DISSERTATION

IMPACT OF ACTIVATED AND RESTING MESENCHYMAL STEM CELLS ON IMMUNE RESPONSES AND GUT MICROBIOME AND IMMUNE RESPONSES TO GUT BACTERIA IN DOGS WITH INFLAMMATORY BOWEL DISEASE

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ABSTRACT

IMPACT OF ACTIVATED AND RESTING MESENCHYMAL STEM CELLS ON IMMUNE RESPONSES AND GUT MICROBIOME AND IMMUNE RESPONSES TO GUT BACTERIA IN DOGS WITH INFLAMMATORY BOWEL DISEASE

Idiopathic inflammatory bowel disease (IBD) is one of the common diseases that causes gastrointestinal tract (GI) disorder and encompasses a group of unknown causes of chronic gastroenteropathies found to have persistent or recurrent GI signs along with intestinal and/or gastric inflammation. Similar to IBD in humans, the pathogenesis of IBD in dogs remains undiscovered, but it is believed to involve an interaction between the abnormal host immune response against intestinal microbiota and predisposing genetic and environmental factors. IBD is mostly incurable with long-term complications despite receiving standard treatments that are typical combinations of food trial, antibiotics, anti-inflammatory and immunosuppressive drugs. However, the therapeutic outcome of medical treatment appears to be multifactorial and inconsistent therapeutic responses ranging from transient recovery to no response have been found. One of the alternative treatments that potentially accelerates therapeutic effects is the use of mesenchymal stem cell (MSC) administration. Therefore, the goal of the research presented in this dissertation was to comprehensively investigate the impact of activated and resting MSCs on immune responses, cells regeneration and gut microbiome for treatment of IBD with a specific emphasis on gaining an improved understanding of the immune responses to the gut bacteria in dogs with IBD.

In the first part of the study, we needed to have better understanding of immunopathogenesis in IBD. Although it is not clear what triggers the intestinal inflammations in IBD affected dogs, we hypothesized that the disease may be mediated, in part, by an abnormal immune response directed against intestinal bacteria. We found the substantially greater percentages and overall binding of IgG and IgA with their intestinal bacteria in IBD dogs than healthy dogs, and the primary production of anti-bacterial antibodies occurs locally in the gut rather than systemically. The IgG-binding bacteria triggered an increase of phagocytosis and pro-inflammatory cytokine production by macrophages. Moreover, Actinobacteria (*Collinsella* genus) was the preferential target for the mucosal IgG immune response to dysbiotic bacteria. We concluded that the mucosal antibody binding to commensal gut bacteria was substantially greater in dog with IBD compared to a healthy, and that the immune response targeted particular bacteria and triggered the pro-inflammatory response in IBD. We noted that the more extensive studies in dogs with IBD and compared to animals with other causes for GI dysfunction may be required.

Then, we focused on the use of MSCs as an alternative treatment for IBD in animals and humans. To address this question, we used a mouse model of IBD to investigate the effectiveness of using 2 types of mesenchymal stem cells (induced pluripotent MSC [iMSC] and conventional adipose-derived MSC [adMSC]) for the treatment of IBD. The impact of MSCs on immune responses, cells regeneration and the gut microbiome were evaluated. We found that iMSC and adMSC treatment effects were equivalent on the basis of significantly improving clinical abnormalities and decreasing inflammation inside the gut. Both types of MSC also stimulated a significant increase in intestinal epithelial cell proliferation and amplified intestinal angiogenesis. Furthermore, the abnormal microbiome found in mice with IBD was returned to nearly normal values in terms of complexity and composition in mice with IBD treated with adMSC or iMSC.

We concluded therefore that the administration of iMSC enhanced the overall intestinal healing, suppressed inflammation, and microbiome restoration with equal effectiveness as treatment using adMSC in a mouse model of IBD. The future studies in animal model including spontaneous IBD in dog or large scale of clinical trial for long-term follow-up to determine iMSC safety and efficacy is required before clinical translation.

Finally, we investigated possible ways to improve the efficacy of mouse and dog MSC treatment by preactivating the MSC with inflammatory cytokines (IFN-g or TNF-a) or TLR agonists (TLR3 or TLR9 agonists). We investigated the response of canine MSCs to the 4 activating stimuli, including measurement of cell surface phenotype and cytokine release. Contrary to previous studies in other species including mouse and man, we found that the pre-activation of dog MSC generally had little effect on either phenotype or function. Therefore, we concluded that the ex-vivo preactivation of canine MSCs by inflammatory cytokines or TLR agonists is not warranted in terms of augmenting the functionality of the cells. We further concluded that dog MSC may be hyporesponsive to preactivating stimuli compared to those of MSC from other species such as mouse and man. Further studies are required for better understanding of the biology of canine MSCs and their responses to immune activation.

Overall, the work described in this dissertation has increased our understanding regarding the immunopathogenesis of the IBD in dogs. The studies have also demonstrated the equivalent activity of iMSC and conventional adMSC for treatment of IBD, and also documented a previously undescribed restorative effect of MSC on the intestinal microbiome. These studies also illustrated species specific differences in the responsiveness of MSC to common immune stimuli. These studies provide a robust foundation for further research and hopefully this work can help stimulate new investigations into alternative treatments for IBD in dogs and humans.

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1.1.1 Pathophysiology and Etiology

1.1.1.2 Demographic and Disease Characteristic of IBD in Dogs

Idiopathic inflammatory bowel disease (IBD) is one of the common diseases that causes gastrointestinal tract (GI) disorder and encompasses a group of unknown causes of chronic gastroenteropathies found to have persistent or recurrent GI signs along with intestinal and/or gastric inflammation [1-4]. Treatment with food trials, antibiotics or immunosuppressive drugs results in various therapeutic responses of transient recovery, including no response [1, 3, 5]. The pathogenesis of IBD in dogs remains poorly understood, but based on analogy to IBD in humans, is believed to involve an interaction between the abnormal host immune response against intestinal microbiota and predisposing genetic and environmental factors [6-9]. The primary features of IBD in dogs mostly resemble those in humans [4, 10], including high genetic sequence similarity [11] and pathogenesis [12], as well as the need for clinical intervention[2, 13]. Thus, spontaneous IBD in dog is considered a potential model for study, with the potential for extrapolation to human IBD.

The etiology of IBD in dogs remains mostly unknown. This disease has some features similar to other GI diseases that have known causative agents, including GI infectious diseases [2]. Additionally, IBD in dogs affects both genders equivalently and mostly is found in middle-aged dogs [14]. Although, several breeds have been documented to have a susceptible gene linked to

intestinal inflammation [11, 14, 15]. IBD potentially develops in most breeds, as it is a multifactorial disease. Exposure to other potential factors may also trigger the disease [6-9].

In human with Crohn's Disease (CD) and Ulcerative Colitis (UC), disease-susceptible genes have been recognized, and mutations have been investigated in particular genes [12, 16-18]. The recent studies in human and genetically engineered animals have documented that abnormal host immune response is potentially influenced by predisposed genes of IBD, including *NOD2/CARD15, IBD5, IL23R*, and *IRGM,* as well as single nucleotide polymorphisms (SNPs)[16, 19-21]. A previous study reported that the German Shepherd dog breed (GSD) has been found to have 16 susceptible genes linked with IBD, such as *Tlr5, IL13* and *IL4* [11, 15]. Some of these genes have been noted to play a role in Th2 pathway cytokines and are involved in UC pathogenesis in humans [18, 22]. In addition, several dog breeds, for example, Basenji and Boxer, are predisposed to specific types of IBD ; however, recent evidence has shown that Granulomatous Colitis in Boxer is associated with invasive *E. coli* in the gut mucosa [23]. Overall, dog IBD mostly resembles human IBD, and it is a multifactorial disease with unclear etiology. Dysbiosis [24], environmental factors and genetics [2, 11] are associated a dysregulated immune system, leading to intestinal injury (described in a later part).

1.1.1.3 Dysregulated Host Immune Response (Innate, Cell-Mediated and Humoral Immunity)

Abnormal innate immunity has a critical role and has long been associated with IBD development [12, 25]. Previous studies have reported an increased upregulation of Toll-like receptors (TLRs) and a higher presence of NF-kB activation, along with alteration of enteric immunity in a tissue biopsy from an IBD dog [15, 26-32]. TLRs on the surface of intestinal epithelial cells are thought to be crucial to maintaining intestinal mucosal tolerance to luminal bacteria [33, 34], and these TLRs,

including TLR2, TLR4, TLR5, and TLR9, were found to upregulate diseased tissues [15, 26]. Genetic abnormalities, including innate allelic variation and polymorphisms of those TLRs are thought to cause a functionally abnormal response to intestinal microbiota [12, 15]. Moreover, the degree of TLR expression in the duodenal tissue from IBD dogs was correlated to the disease activity index [28]. Uncontrolled or inappropriate TLRs activation and the subsequent loss of gut flora homeostasis can lead to the induction of an adaptive immune response and development of inflammation by pro-inflammatory cytokine production [28, 32, 33, 35].

Another significant innate immunity defect in IBD is NOD2, which is expressed in Paneth cells, dendritic cells (DC), macrophages and absorptive intestinal epithelial cell (IECs) [20]. NOD2 senses the bacterial lipopolysaccharide and activates downstream pathways, including NF-kB [21] and mitogen-activated protein kinase (MAPK), leading to the generation of an immune response [16]. Some studies have identified mutations in the NOD2 gene as relevant to CD pathogenesis [16, 19, 21]. Additionally, multiple SNPs were documented in the NOD2 gene found in IBD-affected GSD other breeds, suggesting that NOD2 mutations play an essential role in developing chronic mucosal inflammation in a canine population [36]. Thus, the impaired innate immune system contributes to the defective mucosal barrier and abnormal bacterial defense, contributing to inflammation of gut mucosa.

Dysregulated cell-mediated immune responses contributing to IBD pathogenesis have been reported in human IBD, including abnormal T cell activity; reduced regulatory T cells; abnormal bacterial killing activity; and increased activity of macrophages, lymphocytes, and neutrophils in gut mucosa [37-39]. Similar to observations made in humans, studies in dogs with IBD have shown an increase in specific immune cell infiltration, including IgA+ and IgG+ plasma cells, CD3+, CD4+, and CD8+T cells, macrophages, DC and neutrophils in the affected tissue [27, 31, 40,

[41]. CD4+ T cells are considered to play a key role in disease pathogenesis of IBD by regulating the Th1/Th2 pathway activation. These Th cells exert their effects by secreting proinflammatory (Th1 [IL-2, IL-12, IFN-g, and TNF-a], Th2 [IL-4, IL-5 and IL-6]) or immunomodulatory cytokines (IL-10 and TGF-b) [12, 22, 41-44]. Additionally, previous studies in human and experimental colitis have reported that Th17 cells are potentially involved in the pathogenesis of IBD [42]. The activation of the Th17 pathway results in pro-inflammatory cytokine (IL-17) production with subsequently tissue inflammation[37, 45, 46]. In addition, intestinal macrophages [47, 48], as well as DC [41, 49, 50], play a crucial role in maintaining homeostasis and regulating inflammation [51]. One of their functions is to keep the balance between the immune tolerance to gut flora and mucosal inflammation by balancing the regulatory T cell number and IL-10 production [41-43, 45, 52-55]. Dysregulation of these immune cells, which are associated with an increase in pro-inflammatory stimulation and less counterbalanced regulatory T cells, is generally believed to lead to the development of IBD [22, 37, 41, 43, 47, 50]. On the other hand, a reduction in some inflammatory cells, such as activated macrophage and T helper cells, is associated with clinical improvement in dogs with IBD; however, the CD3+ T cell was unchanged after clinical remission [13], suggesting that mucosal specific adaptive immunity may reside for a long period in the affected tissue [22, 42, 47].

In dogs with IBD, the mucosal cytokine profiles are defined broadly as being mixed, resembling the Th1/Th2 cytokine activation observed in human IBD and experimental colitis [22, 42, 56, 57]. A significant amount of pro-inflammatory cytokines has been reported in CD (described previously). In dogs, increased IL-1B, IL-2, IL-5, IL-12p40, TNF-a and TGF-b have been observed in the mucosal tissue of a dog with IBD [27, 29, 30, 58, 59]. However, similar changes also have been observed in gut inflammation due to Small Intestinal Bacterial Overgrowth (SIBO), suggesting a nonspecific cytokine profile for dog IBD [58]. The reasons behind this could be the variation that

occurs in the cytokine profiles over time in the different stages of disease [30]. In addition, TGF-b and IL-2 are potent immunoregulatory cytokines within the intestinal mucosa, and an increased level in these cytokines may suggest an attempt to downregulate an ongoing inflammatory response; however, no significant change was reported for IL-10 in several dog studies [30, 58].

In IBD, excessive humoral immune (HI) response also plays a crucial role in the induction of chronic active mucosal inflammation[60-67]. The augmented HI response against commensal gut bacteria by increased Immunoglobulin (Ig) binding to gut bacteria was observed in both systemic and local responses in human IBD [63, 65, 67]. In dogs with IBD, increased IgG +ve plasma cells at the lamina propria and increased concentrations of IgG in supernatant samples of colonic lavage indicate an immune activation [27, 64]. Moreover, IBD dogs had significantly higher levels of IgG and IgA bound to gut bacteria than healthy controls, and these antibodies were primarily produced locally in the gut rather than systemically (Soontararak, et.al manuscript submitted). Moreover, Ig-binding bacteria enhanced a proinflammatory effect by increased macrophage phagocytosis and activation, causing elevated TNF-a. This interaction could explain the presence of their commensal bacteria within mucosal macrophage of IBD dogs and remission signs after receiving immunosuppressive drug intervention[41, 68]. Overall, the predisposing genetic and environmental factors that contributed to an abnormal phenotype and impaired function of the mucosal immunity and abnormal host immune response against intestinal microbiota are considered the critical reason for the development of IBD.

1.1.2 Presenting Signs and Diagnosis

Common presenting signs in dogs are signs of chronic or relapsing gastrointestinal upset including vomit, diarrhea, weight loss, and alteration of appetite [2, 13, 69]. The clinical manifestations

of IBD may be diverse, depending on the organ(s) involved, the presentation of active/inactive disease, severity of inflammation and physiologic complications, including protein loss and malnutrition[2, 3, 70, 71]. The duration of sickness is more than 3 weeks, with recovery typically involving treatment with antibiotics, diets and/or immunosuppressants; however, many cases relapse or do not respond [3, 5, 72]. The lesion may involve any portion of the GI tract, including the stomach, small intestine and/or colon. Disease affecting the stomach and duodenum is associated with vomiting and small bowel diarrhea, whereas colonic involvement shows signs of large bowel diarrhea, including mucoid/bloody diarrhea with tenesmus [2, 28, 30]. The extensive lesion along the GI tract results in mixed bowel diarrhea. Importantly, other diseases also mimic IBD symptoms and would be considered for IBD differential diagnosis including food responsive diarrhea (FRD) and antibiotic-responsive diarrhea (ARD) [13, 73]. Additionally, the patient does not experience a full recovery with dietary and antibiotic treatment trial and typically needs immunosuppressants or anti-inflammatory therapy [5, 72, 74, 75]. Therefore, the diagnosis of IBD is likely challenging and requires comprehensive diagnostic tests to rule out other known causes of GI inflammatory disorder.

Several diagnostic tests are necessary to rule out other primary GI diseases because no specific diagnostic test nor pathognomonic marker exists to identify the IBD [69, 76-78]. Not only IBD, but other diseases causing GI signs and intestinal inflammation are in the differential lists, and these include parasitic, bacterial infection, non-GI disorder, exocrine pancreatic insufficiency, and SIBO [78-81]. Fecal examination by wet mount, fecal floatation and IFA are useful for parasitic infection [82]. Fecal culture testing for pathogenic bacteria may be done in the suspicions exist of pathogenic bacterial infection. Routine hematology possibly will reveal systemic inflammation, including neutrophilia with or without left shift. Biochemical analysis may reveal the abnormality

of other organs related to GI signs and patient health assessment including hypoalbuminemia and hyperglobulinemia in dog with protein-losing enteropathy (PLE), which is associated with a negative outcome [71, 73]. In many cases of chronic small bowel disease, serum cobalamin is reduced due to cobalamin malabsorption, and this may cause delayed recovery time and poor prognosis [71]. Abdominal ultrasound is useful for defining the affected area of mucosal disease, including wall thickening, diffused lesion to deeper tissue, or presence of lymphadenopathy and prompt fine needle aspiration for cytology [83].

Endoscopic examination with mucosal biopsy is crucial to confirm the gut mucosal inflammation. Mucosal friability increases granularity, and erosion is reported as an abnormality seen in dog IBD [84, 85] , and the extensive lesion may presence along both duodenal and colonic section [84, 86]. However, a study on dogs with severe clinical illness measured by CIBDAI did not show a correlation to endoscopic score [71].

Histopathologic evaluation of biopsy samples is required for definitive diagnosis of intestinal inflammation [87-89]. Although the histopathologic changes may be found in any GI segment, the small intestine is commonly the predominately affected site [89]. According to WSAVA guidelines for histopathologic evaluation for gastrointestinal inflammation [87], microscopic findings for dog IBD are classified based on the cellular infiltration within the intestinal mucosa and the varying degree of morphologic or cytoarchitectural damage accompanying this infiltration [77]. Although infiltration of inflammatory cells, including lymphocytes, neutrophils, eosinophils, and macrophages, can be found, lymphocytic-plasmacytic enteritis is the most common finding in dog with IBD [73]. Additional techniques, including special stain and fluorescence in situ hybridization (FISH), are considered if neutrophils or macrophages increase with a suspicious infectious process [82].

Similar to the approach used in humans, several clinical parameters are currently used in dogs as potential markers for diagnosis and patient assessment in chronic gastroenteropathy[4, 25, 90-93]. Clinical indices utilize a scoring system evaluated from GI signs alone (CIBDAI) [3] or are incorporated with clinical laboratory testing (CCECAI) [71], and these approaches remain the most widely used tools in the evaluation of disease activity in dog IBD. Because evaluation using disease activity index alone might have limitations, other biomarkers are used in human IBD to identify low-grade inflammation, response to treatment, remission, and relapsing for preemptive treatment[91, 94-96]. Fortunately, some of the human biomarkers are available in dog; however, those biomarkers have shown inconsistent results reports in several dog IBD studies [25, 90, 92, 97]. A variety of noninvasive serologic markers including anti-neutrophil cytoplasmic antibodies (pANCA), matrix metalloproteinases (MMPs), TNF-a, and IL-6 [90, 93, 98] provide an indirect assessment of disease activity and found the lack or weak correlation to the disease activity index, and endoscopic and histopathologic assessments in dog with IBD [86, 98]. Serum C-reactive protein (CRP)[92] and fecal calprotectin [25, 92, 99] were reported to be useful in assessing the response to treatment. However, serum CRP did not show a significant correlation with the CIBDAI and histopathologic lesion score [92]. So far, fecal calprotectin is suggested as a useful candidate biomarker that shows a significant positive correlation to other clinical parameters for dog IBD [92]. In summary, a diagnosis for IBD requires multiple diagnostic tools and likely needs diet and/or antibiotic therapy trials to rule out other known causes of GI inflammation. Idiopathic IBD is applied to dogs with intestinal inflammation confirmed by histological evaluation and that shows an inadequate response to dietary and antibiotic treatment trials. Additionally, no gold standard tool or biomarker is ultimately used for IBD diagnosis. These markers are beneficial for clinician use as adjunctive tools, along with other clinical parameters for diagnosis and therapeutic assessments.

1.1.3 Therapeutic Management and Prognosis

Treatment principles for canine IBD involve combination therapy using dietary and pharmacologic interventions [5, 72, 73, 100]. Although, limited studies with randomized, controlled drug trials have been performed on dogs with IBD, evidence-based observations have shown that feeding an elimination diet and treatment with the and immunosuppressive drug and/or antibiotic trials are useful for IBD treatment and management[5, 72]. Dietary management includes the elimination diet, and novel protein source restriction is used to control the exposure of dietary antigens and may reduce intestinal inflammation[101]. Moreover, a specialized diet with high palatability, high digestibility, and easy absorption potentially treats some abnormal physiological conditions, such as lack of appetite, malnutrition, protein loss[102]. Drug treatment in dog IBD is determined by several factors, including severity and extent of disease, duration of illness, adverse effect of drug, clinician experience and treatment cost [100]. Several immunosuppressive drugs have been reported to treat dogs with IBD effectively, including glucocorticoids [103] , and Cyclosporin[104]. Additionally, a combination of glucocorticoids and antibiotic regimens has been noted for their positive results in several studies[5, 72]. The suppression of gut microbes by antibiotics potentially reduce pathogenic bacterial colonization and attenuate the proinflammatory immune response occurring in the gut mucosa [105]. Moreover, some antibiotics are reported to have an immunomodulatory effect, such as metronidazole[106, 107], and may exert their effect synergistically with immunosuppressive drugs.

In addition, the principal of reintroducing the healthy microbiome into the affected GI tract is considered a promising strategy for preventing, treating and maintaining the GI health [108-110]. IBD treatment using probiotics and/or fecal transplant is currently in focus. Few studies have

confirmed the effect of lyophilized probiotic cocktail to suppress the inflammation in chronic enteropathy [5, 52, 109, 111-113]. However, limited of the results regarding the efficacy of probiotic evaluation have been reported from a clinical trial study of canine idiopathic IBD [109, 114, 115]. Additional prospective clinical study and trials are needed to determine the optimal treatment for dogs with IBD.

The prognosis of IBD is evaluated by responses to treatment that can be observed subjectively and objectively [3, 25, 71, 90, 92, 97]. Several clinicopathologic markers have been useful to assess treatment response and predict prognosis[71, 91, 92, 98]. IBD with complication from protein-losing enteropathy and/or cobalamin deficiency is associated with adverse outcomes and poor prognosis. So, early awareness and sufficient treatment potential prevent those complications. IBD is mostly incurable with long-term complications despite receiving standard treatments that are typical combinations of food trial, antibiotics, anti-inflammatory and immunosuppressive drugs [116]. However, the therapeutic outcome of medical treatment appears to be multifactorial [117]. Finally, the progressive-unresponsive IBD requires intestinal resection together with medication [75] as most patients experience impaired quality of life due to complication and adverse effect from medical and surgical treatment. One of the alternative treatments that potentially accelerates therapeutic effects is the use of mesenchymal stem cell (MSC) administration [118-120] (described later).

1.2 The Role of Gut Microbiota to Intestinal Immunity in Dog IBD

1.2.1 Alteration of Gut Microbiome in IBD

Intestinal microbes substantially maintain host health by providing a defense barrier against pathogens, stimulating the immune system, and supporting the digestion and nutrition for enterocytes [121]. Members of the intestinal microbiota produce various beneficial metabolites; for example, antimicrobial molecules, indole and butyrate affect immunomodulatory properties and protect against colitis [122-125]. Additionally, the crucial contribution of the gut bacteria to the host homeostasis is the development of gut-associated lymphoid tissues (GALTs) generating gut-specific immune responses and the prevention of colonization by pathogens. Studies in animal models demonstrated that intestinal bacteria are required for the progress of intestinal inflammation in IBD [124, 126-130], and the function of mucosal immune cells cannot be disconnected from the microbiota. Therefore, the alteration of bacterial balance secondary to disrupted mucosal homeostasis in the gut plays a pivotal role in IBD pathogenesis, including the loss of immune tolerance to endogenous flora [51, 131, 132] and greater sensitivity to active inflammation [48]. The alteration of the gut environment, as well as dysbiosis during IBD, may allow the commensal bacteria to become opportunistic pathogenic bacteria, contributing to mucosal detriment, inflammation and invasive infection [24, 124]. These alterations are well described in laboratory animal models, humans, and dogs with IBD [10]. For example, adherent-invasive *E. coli* and *Bifidobacteria adolescentis* in humans induce both mucosal and systemic inflammatory Th17 cell [133, 134]. In addition, a study on the GSD breed has identified dysbiosis in association with differential high and low *Tlr4* and *Tlr5* expression compared to that observed in healthy Greyhounds [15, 26]. Therefore, a dysregulated microbial community with genetic and environmental susceptibility affects the gut homeostasis contributing to IBD development.

The interaction between bacteria and their host is crucial in IBD. Great variability exists in the gut microbiota in dogs, and the immunogenicity of the microbiota to the host also varies. The

results from recent studies indicate that the alteration of intestinal microbiota play a role in the pathogenesis of dog IBD [131, 135, 136] rather than particular agents causing IBD. Dogs with IBD have significantly altered microbiota composition either in the luminal or mucosal site compared to normal controls, and the common dysbiotic population corresponds to findings in humans. Most commonly, a decrease in the proportions of *Bacteroidetes,* especially the subsets considered to be a short-chain fatty acid producer [137], is observed to lead to impaired mucosal architecture and immune reactivity. Additionally, studies found an increase in Firmicutes (*Clostridiaceae*) [121, 131, 135] and *Proteobacteria* (*E. coli*) [131, 135, 136, 138] and a high prevalence of invasive *E. coli* strain in the gut mucosa from IBD dogs [59, 131]. However, these observed changes in the microbe community differed over time and stage of the disease, suggesting that a general dysbiosis, rather than infection with invasive pathogens, is associated with IBD. Therefore, a weakened mucosal defense, a reduction in beneficial microbes, and an aberrant immune response lead to perpetuation of intestinal inflammation[131, 136]. Notably, whether the dysbiosis causes disease or whether it is secondary to gut inflammation, is unknown. For these reasons, treatment using antibiotics, including Metronidazole and Tylosin, to attenuate the luminal dysbiosis is found to improve clinical signs in many cases [72, 105, 139]. The dysbiotic population is plausibly controlled by antimicrobial activity per se or by the immunomodulatory effect of those antibiotics [106, 107]. In addition, restoring the intestinal dysbiosis with beneficial bacterial subsets, such as with the introduction of probiotics, therefore may be a reasonable objective in the treatment of canine IBD [121, 130]. For example, treatment of mice with colitis with a probiotic cocktail VSL#3 increased the production of IL-10 and the percentage of TGF-b-expressing T cells [52].

1.2.2 Intestinal Immune Responses in Animals with IBD and Dysbiosis

Mucosal immune response against gut bacteria plays an essential role in mucosal homeostasis. According to current knowledge, the resident microbiota regulates the development of specific immune cell subsets in the gut mucosa, including the Th17, innate lymphoid cells (ILCs), mucosal macrophage and regulatory T cells [124]and the abnormal activation of these cells by dysbiosis potentially contributes to an uncontrolled inflammatory response in IBD cases. For example, a previous study reported that human intestinal mononuclear phagocytes show hyporesponsiveness to microbial stimulation under steady-state conditions, and this tolerance is disrupted in IBD [48]. Additionally, dysbiosis propagates a pro-inflammatory immune response by increased TLRs activation and a reduction in mucosal IgA, leading to more pro-inflammatory cytokine production [26, 28, 48, 140] and immune cell activation, such as increased IgG+ plasma cells in lamina propria [27]. In contrast, the anti-inflammatory cytokine is found to be decreased in dysbiotic condition [141], suggesting that it causes deregulation of regulatory T cell and activates the Th-17 cascade, thereby contributing to a disruption of gut homeostasis, as shown in other studies[46, 142].

Additionally, local humoral immunity plays a crucial role through protective defense mechanisms by IgA binding against commensal bacteria. Previous studies showed that high IgA-sorted bacteria were generally commensal and potentially caused the disease under specific predisposing genetic or environmental conditions [143, 144]. Thus, possibly, some commensal microbial strains are far more effective in inducing mucosal immune response than others under IBD status. For example, phylum *Proteobacteria* is commonly increased in the CD patient [110], and human IBD study showed a higher level of IgG bound to the *Proteobacteria* subset, including *E. coli* [65, 67]. However, in IBD, shifting a common IgA defense mechanism to an IgG-mediated response potential aggravates more inflammation in the gut mucosa in IBD. In comparison with

IgA, the IgG bacteria immune complex triggered more intensive immune response via immune cell activation, phagocytosis and antibody-dependent cell-mediated cytotoxicity [145, 146]. Thus, the IgG is potentially responsible for ongoing proinflammatory milieu in dog IBD.

Overall, the homeostasis of the gut microenvironment requires equity interaction between host immunity and gut flora. The alteration of the bacterial balance potentially causes disrupted mucosal homeostasis, including immune intolerance to endogenous gut flora and susceptibility to response to inflammation. Notably, the breakage of the microbiota balance either could be a cause or a consequence of impaired gut immunity, and future study regarding the interaction between host immunity and the gut microbiome is required.

1.3 Overview of MSCs as a Novel Treatment for IBD

1.3.1 Origins and Characteristics of MSC

MSC is characterized by fibroblast-like-shaped, plastic-adherent, nonhematopoietic, multipotent progenitor cells isolated from an adult or from embryonic tissue [147]. In general, adult tissue-derived MSC can be obtained from a variety of tissues, including bone marrow [148], muscle [148], adipose tissue[148], periosteum [148], umbilical cord [149], umbilical cord blood [150], Wharton Jelly [151], and dermis [152, 153] and more. One of the most common sources of adult stem cell is adipose tissue due to the abundant cell yielded per weight of tissue derived from various anatomic regions [154] and having practical accessibility in a clinic.

Although, the MSC criteria are established, and some markers also have been identified, the actual nature, phenotype, and function are mostly unknown. At present, the MSC phenotype is determined based on the criteria of International Society for Cellular Therapy [155] and includes the

high expression of MSC markers—Sca-1, CD29, CD44, CD73 CD90 and CD105—and weakly or negative leukocyte markers and endothelial markers—CD11b, CD14, CD19, CD31, CD34, CD45 and MHC II. In addition, MSC is considered an immune-privileged cell since the lack of costimulatory molecule CD80 CD86 and MHC II expression [156, 157] prevents them from immune recognition and supports the use of an allogenic MSC source [153]. MSC migrates in response to the chemokines and are able to degrade the basement membrane for extravasation to the sites by activation of matrix metalloproteinases [157]. This feature is useful for MSC to be delivered systemically in clinical application.

MSC can be culture expanded ex vivo in up to 40 to 50 cell doublings without differentiation. Additionally, MSC has superior properties due to its capability to differentiate into various lineages originated from the mesoderm. This capability is controlled by stimulatory factors in the culture conditions, leading to differentiation in cell development, such as bone, cartilage, fat, and other connective tissues. Beyond the mesoderm lineages, previous studies have documented that MSC can differentiate to other lines including hepatocytes, neuron-like cells [158] and pancreatic islet-like cells [159].

One of the crucial features of MSC is their immunosuppressive properties identified in vitro and in vivo against inflammation in various kinds of disease, including SLE, graft-versus-host-disease (GVHD), osteoarthritis and IBD [160-162]. MSC behaves as the potent immunomodulators of the immune response (described later), although the exact mechanism responsible for MSC-mediated immunosuppression remains partly unknown. The suggested mechanisms include soluble factors, as well as contact-dependent mechanisms that support MSC immunomodulatory features.

Overall, the MSC has superior properties, including multilineage potential; self-renewal; and being an easily accessible source of rich tissues that are isolatable and expandable in vitro, with exceptional genomic stability and few ethical issues [160]. These support the MSC as a promising alternative cellular therapy in various immune disorders including SLE, graft-versus-host-disease (GVHD), and osteoarthritis and IBD [160-162].

1.3.2 Therapeutic Effects of MSC in IBD

Cellular therapy with MSC has emerged as a promising new therapeutic strategy for managing inflammatory diseases, including IBD [160, 163, 164], because they have shown the remarkable immunoregulatory property in anti-inflammation and tissue regeneration with few side-effects and long duration of action in both in vivo [165, 166]and clinical trials of IBD [167-169].

In IBD, the number of animal studies have reported the effects of MSC administration by an i.v. or i.p route in ameliorating the inflammation, in both experimental IBD [166, 170-176] and clinical trials [168, 177-180]. The positive responses of MSC therapy in IBD included improved clinical signs, reduced disease activity index and inflammatory score. Additionally, previous documents reported that MSC is the immune privilege cells with the ability to migrate toward the injury sites through chemoattractant gradients [153, 157] demonstrating by fluorescent trafficking study [163, 181, 182]. These studies support that the systemic delivery of MSC could be a useful administration route in clinical application.

Moreover, the recent human clinical trial showed the safety of allogeneic and autologous MSC treatment in chronic relapsing Crohn's disease [179, 180, 183]. Recent clinical studies on IBD using systemic delivery of allogenic MSC showed the safety outcomes in humans [179, 184-187], as well as in other species, including dog [168, 188] and cat [189]. For example, a recent study documented

no detectable infection and adverse effect after MSC treatment in a moderate to severe UC patient in a clinical trial [187]. Additionally, so far, no report has been issued regarding the potential of MSC to cause tumorigenesis after follow-up for approximately 137 months in 41 patients who received MSC treatment [190]. Therefore, this evidence allows systemic delivery of allogenic MSC as practical, convenient and safe for clinical application. However, the risk that MSC will trigger or worsen malignancy from spontaneous malignant tumors and enhanced tumor development[191, 192] is still a concern due to immune suppressive activity and multiple chromosomal aberrations found in a mouse model [193, 194]. Therefore, the need exists for further studies in this regard.

In IBD treatment, MSC has been shown to modulate immune responses and reduce inflammation by suppressing the function of T, B cell, natural killer (NK), and macrophages [171, 195-199]; inhibiting maturation of monocyte to DC [160, 164, 200]; and enhancing regulatory T cell [165, 201], M2 macrophage [164, 202, 203] and IL-10 production [165, 171, 175] at the mucosal sites. Although the actual mechanisms underlying immune modulatory activity of MSC are not fully understood, some of these mechanisms are assumed to require cell-cell contact dependent manners [171, 204] and/or paracrine effect [152] through secretory bioactive mediators including PGE2 [205, 206], IL-10 [165, 171, 175, 207], TGF-β, insulin-like growth factor-1 (IGF-1) [201], indoleamine 2,3 dioxygenase (IDO) [206, 208], cyclooxygenase 2 (COX2) [201] and nitric oxide (NO) [201, 209]. In addition, MSC inhibits the amplification of inflammatory signal by suppression of cytokine production, such as TNF-a, IFN-g, IL-17 and IL-22, therefore leading to less immune cells recruitment to the sites and enhanced regulatory T cell [201, 210].

Although no current evidence shows that MSC could differentiate to target cells, MSC is believed to exert a regenerative effect through secretory mediators, thereby contributing to tissue healing such as by cytokines and growth factors. Beyond the immune modulation, MSC also has

been shown to promote tissue regeneration and mucosal healing for IBD treatment by crypt proliferation as well as neovascularization [163, 203, 211]. Although, the exact mechanism of the re-epithelialization induced by the MSC remains unclear, MSC has been found to secrete significant levels of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), and CCL2 to enhance local neovascularization and healing [152, 157, 203, 212, 213]. Additionally, MSC is believed to have the ability to communicate with the resident tissue stem cells (e.g., intestinal stem cells) through Wingless and INT-1 (Wnt) signaling [214] and enhanced tolerance of mucosal cells against oxidative stress [172]. Moreover, previous studies have shown the effect of MSC treatment to restore microorganism community profiles to resemble the normal healthy state [163]. Therefore, the overall effect of MSC in treatment of IBD could be the summary effect of immunomodulation toward anti-inflammation, together with a regenerative effect to restore the homeostasis of the gut microenvironment, and these effects are essential especially in case of refractory and complicated IBD with fistula [215].

1.3.3 Limitation of Conventional MSC

MSC has advantageous properties that include multilineage potential, self-renewal, and an easily accessible source of rich tissue, that is isolatable and expandable in culture, with exceptional genomic stability and few ethical issues [160]. A number of studies have demonstrated that conventional MSC can be used for multiple purposes including tissue repair, immunomodulation, and drug delivery [163, 164, 216-218]. In IBD, the conventional MSC have been documented for their advantageous features and useful for treatment even in the complicated cases [179, 183-185, 215]. However, some limitations are concerned for conventional MSC transplantation in clinical treatment for IBD including safety, adverse side effects, and consistent efficacy.

The awareness of potential tumorigenicity of transplanted MSC and potential adverse effects have been a drawback for clinical application [157]. Although MSCs are believed to have differential ability, they are restricted to the mesoderm lineage with low risk of tumor formation [157]. The safety of stem cells to treat the patient is based on prioritization of concerns. So far, no evidence regarding tumorigenesis by MSC treatment has been shown [219]. However, the risk that MSC stimulates worsened malignancy by supporting tumor development [191, 192] is still a concern due to their immunosuppressive activity [193, 194]. Additionally, few adverse effects have been documented after MSC administration; however, a limited number of the patients have been approached, and more evidence is needed to confirm this conclusion. Therefore, a continued need exists for further studies in this regard.

A significant limitation of autologous MSC treatment is that it requires a period for cell expansion in the culture that is not convenient and practical for acute injury application. Additionally, some patients might have limited access to their cells, as some diseases [220, 221] and aging potential reduce their stem cell capacity [222], and the MSC derived from younger donors are found to be more active immunologically [223]. Thus, the potential solution would be a source of allogenic MSC, which may be prepared and ready to use. However, the use of conventional allogeneic MSC for larger scale in clinical cellular therapy is also subject to several significant drawbacks, including donor-to-donor MSC variability, the limited proliferative capacity of older MSC [171, 224], the costs for donor screening, and the time and expense associated with expanding MSC in primary culture [225-227]. Therefore, inexhaustible cell sources of MSC with uniform phenotype and standardized function are desirable for the consistent therapeutic outcome.

Another concern is the varying efficacy of treatment by conventional MSC, which ranges from adequate to ineffective outcomes [190], and this is one of major concerns for MSC

transplantation. For example, some of GVHD studies have shown the failure of MSC treatment to protect graft rejection in an in vivo animal model [217, 228]. Moreover, studies in human MSC treatment found no effect of conventional MSC unless those MSC had been preactivated by inflammatory stimuli [229, 230]. Although, the treatment effect probably depends on the experimental designs, including the MSC dosage and administration route [207]. The ineffective result from conventional MSC treatment is probably associated with the acquisition of cytogenetic abnormalities [157], altered phenotype [190] and hypofunction [207], which occur after long-term culture. Thus, modification of MSC that promotes a high quality of MSC with consistent functional activity and stable phenotype is still the ideal for MSC therapy.

Overall, the limitation of using conventionally unstimulated MSC; including safety, quality of cell, genotypic and phenotypic stability, and functional activity for consistency outcome, is a concern when using cellular therapy. Specifically, some limitations (i.e., cell quality, renewal capacity, and functional activity) are probably solved by using modified MSC, such as activated MSC or iPSC-derived MSC (all described later in section 1.4).

1.4 Ex-vivo Activation of MSC to Improve Therapeutic Activity in Inflammatory Diseases

1.4.1 Preactivated MSC by Toll-Like Receptor Agonists and Cytokines

Despite the promising outcome using conventional MSC therapy, challenges remain in the MSC application. One of the issues addressed is the variable or ineffective outcome after MSC administration described previously [217, 228]. For example, studies in human MSC found no effect of conventional MSC in experimental assays, and the immune suppressive effect was retrieved

only in the preactivated MSC by inflammatory stimuli [153, 190, 229, 230]. Therefore, enhancing the capability of MSC by inflammatory mediator activation improves the treatment efficacy.

Previous studies have found an increasing therapeutic effect of MSC by incubating with inflammatory stimuli and/or cross-talking with damaged cells, whereas the nonactivated cells may be incapable [152, 207, 231-233]. The reason is that the final immunomodulatory outcome of MSC is likely influenced by the microenvironment cues at the site of inflammation [234]. Thus, they migrate and get activation by the proinflammatory stimuli including IFN-g [231, 232, 235, 236], TNF-a [236], and LPS (TLR4 agonist) [235, 237], as well as Poly(I:C) (TLR3 agonist) [237]. Previous studies on mouse and human MSC found that the resting MSC did not have significant immunosuppressive activity in the treatment of IBD. Although stimulatory molecules, i.e., LPS, IFN-g, and TNF-a, could activate the NF-kB pathway in MSC within 1 hour [234, 238, 239], their immunomodulatory functions had to be elicited by an incubation time of at least 24 hours to several days [203, 232]. IFN-g- and Poly(I:C)-preactivated MSC showed significant therapeutic effect in the mouse model of colitis [232, 240] in contrast to LPS-treated MSC [147, 240]. Additionally, one study of canine MSC (cMSC) reported that MSC did not affect the unstimulated PBMC because an absence of inflammatory stimuli was present in the culture environment, and cMSC was able to inhibit proliferation in the stimulated PBMC, which was demonstrated by the mixed lymphocyte reaction (MLR) [231]. In addition, preactivated MSC could be induced by a very low concentration of inflammatory cytokines (i.e., 0.4 ng/ml of cytokine), and a high effect was also observed [233]. Thus, the inflammatory milieu even in a low amount of cytokine, plays an important role to determine the final effect of MSC on target cells.

Evidence has been reported that MSC secretes more growth factors after an experience with cytokine milieu, hypoxia and TLRs agonist [207, 241, 242]. IFN-g, LPS, and Poly(I:C) rapidly

induced and activated the NF-kB pathway in human and mouse MSC [237, 239], although resting MSC within the noninflammatory environment could constitutively produce some mediators, as described previously. Preactivated MSC with TNF-a, IFN-g, LPS, Poly(I:C) or IL-1B have been found to produce significant amount of cytokines, growth factors, chemokines and adhesion molecules including IL-6 [208, 231, 242, 243], IL-8 [231, 237, 242, 243], IL-10 [207], HGF [231], TGF-B [242], PGE-2[236], IDO [242, 243], VEGF [231, 244], CXCL2 [245], CCL2[245], CXCR3[207], CCL5[243, 245], CCR10[235], CXCL10[235, 237], ICAM-1[207], and VCAM-1[207]. In addition, upregulated chemotactic ligand induced by IFN-g, TNF-a and IL-1B preactivation enhanced the migratory ability to the affected area, as shown in a tracking study [201, 207] and migratory assay [203, 245]. Importantly, some mediators derived from preactivated MSC likely induce a pro-inflammatory milieu such as IL-6 (neutrophil chemotaxis) and IL-8 rather than anticipated immunosuppressive activity. These effects suggest that the net outcome from preactivated MSC occurring at the inflammatory sites is probably bidirectional activity, representing either inflammation or anti-inflammation, as suggested in several studies [207, 234, 243, 245]. However, no concordance exists in the literature regarding the range of concentration in each stimulus for driving the MSC to immune system inhibitory properties. Therefore, further study to obtain suitable phenotypes and functions for optimal activation when treating diseases remains a challenge and is still a need.

Preactivated MSC with a variety of inflammatory cytokines and TLRs potentially change their phenotype, thereby contributing to an altered function of cells. IFN-g stimulation induces MSC to express PD-L1 [236, 246, 247], and it is thought to be an important surface marker for MSC to suppress T cell proliferation. Moreover, MHC II is upregulated by IFN-g stimulation, suggesting the additional roles of MSC as an antigen-presenting cells [152, 207, 234, 248]. However, the remaining surface markers, including CD34, CD80, CD86, CD105, and CD 106, are not changed by the IFN-

g, LPS, and Poly(I:C) treatments [234, 237]. Additionally, no evidence exists to show that this preactivation alters the MSC Tri-lineage differentiating ability [237].

Most of data regarding the preactivated MSC have been obtained mainly from human and mouse studies. Some significant differences exist between mouse and human MSC in terms of the mediators driving the inflammatory suppression [234]. For example, in human MSC and primate MSC [230, 235, 249], IDO is involved in the suppression of T cell proliferation, and the activated hMSC with IFN-g and TNF-a enhances the degree of suppressing activity [232, 242]. However, mouse MSC have no IDO production, and their immune suppressive activity is mediated by Nitric Oxides (NO) [190, 209, 233, 243]. These data suggest that species variation in the immunosuppression mechanism may be a concern, although majority of MSC action in response to those stimuli is roughly in concordance between interspecies. Moreover, in other species, including dogs, knowledge of the overall alteration of features and function of MSC by preconditioning with those stimuli is still mostly unknown. Therefore, the extensive studies regarding primed MSC and their action to immune system are required for understanding the nature of dog MSC.

Overall, although the immunomodulatory pathway induced by TLR-activated MSC remains controversial, these MSCs potentially enhance the immunomodulatory mediator production and increase the therapeutic effects. Several studies show the increase of anti-inflammation and augmentation of tissue regeneration through the secretory factors, as well as cell-cell contact activity. These approaches would be the way to improve MSC efficacy to obtain a significant effect and consistency result with IBD cellular therapy. However, more studies are required to determine the appropriate way to activation to ensure the beneficial outcome for IBD treatment, especially studies in some species that lack those data such as canine MSC.

1.4.2 Induced Pluripotent Stem Cell-Derived MSCs (iPSC)

MSC exerts beneficial effects that are immunomodulatory and regenerative to treat a variety of injury and inflammatory diseases including IBD. According to their immune privilege and ability to migrate to the inflammatory sites after intravenous administration, the allogeneic transplant of MSC is believed to be useful, convenient and safe for clinical application. However, several essential drawbacks interfere with the practical application of this application at a larger scale, including the limited proliferative capacity of senescence MSC [224], donor variability, donor screening expense, and the time and cost associated with expanding MSC in primary culture [225-227]. Additionally, previous studies have reported inconsistent outcomes of allogenic MSC treatment [190, 217, 228], suggesting from the different phenotype, function, uniformity and cell quality that MSC may be altered in long-term cell culture [157, 190, 207], thereby leading to a reduced therapeutic outcome [250, 251]. Therefore, inexhaustible alternative sources of MSC that are uniform, standardized and renewable, with consistent therapeutic outcomes, are desirable.

Stem cell-based therapy using iPSC-derived MSC (iMSC) is considered a promising therapy. Regarding the limitation of conventional adult stem cells, the iMSC is considered an alternative source that accelerates large-quantity production, with the superior benefit of quality control, greater expandability of homogenous cell population, and less pro-tumor potential [160, 252-254]. iPSC can be generated from an adult somatic cell (i.e., a fibroblast) by reprogramming those cells to a pluripotent state. Thus, iPSCs are similar to embryonic stem cells in term of morphology, gene expression, teratoma formation and in vitro differentiation [153, 255]. After being differentiating to the MSC lineage, iMSC has features that are similar or superior to conventional MSC [253]. For example, iMSC showed an increased proliferation capacity; they achieve their renewal capacity for 40 passages without losing of plasticity or showing senescence [253]. Several studies have

determined that iPSC-derived MSCs are likely similar to conventional MSC regarding phenotype and function [163]. Additionally, iMSC is free of ethical concern and has the potential to be modified for personalized medicine (i.e., drug delivery or anti-inflammatory purposes), as well as being genetically engineered to achieve a safe profile [255].

The ability of iPSC-derived MSC to suppress inflammation in vivo has also been demonstrated in mouse models, including colitis [163], allergic airway disease [256], limb ischemia [253], and myocardium infarction [257]. These studies show that iMSC has equivalent [256, 258] or superior efficacy [253] compared to conventional MSC in the context of features, anti-inflammation and tissue regeneration. Overall, iMSC is a new alternative MSC source that facilitates excellent benefits of more exceptional expandability and easy quality control, which result in consistent therapeutic outcomes in line with the conventional outcome. However, future studies regarding the long-term follow-up of safety and efficacy of iMSC treatment need to be evaluated carefully before clinical translation [153].

1.4.3 Treatment with MSC Secreted Factors

MSCs are multipotent stem cells that have advantages for multipurpose therapies, including tissue repair, immunomodulation and drug delivery [163, 164, 216-218]. These cells are conveniently accessible sources; isolatable; in vitro expandable, with genetic stability; and are free of the ethical issue [160]. In many diseases, including IBD, conventional MSCs have shown their efficacy in treatment even in the complicated cases[179, 183-185, 215]. MSCs have been shown to modulate immune responses and reduce inflammation by suppressing immune cells, and enhanced anti-inflammatory cytokine production. They also support tissue regeneration and neovascularization through secretory mediators, cytokines, chemokines and growth factors.

Although the exact mechanisms underlying MSC function remain to be explored, part of the mechanism is assumed to be operated by secretory mediators and paracrine effect [152].

Some limitations have been noted regarding the use of MSC cellular therapy in IBD. Several studies found inconsistent therapeutic outcome after MSC injection [217, 228], and one possible reason for low efficacy was due to a low number of cells distributed to target tissue [163]. This inefficacy may be caused by an unequal distribution of MSC in some organs and/or inadequate inflammatory signals to attract MSC to sites. Additionally, donor variation and cell damage or senescence before administration are considered the factors of a reduced number of cells at the affected tissue. In addition, the MSC therapeutic effect in IBD does not depend on their full engraftment but mainly on the secretory mediators that contributed to tissue repair [152, 203]. Although, it is suggested that the MSC mechanism requires cell-cell contact interaction, a number of Transwell co-culture systems have demonstrated that the paracrine/endocrine effect provides preferential action and yields a comparable result in the cell-cell contact coculture [209, 235]. Therefore, when therapeutic efficacy in IBD is being augmented, the MSC secretome provide an alternative way for the thorough systemic delivery of active mediators from MSC to the overall body including GI mucosa.

As described previously, MSC secrets secretory bioactive mediators including PGE2 [205, 206], IL-10 [165, 171, 175, 207], TGF-β [152], IGF-1[152, 201], IDO[206, 208], COX2[201], VEGF[231, 244, 259], CXCL2[245], CCL2[152, 245], and NO[201, 209]. These mediators play a role in immune modulation and intestinal healing in IBD. IGF-1 was reported to increase intestinal regulatory T cells, improve intestinal function and promote enterocyte proliferation [201]. The secretome includes the extracellular vesicles and soluble mediators that are constitutively produced by most cell types. The vesicles may contain a variety of bioactive molecules, which include mRNAs, microRNAs, and proteins. Previous

studies have reported the use of secretomes either prepared by MSC condition media or extracted from microvesicles/exosomes in the treatment of colitis. In these studies, the concentrated condition media derived from the MSC culture was administered, and the treatment with secretome ameliorated the colitis, which improved the clinical scores, reduced inflammation, and produced less pro-inflammatory cytokines [203, 260]. One study documented the degree of improvement that was correlated with increased secretome dosage and frequency of injection [203].

The treatment of IBD using the cell-free-based technique is compelling according to in vitro and in vivo models. The MSC-derived secretome overcame some limitations of cellular therapy, including allogenic DNA contamination in the recipient, risk of tumors originating from the transplanted cells, and cell density at the site of action, which resulted in a consistent therapeutic effect. Additionally, the method used to generate the secretome for larger scale usage or clinical applications with high-quality control would be practical. However, some pitfalls must be noted, including the cost of production, frequent administration and stability of mediators. The results of a mouse study showed that nine times more MSC was required for making secretomes compared to the number needed for cell therapy, and multiple doses were needed, which required concentrated filtration to the final optimal concentration [203]. Therefore, the cost of secretome production increased more than the cost of MSC cellular therapy. Additionally, some secretory mediators occurred in low amounts and were unstable or exhibited short-lived action, such as NO [201], so further processing may be required to preserve and maintain the quality. Due to limited knowledge about which secretory molecules are produced by MSC, the secretome composition should be carefully evaluated to ensure a positive result and to prevent the presence of harmful components [201]. Overall, the use of the MSC-derived secretome is a promising treatment option for many diseases, including IBD. The positive therapeutic effects have been demonstrated by

several studies, for example, promoting intestinal re-epithelization and inflammation control. Future studies are needed to identify the secretome composition and assess the safety and side effects of the secretome in the long run.

1.5 Effect of MSC Treatment on Osteoarthritis

1.5.1 Altered Joint Environment in Osteoarthritis

Osteoarthritis (OA) is considered the most common joint disease diagnosed in both humans and animals[261, 262]. OA affects approximately 20% of the dog population [263, 264], and once it is established, life-long management is required. OA in dogs is usually described as a multifactorial disease and considered to largely resemble human OA [265]. Genetics and environmental risk factors including being overweight, pure large breed background, and lifestyle [266-268] considerably affect the development and disease progression of OA [269]. Male [267] and neutered dogs [270] have an observed higher prevalence than females since sex hormones and activity levels are also thought to exacerbate the disease condition. These predisposing factors interact to result in the development of an immune response that destroys the joint microenvironment, including the cartilage, synovium, and subchondral bone [271]. In addition, canine OA is thought to be a degenerative disease that is typically found in middle to senior age dogs [262, 272]. The abnormal signs develop after long-term destruction of the joint, and diagnosis occurs later after deterioration of the affected structure and impaired mobility [273]. Thus, half of diagnosed cases are found within the age range from 8-13 years [263].

Clinical signs are commonly described as lameness, stiffness, painful joint(s) with chronic progressive joint degeneration and an abnormal limb appearance, including cracking joints,

swelling, and muscle wasting [261, 262], contributing to structural and biomechanical joint abnormalities with noticeable gait disturbances. The stifles, hip, and elbow are commonly affected joints based on several studies [261, 262]. OA pathology is characterized by chronic active inflammation with possible findings of cartilage degeneration, an abnormal synovial membrane with inflammatory cell infiltration, subchondral bone thickening, and osteophyte formation [262, 272, 274].

The joint damage is probably triggered by single or chronic physical injury induced by mechanical force to the joint. Affected chondrocytes react to the injury by secreting catabolic enzymes such as matrix metalloproteinase (MMPs) [275-277] and triggering inflammatory cues including TNF-a, IL-1B, and IL-6 with subsequent innate and adaptive immune activation [275-277]. Additionally, IL-1B has been reported to induce apoptosis of chondrocytes and synoviocytes [278], enhancing levels of MMPs including MMP1 and MMP13 and resulting in the proteolysis of collagen type II and proteoglycans, which are significant components of the extracellular matrix in cartilage (ECM) [272, 279] and are replaced by fibrosis via deposition of collagen type I and fibronectin. In contrast, IL-10, IL-13, and TGF-b from regulatory T cells have been shown to have a counterbalancing effect in terms of anti-inflammation, suppression of MMP production and support of collagen type II and aggrecan synthesis [279]. Thus, the inflammatory milieu affects not only reduced synthesis but also accelerated destruction of critical components of the ECM [271].

Adaptive immune cells are activated by pro-inflammatory mediators and migrate to the joint in response to chemokines. A greater number of inflammatory cells, including DCs, NK cells, B cells, Th1 subset cells, CD8+ T cells, and macrophages, are found in synovial fluid and synovium derived from OA tissue [271, 274, 276, 277]. Previous studies have shown that activated immune cells infiltrate sites and release additional IL-1B, IL-2, IL-6, IL8, IFN-g and TNF-a [271,

[274]. These cytokines potentially affect downstream activation of the STAT1/STAT3 and NF-kB pathways [271, 274], leading to the upregulation of proinflammatory genes [279]. In addition, the release of VEGF increases vascular permeability and neovascularization in synovial tissue, resulting in increases in fluid, cell infiltration and pain in the joint. Interestingly, an increase in regulatory T cells was observed in tissue derived from an OA patient. However, low levels of IL-10 were produced by those cells, indicating a regulatory T cell dysfunction [280] and insufficient levels of anti-inflammatory cytokines in the micromilieu. Therefore, disruption of homeostasis toward a catabolic state activates a repetitive cascade of proinflammatory events, resulting in a chronic relapsing course of joint inflammation and destruction [271].

1.5.2 MSC Effects on Cellular Responses in the Joint Microenvironment

OA, also referred as degenerative joint disease (DJD) or osteoarthrosis, is considered one of the most common degenerative diseases caused by multiple risk factors that potentially contribute to the development of chronic inflammation of the joint to culminate in structural damage and painful, impaired limb function (as described previously). Previous studies have reported that the available medications for OA treatment, including nonsteroidal anti-inflammatory drugs (NSAIDs) and analgesics, significantly alleviate the pain and control the inflammation in the joint [281]. However, they are not capable of completely eliminating the inflammation and halting disease progression [264]. A combination of treatment with drugs, food, and physiotherapy is commonly used for OA management in the clinic [282]. To date, no effective therapy has been found to completely treat and restore joint health in OA [283]. Thus, an alternative treatment that is able to modulate immune suppression with potential regenerative effects on joint tissue would be a promising treatment for OA.

A number of MSC studies have shown promising results in the treatment of OA both in animals and humans [284-288]. MSCs exert remarkable immunomodulatory properties in term of anti-inflammation as well as tissue regeneration, as shown by local [285-287] or systemic treatment [287-289]. The beneficial outcomes of MSC treatment include reduced inflammation in the synovial tissue and chondrocytes, increased chondrogenesis [285, 288], and improvements of range of motion [286] and lameness [285, 286]. Regardless of immunomodulation, previous studies have shown other remarkable properties of MSCs, including anti-fibrotic and anti-apoptotic effects and oxidative stress prevention, which affect all cell types in the joint including immune cells, chondrocytes, synoviocytes and fibroblasts [265]. MSCs potentially suppress inflammation by inhibiting the activity of immune cells, including T cells, B cells, and macrophages, and stimulate regulatory T cells to secrete more IL-10 [231, 280]. A reduction of proinflammatory mediators (i.e., IL-1B, IL-6, IL-8, TNF-a and MMPs) with a subsequent increase in chondrogenesis has been reported in OA joints receiving MSC treatment [278].

Previous clinical trials using MSC therapy have demonstrated its safety with rare occurrences of adverse effects and long-lasting treatment effects after MSC transplant [285, 286]. For example, previous studies have reported no severe adverse effects or complications after systemic delivery of MSCs in a clinical trial of spontaneous OA [287]. Additionally, a safety study of intra-articular MSC transplants in dogs and humans reported rare occurrences of side effects (i.e., joint swelling in 5% of cases) [285] as well as no tumorigenicity in long-term follow-up analyses (i.e., 6 months-1 year) [286, 287]. One clinical trial in dogs showed an 80% response rate and the ability to discontinue other medications for one year after intra-articular MSC injection [285]. This evidence supports MSC treatment as practical, effective and safe for OA treatment in the clinic.

Overall, an imbalance toward a pro-inflammatory milieu activates recurring joint destruction with fibrosis, which causes chronic pain, structural deformity, gait disturbance and a reduced quality of life. A comprehensive treatment of OA would function to suppress the inflammation together with regeneration of the vital health of the joint. Of critical importance, MSCs effectively manage the joint microenvironment to achieve a less catabolic state and support the regenerative anabolic state for optimal joint repair.

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CHAPTER 2: HUMORAL IMMUNE RESPONSES DIRECTED AGAINST GUT BACTERIA IN DOGS WITH INFLAMMATORY BOWEL DISEASE (IBD)

2.1 Overview

Background: Inflammatory bowel disease (IBD) in dogs is a clinical disease associated with signs of intestinal inflammation involving the large and small intestines, as well as intestinal dysfunction. Although it is not clear what triggers the intestinal inflammations in IBD affected dogs, we hypothesized that the disease may be mediated, in part, by an abnormal immune response directed against intestinal bacteria.

Objective: To comprehensively investigate the humoral immune response against gut bacteria in dogs with IBD, we conducted studies to determine whether dogs with IBD have more IgG-binding bacteria, and we hypothesized that dogs with IBD have a greater amount of immunoglobulin (Ig) produced against their gut microbiota. The studies also aimed to identify the source of Ig production and investigate the potential consequence of having Ig-binding bacteria to trigger an inflammatory response. We hypothesized that Ig is locally produced and has potential to induce a greater inflammatory reaction by IgG-coated bacteria than bacteria from the healthy gut.

Methods: Stool and serum were collected and processed for flow cytometry to total the binding of IgA and IgG in the evaluation of the humoral immune response to gut bacteria in dogs with IBD. Further investigations of the source of antibody production and potential consequences caused by immune responses were performed via bacterial flow cytometry analysis, macrophage activation and cytokine production. Moreover, the immune targets to specific bacterial subsets were analyzed via sequencing and microbiome analysis.

Results: Dogs with IBD have substantially greater percentages and overall binding of IgG and IgA in their intestinal bacteria than healthy dogs, and the primary production of anti-bacterial antibodies occurs locally in the gut rather than systemically. The IgG-binding bacteria triggered an increase of phagocytosis and pro-inflammatory cytokine production by macrophages. Moreover, *Actinobacteria* (*Collinsella* genus) was the preferential target for the mucosal IgG immune response to dysbiotic bacteria.

Conclusion: In dogs with IBD, the mucosal antibody binding to commensal gut bacteria was substantially greater than it was in healthy dogs, and these Ig-binding bacteria trigger the pro-inflammatory response. Furthermore, anti-bacterial antibody targets a specific phylum, which may play an important role in regulating gut inflammation.

2.2 Introduction

In dogs, inflammatory bowel disease is characterized by clinical symptoms of GI dysfunction (diarrhea, malabsorption, and weight loss) [1, 2] and macrophage and lymphocyte infiltration into the submucosa and mucosa. Studies have demonstrated that the dysbiosis development and changes in the gut environment favor the increased growth of pathogenic bacteria and inflammation, leading to intestinal injury in IBD[3]. Other factors associated with IBD in both humans and dogs include environmental and genetic factors[4, 5]. In humans, inflammation in IBD is considered mediated by both humoral and cellular immune mechanisms[6-8]. Furthermore, the investigations on human IBD (e.g., Crohn's disease and ulcerative colitis) have focused on the functions of immune responses related to commensal gut bacteria rather than the immune responses related to dietary antigens or anti-mucosal antibody[9-12].

Previous studies on dogs with IBD have reported humoral immunity dysregulation specifically with the decreased production of the overall amount of gut IgA[13]. For example, both lowered mucosal IgA production and high pro-inflammatory cytokine productions by mucosal immune cells (T cells and macrophages) have been documented in dogs with IBD[13-15]. However, previous studies have not investigated the specificity of gut Ig in dogs. One study in dog IBD identified a high level of plasma cells, which is in line with the production of local IgG in the lamina propria[16, 17].

Studies in humans showed that there was a substantially high level of fecal bacteria bound to IgG, specifically for patients with ulcerative colitis (UC) and Crohn's disease (CD) [12, 18, 19]. Moreover, studies indicated that some IBD patients have a high level of IgA+ bacteria[12, 20]. Importantly, in CD patients, IgA and IgG antibodies were identified to preferentially bind with specific pathogenic bacteria, such as *Pseudomonas fluorescens, E. coli*, and *Clostridium coccoides*[21-23].

In dogs with IBD, there is a limited understanding of the recognition of antibody in gut bacteria. A previous report found that the microbiome in healthy dogs is very different from that of dogs with IBD and specific phyla are predominant, causing a dysbiosis state[24, 25]. For example, AlShawaqfeh et al. established a dysbiosis index for determining the level of disruption of normal flora by bacterial overgrowth associated with the clinical symptoms of dogs with IBD[26]. Thus, in the current research, the humoral immune responses to gut bacteria in dogs with IBD were investigated compared to those of healthy dogs. To address the evaluation of the quantity of IgA and IgG antibodies bound to the surface of fecal bacteria, flow cytometry was performed. We also examined the existence of circulating anti-bacterial antibodies found in blood using serum incubated with an isolated bacterial strain and quantitate using flow cytometry analysis. To identify

whether the bacterial antibodies presented in dogs with IBD target a particular bacteria, fecal bacteria with high levels of surface IgG were flow cytometry sorted and subjected to 16S rRNA sequencing with subsequent microbiome analysis. Finally, through in vitro assays, we examined the IgG-bound bacteria impact on gut health by affecting the host macrophage activation and innate immune response. This study provides new insights on the pathogenesis of IBD in dogs and proposes that the local humoral immune response against gut bacteria has a significant role in the disease etiology and progression. In addition, this research extends further to expound on the potential value of a spontaneous IBD model in dogs to investigate the microbiome modulation and novel immunotherapies.

2.3 Materials and Methods

2.3.1 Study population

A prospective observational study was conducted at the Colorado State University Veterinary Teaching Hospital (CSU-VTH). All animal studies were approved by the Clinical Review Board (CRB) (#VCS 2016-084), and the Institutional Animal Care and Use Committee (IACUC) at CSU. Dog owners were informed regarding the study protocol, and consent forms were obtained. Twenty-nine dogs, including 20 dogs diagnosed with IBD and 9 healthy control dogs, were evaluated in the study.

2.3.1.1 Dogs

This study recruited twenty dogs (14 males and 6 females) that had IBD with persistent symptoms of gastroenteritis, such as diarrhea, vomiting, and weight loss, for at least 3 weeks. To

confirm the IBD diagnosis and rule out the possibility of intestinal lymphoma, all dogs with IBD underwent an endoscopic examination and biopsy. The majority of the animals had a prior food trial, which was inclusive of a novel protein, hydrolyzed protein, and elimination diet for more than 3 weeks, and no obvious responsive result was found. The dogs did not have a recent history of receiving immunosuppressive medications and were free from other diseases causing chronic GI disease symptoms, including renal disease, metabolic disease, parasitic disease, pancreatic insufficiency, and hepatic disease. The research intentionally omitted German Shepherd dogs because the breed is recognized for being susceptible and prone to defective intestinal IgA and IgG[27, 28].

All IBD dogs received a clinical assessment, including a disease activity index evaluation (Canine Inflammatory Bowel Disease Activity Index; CIBDAI[29] and Canine Chronic Enteropathy Clinical Activity Index; CCECAI[30]), a fecal examination for parasites, a complete blood count (CBC), and a serum chemistry profile. The H&E stained intestinal biopsy specimens from the dogs with IBD underwent a WSAVA histopathologic score evaluation[1, 31] by a board-certified veterinary pathologist. Additional tests performed in IBD dogs included the serum folate concentration and serum cobalamin concentration profile (Gastrointestinal Laboratory, Texas A&M University, TX).

2.3.1.2 Clinical healthy control animals

Nine clinically healthy dogs (4 males and 5 females) age-matched to the IBD dogs were recruited in the study. These dogs were client owned and were assessed for a health checkup at the CSU-VTH. Based on the history and normal physical examination, these animals were considered free from any clinical sign indicating gastrointestinal disease, and the animals had no recent history

of immune-mediated disease or immune suppressive medication usage. All dogs also had a CBC and serum chemistry profile performed, and all evaluations were within the normal limits.

2.3.2 Sample collection

Feces and blood samples from all study dogs were collected and immediately stored at 4°C before sample preparation, which occurred within 4 hours after obtaining the samples. Blood was spun for serum separation, and serum samples were stored at -80°C. Stool samples were obtained by spontaneous defecation and/or rectal palpation. Fresh stool samples were processed to generate a fecal bacteria suspension as previously described[18]. Briefly, 0.5 g of stool was homogenized in 24.5 ml of sterile-filtered phosphate buffered saline (PBS; 0.2 μm-filtered) using vortexing, followed by centrifugation at 700 x G for 5 minutes to separate the large particles from supernatant containing rich bacteria. The fecal bacteria suspension (supernatant with floating bacteria) was collected and stored in 1 ml aliquots at -80°C until use.

2.3.3 Flow cytometry

The fecal bacteria suspension from the freezer was thawed and centrifuged at 10,000 x G for 5 minutes to obtain a bacterial pellet, which was washed with 1 ml of PBS one time. To measure the Ig-binding fecal bacteria, the bacterial pellet was resuspended in 100 μl of rabbit anti-dog IgG-Alexa Fluor 647® conjugate (Jackson ImmunoResearch Laboratories, PA, USA; diluted 1:200 in PBS plus 1% BSA) or with a solution of goat anti-dog IgA-FITC conjugate (Lifespan Biosciences, MA, USA, also diluted 1:200 in PBS plus 1% BSA) and incubated for 30 minutes on ice. The suspensions were pelleted, washed twice, and then fixed for 10 minutes in a solution of 4% paraformaldehyde (PFA). After washing, the bacteria pellets were resuspended in 380 μl of PBS,

plus 20 µl propidium iodine solution (PI; 1 g/ml; Sigma-Aldrich, St. Louis, MO, USA), which was added to each sample immediately prior to flow cytometry analysis. Note that the optimal concentrations of antibodies and PI used in this study were obtained after passing the processes of antibody titration and optimization for the staining condition.

For the detection of serum IgG antibody that is specific to intestinal bacteria, 6 stock bacterial isolates of *Escherichia coli* (*E. coli*) from the stool of healthy dogs (n= 3) and IBD dogs (n= 3) were generated as subsequently described. The bacteria in overnight cultures were collected and incubated with serum from healthy dogs and dogs with IBD for the detection of IgG binding following a previous study method[32]. Briefly, each test serum sample was diluted 1:200 in PBS plus 1% BSA, added to *E. coli* in suspension and incubated for 30 minutes on ice. The samples were subsequently washed twice, incubated with the rabbit anti-dog IgG-Alexa Fluor 647® conjugate, and analyzed by flow cytometry as previously described.

Flow cytometric analysis in this study for IgG and IgA binding bacteria was performed using a Beckman Coulter Gallios flow cytometer (Brea, CA, USA). The analysis was examined on 100,000 PI-positive events gating based on bacterial size and complexity. The purpose of the PI staining was to include bacteria (DNA^+) for analysis and exclude debris without nuclear material (DNA^-). Flow cytometry data were further analyzed using FlowJo Software (Ashland, OR, USA). The analysis included the percentage of positive fluorescent cells and the fluorescence intensity of IgG^+ or IgA^+ cells. The background fluorescence levels were determined using bacteria without the addition of anti-IgG or IgA antibodies. An example of the typical gating scheme is provided in Figure 2.1.

2.3.4 Isolation of E. coli intestinal strains and evaluation of anti-bacterial antibodies present in serum

To evaluate the presence of anti-bacterial antibodies in the serum of dogs with IBD and healthy dogs, six varying isolates of *E. coli* were analyzed, three of which were obtained from the feces of dogs with IBD and the other three from healthy dog feces. Although the study acknowledges that various bacteria species were identified by antibodies through the fecal flow cytometry (see below), we argued that *E. coli* found in the gut might propagate readily in pure culture and overall it could be a great substitution for enteric bacteria in general. Furthermore, the assays were conducted through the pure culture of *E. coli* from the dog GI tract to avoid the confounding effect of IgG that is already bound on the bacteria surface. Moreover, isolates were acquired from healthy dogs and dogs with IBD in case the strains differed based on dog disease status.

To isolate *E. coli*, fresh fecal samples from dogs with IBD and healthy dogs were diluted in PBS and homogenized using a vortex. The bacterial suspension was cultured in Tryptic Soy Broth (TSB) (BD, Franklin Lake, NJ, USA) at 37°C overnight with shaking. The overnight cultured suspension was plated on McConkey agar and incubated in aerobic conditions overnight at 37°C. The next day, the cultured colonies were assessed based on the colony morphology and appearance of agar color, and each *E. coli*-suspected colony was further subcultured onto blood agar as well as a parallel subculture on McConkey agar. The next day, the pure culture isolates were submitted to the CSU-VTH diagnostic lab to confirm the *E. coli* species.

2.3.5 Macrophage isolation and culture

Blood from healthy dogs was processed for macrophage culture as previously described[33]. Briefly, peripheral blood mononuclear cells (PBMC) were separated from EDTA-anticoagulated blood samples by Ficoll-density gradient isolation; the PBMC were washed in PBS, resuspended in complete medium (DMEM, 1% Penicillin-streptomycin, essential and nonessential amino acid) with 1% FBS and plated at a density of $1x10^6$ PBMC/0.5 ml in 48-well polystyrene cell culture plates, followed by incubation for 4 hours at 37°C. After allowing for monocyte adhesion, the non-adherent cells were washed off with PBS twice, refed with complete medium with 15% FBS, supplemented with 10 ng/ml huM-CSF (Peprotech, Rocky Hill, NJ, USA) and cultured for 7 days. The culture medium was changed every 2 days. After 7 days in culture, the monocyte-derived macrophages were used to assess the macrophage activation, phagocytosis and cytokine assays following incubation with fecal bacteria derived from healthy dogs and dogs with IBD (described later).

2.3.6 Macrophage phagocytosis and activation assays

Ten fecal bacteria samples (5 samples per group; IBD and healthy) were used in the macrophage phagocytosis and activation assays. Note that the bacteria used in these assays were nonviable after being processed, frozen and stained with PI. The numbers of bacteria were counted using PI-labeled bacteria relative to a number of counting beads (Invitrogen, Eugene, OR). The final numbers of bacteria in the samples were calculated by comparing the ratio of the bead events to the bacterial cell events according to the manufacturer's datasheet. To assess bacterial phagocytosis, the fecal bacteria at the MOI ratio of 5 bacteria per 1 macrophage were added to the macrophage cultures used. The bacteria were subsequently spun onto macrophages by centrifugation at 2000 x G for 10 minutes and incubated for 2 hours at 37°C. Nonphagocytosed

bacteria in the cultures were then washed, and the macrophage cells were detached and analyzed via flow cytometry analysis. The % of PI+ve macrophages and the PI abundance in macrophages were analyzed.

To evaluate the activation of macrophages by fecal bacteria, macrophages were cultured, and this was followed by a phagocytosis assay; however, rather than detaching the cell to flow cytometry analysis at the end, the macrophages cultured were washed twice with PBS, refed with the new fresh media and cultured for an additional 24 hours. The supernatants were collected for cytokine (TNF-a and IL-10) measurement by ELISA. As a positive control for cytokine release and activation, 10 ng/ml LPS was added to parallel cultures of macrophages. These assays were repeated 3 times using blood from 3 different unrelated donor animals to ensure reproducibility.

2.3.7 Flow sorting and 16S rRNA sequencing

The fecal bacteria from 3 populations were analyzed for the bacterial subset composition using 16S rRNA sequencing and microbiome analysis. The 3 populations consisted of total fecal bacteria from dogs with IBD (n =10), fecal bacteria from healthy dogs (n=10), and bacteria with high levels of bound IgG (IgG^{hi} bacteria) obtained from the feces of dogs with IBD (n = 10) following incubation with anti-dog IgG secondary antibody (as previously described) to enrich for IgG^{+} bacteria. The population of IgG^{hi} bacteria (MFI greater than normal baseline) was sorted by the BD FACSAria sorter. The purity of the sorted product was assessed by flow cytometry and was found to consist of at least 85% IgG^{hi} bacteria. The sorted population was compared to the IBD nonsorted population. The population not subjected to sorting (e.g., sorting IgG negative bacteria) was substantially less because nearly all bacteria in dogs with IBD were IgG+ve.

Bacteria were subjected to 16S rRNA sequencing following DNA extraction using a Mobio PowerSoil DNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted DNA was submitted for 16S rRNA sequencing and analyzed by Novogene Corporation (Chula Vista, CA). The 16S rRNA sequencing was performed as reported in a previous study[34]. Sequence analysis was performed using Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/)[35]. For each representative sequence, Mothur software was performed to compare results against the SSUrRNA database of the SILVA Database (http://www.arb-silva.de/)[36] for species annotation at each taxonomic rank (Threshold: 0.8~1). The OTU abundance data were normalized using a standard of the sequence number of the least sequence sample. Subsequent analyses of the alpha and beta diversity were performed based on these output normalized data. The alpha diversity was calculated using the Shannon diversity index. The beta diversities on both unweighted and weighted UniFrac were calculated by QIIME software (Version 1.7.0).

2.3.8 Statistical analysis

Data were analyzed using Prism 7 software (GraphPad, San Diego, CA, USA). The normality of the data was initially examined using the Shapiro-Wilk normality test. The normally distributed data were reported as the mean ± standard deviation (SD). Data that were not normally distributed were shown as the median (range). Statistical differences between the IBD and healthy dogs were analyzed using the two-tailed unpaired t-test for parametric data and Mann-Whitney test for nonparametric data as indicated in the text. For statistical analysis of the serum IgG response, the % IgG binding *E. coli* were compared using a one-way ANOVA. The results from repeated experiments, including cytokine production from different PBMC donors, were

standardized to the baseline control before analysis. To analyze the association between Ig-binding bacteria and other clinical variables, including the disease activity index and histopathology score, linear regression analysis was performed. The Receiver-Operating Characteristic (ROC) curve was calculated to determine the sensitivity and specificity as a diagnostic ability between IBD and normal dogs. The microbiome analysis was performed by the company as previously described. In all studies, the statistical significance was set at $p < 0.050$.

2.4 Results

2.4.1 Breed characteristics of study dogs

Twenty dogs diagnosed with IBD were enrolled in the study. All dogs showed clinical signs that matched the inclusion criteria as previously described. The demographic and health parameter data of the 20 dogs with IBD enrolled in the study were shown in Table 2.1 and 2.2. The breeds included mixed breeds (n=4), Bernese Mountain Dog (n=4), Yorkshire Terrier (n=2), Labrador Retriever (n=2), Rottweiler, Pug, Cavalier King Charles Spaniel, Boxer, German Shorthaired Pointer, American Eskimo, Siberian Husky, and English Bulldog. The breeds in the healthy control group (n = 9) included Standard Poodle, mixed breed dogs, Cocker Spaniel, Nova Scotia Duck Tolling Retriever, Shih Tsu, English Coonhound, English Setter, and Chihuahua. Overall, there was no significant difference in age between the healthy control dogs and dogs with IBD ($p = 0.42$). However, the body condition score in healthy dogs were greater than IBD dog ($p = 0.01$) which is the common finding since the IBD patients had thin body during episode of illness. In addition, the type of diet that each dog had during the study period was shown in Table 2.2 and 2.3. For disease severity in IBD group, the disease duration was classified as chronic, with a

moderate disease activity index (Table 2.2). Briefly, eleven of the dogs with IBD had moderated lymphoplasmacytic inflammation of the duodenum, in 5 cases associated with mixed eosinophilic/neutrophilic infiltration. Six dogs had mild lymphoplasmacytic inflammation and a half of them were found to have mixed eosinophilic infiltration. Three cases were documented the severe lymphoplasmacytic inflammation and 2 of them were mixed infiltrates. Additional observations included glandular degeneration, crypt abscesses, lacteal dilatation, villous fusion and villous shortening. The evaluated score by endoscopic examination and WSAVA histopathology scores were also reported in Table 2.1 and 2.2.

2.4.2 IgG binding to fecal bacteria in dogs with IBD versus healthy dogs

The IBD dogs had an increased overall degree of the humoral immune response toward their gut bacteria compared to the healthy dogs. The percentage of IgG^+ bacteria in the feces of the dogs with IBD was significantly greater than it was in the feces of the healthy control animals: IBD: 80% ± 15.05; healthy: 47.5% ± 18.35, $p < 0.0001$, (Figure 2.2A). Moreover, the overall amount of IgG bound by bacteria, assessed by the mean fluorescence intensity (MFI), was significantly higher in the dogs with IBD than in the healthy dogs (MFI-IBD: 11,769 ± 6,539 a.u.; MFI-healthy: 6,650 ± 2,687 a.u., $p = 0.005$, Figure 2.2B). The study indicated that there was a substantially higher percentage of IgA^+ bacteria in the IBD dogs than in the healthy dogs; however, the magnitude of the degree difference was less than for IgG binding (IBD: 84.86% ± 9.87; healthy: 73.18% ± 15.83, $p = 0.022$, Figure 2.2C). Nevertheless, there was no significant difference in the total IgA ligand binding with bacteria in both groups of dogs (MFI-IBD: 7,607 (2,834-17,120) a.u.; MFI-healthy: 7,113 (3,280-11,925) a.u., $p = 0.91$, Figure 2.2D).

We also used confocal microscopy to visualize the IgG^+ and IgA^+ populations of bacteria, as well as the potential overlap in the two populations, in the dogs with IBD and healthy dogs (Figure 2.3). The IgG^+ bacteria were visualized in red with rabbit anti-dog IgG-Cy3 conjugate antibody, and the IgA^+ bacteria were visualized in green with goat anti-dog IgA-FITC conjugated antibody. Bacteria expressing both immunoglobulins appeared yellow in merged images. The results indicated that there was a predominant population of the IgA^+ bacteria in the feces of the healthy dogs, with substantially fewer IgG^+ bacteria (Figure 2.3). In the case of the dogs with IBD, many more IgG^+ bacteria were present, as reflected by a large number of dual positive (yellow) bacteria visualized. Linear regression analysis showed a significant correlation between the percentages of IgG^+ and IgA^+ bacteria in the dogs with IBD (R^2=0.45, p = 0.001; Figure 2.4). A similar correlation of the MFI of IgG^+ and IgA^+ bacteria was also noted (R^2=0.48, p = 0.001), which suggests that in IBD cases, increased IgG binding activity was associated with increased IgA binding in terms of both percentages of bacteria bound and in the abundance of IgG and IgA present on the surface of bacteria. However, there was no significant correlation between the IgG and IgA-binding activity found in healthy dogs.

2.4.3 Recognition of fecal bacteria by circulating IgG

To investigate whether the production of IgG bound to gut bacteria occurs primarily in the GI tract or whether it is alternatively produced in tissues of extraintestinal lymphoid and secondarily translocated to the GI tract (e.g., by leakage from intestinal vasculature), investigation assays using the serum and their IgG antibodies with specific binding to the common intestinal bacterium (*E. coli*) were performed as outlined in the methods. Note that because bacteria from

the GI tract of IBD dogs typically contained relatively high levels of IgG binding, these assays were performed using pure cultures of *E. coli* from the GI tract of dogs (total of 6 isolated strains).

We determined that the quantity of the IgG found in the serum bound to *E. coli* did not differ between the dogs with IBD and healthy serum (Figure 2.5). Moreover, there were no differences in the recognition of serum IgG in the different isolated strains of *E. coli* from healthy dogs or dogs with IBD. For example, the percentage of serum IgG-binding *E. coli* was reported as the 4 following groups: 1) IBD serum vs. healthy *E. coli*: 51.54 ± 14.09%, 2) healthy serum vs. healthy *E. coli*: 49.12 ± 9.52%, 3) IBD serum vs. IBD *E. coli*: 44.47 ± 11.07%, and 4) healthy serum vs. IBD *E. coli*: 50.41 ± 9.86%. In addition, we also found the similar recognition of serum IgG toward *Enterococcus* strains as shown in Figure 2.6. Therefore, the study concluded that the production of IgG bound on the fecal bacteria surface occurs locally in the GI tract, instead of systemically. These results were also in agreement with the high levels of plasma cells found in the GI tract of IBD dogs that were previously discussed[16, 17].

2.4.4 Macrophage phagocytosis of fecal bacteria increased in dogs with IBD

With the existence of a substantially high IgG^+ bacteria level in the GI tracts of IBD dogs, we subsequently sought the potential links between this phenomenon and the induction of intestinal inflammation. One outstanding mechanism linking the GI inflammation and IgG^+ bacteria is that the gut bacteria could involve an interaction with phagocytic cells, including macrophages in the lamina propria. Thus, an in vitro system was applied to identify whether gut bacteria from dogs with IBD were inherently more inflammatory than gut bacteria in healthy dogs.

First, we compared the relative phagocytosis abilities of primary macrophages to phagocytose bacteria from health dogs versus IBD dogs (Figure 2.7). The fecal bacteria from dogs

with IBD significantly induced higher macrophage phagocytosis activity than the fecal bacteria from healthy dogs. For example, the percentage of macrophages containing phagocytosed bacteria was 67.91 ± 13.68% in cultures incubated with bacteria obtained from the IBD animals, compared to 55.05 ± 15.48% for bacteria obtained from the healthy dogs (p = 0.023, Figure 2.7B). Furthermore, the mean levels of ingested bacteria for each individual macrophage (reflected by MFI) was significantly high in the macrophages incubated with IBD bacteria (MFI: 2,994 (2,378-3,912)) compared to healthy bacteria (MFI: 2,519 (2,323-3,428), p = 0.005, Figure 2.7C). Therefore, in dogs with IBD, GI bacteria are more likely phagocytosed through macrophages than healthy dogs' bacteria, which suggests more efficient Fc-receptor mediated uptake of the heavily IgG-coated bacteria from dogs with IBD.

2.4.5 Ingestion of bacteria from dogs with IBD triggers greater macrophage inflammatory response

Bacteria ingestion, primarily through Fc receptor-mediated internalization, functions as a critical stimulus of activating macrophages[37]. Thus, the next evaluation was related to how the phagocytosis of fecal bacteria influences the activation of macrophages and the production of cytokines. The study of activated macrophages by bacteria indicated that the expression of costimulatory molecules on macrophages was highly expressed costimulatory molecules (MHCII, CD40) approximately 80-100% of the total cells. However, no significant difference in the costimulatory molecule expressions was observed between activated macrophages incubated with bacteria from IBD and healthy dogs. Importantly, the incubation of macrophages with IBD fecal bacteria yielded higher levels of TNF-a production than the incubation of macrophages with fecal bacteria from healthy dogs (IBD; 332.3 ± 97.28 versus 234.1 ± 30.12 pg/ml, p = 0.04; Figure 2.8).

In contrast, the incubation of macrophages with IBD bacteria led to substantially lower IL-10 (159.3 ± 12.8 pg/ml) than macrophages incubated with healthy dog bacteria (219.3 ± 51.52 pg/ml, $p = 0.03$). Therefore, we concluded that the gut bacteria found in the IBD dogs were integrally more likely to stimulate an inflammatory response than bacteria from the healthy gut, as shown by the capability of activating the production of pro-inflammatory cytokines, such as TNF-a, from activated macrophages.

2.4.6 Microbiome analysis and selectivity of IgG binding

Through sequencing of 16S rRNA metagenomics, the overall gut microbiome complexity and composition in dogs with IBD was compared to that of healthy dogs (Figure 2.9). In dogs with IBD, there was a greater relative abundance of bacteria in the *Proteobacteria* phylum ($p = 0.045$; Figure 2.10), including *Escherichia-Shigella*, and other genera, such as *Clostridium, Blautia, Bifidobacterium, Enterococcus, Pseudomonas, Faecalibacterium,* and *Lactobacillus*, together with a decreased relative abundance of *Bacteroidetes* phyla ($p = 0.02$) and additional genera, *Streptococcus, Fusobacterium, Peptoclostridium,* and *Turicibacter,* compared to the flora present in healthy control dogs (Figure 2.9A). These results are mainly in agreement with previous studies of the microbiome in dogs with IBD and indicate that our study populations were similar to those of other research regarding the diversity of bacteria and abundance difference of the gut microbiome in IBD versus healthy dogs[38-40].

Then, 16S rRNA sequencing studies were conducted to determine whether the increased levels of IgG binding on bacteria found in the feces of dogs with IBD identify exclusively enriched bacteria subsets (e.g., potentially pathogenic bacteria) or whether there was an even distribution in the population of IgG^{hi} bacteria within all major phyla (i.e., no enrichment for specific phyla or

genera). The research rationale is the previous evidence for the selective binding of the immunoglobulin to pathogenic bacteria in CD patients[21, 22].

The bacteria that had the highest degrees of IgG binding were: *Collinsella*, *Faecalitalea, Escherichia-Shigella, Blautia, Bifidobacterium, Clostridium innocuum, Slackia* and *Enterococcus* (Figure 2.9A and Table 2.4). The taxa that had the smallest amount of IgG binding were *Pseudomonas, Clostridium* (sensu stricto) and *Lactobacillus*. According to this study, we found that the predominant fecal bacterial phyla present in the IgG^{hi} bacterial populations were very similar overall to those of nonsorted bacteria from paired IBD samples with the exception that there was a significant enrichment of bacteria in the *Actinobacteria* phylum ($p = 0.036$) in the IgG^{hi} population, compared to the nonsorted IBD bacteria (Figure 2.9B, 2.9C and Table 2.4**)**. Moreover, the most abundant genus in this phylum was *Collinsella,* which was significantly enriched in the IgG^{hi} sorted population of bacteria compared to the nonsorted bacteria. Therefore, these studies indicated that there was a preference in the immune recognition of *Actinobacteria* in dogs with IBD.

2.4.7 Sensitivity and specificity of fecal bacteria IgG assay for the detection of IBD in dogs

The previous results indicated that the quantitation of the relative degree of IgG binding to fecal bacteria might be valuable as a diagnostic test for the detection of IBD in dogs. Thus, this study examined the specificity and sensitivity of the flow cytometric assay for IBD diagnosis in dogs, measuring both the percentage IgG^{+} bacteria and the quantity of IgG bound to each bacterial cell (i.e., MFI). Through receiver operating curves (ROC), it was determined that the area under the curve (AUC) for IgG^{+} bacteria was 0.92 (95% CI: 0.80-1.03, $p < 0.0001$; Figure 2.11). These findings showed that there was a high diagnostic ability for the flow cytometric test utilized with

respect to the percentage of IgG^+ bacteria for distinguishing between healthy dogs and dogs with IBD.

We determined the sensitivity and specificity of the bacterial IgG assay through the cutoff point at the upper limit of the 95% confidence interval established using the range of percentage of healthy dogs, which was identified as 60% IgG^+ bacteria. Using this cutoff, values of 85% sensitivity (95% CI: 62.11-96.79) and 89% specificity (95% CI: 51.75-99.72) were achieved for the detection of clinically apparent IBD in dogs, based on flow cytometric analysis of IgG binding to fecal bacteria. Overall, the fecal IgG test in dogs had a positive likelihood ratio of 7.727 and a negative likelihood ratio of 0.169. Thus, the test showing IgG^+ bacteria above 60% (positive of the test) is moderate to highly suggestive of IBD. However, the IgG^+ bacteria percentage did not correlate with the activity index of disease, including CIBDAI ($p = 0.71$) and CCECAI ($p = 0.55$). Furthermore, the overall endoscopic lesion scores and the histopathologic score had no relationship with the IgG^+ bacteria percentage ($p = 0.28$ and $p = 0.75$, respectively), as determined by linear regression analysis. Thus, the assessment of IgG bound to the surface of bacteria was found to be a very sensitive and specific test for the detection of IBD in dogs, although the test positivity did not correlate with the disease activity or severity. Therefore, it was concluded that the fecal IgG assay had a higher specificity and sensitivity for the diagnosis of IBD than other currently available assays for detecting IBD in dogs, including fecal calprotectin (S100A12), which was found to be 65% sensitive and 84% specific for diagnosing IBD in dogs[41].

2.5 Discussion

The interaction between the gut bacteria and the host immune response is currently considered a primary driver of intestinal inflammation in humans with IBD[6-8]. There is considerable evidence of a greater degree of IgG responses against gut bacteria in CD patients[12, 18, 19]. For example, the abundance of IgG^+ bacteria is shown to be specific for Crohn's disease because increased IgG^+ fecal bacteria levels are not found in patients with coeliac disease[42] and infectious colitis[12, 18]. Similar studies have not previously been conducted in dogs with IBD and to the authors' knowledge, this research is the first study that investigates an immune response against gut bacteria in this disease in the canine population.

The key finding from this study is that dogs with IBD had a significantly higher binding of IgG to gut bacteria than healthy dogs (Figure 2.2). For example, in IBD dogs in general, there was 30% more bacteria bound with IgG than there were in healthy dogs (Figure 2.2A). The numbers are roughly in agreement with the results of the IgG binding bacteria previously described for UC and CD patients[12, 18]. In addition, we further evaluated the IgG-binding in patients with giardia infection represented the model for infectious GI disease. As expected, the % IgG-binding to gut bacteria was lesser than the level found in IBD (p = 0.0002; Figure 2.13) and comparable to the degree from normal dog. This result agreed with a report from infectious colitis study[18] and suggested that the lesser immune response reacted to gut bacteria in case of GI infectious disease. Furthermore, this study also indicates that the anti-bacterial IgG production sources were more likely from local immunoglobulin in the gut instead of systemically released by extraintestinal locations, which is also reported in the investigation of patients with Crohn's disease[7, 12]. Thus, the fecal IgG test would be the potential test indicating the degree of local immunity reaction to gut bacteria which is specific for IBD pathogenesis.

One difference between the immunoglobulin responses against gut bacteria in our study and studies in CD is that there was no heightened IgA ligand binding to bacteria in IBD dogs, while humans exhibited substantially high IgA ligand abundance presented on gut bacteria compared to gut bacteria in healthy patients[12]. The reasons for this variation are unclear; however, it is important to note that the concentration of soluble IgA was reported to be generally lower in the feces of IBD dogs than in healthy dogs[13, 43].

This study also shows a plausible connection between bacterial IgG binding and the induction of intestinal inflammation, which could entail macrophage activation in the gut. For example, the study indicated that macrophage incubation with fecal bacteria from dogs with IBD significantly increased the production of TNF-a and the activation of macrophages than bacteria obtained from the GI tracts of healthy dogs (Figure 2.7, 2.8). In contrast, bacteria from dogs with IBD caused a significantly lower production of IL-10 through macrophages than those from healthy dogs. Therefore, the net impact of the macrophage interactions with IgG bound bacteria in IBD animals could be the activation of increased local inflammation and immune activation. This impact could have interceded, in part, through the interaction of IgG-binding bacteria with triggering Fc receptor activation through macrophages found in the lamina propria or intestinal epithelium[43, 44].

Interestingly, we found that the IgG response against the gut bacteria in dogs with IBD appeared to be directed preferentially toward bacterial groups considered parts of the dysbiotic flora, as described in previous studies[24, 25, 38-40]. Moreover, we found that bacteria in the genera *Collinsella* had the most preferential recognized IgG in dogs with IBD, whereas this organism did not express greater IgG binding in healthy dogs (Figure 2.9A and Table 2.4). Interestingly, this genus was noted in dogs with gastric-dilation and volvulus[45] and has also been reported to be one

of the high IgA binding bacteria detected in patients with CD[11, 46]. While not all dysbiotic flora are considered pathogenic, some of the genera found in dysbiosis (e.g., *Escherichia*, *Clostridium* and *Enterococcus*) have been associated with intestinal infection and invasion[47-50]. These enteroinvasive pathogenic bacteria could trigger greater immune recognition and local antibody production[50-52].

In terms of diagnostic utility, our studies demonstrated that a bacterial flow cytometric IgG assay using fecal samples provided significant sensitivity and specificity to distinguish dogs with IBD from healthy dogs (Figure 2.11). As there is no commercially available assay for accurately identifying dogs with IBD, this bacterial flow cytometric assay may provide a useful and relatively straightforward assay that can be conducted on fresh or frozen fecal samples. For example, we found that the fecal IgG-binding bacteria assay provided consistent results whether the samples were stored at -20°C or -80°C (coefficient of variation = 19.27%).

Our findings did not show an association between the degree of IgG^+ bacteria and clinical parameters associated with IBD, including the disease activity index, the histopathology score, and the endoscopic score. Similar to previous IBD studies in dogs, the clinical parameters showed a lack or weak correlation with other IBD parameters, including serum Ig, C-reactive protein and Calprotectin[13, 53, 54].

However, the relative abundance of the genus *Collinsella*, which was found to have the highest IgG binding in dogs with IBD (Figure 2.9C), showed the highest association with standard IBD clinical parameters, including CCECAI (p = 0.024), CIBDAI (p = 0.032), histopathology scores (p = 0.016) and serum folate concentrations (p = 0.008, Figure 2.12). This finding was in agreement with previous studies in humans and cats[46, 55]. In humans, *Collinsella* is considered one of the taxa used to differentiate between patients with UC and CD[46]. *Fusobacterium* and

Clostridiaceae were also reported to be associated with CIBDAI in dogs[25, 38]; however, in the present study, no association with these taxa and IBD was observed.

Through a culture-independent technique of 16S rRNA metagenomic sequencing for microbiome analysis, this investigation shows a relatively good correspondence to the previous sequencing studies on dogs with IBD focused on mucosa-associated or fecal microbiota[24, 25, 38-40]. For example, a previous study reported dysbiosis of commensal bacteria, including increased *Proteobacteria* (e.g., *E. coli*), *Clostridium,* and *Enterococcus*[40]. In addition, our study extensively identified that these dysbiotic bacteria presented with a high degree of IgG already attached on their surface (Figure 2.9A), leading to a greater overall percentage and increased MFI of IgG-binding bacteria in dogs with IBD (Figure 2.2). However, some of the high IgG binding taxa determined in this study were considered to be non-IBD related taxa in previous studies on IBD dogs, including *Faecalibacterium*, *Allobaculum, Slackia* and *Clostridium*[39].

In our setting, the average age was middle-aged dogs corresponding to the common age for having IBD[56] and was not different from healthy controls, which minimized a potential alteration of the immune response by age[57]. The healthy controls were client-owned dogs (not a laboratory setting) with various home environments and diets. Thus, they were justified as pet dog representatives. As a different diet potentially influences the microbiome, we also reported the type of diet that each dog had during the study period (Table 2.2, 2.3). Although a variety of diets was documented in both groups, the microbiome changing was found to agree with other IBD studies[38-40] suggesting altered microbiome was primary influenced by causative disease rather than diet. In addition, we were unable to breed-matched them with the IBD group because of the small number of controls. One potential limitation of this study is that some dogs with IBD had undergone an antibiotic intervention before they were enrolled in the study. Antibiotic treatment

is known to significantly change the intestinal microbiome in dogs[58, 59]. Nevertheless, there were no substantial changes in the IgG^+ bacteria percentage in the dogs despite their history of antibiotic pretreatment, which implies that there is a minimal discernable effect of antibiotic treatment on the production and function of anti-bacterial IgG in dogs with IBD. This observation is crucial because it indicates that a fecal IgG assay could be moderately resistant to the potential influence of past antimicrobial therapy.

This study demonstrates that a greater percentage of intestinal bacteria were recognized through local IgG produced in the gut of dogs with IBD and that IgG bound bacteria may be relevant to intestinal inflammation. These outcomes must be proven through more extensive studies in dogs with IBD and compared to animals with other causes for GI dysfunction, such as viral infection, dietary changes, and infections with GI parasites (e.g., hookworm, whipworm, or Giardia infection; preliminary result shown in Figure 2.13). Furthermore, the analysis of the serial samples at a later stage after treatment at the various follow-up time points could also be beneficial. These studies could provide additional insights into the role of local gut immune responses against bacteria and the potential role of these processes in mediating GI inflammation in a variety of different GI diseases in dogs.

2.6 Conclusions

This study demonstrated that the gut bacteria found in IBD dogs had a substantially greater degree of IgG binding than gut bacteria in the healthy dogs. In addition, the highest level of IgG binding appeared to be directed against certain phyla of dysbiotic bacteria, with significant high preference for *Actinobacteria*. Furthermore, IgG coated bacteria from IBD dogs significantly

activated higher production of TNF-a by macrophage than bacteria derived from the healthy indicative of the inherently pro-inflammatory effect of high IgG binding to bacteria. These findings suggest that humoral immune recognition of endogenous intestinal bacteria may be an important mediator causing intestinal inflammation in dogs with IBD linking as a crucial part in the immune pathogenesis of certain types of IBD in dogs and human.

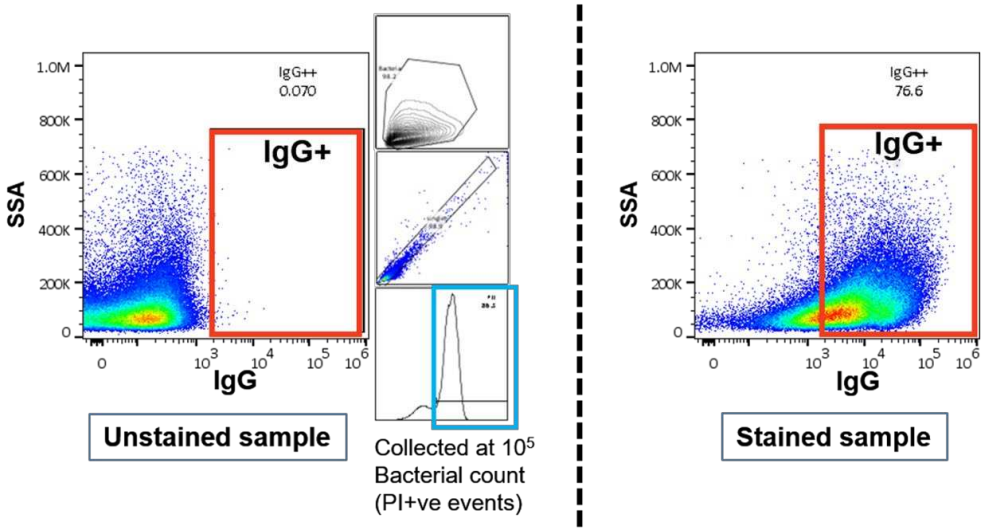

Figure 2.1 Flow cytometry analysis and gating. Fecal bacteria were analyzed based on size and complexity corresponding to bacteria population as well as selective counting of 10^5 bacteria cells. The percentage of positive fluorescence cells of IgG-binding bacteria and fluorescence intensity was analyzed by comparing to background threshold.

Table 2.1 Summary demographic data of study groups.

	IBD	Normal
Sample size	20	9
Gender		
-Male	14	4
-Female	6	5
Age (year)	6.4 ± 3.77	7.6 ± 3.05
Weight (kg)	24.04 ± 13.79	22.73 ± 11.35
BCS (9 scales)	4.1 ± 1.13	5.33 ± 0.86
Disease Duration (month)	4.62 ± 5.27	-
Disease activity index		
-CIBDAI	6.7 ± 3.57	NP
-CCECAI	8.1 ± 4.55	
Endoscopic lesion	100%	NP
Endoscopic score		
-Gastroscopic	1 (0-2)	
-Duodenoscopic	3 (2-6)	NP
-Ileoscopic	3 (1-7)	
WSAVA Histopathology score	5.15 ± 2.68	NP

Data reported as Mean ± SD and Median (range).

CIBDAI = Canine Inflammatory Bowel Disease Activity Index; CCECAI = Canine Chronic Enteropathy Clinical Activity Index; NP = not performed.

Table 2.2 Demographic data, diet and histopathologic evaluation of IBD group.

No.	Breed	Age (yr)	BCS (/9)	CIBDAI	CCECAI	Diet	Histopathology	WSAVA score
1	Yorkshire Terrier	3	4	10	14	Commercial dog food; urinary care formula	1. Mild lymphoplasmacytic enteritis with marked glandular degeneration. 2. Mild lymphoplasmacytic colitis.	6
2	Yorkshire Terrier	2	4	10	13	Commercial dog food with buffalo meat	Mild lymphoplasmacytic enteritis	3
3	Bernese Mountain Dog	6	4	7	9	Any food if dog eats	Mild lymphoplasmacytic and suppurative enteritis with some eosinophils and gland degeneration	6.5

4	Bernese Mountain Dog	9	5	9	9	Commercial dog food with whitefish and any food if dog eats	1. Duodenum: Moderate lymphoplasmacytic and eosinophilic enteritis. 2. Ileum: Mild lymphoplasmacytic and eosinophilic enteritis. 3. Mild lymphoplasmacytic and eosinophilic colitis	7
5	Bernese Mountain Dog	6	5	2	3	Commercial dog food with whitefish and potato	1. Duodenitis, lymphoplasmacytic and eosinophilic, chronic–active, moderate with crypt abscesses, mild lacteal dilation and mild lymphoplasmacytic intraepithelial infiltration 2. Ileitis, lymphoplasmacytic and eosinophilic, chronic, moderate with mild lymphocytic intraepithelial infiltration 3. Colonic inflammation, lymphoplasmacytic and eosinophilic, chronic, mild, with mild lymphocytic intraepithelial infiltration	5
6	Bernese Mountain Dog	2	5	9	11	Homemade diet with lamb and rice	1.Moderate lymphoplasmacytic to eosinophilic duodenitis 2.Mild suppurative colitis with moderate numbers of globular leukocytes	5
7	Mixed breed	6	4	2	5	Commercial dog food with whitefish and potato	1. Duodenum and Ileum: Moderate eosinophilic and lymphoplasmacytic enteritis with villous shortening 2. Minimal lymphoplasmacytic colitis.	6
8	Mixed breed	15	6	3	3	Commercial dog food with rabbit,	Mild lymphoplasmacytic and eosinophilic enteritis	4

						wild bison, grain-free		
9	Mixed breed	12	6	2	2	Commercial dog food with novel protein	Moderate, chronic, regionally extensive lymphoplasmacytic enteritis	1
10	Mixed breed	2	4	3	4	Commercial dog food; high energy for GI	Moderate lymphoplasmacytic enteritis.	5
11	Labrador Retriever	12	4	9	10	Commercial canned dog food with buffalo and salmon	Mild lymphoplasmacytic and eosinophilic enteritis	2
12	Labrador Retriever	5	1	7	8	High caloric commercial puppy food	1. Duodenum: Enteritis, lymphoplasmacytic, chronic, moderate with villus fusion. 2. Ileitis, lymphoplasmacytic and eosinophilic, chronic, severe with villus fusion 3. Colitis, lymphoplasmacytic, chronic, mild with lamina propria fibrosis.	5
13	Pug	10	3	11	13	Homemade diet; chicken, rice and sweet potato	1. Severe catarrhal enteritis in duodenum and ileum, with bacterial colonies on the surface 2. Duodenum, Ileum: increased lymphocytes and plasma cells throughout the lamina propria of the mucosa	12
14	Rottweiler	6	4	14	19	Commercial dog food; weight managemen t formula	1. Duodenum: Severe, interstitial, lymphoplasmacytic and eosinophilic enteritis, with mild villus blunting, mild lacteal dilation, and formation of crypt abscesses 2. Ileum: Mild to moderate, interstitial, lymphoplasmacytic and eosinophilic enteritis,	10.5

							with mild to moderate lacteal dilation 3. Colon: Mild, patchy lymphoplasmacytic colitis	
15	Boxer	4	5	4	4	Commercial dog food with novel protein	Severe lymphoplasmacytic and eosinophilic enteritis	2
16	Cavalier King Charles Spaniel	3	5	2	2	Commercial dog food with turkey and potato	1. Mild to moderate lymphoplasmacytic enteritis with glandular degeneration and villous blunting. 2. Mild lymphoplasmacytic colitis.	6
17	German Shorthaired Pointer	4	4	5	6	Commercial dog food; low fat for GI	Duodenum/Ileum/Colon: Chronic moderate lymphoplasmacytic and mild eosinophilic enterocolitis, few crypt abscesses, and mild lymphangiectasia.	6
18	English Bulldog	7	4	7	8	Commercial dog food with duck, potato	1. Duodenum: Moderate lymphoplasmacytic enteritis with few neutrophils and moderate to marked intraepithelial lymphocytes infiltration. 2. Ileum: Minimal lymphoplasmacytic enteritis with mild intraepithelial lymphocyte infiltration. 3. Colon: Minimal lymphoplasmacytic enteritis.	5
19	American Eskimo	10	3	9	10	Homemade diet with cooked chicken	1. Duodenum: Moderate lymphoplasmacytic duodenitis 2. Ileum: minimal presence of lymphocytes and plasma cells within the lamina propria 3. Colon: Mild lymphoplasmacytic and eosinophilic colitis	3

20	Siberian Husky	4	3	8	9	Commercial dog food with hydrolyzed protein	1. Duodenum: Moderate, diffuse, chronic, lymphoplasmacytic enteritis with moderate villous blunting 2. Ileum: Mild, diffuse, chronic, lymphoplasmacytic ileitis 3. Colon: Mild, diffuse, chronic, lymphoplasmacytic colitis	3

Table 2.3. Demographic data, and diet of normal group.

No.	Breed	Age (yr)	BCS (/9)	Diet
1	English Setter	9	5	Commercial dog food with venison
2	English Coonhound	12	7	Commercial dog food; weight control formula
3	Mixed breed	6	5	Commercial dog food; standard formula
4	Mixed breed	9	6	Commercial dog food; senior formula
5	Cocker Spaniel	5	5	Commercial dog food; standard formula
6	Standard Poodle	6	5	Commercial dog food; standard formula
7	Nova Scotia Duck Tolling Retriever	5	5	Commercial dog food with lamb and rice
8	Chihuahua	12	4	Commercial canned dog food with venison
9	Shi Tsu	4	6	Commercial dog food; standard formula

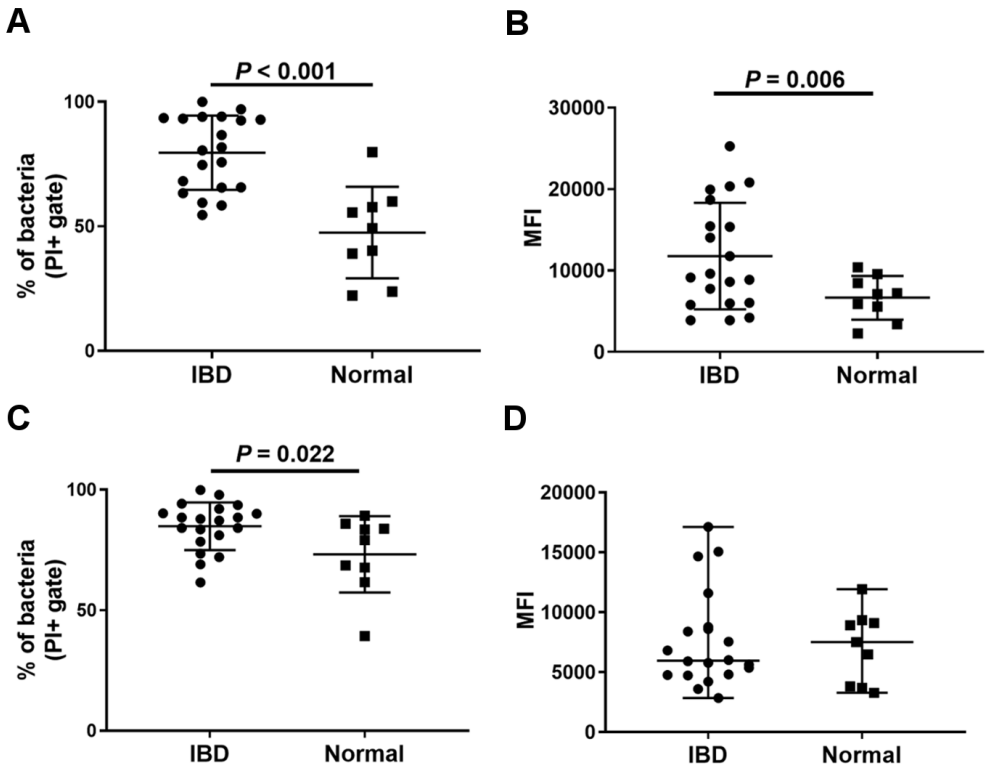

Figure 2.2 IgG$^+$ and IgA$^+$ fecal bacteria in healthy dogs and dogs with IBD. (A) The percentages of IgG+ bacteria are plotted in dogs with IBD versus healthy dogs. (B) The amount of IgG bound to each bacterium (MFI) is plotted for the two groups of animals. IgA binding percentages and total IgA binding to each bacterium are depicted in C and D, respectively. Data are plotted as Mean ± SD. Statistical differences were calculated using two-tailed unpaired t-test (A,B,C) or a Mann-Whitney U test (D).

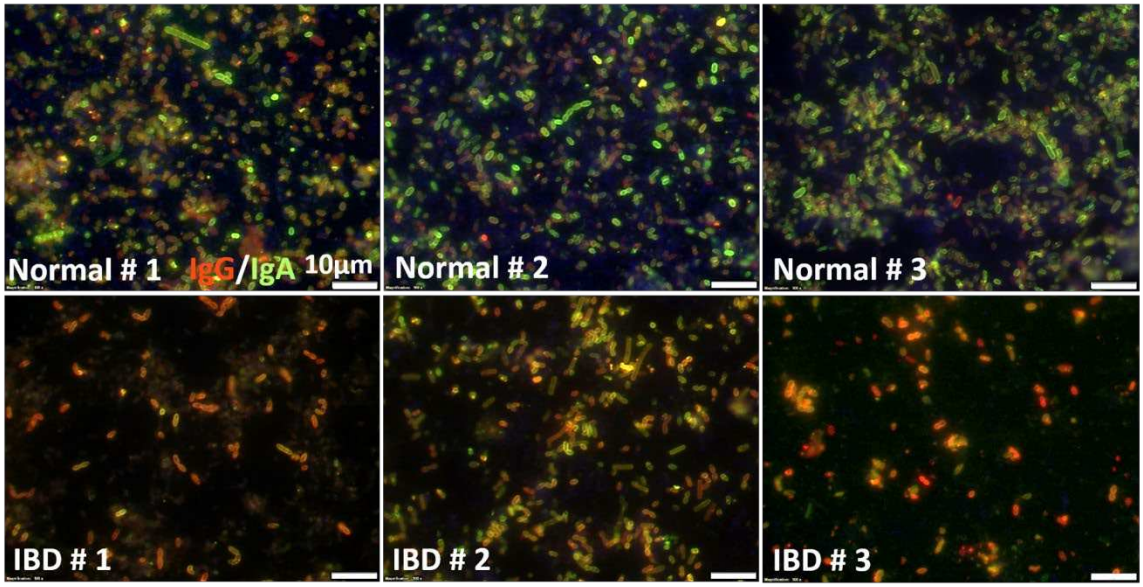

Figure 2.3 Ig-binding fecal bacteria. Immunofluorescence staining and imaging of fecal bacteria from a healthy dog (top row) and from a dog with IBD (bottom row). IgA bound to bacteria indicated as green, while IgG^+ bacteria indicated as red. Bacteria with both bound antibodies show up as yellow images in merged figures. Scale bar indicates 10 µm.

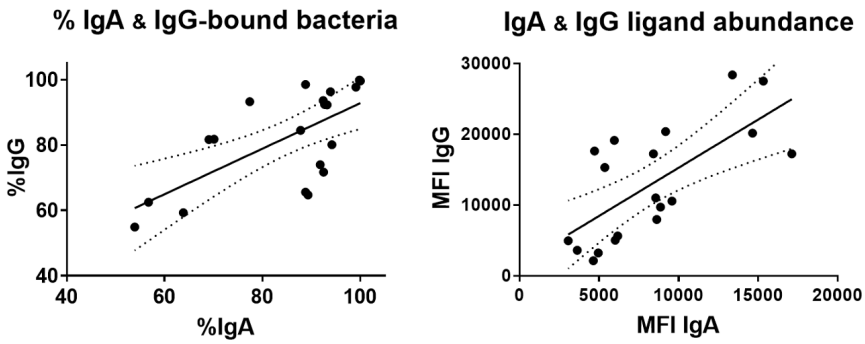

Figure 2.4 Association between IgG and IgA binding to fecal bacteria. Scatter dot plot of (A), percentage of IgA-bound and IgG-bound bacteria and (B) amount of IgG and IgA binding to individual bacteria (MFI) depicted. To analyze the degree of association between IgG and IgA binding, linear regression analysis was performed. The percentage of IgA-bound bacteria was significant correlated with the percentage of IgG-bound bacteria ($R^2 = 0.45$, $p = 0.001$). Also, degree of IgA and IgG binding also showed significant correlation ($R^2 = 0.48$, $p = 0.001$). Dashed lines depict 95% confidence band.

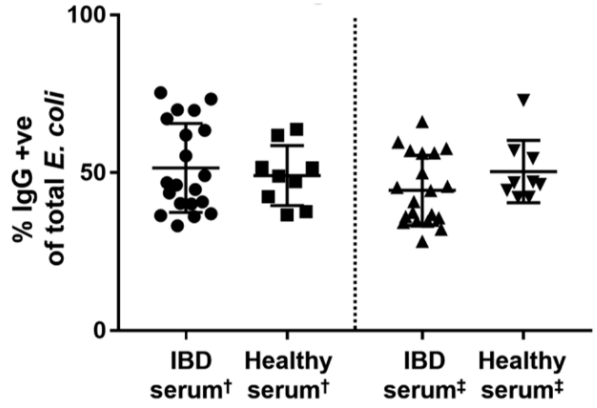

Figure 2.5 Serum IgG recognition of *E. coli* isolated from healthy dogs and dogs with IBD. Six separate fecal isolates of *E. coli* (3 from dogs with IBD and 3 from healthy dogs) were incubated with serum from dogs with IBD (n=20) and healthy dogs (n = 9), and IgG binding to the surface of bacteria was quantitated using flow cytometry, as noted in Methods. Scatter plots depicting IgG^+ bacteria percentages in healthy versus IBD dogs plotted. The percentages of IgG^+ bacteria were not significantly different between the two groups of animal sera (p = 0.29). (†) Indicates *E. coli* isolates from normal dogs, while (‡) indicates *E. coli* isolates from dogs with IBD. Data reported as Mean ± SD and statistical differences were calculated using one-way ANOVA.

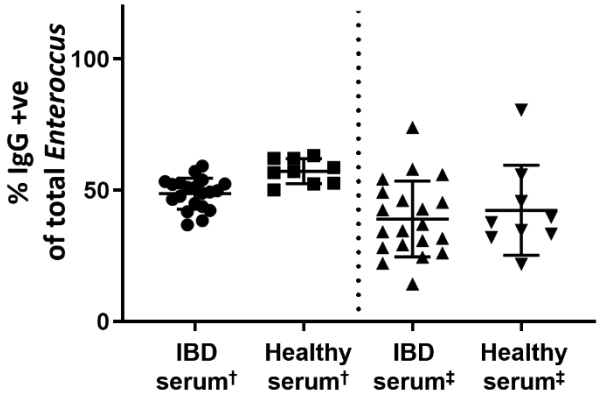

Figure 2.6 Serum IgG recognition of *Enterococcus* isolated from healthy dogs and dogs with IBD. Two separate fecal isolates of *Enterococcus spp.* (from dog with IBD and healthy dog) were incubated with serum from dogs with IBD (n=20) and healthy dogs (n = 9), and IgG binding to the surface of bacteria was quantitated using flow cytometry, as noted in Methods. Scatter plots depicting IgG^+ bacteria percentages in healthy versus IBD dogs plotted. The percentages of IgG^+ bacteria were not significantly different between the two groups of animal sera. (†) Indicates *Enterococcus* isolated from normal dog, while (‡) indicates an isolate from dog with IBD. Data reported as Mean ± SD and statistical differences were calculated using one-way ANOVA.

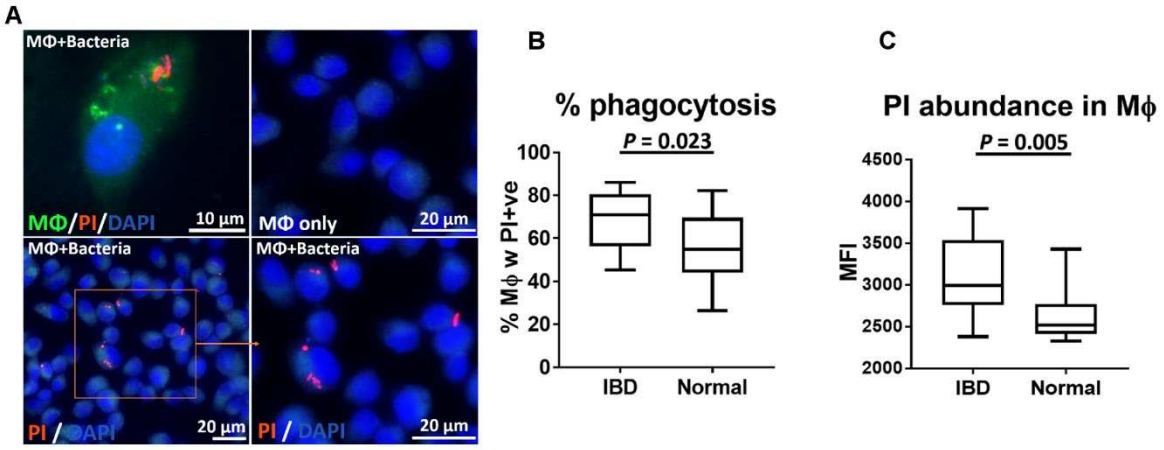

Figure 2.7 Macrophage phagocytosis of fecal bacteria from dogs with IBD versus healthy dogs. (A) Fecal bacteria (PI staining; red) from dogs with IBD and from healthy dogs (n=5 per group) were incubated with primary cultures of canine monocyte-derived macrophages and bacterial uptake was determined using flow cytometry, as described in Methods. Images were obtained using confocal microscopy, with PI stained bacteria visualized as red objects within cultured macrophages. DAPI staining (blue) demonstrates cell nuclei. Similar results were obtained in at least n=3 repeated, independent studies. Box plot comparing the percentage of macrophages containing intracellular bacteria (B) and the relative number of bacteria per macrophage (C), when bacteria from dogs with IBD and healthy control dogs were compared. Statistical differences were calculated using two-tailed unpaired t-tests (B) and by the Mann-Whitney test (C). Scale bar as indicated.

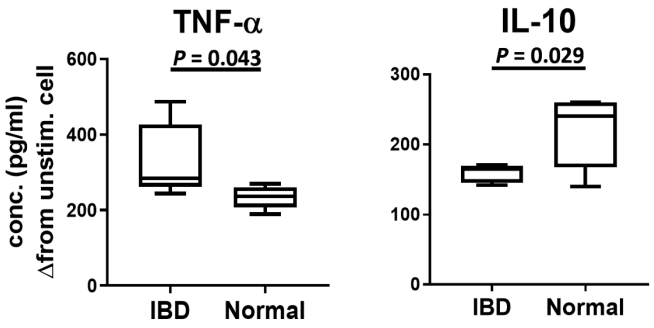

Figure 2.8 Cytokine production by activated macrophages. Canine monocyte-derived macrophages were activated by incubation and phagocytosis of non-viable fecal bacteria obtained from dogs with IBD (n=5) and from healthy normal dogs (n=5), as described in Methods. TNF-a and IL-10 concentrations in media obtained from macrophage cultures 24 hours after bacterial inoculation were measured using commercial canine-specific ELISA. Box plots comparing cytokine concentrations between the 2 groups of fecal bacterial samples are depicted. Statistical differences were calculated using two-tailed unpaired t-tests. The assays were repeated for 3 times, total of 3 different PBMC donors.

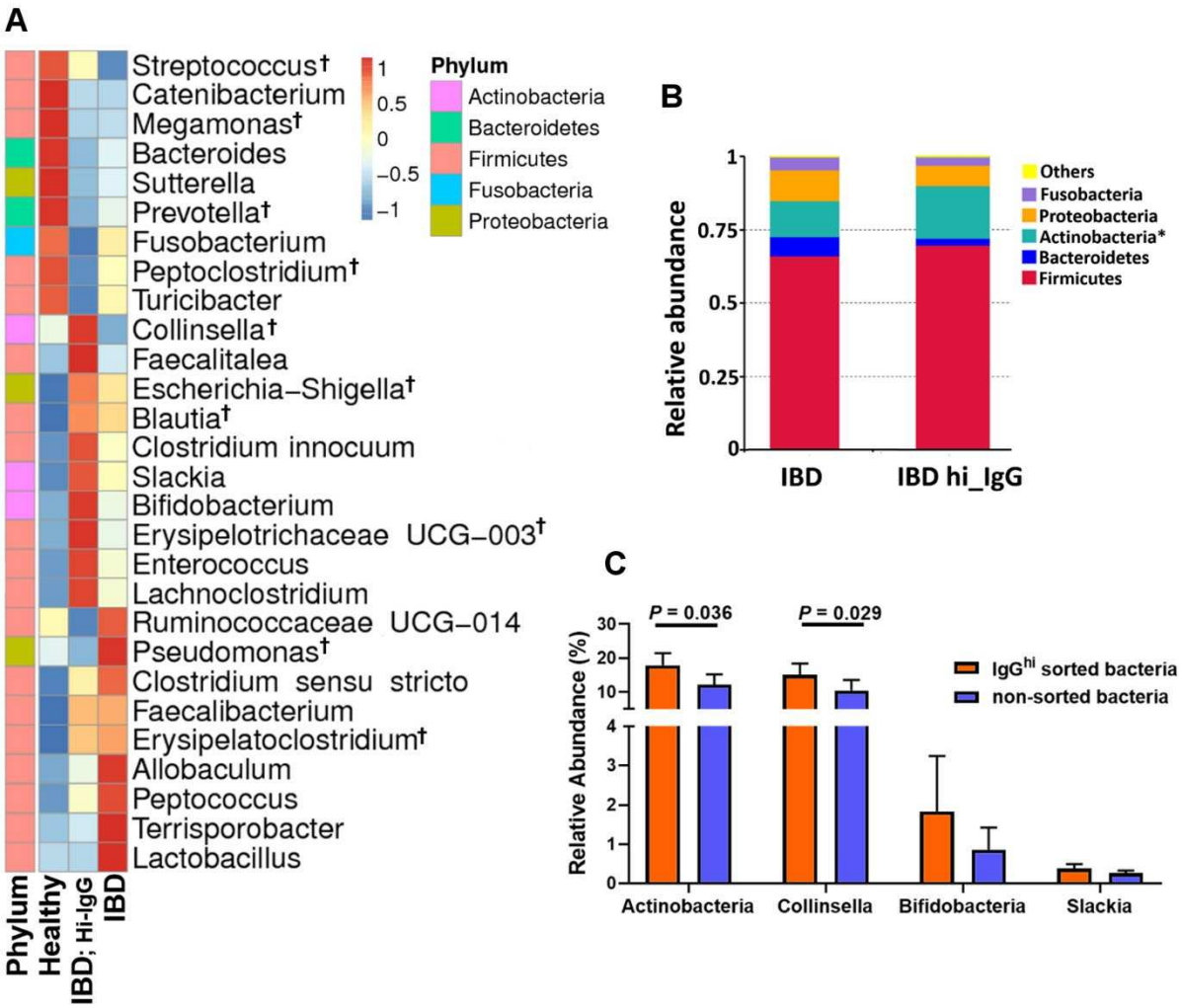

Figure 2.9 Microbiome analysis. IgG^{hi} sorted fecal bacteria from (n=10) dogs with IBD, and non-sorted bacteria (n=10; paired fecal samples from dogs with IBD) and bacteria from healthy control animals (n=10) were analyzed by 16S rRNA sequencing, as described in Methods. (A) Species abundance heat map at taxonomic level representing average differences, with 0 = no difference, -1 and 1 representing maximum differences. (†) Showing the top 10 taxa abundance. (B) Bar graph depicting the relative abundance of 5 major phyla comparing the IgG^{hi} sorted population with non-sorted bacteria, obtained from same dogs with IBD. A significant increased abundance of *Actinobacteria* phyla was found in IgG^{hi} sorted population. (C) Bar graph showed relative abundance comparing between IgG^{hi} sorted and non-sorted bacteria for members of *Actinobacteria* phyla. The data were reported as Mean ± SD, and statistical comparisons were calculated using paired t-test (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$)

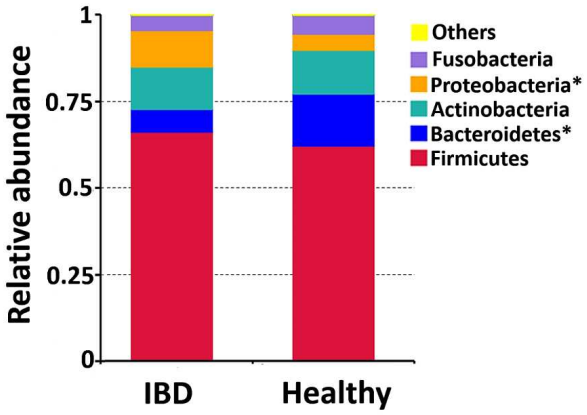

Figure 2.10 Relative abundance of 5 major phyla in dogs with IBD and healthy controls. Significant decrease in *Bacteroidetes* ($p = 0.02$) and increased *Proteobacteria* ($p = 0.045$) were observed in dogs with IBD. Bar graphs depict relative abundance of 5 phyla, and statistical differences calculated using unpaired t-test (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$).

Table 2.4; IgG^{hi}-sorted bacteria abundance.

Taxa	IBD		*p value*
	IgG^{hi} bacteria (n=10)	**Non-sorted bacteria (n=10)**	
Actinobacteria	17.95 ± 11.08	12.23 ± 9.363	**0.036**
Bifidobacteria	0.3 (14.4)	0.25 (5.8)	0.094
Slackia	0.38 ± 0.36	0.26 ± 0.21	0.058
Collinsella	15.1 ± 10.66	10.5 ± 9.75	**0.029**
Bacteroidetes	2.38 ± 1.82	6.64 ± 4.07	0.071
Bacteroides	1.08 ± 0.77	2.43 ± 2.53	0.572
Prevotella	0.34 (2.74)	1.19 (7.56)	0.131
Firmicutes	69.64 ± 10.7	65.99 ± 12.85	0.156
Lachnoclostridium	0.3 (0.3)	0.2 (0.2)	0.176
Megamonas	0.05 (0.78)	0.06 (1.05)	0.77
Faecalitalia	0.3 (0.6)	0.2 (0.4)	0.093
Catenibacterium	0.6 (3.8)	0.75 (7.8)	0.477
Clostridium sensu stricto	0.65 (2.8)	0.7 (6.5)	0.3
Blautia	10.93 (27.03)	9.65 (21.52)	0.492
Enterococcus	1.4 (12.2)	0.8 (4.8)	0.089
Streptococcus	1.17 (19.69)	0.75 (13.74)	0.275
Clostridium innoculum	0.15 (0.8)	0.1 (0.5)	0.140
Lactobacillus	0.9 (4.7)	1.35 (15.1)	0.348
Erysipelotrichaceae	0.14 (18.8)	0.45 (8.43)	**0.002**
Turicibacter	0 (0.1)	0.1 (0.4)	**0.008**
Peptoclostridium	8.0 ± 4.19	10.26 ± 6.78	0.313
Erysipelatoclostridium	1.96 (20.78)	1.63 (26.47)	0.921
Faecalibacterium	0.8 (31.91)	0.7 (31.87)	0.766
Fusobacteria	2.77 ± 2.33	4.41 ± 3.03	0.215
Fusobacterium	2.68 ± 2.31	4.25± 2.94	0.223
Proteobacteria	7.04 ± 6.81	10.61 ± 9.9	0.267
Escherichia-Shigella	2.67 (21.75)	1.57 (13.76)	0.557

Taxa	IBD		*p value*
	IgG^{hi} bacteria (n=10)	**Non-sorted bacteria (n=10)**	
Sutterella	0.05 (0.3)	0.1 (0.2)	0.766
Pseudomonas	0.05 (3.9)	1.18 (31.61)	0.084

Table reported the comparison of % relative abundance between IgG^{hi}-sorted and non-sorted bacteria from IBD group. Data shown in Mean ± SD (if parametric data) and Median (range) (if non-parametric data). The appropriate statistical comparison of 2 groups either paired t-test and Mann-Whitney test was performed corresponding the type of data. *p* value of 0.05 was set.

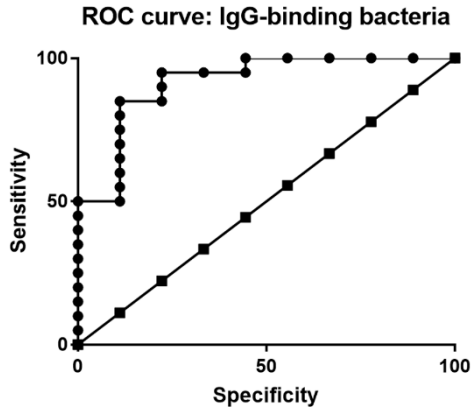

Figure 2.11 Receiver operator curves for bacterial IgG assay. To quantify the diagnostic ability of the bacterial IgG assay to discriminate dogs with IBD (n=20) from normal dogs (n=9) based on percentage IgG-binding gut bacteria, ROC curve analysis was performed. Area under the curve (AUC) was reported as 0.92, SD 0.06, $p < 0.0001$.

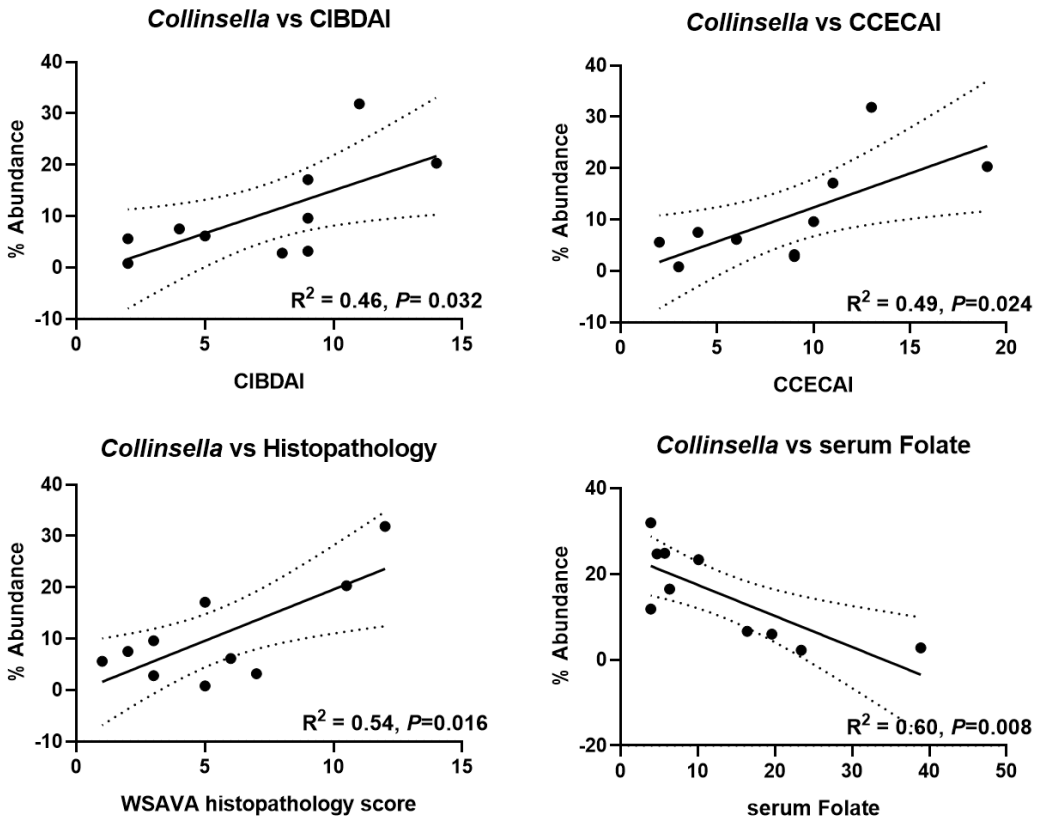

Figure 2.12 Association of *Collinsella* and clinical parameters in IBD. Scatter dot plot of % abundance of *Collinsella* and clinical parameters depicted. Linear regression analysis was performed. The *p* value as stated in the figures. Dashed lines depict 95% confidence band. CIBDAI; Canine Inflammatory Bowel Disease Activity Index, CCECAI; Canine Chronic Enteropathy Clinical Activity Index.

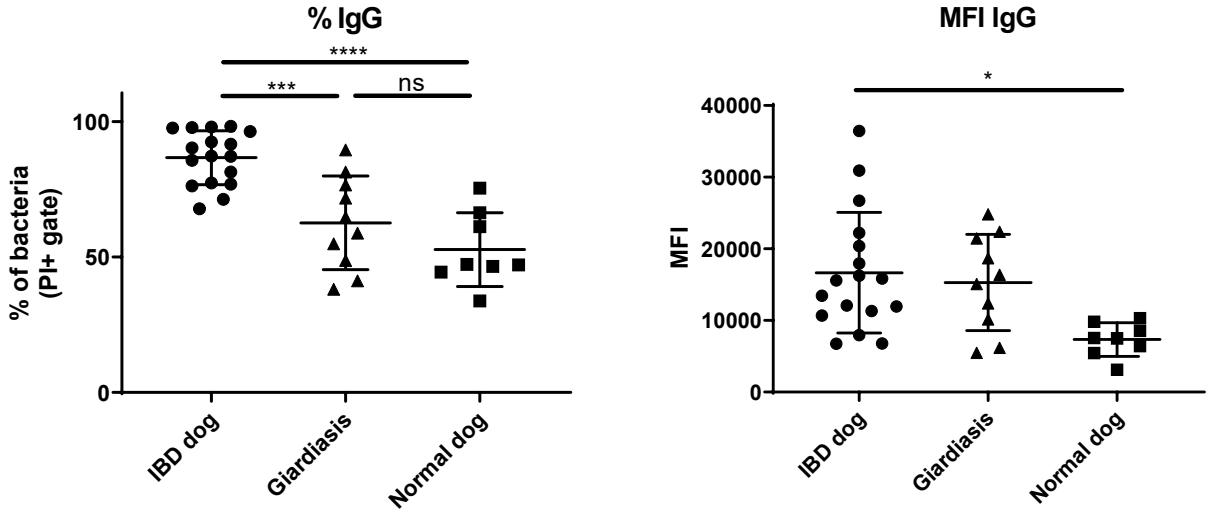

Figure 2.13 IgG$^+$ fecal bacteria in Giardia+ve fecal samples. (A) The percentages of IgG+ bacteria are plotted in dogs with IBD, Giardiasis and healthy dogs. (B) The amount of IgG bound to each bacterium (MFI) is plotted in groups respectively. Data are plotted as Mean ± SD. Statistical differences were calculated using One-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$).

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CHAPTER 3: MESENCHYMAL STEM CELLS (MSC) DERIVED FROM INDUCED PLURIPOTENT STEM CELLS (IPSC) EQUIVALENT TO ADIPOSE-DERIVED MSC IN PROMOTING INTESTINAL HEALING AND MICROBIOME NORMALIZATION IN MOUSE INFLAMMATORY BOWEL DISEASE MODEL[1]

3.1 Overview

Background: The typical MSC therapy that exerts an immunomodulatory effect for suppressing gut inflammation has been evaluated in rodent models of IBD, as well as in clinical trials in humans and animals. Recently, iPSC-derived MSCs (iMSCs) have been suggested to be equivalent to conventional MSCs for the treatment of several inflammatory diseases. However, they have not been studied in IBD.

Objective: The aim was to investigate the effectiveness of using iMSCs and conventional adipose-derived MSCs (adMSCs) for the treatment of IBD in a mouse model of DSS-induced colitis. The comprehensive investigations included the effect of MSCs on inflammatory suppression, intestinal healing and microbiome alteration. We hypothesized that iMSCs and conventional cells are equally effective in ameliorating inflammation and supporting gut healing by tissue regeneration and microbiome restoration.

Methods: Colitis was induced in mice via treatment with a chemical compound, and the mice received intravenous administration of iMSCs or adMSCs for 3 injections with a 2-day interval.

[1] Soontararak S, Chow L, Johnson V, et al. Mesenchymal Stem Cells (MSC) Derived from Induced Pluripotent Stem Cells (iPSC) Equivalent to Adipose-Derived MSC in Promoting Intestinal Healing and Microbiome Normalization in Mouse Inflammatory Bowel Disease Model. *Stem Cells Transl Med.* Apr 10 2018.

The effect of the treatment was assessed using the clinical score, histopathology, immunohistochemistry, and microbiome study, and the stem cell treatments were compared to the group of colitis with no treatment.

Results: The iMSC and adMSC treatment effects were equivalent on the basis of significantly improving clinical abnormalities and decreasing inflammation inside the gut. iMSC administration also stimulated a substantial increase in intestinal epithelial cell proliferation, which was augmented in the Lgr5+ intestinal stem cells and amplified intestinal angiogenesis. Furthermore, the restored microbiome found in mice treated with adMSCs or iMSCs closely resembled those of healthy mice.

Conclusion: The administration of iMSCs enhanced the overall intestinal healing and suppressed inflammation with equal effectiveness as treatment using adMSCs in a mouse model of colitis. This study is also the first report of iMSC effectiveness in IBD treatment and the first description of a unique mechanism of the action concerning microbiome restoration and intestinal healing.

This chapter includes the complete published manuscript[1] for this aim. My contribution to this publication included study design, performing assay and mouse model study, data collection, data analysis, manuscript preparation and submission.

3.2 Introduction

Inflammatory bowel disease (IBD) in humans, including Crohn's disease (CD) and

[1] Soontararak S, Chow L, Johnson V, et al. Mesenchymal Stem Cells (MSC) Derived from Induced Pluripotent Stem Cells (iPSC) Equivalent to Adipose-Derived MSC in Promoting Intestinal Healing and Microbiome Normalization in Mouse Inflammatory Bowel Disease Model. *Stem Cells Transl Med.* Apr 10 2018.

ulcerative colitis (UC), are the results of intestinal inflammation due to an abnormal immune response in the gut[1-4]. These diseases exhibit the following characteristics: inflammatory cell infiltration (activated T cells, neutrophils, monocytes, and macrophages), variable degrees of intestinal injury and occasionally ulceration[5]. Although there has been substantial progress in treating IBD using novel immunomodulatory medicines, there is a necessity for a new approach to the management and treatment of IBD, particularly treatments that have a long action duration and a small number of adverse effects.

Cell-based therapy with mesenchymal stem cells (MSC) that utilizes their immunomodulatory effects has been a new promising therapeutic strategy for managing IBD. First, mesenchymal stem cells are strong immune regulators by suppressing several different inflammatory processes[6-10]. Significantly, it has been shown in a number of current studies in IBD rodent models that systemic MSC administration via i.p. or i.v. routes resulted in considerably improved intestinal inflammation[11-14]. Furthermore, these beneficial effects of systemic MSC application have been noted in individuals suffering from CD and UC treated with cellular therapy, as well as in a canine spontaneous IBD model[15-21]. Thus, there is robust evidence that MSC therapy is likely to be a practical option, even in individuals suffering from drug-refractory IBD.

Although there is convincing evidence of the MSC therapy effectiveness in the management of IBD, our understanding of the mechanisms through which MSC regulates the immunological response and improve clinical abnormalities in the disease remains inadequate. For example, rodent models of IBD (principally the dextran sodium sulfate (DSS)-induced colitis model) have proven that the administration of MSCs can lessen neutrophil, T cell, and macrophage infiltrates and promote the regulatory T gut influx in the gut[13, 22-24]. There are contradictory data on the exact quantity to which MSCs essentially infiltrate to the gut after intravenous delivery,

although the consensus appears to be that general recruitment to the local area in the gut is fairly incompetent[13, 25, 26]. Currently, in IBD, most MSC therapy studies have concentrated on the immunomodulatory effect of MSCs. However, the findings from radiation-induced GI injury models suggest that MSCs may also have a trophic and stimulatory effect on the gut epithelium itself, such as the stimulation of intestinal stem cell proliferation[27-29].

Nevertheless, despite the advantageous MSC therapies, challenges persist in the cellular therapy application as a practical option for IBD management. For example, the MSC source (whether autologous or allogeneic) is the primary variable that affects the therapeutic effectiveness[30-32]. Convincing data indicate that allogeneic MSCs are fully practical in IBD models, and there is significant evidence indicating that MSCs derived from young-aged donors are more immunologically active[32-34]. However, with the use of allogeneic donor MSCs, there is considerable donor-to-donor variability in MSC yields and functionality[35, 36]. Therefore, the regular use of MSC therapy with consistent effectiveness would be derived from a uniform cell source with a stable function and phenotype.

One potential way to solve this problem of a uniform cell origin is to use MSCs derived from induced pluripotent stem cells (iPSCs). Many studies have demonstrated that MSCs derived from iPSCs are similar to the conventional MSCs derived from adipose-tissue or bone marrow regarding both phenotype and function[37-40]. The iPSC-derived MSCs have shown their ability to suppress inflammation in vivo in mouse models with an allergic airway disease[38, 40, 41].

Therefore, in the current study, we examined the iPSC-derived MSC (iMSC) effectiveness for IBD treatment, using a DSS-induced colitis mouse model. Studies were carried out to thoroughly examine the ways by which MSCs may ameliorate the intestinal abnormalities in IBD, including the effects on reduced inflammation, enhanced intestinal healing and restored

microbiome populations. The studies demonstrated that iMSCs were comparably effective to conventional, adipose-derived MSC for the treatment of established and ongoing colitis in mice. Furthermore, these studies also indicated significant indirect trophic effects of MSCs on angiogenesis, intestinal healing, and intestinal microbiome normalization. Overall, the studies provide strong support for the use of iPSC-derived MSCs as a more homogenous and sustainable source of cells for the cellular therapy of IBD.

3.3 Materials and Methods

3.3.1 MSC isolation, culture and tri-lineage differentiation

The MSCs used for this research were isolated and obtained from inguinal and abdominal adipose tissues of 10 week old female CD-1 mice under sterile procedures. Approximately 0.5 grams of fat tissue was cleaned with sterile DPBS (Dulbecco's phosphate-buffered saline; Sigma Aldrich, St. Louis, MO), mechanically chopped using a scalpel and digested by collagenase (Sigma Aldrich; 1 mg/ml) for a period of 30 minutes at a temperature of 37°C. The stromal vascular fraction (SVF) was isolated by centrifugation at 380 g for a duration of 5 minutes. After washing twice with complete culture medium, the SVF was transferred into 75 cm^2 tissue culture flasks (Falcon®, Thermo Fisher, Waltham, MA) and incubated at 37°C, 5% CO_2. After 72 hours, the tissue culture flasks were washed to remove non-adherent cells and were refed with fresh complete medium. Proliferating colonies of adherent cells were allowed to grow to reach 70% confluency, with medium changes every 48 hours. The cells were subsequently detached from flasks for passage by treatment with trypsin (0.25% trypsin; EDTA, Gibco, Carlsbad, CA) and placed in 225

cm^2 flasks for further growth. For the studies described here, adipose-derived MSC (adMSC) were used at passage 3 to 4 and collected when they were approximately 80% confluent.

The iMSC line used in these studies was generated from a CD-1 mouse by the Colorado Denver Charles C. Gates University, Center for Regenerative Medicine and Stem Cell Biology iPSC Core. Transgene integration-free iPS cells were produced from mouse skin fibroblasts via the use of a CytoTune iPS Reprograming kit (Life Technologies Corp., Grand Island, NY) based on the manufacturer's instructions. The capability of adipose-derived iMSCs and MSCs to undergo tri-lineage differentiation was evaluated using a StemPro® differentiation kit (Gibco). The differentiated cell phenotype was assessed by specific cytostaining (adipogenesis: Oil Red O; chondrogenesis: Alcian Blue; and osteogenesis: Alizarin Red).

3.3.2 Flow cytometry

The phenotypic evaluation of adMSCs and iMSCs was performed as previously described. Briefly, the single cell suspensions were prepared at $1x10^6$ cells/ml in FACS buffer, and 1 X 10^5 cells were immunostained in single wells of round bottom 96-well plates. The following antibodies (obtained from San Diego, CA) were used to immunostain the cells: Sca-1-APC (clone eBR2a), CD11b- FITC (clone M1/70), CD29-biotin (clone HMb1-1), CD31-FITC (clone 390), CD44-FITC (clone IM7), CD45-PE (clone 30-F11), CD73-PE (clone eBioTY/11.8), CD90.2-eFlour 450 (clone 53-2.1), and CD106-biotin (clone 429).

For the evaluation of leukocyte populations, the following primary antibodies were used: CD4-FITC (clone GK1.5), CD8-APC-e780 (clone 53-6.7), CD11c- FITC (clone N418), CD45-eFlour450 (clone 30-F11), FOXP3-PE (clone JFK-16s), B220-APC (RA3-682), Ly6G-APC-e780 (clone RB6-8C5), Ly6C-PE (clone HK1.4), and F4/80-APC (clone BM8). Furthermore, after

surface staining was performed, cells were processed for fixation and permeabilization for intracellular staining of anti-FOXP3 antibody. Cells were examined using a Beckman Coulter Gallios flow cytometer (Brea, CA), and data were analyzed using FlowJo Software (Ashland, OR). An example of the gating scheme is shown in Figure 3.1.

3.3.3 DSS-induced colitis model and clinical scoring of disease severity

Mice were induced to have intestinal inflammation via DSS (DSS; Mr ~40,000, Sigma-Aldrich, St. Louis, MO) administered at a 2.5% w/v concentration in the drinking water, as previously described[42]. The amount of drinking water consumed was monitored, and fresh DSS was regularly prepared and changed every 2 days. DSS was administered in the water throughout the study period.

For every study, mice (n = 5 per group) were randomly allocated to the following groups: 1) untreated control group; 2) DSS + adMSC administration; 3) DSS treatment only; and 4) DSS + iMSC administration. The mice were observed on a daily basis for clinical signs, body weight, stool color, and stool consistency. On days 10, 13 and 16 of the study (with DSS administration initiated on day 0), the administration of MSCs was performed via tail vein injection of $1x10^6$ cells per mouse in 200 µl PBS. The control mice (group 1) and DSS only mice (group 3) were administered 200 µl of PBS via tail vein injection.

Clinical scoring was performed with the use of a modified scoring matrix from previous publications[13, 43]. Concisely, the mice were weighed, and stool was collected daily; the mice were also observed daily for clinical signs. The % weight loss was calculated on a daily basis and scored as 0 (no loss), -1 (1-5%), -2 (5-10%), -3 (10-20%) and -4 for greater than 20%. The measure of the fecal occult blood was performed by a test kit (Fisher Healthcare, Houston, TX). A

modification of the color reference guide was employed to quantitate the positive occult blood level in a range of 0 (no blood), -1, -2, -3 and -4 with the darkest blue showing the strongest test kit positive reference color. The fecal consistency score was noted as follows; 0 (normal), -1 (moist), -2 (loose stool), and -3 (watery). The final clinical score was the totality of the scoring from the fecal color, weight, and fecal consistency, and the maximum possible score was -12.

3.3.4 Sample collection for histology and immunofluorescent staining

At 19 days after DSS (3 days after the last MSC injection), the mice were euthanized, and the colon was collected, weighed and measured for the length. A colonic tissue section of 1 cm-long was rapidly frozen at -80°C. One half of the remaining colon was fixed in 10% neutral buffer formalin for processing histology with H&E staining, and the other half was fixed in 1% paraformaldehyde-lysine-periodate (PLP) solution (1% paraformaldehyde in 0.2 M lysine-HCl, 0.1 M anhydrous dibasic sodium phosphate, with 0.21% sodium periodate) for 24 hours at 4°C for immunohistochemical staining. After PLP fixation, the tissue was transferred in a 30% w/v sucrose solution for an additional 24 hours at 4°C, prior to embedding and freezing in O.C.T. compound (Tissue Tek®, Tokyo).

3.3.5 Histopathology

Formalin-fixed paraffin embedded colon tissues were stained routinely with H&E staining for histological assessment. The evaluation of the colonic tissue histopathological scoring was performed in a blinded fashion by a board-certified veterinary pathologist. A previously published scoring system[44] for colonic inflammation was modified to include a category for mucosal inflammation (score 1-6; 2 categories) and damage score (score 1-3) as follows: 1 (leukocyte

infiltration), 2 (mucosal erosion/ulceration +/- colonic gland ectasia and necrosis), and 3 (collapse of mucosal architecture +/- replacement granulation tissue). The final score was the sum of all scoring indexes, with a maximum possible score of 9.

3.3.6 Immunofluorescence staining

For immunofluorescence staining, OCT-embedded frozen tissues were sliced at a 5 μm thickness and mounted on Superfrost slides (Fisher Scientific, Hampton, NH). The following primary antibodies were used: CD3 (clone 145-2C11), F4/80 (clone BM8), CD11b (clone M1/70), CD103 (clone 2E7), CD4 (clone 4SM95; all from eBiocience), CD31 (clone MEC 13.3; BD Pharmingen), cytokeratin-20 (Abcam, Cambridge, MA), FOXP3 (clone JFK-16s), Lgr5 (Abcam), and Ki-67 (clone SolA15; eBioscience); 0.1% Triton X and 0.25% saponin were employed as the permeabilizing agents, with overnight incubation at 4°C. The secondary antibodies were conjugated with Alexa Flour®488 or Cy3 (dilution 1:200 in PBST, Jackson ImmunoResearch Laboratories). The tissue sections were washed twice and counterstained with DAPI (Molecular Probe, Eugene, OR). The slices were subsequently mounted with Prolong® Diamond Antifade medium (Thermo Fisher, Waltham, MA) and placed with a coverslip. Appropriate isotype controls were used for all studies as negative controls. The tissue slides were visualized using an Olympus IX83 confocal microscope and Hamamatsu digital camera.

The slides were examined via confocal microscopy in a total of 10 fields of colonic mucosa starting with a random sample area by the use of DAPI channel; images were subsequently acquired. The positive fluorescent cells expressing CD11b, FOXP3, CD3, F4/80, CD4+ T cells, Ki-67, CD103, CD31 or Lgr5 were enumerated per mm^2 of tissue. For calculation of the microvessel density, the percentage of the CD31 area was divided by the total pixels of the mucosal

area of each image tile, using ImageJ software[45] (Figure 3.2). In this study, MSCs were labeled with fluorescent DiD® or DiR® dye (Invitrogen, Eugene, OR) for tracking, and mice were imaged using an IVIS instrument (PerkinElmer®, Waltham, MA).

3.3.7 Microbiome 16S rRNA sequencing

DNA of the fecal bacteria from all mice was extracted using a Mobio PowerSoil DNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted DNA was submitted to Novogene Corporation (Chula Vista, CA) for 16S rRNA sequencing. Data analysis was also performed by Novogene.

3.3.8 Statistical analysis

In this study, data were analyzed using Prism 7 (GraphPad, San Diego, CA), and the results are shown as the mean ± SD (unless otherwise stated), with the significance set at $p < .05$. The normality of the data was examined using the Shapiro-Wilk normality test. Significant differences among the groups were evaluated using one-way ANOVA with multiple comparisons using Tukey's adjustment or as otherwise stated.

3.4 Results

3.4.1 iMSC and adMSC phenotypic characterization

An evaluation for iMSCs and adMSCs was performed following the mesenchymal stem cell characteristics and identification criteria, as established by the International Society for Cellular Therapy [46]. The flow cytometry analysis indicated that MSC highly expressed the

following markers: CD29, Sca-1, CD44, CD106, and CD73. No leukocyte marker expression was identified, including CD31, CD45, and CD11b (Figure 3.3A). It was noted that while adMSCs had high expression of CD90, the iMSCs were negative for CD90. In addition, the two cell types were positive for CD106 expression, which was demonstrated solely on MSC and not on skin fibroblasts. Tri-lineage differentiation also indicated that both cell types were capable of differentiating into adipocytes, chondrocytes, and osteoblasts (Figure 3.3B).

3.4.2 Effects of MSC administration on clinical signs and colonic lesions in mice with DSS-induced colitis

It was noted that the mice treated with DSS in the drinking water for approximately 7-10 days showed clinical signs that corresponded with progressive colitis, including sustained bloody diarrhea, weight loss, and abnormal fecal consistency, which resulted in reduced clinical scores over time (Figure 3.4). After the signs of colitis developed in all mice, MSCs were administered on days 10, 13 and 16 at a dosage of $1x10^6$ MSCs per mouse per intravenous injection. The mice that were treated with adMSCs or iMSCs were noted to have significantly reduced clinical scores compared to the mice that were treated with DSS without stem cells (adMSC treated mice, p = 0.001 and iMSC-treated mice, p = 0.003) (Figure 3.4A). Within the first day of the first MSC injection, an improvement in the clinical scores was noted and was maintained during the MSC injection period, while the clinical scores continued to become worse in the animals that were in the DSS group without stem cell treatment.

An evaluation of the gross lesion scores and histopathology of the distal colon was performed from the tissues collected in the mice euthanized at 72 hours after the third injection of stem cells (day 19 of DSS treatment). For the mice that were treated with only DSS, the colon was

noted to have shortened and was hyperemic (data not shown). Histologically, colonic tissues from the DSS-treated mice exhibited severe infiltration of inflammatory cells, necrosis, extensive mucosal erosion to ulceration, variable degrees of colonic gland ectasia and occasional complete loss and collapse of the mucosal architecture (Figure 3.4B), consistent with reports from previous studies[42, 44, 47]. In contrast to no stem cell treatment, the colonic tissues from the mice treated with iMSCs or adMSCs showed an overall improvement in the transmural inflammation, with significantly reduced infiltration of inflammatory cells in the lamina propria, decreased mucosal collapse and diminished mucosal ulceration and granulation tissue formation. The overall inflammatory scores assessed by H&E histopathology were considerably improved in the mice treated with adMSCs or iMSCs (adMSC, $p = 0.003$ and iMSC, $p = 0.002$; Figure 3.4C) compared to the DSS only treated group. Thus, these outcomes showed that iMSCs were equivalent to adMSCs in their potency to ameliorate the intestinal inflammation signs induced by DSS treatment in this mouse model of IBD.

3.4.3 Trafficking of MSCs to intestinal and extraintestinal tissues

To demonstrate the homing of MSCs to places of colonic inflammation, MSCs were labeled using DiR® dye immediately before injection. Cell migration was monitored using IVIS live animal imaging. These investigations showed that labeled MSCs were primarily distributed to the lungs at the beginning and were subsequently found in the liver and spleen (Figure 3.5A). However, labeled MSCs could not be detected by IVIS imaging of intestinal tissues, which suggests that few cells migrated to the sites.

To increase the sensitivity of cell detection, mice were injected with DiD®-labeled MSCs (Figure 3.5B) and were then processed for immunohistochemical examination of the tissues from

injected mice (Figure 3.5C-G). These studies showed that labeled MSCs could rarely be identified in the colonic mucosa and submucosa (Figure 3.5D, E), Peyer's patches (Figure 3.5F, G) and mesenteric lymph nodes (Figure 3.5C) of the treated mice. Labeled MSCs were abundant in the spleen (Figure 3.5C) and lung tissues (data not shown). The relative paucity of MSC in colonic tissues following intravenous injection in the DSS model is correlated with some previous studies[13, 22, 48, 49]; however, it differs from reports in other published work in which high numbers of labeled MSCs were identified in intestinal tissues[23, 50, 51]. The very low numbers of MSCs in colonic tissues found in our study imply that the therapeutic benefits of injected MSCs were more likely to have been involved by paracrine secreted factors than by direct cell-to-cell effects between MSCs and colonic epithelial cells.

3.4.4 Effects of MSCs administration on epithelial regeneration

The histologic findings of colonic tissues from MSC-treated mice exhibited a noticeable recovery of the intestinal mucosal integrity (Figure 3.4B). These findings showed that the MSC treatment might have a direct effect on colonic epithelial regeneration. The stimulatory effect on epithelial regeneration could be mediated by several trophic factors, including promoted neovascularization, increased intestinal stem cell numbers, and enhanced epithelial proliferation and differentiation[52, 53]. In this study, we examined the immunohistochemistry of colonic tissue stained by markers regarding these factors indicating epithelial regeneration in mice treated with iMSCs and adMSCs compared to DSS without stem cell treatment (Figure 3.6).

We observed a significant increase in the numbers of Ki-67+ colonic epithelial cells of the iMSC and adMSC treated mice, compared to the numbers of Ki-67+ epithelial cells in the DSS-only treated and untreated control mice (Figure 3.6A, B). Moreover, the number of Lgr5+ intestinal

stem cells had significantly increased in the colonic mucosa of the animals treated with MSCs, compared to the DSS-only treated or control animals (Figure 3.6C, D). Finally, the mice injected with MSCs showed a significant increase in angiogenesis as determined by the higher numbers of CD31+ endothelial cells, with a high mean vessel density and area compared to the control animals (Figure 3.6E, F).

Overall, the results indicate that systemic administration of iMSCs or adMSCs provides a vital trophic effect on intestinal epithelial cells by promoting proliferation and recruiting intestinal stem cells, as well as supporting intestinal blood supply by increased local angiogenesis. Thus, these results suggest the secretion of various trophic mediators by intravenously delivered MSCs. The stem cell tracking data and the limited number of MSCs identified in colonic tissues imply that the trophic factors were likely to have been produced at the remote area far away from the GI tract.

3.4.5 Administration of MSCs reverses microbiome dysbiosis

Alteration of the intestinal microbiome in a mouse model of DSS-induced colitis was observed in a previous study[54]. In our study, we determine the composition of the gut microbiome of DSS-treated mice using 16S rRNA sequencing and microbiome analysis. We also found considerable alterations in the bacterial populations of other important phyla (Figure 3.7). For example, we observed a dramatic increase in *Proteobacteria* (Figure 3.7A), increased *Bacteroides*, and decreased *Firmicutes* in the mice that were treated with DSS, compared to the untreated control animals (Figure 3.7D). In general, the DSS without stem cell treatment had the least microbial community diversity measured within a sample as indicated in an alpha diversity graph (using the Simpson index) (Figure 3.7B). The shared number of bacteria assigned in the groups was generated

according to the clustering of each treatment shown in the Venn diagram (Figure 3.7E). We determined the DSS-only group had the least bacterial sharing with the healthy mice.

Furthermore, the number of bacteria shared between the groups which were generated according to the clustering of each treatment group. We determined that the DSS-only group had the least bacterial group sharing with the healthy mice.

It is important to note that in the mice treated with iMSCs or adMSCs, after 10 days of stem cell treatment with ongoing DSS supplement, the composition of the microbiome in these animals recovered to a population that almost remarkably resembled that of the microbiome of healthy untreated mice (Figure 3.7C). For example, the group of animals treated with iMSCs had a corresponding taxa distribution relative to the healthy control animals, compared to the DSS only animals or the DSS animals treated with adMSC (Figure 3.7D). These findings indicate that treatment with MSCs provides the optimal microenvironment for colonic flora recovery to normal, although the specific mechanism of the effect remains unknown.

3.4.6 Effects of MSC administration on intestinal inflammation.

We examined the effect of MSC injection on inflammatory responses in the colon and regional lymphoid tissues. In the colonic tissues of the DSS-treated animals, increased infiltrates of F4/80+ and CD11b+ macrophages, CD3+, CD4+ T cells, and CD103+ inflammatory monocytes were identified, compared to the colonic tissues of the healthy untreated animals.

In the mice that received an MSC injection, significantly lower numbers of macrophage and monocyte infiltration in the tissue were observed compared to DSS without MSCs (Figure 3.8A). FOXP3+ regulatory T cells were significantly greater in the colonic tissues of the mice treated with iMSCs or adMSCs, compared to the control animals or DSS-only animals (Figure

3.8A). These findings agree with the findings of previous studies[13, 22]. This suggests that MSC treatment inhibits colonic inflammation, as shown by increased regulatory T cells and decreased macrophage and neutrophil infiltration into the lamina propria. However, MSC treatment in our study did not change the numbers of infiltrating T cells in colonic tissues (Figure 3.8A).

Importantly, iMSCs and adMSCs could be compared in their effects on lowering the severity of colonic inflammation. However, there were no changes in the T cell, B cell or myeloid cell populations in the lymphoid tissue (spleen or mesenteric lymph node tissues) of the animals treated with MSCs, compared to untreated colitis (Figure 3.8B). Thus, the administration of MSCs decreased local colonic inflammation, together with stimulating angiogenesis and epithelial cell proliferation. However, it had a small effect on immune cell populations in extraintestinal tissues.

3.5 Discussion

The use of conventional allogenic BMMSCs or adMSCs to treat IBD has resulted in a promising outcome in preclinical studies in laboratory animal models. It is also advantageous in experimental clinical trials in humans[13, 14, 17, 23, 32, 50, 55, 56]. Although positive outcomes using MSCs have been continuously reported, several important drawbacks appear to be the limitations for conventional stem cells, including the limited proliferative capacity of aging MSCs, donor variability, costs and time expense for the selection and expansion of the cells in primary culture[33, 36, 57]. Thus, other inexhaustible cell sources with the constant function of MSCs are required for cellular therapy. In previous studies, the potential advantages of iPSC-derived MSCs (iMSCs) for cellular therapy have been described[37, 38, 41, 58]. However, a lack of studies focus on the use of

iMSCs in IBD models. Therefore, in this study, we have investigated the potential use of iMSCs as an additional cell source for the treatment of IBD.

We found that iMSC were equal to or in some cases better than conventional adMSCs for the treatment of IBD in terms of improving the clinical signs of colitis and stimulating intestinal healing. For example, the general clinical score in iMSC-treated mice was decreased by 26% at day 19, compared to DSS-only mice (Figure 3.4A), while the reduction of the score was 19% in mice treated with adMSCs. Moreover, the intestinal inflammation was equivalently decreased in mice that received an iMSC injection compared to adMSC-treated mice (Figure 3.4C). Furthermore, iMSC administration showed a remarkable stimulation of intestinal epithelial cell proliferation, increased numbers of Lgr5+ intestinal stem cells, and augmented intestinal angiogenic responses (Figure 3.6).

Previous studies have described the anti-inflammatory properties of MSCs that potentially effect and may be responsible for a part of the overall beneficial effect of MSCs on colitis in this study. However, our study suggests alternative processes by which MSCs may result in an equal or greater effect on ameliorating lesions in the gut associated with IBD. For example, stimulation of epithelial regeneration may be necessary for healing when the inflammation is suppressed. The significantly greater number of Lgr5+ stem cells in the intestinal mucosa found in mice treated with adMSC or iMSC, compared to control DSS mice (Figure 3.6C, D), indicates the regenerative activation of the microenvironment via local stem cell recruitment and/or proliferation. In addition, our result shows the remarkable proliferation of mature epithelial cells in the colonic mucosa corresponding to the stimulatory properties of secretory factors derived from MSCs.

Considering the limited MSC distribution to the colonic mucosa and submucosa (Figure 3.5D, E), the MSC effect may be predominately influenced by secretory trophic factors from

resident MSCs at extraintestinal tissues, including the spleen and the lung. Finally, we also found that angiogenic responses were significantly enhanced locally in colonic tissues in mice treated with MSCs, which is consistent with the release of pro-angiogenic cytokines, such as VEGF, as previously reported[59].

Microbiome alterations are considered a second potential mechanism by which MSCs can stimulate intestinal healing. The gut microbiome plays an important role to locally produce a stimulatory effect on immune responses and intestinal integrity[60]. For example, abnormal healing of the intestine has been shown in mice in which the microbiome has been disrupted, as well as in mice unable to detect the intestinal bacteria due to interrupted TLR signaling[61, 62].

In the present study, we observed that the altered microbiome caused by DSS induction was, to a large degree, reversed by the MSC treatment, including the administration of iMSCs or adMSCs (Figure 3.7). For example, the diversity of the gut microbiome taxa was significantly increased in MSC-treated mice, compared to DSS only treated mice (Figure 3.7B). Therefore, these findings suggest that by generating a more diverse microbiome that resembles healthy control animals, thereby leading to an increased production of trophic substances for the support microenvironment, MSC administration improved the overall intestinal health and healing. However, it is not clear whether the changes of the microbiome were due to an improvement in intestinal healing or microbiome restoration causing the intestinal healing. It is also possible that the two mechanisms were likely to be operating with MSC-induced changes in the colonic epithelium causing alterations in the microbiome and subsequently affecting intestinal angiogenesis and epithelial regeneration.

3.6 Conclusion

In summary, our studies show that iMSCs are equally as effective as adMSCs when systemically administered to ameliorate IBD in a mouse model, and they do not induce detectable adverse effects, even when intravenous administration is repeated. Moreover, the effects of MSCs on IBD are likely to be multifactorial regarding the gut homeostasis restoration, including improved intestinal epithelial regeneration, beneficial microbiome alterations, and suppression of intestinal inflammation. For future application, we have also initiated a study to evaluate the safety and efficacy of iMSC treatment in a clinical trial of a spontaneous IBD model in pet dogs based on the early evidence of MSC efficacy in a similar model[16, 63]. Moreover, we have recently conducted safety studies for systemically administered canine iMSCs in dogs and found no evidence of teratoma or tumor formation. Thus, the use of systemically administered iMSCs as a new cellular therapy is an option for the management of refractory IBD, provided regulatory and potential safety issues can be adequately addressed.

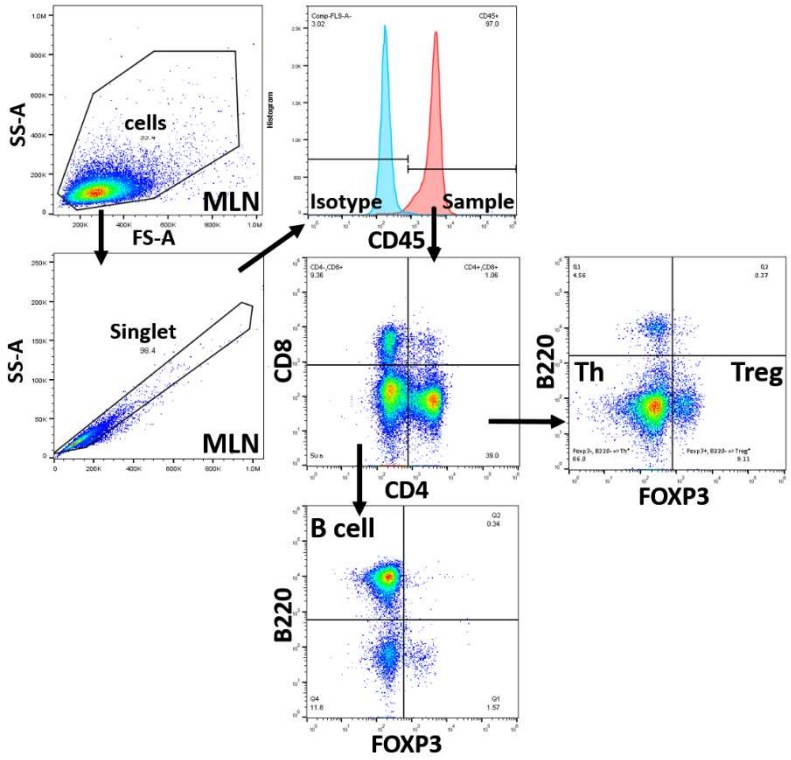

Figure 3.1 Gating strategies for flow cytometry. Gating was done by initially selecting lymphocyte and myeloid cell populations by FS-A and SS-A parameters, then gating for singlets. Next, CD45+ cells were selected for further analysis, which initially included gating for CD4+ and CD8+ events. B cells were selected based on B220 staining, while regulatory T cells were identified from the CD4+ population by intracellular staining for FOXP3. Abbreviations: SS-A, side scatter area; MLN, mesenteric lymph node; FOXP3, foxhead box P3; Treg, regulatory T cell, Th, T helper cell.

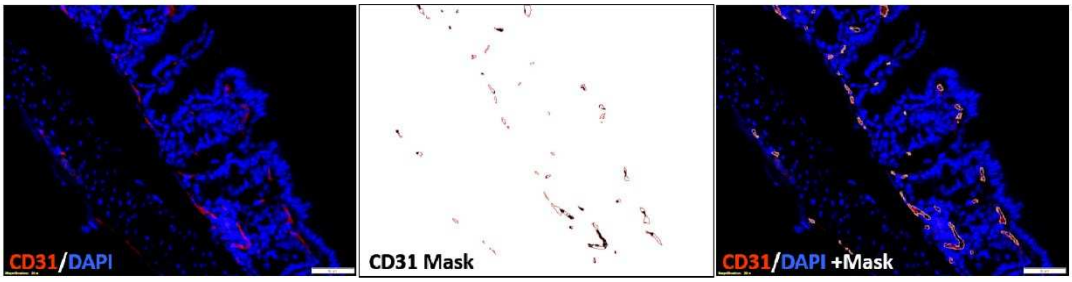

Figure 3.2 Immunofluorescence image analysis by ImageJ software. Quantitative image analysis was done using ImageJ software. A mask layer corresponding to the interested population was created, and the analysis was performed with settings consistently applied through all group comparisons. For example, displayed images represented the area of CD31+ cells which were calculated to generate the microvessel area. Scale bar indicated 50 µm.

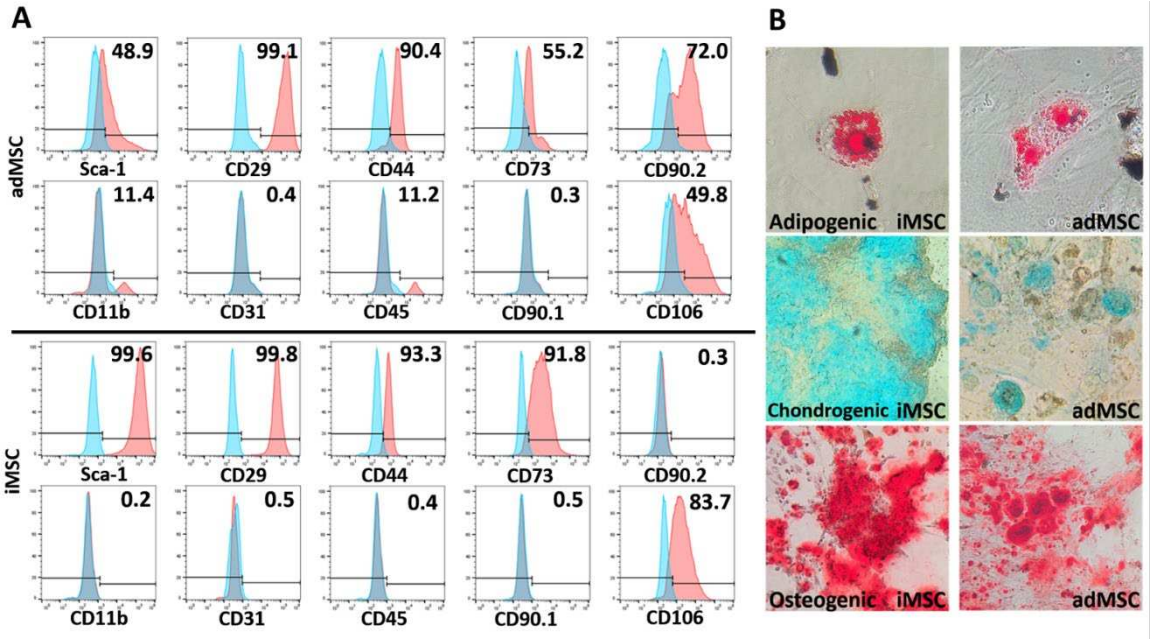

Figure 3.3 Phenotypic characterization of iMSC and adMSC. (A): Expression of cell surface determinants using flowcytometry. Specific antibody staining depicted in red, whereas isotype control staining for each displayed in blue. The percentage of the positive staining for each marker was indicated. (B): Tri-lineage differentiation of passage 3 MSC, as described in Methods. Adipocytes detected using Oil Red O (200X); chondrogenesis by Alcian Blue, and osteogenesis by Alizarin Red staining at day 12 after differentiation. Abbreviations: adMSC, adipose-derived mesenchymal stem cell; iMSC, iPSC-derived mesenchymal stem cells.

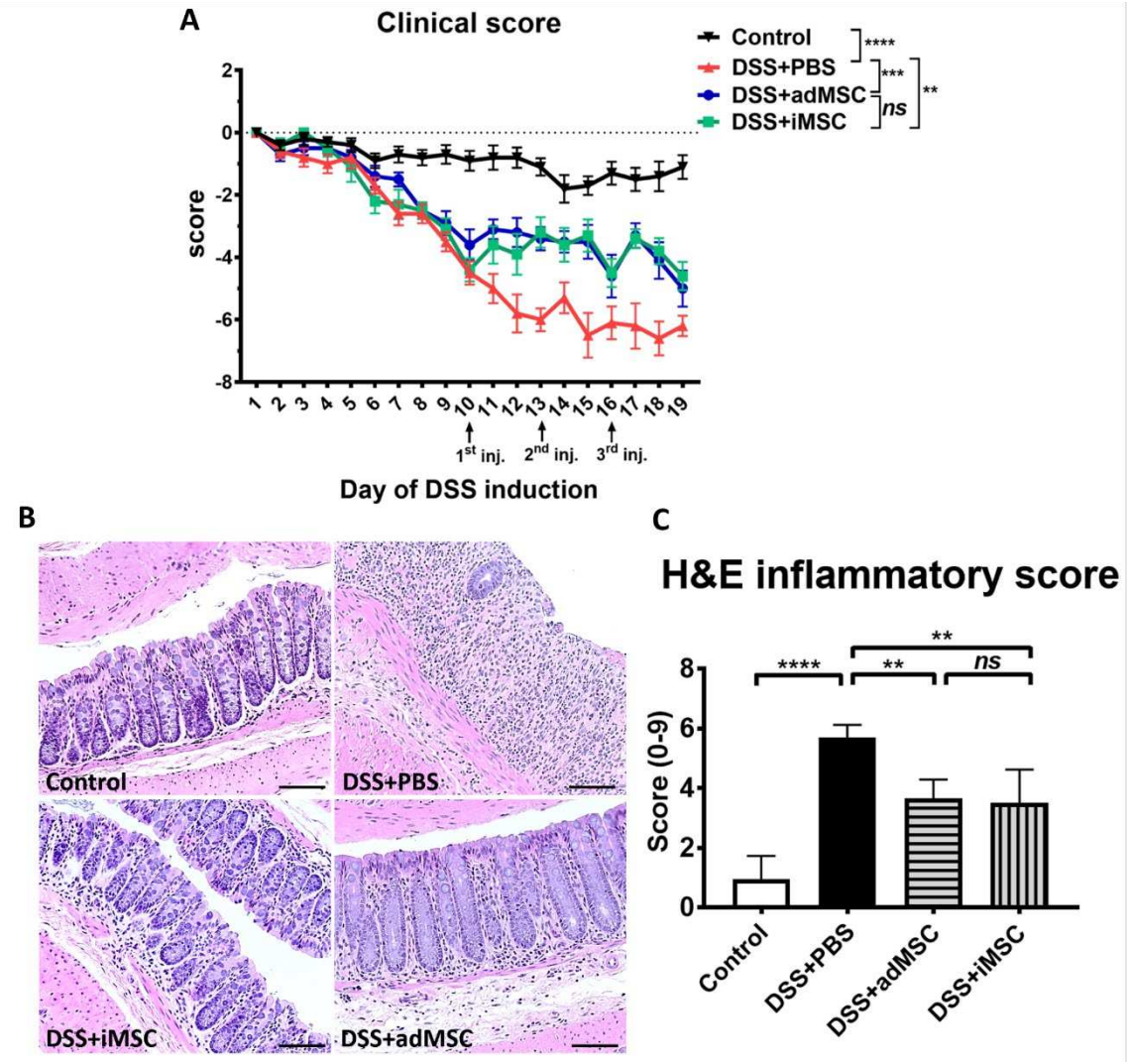

Figure 3.4 Effects of MSC administration on clinical scores and inflammatory score in DSS-induced colitis mice. Mice were monitored for percentage of weight loss, fecal occult blood, and fecal consistency every day during DSS treatment period (19 days) and a total clinical score was calculated. (A): Clinical score over time in 4 groups of mice (n = 5 per group): control, DSS treated + PBS; DSS + iMSC, and DSS + adMSC. MSCs were administered by tail vein on days 10, 13 and 16. (B): Histology of colonic tissue sections from one mouse from each of 4 treatment groups, based on H&E stained sections. (C): Quantitative inflammatory scores assessed by H&E histopathology, as described in Methods. Data was transformed to a normal distribution and the statistical differences were calculated using (A) repeated measures One-way ANOVA with Tukey's adjustment, (B) One-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, **** $p \leq .0001$). Scale bar indicated 50 µm. Abbreviations: inj, injection; ns, not significant.

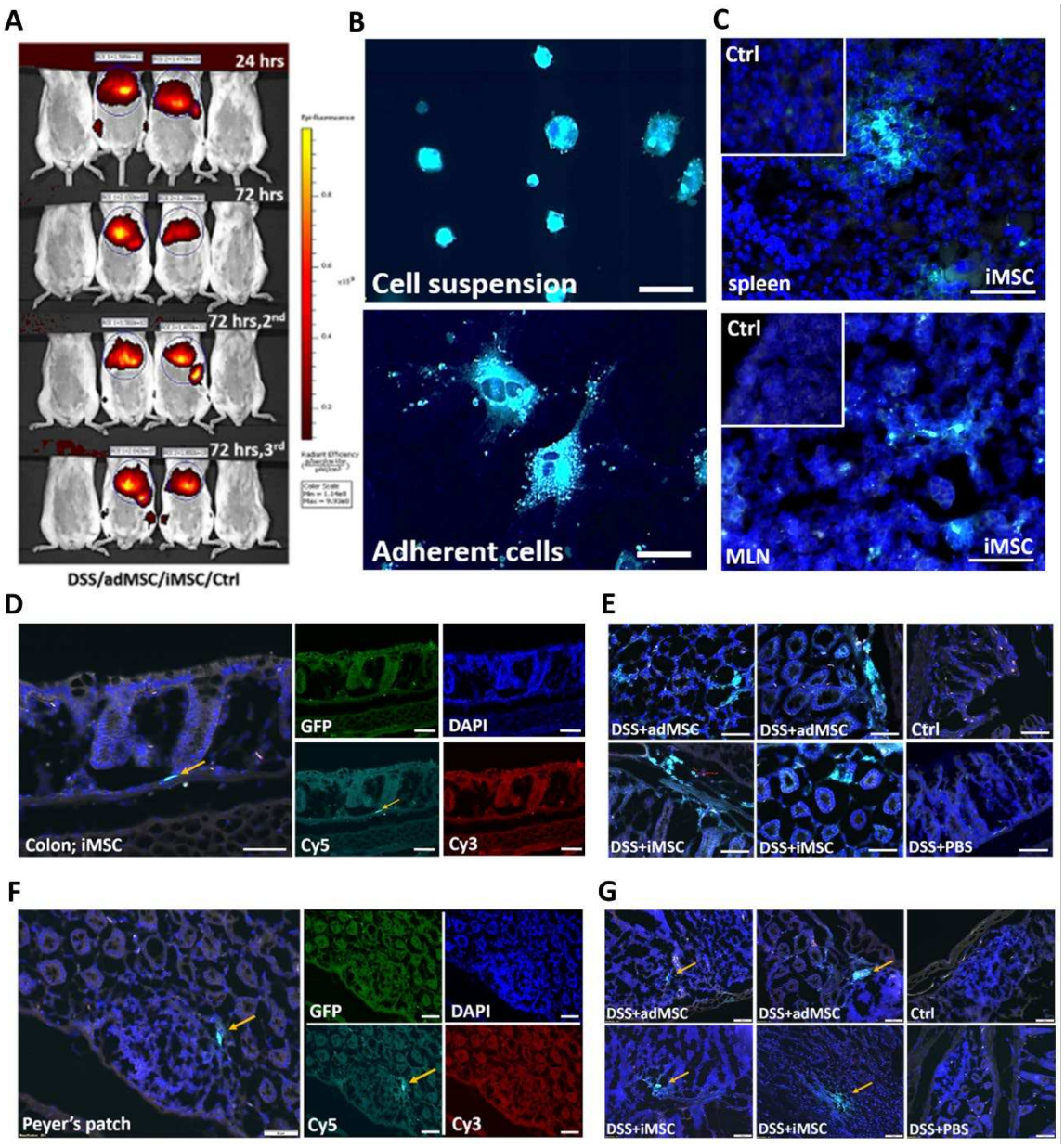

Figure 3.5 Localization of labeled MSC in live animals and tissue sections. (A): For in vivo localization, MSCs were labeled using DiR® dye before injection and mice were monitored by IVIS in-vivo fluorescent imaging at 24 hours after the first injection, 72 hours post-injections and on the day of euthanasia. High concentrations of MSC were found in lung and liver and spleen, but not in intestinal tissues. (B): In vitro labeled MSC using fluorescent labeled (DiD®). Localization of labeled MSCs in colonic mucosa and regional lymphoid tissues, tissues were collected 10 days after MSC administration. (C): Presence of labeled MSCs in spleen and mesenteric lymph node tissues. (D): Labeled MSC in colonic mucosa (arrow) presented in corresponding channel (E): High power views of labeled MSC in colonic mucosa and submucosa. (F and G): Labeled MSC in Peyer's patch of colon (arrow). Scale bar indicated 50 µm in all panels

except panel B (indicated 25 µm). Abbreviations: hrs, hours; Ctrl, control; MLN, mesenteric lymph node.

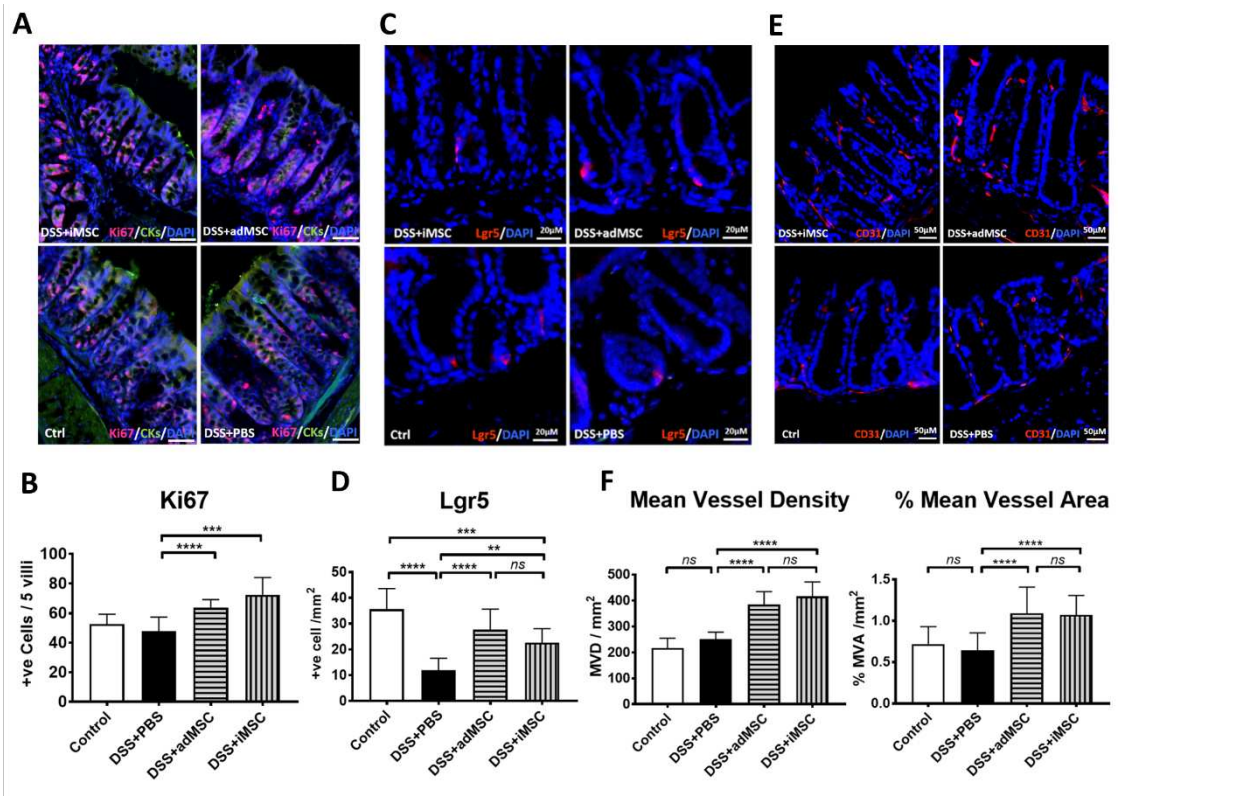

Figure 3.6 Effects of MSC administration on intestinal epithelial cell proliferation, stem cell recruitment, and angiogenesis. (A): Immunoflourescence detection of Ki-67+ (red) cytokeratin+ (green) intestinal epithelial cells in colonic tissues from mice with DSS-induced colitis, with or without MSC treatment. (B): Graphical representation of numbers of Ki-67+ epithelial cells/ 5 villi. (C): Immunofluorescence detection of Lgr5+ intestinal stem cells (red) at the base of colon crypts in colonic tissues from control and MSC-treated mice. (D): Graphical representation of numbers of Lgr5+ stem cells/mm^2 tissue. (E): Immunofluorescence detection of CD31+ neoangiogenic cells in colonic tissues of control and MSC treated mice. (F): Graphical representation of the mean vessel density and mean vessel area / mm^2 of mucosa, as determined as described in Methods. The statistics reported as mean ± SD, statistical differences were calculated using One-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$). Scale bar indicated 50 µm in all panels. Abbreviations: CKs, cytokeratin; DAPI, 4',6-diamidino-2-phenylindole; Lgr5, leucine rich repeat containing G protein-coupled receptor 5; ns, not significant.

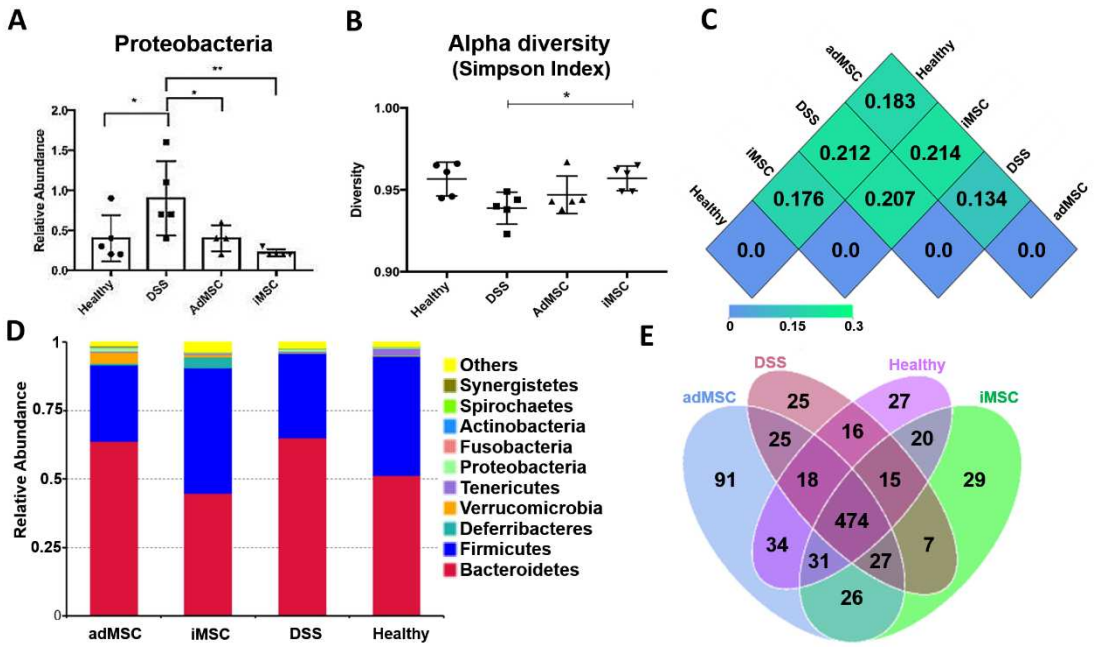

Figure 3.7 Effects of MSC administration on gut microbiome. Fecal pellets were collected at 2 days after the last MSC injection from control and MSC-treated animals (n = 5 per group) and analyzed by 16s rRNA sequencing as determined as described in Methods. (A): Relative abundance of Proteobacteria presented in each treatment group. (B): Microbial diversity within treatment groups (Simpson alpha diversity index) calculated with QIIME (Version 1.7.0). (C): Comparative analysis of differences between treatment groups (beta diversity), heat map represents average differences, with 0 as no difference, 0.3 is maximum differences. Scale bar shown in bottom left. (D): Relative abundance of top 10 phyla presented in each treatment group. (E): Venn diagram generated according to the Operational Taxonomic Unit clustering of each treatment group, each number represents number of bacterial species, either shared or unique to treatment groups. The statistics reported as mean ± SD, statistical differences were calculated using One-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$).

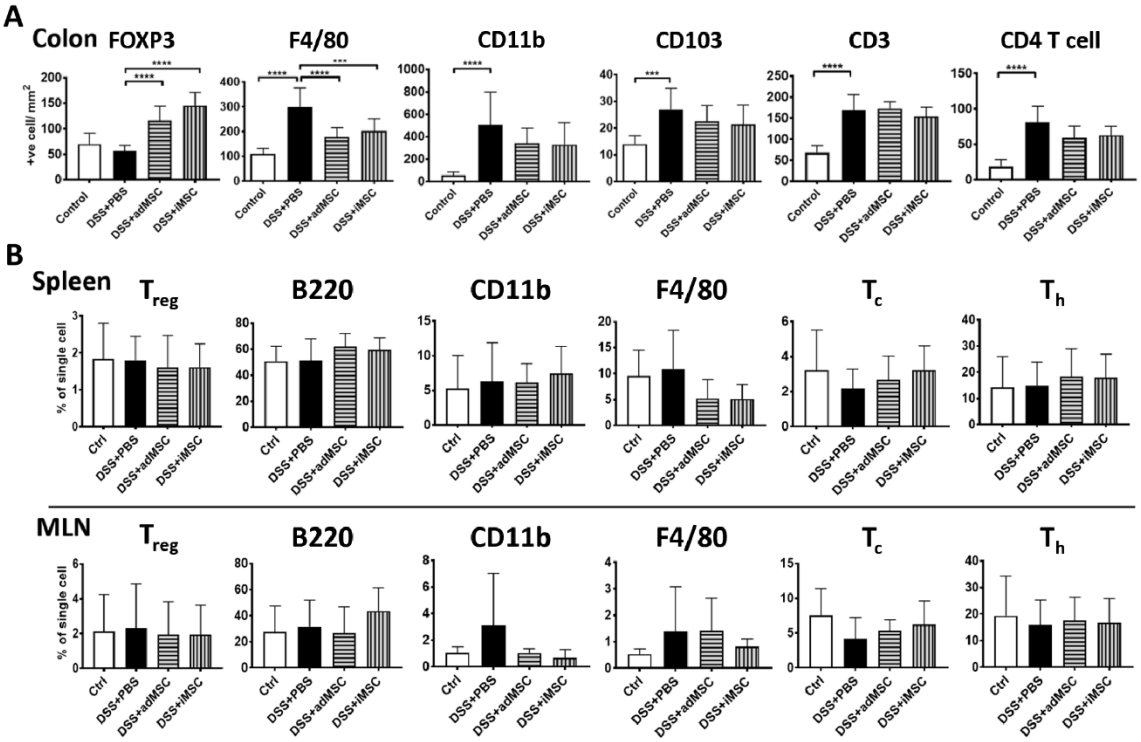

Figure 3.8 Effects of MSC administration on colonic inflammation. Tissue sections were immunostained with the indicated antibodies, as noted in Methods, and imaged using a confocal microscope. Quantitative image analysis was used to quantitate the density of inflammatory cells (see Methods). (A): Distribution of FOXP3, F4/80, CD11b, CD3, CD4 T cell and CD103+ cells in colonic tissues. (B): Leukocyte populations in spleen and mesenteric lymph node tissues as assessed by flow cytometry (see Methods). Statistical differences were calculated using One-way ANOVA with Dunnett multiple comparison to DSS treated group *(*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$).* Abbreviations: FOXP3, Foxhead Box P3; DAPI, 4',6-diamidino-2-phenylindole; Treg, regulatory T cell; Tc, cytotoxic T cell, Th, T helper cell.

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CHAPTER 4: RESPONSE OF CANINE MSC TO INFLAMMATORY CYTOKINES AND TOLL-LIKE RECEPTOR (TLR) AGONISTS AND THEIR INTERACTION WITH IMMUNE CELLS AND CELLS FROM JOINT MICROENVIRONMENT

4.1 Overview

Background: Inconsistent therapeutic effect using the conventional MSCs have been reported, and MSCs activation by inflammatory cytokines and TLR agonists has been reported in species other than the dog to improve the efficacy of MSCs treatment. However, the overall alteration of features and functions of canine MSCs by preconditioning with those stimuli is mostly unknown. Therefore, the studies described here investigating the effect of preactivated MSCs and their interactions with other cells.

Objective: This study comprehensively investigated the response of canine MSCs to inflammatory stimuli and their interactions with immune cells and cells from the joint microenvironment. We hypothesized that IFN-g and Poly(I:C) would more potently activate dog MSC than the other stimuli.

Result: Stimulation of dog MSC with inflammatory stimuli elicited little response in terms of either cell surface phenotype changes or immune functional changes, indicative of a hypo-responsive state. The only detectable changes were an increase in PD-L1 expression following IFN-g exposure, and increased secretion of IL-8 following Poly(I:C) exposure. Pre-activation also had no effect on the interaction of MSC with macrophages or with fibroblasts.

Conclusion: Dog MSC appeared to be hypo-responsive to activation with key inflammatory cytokines or TLR agonists, relative to other species. The reasons for this lack of responsiveness

are not known but could reflect differences in receptor signaling strength or expression, in intracellular signaling processes, or in strength of cellular responses to activation.

4.2 Introduction

MSCs have advantageous properties including multi-lineage potential, self-renewal, rich tissue source with easy accessibility, isolatable and expandable in vitro with exceptional genomic stability and few ethical issues[1]. MSCs are conveniently accessed from several sources from adult tissue including bone marrow[2] , adipose tissue [2], dermis[3, 4] and more. MSCs are also considered the immune-privilege cells since the lack of co-stimulatory molecule expression[5] prevents them from immune recognition and support the use of allogenic MSC source [4]. MSCs are also well-known in their anti-inflammation effect to immune cells (as described previously). These support the MSCs as a promising alternative cellular therapy in various immune disorders including SLE, graft-versus-host-disease (GVHD), osteoarthritis (OA) and IBD [1, 6, 7].

The conventional MSCs have been documented for their advantageous features, and effective for treatment in inflammatory diseases[1, 6, 7]. However, inconsistent efficacy using the conventional MSCs in several studies have been reported ranging from less effective to ineffective outcomes[8]. For example, studies in human MSC treatment found no effect of conventional MSC unless preactivation the MSCs by inflammatory stimuli [9, 10] prior treatment. Additionally, the final immunomodulatory outcome of MSC is influenced by the microenvironment cues at the site of inflammation [11]. Several studies indicated that MSCs are attracted to the sites and become activation by inflammatory stimuli including IFN-g [12-15], TNF-a [15], and LPS [14, 16]. Previous studies in human and mouse documented an increase of MSC efficacy by those stimulations, despite the short period of incubation time (i.e., 1 hour[17]), which are postulated to resemble the endogenous

response occurring to MSCs at the site of inflammation[17] in vivo. These pieces of evidence suggest the plausible way to improve the efficacy of MSC treatment by preactivation the stem cells by inflammatory cytokines and TLR agonists in vitro prior administration for treatment.

Precondition MSCs with inflammatory cytokines and TLR agonists showed higher effective function of MSCs both in immunomodulatory and tissue regeneration effect[15, 18-21]. Several studies have been reported the increase of anti-inflammation and augmentation of tissue regeneration through the secretory factors produced by preactivated MSCs, as well as cell-cell contact activity[13, 15, 18-23]. Preactivated MSCs with TNF-a, IFN-g, LPS, Poly(I:C) or IL-1B have been noted to produce a significant amount of cytokines, growth factors, chemokines and adhesion molecules including IL-10 [24], HGF [13], TGF-b [25], PGE-2[15], IDO[25, 26], VEGF [13, 27], CCL2[28], and VCAM-1[24]. Some of these mediators were reported to enhance the migratory ability of MSC to the affected area[22, 24] as well as ability to support the growth of cells including fibroblasts[28, 29], epithelium and synoviocytes. Moreover, they become more suppressive phenotype inhibiting the immune cell activity including suppressed T cell proliferation and macrophage polarization (as described in a previous section) which are crucial for treating inflammatory diseases.

Although previous studies found that preactivation of MSCs showed an increased efficacy, the actual effects derived from each stimulus remain controversial and potential a bidirectional activity[11, 24, 26, 28], either immunosuppressive or pro-inflammation. For example, an increase of IL-8 was found in Poly(I:C) activated MSCs in one study[20] but not in another study[25], even the same stimulation dosage and incubation period. Also, no agreement exists in other studies regarding the optimal protocol to yield the best stimulation effect. Various combination of time and dosage have been reported to be useful for MSCs activation [15, 16, 18-21, 30, 31]. For example, the effective doses from 2ng [30]-100ng/ml[29] and various incubation times (i.e., 1 hour [20, 25], 24 hour[31] to 5 days[16]) have

been reported for IFN-g activation. Thus, the optimal activation for proper MSCs function remains a challenge and crucial for determining the ideal outcomes.

At present, most of the results regarding preactivation MSCs have been reported from mouse and human. In particular, TLR-mediated responses are considered both species-specific and cell-type specific[32]. Very limited studies have investigated the effect of MSCs activation in dog and some significant differences between interspecies have been considered. For example, previous studies in murine and human found that IL-10 was not constitutively produced by resting MSCs however, only human MSCs was shown an increase of IL-10 production in response to Poly(I:C) activation [15]. Unfortunately, the knowledge on overall alteration of features and function of MSCs by preconditioning with those stimuli has been mostly unknown in dog MSCs. Therefore, the extensive studies regarding preconditioning MSCs in dog is required.

This study investigates the response of canine MSCs to inflammatory cytokines and TLR agonists and their interactions with immune cells and cells from the joint microenvironment. The results of stimulation with following stimuli; IFN-g, TNF-a, Poly(I:C) and LPS; that effect to the immune cells and cells from joint microenvironment are investigated and compared to unstimulated MSCs. Preactivation MSCs with inflammatory cytokines and TLRs agonists generally showed the equivalent effects to the resting MSCs in the most parts except increased PD-L1 expression by IFN-g and unique high IL-8 secreted by Poly(I:C) activation. Alternative macrophage phenotype was induced by most of the stimuli as well as unstimulated MSCs. Significant regenerative responses of fibroblast and synoviocytes were stimulated by MSC-CMs with the comparable effect between intact and unstimulated cells. Compared to other species, canine MSCs showed the hyporesponsive response to inflammatory stimuli suggesting the interspecies variable of cytokines and TLR-mediated response.

4.3 Materials and Methods

4.3.1 MSCs isolation, culture and stimulation procedures

The MSCs used in this study were derived from frozen-stored fat tissue of healthy dog donors. Briefly, 0.5 gram of fat tissue was thawed and washed with sterile PBS, then mechanical minced by scalpel and digested by collagenase for 30 minutes at 37°C. The stromal vascular fraction was isolated by centrifugation at 380xg for 5 minutes. After twice washes with complete medium (DMEM with 10% FBS, 1% Penicillin-streptomycin, essential and non-essential amino acid), SVF was transferred to 75 cm^2 tissue culture flask. The cells were incubated at 37°C, 5% CO_2 and allowed 72 hours for cell adhesion to plastic. After 72 hours, the media was removed and refed with the fresh complete media. Proliferating cells were allowed to grow and reach 70% confluency with media change every 48 hours. The adherent cells were passaged according to confluent. Trypsin was used to detach the cells from flasks, and the cells were washed and transferred to 225 cm^2 tissue culture flask. In this study, 6 MSC lines were derived from adipose tissue from 6 dogs, and 4-6 MSCs were used in each experiment implied that 4-6 biological replicates were done. All MSCs used in this study were within passage 3-6.

Total of 5 MSC-conditioned media (MSC-CMs) derived from different treatments as following;1) IFN-g-MSC-CM, 2) TNF-a-MSC-CM, 3) Poly(I:C)-MSC-CM, 4) LPS-MSC-CM, 5) Ctrl-MSC-CM were examined. Briefly, MSCs were detached and obtained the cell suspension, washed and pelleted by centrifugation. The MSCs were stimulated by one of following reagents; recombinant canine IFN-g (10ng/ml; R&D Minneapolis, MN), TNF-a (10ng/ml; R&D), Poly(I:C) (10ug/ml; Sigma-Aldrich, St. Louis, MO), LPS (1 ug/ml; Sigma-Aldrich). Those prepared reagents

were added to the fresh complete media, then resuspended the cells with conditioned media to get the optimal concentration at 1 million cells/ml. MSCs were incubated for 1 hour at 37°C, 5% CO_2 with often shaking every 10 minutes. After 1 hour of stimulation, MSCs were washed twice, refed with the new fresh condition medium at a concentration of 1 million cells/ml, and plated in 24-well plate with 0.5 ml/well. The unstimulated cells were processed the same incubation method with the control complete media. The plates were incubated further 24 hours and the supernatants from each treatment were collected and centrifuged to separate the dead cells and debris at 400xg 5 minutes, then transferred to new eppendorf and stored at -80°C until used. Most of the downstream assays were used supernatant derived from MSCs (MSC-CMs); otherwise, the stimulated (or preactivated) cells were used instead of MSC-CMs for assay of T cell suppression test as indicated in the text.

4.3.2 Phenotype assessment by flow cytometry.

The phenotypic evaluation of preactivated MSC and control MSC were performed as described previously[33]. Briefly, after MSC-CM was collected, the MSCs were detached and processed for the single cell suspensions ($1x10^6$ cells/ml) in FACS buffer and 1 X 10^5 cells were immunostained in single wells of round bottom 96-well plates. The following antibodies (all from San Diego, CA) were used for immunostaining; MHCII-FITC (YKIX334.2), CD73-PE (clone TY/11.8), CD90-APC (clone YKIX337.217), purified PD-L1 (clone 4F2). The cells with primary antibody were incubated for 30 minutes at room temperature and shielded the light. After complete incubation, the cells were washed twice with FACS. For PD-L1 staining, the donkey anti-mouse Cy3 conjugated antibody was used as the secondary antibody. For phenotypic evaluation of

activated macrophage, macrophages obtained after MSC-CMs incubation were detached and processed immune staining with MHCII (same staining method as described previously).

Phenotype evaluation of synoviocytes were processed with the same protocol. Briefly, synoviocytes were plated in 48-well plates followed by MSC-CM incubation for 24 hours. Then, the cells were harvested and immunostained. Anti PD-L1 and MHC II used in the tests also the same clone. Cells were examined using Beckman Coulter Gallios flow cytometer (Brea, CA) and data were analyzed using FlowJo Software (Ashland, OR).

4.3.3 ELISA for measured the cytokines produced by preactivated MSC

The concentration of cytokines produced from preactivated MSCs were measured by sandwiched ELISA (DUOSET; R&D) followed manufacturer guidelines. The cytokines included IL-6, IL-8, VEGF, IL-1B, MCP-1, IL-10 and TNF-a. Also, the supernatant derived from macrophage activation assay was measured for TNF-a and IL-10 production. The ELISA has performed in total at least 3 independent MSC donor with triplicates. The readouts parameters were reported both protein concentration (pg/ml) and the ratio from unstimulated cells.

4.3.4 T cell proliferation suppression test with EDU

Blood from healthy dogs was processed to obtaining peripheral blood mononuclear cells (PBMCs). Briefly, EDTA-anticoagulated blood was processed the separation of PBMC by Ficoll-density gradient isolation according to the manufacture's protocol. The PBMCs were washed and resuspended with complete medium (RPMI with 15% FBS, 1% Penicillin-streptomycin, essential and non-essential amino acid) at the concentration 1 million cells /ml.

This assay used the activated MSCs (not MSC-CMs) and co-incubated with the PBMCs since the preliminary study showed that the MSC-CMs of dog could not suppress T cell proliferation. MSCs were stimulated by the stimuli as described previously. Moreover, the preactivated cells were detached and washed twice with PBS and resuspended with the complete media. The MSCs were counted, and 20,000 cells were plated to each well of 96-well plate and allow the cells adhered to the plastic for 2 hours. Then aspirate the media out and put the 200 ul of PBMC suspension (prepared as previously described) into each well so the proportion of MSCs vs PBMCs was 1:10. The T cell proliferation with the addition of preactivated MSCs was analyzed using EDU (5-ethynyl-2′-deoxyuridine). Briefly, the experimental wells (direct culture of PBMCs and MSCs) were activated using Concavalin A at 10ug/ml. After 48 hours of reaction, 10uM EDU (Life Technologies Corp. Grand Island, NY) was added to the co-culture. Next 24 hours, the non-adherent cells were collected and immune stained for the detection of canine CD5+ cells (clone YKIX322.3, ABD Serotech. Raleigh, NC). Then, the cells were fixed with 4% PFA for 10 minutes and permeabilized with saponin-based permeabilization reagent (Life Technologies Corp. Grand Island, NY). The reagent of staining cocktails containing 1mM Copper (II) sulfate pentahydrate ($CuSO4 \cdot 5H_2O$), 50mM L-Ascorbic acid (Sigma-Aldrich, St. Louis MO). 1 uM Sulfo-Cyanine3 (Cy3) azide (Lumiprobe Corporation, Hallandale Beach, FL) was added to each well and incubated for 30 minutes at room temperature. After complete staining, cells were washed and evaluated using the flow cytometry. Percentage of T cell proliferation and mean fluorescent intensity (MFI) of EDU were analyzed using Flowjo software.

4.3.5 Macrophage activation with LPS

To investigate the effect of MSC-CMs on macrophage activation, the monocyte-derived macrophages were set up. Briefly, PBMC from a healthy donor was processed as described previously. Then, PBMCs were resuspended with complete medium and plated at a density $1x10^6$ PBMC/0.5 ml in 48-well polystyrene cell culture plates, incubated for 4 hours at 37°C. After allowing for adhesion, the non-adherent cells were washed twice and refed with complete medium with 15% FBS, supplemented with 10 ng/ml huM-CSF (Peprotech, Rocky Hill, NJ, USA). The medium was changed every 2 days, and after 7 days in culture, the macrophages were stimulated with LPS (100ng/ml) for 2 hours then adding fresh medium alone (control) or medium plus 50% of MSC-CMs for 2 hours. After complete incubation, the culture macrophages were washed twice with PBS and refed with 500 ul of fresh media and culture for an additional 24 hours. The next day, macrophage supernatant was collected, and macrophages cells were detached and prepared for phenotype assessment (described previously).

4.3.6 Macrophage polarization evaluated by immunocytochemistry (ICC)

Monocyte-derived macrophages were cultured in 8-well chamber slides for 7 days. Then, the culture media was aspirated and refed with half of fresh media and half of MSC-CM from each condition per well. Also, the standard polarization of macrophages (M0, M1, M2) were used as the control. M0 stage was the cultured macrophage in fresh medium. M1 were obtained by adding 20ng/ml of canine recombinant IFN-g in the media. M2 were derived from macrophage incubated with a mixture of recombinant canine IL-4 and IL-13 (20ng/ml each; R&D). All treated wells were incubated for a total 24 hours and then fixed with 2% PFA (Thermo Fisher, Waltham, MA). After washed twice PBS, the slices were prepared for immunofluorescence staining. Briefly, the permeabilization buffer using 0.1% Triton X was added and incubated for 15 minutes following

with blocking step for 1 hour and washed twice. The anti-iNOS (polyclonal, dilution 1:200) antibody and CD206 (clone 3.29B1.10, dilution 1:20) were incubated overnight with 0.25% saponin at 4°C. The next day, the slices were washed twice followed by secondary antibody staining. Secondary antibodies (Alexa Flour®647 and Cy3; dilution 1:200 in PBST, Jackson ImmunoResearch Laboratories) were incubated for 30 minutes at room temperature. After twice wash, the slices were counterstained with DAPI (Molecular Probe, Eugene, OR), and subsequently mounted with Prolong® Diamond Antifade medium (Thermo Fisher, Waltham, MA). Appropriate isotype control were used for all studies as negative control. The macrophage slides were visualized using an Olympus IX83 confocal microscope. For image analysis of macrophage polarization, 10 random fields of 20x magnification were captured. The fluorescent intensity and number of positive cells corresponding to each marker were counted using ImageJ software. The results were reported both MFI for each marker and the ratio of CD206/iNOS (M2/M1 ratio).

4.3.7 Scratch assay for Fibroblast migration

Canine fibroblasts were plated in 96 well-plate at 30,000 cells/well and cultured for 12-18 hours to allow the cells adhesion to the bottom. Then, the monolayer of fibroblast was scratched using the WoundMaker™. The wells were washed twice with media and refed with half of fresh media and half of MSC-CMs. For control well without CMs, half of fresh media and half of DMEM were fed in a total of 150 ul. The plated were cultured for 72 hours in the IncuCyte™ machine to assessing the migratory ability of fibroblast to cover the scratch area. The machine was programmed to assess the wound area by photo capture and serial image analysis every 3 hours. The parameters reported from the software including the wound width, % relative wound density, % relative wound confluence. Wound width is the area measured the gap width of scratch. %

relative wound density is the density of the cells inside the wound area relative to the area outside the wound. % wound confluence is the cell confluence within the wound region.

4.3.8 MTT assay

The cells (synoviocytes or fibroblasts) were plated into 96 well plates at the density of 20,000 cells/well and incubated for 4 hours to allow the cells adhesion. The MSC-CMs were added with the fresh medium at ratio 1:1 and incubated for 48 hours. After 2 days, 10 ul of MTT (5mg/ml, Sigma, USA) was added to each well and the mixture was incubated for another 4 hours. Then, the solubilizing solution was added and vigorous mix. The optical density (OD) value of the samples was measured at 570 nm with a microplate reader.

4.3.9 Statistical Analysis

Data were analyzed using Prism 7 software (GraphPad, San Diego, CA, USA). The normality of the data was initially tested by Shapiro-Wilk normality test and parametric data were reported as mean ± standard deviation (SD). Data which were not normally distributed were shown in median (range). In parametric data, the statistical differences across all groups compared were analyzed using One-way ANOVA with Dunnett correction. In some analysis, the results were calculated as the ratio from unstimulated MSC group and Kruskal-Wallis test with Dunn's correction was used (as stated in the text). For the fibroblast migration over times, data was analyzed using 2-way ANOVA with Dunnett correction. Also, the results from repeated experiments including cytokine productions from different MSC donors were standardized to baseline control before analysis. In all studies, the statistical significance was set at $p < 0.050$.

4.4 Results

4.4.1 IFN-g induced a higher number of PD-L1+ve MSCs

We examined the effect of preactivation on PD-L1, MHC II, CD73 and CD90 expression by flow cytometry analysis. The percentage of cells expressing each marker and their ratio to control MSCs have shown in Figure 4.1. We found that PD-L1, MHC II, CD73 were not constitutively expressed by resting MSCs and very small number of MSCs (roughly 0.5-1%) showed to have these markers on their surface. IFN-g stimulation increased the number of MSCs expressing PD-L1 for approximately 1.4 times (ratio; 1.044 - 1.8; $p = 0.022$) compared to resting MSCs (Figure 4.1A). We also found that resting MSCs started upregulating constitutive PD-L1 after 24 hours in culture leading to the significant number of PD-L1+ve cells and the most drastic effect was found at 72 hours with the stimulus (Figure 4.2). CD90 was constitutively expressed on resting MSCs (69.83 ± 17.45%). There was no change in the ratio of CD90, MHC II and CD73 expression in response to any pretreatment indicating no detectable effect from those preconditions on the expression of MHC II, CD73. In addition, we found that MHC II was intrinsically upregulated for 10% of the cells after 72 hours of culture (Figure 4.2) suggesting that MSCs became more immune recognizable in long term culture of MSCs.

4.4.2 Enhance of cytokines production by Poly(I:C)

The cytokines production in response to inflammatory cytokines and TLRs activation was evaluated using ELISA. In our study, we found that resting MSCs constitutively produced IL-1B, IL-6, IL-8, MCP-1, and VEGF (Figure 4.3). Specifically, the level of pro-inflammatory cytokines including IL-6 and IL-8 were approximately 100-200 pg/ml (IL-6; 170 ± 154.4, and IL-

8; 72.84 ± 26.17), and level for IL-1B was less than 10 pg/ml. In contrast, resting MSCs intrinsically produced a large amount of VEGF (1,575 ± 861.8 pg/ml) and MCP-1 (10,100 ± 2,401 pg/ml). Interestingly, Poly(I:C)-MSC secreted an extreme amount of IL-8 (1,538 ± 538.9 pg/ml) which was approximately 20-40 times greater than the amount produced in resting state (p <0.0001) as shown in Figure 4.3. However, the level of MCP-1, VEGF, IL-6, and IL-1B were unchanged after stimulation indicating no detectable effect from those preconditions on the production of those cytokines. Additionally, the level of IL-10 and TNF-a in MSC-CMs were very low and undetectable by the kits indicating that preactivation on canine MSCs did not affect the production of these cytokines.

4.4.3 Impact of MSC activation on T cell and macrophage activity

To investigate whether the activation of MSC alters their immune suppressive properties, the activity of immune cells in response to MSC activation were determined using T cell proliferation and macrophage activation assay. As shown in Figure 4.4A, the MSCs, either intact or activated cells, were able to suppress T cell proliferation approximately 50% compared to T cell treated with Concavalin A only. However, the degree of inhibition of T cell proliferation was not altered by MSC activation. Noteworthy, this effect required cell-cell contact since the assay using MSC-CMs failed to suppress the proliferation (data not shown).

Additionally, MSC-CMs showed an inability to inhibit macrophage activation since there were no differences in MHC II expression and cytokines productions from LPS-activated macrophages despite treated with MSC-CMs (Figure 4.4B, C). We also observed the slightly reduced TNF-a production when activated macrophages were incubated with unstimulated MSC-

CM (p = 0.063; Figure 4.4C). However, no change was observed in IL-10 production by LPS-activated macrophages incubated with MSC-CMs.

4.4.4 Activated and resting MSCs drive macrophage polarization toward the alternative type (M2)

We investigated the effect of preactivated MSC-CMs on macrophage polarization. Macrophages incubated with MSC-CMs were stained for CD206 and iNOS. The expression of the markers (MFI) was analyzed using image analysis, and the M2/M1 ratio were calculated. The marker expression was shown in the graph either with standard polarization or ratio to unstimulated CM (Figure 4.5). In general, M2 macrophage has been described to upregulate CD206 and less iNOS production[34]. We observed that Ctrl-MSC-CM and activated MSC-CMs except for Poly(I:C) group influenced the macrophage polarization toward M2-like phenotype indicating as $CD206^{hi}$, $iNOS^{lo}$ compared to standard (Figure 4.5A, B). However, Poly(I:C) stimulation affected the macrophage phenotype to become $CD206^{int}$, $iNOS^{lo}$. Comparing M2/M1 ratio among CMs treatment group, control MSC-CM was superior to others in M2 macrophage polarization since a significant greater M2/M1 ratio ($p < 0.001$) was observed as shown in Figure 4.5C.

4.4.5 Activated and resting MSCs support fibroblast proliferation and migration

The effect of MSC-CMs on fibroblast proliferation was evaluated using the MTT assay. Treatment with MSC-CMs showed a significant cell proliferation compared to untreated fibroblast (p <0.001, Figure 4.6A). Comparing to unstimulated cells, the preactivation of MSC slightly increased proliferation of fibroblast; however, there was no statistical difference effect by preactivation.

The migration of fibroblast cells corresponding to MSC-CMs was measured by a scratch assay. At 48 hours, all MSC-CMs group showed an increase of fibroblast migration compared to untreated fibroblast, and some wells with CMs treatments started the closure of the scratch gap. The percentage of relative wound width reducing over times was shown in Figure 4.6B and summary values at 48 hours were reported as following; Ctrl-MSC-CM; 62.25 ± 12.29%, IFN-g-MSC-CM; 80.55±18.62%, TNF-a-MSC-CM; 76.4±13.63%, Poly(I:C)-MSC-CM; 75.61±10.62%, LPS-MSC-CM; 75.04±10.40%. Unstimulated and preactivated MSC-CMs showed the significant wound reduction compared to untreated fibroblast at 48 hours, and the effect of preactivated CMs became overt at 36 hours of incubation. The effect of resting MSC was comparable to preactivated MSC. We also found that TNF-a significantly reduced wound width compared to Ctrl-MSC-CM (p = 0.017) at 36 hours suggesting the superior effect on fibroblast migration induced by TNF-a activated MSCs.

At 36 and 48 hours, treatment with MSC-CMs showed a significant increase of wound confluence indicating greater cell confluence in the wound regions compared to untreated fibroblast (p <0.001, Figure 4.6C). However, when compared the efficacy among the CMs treatment group, there was not different between the activated CMs and unstimulated CM. At 48 hours, the relative wound confluences were reported as following; Ctrl-MSC-CM; 69.63 ± 7.93%, IFN-g-MSC-CM; 75.64 ± 7.35%, TNF-a-MSC-CM; 73.91 ± 9.78%, Poly(I:C)-MSC-CM; 72.44 ± 8.52%, LPS-MSC-CM; 72.07 ± 6.39%. Interestingly, the effect of preactivated CMs including a group of IFN-g, TNF-a and LPS became overt earlier at 24 hours of incubation indicating more rapid effect than unstimulated CM. In addition, we also assessed the relative wound density; however, MSC-CMs were not different compared to no CMs treated-group (data not shown).

4.4.6 Effect of preactivation of MSC on the phenotype of synoviocytes and proliferation.

We investigated the effect of MSC-CMs on synoviocytes activities in term of phenotype and proliferation. Synoviocytes treated with MSC-CMs have noticeable changes of the phenotype by upregulation MHC II and PD-L1 compared to untreated synoviocytes (Figure 4.7A, B; left panel). Also, IFN-g-MSC-CM treated synoviocytes showed 1.5 times increasing number of cells expressing PD-L1 compared to unstimulated MSC-CM effect ($p = 0.042$, Figure 4.7B; right panel). Also, MSC-CMs stimulated significant proliferation of synoviocytes compared to untreated cells measured by MTT assay (Figure 4.7C); however, there was no different effect among different CMs group.

4.5 Discussion

MSC treatments are promising in regenerative medicine because of several advantageous features including multipotency, self-renewable, accessible, and practical for clinical application. However, treatment with conventional MSCs has been reported to have ineffective and variable outcomes[35-37] possibly because injected MSCs encounter insufficient concentrations of proinflammatory cytokines for full activation, or because of a biased cytokine milieu in vivo. Thus, reactivation the MSCs with inflammatory cytokines and TLR agonists has the potential to augment the immunomodulatory effects of MSC and to enhance tissue regeneration following MSC injection. Also, the overall effects of preconditioning with these stimuli are mostly unknown in canine MSCs. Therefore, we investigated the effect of pretreatment of canine MSCs with inflammatory cytokines and TLR agonists and compared their features and functions to that of resting canine MSCs.

In our study, preactivation of MSCs with inflammatory cytokines and TLRs agonists had little effect on either the phenotype or function of the MSC. Resting canine MSCs were found to have unique features and functions including, lack of MHC II and PD-L1 expression, absence of IL-10 and TNF-a production consistent with other studies[38]. In our study, exposure of MSCs to inflammatory stimuli for 1 hour[17] only activated two detectable responses: upregulation of PD-L1 expression (IFN-g activation) and secretion of IL-8 (Poly(I:C) activation). However, most of the other functional assays for MSCs; including suppress of T cell proliferation, inhibit of macrophage activation by LPS; showed the comparable results between primed MSCs and resting MSC (Figure 4.4). These results are different than those published by other groups studying mouse and human MSC in which they have observed significant immunosuppressive activity in human[20, 25] and murine MSCs[19, 39]. Additionally, in murine and human MSCs activation with TLR agonists (i.e., Poly(I:C) and CpG oligonucleotides) has been shown to induce a high level of cytokines such as IL-10, IL-6, MCP-1[20, 25]. However, in dogs none of these stimuli activated the MSC (Figure 4.3). We included studies with mouse MSC to point out the differences between preactivation of dog MSC versus mouse MSC in Figure 4.8. These pieces of evidence suggest the existence of a unique hypo-responsive state in canine MSC.

MSCs also secrete factors that regulate macrophage function and their effects on suppressing inflammation and tissue remodeling. In our studies, we observed that conditioned medium (CM) from dog MSC altered the phenotype of dog monocyte-derived macrophages (upregulating CD206 expression and downregulating iNOS expression) (see Fig 4.5). However, CM from pre-activated dog MSC had no additional effect on the phenotype of dog macrophages when compared to CM from dog MSC that were not pre-activated. Thus, in terms of MSC effects

on macrophage phenotype and function, dog MSC also appear hypo-responsive to inflammatory stimuli, relative to MSC from other species.

Besides immunosuppressive activity, MSCs have been shown to influence tissue healing for support the treatment of chronic inflammatory disease including OA and IBD. In this study, we examined the effect of CMs rather than cell-cell contact because the therapeutic effect is believed to achieve by paracrine and soluble factors[37] since the very rare number of MSCs was found at the target tissue [40, 41] (i.e., joint and gut mucosa). As shown in our study, MSC-CM increased cell viability and proliferation of the cells in the joint environment including fibroblast and synoviocytes although the comparable effect was observed among all treatments, rapid onset was observed specifically in preactivated CMs (Figure 4.6, 4.7). However, as before there was no effect of MSC pre-activation on the secretion of factors that alter fibroblast proliferation or migration.

There may be several explanations for the hypo-responsiveness of dog MSC to activation. For one, our study investigated the effect of short time activation of canine MSCs by inflammatory stimuli. Dosage and period of activation have been postulated to mimic the physiological state [25] of MSCs in vivo and has been documented to achieve the activation of MSCs leading to downstream action including the NF-kB activation[20, 25]. However, previous studies in other species of MSC demonstrated that 1 hour was sufficient time for activation. Moreover, our results with dog MSC also showed that 1-hour exposure to inflammatory stimuli was sufficient for immune activation. Thus, the length of time of pre-activation is unlikely to explain our findings. Contrary to our findings, the significant increase of IL-6, IL-10 and immune cell migration by Poly(I:C) and LPS activation were reported in that human MSCs studies[20, 25, 42]. These pieces of evidence suggested the interspecies variability of TLR response[32] leading to a hypo-responsive feature of

canine MSCs. It should be noted that although there was a consistency effect in unique high IL-8 observed in Poly(I:C) activation, we did observe donor variability influencing these patterns suggesting the different TLR activity in a different donor. However, very limited data regarding preactivated MSCs in canine species as well as no report of a clinical trial using the preactivated MSCs have been documented so far. Thus, more studies regarding alteration of features and function of preconditioning MSCs in dog are required. Also, further investigation on optimal activation to achieve an effective MSCs remains a challenge and would be of interest for clinical application.

4.6 Conclusion

Preactivation of canine MSCs by inflammatory cytokines and TLR agonists exerts little effect on either phenotype or function. These findings suggest that dog MSC are hypo-responsive to inflammatory stimuli, and provide little justification for using pre-activation as a strategy to improve the effectiveness of dog MSC in clinical studies. Further studies are required to better elucidate the nature of the apparent hypo-responsive state of dog MSC to immune stimuli.

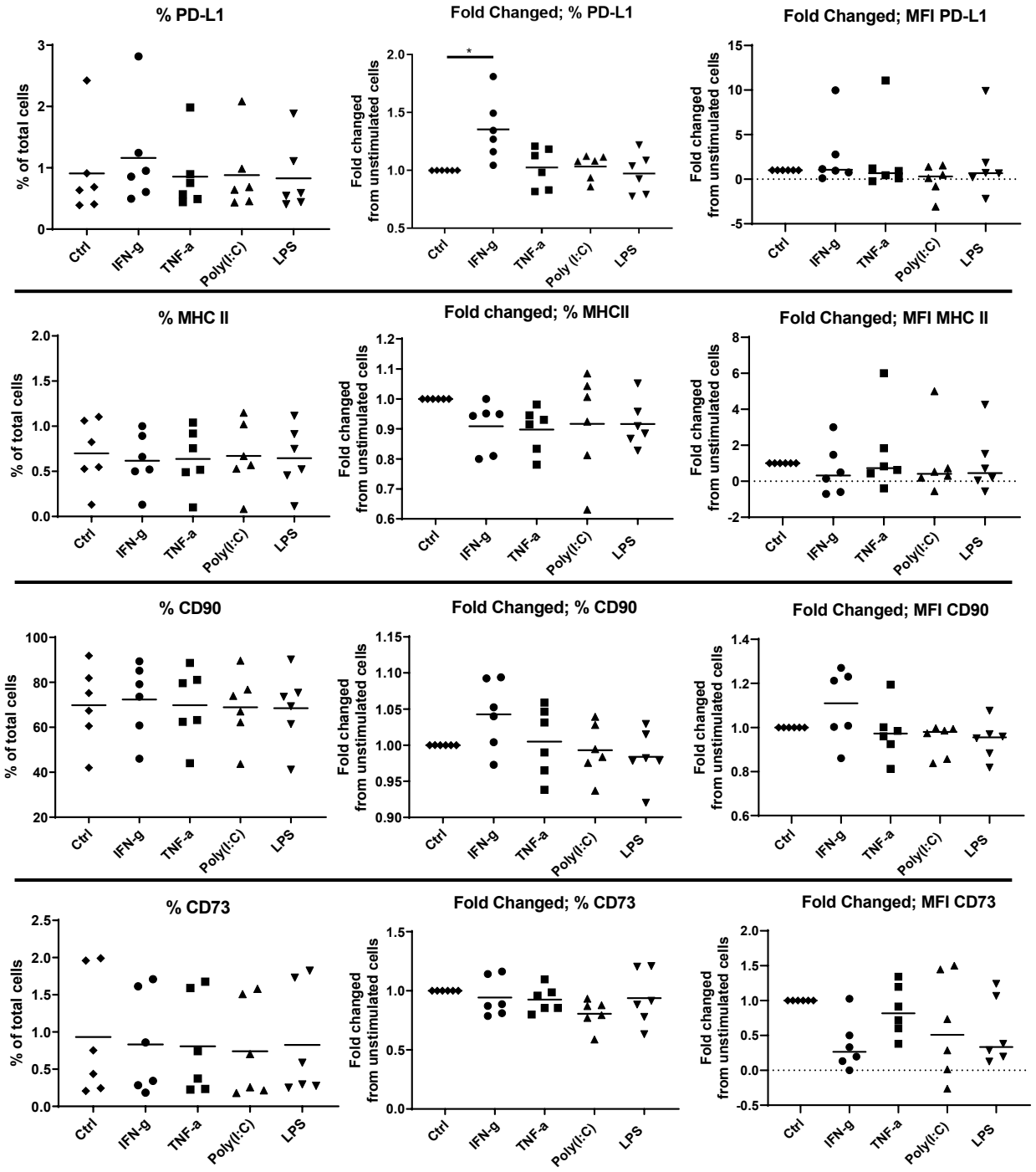

Figure 4.1 Phenotypic evaluation of MSCs in response to the inflammatory cytokines and TLRs. Dot plot depicted the expression of surface markers; PD-L1, MHC II, CD 90, and CD73 (one marker per row). In same row, the % of positive cells, Ratio of % positive cells to resting MSCs, and Ratio of MFI to resting MSCs were shown respectively. The statistics reported as mean, statistical differences were calculated using Kruskal-Wallis with Dunn's multiple comparison (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Abbreviations: PD-L1, Programed death-ligand 1; MHC II, major histocompatibility class II; CD, cluster of differentiation.

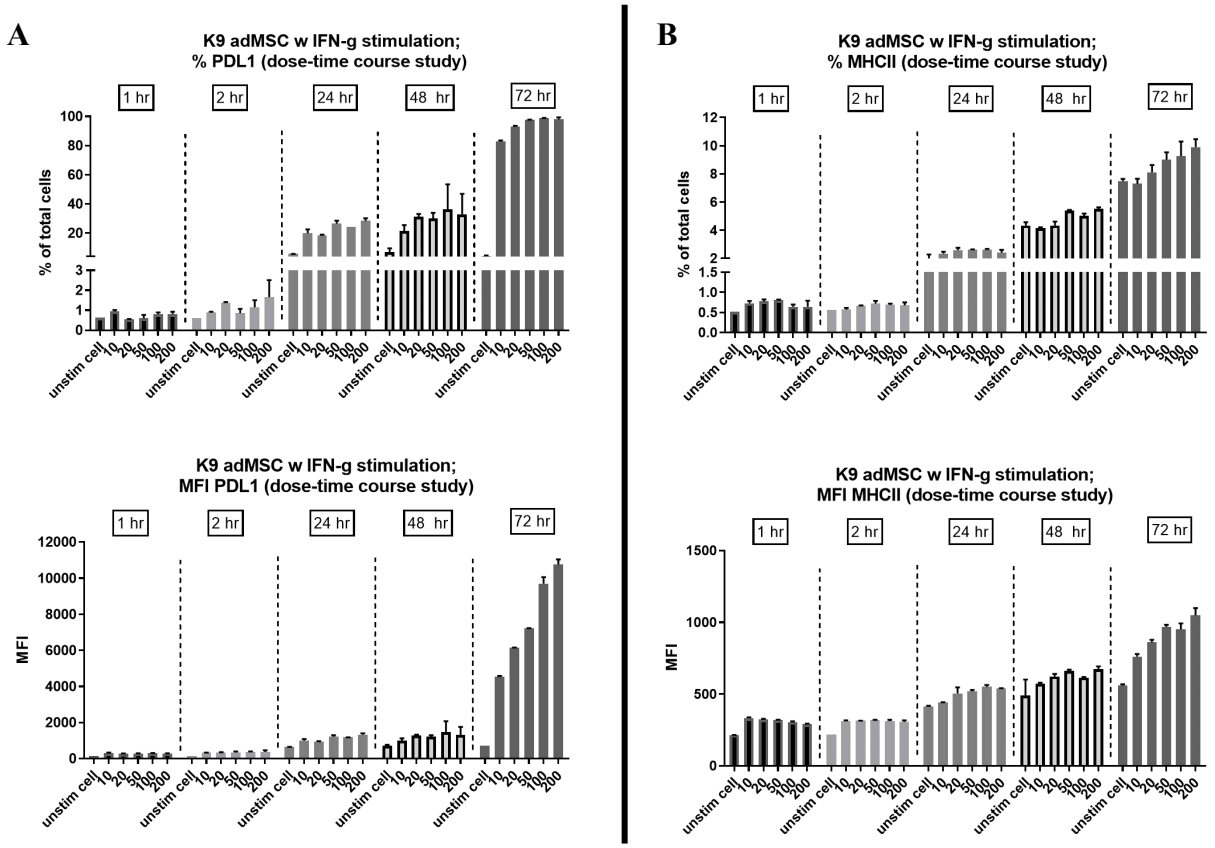

Figure 4.2 IFN-g activation on MSCs; Dose-time course study. MSCs were incubated with multiple dose of IFN-g and cells were harvested on different incubation time to measure the expression of PD-L1 and MHC II. Bar graph depicted time course study of (A) PD-L1 and (B) MHC II. % of positive cells in upper panel and MFI in lower panel.

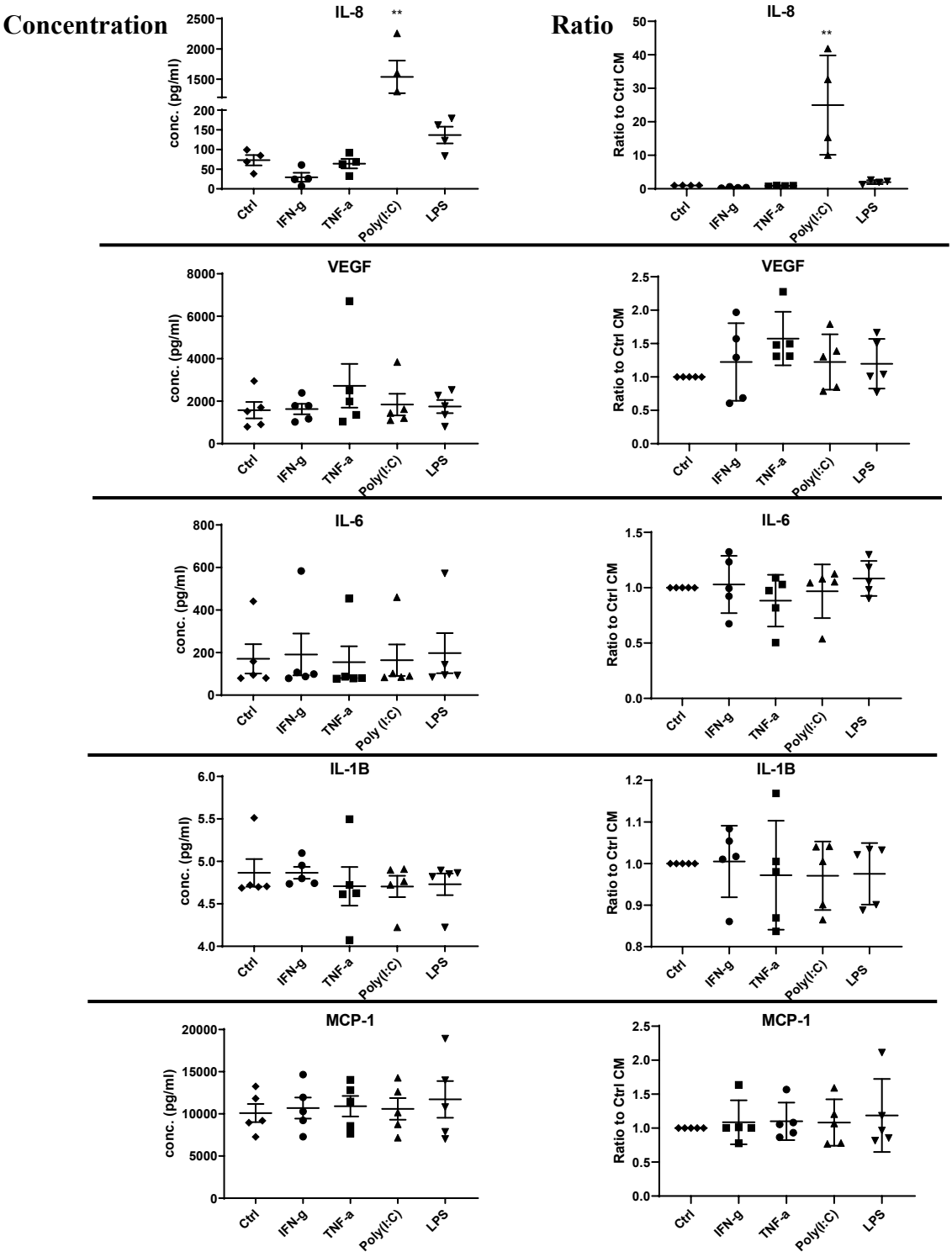

Figure 4.3 Cytokines production of preactivated MSCs. Dot plot depicted the concentration of cytokines (pg/ml) and the ratio to control MSCs. The statistics reported as mean ± SD, statistical differences were calculated using Kruskal-Wallis with Dunn's multiple comparison (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Abbreviations: IL-6, Interleukin 6; IL-8, Interleukin 8; IL-1B, Interleukin 1 beta; VEGF, vascular eudothelial growth factor; MCP-1, Monocyte chemoattractant protein 1.

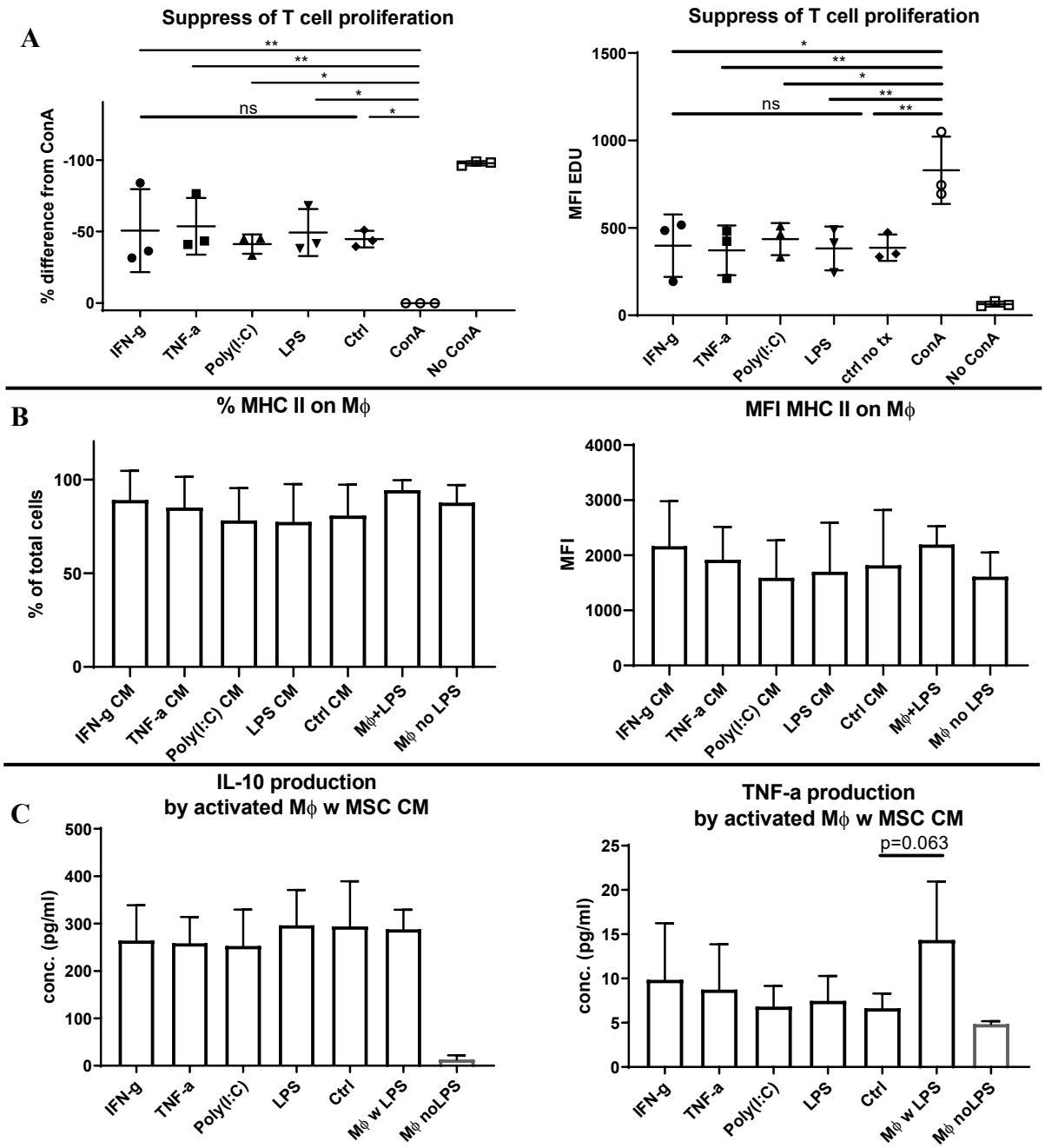

Figure 4.4 Impact of MSC activation on T cell and macrophage activity. (A) Dot plot depicted the suppression of T cell proliferation in response to precondition stimuli; (Left) % suppression and (Right) MFI EDU. (B) Bar graph depicted the expression of MHC II on LPS-activated macrophage incubated with activated MSC-CMs; (Left) % of positive cells and (Right) MFI of MHC II. (C) Bar graph depicted level of cytokines released by LPS-activated macrophage with preconditioning MSC-CMs; (Left) IL-10 and (Right) TNF-a. The statistics reported as mean ± SD, statistical differences were calculated using One-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Abbreviations: MHC II, major histocompatibility class II; IL-10, Interleukin 10; IL-8, Interleukin 8; TNF, tumor necrotic factor; MSC, mesenchymal stem cell; CM, conditioned media

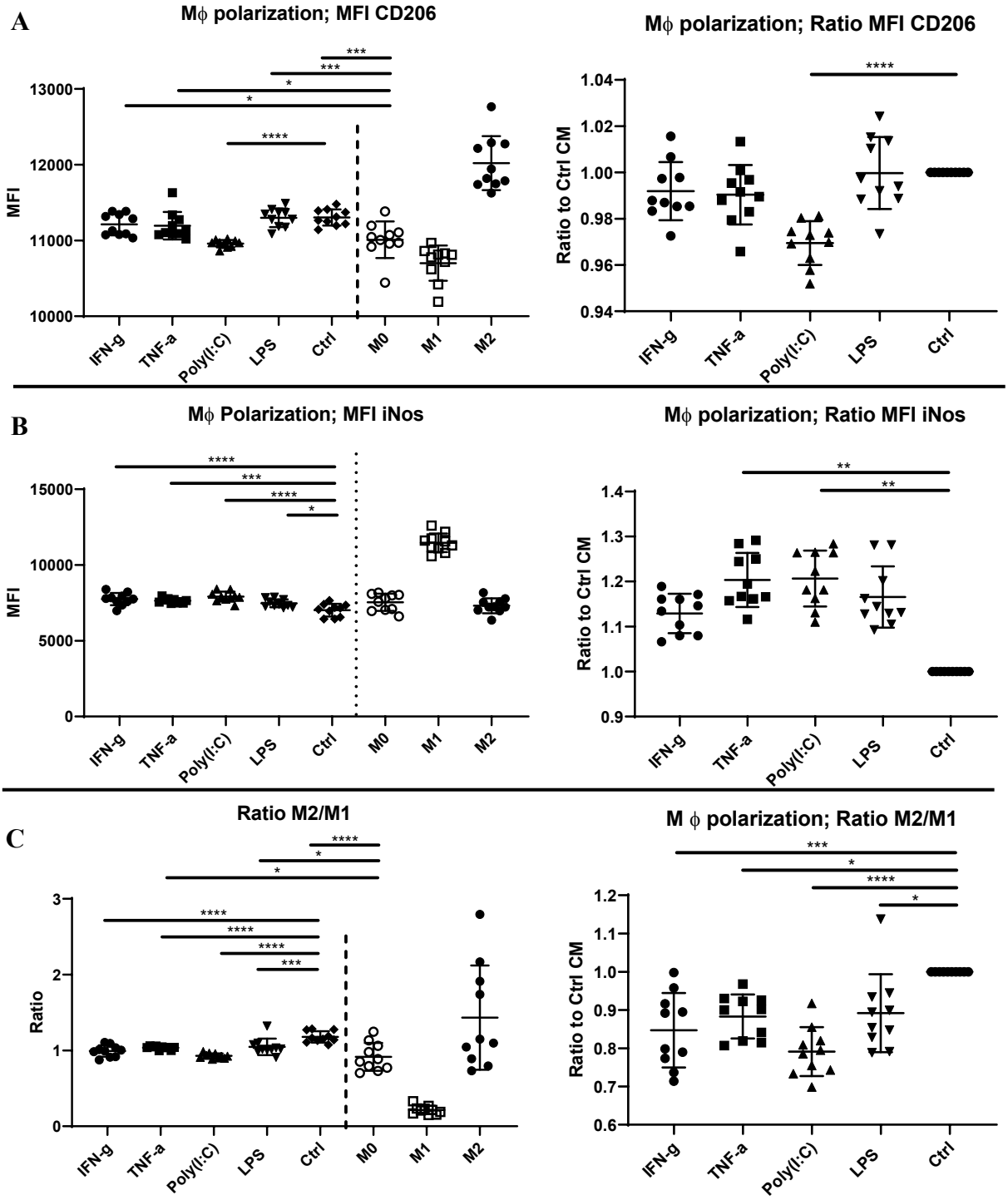

Figure 4.5 Macrophage polarization by preactivated MSC-CMs. (A, B) Dot plot showed the expression of surface markers of macrophage (A) CD206; (Left) MFI and (Right) Ratio of MFI to unstimulated MSC-CMs, (B) iNOS; (Left) MFI and (Right) Ratio of MFI to unstimulated MSC-CMs. (C) Dot plot depicted the ratio of CD206/iNOS cells (M2/M1), and M2/M1 Ratio compared to Ctrl-CMs (Right). The statistics reported as mean ± SD, statistical differences were calculated using (A,B,C; Left panel) One-way ANOVA with Tukey's adjustment, and (A,B,C; Right panel)

Kruskal-Wallis with Dunn's multiple comparison (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$). Abbreviations: Mφ, macrophage; MFI, mean fluorescence intensity; CD, cluster of differentiation, iNOS, induced nitric oxide synthase.

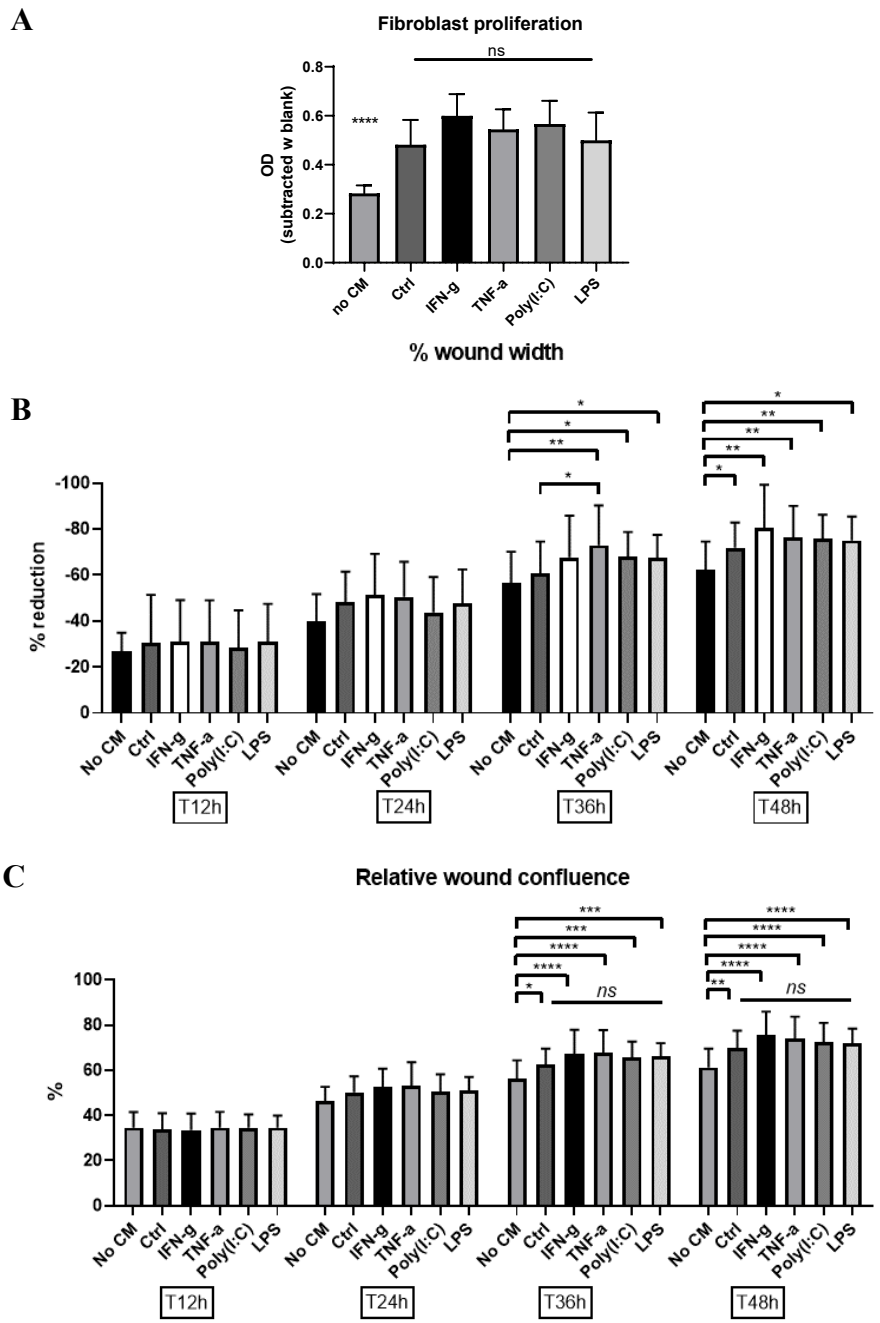

Figure 4.6 MSC-CMs support fibroblast proliferation and migration. (A) Bar graph showed the fibroblast proliferation in response to preconditioned MSC-CMs measured by MTT assay, (B) % wound width and (C) % relative wound confluence over times. The statistics reported as mean ± SD, statistical differences were calculated using Two-way ANOVA with Tukey's adjustment (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$).

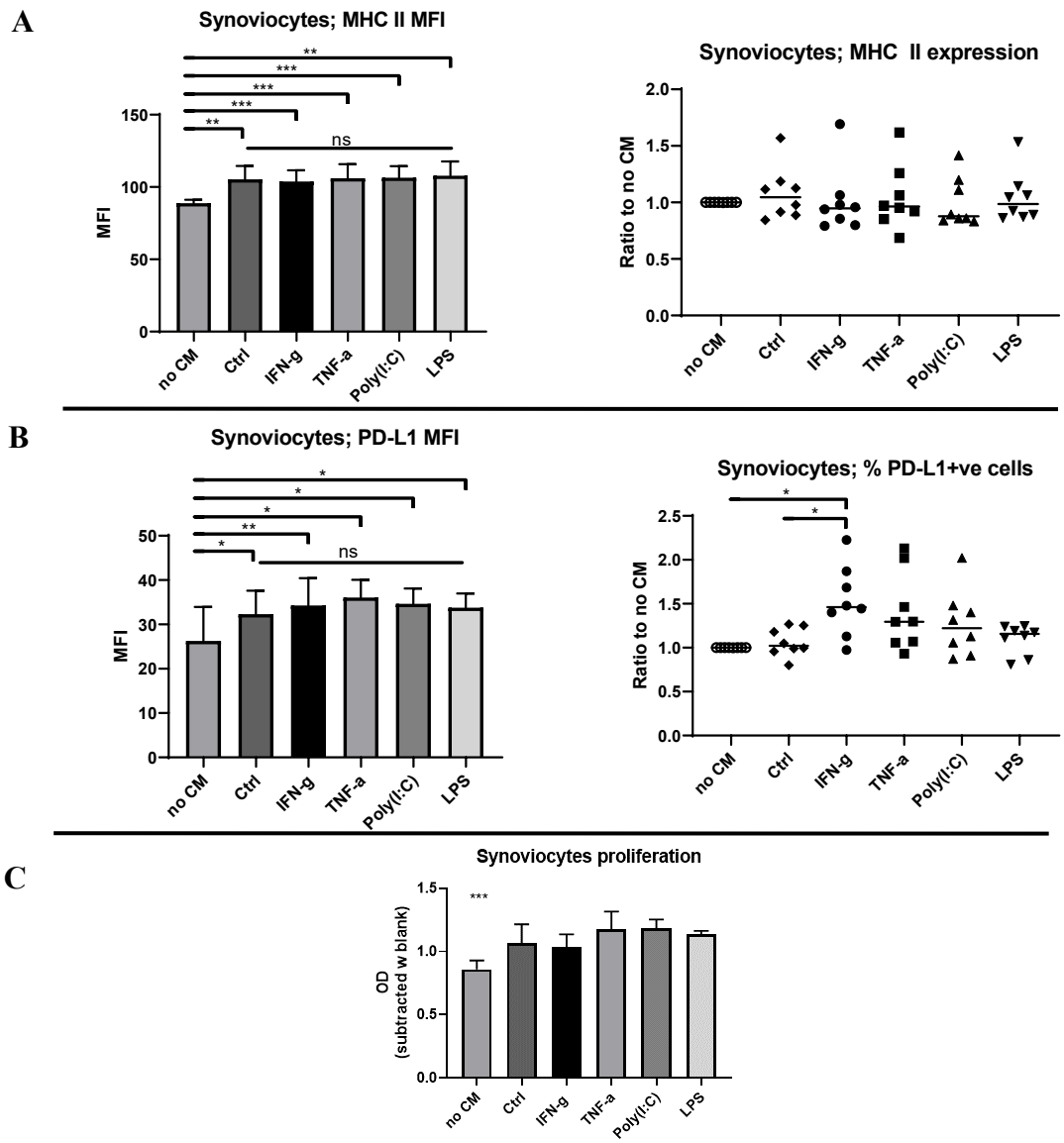

Figure 4.7 MSC-CMs effect on phenotype of synoviocytes and proliferation. Bar graph showed the expression of surface markers; (A) MHC II, (B) PD-L1 of synoviocytes incubated with MSC-CMs for 2 days. Dot plot depicted the ratio compared to untreated synoviocytes; (A) MHC II expression, (B) ratio of % PD-L1+ve cells to untreated cells. The statistics reported as mean ± SD, statistical differences were calculated using (A,B; Left panel, and C) One-way ANOVA with Tukey's adjustment, and (A,B; Right panel) Kruskal-Wallis with Dunn's multiple comparison (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$). Abbreviations: MFI, mean fluorescence intensity.

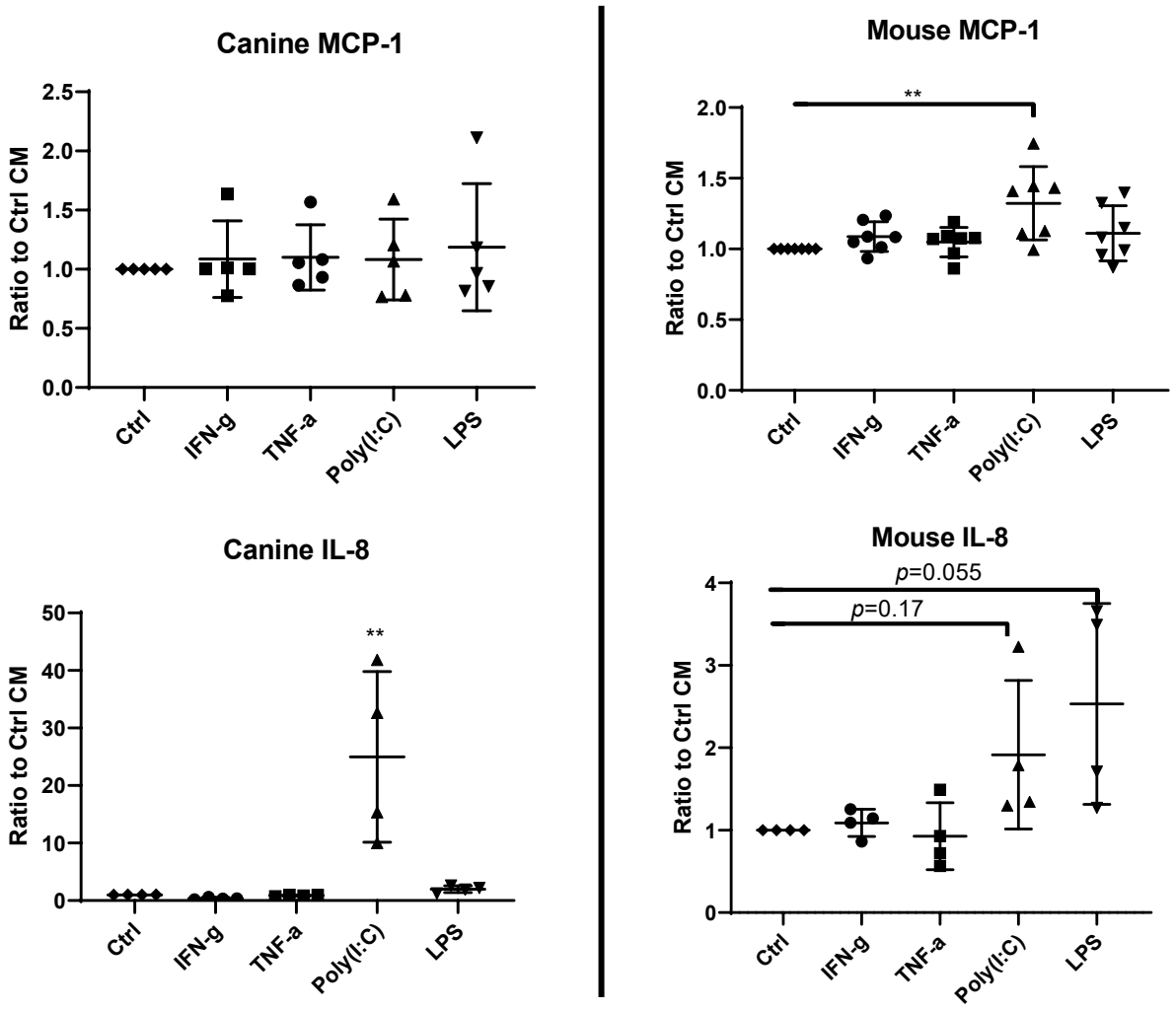

Figure 4.8 Cytokines production differences between mouse and canine MSC. Dot plot depicted cytokine production ratio to control MSCs. Activation of mouse MSC with the same dosage and time of activation showed more response to activation by Poly(I:C) and LPS compare to canine MSC. The statistics reported as mean ± SD, statistical differences were calculated using Kruskal-Wallis with Dunn's multiple comparison (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Abbreviations: MCP-1, Monocyte chemoattractant protein 1; IL-8, Interleukin 8.

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CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 Significance of Work

The goal of the research presented in this dissertation was to comprehensively investigate the impact of activated and resting MSCs on immune responses, cells regeneration and gut microbiome for treatment of IBD with a specific emphasis on gaining an improved understanding of the immune responses to the gut bacteria in dogs with IBD. IBD in dogs represent a significant chronic inflammatory condition affecting the GI in humans known as Crohn's disease and Ulcerative Colitis. The disease potential affects 100-200 per 100,000 people in North America and it is emerging to the other parts of the world including Asia[1]. Not only humans, IBD dogs also suffer from this chronic disease and most of them experienced impaired quality of life due to the complication and adverse effect from medical and surgical treatment. Thus, the MSCs is promising to be an effective treatment with safe and less side-effect for IBD treatment. Additionally, the primary features of IBD in dogs mostly resemble those in humans [2, 3], including unknown cause [4-6], high genetic sequence similarity [7] and pathogenesis [8], as well as the need for clinical intervention[9, 10]. Thus, spontaneous IBD in dog is considered a potential model for study, with the potential for extrapolation to human IBD.

This work was the comprehensive studies that integrated the works starting from a basic science (i.e., cell cultures) to laboratory animal model (i.e., mouse model of colitis) and the knowledge obtained from this work eventually have a potential to apply on clinic either veterinary or human medicine. Hopefully, some aspects of this research could impact on the current treatment of IBD and potential draw the further investigations in the future. In chapter 2, we investigated the

humoral immune response against gut bacteria in dogs with IBD. In this part, we determined whether dogs with IBD have more IgG-binding bacteria and the potential consequence to trigger GI inflammation. Also, Chapter 3, we investigate the effectiveness of using iMSCs and conventional adipose-derived MSCs for the treatment of IBD in a mouse model of DSS-induced colitis. In chapter 4, we investigate the response of canine MSCs to inflammatory stimuli and their interactions with immune cells and potential regenerative effect on cells from joint microenvironment. This would be the way to improve the efficacy of conventional MSCs treatment. Based on the collective finding of this work we can conclude that treatment with MSCs is a promising treatment for IBD since they have shown the remarkable properties in anti-inflammation, tissue regeneration as well as microbiome restoration.

By investigating an immune response to gut bacteria in chapter 2, we also conclude that the mucosal antibody binding to commensal gut bacteria was substantially greater in dog with IBD compared to a healthy, and the immune targeted particular bacteria and triggered the pro-inflammatory response. As IBD caused by dysregulation of immune and the over response to their gut bacteria as we found from chapter 2.

Based on the findings from chapter 3, we conclude that MSCs are effective for IBD treatment and we support the use of either iMSCs or adMSCs since they were mostly equivalent. However, conventional MSCs has known to have several limitations including the variable treatment effect caused by un-uniform cell source and loss of potency by senescence. Thus, iMSCs is probably the better choice, however, further study regarding safety need to be done.

Also, we examine the impact of precondition MSCs compared to resting MSCs in chapter 4. We conclude that preactivation of MSCs potentially induced a few changes of phenotypes and functions and most of the immune response were comparable to a resting MSCs. Further

investigation on optimal activation to achieve an effective MSCs remains a challenge and would be of interest for clinical application.

5.2 Future Directions

Based on the findings and our conclusion, iMSC is effective in treatment of IBD and has advantageous properties for the convenient use and potential for clinical application including exceptional expandability and easy quality control. However, several essential drawbacks must be concerned including safety, genetic stability, consistency of phenotype and function. Also, iMSCs is novel and has been recently developed. There is the limited resources and small number of back up evidence. Most of the studies have been reported from the side of pure science evidence, very few studies was conducted in animal model or safety study[11] and no clinical trial has been documented. Thus, future studies in animal model including spontaneous IBD in dog or large scale of clinical trial for long-term follow-up to determine iMSC safety and efficacy is required before clinical translation.

Our work also supports the use of conventional MSCs for treatment of inflammatory diseases and allogeneic source is convenient, safe and practical. However, the previous studies have reported inconsistent outcomes of allogenic MSC treatment [12, 13], suggesting from the different phenotype, function, uniformity and cell quality that MSC may be altered in long-term cell culture [12, 14, 15], thereby leading to a reduced therapeutic outcome [16, 17]. Therefore, we investigated the effect of preconditioned MSCs in vitro (chapter 4). Although the effect of preactivated MSC was barely enhanced by an activation contrary to the findings from other species. This low response may be caused by interspecies difference in TLRs response. Study

focused on the combination of stimuli, variety of dose and time for activation would be useful and potential to develop the standard protocol for precondition the MSCs. The same setting can apply to test on iMSC as well. Therefore, further study for optimal activation to obtain suitable phenotypes and functions corresponding to specific disease remains a challenge and is still a need.

Although this study has shown the effective of MSCs in treatment of mouse model of colitis. The similar outcome when apply the treatment to larger animal or in clinical trial cannot be ensured. For example, several studies have reported the failure of MSCs engraftment although the promising result were obtained during the experiment in animal model[13, 18]. Therefore, clinical trial study in larger population for investigate the efficacy and safety of MSCs in IBD treatment is ultimately required. Also, various trial designs may be useful to evaluate the optimal method or standard protocol for the maximum therapeutic efficacy.

The MSC therapeutic effect in IBD does not depend on their full engraftment but mainly on the secretory mediators. Many assays included in this study, we have been using the condition media/secretome of MSCs to induce the response of immunomodulation and cell regeneration. We also found that secretome is likely effective as cellular therapy but more convenient, easy to use and long-term store. However, concerns of the duration of action and cost of production were described in previous studies. The study to investigate the use of MSC secretome and their effect would be developed.

Our findings from chapter 2 indicated that a bacterial flow cytometric IgG assay using fecal samples provided significant sensitivity and specificity to distinguish dogs with IBD from healthy dogs. These outcomes must be proven in larger population in dogs with IBD and compared to animals with other causes for GI dysfunction, including viral infection, and infections with GI

parasites. Furthermore, the analysis of the serial samples at a later stage after treatment at the various follow-up time points could also be beneficial.

Overall, we finally need to develop our comprehensive understanding regarding the immunopathogenesis of the IBD and how the MSCs effect in that process. Also, thoroughly know the most parts of pro and con of MSCs would be essential. Therefore, there are the great opportunities for continued study in these areas, and the studies described in this dissertation provides a robust foundation for further research.

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