IMMUNOMODULATORY PROPERTIES OF FELINE MESENCHYMAL STEM CELLS AND THEIR CLINICAL APPLICATION IN TREATMENT OF FELINE CHRONIC IDIOPATHIC CYSTITIS

By

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ABSTRACT

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Mesenchymal stem cells (MSC) offer great promise for treatment of inflammatory disorders. Cats spontaneously develop a variety of disorders that can serve as translational model for human diseases. One such disorder is chronic feline idiopathic cystitis, which closely resembles human Interstitial Cystitis/ Painful Bladder Syndrome. These cystopathies in both cats and humans are characterized by inflammatory cell infiltrate within bladder wall. Since MSC have been reported to migrate to sites of inflammation, bladder wall inflammation can potentially be targeted by MSC, whose immunomodulatory properties could beneficially affect disease expression.

One of the initial steps needed to evaluate use of feline MSC, as a potential therapy is to investigate similarities between feline and human MSC in their immunomodulatory properties. We investigated expression of genes involved in MSC immunomodulation and how they react to stimulation with two cytokines INFγ and TNFα. Unstimulated MSC express similar immunomodulatory genes as human MSC except for FASL and IL10. The reaction of feline MSC to cytokine stimulation is similar to that of human MSC, including the upregulation of IDO in reaction to INFγ stimulation. Other genes upregulated by stimulation included IL-6, PD-L1 and HGF. IL-6 was also significantly upregulated at protein level after stimulation. However levels of HGF were lowered in cell culture supernatants under some conditions. Increased IDO expression was further confirmed through increase in kynurenine, a tryptophan degradation

product. TNFα stimulation resulted in strong upregulation of IL-6 gene and protein expression. Interestingly PGE2 levels remained unchanged after stimulation, although basal expression of this factor was high relative to published human data. Feline MSC were also capable of blocking proliferation of activated peripheral blood mononuclear cells.

Route of injection of MSC can potentially affect the efficacy of MSC therapy. To avoid pulmonary vascular trapping after IV injection we have investigated the intraperitoneal route of MSC injection, as an alternative route of MSC administration. Our studies show intraperitoneal administration of MSC is safe and associated with only mild short-term adverse effects.

Reliable biomarkers are not currently available for FIC. This makes diagnosis dependent on exclusion of other lower urinary tract disorders and evaluation of patient responses difficult. We investigated urine and serum cytokine concentration as biomarkers. We have identified that FIC affected cats have increased urine concentrations of IL-6 and sFAS and increased serum IL-12, IL-18, SDF1 and FLT3.

Lastly, we performed a pilot study to evaluate MSC based therapy for chronic FIC. Unfortunately, only one patient met inclusion criteria and received autologous MSC by IP injection. While reportedly showing an initial favorable response for the first 10 days, clinical signs returned and were progressive. This initial experience was very informative with regard to the many practical difficulties of assessing the effects of such a treatment in this complicated disease.

This dissertation is dedicated to my wife, Magdalena Parys, my parents, Piotr and Teresa Parys as well as my grandfather Eugeniusz Parys.

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KEY TO ABBREVIATIONS

MSC	Mesenchymal Stem Cells
IC/PBS	Interstitial Cystitis/ Painful Bladder Syndrome
INFγ	Interferon Gamma
TNFα	Tumor Necrosis Factor Alpha
FASL	FAS ligand
IDO	Indoleamine 2,3-Dioxygenase
IL-6	Interleukin 6
PD-L1	Programmed Death Ligand 1
HGF	Hepatocyte Growth Factor
PGE2	Prostaglandin E2
FAS	Fas Cell Surface Death Receptor
IL12 (p40)	Interleukin 12 subunit p40
IL-18	Interleukin 18
SDF1	Stromal Derived Factor 1
FLT3L	Fms-Related Tyrosine Kinase 3 Ligand
FIC	Feline Idiopathic Cystitis
CD105	Endoglin
CD90	Thy-1 Cell Surface Antigen
CD73	Ecto-5-Nucleotidase
Th2	T-helper 2 cells
TGFβ	Transforming Growth Factor Beta
iNOS	Inducible Nitric Oxide Synthase
GCN2	General Control Nonderepressible 2

ISR	Induced Stress Response
mTOR	Mechanistic Target Of Rapamycin
PKCθ	Protein Kinase C Theta
NO	Nitric Oxide
EP2	Prostaglandin E Receptor 2
EP4	Prostaglandin E Receptor 4
cAMP	Cyclic Adenosine Monophosphate
TCR	T-cell Receptor
Src	Proto-oncogene Tyrosine-protein Kinase Src
Lck	Lymphocyte-specific Protein Tyrosine Kinase
STAT5	Signal Transducer And Activator Of Transcription 5
CCL2	Chemokine (C-C Motif) Ligand 2
CCR2	Chemokine (C-C Motif) Receptor 2
STAT3	Signal Transducer And Activator Of Transcription 3
MMP	Matrix Metallopeptidase
PD1	Programmed Death 1 Receptor
Treg	T regulatory cell
HMOX1	Heme Oxygenase 1
SnPP	Tin protoporphyrin IX
Tr1	Type 1 regulatory cells
Th3	T helper 3 cells
COX2	Cyclooxygenase-2
CD25	Cluster of differentiation 25 (IL2 receptor alpha chain)
CTLA4	Cytotoxic T-lymphocyte-associated Protein 4
OX40	Tumor necrosis factor receptor superfamily, member 4

GITR	Glucocorticoid-induced TNFR-related protein
HELIOS	Zinc Finger Protein, Subfamily 1A, 2
FOXP3	Forkhead Box P3
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog
PTEN	Phosphatase And Tensin Homolog
TSDR	Treg Specific Demethylation Region
IL2	Interleukin 2
NK	Natural Killer Cells
NKp30	Natural Killer Cell P30-Related Protein
NKp44	Natural Killer Cell P44-Related Protein
NKG2D	NK Cell Receptor D
BLIMP-1	Beta-Interferon Gene Positive-Regulatory Domain I Binding Factor
PAX5	Paired Box 5
NZB	New Zealand Black
NZW	New Zealand White
IL-10	Interleukin 10
JAK	Janus Kinase
STAT	Signal Transducer And Activator Of Transcription
CCR7	Chemokine (C-C Motif) Receptor 7
IL-1Ra	Interleukin 1 receptor alpha
IL-1b	Interleukin 1 beta
IV	Intravenous
IBMIR	Instant Blood-Mediated Inflammatory Reaction
IP	Intra-peritoneal
MRI	Magnetic resonance imaging

PET	Positron emission tomography
SPECT	Single-photon emission computed tomography
CD44	Hematopoietic Cell E- And L-Selectin Ligand
MHCII	Major histocompatibility complex II
PBMC	Peripheral Blood Mononuclear cells
FBS	Fetal Bovine Serum
KNAC	Keratinocyte SFM medium supplemented with N-acetyl-L-cysteine and ascorbic acid 2- phosphate
DMSO	Dimethyl sulfoxide
PTGES1-3	Prostaglandin E Synthase 1-3
CK	Creatine kinase
FeLV	Feline Leukemia Virus
FIV	Feline Immunodeficiency Virus
NSAIDs	Non-steroidal anti-inflammatory drugs
sFAS	Soluble Fas Cell Surface Death Receptor
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-13	Interleukin 13
IL-18	Interleukin 18
KC	Chemokine (C-X-C motif) ligand 1
MCP-1	Monocyte Chemotactic Protein 1
PDGF-BB	Platelet-Derived Growth Factor Receptor, Beta Polypeptide
RANTES	Chemokine (C-C Motif) Ligand 5

SCF	Stem Cell Factor
SD	Standard deviation
BCG	Bacillus Calmette–Guérin
Th1	T-helper 1
CXCL12	Chemokine (C-X-C Motif) Ligand 12
CXCR4	Chemokine (C-X-C Motif) Receptor 4
FLT3	Fms-Related Tyrosine Kinase 3
APF	Antiproliferative Factor
CXCL-1	Chemokine (C-X-C motif) ligand 1
CXCL-10	Chemokine (C-X-C motif) ligand 10
BB-CLIA	Bead based chemiluminescent immunoassay
EGF	Epidermal Growth Factor
ELISA	enzyme-linked immunosorbent assay
GP51	Urinary Glycoprotein (51 kDa)
LC-MS/MS	Liquid chromatography tandem mass spectroscopy
IRMS	Infrared microspectroscopy
NMR	Nuclear magnetic resonance
Q-TOF-MS	Quadrupole-time-of-flight mass spectrometry
qPCR	Quantitative PCR
MALDI-TOF-MS	Matrix Assisted laser desorption ionization time of flight mass spectroscopy
MSU-VMC	MSU Veterinary Medical Center
PDS	Poly (p-dioxanone)
PLGA	Poly(lactic-co-glycolic acid)
gp130	Glycoprotein 130

IL6ST	Interleukin 6 signaling transducer
LIF	Leukemia Inhibitory Factor
IL-11	Interleukin 11

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Mesenchymal stem cells and their immunomodulatory capacities: an overview

Mesenchymal stem cells (MSC) are of growing interest in both human and veterinary medicine. The current most common uses for MSC are tissue engineering/regenerative medicine[1] and immunomodulation . This review pertains to the immunomodulatory properties of mesenchymal stem cells. These cells were initially identified in bone marrow as colony forming fibroblasts with potential for differentiation into various tissues [2-4]. Subsequently MSC were isolated from adipose tissue, amniotic fluid, and other tissues [1,5]. Their ease of isolation, characterization, and expansion [1,3,6] make these cells an ideal candidate for cell-based therapies. MSC identification is based on characterization of their multipotency and expression of specific surface markers [7]. To meet criteria proposed by the International Society for Cellular Therapy, MSC should be capable of differentiation into osteocytes, chondrocytes and adipocytes as well as display expression of surface markers CD105, CD90, CD73 and lack of expression of hematopoietic makers [1,6,7]. Investigations into the role of MSC in bone marrow homeostasis have led to the study of their potential for supporting bone marrow transplantation [8,9] and subsequently, to study of their immunomodulatory properties. Both *in-vitro* and *in-vivo* studies have shown that MSC are capable of inducing anti-inflammatory phenotype and/or blocking effector function of majority of immune cells in the organism including T-cells, NK cells, B-cells, dendritic cells, macrophages or mast cells [10-16]. Many investigators initially studied interactions between MSC and T-cells. MSC are capable of inducing T-cell anergy [17], suppressing expansion of T-cells [18] and priming of T-cells by dendritic cells [19] as well as inducing T-cell apoptosis [20]. Despite extensive study, the specific mediators of these events have yet to be fully characterized. Initial immunologic studies suggested a

role for HGF and TGFβ[18]. Both HGF and TGFβ are known immunomodulatory factors. However, the effect of HGF on T-cell function is still under debate. Results of some studies indicate that HGF is capable of reducing proliferation of CD4 cells and inducing Th2 response, as well as decreasing formation and effector function of CD8 cells [21,22]. Other studies suggest an indirect HGF-induced effect on antigen presenting cells and induction of a more tolerogenic phenotype [23,24]. [21]. TGFβ has multiple effects on T-cells. This cytokine is important for maintenance of immune tolerance and TGFβ knockout mice experience early death due to autoimmune disease [25,26]. Depending on other microenvironmental stimuli, TFGβ can reduce T-cell proliferation or support T-cell expansion [27,28]. This cytokine is also important for survival and generation of T-regulatory cells [29-31].

Subsequent studies investigated IDO as modulator effects of human MSC effects on T-cells [32] and iNOS as mediator of immunosuppressive effects on murine T-cells [33-35]. Genes for both factors are expressed at low levels in MSC of their respective species, but are inducible by INFγ. IDO mediates its immunomodulation through reduction of tryptophan, which is an essential amino-acid necessary for cell proliferation, as well as through increasing concentrations of the metabolite-kynurenine, that exerts antiproliferative effects on T-cells [32]. Mechanistically the reduction of tryptophan leads to activation of stress-sensing GCN2 kinase in T-cells, which in turn activates the downstream integrated stress response (ISR) pathway and effectively blocking proliferation and inducing anergy in T-cells [36]. IDO mediated tryptophan depletion also results in repression of mTOR and PKCθ pathways, which are necessary for T-cell growth and function [37,38]. IDO has been shown to be induced by inflammatory environment in humans, while in mouse nitric oxide (NO) plays this role and blocking studies have shown its important role in suppressing T-cell responses and

induction of IDO is lacking [33-35]. Nitric oxide has been shown to block phosphorylation of STAT5, which suppresses the proliferation of T-cells [34,39]. Interestingly the effect of NO is dose dependent and increasing concentrations of NO result in decrease of INFγ production, thus reducing activation of Th1 cells, whereas low levels of NO resulted in activation of T-cells [40]

PGE2 has been identified as another factor produced by MSC, which has been shown to be involved in MSC-based immunomodulation [32,39,41,42]. This factor decreases the amount of INFγ produced by stimulated T-cells in co-culture experiments [41]. Blocking of cyclooxygenases with selective inhibitors has identified PGE2 as one of the necessary factors for anti-inflammatory effects of MSC [32,39,41,42]. PGE2 exerts its immunosuppressive effect mainly through EP2 receptors and to a lesser extent EP4 receptors on surface of T-cells, both of which are G-coupled receptors [43]. Multiple molecular mechanisms have been proposed to be responsible for effects of PGE2 on T-cells. These include reduction of cellular cAMP-mediated expression of IL2 [44], IL2R [45] and NfκB [46]. In addition, PGE2 appears to affect T-cell receptor (TCR) mediated activation of T-cells through inhibition of one of the Src family kinases- Lck [47,48].

CCL2 is a chemokine that is expressed by MSC and induced under inflammatory conditions[49]. Interestingly, after release by MSC, CCL2 undergoes a MMP-mediated cleavage that modifies chemokine into a CCR2 blocking ligand resulting in suppression of STAT3 phosphorylation in-vitro in activated splenocytes [50]. CCR2 is a crucial factor involved in cell migration and blocking of this receptor with MMP-cleaved CCL2 results in decreased immune cell migration, which is an important element of inflammatory response [51].

The aforementioned factors –HGF, TGFβ, PGE, iNOS, IDO and CCL2, are soluble factors, but it is known that direct contact of MSC and target cells also plays an

important role. Two immunosuppressive surface proteins expressed by MSC include PD-L1 and FASL [20,52-54]. PD-L1 acts upon target cells via its receptor- PD1, inducing a block in T-cell proliferation and production of cytokines [54]. T-cells, B-cells as well as other cells express PD1 receptor [55]. Expression of PD-L1, an important factor for immune cell homeostasis, has been identified in T-cells and antigen presenting cells [55,56]. Mesenchymal stem cells have been shown to upregulate their PD-L1 expression upon co-culture with stimulated T-cells and splenocytes [52,54,57]. Furthermore, experiments utilizing blocking antibodies and siRNA have provides additional evidence supporting the importance of PD-L1 in mediating the immunomodultory effects of MSC [54,57,58]. However, other studies did not detect expression of PD-L1.

FASL has also been implicated as a factor involved in immunomodulatory role of MSC[20] as a single factor or coexpressed with PD-L1 [57]. FASL binds to FAS (CD95) and this interaction leads to activation of T-cell apoptosis [59]. MSC-expressed FASL is capable to induce apoptotic death of T-cells when cultured in in-vitro [59] as well as in in- vivo experiments [20].

Another important feature of MSC is their role in induction of T-regulatory cells (Tregs), which have been identified both *in-vitro* as well as *in-vivo* [60-64]. Different types of Tregs have been reported including nTregs (natural Tregs) as well as iTregs (induced Tregs) [61]. nTregs are produced in the thymus while iTregs are derived from peripheral blood T-cells. These two forms of Tregs differ in expression of their cell surface markers (CD25, FOXP3, CTLA4, OX40, GITR, HELIOS), which allows for identification of cell subsets [65]. Each subset of T regulatory cells release various immunosuppressive cytokines including TGFβ1 and IL-10 [65]. In addition, these cells are capable of blocking proliferation through cell contact dependent mechanisms [66]. Multiple factors have been implicated in Treg induction by MSC [60-62]. One of the

genes involved in formation of these regulatory cells is HMOX1, encoding an enzyme involved in hemoglobin degradation [61]. Experiments using HMOX1 specific inhibitor SnPP have implicated a role of this enzyme in formation of iTregs, specifically Tr1 and Th3 [61]. Simultaneous inhibition of COX2 and TGFβ1 has significantly reduced CD4 T-cell expression of FOXP3, a crucial transcription factor involved in formation of Tregs [62]. Similar results have been obtained in transwell system preventing cell-to-cell contact [62]. In other studies, PD-L1 has been implicated in induction of iTregs in the periphery through reduction of phosphorylation of Akt and mTor, while increasing phosphorylation of PTEN [67]. Methylation of FOXP3 Treg specific demethylation region (TSDR) is an important marker for distinguishing between nTreg and iTregs [68]. Evidence suggests that MSC are capable of inducing iTreg phenotype, with strongly methylated FOXP3 TSDR, but also to a lesser extent, promoting IL2 dependent proliferation of nTregs [60]. *In vivo* studies have confirmed *in vitro* observations and increases in Tregs cells have been identified both preclinical models [64] as well as human clinical trials [69].

Natural Killer (NK) cells are also a target of two MSC produced factors- PGE2 and IDO [70]. These factors block proliferation of NK cells and decrease expression of NKp30, NKp44 and NKG2D, which are NK cell activating receptors, as well as reduce cytotoxicity and INFγ production by these cells [70]. The effect of MSC on NK cell proliferation depends on cell dose and MSC are subject to NK cell mediated lysis [71].

B-cells are also differently affected by MSC. Contrary to other adaptive immunity-related cells, MSC promote proliferation of B-cells. However, MSC can block or reverse B-cell differentiation into plasma cells [72,73]. MSC-derived and cleaved CCL2 is considered to play an important role in B-cell proliferation and dedifferentiation[73]. Modified CCL2 binding to B-cell's CCR results in decrease

phosphorylation of AKT and STAT3, which down-regulates BLIMP-1 and upregulates PAX5 [73]. This pathway modification leads to de-differentiation of plasma cells and inhibiting immunoglobulin production. Authors of the study suggest that the proposed model may affect only CCR+ subset of B-cells [73]. Other studies of human MSC have suggested that MSC promote the expansion as well as differentiation of B-cells through direct cell contact [74]. Subsequent studies in a mouse lupus model (NZBx NZW mouse) confirmed the findings that MSC can worsen not only disease progression, but also increase kidney damage and proteinuria [75]. These findings emphasize the necessity for future studies investigating the safety of MSC-based treatment of Th2 type of disorders with strong B-cell involvement. Conversely, it has been recently shown that MSC are capable of forming B-regulatory cells, both *in-vitro* and *in-vivo* [76,77]. Induction of these IL-10 producing cells occurs through cell contact-dependent mechanisms and IDO activity has been identified as being partially responsible for this activity in co-culture experiments [76,77].

Antigen presenting-cell interactions with MSC have been intensively studied. It has been shown that PGE2 induced IL6 expression affects dendritic cell (DC) maturation [78]. JAK- STAT is thought to be involved in this mechanism and subsequent induction of IL10 expression [79,80]; however, some authors suggest a contact dependent mechanism rather than soluble factor involvement [80]. Notch signaling has also been implicated the in the effect of MSC on DC maturation. Co-culture experiments of MSC and DC resulted upregulation of expression of Notch receptors and subsequent blocking experiments resulted in reversal of immature phenotype [81]. MSC also decrease migration of DCs and T-cell priming [82,83]. DCs exposed to MSC decrease expression of CCR7, an important gene involved in homing of DCs to the lymph nodes, increase production of anti-inflammatory cytokines IL-10, IL-1Ra, and decrease release of

proinflammatory cytokines IL-12 and IL-1b [82,84]. Similar MSC-induced effects were observed in macrophages in *in-vitro* and *in-vivo* [85,86].

MSC produced PGE2 was also involved in mast cell function inhibition including degranulation, migration and TNFα production [87]. These effects were obtained in both *in-vitro* as well as *in-vivo* setting and it's mediated through EP4 receptor expressed on surface of Mast cells.

1.2 Sources of MSC

Mesenchymal stem cells can be isolated from various tissues including bone marrow, adipose tissue, placenta, Wharton Jelly as well as other sources [1,6,88,89]. The most commonly used tissue sources are bone marrow and adipose tissue. The main advantage of using adipose tissue over bone marrow as source of MSC in small animal medicine is the ease of MSC isolation and the ability to obtain numbers of MSC sufficient for subsequent injection [6]. Limited information is available on differences between the immunomodulatory properties of MSC derived from adipose tissue or bone marrow. A recent report suggested that adipose tissue-derived MSC release significantly higher concentrations of immunomodulatory factors than bone marrow MSC [90]. However, in another study, significant differences were not observed between the immunomodulatory properties of bone marrow- and adipose tissue-derived MSC [91]. The reason for differences between two studies is unclear. Increased passaging has been shown to affect MSC gene expression and effector functions [92,93] and it is possible that cells at different passage levels were utilized. However, specific cell passage levels used in either study were not reported. [90, 91]. Bone marrow derived MSC have been shown to undergo senescence earlier then adipose tissue derived MSC in a single study[94].

1.3 Delivery routes of MSC for treatment

The optimal route for delivery of any cellular therapy should maximize the number, survival, and localization of transplanted cells, while minimizing adverse effects to the patient. Depending on the goal of the treatment, MSC have been delivered by a variety of routes. For the purpose of systemic immunomodulation, the most common route of administration is by intravenous (IV) injection. This route has been utilized in multiple disease models as well as clinical trials in humans, cats and dogs [95-98]. Although generally considered to be safe, in some companion animal studies, IV injection of MSC was associated with adverse effects such as vomiting, possible thromboembolism and pulmonary edema and hemorrhage [96,99]. MSC injected IV are also known to survive only short term and the majority of them remain within the pulmonary vasculature [100]. Although MSC express Factor H, a known inhibitor of complement cascade [101], these cells are also capable of activating complement [102]. Complement activation is related to expression of Tissue Factor by MSC and leads to emboli formation by the transplanted cells through a phenomenon known as the Instant Blood-Mediated Inflammatory Reaction (IBMIR) [103,104]. Although emboli are potentially detrimental, formation of emboli and IBMIR has been suggested to have a beneficial role in MSC immunomodulation [105]. MSC grown in spheres increase production of PGE2 and this allows for thrombi-infiltrating macrophages to switch from M1 to M2 phenotype resulting in immunomodulation [105,106].

An alternative delivery route for MSC is intraperitoneal (IP) injection [107]. One major advantage of IP over IV administration is the capacity to administer larger numbers of cells without risk of inducing potential life threatening pulmonary thromboembolism. Another advantage, especially for the disorders localizing to the peritoneal cavity, is the close proximity of MSC to the target organ, which allows for a

more efficient migration and cell contact-dependent immunomodulation. Recent studies comparing IV vs. IP injection of MSC in a rat colitis model have shown advantage of the latter [108]. Intranasal, intrathecal and intra arterial injections have been also described for treatment of various disorders [109,110].

1.4 Monitoring the delivery of MSC – challenges and future directions

Accurate tracking of MSC after delivery is desirable to document migration and distribution of cells. An ideal marker for this purpose should exhibit multiple characteristics such as lack of toxicity to the cells and organism, ease of detection, and reliable detection post delivery. Many imaging techniques have been utilized for in vivo cell tracking including MRI, scintigraphy, PET and SPECT [111-114]. Each of these modalities has their limitations that include inclusion of radioactive agent (PET, scintigraphy) potentially decreasing cellular viability, uptake by other cell types after cell death and subsequent non-specific labeling and tracking, as well as the need for highly specialized equipment and knowledge.

MRI-based tracking has several advantages over other types of imaging modalities. One major advantage is lack of exposure to ionizing radiation, which allows for investigating cell migration over time. This technique most commonly utilizes nanoparticles of various sizes and coatings with an iron-oxide core that allows for their detection by MRI imaging through T2* due to susceptibility artifact caused by the iron oxide containing particles [111]. MRI imaging using nanoparticles allows resolution to a single labeled cell [115]. One disadvantage of this technique is that if cells die or are phagocytized, the Fe particle based MRI signal still remains giving a potentially confounding result pertaining to the migration and survival of the cells [116,117]. Effects of iron oxide nanoparticles on MSC have been previously studied. After

nanoparticle loading, MSC are still capable of differentiation into all three cell lineages [118,119]. Similarly, expression of various cytokines were not altered when MSC were exposed to the concentration of nanoparticles necessary for tracking [119]. However, studies in other cell systems suggest that iron particle loading can result in changes in expression of genes involved in metal handling as well as those involved in detoxification and lysosomal function [120]. Loading of macrophages with iron oxide particles resulted in more tolerogenic phenotype as it increased secretion of IL10 and decreased production of TNFα [121]. Further studies are necessary in order to better characterize how iron oxide particles affect cytokine-activated MSC.

1.5 Feline Idiopathic Cystitis

Feline Idiopathic Cystitis is the most common lower urinary tract disorder in domestic cats, affecting a quarter to a half million cats annually in North America. Clinical symptoms include bloody urine (hematuria), straining to urinate (stranguria), painful urination (dysuria), increased frequency (pollakiuria) and urinating outside of the litter box (periuria) [122]. Multiple forms of FIC have been described. In majority of cases (approximately 80%), FIC is an acute self-limiting disorder in which clinical signs last for approximately a week and then spontaneously subside, with or without therapy. Cats that experience an FIC episode are at a high risk of recurrence of clinical signs [123,124]. In severe cases, affected cats never fully recover from the disorder and have frequently recurring or constant clinical signs. These cats are classified as having chronic idiopathic cystitis [122]. Although the clinical features of acute and chronic FIC are well characterized, the underlying causative factors in the pathogenesis of the disorder have not been defined. Likewise, there is no specific diagnostic marker or consistently effective means of treatment and prevention of chronic FIC [122]. Lack of

validated biomarkers to establish a diagnosis and differentiate various disease subtypes, has hampered design and execution of clinical studies investigating the biological behavior, pathogenesis, treatment, and prevention of this disorder. Owner observations of changes in urination behaviors are currently the primary means of assessing outcomes of therapeutic interventions. This imposes a potentially strong bias in the study and potential for placebo effect. Identification of sensitive and specific biomarkers can significantly affect the research of this disease and influence the future development of therapeutic interventions.

The clinical and morphologic features of FIC are strikingly similar to those of an idiopathic cystopathy of humans called interstitial cystitis/painful bladder syndrome (IC/PBS; as summarized in Table 1). In 2011, interstitial cystitis was estimated to affect from 3.3 to 7.9 million women in the United States alone [125]. Previous observations suggest that chronic FIC is associated with various clinical and pathologic phenotypes, which include ulcerative inflammatory and nonulcerative noninflammatory forms.[126-129] The last two phenotypes appear to be similar to nonulcerative (Type I) and ulcerative (Type II; classic) forms encountered in people with IC/PBS. [122,130]. Differences between human and feline cystitis include: differences in presentation (acute as well as chronic hyperproliferative forms have not been reported in humans), age of onset (young to middle aged cats vs. middle aged and older people), different distribution of pathological changes (in FIC changes are usually confined to the urothelium and suburothelial space rather than the entire bladder wall as observed in affected people.

Despite these differences, FIC shares some remarkable similarities with IC/PBS. FIC represents the only spontaneously occurring disease model of human IC/PBS. Multiple induced models of IC/PBS have been described in mouse, rat, rabbits and

monkeys [131]. These include cyclophosphamide induced cystitis [132], uroplakin autoimmune model [133] or acetone treatment[134]. However, most of these models characterize acute changes, which can limit their applicability to IC/PBS. In contrast, the spontaneous occurrence of chronic disease makes FIC an excellent translational model for novel therapies such as MSC based approaches, which will be discussed in this dissertation.

APPENDIX

Figure 1.1 Soluble and membrane bound factors expressed by Mesenchymal Stem Cells affect a wide variety of immune cells in the body

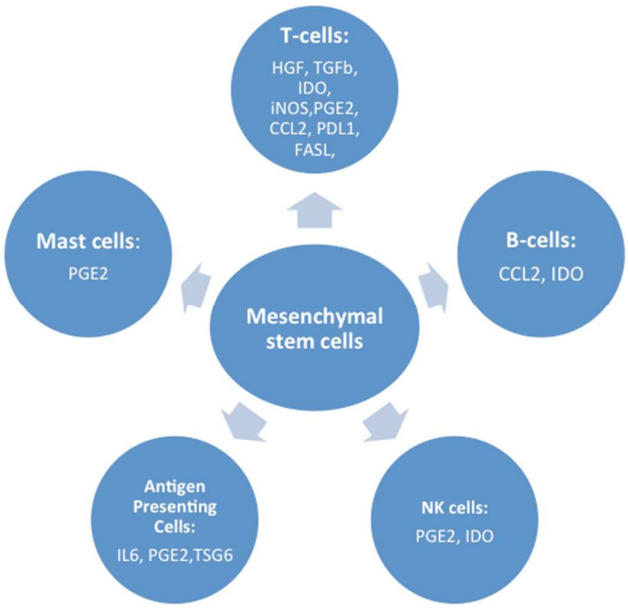

Table 1.1. Comparison of various features of feline idiopathic cystitis and human interstitial cystitis.

	Feline Idiopathic Cystitis		Human Interstitial Cystitis/Painful bladder Syndrome	
	Acute	Chronic	Ulcerative	Non ulcerative
Presentation	- Clinical signs < 7 days - Self limiting - Male cats- at risk for urinary tract obstruction - Relapses in up to 65% of cases [122,135]	- Clinical signs persist or frequently relapse - Three forms: ulcerative, non ulcerative and hyperplastic. [127,128,136]	- Chronic disease - Clinical signs: pain, urgency, frequency - Clinical signs lasting for at least 6 months - Clinical signs more severe in ulcerative form [137,138]	
Diagnosis	- Based on exclusion of other potential causes - Cystoscopy rarely performed [139]		- Based on exclusion of other potential causes of the disease - +/- cystoscopic identification of bladder lesions(gromerulations and/or Hunner's lesions), - Histopathological confirmation of changes [137,138]	
Sex predisposition	- No apparent sex predisposition [140]		- More common in women, - Potentially under/mis-diagnosed in men [137,138,141]	
Age	- Young to middle aged animals [123,140,142]		- Middle-aged and older [141]	

Table 1.1 (cont'd)

	Feline Idiopathic Cystitis		Human Interstitial Cystitis/Painful bladder Syndrome	
	Acute	Chronic	Ulcerative	Non ulcerative
Urothelial changes	- NR	- Variable loss of urothelial barrier [143] - Increased permeability [143] - Disruption of tight junctions - Changes in urothelial glycocalyx[143,144] - Loss of umbrella cells [145] - Decrease in bladder volume	- Variable loss of urothelium [146]	- Frequently complete denudation of mucosal surface [147]
			- Variable results of permeability studies [145] - Changes in expression of proteins involved in urothelial barrier [148] - Decrease in bladder volume	
Mat cell infiltration	- NR	- Present in the mucosal and submucosal layer - Mixed reports concerning detrusor muscle [129,136]	- Present in mucosal stroma and detrusor [146] - Absent in urothelium layer[149]	- Present in all layers: mucosal stroma, detrusor and urothelial layer [149]
Inflammation	- NR	- Mononuclear and mixed cell infiltrate frequently present in mucosal and submucosal layer [127]	- Present in small amount - Detrusor normal [147]	- Strong inflammatory response present in all layers [147]
Fibrosis		- Present in submucosal surface [129]	- Absent [146]	- Present in detrusor [147]

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CHAPTER 2

EVALUATION OF IMMUNOMODULATORY PROPERTIES OF FELINE MESENCHYMAL STEM CELLS

2.1 Introduction

Mesenchymal stem cells (MSC) have been intensively studied since their first characterization in 1970s [1]. The more recent discovery that MSC are capable of modulating a wide array of immune cells including T-cells, natural killer (NK) cells, dendritic cells, macrophages, B-cells, mast cells and neutrophils [2-9] and induction of T-regulatory and B-regulatory cells [10,11] has led to interest in their use in patients with inflammatory disorders. In the settings of both induced inflammatory disorders in rodents,[12,13] as well as spontaneously occurring disorders [11,14,15] the MSC-based therapy has been successfully implemented and are approved for use in some patients with GVHD in New Zealand and Canada. Many questions still remain unanswered such as optimal route of injection and dose to be used and how these will impact diseases that can be potentially targeted. Use of large animal models such as cats may assist in the preclinical phase of investigations for humans.

Cats spontaneously develop a variety of inflammatory disorders such as asthma [14], chronic idiopathic (interstitial) cystitis [16], chronic pancreatitis [17], chronic kidney disease [18] or inflammatory bowel disease [19], which can serve as potential translational models for cell based therapies.

Many inflammatory mediators have been implicated in immunomodulatory properties of MSC. These factors include IDO, iNOS, PGE2, IL10, HGF, TGF-beta, IL6, PD-L1, FASL, HMOX1 [3,4,8,15,20-24]. Interspecies differences exist in immunomodulation between human MSC and murine MSC [20,21,25]. In mice, the immunomodulation is considered to be iNOS dependent [26], while in humans it is IDO1 dependent and iNOS is expressed at very low levels [20,25]. Information on expression of immunomodulatory genes in cats is lacking. Therefore, the current study was undertaken to characterize the immunomodulatory properties of feline MSC.

2.2 Materials and methods

2.2.1 Isolation of feline MSC

MSC were isolated from adipose tissue of healthy adult research cats [20.7 (±5.7) months] as previously described by our group for canine MSC [27]. Tissues were collected from animals under approved procedures of the Institutional Animal Care and Use Committee at Michigan State University (AUF#: 09/12-171-00). In brief, adipose tissue (2-4 grams) was sterilely excised from subcutaneous tissues, minced and incubated in Collagenase I (Sigma-Aldrich, St.Louis, MO) at a concentration of 1 mg/ml in HBSS (Sigma-Aldrich, St.Louis, MO) for 2 hours at 37°C with 5% CO_2. The digested tissue was then passed through 70µm cell strainer in order to create a single cell suspension and washed in sterile PBS (Sigma-Aldrich, St.Louis, MO). The cell pellet was then suspended in KNAC medium [Keratinocyte SFM medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) supplemented with 2mM of N-acetyl-L-cysteine (Sigma-Aldrich, St.Louis, MO) and 0.2 mM of L-ascorbic acid 2-phosphate (Sigma-Aldrich, St.Louis, MO)] with 5% of MSC-grade FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) and plated in a T25 flask. Cells were trypsinized with 0.05% trypsin after reaching 80% confluency and subsequently passaged using KNAC medium. At each passage, half of the cells were cryopreserved utilizing Basal Medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) supplemented with 20% of FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) and 10% DMSO (Sigma-Aldrich, St.Louis, MO). Cells utilized in the experiments described in the manuscript were from passages 3-8.

2.2.2 Phenotypic characterization

All MSC cell lines were characterized for tri-lineage differentiation as well as expression of CD90, CD44 and CD105 on cell surface of the cells. Trilineage

differentiation was accomplished through utilization of Stempro (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) differentiation medium specific for each lineage of differentiation. Experiments were carried out according to manufacturers recommendations in 24 well plates (USA Scientific, Ocala, FL) for adipogenesis and osteogenesis. For chondrogenesis, instead of using a plate, a 15 ml spinning tube was utilized to form micromass of $1x10^6$ cells/tube. Cells were incubated in differentiation media for at least two weeks before staining for adipogenesis, utilizing Oil-o-red stain. Two-week incubation period was also used for characterization of chondrogenesis and subsequent Alcian Blue staining. Cells in the osteogenesis group were incubated for 3 weeks and stained with Alizarin red stain.

Expression of cell surface markers CD90, CD44, CD105, MHCII was analyzed using flow cytometry, using previously described [28,29] antibodies: CD90 (clone 5E10, BD Biosciences, San Jose, CA), CD44 (clone IM7.8.1, Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY), CD105 (clone SN6, Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY), MHCII (clone Tu39, BD Biosciences, San Jose, CA). All samples were characterized using LSRII flow cytometer (BD Biosciences, San Jose, CA). Data was then analyzed using commercially available software (FlowJo, LLC, Ashland, OR).

2.2.3 PBMC isolation

Peripheral blood was collected into EDTA tubes. Peripheral blood mononuclear cells (PBMC) were isolated using gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St.Louis, MO) in Accuspin tubes (Sigma-Aldrich, St.Louis, MO) according to manufacturer recommendations. Collected cellular interface was washed twice with PBS (Sigma-Aldrich, St.Louis, MO) and cryopreserved in Basal Medium (Gibco, Life

Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) supplemented with 20% of FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) and 10% DMSO (Sigma-Aldrich, St.Louis, MO).

2.2.4 INFγ and TNFα stimulation

In order to evaluate the effects of inflammatory cytokines INFγ and TNFα on gene expression of feline MSC, cells were plated in a 6 well plate at $2x10^5$ in KNAC medium and allowed to attach overnight and then stimulated with increasing concentrations of feline recombinant INFγ (R&D Systems, Minneapolis, MN), ranging from 0.25 to 50 ng/mL, or of feline recombinant TNFα (R&D Systems, Minneapolis, MN), ranging from 0.25 to 10 ng/mL. Cells were incubated for 24 hours. Both cells and supernatant were collected and frozen for subsequent analysis at -80° C.

For protein/metabolite analysis cells were stimulated with a single dose of INFγ (50 ng/mL), TNFα (25 ng/ mL) or both INFγ (50 ng/mL) and TNFα (25 ng/ mL).

2.2.5 Quantitative Real Time PCR

For gene expression analysis, RNA was isolated using mirVana Isolation kit (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY). RNA was quantified on Qubit 2.0 Fluorometer using Qubit RNA HS assay kit (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY). Quality of RNA was evaluated using Bioanalyzer (Agilent, Santa Clara, CA) or Caliper LabChip GX (PerkinElmer, Waltham, MA). Only RNA with RNA integrity number higher then 8 was used for subsequent analyses.

Reverse transcription was accomplished using Superscript III reverse transcription kit (Invitrogen, Thermo Fisher Scientific Inc., Grand Island, NY) and random primer hexamers (Promega, Fitchburg, WI).

QPCR was performed using custom designed TaqMan primers (Life

Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) and TaqMan Universal Master Mix (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) on ABI 7900 HT RT-PCR system or StepOnePlus system (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY). QPCR analysis was performed using ΔΔCT method.

2.2.6 Protein quantification

PGE2 was quantified in cell culture medium using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI). IL10 and HGF concentrations were assed using feline specific ELISA kits (R&D Systems, Minneapolis, MN and MyBioSource, Inc., San Diego, CA respectively). Feline IL6 and multispecies TGFβ1 were used to quantify the respective proteins using Milliplex magnetic bead based assays (Millipore Billerica, MA) according to manufacturer recommendations. ELISA plates were analyzed using a plate reader (PerkinElmer, Waltham, MA), while bead based assays were analyzed using Luminex 100 machine (Luminex Corporation, Austin, TX). For each assay samples were run at least in duplicates.

2.2.7 IDO activity measurement

$5x10^5$ MSC were plated in a T25 flask in KNAC medium with 5%FBS and allowed to attach overnight. Cell culture medium was then changed to KNAC medium with 5% FBS supplemented with 100μM Tryptophan (Sigma-Aldrich, St.Louis, MO). After 3-day incubation, the cell culture supernatant was collected and analyzed. To measure the IDO mediated tryptophan catabolism, a spectrophotometric method was utilized to detect kynurenine, a stable catabolite of IDO, as previously described [30] with slight modifications. Cell culture medium was mixed with 30% trichloroacetic acid at a 2:1

ratio, mixed and spun down at 10000 rpm. Supernatant was then mixed in equal volume with Ehrlich reagent in a 96 well microplate (R&D Systems, Minneapolis, MN). Optical density was then read at 492 nm utilizing a plate reader (PerkinElmer, Waltham, MA).

2.2.8 *Co-culture experiments*

MSC were plated at a density of 10^4 cells per well in KNAC medium with 5% FBS and irradiated with 30Gy using X-RAD320 irradiator (Precision X-ray, North Branford, CT) in order to stop proliferation. Isolated PBMCs were thawed, washed in PBS and quantified utilizing Countess cell counter (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY). PBMCs were then suspended in RPMI1640 (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) with 10% FBS (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) and added at ratio of 1:5 of MSC to PBMC. Proliferation of lymphocytes was induced by 5 ng/mL of ConA (Sigma-Aldrich, St.Louis, MO). Cells were pulsed with EdU (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY) 24 hours before the end of 3-day incubation period. Fluorescence intensity was then measured using fluorescent plate reader (PerkinElmer, Waltham, MA). Background fluorescence assessed from wells containing only MSC was subtracted from the total fluorescent values in co-culture wells and compared to wells containing only stimulated PBMCs.

2.2.9 Statistical analysis

For gene expression studies ΔCT values from all samples for each concentration of the stimulant were compared using ANOVA. Post hoc analysis was performed using Dunett test. In protein assays comparisons between concentrations of proteins detected in supernatants coming from unstimulated and stimulated cells were

compared using paired t-test or non-parametric Wilcoxon signed rank test. All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA)

2.3 Results

2.3.1 Isolation and characterization of MSC

All MSC cells isolated expressed high levels of the cell surface markers CD90, CD105 and CD44, as well as did not express MHCII. They also underwent trilineage differentiation into chondrocytes, adipocytes and osteocytes as previously described by our group [31].

2.3.2 Quantitative real time PCR

All cell lines constitutively expressed IDO1, PTGES1, PTGES2, PTGES3, HGF, TGFβ1, IL6, PD-L1, and HMOX1. IL10 was undetectable at RNA level, while levels of iNOS and FASL were low or undetectable. Stimulation with varying concentrations of INFγ resulted in increased expression of IDO1 (8.5 to 12.3 fold increase, $p<0.0001$, ANOVA), which reached plateau at concentrations as low 5 ng/ml of stimulating cytokine (Figure 2.1). Expression of IL-6 was upregulated after exposure to INFγ as well (Figure 2.2, 0.3 to 2.1 fold increase, $p<0.0001$, ANOVA), with increased expression being statistically significant at levels as low as 1 ng/ml of cytokine. Concentrations of INFγ as low as 0.1 ng/ml significantly upregulated expression of PD-L1 (Figure 2.3, 1.5 to 3.7 fold increase, $p<0.0001$, ANOVA), while expression of HGF rose gradually with cytokine concentration increment (Figure 2.4, 0.29 to 1.8 fold increase, $p<0.0001$, ANOVA), with statistically significant result at as low 1 ng/ml of INFγ.

Stimulation of MSC with as low as 0.25 ng/ml of TNFα resulted in significantly increased expression of IL6 (Figure 2.5, 2.7 to 5.2 fold difference, $p<0.0001$, ANOVA).

2.3.4 Protein quantification

Levels of IL6 were significantly higher when cells were stimulated with a single dose of TNFα ($p<0.0001$, t-test), INFγ ($p=0.0038$, t-test) or both TNFα and INFγ ($p=0.000696$, t-test), while no change was detectable between unstimulated and stimulated cells after 24 hour incubation with INFγ only (Figure 2.6).

IL10 values were below the detection limit of the only commercially available assay for feline IL10 (Figure 2.10). No changes were detectable in levels of PGE2 and TGFβ1 between treatments (Figure 2.10).

Interestingly, the concentration of HGF in the supernatant significantly decreased after 24-hour stimulation with INFγ ($p=0.00956$) or both INFγ and TNFα ($p=0.00019$) (Figure 2.7).

2.3.5 IDO activity

INFγ stimulation significantly increased kynurenine levels in cell culture supernatants after 3 days of incubation compared to unstimulated cells ($p<0.0001$, t-test, Figure 2.8).

2.3.6 Co-culture experiments

In order to assess the capability of MSC to block proliferation of stimulated PBMC a co-culture experiment was performed. Co-culture of irradiated MSC with ConA stimulated PBMCs at 1:5 ratio significantly blocked the proliferation of PBMCs as measured by EdU incorporation assay (Figure 2.9). To investigate if inflammatory cytokines will enhance the proliferation blockage of MSC on PBMC, MSC were prestimulated for 2 hours prior to addition of PBMCs. Prestimulation of MSC with either INFγ, TNFα or with both INFγ and TNFα, did not change the effect of MSC on PBMCs in co-culture experiment (Figure 2.9).

2.4 Discussion

The data presented in this study documents that feline MSC constitutively express IDO1, PTGES1, PTGES2, PTGES3, HGF, TGF-beta, IL6, PD-L1, and HMOX1. Significantly, stimulation with INFγ for 24hrs, resulted in up to 12-fold increase in IDO1 expression in MSC, in a manner similar to those observed in human MSC upon stimulation with this cytokine [32]. The upregulation was confirmed through measurement of kynurenine, an IDO1-mediated tryptophan metabolite. The increase in this metabolite was clearly detectable after 3-days of INFγ incubation. Interestingly, IDO1 is strongly upregulated and functional in human MSC [20,32], whereas in the mouse, this gene is not responsive to INFγ [3,21,25,33]. Mouse MSC induce iNOS upon stimulation with INFγ, one of the key molecules involved in murine immunomodulation [26,34]. Expression of iNOS by feline MSC in our experimental conditions was low or undetectable, which closely parallel human MSC [25].

Expression of IL6 was upregulated by both INFγ and TNFα stimulation at the RNA level, similarly the protein measurement using ELISA, detected induction of IL6 in both inflammatory conditions. Constitutive expression of IL6 is characteristic for MSC, but to our knowledge no species related differences have been described related to this cytokine thus far.

FASL and IL10 transcripts were undetectable in the feline MSC and stimulation using INFγ and TNFα did not change this. FASL expression has been recently put forth as being important for MSC mediated T-cell apoptosis [15]. Interestingly the authors of that study did not detect expression of PD-L1, however, we have observed strong induction of PD-L1 after stimulation with INFγ. Our findings are concordant with data reported from placenta derived MSC [35,36], where expression of PD-L1, but not FASL, has been reported Thus, MSC may use different effectors to induce apoptosis of T-cells.

The differences may arise from culture conditions, source of MSC (adipose, placenta, bone-marrow). In previously described studies, bone marrow derived [15,36] and placenta derived cells were utilized [22]. Expression of IL10 by feline MSC was not detectible in our study at the gene or protein expression level. At the protein level, the signals generated were below the limit of detection of the commercially available feline IL10 assay (<125 pg/ml). Reports on the production of IL10 by MSC are not fully concordant. In some studies with human MSC, IL10 was detectable at the gene expression level [37] and at the protein level [22,33], while in others, no IL10 was detected [3,38]. Both the source of MSC and culture conditions may affect the production of IL10.

Interestingly, although all of the PGE2 synthases were strongly expressed in our data set, we also did not identify increase in PGE2 production after stimulation of MSC (Figure 2.10). The amount of PGE2 in cell culture supernatants were similar to previously described quantities for adipose tissue derived MSCs from mouse. [39] However, in our system, amounts PGE2 released did not increase with dual stimulation with both INFγ and TNFα. We hypothesize that strong expression of PGE2 syntheses in our experimental setting have precluded additional induction through stimulation. One of the explanations could be utilization of different cell culture medium compared to other studies as well as differences in amount of cells plated.

Release of HGF by feline MSC into the supernatant was significantly decreased after stimulation with INFγ or both INFγ and TNFα. In a study with human MSC, 24 hr. stimulation with INFγ was reported to increase HGF release by 10 times [40], however, the INF γ concentration used was four times higher (200ng/ml) as compared to our study. Thus, different doses of INFγ may induce release of HGF at different time points.

Further studies at different time points and different doses of INFγ need to be performed to further address this question.

Interestingly the levels of TGFβ1 in our data set remained unchanged after stimulation at both gene expression as well as protein levels. In one study on human MSC the levels of TGFβ1 almost doubled after stimulation with INFγ [40]. In another study, stimulation of human MSC with INFγ and/or TNFα resulted in decreased production of TGFβ1[41].

Feline adipose derived MSC significantly blocked proliferation of ConA stimulated feline PBMCs both in naïve state as well as post prestimulation with INFγ, TNFα or both. No difference was noted between the prestimulated and naïve cells in our experiments and mixed results have been previously reported in this aspect [26,40,42]. Significant reduction of stimulated PBMCs seen in co-culture studies described above, along with the gene expression of immunomodulatory molecules documented, provides a strong rationale for clinical utilization of MSCs in treatment of feline inflammatory disease as well as for the utilization of cats in translational studies.

The current study documents that feline MSC share many similarities in immunomodulatory properties as human MSC and adds to the limited information available from large animal systems. Spontaneously occurring chronic inflammatory disorders in cats and dogs may serve as translational models of MSC-based therapy of human diseases [43]. Studies of MSC in mice have previously reported differences in mice as compared to humans especially with regard to expression of INOS vs. IDO [21,25]. The appropriateness of mice models for human immune disorders has recently been challenged [44-46]. Although mouse models will remain important tools in the biomedical field [47], use of spontaneously occurring disorders in cats can potentially

successfully fill the gap between mouse and human in development of new treatment strategies for a variety of disorders[43,48].

The immunomodulatory properties of feline adipose derived MSC documented in this study provide the rational for the utilization of MSC based approaches to feline diseases with inflammatory components for the treatment of both the feline disease as well as an informative translational system, valuable for development of human therapies.

APPENDIX

Figure 2.1. Stimulation with as low as 0.1 ng/ml of INFγ resulted in significant upregulation of IDO1 expression, which reached plateau at concentration of 5 ng/ml of stimulating cytokine. n=5 each assay was run in triplicate.

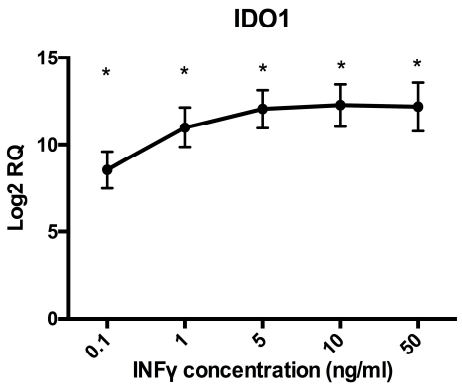


Figure 2.2. Increasing concentrations of INFγ increased the expression of IL6, reaching statistical significance at INFγ concentrations as low as 1 ng/ml. n=5 each assay was run in triplicate.

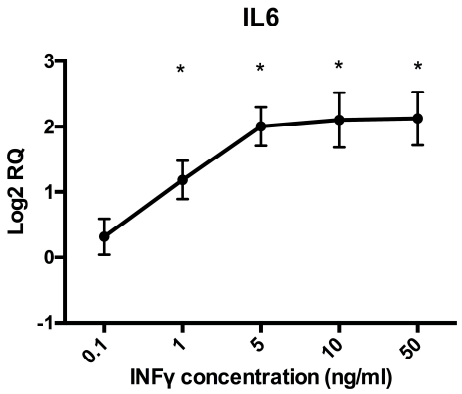


Figure 2.3. PD-L1 was significantly upregulated through stimulation with as low as 0.1 ng/ml of INFγ. n=5

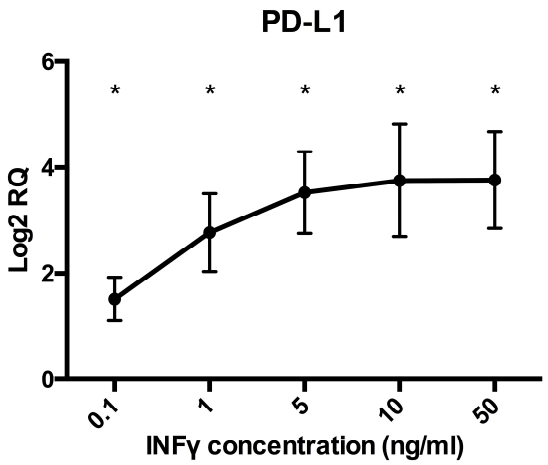


Figure 2.4. HGF was significantly upregulated after stimulation with 1 ng/ml of INFγ and gradually increased in concentration in dose dependent fashion. n=5

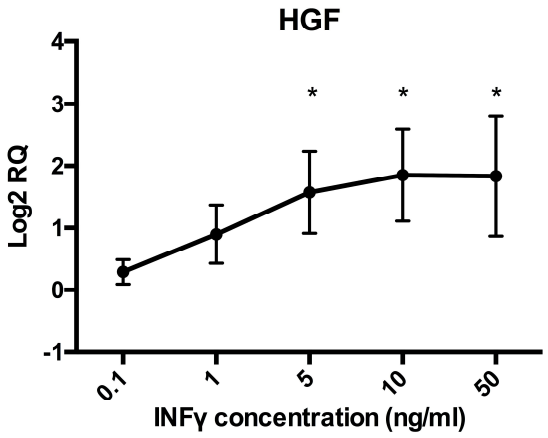


Figure 2.5. Stimulation with TNFα significantly increased the expression of IL6 at concentration as low as 0.25 ng/ml of cytokine. n=5 each assay was run in triplicate.

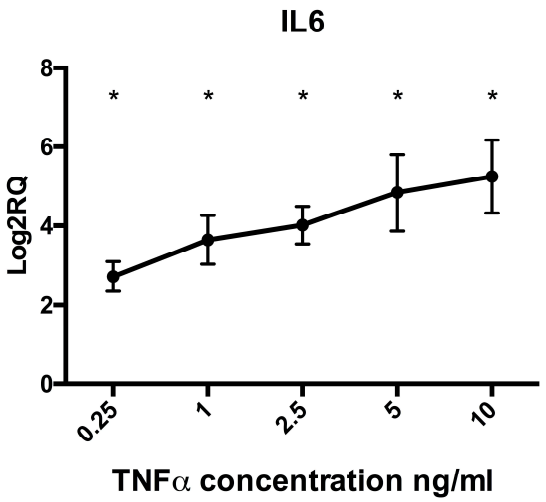


Figure 2.6. IL6 concentration in cell culture medium as measured by ELISA. Note that stimulation with a single dose of TNFα, INFγ or both TNFα and INFγ resulted in significant upregulation of IL6 production. n=5 per group, each assay was run in triplicate. * p<0.01

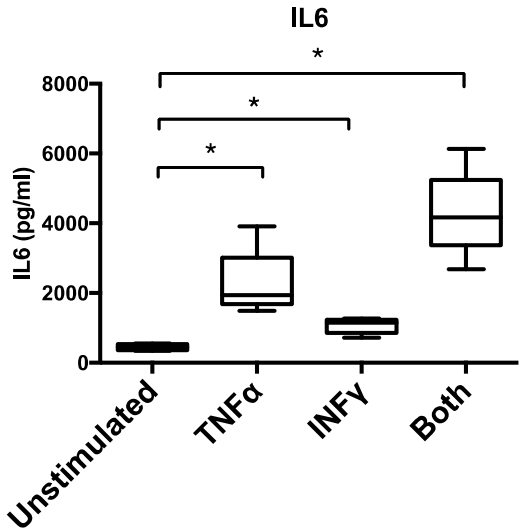


Figure 2.7. Significant decrease in HGF concentration in cell culture medium after stimulation with INFγ or both INFγ and TNFα. n=5, p<0.01

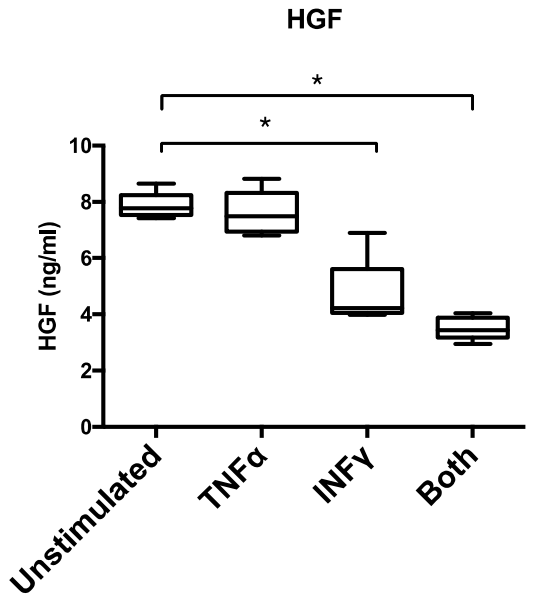


Figure 2.8. Cells incubated for 3 days with 50 ng/mL of INFγ in medium supplemented with tryptophan significantly increased the amount of kynurenine in the cell culture medium. n=3

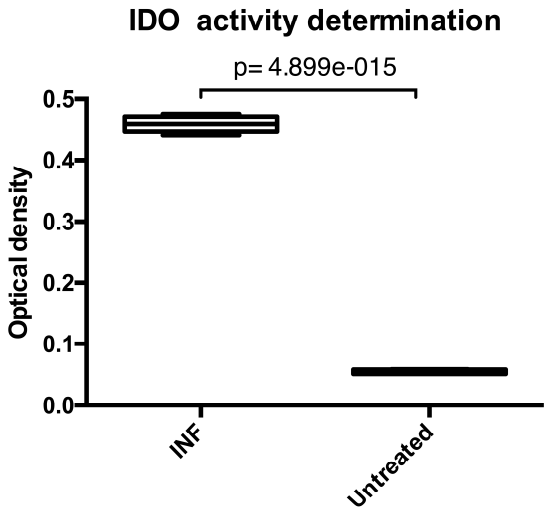


Figure 2.9. Co-culture experiment revealed a strong decrease in PBMC proliferation at 1 to 5 ratio as measured by fluorescence intensity after subtraction of background proliferation of MSC. n=4, each assay was run in triplicate. $p<0.0001$

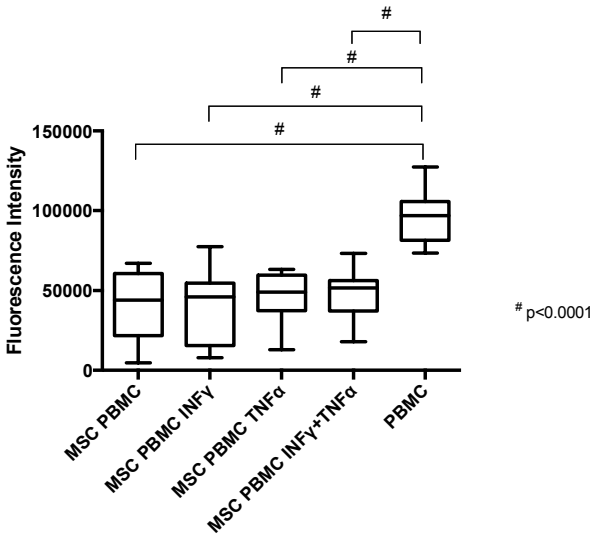
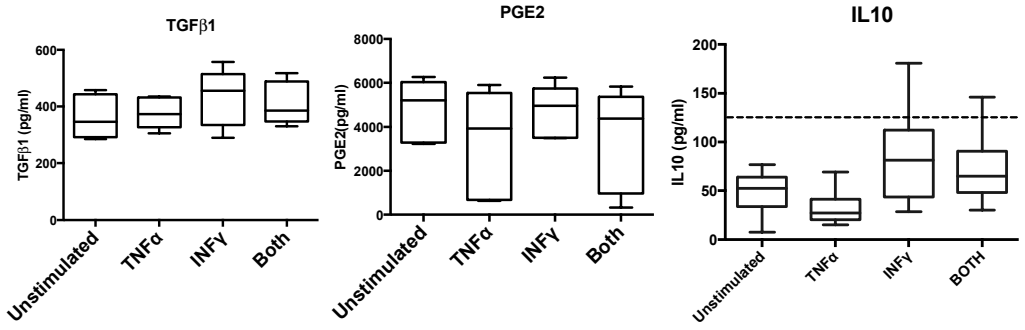


Figure 2.10. Concentrations of TGFβ1 and PGE2 remained unchanged after stimulation, while concentration of IL10 was below the limit of detection (dotted line, 125 pg/ml)



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CHAPTER 3

SAFETY OF INTRAPERITONEAL INJECTION OF ADIPOSE TISSUE-DERIVED AUTOLOGOUS MESENCHYMAL STEM CELLS IN CATS

Parys, M., Nelson, N., Koehl, K.,Miller, R.,Kaneene, J. B., Kruger, J. M.,Yuzbasiyan-Gurkan, V. Safety of Intraperitoneal Injection of Adipose Tissue-Derived Autologous Mesenchymal Stem Cells in Cats; Journal of Veterinary Internal Medicine, Volume 30, Issue 1, pages 157–163, January/February 2016

3.1 Introduction

Administration of mesenchymal stem cells (MSC) has been recently proposed as an alternative therapeutic modality for management of chronic inflammatory conditions in cats and other species.[1-3] MSC express a myriad of factors such as IDO, IL10, HGF, PGE2, TGF-beta, IL6, PD-L1, FASL or HMOX1[4-15] and affect effector functions of many types of immune cells.[12,16-20] Interestingly, MSC are capable of inducing T-regulatory cells, which in turn can provide a long-lasting immunomodulation.[21] *In-vitro* experiments suggest that MSC need to be in close proximity to target cells and in sufficient quantity to induce a therapeutic effect.[11,13,14,18,22] In addition, *in-vivo* experiments also suggest paracrine effects of MSCs on other cells are also important.[15,23,24]

Multiple routes of injection of MSC are described and include intravenous (IV), intraperitoneal (IP) as well as intra-articular, cardiac, hepatic or nasal.[1,24-27] IV injection is a frequently reported route of MSC administration for the purpose of immunomodulation.[1,2,15,22,24-29] However, IV administration of MSC has potential limitations for treatment of inflammatory conditions localized to the peritoneal cavity. Exposure of intravenously administered MSC to plasma complement reduces cell survival.[30] Retention of MSC in pulmonary vasculature reduces the number of cells reaching the target and increases the risk of pulmonary microthrombus formation.[31,32] Pulmonary thrombosis is a potential serious complication and might increase morbidity and mortality.[2] Furthermore, instant blood mediated inflammatory reaction (IBMIR) occurs after injection of MSC and can be cause of decreased cell survival and increased risk of thromboembolism.[33-36] Thus, IV injection can potentially limit the availability of MSC and their migration capabilities, decrease the overall cell survival, and increase risk of adverse effects.[30]

Intraperitoneal injection is potentially a safer and more effective route of MSC administration for disorders within the abdominal cavity. IP injected MSC are placed in close proximity to the target organ and could reach the target in greater numbers and subsequently effect in a better outcome. IP injection is simple to perform and has the promise of widespread adoption in clinical settings and high impact on patient management. IP injection of MSC was significantly more effective than IV administration for ameliorating clinical signs in a rodent model of inflammatory bowel disease.[37] MSC, administered IP have been studied also for their regenerative potential[38] and ability to deliver gene therapy.[39,40]

The safety of IP injection of MSC has not been studied in the cats. This study was undertaken to test the hypothesis that IP injection is a safe route of autologous MSC administration in cats.

3.2 Materials and methods

3.2.1 Animals

Ten purpose-bred mixed breed, intact female cats were obtained from the MSU Comparative Ophthalmology Cat Colony and from a licensed commercial vendor (Liberty Research, Inc., Waverly, NY), five from each source. The mean age (±SD) at the time of injection of the cats was 20.7 (±5.7) months and the mean (±SD) weight was 3.79 (±0.5) kg. Animals were housed under standard conditions at the Michigan State University College of Veterinary Medicine Vivarium, fed a commercial diet (Adult Optimal Care, Hill's Pet Nutrition, Inc., Topeka, KS) and received environmental enrichment during the study period. Cats were determined to be healthy on the basis of physical examination. The study was approved by the Michigan State University

Institutional Care and Animal Use Committee.

3.2.2 Isolation and characterization of mesenchymal stem cells

Subcutaneous adipose tissue (2-4 gram) from the ventral midline abdomen was collected during ovariohysterectomy was placed in KNAC medium (Keratinocyte SFM medium supplemented with 2mM of N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO) and 0.2 mM of L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO) with 5% of MSC-grade FBS (Thermo Fisher Scientific Inc., Grand Island, NY) in 50ml tube for transfer to the laboratory for processing. Each tissue sample was finely minced with sterile scalpel blade and incubated in 1 mg/ml of Collagenase I (Sigma-Aldrich, St. Louis, MO) in HBSS (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C with 5% CO_2. After incubation, the cell suspension was repeatedly aspirated with sterile serological pipette in order to facilitate dissociation and passed through a 70 μm cell strainer (Thermo Fisher Scientific Inc., Grand Island, NY) to remove excess tissue stroma. The resulting cell suspension was washed twice in sterile PBS by centrifugation at 1200 rpm for 5 minutes at room temperature. Cells were then re-suspended in 5 ml of KNAC medium with 5% of MSC-grade FBS (Thermo Fisher Scientific Inc., Grand Island, NY) and plated in T-25 plastic tissue culture flask (USA Scientific, Ocala, FL). After reaching 80% confluency, cells were trypsinized with 0.05% trypsin (Thermo Fisher Scientific Inc., Grand Island, NY), resuspended in 20 ml of medium and divided between two T-75 plastic tissue culture flasks (USA Scientific, Ocala, FL). All cell cultures were subsequently passaged in a similar fashion until sufficient quantities of cells were available for injection and characterization, which took approximately 3 weeks.

Adherent cells were characterized for cell surface expression of CD90 (Anti-human CD90, clone 5E10, BD Biosciences, San Jose, CA), CD105 (Anti-human CD105,

clone SN6, Invitrogen, Grand Island, NY), CD44 (Anti-mouse CD44, clone IM7.8.1, Invitrogen, Grand Island, NY), MHCII (Anti-human HLA-DR, clone Tu39, BD Biosciences, San Jose, CA) and for differentiation into osteocytes, adipocytes and chondrocytes.[1,41,42] Cell membrane marker expression was analyzed by flow cytometry (BD Biosciences, San Jose, CA) and analyzed using commercial software (BD Biosciences, San Jose, CA). Differentiation of cells was achieved through use of commercial osteogenesis, adipogenesis and chondrogenesis kits (StemPro Chondrogenesis, Adipogenesis, Osteogenesis Differentiation Kit, Gibco, Grand Island, NY) according to manufacturer's recommendations. For osteogenesis and adipogenesis experiments, 1 x 10^4 and 1 x 10^5 cells per well were plated in 24-well tissue culture plates containing KNAC medium with 5% FBS and incubated at 37°C for 24 hours to allow cells to attach. After incubation, the medium was changed to the specific differentiation medium and cultured for 3 weeks in 37°C with 5% CO_2 with medium being changed twice a week. Chondrogenic differentiation was accomplished through culture of at least 1 x 10^6 cells in micromass. Cells were re-suspended in 1 ml of cell culture medium and spun down at 1000 rpm for 5 minutes. Subsequently the medium was carefully exchanged with chondrogenesis medium, in order not to disrupt the pellet. Cells were then cultured for three weeks at 37°C with 5% CO_2, with semiweekly medium changes. After 3 weeks of differentiation the cells were stained with Alizarin Red (Sigma-Aldrich, St. Louis, MO) to assess osteogenic differentiation, Alcian Blue (Sigma-Aldrich, St. Louis, MO) to assess chondrogenesis and Oil-o-Red (Sigma-Aldrich, St. Louis, MO) stain to assess adipogenesis.

3.2.3 Procedures

All cats underwent a routine ovariohysterectomy, at which time a 2-4 gram sample of ventral midline subcutaneous fat was obtained and processed for MSC isolation and

propagation. For ovariohysterectomy, animals were sedated with acepromazine (0.05 mg/kg, Boehringer Ingelheim, Ridgefield, CT) and buprenorphnine (10 mcg/kg, Reckitt Benckiser, Hull, England), anesthetized with isoflurane (0.25-3% isoflurane in 100% oxygen in an induction chamber, IsoFlo, Abbott Laboratories, Abbott Park, Illinois). For subsequent examinations (ultrasound, blood draw, cystocentesis) all cats were sedated or anesthetized for all procedures with either acepromazine (0.05 mg/kg, Boehringer Ingelheim, Ridgefield, CT) and buprenorphnine (10 mcg/kg) or isoflurane (0.25-3% isoflurane in 100% oxygen in an induction chamber, IsoFlo, Abbott Laboratories, Abbott Park, Illinois). Five cats (three MSC-treated and two sham-injected) were sedated using acepromazine and buprenorphine and five cats (two MSC-treated and three sham-injected) were anesthetized with isofluorane.

After a three-week recovery period from ovariohysterectomy, all cats were evaluated at baseline (pretreatment) with a complete physical examination, a CBC, serum chemistry profile, urinalysis, and abdominal ultrasonography. Cats were then randomly divided into two groups of five animals each. Cats from both sources were represented in each of the groups. Group 1 cats received an ultrasound-guided injection of $1x10^6$ autologous MSC/kg of body weight; Group 2 control cats received an equivalent intraperitoneal sham injection of sterile phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO). MSCs were suspended in 4mL of saline for IP injections. Isolated MSCs in passage 4 were grown to about 80-90% confluence, detached using 0.05% trypsin for 10 minutes, washed twice using PBS and quantified using an electronic cell counter (Thermo Fisher Scientific Inc., Grand Island, NY) and resuspended in 4 mL of PBS for injection. The suspension was then transferred into a 20cc syringe (BD Medical, Franklin Lakes, NJ) with a 22F needle (BD Medical, Franklin Lakes, NJ) for injection. After injection of MSC, cats were monitored for 6 weeks with

daily physical examinations and weekly clinicopathological evaluations (CBC, serum chemistry profiles, and urinalyses; DCPAH, Michigan State University, East Lansing, MI). Abdominal ultrasonography was repeated at one and five weeks post- injection (Veterinary Medical Center, Michigan State University, East Lansing, MI) with the ultrasound examiner being blinded to each cat's treatment status.

For ultrasonographic examinations, cats were placed in dorsal recumbency and the abdomen was imaged using a linear array multifrequency transducer (GE Logiq 9, General Electric, Princeton, NJ). The frequency used varied between 10 and 13 MHz, with the highest frequency chosen, which allowed complete organ evaluation. During each ultrasound evaluation, the entire abdominal contents were surveyed, including the liver, spleen, kidneys, bladder, adrenal glands, pancreas, stomach and small intestines. The medial iliac and jejunal lymph nodes were also imaged at these time periods, and their long axis (cranial to caudal length) and short axis (dorsal to ventral height) were measured. For statistical comparisons, lymph node size was calculated as the multiplication of length times width. At five weeks post-injection, fine needle aspirate biopsies of selected abdominal lymph nodes were obtained from 3 MSC-treated cats under ultrasound guidance using a 22g 1.5 inch needle.

3.2.4 Statistical analyses

Mean values with standard deviation were calculated for MSC-treated and control cats at each sampling time for data from physical examination, clinicopathological evaluations and ultrasound measurements. Comparison between the before and after treatment results within each group were performed using paired t-test. Continuous quantitative variables that failed normality testing were evaluated with nonparametric analyses. Nonparametric Wilcoxon signed-rank tests were used for

analyses of outcome variables for significant differences between treatment groups. In addition, multivariable ANOVA (MANOVA) was used to evaluate the linear changes over time. All data were analyzed with a commercially accessible statistical software package (SAS 9.3, SAS Institute Inc., Cary, NC). Results with $p<0.05$ were considered statistically significant.

3.3 Results

Each cell preparation displayed the expected MSC-phenotype. Cells underwent differentiation into adipocytes, chondrocytes and osteocytes (Figure 1) and strongly expressed the surface markers CD90, CD44, CD105 (Figure 2).

Severe adverse effects were not observed in any cat after injection of MSC. There were no significant differences in mean temperature, respiratory rate, heart rate, and body weight between groups over the course of the study. Two MSC-treated cats were lethargic and were less interactive with their caretakers immediately after injection. No pain was elicited by abdominal palpation in either cat. Both cats spontaneously recovered within 1 to 3 days post-injection of cells.

Results of clinicopathologic evaluations revealed significant differences between study groups for creatine kinase (CK) enzyme activity. CK activity was significantly higher in MSC-injected cats on weeks one [271.4 +/-154.36 IU/L vs. 150.6 +/- 54.79 ; p=0.047,Wilcoxon] and three [196 +/- 49.75 vs. 125.8 +/- 34.72; normal reference range = 59-354 IU/L; p=0.047 ,Wilcoxon]. Individual cat CK values were above the reference range at various times in three MSC injected cats and two sham-injected cats. Abdominal ultrasonography revealed a significant increase in jejunal lymph node size in MSC-injected cats compared to controls at weeks 1(1.38 ± 0.25 cm^2 vs. 0.88 ± 0.25 cm^2;

p=0.036) p= 0.036, Wilcoxon) and 5 (1.75 ± 0.82 cm^2 vs. 0.79 ± 0.12; p= 0.047, Wilcoxon) after cell injection (Figure 3). A significant increase in lymph node size over time was also identified in MSC-injected cats when comparing the before injection lymph node size with size at week 5 (1.1 ± 0.52 cm^2 before injection vs. 1.75 ± 0.82 cm^2 after injection; p=0.033). A hyperechoic renal segmental cortical lesion consistent with a renal infarct was identified sonographically in one MSC-injected cat 1 week after injection. Cytologic examination of lymph node aspirates obtained from three MSC injected animals revealed normal cell populations consisting primarily of small lymphocytes with low numbers of medium to large lymphocytes and occasional plasma cells and macrophages; there was no cytologic evidence of disease.

3.4 Discussion

This study evaluated the safety of intraperitoneal injection of autologous mesenchymal stem cells in cats. Our findings show that IP administration of autologous MSCs appears to be a safe and technically feasible approach to cell-based therapy in cats. Limited adverse side effects were observed, manifesting as transient lethargy and decreased activity in two cats. The cause of these behaviors in uncertain, but might be related to discomfort associated with a host response to MSC or other components of the cell preparation. Although abdominal pain was not elicited during palpation, increased abdominal lymph-node size in MSC-injected cats is consistent with a heightened host response to MSC injection. Alternatively, this reaction might have been caused by the sedative or anesthesia used. However, the fact that this behavior was observed only post cell injection and not at other times the cats underwent anesthesia, suggests that an adverse anesthetic reaction is less likely the cause of lethargy.

All clinicopathological results were similar between MSC injected and sham injected animals with the exception of CK. Importantly, CK values were only slightly outside of the reference ranges. Activity of this enzyme can be induced by multiple factors. Cats in this study received intramuscular injections of sedatives, which can increase the CK values.[43] Some animals required additional physical restraint, which could result in increase of CK as well.[43]

A hyperechoic renal segmental cortical lesion was observed in one MSC-injected cat one week after injection, which was assumed to be a renal infarct. These changes are incidentally encountered in cats in the clinical practice setting and have been observed more frequently in Ragdoll cats and cats with cardiomyopathy.[44,45] In the MSC-injected cat with the renal segmental cortical lesion in the present study, no heart murmur was identified on cardiac auscultation. An echocardiogram was performed to investigate for cardiomyopathy at a recheck appointment one year after completion of this study and no abnormalities were noted within the heart by a board certified cardiologist. Interestingly the animal developed other similar infarcts in the other kidney at the 1 year follow up which suggests that this animal is prone to this kind of changes and suggest that the infarcts were not related to the injected MSCs.

We also observed a significant increase in the size of jejunal lymph nodes in MSC-injected cats over the course of the study. We speculate that secretion of IL6 by MSC[46], which is known to cause T-cell expansion, may be responsible for the differences in lymph node size observed between the two groups. Cytological examination of lymph nodes of a limited number of MSC-injected animals did not reveal any pathological process. However, proof of that hypothesis would require histopathological evaluation of the jejunal lymph nodes. Necropsy examinations were not part of the experimental design so it could not be assessed. Another explanation

might be a complement-mediated reaction to injected MSCs, similar to IBMIR reaction to injected cells and subsequent activation of innate immune system. Elements of complement are constitutively expressed by mesothelial cells.[47] C3 complement component expressed by peritoneal mesothelial cells has been previously implicated to be responsible for IBMIR reaction post MSC IV injection *in vivo* and subsequent activation of innate immune system.[48] Interestingly, the complement binding also enhances the immunomodulatory properties of MSCs.[48] Regardless, follow-up ultrasonograms performed on 2 MSC-injected cats approximately 12 months after injection, revealed that all abdominal lymph nodes had returned to pre-injection size (data not shown). These observations suggest that abdominal lymphadenopathy associated with MSC injection is be transient in nature. Further studies are needed to determine the precise cause of the increased lymph node size observed in MSC-injected cats.

A limitation of this study is that only a single dose of MSC was evaluated. Responses to IP MSC-injection may vary by dose and frequency of administration, as suggested by *in vitro* studies in other species.[12,18,46,49-51] Future studies evaluating a higher dose of MSC than the one evaluated in this study may be necessary to fully evaluate the safety profile of the IP injection in cats.

Although the efficacy of IP MSC injection has been previously studied in a variety of species and disease models[37-40,52], there have been few studies reported that comprehensively investigate the safety of IP administration of MSC in healthy control animals. In one study evaluating the safety and distribution of amniotic fluid stem cells injected IP in neonatal rats[53], no adverse effects were noted in MSC-injected rats during the 21-day post-injection observation period. However, neither lymph node size nor behavioral changes were investigated in the rat study.

We conclude that IP injection of autologous MSC in cats is overall safe and is associated only with mild, self-limiting, and short-lasting adverse effects and provides a safe alternative route of administration for cell-based therapy in cats. Given the apparent safety of IP administration of MSC and the potential of IP administered MSC to reach intra-abdominal target sites, the effectiveness of the IP route for MSC-based therapy of chronic inflammatory disorders of the peritoneal cavity such as chronic idiopathic cystitis, hepatitis, pancreatitis or inflammatory bowel disease warrant further investigations.

APPENDIX

Figure 3.1. Representative photomicrographs illustrating differentiation characterization of a MSC cell line isolated from adipose tissue obtained from a 2 year old, female mixed breed cat (magnification 40X). A) Lipid droplets in differentiated cells are stained red with Oil-o-red demonstrating adipogenesis, B) Red colored calcium deposits inside cells stained with Alizarin red stain demonstrating osteogenesis, C) Blue colored in glycosaminoglycan deposits in cells stained with Alcian Blue stain after chondrogenic differentiation. D,E,F) Control photomicrographs after incubation of the same cell line in KNAC medium and stained with Oil-o-red (D), Alizarin red (E) Note lack of uptake of the stains in both micrographs and no blue staining extracellular matrix in the chondrogenic control stain (F).

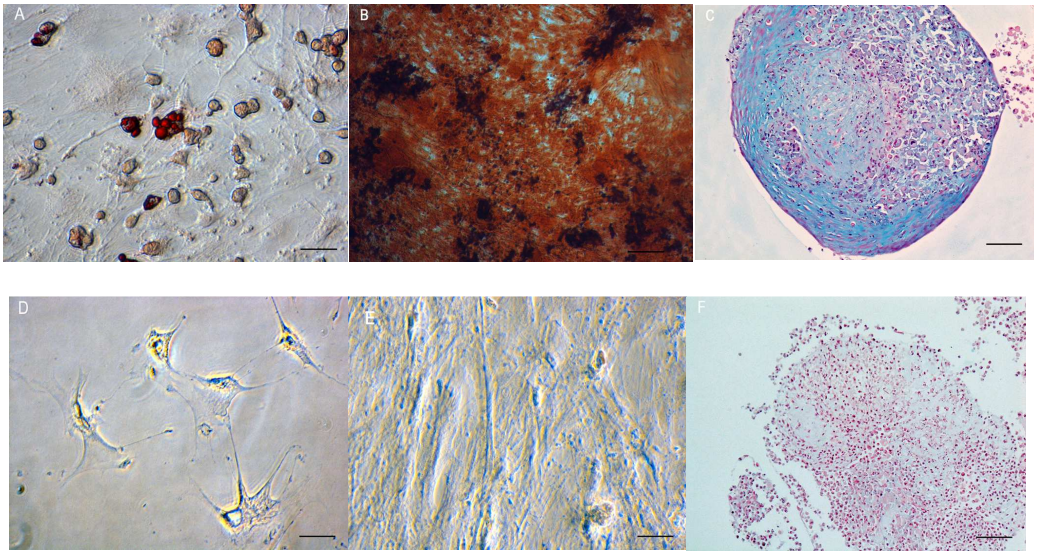


Figure 3.2. Flow cytometric analysis of surface markers expressed by a representative cell line from the same Animal 1 as in Figure1. Grey lines represent the negative controls, while in black is the population of cells stained with antibody specific for each epitope. The X-axis represents the fluorescence intensity of the fluorophore (APC or PE) while the Y-axis represents the cell counts. Note strong expression of all markers a) CD90, b) CD105, c) CD44 in each cell line (continuous line), compared to negative control (dashed line)

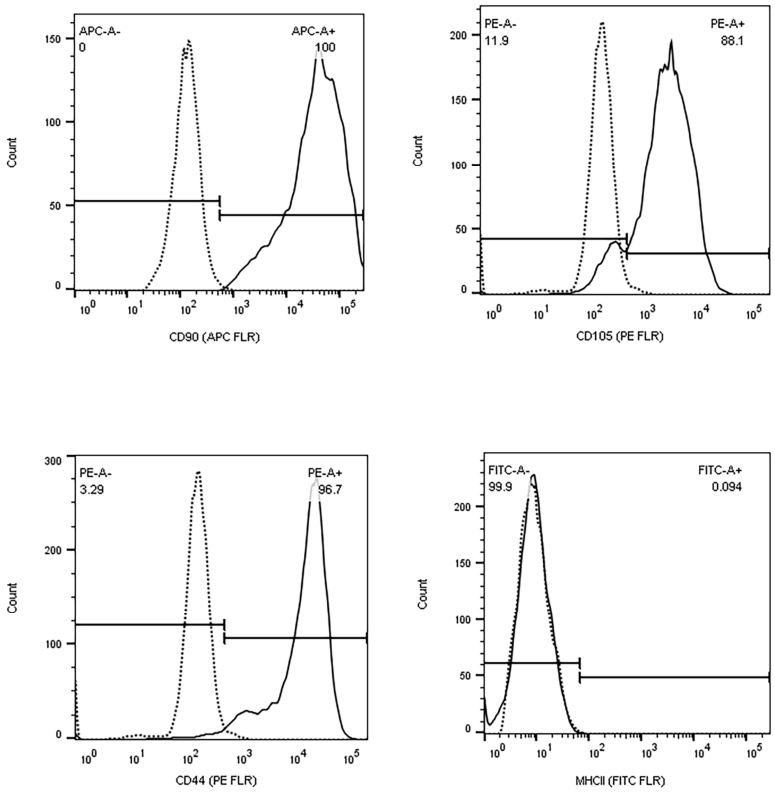
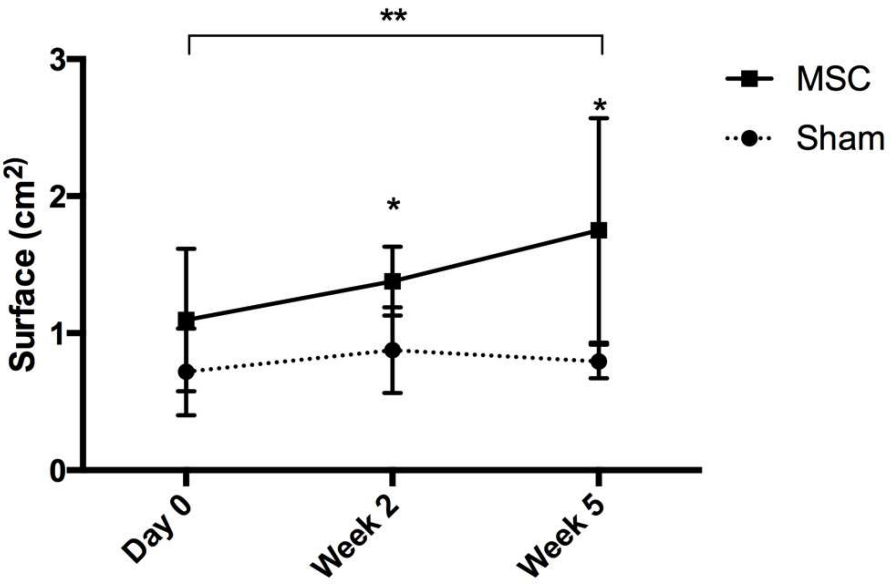


Figure 3.3 Changes in mean (±SD) jejunal lymph node size in cats injected intraperitoneally with either $1x10^6$ MSC (n=5) or a sham preparation (n=5) over the course of study.

* Statistically significant difference between treatment groups (MSC treated vs. sham treated animals) at the weeks indicated ($p<0.5$)

** Statistically significant difference between pre-injection and 5 week post-inject lymph node size (as presented by the black line over the graph) within the group treated with MSCs ($p=0.033$)



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CHAPTER 4

SERUM AND URINARY CYTOKINE PROFILING IN FELINE IDIOPATHIC CYSTITIS

4.1 Introduction

Feline Idiopathic cystitis (FIC) is the most common lower urinary tract disorder in domestic cats and is characterized by clinical signs of dysuria, pollakiuria, stranguria, hematuria and in males urinary tract obstruction. [1,2]. The clinical and morphologic features of FIC are strikingly similar to those of an idiopathic cystopathy of people called interstitial cystitis/painful bladder syndrome (IC/PBS) [3-7]. Our observations and those of others suggest that FIC is associated with different clinical and pathologic phenotypes which include two forms; an acute self-limiting form in which signs resolve spontaneously in 3 to 7 days regardless of therapy, and a chronic form in which clinical signs persist or recur frequently for weeks or months [8]. Chronic FIC can be further subcategorized into ulcerative inflammatory and nonulcerative noninflammatory forms [5,6]. These chronic FIC phenotypes appear to be similar to nonulcerative (Type I) and ulcerative (Type II; classic) forms encountered in humans with IC/PBS [9]. As of yet, there are no consistent specific diagnostic markers that establish a diagnosis of FIC or IC/PBS, or differentiate between various disease phenotypes. In the absence of specific biomarkers for FIC, virtually all clinical studies to date have relied upon subjective caregiver observation, interpretation, and quantification of clinical signs that typify FIC. However, the ability of caregivers to reliably quantify urination patterns and behaviors is inconsistent at best [10]. Over the past several decades, a large number of potential biomarkers have been identified and evaluated in people with IC/PBS; however, relatively few have been evaluated in cats with FIC (Table 4.1, adapted from [11]). Inflammation appears to be involved in the pathogenesis of some forms of FIC and mast cells as well as mononuclear cell infiltrates have been previously described in cats with chronic FIC [3,5,12]. Cytokines and chemokines are factors important for migration, maturation as well as proliferation of immune cells. In IC/PBS several cytokines and

chemokines have been intensively studied as potential biomarkers of the disorder (Table 4.1) [13-18].

The objective of this study was to investigate the concentrations of 19 cytokines in serum and urine of cats with acute or chronic FIC and unaffected healthy control cats to identify potential new biomarkers of the disease.

4.2 Materials and methods

4.2.1 Urine and Serum samples

Specimens included archived frozen urine and serum samples collected from cats with clinical diagnosis of nonobstructive acute or chronic FIC that were enrolled as part of previous studies, or cats enrolled in a parallel study pursued by the authors. All animals were evaluated at Michigan State University Veterinary Medical Center. Control samples were obtained from clinically healthy client-owned, blood donor or research colony cats. All cats were evaluated with a standardized medical history, a complete physical examination, and a complete urinalysis, a urine culture for aerobic bacteria, and an abdominal ultrasound in order to exclude other potential caused of their lower urinary tract signs. Additionally all FIC cats had serum chemistry profiles, complete blood count, and FeLV/ FIV testing performed. Cats with acute FIC were excluded from the study if they had another detectable disease process, had concurrent urethral obstruction, or had been treated with antimicrobials, antihistamines, corticosteroids, NSAIDs, anticholinergics, antidepressants, urinary acidifiers, glycosaminoglycans, diuretics, dimethyl sulfoxide, cyclophosphamide, or any other medication used to treat IC/PBS. Similarly, cats with chronic FIC were excluded from the study if they had any other concurrent disease process, undergone cystotomy or cystoscopy within the last

year, had urinary tract obstruction within past year, or received any corticosteroids, NSAIDs, antidepressants, antihistamines or glycosaminoglycan preparations.

Urine samples were obtained via cystocentesis and the blood samples through venipuncture. Collected samples were spun down at 1000g for 10 minutes. Serum and urine supernatants were subsequently cryopreserved at -80° C.

All samples utilized in this study were obtained under informed consent from the owners and with the approval of the Michigan State University Institutional Animal Care and Use Committee.

4.2.2 Multiplex cytokine assays

Milliplex MAP Feline Cytokine/Chemokine Magnetic Bead Premixed Panel (Millipore, Billerica, MA) was utilized allowing for simultaneous measurement of 19 cytokines: sFas, Flt-3 ligand, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12 (p40), IL-13, IL-18, KC, MCP-1, PDGF-BB, RANTES, SCF, SDF-1, TNF-α. Serum samples were assayed according to manufacturer recommendations. Urine cytokine concentrations were assayed using a modification of the serum protocol. Briefly urine samples were diluted 1:1 with assay buffer and incubated for 10 minutes prior to assay commencement in order to equilibrate the pH. All subsequent steps were carried out according to standard manufacturer recommendations. Samples were analyzed on Luminex 100 (Luminex Corporation, Austin, TX) machine with Bio-Plex software (Bio Rad, Hercules, CA). All assays were run in duplicate.

4.2.3 Statistical analysis

Statistical analysis was performed using commercially available statistical software (GraphPad Prism 6.0). D'Agostino & Pearson omnibus test was used to

evaluate normality of data. Comparisons of cytokine concentrations between groups with normal distribution were performed using student t-test , otherwise non-parametric Mann-Whitney U test was performed. Pearson's r correlation test was used to investigate potential correlations between the cytokines that were significantly increased in FIC patient's urine and those increased in the serum P-value of <0.5 was considered statistically significant.

4.3 Results

4.3.1 Animals

Samples from a total of 15 affected animals and 13 control animals were utilized in the study. The FIC group included 6 female and 9 male cats, while control group included 5 female and 8 male cats. Average age of affected cats was 60.64 months (SD±22 months, range 17 to 84 months) and 61.72 months (SD±22.7, range 12 to 86 months) for control cats. Within the FIC group, 12 cats had clinical signs consistent with acute cystitis, with clinical signs lasting an average of 3 days (SD±2.21 days, range 1 to 8 days) and three cats had chronic cystitis with clinical signs persisting for more than 3 weeks. The mean urine specific gravity of FIC cats was significantly lower than the control cats ($p=0.0084$, t-test; FIC: 1.049 ±0.011 vs. control 1.060 ± 0.0095). Data from urinalysis is summarized in table 4.3.

Within the FIC group, some cats had mild abnormalities in their blood chemistry and CBC values. These abnormalities included hyperglycemia (4/15), increase in CK activity (10/15), hypo (1/15) or hypernatremia (1/15), hypochloremia (1/15), hyperglobulinemia (6/15), hypoglobulinemia (1/15), decreased amylase activity (1/15), hypocholesterolemia (2/15), increased MCHC (8/15), hypomagnesaemia (2/15), hypoferritinemia (2/15), decreased MCV (3/15), increased TCO2 (1/15),

increased AST activity (2/15), increased ALT activity (1/15), hypophosphatemia (1/15), decreased Hct and Hgb (2/15) as well as decreased RBC counts (1/15). All other values were within reference ranges.

4.3.2 Serum cytokine/chemokine concentration

Thirteen FIC and 13 control serum samples were available for evaluation at the time of experiments. Twelve of 13 FIC serum samples were obtained from cats with acute disease; one sample was from a cat with chronic FIC.

Four of 19 analytes were undetectable or detectable in insufficient number of samples to make a meaningful comparison and were excluded from analyses. These analytes were GM-CSF, IL-1b, IL-2 and PDGFBB (Table 4.2). Concentration of four cytokines were significantly increased in serum of FIC cats compared to controls: **IL-12 (p40)** ($p<0.0001$ Mann-Whitney test; FIC: 1191± 484.9 pg/mL vs. control: 219.6 ± 393.3 pg/mL), **SDF1** ($p=0.0024$, Mann-Whitney test; FIC: 2015± 1131 pg/mL vs. control 528.7 ± 1002 pg/mL), **IL-18** ($p=0.0202$, Mann-Whitney test; FIC: 483.8±468.8 pg/mL vs. control: 92.03 ± 272.6 pg/mL) and **Flt3L** ($p=0.0015$, Mann-Whitney; FIC: 358 ± 104.3 pg/mL vs. control: control: 267.9 ± 290.2 pg/mL Table 4.2, Figure 4.1).

4.3.3 Urine cytokine/chemokine concentration

Urine specimens from 15 FIC cats and 12 controls were available for evaluation at the time of the experiment. Twelve of 15 FIC urine samples were obtained from cats with acute disease; three samples were from cats with chronic FIC. Nine analytes were undetectable or detectable in insufficient number of samples to make a meaningful comparison. These analytes were IL-1b, PDGFBB, IL-13, KC, RANTES, MCP-1, INFγ (Table 4.2). Of the remaining 10 cytokines, concentrations of two were significantly

increased in urine of FIC cats compared to unaffected controls: **IL6** (p=0.0011, Mann-Whitney test; FIC: 236.2± 189.3 vs. controls 39.71± 51.46 pg/mL) and **sFAS** (p=0.0299, t-test; FIC: 13.46± 6.07 vs. control 9.03± 3.033 pg/mL; Table 4.2, Figure 4.2). In addition, urine concentrations of IL-2 (p=0.0646) and IL-8 (p= 0.0529) were substantially increased in FIC cats, but differences did not achieve statistical significance. Interestingly, the 3 cats with chronic disease had relatively low levels of most cytokines compared to cats with acute FIC (Figure 4.2)

No significant correlation was found between urine concentrations of IL-6, sFAS and serum concentrations of IL12 (p40), SDF1 and IL18.

4.4 Discussion

To our knowledge this is the first study investigating serum and urine cytokines and chemokine concentrations in cats with FIC. We have identified a total of five potential inflammatory biomarkers, two in urine (IL-6 and sFAS) and four in serum (IL-12 (p40), SDF-1, IL-18, Flt3L) that were significantly increased in FIC affected cats. Two additional cytokines detected in urine (IL-2 and IL-8 were also substantially increased and trending towards significance.

IL-6 is a cytokine with pleiotropic effects, which can be produced by a wide variety of cells [15] and is important for acute inflammatory responses[19] as well as in chronic disease. IL-6 promotes T-cell proliferation and survival [20,21]. IL-6-induced cellular responses are initiated by binding to the soluble or membrane bound receptor IL-6R and co-receptor gp-130, and subsequent activation of the JAK-STAT pathway [22]. Depending on the type of receptor binding, IL-6 can play proinflammatory or anti-inflammatory /reparative roles [23]. Similar to increases in urine IL-6 observed in our FIC cats, IL-6 is increased in urine of people with IC/PBS [14,15,24]. IL-6 in conjunction

with another mast cell released factor methylhistamine has been shown to have sensitivity of 70% sensitive and specificity of 72.4% for diagnosis of PBS/IC [24]. In addition, increased amounts of IL-6 in urine of humans with IC/PBS have been shown to correlate with patient pain scores [15] and nocturia [14]. Increased IL-6 in urine and increased gene expression in bladder tissue were associated with decreased bladder volume in human patients [25,26]. Interestingly, in our study we did not detect increase in serum IL-6 ($p= 0.26$) concentrations in FIC affected cats, whereas studies in human patients identified significant increases in serum concentrations of this proinflammatory cytokine [27]. The reasons for this disparity are unknown but may be related to differences in patient populations. People with IC/PBS typically have chronic disease with at least 6 months of clinical signs [28], while the majority of cats investigated in the current study were patients with acute disease with clinical signs of only several days duration. Another possible explanation for differences between serum and urine concentrations may be related to differences in the distribution of cells producing IL-6. Previous studies in humans have identified urothelium, suburothelium and blood vessels as layers actively expressing IL-6 as determined by in situ hybridization [15]. However, studies investigating the distribution of IL-6 expressing cells in cats have not been reported. It is conceivable that cats may have a different distribution of IL-6-producing cells than people. Some human studies have investigated IL-6 as a potential measure of response to therapy. In one study, IC/PBS patients in clinical remission after intravesical injection of BCG had levels of IL-6 similar to unaffected controls, which may suggest utilization of this marker as potential clinical response measure [29]. Finally while we were able to detect IL-6 in both normal and FIC affected cats, another study investigating urinary cytokines in feline chronic kidney

disease was unable to detect IL-6 in both affected as well as normal cats [30]. The reason for that is unknown, but may be related to differences in types of assays utilized.

Fas is a receptor involved in the extrinsic apoptotic pathway, which has been previously shown to be activated in people with IC/PBS [31]. sFas investigated in this study is an alternatively spliced form of Fas, which exerts protective, anti-apoptotic function [32]. To our knowledge, studies investigating levels of sFas in urine of patients with IC/PBS have not been reported; however it has been investigated in chronic kidney disease [33] as well as bladder cancer [34]. In the present study, sFas was increased in urine, but not serum, of cats with FIC reflecting a more localized response.

We have identified four cytokines/chemokines that were significantly higher in serum of FIC-affected cats compared to controls (IL-12 (p40), SDF-1, IL18, Flt3L). To our knowledge, studies investigating serum concentrations of these cytokines in cats with FIC or people with IC/PBS have not been reported.

Interleukin 12 is a proinflammatory cytokine composed of two subunits p35 and p40 [35]. Antigen-presenting cells are the main source of IL-12 in the body and IL-12 is an important mediator of Th1 responses and induction of INFγ production [35-37]. Limited information is available on role of IL-12 in human IC/PBS. This cytokine was not significantly increased in bladder biopsies of patients [38]. However, urinary IL-12 was increased in human patients with overactive bladder syndrome [39]. Increased RNA expression of IL-12 (p40) within bladder tissues was also identified in mice with experimental autoimmune cystitis [40]. Interestingly, human patients receiving intra-vesicular instillation of IL-12 for treatment of superficial bladder transitional cell carcinoma had side effects that were similar to IC/PBS such as pain, dysuria or increased frequency [41]. In the present study, IL-12 was increased in serum, but not

urine, of cats with FIC. The reason for this disparity is unknown, but may also be related to a more systemic response rather then a localized one.

Interleukin 18 is another Th1 proinflammatory cytokine, and significantly increased serum concentrations were observed in our cohort of FIC affected patients. It is well established that IL-18 and IL-12 work in synergy to induce of INFγ expression in T-cells, NK cells and dendritic cells [36,42,43]. IL-18 was observed to be significantly increased in bladder tissues of IC/PBS- affected patients. Furthermore, decreasing urine concentrations of this cytokine in IC/PBS patients have correlated with clinical improvement post hydrodistention therapy [38]. In the present study, IL-18 was increased in serum, but not urine, of cats with FIC. Like IL-12, reasons for this disparity are unknown, but may reflect a systemic response rather then a localized one.

SDF1 (also known as CXCL12) is a chemokine, which induces migration of various cells such as T-cells, B-cells, macrophages and dendritic cells through its receptor CXCR4 [44,45]. We observed significantly increased concentrations of SDF1 in serum, but not urine, of cats with FIC. Studies investigating the role of this chemokine in human or feline cystopathies have not been reported. However, increased levels of SDF1 in the bladder tissue was documented in cyclophosphamide-induced bladder inflammation rat model [46] . Pain is one of the elements of both FIC and IC/PBS and the chemokine SDF1 has been shown to directly affect nociceptive neurons and induce pain [47]. In the present study, SDF1 was increased in serum, but not urine, of cats with FIC. The reason for this disparity is unknown, but may also be related to distribution of SDF1 producing cells and release of the factor to the vasculature rather then into the urine. SDF1 expressing cells are present in most organs in the body [48,49] including urothelium [50] while the exact distribution in cats in unknown.

Another significantly increased cytokine in the serum of FIC cats was Flt3L.The receptor for this ligand, Flt3 is solely expressed in hematopoietic progenitor cells and is crucial for development of dendritic cells and enhances production of lymphoid progenitors [51-53]. Flt3L has been implicated as a proinflammatory factor in the pathogenesis of a variety of inflammatory diseases [54,55]. It has been also shown to have a protective anti-inflammatory role in some animal models [56]. One proposed mechanism for this action of Flt3L has been the induction of T-regulatory cells [57]. The exact reason for increased Flt3L in our cohort of FIC-affected cats is unknown. To our knowledge studies investigating the role Flt3L in human IC/PBS have not been reported. The exact pathogenesis of FIC is still largely unknown and multiple potential causes have been proposed [8]. One could speculate that the reason for increased serum Flt3L is in response reaction to infection, as previously it has been noted that Flt3L is transiently induced upon murine CMV infection or exposure to TLR agonists [58]. The potential role of viral infection in the pathogenesis of FIC has been previously investigated [59-63] but the results do not explain all of the cases of the disorder. The hypothesis that increased Flt3L is a reaction to an infectious agent is further supported through concurrent increase of other cytokines important for function of innate immune system and Th1 responses- IL12 and IL18. In the present study, Flt3L was increased in serum, but not urine, of cats with FIC. The reason for this disparity is unknown, but may be related to the tissue source of this factor. Flt3L can be produced by a wide variety of cells in the body both immune cells as well as non immune cells such as fibroblasts or endothelial cells [64,65] and NK cells are the major source of Flt3L in the body [64]. Interestingly Flt3L can be also released by mast cells after exposure to uric acid [66] and as urothelial barrier is damaged in FIC cats [7] infiltration of uric acid into the bladder wall could be a factor.

Two additional cytokines trending towards significance in urine of FIC affected cats were IL-2 and IL-8. Urine IL-8 was previously studied in patients with IC/PBS. Activated mast cells release IL-8 [67] and can stimulate urothelium to do so as well [68]. Urine concentration of IL-8 were previously correlated with mast cell counts [16]. In another study urine concentrations of IL-8 were not significantly increased compared to controls, except for small subset of patients [14]. IC/PBS patients, who were in disease remission after BCG treatment had decreased levels of IL-2 and IL-8 compared to patients with active disease [29]. Serum levels of IL-8 in patients with IC/PBS were previously reported being increased compared to controls [27], which was contrary to the results of our study.

We have identified multiple potential biomarkers of FIC. However this study has some limitations including: the relatively low sample size, the limited numbers of cats with chronic disease, the lack of histologic phenotyping by light microscopy, and the retrospective nature of the study. In addition, future studies should incorporate additional control populations of cats with non-FIC lower urinary tract diseases such as urolithiasis or bacterial urinary tract infection to assess the specificity of changes in cytokine and chemokine concentrations. The limited number of patients decreased the statistical power to detect more subtle differences in cytokine concentration and increased the risk of type II statistical error in our data set. Only 3 cats included in our study had chronic FIC while the majority cats were diagnosed with acute disease. That raises the question whether significant differences in cytokines observed in the present study would be similar in larger cohort that included a greater number of chronic cases. Most of urine concentrations of cytokines observed in the three chronic FIC cases were interestingly at the lower end of the data set and similar to levels observed in controls (Figure 4.2, labeled as stars). The limited number of chronic cases precluded us from

running statistical analyses. This emphasized the need for future studies analyzing cytokines in cohort of patients with chronic FIC, as these cats would especially benefit from sensitive and specific biomarkers to measure treatment responses. FIC cases in general and chronic FIC in particular, would benefit from thorough phenotyping including cystoscopy and bladder biopsies in order to be able to fully assess the diagnostic utility of cytokines in these cases. Our group [5] and others [12] have previously identified that a subset of cats with chronic FIC has hyperproliferative changes in the urothelium. It is unknown whether functional changes are associated with this type of FIC and how it would affect urinary cytokine concentrations.

Future studies are needed to determine the optimal methods for preparation and storage of feline urine samples for cytokine/protein analysis. We utilized a 1:1 dilution of urine with sample buffer as suggested by manufacturer, while other investigators have concentrated urinary proteins for cytokine analyses [30]. In addition, the stability of cytokines in feline urine remains incompletely understood. We have identified that many of the cytokines undergo degradation with just a single freeze thaw cycle (supplementary data).

This is the first study investigating cytokine serum and urine concentrations in cats with FIC. It creates a strong foundation for future studies investigating the role of cytokines in FIC and their utilization as biomarkers for diagnosis, differentiating disease phenotypes, and monitoring therapeutic outcomes in different forms of the disease. Future studies are needed to optimize sample preparation and storage, and to evaluate the specificity, sensitivity and clinical utility of these serum and urine cytokines as noninvasive biomarkers for FIC.

APPENDIX

Table 4.1. Potential serum and urine biomarkers for idiopathic cystopathies in people and cats.

Marker	Sample	Method	Species Investigated		References
			Human	Cat	
Antiproliferative Factor (APF)	U	Bioassay	X	NR	[69-71]
Chemokines; CXCL-1, -10	U	BB-CLIA	X	NR	[13]
Epidermal Growth factor (EGF)	U	ELISA	X	NR	[14,72]
Genomic Profile	T/C	DNA sequencing	X	NR	[73-75]
GP51	U	ELISA	X	X	[76]
Interleukins: IL-1β, -2, -6, -8, -12, -18	U, S	ELISA, BB-CLIA	X	X	[13,14,27,38,77]
Lipidomic Profile	T	LC-MS/MS	ND	X	[78,79]
Metabolomic Profile	U, S	IRMS, LC-MS/MS, NMR, Q-TOF-MS	X	X	[75,80-83]
MicroRNAs	T	qPCR	X	X	[84-86]
Nerve Growth Factor (NGF)	U, S, T/C	ELISA, BB-CLIA	X	NR	[13,71,77,87-89]
Prostaglandin E2 (PGE2)	U, T/C	ELISA, LC-MS	X	X	[79,88,89]
Proteinomic Profile	U, S	LC-MS/MS, MALDI-TOF-MS NMR, Protein array	X	X	[75,81,83,90,91]
Stromal Cell-Derived factor-1 (SDF-1)	U, S, T	BB-CLIA	X	X	[38,86]
Tumor Necrosis Factor alpha (TNFα)	U,S,T/C	ELISA, BB-CLIA	X	X	[27,74,77]
Transcriptomic Profile/Gene Expression	T/C,US, PBMC	RNA sequencing, Gene array, qPCR	X	X	[17,75,92,93]
Urinary Trefoil Factor 2	U	MALDI-TOF-MS	NR	X	[91]

BB-CLIA= bead-based chemiluminescent immunoassay; CE-MS= capillary electrophoresis mass spectroscopy; DEAEC-C= Diethylaminoethyl cellulose ion exchange chromatography; DESI-MS= desorption electrospray ionization mass spectroscopy; ELISA= enzyme-linked immunosorbent assay; GWAS= genome-wide association study; IRMS= infrared microspectroscopy**;** LC-MS/MS= liquid chromatography tandem mass spectroscopy; MALDI-TOF-MS= Matrix-assisted laser desorption ionization time-of-flight mass spectroscopy ; NR= not reported; PBMC= peripheral blood mononuclear cells; P=plasma; qPCR=quantitative real-time polymerase chain reaction; Q-TOF-MS=quadrupole time-of-flight mass spectroscopy; RFLP= restriction fragment length polymorphism; RIA= radioimmunoassay; SELDI-MS= Surface-enhanced laser desorption/ionization mass spectroscopy; S=serum; SM= stone matrix; T/C= tissue or cells; TRAP= telemetric repeat amplification protocol; U= urine

Table 4.2 Mean serum and urine concentrations of 19 cytokines/chemokines in cats with FIC vs. healthy control cats. In bold are cytokines significantly different between two groups

	Serum Concentration (mean±SD)			Urine Concentration (mean±SD)		
Cytokine	FIC	Control	P value	FIC	Control	P value
sFas	15.55 ± 44.25	35.78 ± 48.35	0.3014	**13.46 ± 6.07**	**9.03 ± 3.033**	**0.0299**
Flt3L	**358 ± 104.3**	**267.9 ± 290.2**	**0.0015**	622.1 ± 419.1	402.3 ± 203.8	0.4271
GM-CSF	ND	ND		54.93 ± 71.47	72.05 ± 76.72	0.4427
INF	463.8 ± 462.8	2293± 5290	0.2850	ND	ND	
IL-1b	ND	ND		ND	ND	
IL-2	ND	ND		120.8 ± 111	39.7 ± 70.7	0.0646
PDGFBB	ND	ND		ND	ND	
IL-12 (p40)	**1191 ± 484.9**	**219.6± 393.3**	**< 0.0001**	105.5 ± 100.8	97.07 ± 67.07	0.8123
IL-13	37.47 ± 49.42	64.94± 80.49	0.5513	ND	ND	
IL-4	537.8 ± 521.4	1397± 1244	0.1477	147.3 ± 205.6	39.42 ± 46.87	0.1207
IL-6	248.6 ± 369.4	2245± 6052	0.1561	**236.2± 189.3**	**39.71 ± 51.46**	**0.0011**
IL-8	30.81 ± 39.78	123.6± 184.1	0.1374	662.8 ± 1036	127.2 ± 85.1	0.0849
KC	101.1 ± 143	30.23± 37.86	0.1183	ND	ND	
SDF1	**2015 ± 1131**	528.7**±** 1002	**0.0024**	4092 ± 2055	4712 ± 2301	0.7189
RANTES	141 ± 87.49	129.9 ± 171.1	0.1857	ND	ND	
SCF	237.3 ± 129.4	340.6 ± 387.2	0.8798	207.1 ± 207.4	319.1 ± 352.9	0.6015
MCP-1	2024 ± 2756	6047± 9604	0.2713	ND	ND	
TNFa	133.0 ± 279.3	717.7± 2048	0.6646	12.24 ± 18.96	9.33 ± 23.15	0.3728
IL18	**483.8± 468.8**	**92.03 ± 272.6**	**0.0202**	42.42 ± 89.72	9.891 ± 18.12	0.6458

Table 4.3 Urinalysis results of 15 cases of FIC and 13 healthy controls utilized in this study

FIC	Specific gravity	RBC	WBC	Crystals	pH	Controls	Specific gravity	RBC	WBC	Crystals	pH
1	1.044	TNTC	<2	None	6	1	1.073	0-5	0-1	None	6.5
2	1.021	<1	<1	None	6.1	2	1.064	0	0	Many struvites	7.3
3	1.048	<1	<1	None	6.4	3	1.05	0	0-3	Many struvite, few amorphous	7.2
4	1.052	5-10	<1	None	6.1	4	1.072	10-20	0-1	None	5.8
5	1.051	<2	<2	None	6.1	5	1.057	0-2	0-2	None	7.1
6	1.057	5-10	<1	Occasional struvite	7.4	6	1.061	0	0-1	Many struvite, few amorphous	7.2
7	1.028	30-40	2-5	None	6.8	7	1.069	1-3	0	Few struvites	7.3
8	1.052	TNTC	2-4	Many struvites	7.3	8	1.051	0	0-1	None	5.9
9	1.06	40-50	1-3	Moderate struvites	7.4	9	1.051	0	0	None	7.2
10	1.045	>60	0	None	6.8	10	1.057	TNTC	0	None	7
11	1.054	TNTC	2-4	None	6.5	11	1.044	0	0	None	6.1
12	1.059	TNTC	1-2	None	6.3	12	1.072	0	01	None	6
13	1.042	5-10	0	None	6.1	13	1.06	0	0	None	6.7
14	1.064	<2	0	None	6.2						
15	1.051	0	0	None	6						

Figure 4.1 Concentrations of IL12 (p40), SDF1, Flt3L and IL18 in serum of 13 FIC cats were significantly higher then in 13 healthy control cats. A single chronic FIC patient is labeled as a star.

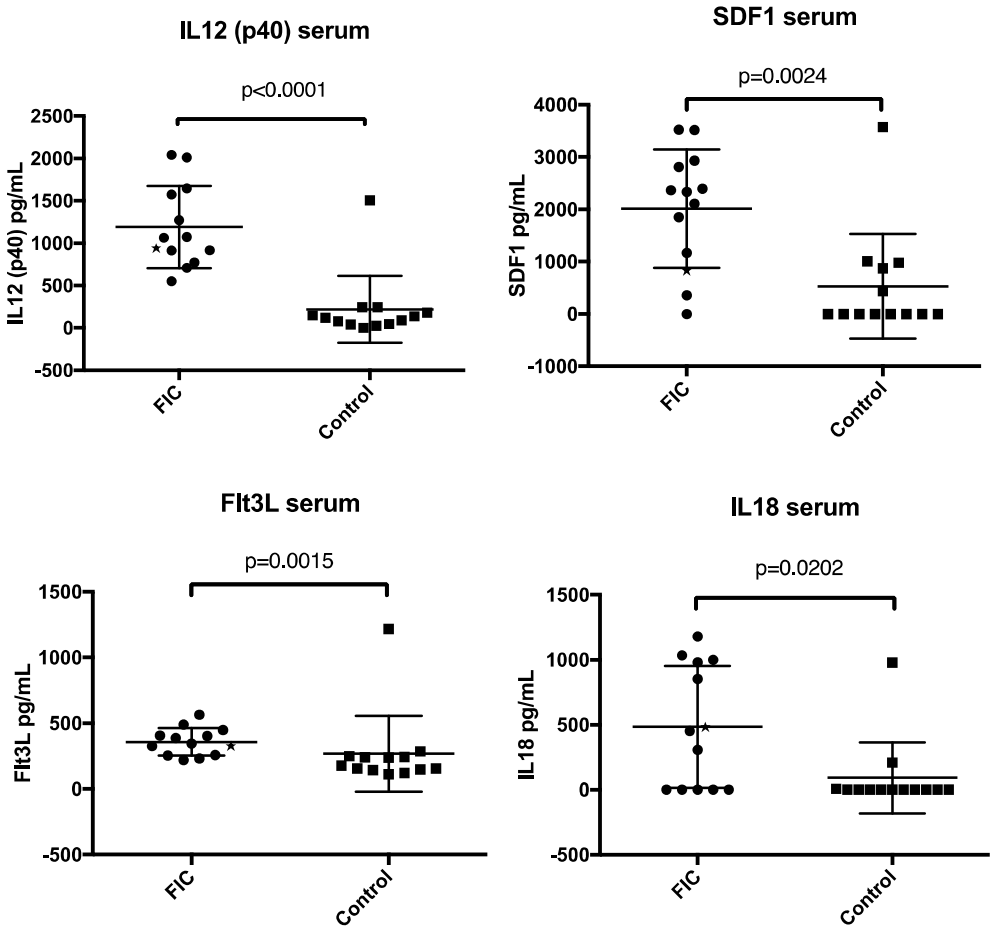
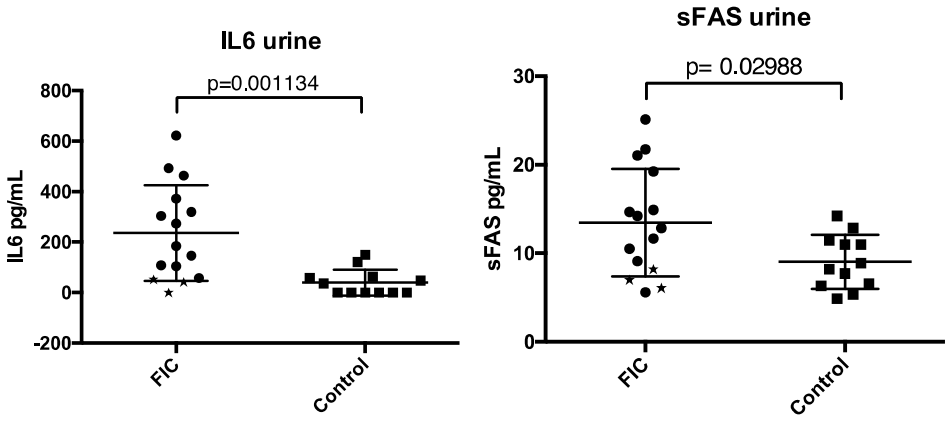


Figure 4.2 Concentrations of IL6 and sFAS in urine of 15 cats with FIC were significantly higher then in 13 unaffected cats. Chronic FIC cats are labeled as stars.



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CHAPTER 5

INVESTGATING THE USE OF MESENCHYMAL STEM CELLS FOR TREATMENT OF CHRONIC FELINE IDIOPATHIC CYSTITIS – A PILOT STUDY

5.1 Introduction

Feline idiopathic cystitis (FIC) is estimated to affect hundreds of thousands of animals annually in the US[1]. Clinical signs in affected cats include pollakiuria, periuria, dysuria, and stranguria [1,2]. While most of cases of FIC are self-limiting within approximately one week, a subset of affected cats experience clinical signs that persist for months or even years [1]. These cats are classified as having chronic FIC. Chronic or frequently recurrent form of the disease accounts for approximately 15% of all affected cats [1]. The exact pathogenesis of FIC remains unknown, although several theories have been proposed including neuroinflammation, urothelial dysfunction, stress or infection [1-10]. Histopathological and immunohistochemical analyses of bladders of cats with chronic FIC performed by one of the member of our group, have revealed that T-lymphocyte and mast cell infiltration exists in majority of cases of chronic FIC [7,8]. The clinical and morphologic features of chronic FIC are strikingly similar to those of an idiopathic cystopathy of humans called interstitial cystitis/painful bladder syndrome (IC/PBS)[7,8,11-13].

A wide variety of treatments have been suggested over the years; however, none of these therapies have been shown to be consistently effective for management and prevention of chronic FIC [1,2,5,14-16]. The inability to successfully treat chronic FIC is often the reason that many affected cats are surrendered, abandoned or euthanized.

Mesenchymal stem cells (MSC) have recently emerged as potent immunomodulators that have the potential to treat a variety of chronic inflammatory conditions [17-19]. MSC express a variety of soluble and membrane bound factors, such as indoleamine 2,3-dioxygenase (IDO), transforming growth factor beta (TGF-beta), interleukin 10 (IL10), prostaglandin E-2 (PGE2) or programmed death ligand 1(PD-L1) that modulate the function of inflammatory cells in the body [16,17,20-24]. Exposure to

MSC slows down T cell proliferation and induces formation of T-regulatory cells, which leads to long-term immunomodulation [25,26]. Mast cell degranulation is decreased after exposure to MSC [27]. MSC also release several growth factors that could assist with restoration of urothelial barrier function [28].

We hypothesized that injection of autologous MSC would alleviate the clinical signs of chronic FIC by reducing inflammation within the bladder wall and supporting urothelial regeneration. The goal of this pilot study was to evaluate the efficacy of intraperitoneally injected MSC to eliminate or reduce the signs associated with chronic FIC. Assessment of migration of these MSC towards the bladder was performed using iron particles with which MSC were labeled prior to injection and were detected using MRI.

5.2 Materials and methods

The study design was an open prospective pilot study of use of autologous MSCs for treatment of cats with chronic FIC. Cats with a history of chronic FIC that have failed conventional therapy were recruited through local veterinarians. All cats were examined at the Michigan State University Veterinary Medical Center (MSU-VMC) and were evaluated with a standardized medical history, a complete physical examination, and a routine health screen consisting of a serum chemistry profile, a complete blood count, FeLV antigen and FIC antibody testing, a complete urinalysis, a urine culture for aerobic bacteria, and an abdominal ultrasound. Inclusion criteria included: a) two or more lower urinary tract signs (pollakiuria, periuria, dysuria, macroscopic hematuria, or stranguria); b) clinical signs persisting for more than three weeks or recurring frequently (i.e. three or more episodes in three months); c) between 1 and 8 years of age; d) housed indoors exclusively; e) households with a maximum of one other cat, and

f) failure of currently recommended treatment for FIC (dietary, pharmacologic, and environmental management therapies). Cats were excluded from the study if they: a) had any other concurrent disease b) had undergone cystotomy or cystoscopy within the last year, c) had urinary tract obstruction within past year, or d) had received any corticosteroids, NSAIDs, antidepressants, antihistamines or glycosaminoglycan preparations within four weeks prior to enrollment. All owners were required to review and sign an informed consent form. This study was approved by the MSU Institutional Animal Care and Use Committee

Once enrolled, lower urinary tract signs were quantified and scored by the owner for a pre-treatment period of 8 weeks using a daily log and standardized scoring system (see APPENDIX). Daily owner observations included: difficulty of urination, blood in urine, urination outside of the box, frequency and straining. Each clinical sign, except for straining, was scored for severity where 1 was mild, 2 moderate, 3 severe. These observations were intended to serve as baseline measurements for assessing response to MSC therapy. During this baseline period and throughout the remainder of the study, cats were continued on their current diet, medications, and environmental enrichment.

After baseline observations, cats returned to the MSU-VTH for harvesting and processing of MSC. Subcutaneous adipose tissue was harvested from the ventral abdomen by sterile surgical excision. Animals were premedicated with acepromazine (0.05 mg/kg IM) and butorphanol (0.1-0.4 mg/kg IM), induced with propofol (up to 5 mg/kg), and maintained with isoflurane (0.25-3% in 100% oxygen). This anesthetic regimen was also utilized for subsequent MRIs. The surgical site was clipped and prepped using alternating chlorhexidine and alcohol scrub. A 1.5 cm skin incision was made in close proximity to the umbilicus and 3-5 g of fat was excised. The wound was

then closed with an intradermal pattern of 4-0 PDS, burying the knots at the beginning and end. Post surgical analgesia was provided using opioids (buprenorphine 0.015mg.kg buccally q8 to 12 hrs). MSC were subsequently isolated from adipose tissue, expanded and characterized as previously described [29,30].

Prior to injection, MSC were quantified and half of the cells were incubated with PLGA-coated iron nanoparticles at a density of 10 pg of iron per cell (20-30 nonoparticles), which has been previously shown to be sufficient to acquire an MRI signal. PLGA-coated iron particles were kindly provided by Dr. Erik Shapiro and were prepared as previously described [31]. After loading with nanoparticles, cultured MSC were trypsinized, washed three times, quantified and re-suspended in PBS for injection.

Two weeks after adipose tissue collection, cats returned to the MSU-VMC for pre-injection evaluation with a CBC, chemistry panel, urinalysis, and urine culture. All cats were anesthetized and a pre-injection abdominal MRI was performed. A total of $1x10^6$ MSCs per kg of body weight were then injected intraperitoneally in close proximity to the bladder under ultrasound guidance. Immediately after injection, an abdominal MRI was repeated. MRI scans were performed at MSU-VMC Diagnostic Imaging section using 1.5T Siemens MRI.

After recovery from anesthesia, cats were discharged from the hospital and owners were asked to quantify post-treatment lower urinary tract signs using the same daily symptom log used for baseline observations. A CBC, serum chemistry profile, urinalysis, and urine culture were scheduled to be repeated after one week and at one, two and six months post injection. An abdominal MRI was repeated at one week post-injection to assess MSC migration.

5.3 Results

5.3.1 Patient population

A total of 5 FIC affected cats were screened as potential candidates. Two animals were excluded from the study due to insufficient severity of the disease or previous treatments did not exhaust other possible options. One animal was excluded due to concurrent liver disease and one due to concurrent urinary tract infection. A single cat fit all the criteria and was included in the study.

5.3.2 Case report

A 5-year-old male castrated Domestic Short Hair cat, weighing 8.3 kg, was presented to the MSU-VMC with a one year history of persistent pollakiuria, periuria, dysuria, and stranguria. One month prior to presentation, the cat developed gross hematuria that coincided with moving to a new home. The cat was housed indoors and lived with one other cat and one dog. The cat was fed a commercially available dry obesity management food. The cat had been unresponsive to treatment with alprazolam, glucosamine, chondroitin sulfate, robenacoxib, cold laser therapy, and a variety of antimicrobics. At initial evaluation, physical examination revealed no significant abnormalities with the exception of obesity (BSC 9/9). Results of a CBC and serum chemistry profile were within normal limits (Table 5.1 and 5.2). Urinalysis revealed concentrated acidic urine with mild microscopic hematuria (
Table 5.3). Urine culture for aerobic bacteria was negative. Abdominal ultrasonography detected small renal infarctions in both kidneys, no additional abnormalities were observed. Over the next 6 weeks of preinjection observations, the cat had an average weekly clinical severity score of 1.75 (±0.58, Figure 5.1).

On week 6, the cat returned to the MSU-VTH for collection of adipose tissue as described above. Physical examination was unremarkable. The cat was anesthetized

and two adipose tissue specimens were harvested from the right ventral abdomen. The cat recovered uneventfully and was discharged from the hospital with opioid analgesia (buprenorphine 0.015mg/kg buccally q8 to 12 hrs.) for 3 days.

On week eight, the cat returned to MSU-VMC for MSC administration. Clinical signs had remained unchanged. Physical examination and results of a CBC, serum chemistry profile, urinalysis and urine culture were within normal limits (Tables 5.1-5.3). The cat was anesthetized and a preinjection MRI was performed. The cat then received $1x10^6$ iron labeled MSC per kg intraperitoneally under ultrasound guidance in close proximity to the bladder. An MRI was immediately repeated post injection. T2 weighted MRI images revealed a hypointense signal due to iron particle loaded MSC adjacent to the apex of the urinary bladder (Figure5.2A). The cat recovered uneventfully with no observable adverse effects.

The cat returned to the MSU-VMC at 7 days post-injection. The owner reported a dramatic decrease in clinical signs over the past week with only one episode of periuria and hematuria observed immediately after returning home from the previous visit (Figure 5.1). No adverse effects were observed by the owner. Physical examination and results of a CBC, serum chemistry profiles were within normal limits (Table 5.1-5.3). Urinalyses revealed concentrated acidic urine and mild microscopic hematuria. Urine culture was negative. Abdominal ultrasonography revealed that renal lesions were unchanged. The cat was anesthetized and an abdominal MRI was performed. T2 weighted MRI images did not allow definitive localization migrating MSC to the bladder wall. (Figure 5.2B)

Approximately 10 days post MSC injection, the owner reported a sudden relapse of clinical signs including pollakiuria, periuria, and hematuria. Signs were persistent and increasingly severe (Figure 5.1). The cat returned to the MSU-VMC at 21 days post

MSC injection for an unscheduled re-evaluation. Physical examination was unremarkable. Urinalysis revealed concentrated acidic urine with mild microscopic hematuria (Table 5.3). Urine culture for aerobic bacteria was negative, Abdominal ultrasonography did not identify any additional abnormalities compared to the previous visit. The cat was discharged and buprenorphine (0.015 mg/kg 2-3 times daily) and oxybutinin (0.2 mg/kg 2 times daily) were prescribed for symptomatic treatment of pain and urinary urgency.

At 8 weeks post MSC injection, the cat returned to the MSU-VMC for follow-up evaluation. The owner reported persistent lower urinary tract signs that were increasing in frequency and severity. In addition, the owner observed behavior changes including urine spraying in vertical surfaces and destructive chewing. The behavior changes coincided with another person joining the household. Physical examination was unremarkable and results of a CBC and serum chemistry profile were within normal limits (Table 5.1 and 5.2). Urinalysis revealed concentrated acidic urine with mild microscopic hematuria (Table 5.3). Urine culture for aerobic bacteria was negative, abdominal ultrasonography did not identify any additional abnormalities compared to the previous visits. The cat was discharged with a prescription for venlafaxine (5 mg q24 hrs.) for anxiety and neuropathic pain. Unfortunately, the Patient was then lost to follow up. Future communication with referring veterinarian's clinic revealed that the cat was later euthanized due to severity of clinical signs.

5.4 Discussion

Mesenchymal stem cells have emerged as potent immunomodulators that have the potential to treat a variety of chronic inflammatory conditions [18,19,32,33]. To our knowledge, this is the first study investigating the use of MSC for treatment of chronic FIC. Although only a single cat completed therapy, our observations provide important insights on potential adverse effects associated with intraperitoneal administration of MSC to cats with chronic FIC. Interesting, the one cat completing therapy initially experienced a dramatic reduction in the frequency and severity of clinical signs after MSC administration. Unfortunately, remission of signs was short-lived and approximately 10 days after injection, the owners reported a severe relapse of lower urinary tract signs in conjunction with other behavioral changes.

The causative factors precipitating the relapse of clinical signs are unknown. However, they may be related to administration of MSC, environmental changes associate with increased stress, or other co-morbid conditions that were not detected by follow-up evaluations.

Multiple mechanisms can be proposed as the reason of the adverse effects observed in the MSC treated cat. Our study investigating potential biomarkers for FIC described in chapter 4 of this dissertation identified increased SDF1 levels in serum of cats with FIC. MSC are known to express CXCR4 which is the receptor for the SDF1 ligand [34] and supports the notion that it is likely that MSC migrated to the bladder of the affected animal. Feline MSC are capable of producing large quantities of IL-6 in vitro (Chapter 2 of this dissertation). Similarly, a recent study investigating MSC-based treatment of chronic gingivostomatitis identified increased levels of IL-6 in serum of cats undergoing cellular therapy [19]. It is tempting to speculate that MSC localizing to the bladder wall may have released IL-6 within the bladder leading to further activation

of IL-6 signaling. Our preliminary RNA sequencing project on bladder tissue samples from cats with chronic FIC (Parys et al unpublished data) suggests enrichment of JAK-STAT signaling pathway within the urothelium of FIC cats. One gene in particular, gp130 (IL6ST) was significantly increased in affected cats. Gp130 is a co-receptor for IL-6R and is necessary for downstream signaling of both IL-6 as well as other cytokines including LIF and IL-11 [35], both of which can also be released by MSC [36,37]. With upregulation of this co-receptor, it is conceivable that MSC migrating to the bladder wall may have further potentiated the effects of IL-6 signaling and exacerbated clinical signs. This hypothesis is in concordance with previously published data on human IC/PBS where increased IL-6 concentrations correlated with urinary pain scores and clinical signs [38]. However, proof of this hypothesis would require additional studies. Unfortunately, bladder biopsy samples from our MSC-treated cats were not available.

Another possible explanation for the relapse of signs could be related to MSC-enhance expansion or increased maturation of B-cells into plasma cells and subsequent production of antibodies and immune-mediated inflammatory responses. Mixed mononuclear cell infiltrates, many including B-cells were frequently identified in bladder biopsy samples from cats with chronic FIC [7,8]. However, there is considerable debate regarding the effects of MSC on B-cells. In studies investigating the use of MSC in an animal model of lupus, MSC appeared to potentiated the disease process [39], whereas in another studies MSC ameliorated the disorder[40]. Proof of whether MSC-altered B-cell response could be related to the relapse observed in our patient would require further investigations.

The MSC used in this study were labeled with iron nanoparticles and it is conceivable that labeling altered the properties of MSC. However different types of iron particles have not been shown to affect viability or differentiation of MSCs [31,41] as

well as their immunomodulatory properties [42], thus it is unlikely that the side effect noted could be related to the iron particles.

Stress has been implicated as a risk factor in the pathogenesis of FIC [6]. Prior to enrollment, the owners reported worsening of clinical signs following a move to a new house. Interesting, relapse of lower urinary tract signs and appearance of additional behavior changes after MSC administration appeared to coincide with the owner's partner moving into the home. It is possible that these environmental changes could have led to exacerbation of the disease and abnormal urination patterns. In addition, we cannot completely exclude the possibility that travel, examinations, and anesthetic and surgical events associated the study could have been additional stressors.

Although the adverse effects observed in the single case described in this study should not completely exclude future trials investigating use of MSC for treatment of chronic FIC, they certainly indicate that a more cautious approach is warranted and emphasize the need for further studies characterizing the safety and efficacy of this treatment modality. Future studies should give consideration to including cystoscopy and bladder biopsy evaluations before and after MSC treatment. In addition, alternative route of administration should be considered. We have opted to use intra-peritoneal (IP) route based on the rationale that IP injection potentially results in increased survival and migration of MSC to the target organ allowing MSC membrane-bound factors to compliment the effects soluble factors. We previously have shown the safety of this route of MSC administration in healthy cats [29]. However, it is conceivable that an intravenous (IV) route of administration would be safer and equally effective in affected cats. With IV injection, MSC would not directly affect the bladder structures and infiltrating cells as they would mostly remain within the lung parenchyma [43], but

could indirectly provide anti-inflammatory effect through T-regulatory and B-regulatory cells [44,45].

This study illustrates the many challenges of implementing MSC-based therapy trials for this and other chronic inflammatory disorders encountered in cats. Further studies are necessary to investigate the safety and efficacy of MSC-based therapies for management of chronic FIC as well as to potentially explain the adverse effects that occurred in the patient described in this study.

APPENDIX

Table 5.1 Chemistry panel results of 5-year-old castrated male domestic shorthair cat with chronic FIC treated with intraperitoneal injection of autologous MSC. No significant changes were observed over the course of the study

	Screening	Preinjection	Week post injection	8 weeks post injection
Lipemia Chem	Normal	Normal	Normal	Normal
Icterus Chem	Normal	Normal	Normal	Normal
Hemolysis Chem	Normal	Normal	Normal	Slight
Urea Nitrogen	19	28	24	27
Creatinine	1.2	1.2	1.3	1.2
Sodium	149	155	155	151
Potassium	3.5	4.3	4.3	4.4
Chloride	115	117	119	119
TCO2	17	19	18	16
Na/K Ratio	43	36	36	34
Anion Gap	21	23	22	20
Osmolarity Calc	313	**327 H**	325	319
Calcium	9.9	10.1	9.8	9.8
Phosphorus	2.9	3.6	3.4	3.4
Magnesium	2	2.2	2.2	2.2
Iron	96	137	127	145
Total Protein	7.5	7.5	7.6	7.7
Albumin	3.8	3.6	3.7	3.8
Globulin Calc	3.7	3.9	3.9	3.9
Glucose	150	131	109	137
Amylase	1307	1216	1149	1213
Total Bili	0.1	0.1	0.2	0.1
Direct Bili	0	0	0	0
Indirect Bili	0.1	0.1	0.2	0.1
ALP	35	34	30	32
ALT	39	45	54	42
AST	22	18	21	19
CK	166	128	119	102
Chol	111	128	134	141

Table 5.2 Complete blood count results of 5-year-old castrated male domestic shorthair cat with chronic FIC treated with intraperitoneal injection of autologous MSC. Note mild monocytosis one-week post injection as well as gradual increase in number of band neutrophils; however all results remained in normal reference intervals.

	Screening	Preinjection	Week post injection	8 weeks post injection
Hemolysis	Normal	Normal	Normal	Normal
Lipemia	Normal	Normal	Normal	Normal
Icterus	Normal	Normal	Normal	Normal
Tot Protein	8.3	8.4	8.4	8.5
RBC	8.2	9.3	8.6	9.4
Hgb	13.3	15.1	13.9	15
Hct	40	43	39	44
HCT Spun	42	44	39	45
MCV	49	46	46	47
MCH	16	16	16	16
MCHC	33	36	35	34
CHCM	34	35	34	32
RDW	15	16	16	15
Platelet	**38 L**	323	331	329
MPV	12.7	12.2	13	11.7
WBC	9.1	5.8	6.4	7.4
Diff Type	Manual	Auto	Auto	Auto
Seg Neut # Manual	6.6	3.1	2.9	4.7
Band Neut # Manual	0	1.4	1.8	2
Lymphocyte # Manual	1.7	0.1	0.2	0.2
Monocyte # Manual	0.5	1.3	**1.6 H**	0.5
Eosinophil # Manual	0.3	0	0.01	0

Table 5.3 Urinalysis results of 5-year-old castrated male domestic shorthair cat with chronic FIC treated with intraperitoneal injection of autologous MSC. No significant changes were observed over the course of the study.

	Screening	Preinjection	Week post injection	3 weeks post injection	8 weeks post injection
UA Color	Yellow	Yellow	Yellow	Yellow	Yellow
UA Appear	Clear	Mild Turbidity	Mild Turbidity	Mild turbidity	Mild Turbidity
UA Spec Grav	1.042	1.045	1.048	1.049	1.043
UA pH	6.1	6	6.1	6	5.8
UA Protein	Negative	Negative	Negative	Negative	Negative
UA Glucose	Negative	Negative	Negative	Negative	Negative
UA Ketones	Negative	Negative	Negative	Negative	Negative
UA Heme	Large	Large	Moderate	Large	Moderate
UA Bili	Negative	Negative	Negative	Negative	Negative
UA WBC	None Seen	None Seen	0-2	None seen	None Seen
UA RBC	5-10	30-40	30-40	10-15	15-20
UA Epi Cells	None Seen	None Seen	None Seen	None Seen	None Seen
UA Casts	None Seen	None Seen	None Seen	None Seen	None Seen
UA Bacteria	None Seen	None Seen	None Seen	None Seen	None Seen
UA Sperm	None Seen	None Seen	None Seen	None Seen	None Seen
UA Crystals	None Seen	None Seen	None Seen	None Seen	None Seen
UA Fat Droplets	Few	Few	Moderate	Few	Moderate

Figure 5.1 Mean weekly clinical sign severity scores for a 5-year-old neutered male mixed-breed cats with chronic FIC treated with intraperitoneal administration of autologous mesenchymal stem cells. The cat's daily clinical sign logs were not maintained by the owner after study week 10. Clinical sign scores for study weeks 11 through 17 were estimated from owner's verbal description of the frequency and severity of signs. Green arrow points to the time of adipose tissue collection, while the blue arrow is the MSC injection date.

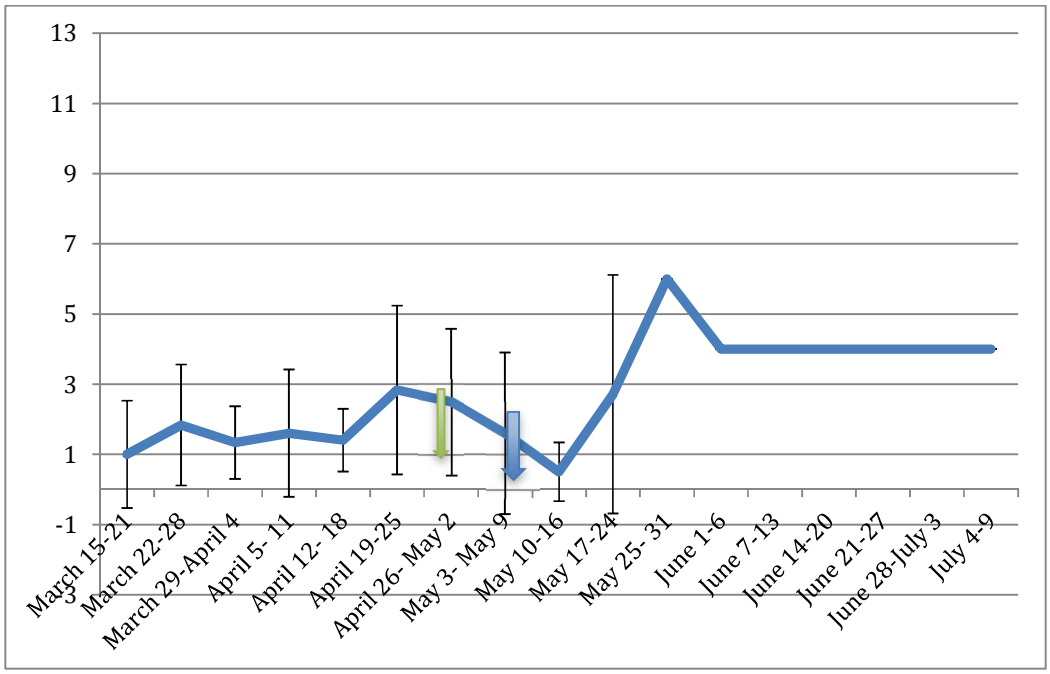
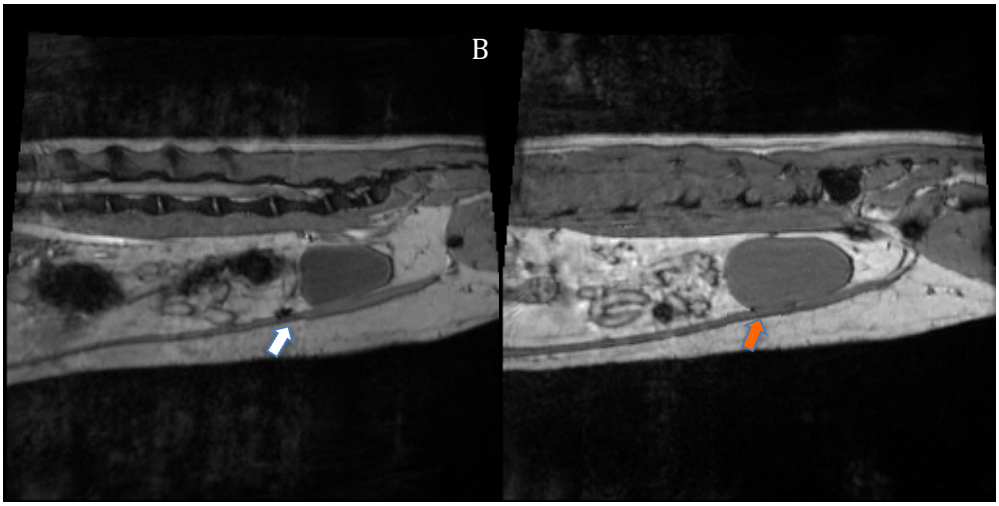


Figure 5.2 T2 weighted MRI image just after the injection (A) and a week post injection (B). Note hypointense signal due to iron particle loaded mesenchymal stem cells (white arrow in picture A). One week after injection (B) no precise recognition of putative migrating MSCs were possible, although one cannot completely exclude a site within the bladder wall (orange arrow).



Owner Clinical Signs Log Instructions

Throughout this trial, you are asked to monitor five clinical signs related to FIC. This will be accomplished by completing a daily log in paper form. The five clinical signs you are asked to look for are:

- Difficulty (dysuria) – difficulty passing urine, straining, and/or vocalizing due to pain on urination
- Blood (hematuria) – presence of pink urine or overt blood in the urine
- Outside box (periuria) – urinating in inappropriate places in the house, outside of the litter box
- Frequent (pollakiuria) – increased frequency of urination above the individual cat's normal behavior
- Straining (stranguria) – persistent but completely non-productive straining or posturing to urinate (the cat positions to urinate, but no urine is produced)

Please record the presence or absence of these signs by your cat on the attached sheets by circling one of the following responses for each sign:

- N/O = Not observed, you weren't around while your cat used the litter box/urinated
- Y = Yes, you observed this clinical sign today
- N = No, you were around while your cat used the litter box/urinated and you did not observe the sign

Anytime you observe a sign (circling Y), please further evaluate the Yes response in the comments section below that week using the following scale:

KEY	**None/Normal**	**1- Mild**	**2-Moderate**	**3-Severe**
Difficulty	Never occurs	1-34% of the time	35-70% of the time	>70% of the time
Blood in Urine	Never occurs	1-34% of the time	35-70% of the time	>70% of the time
Outside Box	Never occurs	1-2x	3-6x	>6 times
Frequent	2-3x a Day	4-6x a day	7-10x day	>10x a day
*Straining	Never occurs			

*Note: no severity clarification is needed for stranguria. This sign is either present or not.

At each office visit, we will collect this form for our records, so please keep it in your Owner's Folder and remember to bring it with you. At the final visit of the trial, you will return all of the original, completed paper logs to us. Contact us with any questions regarding scoring of clinical signs.

****IMPORTANT: If your cat experiences any clinical signs, please contact us within 24-48 hours to inform us of the recurrent episode. Upon consultation with you, we will determine if your cat needs to be examined**.

Clinical Signs Log

Record daily for each of the five signs if the sign was present, absent, or not observed. If Y is selected, please record in the space provided a severity score (1 = Mild, 2 = Moderate, 3 = Severe). No score is needed if straining is observed. See the cover instruction page for clinical signs definitions.

		Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
	Date:						
☐	**Difficulty**	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Blood	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Outside Box	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Frequent	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Straining	N/O N Y	N/O N Y	N/O N Y	N/O N Y	N/O N Y	N/O N Y

	Date:						
☐	**Difficulty**	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Blood	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Outside Box	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Frequent	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___	N/O N Y___
	Straining	N/O N Y	N/O N Y	N/O N Y	N/O N Y	N/O N Y	N/O N Y

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CHAPTER 6

FUTURE DIRECTIONS

The results of these studies underscore the great potential of using cats as translational models for MSC based therapies as feline MSC share many similarities with human MSC. Thus future studies can evaluate the use of cat MSCs in cats with FIC as well as other inflammatory diseases such as asthma, IBD, chronic pancreatitis or hepatitis.

The optimal injection route for MSC should be considered on a disease-by-disease basis and require further studies. Comparison of IV vs. IP route of injection in the clinical setting is necessary in order to establish the optimal route of MSC administration for specific diseases. Intraperitoneal delivery of MSC can be also evaluated in gene therapy approaches, when MSC are employed as gene transfer means, for example for cancers localizing to the peritoneal cavity, such as intestinal adenocarcinomas, or for delivery of deficient proteins such as erythropoietin in cases of advanced chronic kidney disease.

Results of this study provide the foundation for future work on cytokines as biomarkers of FIC. Several candidate cytokines have been identified in this study. However, they should be further evaluated prospectively in larger cohort of patients prospectively in order to ensure that our findings are clinically relevant and can be utilized as a response biomarker in this disorder.

Larger studies are needed to assess if MSC based treatment is safe and effective therapy for FIC and whether MSC could be a therapeutic option for IC/PBS. Better characterization of the phenotype of each FIC patient by cystoscopy and biopsy would facilitate selection of patients who would most benefit from MSC therapy. Furthermore one should consider investigating alternative routes of injection including both IV as well as IP routes to determine optimal route for FIC.