ABSTRACT

Myeloid-derived suppressor cells (MDSCs), characterized by the co-expression of CD11b and Gr1, are a heterogeneous population of immature myeloid cells that exhibit strong suppressive functions against T cell responses. In inflammatory conditions like IBD, there is an increase in MDSCs but this is not sufficient to improve intestinal inflammation in IBD. Herein, we investigated the expansion of MDSCs in TNBS-induced acute colitis and whether the adoptive transfer of in vitro generated MDSCs ameliorated intestinal inflammation. We found that CD11b⁺Gr1⁺ MDSCs were significantly increased in experimental colitis. Further, this increase correlated to some extent with the severity of the disease. As per our protocol, MDSCs were generated from bone marrow cells co-cultured with hepatic stellate cells (HSCs), an essential cell type to obtain functional MDSCs in vitro. Adoptive transfer of HSC-induced MDSCs improved body weight loss and significantly downregulated inflammatory cytokines TNF, IFN-γ, and IL-17 in colonic tissue. Our results indicate MDSCs are Immunoregulatory players in intestinal inflammation and that the adoptive transfer of in vitro generated MDSCs may provide a novel therapeutic approach for inflammatory bowel disease.
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To the late Doris Geodeke
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<td>Alpha-Smooth Muscle Actin</td>
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<td>Antigen-Presenting Cells</td>
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<td>ATG16L1</td>
<td>Autophagy-Related 16-Like 1</td>
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<td>BM</td>
<td>Bone Marrow</td>
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<td>CARD15/NOD2</td>
<td>Caspase Recruitment Domain-Containing Protein 15/Nucleotide-Binding Oligomerization Domain-Containing Protein 2</td>
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<td>DCs</td>
<td>Dendritic Cells</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>G-CSF</td>
<td>Granulocyte-Colony Stimulating Factor</td>
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<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
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<td>Hematoxylin and Eosin</td>
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<td>IMCs</td>
<td>Immature Myeloid Cells</td>
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<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<td>LPMC/s</td>
<td>Lamina Propria Mononuclear Cells</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
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<td>MDSCs</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
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<td>NK</td>
<td>Natural Killer Cell</td>
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<td>NKT</td>
<td>Natural Killer T Cell</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<td>Th</td>
<td>Helper T Cells</td>
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<td>TLRs</td>
<td>Toll-Like Receptors</td>
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<td>TNBS</td>
<td>2,4,6,-Trinitrobenzene Sulfonic Acid</td>
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<td>Treg</td>
<td>Regulatory T Cell</td>
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MYELOID-DERIVED SUPPRESSOR CELLS AS REGULATORS OF THE IMMUNE SYSTEM

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Figure A. Origin of MDSCs

Figure B. Suppressive mechanisms of MDSCs
INTRODUCTION

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory condition of the gastrointestinal tract, comprised mainly of Crohn’s disease and ulcerative colitis, generally characterized by clinical symptoms including fatigue, weight loss, diarrhea, bloody stools, and intestinal inflammation. Typically, inflammation in Crohn’s disease is transmural and in ulcerative colitis it is confined to the mucosa. The former is identified by the enhanced production of T helper 1 (Th1) and T helper 17 (Th17) proinflammatory cytokines, which can affect any area of the bowel but most commonly the small intestine and the colon in a fragmented fashion. In contrast, the latter is vaguely associated with T helper 2 (Th2) responses, mainly affecting the colon.

Epidemiology

Epidemiological studies have revealed the highest incidence and prevalence rates of IBD in industrialized or “Westernized” regions of the world such as northern Europe, the United Kingdom, and North America. Interestingly, disease emergence has been reported in recent years in places where it was thought to be formally uncommon like southern or central Europe, Asia, Africa, and Latin America, hence, making IBD a worldwide health concern. It is estimated that 1.4 million individuals suffer from IBD in the United States and another 2.2 million in Europe. In Canada,
approximately 170,000 persons (0.5% of the entire population) have IBD, with usual high rates in Manitoba, making Manitoba home of the highest incidence rates in the world. Crohn’s disease and ulcerative colitis affect the young and adults, both men and women. It has, however, been observed that Crohn’s disease is slightly more predominant in females and ulcerative colitis in males, both with an overall age-specific incidence peak in the third decade of their lives.  

Pathogenesis

Although the etiology or pathogenesis of IBD still remains unknown, significant progress has been made in elucidating the players that orchestrate the development of intestinal inflammation. Substantial evidence indicates IBD is the result of complex interactions between environmental factors and aberrant immunological responses to enteric bacteria in genetically susceptible individuals.

Environmental Factors

As communities become more educated about health issues and improve their living conditions, their lack of proper exposure to infectious agents in early childhood renders them more susceptible to these organisms when encountered later in life, developing abnormal immune responses. This “Westernization” of lifestyle in either developing countries or amongst the offspring of migrants to industrialized nations could help explain how the environment contributes to the development of IBD.

Smoking. Cigarette smoking has been well established as a contributor to an increased risk of Crohn’s disease development while conferring partial protection against ulcerative colitis. A meta-analysis of selected studies reported that current smokers
more than double their risk for developing Crohn’s disease, and former smokers are at a greater risk than nonsmokers. Inversely, current smokers are 40% as likely to acquire ulcerative colitis as nonsmokers, and former smokers are at a greater risk than nonsmokers.\textsuperscript{32} The mechanisms of protection against ulcerative colitis in smoking individuals are not known, however, special attention has been given to nicotine.\textsuperscript{33, 34} A study showed nicotine \textit{in vivo} inhibits Th2 responses mainly seen in ulcerative colitis, while no effect is noted on the inhibition of Th1 cytokines predominating in Crohn’s disease.\textsuperscript{35}

\textit{Diet.} The intake of food may alter enteric flora or the functional properties of the epithelium. Over the last two decades, the Japanese population has experienced an increase in IBD, regardless of lacking a genetic mutation, NOD2, which has been associated with disease susceptibility.\textsuperscript{36} This increase in IBD may be due to the change in diet, which includes products richer in linoleic acids (beef and pork) than ω-3 fatty acids (fish).\textsuperscript{37} Also, it has been hypothesized that the excessive intake of sugar (Fermentable Oligo-, Di- and Monosaccharides and Polyols) promotes the expansion of the flora in the gut and increases the permeability of the intestine perhaps by hindering barrier function, thus, predisposing genetically susceptible individuals to Crohn’s disease.\textsuperscript{38}

\textit{Appendectomy and other factors.} Removal of the appendix appears to provide a protective effect for ulcerative colitis. Duggan et al indicated the removal of the organ in individuals younger than 20 years of age provides the greatest protection against the disease.\textsuperscript{39} Several other environmental factors such as vaccination, occupation, stress, and oral contraceptives have been suggested to increase the risk of IBD; however, further
evidence is required to establish a strong association between these environmental factors and the disease.\textsuperscript{40}

**Immune Dysfunction**

The intestinal epithelium serves as a physical barrier to prevent the entry of harmful substances from the lumen into the lamina propria. Intestinal epithelial cells are responsible for the sampling of gut microbiota via pattern-recognition receptors (PRRs) such as TLR and NOD, and for the secretion of mucus (goblet cells) and anti-microbial peptides (Paneth cells). Furthermore, innate and adaptive immune cells also play a key role in the regulation of intestinal microbiota. Through a complex network of interactions and signals, these cell populations and their products maintain a tolerogenic tone against non-pathogens in the normal intestine. However, in IBD, this tolerant state is lost and abnormal immune responses develop against the microbiota, resulting in chronic inflammation and subsequent tissue damage.\textsuperscript{41-44}

*Innate immunity.* The innate immune system is designed to provide an initial and quick response to pathogens in a non-specific fashion. Defects in the intestinal epithelium may allow exposure of TLR-expressing cells to excessive luminal antigens, triggering the innate arm of the immune system.\textsuperscript{43} Innate cells like dendritic cells (DCs) and macrophages increase in numbers and upregulate the expression of TLR, recognizing molecular patterns from microbes. In IBD, TLR-activated gut DCs and macrophages stimulate the activation of the NF-\(\kappa\)B protein complex, which then turns on the transcription of genes responsible for the increased production of proinflammatory cytokines such as IL-12/IL-6 and IL-23/TNF-\(\alpha\), respectively.\textsuperscript{45,46} The aforementioned proinflammatory cytokines along with chemokines (e.g. CCL20),\textsuperscript{47,48} adhesion molecules
(e.g. I-CAM), and co-stimulatory molecules (e.g. CD40) become critical for the amplification and maintenance of intestinal inflammation through the recruitment and activation of local and peripheral immune cells. These innate immune responses set the stage for the adaptive response.

Adaptive immunity. The adaptive immune system evolved to provide a more specific response to antigens, exhibiting diversity and memory. CD4+ T cells are central regulators of adaptive immune responses in IBD and their differentiation is greatly influenced by the environment and cytokine milieu.

In Crohn’s disease, both Th1 and Th17 cells have been implicated as the key mediators driving the pathogenesis of the disease. The sustained activation of these cells leads to the inappropriate secretion of cytokines and other mediators that exhibit inflammatory functions resulting in tissue damage. Accumulated evidence supports the expansion of Th1 and Th17 cell populations and their cytokine profiles in Crohn’s disease patients. The Th1 cell subset is regulated by T-bet in the presence of IL-12 (composed of p40 and p35 subunits) which is a cytokine typically produced by APCs activated in response to intracellular pathogens. In Crohn’s disease, Th1 cells produce enhanced amounts of TNF-α, IFN-γ, and IL-2 cytokines, which may cause tissue injury or recruit other inflammatory cells such as NK cells and macrophages. These recruited inflammatory cells may also contribute to tissue destruction via IFN-γ and TNF-α production, respectively. Conversely, Th17 cells are driven by RORγt in the presence of IL-6 and TGF-β, and are further expanded by IL-23 (p40/p19 heterodimer). The increased expression of Th17 cytokines in Crohn’s disease such as IL-17, IL-21, IL-6, and TNF-α may also promote the destruction of tissue and enhance T-cell resistance to
apoptosis. The differentiation of Th1 and Th17 cells is negatively cross-regulated by each other.\textsuperscript{51}

In ulcerative colitis, Th2 responses are thought to mediate the disease. Though IL-4 is absent in ulcerative colitis tissues, increased levels of IL-5, IL-13, and IFN-\textgamma have been observed in the mucosa of these patients.\textsuperscript{41,42} The overexpression of IL-13, which is also produced by other non-T cells (e.g. NK T cells), may cause epithelial cells to become dysfunctional.\textsuperscript{41} Lastly, Th2 cell differentiation is inhibited by Th1 cytokines and vice versa.

In healthy individuals, these Th cell responses are counterbalanced by regulatory T cells (Treg) via the production of anti-inflammatory mediators such as IL-10, TGF-\textbeta, and IL-35 or through the induction of apoptosis.\textsuperscript{54} However, in IBD, Treg cells are downregulated or absent in the lamina propria and Th cells become resistant to apoptosis.\textsuperscript{55} In IBD experimental models, IL-10-deficient mice develop spontaneous enterocolitis as they produce insufficient levels of IL-10, a cytokine known for its anti-inflammatory and immunoregulatory functions.\textsuperscript{56}

**Enteric Microbiota**

More than $10^{14}$ microorganisms reside within the gastrointestinal tract (mostly in the colon) consisting of more than 1000 different species and their numbers are greater than the number of cells in an individual.\textsuperscript{54,57} The composition of the intestinal microbiota includes Bacteroidetes (gram-negative bacteria), Firmicutes (gram-positive bacteria), Proteobacteria, Actinobacteria, viruses, protists, and fungi; over 90% of these microorganisms belong to the first two categories or phyla.\textsuperscript{56,58} Commensal microflora defends the host from enteric pathogen colonization and contributes to the development
of the immune system, yet it is possible it contributes to IBD as well. It is unknown, however, whether the immune system responds to triggers from selected microbes or the entire enteric microbial community. Studies have reported that the administration of probiotics to IBD patients improved the disease. On the other hand, pathogenic agents have also been implicated in the pathogenesis of IBD such as *Mycobacteria paratuberculosis*, *Listeria monocytogenes*, measles virus, and specific strains of *Escherichia coli*, but none of these have been shown to be essential for the development of the disease. Adherent-invasive E. coli, however, has been strongly associated with the pathogenesis of Crohn’s disease as it is able to adhere and penetrate the epithelium.

Finally, studies involving IBD experimental models have reported that mice kept in a sterile germ-free environment do not develop inflammation in the intestines suggesting a relationship between enteric microbes and the pathogenesis of IBD.

**Genetic Factors**

Genetic abnormalities such as CARD15/NOD2 and ATG16L1 mutations have been strongly associated with the development of IBD in genetically susceptible individuals.

First, the CARD15 gene encodes NOD2, a cytosolic receptor expressed in epithelial cells, Paneth cells, macrophages, DCs, and endothelial cells. DCs in the lamina propria sense peptidoglycan (PGN) via TLR2 and become activated, leading to the activation of NF-κB and resulting in the production of antimicrobial peptides and cytokines that promote the differentiation of IFN-γ and IL-17 producing cells. NOD2, on the other hand, becomes activated upon the recognition and ligation of a peptide found in PGN, muramyl dipeptide (MDP), suppressing the activation of NF-κB mediated by PGN.
The inhibition of NF-κB by NOD2 downregulates the secretion of cytokines responsible for the induction of proinflammatory cells. However, the function of NOD2 may be impaired in Crohn’s disease patients, resulting in the enhanced production of proinflammatory cytokines. Individuals affected by Crohn’s disease carrying a dysfunctional NOD2 protein (20 to 30% of patients) show declined expression of antimicrobial peptides like α-defensins by paneth cells, allowing bacteria to thrive; thus, leading to inadequately prolonged immune responses. Similar findings showing decreased α-defensin production by Paneth cells have been reported in Nod2-/- mice studies.

Second, ATG16L is a gene associated with autophagy regulation. Autophagy is a catabolic process with the ability to degrade organelles within cells in response to cellular stress, leading to cell death when prolonged. Crohn’s disease patients expressing the ATG16L1 mutation have defective Paneth cells; as a result, paneth cells may fail to effectively regulate enteric microbes via α-defensin production.

**Therapeutic Strategies for IBD**

The design of an ideal therapy that would rebalance gut immune responses in IBD patients consists in achieving and maintaining remission free of side effects and the least possible surgical interventions. Commonly, as IBD worsens it requires a shift from anti-inflammatory drugs, to immunosuppressant regimens, or to biological agents. Current biological therapies with proven efficacy used in the clinic for IBD target proinflammatory cytokines or block selective adhesion molecules important for the recruitment of leukocytes. Other promising therapeutic strategies include the blockade
of receptors involved in the activation and differentiation of T-cells, induction of anti-inflammatory cytokines, and cell-based therapies.\textsuperscript{69,70}

Current Biological Therapies

\textit{Anti-TNF monoclonal antibody}. TNF-\(\alpha\) is a proinflammatory cytokine that plays a critical role in IBD. Currently, three anti-TNF-\(\alpha\) monoclonal antibodies are used to treat IBD patients: infliximab, certolizumab pegol, and adalimumab. Infliximab, a chimeric monoclonal antibody, has been shown effective in treating refractory Crohn’s disease and ulcerative colitis;\textsuperscript{71,72} however, some patients generate antibodies against the agent.\textsuperscript{73} Certolizumab pegol, a humanized monoclonal antibody, has shown high remission rates in individuals with Crohn’s disease. Patients treated with certolizumab pegol develop antibodies to the agent too, but these are fewer than those treated with infliximab.\textsuperscript{74} Finally, adalimumab, a fully human monoclonal antibody, has also been reported to induce remission in Crohn’s disease individuals with moderate to severe illness.\textsuperscript{75} As a final note, all these anti-TNF monoclonal antibodies promote apoptotic cell death in T cells in the intestine.\textsuperscript{76}

\textit{Selective adhesion molecule inhibitors}. The interaction between immune migratory cells and endothelial cells is crucial for the homing of leukocytes to the site of injury or insult. Agents like Natalizumab and MLN-0002 have been used to prevent the accumulation of inflammatory cells in the intestines of IBD patients. Natalizumab, for instance, binds \(\alpha_4\) integrin and interferes with the adhesion and transmigration of leukocytes to the gut.\textsuperscript{68} This agent has been shown to be effective for achieving remission in Crohn’s disease in a couple of phase-II clinical trials.\textsuperscript{77} Though recalled once for safety evaluations, Natalizumab is back in the market for treating Crohn’s disease and other
autoimmune conditions. On the other hand, MLN-0002 binds leukocyte $\alpha 4/\beta 7$ integrin, preventing leukocyte adhesion to MAdCAM-1 specifically in the intestines. Lastly, this agent has demonstrated its efficacy in ulcerative colitis.

**Promising Therapies**

*Anti-IL-12/IL-23 p40 antibodies.* ABT-874 and ustekinumab target the p40 subunit shared by IL-12 and IL-23, which are essential cytokines for the differentiation and survival of Th cells implicated in intestinal inflammation. A trend for remission was observed in Crohn’s disease patients receiving ABT-874, which was characterized by lower levels of IL-12, IFN-$\gamma$, and TNF in the colon. A substantial number of patients, however, experienced reactions at the injection site and a small number of them developed antibodies against the agent.

*Recombinant human cytokines.* The local administration of anti-inflammatory agents like IL-10, which blocks the production of inflammatory mediators by macrophages, has shown to increase IL-10 levels in the intestinal mucosa of mice. This therapy has achieved encouraging results in a phase I study in patients with Crohn’s disease and the same approach is being explored in ulcerative colitis.

*Cell-based therapy.* Another attractive strategy being explored aims at “resetting” the immune system through Hematopoietic cell transplantation (HCT). The majority of patients with Crohn’s disease who have undergone allogeneic or autologous HCT have achieved remission, thus, establishing the effectiveness of HCT. However, questions such as HCT conditioning, transplant related mortality, and the transfer of known susceptible genes from allogeneic donor to recipient are of great importance. Tolerogenic DCs and Treg cells are also being considered as immunotherapeutic tools to restore tolerance.
Tolerogenic DCs are characterized by their ability to produce immunosuppressive cytokines and downregulate their own expression of co-stimulatory molecules, inducing and expanding Treg cells as their key mechanism for the maintenance of tolerance.\textsuperscript{82} Genetically modified tolerogenic DCs have been shown to prevent and suppress experimental arthritis, but at the same time they have been shown to prime allogeneic responses in transplantation, resulting in a faster rejection of the allograft.\textsuperscript{85} Similarly, \textit{in vitro} expanded Treg cells prevented and reversed diabetes in diabetes-susceptible NOD mice,\textsuperscript{86} however, when Treg cells from diabetic patients were expanded \textit{ex vivo} these mutually expressed TNF-\(\alpha\) and IL-17.\textsuperscript{69}

Finally, though therapies currently available in the market have demonstrated acceptable efficacy, concerns such as elevated costs, immunosuppression, development of antibodies against used agents, and side effects urgently call for more effective therapeutic options.

**Myeloid-Derived Suppressor Cells**

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells (IMCs), consisting of myeloid progenitor cells, which exhibit strong immunosuppressive functions against T cell responses. MDSCs were described more than two decades ago as a splenic macrophage-like suppressive cell population found responsible for the suppression of antitumor cytotoxicity in a MOPC-315 tumor model.\textsuperscript{87} Young et al. also reported the appearance of a bone marrow derived suppressor cell population termed “natural suppressor cells,” which accumulated in response to soluble factors produced by tumor cells in tumor-bearing mice\textsuperscript{88} and in cancer patients.\textsuperscript{89}
Further studies identified the expansion of a CD11b+/Gr1+ cell population with similar functional characteristics as “natural suppressor cells” and were speculated to be responsible for the decline of antigen tumor T cells in immunized mice. In recent years, this attractive cell population, MDSCs, has gained special attention due to its strong potential for immunosuppression observed not only in cancer settings but also in non-tumor diseases.

**Origin of MDSCs**

In the bone marrow, the natural process of myelopoiesis, which is regulated by cytokines and soluble factors, generates IMCs. Under physiological conditions, these IMCs differentiate into mature DCs, granulocytes, or macrophages; however, in pathological settings, the differentiation of IMCs is partially inhibited, resulting in the expansion and activation of a cell population with strong immunosuppressive features collectively known as myeloid-derived suppressor cells (Fig. A).

**Identification of MDSCs**

The description of MDSCs in tumor settings has facilitated their uniform characterization across different diseases in mice, but not in humans. In mice, MDSCs lack the expression of mature myeloid cell markers and are consistently identified by the non-specific co-expression of both CD11b and Gr1 antigens. CD11b, an αM integrin, is mainly expressed on myeloid cells (monocytes and macrophages, granulocytes, DCs) and lymphocytes (T and B cells). Gr1 (Ly6G and Ly6C), a myeloid differentiation antigen, is expressed by myeloid precursor cells and granulocytes. Monocytes also express Gr1 but
**Figure A.** Origin of MDSCs. 

**a** | Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, which takes place in the bone marrow and is controlled by a complex network of soluble factors. **b** | Normally, IMCs migrate to different peripheral organs, where they differentiate into macrophages, dendritic cells or granulocytes. However, factors that are produced during acute or chronic infections, trauma or sepsis, inflammation, and in the tumor microenvironment promote the accumulation of IMCs at these sites, prevent their differentiation and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSCs).
transiently. Two MDSC subsets have been characterized: CD11b\(^+\)Gr1\(^{\text{high}}\) (or CD11b\(^+\)Ly6G\(^+\)) granulocytic and CD11b\(^+\)Gr1\(^{\text{low}}\) (or CD11b\(^+\)Ly6C\(^+\)) monocytic. While the granulocytic group (70-80%) is more numerous than the monocytic group (20-30%), the second one is more suppressive. Granulocytic MDSCs produce higher amounts of ROS and lower levels of NO than monocytic MDSCs, while both express arginase 1. Even though granulocytic and monocytic MDSCs share morphological features between polymorphonuclear neutrophils (PMNs) and monocytes, respectively, their functional aspects are different. For example, granulocytic MDSCs are immunosuppressive and PMNs are not. Also, granulocytic MDSCs produce higher amounts of ROS and arginase 1, and are less phagocytic than PMNs. The second MDSC subtype, monocytic, is more suppressive and produces higher amounts of iNOS and arginase 1 than monocytes. In humans, MDSCs from cancer patients also lack the expression of mature myeloid cell markers, but their phenotype and subsets are rather vaguely described and may vary with disease type. In malignant settings, MDSCs were originally characterized as LIN\(^-\)HLA-DR CD33\(^+\) or CD11b\(^+\)CD14\(^-\)CD33\(^+\). Still, the hallmark and most reliable marker for the identification of MDSCs in either mice or humans is their own suppressive function.

Expansion and Activation of MDSCs

In the bone marrow of normal mice, CD11b\(^+\)Gr1\(^+\)-expressing cells account for approximately 20%, while 2-4% of these cells are found in the spleen with no detectable suppressive activity (IMCs make up 0.5% of peripheral blood mononuclear cells in healthy persons). Nevertheless, in tumor-bearing mice, this cell population expands in the spleen 20-40%, acquiring immunosuppressive functions. The expansion of MDSCs is
promoted by factors like GM-CSF, G-CSF, M-CSF, SCF, VEGF, and IL-13, which are produced during pathological conditions through the stimulation of myelopoiesis and by inhibiting their differentiation into mature myeloid cells. These factors elicit signaling pathways in MDSCs that lead to the persistent activation of STAT3, which is involved in preventing differentiation and apoptosis and promoting survival and proliferation of MDSCs. The activation of MDSCs, dependent on STAT6 and STAT1 signaling, is influenced by factors released by activated T cells and tumor cells such as TGFβ, IFN-γ, IL-4, IL-13, and ligands for TLRs; and the activation of these two signaling pathways, along with STAT3, contributes to the upregulation of immunosuppressive mediators in activated MDSCs.1

Suppressive Mechanisms of MDSCs

*In vitro* and *in vivo* studies have reported mouse MDSCs suppress CD8+ T-cell responses in an antigen-specific fashion, perhaps through the uptake and presentation of soluble or tumor antigens, and require cell-cell contact. Whether CD4+ T-cell suppression by MDSCs is antigen-specific is still under investigation. Nonetheless, MDSC suppression in peripheral lymphoid organs may be antigen-specific in order to avoid systemic immunosuppression and to allow T cells to respond to other antigens. The mechanisms of suppression by activated MDSCs include the production of arginase 1, iNOS, ROS, peroxynitrite, and the induction of Treg cells (Fig. B). First, arginase 1, produced by both MDSC subsets, metabolizes L-arginine found in the local microenvironment. The reduction or depletion of L-arginine negatively influences the expression of CD3ζ in T cells and impairs their function. Second, iNOS, which is
mainly produce by monocytic MDSCs, generates NO, which then disrupts JAK3 and
STAT5 signaling in T cells and inhibits their function. Third, ROS is mainly produced
by the granulocytic group, and it also contributes to the downregulation of the CD3 chain expression in T cells and suppresses their antigen-specific responses. Fourth, peroxynitrite produced by MDSCs during cell-cell contact nitrates the CD8+ T cell receptor, making T cells unresponsive to specific antigen. Fifth, MDSCs also promote the differentiation of CD4+ T cells into Treg cells, perhaps through interactions of cell-surface co-stimulatory molecules.

MDSCs in Different Pathological Conditions

Since their initial identification in malignant settings more than two decades ago, MDSCs have also been recently described in different non-tumor disorders. For instance, studies have reported the significant expansion of a CD11Gr1 cell population upon parasitic infection with Leishmania major, Trypanosoma brucei, T. cruzi, and Plasmodium chabaudi, which mediated T cell hyporesponsiveness via NO. During a viral infection, the generation of large numbers of CD11b LyC cells suppressed immune responses to Theiler’s murine encephalomyelitis virus (TMEV), leading to the establishment of continuous infection in the CNS; however, the depletion of these cells resulted in the improvement of demyelinating disease that was associated with an increase of antigen-specific T cell responses. In allergic airway inflammation, three CD11b Gr1 subsets were identified in which Ly6C Ly6G and Ly6C Ly6G showed anti-inflammatory activity, while Ly6C Ly6G showed proinflammatory activity. The increase of a heterogeneous and immature IL-10-producing cell population in a
Figure B. Suppressive mechanisms of MDSCs. Myeloid-derived suppressor cells (MDSCs) consist of two main subsets: monocytic MDSCs, which have a CD11b^+LY6G^-LY6C^hi phenotype, and granulocytic MDSCs, which have a CD11b^+LY6G^+LY6C^low. In most tumour models, it is predominantly (70-80%) the granulocytic subset of MDSCs that expands. We suggest that the granulocytic subset of MDSCs has increased activity of signal transducer and activator of transcription 3 (STAT3) and NADPH, which results in high levels of reactive oxygen species (ROS) but low nitric oxide (NO) production. ROS and, in particular, peroxynitrite (the product of a chemical reaction between superoxide anion and NO) induces the post-translational modification of T-cell receptors and may cause antigen-specific T-cell unresponsiveness. The monocytic MDSC subset has upregulated expression of STAT1 and inducible nitric oxide synthase (iNOS), and increased levels of NO but low ROS production. NO, which is produced by the metabolism of L-arginine by iNOS, suppresses T-cell function through various different mechanisms that involve the inhibition of Janus kinase 3 and STAT5, the inhibition of MHC class II expression and the induction of T-cell apoptosis. Both subsets have increased levels of arginase 1, which causes T-cell suppression through depletion of L-arginine. Only monocytic MDSCs can differentiate into mature dendritic cells and macrophages \textit{in vitro}. 
polymicrobial sepsis mouse model, which required signaling through MyD88 for its expansion, inhibited CD8+ T cell responses and was observed to also orchestrate the characteristic shift from Th1 to Th2 polarization in sepsis. Also, a monocytic CD11b+Ly6C+Ly6G- cell population with regulatory functions was reported to efficiently suppress both CD4+ and CD8+ T cell proliferation through NO production in experimental autoimmune encephalomyelitis (EAE).

Cancer

In recent years, the study of MDSCs in tumor settings has led to the identification of additional markers for this cell population. Blood samples from patients with colon cancer, breast cancer, and kidney cancer have shown the expansion of LIN-HLA-DR CD33+CD11b+ MDSCs. Similarly, peripheral blood from individuals with different types of cancer revealed a defective and immature DC population that lacked the expression of HLA-DR (MHC-II) and co-stimulatory molecules, exhibiting inhibitory activities against antigen-specific T cell responses in vitro; nevertheless, the addition of GM-CSF and all-trans-retinoic acid (ATRA) promoted the differentiation of these cells into mature DCs, restoring their capacity to stimulate an immune response. In mice, CD11b+Gr1+ cells were found to facilitate tumor progression via TGF-β production in a 4T1 mammary tumor model. Additionally, IFN-γ-stimulated Gr1+CD11b+ myeloid suppressor cells inhibited T cell proliferation in vitro and induced Treg cells via IL-10 and TGF-β secretion in tumor-bearing mice.
Inflammatory Bowel Disease

To date, Haile and colleagues have recently reported the appearance of an MDSC-like cell population in a particular transgenic mouse model of experimental colitis. Colitis in mice harboring enterocyte-specific hemagglutinin (HA) was induced by a single transfer of HA-specific CD8+ T cells. Interestingly, three HA-specific CD8+ T cell transfers induced a substantial expansion of CD11b^+Gr1^+ cells in both spleen and gut. These CD11b^+Gr1^+ MDSCs were found to suppress the proliferation of CD8+ T cells when assayed ex vivo. Furthermore, co-transfer of splenic MDSCs together with HA-specific CD8+ T cells impaired inflammation in HA-transgenic mice, thus, demonstrating that MDSCs provide protection. Finally, this same group also described the expansion of a cell population exhibiting a phenotype suggestive of MDSCs in peripheral blood of IBD patients.

Adoptive Transfer of In Vitro Generated MDSCs

Haile et al. demonstrated that the adoptive transfer of MDSCs isolated from the spleen of colitis mice ameliorates inflammation; nonetheless, the method for acquiring MDSCs is not practical for research and clinical practice. A recent study reported the effectiveness of co-transplanted MDSCs generated in vitro in the protection of islet allografts from host immune attacks during the entire course, resulting in long-term survival in more than 60% of islet allografts in the absence of immunosuppressant agents. This finding is further supported by another study in which co-transfer of in vitro generated MDSCs significantly increased the percentage of long-term survivors in about 75% of mice transplanted with allogeneic pancreatic islets for the entire 200-day
observation period.\textsuperscript{121} In GVHD, this approach successfully prevented the disease,\textsuperscript{122} leading to long-term survival in about 82\% of the recipients.\textsuperscript{123} These observations indicate the potential role of MDSCs in the long-term inhibition of immune responses.

**Generation of MDSCs In Vitro**

The generation of MDSCs _ex vivo_ and their subsequent administration into patients manifesting diseases characterized by prolonged and abnormal activation of T-cell responses may potentially improve the patient’s condition by inhibiting these aberrant immune responses. Different approaches have been explored for generating MDSCs _in vitro_ via co-culture systems using bone marrow,\textsuperscript{124} or peripheral blood,\textsuperscript{125} or embryonic stem cells\textsuperscript{123} with a combination of cytokines and growth factors or hepatic stellate cells.\textsuperscript{126}

**Growth Factors/Cytokines**

Several factors have been reported to promote the expansion of MDSCs _in vitro_. Combined LPS and IFN-\(\gamma\) treatment generates MDSCs from bone marrow progenitor cells under GM-CSF conditions, blocking the development of DCs.\textsuperscript{124} A cocktail of cytokines generates functional MDSCs from embryonic stem cells capable of inducing CD4\(^+\)CD25\(^+\)Fox3\(^+\) Treg cells and suppressing T cell activation _in vitro_ and _in vivo_ in a GVHD model.\textsuperscript{123} GM-CSF alone or in combination with a mixture of cytokines promotes the generation of CD33\(^+\) MDSC-like cells from PBMCs isolated from healthy donors.\textsuperscript{125} Also, IL-13\textsuperscript{122}, or GM-CSF and IL-6, or GM-CSF and G-CSF, induce the differentiation of mouse and human bone marrow precursor cells into immunosuppressive MDSC.\textsuperscript{121}
Hepatic Stellate Cells

Stellate cells are retinoid-storing cells found in the pancreas, lung, kidney, intestine, and liver.\textsuperscript{127} Extrahepatic stellate cells exhibit a fibroblast morphology, while hepatic stellate cells (HSC) display a star-like shape, playing non-immunological and immunological functions.\textsuperscript{128} Of importance, HSC participate in the healing process after liver injury, possess strong inhibitory functions against T-cell responses, and have been shown to potently induce MDSCs \textit{in vitro} and \textit{in vivo} through IFN-\(\gamma\) and complement 3 (C3) production.\textsuperscript{126}

When HSCs are in a dormant state, they express desmin and glial fibrillary acidic protein (GFAP) and contain lipid droplets in the cytoplasm.\textsuperscript{129} However, during liver injury, HSCs transition into an activated state changing their morphology to that of fibroblast-like cells or myofibroblasts, lose retinoids, decrease GFAP expression, upregulate the expression of desmin and alpha-smooth muscle actin (\(\alpha\)-SMA), and secrete multiple factors.\textsuperscript{129-131} Similarly, \textit{in vitro} cultures of primary HSCs adhere to the surface of uncoated plastic and become activated, change their morphology, lose characteristic droplets, and upregulate the expression of \(\alpha\)-SMA.\textsuperscript{131,132} And, though not specific, \(\alpha\)-SMA is the most dependable marker for the identification of HSCs \textit{in vitro} as other local cells do not express it with the exception of smooth muscle cells enclosing large vessels.\textsuperscript{131}

The co-transplantation of HSCs has been shown to protect islet allografts from being rejected by inducing T cell hyporesponsiveness\textsuperscript{133} and Treg cell development,\textsuperscript{134} and by promoting MDSC generation.\textsuperscript{126} This co-transplantation needs to be autologous as allogeneic HSCs provide limited protection,\textsuperscript{135} nonetheless, large numbers of HSCs are
needed for co-transplantation and such obtainment could add risks to the recipient. Alternatively, substantial numbers of MDSCs can be generated \textit{in vitro} from the recipient’s bone marrow-derived myeloid precursor cells in the presence of small amounts of HSCs, which can be obtained from discarded liver donors or surgical specimens.\textsuperscript{136} \textit{In vitro} generated MDSCs have been shown to suppress effector T cell responses \textit{in vivo}, thereafter, substituting HSCs for protecting cell transplants from immune attack.\textsuperscript{126} In addition, this same group recently demonstrated that MDSC propagation by HSCs is not major histocompatibility complex (MHC) restricted;\textsuperscript{136} that is, allogeneic HSCs can be used to induce recipient’s bone marrow-derived MDSCs. Collectively, these exciting findings demonstrate that generating MDSCs \textit{in vitro} is feasible.
RATIONALE

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory condition of the gastrointestinal tract characterized by exaggerated Th1 and Th17 cell responses and deficient Treg cell functions. In IBD, a defective innate immune system fails to control bacterial entry into the gut mucosa, leading to the abnormal activation of adaptive immunity, which results in substantial intestinal damage. In particular, over-activated Th cells from the adaptive immune system secrete proinflammatory cytokines such as TNF, IFN-γ, and IL-17, which exacerbate inflammation causing bowel tissue destruction. Though other immune cells also contribute to the production of these cytokines, the inflammatory cytokine secretion especially from T cell sources has been found to have deleterious effects in autoimmune diseases and to also recruit and activate other inflammatory cells.

Current biological therapies aim at blocking the aforementioned proinflammatory cytokines after being released, often targeting a single molecule (e.g. TNF); however, their effectiveness is limited, among other reasons, due to the heterogeneity of IBD, which involves multiple cytokines and immune cells for its development. A commonly used therapy in clinical practice that has achieved great success is infliximab, an anti-TNF mAb, but its drawbacks such as high costs ($27,000/year) and the large proportion of individuals that develop antibodies against the agent outweigh its benefits. Alternatively, cell-based therapies like Hematopoietic cell transplantation (HCT) have
attempted to “reset” the immune system through hematopoietic cell infusion, demonstrating great promise by inducing clinical remission in Crohn’s disease patients. Nonetheless, HCT requires rigorous conditioning, including high doses of chemotherapy, and it also involves the potential for transplant-related mortality and the transfer of known susceptible genes between allogeneic donor and recipient, predisposing recipients to other diseases. Other attractive therapeutic approaches in mouse models have shown the potential of tolerogenic dendritic cells (DCs) and regulatory T cells (Treg) to restore tolerance. Genetically engineered tolerogenic DCs have been shown to prevent and suppress experimental arthritis, but at the same time they have been shown to prime allogeneic responses, resulting in a faster rejection of the allograft. Such DCs exert their tolerogenic effects via the induction of Treg cells, hence their persistence could lead to immunosuppression. Similar to tolerogenic DCs, Treg cells have been shown to revert diabetes in mice, but their inherent instability and plastic phenotype, such as the concomitant expression of TNF-α and IL-17, could be detrimental. Thus, the urgent need for novel and more effective therapies is eminent.

Myeloid-derived suppressor cells (MDSCs) exhibit intrinsic suppressive functions against multiple Th cell responses and also promote the development of Treg cells. MDSCs are expanded by factors produced during a pathogenic state which include cyclooxygenase 2, prostaglandins, stem-cell factor, and macrophage-colony stimulating factor, triggering signaling pathways like STAT3 that prevent their differentiation; the activation of MDSCs is carried out by factors produced mainly by T cells, such as IFN-γ, ligands for Toll-like receptors, and IL-4, which also activate signaling pathways like
STAT6, STAT1, and nuclear factor-κB involved in the upregulation of short-lived soluble mediators. These soluble mediators include arginase 1, iNOS, ROS, and peroxynitrite through which MDSCs mediate their immunosuppressive activities. The initial identification of MDSCs in cancer has led to their recent description in other conditions like viral and parasitic infections, allergy, sepsis, encephalomyelitis, and experimental colitis.

At the present time, little is known about MDSCs in IBD. To address this, we sought to determine the role of MDSCs in a TNBS-induced colitis mouse model, which is commonly used for IBD research due to its immediate onset colonic inflammation and absence of genetic manipulations typically not found in IBD patients. MDSCs play multiple roles in the immune system depending on the context that they are found in. For example, in tumor settings, they assist malignant cells to evade immune surveillance, while in autoimmune diseases like IBD, MDSC-mediated Th cell inhibition may protect the host. Though MDSCs are expanded in inflammatory conditions like IBD, their numbers are not sufficient to help ameliorate intestinal inflammation. Strong evidence from animal models of graft-versus-host disease (GVHD) and enterocolitis suggest that the adoptive transfer of MDSCs, either generated in vitro or isolated ex vivo, significantly improves immune-mediated injury. Several systems have been developed to generate MDSCs in vitro through cultures of bone marrow progenitor cells together with a cocktail of cytokines or hepatic stellate cells (HSCs). Here, we used HSCs as they have been shown to propagate large numbers of MDSCs in vitro. Therefore, an adoptive cell therapy with MDSCs could target the proliferation and effector functions of Th cells, limiting or even preventing the release of multiple inflammatory cytokines (e.g. TNF,
IFN-γ, and IL-17) that cause intestinal tissue destruction, and induce Treg cells to restore and maintain tolerance. Questions whether MDSC persistence would predispose IBD patients to cancer are of great importance; nevertheless, MDSCs have been shown to disappear upon disease improvement.\textsuperscript{126,142} Hence, \textbf{we hypothesize that TNBS-induced colitis promotes the expansion of MDSCs, and that the adoptive transfer of \textit{in vitro} generated MDSCs can ameliorate murine acute colitis via Th1 and Th17 cell suppression and through the induction of Treg cells.}
SPECIFIC AIMS

1. To identify myeloid-derived suppressor cells in mice with TNBS-induced acute colitis.

2. To establish the technique for generating hepatic stellate cell-induced myeloid-derived suppressor cells (H-MDSCs) \textit{in vitro} from bone marrow progenitor cells

3. To explore whether adoptive transfer of hepatic stellate cell-induced myeloid derived suppressor cells (H-MDSCs) can improve TNBS-induced acute murine colitis.
MATERIALS AND METHODS

Animals

Female BALB/c mice (7-8 weeks old) were purchased from Charles River Laboratories (Saint-Constant, PQ, Canada) and kept at the Central Animal Care Services, University of Manitoba. The University Animal Ethics Committee approved all protocols used.

Induction of Colitis

A commonly used mouse model of IBD is colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), which haptinizes colonic proteins leading to the development of a delayed-type hypersensitivity reaction characterized by Th1 responses. This hapten-induced colonic inflammation mimics human Crohn’s disease characterized by transmural mononuclear cell infiltrates, abnormal crypt architecture, loss of goblet cells, and ulcerations. Here, mice were lightly anesthetized with isoflurane, and then intrarectally challenged with TNBS (Sigma-Aldrich) via a 3.5 F catheter affixed to a 1-mL syringe. The catheter was inserted through the rectum until the tip was advanced 4 cm into the colon, and a total of 100 µl of TNBS (1.8 mg in 50% ethanol) was injected. To ensure retention of TNBS within the entire colon and cecum, mice were held in a vertical position for 40 seconds after the administration. For the development of acute colitis, mice were administered TNBS twice at days 1 and 8. All animals were sacrificed.
4 days after the second TNSB challenge.

**Body Weight Monitoring**

Mice were monitored twice a day for the first 48 hours and once a day thereafter. All animals, except normal group, received 1 mL of saline 24 hours after each TNBS challenge as they dehydrate and sometimes present symptoms of diarrhea. Mice were weighed daily in the mornings prior to either TNBS or saline administration. Mice that reached a humane end point at 20% body weight loss were sacrificed and their body weight values excluded from the analysis.

**Preparation of HSCs**

HSC were isolated from normal mouse livers by perfusion, via subhepatic vena cava, with 20 mL of a solution (I) containing Ca$^{2+}$ and Mg$^{2+}$–free Hank’s balanced salt solution (HBSS; Sigma Chemical Company), 283 g/L HEPES, 35 g/L NaHCO$_3$, 90 g/L glucose, 8.5 g/L EGTA, pH 8, followed by 20 mL of a solution (II) containing 0.15 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN). The liver was then excised, mashed, and incubated at 37°C for 20 minutes in a suspension solution (III) containing 0.15 mg/mL collagenase D, 0.4 mg/mL pronase (Roche Diagnostics, Indianapolis, IN), 20µg/mL DNase (Roche Diagnostics, Indianapolis, IN). Both solutions II and III were dissolved in Ca$^{2+}$ and Mg$^{2+}$–free Hank’s balanced salt solution (HBSS; Sigma Chemical Company), 283 g/L HEPES, 35 g/L NaHCO$_3$, 90 g/L glucose, 110 g/L CaCl$_2$). The resulting homogenate was filtered and then centrifuged on a density gradient cushion. HSC were collected from the upper layer and cultured in 20% FCS in 5% CO$_2$ in air at
37°C for 7-14 days. The purity of HSC was > 95% as determined by the expression of α-SMA and their star-like shape appearance examined under typical light.126

**LPMC Isolation**

LPMC were isolated from freshly removed colon tissue. Colon samples were washed in Ca²⁺ and Mg²⁺–free Hank’s balanced salt solution (HBSS; Sigma Chemical Company), opened longitudinally, cut into 5 mm pieces, and incubated in HBSS containing EDTA (0.37 mg/mL) and DTT (0.145 mg/mL) at 37°C for 30 minutes in a shaking incubator (this releases intraepithelial lymphocytes and epithelial cells). The tissue specimens were then digested in Ca²⁺ and Mg²⁺–free HBSS containing 0.5 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN) and 0.01mg/mL DNase (Roche Diagnostics, Indianapolis, IN) in a shaking incubator for 1.5 hours at 37°C. The released cells were centrifuged on a 40-100% Percoll gradient (Pharmacia). LPMC were collected at the 40-100% interface.

**Splenocyte Isolation**

The spleen was removed and mashed with a plunger through a cell strainer. Splenocytes were lysed of red blood cells, washed in phosphate buffer solution (PBS), and resuspended in RPMI-1640.

**HSC-Induced MDSC (H-MDSC) Generation**

Bone marrow (BM) cells were isolated from normal mouse tibias and femurs, lysed of red blood cells, and 2 X 10⁶/well cultured in 10% FCS in the presence of 15
ng/mL GM-CSF. HSCs were added at the beginning of culturing (HSC:BM cell ratio of 1:50). The BM cell culture without HSC was used as control cells (cells were exposed to 1 µg/mL LPS for the last 18-hour culture to generate mature DCs). 5-6 days later, both H-MDSC and DC cultures were harvested.

**Adoptive Transfer**

The following four groups were used for this experiment:

A) H-MDSCs (received TNBS and H-MDSCs; n=8)

B) DC (received TNBS and DCs; n=7)

C) Saline (received TNBS and saline; n=7)

D) Normal (received neither TNBS nor H-MDSCs; n=4)

A total of 1 X 10⁶ in vitro generated H-MDSC were adoptively transferred into mice in the H-MDSC group. 1 x 10⁶ mature DCs were injected into mice in the DC group. Mice in the saline group received 100 µL saline. All mice in these three groups received intravenous injections through the tail vein once on day 1, 4 hours prior to the first TNBS administration. TNBS was given on day 8 again. All mice were sacrificed four days after the second TNBS challenge and the spleen and colon were removed for analysis.

**Cytokines Measured by ELISA**

Frozen colonic specimens were homogenized in a buffer consisting of 1M Tris-HCl, 3M NaCl, and 10% Triton supplemented with protease cocktail (Sigma-Aldrich, St. Louis. MO). Then, samples were frozen at -70°C and thawed in a water bath at 37°C.
This process was repeated three times and was followed by centrifugation at 2,500 *rpm* for 10 minutes at 4°C. The supernatants were immediately used to measure the concentrations of TNF, IFN-γ, and IL-17 (BD Bioscience) by ELISA or frozen at -70°C until assayed. ELISA measurement was performed according to the manufacturer’s instructions.

**Flow Cytometry Analysis**

PE-Cy7-anti-CD11b, PE-anti-Gr1, FITC-anti-Ly6C, and APC-anti-Ly6G monoclonal antibodies were purchased from Biolegend. 1 X 10^6 cells were stained with labeled antibodies against CD11b, Gr1, Ly6C, Ly6G, and incubated on ice for 30 minutes and then washed with FACS buffer. Cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

**Histology**

The intestine was removed, sectioned, and fixed in 10% formalin. The tissue sections were embedded in paraffin blocks and cut longitudinally 6-μm thick. Slides were stained with hematoxylin and eosin (H&E) for inflammation assessment. A pathologist, blinded to the type of treatment, examined the histological sections according to the following parameters: a) severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0-3: none, slight, moderate, severe), depth of injury (0-3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0-4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and
epithelium lost). Mice received scores individually, all of which were added to a maximum of 10.

**Immunofluorescence Staining**

Cells were grown on uncoated 8-well coverslip dishes and fixed in ice-cold 4% paraformaldehyde at room temperature for 30 minutes. Cells were then permeabilized in blocking solution (Amresco, United States) containing 0.5% Triton X-100 and incubated at 4°C for 1 hour with FITC-labeled anti-α-SMA (1:200) antibody in 1%BSA solution. Cells were then stained with Hoechst (1:200) and incubated at room temperature for 5 minutes. The reaction was evaluated using fluorescent microscopy.

**Statistical Analyses**

All experiments were performed more than two times, and values expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test or Student’s t test using GraphPad Software (San Diego, California, USA). *P* < 0.05 was considered statistically significant.
RESULTS

**CD11b^+Gr1^+ MDSCs Are Expanded in Experimental Colitis**

To address the question whether MDSCs accumulate in TNBS-induced acute colitis, we detected CD11b^+Gr1^+ MDSCs in BALB/c mice with colitis. Mice were intrarectally challenged twice with TNBS to establish the development of intestinal inflammation. All mice were sacrificed four days after the second TNBS administration and their spleens and colons were examined. Spleens from normal mice contained 4.34±0.41% of CD11b^+Gr1^+ cells (Fig. 1A); however, a significant increase in the number of CD11b^+Gr1^+ MDSCs was observed in mice with colitis (Fig. 1A). We further characterized CD11b^+Gr1^+ MDSCs into their two well-defined subsets, monocytic and granulocytic, based on their phenotypic expression CD11b^+Ly6C^+ and CD11b^+Ly6G^+, respectively. Both monocytic and granulocytic subtypes were significantly increased in the colitis group when compared with normal mice (Fig. 1B and C). In addition, we analyzed whether the expansion of CD11b^+Gr1^+ MDSCs in the spleen and LPMC is correlated with the severity of the disease. In a normal mouse, 0.27% of LPMCs and 4.77% of splenocytes were CD11b^+Gr1^+ MDSCs (Fig. 2, far left panel). The frequency of this cell population was relatively higher in animals with moderate inflammation or severe inflammation. In a colitis mouse with moderate inflammation, CD11b^+Gr1^+ MDSCs were found to be 1.57% of LPMCs and 10.29% of splenocytes (Fig. 2, middle panel), while in a colitis mouse exhibiting severe inflammation, CD11b^+Gr1^+ MDSCs
were found to be 12.5% of LPMCs and 59% of splenocytes (Fig. 2, far right panel). The frequency of CD11b+Gr1+ MDSCs in each mouse was higher in spleen than LPMCs (Fig. 3). Taken together, these findings indicate CD11b+Gr1+ MDSCs are significantly increased in experimental colitis and that this expansion in the spleen and LPMCs correlates with the severity of intestinal inflammation.

**Figure 1.** CD11b+Gr1+ MDSCs are increased in the spleen of TNBS-induced acute colitis. BALB/c mice were intrarectally challenged with TNBS twice at a one-week interval. Four days after the second TNBS administration, mice were sacrificed. Splenocytes were isolated from normal and colitis mice, stained for markers expressed by MDSCs and their two subsets, CD11b, Gr1, Ly6C, and Ly6G, and analyzed by flow cytometry. (A) Colitis mice showed a significant increase in CD11b+Gr1+ MDSCs when compared to naïve mice. This same expansion was seen when CD11b+Gr1+ MDSCs were further characterized into their respective (B) monocytic and (C) granulocytic subsets. **P < .01**
Figure 2. Expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs in LPMCs and spleen is correlated to some extent with the severity of acute colitis. Representative analysis of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs in LPMCs and spleen of colitis mice shows the recruitment of this cell population is highest in a mouse with severe disease (far right panel). Also, the expansion of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs in LPMCs and spleen is higher in a mouse with severe inflammation (middle panel) than in the naïve mouse (far left panel).
Figure 3. The frequency of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs is higher in spleen than LPMCs. All three mice expressed higher percentages of CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs in the spleen (right) than LPMCs (left), but these were highest in the mouse with severe inflammation than in the mouse with moderate inflammation and naïve mouse.
**HSCs Promote the Development of MDSCs In Vitro**

To generate MDSCs in vitro, we first isolated and cultured HSCs from mouse liver for 7-14 days. HSCs adhered to the bottom of the 6-well plate, proliferated, and lost their intracytoplasmatic lipid droplets as they became activated by day 9. HSCs transitioned from a quiescent state noted by their star-like shape to an activated state observed by a myofibroblast morphological change (Fig. 4A). HSCs were identified by their morphological characteristics under light microscopy and by the expression of \( \alpha \)-SMA (Fig. 4B), a marker for activated HSCs, using fluorescent microscopy. We then isolated bone marrow cells from normal mouse femurs and tibias and cultured them. HSCs were added at the beginning of culture at a ratio of 1:50 (HSC:BM), in the presence of GM-CSF, for 5 days to generate HSC-induced MDSCs (H-MDSCs). Cell culture without HSCs was used as control cells and LPS was added during the last 18 hours of culture to drive the maturation of DCs. These cultures were stained with labeled antibodies against CD11b, CD11c, Gr1, Ly6C, and Ly6G, and analyzed using flow cytometry. 90.1% of H-MDSCs were CD11b\(^+\)CD11c\(^-\) and 62.6% of DCs were CD11b\(^+\)CD11c\(^+\). In H-MDSC cultures, 47.7% of cells expressed CD11b and Gr1 molecules; while in DC cultures, 33.2% of cells expressed CD11b and Gr1 molecules. H-MDSCs contained only 1.14% of CD11b\(^+\)CD11c\(^+\) cells, whereas DCs contained 25.4% of CD11b\(^+\)CD11c\(^+\) (Fig. 5), indicating the inhibition of DC development in the CD11b\(^+\)Gr1\(^+\) H-MDSCs culture.

To further characterize these in vitro generated MDSCs into their respective subsets, granulocytic and monocytic, cell cultures were gated on CD11b\(^+\) and analyzed for the expression of Ly6C and Ly6G. Here, 43.1% of H-MDSCs were Ly6G\(^+\)Ly6C\(^-\)
granulocytic and 0.289% were Ly6G\(^+\)Ly6C\(^+\) monocytic; while 20.4% of DCs were Ly6G\(^+\)Ly6C\(^-\) and 3.79% were Ly6G\(^-\)Ly6C\(^+\) (Fig. 6). Our results indicate HSCs induce high levels of CD11b\(^-\)Gr1\(^+\) MDSCs, and that most of these H-MDSCs express markers that resemble the granulocytic subset, Ly6G\(^+\)Ly6C\(^-\).

**Figure 4.** Morphology of HSC culture at day 9. (A) HSCs were isolated from mouse livers and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum for 7-14 days. The purity of HSC preparation was > 95% as determined by their typical appearance under a light microscope and by their expression of α-SMA. (B) The expression of α-SMA by HSCs was analyzed by fluorescent microscopy. HSCs were grown on uncoated 8-well coverslip dishes, fixed in ice-cold 4% paraformaldehyde, and stained with FITC-labeled antibody against α-SMA and Hoechst for the nuclei at a dilution of 1:200. The green color shows α-SMA and the blue color shows the nuclei. Magnification (X 400).
**Figure 5.** *In vitro* generation of H-MDSCs from bone marrow progenitor cells. 2 X 10⁶ bone marrow cells were co-cultured with HSCs at a ratio of 50:1 in the presence of GM-CSF for 5 days to generate CD11b⁺Gr1⁺ H-MDSCs (bottom left). For control cells, CD11b⁻CD11c⁺ DCs, LPS was added during the last 18 hours of culture (upper right). Cells were stained against CD11b, CD11c, Gr1, Ly6C, and Ly6G, and analyzed using flow cytometry.
Figure 6. Characterization of \textit{in vitro} generated H-MDSCs. Cells were stained against CD11b, CD11c, Gr1, Ly6C, and Ly6G, and analyzed using flow cytometry. H-MDSCs (left) and DCs (right) were gated on CD11b$^+$ and further characterized into monocytic and granulocytic subsets based on the expression of Ly6C and Ly6G.
Adoptive Transfer of H-MDSCs Ameliorates Experimental Colitis

Our lab has previously investigated the suppressive activity of in vitro generated H-MDSCs and found H-MDSCs inhibit the differentiation of Th17 cells and induce Treg cell differentiation in an in vitro system. Hence, we investigated the effects of in vitro generated H-MDSCs in an in vivo system using TNBS-induced acute colitis mice by adoptive transfer. In this preventive study, mice were injected 1 X 10^6 H-MDSC or control cells intravenously (tail vein) on day 1. Four hours later, mice received the first TNBS administration and a second dose on day 8 (1.8 µg in 50% ethanol). The transfer of H-MDSC resulted in mild colitis as indicated by the improvement in body weight loss after day 6 and by the fast recovery following the second TNBS dose (Fig. 7). A maximum of 4.17% loss in body weight was seen in the treated mice after the second TNBS administration, while the positive control groups, saline and DC, showed a maximum of 9.08% and 9.85% loss in body weight, respectively (Fig. 7). We also assayed for inflammatory cytokines known to play a central role in the development of inflammation in TNBS-induced colitis such as TNF, IFN-γ, and IL-17. As expected, colonic TNF, IFN-γ, and IL-17 cytokine levels were significantly downregulated in the group treated with H-MDSCs when compared to DC and saline groups (Fig. 8). The improvement of intestinal inflammation in the H-MDSC treated group was further confirmed by histological analysis of the colonic tissue. H&E staining indicated that mice receiving H-MDSCs had a more preserved intestinal structure and slight neutrophil infiltrates, correlating these changes with a 3.75 inflammation score. In contrast, colonic tissue sections from the saline and DC groups showed the presence of basal two-thirds crypt damage and moderate neutrophil infiltrates, correlating with a 6.25 and 5.8
inflammation score, respectively (Fig. 9). Our data suggest adoptive transfer of H-MDSC improves body weight loss and downregulates inflammatory cytokines in colonic tissue, thus, ameliorating intestinal inflammation.

Figure 7. Adoptive transfer of H-MDSCs improves body weight loss. Mice were intravenously injected once with 1 X 10⁶ H-MDSCs, or 1 X 10⁶ DCs, or 100 mL of saline. Four hours later, the first TNBS challenge was administered (1.8 µg in 50% ethanol) followed by a second dose on day 8. Four days after the second TNBS administration, mice were sacrificed and their spleen and colon were removed for analysis. As expected, mice treated with H-MDSCs showed improved body weight loss when compared to the saline and DC groups.
Figure 8. Adoptive transfer of H-MDSCs downregulates colonic levels of inflammatory cytokines. Splenocytes were stained for TNF, IFN-γ, and IL-17, and analyzed by ELISA. Th cell inflammatory cytokines levels were significantly downregulated in mice treated with H-MDSCs compared to saline and DC groups. *$P < 0.05$, **$P < 0.01$
**Figure 9.** Adoptive transfer of H-MDSCs improves intestinal inflammation. Colonic samples were stained with H&E and analyzed by a histopathologist blinded to the different treatment groups. The inflammation scores were assigned based on three different parameters: severity of inflammation, depth of injury, and crypt damage. Magnification (X 100), *P < .05*
DISCUSSION

The secrets of IBD remain to be unlocked; nonetheless, substantial evidence supports the theory of a multi-factor phenomenon versus a single component as the causation of the disease.\textsuperscript{3} Though it is widely accepted that the combination of environmental factors, genetic factors, enteric microflora, and abnormal immune responses contribute to the development of IBD, the last one is implicated to directly induce tissue damage. Aberrant immune responses are explicitly seen in both forms of IBD, Crohn’s disease and ulcerative colitis. Crohn’s disease has been characterized by the enhanced expression of Th1 and Th17 cell cytokines, while ulcerative colitis exhibits a moderate Th2 cytokine profile.\textsuperscript{143} It is important to note that although the main inflammatory cytokines involved in the pathogenesis of IBD are characteristic of the aforementioned Th cell subsets, other immune cells also secrete these cytokines, thus, contributing to the development of the disease. For example, in Crohn’s disease, Th1 cells produce TNF and IFN-γ and Th17 cells secrete IL-17. Similarly, macrophages/NK cells produce TNF and IFN-γ and neutrophils/CD8+ T produce IL-17.\textsuperscript{41,144} Th2 cells make IL-13, and NK T cells also produce this cytokine in ulcerative colitis.\textsuperscript{145} Hence, the targeting of a single cytokine or immune cell is unlikely to effectively treat the condition, as IBD is a heterogeneous disease that relies on multiple cytokines and immune cells for its development.
MDSCs are an attractive group of cells exhibiting strong suppressive functions against various types of T cell responses in pathological states,\textsuperscript{1} although their physiological role still remains a mystery. A great wealth of evidence indicates the expansion of MDSCs in cancer settings promotes tumor escape from immune surveillance;\textsuperscript{96} however, the immunoregulatory role played by MDSCs may be beneficial in autoimmune and inflammatory diseases like IBD. Herein, we detected CD11b\textsuperscript{+}Gr1\textsuperscript{+} MDSCs in both normal and TNBS-induced colitis mice. Splenocytes and LPMCs of colitis mice were stained against CD11b and Gr1, and analyzed using flow cytometry. As expected, both the spleen and LPMCs of colitis mice showed a significant increase of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells when compared to normal mice. Our data are supported by another experimental colitis study where the expansion of CD11b\textsuperscript{+}Gr1\textsuperscript{+} MDSCs was reported in the spleen and MLN of antigen-specific enterocolitis transgenic mice.\textsuperscript{100} A caveat to MDSC identification is the lack of specific markers as other immune cells also co-express CD11b and Gr1; thus, the assessment of inhibitory functions by CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells is important to indicate whether these CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells are MDSCs. Although the immunosuppressive properties of expanded CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells were not explored in this study, my colleagues did perform functional assays and found that CD11b\textsuperscript{+}Gr1\textsuperscript{+} isolated from colitis mice could inhibit the proliferation of splenocytes \textit{in vitro} when compared with control cells (manuscript submitted). Hence, based on their expression of cell surface markers and their inhibitory activity \textit{in vitro}, these expanded CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells in TNBS-induced colitis mice are therefore regarded as MDSCs.

In addition, we investigated the increase in frequency of CD11b\textsuperscript{+}Gr1\textsuperscript{+} MDSCs in relation to the severity of the disease in three mice representative of normal, moderate
inflammation, and severe inflammation. As anticipated, the mouse affected by severe inflammation expressed the highest percentage of CD11b⁺Gr1⁺ cells in the spleen and LPMCs compared to the mouse with moderate inflammation and the naïve mouse, respectively. Although these results came from a small sample analyzed at one time point only, our lab (manuscript submitted) and others have reported the kinetics of MDSC accumulation in colitis and prostate inflammation, respectively, showing the highest frequency of MDSCs at the peak of acute inflammation.

Though MDSCs are expanded in experimental colitis, their numbers are not sufficient to improve the disease. Thus, the adoptive transfer of MDSCs generated in vitro may be required to regulate inflammatory responses. Here, we used HSCs due to their strong potential for promoting MDSC generation in vitro and in vivo. Additionally, our lab has previously compared the suppressive activity of in vitro generated MDSCs using three different approaches by co-culturing BM cells together with IL-6/GM-CSF, or LPS/GM-CSF, or HSCs, and found H-MDSCs to exhibit the strongest effects for promoting Treg cell differentiation and for inhibiting Th17 cell differentiation than the other two approaches (unpublished data). Therefore, we isolated HSCs and identified them by their typical morphological appearance and by the expression of α-SMA (purity > 95%). Next, we co-cultured BM cells together with HSCs to generate H-MDSCs in vitro. Approximately 50% of CD11b⁺ cells from the H-MDSC culture were Gr1⁺; while the frequency of control cells expressing CD11c was ~25%. Thus, it is necessary to improve culturing techniques in order to obtain a higher frequency of CD11b⁺Gr1⁺ H-MDSCs and a higher purity of DCs (CD11c⁺). Also, in vitro
generated H-MDSCs will require further assessment to explore whether they mediate suppression through the production of iNOS and arginase 1.

To investigate whether in vitro generated H-MDSCs improved intestinal inflammation, we adoptively transferred H-MDSCs into TNBS-induced colitis mice, as this mouse model resembles close immunological characteristics of Crohn’s disease. Mice receiving H-MDSCs showed improved body weight loss, reduced levels of colonic Th cell inflammatory cytokines (TNF, IFN-γ, and IL-17), and a more preserved intestinal structure. Other groups have also demonstrated that adoptive transfer of in vitro generated MDSCs potently inhibit unwanted immune responses in transplantation and GVHD. Although our findings show that H-MDSCs provided greater protection in the maintenance of intestinal mucosa over mature DCs, perhaps by downregulating Th cell cytokine levels, the use of other control cells such as immature DCs and macrophages need to be considered in future experiments. Also, Treg cell cytokines such as IL-10 and TGF-β need to be assessed in mice treated with H-MDSCs, as these were not evaluated in this study.

Now, the adoptive transfer of MDSCs and the potential local or systemic persistence of immunosuppressive MDSCs might raise concerns that they could create a favorable environment for tumor growth, increasing the risk for cancer development. This situation, however, seems unlikely to occur as the recruitment and activation of MDSCs takes place exclusively during pathological settings in response to inflammation- and cancer-released mediators. Reports have shown that MDSCs disappear or decline in numbers upon disease improvement. Thus, the role of MDSCs should be investigated in inflammation-related tumorigenesis using the DSS-induced colitis-
associated colon cancer model\textsuperscript{146} to explore whether treatment with MDSCs contributes to tumor growth and progression.

A cell-based therapy using MDSCs seems attractive for the potential treatment of IBD; however, questions related to safety, stability, dosage, route of administration, and the predisposition to cancer are of great importance. Little is known about B cells in IBD; thus, it would be interesting to explore whether MDSCs also influence the expansion of the CD1d\textsuperscript{+} B cell subpopulation, which has been reported to exhibit regulatory functions in a diabetes mouse model.\textsuperscript{147} Future studies will require clarification on the mechanisms of MDSC-mediated Th cell suppression in non-cancer pathogenic conditions as most of the existing knowledge on the MDSC field has come from tumor setting reports in which the expression of MHC II by MDSCs was absent or substantially low.\textsuperscript{92}
CONCLUSION

Herein, we reported for the first time a potential preventive role of MDSCs in TNBS-induced colitis, a murine model that resembles Crohn’s disease in several aspects, including cytokine dysregulation and no genetic modifications normally not found in IBD patients. In this study we showed that CD11b\(^{+}\)Gr1\(^{+}\) MDSCs are expanded in TNBS-induced murine colitis, but such increase in MDSC numbers is not sufficient to prevent the disease. Our findings demonstrated that \textit{in vitro} generated H-MDSCs are effective in the amelioration of TNBS-induced colitis. Adoptive transfer of H-MDSCs alleviated intestinal inflammation as indicated by the improvement in body weight loss, downregulation in Th cell inflammatory cytokine levels in the colon, and reduction of inflammation scores, suggesting that H-MDSCs exhibit an immunoregulatory role \textit{in vivo}. Our work differs from the one published by Haile et al.,\(^{100}\) mainly due to the fact that they analyzed the role of MDSCs in a colitis model of IBD using transgenic mice that expressed hemagglutinin in enterocytes and their method for acquiring MDSCs is not practical for research and clinical practice as they isolated MDSCs from the spleen; hence, we believe our model better addresses these potential issues, making it more suitable for translation research. Other groups have shown the expansion of MDSCs \textit{in vitro} from human peripheral blood\(^{125}\) and bone marrow,\(^{121}\) but further research is needed to better characterize these cells in non-malignant settings and to generate MDSCs that are suitable for safe administration to individuals with autoimmune diseases like IBD. A
cell-based therapy with MDSCs has great potential for the effective treatment of IBD, one that could prove to be superior to current strategies by directly preventing Th cell inflammatory cytokine secretion instead of attempting to block individual molecules after they have already been released.
REFERENCES


