-µm cut tissue sections were placed on microscope slides, dewaxed, and rehydrated for routine hematoxylin and eosin staining. Gross histologic observations were performed using light microscopy (Olympus DP70 digital microscope).

2.10. Immunocytochemistry

Immunostaining was performed as described in Altuntas et al., 2006(Altuntas, Johnson et al. 2006). Briefly, unmasked in 1 mM EDTA and blocked formalin-fixed paraffin-embedded 5-µm tissues sections were treated with a 1:250 dilution of rat antimouse CD3 (Novacastra, Newcastle Upon Tyne, UK) followed by a 1:100 dilution of

mouse-adsorbed biotinylated goat anti rat immunoglobulin (IgG) (BD Biosciences).Slides were treated with 1.5% H_2O_2 in methanol and then the slides were developed conventionally using streptavidin-horseradish peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA), 3, 3'- diaminobenzidine chromogen solution and H2O2 substrate (Biognex, San Ramon, CA, USA). The slides were stained with H&E and dehydrated in an ascending gradient of ethanol followed by xylene. The slides were examined by light microscopy (Olympus DP70 digital microscope).

2.11. Urinary Frequency-Volume Assessment (FVC)

Twenty four hr earlier to FVC assessment, solid food was eliminated from cages and supplied with lactose-free milk to minimize the frequency and mass of the feces produced at the time of examination(Liu and Daneshgari 2006). 24-hr micturition and sipping behaviors of mice were measured by placing each mouse individually in metabolic cages (MED-CYT-M; Med-Associates, St. Albans, VT). Urine was collected in a plastic tray located on an analytical balance (VI- 3 mg; Acculab, Huntingdon Valley, PA) set directly underneath of each cage. Balances were linked to a data acquisition software program advanced by manufacturer, which measure the weight of urine collection along the defined period of time. Throughout testing, mice were provided with free access to lactose-free milk and water, and the testing room was maintained on a light/dark cycle alike usual housing environments. At end of testing, milk and water bottles were examined to quantity consumption of fluid over the 24 hr period. The data is analyzed with OriginLab Data Analysis and Graphic Software.

2.12. Pain Assessment

We assessed the pelvic pain response of mice by using calibrated von Frey monofilaments after the 5 weeks of immunization and after adoptive of CD4+ T cells, CD8+ T cells, serum, and B cells at day 5, 10, 20, 30. We tested pelvic pain by using quantitative behavior testing methods to assess tactile sensitivity of pelvic region (Rudick, Schaeffer et al. 2008; Rudick, Schaeffer et al. 2009).The von Frey filaments applied to the pelvic region of the BALB/c mice. The withdrawal frequency was calculated in percentage out of 10 times pocked mice with each of the von Frey filaments. The measurement was assessed with 12 von Frey hairs using the sizes 1.65, 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31, 4.56, 4.74 with the forces (gr) 0.008, 0.02, 0.04, 0.07, 0.16 0.4, 0.6, 1, 1.4, 2, 4, 6 respectively. The each of the filaments was employed for 1-3 second in an order beginning from the thinner to thicker with a 5-8 second intervals between two stimuli, completing to 10 times pocking.

2.13. Adoptive Transfer of EAC

To determine whether EAC (transferring the immunologic functionality and features into naïve host) could be transferred into naïve BALB/c mice, the isolated and activated CD4+ T cells, CD8+ T cells, B-Cells and also collected Sera from immunize groups were used. 6- to 8-week-old BALB/c female mice were immunized s.c. in the abdominal flank on day 0 with either 200 μg Ovalbumin (OVA; Sigma) or 200 μg UPK3A 65-84 in 200 μl of an emulsion of equal volumes of water and Freund's adjuvant (Difco)and 400 μg Mycobacteria tuberculosis H37RA (Difco, Detroit, Michigan).

Ovalbumin was used as a control. Lymph node cells were taken at day 8-10 after immunization and LNCs were stimulated with immunogens (50 μg/ml) in vitro for 96 hours, and the isolated and purified $2x10^7$ CD4+ or CD8+ T cells by magnetic bead separation were transferred into naive mice by i.v tail vein injection ($2x10^7$ cells in PBS, 200 μl per mice/naïve recipient) and the development of EAC phenotype was assessed by measuring micturition frequency by FVC at day 20, and referred pelvic pain by von Frey Filaments at day 5, 10, 20, 30. Correspondingly, B-Cells were isolated from spleens of immunized mice 5 weeks after injection with peptides (UPK3A 65-84, or Ovalbumin) with magnetic bead separation using anti-CD45R (B220) Micro Beads, mouse, (Miltenyi Biotec). The isolated B-Cells were transferred into syngeneic naïve recipient by i.v. tail vein injection at $2x10^7$ cells/per mice ($2x10^7$ cells in PBS, 200 μl per mice/naïve recipient). Likewise, serum samples taken from at 5 weeks after immunization with immunogens (UPK3A 65-84 or Ovalbumin), the collected and pooled sera were injected (3 injections of 200 μl per mice, i.v, every other day) into naive recipient BALB/c female hosts for determining their ability to mediate disease.

2.14. Cystometrogram (CMG)

CMG testing was performed as previously described (Kim, Huang et al. 2007; Liu, Lin et al. 2010). 5 weeks after immunization mice were put in particular metabolic cages for 2–4 h. During this time, the PE-10 catheter was attached via a stopcock to both a pressure transducer and a flow pump. The bladder was then filled with room temperature saline (1 ml/h) via the catheter, while bladder pressure was recorded. The animals were conscious and could micturate through the urethra during the study. Urine

was collected in a beaker on a force transducer (Model PT5, Astro-Med) set underneath of every cage. The pressure and force transducers were connected to an amplifier, chart recorder, and a computer for recording data. With CMG data the following measurements were performed: threshold pressure, peak voiding pressure, resting pressure, compliance, and mean inter contractile intervals. Peak voiding pressure was described as the highest pressure throughout micturition. Threshold pressure was the pressure just before the initial micturition phase. Resting pressure is the pressure right after the micturition. Compliance was calculated by dividing the voided volume by the variation in bladder pressure throughout the voiding phase (threshold pressure minus the resting pressure). The mean intercontractile interval is the time between the start of the one voiding cycle (initial resting pressure) to the end of that voiding cycle (end of the micturition phase).

2.15. Statistical analysis

The unpaired Student t-test was used to analyze differences in micturition frequency, mean urine output/micturition ratio, and bladder weight/body weight ratio between UPK3A 65-84 and control-immunized mice. For RT-PCR experiments, statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's post hoc test for comparisons between groups. The pain differences between groups were analyzed by a one-way ANOVA followed by a posthoc test comparison using Dunnet's multiple comparisons. A value of $p<0.05$ was considered statistically significant.

CHAPTER III

RESULTS

3.1. Immunogenic Peptides Selection and Prediction Based on MHC motifs

The online database (http://www.syfpeithi.de/) (Hans-Georg Rammensee 1999) research of MHC and their recognized peptide motifs gave us potentially higly immunogenic peptides of known sequences of bladder-specific Uroplakins for BALB/c mice. For the SWXJ mice, peptides were derived from the known sequence of Uroplakins were selected based on having the -KXXS- tetrapeptide-binding motif for IAs and IAq MHC class II molecules expressed in SWXJ mice(Table I). The KXXS motif has been used for immunogenic peptide selection in many studies(Table II) such as experimental autoimmune encephalomyelitis (EAE) in SWXJ, SWR/J and SJL/J mice(Tuohy and Thomas 1995; Tuohy, Thomas et al. 1995), experimental autoimmune myocarditis and autoimmune sensorineural hearing loss in SWXJ mice(Jane-wit, Yu et al. 2002; Solares, Edling et al. 2004). We identified that UPK3A 65-84, 20-mer peptide derived from the mouse bladder specific Uroplakin 3A protein, contain -SXXVXV- binding motif for IA^d MHC class II molecules expressed in BALB/c mice was predicted the uppermost

Immunogenic according to online database and [methyl-3H] thymidine incorporated proliferation assays.

Immunogenic Peptide	Amino Acid Sequence **-KXXS-** Motif	Reference
PLP 104-117	KTTICG **-KGLS-**ATVT	(Tuohy and Thomas 1995)
MOBP 37-51	REIVD-**RKYS-**ICKSGC	(Amor, Groome et al. 1994)
MOG 92-106	DEGGYTCFF-**RDHS-**YQ	Holz, Bielekova et al. 2000)
CAMHC 406-425	KVGNEYVT-**KGQS-**VQQVYYSI	(Jane-wit, Yu et al. 2002)
CAMHC 1631-1650	LSQAN-**RIAS-**EAQKHLKNSQA	(Jane-wit, Yu et al. 2002)
Coch 131-150	STQEATG-**RAVS-**TAHPPSGKR	(Solares, Edling et al. 2004)
β-tectorin 71-90	FVIPDLSP-**KNKS-**YCGTQSEY	(Solares, Edling et al. 2004)
Inα 215-234	FLVAHTRA-**RAPS-**AGERARRS	(Altuntas, Johnson et al. 2006)

Table 2. Mostly used immunogenic peptides with -KXXS- MHC motif sequences.

3.2. Immunogenic UPK3A 65-84 Peptide Elicited Proliferative Response in BALB/c mice.

UPK3A 65-84, 20-mer peptide derived from the mouse bladder specific Uroplakin 3A protein, contain -SXXVXV- binding motif for IA^d MHC class II molecules expressed in BALB/c mice was predicted highly immunogenic according to online database (http://www.syfpeithi.de/). The relatively highest immunogenicity of this peptide among the other peptides was confirmed with [methyl-3H] thymidine incorporated proliferation assays. Lymph node cells (LNC) taken 10 days after immunization with UPK3A 65-84 showed antigen specific proliferative responses to UPK3A 65-84 but were unresponsive to Ovalbumin as a control (Figure 1a-b). UPK3A 65-84 was able to elicit extensive recall proliferative responses whereas the rest of the peptides were relatively non-immunogenic when assessed through stimulation index or with cpm.

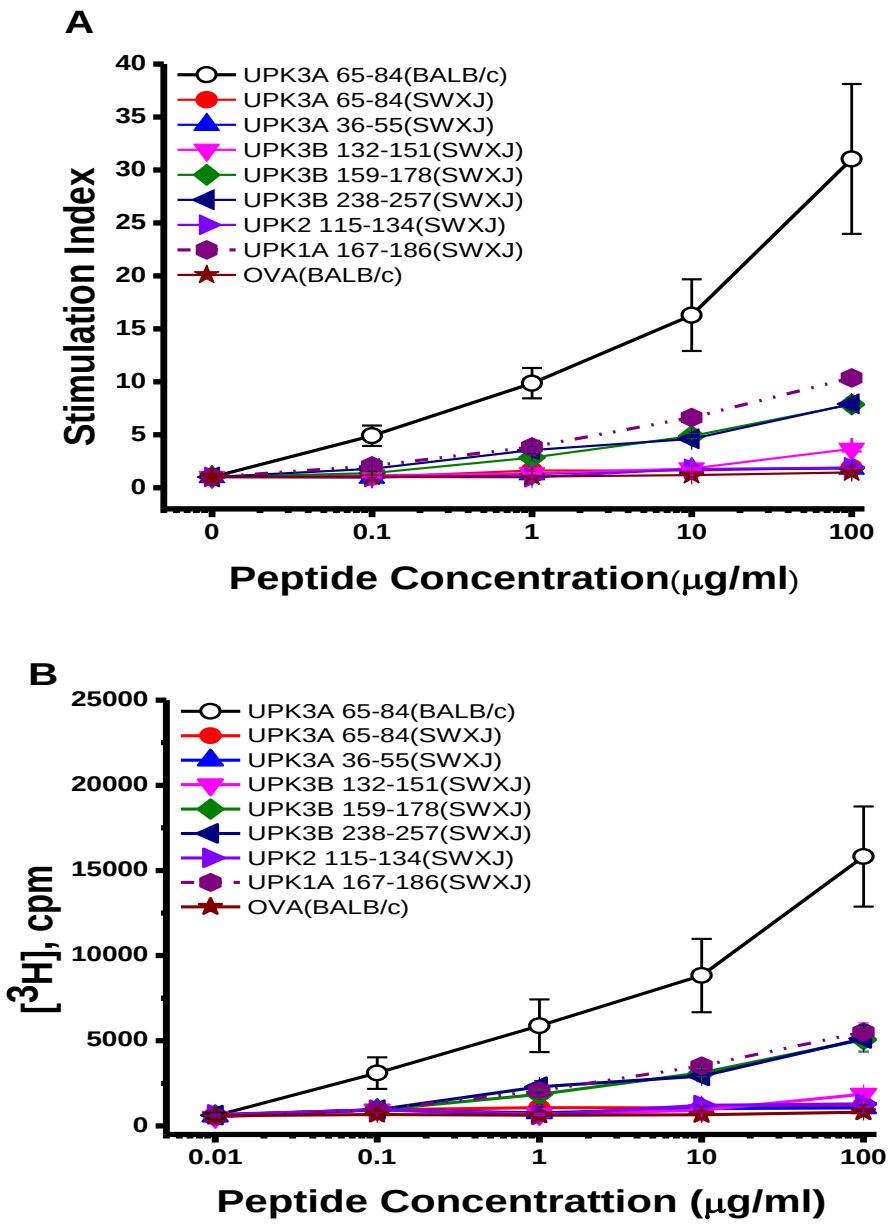


Figure 1. UPK3A 65-84 is Higly Immunogenic in BALB/c mice. 20-mer peptide derived from Uroplakin proteins, contain -SXXVXV- binding motif for IA^d MHC class II molecules expressed in BALB/c mice, and for the SWXJ mice, peptides were derived from the known sequence of Uroplakins were selected based on having the -KXXS- tetrapeptide-binding motif for IA^s and IA^q MHC class II molecules expressed in this mice. Characterization of the Immune Response to UPK3A 65-84- Lymph node cells (LNC) taken 10 days after immunization with UPK3A 65-84 and other peptides showed: (A-B) antigen specific recall proliferative responses to UPK3A 65-84 but not to Ovalbumin, and other Uroplakin derived peptides (n=5), expressed results are the mean of triplicate cultures pulsed with [methyl-3H] thymidine graphed based on the stimulation index (A) and radioactivity in cpm (B**).**

3.3. Th1-like Proinflammatory Phenotype was revealed to UPK3A 65-84 in immunized mice.

ELISA analysis of 48 hour supernatants of cultured and peptide induced LNCs from 8-10-day after immunization showed that the recall responses to UPK3A 65-84 was a proinflammatory type-1 cytokine response characterized by enhanced expression of IFNγ and IL-2 and nearly no production of IL-4, IL-5 and IL-10 (Figure 2).

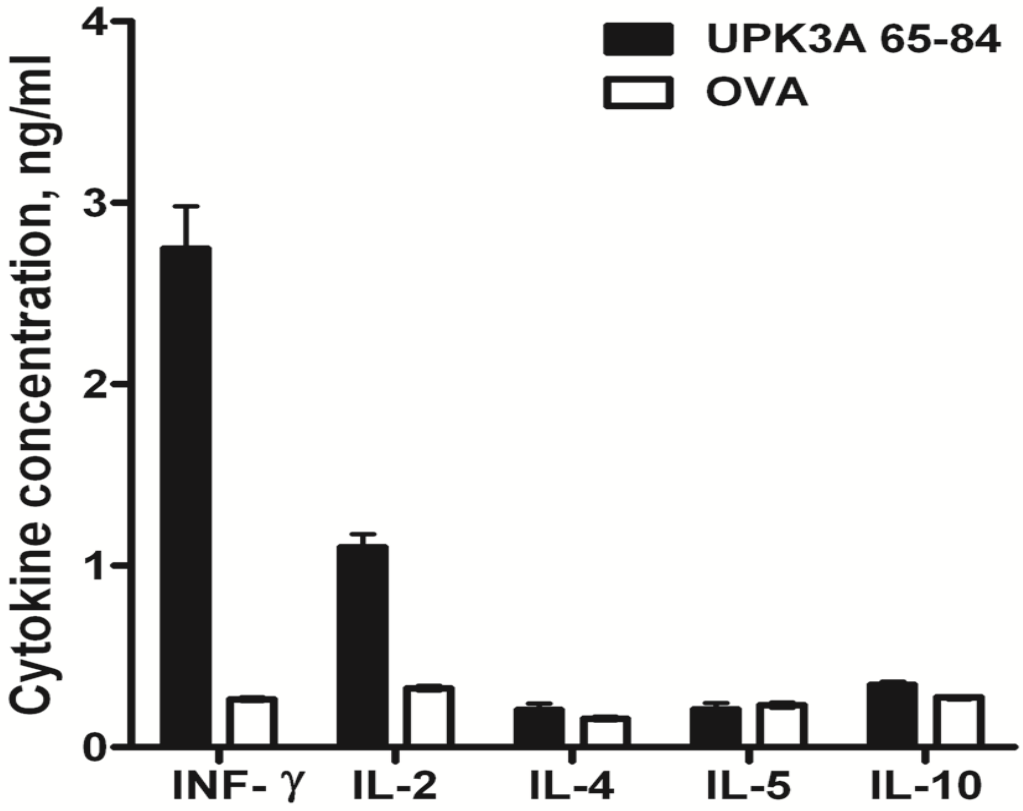


Figure 2. UPK3A 65-84 Induces a Th1-like Proinflammatory Response. ELISA analysis of cytokines from the 48-hour supernatant of cultured LNCs demonstrated the response to UPK3A 65-84, a proinflammatory type-1 phenotype with high production of IFNγ and IL-2 and low production of IL-4, IL-5 and IL-10 in response to UPK3A 65-84, (n=8).

3.4. **UPK3A 65-84 Activates CD4+ Proinflammatory Th1-Like T Cells in an IAd-restricted Manner**

UPK3A 65-84 was capable of inducing CD4+ T –cell proliferative response. Its responsiveness was elicited from purified (>90%) CD4+ T cells and LNC but not CD8+ T cells (Figure 3). CD4+ T cells and CD8+ T cells were purified from whole LNC by magnetic bead separation and reactivity to UPK3A 65-84 at 50µg/mL was examined in culture with gamma-irradiated syngeneic splenocyte feeders. The CD4+ enriched T-cells demonstrated enhanced proliferation to UPK3A 65-84 compared to the enriched CD8+ T-cells and LNC at 8-10 days after immunization (Figure 3). This confirms that UPK3A 65-84 preferentially actuates CD4+T cells restricted to IA^d of MHC class II molecules in BALB/c mice.

3.5. High Titer Antibody Response to UPK3A 65-84 along with Type 1 antibody isotype was showed in Immunized mice.

All sera collected from mice at 5 weeks after immunization and serially diluted antibody isotype titers to UPK3A 65-84 were determined. The high titer systemic serum antibody response to UPK3A 65-84 peptide was observed (1/12,800) (Figure 4). In some mice immunoreactivity the titer was clearly detectable at dilutions exceeding 1/12,800. The predominant antibody isotypes to the UPK3A 65-84 peptide were IgG2a and IgG3, which are known to be induced by IFN-γ, and inhibited by IL-4. This finding indicated that the response to peptide is Th1-associated Ab response with elevated production of IgG2a and IgG3, low production of IgG1, and IgG2b (Figure 5).

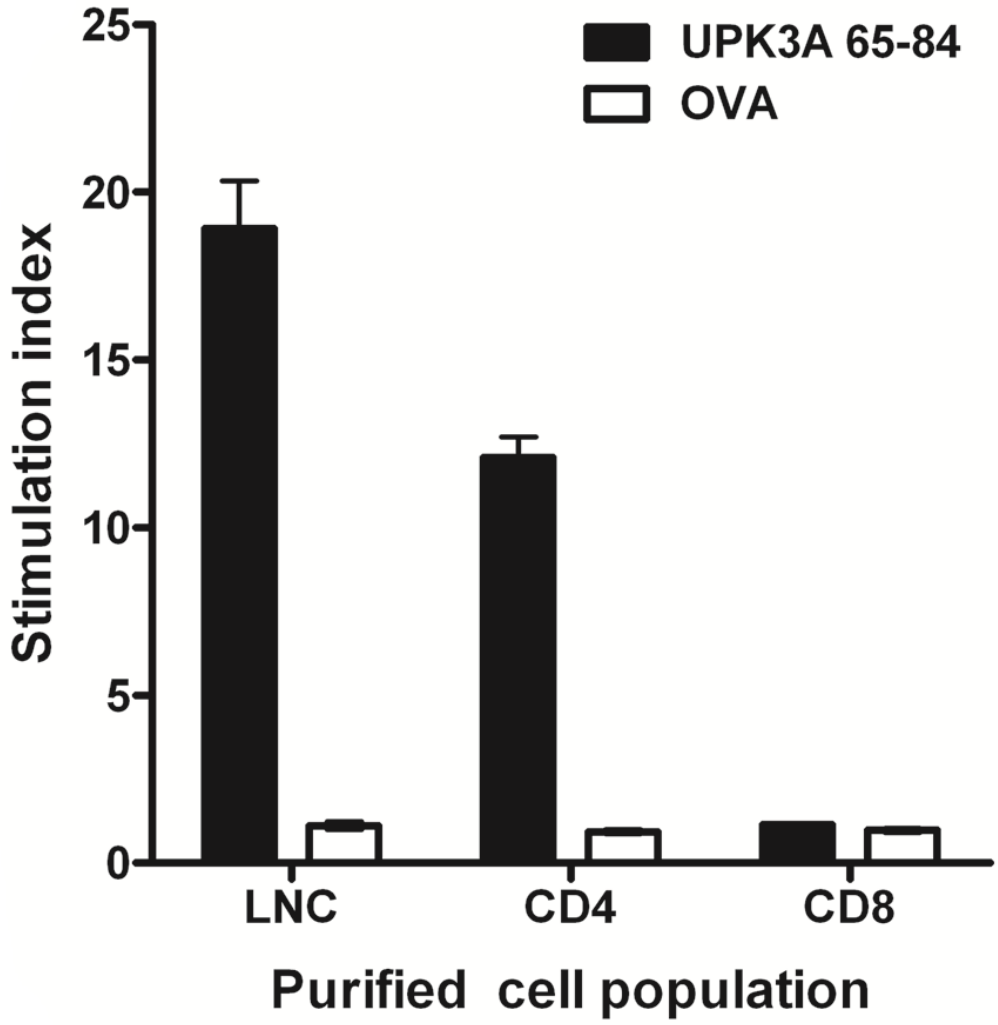


Figure 3. CD4+ T cell Reactivity to UPK3A 65-84. The high recall proliferative response to LNC and from CD4+ T cells, but not CD8+ T cells, purified (>90%) by magnetic bead separation, (n=5), results are the mean of triplicate, after 96 hour, wells were pulsed with [methyl-3H] thymidine and harvested 16h later by aspiration onto glass fiber filters. The incorporated radioactivity was determined with scintillation spectrometry. All error bars show ±SE.

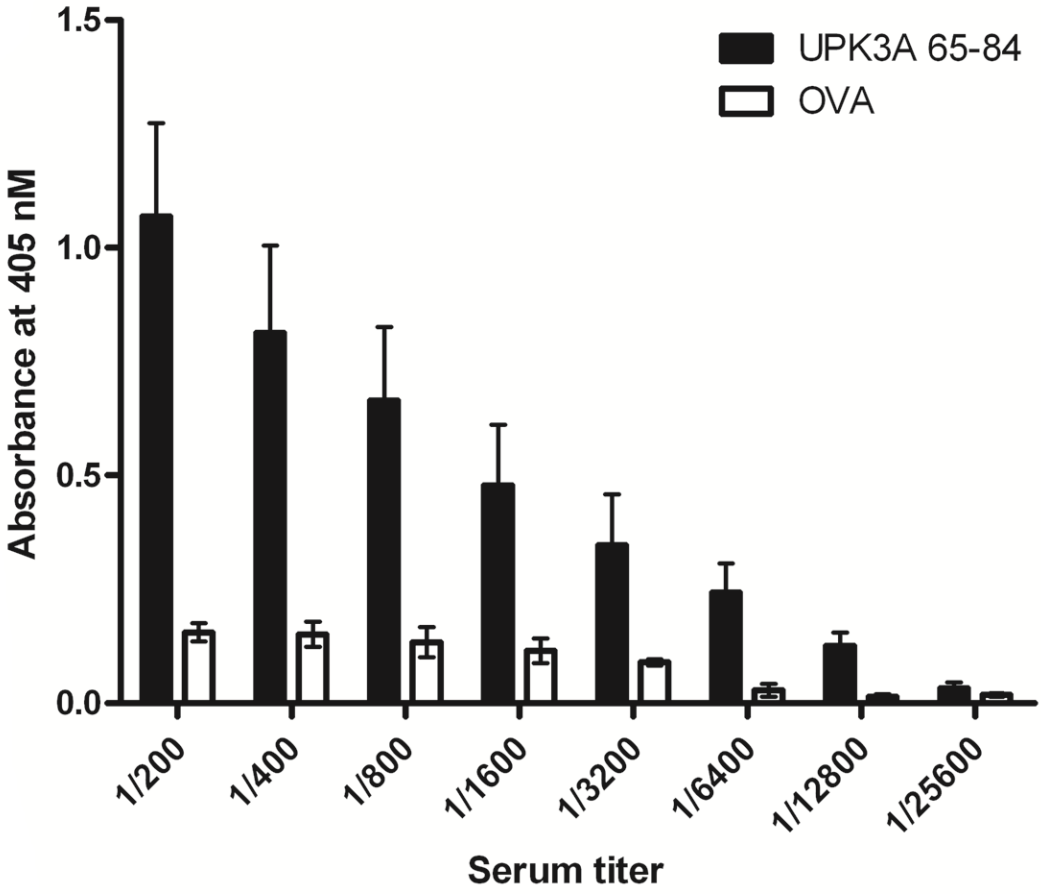


Figure 4. High Antibody Response to UPK3A 65-84. ELISA analysis of sera taken 5 weeks after immunization with UPK3A 65-84 showed high titer serum antibody responses to UPK3A 65-84 but not Ovalbumin (n=5).

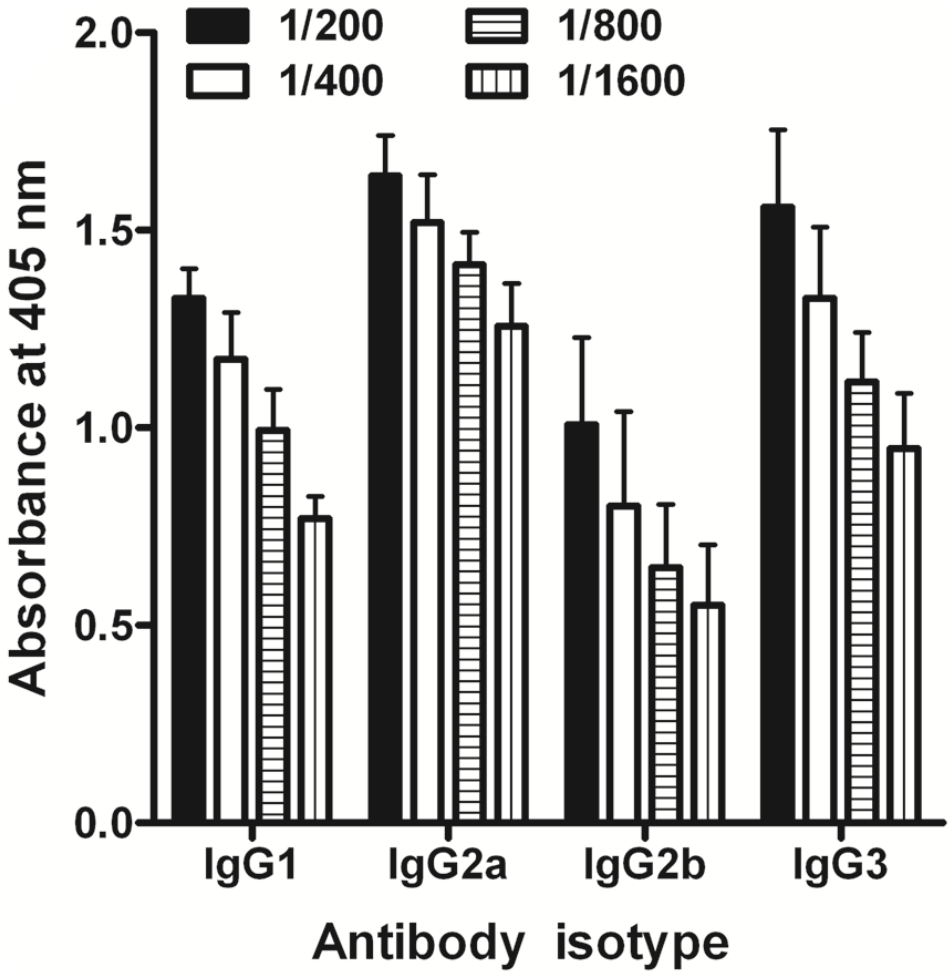


Figure 5. Type 1 Antibody Isotype Response to UPK3A 65-84. The antibody response to UPK3A 65-84 was predominantly a type-1 response involving high production of IgG2a and IgG3, low production of IgG2b and IgG1 (n=4). All error bars show ±SE.

3.6. Active Induction of EAC in BALB/c mice Immunized with UPK3A 65-84

Active immunization of BALB/c mice with 200 µg of UPK3A 65-84 in CFA resulted in induction of experimental autoimmune cystitis 5 weeks after immunization. Immunocytochemical staining for T-cells determinants in the bladder tissue after 5 weeks after immunization, showed that the bladder infiltrating cells were predominantly T cells (Figure 6a, right) and that such T cell infiltration was not evident in bladders from control mice (Figure 6a, left). Hematoxylin and Eosin(H&E) stained bladder sections taken 5 weeks after immunization with UPK3A 65-84 showed extensive perivascular infiltration (Figure 6b, right) that was not evident in any sections taken from CFA immunized control mice (Figure 6b, left). The bladder T cell accumulation in UPK3A 65-84 immunized mice was predominantly located under the urothelial epithelium, the area of lamina propria.

Bladder specific inflammation was demonstrated in mice immunized with UPK3A 65-84. qRT-PCR analysis showed significantly elevated gene expression levels of the inflammatory cytokines, TNFα, IL-17a, IFNγ, and IL-1β in the bladder but not in the kidney, ovary, uterus, and liver of immunized mice with UPK3A 65-84 compared to tissues taken from age- and sex-matched naive mice or control mice immunized with CFA($P<0.001$ in all cases) (Figure 7). We clearly determined that the inflammation is confined to bladder tissue of EAC mice.

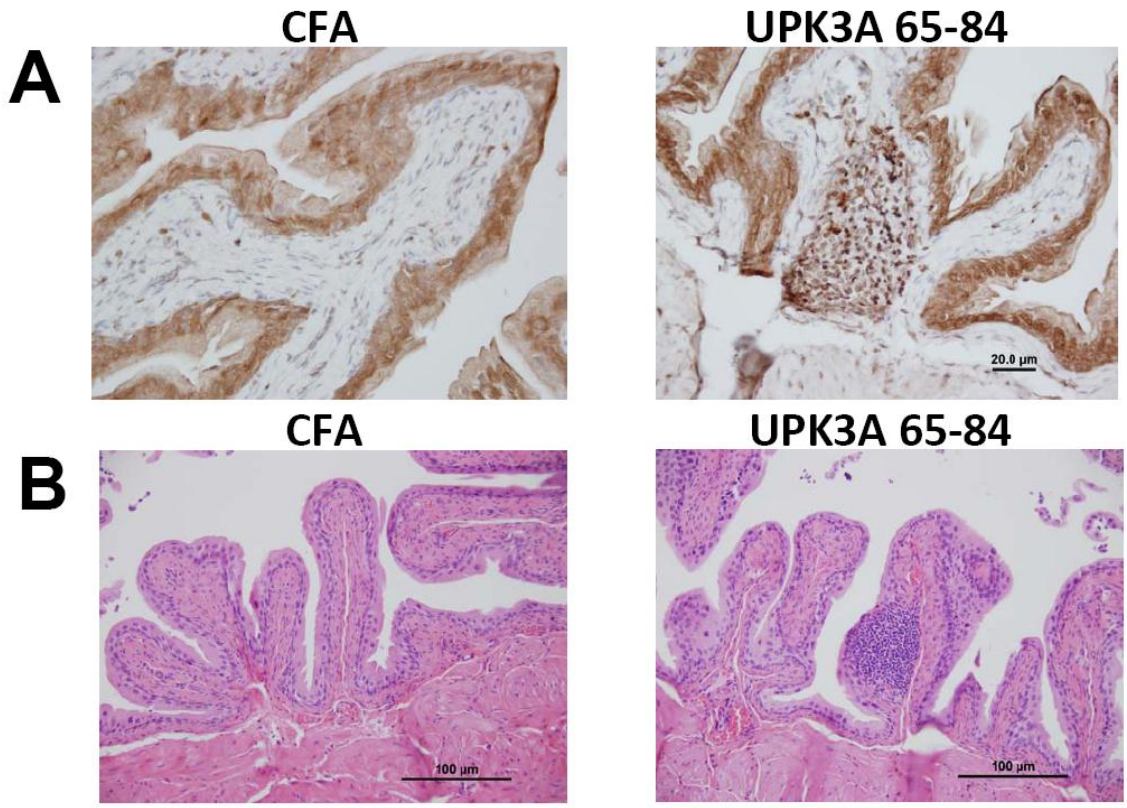


Figure 6. H&E and Immunocytochemical Staining of Bladder Tissues
(A) Immunocytochemical analysis of bladder tissue Immunostaining with CD3 antibody showed a predominance of T cells in the bladder infiltrates of mice immunized with UPK3A 65-84 (right panel) not evident in anti-CD3 stained sections from control mice immunized with CFA (left panel). Solid bar = 20 µm for sections **(B)** Hematoxylin and Eosin stained bladder sections taken 5 weeks after immunization with UPK 3A 65-84 showed extensive perivascular infiltration (right panel) not evident in sections taken from CFA immunized control mice (left panel). Solid bar =100 µm for sections.

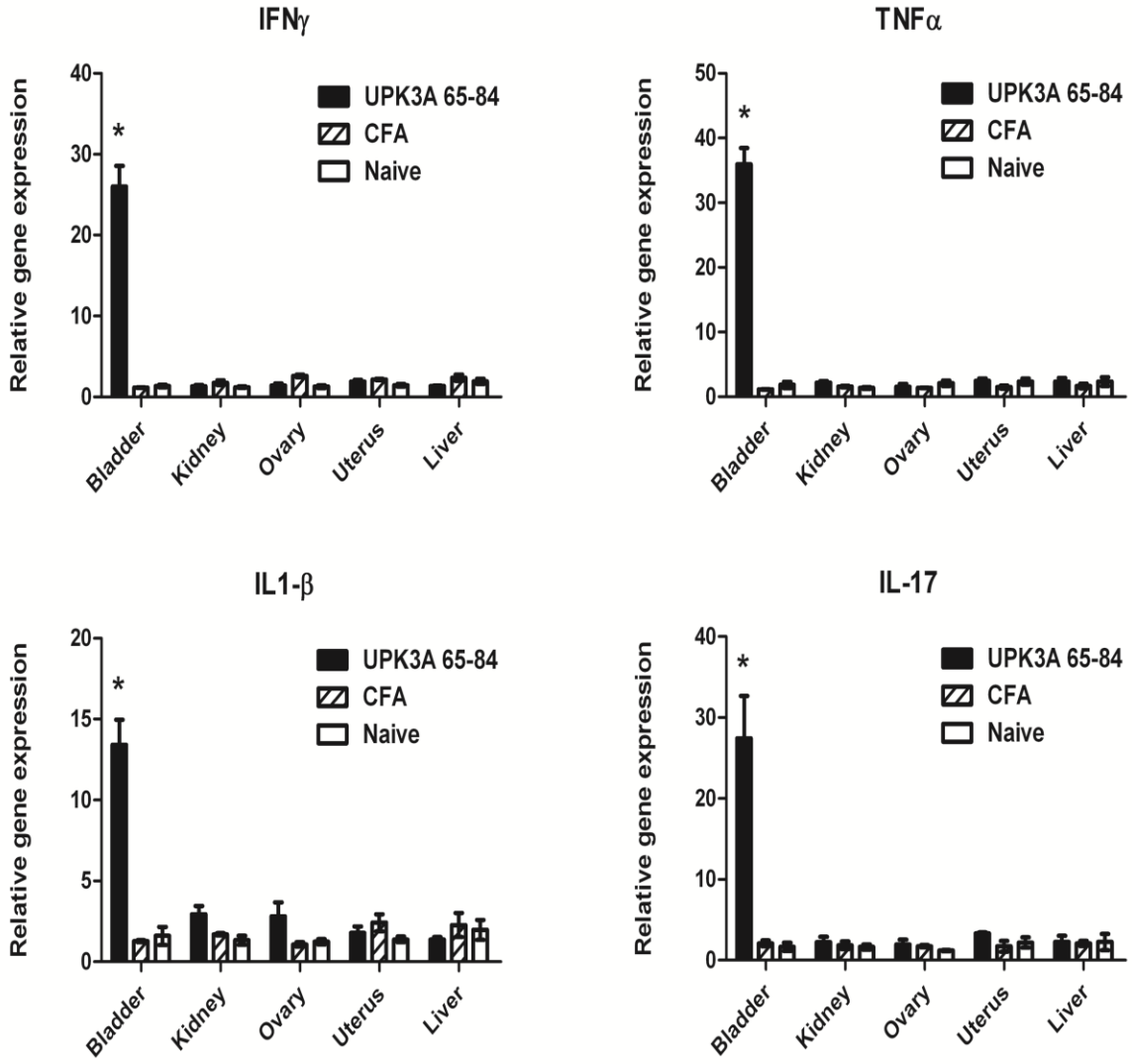


Figure 7. Bladder Specific Inflammatory Response. Real-time-PCR analysis showed significantly elevated ($P<0.05$ in all cases) expression levels of IFNγ, TNFα, IL-1β, and IL-17A in the bladder but not in the kidney , ovary, uterus, and liver of mice immunized with UPK3A 65-84 compared to tissues taken from age- and sex-matched naive mice or control mice immunized with CFA. Error bars show ±SE.

3.7. Bladder Dysfunction, IC/PBS phenotypical characterization following active immunization with UPK3A 65-84

To find out the whether UPK3A 65-84 were able to induce an experimental autoimmune cystitis, female BALB/c mice were immunized with this peptide and bladder dysfunction by FVC and pelvic pain were tested 5 weeks later. Functional analysis showed that 5 weeks after immunization with UPK3A 65-84, BALB/c female mice showed significantly increased micturition frequencies (Figure 8a) and significantly decreased mean urine outputs per micturition (Figure 8c) ($P<0.0001$). Figure 8b-d-shows the urine production and its frequency of an individual immunized mouse with UPK3A 65-84 and immunized mice with CFA respectively. This observation correlates the phenotypical features, which are urinary frequency and urgency, seen in human IC/PBS. The pelvic pain assessment with the most popular non-invasive von Frey Filaments on the pelvic region revealed that immunized mice with UPK3A 65-84 developed pelvic pain with the increase response to noxious stimulus as the meaning of decreased threshold of pain response on the pelvic region as an indicator of referred pain of bladder organ($p<0.05$)(Figure 9).

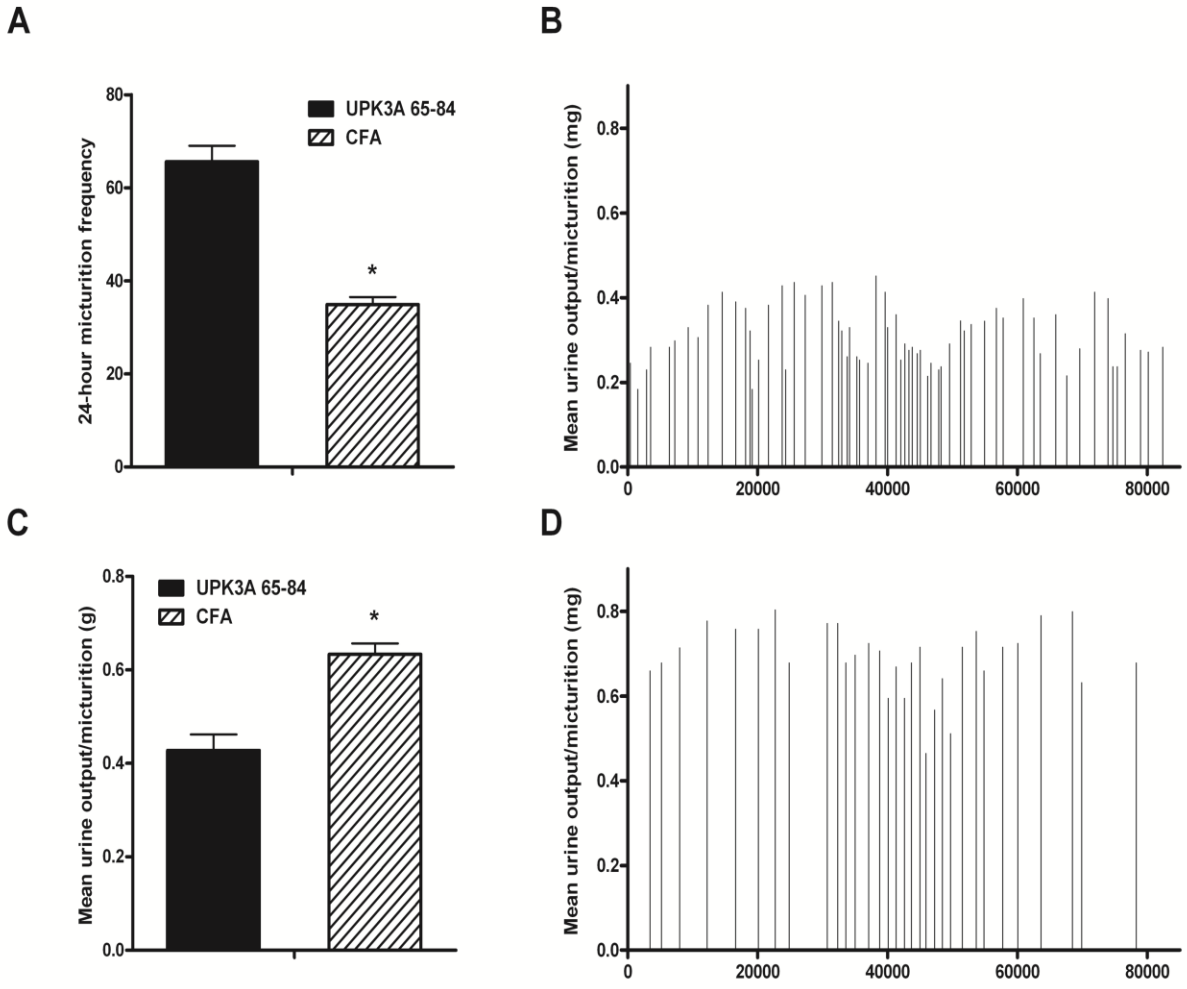


Figure 8. Bladder Dysfunction in Mice Immunized with UPK3A 65-84. (A) 24 hour micturition frequencies were significantly higher 5 weeks after immunization of BALB/c female mice with UPK3A 65-84 compared to control mice immunized with CFA. **(B)** Inversely, urine output/micturition was significantly lower in mice immunized with UPK3A 65-84 compared to control mice immunized with CFA.* indicates the P value<0.0001 significantly difference. Error bars show±SE. **(C-D)** The representative graphs show the urine production and its frequency of an individual immunized mouse with UPK3A 65-84 and immunized mice with CFA.

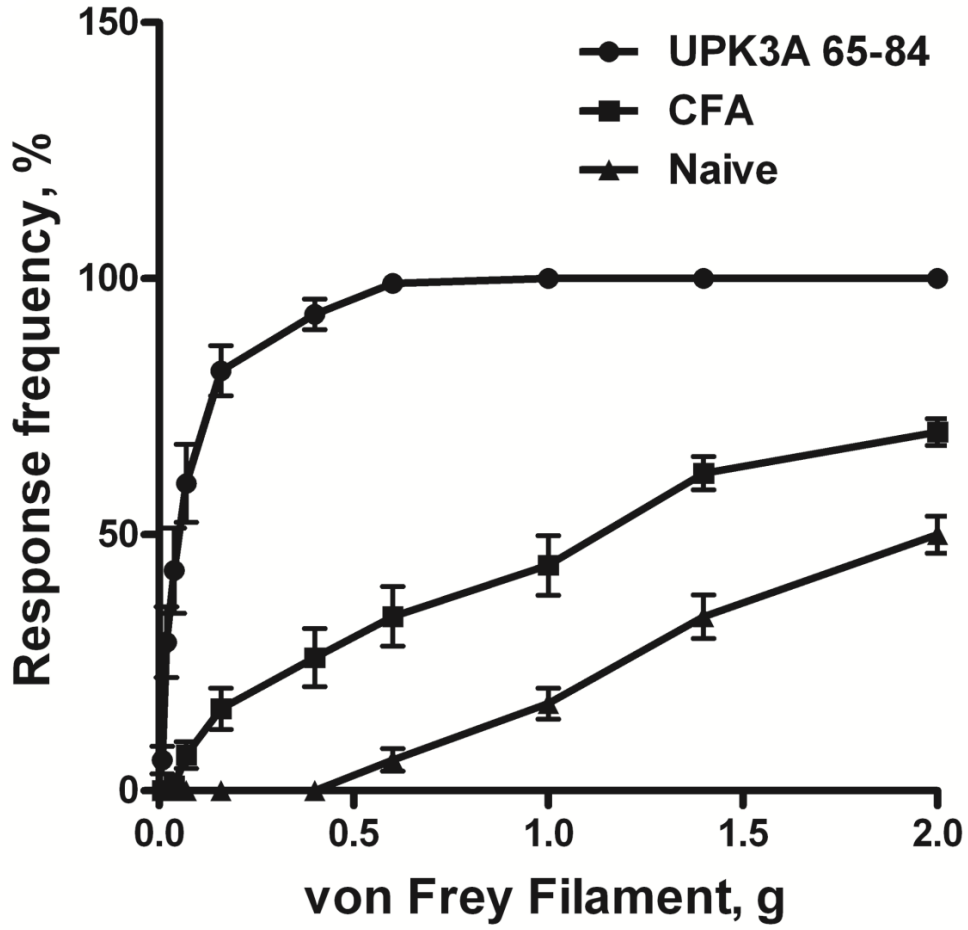


Figure 9. Increased Pelvic Pain Response in EAC. The referred hyperalgesia was examined with von Frey filaments applied to the pelvic region (suprapubic) 5 week after immunization (n=10). The behaviors that were considered to be as a positive response are: 1) sharp withdrawal of the abdomen; 2) instant licking and scratching; 3) jumping. The response frequency was calculated for each filament as the percentage of positive responses out of 10 stimuli. Each filament was performed for 1-3 seconds with 5-8 seconds intervals between each stimulus of total 10 times. Immunized mice with UPK3A 65-84 developed pelvic pain with the elevated response to stimulus as the meaning of decreased threshold of pain response on the pelvic region as an indicator of referred pain of bladder organ($p<0.05$) compared to control CFA and naïve(baseline) control.

3.8. Immunization with UPK3A 65-84 Increases Bladder Weights.

5 five after immunization the bladder weights were measured from BALB/c mice immunized with UPK3A 65-84 or CFA alone. UPK3A 65-84 immunized mice showed significantly increased ($P<0.001$) bladder weight (mg)/body weight (mg) ratios, a simple but reliable measure of organ inflammation (Figure 10).We observably confirm that active immunization with UPK3A 65-84 peptide is capable of inducing autoimmune cystitis in BALB/c female mice.

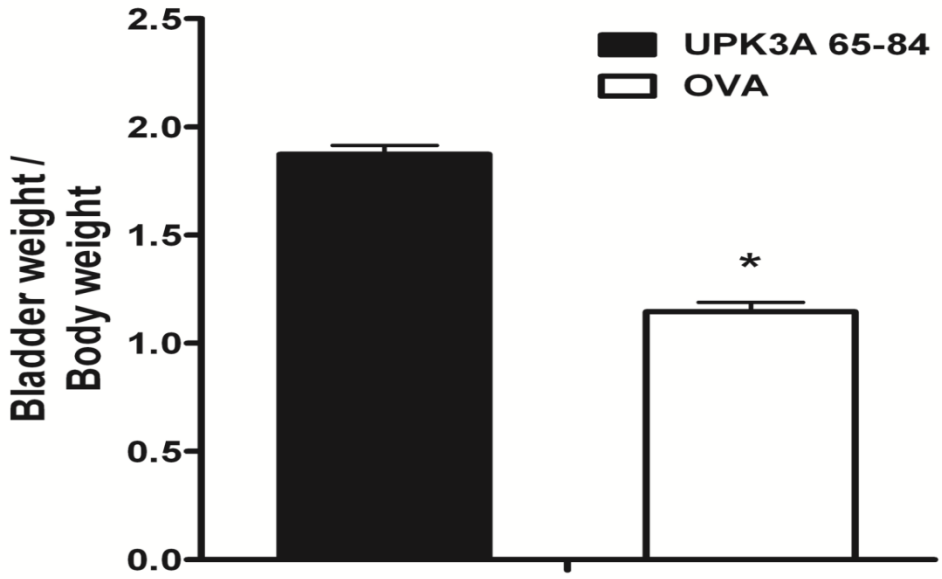


Figure 10. Immunization with UPK3A 65-84 Increases Bladder Weights. Bladder weight (mg)/body weight (mg) ratios were significantly higher ($P<0.001$) in mice immunized with UPK3A 65-84 compared to controls. Error bars show ±SE.* indicates P value<0.05.

3.9. Cystometrogram Analysis of Bladder Dysfunction in EAC Mice

The bladder threshold pressure, peak voiding pressure, resting pressure, compliance, and mean inter contractile interval were measured during micturition. Figure 11 and 12 shows the representative CMG data of UPK3A 65-84 immunized mice and CFA alone. Table III shows the CMG analyzed data. Peak voiding pressure which is the highest pressure throughout micturition was decreased significantly in UPK3A 65-84 immunized mice after 5 weeks immunization compared to CFA injected group. Threshold pressure, which is the pressure at the beginning of micturition phase, was also decreased significantly compared to control group. Resting pressure, which is the pressure after micturition, was significantly decreased in UPK3A 65-84 immunized mice. The significant decreased bladder compliance, which is calculated by dividing the voided volume by the variation in bladder pressure throughout the voiding phase, was observed in UPK3A 65-84 immunized mice. The mean intercontractile intervals were decreased in EAC mice compared to CFA injected control group.

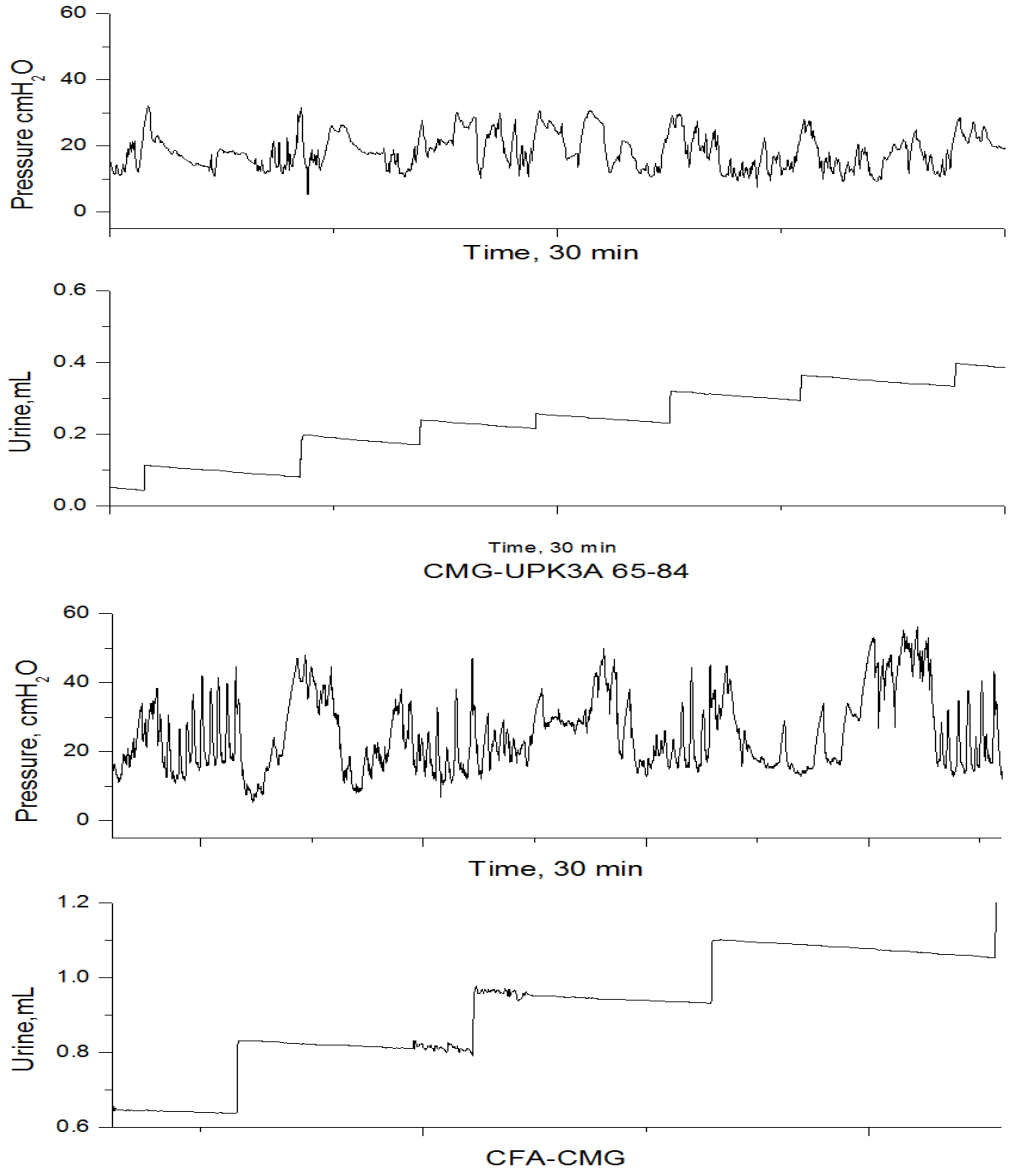


Figure 11. Representative Cystometrogram for EAC and Control Group.
Representative images of Cystometrogram of UPK3A 65-84 immunized mice and CFA alone 5 Week after Immunization. Saline Infusion rate mL/s=0.000277778 or Infusion rate mL/hr=1.

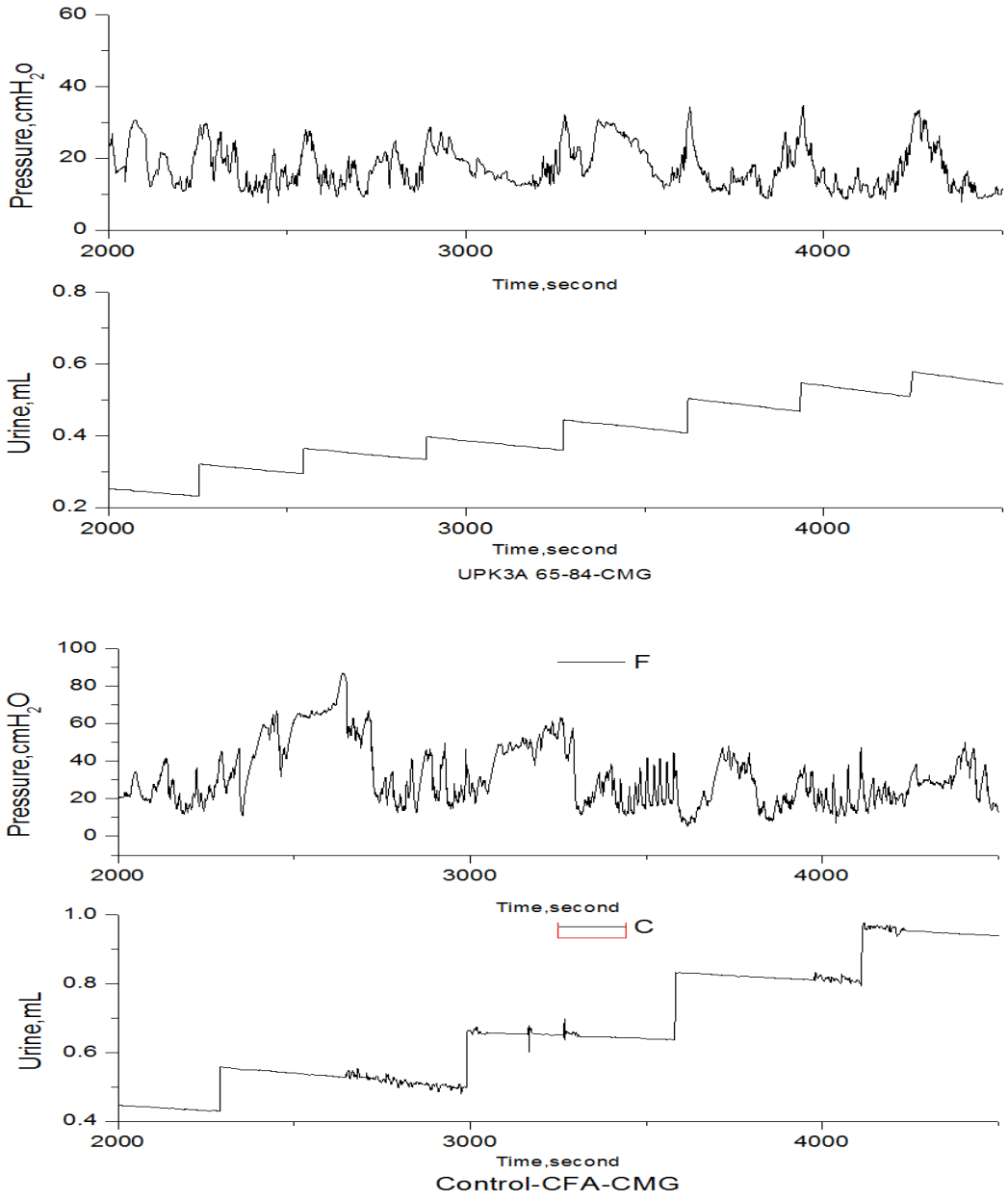


Figure 12. Representative Cystometrogram of EAC vs Control. Representative images of Cystometrogram of UPK3A 65-84 immunized mice and CFA alone 5 Week after Immunization. Infusion rate mL/s=0.000277778 or Infusion rate mL/hr=1.

Cystometry Results of EAC mice

	Resting Pressure,cmH2O			
	Mean	**SD**	**SEM**	**P value**
UPK3A 65-84 Immunized Group	**10.84796**	**3.05511**	**0.3168**	0.032
Control-CFA Group	12.02088	2.54372	0.43624	
	Peak Voiding Pressure, cmH2O			
UPK3A 65-84 Immunized Group	**39.86806**	**11.2975**	**1.1715**	0.001
Control-CFA Group	46.57529	5.94939	1.02031	
	Threshold Pressure, cmH2O			
UPK3A 65-84 Immunized Group	**22.86892**	**5.40327**	**0.56029**	0.00007
Control-CFA Group	27.53118	6.45137	1.1064	
	Bladder Capacity, mL			
UPK3A 65-84 Immunized Group	**0.0651**	**0.03558**	**0.00388**	0.00001
Control-CFA Group	0.14652	0.07024	0.01282	
	Compliance, mL/cmH2O			
UPK3A 65-84 Immunized Group	**0.00575**	**0.00331**	**3.61E-04**	0.0005
Control-CFA Group	0.01104	0.00734	0.00134	
	Mean Intercontractile intervals, second			
UPK3A 65-84 Immunized Group	**234.35646**	**128.07065**	**13.97365**	0.0000007
Control-CFA Group	534.15138	254.64703	47.28677	

Table 3. Comparisons of Cystometry Results. Cystometry results of EAC mice.5 week after immunization conscious CMG was performed. Bladder resting pressure, bladder threshold pressure, peak voiding pressure, bladder capacity, compliance and mean intercontractile intervals were analyzed on cystometry data of mice immunized with UPK3A 65-84(n=6), and mice immunized with CFA(n=5). Infusion rate mL/s=0.000277778 or Infusion rate mL/hr=1.

3.10. IC/PBS phenotype occurred following adoptive transfer of CD4+ T-cells from UPK3A 65-84 Primed Mice.

We determined EAC (transferring the immunologic functionality and features into naïve host) induction capability of adoptive transferring of isolated CD4+ T cells, CD8+ T cell, B cells, and serum from immunized mice into naïve BALB/c mice. 10 days after immunization with UPK3A 65-84 peptide or OVA, CD4+ and CD8+ T cells were isolated and enhanced proliferation with the immunogens, and transferred into naïve recipients. After 20 days of adoptive transfer, the phenotype of EAC bladder dysfunction tested with FVC showed that 24 hour micturition frequencies were significantly higher in mice with peptide specific CD4+ T cells-transferred compared to control-OVA induced T-cell transferred. Inversely, urine output/micturition was significantly lower in mice with peptide specific CD4+ T cells-transferred compared to control mice (Figure 13a-b). However, no phenotypical signs of IC/PBS occurred in FVC measurements after transferring the peptide induced CD8+ T cells into naïve BALB/c mice recipients compared to control OVA- mediated CD8+ T cells transferred (Figure 14). Similarly, the transfer of collected sera from 5 weeks after immunized mice with UPK3A 65-84 or Ovalbumin, no micturition abnormalities associated to EAC phenotype observed compared to control (Figure 15). The passive transfer of B-Cells from immunized mice with UPK3A 65-84 did not show differences in voiding frequency and micturition output/voiding compared to control(P values 0.77, and 1 respectively)(Figure 16).

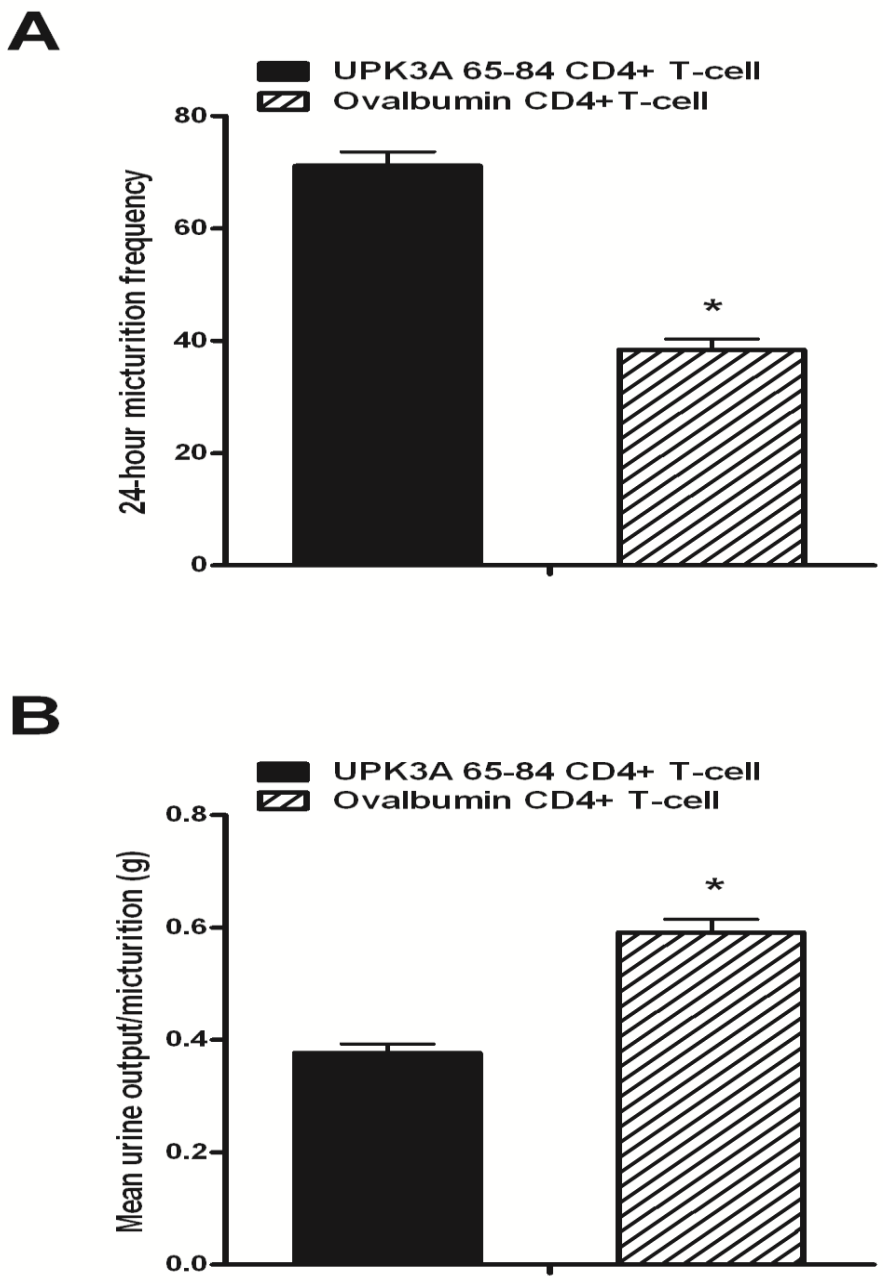


Figure 13. Adoptive Transfer of enriched CD4+T cells developed EAC. The phenotype of EAC development was tested with FVC after 20 days of adoptive transfer (A) Bladder dysfunction resembling IC/PBS phenotype in mice with peptide specific CD4+ T cells-transferred showed that 24 hour micturition frequencies were significantly higher compared to control-OVA induced T-cell transferred(n=10). (B) Inversely, urine output/micturition was significantly lower in mice with peptide specific CD4+ T cells-transferred compared to control mice.

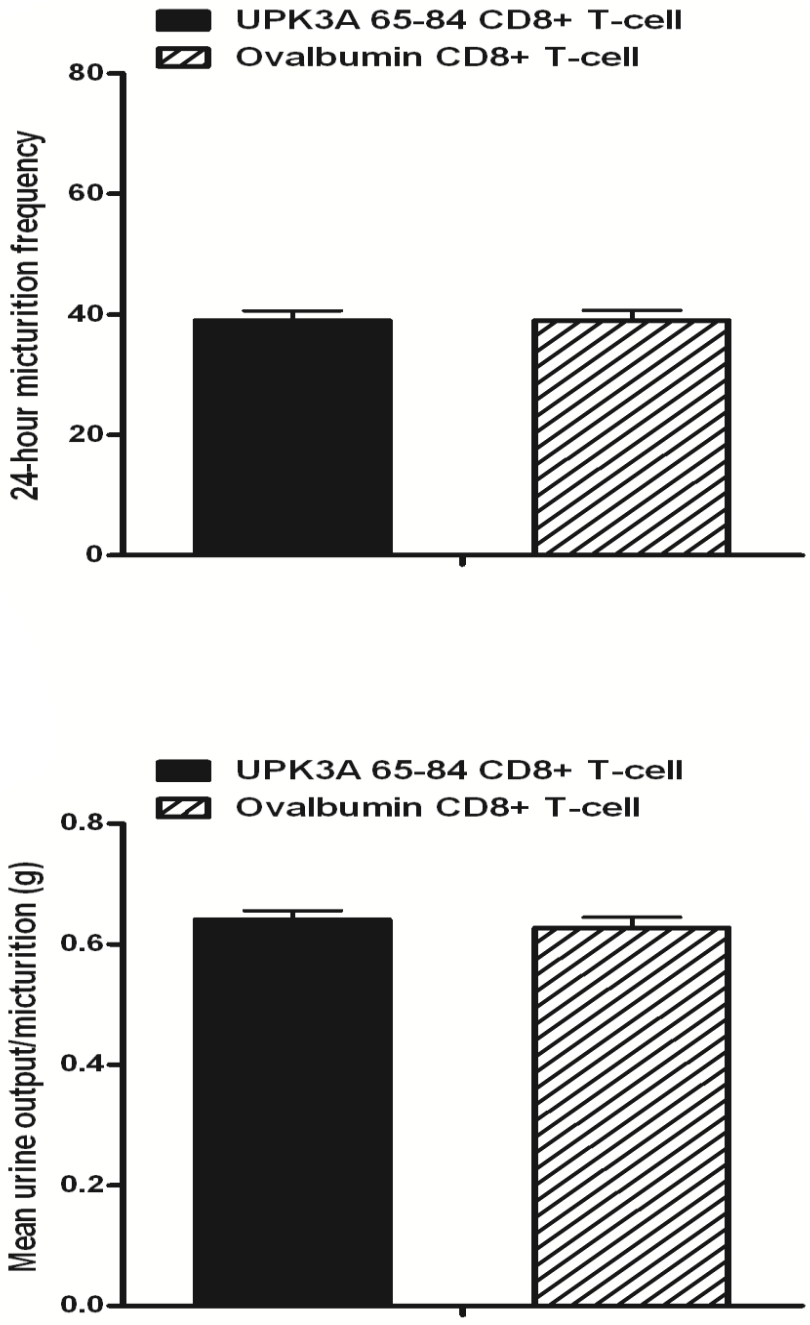


Figure 14. Adoptive Transfer of CD8+T cells, no Phenotypical Changes in FVC. No phenotypical signs of IC/PBS occurred in FVC measurements after transferring the peptide induced CD8+ T cells into naïve BALB/c mice recipients compared to control OVA- mediated CD8+ T cells transfer.

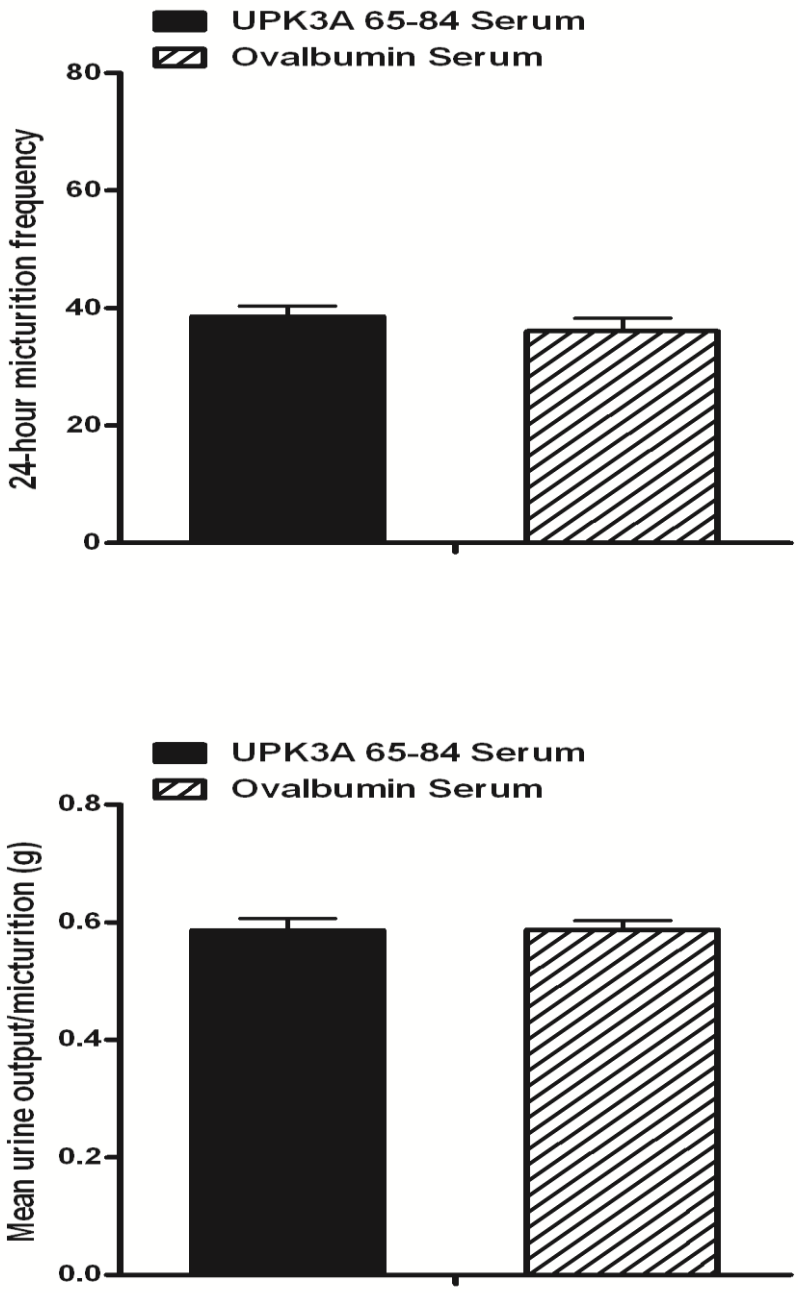


Figure 15. Sera Transfer from Immunized Mice to Naive Host. 5 weeks after immunization collected and pooled sera from immunized mice with UPK3A 65-84 or Ovalbumin, transferred into naïve BALB/c recipients and the 20 days later FVC measured showed no differences between groups.

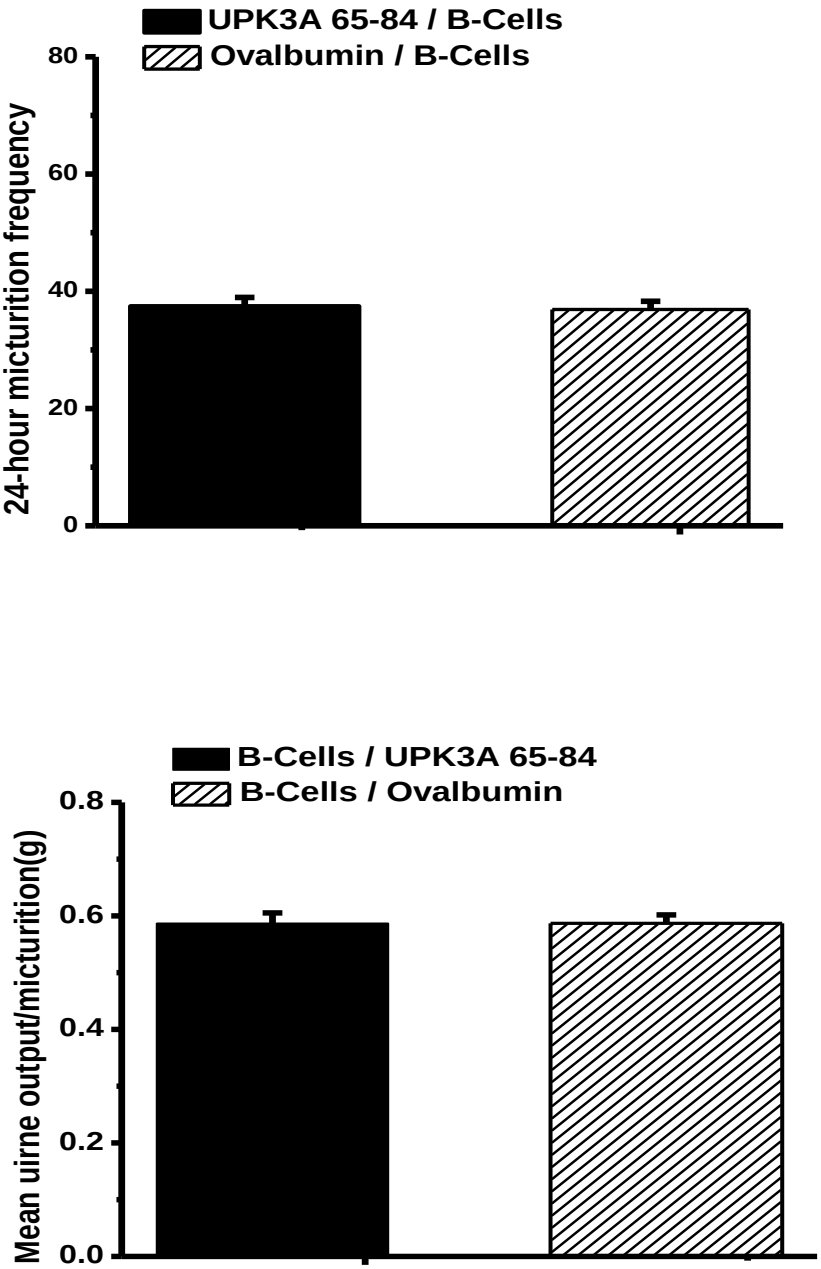


Figure 16. Passive Transfer of B-cells from Mice Immunized with UPK3A 65-84 to Naive Recipient. 5 weeks after immunization with UPK3A 65-84 or Ovalbumin, the purified B-cells transferred into naïve BALB/c recipients and the 20 days later FVC measurement showed no differences between groups.

3.11. Pelvic Pain Response Following Adoptive Transfer of CD4+ T-cells from UPK3A 65-84 Primed Mice.

Pelvic pain which is the most prominent phenotype of IC/PBS, was examined with von Frey filaments by administration onto the pelvic region (suprapubic) at day-5-10-20-30 after passive transfer of the UPK3A 65-84 primed CD4+ and CD8+ T cells, B cells and sera comparing with OVA primed CD4+ and CD8+ T cells, and sera transferred group, into naïve BALB/c recipients. UPK3A 65-84 primed CD4+ T cells revealed increase pain response to stimulus compared to control mice transferred with CD4+ T cells specific for OVA, as the indicator of decreased threshold of referred pain of bladder organ on the pelvic region (Figure 17). On the other hand, the passive transfer of peptide induced CD8+ T cells into naïve BALB/c mice recipients showed no difference in pain assessment compared to control OVA- mediated CD8+ T cells transfer (Figure 18). The pooled sera taken from 5 weeks after immunization of mice with each immunogens were injected into naive recipient BALB/c female hosts. The pain measurements at 5, 10, 20, 30 days were not different compared to control groups (Figure 19). The passive transfer of B-Cells from immunized mice with UPK3A 65-84 did not show differences in pelvic pain responses compared to control groups (Figure 20).

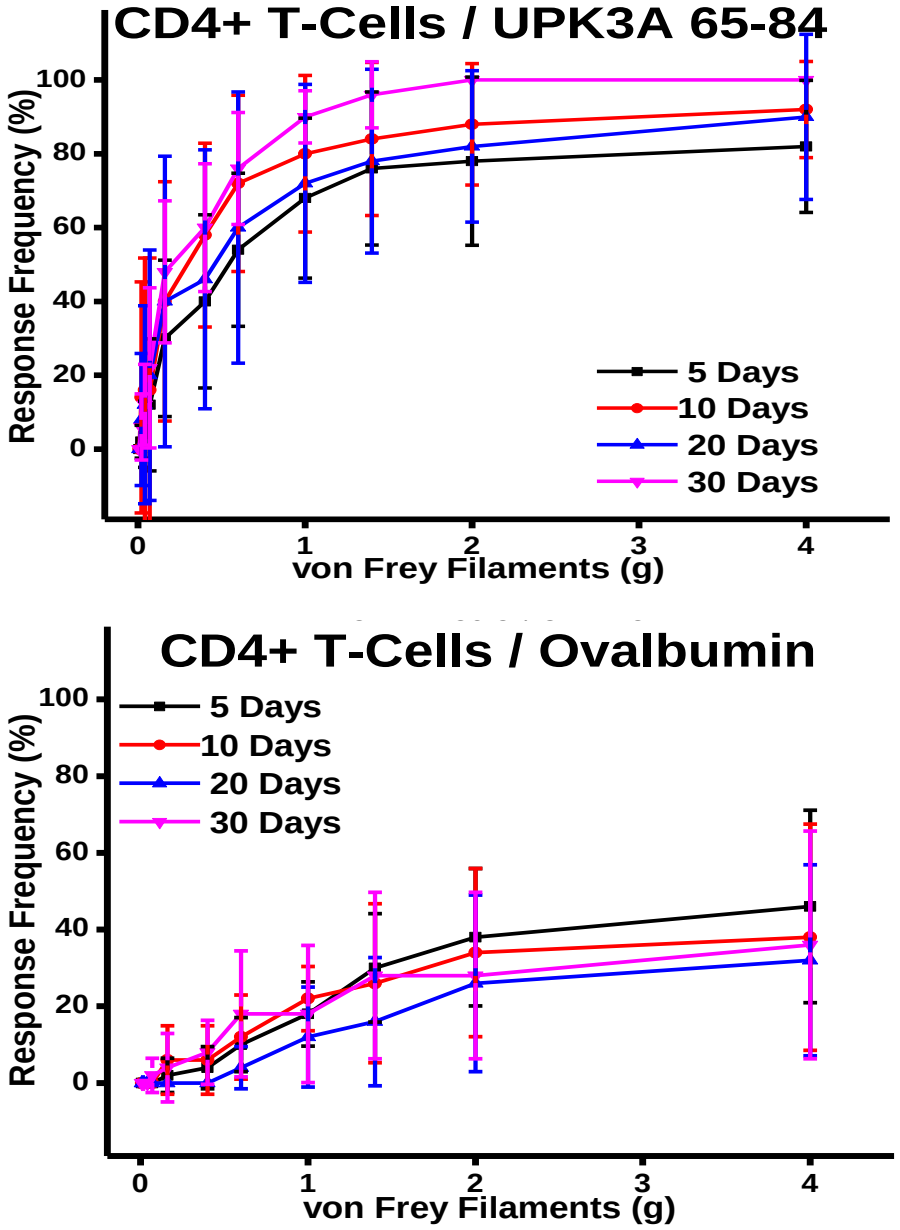


Figure 17. Adoptive Transfer of enriched CD4+ T cells from UPK3A 65-84 immunized mice revealed increase pelvic pain response. Pelvic pain which is the most prominent phenotype of IC/PBS, was examined with von Frey filaments applied to the pelvic region (suprapubic) at day 5, 10, 20, and 30 after transferring the UPK3A 65-84 primed CD4+ and CD8+ T cells and serum comparing with OVA primed CD4+ and CD8+ T cells, and sera transferred group into naïve recipients(n=10). Adoptive transfer of UPK3A 65-84 primed CD4+ T cells revealed increase response to stimulus after 5, 10, 20 and 30 days, compared to control mice transferred with CD4+ T cells specific for OVA, as the indicator of decreased threshold of referred pain of bladder organ on the pelvic region.

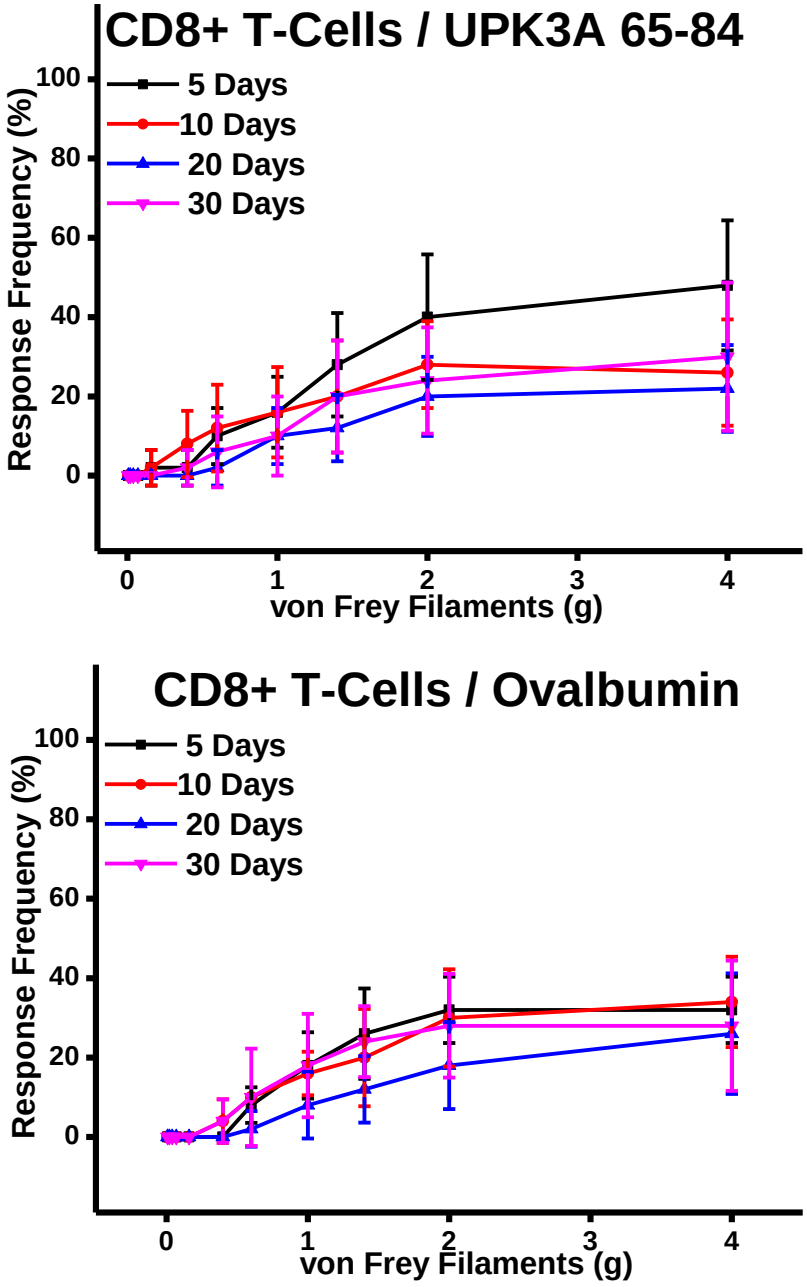


Figure 18. There is no pelvic pain response after adoptive transfer of CD8+ T cells. The pelvic pain phenotype of IC/PBS was not occurred by testing von Frey filaments on the referred area of bladder pain (suprapubic) at day 5, 10, 20, and 30, after transferring the peptide induced CD8+ T cells into naïve BALB/c mice recipients compared to control OVA- mediated CD8+ T cells transfer.

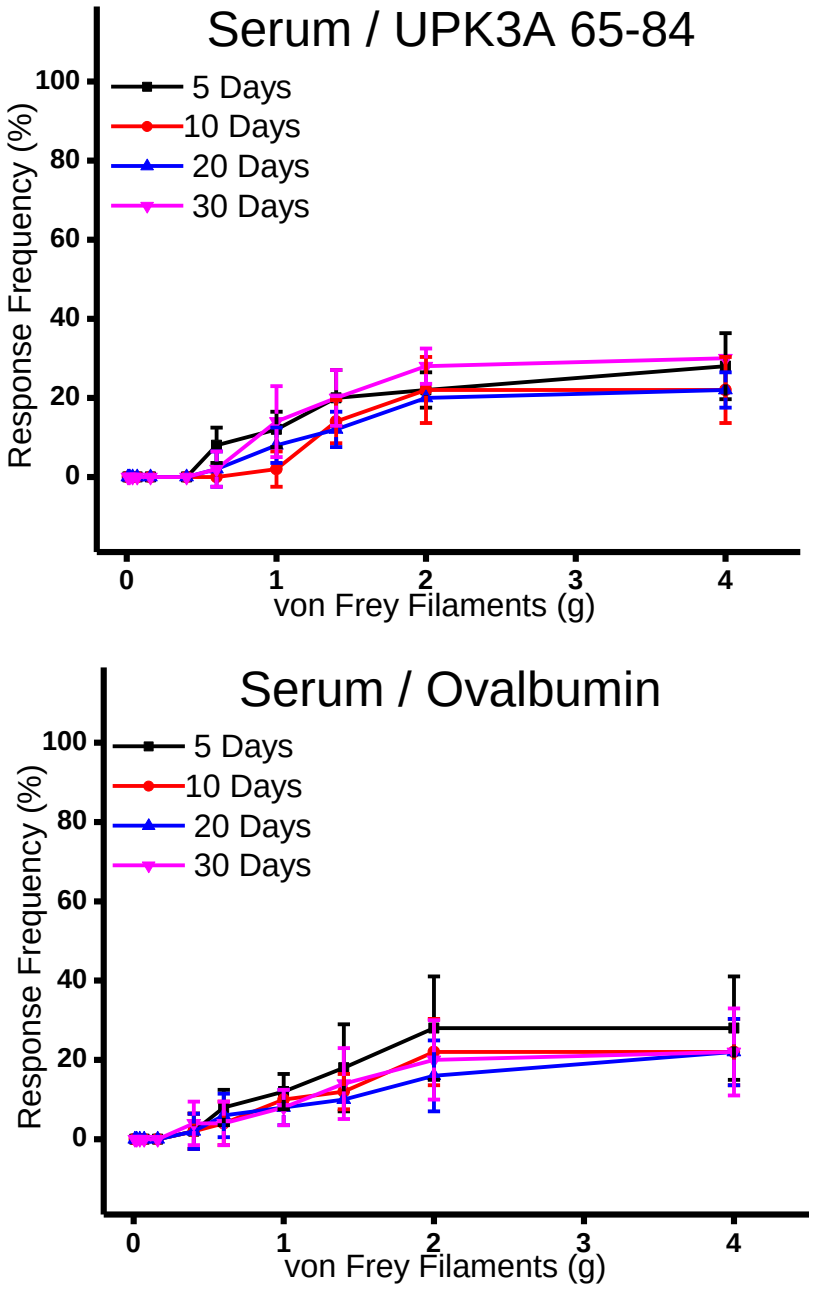
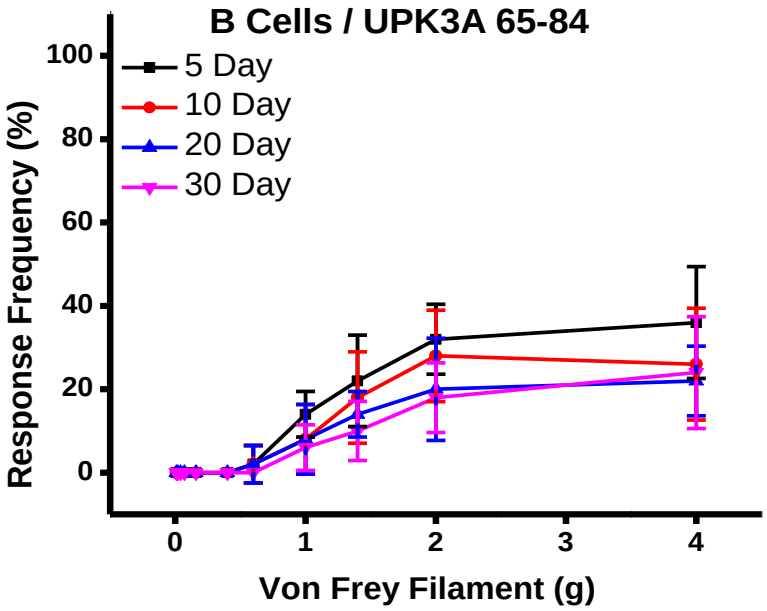


Figure 19. There is no pelvic pain response after sera transfer from mice immunized with UPK3A 65-84. The sera samples taken from 5 weeks after immunization with each immunogenic peptide (Ovalbumin and UPK3A 65-84), the collected and pooled sera were injected [3 injections of 200 µl per mice, i.v/i.p injection, every other day] into naive recipient BALB/c female hosts for determining the ability to mediate EAC. After the 5, 10, 20, 30 days pain measurements, no difference was observed to stimulus in transferred groups compared to control groups.



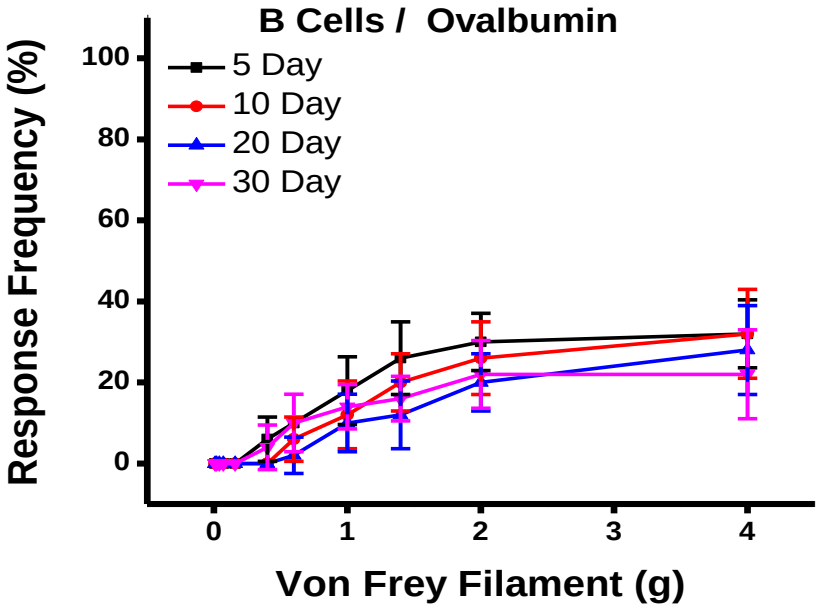


Figure 20. Passive Transfer of B-cells from mice immunized with UPK3A 65-84 into naive host did not developed pelvic pain response. Passive Transfer of B-cells from UPK3A 65-84 immunized mice into naïve BALB/c host did not revealed pelvic pain response by testing von Frey filaments on the referred area of bladder pain at day 5, 10, 20, and 30 compared to control groups.

Thus, the passive transfer of CD4+ T cells, isolated from LNCs of UPK 3A 65-84 immunized mice, developed EAC in naïve host BALB/c mice showing the phenotypical features of IC/PBS; frequent urination, decrease output per micturition and increase pelvic pain response to von Frey monofilament stimulus.

CHAPTER IV

DISCUSSION

Our study reveals that an UPK3A peptide specific CD4+ T-cell autoimmunity mediates painful bladder dysfunction in mice that mimics human IC/PBS. The pathophysiology of IC/PBS remains puzzling. Advancement in addressing IC/PBS has been overwhelmingly slow due to a lack of understanding of the underlying pathophysiology and lack of markers for the disease. The current approaches in our scientific area mostly focus on merge clinical practice and translational thus stressing the significance of translational models. Almost 20 animal models have been scrutinized for two decades which resembles somewhat IC/PBS phenotype (Westropp and Buffington 2002). One of the limitations in IC/PBS research has been the lack of an appropriate animal model. The NIH (National Institutes of Health) has launched a number of initiatives in both clinical and basic science of IC/PBS over the past 15 years without making a meaningful impact. Suffice it to say that IC/PBS remains one of the top priorities for NIH-NIDDK as a disease that could benefit from a realistic animal model and translational research.

The earlier animal models have been developed through different approaches such as inducing inflammation by transurethral application of irritant or immune stimulants into urinary bladder, systematic and environmentally caused irritations (e.g., acetone, glycerol, mustard oil, acetic acid, cyclophosphamide [CYP]), autoimmune strategies (bladder homogenate, URO-OVA or URO-OVA/OT-I, UPK2 protein)(Liu, Evanoff et al. 2007; Altuntas, Daneshgari et al. 2008; Lin, Liu et al. 2008; Liu, Deyoung et al. 2008; Rudick, Schaeffer et al. 2008; Altuntas, Daneshgari et al. 2011). Transgenic EAC model of URO-OVA mice fabricate a membrane structure of Antigen-Ovalbumin (OVA) like a self-antigen on the urothelium derived by Uroplakin II gene promoter and then the bladder autoimmune cystitis was generated through the transfer of OVA-specific OT-I $CD8^+$ T-cells to this mouse (Liu, Evanoff et al. 2007; Liu, Deyoung et al. 2008). The disadvantage of this model is that OVA is not endogenous antigen of bladder and the urodynamic assessment of the bladder or bladder dysfunction, and pelvic pain associated to IC/PBS phenotype have not been characterized and demonstrated. Even though CYP- employed cystitis model is well-described and broadly study for chronic cystitis, the pathogenesis does not fit the IC/PBS of human(Vera, Iczkowski et al. 2008). A spontaneous model of IC/PBS was described in feline(Buffington 2004), however this was not applied extensively in scientific community due to cost and handling problems.

Autoimmunity of IC/PBS has been suspecting due to concomitant occurrence with some autoimmune disorders for instance; systemic lupus erythematosis(SLE), rheumatoid arthritis, ulcerative colitis, thyroiditis, Sjögren syndrome and fibromyalgia syndrome (Keay, Zhang et al. 1997; Ochs 1997; Lorenzo Gomez and Gomez Castro

2004; van de Merwe 2007). And also the relatively high occurrence of antibodies in patients with IC (van de Merwe 2007), the increase demonstration of CD4 and CD8 T-Cells, B cells, plasma cells(MacDermott, Miller et al. 1991; Christmas 1994), and inappropriate expression of HLA-DR in the bladder biopsies of IC patients(Christmas and Bottazzo 1992).

The strategy of mounting an inflammatory T-cell response against carefully selected antigens that are expressed only in the targeted organ has utilized to the establishment of practical experimental autoimmune animal models for studying a variety of human diseases including autoimmune encephalomyelitis(EAE)-Multiple Sclerosis (Tuohy, Lu et al. 1989; Yu, Johnson et al. 1996), autoimmune inner ear disease (Solares, Edling et al. 2004), autoimmune oophoritis -premature ovarian failure(Altuntas, Johnson et al. 2006) , and autoimmune myocarditis-dilated cardiomyopathy (Jane-wit, Yu et al. 2002).

The similar strategies mounting an inflammatory T cell response to targeted tissues were reported in the employment of bladder homogenate and bladder specific protein UPK2 (Lin, Liu et al. 2008), (Altuntas, Daneshgari et al. 2011) showing induction of autoimmune mediated EAC in SWXJ mice resembling the human IC urodynamicaly. The disadvantage of immunization with bladder homogenate is the possible induction of non-specific and systemic immune reaction alongside the bladder tissue because of the composition of bladder non-specific or specific antigens. The IC mice model immunization with bladder specific recombinant UPK2 protein mice exhibited bladder specific T cell mediated autoimmunity and significant proof bladder dysfunction in regard to increased frequency of urination and decrease urine output per voiding (Lin, Liu

et al. 2008; Altuntas, Daneshgari et al. 2011); however, this EAC mouse model was not revealed pelvic pain response to noxious stimulus with von Frey Filaments, that missing one of the major symptoms of IC/PBS phenotypes. These observed phenotypical characteristics and urodynamic abnormalities in those mice models were somewhat similar with IC/PBS symptoms seen in human, thus we aimed to create more specific autoimmunity targeting a bladder specific protein, which could induce all major phenotypical features of IC/PBS, especially the pelvic pain phenotype. In view of that, it is necessary for an animal model that merges all phenotypical properties as observed in human IC/PBS together with frequency, urgency, pelvic pain along with bladder specificity and high induction rate of IC/PBS phenotype.

In our present study, we evaluated immunogenicity of the peptides derived from bladder specific Uroplakin proteins in different mice strains. We found that a 20-mer peptide from UPK3A is highly immunogenic and specifically elicits CD4+ T-cell mediated Th1 immune response in BALB/c mice inducing EAC along with bladder confined inflammation. We verified inflammation in bladder by revealing the increase expressions of proinflammatory cytokines and the presence of clusters of T-cells in the bladder following immunization. Moreover, the significantly increased bladder weight to body weight ratios are basic and simple indicator for inflammation. These findings point out that immunization of BALB/c mice with UPK3A 65-84 incites an organ specific autoimmune disorder described through inflammation restricted to the bladder that continually directs to bladder dysfunction parallel to that examined in Uroplakin III or II-null mice (Aboushwareb, Zho et al. 2007). Subsequently, we tested bladder function and pelvic pain response whether active immunization with UPK3A 65-84 can induce or

initiate an IC/PBS phenotype. The phenotypical characteristics of our current model were seen 5wk after active immunization with UPK3A 65-84. The mice immunized with UPK3A 65-84 showed significantly elevated urinary frequencies, decreased urine outputs per void, and increased pelvic pain response, which resemble all three major symptoms of IC/PBS. Moreover, decrease intercontractile intervals and bladder compliance that were observed on CMG measurement in EAC model, also correlate with the physiological alterations seen the human IC/PBS. Correspondingly, the passive or adoptive transfer of UPK3A 65-84 stimulated CD4+ T-cell mediates EAC in BALB/c naïve recipients manifesting significantly detectable pelvic pain response, increased micturition frequency, and decreased output per micturition, similar to the symptomatology observed in IC/PBS. Our data indicates that the bladder dysfunction and increase pelvic pain response occurring in EAC is due to CD+4 T-cell mediated autoimmune inflammation of the bladder tissue. We elucidated a potential move toward to examine referred hyperalgesia by von Frey Filaments on pelvic region (Laird, Martinez-Caro et al. 2001). 5 weeks after active immunization and 5, 10, 20, 30 days after the adoptive transfer of CD+4 T-cells, for pelvic and urinary bladder pain assessment, we found an enhanced response to a painful stimulus and a painful reaction to a normal stimulus.

The significance of our model is that just one single peptide, UPK3A 65-84, induces T-Cell dependent autoimmune mediated EAC with higher bladder specificity, accurately reflecting the urodynamic symptoms and chronic pelvic pain of IC/PBS phenotype. The further characterization of the pelvic pain mechanism in this model is needed to investigate. The role of mast cells in the induction of cystitis pain has already

revealed (Rudick, Bryce et al. 2008; Rudick, Schaeffer et al. 2009). For future studies the mast cells and T-cells involvement in pelvic pain development in IC/PBS model could be investigated.

In this mouse model, we clearly characterized and showed a targeted autoimmunity against bladder specific protein, UPK IIIA, induces a new type of EAC. This bladder specific autoimmunity can be used in an actively way to prevent the development of bladder cancers and retardation of existing tumors. It is already known that the highly expressed antigens in tumor are appropriate target for cancer immunotherapies. It was also suggested that the organ or tissue specific antigens that are produced through normal tissue or their originated cancer cells could be targeted for cancer vaccination (Jaini, Kesaraju et al. 2010; Tuohy 2010; Altuntas, Jaini et al. 2012). It was proposed that peripheral self-tolerance mechanisms possibly hinder the endogenous immune reaction to cancer, and it is essential to breakdown to elicit clinically applicable antitumor response (Dudley, Wunderlich et al. 2002; Dudley, Wunderlich et al. 2005). The effect of immunotherapy and autoimmunity regarding to tumor retardation and autoimmune reactivity to normal tissues were studied in some clinical immune-mediated treatments, and also in pre-clinical immunotherapy models (Huber and Wolfel 2004; Dudley, Wunderlich et al. 2005) (Overwijk, Lee et al. 1999; Bettinotti, Panelli et al. 2003; Phan, Yang et al. 2003). It was tested in mice that the targeted autoimmune response to organ specific proteins can hinder or treat tumor development in that organ or tissues. The study in autoimmune mice models, the tumor development of autochthonous ovarian granulosa cell tumors in SJL.AMH-SV40Tag transgenic mice was hindered in immunized group with Inhibin alpha 215-234 peptide and also, the therapeutically

vaccinated group that had ovarian granulosa cell tumors showed significantly reduction of tumor growth(Altuntas, Jaini et al. 2012). In another study, breast autoimmunity was developed immunization with the recombinant mouse mammary-specific differentiation protein antigen (α-lactalbumin), and assessed the effectiveness of immunization with α-lactalbumin on cancer development in prophylactic and therapeutic approaches. It was demonstrated that immunoreactivity against alpha-lactalbumin resulted in significant prevention and treatment against autochthonous tumor development in breast cancer mouse models, and against 4T1 transplantable breast tumors in BALB/c mice (Jaini, Kesaraju et al. 2010). Recently, the Tumor vaccine development trials with immunogenic cancer/testis (CT) antigens produced in normal gametogenic tissues and in various cancers, MAGE-A3 (melanoma antigen A3) and NY-ESO-1 have been conducted, and suggested that these antigens could be used for antigen-specific adoptive T-cell transfer for the cancer immunomodulation (Parmigiani, Bettoni et al. 2006; Caballero and Chen 2009). NCI (National Cancer Institute) Researchers revealed the success of immunotherapy method for melanoma treatment showing significant shrinkage in the tumor size of melanoma patients who have progressive melanoma unresponsive to former treatments and autoimmunity targeted against normal melanocytes was detected in 75% of the adoptively T-cell treated patients who showed tumor inhibition (Dudley, Wunderlich et al. 2005).

Uroplakins (UPKI, II, and III) are integral membrane proteins uroepithelium (Kong, Deng et al. 2004) vastly and specifically produced in bladder tissue. Uroplakins might be effective as an anti-cancer vaccine development for targeting an autoimmune attack against bladder cancers. The expression of UPK III bladder cancer were showed

that there is 60% (21/35) in the primary tumors and 53% in (17/32) the metastatic tumor of human urothelial carcinomas (Kaufmann, Volmerig et al. 2000). Several studies have validated the stable expression of UPKII in human bladder cancer cell lines (Wu, Kakehi et al. 2005). UPKII promoter driven expressions of SV40 led urothelium cancer in the bladder of transgenic mouse (Saban, Towner et al. 2007). It was suggested that Uroplakins are also able to be suitable for creating tissue specific vectors designed for bladder cancer gene therapy (Zhu, Zhang et al. 2004; Zhu, Zhang et al. 2004).

Overall, we have established a bladder-specific autoimmunity of UPK3A 65-84 immunized mice with a high induction rate of EAC, and propose that our model is a remarkably practical model for future exploration of mechanistic event in pathogenesis and therapeutic intervention of IC/PBS and the facts gained in this model gives further vision to test different strategies for autoimmune mediated regulation of bladder cancer.

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