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CCL2 (MCP-1) Mediates Chronic Pelvic Pain Through Mast Cells in Experimental Autoimmune Cystitis

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CCL2 (MCP-1) MEDIATES CHRONIC PELVIC PAIN THROUGH MAST CELLS IN EXPERIMENTAL AUTOIMMUNE CYSTITIS

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DEDICATION

To My Mother-Rukiye Bicer, My Father- Ismet Bicer, My wife- Hacer Guvenc Bicer, My sister's husband- Sait Hakverir, My Sisters, My Brothers (especially Murat Bicer) and My all supporters

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CCL2 (MCP-1) MEDIATES CHRONIC PELVIC PAIN THROUGH MAST CELLS IN EXPERIMENTAL AUTOIMMUNE CYSTITIS

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ABSTRACT

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a chronic inflammatory bladder condition with unknown pathophysiology. Chronic pelvic pain is one of the most important and disturbing symptoms of IC/PBS, beside urinary frequency, urgency, and nocturia. Increased amounts of mast cells (MCs) and CCL2 have been found in bladder tissue and urine, respectively. Whether or not there is a causal relationship between CCL2, MCs, and pelvic pain in IC/PBS is unknown.

Methods: Female BALB/c mice were immunized with a peptide consisting of residues 65-84 of the bladder urothelium-specific protein uroplakin 3A to induce experimental autoimmune cystitis (EAC), a model for human IC/PBS. Referred visceral pelvic pain was measured using von Frey monofilaments at different times after immunization. Lidocaine was instilled into the bladder, colon and uterus to locate the source of the pelvic pain. CCL2 expression in tissues was measured by qRT-PCR and ELISA, and MCs in the bladder were quantified by toluidine blue staining. An MC stabilizer (cromolyn sodium), histamine H1 receptor blocker (cetirizine), and histamine H2 receptor blocker (ranitidine) were administered orally to show the relation of MCs with the pain. $CCL2^{-/-}$ and $CCR2^{-/-}$ mice, and CCR2 (CCL2 receptor) antagonist treatment were used to delineate the causative effect of CCL2 on MC accumulation and chronic pelvic pain.

Results: All mice immunized with the uroplakin 3A peptide developed pelvic pain within 5 days and up to 40 days after immunization. Lidocaine alleviated the pain only when it was installed into the bladder of EAC mice, confirming the bladder origin of the pain. The amounts of CCL2 mRNA and protein, and the numbers of MCs were markedly increased in bladder tissue up to 40 days after immunization with peptide compared with controls. Administrations of cromolyn sodium and ranitidine significantly decreased pelvic pain in the model. Moreover, immunization did not establish chronic pelvic pain or accumulation of MCs in MCP-$1^{-/-}$ or $CCR2^{-/-}$ mice, compared with wild type mice.

Conclusion: CCL2 has a vital role in causing chronic pelvic pain through accumulation of MCs in this newly created EAC mouse model, and might be an essential player in the pathogenesis of human IC/PBS.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AUA	American Urologic Association
APF	Antiproliferative Factor
BALB/cJ mouse	Balb/c background strain mouse
BCG	Bacillus Calmette-Guerin
BSA	Bovine Serum Albumin
BMMCs	Bone marrow-derived mast cells
CCL2	Chemotactic C-C ligand 2
CCR2	chemokine (C-C motif) receptor 2 (a chemokine receptor)
CCR2 KO mice	chemokine (C-C motif) receptor 2 Knockout
CCR2 -/- mice	chemokine (C-C motif) receptor 2 Knockout
CXCR2	Chemokine (C-X-C motif) receptor 2
CFA	Complete Freund’s adjuvant
CD117	c-Kit receptor
cDNA	Complete Deoksiribose Nucleic Acid
CGRP	Calcitonin Gene-Related Peptide

CNS	Central Nervous System
CPPS	Chronic Prostate Pelvic Pain
CYP	Cyclophosphamide
EAC	Experimental Autoimmune Cystitis
EAE	Experimental Autoimmune Encephalomyelitis
EAMC	Experimental Autoimmune Myocarditis
EAO	Experimental Autoimmune Oophoritis
EUA	European Association of Urology
ELISA	Enzyme Linked Immunosorbent Assay
ESSIC	European Society for the Study of IC/BPS
ddH2O	Double Distilled Water
DMSO	Dimethyl sulfoxide
FceRII	Anti-Mouse Fc epsilon Receptor II alpha
FDA	Federal Drug Administration
FVC	Frequency Voiding Charts
G (g)	Gram
GAG	Glycosaminoglycan
H1	Histamine receptor 1
H2	Histamine receptor 2

H3	Histamine receptor 3
H4	Histamine receptor 4
HLA	Human Leukocyte Antigen
HPLC	High Pressure Liquid Chromatograph
IBS	Inflammatory bowel diseases
ICAM	Intercellular Adhesion Molecule
IC	Interstitial Cystitis
IC/PBS	Interstitial Cystitis/Painful Bladder Syndrome
IC/BPS	Interstitial Cystitis/ Bladder Pain Syndrome
ICS	International Continence Society
IgE	Immunoglobulin E
IFN-γ	Interferon-Gamma
MC	Mast cell
MCp	Mast cell progenitor
MCP-1	Monocyte Chemoattractant Protein 1
MCP-1 KO	Monocyte Chemoattractant Protein 1 Knockout
MCP-1 -/-	Monocyte Chemoattractant Protein 1 Knockout
MCs	Mast cells
MC-T	Mast cell –containing trptase

MC-TC	Mast cell -containing trptase, chymase, and carboxypeptidase A
mMMC	Mouse Mucosal Mast cell
mCTMC	Mouse Connective tissue type mast cells
IL	Interleukin
ISAP	International Association for Study of pain
LL-37	Leucine leucine-37
LPS	Lipopolysaccharide
LT	Leukotriene
MadCAM-1	Mucosal addressing cell adhesion molecule-1
MHC	Major Histocompatibility Complex
MIP-1 alpha	Macrophage Inflammatory Proteins 1 alpha
MS	Multiple Sclerosis
NGF	Nerve Growth Factor
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
PAF	Platelet-Activating Factor
P2X3	Purinergic receptor
PDGF	Platelet Derived Growth Factor

PG	Prostaglandin
PPS	Pentosan polysulfate sodium
PST	Potassium sensitivity test
RA	Rheumatoid Arthritis
RANTES	**R**egulated on **A**ctivation **N**ormal **T**-cell **E**xpressed and **S**ecreted
RNA	Ribose Nucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	stem cell factor=kit ligand
SDF-1	Stromal cell-derived factor-1
Th1	T helper Cell Type 1
Th2	T helper Cell Type 2
THP	Tam Horsfall Protein
TGF-β	Tumor Growth Factor Beta
TNF	Tumor Necrosis Factor
TLR	Toll-Like Receptor
UPK	Uroplakin
UPK3A	Uroplakin 3A
UP	Uroplakin
OVA	Ovalbumin

WT	Wild Type
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular Endothelial Growth Factor
$	Dollar
i.e.	That is; in other words; that is to say
-/-	Knockout

LIST OF FIGURES

CHAPTER I

I.BACKGROUND AND SIGNIFICANCE

Interstitial Cystitis/Painful Bladder Syndrome (IC/PBS)

The International Continence Society (ICS) calls the disease as IC/PBS **(**Interstitial Cystitis/Painful Bladder Syndrome). On the other hand, The European Society calls it as IC/BPS (Interstitial Cystitis/bladder pain syndrome) [1]. IC/PBS is a chronic inflammatory condition of the bladder characterized by symptoms of urinary frequency, urgency and pelvic pain [2]. In IC/PBS, unlike other bladder inflammations caused by infections, there is no bacterial infection and pathological agent in bladder and urinary track [3]. Women are mainly affected by IC/PBS, but men also suffer from it.

Definition

In the 1990, according to National Institute for Diabetes and Diseases of the Kidney (NIDDK) Criteria (this criteria mostly used in research studies, not used for clinic area) [4]; A diagnosis criterion for IC can be made when patients have bladder pain or urinary urgency and glomerulations or Hunner's ulcer during cystoscopy/hydrodistension and except criterions listed in a Table 1.

Table 1: Except diagnostic criterions for Interstitial Cystitis

Awake cystometric capacity >350 mL using a fill rate of 30-100 mL/minute
Absence of intense urge to void at 100 mL gas or 150 mL liquid
Involuntary detrusor contractions on cystometry
Urinary frequency less than 8 voids per day
Absence of nocturia
Duration of symptoms less than 9 months
Cystitis (bacterial, chemical, post-irradiation), prostatitis, vulvitis (herpes) or vaginitis
Bladder, uterine, cervical, vaginal or urethral cancer
Bladder or lower urethral calculi
Age less than 18 years
Urethral diverticulum

The definition of IC has been widely varied over the past few decades. Before 2002, IC was defined as *"An unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptoms of more than six weeks duration, in the absence of infection or other identifiable causes"* according to the criteria of the National Institute for Diabetes and Diseases of the Kidney (NIDDK), it is the so-called "NIDDK criteria"[4-6]. This definition was too restrictive for general use. Therefore, in the 2002, IC/PBS was

defined, as *"Painful bladder syndrome is the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and nighttime frequency, in the absence of proven urinary infection or other obvious pathology"*. In 2006, the European Society for the Study of IC/BPS (ESSIC) defined another system that also became reliable [7]. The presence of pain related to urinary bladder and at least one other urinary symptom is used for the diagnosis of Bladder Pain Syndrome (BPS), distinct from PBS. Other diseases that have similar symptoms need to be excluded by cystoscopy with hydrodistension and biopsy (if needed). It seems that there would be further definition during the coming years.

Prevalence

There are many variable diagnostic criteria for IC/PBS. Therefore, there have been shown various prevalence rates in different studies. In the United States, it is estimated that there are 1.2 million women and 82,000 men suffering from IC/PBS and a total cost of IC/PBS patients in the US was nearly $65.9 million in 2000 [8, 9]. Between 1994 and 2000, annual national expenditures for interstitial cystitis are about $37 million. However, annual costs for painful bladder syndrome significantly enlarged from $481 million to $750 million[8]. Some other epidemiological studies showed; there are reported incidence rates of 10 to 865 cases per 100.000 in term of population-based studies [10, 11]; a survey from the United States Nurses' Health Studies reported a prevalence of 52 to 67 cases per 100,000 women[12].

A diagnostic survey from out of the United State of America done by urologists showed that 2.8 percent of patients examined during in first two week period were diagnosed with IC (7.9 % female/0.4% male≈8: 1) [13]. Another group reported a prevalence of 197 cases per 100,000 women and 41 per 100,000 men in a primary care population[14]. However, the same population has much higher

prevalence of IC/PBS symptoms such as 11 percent of women and 5 percent of men[15].

Some investigators say that if women have chronic pelvic pain, they may actually have high incidence of IC as well as men who have lower urinary tract infection or prostatitis may mostly have IC [16-18].

Epidemiology

IC/PBS was mostly diagnosed in women according to many studies [4], the ratio of disease in female and male population is reported as 4.5 to 9 females to one male [10, 13, 19]. Most patients are probably diagnosed about age of 42 to 45 years. Sometimes physician diagnosed them even in childhood terms [11, 20, 21]. Monozygotic twin is more prone to IC than dizygotic twin pairs. That shows there can be a genetic susceptibility to IC[22].

In a population-based study, the women who have history of lower urinary tract infection, depression, some cardiac and thyroid related medication usage, and hysterectomy are more prone to have IC/PBS [23]. Men who have depression are more prone to IC/PBS[23].

Etiology and Pathogenesis

The etiology and pathogenesis of IC/PBS is still obscure, although several theories have been described. One of the most popular hypotheses was developed due to the urothelium damage caused by inflammatory or autoimmune conditions; therefore the bladder is permeable to anything in the urine [24, 25]. An alternative theory suggests that initial urinary infections start and causes a series of reactions in the bladder such as nerve stimulation. This stimulation triggers an inflammatory response, or neurogenic inflammation. After that, release of substances such as MCP-

1 may cause the secretion of mast cell mediators, histamine and leukotrienes resulting in inflammation and tissue damage [25, 26].

In addition, the other etiologic theories are respectively activation of bladder mast cells, neuropathic changes, neurogenic inflammation, some toxic substances in the urine flow, and unknown infection [27].

On the other hand, many studies showed that IC patients have urothelial abnormalities in bladder biopsies. However, It is unknown those abnormalities is primary phenomena or secondary phenomena. Those abnormalities can be: different expression level of HLA class I and II antigens in urothelium, changes of glycosaminoglycan (GAG) layer, the transition of urothelium cell to squamous cell, and low level of uroplakin expression and chondroitin sulfate [28-33]. In IC patients, NFkB gene activation is increased, and interleukin-6 and P2X3 ATP receptor are more expressed compared the control group. Some patients have abnormality in Tamm-Horsfall Protein (THP) [34].

The glycosaminoglycan layer that protects the surface from irritants in urine normally covers the urothelial surface of bladder. When GAG layer is damaged, the irritants penetrate into urothelium and affect inside nerve and muscle tissues [35]. Therefore, further tissue damage, pain, and hypersensitivity are inevitable. In addition to that, mast cell also propagates those damages [36, 37].

One study reported that Antiproliferative factor (APF) may also have a pathogenetic role in the generation of IC symptoms. The urothelium of IC patients express APF as known a lipoglycopeptide [38, 39]. APF can play a role in urothelial activity in terms of growth and function [40].

Neurologic changes in bladder accompanied to central sensitization and increased activation of bladder sensory neurons have an effect on the generation and maintenance of IC symptoms during the normal periods [41, 42]. This increased sensitivity may be due to bladder abnormalities or central nervous issues. New pathways can be involved or there can be increased activity of the central nervous system. Some animal models showed other diseases (placed in pelvic area) that cause hypersensitivity may have a role in IC patients [43, 44]. Those findings guide us bladder afferents can overlap with other afferents comes from somatic parts [45, 46].

Recent studies have shown that there are autoantibodies, which recognize urothelium cells as targets in the urea and serum of IC patients [47, 48]. In addition, according to the many studies, activated mast cells (MCs) have an important role in the formation of IC/PBS [49-51]. Detrusor MC density, sub-mucosal MC density, and the MC found in the mucosa were increased during IC/PBS in bladder [52-54]. In most cases of IC/PBS, it was observed that activated MC numbers increase as much as 10 fold in bladder[49]. In our recent study, our colleagues showed autoimmunity against the bladder epithelium in a mice study. Our laboratory immunized mice with peptide derived from bladder specific protein-Uroplakin 3A (Uroplakin 3A 65-84 peptide). After this immunization, the created this outstanding model characterized in term of both immunologic and urodynamic phenotype. These mice have increased levels of antibodies against this peptide. In this model, it has been shown that there is a CD4+ T cell mediated Th1 response. This mouse model mainly mimics the human IC/PBS disease and significantly increased the pelvic pain response on suprapubic area were demonstrated [55].

Clinical Findings

The clinical presentation of IC/PBS is common and variable [56, 57]. Pain is associated with all patients with PBS/IC. The pain is related to bladder filling and/or emptying, and usually accompanied by urinary frequency, urgency, and nocturia. The pain is normally placed suprapubic area originated from bladder, but sometimes it patters as unilateral lower abdominal area or low back area [58-61]. The severity of pain was changing due to bladder filling situation from mild to severe pain. Most patients fell well when the bladder is voiding. This clinical features gives physicians clue to think IC/PBS disease [62]. Some patients could have other kind of diseases at the same time that has also pain symptom such as irritable bowel syndromes, uterine related diseases, vulval problems and fibromyalgia [63, 64].

The exacerbation of IC/PBS changes term to term and can be affected by diets (coffee, alcohols, and some fruits) or the present condition that patients are in such as exercises [1, 65, 66].

The miserable symptoms such as pain and frequent voiding make patients very uncomfortable during working, and sexual relation. Having voiding many times in a day affects on the comfort of life style that ruins the life. Sometimes, patients can stay on the toilet for hours. In surveys, 50 percent of patients are unable to work full-time, 75 percent of patients have dyspareunia, 70 percent of them have reported sleep disturbance [67, 68]. One of the most important problems in IC/PBS patients are sexual dysfunction such as lack of interest (31%), arousal difficulties (19%), inability to achieve orgasm (25%), performance anxiety (12%), pain (15%), and lack of pleasure (23%) [68-70].

Diagnostic Evaluation

The guideline was published about IC/PBS by the American Urologic Association (AUA) [71] and European Association of Urology (EUA) [72]. The diagnosis of IC/PBS based on the presence of characteristic symptoms in both genders. Other disease such as urogenital cancers, urinary tract stones and infection, and irritable bowel syndrome should be excluded carefully by desired applications. Those applications;

1- **Physical examination** [71]: It is very vital method to diagnose IC/PBS or to exclude other problems, which is not IC/PBS. Even with small observation of patients would give physician clues to diagnose. Physician can evaluate bladder and any other around organs through abdominal wall to look at their present conditions. In case of bladders problems, the physician can feel the tenderness of pelvic organs due to sensitization of afferent nerve fibers in the dermomyotomes to which the bladder refers. In addition, vaginal speculum and bimanual examination can be used to examine the region, but sometimes it can be difficult to use this method due to tenderness. Ultrasonography is other a useful technique to evaluate the area. Sometimes physical examination cannot be enough to diagnose due to allodynia that can be source from other organs. In this case, empiric treatment would be applied. If the symptom were still continuing even with empiric medication, the diagnosis would be reevaluated.

2- **Laboratory tests:** Urine is the most essential sample to be used in laboratory procedures such as checking infection and hematuria by microscopy, culture, and some basic techniques. Urine cytology and cystoscopy are also need to be performed in some patients. A post-void residual urine volume can be measured manually. In mice studies, the laboratory tests that have been used for IC/PBS

evaluation are ELISA, western blot, RT-PCR, Immuno-staining methods, Frequency voiding chart (FVC) and so on[73].

3- Hydrodistension: Bladder is filled with water until 70 cm pressure is reached. During the procedure, bladder is examined in term of characteristic findings of IC such as Hunner's ulser and glomerulations. However, it is not e requirement technique for diagnosis of IC/PBS[74, 75].

4- Bladder biopsy: Urologists can use bladder biopsy to distinguish other disorders from IC/PBS. It is not required for a diagnosis of PBS/IC. Bladder biopsy has been done to 204 patients with clinical diagnosis of IC [76]. It has been shown that only 50 percent of patients have an increase in mast cell in the bladder lamina propria, 11 percent of then has damage of urothelium (ie, an ulcer), 14 percent has granulation tissue in lamina propria, and vascular density and submucosal hemorrhage occur in 67 percent [77].

5- Potassium sensitivity test: Some researcher used the potassium sensitivity test (PST) for diagnosis of PBS/IC[78], but is not suggested for routine use for PBS/IC [79]. During this test, he bladder is filled with potassium chloride and then occurring pain would be an indicative bladder hypersensitivity and suggestive of PBS/IC.

6- Biomarkers: There have been markers being proposed for diagnosis of IC/PBS. One of them was anti proliferative factor (APF) that is a promising marker. One study showed that the sensitivity and specificity of APF is very high in IC patients versus control groups. However, this marker need to be further investigated before recommendation for being a diagnostic marker[38].

Treatment of Painful Bladder Syndrome/Interstitial Cystitis

There is still no any promising treatment for IC/PBS because of many reasons such as unknown clear etiology, pathophysiology, and most importantly unqualified designed clinical trials for treatment [56, 80]. There are many treatment diversities of IC patients due to lack of understanding the treatment of the syndrome. In those trials, physicians treated 581 IC patients with 183 different type of therapy. That is why, treatment of IC is still a big problem in clinicians [81]. Most patients get empiric therapy rather than actual therapy [27].

According to the American Urologic Association, the ideal treatment approach to IC/PBS patients from at the beginning of disorder should be the least invasive and toxic treatments and later more invasive unless there is no effective response [71]. Therapies are explained below;

A- Nonspecific Therapies

There are many nonspecific ways have been used for IC/PBS patients such as psychosocial therapy, referring pain management specialties, avoiding activities associated with flares, and behavioral therapy.

1- Psychosocial support

It is very common method that has been using in chronic pain disorders [82, 83]. There are two groups such as the Interstitial Cystitis Society and the Interstitial Cystitis Network to help patients to overcome their uncomfortable situation. Those types of centers can refer to a psychologist for counseling of the disease. Depression related chronic pelvic pain should be referred to a professional health specialty.

2- Referral to pain management specialists

In any case of unresponsive of pain should be evaluated by pain management specialty. Referral to specialists in pain management should be considered if the full range of pain management options is not available within the practice.

3- To treat flares that aroused from activities of IC/PBS patients

Some human activities can cause trouble situation such as sexual activities, recreational activities when the person has IC/PBS disorder [68, 84-86]. In addition, some food mentioned previously may cause uncomfortable feeling. To avoid from those activities and a certain foods consuming can help patients to live more comfortable and relief [27, 87-89].

4- Behavioral therapy

Sometimes, educating the patients help many diseases in term of therapy. For example, IC/PBS patients can have diminished functional bladder capacity. Those patients can be educated to be expanded function of bladder [90, 91]. For instance, the voiding interval first week can be 30 min, second week 1 hour, and it can keep being longer and longer. Only one study has shown that the timed voiding in IC patients reported 15 of 21 patients experienced a 50 percent decrease in their IC symptoms [92].

B- Specific Therapies

1-Pentosan polysulfate sodium (PPS)

PPS is the only oral medication approved by the United States Food and Drug Administration (FDA) for treatment of IC [93]. PPS is a kind of protein that may be rebuilt the damaged of glycosaminoglycan [94]. PPS is more effective than placebo for treatment of IC that shown in one study [95].

2-Intravesical heparin and lidocaine

Some investigators showed those therapies have improvement effect on IC/PBS [96-98]. For example, one of them is showed that 65 % percent of 23 IC patients were improved on the Patient Objective Rating of Improvement of Symptom scale [98].

3-Intravesical-dimethyl sulfoxide (DMSO)

FDA also approved DMSO for use in IC in 1997 [93, 99]. It may have some nonspecific effects such as antiinflammatory, analgesic, smooth muscle relaxing, and mast cell inhibiting effects [100, 101].

4-Intravesical-botulinum toxin

FDA is not approved botulinum toxin for the treatment of IC. This therapy is based on the ability of botulinum toxin to modulate sensory neurotransmission. Some researchers showed the benefit of botulinuim toxin on IC/PBS [102, 103].

C- Neuromodulating therapies

IC/PBS is a kind of disorder that has more neurological involvements. That is why, there are been many treatments have been proposed for use in IC/PBS treatment that is related with Neuronal issues. These include:

1- Amitriptyline

This tricyclic antidepressant is used in many problems such as post-herpetic neuralgia, diabetic peripheral neuropathy, and chronic low back and pelvic pain [104-109]. Dr. Hanno et al. used amitriptyline using for IC/PBS resulted in a significant decrease in daytime frequency and pain [110]. However there are many side effects of amitriptyline include anticholinergic effects, sedation, weight gain, orthostatic hypotension, and conduction abnormalities [111].

2- Gabapentin

Gabapentin is an antiepileptic agent that can be used treatment of painful disorders [112]. Some studies showed that a group of patients that have urogenital pain get benefit from the treatment of gabapentin [112, 113]. There is also an animal study that showed the effectiveness of gapapentin on pain related from pelvic area [114].

D- Electrical stimulation therapy

This FDA approved method used in some patients for the treatment of urinary and urgency. The results were effective; however, FDA approval is not specifically for treatment of PBS/IC. This device placed on S3 sacral nerve root and attached to the special designed power [115]. Some studies proposed that this technique improves

the dysfunctional voiding and pelvic pain in patients with interstitial cystitis [116-118].

E- Physical therapy

Some patients get benefits from physical therapy such as myofascial physical therapy, manual physical therapy, and pelvic floor massage [119-121].

F- Mast cell related therapies

Mast cell has a critical role in the development and maintenance of IC/PBS symptoms in both human and in animal models [122, 123]. Some direct and indirect mast cell function related drugs were used for treatment of IC such as H2 receptor blocker, and montelukast. The treatment with cimetidine, H2-receptor blocker, has benefits on IC, but it is not commonly used [124-126]. Montelukast treatment showed an improvement of frequency and pain of interstitial cystitis [127, 128].

G- Immunomodulatory treatments

Some immunomodulators has an effect on the treatment of IC/PBS patients such as cyclosporine A, bacillus Calmette-Guerin (BCG) [129-133].

H- Surgery

Sometimes, urologists cannot find a solution to the pain originated from pelvic area. At this point, the surgical procedure needs to be applied in same case [134].

It seems that the therapy for IC has been in wide range due to many reasons such as unknown pathopyhsiology, not easy diagnosable disease, very common symptoms with other disease, not so much research has been done so far.

MAST CELLS

Development, identification, and physiologic roles

Dr. Ehrlich et all firstly described the mast cells “Mastzellen” in 1879 when he was in medical school [135]. Mast cells are placed in almost all tissues of body. Most of them are positioned in main entrance of body such as urogenital system, gastrointestinal system, and respiratory system and in dermal and articulation tissues [136-142]. They are also seen in heart and vertebras [143-145].

It is been long time Mast cells have been acknowledged for the involvement in many diseases that involve asthma, rhinitis, atopic dermatitis, anaphylaxis, urticaria, and conjunctivitis. They also play a role in regeneration and protection of body [145-147].

Anatomic Locations of Mast Cell

In normal tissues, Mast cells mostly place in connective tissues of organs and vascularized organs. Mast cells play an important role in communication of external and internal environment in terms of foreign organisms and antigens recognition [148-150]. The places Mast cells reside are the dermis, intestinal mucosa and submucosa, conjunctiva, and respiratual mucosa [136, 151-154]. One study showed that Mast cells accumulate in dermal tissue within communication of blood vessels, nerves, and lymphatics. There has been shown huge number of accumulation of Mast cells per mm^3 of skin [154, 155]. Mast cells may be having effect on vascular structure, blood supply with their released mediators within those locations [156]. Mast cell also found in central nervous system [157].

Amount of mast cells are increased in a plenty of inflammatory diseases such as rheumatoid arthritis, psoriasis, asthma, pulmonary fibrosis, inflammatory bowel

diseases, and cardiovascular diseases. However, their roles still not clear [141, 158-162]. Another famous disease, Mastocytosis is also kind of skin disease that has huge accumulation of Mast cells [163].

Collection and Culture of Mast cell

Mast cells are very hard to work in vitro [164]. MC are the multifunctional immune cell source from multi-potent hematopoietic progenitor in the bone marrow, which are produced as undifferentiated MC and they become mature and acquire their characteristics in the tissues they are sent [165-169]. Mast cells can be study in vitro condition by getting with different techniques. Those ways can be from bone marrow, solid tissues, human cord blood, and peripheral blood [170-174]. When they are used for in vitro experiments, those types of mast cell collection can have some problems such as different functionality, abnormality and natural visibility rather than natural tissue mast cell [175, 176]. For example, sometimes for mast cell culturing, co-culture of fibroblast and mast cell help to get similar mast cell condition to natural mast cell [176].It is also very hard to isolate from tissues [177]. Other problem is that murine mast cell can be different than human mast cell in terms of phenotype and distribution.

Because of the difficulties in collecting human mast cells, mast cells from mice are often utilized instead. However, the distribution and phenotype of murine mast cells are substantively different from that of human cells [164].

Development of Mast cell

Mast cells originate from pluripotential CD34+ stem cells that are inherent in the bone marrow and spleen [178-180]. Myeloid pathway is common in almost hematopoietic cells, and sharing similar mediators with monocyte/macrophages and

granulocytes. Mast cells leave the hematopoietic tissues as committed progenitors [178, 181, 182].

SCF (stem cell factor=kit ligand) and IL 4 have very important role in mast cell development and activation [183-185]. SCF that binds to the c-Kit receptor (CD117) works as an important growth factor in hematopoiesis, spermatogenesis, and melanogenesis [186]. The receptor tyrosine kinase c-kit, receptor for SCF, expressed by most hematopoetic cell continues along with myeloid differatation. It is used an early marker for mast cell precursor. Mast cells continue express this marker during its whole life rather than basophils [137, 181, 187].

Migration into tissues

This process can be depend upon the interaction of endothelial cells and mast cells initially, and then, many tissue sourced mediators such as adhesion molecules, chemokines, cytokines, integrins and immunoglobulin and receptors have been proposed play a role in mast cell regulation [188-191]. As an example, stromal cell-derived factor-1 (SDF-1) may be regulate mast cell migration and mediator production [192]. Other example, IL 3 dependent murine bone marrow-derived mast cells (BMMCs) may be more adhesive and proliferative in tissues when they come across with the matrix protein, and vitronectin [193].

It seems every organs can has their own mast cell progenitors based on limited studies [179, 194]. When they migrate the tissue, they can undergo differentiation by hosting tissue [195]. For instance, MCp expression of the integrin alpha4beta7 (a4b7) can help mast cell progenitors migrates into the tissue in an animal study. The integrin alpha4beta7 is doing this movement by binding to mucosal addressing cell adhesion molecule-1 (MadCAM-1) and vascular cell adhesion molecule-1 (VCAM-1) present

on vascular endothelium expressed in Peyer's patches and lamina propria. IL-8 receptor on both mast cell progenitors and the vascular endothelium causes specific migration of the cell into intestinal mucosa [196, 197].

In a mouse lung study, they showed that mast cell accumulation in submucosa is less during absence of inflammation. However, during allergic inflammation, mast cell progenitors significantly increased about >25 fold in the mouse lung tissue by interaction of a4b1 and a4b7 integrins on the MCps and VCAM-1 and CXCR2 on the endothelium [198, 199].

Chemoattractants

Mast cell may migrates to tissues under a variety of chemokine, cytokine, and growth factors such as SCF, MCP-1, and MIP-1 alpha and IL-3. The C-C chemokines, RANTES (**R**egulated on **A**ctivation **N**ormal **T**-cell **E**xpressed and **S**ecreted) and monocyte chemotactic protein (MCP-1) has a role in migration of human cultural mast cell and mouse bone marrow-derived mast cells on vitronectin and laminin [200-204].

Phenotypes of mature mast cell

Mast cell related sophisticated studies have been done on animal mouse models. There are described a few mature mast cell subtypes in terms of their location, granule contents, mediators production and behaviors [137, 205]. Two subtypes were described in the mouse: murine mucosal mast cells and murine connective tissue mast cells, the difference are their secretions and their environments changes [206-208]. Another study showed that mucosal mast cells secrete both prostanoides and cysteinyl leukotrienes, connective tissue mast cells mainly secrete prostanoids [209].

In the same way, mast cell types in human studies have been categorized based on their secretion from the granules of mast cell. One of them is the one that has more tryptase is called MC-T (mostly located in mucosa), other one is containing trptase, chymase, and carboxypeptidase A is called MC-TC (mostly located in submucosa and connective tissues) [209, 210].

Thus, the MC-T and MC-TC are comparable to mMMC and mCTMC, although due to the plasticity of this cell and differences in the proteases between the two species, this is an oversimplification and caution is advised in drawing too strong an analogy between the murine and human systems.

1-Mucosal mast cells

They are mostly placed in mucosa of intestinal and respiratory epithelial cells, which involved in inflammation associated with Th2 responses. Allergic diseases specifically related with respiratory system and intestinal inflammations increases the numbers of mucosal mast cells [211-213]. The differentiation of mast cell depends on micro-environmental changes of tissues that mast cell located[206].

2-Connective tissue mast cells

Connective tissue mast cells are located in submucosal areas that have a variety of connective tissues[214]. Those type of cell is dependent to Th1 cell response [214].

Another study classification for MCs is shown. There are two type of MC is described according to their granules and degranulation of them in cytoplasm. One of them is called activated MCs show degranulation and release of toluidine blue–positive remnants. Other one is resting MCs show intense granule store in cytoplasm.

There is more intracellular and intravesicular accumulation of toluidine blue in resting MCs [215].

As mentioned previously, mast cell significantly increases in some diseases such as asthma, parasitic infections, rheumatoid arthritis, scleroderma, mastocytosis, atopic rhinitis, atopic eczema, dermatitis, psoriasis, acute allergic reactions, urticarial diseases, conjunctivitis, neoplastic diseases chronic renal and lever diseases, and intestinal inflammatory diseases [216-218].

Cellular Identification of Mast cell

Mast cells are mostly round mononuclear cells, which have many types of cytoplasmic morphology. There is two type of mast cell in terms of secretion condition such as activated mast cell that has no or a few granules in the cytoplasm and resting mast cell that has a lot of granules in cytoplasm while staining with toluidine blue (Figure 5). Cell diameter ranges from 8 micron to 20 [219, 220].

Staining properties of Mast cell

The mast cell has various metachromatic cytoplasmic granules that almost occupy 50-55% of the cytoplasm [221, 222]. The surface of mast cell is scanned by electron microscopy that show mast cell has numerous short and long microvilli and bulges on its surface, which is has connection with inside granules [222]. The diameter of those granules is about 0.5-0.7 μm [222]. Mast cell is stained to red-purple with the basic dye toluidine blue. The color is due to granule contents that has highly sulfated, anionic proteoglycans [223].

Some investigators worked with electron microscopy showed that there are two different structure of mast cell described. One of them is the scroll-like structure related with mucosal mast cell, which represents tryptase phenotype. Other one is a

lattice-like granting pattern related with submucosal mast cell, which represents tryptase/chymase phenotype [224-226].

Physiologic Roles of Mast cell

Mast cells have roles in both physiologic and pathogenic processes. Mast cells play very important role in defense mechanism against bacteria, parasites, and viruses [227-234]. Mast cell is important in both innate and adoptive immunity [228, 233, 235]. Specifically, mast cells recognize pathogenic organism from their surface receptor, after that MCs call and induce leukocytes and other cell that involve in defense mechanism. In this process, mast cell is a key cell against pathogens and tissue repair [228, 230].

Mast cell plays very important essential role in innate immunity [228]. Mast cells are defending host body against pathogens by releasing some products such as chemokine, cytokines, lipopolysaccharide, and peptidoglycan [228]. In addition to that, toll-like receptor (TLR) on mast cell is also significant in terms of activation of mast cell [233, 236, 237]. Mast cell can work as a phagocytosis cell that involve in Salmonella typhi and Escherichia coli [238]. Mast cell can produce cathelicidins that causes bacterial cell (such as streptococci), and tumor cell lysis [239, 240]. A mast cell-deficient mice are more susceptible to toxin such as snake and honeybee venoms [241].

Mast cell-derived cytokines are very important in terms of hematopoetic cell recruitment into infection area [242, 243]. Specifically, mast cell derived tumor necrosis factor (TNF) plays a role in recruitment of dendritic cells [243].

Mast cell in Acquired immunity

Mast cells play very important role against parasitic infections, even though its mechanisms is not fully understood [244-246]. IgE is an important antibody against intestinal parasitic infestation [244]. Mast cell plays roles in both Th1 and Th2 response [214, 247].

Mast cells have been implicated in various physiologic and regenerative processes including; some intestinal, respiratory functions that have protective effect against invaders pathogens. They also consider response reactions against allergic diseases [230, 248]. Mast cell is effective in wound healing and scar formation by stimulating fibroblast proliferation and collagen synthesis that also limits infections [249, 250].

Mast cells in allergy causes two step reaction that categorized early phase response and delayed phase response. Early phase response, which happens within 10 minutes, is due to histamine release from mast cell that causes skin lesions such as edema, itchiness symptoms that represent in allergic disease [142, 251]. After early phase, late phase response will start in some patient that is characterized with cutaneous erythema, warmth, and induration. This phase takes longer than early phase about 6 – 8 hours, which is more related with other cell accumulation such as mononuclear cells, eosinophils, and neutrophils [251, 252].

Mast cell derived mediators

As I mentioned previously, mast cells release a variety of mediators that can be divided into three overlapping categories such as preformed mediators, newly synthesized lipid mediators, and cytokines and chemokines.

A- Preformed Mediators

Those mediators are histamine, neutral proteases, proteoglycans, and some cytokines, such as TNF-alpha (TNF-α), which is released into environment upon cell stimulation. Those mediators are secreted rapidly and mediate same allergic reactions such as edema, bronchoconstriction, and increased vascular permeability.

1-Histamine is one of the most important neurotransmitter that is secreted from mast cell [253-256]. The mean level of histamine in human cutaneous mast cells are measured as showed 1.9±0.8 µg 10^6 cells [256]. Histamine involves in both local immune response and physiological function in organs [253, 257]. It also plays a role in pain IC/PBS patients. High levels of histamine metabolites are detected in IC patients [77, 258, 259]. Histamine shows it effect through several receptors; H1, H2, H3, and H4 located on target cells [260, 261].

H1 receptor distributes in organs that involve smooth muscle, endothelial cells, adrenal medulla, heart, CNS [260]. H1 receptor stimulation cause many type of reactions such as systemic vasodilatation, bronchial and intestinal smooth muscle contraction, modulate circadian cycle, flushing, itching hematopoietic cell chemotaxis, and pain regulations [230, 262, 263]. As an example, H1 receptor knockout mice showed less pain perception versus control group [263] and reduce lung inflammation by decreasing t cell migration [262].

H2 receptor distributes in organs that involve Gastric parietal cells, vascular smooth muscle, suppressor T cells, neutrophils, CNS, heart, uterus. The effects that occurred by inducing H2 receptor are gastric acid secretion, smooth muscle relaxation, increased vascular permeability, airway mucus production,

hyperpolarization or facilitation of signal transduction in CNS, block of Ca^{2+}-dependent potassium conductance, and inhibition of lymphocyte function [260, 264].

H3 receptor is located on CNS, peripheral nerves, endothelium, and enterochromaffin cells. The functions induced by H3 receptor stimulation are gastric acid inhibition in dog, inhibition of neurotransmitter release, relaxation of middle cerebral artery of rabbit, increase current of Ca dependent channel in smooth muscle, and inhibition of neuronal stimulation [260, 265].

H4 receptor is highly expressed in bone marrow, CNS and white blood cells and regulates neutrophil release from bone marrow [266, 267]. Histamine H4 receptor has a role in chemotaxis and calcium mobilization of mast cell [261]. H4 -/- mice showed less infiltration of eosinophils and lymphocytes [268].

In general, Histamine increased mast cell density and has effect on mast cell degranulation, also induce neuronal density and depolarize neuronal cells [269-272]. Histamine also mediates pelvic pain [273]. Histamine receptors are expressed on neuron and mast cell [274-276].

2- Serotonin release was showed in some term life of human being such as during fetal development and in some adult life condition [277, 278].

3- Proteoglycans are heparin and chondroitin sulfate that cause mast cell stained as metachromatic. These are composed of a peptide core, serglycin, complexed to glycosaminoglycans[279, 280].

4-Tryptases and other proteases such as chymases, cathepsin G, renin, and carboxypeptidase A are important other products released by mast cell [281, 282].

Tryptases are the one which produces much more than other proteases [283, 284]. Some of them are related with pain. As an example, tryptase causes hyperexcitability of guinea-pig submucosal neurons [285]. Chymase can indirectly induce pain by recruit neutrophils, eosinophils and other inflammatory cells [286].

B- Newly Synthesized Lipid Mediators and NGF

Mast cells can synthesis eicosanoids derived from arachidonic acid such as prostaglandins (PG), leukotrienes (LT), and growth factor as NGF and platelet-activating factor (PAF) [287-289]. Those mediators releases 15 minutes to one hour after the cell activation. They are mostly involved in allergic symptoms [290, 291].

1-Prostaglandins

Mast cells mainly produce prostaglandin D2 (PGD2) that involve in respiratory problems such as asthma [292, 293]. As an example, PGD2 receptor deficient mice decreases airway reactivity in asthmatic mouse model [294].

2- Cysteinyl leukotrienes

Activated human mast cells can release the cysteinyl leukotrienes, LTC4, and lesser amounts of LTB4 [295]. The cysteinyl leukotrienes play a role in microvascular permeability and are also responsible for wheal and flare responses in diseases [295]. Most of them play a role in allergic diseases such as asthma, IC/PBS, irritable bowel disease and rhinitis [296-298].

3- Platelet activating factor

Mouse and human mast cell can produce platelet activating factor (PAF) [299, 300]. PAF has a chemotactic role in some hematopoetic cells [301, 302]. PAF one of the important factor involve in pain such as induced pain hypersensitivity and role in pain signaling [303].

4-NGF

There are many study showed that NGF significantly involve in IC /PBS pain and other pain related disease. NGF is very important for sympathetic and sensory neurons' maintenance and survival [304]. NGF increases in IC, it stimulates mast cell. And then mast cells secrete cytokines that play role in neuronal sensitization and pelvic pain. NGF is also directly secreted by Mast cell [305]. Dr. Lewin et al showed NGF has important role effect on A- delta and C fibers in term of pain [306]. NGF is also a trigger on both neural excitability and neural density in peripheral tissues [307]. NGF play a role in VEGF production that induce angiogenesis, and then neuronal tissues density [307].

C- Cytokines and Chemokines

Mast cell can produce many cytokines and chemokines that involve in other inflammatory cell migration [289, 308]. Those cytokines are IL-4, IL-5, TGF-beta (transforming growth factor), and TNF-alpha (Tumor necrosis factor-alpha) [161, 308-311]. Mast cell secreted chemokines mostly belong to the CC chemokines (macrophage chemotactic protein-1 =MCP-1 or CCL2) and the CXC chemokines (IL-8) families [308, 312-314]. IL-1b, IL-3, and platelet derived growth factor (PDGF) can be produced by mast cell [315, 316].

Mast cell play pivotal role in IC/PBS and pain related hyperalgesia and neurogenic inflammatory diseases by their products [317-320]. As an example, MC can produce adenosine phosphates, bradykinin, histamine, leukotrienes, potassium, lymphokines, tumor necrosis factor (TNF), and prostaglandins in IC, those products can cause many symptoms observed in IC. Most common one is pain [321]. Other example, Histamine, PGD2 and proteases are important mediator for pain [146]. It seems that MCs has a crucial role in pain, which highlights need for further investigation.

Chronic pelvic pain

Pain is a feeling of unpleasant sensation resulting of many types of diseases. In 1994, Dr. Merskey and Bogduk classified pain according of the International Association for Study of pain (ISAP) into different groups such as based on: intensity and time (mild, moderate, and severe; acute and chronic pain), body part involved (abdominal pain, back pain, pelvic pain, headache), etiology (cancer and non-cancerous), and systems whose dysfunction may be causing pain (gastrointestinal, nervous) [322, 323]. Chronic pelvic pain has long duration and is restricted to the pelvic area. Mostly genital-tract based problems such as bladder and uterus causes chronic pelvic pain. One of the most prominent cause of chronic pelvic pain is IC/PBS [324].

Mast cells (MCs) and the Role of MCs in IC/PBS

MCs are the multifunctional immune cell source from multi-potent hematopoietic progenitor in the bone marrow, which are produced as undifferentiated MC and they become mature and acquire their characteristics in the tissues they are sent [165-169]. MCs produce many potent inflammatory mediators such as histamine, tryptase, cytokines, and chemokines (especially MCP-1) [325, 326]. MCs

express high affinity immunoglobulin E receptors (FceR1) [327]. It has found that these secreted products from MC directly or indirectly have effects on IC/PBS [27, 328-330]. Mast cell amount increased 10 fold in the lamina propria of IC bladder biopsies [49, 77, 258]. It is still not clear why MC migrates to the bladder and proliferates in the bladders of IC/PBS patients. One possible explanation is that damaged urothelium cells secrete cytokines and growth factors that cause migration of the MCs to the bladder and their activation [49, 325, 331-334]. MC plays roles in allergic reactions, innate immunity and autoimmunity [123, 165, 166].

Mast cell and Chronic Pelvic Pain of IC/PBS

There is a communication between MCs and neurons through mediators. MCs can induce neuronal activity by releases of histamine [335, 336]. Mast cells mostly accumulate at the end of nerve endings in many different tissues, including the epithelium [335, 337, 338]. Secretions of the activated MCs cause neural sensitization and more secretion of mast cell activators and it is believed that this vicious cycle may contribute the pain in IC/PBS [33, 38]. MCs play fundamental role in numerous pain related diseases such as IC/PBS, IBS, postoperative and neuropathic pain [114, 123, 339, 340]. Other study showed mast cell along with pain is most important sign of IC/PBS [341, 342]. Those results show that MC have a central role in the IC/PBS pathogenesis via stimulation of neuronal systems or other unknown mechanism [336].

Histamine and the Role of Histamine in IC/PBS

Histamine is an organic nitrogen compound (Figure 1), a neurotransmitter [253, 343] and is produced by MCs and neurons [271, 344]. Neurons and MCs have histamine receptors on their surfaces [274, 276]. Histamine released from MCs play an important role in local immune responses and physiological functions in tissues [253]. MCs release histamine which induces degranulation of itself [270]. Histamine

induces neuronal hypertrophy and mast cell density [269, 271]. Histamine depolarizes neurons [272] and mediates pelvic pain [273]. Some investigators have shown that IC/PBS patients have increased levels of urinary histamine metabolites [77, 258, 259].

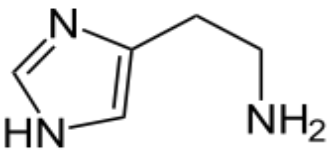

Figure 1: The chemical structure of Histamine.

Histamine receptors

Histamine receptors were described in previous paragraphs. Previously, four types of histamine receptors (histamine receptor type 1 to type 4(H1- H4) have been described [345].

Histamine H1 receptors are dispersed in a broad variety of organs, e.g. central nervous system [345], smooth muscle, gastrointestinal tract, cardiovascular system, endothelial cells and lymphocytes [346]. Histamine H2 receptors are found in a variety of tissues including brain, gastric parietal cells and cardiac tissues [260].

The role of MCP-1 and its receptor CCR2 on MCs and Pain

Monocyte chemoattractant protein MCP-1 (CCL2) is a member of the C-C chemokine family and may has chemotactic effect on monocyte and MC [347]. MCP-1 also itself may plays a role in degranulation of mast cell [348]. MCP-1 suggests as a biomarker for the CPPS and IC/PBS [349, 350]. MCP-1 and its -chemokine-receptor-2 (CCR2) have been shown that they can cause neuronal hyperexcitability after chronic nerve compression injury [351]. MCP-1 and CCR2 play a role in microglial activation and neuropathic pain. Administration of MCP-1 to rat causes tactile allodynia. In the contrast, MCP-1 neutralizing antibody reduced neuropathic pain

[352]. IC patients exhibited increased levels of MCP-1 in urine, and bladder tissue and the expression of MCP-1 is correlated with IC [350]. MCP-1 can also be a good therapeutic biomarker for IC/PBS and CP/CPPS [353].

IC/PBS and CP/CPPS meaningfully overlap with the symptoms of pelvic pain and other common conditions therefore; MCP-1 can be a therapeutic biomarker for CP/CPPS and IC/PBS [354].

Referred Visceral Pain

Referred visceral pain is the kind of unpleasant feeling, when internal organs are damaged or injured [355]. It is one of the most common symptoms caused by diseases of internal organs; this symptom is one of the most common reasons that brings patient to the clinic [324, 356]. Referred visceral pain sources from internal organs (bladder, colon, ovary, uterus liver and so on), but it is perceived from at different skin locations other than stimulus region [357]. There is a very famous theory for the mechanism of referred visceral pain; this pain is due to a convergence of somatic and visceral fiber at the level of spinal cord [355]. There are many studies showing that referred visceral pain can be measured by von Frey filaments [114, 358-360], one of the most popular non-invasive techniques used in urology for prototyping. This Filament was calibrated at different forces from 0.008g to 300g that assess pain. Pain measurement location is the place that is signed with red circles (Figure 2).

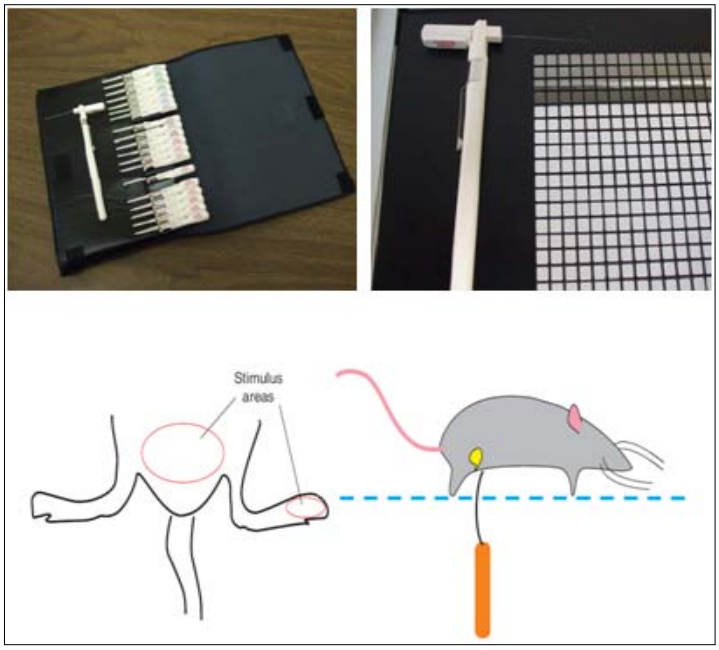

Figure 2: Von Frey Filaments and measurement locations.

Both pictures at top are a von Frey filament forces that use for pain assessment. The red circle at the bottom left panel shows where we poke for referred visceral hyperalgesia measurement, and the bottom right panel represents what kind of place we used for the measurement. It has a chamber with a mesh floor (right top) [273, 361].

Animal Models

We have been lack of animal models of IC/PBS a long time to discover the pathophysiology of the disease. That is why, many studies has recently been proposed to get an animal model. Those are respectively described below.

In 1992, an animal model was reported by induction of bladder inflammation with syngeneic bladder antigen [362]. BALB/cJ mice were used for that. It was a bladder specific and cell-mediated autoimmune response model [362].

A transgenic model was designated as URO-OVA and URO-OVA/OT-I mice [363]. In this model, the bladder epithelium actively produces self-Ag (OVA Ag) to the immune system. After that the antigen induces CD8 (+) T cell tolerance, activation, and autoimmune response [363].

Dr. Christensen et al. were created an animal model in a guinea pig in which bladder was induced by a protein that had been previously used to the animal resulted in bladder inflammation [364].

Many researchers used feline interstitial cystitis model. This model is creating a spontaneous idiopathic cystitis in domestic cats [365-367]. The models demonstrates urinary urgency, frequency, and pain with sterile urine, bladder mastocytosis, increased histamine excretion, increased bladder permeability, decreased urinary GAG excretion [368]. Beside cats, some researcher also used African monkeys to create a model that mimics the symptoms of IC/PBS by using acetone intravesically [369]. Many rat models were also created such as rat bladder induce by hydrochloric acid and bladder induced by virus for neurogenic cystitis [370].

In 2011, Dr. Oottamasathien et al. used C57B/L6 mice to create a new model for bladder inflammation by using LL-37 peptide. LL-37 is a urinary defense peptide works as an antimicrobial [371].

Lastly, our laboratory produced a novel experimental autoimmune cystitis mouse model that represents all symptoms of human IC except pelvic pain by using recombinant uroplakin II protein injection [372]. Other model is created by same group is that this EAC group were immunized with mouse bladder homogenate in complete Freund's adjuvant (CFA) and a control group immunized with CFA alone [55, 373].

There are over than 15 interstitial cystitis models have been proposed so far [374]. Some of them are induced by intravesical irritants and immune substances to produce inflammation. Some examples of substances are those (e.g.,

cyclophosphamide, hydrochloric acid, protamine sulphate, mustard oil, turpentine, and lipopolysaccharide) used for induction of Cystitis [374, 375]. Moreover, some of them were produced genetically. However, most of them were not too specific to bladder as human IC/PBS itself. In addition, those models can not represent the human IC/PBS symptoms. Because of that, we still need to have very specific model for human IC/PBS in animal [374]. Although animal models can yield clues to etiology, all theories must ultimately be tested in humans with the disease.

Experimental autoimmune cystitis (EAC) mouse model

Our laboratory, in the Department of Urology at Case Western Reserve University, recently created an experimental autoimmune cystitis (EAC) mouse model (BALB/cJ) that mimics exactly human IC/PBS patients in term of both immunologic and urodynamic phenotype. Many animal models have been created by the same autoimmune mediated targeted techniques used such as autoimmune myocarditis, autoimmune oophoritis, and autoimmune encephalomyelitis as models of autoimmune diseases, and then they were used successfully for investigation of diseases [376, 377]. In this model, we showed a chronic pelvic pain phenotype at different times (early onset and persistence of pain). Moreover, we showed a MC specific marker (FceRI), and increased MCP-1 transcription level in this model. Those new findings give us a piece of mind to study the mechanism of chronic pelvic pain in this model by targeting the role of MCP-1, CCR2 and MCs.

The Significance

The mechanism of pelvic pain in IC/PBS is unknown. The determination of the mechanistic role of MCP-1, its receptor CCR2, and MC histamine, in chronic pelvic pain of experimental autoimmune cystitis (EAC) mouse model, could improve our understanding of the pathogenesis of IC/PBS for new therapeutic approaches. This study will give us better understanding of IC/PBS pathophysiology.

CHAPTER II

II.GENERAL MATERIALS AND METHODS

Mice and immunization

Female BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Adult female and male MCP-1-deficient (MCP-1$^{-/-}$) mice [378] and CCR2-deficient (CCR2$^{-/-}$) mice [379] on the BALB/c background were obtained from Dr. B. Rollins (Harvard Medical School, Boston, MA) [380] and Dr. N. Mukaida (Kanazawa University, Kanazawa, Ishikawa, Japan) [381]. Knockout mice were bred and all mice were maintained on a regular 12:12 hour light:dark cycle with ad libitum food and water in the Animal Resource Center of Case Western Reserve University. At 8-10 weeks of age, mice were injected subcutaneously in the upper abdominal flank with 200 µg of mouse uroplakin 3A 65-84 (UPK3A 65-84) peptide 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, MI), or with CFA and water alone. This maximum concentration of UPK3A 65-84 peptide was chosen based on our previous studies [382]. Mice were euthanized by

asphyxiation with carbon dioxide followed by cervical dislocation 10, 20, or 40 days after immunization. All protocols were preapproved by the Institutional Animal Care and Use Committee of Case Western Reserve University in conformity with the Public Health Service policy on humane care and use of laboratory animals.

Peptide

Uroplakin 3A 65-84 peptide was used to create EAC mouse model on BALB/cJ background mice in our laboratory [73, 383]. This peptide was purchased from Cleveland Clinic at Molecular Biotechnology/ Peptide Synthesis core facility of Lerner Research Institute. They used standard solid phase methodology and FMOC side chain-protected amino acids. Peptide was purified >97% by reverse phase HPLC and amino acid composition was confirmed by mass spectrometry. This peptide sequence were selected based on immunogenicity on BALB/cJ mice [73].

Pain assessment

Tactile sensitivities of the suprapubic and hindpaw regions of mice, with the former considered a surrogate for pelvic visceral pain [384], were measured using a series of 14 von Frey filaments with increasing calibrated forces from 0.008g to 10.0g (Stoelting Co., Wood Dale, IL). These filaments provide an approximately logarithmic series of forces and a linear scale of perceived intensity. Beginning with the smallest filament, each filament was applied a total of 10 times for 3 seconds, with intervals of 8 seconds between each stimulus. The behaviors that were considered to be a positive response were: 1) sharp retraction of the abdomen or hindpaw, 2) instant licking and/or scratching of the stimulated area, or 3) jumping. The results are expressed as the percentage of positive responses out of 10 trials (response frequency) for each monofilament. In addition, the von Frey force defined as that which would elicit a response 50% of the time (50% threshold) was calculated for each mouse from

the regression line drawn through the linear portion of a plot of response frequency vs. log of the von Frey force, using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Lidocaine instillation

On the 40th day after immunization, while the mice were under isoflurane-anesthesia, the local anesthetic lidocaine (Sigma-Aldrich; 50 µl of a 2 % solution in sterile water) was instilled into the bladder, colon, or uterus of UPK3A 65-84-immunized mice using a 24 gauge, 0.7×19mm catheter (BD Angiocath™ Autoguard™, BD Medical Systems, Sandy, UT, ref. #381700). Saline was administered to CFA-injected control mice. Pain assessment was performed using von Frey filaments one hour after drug administration.

Mast cell staining and counting

Mouse bladders were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned using a microtome. For staining of MCs, bladder cross sections were deparaffinized, hydrated sequentially with xylene, alcohol and dH_2O, and stained in 0.1% toluidine blue working solution for 2-3 minutes. After rinsing with dH_2O and dehydration, slides were mounted with coverslips using Cytoseal XYL mounting medium solution (Richard-Allan Scientific). Activated and resting MCs were distinguished under a light microscope at 40x magnification and counted separately. MCs were counted in eight different toluidine blue-stained sections of the bladder of each mouse.

Reverse transcription and quantitative real time PCR (qRT-PCR)

Bladders of UPK3A 65-84-immunized and control mice were harvested 10, 20, and 40 days after immunization and cut in half. One half was frozen and stored at –80°C for ELISA; the other half was homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated. cDNA was synthesized from the RNA using a Super Script III cDNA synthesis kit with random hexamer primers (Invitrogen). The cDNAs were analyzed for expression of the genes for MCP-1, Fc epsilon receptor 1 alpha (FceRI), and β-actin (loading control) by qRT- PCR, using gene-specific primer pairs designed with the online Universal Probe Library Assay Design Center (Roche, Mannheim, Germany). The sequences of the primer pairs are: MCP-1, CACAGTTGCCGGCTGGAGCAT (sense) and GTAGCAGCAGGTGAGTGGGGC (antisense); FceRI, TGGGGGCTCAGTGCCTGTCA (sense) and CCAAGGCTTCGGGATGCTTGAGG (antisense); β-actin, GGTCATCACTATTGGCAACG (sense) and ACGGATGTCAACGTCACACT (antisense). qRT-PCR was performed using a SYBR Green PCR Master kit with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). MCP-1 and FceR1 gene expression levels were normalized to expression of the β-actin gene and calculated relative to the average level in CFA-immunized mice by the comparative C_T method [385].

Treatment with sodium cromolyn

Sodium cromolyn (Sigma Aldrich, St. Luois, MO) is used as a mast cell stabilizer. Each mouse was treated with sodium cromolyn orally as 10mg/kg by using a Hamilton syringe with rounded tip needle which is 2.5cm long[386]. The drug was given 3 times a day with 2-hour intervals. Pain assessment was done using von Frey

ligament one hour after the last drug administration. The control mice were administered with saline.

Treatment with a histamine receptor 1 antagonist (cetirizine) and a histamine receptor 2 antagonist (ranitidine)

Each mouse was treated separately with cetirizine and ranitidine (Sigma Aldrich, St. Luois, MO) orally as 10mg/kg by using Hamilton syringe with rounded tip needle which is 2.5cm long [387, 388]. The drug was given 3 times a day with 2-hour intervals. Pain assessment was done using von Frey ligament one hour later after the last drug administration. The control mice were administered with saline.

Treatment of CCR2b antagonist

The CCR2b antagonist RS 102895 [389] (all from Sigma-Aldrich, St. Louis, MO) is a potent antagonist of MCP-1(CCL2) receptor and a member of the spiropiperidine. Each mouse was orally administered with 10mg/kg/day starting from day 1 until day 20 [390]. After 20 days treatment, mice were assessed in term of pelvic pain with von Frey filament graded from 0.004g to 4g. On the same day after pain assessment, all mice (Saline given-CFA immunized mice, n=10; CCR2b-treated UPK 3A immunized mice, n=10; Saline given-UPK 3A immunized mice, n=10) were scarified to harvest their bladder for mast cell counting by staining of toluidine blue.

MCP-1 and IgE ELISA

From the same mice from which bladders were harvested for qRT-PCR and ELISA, with half of each bladder stored at −80°C for ELISA, blood was collected, and serum was separated and stored at −80°C. In addition, six other tissues (uterus,

ovary, colon, liver, kidney, and lung) were harvested from UPK3A 65-84-immunized mice 40 days after immunization and stored at −80°C. The tissues were homogenized with a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA), and protein concentrations were determined by the method of Bradford (Bio-Rad Protein Assay, BioRad Laboratories, Hercules, CA). MCP-1 and IgE antibody concentrations were measured in the tissues and serum, respectively, using ELISA kits (MCP-1, Ray Biotech, Inc., Norcross, GA; IgE, BioLegend, Inc., San Diego, CA) according to the manufacturer's instructions. Absorbances at 450 nm were read in a Versamax ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Approaches

Comparisons of log transformed 50% pain threshold forces, MC numbers, MCP-1 and FceR1 mRNA levels, and MCP-1 protein levels between two groups of mice (e.g., UPK3A 65-84- vs. CFA-immunized mice, drug- vs. saline-treated mice, and wild type (WT) vs. MCP-$1^{-/-}$ mice) were performed using the unpaired, two-tailed Student's t test, with Welch's correction in cases where the variances were significantly different. Comparisons of 50% pain threshold forces in immunized mice at different time points with naïve mice, and MCP-1 protein levels in multiple tissues with the level in bladder, were performed using one way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Comparisons of MC numbers among wild type and knockout mice immunized with UPK3A 65-84 or CFA were performed using one way ANOVA with Tukey's multiple comparison test. Values of $p<0.05$ were considered statistically significant.

CHAPTER III

III.RESULTS

1- Early onset and persistence of pain in EAC mice

We used our previously created model of EAC in female BALB/cJ mice, in which the bladder-specific UPK3A 65-84 peptide induced a CD4+ T cell-mediated autoimmune response that mimicked the major symptoms of human IC/PBS, including elevated urinary frequency, decreased urine output per void, and increased pelvic pain responses (K. Izgi, et al., submitted). In this study, we evaluated the onset, duration, and specificity of pain induced by UPK3A 65-84 in comparison to CFA-immunized mice (control) and uninjected naïve mice (baseline). Mechanical stimulation of UPK3A 65-84-immunized mice on both the suprapubic region (Fig. 3A) and the hindpaw (Fig. 3D) with von Frey filaments of increasing size resulted in significantly greater pain sensitivity relative to naïve mice within 5 days of immunization and persisting through day 40, at which time the experiment was terminated. Much smaller increases in pain sensitivity relative to naïve mice were observed in CFA-immunized mice stimulated on both the suprapubic region (Fig. 3B) and the hindpaw (Fig. 3E) 5 to 40 days after immunization. Calculation of 50%

threshold forces revealed significantly lower pain thresholds in UPK3A 65-84-immunized mice stimulated on both the suprapubic region (Fig. 3C) and the hindpaw (Fig. 3F) relative to CFA-immunized mice at all time points. Comparison of results from CFA-injected mice at different time points with naïve mice by one way ANOVA with Dunnett's multiple comparison test revealed barely significant decreases in 50% thresholds on the suprapubic region 20 and 30 days after immunization, and moderately significant decreases in 50% thresholds on the hindpaw 20, 30, and 40 days after immunization.

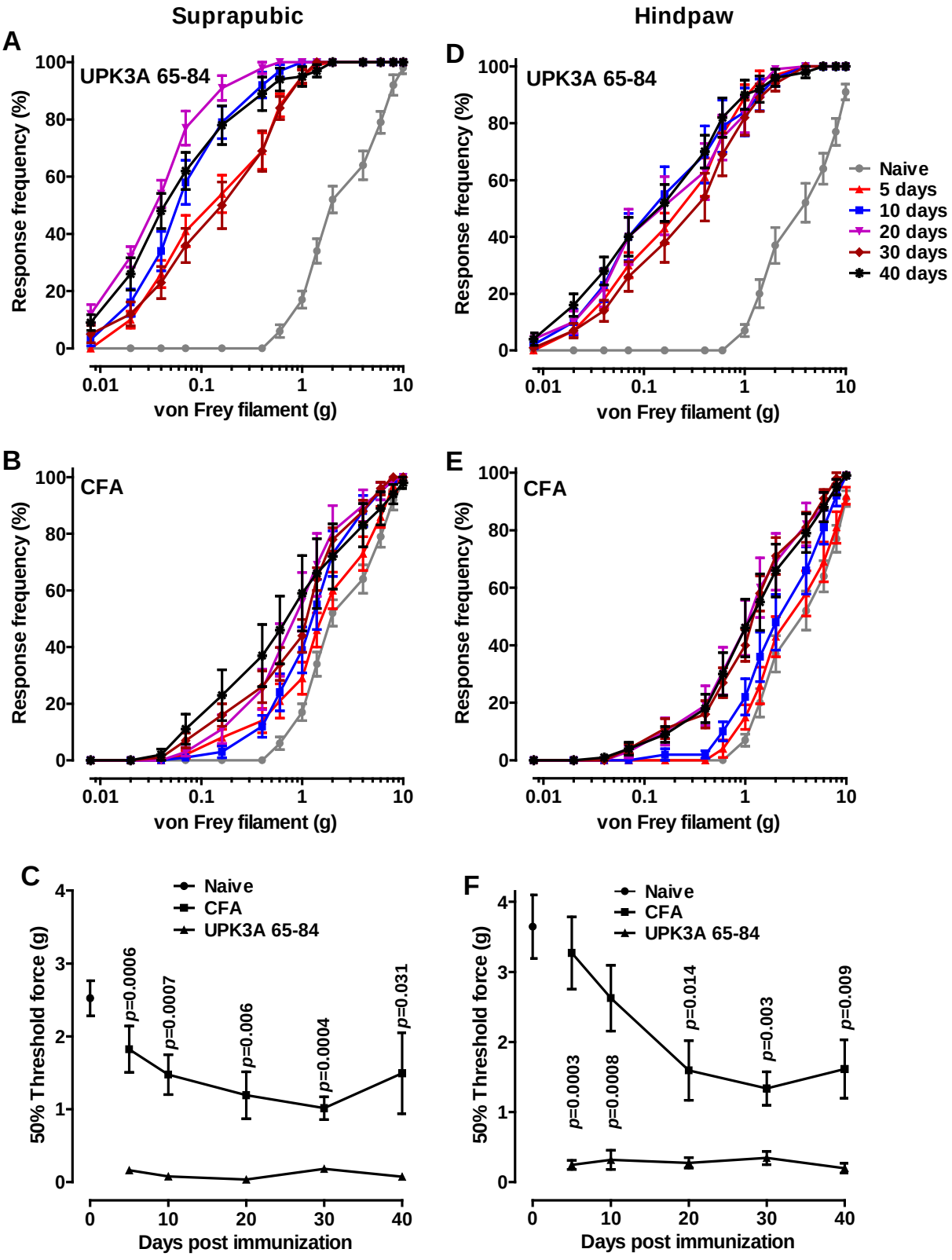
Suprapubic
Hindpaw
A
UPK3A 65-84
Response frequency (%)
von Frey filament (g)
D
UPK3A 65-84
Naive
5 days
10 days
20 days
30 days
40 days
B
CFA
E
CFA
C
Naive
CFA
UPK3A 65-84
50% Threshold force (g)
p=0.0006
p=0.0007
p=0.006
p=0.0004
p=0.031
Days post immunization
F
p=0.0003
p=0.0008
p=0.014
p=0.003
p=0.009

Figure 3: Immunization with UPK3A 65-84 peptide causes early and chronic pelvic pain in female BALB/cJ mice.

Mice were assessed for referred visceral hyperalgesia by applying a series of 14 von Frey filaments of increasing forces to the suprapubic region *(A-C*) and hindpaw (*D-F*). Each data point in *A*, *B*, *D*, and *E* indicates the mean response frequency (percentage of responses out of 10 trials) plus and minus the standard error of the mean (SEM) on the indicated number of days after immunization with UPK3A 65-84 (*A*,*D*) or CFA (*B*,*E*), or in naïve mice (n=10 mice per group). Data points in *C* and *F* indicate the mean 50% threshold forces ± SEM determined by linear regression of response frequency vs. log of the filament force for the three groups at the indicated time points. UPK3A 65-84-injected mice had substantially increased pain on both the suprapubic area (*A*) and hindpaw (*D*) compared to naïve mice. Much smaller increases in pain responses were observed in CFA-injected mice in the suprapubic (*B*) and hindpaw regions (*E*) compared to naïve mice. At every time point, the mean 50% threshold in mice immunized with UPK3A 65-84 was significantly lower than in CFA-injected mice on both the suprapubic region (*C*) and hindpaw (*F*), as determined by unpaired t tests of log transformed 50% pain thresholds with Welch's correction; levels of significance are indicated on the graphs. Comparisons of log transformed 50% thresholds in immunized mice at different time points with naïve mice by one way ANOVA with Dunnett's multiple comparison test revealed significant decreases in UPK3A 65-84-immunized mice on both the suprapubic and hindpaw regions at all time points ($p<0.001$), while CFA-injected mice exhibited barely significant decreases on the suprapubic region 20 and 30 days after immunization ($p<0.05$), and moderately significant decreases in 50% thresholds on the hindpaw 20, 30, and 40 days after immunization ($p<0.01$).

2- Chronic pelvic pain in EAC mice emanates from the bladder.

To determine whether the pain in EAC mice emanated from the bladder or not, lidocaine was instilled directly into the bladder under anesthesia of mice for 60 minutes by using 24GA 0.75 IN 0.7x19mm catheter (BD Angiocath Autoguard, Utah-USA, ref#381700). After waking up, each mouse was assessed in terms of referred visceral hyperalgesia by using von Frey fibers to show the difference between three groups: the peptide-immunized mice with lidocaine-treatment, the peptide-immunized mice with saline-treatment and CFA immunized mice with saline-treatment. Installation of lidocaine into the bladder significantly reduced pelvic pain in both on

suprapubic region (Fig. 4A) and on paw region (Fig. 4B). The same procedure for instillation of lidocaine into colon had no effect on the pelvic pain in both on suprapubic region (Fig. 4C) and on paw region (Fig. 4D). The same procedure for instillation of lidocaine into uterus had no effect on the pelvic pain in both on suprapubic region (Fig. 4E) and on paw region (Fig. 4F).

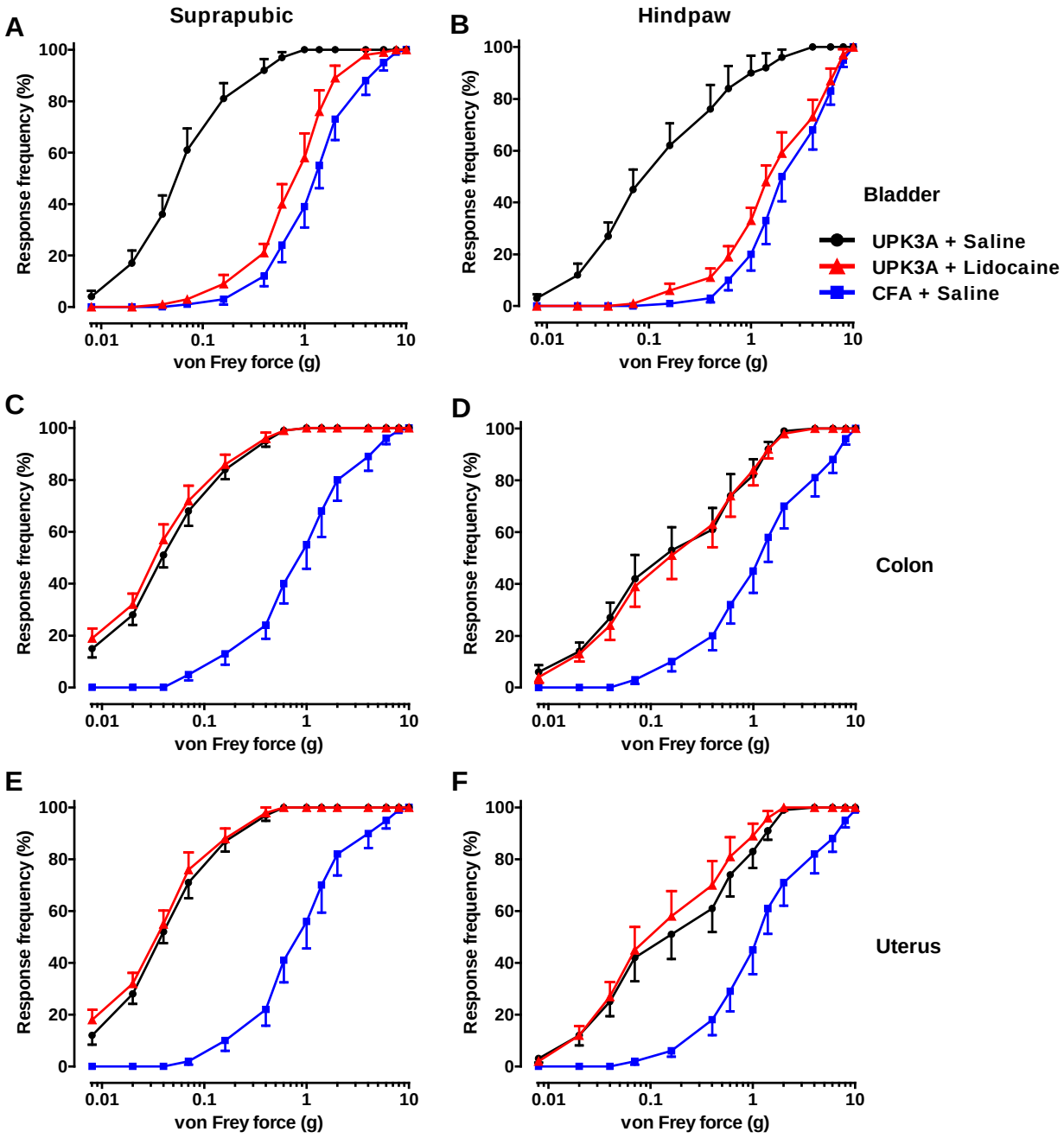

Figure 4: Lidocaine reduces pain of UPKA3A 65-84-immunized mice when instilled into the bladder.

Instillation of lidocaine into the bladders of UPKA3A 65-84-immunized mice resulted in significantly increased 50% pain thresholds of von Frey filaments applied to the suprapubic region (*A*, p=0.0006 by unpaired t test of log transformed 50% pain thresholds with Welch's correction) or hindpaw (*B*, p=0.0004), to levels that were not significantly different from control mice injected with CFA + saline (n=10 per group). On the other hand, instillation of lidocaine into the colon or uterus had no effect on 50% pain thresholds on either the suprapubic region (C,E) or hindpaw region (D,F). Data points indicate the mean response frequencies plus or minus SEM.

3-The EAC mouse model has increased MC in the bladder

We examined MC amount as well as the activation status of MC. There are two types of MC status: resting and activated MC in the tissues. Activated MC are granulated (more intense staining) and resting MC have a few or no granules in the cell boundary, when the bladder is stained with toluidine blue (Figure 5A). Figure 5B showed a representative staining of bladder of immunized mice with CFA control mice versus UPK3A 65-84 immunized mice. We can easily say that there is a significant accumulation of MC in the peptide-immunized mouse (Figure 5B). The number of total MC significantly increased in UPK3A 65-84 peptide immunized mice compared to CFA-immunized mice alone at different time points by staining with toluidine blue (Figure 5E). The number of activated mast cells was more than the number of resting MCs. Both of them significantly increased in the peptide-immunized mice compared with CFA-immunized mice alone on 10^{th}, 20^{th}, and 40^{th} days (Figure 5C, D). We also showed that FceRI gene expression significantly increased on 10^{th}, 20^{th}, and 40^{th} days in immunized mice compared control group (Figure 5F).

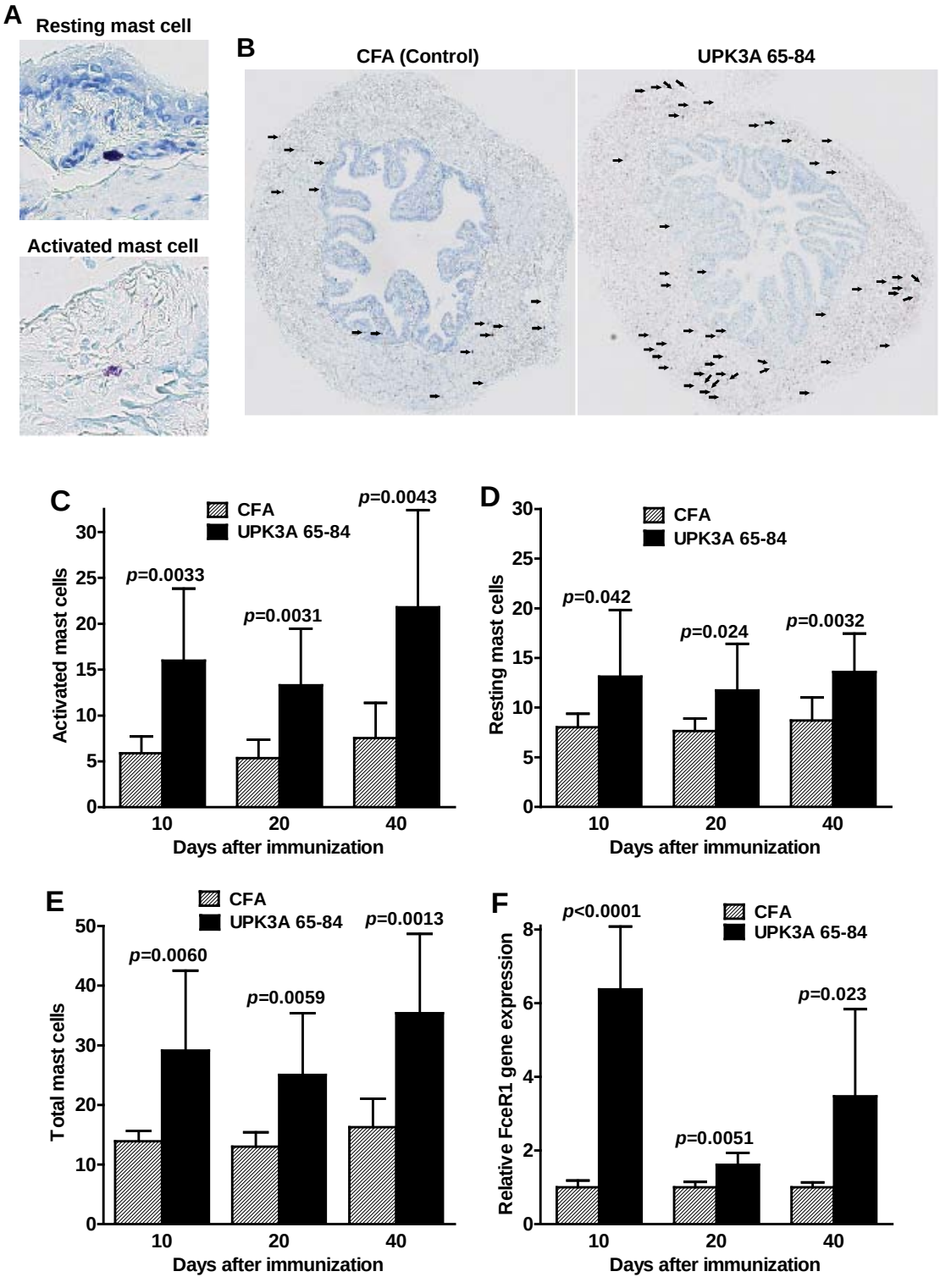
A
Resting mast cell
Activated mast cell
B
CFA (Control)
UPK3A 65-84
C
CFA
UPK3A 65-84
Activated mast cells
p=0.0033
p=0.0031
p=0.0043
Days after immunization
D
CFA
UPK3A 65-84
Resting mast cells
p=0.042
p=0.024
p=0.0032
Days after immunization
E
CFA
UPK3A 65-84
Total mast cells
p=0.0060
p=0.0059
p=0.0013
Days after immunization
F
CFA
UPK3A 65-84
Relative FceR1 gene expression
p<0.0001
p=0.0051
p=0.023
Days after immunization

Figure 5: Increased MC accumulation in bladders of mice immunized with UPK3A 65-84 peptide.

MCs in bladder cross sections from UPK3A 65-84- and CFA-immunized mice were stained with toluidine blue (*A*), then activated MCs with less visible cytoplasmic granules and resting MCs with more granules (all cytoplasm is stained due to invention of granules) were counted separately under a light microscope at 40x magnification (*B*, MCs are indicated by arrows). MCs were counted in eight different sections of each bladder. At 10, 20, and 40 days after immunization, bladders from UPK3A 65-84-immunized mice contained significantly more activated (*C*), resting (*D*), and total (*E*) MCs compared with CFA-injected mice (n=10 mice per group at each time point). Also, FceRI gene expression was significantly increased in UPK3A 65-84-immunized relative to CFA-injected mice 10, 20, and 40 days after immunization (n=8 mice per group-time point) (*F*). Each column indicates the mean plus standard deviation (SD); *p* values indicated on the graphs are results of unpaired t tests, all with Welch's correction.

4-The administration of ranitidine, cetirizine and cromolyn sodium attenuated chronic pelvic pain in EAC model

Many drugs such as cetirizine, ranitidine and cromolyn sodium stop MC related effects such as pain. That is why we used this drug to show the role of MC in pelvic pain. The MC stabilizer, Cromolyn sodium, significantly reduced pelvic pain in both on suprapubic region (Figure 6A) and on paw region (Figure 6D) by giving the drug 3 times a day with 2 hours interval. The antihistamine specific H1 receptor blocker, Cetirizine, reduced pelvic pain in both on suprapubic region (Figure 6B) and on paw region (Figure 6E) by giving the drug 3 times a day with 2 hours interval. Furthermore, the antihistamine specific H2 receptor blocker (Ranitidine) significantly reduced pelvic pain in both on suprapubic region (Figure 6C) and on paw region (Figure 6F) using the same protocol. Pain was measured for each trial after one hour

by the end of last dosage administration (10 mg/kg). Finally, Those results show that mast cell products especially histamine has very important role in the pelvic pain.

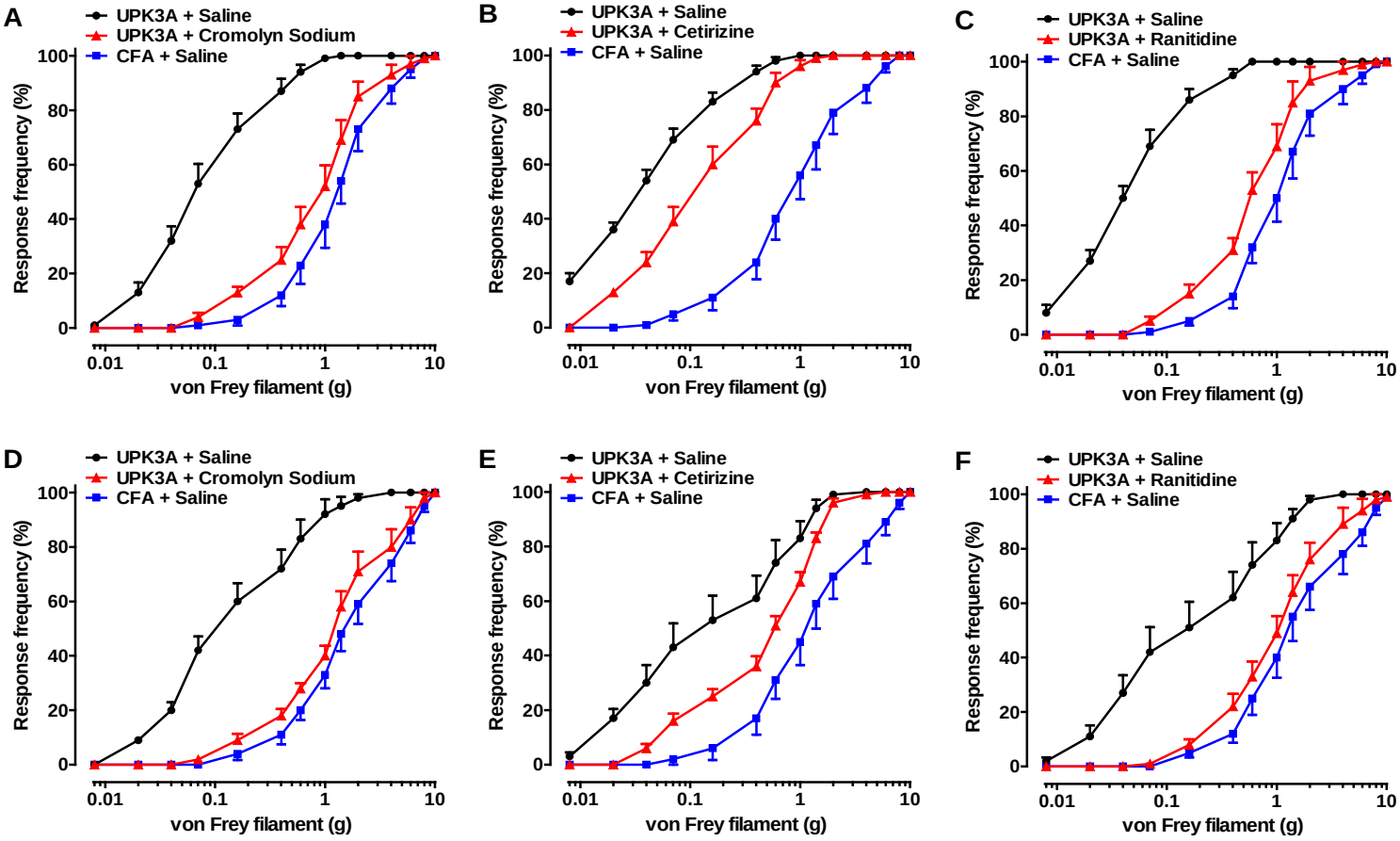

Figure 6: MC stabilizer and antihistamines reduce pain in UPK3A 65-84-immunized mice.

Oral administration of the MC stabilizer cromolyn sodium to UPK3A 65-84-immunized mice resulted in significantly higher 50% pain thresholds with von Frey filaments applied to both the suprapubic region (*A*, p=0.0004) and hindpaw (*D*, p=0.0014), to levels that were not significantly different from control mice injected with CFA + saline. Antagonists of histamine receptors 1 (cetirizine) and 2 (ranitidine) significantly reduced pain (increased 50% thresholds) of von Frey filaments applied to the suprapubic region (*B*, cetirizine, p=0.0025, and *C*, ranitidine, p=0.0025 compared to saline vehicle-treated, UPK3A 65-84-immunized mice). However, while ranitidine reduced hindpaw pain responses significantly (*F*, p=0.0072), cetirizine did

not (*E*). Of the two histamine receptor blockers, only ranitidine reduced pain to levels that were not significantly different from CFA + saline control mice, on both the suprapubic (*C*) and paw regions (*F*). Drugs were administered and pain assessment was performed as described in Materials and Methods. n=10 mice per group; *p* values are results of unpaired t-tests of log transformed 50% pain thresholds, with Welch's correction in cases of significantly different variances. Data points indicate the mean response frequencies plus or minus SEM.

5- MCP-1 level specifically increased in bladder of EAC mice that have the same amount of IgE compared with CFA-Control group.

MCP-1 levels were quantified both transcription level and translational level in bladder of EAC mice. Bladders of both control (CFA) and immunized mice were harvested, and bladders were cut in the middle of it. One-half was kept for RT-PCR, and other-half was kept for ELISA. MCP-1- mRNA and protein level in UPK 3A 65-84 immunized mice is significantly higher than CFA immunized mice alone at 10^{th}, 20^{th}, 40^{th} day (Figure 7A, B). MCP-1 specifically increased in bladder of EAC mice other than the organs showed in the graph (Figure 7C). IgE level of EAC mice in serums is almost same with Control –CFA immunized group (Figure 7D). To sum up, these outstanding results demonstrate that MCP-1 level is consistently elevated in bladder of EAC mice. It also correlates with MC accumulation in terms of time points (Figure 5).

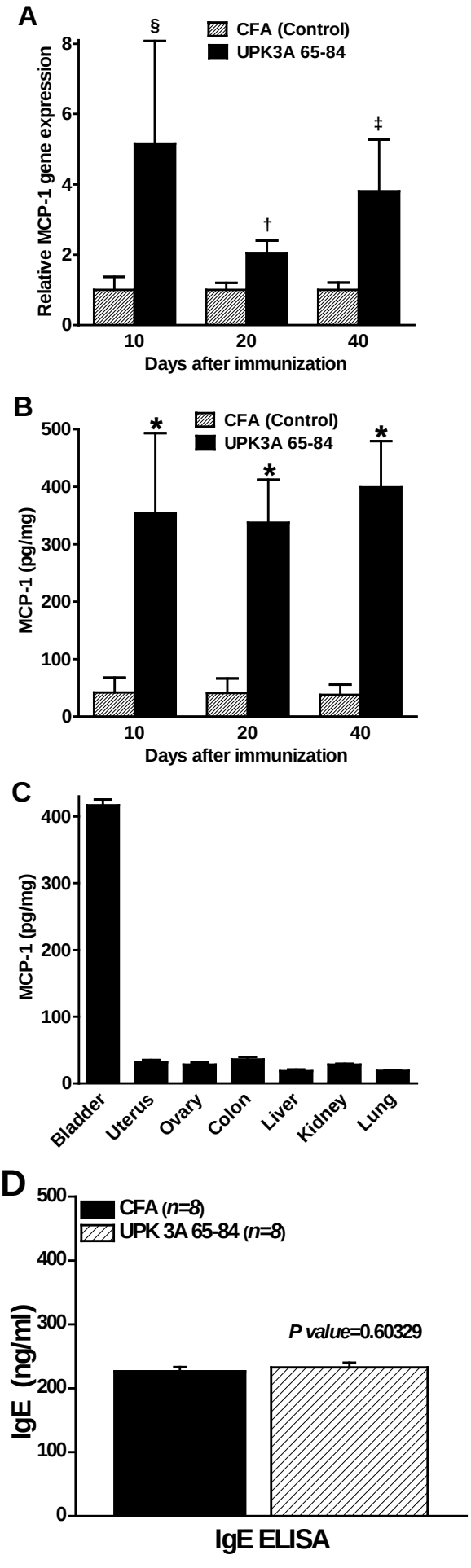
A
§
CFA (Control)
UPK3A 65-84
Relative MCP-1 gene expression
8
6
4
2
0
†
‡
10
20
40
Days after immunization
B
CFA (Control)
UPK3A 65-84
*
*
*
MCP-1 (pg/mg)
500
400
300
200
100
0
10
20
40
Days after immunization
C
MCP-1 (pg/mg)
400
300
200
100
0
Bladder
Uterus
Ovary
Colon
Liver
Kidney
Lung
D
CFA (n=8)
UPK 3A 65-84 (n=8)
IgE (ng/ml)
500
400
300
200
100
0
P value=0.60329
IgE ELISA

Figure 7: Immunization with UPK3A 65-84 peptide induces MCP-1 production in the bladder. There is no significant difference amount of IgE between peptide immunized mice and CFA immunized mice.

UPK3A 65-84-immunized and CFA control mice were sacrificed and bladder and other organs were harvested and processed 10, 20, and 40 days after immunization. MCP-1 mRNA (*A*) and protein (*B*) levels quantified by reverse transcription/qRT-PCR and ELISA, respectively, were significantly higher in UPK3A 65-84-immunized mice at all three time points. §n=8 per group, p=0.005; †n=8 per group, p<0.0001; ‡n=5 per group, p =0.013; and *****n=10 per group, p<0.0001 by unpaired t tests, using Welch's correction for all except MCP-1 gene expression at 20 days. *C*. The level of MCP-1 protein was substantially higher in the bladder compared to six other organs in EAC mice 40 days after immunization with UPK3A 65-84 peptide (n=3 per group, p<0.001 comparing each organ to bladder by one way ANOVA with Dunnett's multiple comparison test). *D.* There is no difference amount of IgE in serum of immunized and control mice by using two sample *t*-test. Each column indicates the mean plus SD.

6-MCP-1 -/- mice decreased suprapubic pelvic pain.

MCP-1 -/- mice was obtained as a gift from Dr. Barrett Rollins (Harvard Medical School, Boston, MA). Autoimmunity against UP3A 65-84 peptide causes chronic cystitis in WT BALB/cJ mice resulting in chronic pelvic pain (figure 8B). However, immunization MCP-1-/- BALB/cJ mice (Figure 8A) were not showed any pelvic pain development at time courses versus WT peptide immunized mice (Figure 8B). Peptide immunized MCP-1 -/- mice (Figure 8A) have the same pain responses with WT CFA immunized control group (Figure 8D) which also showed MCP-1 is again very important in pelvic pain development. Mechanical stimulation of suprapubic area by the filaments resulted in non-significant increment between CFA immunized mice groups compared to baseline mice (without any injection) on the suprapubic region (Figure 8D) during the 40 days of the experiment. In contrast, there was a significant difference between naive (baseline) mice and peptide-immunized

mice in terms of pelvic pain percentage response at all fibers on suprapubic region (Figure 8B). There was no any significant difference between naive (baseline) mice and peptide-immunized MCP-1 -/- mice in terms of pelvic pain percentage response at all fibers on suprapubic region (Figure 8A) during the 40 days of the experiment. 50% threshold of immunized mice is significantly decreased compared MCP-1 -/- mice due to being more sensitive to the pain (Figure 8C). However, 50% threshold of CFA mice is significantly almost same compared MCP-1 -/- except 40 day due to WT mice can be more sensitive than the immunized KO mice (Figure 8F). Those data showed that MCP-1 plays very important role in chronic pelvic pain development of EAC mice model. Based on these studies, we can use MCP-1 as a target in term of further investigation and therapeutic approaches of IC/PBS patients.

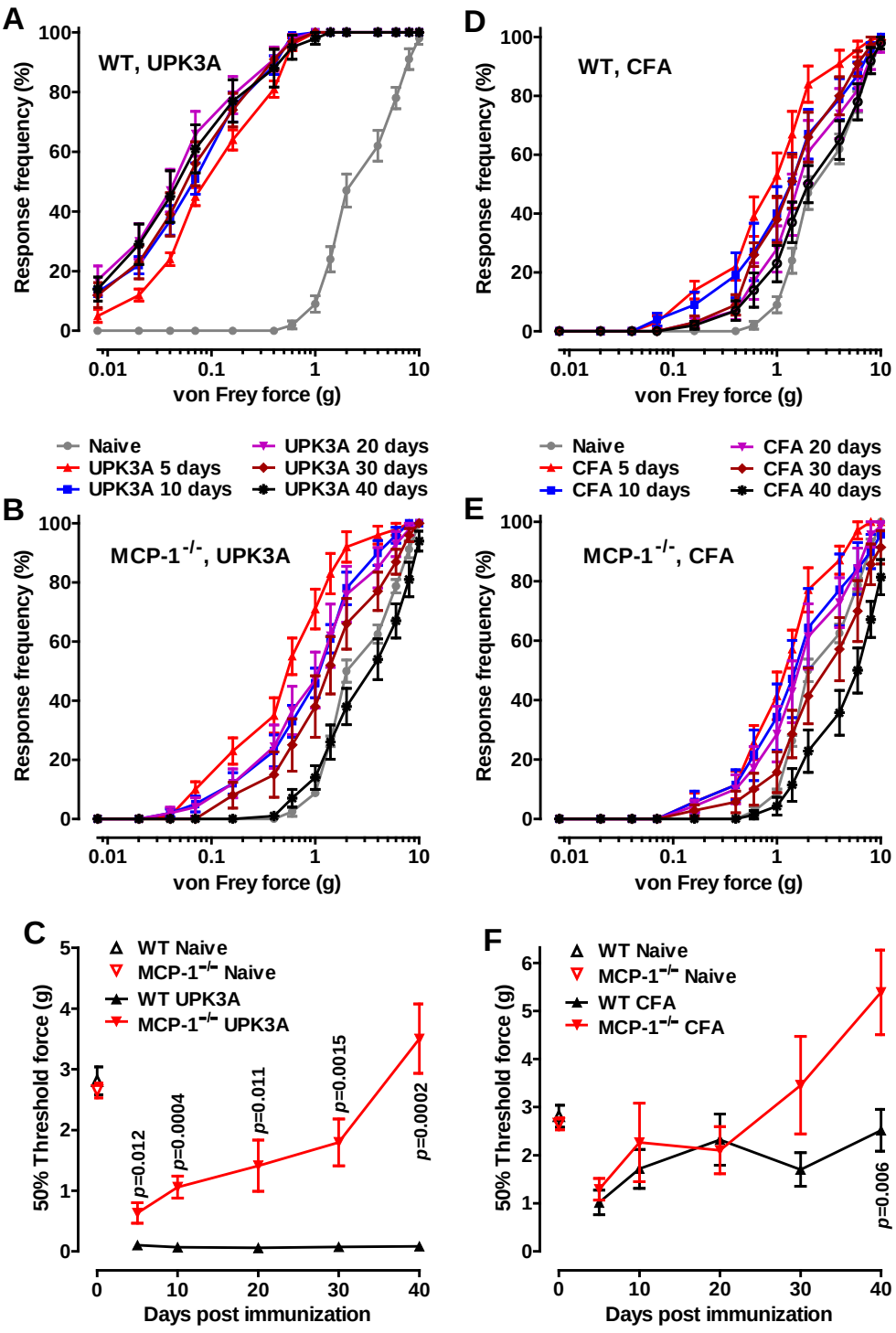
A
WT, UPK3A
Response frequency (%)
von Frey force (g)
D
WT, CFA
Naive
UPK3A 5 days
UPK3A 10 days
UPK3A 20 days
UPK3A 30 days
UPK3A 40 days
CFA 5 days
CFA 10 days
CFA 20 days
CFA 30 days
CFA 40 days
B
MCP-1-/-, UPK3A
E
MCP-1-/-, CFA
C
WT Naive
MCP-1-/- Naive
WT UPK3A
MCP-1-/- UPK3A
50% Threshold force (g)
Days post immunization
p=0.012
p=0.0004
p=0.011
p=0.0015
p=0.0002
F
WT CFA
MCP-1-/- CFA
p=0.006

Figure 8: MCP-1$^{-/-}$ mice exhibit significantly less suprapubic pelvic pain compared with wild type (WT) BALB/cJ mice after immunization with UPK3A 65-84.

Mice were assessed for referred visceral hyperalgesia 5, 10, 20, 30, and 40 days after immunization by applying von Frey filaments of increasing forces to the suprapubic region as described in Materials and Methods. Mean response frequencies ± SEM were plotted against the log of the von Frey force (*A*, WT naïve and UPK3A 65-84-immunized mice; *B*, MCP-1$^{-/-}$ naïve and UPK3A 65-84-immunized mice; *D*, WT naïve and CFA-immunized mice; *E*, MCP-1$^{-/-}$ naïve and CFA-immunized mice; n=10 mice per group). In addition, 50% thresholds were calculated as described in Materials and Methods; data points in graphs *C* and *F* indicate the mean 50% thresholds ± SEM at the indicated time points. UPK3A 65-84 immunization yielded significantly less suprapubic pain in MCP-1$^{-/-}$ mice than it did in WT mice at all time points (*C*; *p* values on the graph are results of unpaired t tests of log transformed 50% pain thresholds with Welch's correction). CFA (control) immunization yielded significantly less suprapubic pain in MCP-1$^{-/-}$ mice than it did in WT mice 40 days after immunization, but not at any other time point (*F*; *p* value on the graph is the result of an unpaired t test). Furthermore, 50% thresholds were not significantly different in MCP-1$^{-/-}$ mice injected with UPK3A 65-84 compared with MCP-1$^{-/-}$ mice injected with CFA at any time point (compare red lines in *C* and *F*).

7-MCP-1 -/- mice decreased paw pain

MCP-1 -/- mice was obtained as a gift from Dr. Barrett Rollins (Harvard Medical School, Boston, MA). Autoimmunity against UP3A 65-84 peptide causes chronic cystitis in WT BALB/cJ mice resulting in pain of paw region (Figure 9A). However, immunization MCP-1 -/- BALB/cJ mice (Figure 9B) were not showed any paw pain development at time courses versus WT peptide immunized mice (Figure 9A). Peptide immunized MCP-1 -/- mice (Figure 9B) have the same pain responses with WT CFA immunized control group (Figure 9D) which also showed MCP-1 is again very important in pelvic pain development. Mechanical stimulation of paw area by the filaments resulted in non-significant increment between CFA immunized mice groups compared to baseline mice (without any injection) on the paw region (Figure 9D) during the 40 days of the experiment. In contrast, there was a difference between naive (baseline) mice and peptide-immunized mice in terms of percentage of paw pain

response at all fibers on paw region (Figure 9A). There was no any significant difference between naive (baseline) mice and peptide-immunized MCP-1 -/- mice in terms of percentage of paw pain response at all fibers on paw region (Figure 9B) during the 40 days of the experiment. 50% threshold of immunized mice is significantly decreased compared MCP-1 -/- mice due to being more sensitive to the pain (Figure 9C). However, 50% threshold of CFA mice is almost same compared immunized MCP-1 -/- (Figure 9F). Those data showed that MCP-1 plays a crucial role in paw area related pain development of EAC mice model. Based on these studies, we can use MCP-1 as a target in term of further investigation of pain.

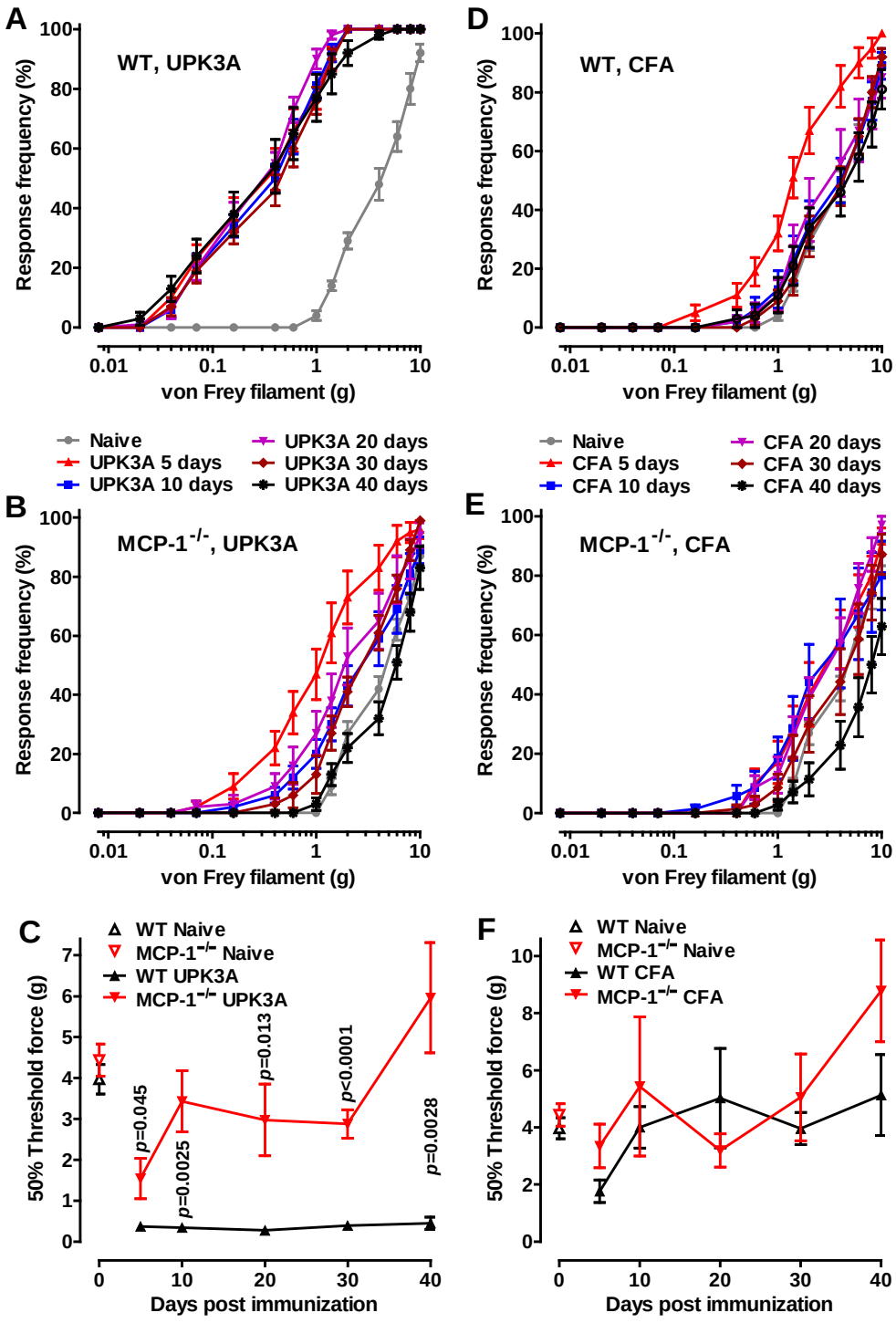
A
WT, UPK3A
Response frequency (%)
von Frey filament (g)
D
WT, CFA
Naive
UPK3A 5 days
UPK3A 10 days
UPK3A 20 days
UPK3A 30 days
UPK3A 40 days
CFA 5 days
CFA 10 days
CFA 20 days
CFA 30 days
CFA 40 days
B
MCP-1-/-, UPK3A
E
MCP-1-/-, CFA
C
WT Naive
MCP-1-/- Naive
WT UPK3A
MCP-1-/- UPK3A
p=0.045
p=0.0025
p=0.013
p<0.0001
p=0.0028
50% Threshold force (g)
Days post immunization
F
WT CFA
MCP-1-/- CFA

Figure 9: MCP-1$^{-/-}$ mice exhibit significantly less hindpaw pain compared with WT BALB/cJ mice after immunization with UPK3A 65-84.

The mice used to generate the data for Figure 8 were also assessed for referred visceral hyperalgesia by applying von Frey filaments to the hindpaw. Mean response frequencies ± SEM was plotted (*A, B, D, and E*) and mean 50% thresholds ± SEM were calculated and plotted (*C, F*) as in Figure 8. UPK3A 65-84 immunization yielded significantly less hindpaw pain in MCP-1$^{-/-}$ mice than it did in WT mice at all time points (*C*; *p* values on the graph are results of unpaired t tests of log transformed 50% pain thresholds with Welch's correction). Hindpaw pain responses were not significantly different in CFA-immunized MCP-1$^{-/-}$ mice compared to CFA-immunized WT mice at any time point (*F*). Furthermore, 50% thresholds were not significantly different in MCP-1$^{-/-}$ mice injected with UPK3A 65-84 compared with MCP-1$^{-/-}$ mice injected with CFA at any time point (compare red lines in *C* and *F*).

8- MCP-1 is required for MC accumulation and activation in bladder in response to UPK 3A 65-84 immunization.

MCP-1 is a member of chemokine family. The aim is to show whether the MCP-1 is effective to decrease MCs accumulation. MCP-1 -/- mice was obtained as a gift from Dr. Barrett Rollins (Harvard Medical School, Boston, MA). After all groups' immunization, all mice (CFA injected WT mice, n=10; UPK 3A 65-84 injected WT mice, n=10; UPK 3A 65-84 injected MCP-1 KO mice, n=10; CFA injected MCP-1 KO mice, n=10) were scarified to harvest their bladder for mast cell counting after staining with toluidine blue. The results showed that total mast cell decreased in MCP-1 -/- mice bladder compared to peptide immunized WT mice. The same correlation also occurred in resting and activated mast cell accumulation. All results are significantly different (*****p<0.001).

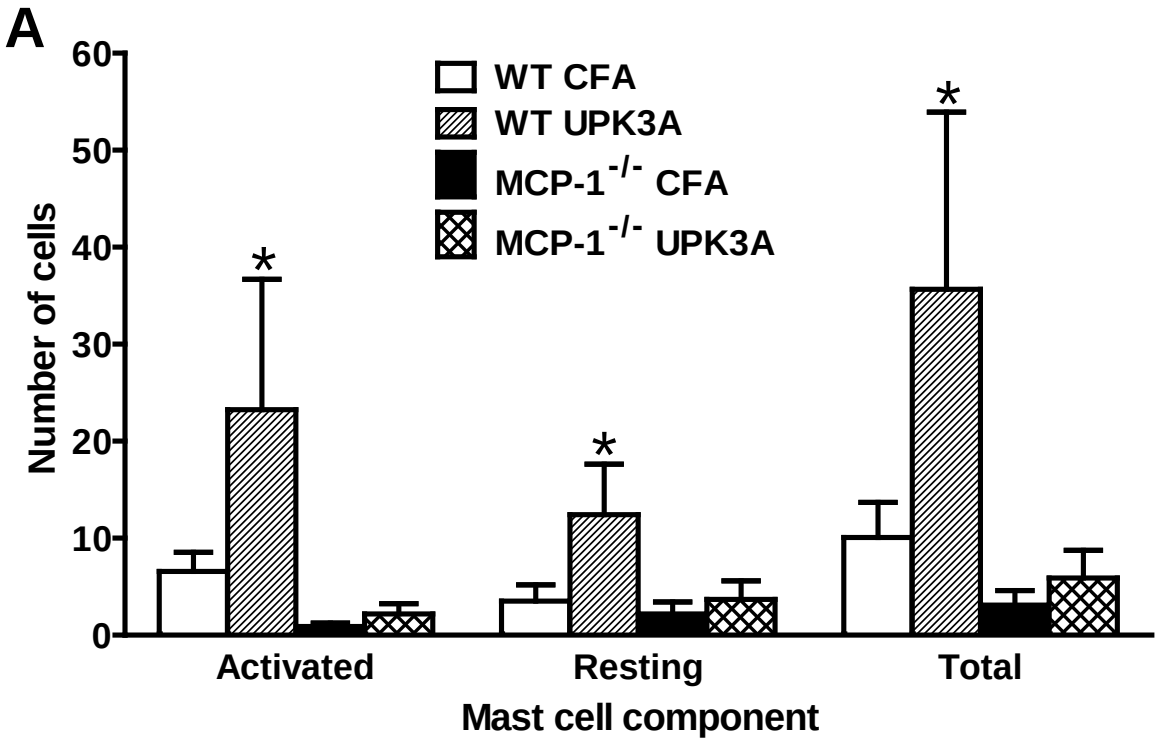

Figure 10: MC accumulation and activation in the bladder in response to immunization is markedly attenuated in MCP-1$^{-/-}$ mice.

On the 40th day after immunization, all mice in which pain was assessed for Figures 8,9 were sacrificed one hour after pain assessment, then bladders were harvested and processed for toluidine blue staining of MCs as described in Materials and Methods. Activated and resting MCs were counted in bladder cross sections from UPK3A 65-84- and CFA-immunized WT and MCP-1$^{-/-}$ mice (n=10 mice per group). Numbers of activated, resting, and total MCs were significantly higher in bladders from UPK3A 65-84-immunized WT mice compared to MCP-1$^{-/-}$ mice immunized with either UPK3A 65-84 or CFA and to CFA-immunized WT mice (*A*; ***p*<0.001 by one way ANOVA with Tukey's post test comparing UPK3A 65-84-immunized WT mice with every other group; no other pairs of groups differed significantly from each other). Each column indicates the mean plus SD.

9-CCR2b antagonist decreases pelvic pain

CCR2 is a receptor of MCP-1. This antagonist property on the receptor was shown in rodents [390]. The aim is to show whether the CCR2b antagonist is effective to stop MC accumulation and pelvic pain. CCR2b antagonist (RS 102895) was purchased from Sigma (St Louis, MO. RS 102895) which is a potent antagonist of MCP-1 (CCL2) receptor and a member of the spiropiperidine. Each mouse was orally administered RS 102895 10mg/kg/day starting from day 1 until day 20. [390]. After 20 days treatment, mice were assessed in term of pelvic pain with von Frey filament graded from 0.008g to 4g. The results showed decreased pelvic pain in the peptide-immunized mice that were administered with this compound compared to the peptide-immunized mice and CFA-immunized mice that were not administered with this compound. The pelvic pain decreased in both on suprapubic region (Figure 11A) and on paw region (Figure 11B). After 20 days all mice (CFA alone immunized mice, n=10; Treated UPK 3A immunized mice, n=10; Untreated UPK 3A immunized mice, n=10) were sacrificed to harvest their bladder for mast cell counting by staining of toluidine blue. The results showed that total mast cell decreased in CCR2b antagonist-treated mice compared to other two control groups and number of activated mast cell is more than the number of resting mast cell (Figure 11C).

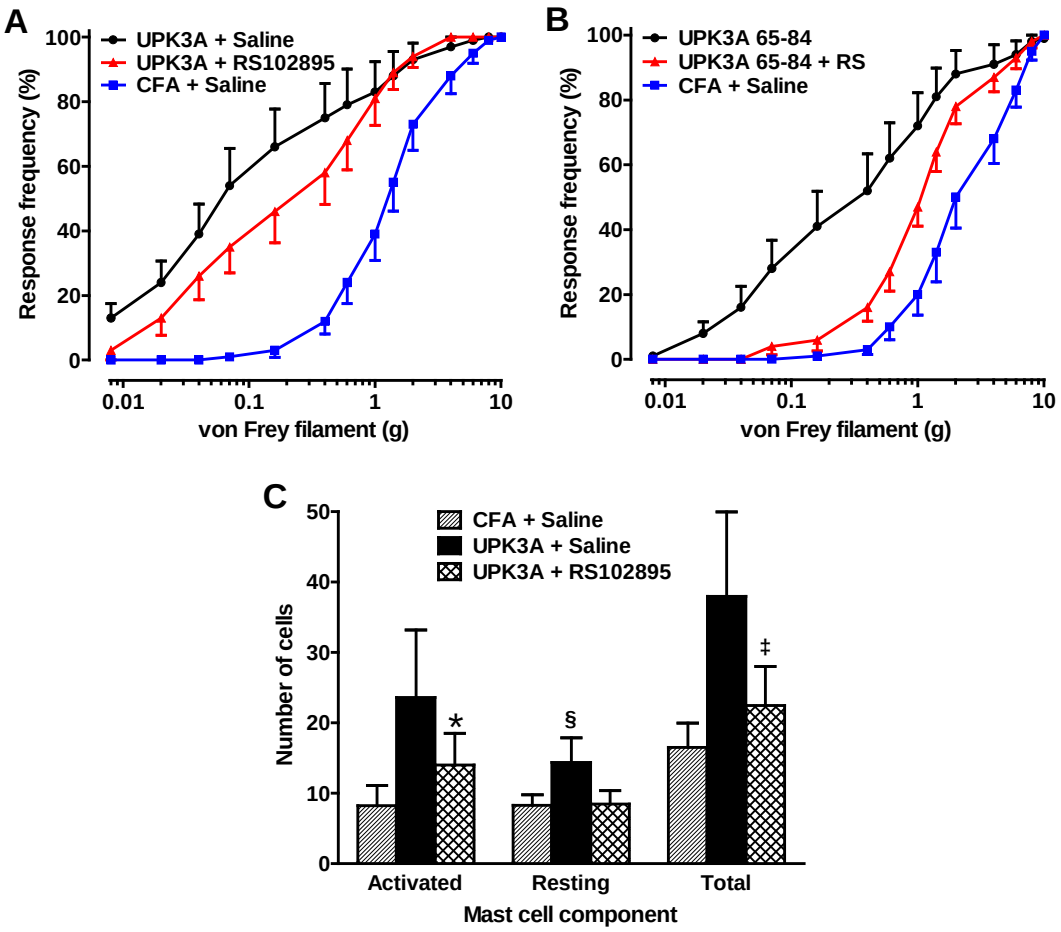

Figure 11: CCR2b antagonist decreases pain and has less mast cell accumulation in bladder of UPK3A 65-84-immunized mice.

Oral administration of the CCR2b antagonist RS 102895 to UPK3A 65-84-immunized mice resulted in significantly higher 50% pain thresholds with von Frey filaments applied to the hindpaw (*B*) (p=0.027 by unpaired t test of log transformed 50% thresholds using Welch's correction), but not to the suprapubic region (*A*), compared with saline-treated UPK3A 65-84-immunized mice (n=10 mice per group). Data points in *A* and *B* indicate the mean response frequencies plus or minus SEM. On the same day after pain assessment, all mice were sacrificed, bladders were harvested and processed, and MCs were stained with toluidine blue and counted as described in Materials and Methods (*C*). The results showed significant decreases in activated (*p=0.014), resting ($^{\S}p$ =0.0002), and total ($^{\ddagger}p$=0.003) MCs in CCR2b antagonist-treated compared to saline-treated UPK3A-immunized mice, by unpaired t tests with (activated, total) or without (resting) Welch's correction. The decrease in resting MCs was to a level not significantly different from saline-treated CFA control mice. Each column indicates the mean plus SD.

10-CCR2 -/- mice decreased suprapubic pelvic pain.

CCR2 -/- mice were obtained as a gift from Dr. Naofumi Mukaida Cancer Research Institute, Kanazawa University (Kanazawa, Japan). Autoimmunity against UP3A 65-84 peptide causes chronic cystitis in WT BALB/cJ mice resulting in chronic pelvic pain (Figure 12A). In contrast, immunization CCR2-/- BALB/cJ mice (Figure 12B) were not showed any pelvic pain development at time courses versus WT peptide immunized mice (Figure 12A). Peptide immunized CCR2 -/- mice (Figure 12B) have the same pain responses with WT CFA immunized control group (Figure 12D) which also showed CCR2 -/- mice is again very important in pelvic pain development. Mechanical stimulation of suprapubic area by the filaments resulted in non-significant increment between CFA immunized mice groups compared to baseline mice (without any injection) on the suprapubic region (Figure 12D) during the 40 days of the experiment. In contrast, there was a significant difference between naive (baseline) mice and peptide-immunized mice in terms of pelvic pain percentage response at all fibers on suprapubic region (Figure 12A). There was no significant difference between naive (baseline) mice and peptide-immunized CCR2 -/- mice in terms of pelvic pain percentage response at all fibers on suprapubic region (Figure 12B) during the 40 days of the experiment. 50% threshold of immunized mice is significantly decreased compared CCR2 -/- mice due to being more sensitive to the pain (Figure 12C). However, 50% threshold of CFA mice is almost same compared immunized CCR2 -/- (Figure 12F). Those data showed that CCR2 plays very important role in chronic pelvic pain development of EAC mice model. Based on these studies, we can use CCR2 as a target in term of further investigation and therapeutic approaches of IC/PBS patients.

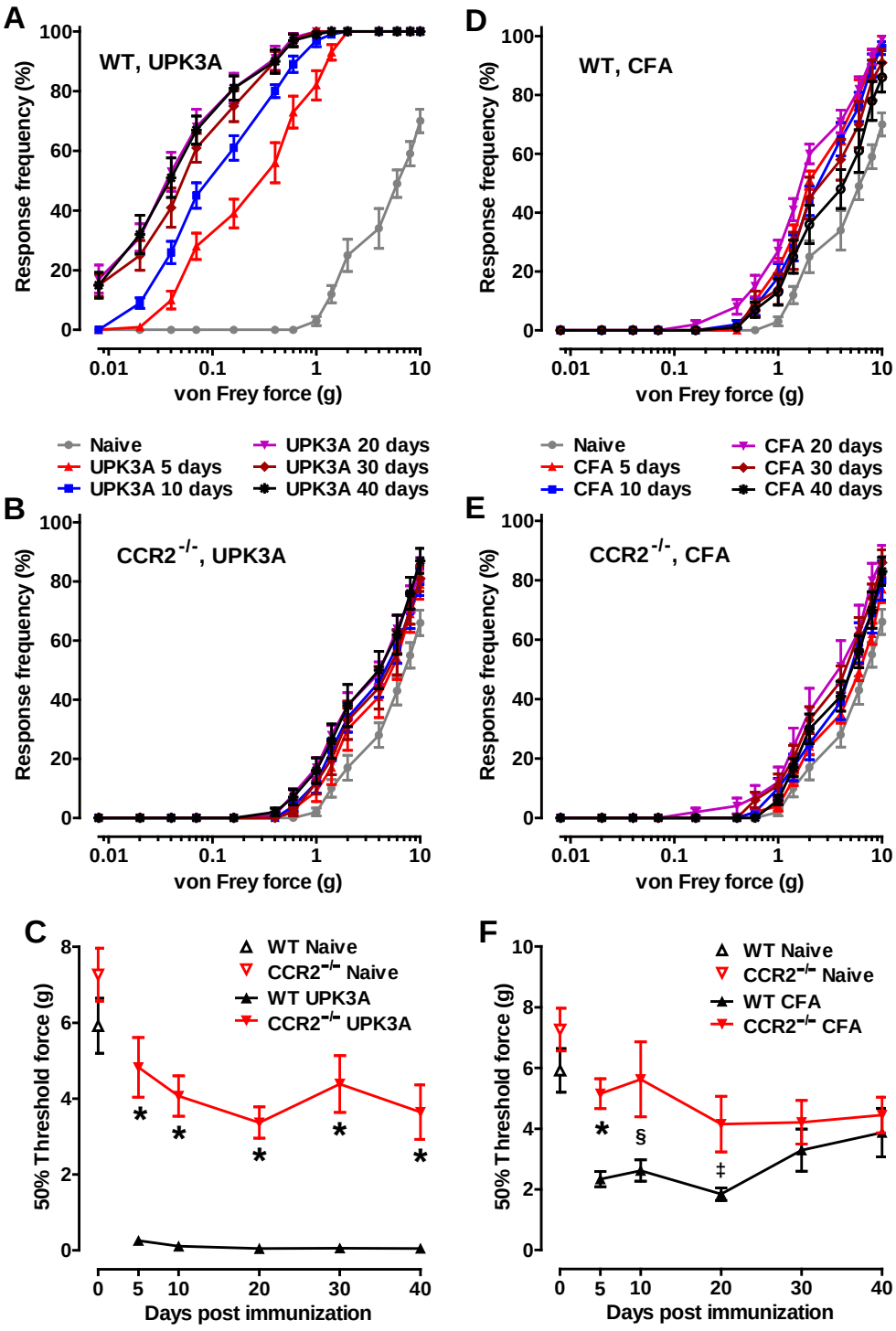
A
WT, UPK3A
Response frequency (%)
von Frey force (g)
Naive
UPK3A 5 days
UPK3A 10 days
UPK3A 20 days
UPK3A 30 days
UPK3A 40 days
D
WT, CFA
Naive
CFA 5 days
CFA 10 days
CFA 20 days
CFA 30 days
CFA 40 days
B
CCR2-/-, UPK3A
E
CCR2-/-, CFA
C
50% Threshold force (g)
Days post immunization
WT Naive
CCR2-/- Naive
WT UPK3A
CCR2-/- UPK3A
F
WT CFA
CCR2-/- CFA

Figure 12: CCR2$^{-/-}$ mice exhibit significantly less suprapubic pelvic pain compared with WT BALB/cJ mice after immunization with UPK3A 65-84.

Mice were assessed for referred visceral hyperalgesia 5, 10, 20, 30, and 40 days after immunization by applying von Frey filaments of increasing forces to the suprapubic region as described in Materials and Methods. Mean response frequencies ± SEM were plotted against the log of the von Frey force (*A*, WT naïve and UPK3A 65-84-immunized mice; *B*, CCR2$^{-/-}$ naïve and UPK3A 65-84-immunized mice; *D*, WT naïve and CFA-immunized mice; *E*, CCR2$^{-/-}$ naïve and CFA-immunized mice; n=10 mice per group). In addition, 50% thresholds were calculated as described in Materials and Methods; data points in graphs *C* and *F* indicate the mean 50% thresholds ± SEM at the indicated time points. The reductions in suprapubic pain responses in CCR2$^{-/-}$ mice compared with WT mice after immunization with UPK3A 65-84 were highly significant at all time points (*C*; **p*<0.0001 by unpaired t tests of log transformed 50% pain thresholds). CCR2$^{-/-}$ mice immunized with CFA exhibited significantly less suprapubic pain than WT CFA-injected mice 5, 10, and 20 days after immunization (*F*; **p*<0.0001 and $^{\S}p$=0.017 by unpaired t tests; $^{\ddagger}p$=0.031 by unpaired t test with Welch's correction). Furthermore, 50% thresholds were not significantly different in CCR2$^{-/-}$ mice injected with UPK3A 65-84 compared with CCR2$^{-/-}$ mice injected with CFA at any time point (compare red lines in *C* and *F*).

11-CCR2 -/- mice decreased paw pain

CCR2 -/- mice were obtained as a gift from Dr. Naofumi Mukaida Cancer Research Institute, Kanazawa University (Kanazawa, Japan). Autoimmunity against UP3A 65-84 peptide causes chronic cystitis in WT BALB/cJ mice resulting in pain of paw region (Figure 13B). However, immunization CCR2-/- BALB/cJ mice (Figure 13A) were not showed any paw pain development at time courses versus WT peptide immunized mice (Figure 13B). Peptide immunized CCR2 -/- mice (Figure 13A) have the same pain responses with WT CFA immunized control group (Figure 13D) which also showed CCR2 -/- mice is again very important in pelvic pain development. Mechanical stimulation of paw area by the filaments resulted in non-significant increment between CFA immunized mice groups compared to baseline mice (without any injection) on the paw region (Figure 13D) during the 40 days of the experiment. In contrast, there was a difference between naive (baseline) mice and peptide-

immunized mice in terms of paw pain percentage response at all fibers on paw region (Figure 13B). There was no significant difference between naive (baseline) mice and peptide-immunized CCR2 -/- mice in terms of paw pain percentage response at all fibers on paw region (Figure 13A) during the 40 days of the experiment. 50% threshold of immunized mice is significantly decreased compared immunized CCR2 -/- mice due to being more sensitive to the pain (Figure 13C). However, 50% threshold of CFA mice is almost same compared CCR2 -/- (Figure 13F). Those data showed that CCR2 plays important role in paw area related pain development of EAC mice model. Based on these studies, we can use CCR2 as a target in term of further investigation of pain.

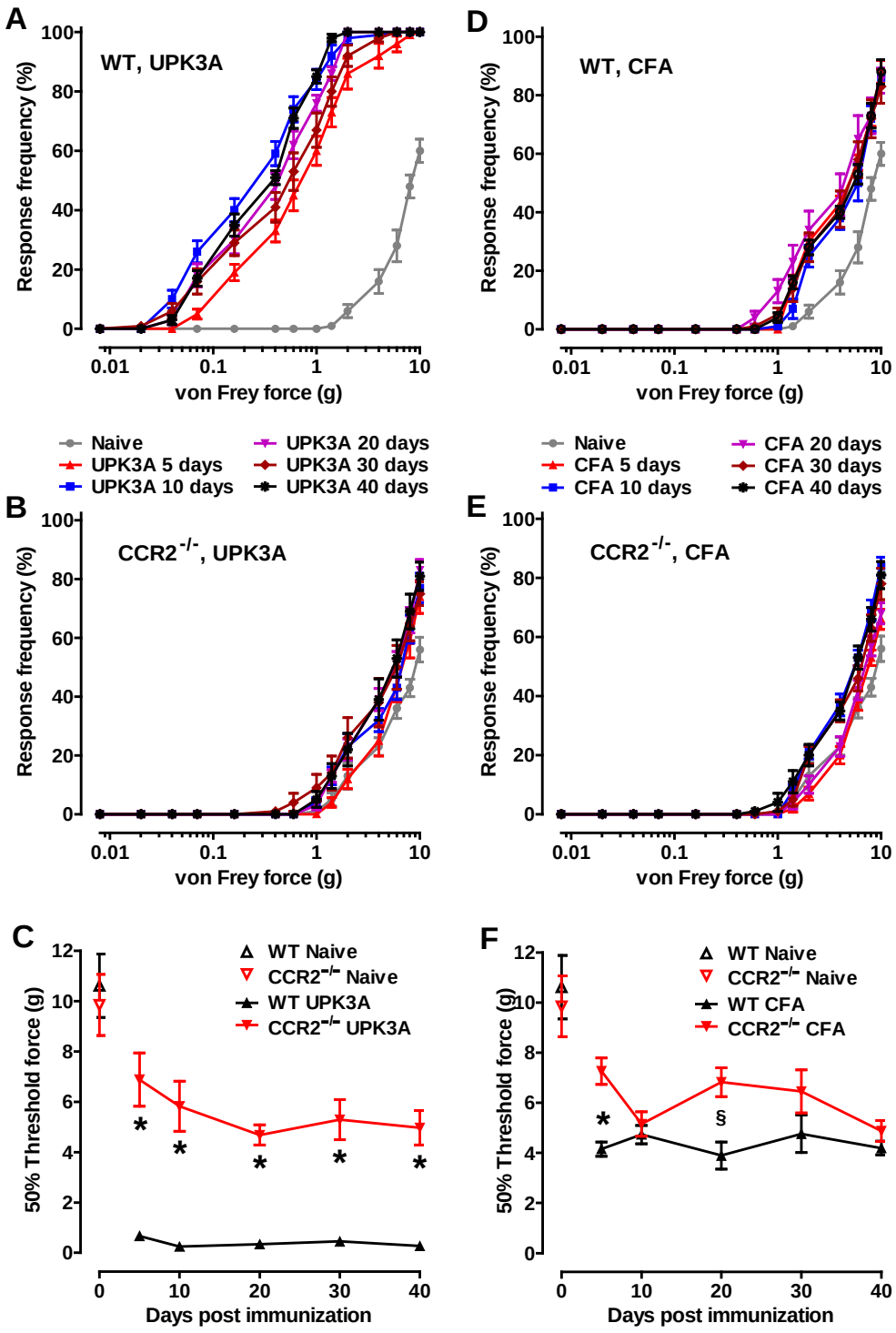
A
WT, UPK3A
Response frequency (%)
von Frey force (g)
Naive
UPK3A 5 days
UPK3A 10 days
UPK3A 20 days
UPK3A 30 days
UPK3A 40 days
D
WT, CFA
CFA 5 days
CFA 10 days
CFA 20 days
CFA 30 days
CFA 40 days
B
CCR2-/-, UPK3A
E
CCR2-/-, CFA
C
50% Threshold force (g)
Days post immunization
WT Naive
CCR2-/- Naive
WT UPK3A
CCR2-/- UPK3A
F
WT CFA
CCR2-/- CFA

Figure 13: CCR2$^{-/-}$ mice exhibit significantly less hindpaw pain compared with WT BALB/cJ mice after immunization with UPK3A 65-84.

The mice used to generate the data for Figure 12 were also assessed for referred visceral hyperalgesia by applying von Frey filaments to the hindpaw. Mean response frequencies ± SEM were plotted (*A*, *B*, *D*, *E*) and mean 50% thresholds ± SEM were calculated and plotted (*C*, *F*) as in Figure 12. UPK3A 65-84 immunization yielded significantly less hindpaw pain in CCR2 $^{-/-}$ mice than it did in WT mice at all time points (*C*; *****p<0.0001 by unpaired t tests of log transformed 50% pain thresholds). CCR2$^{-/-}$ mice immunized with CFA exhibited significantly less hindpaw pain than WT CFA-injected mice 5 and 20 days after immunization (*F*; *****p<0.0001 and $^{\S}p$=0.003 by unpaired t tests). 50% thresholds in CCR2$^{-/-}$ mice injected with UPK3A 65-84 were significantly lower than in CCR2$^{-/-}$ mice injected with CFA 20 days after immunization (p=0.006 by unpaired t test), but not at any other time point (compare red lines in *C* and *F*).

12-CCR2 receptor plays very important role in MCs accumulation in balder that leads pelvic pain.

CCR2 is a receptor for MCP-1. The aim is to show whether the CCR2 involves in MC accumulation. CCR2 -/- mice were obtained as a gift from Dr. Naofumi Mukaida Cancer Research Institute, Kanazawa University (Kanazawa, Japan). After all groups' immunization, all mice (CFA injected WT mice, n=10; UPK 3A 65-84 injected WT mice, n=10; UPK 3A 65-84 injected CCR2 KO mice, n=10; CFA injected CCR2 KO mice, n=10) were scarified to harvest their bladder for mast cell counting by staining of toluidine blue. The results showed that total mast cell decreased in CCR2 -/- mice bladder compared to peptide immunized WT mice. The same correlation also occurred in resting and activated mast cell accumulation. All results are significantly different (*****p<0.001, $^{\S}p$<0.01).

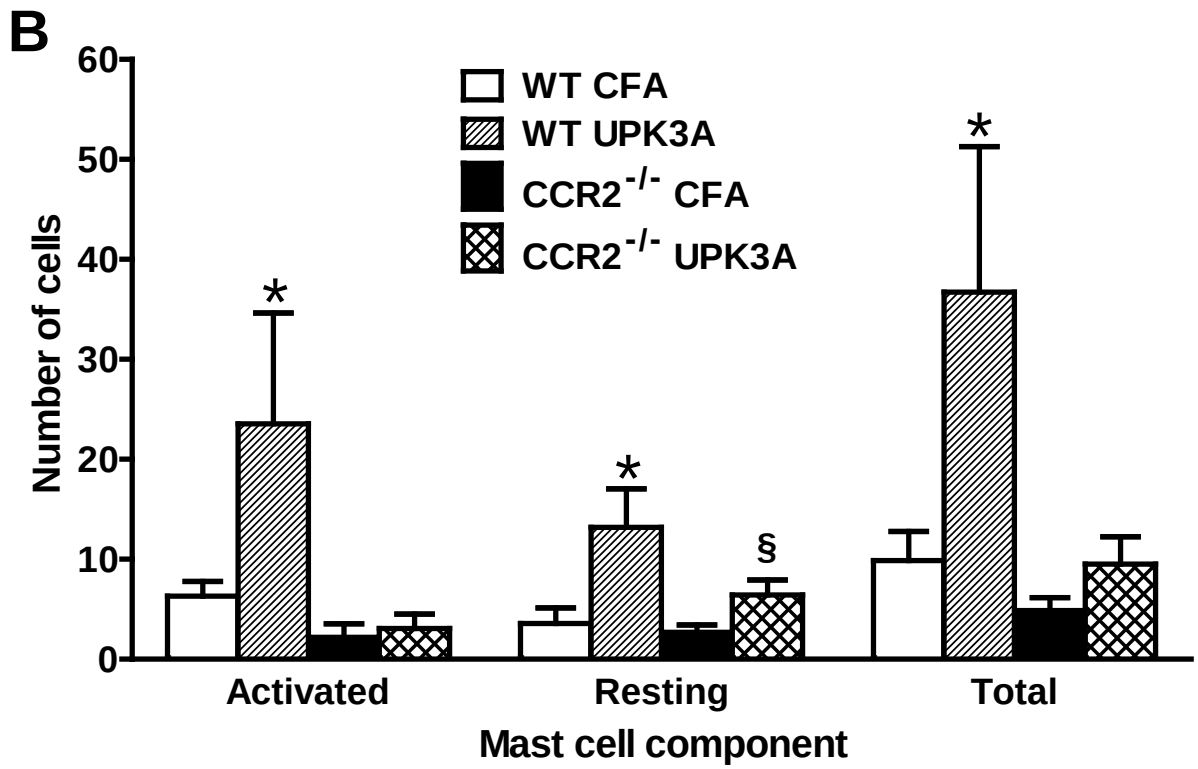

Figure 14: MC accumulation and activation in the bladder in response to immunization is markedly attenuated in CCR2$^{-/-}$ mice.

On the 40th day after immunization, all mice in which pain was assessed for Figures 12,13 were sacrificed one hour after pain assessment, then bladders were harvested and processed for toluidine blue staining of MCs as described in Materials and Methods. Activated and resting MCs were counted in bladder cross sections from UPK3A 65-84- and CFA-immunized WT and CCR2$^{-/-}$ mice (n=10 mice per group). Numbers of activated, resting, and total MCs were significantly higher in bladders from UPK3A 65-84-immunized WT mice compared to CCR2$^{-/-}$ mice immunized with either UPK3A 65-84 or CFA and to CFA-immunized WT mice (*A*; ***p*<0.001 by one way ANOVA with Tukey's post test comparing UPK3A 65-84-immunized WT mice with every other group; no other pairs of groups differed significantly from each other). except a significant increase in number of resting mast cells was also found in UPK3A 65-84-immunized vs. CFA-immunized CCR2$^{-/-}$ mice ($^{\S}p$<0.01 by Tukey's post test comparison). Each column indicates the mean plus SD.

CHAPTER IV

IV.DISCUSSION

In the United States, it is estimated that approximately 1.2 million women and 82,000 men suffering from IC/PBS and the economic burden of IC/PBS patients in the US was nearly $65.9 million in 2000[8]. The etiology of interstitial cystitis disease has not been revealed yet[25]. The major symptoms of IC/PBS are urinary frequency, urgency and pelvic pain[391]. Pelvic pain is the most common symptom of IC/PBS, and also the most annoying reason that brings the patients to the physician[324]. Numerous animal models have been developed to investigate the pathogenesis of IC/PBS. The mouse model, which is generated by bladder specific Uroplakin derived UPK3A 65-84 peptide induced experimentally autoimmune mediated cystitis, presents the all three major phenotypical characteristic of IC/PBS seen in patients in terms of urodynamic alterations and particularly pelvic pain. UPK3A 65-84 induced EAC model is very potent in terms of bladder specific inflammation among the other created mouse models.

The various studies have been projected that there could be a relation and correlation between mast cell and chronic pelvic pain in some diseases such as IBS, IC, and prostatitis [273, 392].

It has found that secreted products from MCs directly or indirectly have effects on IC/PBS [328, 329]. Secretions of the activated MCs cause neural sensitization and more secretion of mast cell activators and it is believed that this vicious cycle may contribute the pain in IC/PBS [33, 38].It is still not clear why MCs migrates to the bladder and proliferates in the bladders of IC/PBS patients. One possible explanation is that damaged urothelium cells secrete cytokines and growth factors that cause migration of the MCs to the bladder and own activation [49, 325, 331-334].

Moreover, it was also shown that MCs have a pivotal role in the IC/PBS pathogenesis via stimulation of neuronal systems or other unknown mechanism [336]. It was demonstrated that there is a communication between MCs and neurons through mediators [335, 393]. Mast cells mostly accumulate at the end of nerve endings in many different tissues, including the epithelium [335, 337, 338]. Neurons and MCs have histamine receptors on their surfaces [274, 276]. MCs can induce neuronal activity by releases of histamine [335]. MC releases histamine which induces degranulation of itself [270]. Histamine depolarizes neurons [272] and mediates pelvic pain [273]. CGRP (calcitonin gene-related peptide), substance P released from neurons can activate MCs [335, 336].

Furthermore, a study revealed that IC patients exhibited increased levels of MCP-1 chemokine in urine, and bladder tissue and the expression of MCP-1 was correlated with IC[350]. MCP-1 has been suggested as a biomarker for the CPPS and

IC/PBS [349, 350]. The effect of MCP-1 and its -chemokine-receptor-2(CCR2) on causing neuronal hyperexcitability after chronic nerve compression injury was shown [351]. MCP-1 itself plays a role in degranulation of mast cell[348]. The roles of the MCP-1 and CCR2 in microglial activation and neuropathic pain have been shown [394]. In another study, the administration of MCP-1 to rat induced tactile allodynia, in the contrast, MCP-1 neutralizing antibody reduced neuropathic pain[352].

Our first report is to show the model develop chronic pelvic pain sourced from bladder. Secondly, we showed MC accumulates in bladder. Even though some investigator showed mast cell product such as histamine increased in human IC/PBS[327] and expression of monocyte chemoattractant protein (MCP-1) was increased in IC/PBS patients[350], but MC was not showed in transcription and tissue level.

In our study, we showed that UPK3A 65-84 peptide induced EAC mouse model develops chronic pelvic pain at different time points. The localization, modulation and regulation of pelvic pain are characterized in this cystitis model. We showed this chronic pelvic pain development in detail, because in our first study, pelvic pain has not been systematically characterized in EAC model[395]. Pelvic pain in this model remains continual that closely mimic pelvic pain of IC/PBS seen in human. The pain that reflected on suprapubic region sourced from bladder is proofed by lidocain installation. The pelvic pain was pharmacologically decreased by histamine related blockers; ranitidine, cetirizine and cromolyn sodium. In addition to that, mast cell related blockers showed that the pain in this cystitis mouse model is due to mast cell accumulation and its secretions.

We pharmacologically, genetically and at immunostaining level showed that MCP-1 mediated Mast cell accumulates in bladder that correlate exactly with pelvic pain at different time points. MCP -1's translational and transcription level and the amount of mast cells in bladder significantly increased from 5 days after the peptide induction to 40 days in BALB/c mice.

We also investigated the role of CCR2, which is also called MCP-1 receptor, in pathogenesis of chronic pelvic pain of EAC. The first step is that we used CCR2 (MCP-1 receptor) antagonist that significantly decreased pelvic pain and MC accumulation into bladder by time points. Chemokine (C-C motif) receptor 2 [CCR2], a well-known MCP-1 receptor, is located on the surface of MCs and has a role in migration of MC [203].

The best way to understand the role of MCP-1 and its receptor (CCR2) in this EAC model is to use their knockout in same strain-BALB/cJ. The result of knockout studies showed that the peptide immunized MCP-1 and CCR2 KO mouse did not develop the pelvic pain and also has almost same or less amount of MC counts in bladder compared to CFA-control immunized WT BALB/c mice. It seems that MCP-1 and CCR2 has a pivotal role in development of cystitis and chronic pelvic pain.

We report MCP-1 and its receptor (CCR2) mediated MCs have a central role in pathogenesis of chronic pelvic pain of EAC as a model of IC/PBS. The increase of MCP-1 in the bladder of EAC model is the most important factor that causes accumulation MCs in the bladder. While the immune response have obviously crucial role, the MC and MCP-1 and its receptor (CCR2) might have an essential consequences for the development and progression of pain symptoms in EAC under the light of our findings.

CONCLUSION

MCP-1 mediates chronic pelvic pain through mast cell accumulation in bladder of EAC mouse model. Our findings on this model will contribute a lot of beneficial aspects to unknown IC/PBS etiology and pathogenesis. With these new discoveries for the disease, we can say that MC and MCP-1 is going to be good candidate in term of therapeutical approaches for pelvic pain treatment clinical trials and also they can be investigate in any other disease related with pain.

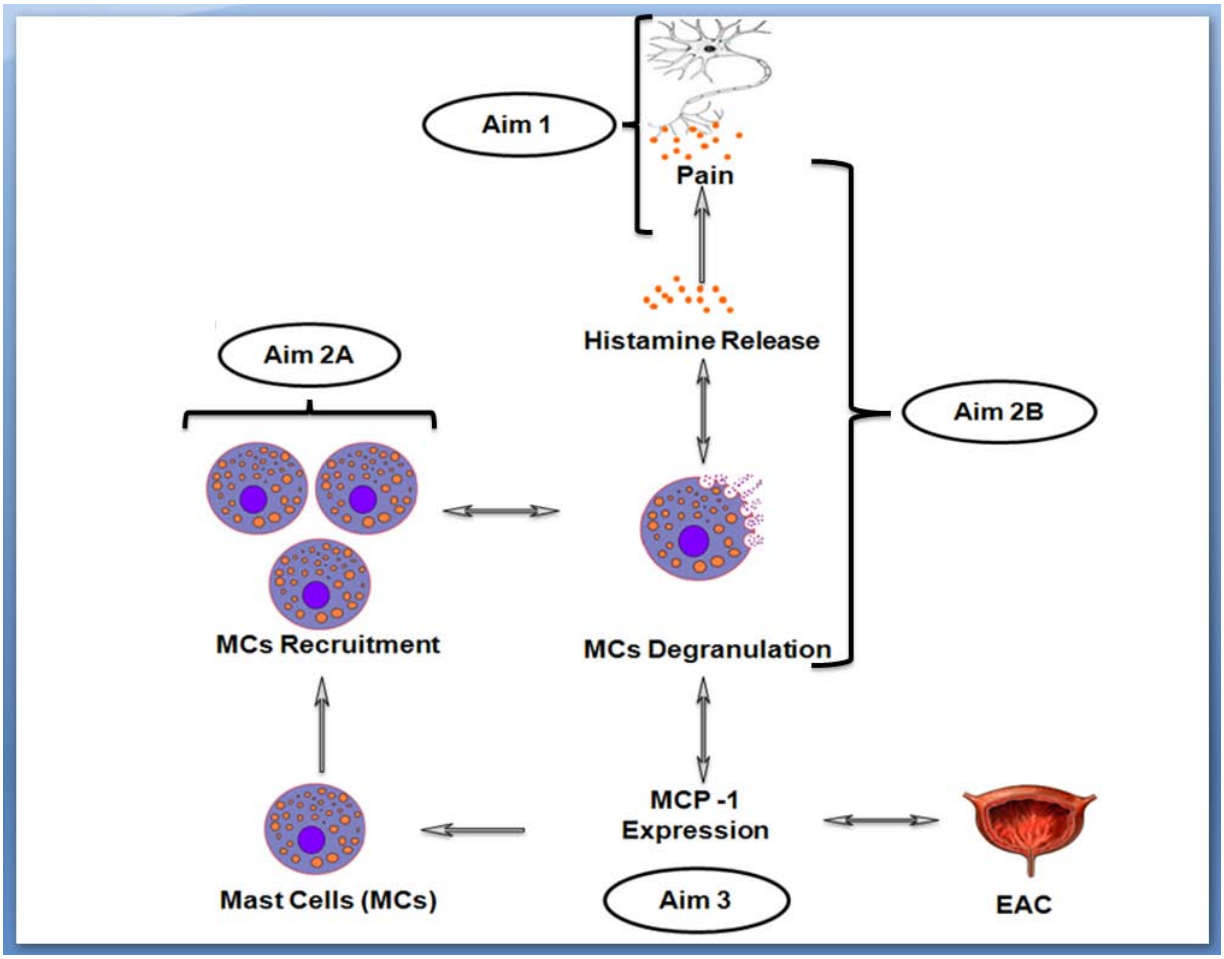

Figure 15: We confirmed all specific aims by the experiments in the figure that shown above. EAC mice developed chronic pelvic pain (1). Mast cell accumulations are increased in bladder of EAC mice (2). Finally, it has been shown that MCP-1 plays a role in chronic pelvic pain of EAC through Mast cells (3).

FUTURE DIRECTIONS

We are going to have further discoveries in our next studies under the light of our new findings with such a wonderful mouse model. We are planning to use this model for immunotherapy of bladder cancer by immunization methods. Gene therapy can be applied in bladder cancer to use MCP-1 gene as a target. New treatment methods can be pharmacologically created in this disease to target MCs, MCP-1, and CCR2.

In bladder cancer, a couple of studies showed that Bacillus Calmette–Guérin (BCG) can be used for immunotherapy of bladder cancer to trigger the inflammation in bladder [396, 397]. In our case, we can increase inflammation in bladder of mice by UPK 3a 65-84 peptide while mouse has bladder cancer. This type of method used in many type of cancer such as prostate, breast, ovarian cancers and other tumors [398, 399]. Other method is a gene therapy that would be constructed plasmid that contain MCP-1 gene. We will install this plasmid into bladder to trigger inflammation while the bladder has cancer. Accumulated white based blood cell will attack the cancer cell to struggle with condition in better bay. For example, in prostate cancer, prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and prostate-specific membrane antigen were used as a target to produce a new vaccine for the disease [400, 401]. One of them is Sipuleucel-T [402, 403]. This drug is in clinical phase trial III and an immunotherapeutic cellular product, which includes autologous dendritic cells pulsed ex vivo with a recombinant fusion protein (PA2024) consisting of granulocyte macrophage colony-stimulating factor and PAP. While applying the drug in prostate cancer patients, T cell will be activated to attach the prostate cancer cell which is expressed PAP [403].One study showed that, monocyte chemoattractant

protein-1 (MCP-1) can play a role in monocyte/macrophage infiltration and enhance their phagocytosis in cervical cancers. With this perspective, we can target MCP-1 as gene therapy for bladder cancer [404].

In addition of that, MCs, MCP-1 and CCR2 can be good candidates in term of new drug discovery for IC/PBS patients. We can pharmacologically design new compound that block the effects of those targets in term of IC/PBS treatment.

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