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Mesenchymal stem cells: mechanisms involved in the treatment of Inflammatory Bowel Disease

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I. INTRODUCTION

1.Inflammatory bowel disease from epidemiology to malfunction of the immune system

1.1 Epidemiology and Environmental factors

Inflammatory bowel disease (IBD) is defined as an inappropriate immune response that occurs in genetically susceptible individuals as the result of a complex interaction among environmental factors, microbial factors, and the intestinal immune system. As a result, chronic intestinal inflammation involving a pathological response in both the innate and adaptive immune systems is produced. Crohn's Disease (CD) and ulcerative colitis (UC) represent the two main forms of IBD. Even though CD and UC represent two distinct forms of chronic inflammation of the gastrointestinal tract and therefore have different causes and different pathogenic mechanisms, the factors underlying the appearance of both CD and UC are roughly the same, and ultimately lead to an abnormal immune reactivity which is responsible for damaging the gut and causing clinical manifestations [1]. Race and ethnic origin seem to represent risk factors for the development of IBD, since a study shows racial differences in disease location and extraintestinal disease complications. In particular, it was observed that Jewish people are more susceptible to both CD and UC. Epidemiologically, northern Europe, the UK and North America display the highest incidence rates and prevalence of CD and UC, while South America, southeast Asia, Africa and Australia are considered low incidence areas [2]. The demography and clinical characteristics of these diseases in Asia show both similarities and differences to IBD in western populations: UC is more common than CD, and the clinical course tends to be milder, with fever complications and less need for surgical procedures. The peak age of onset of UC and CD is between 15 and 30 years. A second peak occurs between the ages of 60 and 80. The male to female ratio for UC is 1:1 and for CD is 1.1 -1.8:1. Since many areas with low incidence rates include developing countries, these data could indicate that variation in access to healthcare and its

quality, as well as different extents of industrialization, sanitation and hygiene together with other environmental factors may play a pivotal role in the eziopathogenesis of IBD. However, the key question in regard to IBD epidemiology that remains still unresolved is what causes favor the emergence of IBD in new areas of the world [3]. A large number of risk factors have been proposed including cigarette smoking, diet, oral contraceptives, appendectomy, infections and vaccinations, and perinatal and childhood factors. However, with the exception of cigarette smoking, none of the other factors are supported by enough direct evidence to be considered true risk factors [3]. The highest mortality in IBD patients is during the first year of disease. Moreover in long-term disease, IBD-associated chronic inflammation increases the risk of dysplasia and colon cancer [4].

1.2 Pathogens and Commensal Intestinal flora

During microbial colonization, the mucosal immune system matures, and it is during this time that immune, or oral, tolerance is established. In IBD patients, oral tolerance to the microbiota is altered [5].

Over the past years classical infectious agents such as Chlamydia tracomatis, Escherichia coli, Cytomegalovirus, Saccharomyces cerevisiae, as well as others, have been proposed as causes of IBD, but to date this hypothesis is rather weak. In particular, Mycobacterium paratuberculosis as the agent of CD received considerable attention [6], but follow up studies attempting to confirm its presence by histological examination have all yielded conflicting and inconclusive results. Subsequently the finding of paramixovirus-like particles in CD endothelial granulomas led to the suggestion that CD could be a form of chronic vasculitis caused by the persistence of the measles virus in the mucosa [7], but subsequent studies failed to confirm this association. While the hypothesis of pathogen-associated IBD was failing, evidence continued to mount indicating that the indigenous commensal flora of the gut is the target of the immune response in IBD. Under normal circumstances there is an intimate interaction between commensal intestinal bacteria and the

immune system and this complex crosstalk is under the control of immune tolerance.

A large body of data coming from animal models of IBD indicates that the normal enteric flora is needed to develop experimental colitis [8, 9]. Thus, the paradigm "no bacteria, no colitis" was created to underscore the central role of the intestinal microbiota in IBD pathogenesis. This paradigm is supported by the observation of an increased number of bacteria in close contact with the mucosa in IBD patients [10]. Furthermore, IBD lesions occur preferentially in segments with the highest concentrations of bacteria, and surgical diversion of the fecal stream prevents reappearance of CD whereas restoration of the fecal flow induces disease recurrence [11]. Finally, modulation of the enteric flora with antibiotics and probiotics attenuates inflammation in IBD patients, most of whom show also an enhanced systemic and mucosal immunological reactivity against gut bacterial antigens [12]. It has been proposed that this abnormal immune reactivity is the consequence of a loss of tolerance towards the autologous enteric flora, resulting in an inappropriate immune response in the mucosa that is manifested by the chronic inflammatory process typical of CD and UC [13]. Why tolerance is lost and an abnormal response to otherwise normal gut bacteria develops in IBD is still not entirely clear.

1.3 Genetic factors

Technological advances in DNA analysis and sequencing and the use of multicenter databases have allowed screening for IBD-associated genetic mutations, confirming the theory of genetic susceptibility of both CD and UC. A number of studies has demonstrated clustering of cases of UC or CD within the same family, suggesting that patients share a genetic background. In the last years, several studies have been carried out to reveal the frequency of familial occurrence of IBD and the prevalence of IBD among first relatives. Epidemiological studies have shown that, in 75-80% of families with members affected by the disease, affected individuals are concordant for disease type, with all affected individuals having CD or, in distinct families, all ulcerative colitis affected individuals. The

remaining 20% of multiple affected families are mixed in which one member has CD and the other member has UC [14]. These findings suggest that a subset of genes associated with IBD would be common to both CD and UC, and others would be found only in one of the two diseases. It was recently discovered that 10-15% CD patients carry homozygous mutations in the CARD15 gene [15, 16]. Strictures, early onset of disease and/or fistulas are more common in patients carrying these mutations. CARD15 gene encodes a protein (nucleotide-binding-oligomerization-domains 2, NOD2) involved in bacterial recognition. Thus, defective mechanisms of bacterial sensing (i. e. due to mutations in CARD15 gene which result in a protein product that no longer interacts with muramyl dipeptide (MDP) [17]) could represent the link between the gut flora and altered immune response found in IBD [18]. However, CARD15 represents only one of the genes underlying IBD susceptibility loci that were identified. Indeed, it has been demonstrated that genetic susceptibility is also important both in initiating and perpetuating chronic inflammation, which characterizes CD and UC. Besides CARD15, other genes, recently reported to be linked with the onset of IBD, are represented by MDR-1, SLC22A4/5 (which encode OCTN1/2) and DLG5. Furthermore, results from genome wide scans have evidenced a strong gene association in both forms of IBD with the gene encoding IL-23R. The engagement of IL-23 by its receptor, results in the activation and nuclear translocation of STAT3 transcription factor as well as STAT4 and STAT5 which have central roles in the differentiation of Th17 and Th1 cells [19]. The characterization of additional IBD susceptibility genes could potentially lead to the identification of novel therapeutic agents for IBD, and potentially allow for the molecular reclassification of the disease, and increase the understanding of the environmental factors contributing to intestinal inflammation.

1.4 Altered intestinal homeostasis

Increasing evidences have revealed that both human IBD and experimental colitis in mice are associated with immune activation in all gut-associated lymphoid tissue (GALT) organs

at very early stages of disease, suggesting a predominant role of these structures during the inflammatory process. The GALT consists of different organs, including Peyer's patches (PP), isolated lymphoid follicles (ILF), cryptopatches (CP) and mesenteric lymph nodes (MLN). There is endoscopic and histological evidence that the earliest observable lesions in ileal CD are located in the follicle-associated epithelium (FAE) overlying lymphoid follicles and PPs, which are an inductive site of the mucosal immune system [20]. Therefore, PPs and Microfold cells (M cells), which transport organisms and particles from the gut lumen to immune cells across the epithelial barrier and thus stimulate mucosal immunity, have been regarded as potential sites of the inflammatory onset in CD [21]. Thus, interaction between luminal antigens and microorganisms with epithelial cells and dendritic cells at the FAE may be a crucial step in the initiation of the inflammation in CD. Preliminary findings suggest increased transmucosal passage of nonpathogenic E. Coli in the FAE of non-inflamed ileum of CD, despite a normal permeability to protein antigens [22]. A previously unrecognized specific defect in the barrier to commensal bacteria may lead to increased crosstalk between luminal bacteria and the inductive sites of the mucosal immune system. The universal barrier dysfunction seen in CD and discussed later in this paragraph, may be generated by signals initiated by immune-antigen interactions in the FAE. Therefore, the observed diminished barrier function in the FAE may represent a very early step in mucosal inflammation, leading to the initiation of CD.

There is a reduction in epithelial resistance and an increase in permeability of the inflamed and non-inflamed mucosa in both CD and UC [23]. In physiological conditions, the intestinal epithelium must function as a selective barrier to limit penetration of antigens to the mucosal immune system for the purpose of generating oral tolerance responses to food antigens or commensal organisms and host defense responses against pathogens. Epithelial cells lining the gastrointestinal tract are held together at the apical and basal poles by tight junctions that form intimate contacts to restrict the passive flow of molecules between these

cells. The increased epithelial permeability observed in IBD could be a consequence of epithelial cell apoptosis or disruption and/or down-regulation of tight-junction proteins exerted by activated T cells and pro-inflammatory cytokines [24-26]. In addition to increased permeability, epithelial cells display altered innate immune mechanisms which in turn reduce their ability to eliminate invasive and pathogenic microbes and lead them to acquire an activated phenotype, able to induce effector T cell responses [27-29]. Interestingly, these over-reactive or auto-reactive T cells do not undergo apoptosis once activated. Furthermore, mucosal dendritic cells (DC) of IBD patients lose their regulatory capacity, incorrectly recognizing commensal bacteria and activating immune responses normally directed at pathogens [30, 31].

1.5 Adaptive immune system

After the identification of T-cells as central effector cells, and of their soluble mediators as key modulators of immunity, the focus of immune investigation in IBD shifted to T helper (Th) cell subsets and the soluble mediators that they produce. In fact in IBD, the balance of regulatory and effector T cells is altered. When the disease is active, effector T cells predominate over regulatory T cells [32, 33]; whereas in CD, intestinal CD4+ T cells differentiate predominantly into Th1 phenotype producing a large amount of INF-γ, IL-12, IL-18, which stimulate mucosal macrophages to release IL-1, TNF-a and IL-6 [34-38]. Th1 differentiation is mediated in these patients by the marked over-expression of transcription factor, T-bet and the IL-23 [7, 39]. Furthermore, a persistence of auto-reactive T-cell populations has been reported in both thymus and colon indicating a failure to undergo apoptosis suggesting a loss of central and peripheral tolerance [40]. In contrast, patients with UC show an enhanced Th2 immune response with increased amounts of IL-13 and IL-5 [41]. A few years ago IL-17-producing Th17 cells were identified as a new subset of T cells capable of promoting immune-mediated inflammatory responses in various tissues including the intestinal mucosa [42]. The discovery that this new T-cell subset drives

immune-mediated pathology in the gut, and that interleukin (IL)-23 amplifies Th17 cell responses and gut inflammation, has contributed to elucidate new pathways of tissue damage as well as open new avenues for the development of therapeutic strategies in IBD [43]. Nonetheless, it has been recently shown that Th17-related cytokines, such as IL-17A and IL-22, can exert protective rather than detrimental effects in the gut. Numerous studies in IBD patients and in animal models of colitis have demonstrated that the increased inflammatory infiltrate in the lamina propria (LP) reflects a consistently increased production of chemokines and an up-regulation of their receptors [44-53]. An array of chemokines have been found to be up-regulated in both CD and UC which are involved in the recruitment of different leukocyte subtypes into the gut mucosa, and have been proposed as indicators of acute phase reactivity [47, 54]. Among these chemokines, particularly important are IL-8, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, and MCP-3, whose levels are elevated both locally and systemically, and induce conformational changes in adhesion molecules on lymphocytes and granulocytes.

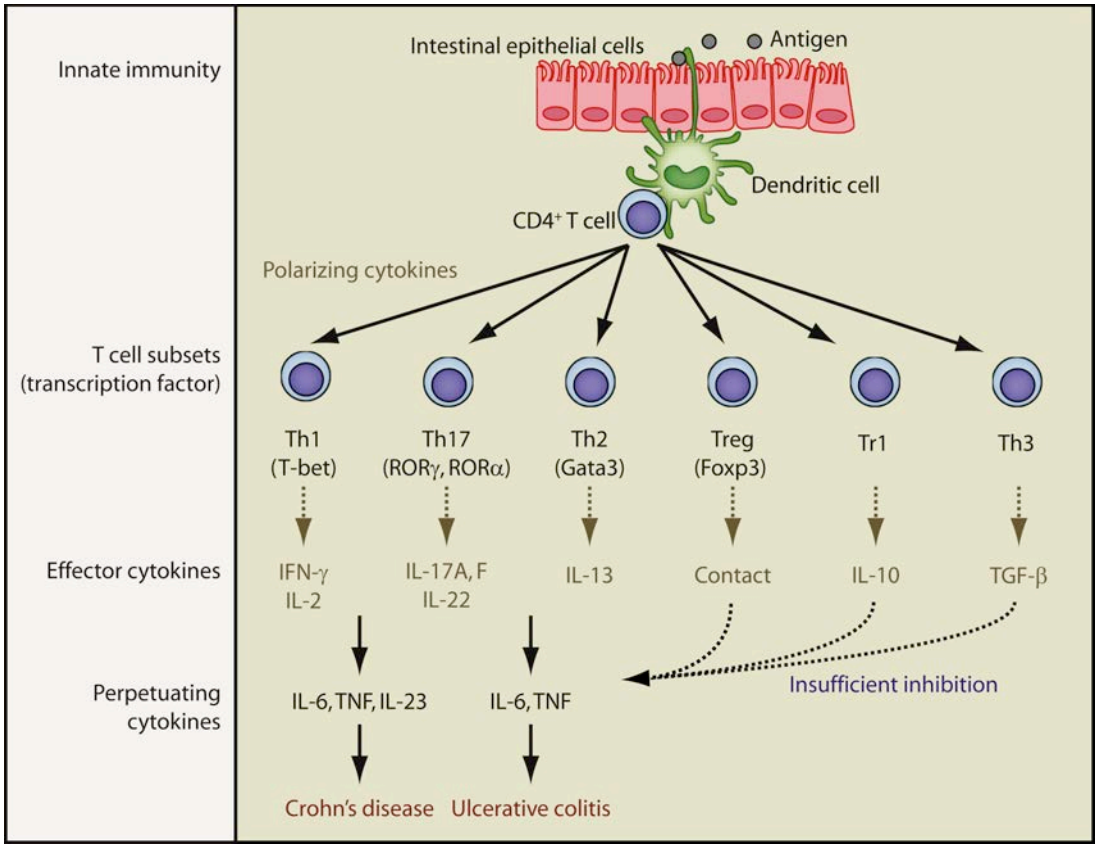

Figure 1: Cytokines and T Cell Subsets in the Pathogenesis of IBD

The T cell fate below the mucosal surface in IBD is determined by a complex interplay between bacterial antigens and innate immune mechanisms. Intestinal epithelial cells and DC modulate the activation of the mucosal immune system by producing various cytokines and regulatory proteins. Based on these, signal effector T cells may differentiate into Th1, Th2, or Th17 effector T cells that are characterized by specific signature cytokines and transcription factors. Current evidence suggests that Crohn's disease is associated with an augmented Th1 and Th17 cell cytokine response, whereas ulcerative colitis is characterized by the production of some Th2 and Th17 cell cytokines. The effector T cell response in IBD is augmented by disease-perpetuating cytokines such as IL-6 and TNF that induce T cell activation and prevent T cell apoptosis. The aggressive T effector cell activation is not sufficiently counteracted by regulatory and anti-inflammatory T cells (Treg, Tr1, Th3 cells), thereby leading to mucosal inflammation and tissue destruction. Neurat M. F., Immunity, 2009.

2. Dextran sodium sulphate (DSS): experimental model of colitis

IBD is a complex multifactorial disease, as its pathogenesis is not limited to a unique factor, and cannot be reproduced in cell culture systems. In recent years the development of experimental animal models of IBD have significantly contributed to the present understanding of IBD, as they have provided a platform through which some of these complex mechanisms can be systematically investigated [55, 56]. There are several experimental models of colitis which provide different conditions for the study of factors involved in the pathogenesis of these diseases such as: environmental factors, the role of specific immune and genetic factors, and therapeutic options in IBD. They can be classified into four main categories Firstly, intestinal inflammation and tissue damage can be induced in mice via the administration of specific chemical agents: acetic acid, dextran sodium sulphate (DSS) [57], trinitrobenzene sulfonic acid (TNBS) and oxazolone [58, 59]. Secondly, colitis develops spontaneously in a few naturally occurring mutant mouse strains: C3H/HeBir and SAMP1/Yit [60]. Thirdly, several gene-knockout and transgenic mouse strains develop colitis [61-63]. Fourthly, intestinal inflammation is observed following reconstitution of immunodeficient mice with $CD4^+$ T cells [64]. The DSS model of colitis is the most common, quick and easily reproducible chemically induced colitis model, it has similarities to clinical and histological features of human IBD with UC characteristics [65]. The colitis is induced by the addition of DSS in drinking water. As in ulcerative colitis, DSS-induced colitis occurs in the distal tract of the colon provoking bleeding, diarrhoea and weight loss. The severity of the disease is assessed in a similar approach that used in humans: a disease activity index (DAI), which takes into consideration weight loss, occult blood in stool and stool consistency. The onset and severity of these features depends on the animal species, the concentration of DSS and the duration of treatment. DSS colitic mice show shortened oedematous colon with areas of haemorrhage and ulceration. Histologically, the colon presents a superficial inflammation, mainly affecting the mucosa, but may extend

to the submucosa and the muscularis mucosa. Superficial ulcers, goblet cell loss, crypt distortion and abscesses with inflammatory cell infiltration further characterize the inflamed mucosa of colitic mice. Colonic damage can also be macroscopically observed by endoscopy evaluating the presence of bleeding mucosa, altered vascular patterns, change in colon wall thickness and deposition of fibrin. The exact mechanism though which DSS initiates colitis is still unknown. One possible mechanism may be the direct alteration of gut permeability. Tight junction proteins responsible for paracellular permeability were directly reduced during DSS treatment as early as day 1, leading to the gradual increase in colonic inflammation. Another possible suggested mechanism is the cytotoxicity of DSS directly on the colonic mucosa, leading the alteration of the interaction between epithelial cells and γδ-intraepithelial T cells [65].

3. Therapeutic treatment of IBD

The quality of life for IBD patients is lower compared to the normal population and is inversely related to active disease, hospitalization and surgery . Drug therapy has been limited to immunosuppressant and corticosteroid use until recently, with many adverse effects or complaints from patients. Absence of well-defined aetiological factors and unclear mechanisms has limited the development of new therapeutic tools. In the last years many studies have expanded the understanding of these diseases and fortunately new targets have been identified as possible new therapies in an attempt to avoid chronic steroid use, to prevent disease progression and to eliminate the need for surgery. Novel therapies now consist of monoclonal antibodies, small molecule inhibitors, peptides, and vaccines . These agents, collectively known as "biological" therapeutics, include recombinant peptides or proteins, antibody-based therapy, nucleic acid-based therapies, and cell and gene therapies [66]. Biological agents can be classified as inhibitors of key molecules, such as proinflammatory cytokines (including TNF-α), anti-inflammatory cytokines, blockers of cell adhesion molecules (CAMs), anti-leukocyte molecules, growth factors and immunostimulators, or act as inhibitors of pathologic mechanisms including Th1 polarization, T cell activation and proliferation. Although effective in producing clinical response, the last approach leads to serious adverse events, and it has been therefore abandoned [66]. Even if several anti-tumor necrosis factor α therapies have been developed (Infliximab, Adalimumab, CDP571, Certolizumab) by now the only approved biologics for the treatment of inflammatory bowel diseases are only two, the humanized monoclonal antibody Infliximab and the fully human antibody Adalimumab, both used for the treatment of Crohn's disease and Infliximab for the treatment of ulcerative colitis [66]. In addition to TNF-α, other pro-inflammatory cytokines (IL-12/IL-23 and IFN-γ), or cytokine receptors (IL-2R) [67-70], or downstream signaling pathways mediated by cytokines (Janus Kinase (JAK) 3) [71, 72] have been recently targeted, but data coming from ongoing clinical trials

demonstrate that their efficacy is limited. A great interest for drugs targeting other pathways is rinsing: Among them, the use of anti-inflammatory cytokines, like IL-10, IL-11 and IFN-β, was tested in clinical practice, but it did not produce satisfactory results [73-75]. Conversely, blockers of CAMs, such as natalizumab, gave rise to great expectations, since they were shown to strongly inhibit the leukocyte infiltration process [76]. However, their clinical use has been limited, due to the increased risk of developing progressive multifocal leukoencephalopathy (PML) [77]. Additionally, one recent approach to the treatment of IBD consists of stimulators of the innate immune system, such as granulocyte colony-stimulating factor (G-CSF), which addresses the impaired acute inflammatory response that results in delayed clearance of bacteria that penetrate the gut wall [78, 79]. Biologicals have been mainly tested in randomized controlled trials with different study designs, patient populations and outcome measures, making comparisons between them difficult (fig 2). Only a relatively small number of these biological agents have demonstrated any efficacy. Despite these advances, however, only a small percentage of patients benefit from these novel therapies, and clear limitations of the new therapeutic approaches exist. However, none of these treatments are curative for IBD, and a relevant number of patients are refractory or intolerant to many pharmacological approaches. Altered control of intestinal immune cell function and turnover appear to be crucial factors responsible for the dysregulated inflammatory response in IBD patients. Indeed, immunosuppression is a central component of inflammatory bowel disease treatment.Stem cell-based therapy, particularly that consisting of mesenchymal stem cells (MSC) transplantation, appears promising. Indeed, data coming from ongoing clinical trials demonstrate that intrafistular injections of MSC resulted in sustained complete closure as well as a reduction in CD activity [80]. Similarly, the use of adipose-derived MSC with fibrin glue in perianal fistulas promoted fistulas healing in most treated patients [81]. Despite the strong efficacy of MSC delivered locally in the treatment of refractory CD, therapeutic efficacy of MSC

systemically administered needs further investigation.

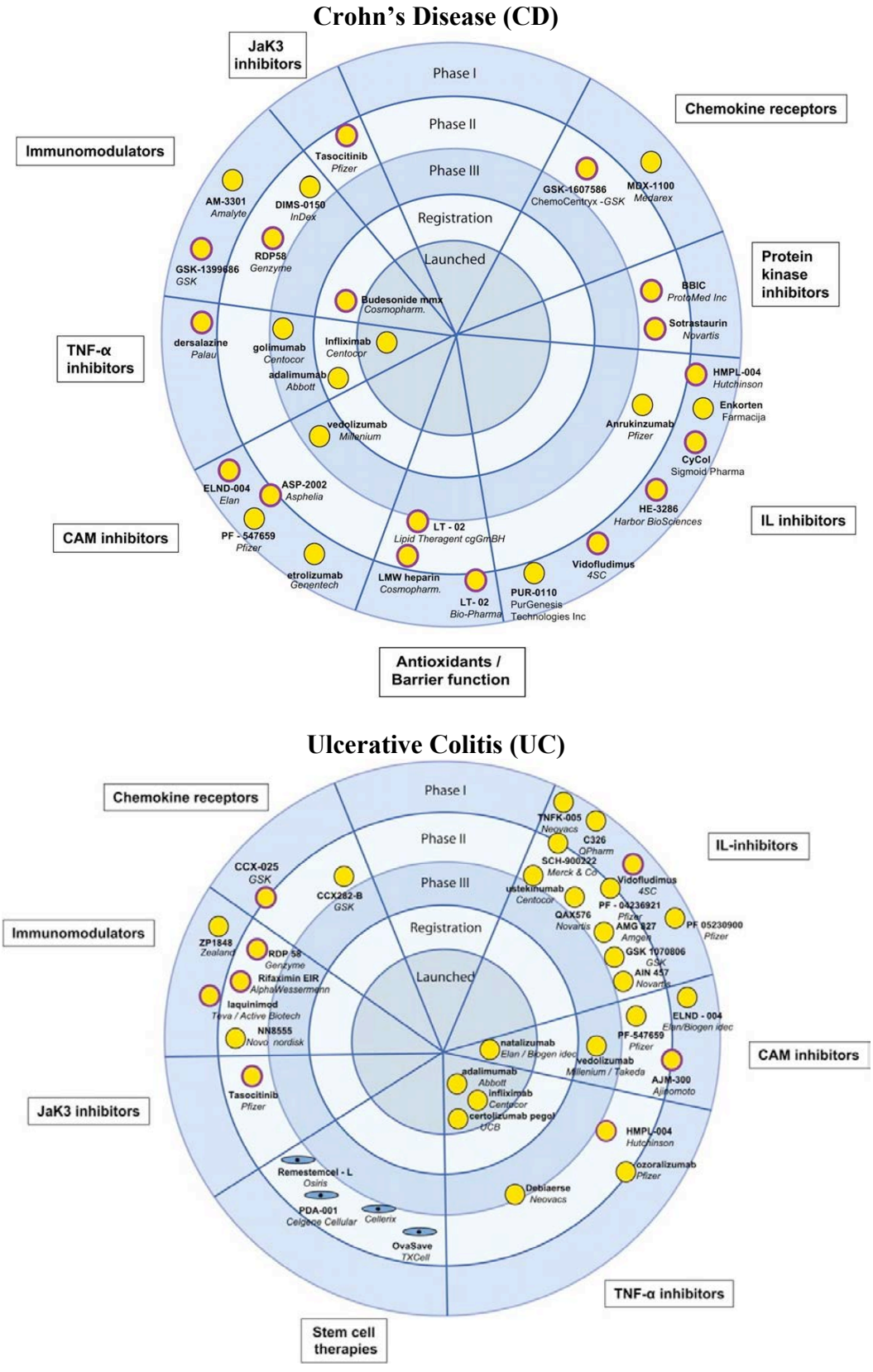

Figure 2: The therapeutic pipeline in IBD.

Drugs are categorized based on the mechanism of action. Purple symbols indicate oral drugs. Danese S Gut 2012

4. Mesenchymal stem cells

Adult mesenchymal stem cells (MSC) are an heterogeneous population of pluripotent progenitor cells that can be isolated and expanded in vitro from different tissues such as bone marrow, cartilage, muscle, tendon, fat and gingiva. The differentiation capacity of MSC along mesenchymal lineages and immunomodulatory proprieties has risen to enormous potential of these cells for cell-based regeneration strategies for mesenchymal tissue injuries and for immune disorders. Although there is a growing body of research focused on the potential therapeutic activity of MSC, several aspects related to their biological properties still remain to be elucidated.

Adult MSC were first discovered by Friedenstein and co-workers more than 40 years ago, when observing bone marrow cells, the researchers have noticed within hematopoietic non-adherent cells the presence of a rare population of plastic-adherent cells (approximately 1 in 10000 nucleated cells in the bone marrow). The initial clones of adherent cells expanded into round-shaped colonies composed of fibroblastoid cells, thus the term of Colony Forming Unit- fibroblasts (CFU-f) [82, 83]. Other groups then extended these initial observations, studying CFU-f proliferative abilities and phenotypic characteristics, and it was found that these cells were multipotential and could differentiate into osteoblasts, chondrocytes, adipocytes, and even myoblasts [84-86]. Importantly, each bone marrow donor shows a specific frequency of CFU-f, which is dependent on the age and health of the donor. Initially these cells were called either mesenchymal stem cells (MSC), for their ability to differentiate into cells of the mesenchymal lineages [87], or stromal cells, for their stromal origin. Afterwards it has been discovered that MSC cultures are heterogeneous displaying various grade of stemness, therefore these cells have termed multipotent mesenchymal stromal cells" [88, 89]. Despite years of intense investigation, the location and role of the native MSC within their tissue of origin *in vivo* are not completely defined, mainly because of the lack of specific markers allowing their unambiguous identification

[88, 90, 91]. Thus, the possibility exists that MSC phenotype and properties vary between *in vivo* and *in vitro* settings due to the removal from their natural environment and the use of chemical and physical growth conditions that might alter their characteristics.

4.1 Phenotype of mesenchymal stem cells

The lack of specific markers that can unequivocally identify MSC and distinguish them from other cell types, leads in 2006 the international Society for Cell Therapy to propose the following criteria for the minimal identification of human MSC [92]: they have to adhere to plastic under standard culture conditions; they have to be positive for CD73, CD90, CD105 and negative for CD34, CD45, HLA-DR, CD14, CD11b, CD79a, CD19 by flow cytometry; they have to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts. Although these criteria have started paving the way for homogeneous and unequivocal definition of MSC cultures, they allow once more a retrospective definition of putative MSC but do not allow their prospective purification. In addition, these criteria are not entirely valid across and intra species. Indeed, murine MSC differ frequently not only from the human MSC, but also among strains in marker expression and behavior in culture. To date it is widely accepted that human MSC express CD29, CD44, CD73, CD90, CD105, CD146 and CD166, while they are negative for the hematopoietic markers CD14, CD34, CD45 and CD133. In addition to these well-known markers, it was recently discovered that human MSC are also positive for CD54, CD56, CD61, CD63, CD71, CD97, CD98, CD99, CD106, CD112, CD155, CD276, CD304, CD325 [93], CD271 (LNGFR) and CD49a [94]. On the other hand, murine MSC appear negative for both the hematopoietic surface markers CD11b, CD34, CD45, CD117 (c-Kit), CD135 and the endothelial surface marker CD31 (PECAM-1), while they are positive for CD29, Sca-1 and CD44. Of note, expression levels of CD90 (Thy-1) and CD106 (VCAM-1) remain controversial, since MSC both positive and negative for these surface markers have been reported [95, 96]. In addition, slight differences have

been reported in murine MSC phenotype depending from the strain and the age of mice employed, the MSC tissue source, the isolation protocol and the culture conditions used. Importantly, since culture conditions for derivation and expansion of MSC were demonstrated to maintain MSC typical differentiation potency but do not preserve what is currently considered to be the native MSC phenotype, they seem to exert the strongest influence on the expression levels of MSC surface markers [91]. In addition to different culture conditions, mice strains from which cells are isolated deeply influence MSC phenotype. Indeed, it has been demonstrated that murine MSC isolated from 4 inbred strains show different expression levels of CD34, Sca-1 and CD106. In particular, C57Bl/6 (Bl/6) MSC expressed high levels of CD34, FVB/N MSC express moderate levels, and both Balb/C and DBA1 MSC expressed low levels.

Many conflicting data have been published regarding the effects produced by MSC administration in different experimental models of human pathologies, and also data coming from ongoing MSC clinical trials are sometimes controversial. Further investigations are therefore necessary to identify specific, better unique MSC markers, which allow to obtain standardized MSC cultures, with well-defined *in vitro* characteristics that lead to predictable effects once used *in vivo*.

4.2 Source and characteristics of mesenchymal stem cells

MSC main source was represented by bone marrow. However, in the last few years many other sources were identified and successfully employed for MSC isolation, such as peripheral blood [97], cord blood [98], cord Wharton's jelly [99], adipose tissue [100], amniotic fluid [101], compact bone [102], periosteum [103], synovial membrane [104], synovial fluid [105], articular cartilage [106] and foetal tissues [107]. Despite this abundance of tissue sources, MSC, especially human MSC, are mostly isolated from bone marrow and adipose tissue. In particular, bone marrow MSC (BM-MSC) derived from

aliquots of bone marrow obtained from normal donors undergoing marrow aspiration for purposes of allogeneic marrow transplantation, while adipose-derived MSC (A-MSC) derived from raw human lipoaspirates, which were digested with collagenase before plating in order to disaggregate tissue and obtain a single cell suspension. Importantly, since adipose tissue is much more accessible and abundant than bone marrow, purification of A-MSC is easier compared to that of BM-MSC and, most important, less painful for the donor. For this reason, research on A-MSC properties both *in vitro* and *in vivo* has gained much more attention in recent years.

BM-MSC are made up of at least 2 different subsets of cells: a population of small and agranular cells (RS-1) with a low proliferation capacity, and a more abundant, fast-growing population. It has been hypothesized that the former is represented by uncommitted progenitors while the latter is made up of more mature, committed progenitors. These RS-1 cells seem to maintain the expansion potential of committed progenitors and start proliferating in response to factors secreted by them. Thus, BM-derived MSC cultures consist of an ensemble of uncommitted and committed progenitors with different degrees of stemness [108]. Interestingly, it was observed that bone marrow stroma feeds them into distant mesenchymal tissues [109]. The uncommitted MSC must leave the marrow stroma as such or after undergoing either self-renewal or commitment, then they should transit in the peripheral blood in the search of their final destination: a proper microenvironment in a distant tissue where they can home, expand, and further differentiate [108]. This process occurs during the growing period of an organism [110, 111] as well as in adult life, during tissue remodeling and repair in case of injury or disease [112-114]. Importantly, it has been shown that stem cells are usually found within specific compartments, whose maintenance ultimately depends on cell autonomous regulators modulated by external signals. Such intrinsic regulators include factors controlling cell proliferation and expression of genes related with the uncommitted and committed stages. In turn, extrinsic signals that control

stem cell fate collectively make up the stem cell microenvironment or niche, which seems to be essential to provide and maintain *in vivo* stem cells characteristics and properties [115]. This niche involves a complex interplay of short- and long-range signals between uncommitted and committed stem cells and between them and neighboring cells. Distinct niches were identified within the bone marrow that support hematopoietic stem cells (HSC) survival and growth. Indeed, bone marrow niches provide the requisite factors and adhesive properties to maintain HSC viability, while facilitating an appropriate balanced output of mature progeny for the lifetime of an organism. Current models of HSC niche advocate two overlapping domains: the endosteal niche near bone surfaces as the primary location of dormant, quiescent HSC, and the (peri)vascular niche associated with the sinusoidal endothelium as the primary site of dividing, self-renewing HSC [116]. In the bone marrow niche, MSC appear tightly associated with HSC and sympathetic nerve fibres [117].

4.3 Isolation method

Independently from tissue sources, MSC isolation takes advantage of their capability to adhere to plastic when maintained in culture [118]. However, once more huge differences exist between human and murine MSC. Indeed, while the human MSC are easily isolated and extensively expanded in culture, the murine cells are far more difficult both to isolate and to expand. In contrast with human MSC, murine MSC cultures, especially those isolated from the bone marrow, are frequently contaminated by hematopoietic progenitors that overgrow the cultures. Furthermore, even after purification murine MSC cultures expand poorly [119-121]. Thus, many efforts have been made to set up new protocols for the isolation of murine MSC. Among them the use of monoclonal antibodies in order to pre-select cells with a MSC surface phenotype was employed. The methods vary from negative selection, where other cell types, such as hematopoietic cells, are removed [96], to positive selection, when MSC are directly enriched from a pool of other cells in which they are

known to be present [94]. However, the phenotypic plasticity that MSC demonstrate in vitro which was previously discussed, once more point out the need for specific marker sets in order to obtain enrichment and, ideally, purification of MSC subsets maintaining native properties.

4.4 Immunomodulatory properties of mesenchymal stem cells

Beside to display multilineage differentiation capacity, MSC exert strong anti-inflammatory and immunosuppressive effects on the main immune cell subsets through production of various soluble factors or by directly interacting with target cells. Both murine and human MSC derived from different tissue sources are considered tolerogenic, since they express MHC class I but not MHC class II antigens, and they lack the expression of costimulatory molecules, such as CD40, CD80 and CD86 [122, 123]. However, recent studies have demonstrated that MSC expression of MHC class II can be modulated by IFN-γ both *in vitro* and *in vivo*, but whether this results in an up-regulation or a down-regulation of the molecule is still unclear. Indeed, many authors reported an increase of MHC class II upon IFN-γ stimulation, which could elicit alloreactive lymphocyte proliferative responses [124, 125], while others showed the opposite, with high levels of IFN-γ decreasing MSC expression of MHC class II thereby causing loss in the ability of these cells to act as antigen presenting cells [126]. Thus, it is possible that MSC can change their immune suppressive functions according to their microenvironments, acting as immune suppressors or stimulators. It has been widely demonstrated that MSC exert immunomodulation by inhibiting the proliferation of IL-2 and IL-15 activated NK cells [127] and that of allogeneic lymphocytes. Indeed, MSC suppress the *ex vivo* expansion of γδ T cells without affecting their cytotoxic activity [128]. Furthermore, they can selectively abrogate the proliferation of CD4+ (helper) and CD8+ (cytotoxic; CTL) T cells and anti-CD40 or IL-4 stimulated B cells [129, 130]. Importantly, this inhibitory effect is ratio-dependent and seems to be mediated

mainly by production and secretion of soluble factors, such as IFN-γ [131]. Alternatively, it was proposed that MSC render lymphocytes anergic by a cell-to-cell contact mechanism via activation of PD-1/PD-L1 or PD-L2 pathway, which ultimately leads to apoptosis of T and B cells [132]. However, this hypothesis was subsequently contradicted by the observation that the proliferation of T cells efficiently resumed when restimulated with cellular or humoral activators in the absence of MSC [129]. Thus, so far soluble factors remain the most important mediators of MSC induced lymphocytes anergy. In addition to immunosuppression, MSC have been proved to efficiently modulate the functions of both T and B lymphocytes. Indeed, MSC can affect the production of both pro- and anti-inflammatory cytokines of CD4+ and CD8+ T cells, inhibiting TNF-α and IFN-γ secretion, restoring IL-4 and IL-5 expression and up-regulating IL-10 expression [133]. Interestingly, MSC are able to suppress the differentiation of CTL precursors into CTL effectors in a dose and time dependent manner. Indeed, it was demonstrated that addition of MSC to mixed lymphocyte culture late, after CTL have reached their cytotoxic phase, fails to produce suppressive effects [134]. In addition, MSC were also proved effective in abrogating NK cell proliferation and NK cell-mediated cylolysis induced by IL-2 by down-regulating their surface expression of the activating receptors NKp30, NKp44 and NKG2D [127, 135]. Of note, since MSC express MHC class I molecule, they can be recognized as targets by activated CTL and NK cells. However, data regarding MSC susceptibility to be lysed by them are extremely controversial, since many authors demonstrated that MSC are not lysed by CTL and IL-2/IL-15 activated NK cells [134], while others proved the contrary [136]. More research is therefore necessary to investigate the susceptibility of MSC for lysis by immune cells, since it is essential for the efficacy and the safety of MSC therapy. As previously mentioned, most of MSC immunosuppressive functions are ultimately mediated by soluble factors, which are produced following the activation of MSC by immune cells. In addition to TNF-α and LPS, MSC are activated by IL-1β and IFN-γ, which are produced by

monocytes and activated lymphocytes or NK cells, respectively [137]. Although new insights in this field have been gained, the identity of soluble factors produced by MSC as well as the mechanisms through which they act remain unclear. Among factors with immunomodulatory properties, PGE_2, IDO, IL-10, M-CSF, IL-6, NO, HLA-G5, HGF and TGF-β1 have been recently identified. Interestingly, TGF-β1 and HGF were demonstrated to work in a synergistic manner, blocking T cell proliferation and therefore causing immunosuppression [138]. Similarly, IL-10, PGE_2 and NO were shown to produce immunosuppressive effects. In addition, PGE_2 also strongly inhibits dendritic cells (DC) differentiation [139]. IL-6 and M-CFS were shown to retain the monocyte or macrophage immunophenotype (high CD14 levels and low CD1a levels), blocking their differentiation into DC [140]. Furthermore, the soluble isoform of the nonclassical MHC class I molecule HLA-G (HLA-G5) secreted by MSC was shown to inhibit the cytolytic activity of NK cells and CTL, to shift the T cell response to Th2 cytokine types and to induce the expansion of regulatory T cells (T reg) [141]. Interestingly, T reg (CD4+ CD25+ FoxP3+) represent specialized subsets of T cells with the capacity to suppress T cell responses, and are involved in both autoimmune reactions and graft rejection. MSC were demonstrated to increase the number of T reg both *in vitro* and *in vivo* probably through secretion of soluble factors. However, it is still not clear whether MSC contribute to the expansion of the already existing T reg, or induce new regulatory cell populations from the progenitor naïve T cells [122]. One of the most characterized immunosuppressive factor produced by MSC is represented by the enzyme indoleamine 2,3-dioxygenase (IDO). This enzyme degrades tryptophan, which is crucial for cell proliferation. Thus, IDO produced by MSC was demonstrated to block the proliferation of both T cells and NK cells, the last acting synergistically with PGE_2 [135, 142]. Interestingly, recent findings suggest that this enzyme also induces generation of T reg [143]. Importantly, even though the aforementioned soluble factors were demonstrated to be crucial, it cannot be excluded that cell-to-cell contact also

contributes to provide MSC immunosuppressive functions. Indeed, it was recently demonstrated that the immunomodulatory effects of MSC on DC during LPS activation or antigen loading are dependent on cell-to-cell contact interactions. Furthermore, MSC were shown to down-regulate the expression of MHC class II and the costimulatory molecules CD80 and CD86, and decrease the secretion of IFN-γ, TNF-α, IL-2 and IL-12 by mature DC. This ultimately results in the modulation of the balance between Th1 and Th2 cells in favor of the latter [140]. It is still not clear whether these effects are produced by merely interaction of MSC with DC or soluble factors are also required. Of note, DC are the gatekeepers to the immune system of the human body, thus it seems likely that by suppressing the function of DC through the limitation of antigen uptake, processing or presentation, it could rapidly achieve a strong immunosuppression, blocking the activity of ideally all effector cells. Importantly, the effects of MSC on DC are reversible and thus avoid the complications of long-lasting hypoimmune competence following MSC transplantation [122].

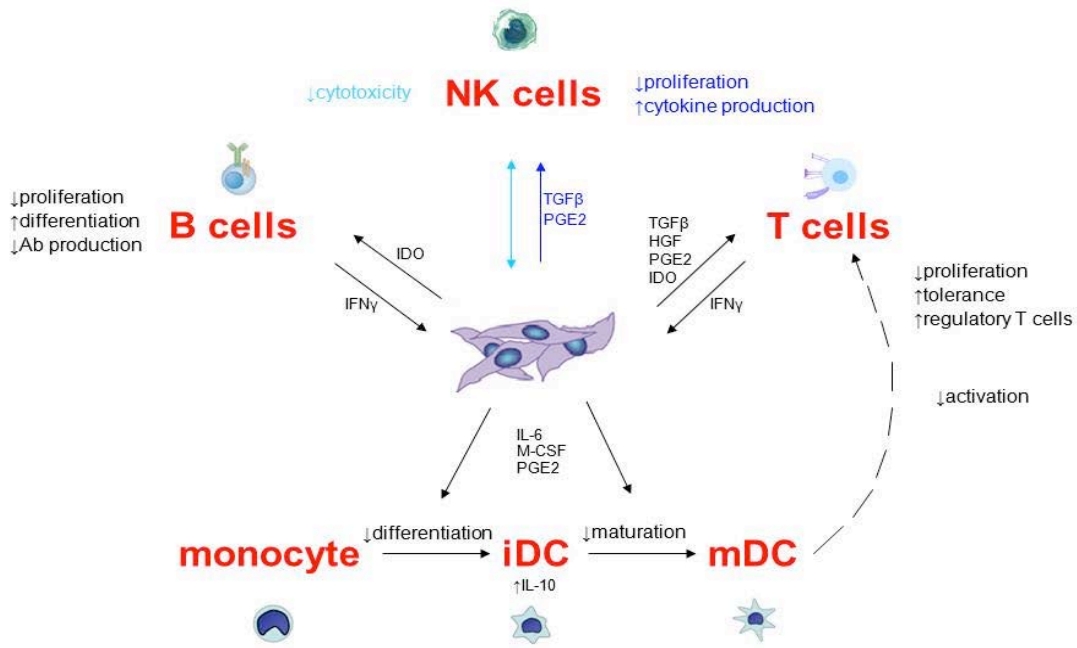

Figure 3: Mechanisms of MSC immunomodulation.

Schematic representation of MSC immunomodulatory properties. Adapted from Alma J Blood 2007.

4.5 The use of mesenchymal stem cells for therapy

Since MSC have the capacity to promote angiogenesis, differentiate to produce multiple types of connective tissue and down-regulate an inflammatory response, MSC have been extensively studied as a new therapeutic approach for many disorders. Initially MSC have been used in HSC transplantation, for enhancement of hematopoietic engraftment and for treatment/prevention of graft versus host disease [144]. Progressively after *ex vivo* expanding, MSC have been explored to regenerate damaged tissue and treat inflammation, resulting from cardiovascular disease and myocardial infarction, pulmonary diseases, brain and spinal cord injury, stroke, diabetes, pancreatitis, cartilage and bone injury.

Despite the beneficial properties, for clinical application, several potential risks should be considered, such as the immunogenicity of the cells, the biosafety of the medium components, the risk of ectopic tissue formation, and the potential *in vitro* transformation of the cells during expansion. Although MSC are considered to be immunoprivileged, infusion of allogeneic MSC into MHC-mismatched mice was recently demonstrated to induce an immune response, resulting in their rejection [145]. On the contrary, the infusion of syngeneic host-derived MSC resulted, in the same mouse model, in enhanced engraftment of stem cells [145]. Moreover, experimental evidences suggest that IL-2-activated autologous and allogeneic NK cells are capable of effectively lysing MSC, since the latter display ligands that are recognized by activating NK receptors that, in turn, trigger NK alloreactivity [146]. Despite these results, the majority of the clinical reports have suggested low immunogenicity of transplanted MSC in humans [80, 144]. However, on the basis of these experimental and clinical findings, some fundamental issues should be taken into consideration when determining the clinical application of MSC, including whether autologous or allogeneic cells should be employed, the state of immune competence of the patient at time of infusion, and the number of infusions needed to treat the patient. Importantly, since fetal calf serum (FCS) commonly used for *ex vivo* expansion of MSC has

been associated with the risk of transmission of zoonoses and potential immune reactions in the host [147, 148], alternative animal-free additives have been considered for clinical-grade expansion of MSC, such as platelet lysate (PL)/platelet rich plasma (PRP) [149]. One of the potential risks of MSC treatment involves the formation of mesenchymal tissues at ectopic sites, as observed in an experimental model of . myocardial infarction [150], glomerulonephritis [151], and GvHD [152]. Beside formation of mesenchymal tissues, intracerebroventricular (ICV)-transplanted MSC in experimental autoimmune encephalomyelitis (EAE) were found migrating into the brain parenchyma and, depending on their density, forming cellular masses characterized by focal inflammation, demyelination, axonal loss and increased collagen-fibronectin deposition. These masses, formed by MSC at inner layers, T cells and B cells, were present only inanimals ICV MSC-transplanted which carried severe EAE. Importantly, the mechanism underlying the formation of these masses involved massive migration and subsequent accumulation at high density of ICV transplanted MSC in the brain parenchyma. Here, they may determine the excessive host reaction, which ultimately leads to masses formation [153]. However, despite these worrying results, no ectopic tissue formation has been observed following MSC transplantation in clinical trials, even if a strict and long-term follow-up of patients treated with MSC still lack. Fortunately, it was demonstrated that human MSC of various tissue origin can be cultured for long period without losing their usual phenotypical/functional characteristics and without developing chromosomal aberrations. Indeed, MSC malignant transformation in *ex vivo* expanded human MSC is likely to be an extremely uncommon event, estimated to be in the frequency of $<10^{-9}$ [154]. However, a genetic characterization of MSC through conventional/molecular karyotyping should be considered before release of them for clinical application.

4.6 Proposed mechanisms of action involved in the efficacy of mesenchymal stem cells therapy

Acute myocardial infarction

One of the most well characterized field of application of MSC therapy is represented by acute myocardial infarction (MI), in which systemically delivered MSC were demonstrated to specifically home to injured site via SDF-1-CXCR4 interaction [155]. Indeed, CXCR4 expressed on MSC surface binds to SDF-1, whose expression is up-regulated in ischemically damaged myocardium. Once engrafted, MSC strongly improved heart function through a number of different mechanisms operating alone or in concert. Indeed, MSC were shown to restore the depleted stem cell pool at the site of injury; to differentiate into myocytes, endothelial cells and vascular smooth muscle cells; to enhance the signaling from the injury site to attract more endogenous cardiac stem cells; to induce mechanical strengthening of the infarct scar, preventing on-going left ventricular (LV) remodeling and allowing endogenous repair mechanisms to improve LV function; to modulate the inflammatory response to acute MI, thereby limiting the amount of initial tissue damage [155]. In addition, MSC were shown to produce a variety of cardio-protective signaling molecules. Among them, insulin-like growth factor (IGF-1) released by MSC was shown to enhance c-Kit expression in neonatal cardiomyocytes (NCM), leading to an increase in NCM proliferation potential, which ultimately contributes to myocardial regeneration [155].

One recent study demonstrated that part of the therapeutic effects which MSC exert in MI could be due to their production and secretion of TNF-α stimulated gene/protein 6 (TSG-6), which ameliorated the clinical course of MI by decreasing both pro- and active MMP9 levels, which leads to the reduction in granulocyte and monocyte infiltrating the ischemic heart [156].

Pulmonary diseases

It has demonstrated that systemically delivered MSC could be beneficial in the treatment of an experimental mouse asthma model, which is characterized by an allergic, Th2 dominant environment [157]. Indeed, when injected at the time of antigen challenge, MSC inhibited eosinophil infiltration and excess mucus production in the lung, decreased levels of Th2 cytokines (IL-4, IL-5 and IL-13) in bronchial lavage, and lowered serum levels of Th2 immunoglobulins (IgG1 and IgE). Interestingly, these effects were mediated by IL-4 and/or IL-13 activation of the STAT6 pathway in MSC, which results in an increase of their TGF-β production. In addition, MSC were also able to specifically recruit T reg in inflamed lung , which contribute to dampen the allergic response [157]. Recently, MSC were also found effective in the treatment of acute lung injury (ALI) [158]. In ALI experimental model, the treatment with MSC significantly reduced the expression of pro-inflammatory cytokines and chemokines, neutrophil counts and total protein in bronchoalveolar lavage (BAL) 24 and 48h after LPS exposure, which leads to a reduction in pulmonary edema. Importantly, the anti-inflammatory effects of MSC in this experimental model of lung injury were not dependent on their localization to the lung, as when administered via ip they were also able to attenuate ALI, but on TSG-6 secretion. Indeed, knockdown of TSG-6 expression in MSC abrogated most of their anti-inflammatory effects, while intra-pulmonary delivery of recombinant human TSG-6 reduced LPS-induced inflammation in the lung similarly to MSC administration [158]. Finally, in a bleomycin-induced lung injury model, intravenous infusion of murine MSC decreased the inflammatory response to bleomycin and prevented the lungs from developing fibrosis [159]. The beneficial effects of MSC were largely explained by the cells being activated to secrete IL-1 receptor antagonist (IL-1ra), which inhibited the production of TNF-α by IL-1a activated macrophages. Importantly, MSC were effective only when administered at the same time as the bleomycin and not at later time points, since their action was exerted in the initial phases of the injury [159].

Kidney diseases

MSC were also successfully employed for the treatment of kidney diseases. Indeed, in an experimental model of acute kidney injury (AKI), administration of MSC six hours after damage determine a reduction in the serum creatinine compared to non-treated animals [160]. Furthermore, the improvement in renal function was followed by a lower expression of IL-1β, IL-6, TNF-α and higher expression of IL-4 and IL-10, which indicates that MSC treatment promoted the shift from a Th1 to a Th2 inflammatory profile. Although these effects seem to be mediated by paracrine factors secreted by MSC, their identity remains unknown. Interestingly, the immunomodulatory effects exerted by MSC occur at very early time point, that is within 24h after their injection. Thereafter, their therapeutic efficacy progressively reduced, until coming to exhaustion [160]. Another study demonstrated that systemically injected MSC were able to induce kidney allograft tolerance [143]. Thus, Balb/C recipients were administered with MSC 24h after receiving a life-supporting orthotopic C57Bl/6 renal graft. MSC injection was proved to be effective in maintaining a normal histology of the transplanted kidney and undetectable antidonor antibody levels. Furthermore, tolerant recipients demonstrated increased circulating kynurenine levels, significantly high frequencies of tolerogenic DC, strongly impaired CD4+ T-cell responses and Th2-dominant cytokine shift. In addition, high frequencies of T reg were found both in recipient spleens and in donor grafts. Importantly, since MSC isolated from IDO knockout mice failed in achieving allograft tolerance, this soluble factor was supposed to be responsible, at least in part, for reduction of graft rejection, possibly through generation of T reg [143].

Rheumatoid arthritis

In an experimental model of rheumatoid arthritis, the collagen-induced arthritis (CIA), human MSC delivered systemically were tested both in a prevention and treatment setting [161]. In both cases, MSC significantly reduced the incidence and severity of experimental

arthritis, inhibiting the production of various inflammatory mediators, decreasing antigen-specific Th1/Th17 cell expansion, and inducing IL-10 secreting T reg, which, in turn, suppressed the self-reactive T cells. Interestingly, this study is consistent with others which demonstrated that after systemic administration MSC did not localize to the joints, but exert therapeutic effects at distance by inducing the "reset" of the immune system [161].

Multiple sclerosis

In a recent study, the therapeutic effects of human MSC derived from placenta, was evaluated in experimental autoimmune encephalomyelitis (EAE) [162]. In this murine model of MS, MSC were intracerebrally transplanted 5 or 10 days after EAE induction and then monitored for a month. Transplanted animals displayed an increase in the survival and a reduction in the disease severity, compared to controls. *In vitro* experiments demonstrated that conditioned media from LPS-activated astrocytes, as well as TNF-α or IL-1β, stimulated MSC to express TSG-6, which could be responsible for the observed therapeutic effects *in vivo* [162]. In order to efficiently treat nervous system injury, a recent study investigated the possibility to enhance human MSC trophic effects by forcing them to adopt *in vitro* characteristics of Schwann cells (SC), which are known to provide trophic support for regenerating axons [163]. The induced MSC (sMSC) adopted a SC-like morphology, expressed SC-specific proteins and secreted higher amounts of several growth factors, such as HGF and VEGF when compared with uninduced MSC. Importantly, transplantation of sMSC in an *ex vivo* model of spinal cord injury, dramatically enhanced axonal outgrowth, which was mediated by HGF and VEGF secretion, and also decreased cell death [163]. These results demonstrated that *in vitro* manipulation of MSC could be a feasible approach to improve their therapeutic properties and therefore to better target specific pathologies.

Corneal injury

MSC treatment was successfully employed in a chemical and mechanical model of sterile injury to the cornea, where MSC administered both iv and ip were shown to reduce

inflammation, by decreasing neutrophil infiltration, production of pro-inflammatory cytokines, and development of the opacity in the cornea [164]. Importantly, MSC reduced inflammatory damage without engrafting into the tissue, but secreting TSG-6. Indeed, MSC with an siRNA knockdown of the TSG-6 gene were not effective, while systemically administration of recombinant human TSG-6 largely duplicated the beneficial effects of MSC [164].

Pancreatic diseases

The therapeutic potential of this class of stem cells was also investigated in diabetes, in which pancreatic β islets co-cultured *in vitro* with either MSC or MSC conditioned medium (MSC-CM) demonstrated lower ADP/ATP ratios, higher glucose stimulated insulin secretion (GSIS) indexes and increased viability [165]. *In vivo*, diabetic mice that received islet transplants cultured in MSC-CM for 48h showed significantly lower blood glucose levels and enhanced blood vessel formation. Importantly, MSC were demonstrated to increase islet survival and function after transplantation by secreting a variety of trophic factors. Indeed, besides IL-6, IL-8 and TGF-β, which are mainly involved in immunomodulation, high levels of VEGF-A and HGF were also detected in MSC-CM [165]. In the field of pancreatic disorders, MSC were also studied as a stem cell-based therapy in an experimental model of edematous and necrotizing acute pancreatitis (AP) in rats [166]. Infusion of MSC 24h after the induction of the AP dampens inflammation at the level of histomorphology, edema, activation of digestive enzymes, and infiltration of inflammatory cells into the pancreas. *In vivo* tracking techniques revealed active homing of MSC to the injured pancreas, that strongly correlated with the severity of the pancreatitis. The mechanism proposed for such a therapeutic efficacy involved multiple aspects of the inflammatory response. Indeed, transfusion of MSC significantly reduced production of pro-inflammatory cytokines and increased the production of anti-inflammatory ones, both locally and systemically. Furthermore, MSC specifically recruited T reg to the inflamed

pancreas, which are ultimately considered the mediators of the MSC induced immune modulation during AP [166].

Peritoneal injuries

The possibility to apply MSC therapy to an experimental model of peritoneal injury, induced by mechanical scraping of the cecum surfaces, was also addressed [167]. Here, systemically injected rat MSC 24h after damage reduced adhesion formation, infiltration of neutrophils, macrophage proliferation and stimulated the repair of peritoneal mesothelial cells. Since MSC-conditioned medium reproduced the therapeutic effects of the cells, and MSC injected via iv mainly accumulated in the lungs, it was clear that production and secretion of soluble factors instead of migration and differentiation into injured cells was the mechanism through which MSC exert their effects. In particular, TSG-6 was identified as the factor that made a major contribution to the therapeutic benefits of MSC in this experimental model [167]. MSC therapeutic efficacy was studied also in the context of systemic inflammation. In particular, therapy with systemically delivered MSC was proved to be beneficial in the treatment of an experimental model of sepsis, induced by cecal ligation and puncture (CLP) [168]. Indeed, MSC administration to mice before or shortly after inducing sepsis reduced mortality and improved organ function. Importantly, also in this model MSC delivered via iv mainly localized to the lungs, where they became activated by TNF-α to secrete prostaglandin E_2 (PGE_2). PGE_2, in turn, specifically interacted with prostaglandin EP2 and EP4 receptors expressed on macrophages, reprogramming them to a less inflammatory, IL-10 secreting phenotype [168]. In an experimental model of zymosan-induced peritonitis, systemically administered human MSC were shown to significantly decrease the amount of neutrophils and monocytes/macrophages in the peritoneal cavity [169]. These anti-inflammatory effects were due to MSC secretion of TSG-6, which interacted with CD44 receptor on resident macrophages, decreasing zymosan/TLR2-mediated nuclear translocation of the NF-kB, and the subsequent production and secretion

of the pro-inflammatory cytokines TNF-α and IL-1a. Since macrophages were the first cells activated to produce pro-inflammatory cytokines, their blockage by MSC through TSG-6 production created a negative feedback loop, which attenuated the inflammatory cascade that is initiated by resident macrophages and then amplified by mesothelial cells and probably other cells of the peritoneum [169].

Intestinal diseases

MSC treatment was demonstrated effective in increasing and accelerating the recovery of the small intestine with reversible alterations and extended the life of animals developing irreversible gastrointestinal damages [170]. Importantly, MSC effects were a consequence of their ability to enhance or maintain the re-epithelization process, by both increasing endogenous proliferation and inhibiting apoptosis of intestinal epithelial cells. Furthermore, MSC brought about fast recovery of epithelial secretory and absorptive response, which were both strongly damaged by radiation, leading to the re-establishment of an anionic gradient across the epithelial membrane [170]. In line with this, a second study showed that both human and murine adipose-derived MSC significantly ameliorated the clinical and histopathological severity of DSS-induced colitis, abrogating weight loss, diarrhea and inflammation, and increasing survival [171]. These therapeutic effects were associated with downregulation of the Th1-driven inflammatory responses. Indeed, MSC diminished inflammatory infiltration in the mucosa and induced monocytes/macrophages to change their phenotype in a less inflammatory one, resulting in a local reduction of pro-inflammatory cytokines and chemokines and in an increase of IL-10 production. Importantly, MSC were also demonstrated to induce IL-10-secreting T reg. These therapeutic effects seem to be completely independent from MSC homing to inflamed gut, since it was detected only a transient engraftment of injected cells in the intestine that peaked at day 2 post-injection, with 15 transplanted MSC/mg of tissue [171].

4.7 Mechanisms to increase gut homing of mesenchymal stem cells

Thus, increasing data demonstrated that systemically delivered MSC poorly engrafted into injured gut, exerting therapeutic efficacy via production and secretion of still unidentified soluble factors, which exert anti-inflammatory and/or trophic activities. These factors are produced by systemically delivered MSC far away from injured sites, especially in the lungs, and then reached damaged intestine via bloodstream. However, whether injected MSC could be specifically directed to gut, in situ production of therapeutic factors would probably increase both their safety and therapeutic efficacy. In particular, it was demonstrated that MSC cultured under sublethal hypoxic conditions increased survival and upregulated CXCR4 expression [172]. Since CXCL12, the ligand of CXCR4, is expressed in the intestine and CXCR4-expressing cells are recruited during inflammation in IBD, this approach could be effective in enhancing MSC gut homing *in vivo*. Alternatively, it was shown that MSC engraftment into intestine could be induced by pre-coating cells with antibodies [173]. In this study, MSC were treated with palmitated protein G and then coated with anti-VCAM-1 or MadCAM-1 antibodies. When injected into DSS-treated mice, an increase in VCAM-1-coated MSC engraftment occurred in the colon. Moreover, both MadCAM-1-coated and VCAM-1-coated MSC significantly increased survival of colitic mice compared to no treatment, MSC alone, or MSC co-injected with free MadCAM-1 or VCAM-1 antibodies. Another valid approach, which removes the need for cell homing to injured site avoiding *in vitro* cells manipulations, is represented by local administration of MSC. Indeed, MSC injected into the colonic submucosa of rats previously injured with TNBS, were shown to decrease lesion size compared to controls [174]. Furthermore, in a model of ischemia-reperfusion injury, local MSC administration was shown to decrease pathology scores and bacterial translocation in rats [175]. In addition, local MSC injection in mice damaged by irradiation was found to increase their survival and histological scores [176]. Finally, data supporting the efficacy of local MSC transplantation for the treatment of

intestinal diseases come also from clinical trials, as previously described [81]. Interestingly, it was recently demonstrated that MSC culture conditions could also strongly influence their therapeutic potential. Indeed, human MSC cultured as 3D aggregates or as spheroids were self-activated to express anti-inflammatory proteins, such as TSG-6, stanniocalcin-1 and PGE_2, and three anti-cancer proteins, that is IL-24, TNF-α related apoptosis inducing ligand (TRAIL) and CD82 [177]. Furthermore, the spheroid MSC were demonstrated to be more effective than MSC from adherent monolayer cultures in suppressing inflammatory responses both *in vitro* and *in vivo*. In addition, thanks to their reduced volume compared to MSC from adherent cultures, they displayed lower entrapment in the lungs after iv infusion, and were therefore recovered in spleen, liver, kidney and heart [177]. Thus, culturing MSC as spheroids could represent a valid approach to enhance both their anti-inflammatory properties and their engraftment into tissues. Finally, time also exert a deep impact on MSC therapeutic efficacy. Indeed, MSC treatment was shown to be effective in many different experimental models of human pathologies, including gut disorders, only when delivered soon after injury, since they are supposed to modulate/block the early inflammatory response, avoiding its propagation and perpetuation. In addition, MSC therapeutic effects were exerted within few hours after their injection, then they progressively reduced, until coming to exhaustion. Thus, despite many advances have been made in the field of MSC-based therapy, we are still far from a detailed knowledge of MSC *in vitro* properties and *in vivo* behavior. One of the most important outstanding question regards the long-term fate of these cells once implanted, which strongly correlates with both their safety and efficacy. Importantly, the recent discovery that MSC therapeutic efficacy could be linked to production and secretion of soluble factors, instead of migration and differentiation into injured cells, gives rise to the possibility to move from a MSC-based therapy towards a factor(s)-based therapy. The latter approach would be more and more useful, since it would allow to set up a specific therapeutic regimen, formed by specific factor(s), depending on

the pathology, and to better predict both its therapeutic efficacy and its side effects. Furthermore, the half-life of the factor(s) in an organism would be easily determined, which make this kind of therapy much safer than a cell-based one. Thus, few years ago it was thought that MSC-based therapy would have represented the future, however the possibility that they could only represent a tool for the identification of new potent anti-inflammatory molecules which may be efficiently and safely used in therapy is now becoming more and more convincing.

II. OBJECTIVES

Although the etiology is still unknown, studies have provided evidence that both forms of IBD are a result of a genetic predisposition that leads to a mucosal immune regulatory cell defect, barrier defects and susceptibility to environmental triggers, including luminal bacteria and specific antigens which induce to chronic gut inflammation [1, 178, 179]. The quality of life for IBD patients is lower compared to the normal population and is inversely related to active disease, hospitalization and surgery. Drug therapy has been limited to immunosuppressant and corticosteroid use until recently, with many adverse effects or complaints from patients. Absence of well-defined aetiological factors and unclear mechanisms have limited the development of new therapeutic tools. In the last years many studies have expanded the understanding of these diseases and fortunately new targets have been identified as possible new therapies in an attempt to avoid chronic steroid usage, to prevent disease progression and to eliminate the need for surgery [180]. Novel therapies now consist of monoclonal antibodies, small molecule inhibitors, peptides, and vaccines [181]. Despite these advances, however, only a small percentage of patients benefit from these novel therapies, and clear limitations of the new therapeutic approaches exist. However, none of these treatments are curative for IBD, and a relevant number of patients are refractory or intolerant to many pharmacological approaches. Altered control of intestinal immune cell function and turnover appear to be crucial factors responsible for the deregulated inflammatory response in IBD patients. Indeed, immunosuppression is a central component of inflammatory bowel disease treatment [1, 182, 183]. Clinical studies conducted in CD patients refractory to pharmacological treatments have demonstrated that a high dose of immune ablation followed by hematopoietic stem cell transplantation (HSCT) induced and maintained the clinical remission in these patients [184-189]. Although clinical reports are encouraging, however, this approach remains invasive for the patients.

Furthermore, it is important to consider the profound alterations in barrier function that can also occur as a consequence of transplantation procedures and are associated with colitis might predispose individuals to a more severe systemic sepsis [190]. Treatment with mesenchymal stem cells, on the contrary, is considered an innovative, safe, non-invasive, non myeloablative therapeutic approach for IBD [191, 192]. MSCs maintaining the pluripotent cellular capacity of multilineage differentiation, possess valuable characteristics for tissue repair or regeneration [170, 193]. These cells, indeed, have been shown to functionally integrate and remodel bone, cartilage and myocardial tissues [194-196]. In addition, MSCs are not immunogenic, possess the ability to modulate the immune response and inhibit T-lymphocyte activation [142, 197]. Accumulated data indicates that in contrast to the strong immunosuppressive effects induced by drugs or myeloablation, MSCs do not completely inhibit immune cell function. These properties could enable MCS-based therapy as an alternative therapeutic approach to HSC transplantation in IBD treatment.

Results from the phase 2 clinical trials indicate that MSC treatment reduces the disease severity in Crohn's disease patients with a positive correlation between dose and response. Furthermore MSCs injected directly in the site of the lesions have shown a complete closure of the wound without any adverse events [191, 198]. Although these clinical results are encouraging and promising for a future cellular therapy in the treatment of IBD, the molecular mechanisms underlying MSC beneficial effects have not been clarified. The results from multiple clinical trials using systemically administered MSC give rise to critical challenges that must be addressed to better understand the therapeutic potential of MSC. Based on this background, the objective of my thesis was to explore in experimental model of IBD the mechanisms through which MSC exert therapeutic efficacy in inflamed intestine.

In order to assess the function of MSC in the treatment of IBD, I decided to divided this aim in two main tasks:

1. Isolation and in vitro characterization of MSC.

2. Investigation of the mechanisms involving the therapeutic effects of mesenchymal stem cells in the inflamed gut.

1. Isolation and in vitro characterization of MSC

The first task of this study was to set up and standardize a new protocol for murine MSC isolation for obtaining a pure MSC population. The isolation protocols already existing and described in literature are based on MSC property to adhere to plastic [199, 200]. Unfortunately in our hand once put in culture, these cells appeared as mixed cultures, in which MSC coexisted with HSC, therefore the first step was to resolve this aspect optimizing an efficient and alternative method. So far, bone marrow represents the main source of MSC, even if many others have been discovered and exploited in the last years, as previously described [97-107]. In particular, the main disadvantage of using bone marrow to isolate MSC is represented by the very low frequency of the cells within it (1 MSC every 100000/1000000 bone marrow cells). Since the adipose tissue provides one of the most tempting alternative source to bone marrow thanks to its accessibility, the second step of this task was to isolate both bone marrow (BM) and adipose (A) MSC taking advantage of the new protocol, and compare their in vitro properties and in vivo therapeutic effects, in order to choose the "best" MSC line in term of phenotype, differentiation potential, immunosuppressive properties and therapeutic efficacy in the treatment of experimental colitis.

2. Investigation of the mechanisms involving the therapeutic effects of mesenchymal stem cells in the inflamed gut

Given that MSC therapeutic potential is attributed to their property of specifically homing to damaged tissues and inhibition of inflammatory responses at target sites facilitating repair of

the damaged tissue [155, 166], the aim of this task is to elucidate whether MSC exert therapeutic efficacy by migrating into inflamed gut and differentiating into damaged cells therefore promoting tissue repair, or by producing and secreting soluble factors with trophic and/or anti-inflammatory effects, or both.

III. RESULTS

AIM 1. ***Isolation and in vitro characterization of MSC***

Isolation of mesenchymal stem cells

MSC isolation protocols already existing take advantage of their capability to adhere to plastic when maintained in culture [118]. Unfortunately once put in culture, these cells appeared as mixed cultures, in which MSC coexisted with HSC, therefore the first step was to resolve this aspect optimizing an efficient and alternative method. The possibility to use monoclonal antibodies in order to pre-select cells with a MSC surface phenotype by flow cytometric cell sorting was already explored. In particular the methods vary from negative selection, where other cell types, such as hematopoietic cells, are removed [96], to positive selection, where MSC are directly enriched from a pool of other cells in which they are known to be present [94]. The idea of combining negative and positive selection in a new cell sorting protocol comes from the need to obtain MSC culture with high degree of purity. For this reason MSC were sorted for Lin- and CD31- in order to eliminate hematopoietic and endothelial contaminants, respectively, and for Sca-1+ to enrich the culture of stem cells. The only concern was represented by the high mortality rate of this technique [201]. However, in our experience MSC isolated utilizing this new protocol appeared vital, and displayed a typical MSC phenotype and bi-lineage differentiation potential. Therefore, cell sorting is a valid approach to isolate MSC, and the combination of positive and negative selection represents a successful strategy in order to obtain highly pure MSC culture.

MSC isolated from bone marrow and adipose tissue displayed similar phenotypes but different morphology and differentiation capacity

MSC were isolated from both bone marrow and adipose tissue of 4 to 6 week-old

C57BL/6N and C57BL/6-Tg(UBC-GFP)30Scha/J mice, expanded in complete MSC expansion medium and sorted for Lineage Cell Detection Cocktail-, CD31- and Sca-1+. Before sorting, the mixed population of bone marrow cells displayed high contamination of both hematopoietic (Lineage+) and endothelial (CD31+) cells and a very low percentage of MSC (0.6%, Figure 1A). On the contrary, the mixed population of adipose-tissue derived cells contained 96.7% of MSC and low hematopoietic and endothelial cell contamination (Figure 1B).

Since it is still not possible to unequivocally identify MSC with a single surface marker, both sorted cells were further phenotypically characterized for surface-marker expression levels of a selected protein panel by flow cytometry [95, 96]. Sorted BM- and A-MSC expressed neither the hematopoietic surface markers Lineage Cell Detection cocktail, CD45, CD34 and CD117 (C-kit), nor the endothelial surface marker CD31, while both lines stained positive for Sca-1, CD44 and CD106 (VCAM-1) as it is possible to observe in Figure 1A and 1B. Interestingly, CD90 (thymocyte differentiation antigen 1 (Thy-1) marker was higher in A-MSC than in BM-MSC (72.3% versus 13%, respectively).

BM- and A-MSC used for the experiments were from early passages after sorting, to avoid cellular senescence and consequent alteration of their properties [202]. Confocal analysis of BM- and A-MSC grown on PolyLysine pre-coated glass coverslips demonstrated that they displayed different morphological characteristics in terms of size and shape (Figure 1C). Furthermore, once put into culture and prompted to differentiate, A-MSC efficiently generated adipocytes after few days of culture under standard in vitro differentiating conditions, but they poorly differentiated into osteoblasts, even after 3 weeks of culture. On the contrary, BM-MSC were equally able to generate osteoblasts and adipocytes (Figure 1C).

BM-MSC ameliorated DSS-induced acute colitis more efficiently than A-MSC

MSC have already been shown to exert therapeutic efficacy in many experimental models for human pathologies, including chronic and acute colitis [171]. In order to compare the therapeutic efficacy of the two MSC lines isolated in the treatment of DSS-induced acute colitis, colitic mice were injected intraperitoneally with $3x10^6$ of either BM- or A-MSC 5 days after the beginning of DSS treatment. Colitic mice injected with saline and healthy mice injected with either BM- or A-MSC at day 5, respectively, represented positive and negative controls. BM-MSC administration considerably reduced body weight loss of colitic mice and significantly improved their disease activity index (DAI) starting from 48 hours after the injection (day 7) compared to positive control mice. Although A-MSC injection also promoted a reduction of body weight loss of colitic mice and an improvement in their DAI, it appeared less effective in ameliorating DSS-induced acute colitis compared to BM-MSC administration. No clinical effect in terms of both body weight changes and DAI were detected in the negative control group (Figure 2A and 2B). At day 10 mice were sacrificed and colon length measurements revealed a reduction in colon shortening in colitic mice treated with BM-MSC compared to positive control mice, while colons of colitic mice injected with A-MSC displayed an intermediate length between saline and BM-MSC treated group (Figure 2C). A blinded pathologist scored histological sections of formalin-fixed and paraffin-embedded colons stained with hematoxylin and eosin. BM-MSC administration strongly reduced the inflammation score and the extension of DSS-induced injuries of the colon, while A-MSC injected mice once more displayed an intermediate inflammation score and grade of involvement between saline and BM-MSC treated group (Figure 2D). Representative images of colon sections stained with hematoxylin/eosin for each group not only confirmed the differences in the extension of DSS-induced injuries of the colons but also revealed that epithelial organization into crypts of colitic mice treated with BM-MSC was partially maintained, due to the presence of less inflammatory cells infiltrating the

tissue (Figure 2D). Although no significant differences were detected among groups in terms of cytokine (IL-6, TNF-α, IFN-γ, IL-10) and chemokine (RANTES, MIP-1α, KC) levels, BM-MSC administration partially reduced both IL-6 and KC production (Figure 2E). These results suggested that although phenotypically similar, MSC isolated from bone marrow exert in vivo more immunomodulatory effects in the treatment of experimental colitis compared those from adipose tissue.

BM-MSC displayed *in vitro* a stronger immunosuppressive activity compared to A-MSC

It was demonstrated that MSC exert immunomodulatory properties *in vitro* [122]. Therefore, BM- and A-MSC were tested *in vitro* for their immunosuppressive properties by performing co-culture experiments with activated splenocytes (SPLs) at different SPLs:MSC ratios. BM-MSC efficiently inhibited proliferation of activated SPLs even when highly diluted (SPLs:MSC ratio 50:1), while A-MSC lost their immunosuppressive effects at a SPLs:MSC ratio of 10:1 and 50:1.

Altogether the results of this aim demonstrate firstly that the combination of positive and negative selection allows for the obtainment of a pure and viable population of MSC without altering their properties. Secondly, the expression of positive marker surfaces including Sca-1, CD90, CD106 and CD44 on MSC is not related to activities or properties of MSC. Finally, the isolation source of MSC affects *in vivo* and *in vitro* immunomodulatory properties indicating thus that the therapeutic efficacy of these cells can be source-dependent. Therefore, Since BM-MSC appeared more effective than A-MSC in the treatment of DSS-induced colitis, we decided to focus on BM-MSC in order to clarify the mechanisms underlying their therapeutic efficacy.

AIM 2. ***Investigation of the mechanisms involving the therapeutic effects of mesenchymal stem cells in the inflamed gut***

BM-MSC did not engraft into the inflamed colon but remained in the peritoneal cavity forming cellular aggregates

In order to follow the movements of MSC and shed light on the mechanisms by which MSC exert therapeutic efficacy in the treatment of DSS-induced colitis, a BM-MSC line was generated from C57BL/6-Tg(UBC-GFP)30Scha/J MICE. Healthy and colitic mice at day 5 of DSS treatment were injected intraperitoneally with $3x10^6$ GFP-MSC. Colons were collected 24, 48, 96 and 120 hours after the injections from all mice and analyzed for the expression of GFP by flow cytometry. No GFP positive cells were detected in the colon of colitic mice treated with saline or in the colon of healthy mice treated with GFP-MSC. Importantly, GFP-MSC positive cells appeared in the inflamed colon of colitic mice only at 48 hours after injection and with a very low frequency (<1%). Furthermore, no GFP-MSC positive cells were found in the inflamed colon at the other time points tested (Figure 4A). Representative flow cytometry dot-plots at 48 hours of GFP-MSC distribution in the colons for each group are reported in Figure 4A. In a parallel experiment, MLNs, liver spleen and lungs were also investigated for the presence of engrafted GFP-MSC 24, 48, 96 and 120 hours after the injection, but no GFP+ cells were detected at any time point tested. Poor engraftment of GFP-MSC into the inflamed colon was further confirmed by immunohistochemical analysis. Colon sections of colitic mice were stained with an antibody directed against GFP. Immunohistochemical staining showed that only a small amount of GFP+ cells were able to engraft into the inflamed gut, where they specifically localized either in the epithelial (Figure 4B) or endothelial cell layers (Figure 4C).

Once the low frequency of MSC in the inflamed colon was assessed, our principal concern was to elucidate the MSC fate after intraperitoneal administration. For this purpose, GFP or

DilC18(3)-DS pre-stained MSC were injected in colitic and healthy mice. After 24, 48 and 72 hours of injection, we performed a whole mount staining of the entire colon of treated mice. We observed that DilC18(3)-DS pre-stained MSC (red signal) did not egress from the peritoneal cavity but they aggregated within 72 hours and remained outside of the bowel wall vessels (in green CD31 endothelial marker) generating small aggregates. A representative image of a 72 hour structure is reported in Figure 5A. MSC-aggregates were recovered from colitic mice by peritoneal lavages 5 days after MSC administration, and they appeared as small high-density cell aggregates that resembled lymphoid organization both in shape and dimension (Figure 5B). The number and size of these aggregates were variable and not dependent on the inflammatory state. In order to better study their three-dimensional organization and cellular composition, we performed a whole mount staining of a single MSC aggregate. This analysis revealed that GFP-MSC formed the internal core of the MSC-aggregates surrounded by immune cells in particular macrophages (CD68 positive) and lymphocytes (CD3 positive) as reported in Figure 5C. FACS analysis of these aggregates has further clarified the cellular types recruited from MSC. Indeed, GFP-MSC represented the most abundant population (74%), followed by F4/80+ macrophages (14.2%), B220+ B lymphocytes (9.2%), CD3+ T lymphocytes (7%) and Ly6G+ polymorphonuclear cells (PMN; 6.6%; Figure 5D). In order to exclude that once injected in the peritoneal cavity the low migratory capacity of MSC was related to their poor viability, and that consequently the MSC-aggregates were the result of cellular necrosis, we stained the MSC-aggregates by both fluorescein diacetate and ethidium bromide staining, which mark in green viable cells and in red those dead (Figure 5E). The viability of cells within the MSC-aggregates was further confirmed by the observation that once put in culture, the cells spontaneously detached from aggregates and adhered to culture plates as demonstrated by FACS analysis performed on these cells (Figure 5F-G). Indeed, gating on Lin-, CD45- and CD31- to exclude hematopoietic and endothelial cells, respectively, more than 50% of cells detached

from the structure and adhered to the plate were GFP-MSC (Figure 5G).

In order to characterize the structure, MSC-aggregates were analyzed by two-photon confocal microscopy. Interestingly, this analysis revealed a strong deposition of extracellular collagen fiber within the MSC-aggregates as emerged from second harmonic generation signals (Figure 5H). The presence of collagen was further confirmed also by Sirius Red staining as reflected by red collagen fibers in Figure 5I.

Interestingly, MSC-aggregates were observed also in healthy mice and their organization as well as composition was similar to those of colitic mice.

BM-MSC expressed low levels of chemokine receptors and high levels of cell adhesion molecules

To verify whether BM-MSC were not successful in performing gut homing for the lack of expression of chemokine receptors and/or adhesion molecules, we analyzed the chemokine receptor expression profile of MSC by RT- qPCR and the expression levels of adhesion molecules by flow cytometry and immunofluorescence. Interestingly, MSC expression levels of CCRs receptors (from 1 to 9), as well as CXCRs receptors (from 1 to 7) and XCR1, CX3CR1 were very low compared to those of macrophages used as a positive control in this experiment (Figure 6A).

However, FACS analysis together with immunofluorescence staining demonstrated that BM-MSC expressed high levels of the adhesion molecules JAM-A, CD106, CD44 and CD29. A weak positivity was also detected for CD90, while negative for CD54 (Figure 6B).

BM-MSC therapeutic efficacy in DSS-induced acute colitis: action at a distance without significant engraftment into the inflamed colon

To investigate whether MSC had to be injected near the inflamed tissues in order to be therapeutically efficient, we administrated $3x10^6$ GFP-MSC subcutaneously (sc) on the

dorsal surface of mice after 5 days of DSS treatment; colitic mice injected with saline were used as a positive control. MSC administration via sc considerably reduced body weight loss of colitic mice (Figure 7A) and significantly improved their DAI (Figure 7B) starting from 48 hours after the injection (day 7) compared to positive control mice. At day 10 mice were sacrificed and MSC-aggregates were recovered from the subcutaneous cavity of MSC treated mice. FACS analysis revealed that their cellular composition was similar to that of MSC-aggregates recovered from the peritoneum, with 68% of GFP positive cells (Figure 7C). Representative hematoxylin eosin staining of these aggregates was reported in Figure 7D.

The treatment with encapsulated BM-MSC ameliorated DSS-induced colitis improving clinical parameters

To finally corroborate that MSC therapeutic efficacy was completely independent from their homing capability to the inflamed gut, we encapsulated MSC into microcapsules of barium alginate and evaluated their effectiveness compared to free MSC injection in the treatment of DSS-induced acute colitis. $3x10^6$ of either encapsulated or free MSC were implanted into the peritoneum of colitic mice 5 days after the beginning of DSS-treatment; colitic mice implanted with empty microcapsules were used as a positive control.

An electron microscope image of encapsulated MSC was reported in Figure 7E. Before implantation, viability of cells entrapped within the capsules was assessed by fluorescein diacetate and ethidium bromide staining (Figure 7F) that marks in green viable cells and in red those dead. Encapsulated cells maintained their viability until day 10, when mice were sacrificed and microcapsules were recovered from their peritoneum (Figure 7G). Both encapsulated and free MSC promoted survival of colitic mice (Figure 7H), reduced their body weight loss (Figure 7I), DAI (figure 7J), and colon shortening (Figure 7K) compared to positive control mice. Interestingly, comparable clinical effects have been observed

between the group of mice treated with encapsulated MSC and that of mice receiving free MSC.

BM-MSC expressed and secreted high levels of TNF-α stimulated gene/protein 6 *in vitro*

The efficacy of MSC treatment in our experimental model of colitis appeared completely independent from their homing capability to the site of inflammation. Therefore, one or more soluble factors produced and secreted by MSC were hypothesized to be responsible for their effectiveness. Thus, we tested MSC for the expression levels of many genes, which were known to provide their therapeutic efficacy in pathologies other than colitis [143, 156-159, 162, 164, 167-169]. We discovered that our MSC at baseline without any stimulation expressed high transcript levels of TNF-α stimulated gene/protein 6 (TSG-6) (Figure 8A), which is a ~35 kDa secreted protein known to be involved in cell migration and extracellular matrix stability and remodelling by enhancing the serine protease inhibitory activity of inter-alpha-inhibitor [203]. TSG-6 protein expression was also confirmed by immunofluorescence and western blot analysis as reported in Figure 8B.

Furthermore, ELISA assay, performed on supernatant collected from MSC culture, demonstrated that MSC were able not only to synthesized TSG-6, but also to secrete the protein in their surrounding environment (Figure 8B).

Treatment with MSC increased serum levels of TSG-6

Serum samples of colitic mice treated either with MSC or saline were collected 48 and 72 hours after injection and were analyzed for the presence of TSG-6 by ELISA. We found an increase of TSG-6 in MSC treated mice serum compared with untreated colitic mice at 48 hours, which became significant at 72 hours (Figure 8C).

MSC-aggregates released *in ex vivo* high amounts of TSG-6

Although we do not have any direct evidence of the source of TSG-6, we suppose that MSC once implanted release TSG-6 *in vivo*. To test in part this hypothesis, MSC-aggregates were recovered from the peritoneal cavity of colitic mice and either stained for TSG-6 directly or put in culture to evaluate secreted TSG-6 levels in their supernatant. Whole mount staining of MSC-aggregates revealed a strong positivity for TSG-6, in particular in the internal core of these aggregates as shown in the Z-stack images of MSC-aggregates (Figure 8D-E). Furthermore, ELISA assay performed on organ culture supernatants of MSC-aggregates collected 48 and 72 hours after MSC injection, showed a high secretion of TSG-6 at 48 hours, which was partially maintained at 72 hours (Figure 8F).

Exogenous TSG-6 administration ameliorated DSS-induced colitis improving clinical parameters

In order to test whether TSG-6 could be considered one of the responsible soluble factors of the beneficial effects of MSC in the treatement of DSS-induced acute colitis, recombinant murine TSG-6 (rmTSG-6) was delivered intraperitoneally to colitic mice and its therapeutic efficacy was compared to that of MSC administration. After several experiments for setting the timeline and the right dose of TSG-6, colitic mice were injected daily with 4 μg rmTSG-6 intraperitoneally starting from day 5 of DSS treatment until day 9, while MSC were administered as a single intraperitoneal dose of $3x10^6$ cells at day 5. Colitic mice injected with saline represented positive control. Although both TSG-6 and MSC administration considerably improved survival of colitic mice (Figure 9A), reduced their body weight loss (Figure 9B) and ameliorated their DAI (Figure 9C) compared to positive control mice, mice injected with rmTSG-6 displayed a much less severe clinical profile. Furthermore, after 10 days of DSS treatment, positive control mice appeared physically exhausted, due to systemic inflammation derived from severe colon damage caused by DSS treatment (Figure

9D). On the contrary, colitic mice injected with rmTSG-6 appeared healthy and vital (Figure 9D). We therefore hypothesized that TSG-6 not only locally reduced colon inflammation, but avoided its propagation into the entire organism. At day 10 colonscopic analysis of all groups was performed. In line with clinical parameters, colons of TSG-6 treated mice displayed less mucosal hyperemia, thickening and ulcerations compared to colons of positive control mice; group of mice injected with MSC showed an intermediate level of inflammation (Figure 9E). Furthermore, mice treated with both TSG-6 and MSC displayed lower histological score together with less infiltrating inflammatory cells compared to positive control mice (Figure 9E). Representative colonoscopy and histological images were shown in Figure 9E. Taken together these results clearly demonstrated that rmTSG-6 administration was strongly effective in the treatment of DSS-induced acute colitis and in the attenuation of systemic effects produced by exacerbated colonic damage.

rmTSG-6 administration reduced the inflammatory immune response in mice with DSS-induced colitis

Pro-inflammatory cytokine and chemokine levels were evaluated in serum, colon and mesenteric lymph nodes of colitic mice treated with either rmTSG-6 or saline after 10 days of DSS treatment. As reported in Figure 10, TSG-6 injections reduced markedly the serum levels of pro-inflammatory cytokines of Th1 and Th2 pathways such as IL-6, IFN-y, IL-5 and IL-13, and chemokine MCP-1. No significant differences were detected between TSG-6 and saline-treated mice for IL-12p40, IL-12p70, IL-4, IL-17a, TNF-α and KC levels. Interestingly, the treatment with TSG-6 demonstrated different patterns of serum cytokine expression compared to MSC-treated mice. Indeed, MSC-treated mice had lower serum levels of TNF-α, IL-17a and KC and significantly higher levels of IL-10 than TSG-6 treated mice (Figure 10).

Anti-inflammatory and pro-inflammatory cytokine patterns were analyzed in colonic

mucosa and mesenteric lymph nodes of all groups by RT qPCR.

Although no significant differences were found between treated and untreated-mice, the groups treated with TSG-6 and MSC displayed a trend of reduction in typically Th1 cytokine IL-6 and an increase in Th2 cytokines such as IL-5 and IL-13 compared to saline treated-mice in the colon (Figure 11), while no changes were observed in the mesenteric lymph nodes except for TGF-β level which was significantly higher in MSC treated mice compared to TSG-6 and saline injected mice (Figure 12).

Furthermore, both TSG-6 and MSC administration reduced transcript levels of the two chemokines MCP-1 and MIP-1α in the colon of colitic mice compared to untreated mice. However, while MSC treatment only produced a trend of reduction in the transcript levels of these two pro-inflammatory chemokines, TSG-6 induced statistically significant effect in reducing MCP-1 and MIP-1α levels as well as in the serum (Figure 11).

In line with reduced levels of pro-inflammatory cytokines, TSG-6 and MSC treated mice showed lower percentage of CD45 positive leukocytes infiltrating colonic mucosa compared to untreated mice (Figure 13A). Interestingly, TSG-6 administration displayed a trend of reduction in the percentage of polymorphonuclear leukocytes (PMN) evaluated as percentage of Ly6G positive cells gated on CD45 positive cells, and a trend of increase in the percentage of T regulatory cells (Treg) evaluated as percentage of CD4 positive CD25positive FoxP3 bright regulatory T cells infiltrating the mucosa. In addition, TSG-6 administration also produced an increase in the percentage of colonic macrophages evaluated as percentage of F4/80 positive cells gated on CD45 positive cells, which was significantly higher compared to saline and MSC-treated mice (Figure 13A).

rmTSG-6 decreased pro-inflammatory proteases in mice with DSS-induced colitis

Emerging studies have been shown that in many experimental models of human pathologies the anti-inflammatory properties of TSG-6 were due at least in part to its capability to

enhance the serine protease inhibitory activity of inter-alpha-inhibitor (IαI), blocking the subsequent activation of matrix metalloproteinases (MMPs) [156, 164, 167]. To test whether the protective effects mediated by TSG-6 or MSC treatment inhibited mucosal MMP-activation, the expression of MMP-3, MMP-9 and MMP-12, which are overexpressed in the inflamed mucosa of IBD patients, was analyzed. Transcript MMP levels showed a significant decrease of MMP3 and MMP9 specifically in colons of TSG-6 and MSC injected mice compared to untreated mice (Figure 13B), while no statistically difference was detected between all groups for MMP12. In order to confirm the results obtained by RT-PCR on protein level, proteins were isolated from the same tissue samples. Using specific antibodies against MMP3 and 9 a distinction between the latent pro-form and the active form of the MMPs was possible. Results of western blots reported in Figure 13C demonstrated a marked reduction in MMP3 and MMP9 active form in treated mice, which is strong in TSG-6 and only moderate in MSC injected-mice. These results indicated that MMP3 and MMP9 activities are modulated by both TSG-6 and MSC treatment.

IV. FIGURES

FIGURE 1.

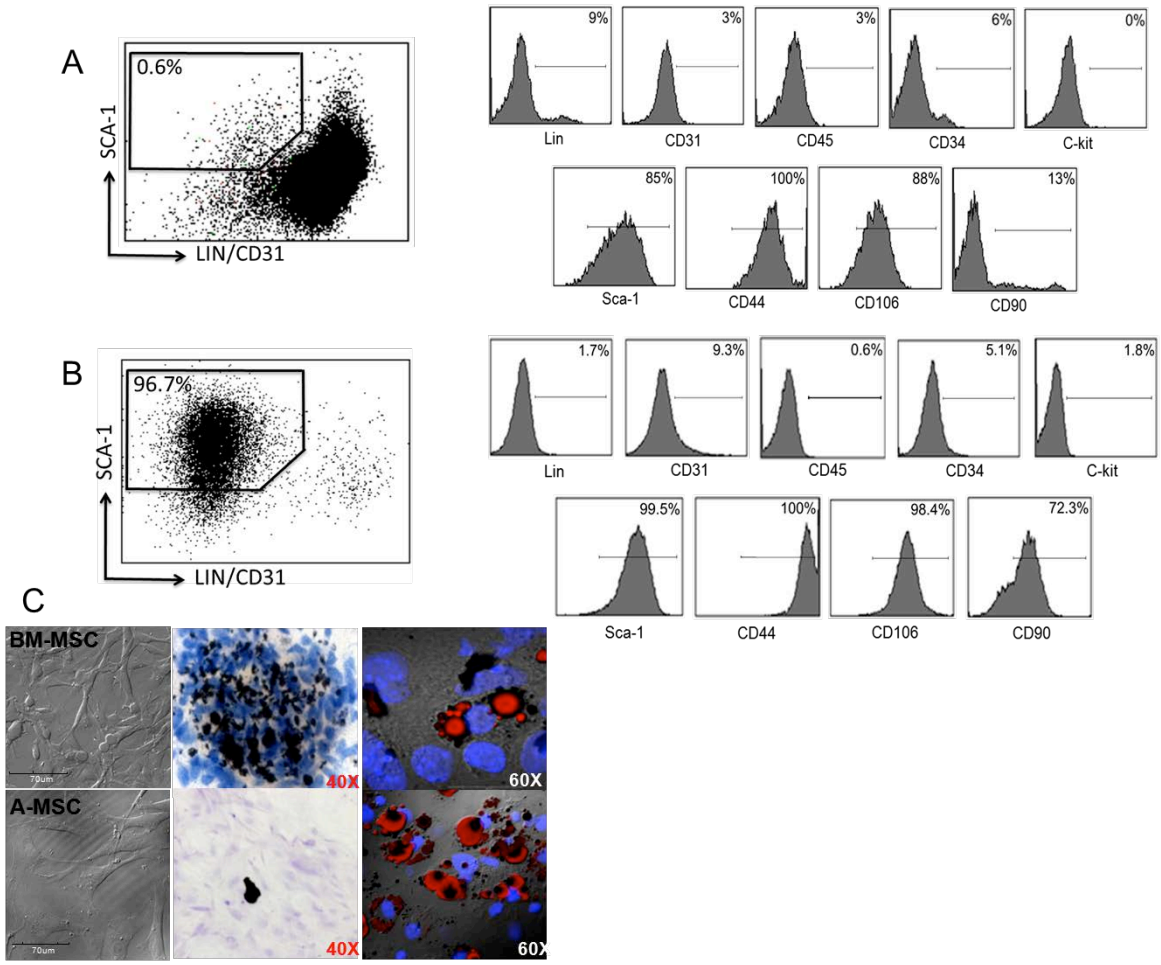

Figure 1. Purification and characterization of BM- and A-MSC phenotype.

(**A**) According to their cell surface marker expression (Lineage-, CD31- and Sca-1+, left panel), BM-MSC were FACS-sorted from a mixed bone marrow cell culture, and their percentage (0,6%) is reported. Sorted BM-MSC were then expanded and phenotypically characterized by FACS for the presence and the absence of MSC surface markers and the percentage of positivity for each marker is reported (right panel). (**B**) The same protocol was used to isolate A-MSC from subcutaneous abdominal fat, and the percentage of A-MSC obtained is reported (left panel). A-MSC were also phenotypically characterized and the percentage of positivity for each surface marker is reported (right panel). (**C**) Representative confocal images of BM- and A-MSC before (left panel) and after differentiation into osteoblasts (central panel, bright field images) and adipocytes (right panel, confocal images). Lipid vacuoles accumulation (red) is typical of differentiated adipocytes (right panel), while extracellular calcium deposition (black, central panel) is typical of differentiated osteoblasts. Nuclei were counterstained with either hematoxylin (light blue, central panel) or DAPI (blue, right panel), respectively.

FIGURE 2.

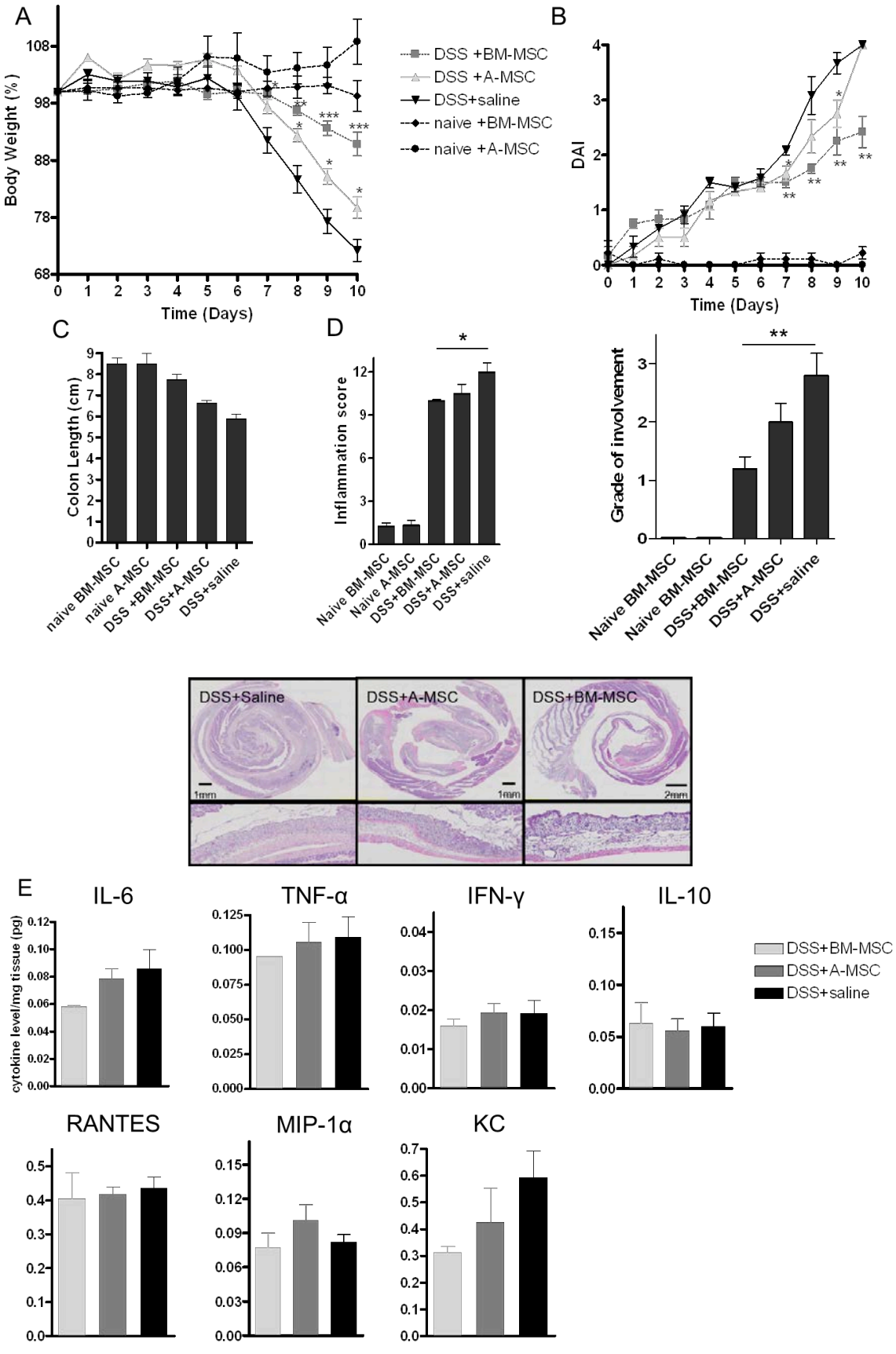
A
Body Weight (%)
Time (Days)
DSS +BM-MSC
DSS +A-MSC
DSS+saline
naive +BM-MSC
naive +A-MSC
B
DAI
Time (Days)
C
Colon Length (cm)
naive BM-MSC
naive A-MSC
DSS +BM-MSC
DSS+A-MSC
DSS+saline
D
Inflammation score
Naive BM-MSC
Naive A-MSC
DSS+BM-MSC
DSS+A-MSC
DSS+saline
Grade of involvement
Naive BM-MSC
Naive BM-MSC
DSS+BM-MSC
DSS+A-MSC
DSS+saline
DSS+Saline
DSS+A-MSC
DSS+BM-MSC
E
IL-6
TNF-α
IFN-γ
IL-10
cytokine level/mg tissue (pg)
DSS+BM-MSC
DSS+A-MSC
DSS+saline
RANTES
MIP-1α
KC

Figure 2. Comparison of BM- and A-MSC therapeutic efficacy in the DSS experimental model of acute colitis.

Colitic mice in a C57BL/6N genetic background, were ip injected with $3x10^6$ of either BM- or A-MSC. Healthy mice receiving BM- or A-MSC and colitic mice injected only with saline were used as negative and positive controls, respectively. Body weight loss (**A**) and disease activity index (DAI) (**B**) were monitored daily during the entire experiment. After 10 days of DSS treatment, mice were sacrificed, and colons were extracted for length measurements (**C**) and histological analysis (**D**). In **D** histological data are reported as inflammatory scores and grade of inflamed tissue involved (upper panel), while representative Hematoxylin and Eosin (H&E) images of inflamed colon 10 days after DSS treatment, are reported the lower panel. (**E**) Part of the extracted colons were homogenized in lysis buffer and analyzed for cytokine and chemokine expression, by ELISA. Chemokine and cytokine concentrations were normalized per mg of tissue. Values are expressed as mean ± SEM; n=4 mice for DSS groups; n=3 for healthy groups. *P< 0.05; **P<0.01; ***P<0.001.

FIGURE 3.

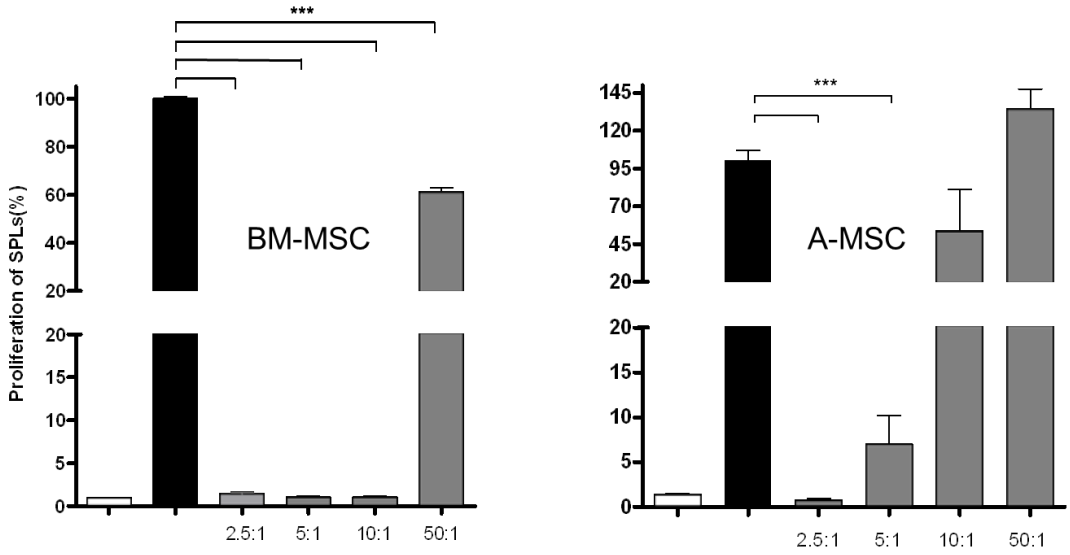

Figure 3. *In vitro* BM- and A-MSC immunomodulatory properties

Proliferation of CD3ε/CD28-activated splenocytes (SPL) co-cultured with BM-MSC (left panel) and A-MSC (right panel) at different ratio (SPL:MSC, 2.5:1; 5:1; 10:1 and 50:1) (grey bars) was evaluated. Naïve SPL (white bars) and CD3ε/CD28-activated SPL alone (black bars) were used as negative and positive control, respectively. Results were expressed as percentage of SPL proliferation. Values are expressed as mean ± SEM; n=3 wells for each group.*** P<0.001.

FIGURE 4.

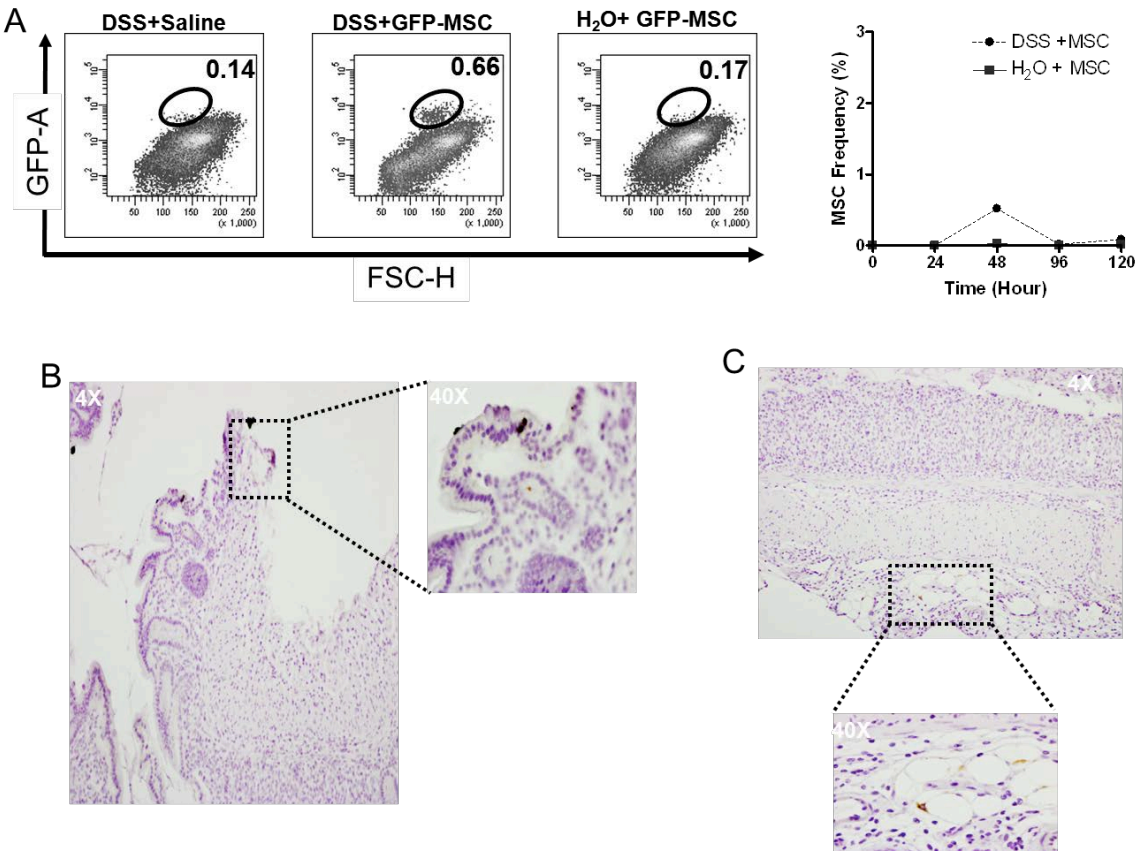

Figure 4: Capability of intraperitoneally delivered BM-MSC to engraft into inflamed colon

Healthy and colitic mice were ip injected with $3x10^6$ GFP-MSC at day 5 of DSS treatment and colons were collected 24, 48, 96 and 120 hours after the injection to visualize GFP+ cells by FACS. Colitic mice ip injected with saline were used as negative control. (**A**) Representative Dot Plots at 48 hours after the injection (left panel) and GFP+ MSC frequency expressed as percentage in healthy and colitic colons at all time points (right panel) are reported. All frequencies are referred to viable cells. (**B,C**) After 10 days of DSS treatment, GFP+ MSC were visualized by immunohistochemistry on paraffin-embedded sections of colitic mice. Nuclei were conterstained with hematoxylin (light blue). Representative images of GFP+ cells engrafted into epithelium (**B**) and endothelium (**C**) are shown.

FIGURE 5.

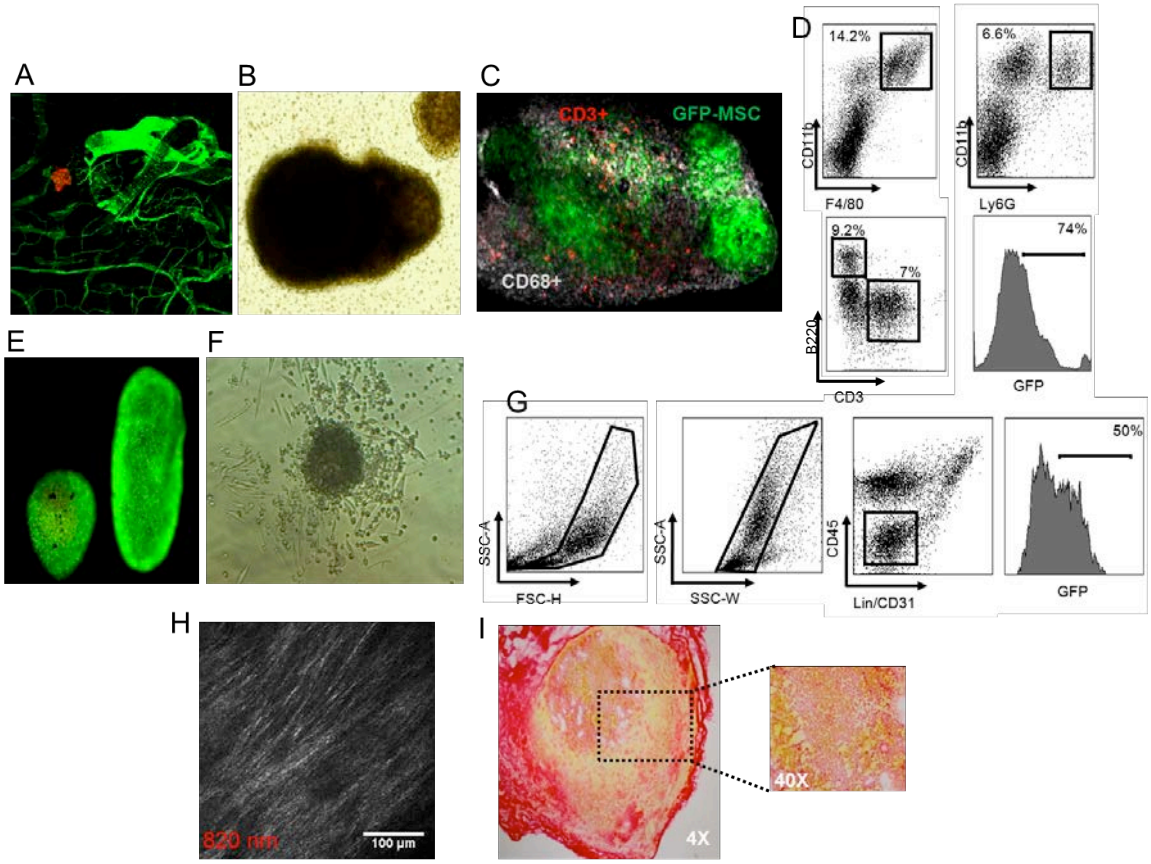

Fig. 5: Intraperitoneally delivered BM-MSC remained in the peritoneal cavity forming cellular aggregates

MSC ($3x10^6$) pre-stained with DilC18 (3) were injected in colitic mice and 24, 48 and 72 hours later colons were extracted and stained for CD31, by Whole Mount technique. (**A**) Representative image of MSC (red) aggregating outside the bowel wall vessels (green) 72 hours after the injection is reported. (**B**) After 10 days of DSS treatment, both healthy and colitic mice injected with $3x10^6$ GFP-MSC at day 5 were sacrificed and peritoneal lavages were performed. Representative phase-contrast image of structures recovered from the peritoneal cavity is shown. (**C**) Recovered structures were either fixed in PFA 4% and whole mount stained for CD3 (T lymphocytes; red signal) and CD68 (macrophages; grey signal). (**D**) Recovered structures were digested and analyzed by flow cytometry. CD45+ cells (leukocytes) were double-stained for CD11b and F4/80 (macrophages), CD11b and Ly6G (PMN), CD3 (T lymphocytes) and B220 (B lymphocytes). Representative Dot Plot of CD45+/CD11b+ cells stained for F4/80 or Ly6G, and representative Dot Plot of CD45+ Side Scatter (SSC) low cells stained for CD3 and B220 are shown. All cell frequencies were referred to CD45+ cells, while the frequency of GFP-MSC was referred to total viable cells. (**E**) Viability of cells within the structures was assessed by fluorescein diacetate and ethidium bromide staining; green signal represented viable cells, while red signal represented dead ones. (**F**) Recovered structures were also collected and cultured. After 3 days in culture many cells detached from the structures, adhering to the plate. Representative phase-contrast image of a cultured structure, with detached cells. (**G**) The phenotype of cells detaching from the cultured structures was analyze by FACS for Lineage-, CD31-, CD45- and GFP+ (**G**) and relative Dot Plots are reported. (**H**,**I**) Deposition of collagen into the structures was analyzed by both two photon microscopy and Picro-Sirius Red staining. Representative two photon microscopy image of second harmonic generation signal derived from collagen fibers (**H**) and representative bright field image of Picro-Sirius Red staining (**I**, collagen in red) are reported.

FIGURE 6.

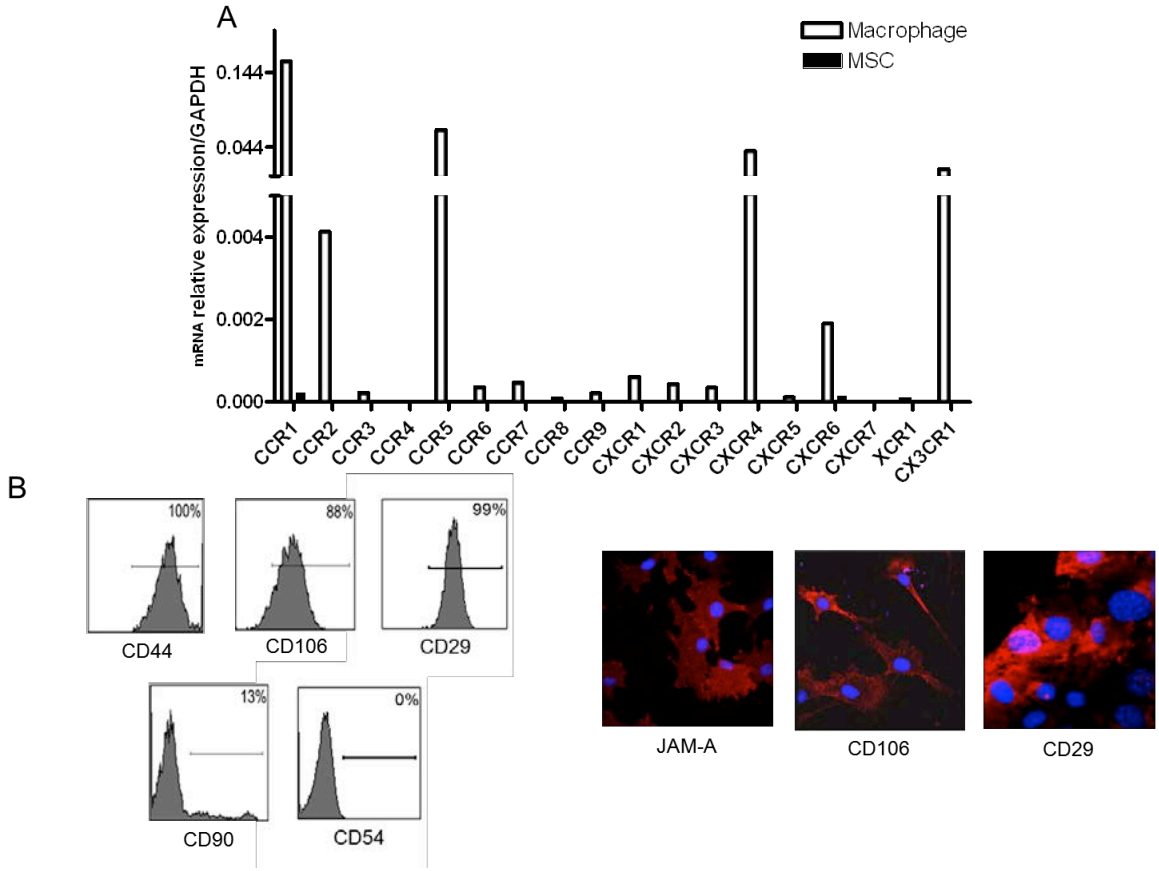

Figure 6: BM-MSC expression levels of chemokine receptors and cell adhesion molecules (CAMs)

(**A**) The expression profile of chemokine receptors was analyzed in PEC macropheges (positive control) and MSC, by real-time qPCR. Results are expressed as mRNA levels relative to GAPDH expression levels. (**B**) Expression levels of CAMs and surface molecules involved in the interaction between cells and activated endothelium and/or extracellular matrix were quantified by flow cytometry (left panel) and/or immunofluorescence (right panel). Percentage of positive cells for each molecule is reported. In immunofluorescence images red signal represents positivity for CAM, while nuclei were counterstained with DAPI (blue).

FIGURE 7.

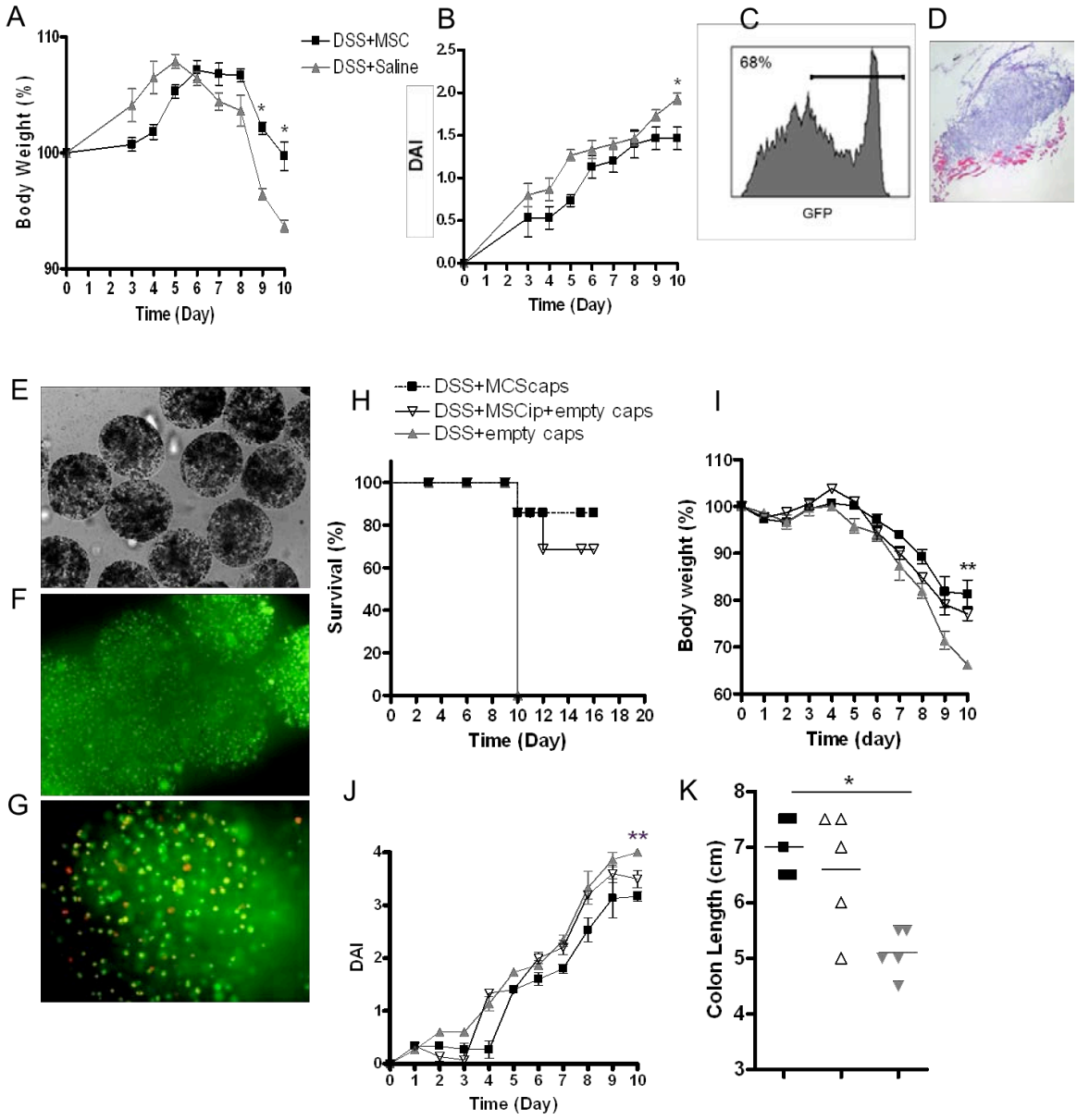

Figure 7: BM-MSC capability to exert therapeutic efficacy at distance in the DSS experimental model of acute colitis.

MSC ($3x10^6$) or saline (positive control) were administered subcutaneously to colitic mice after 5 days of DSS treatment. Loss of body weight (**A**) and DAI (**B**) were monitored daily during the entire experiment. (**C**) At day 10 after DSS treatment mice were sacrificed and structures recovered from their subcutaneous cavities and analyzed for GFP expression by FACS. Percentage of GFP+ cells is reported. (**D**) These structures were also paraffin-embedded and stained for histologic examination. (**E-G**) MSC migration was blocked by encapsulation into barium alginate microcapsules. An electron microscopy image of encapsulated cells is shown (**E**). Fluorescein diacetate and ethidium bromide staining was employed to verify the viability of cells entrapped within the capsules both before implantation (**F**) and after 5 days into the peritoneal cavity (**G**). (**H-K**) $3x10^6$ of MSC either encapsulated or not were implanted into the peritoneal cavity of colitic mice 5 days after the DSS treatment. Colitic mice implanted with empty capsules were used as positive control. Survival (**H**), loss of body weight (**I**) and DAI (**J**) were monitored daily during the entire experiment, while colon length was measured at the end of the experiment, when mice were sacrificed (**K**). Values are mean ± SEM; n=5 mice for all groups. *P< 0.05; **P<0.01.

FIGURE 8

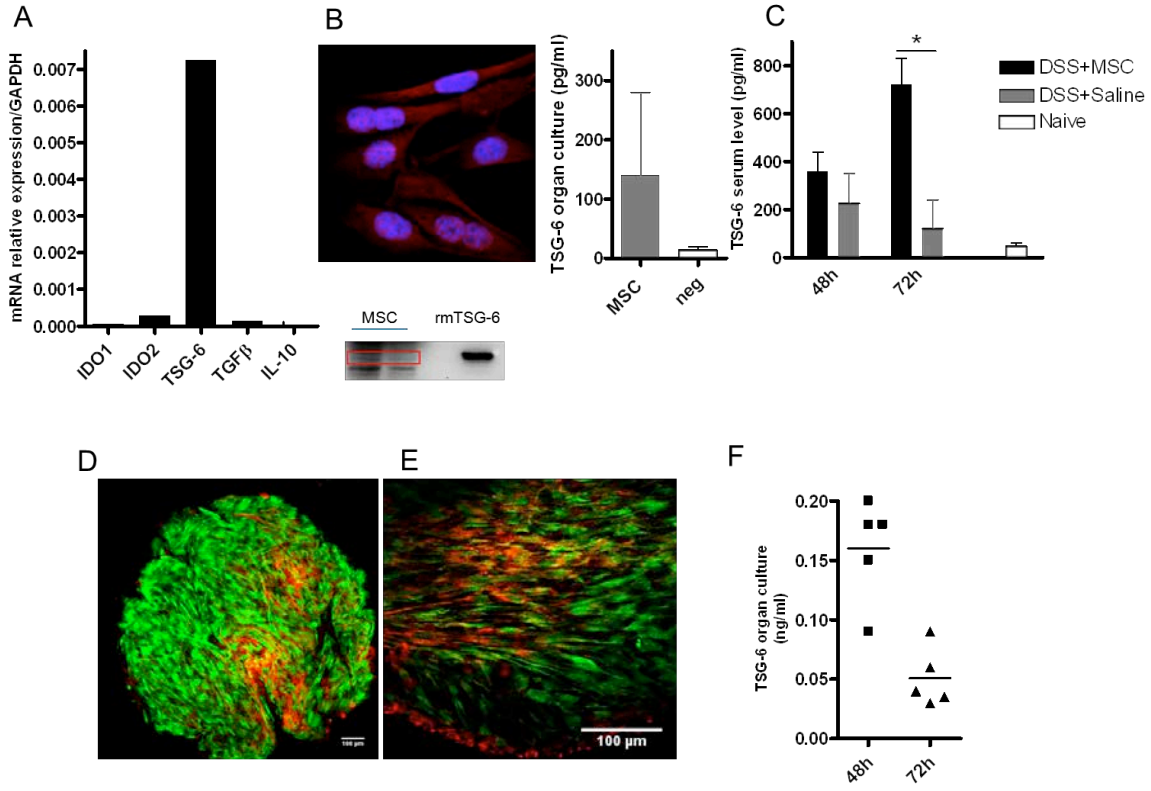

Figure 8: TNF-α-stimulated gene/protein 6 (TSG-6) production and secretion by BM-MSC *in vitro*, *in vivo,* and *ex vivo*

(**A**) MSC were analyzed for transcript expression levels of anti-inflammatory factors known to be responsible for their therapeutic efficacy in many pathologies other than colitis, by RT qPCR. (**B**) Among all factors analyzed, MSC expression levels of TSG-6 protein was investigated by both immunofluorescence (upper panel) and western blot (lower panel). Red signal represented positivity for TSG-6 signal, while nuclei were counterstained with DAPI (blue); recombinant murine TSG-6 was used as positive control in western blot. Levels of secreted TSG-6 on supernatants collected from MSC culture, were quantified by ELISA and expressed as pg/ml. (**C**) GFP-MSC ($3x10^6$) or saline were ip injected into colitic mice 5 days after DSS treatment, while naïve mice were employed as control. Serum levels were then collected 48 and 72 hours after the injection and analyzed for TSG-6 levels by ELISA. (**D,E**) Ten days after DSS treatment, mice were sacrificed, structures were recovered from their peritoneum and whole mount stained for TSG-6 (red). GFP-MSC are visualized in green. (**F**) Structures isolated from the peritoneum of colitic mice at 48 and 72 hours after GFP-MSC injection were cultured for 24 hours and their supernatants were analyzed for TSG-6 secretion levels, by ELISA. Values are expressed as mean ± SEM; n=5 mice for all groups. *P< 0.05.

FIGURE 9

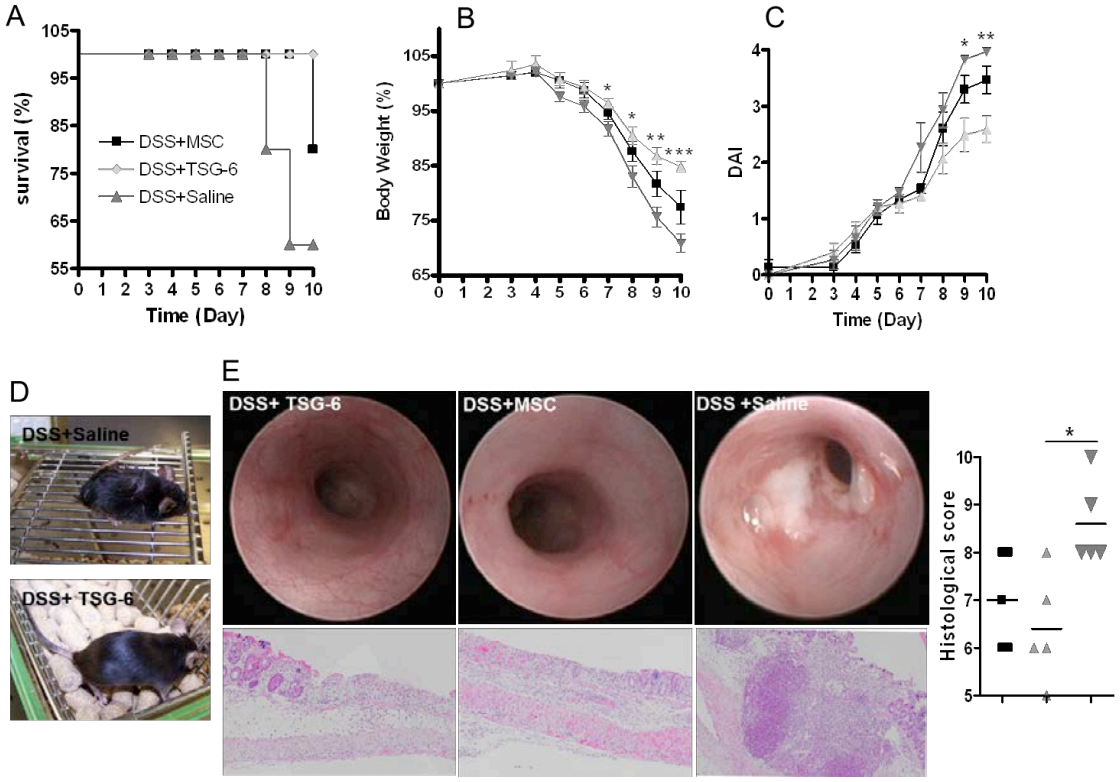

Figure 9: Therapeutic efficacy of recombinant murine TSG-6 (rmTSG-6) administered via ip in the treatment of DSS-induced acute colitis

Colitis was induced in C57BL/6N mice by administration of 3% DSS in drinking water ad libitum for 10 days. Mice were divided into 3 groups. In the first group colitic mice received $3x10^6$ MSC intraperitoneally 5 days after DSS treatment; in the second group colitic mice were administered daily with rmTSG-6 (4µg/mouse) starting from day 5 until day 9 after DSS treatment. Positive control group was represented by colitic mice receiving saline. Survival (**A**), loss of body weight (**B**) and DAI (**C**) were monitored daily during the entire experiment. (**D**) Pictures of mice injected with either saline (upper panel) or rmTSG-6 (lower panel) are reported to show their different physical condition after 10 days of DSS treatment. (**E**) Before sacrifice, colonscopic analysis was performed for each group, while after sacrifice colons were formalin-fixed and paraffin-embedded for histologic examination. Representative endoscopical (upper panel) and histological (lower panel) images together with the histological score (right panel) are shown Values are expressed as mean ± SEM; n=5 mice for all groups. *P< 0.05; **P<0.01; ***P<0.001.

FIGURE 10

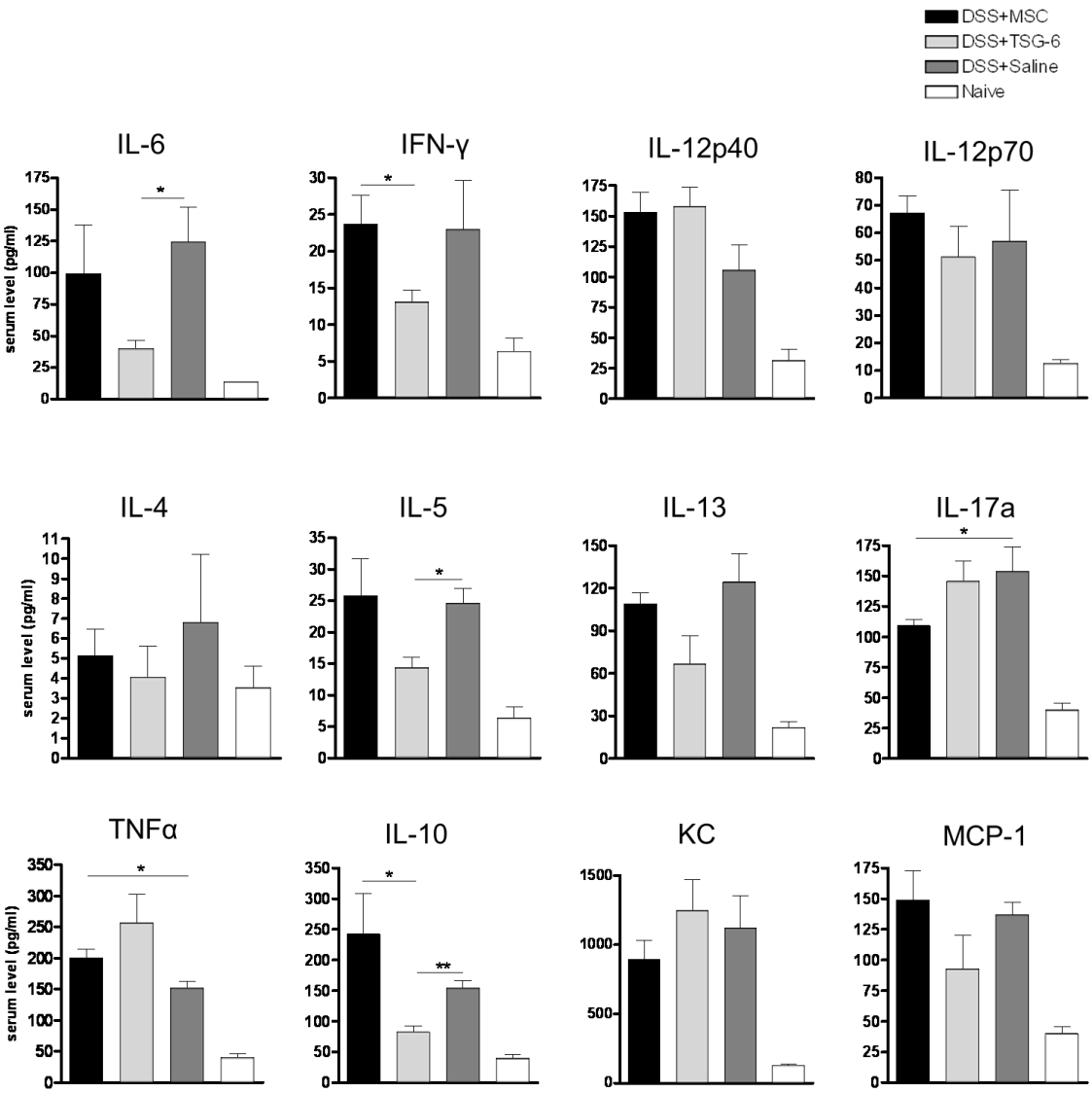

Figure 10: Systemic effect of rmTSG-6 treatment on chemokine and cytokine serum levels.

Serum samples from colitic mice intraperitoneally injected with either MSC ($3x10^6$) or rmTSG-6 were collected after 10 days of DSS treatment and analyzed for IL-6, IFN-γ, IL-12p40, IL-12p70, IL-4, IL-5, IL-13, IL-17a, TNF-α, IL-10 cytokine levels and KC, MCP-1 chemokine levels by Bio-Plex assay. Positive and negative controls were represented by colitic mice injected with saline and healthy mice, respectively. Values are expressed as mean ± SEM; n=5 mice for all groups. *P< 0.05; **P<0.01.

FIGURE 11

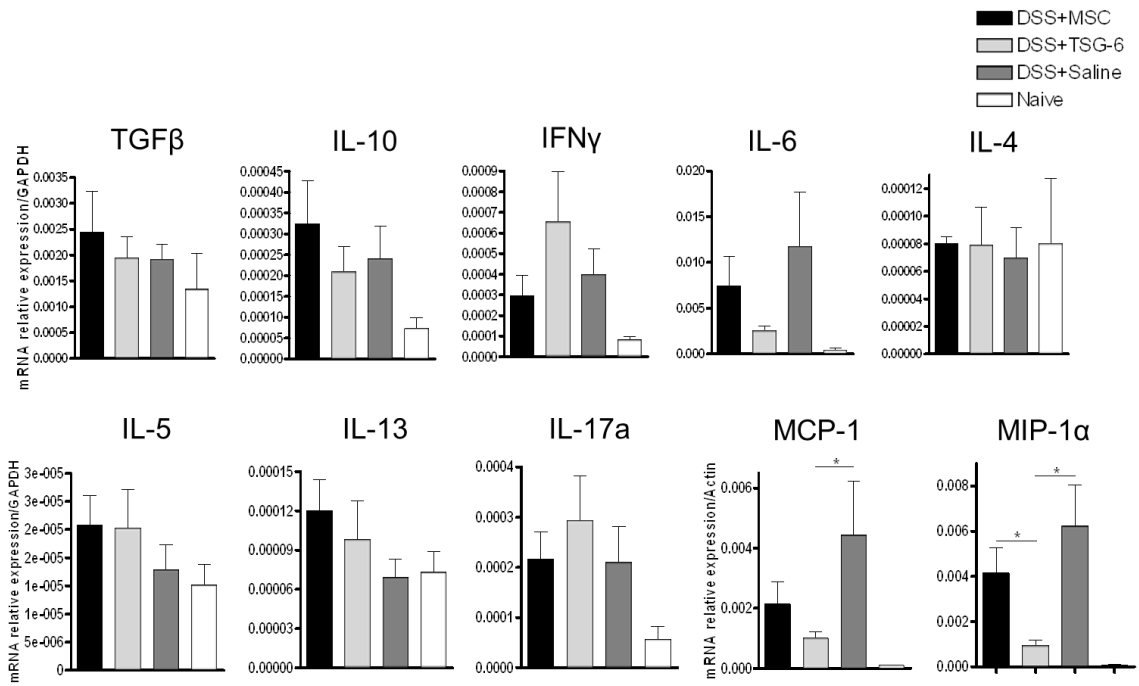

Figure 11: Analysis of cytokine/chemokine expression profile in colon of rmTSG-6-treated mice

Colitic mice were intraperitoneally injected with either MSC ($3x10^6$) or rmTSG-6 and after 10 days of DSS treatment colons were collected. Positive and negative controls were represented by colons recovered from colitic mice injected with saline and from healthy mice, respectively. After RNA extraction and retrotranscription, expression of anti-inflammatory cytokine TGF-β and IL-10 together with pro-inflammatory cytokine IFN-γ, IL-6, IL-4, IL-5, IL-13, IL-17a as well as chemokines MCP-1 and MIP-1α was determined by RT-qPCR. For each molecule, results are expressed as relative mRNA expression levels over the total amount of GAPDH or Actin. Values are mean ± SEM; n=5 mice for all groups. *P< 0.05

FIGURE 12

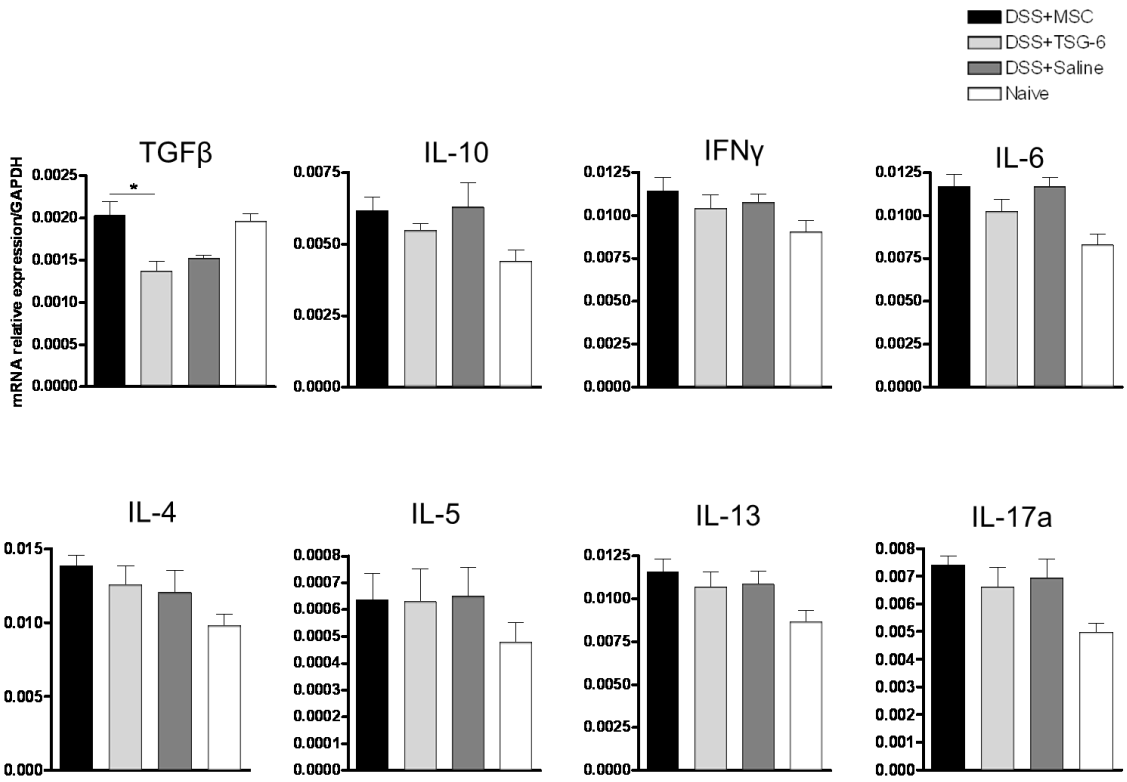

Figure 12: Analysis of cytokine expression profile in MLNs of rmTSG-6 treated mice

Colitic mice were intraperitoneally injected with either MSC ($3x10^6$) or rmTSG-6 and after 10 days of DSS treatment MLNs were collected. Positive and negative controls were represented by MLNs recovered from colitic mice injected with saline and from healthy mice, respectively. After RNA extraction and retrotranscription, expression of anti-inflammatory cytokine TGF-β and IL-10 together with pro-inflammatory cytokine IFN-γ, IL-6, IL-4, IL-5, IL-13, IL-17a was determined by RT-qPCR. For each molecule, results are expressed as relative mRNA expression levels over the total amount of GAPDH. Values are mean ± SEM; n=5 mice for all groups. *P< 0.05

FIGURE 13

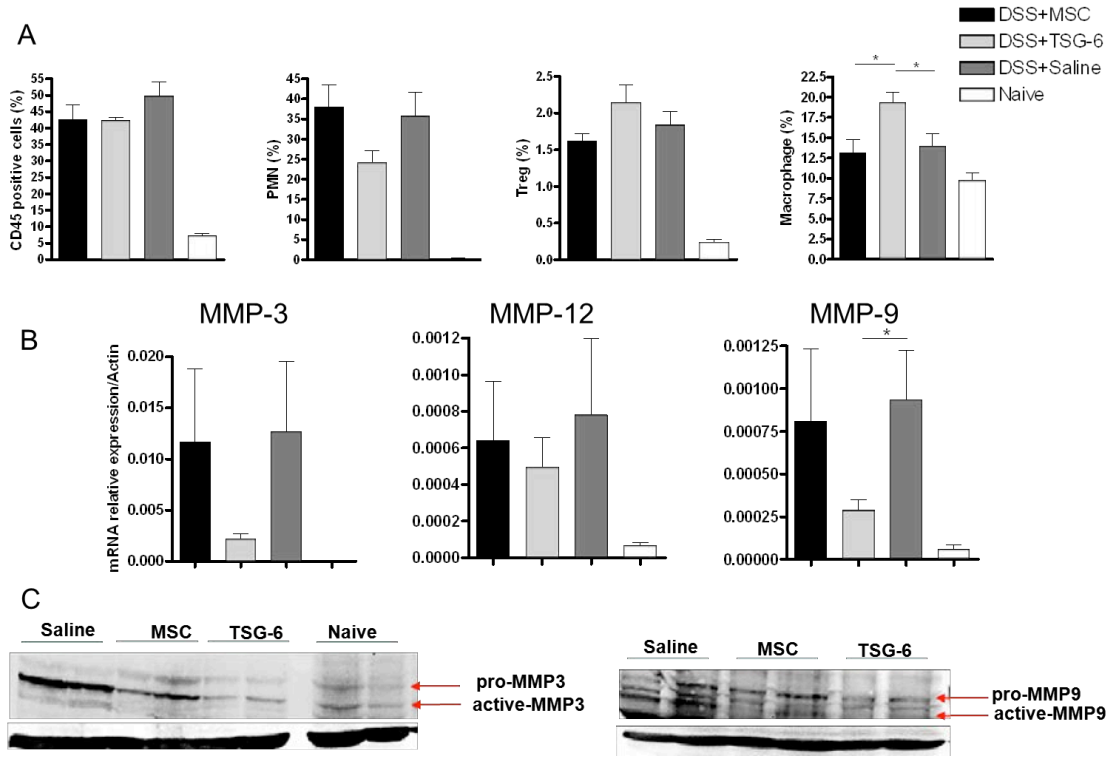

Figure 13: Analysis of inflammatory cells and matrix metalloproteinases (MMPs) expression in colons of colitic mice treated with rmTSG-6

Colitic mice were intraperitoneally injected with either MSC ($3x10^6$) or rmTSG-6 and after 10 days of DSS treatment colons were collected. Positive and negative controls were represented by colons recovered from colitic mice injected with saline and from healthy mice, respectively. (**A**) After digestion, colons were analyzed for the presence of infiltrating inflammatory cells (CD45+), polymorphonuclear cells (PMN), regulatory Tcells (Treg) and macrophages, by FACS. Frequency of CD45+ cells was referred to total viable cells, while all the other percentages were referred to CD45+ cells. (**B**,**C**) From the same colons RNA and proteins were simultaneously extracted and expression levels of matrix metalloproteinases MMP3, MMP9 and MMP12 were determined by both RT qPCR (**B**) and western blot (**C**). Actin was used as loading control for protein expression levels. RT-qPCR results are expressed as relative mRNA expression levels over the total amount of GAPDH or Actin. Values are mean ± SEM; n=5 mice for all groups. *P< 0.05.

V. DISCUSSION

Cellular therapy with mesenchymal stem cells is a promising new approach capable of addressing yet unmet medical needs for the treatment of inflammatory bowel diseases, which are characterized by sterile inflammation leading to loss of intestinal function [1]. The considerable excitement surrounding the MSC field was initially based on the unique biological properties of these cells and their capacity to self-renew and regenerate tissue and organ systems [84-86]; later, the immunomodulatory ability of stem cell therapy has become also apparent. Indeed, they were also demonstrated to be tolerogenic, and to modulate the immune system, dampening the inflammation and restoring the balance between regulatory and effector T cell response [122]. Results from murine models of colitis and ongoing clinical trials are encouraging, suggesting that MSC therapy is safe and effective in ameliorating experimental colitis and promoting clinical remission in most treated patients [81, 171]. However, the mechanism(s) through which these cells exert therapeutic efficacy in the intestine remain unclear, basic science and animal experiments continue to play an important role in the understanding of the mechanisms involved. Mesenchymal stem cells coexist with other cell types depending on source. In the bone marrow they coexist with hematopoietic stem cells and their frequency is very low (1 MSC every 100000/1000000 bone marrow cells) [82, 83]. Since the low frequency and the absence of specific markers for their characterization, the first critical and important step in the study of MSC activities is represented by their purity. Most of isolation protocols are based on MSC property to adhere to plastic once put in culture [118], but these methods lead to the obtainment a MSC population contaminated. In order to shed light on the mechanisms underlying the beneficial effects of MSC in the IBD, I set up in the first part of my study a new isolation protocol to obtain a pure population of MSC. This new purification method took advantage of negative selection for endothelial and hematopoietic markers such as CD31 and Lineage, and positive selection for Sca-1, marker with high

grade of stemness. The double selection permitted to obtain high purity of MSC population without affecting their properties, as demonstrated by our results. MSC can be isolated from different adult tissues such as bone marrow, cartilage, muscle, tendon and fat. In order to investigate the mechanisms of MSC-based therapy for IBD, it is necessary to identify the best cell source in terms of frequency of cells, low risks for the patients and cell activity. Although bone marrow remains the main source of MSC for clinical studies, adipose tissue is considered an ideal source due to accessibility. Therefore I decided to compare in terms of phenotype, properties and therapeutic efficacy MSC isolated from both bone marrow (BM) and adipose tissue (A). Importantly, mice of 4 to 6 week-old were used, as MSC frequency was demonstrated to inversely correlate with the age of mice [204]. Surprisingly, the frequency of sorted MSC cells was completely different depending on tissue source, going from 0.6% in the bone marrow to 96.7% in the adipose tissue. Analysing the quality of cultures generated by both sorted cells, I found that they consisted of pure populations of MSC, as demonstrated by the pattern of surface markers expressed by them. Interestingly, BM- and A-MSC displayed similar phenotype, differing only for the expression level of CD90, which was almost absent on BM-MSC surface, while strongly expressed by A-MSC. CD90, also known as Thy-1, is commonly used as a marker for a variety of stem cells and for the axonal processes of mature neurons. The function of CD90 has not yet been fully elucidated. It seems to play role in cell-cell and cell-matrix interactions, and to be implicated in neurite outgrowth, nerve regeneration, apoptosis, metastasis, inflammation and fibrosis [205]. Its specific function on MSC surface remains unknown. Importantly, it was demonstrated that while CD90 expression level is high on human MSC surface, it can vary on murine MSC, depending also on the strain of mice from which they are isolated as well as on the tissue source [119]. Notably, when administered to colitic mice at day 5 of DSS treatment, BM-MSC appeared much more effective than A-MSC in ameliorating both their clinical and histological scores, and in reducing the amount of pro-inflammatory

cytokines and chemokines in the inflamed colon. These *in vivo* differences between the two stem cell lines could be related to different *in vitro* properties that they exerted. Indeed, beside a different morphology, BM- and A-MSC differed for differentiation potential, with the latter being much more prone to originate adipocytes than osteoblasts. This could reflect a different composition and grade of stemness of the two cultures, with BM-MSC mainly constituted by uncommitted "bona fide" stem cells, and A-MSC formed by committed, more differentiated progenitors. As a consequence, the regenerative potential of the two stem cell lines was expected to be different also *in vivo*, possibly affecting their therapeutic efficacy. A second explanation for BM-MSC being more effective in the treatment of DSS-induced colitis, could be represented by their capability to strongly inhibit the proliferation of activated splenocytes (SPLs), also if highly diluted. On the contrary, A-MSC were able to suppress splenocytes proliferation only in cultures characterized by low A-MSC-SPLs ratio. Thus, whether reduced immunomodulatory properties, or regenerative potential, or both were the cause of reduced A-MSC therapeutic efficacy compared to BM-MSC in DSS model of colitis, remains to be clarified. These data demonstrate that MSC cultures established from different tissue sources are not equal in terms of both *in vitro* and *in vivo* properties, and could therefore display different effects once translated to the clinical practice. Given that BM-MSC display a higher efficacy in ameliorating intestinal inflammation than A-MSC, the second part of my thesis has been focused on investigating the fundamental mechanisms by which BM-MSC exerted therapeutic efficacy in experimental model of colitis. There are several experimental models of colitis which provide different conditions for the study of factors involved in the pathogenesis of inflammatory bowel diseases such as: environmental factors, the role of specific immune and genetic factors, and therapeutic options in IBD. For the aims of this thesis I used the DSS model of colitis that is the most common, quick and easily reproducible chemically induced colitis model. It has similarities to clinical and histological features of human IBD

with UC characteristics [65].

Data coming from experimental models suggest the existence of two alternative mechanisms explaining the beneficial effects of MSC. The first mechanism proposed that after systemic delivery, MSC migrated to the site of inflammation where they both differentiated into injured cells and locally modulated the inflammatory response [155, 166]. Alternatively, MSC were identified as a natural source of trophic and anti-inflammatory factors, which they produced at distance upon stimulation, without engrafting into tissue [206]. In order to address which of them proposed mechanisms were involved in intestinal diseases, BM-MSC from GFP mice were intraperitoneally injected, and their presence in the colon and MLNs was evaluated at different time points using different approaches.

Surprisingly, we found that MSC did not successfully engraft either MLNs or colon at any time points tested, with a frequency of less than 1% in the latter 48h after the injection. Other organs, such as liver, spleen and lungs were therefore investigated at different time points for MSC presence, but no GFP+ cells were detected. The work hypothesis was that MSC after intraperitoneal injection entered the lymphatic and/or blood circulation, migrated throughout the body and homed to inflamed gut via activation of specific chemokines/chemokine receptors axis. In order to verify whether the absence of MSC in the peritoneal organs was due to a defective expression of chemokine receptors and/or adhesion molecules in these cells, we analyzed the expression of a panel of chemokines/chemokine receptors and integrins/adhesion molecules could be involved in the migratory activity of MSC to the gut. Interestingly, BM-MSC expressed very low levels of chemokine receptors particularly of those involved in migration towards intestine (CCR9) and secondary lymphoid organs, such as MLNs (CCR7), but were strongly positive for most of the integrins and adhesion molecules tested. Therefore, MSC administered via ip not only failed to engraft peritoneal organs, but were not able to enter the circulatory system, remaining in

the site of injection. Indeed, MSC aggregated outside the bowel wall vessels of the distal colon together with immune cells, especially macrophages, T and B lymphocytes and polymorphonuclear cells (PMN), generating MSC-aggregated. These structures appeared after 48 hours of MSC administration, and increased their size day-by-day, resembling lymphoid structures either for shape and size. Importantly, cells contained within the structures were vital, as demonstrated not only by fluorescein diacetate and ethidium bromide stain, but also by their capability to detach from the structures and adhere to plastic once put in culture. In addition, it was found that structures were not simple cell aggregates, but organized units, characterized by deposition of an extracellular matrix of collagen, an internal MSC core and immune cells on their surface. This organization, together with their cellular composition, resemble that of tertiary lymphoid structures (TLS), which are inducible non-capsulated lymphoid aggregates, capable of ectopic immunological activity, that usually originate in inflamed tissues as a result of immune infiltration [207]. Development of TLS was well characterized in the synovium of rheumatoid arthritis patients, where the initial recruitment of lymphocytes displays a disorganized accumulation of T and B cells around a central blood vessel. The increasing size of the aggregate is accompanied by variable upregulation of key molecules regulating lymphoid organogenesis and homeostasis in physiological conditions, such as LTβ and lymphoid chemokines CXCL13, CCL21 and CCL19. Expression of these chemokines leads to initial T/B cells compartmentalization, and to increased accumulation of LTβ+ hematopoietic cells promoting further differentiation/proliferation of stromal and vascular cells. Amplification of these processes leads to the formation of functional germinal centre (GC) inside these structures, where differentiated follicular dendritic cells (FDC) accumulate and give rise to a network which support expression of activation-induced cytidine deaminase (AID) in B lymphocytes. In this environment B cells undergo Ig repertoire diversification and affinity maturation, suggesting that these lymphoid aggregates may function as “protective niches”

for local B cell differentiation and auto-antibody production, with the potential of directly contributing to the immune response. However, it is still debated whether they directly support local pro-inflammatory activity by facilitating cell cooperation or play a regulatory, counter-inflammatory role, since evidences supporting both theses exist. Recently, it has been proposed that formation of ectopic lymphoid aggregates in an inflamed tissue is not necessarily detrimental or beneficial *per se*, but their specific physiopathological effect might be dynamically or spatially dependent on their functional status and the characteristics of the surrounding environment [207]. Thus, further investigations are necessary to assess whether structures identified in the peritoneal cavity of mice injected with MSC are *bona fide* TLS or not. The solid evidence is that MSC administration is effective in ameliorating DSS-induced colitis, probably via secretion of soluble factor(s), but we still have to verify whether MSC organization into structures together with immune cells is necessary for their therapeutic efficacy. In this regard, it was recently demonstrated that MSC cultured as 3D aggregates or as spheroids were self-activated to express anti-inflammatory proteins and appeared more effective than MSC from adherent monolayer cultures in suppressing inflammatory responses both *in vitro* and *in vivo* [177]. Thus, considering that MSC are supposed to exist in adult tissue organized into niche [117], we can speculate that, alternatively to TLS, MSC aggregates could represent a reproduction of their original niche, which MSC reconstitute once injected in order to reproduce their natural environment, where their beneficial effects are maximized. Surprisingly, structures were found not only in the peritoneum of colitic mice, but also in that of healthy mice administered with MSC, suggesting that these cells are able to aggregate and recruit immune cells *per se*, independently from the presence of an inflammatory environment surrounding them. As a consequence, it might be hypothesized that the generation of organized structures prompt by MSC do not need their previous activation by proinflammatory cytokines, but happens spontaneously after their injection. This could be

probably due to *in vitro* MSC handling before injection, which might promote and maintain these cells in a constant activation state. Indeed, in order to increase MSC number, they are cultured in specific media rich of factors that stimulate cells proliferation and block their differentiation. Beside these effects, it is possible that the same factors induce a constitutive activation in MSC. Recently, it has been demonstrated that in experimental autoimmune encephalomyelitis MSC aggregate with immune cells *in vivo*[153]. The authors of this study have reported that intracerebroventricular (ICV)-transplanted MSC migrated into the brain parenchyma and, together with T and B cells, formed cellular masses. However, these masses appear different from MSC-aggregates that I observe in the peritoneal cavity. Indeed, they were observed only when locally injected MSC accumulated at high density in the brain parenchyma, which depend on the number of ICV-transplanted cells and environmental factors, such as the disease activity. In my case accumulation of MSC at high density was unlikely, since intraperitoneally administered cells spread throughout the peritoneum. In addition, it was reported that the probability to develop masses into the brain directly correlated with the severity of the EAE, while I observed structures formation independently on the presence of inflammation, both in colitic and healthy mice. Finally, MSC entrapped into masses instead of producing therapeutic effects, generated focal inflammation, demyelination, axonal loss and increased collagen-fibronectin deposition.

The final confirmation that MSC therapeutic efficacy occurs via production and secretion of soluble factors, without the need of gut homing was obtained by observing that MSC, encapsulated into alginate-based microcapsules and implanted into the peritoneum of mice, were as effective as free MSC in ameliorating DSS-induced colitis. Importantly, these microcapsules entrapped the cells inside but are completely permeable to soluble factors. Of note, MSC microcapsules could be approximately considered as small structures, since cells are forced to stay in close contact and therefore they can interact each other. This reinforces the idea that MSC need to aggregate in order to be able to exert their therapeutic efficacy, as

previously discussed. However, it has to be taken into consideration that MSC entrapped within microcapsules cannot directly interact with immune cells as happened in the structures. This observation gives rise to many doubts regarding the contribution exerted by immune cells in the structures, since their absence in MSC microcapsules does not prevent MSC therapeutic efficacy. Thus, even if the capability of MSC to exert therapeutic efficacy in the treatment of DSS-induced colitis without homing to inflamed intestine has been definitively demonstrated, further investigation are necessary to better characterized the function of the structures, especially the role of immune cells which take part in their formation.

Therefore, MSC administered intraperitoneally remained in the site of injection, where they aggregated with immune cells. However, even if MSC did not migrate towards inflamed gut, they stayed close to the inflamed area, that is in the peritoneum. To exclude the possibility that in order to be therapeutic MSC had to be administered closed to the site of inflammation, I tested the therapeutic efficacy of MSC subcutaneously injected in the treatment of colitis. Surprisingly, subcutaneous injections of MSC back of arms were as effective as ip administered MSC in reducing body weight loss of mice and ameliorating their DAI. Importantly, the observation that subcutaneously injected MSC aggregated along with immune cells generating MSC-aggregates, further reinforce the idea that MSC aggregation is a pivotal process for their therapeutic efficacy.

Thus, MSC therapeutic efficacy in the treatment of DSS-induced colitis is probably related to their capability of generating MSC-aggregates, where they may cross-talk each other and with immune cells, giving rise to a well-organized microenvironment in which they start producing and secreting soluble factor(s). Many soluble factors have been identified as responsible for MSC therapeutic effects in pathologies other than colitis, as previously discussed, and among them I discovered that my MSC expressed and secreted TSG-6 both *in vitro* and *in vivo* TSG-6 is a ~35kDa secreted protein composed mainly of contiguous

Link and CUB modules [203]. By its Link domain, TSG-6 binds to many components of the extracellular matrix (ECM), such as hyaluronan (HA), chondroitin-4-sulphate (C4S) and aggrecan. Its CUB module is highly conserved between species and occurs in a wide range of proteins involved in fertilization and development, suggesting a role for TSG-6 in these processes. Although there is little or no constitutive expression of TSG-6 in unstimulated tissues, it is produced by a variety of cells in response to a wide range of pro-inflammatory factors, such as TNF-α, IL-1 and LPS, as well as various growth factors, like TGF-β, FGF and EGF. Once produced and secreted, TSG-6 has been demonstrated to exert anti-inflammatory effects. In particular it has been shown that it is implicated in several processes, which take place during inflammation, in particular in cell proliferation, and leukocytes homing to inflamed tissues. The mechanisms underlying these effects are mostly unknown, but they seem to involve TSG-6 capability to interact with either HA or inter-α-inhibitor (IαI), which is a serine protease inhibitor, or both. Indeed, it was recently suggested that TSG-6 might somehow participate in the formation of HA·IαI complexes, and thus be important for regulating ECM remodelling and/or assembly. In addition, TSG-6 non-covalent interaction with IαI enhances the anti-plasmin activity of the latter, causing a decrease in the activation of MMP, which normally occurs via pro-MMP cleavage exerted by plasmin. This blocks MMP degradation of the ECM, a common denominator of any inflammatory process. As a consequence, the capability of leukocytes, especially of neutrophils, which are the firsts recruited to inflammatory sites, to infiltrate damaged tissues is extremely reduced. Alternatively, TSG-6 might limit the extravasation of leukocytes by binding HA, which is overexpressed on the activated vascular endothelium. Indeed, HA bound to TSG-6 is no more available for interaction with CD44 on leukocytes, which normally mediated their extravasation [203]. As anticipated before, MSC *in vitro* express high transcript and protein levels of TSG-6, and were also able to secrete this protein in their supernatant. Surprisingly, MSC did not require to be stimulated by TNF-α or other

pro-inflammatory cytokines to express and secrete TSG-6 as normally occurred, but they were already activated to produce it at the baseline. This finding further reinforces our hypothesis that MSC *in vitro* culture conditions exert a deep impact on their activation state. In particular, as mentioned above, media for MSC culture are rich of factors that beside stimulating cells proliferation and blocking their differentiation, might render them constantly activated. *In vivo*, colitic mice administered with MSC displayed an increase in TSG-6 serum levels especially 72h after injection compared to saline treated mice and naive mice. The whole mount stain for TSG-6 performed on structures recovered from the peritoneal cavity of colitic mice 48 and 72h after GFP-MSC injection, demonstrated that MSC produced *ex vivo* TSG-6 and secreted it in the extracellular space, where it could contribute to organize the extracellular matrix. In addition, the organ culture of the same structures revealed secreted TSG-6 also in their supernatant, whose levels seem to correlate with those present in the serum of MSC treated mice. Indeed, if we assume that TSG-6 secreted by MSC organized into structures requires more or less 24h to be absorbed and to appear in the bloodstream, the high levels of TSG-6 secreted *ex vivo* by the 48h structures, could produce *in vivo* the observed increase in TSG-6 serum levels 72h after MSC injection. However, this represents only an hypothesis, since the *ex vivo* model of structures' organ culture could not truly reproduce their *in vivo* behavior, and we cannot be sure that the increase in TSG-6 serum levels observed in MSC treated mice is exclusively due to MSC contribution, and not to other cells prompt by MSC to secrete TSG-6. In this regard, isolation of MSC from mice knock out for this protein could shed light on both the role played by these cells in providing the enhancement of TSG-6 serum levels once injected into colitic mice, and its relevance in determining MSC therapeutic efficacy in the treatment of DSS-induced colitis. However, assuming TSG-6 as the soluble factor secreted by MSC responsible for most of their therapeutic effects in the treatment of DSS-induced colitis, the trend of TSG-6 secretion by structures observed *ex vivo*, demonstrated that 48h after

injection these cells produced the highest amount of TSG-6, which appeared strongly reduced at 72h. This is in line with previous findings, which suggest that MSC effects are generated soon after their injection that is within 72h and thereafter come to exhaustion. As a consequence, in order to prolong their beneficial effects, especially in long-lasting experimental models of human pathologies, multiple administrations seem to be necessary. To confirm that TSG-6 is therapeutic in the treatment of DSS-induced colitis, rmTSG-6 was administered to colitic mice intraperitoneally, and its effects were compared to those exerted by MSC. Data coming from *ex vivo* organ culture of MSC organized into structures suggested that they might *in vivo* continuously release TSG-6 in the peritoneum. This process seemed to occur with high efficiency within few hours from their injection, and progressively reduced thereafter. In order to reproduce the kinetic of TSG-6 release by MSC, multiple administration of rmTSG-6 were performed. Interestingly, exogenous TSG-6 strongly promoted survival of colitic mice, reduced their body weight loss and ameliorated their DAI. Surprisingly, after 10 days of DSS treatment, TSG-6 injected mice appeared healthy and vital and displayed reduced colon inflammation compared to saline injected mice, as demonstrated by endoscopic and histological analysis. In this study we demonstrated that the impressive clinical amelioration of colitis produced by TSG-6 was related to its capability to locally reduce colon inflammation, avoiding its propagation into the entire organism. Indeed, TSG-6 treated mice displayed reduced levels of both total and active MMP3 and MMP9 in the inflamed colon, which in turn decreased the ECM degradation and the subsequent leukocytes infiltration. Notably, among leukocytes infiltrating inflamed gut, PMN exert detrimental effects, since they are recruited in the early phase of colon damaged and promote both the perpetuation and the propagation of the inflammatory process [208]. Treatment with rmTSG-6 was demonstrated to specifically reduce the percentage of PMN infiltrating the inflamed colon, which resulted in the reduction of colon inflammation, as demonstrated by the reduced levels of local pro-

inflammatory cytokines (especially IL-6) and chemokines (MCP-1 and MIP-1α).

In CONCLUSION, MSC-based therapy is a promising new approach for the treatment of IBD. The results of my thesis demonstrate that the isolation source, procedure, culture manipulation as well as species from which MSC are purified could also play an important role in the therapeutic efficacy of these cells. Furthermore these data show that once injected, at least in DSS experimental model of colitis, MSC do not engraft the inflamed colon, but remain in the site of injection where aggregate with immune cells. Overall, my study indicates that MSCs do not need to reach the inflamed colon for exerting immunomodulatory effects, but dampen the mucosal inflammatory response at distance by releasing a soluble factor as potent anti-inflammatory protein TSG-6.

VI. METHODS

Isolation of murine BM-MSC and A-MSC

BM- and A-MSC were isolated from both C57BL/6 mice and C57BL/6-Tg(UBC-GFP)30Scha/J mice of (Charles River). BM-MSC were isolated by flushing femurs and tibias of mice. The cells obtained were plated at the concentration of $1x10^6$cells/cm^2 in complete MSC expansion medium (StemCell Technology). A-MSC were isolated from abdominal subcutaneous fat of mice by digestion with 1mg/ml Collagenase type I (Worthington) for 45 min at 37°C. Cells were then filtered and centrifuged before growing in MSC expansion medium. Non-adherent cells were removed from BM-MSC culture 72h after plating, while from A-MSC culture after 24h. At passage 3 both BM- and A-MSC were sorted with FACSAria II flow cytometer (BD Biosciences) selecting Lineage Cell Detection Cocktail-, CD31- and Sca-1+ cells.

Morphologic characterization and differentiation potential of murine BM- and A-MSC

BM- and A-MSC were seeded on PolyLysine (1:10 in PBS; Sigma-Aldrich) on glass coverslips (13mm diameter; Carolina Biological Supply), which were either fixed with pre-warmed paraformaldehyde (PFA) 2% for 10 min at 37°C to analyse their morphology or cultured with specific differentiation media to differentiate cells into osteoblasts and adipocytes. Osteoblastic differentiation was achieved by culturing both cell lines in complete DMEM supplemented with indomethacin 0.14mM (SIGMA), ascorbic acid 0.284mM (SIGMA) and dexamethasone $1x10^{-4}$mM (MP Biomedicals), while adipogenic differentiation was obtained by using complete DMEM supplemented with glycerol phosphate 10mM (SIGMA), dexamethasone $1x10^{-4}$mM and ascorbic acid 0.284mM. After 3 weeks differentiated adipocytes were checked by lipid vacuoles presence visualized by Oil Red O staining while the differentiated osteoblasts were detected by deposition of

extracellular calcium visualized by Von Kossa staining. Confocal images were acquired with an oil immersion objective (60x, 1.4 NA Plan-Apochromat; Olympus). Bright field images were acquired with a 40x objective (IX51 inverted microscope, Olympus).

Immunosuppression assay

Total splenocytes (SPL) were isolated from spleen of C57BL/6N mice. 200,000 SPL/well were seeded in a 96-well plate in complete RPMI supplemented with β-mercaptoethanol 50 μM and co-cultured with BM- or A-MSC at ratio MSC:SPL of 1:2.5, 1:5, 1:10 and 1:50. Stimulation of T lymphocytes was induced by adding 1 μg/ml of purified Anti-Mouse CD3ε (BioLegend) and 0.5 μg/ml of purified Hamster Anti-Mouse CD28 (BD Biosciences) antibodies to the co-culture. After 48h, cells were incubated with [^{3}H]thymidine (0.01μCi/μl) for 16h. A scintillation beta-counter was used to measure the incorporation of [^{3}H]thymidine and results were expressed as percentage of SPL proliferation.

MSC administration in experimental model of colitis

Colitis was induced in eight- to twelve- week- old female C57BL/6 mice by administration of 3% DSS in drinking water *ad libitum* for 10 days. 3x10^6 of MSC/GFP-MSC were injected either intraperitoneally or subcutaneously at day 5 of DSS treatment. In selected experiments, MSC were implanted encapsulated into microcapsules of barium alginate at day 5 of DSS treatment in the peritoneum. Sorted MSCs were used in the experiments at passage ranking 7 to 10. Saline was administered as control. In parallel experiments, 4 μg/mouse of Recombinant Mouse TSG-6 (R&D) was injected intraperitoneally to colitic mice starting from day 5 of DSS treatment to day 9. Survival, body weight and disease activity index (DAI) were evaluated daily as previously reported [209]. At day 10 damage to the mucosa of the colon was evaluated in vivo using the experimental Colorview

endoscopy system (Karl Storz Veterinary). Mice were then sacrificed, colon excised and colon length recorded. Colons were then Swiss-rolled and fixed in formalin 4% for 24 hours, paraffin-embedded and sectioned (4μm). H&E staining was performed to evaluate tissue damage, which was scored by a blinded pathologist as described previously [210]. Animal experiments adhered to the requirements of the Commission Directive 86/609/EEC and to the Italian legislation (Decreto Legislativo 116; 27 January 1992). The studies were approved by the Animal Care and Use Committee (Istituto Clinico Humanitas, Milan, Italy).

Encapsulation of MSC

Encapsulation of MSC into microcapsules of barium alginate was performed in collaboration with Prof. Calafiore, Department of Internal Medicine, University of Perugia, Perugia, Italy, as previously described [211], resulting in the production of microcapsules measuring 400-500 μm in equatorial diameter, with no loss of MSC function and morphological properties, either *in vitro* and *in vivo*. The viability of cells entrapped within the capsules was assessed by fluorescein diacetate and ethidium bromide staining either before the implantation or 5 days after, when they were recovered from the peritoneal cavity of mice.

Colons and structures digestion

Colons and structures from both the peritoneal and subcutaneous cavity of colitic mice were recovered at day 10 of DSS treatment, rinsed in PBS w/o calcium and magnesium supplemented with penicillin, streptomycin and amphotericin B (Cambrex). Before digestion, colons were incubated 5 min at RT with 1mM dithiothreitol (DTT; Sigma) in HBSS w/o calcium and magnesium supplemented with antibiotics and cut into small pieces. Colon fragments and structures were digested for 1hour at 37°C with constantly agitation in

RPMI medium 1640 supplemented with 10% FCS, 20µg/ml DNASE I (ROCHE), 0.5 mg/ml Collagenase NB4 Standard Grade (SERVA), 5mM $CaCl_2$. Cell suspension obtained after digestion was then filtered through cell strainer with a mesh size of 100 µm, while tissue fragments were smashed on it. The cell strainer was carefully washed with RPMI medium 1640 supplemented with 10% FCS and the washing solution obtained was pulled together with the cell suspension and filtered through cell strainer with a mesh size of 70 µm, centrifuged at 1500 rpm for 5 min at 4°C. After a final wash in PBS w/o calcium and magnesium supplemented with antibiotics, cells were ready for subsequent manipulations.

Flow Cytometry

MSC or cells coming from digestion of colons and structures were divided into FACS tubes at a density of $1x10^6$ cells/tube, and incubated with the selected primary antibody in FACS buffer (PBS w/o calcium and magnesium supplemented with 1% FCS) for 20 min at 4°C. Table 1 shows in detail the list of the used antibodies. Alexa Fluor 647 streptavidin (1: 500; Invitrogen) was used to detect the expression levels of biotinylated antibody (Lineage Cell Detection Cocktail and CD31), while Alexa Fluor 647 goat anti-mouse (1:1500; Invitrogen) was employed for purified primary antibodies (CD106, CD90, CD34 and CD54). Viability of the cell suspension was assessed by the staining with Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen). Afterwards, all samples were fixed with 1% paraformaldehyde, while intracellular staining for FoxP3 was performed using the FoxP3 Staining Buffer Set (eBioscience). Samples were analysed on a FACScalibur flow cytometer (BD Biosciences).

Table 1.

ANTIBODY	ISOTYPE AND REACTIVITY	DILUTION	COMPANY
CD11b	rat anti-mouse	1:80	eBioscience
F4/80	rat anti-mouse	1:10	AbD Serotec
Ly/6G	rat anti-mouse	1:30	BD Pharmingen
CD45R/B220	rat anti-mouse	1:50	Biolegend
CD3	rat anti-mouse	1:100	BD Pharmingen
CD45	rat anti-mouse	1:100	BD Pharmingen
CD45.2	mouse anti-mouse	1:300	eBioscience
Lin Cell Det Cocktail biotin	rat anti-mouse	1:10	Miltenyi Biotec
CD31 biotin	rat anti-mouse	1:10	BD Pharmingen
CD44	rat anti-mouse	1:100	Biolegend
CD106 (VCAM-1)	rat anti-mouse	1:80	BD Pharmingen
CD29	Armenian Hamster anti-mouse	1:100	Biolegend
CD90	rat anti-mouse	1:50	BD Pharmingen
CD54 (ICAM-1)	Armenian Hamster anti-mouse	1:80	BD Pharmingen
CD25	rat anti-mouse	1:200	eBioscience
CD4	rat anti-mouse	1:200	BD Biosciences
FoxP3	rat anti-mouse	1:50	eBioscience
Sca-1 (Ly-6A/E)	rat anti mouse	1:100	eBioscience
CD117 (C-kit)	rat anti mouse	1:20	Miltenyi Biotec
CD34	rat anti mouse	1:50	BD Biosciences

Immunohistochemistry

Immunohistochemical staining for GFP was performed as previously reported [212]. Colons of colitic mice injected with $3x10^6$ GFP-MSC 5 days after the beginning of DSS treatment were collected at day 10, fixed in 4% formalin and paraffin-embedded. Sections were cut at 3 μm thickness, deparaffinized, hydrated and subsequently subjected to microwave epitope enhancement using citrate buffer 10 mM retrieval solution. A subsequent block for endogenous peroxidase with 0.3% H_2O_2 in block buffer (PBS w/o calcium and magnesium supplemented with 0.02% NP-40 (Calbiochem) and 1% Bovine Serum Albumine (BSA;

VWR International SRL) was performed. Sections were then incubated with the primary antibody anti-GFP, biotin-conjugated (1:500, o.n. at 4°C, Invitrogen) in block buffer. Detection was achieved using a standard streptavidin-biotin system (Vector Laboratories), and antigen localization was visualized with 3′-3-diamino benzidene (Vector Laboratories).

Immunofluorescence

MSC were seeded on PolyLysine on glass coverslips as previously described and then fixed with pre-warmed paraformaldehyde (PFA) 2% for 10 min at 37°C. Cells were permeabilized with PBS containing 0.05% Tween20 (MERK), 3% BSA (VWR International SRL), 0.1% Triton X-100 (Sigma) for 20 min at RT and then blocked with PBS containing 0.05% Tween20; 3% BSA; 5% donkey serum (Jackson ImmunoResearch) or goat serum (DAKO) for 45 min at RT. Afterwards, MSC were incubated o.n at 4°C with the following primary antibodies: rat anti-mouse JAM-A (BV12, 1:20; a gift from E. Dejana, FIRC Institute of Molecular Oncology (IFOM), Milan, Italy), rat anti-mouse CD106 (1:80; BD Pharmingen), rabbit anti-mouse CD29 (1:100; Abcam) and rabbit anti-mouse RAM-1 TSG-6 (1:250; produced by AJ Day's group, Department of Biochemistry, University of Oxford, Oxford, United Kingdom). Cells were washed three times in PBS 0.05% Tween 20 and then incubated for 30 min at RT with Alexa Fluor 647-conjugated donkey anti-rabbit or Alexa Fluor 594-conjugated goat anti-rat antibodies (1:1000; Invitrogen), followed by incubation for 10 min at RT with DAPI (1:25000; Invitrogen). Finally, glass coverslip were mounted with Prolong* Gold Antifade Reagent (Invitrogen).

Whole Mount staining

Whole mount staining was performed in colon of healthy and colitic mice, injected intraperitoneally at day 5 with MSC pre-stained with $DilC_{18}(3)$-DS (Invitrogen). Briefly, MSC were collected, resuspended in PBS w/o calcium and magnesium supplemented with

0.1% FCS and 1μM $DilC_{18}(3)$-DS, incubated first 5 min at 37°C and then 15 min at 4°C immediately before the injection. 24, 48 and 72 hours after the injection, mice were perfused with 1% PFA, colons were collected, opened and cut into three pieces (rectal, proximal and distal colons), mounted on a hard silicon base and incubated with rat anti-mouse CD31 (1:150; BD Pharmingen) in PBS containing 2% BSA; 0.01% NaN_3; 0.3% Triton X-100; 5% goat serum o.n. at 4°C. Afterwards, colons were incubated with Alexa Fluor 488-conjugated goat anti-rat (1:1000; Invitrogen) o.n. at 4°C and mounted with Prolong* Gold Antifade Reagent (Invitrogen). The same protocol was used for whole mount staining of colons, in which GFP-MSC were injected intraperitoneally at day 5 of DSS treatment. After 5 days from the injection, mice were sacrificed, structures were recovered, fixed in PFA 4% o.n. at 4°C and stained with rat anti-mouse CD68 (1:200; BD Pharmingen), rabbit anti-mouse CD3 (1:100; DAKO) and rabbit anti-mouse TSG-6 o.n. at 4°C. A final incubation with Alexa Fluor 594-conjugated chicken anti-rat and/or Alexa Fluor 647-conjugated donkey anti-rabbit (1:1000; Invitrogen) o.n. at 4°C was performed before mounting the structures with Prolong* Gold Antifade Reagent (Invitrogen).

Real-Time PCR

RNA from MSC and macrophages was extracted using the commercially available RNeasy Mini kit (Qiagen), while RNA from colons and MLNs was extracted using RNeasy Lipid Tissue Mini kit (Qiagen) according to manufacturer's instructions. First-strand cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems) from 2 μg of total RNA was conducted according to the manufacturer's instructions. Then quantitative real-time PCR was performed using SyBr Green PCR Master Mix (Applied Biosystems) or TaqMan Gene Expression Master Mix (Applied Biosystems) and detected with 7900HT Sequence Detection System (Applied Biosystems). Primer sequences or probe assay ID/part numbers are reported in Table 2a and 2b, respectively. The relative mRNA abundance of all

transcripts was calculated as $2^{-\Delta Ct}$ (where ΔCt is equal to Ct sample - Ct housekeeping) analysed over the total amount of GAPDH or Actin.

Table 2a.

GENE	PRIMER SEQUENCES
CCR1	Forward, 5'- CTG CCC CCC CTG TAT TCT CT-3' Reverse, 5'- GAC ATT GCC CAC CAC TCC A-3'
CCR2	Forward, 5'- CTA CGA TGA TGG TGA GCC TTG TC-3' Reverse, 5'- AGC TCC AAT TTG CTT CAC ACT G-3'
CCR3	Forward, 5'- AGT GGG CAC CAC CCT GTG-3' Reverse, 5'- GCC ATG ACC CCA GCT CTT T-3'
CCR4	Forward, 5'- ACG AAA GCA TGC CAA AGC C-3' Reverse, 5'- CCC CAA ATG CCT TGA TAC CTT-3'
CCR5	Forward, 5'- TCA GCA CCC TGC CAA AAA AT-3' Reverse, 5'- CAG GAG CTG AGC CGC AAT-3'
CCR6	Forward, 5'- GGC CTG TAT CAG CAT GGA CC-3' Reverse, 5'- GAT TTG GTT GCC TGG ACG AT-3'
CCR7	Forward, 5'- TGG TGG TGG CTC TCC TTG TC-3' Reverse, 5'- CCT CAT CTT GGC AGA GAA GCA CA-3'
CCR8	Forward, 5'- ACC CTG ATT TCT TCA CCG CC-3' Reverse, 5'- TGC CCC TGA GGA GGA ACT CT-3'
CCR9	Forward, 5'- TGA TGC CCA CAG AAC TCA CAA-3' Reverse, 5'- TGA AGT CAT CAA ACA TGC CAG G-3'
CXCR1	Forward, 5'- TCC TGA GGT GAC TTT GAG AAA G-3' Reverse, 5'- GGC AGC ATT CCC GTG ATA TTT-3'
CXCR2	Forward, 5'- GTC ATC TTC GCT GTC GTC CTT-3' Reverse, 5'- GTT GTA GGG CAG CCA GCA G-3'
CXCR3	Forward, 5'- TGG AAA ACA GCA CCT CTC CC-3' Reverse, 5'- AGA AGT CGC TCT CGT TTT CCC-3'
CXCR4	Forward, 5'- CCT GCT TCC GGG ATG AAA A-3' Reverse, 5'- TGG TGG GCA GGA AGA TCC TAT-3'
CXCR5	Forward, 5'- ACT CGG AGC TCA ACC GAG AC-3' Reverse, 5'- AAG GTC GGC TAC TGC GAG G-3'
CXCR6	Forward, 5'- TAC GAT GGG CAC TAC GAG GG-3' Reverse, 5'- ATC ACT GGA ATT GTT GAA GAG CC-3'
CXCR7	Forward, 5'- TGT AAC AGC AGC GAC TGC ATT-3' Reverse, 5'- CAT GGT GGG ACA CTG CAC AG-3'
XCR1	Forward, 5'- TCT TCA CCG TCG TGG TAG CA-3' Reverse, 5'- TGA GGT TGT AGG GAG CCC AG-3'
CX3CR1	Forward, 5'- CTG TCC GTC TTC TAC GCC CT-3' Reverse, 5'- CAG ATT TCC CAC CAG ACC GA-3'
IDO 1	Forward, 5'- TGG CGT ATG TGT GGA ACC G-3' Reverse, 5'- CTC GCA GTA GGG AAC AGC AA-3'
IDO 2	Forward, 5'- AAG GCC AAC CCC AAA AGG TG-3' Reverse, 5'- ACC AGG ATA GGC GGG AGT C-3'
TSG-6	Forward, 5'- GGC TGG CAG ATA CAA GCT CA-3'

	Reverse, 5'- TCA AAT TCA CAT ACG GCC TTG G-3'
TGF-β	Forward, 5'- ATC CTC AAG TTG CAC CCT TAT CT-3' Reverse, 5'- AAA GAG CCT TCG GTG GAT TGC-3'
IL-10	Forward, 5'- GCT CTT ACT GAC TGG CAT GAG-3' Reverse, 5'- CGC AGC TCT AGG AGC ATG TG-3'
IFN-γ	Forward, 5'- GGA TGG TGA CAT GAA AAT CCT GC-3' Reverse, 5'- TGC TGA TGG CCT GAT TGT CTT-3'
IL-6	Forward, 5'- TAG TCC TTC CTA CCC CAA TTT CC-3' Reverse, 5'- TTG GTC CTT AGC CAC TCC TTC-3'
IL-4	Forward, 5'- CCA TAT CCA CGG ATG CGA CAA-3' Reverse, 5'- CCT CGT TCA AAA TGC CGA TGA T-3'
IL-5	Forward, 5'- ACT GTC CGT GGG GGT ACT G-3' Reverse, 5'- AGG AAC TCT TGC AGG TAA TCC A-3'
IL-13	Forward, 5'- CAG CCT CCC CGA TAC CAA AAT-3' Reverse, 5'- GCG AAA CAG TTG CTT TGT GTA G-3'
IL-17a	Forward, 5'- TCA GCG TGT CCA AAC ACT GAG-3' Reverse, 5'- GAC TTT GAG GTT GAC CTT CAC AT-3'
MMP12	Forward, 5'- GAG TCC AGC CAC CAA CAT TAC-3' Reverse, 5'- GCG AAG TGG GTC AAA GAC AG-3'
GAPDH	Forward, 5'- AGG TCG GTG TGA ACG GAT TTG-3' Reverse, 5'- TGT AGA CCA TGT AGT TGA GGT CA-3'

Table 2b.

GENE	ASSAY ID/ PART NUMBER	COMPANY
MMP3	Mm00440295_m1	Applied Biosystems
MMP9	Mm00442991_m1	Applied Biosystems
MCP1 (CCL2)	Mm00441242_m1	Applied Biosystems
MIP1α (CCL3)	Mm00441259_g1	Applied Biosystems
Actin (ACTB)	4352341E	Applied Biosystems

Structures and MSC Organ Culture

Structures were recovered from the peritoneal cavity of colitic mice 48 and 72h after MSC injection. The structures were carefully washed in cold PBS supplemented with antibiotics and then incubated at 37°C and 5% CO_2 in DMEM medium supplemented with 0.1% FCS and antibiotics. After 24 hours, supernatant fluid was collected, centrifuged and stored at -20°C for subsequent analysis, while cells that had detached from the structures and adhered to the dish surface were collected and analysed by flow cytometry. In parallel experiments, MSC were seeded at a density of 10000 cells/cm^2 in a 96-well plate in complete MSC

expansion medium and when confluence was reached, supernatant fluid was collected, centrifuged and store at -20°C. Since MSC expansion medium contained unknown growth factors and cytokines, an aliquot of this culture medium was used as negative control in further analysis.

Bio-Plex Assay and ELISA

The concentration of TSG-6 was evaluated in the supernatants obtained from Organ Culture of MSC and in the serum of healthy and colitic mice injected or not with MSC, 48 and 72 hours after the injection. The quantification was assessed following the manufacture's instruction of the ELISA kit for Tumor Necrosis Factor Alpha Induced Protein 6 (TNFaIP6) (Life Science Inc). In parallel experiments, serum samples were obtained from healthy and colitic mice injected with either MSC, Recombinant Mouse TSG-6, or saline and sacrificed 5 days after the injection. The concentration of IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17a, IFN-γ, KC, MCP-1 (MCAF) and TNF-α was detected using Bio-Plex Mouse Cytokine Group I 12-plex Assay (Biorad). Naïve mice were used as negative control. Finally, protein colon lysates of mice injected with either BM-or A-MSC were prepared by homogenization in Lysis Buffer (50mM Tris-HCl pH 7.4; 1mM EDTA pH 8; 150mM NaCl; 0.1% Triton X-100; 1X Protease Inhibitor cocktail (Roche); 1X PMSF (SIGMA) with TissueLyser II (Qiagen) and analysed for the concentration of IL-6, TNF-α, IFN-γ, Il-10, RANTES (CCL5), MIP-1α (CCL3) and MCP-1 (CCL2) using commercially available ELISA kits (R&D).

Western Blot

Protein lysates were collected from MSC, as previously described. Protein samples were separated by 10% SDS-PAGE under reducing conditions by using Tris-glycine running buffer. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF)

membrane (Biorad). Membrane was blocked in 5% milk in PBS for 1 hour and then incubated with rabbit anti-mouse RAM-1 TSG-6 (1:500) in PBS supplemented with 0.1% Tween20 and 5% milk o.n. at 4°C. Membrane was washed and incubated first for 1 hour with HRP-conjugated anti-rabbit IgG (VWR International) in PBS containing 0.1% Tween20 and then with Immobilon Western Chemiluminescent HRP Substrate (Millipore) for 1 minute. Antigen-antibody complexes were detected by using ChemiDoc XRS (Biorad). MMP3 and MMP9 protein levels were analysed on colon lysates of healthy and colitic mice injected with either MSC, Recombinant Murine TSG-6 or saline using the following antibodies: rabbit anti-mouse MMP3 (1:1000; abcam) or rabbit anti-mouse MMP9 (1:800; Aviva Systems Biology) as primary antibodies; HRP-conjugated anti-rabbit IgG as secondary antibody. The filter was then stripped with buffer Restore (Pierce) and reprobed with an anti–β-actin antibody to control for protein loading.

Analysis of collagen deposition within the structures

Structures were recovered from the peritoneal cavity of colitic mice, fixed in PFA 4% o.n. at 4°C and mounted with Prolong* Gold Antifade Reagent on agarose-coated glass slide to maintained their 3D organization. Type I collagen deposition inside structures was visualized by Two Photon Excitation Microscopy (TPEM) and Second Harmonic Generation (SHG) signals for every chosen layer was recorded. The received pictures of type I collagen fibres were then assembled and analysed. The excitation wavelength of two photon laser was tuned to 820 nm. Alternatively, structure frozen sections of 20µm thickness were fixed in 37% formaldehyde and stained with Picro-Sirius Red to visualize collagen type I and III fibers. Briefly, sections were incubated with Picro-Sirius Red stain solution (0.1% direct red 80 (Sigma) and 0.1% fast green FCF (Sigma) dissolved in saturated aqueous picric acid (1.2% picric acid (Sigma) in water) for 60 min at RT. After rinsing in distilled water and HCl 0.01M (PH=2) for 2 min at RT, sections were dehydrated

and mounted in xylene. Bright field images were acquired with a 4x and 40x objectives (IX51 inverted microscope, Olympus).

Statistical Analysis

Data were analysed using GraphPad software. The Student *t* tast was used for comparisons between groups. Statistical significance was set at $P<0.05$.

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