THE ISOLATION, CHARACTERIZATION AND APPLICATION OF A POPULATION OF HIGHLY IMMUNOSUPPRESSIVE BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS IN AUTOIMMUNE DISEASE

Ding Yuan Oh

Doctor of Philosophy

School of Applied Science and Engineering, Faculty of Science,

Monash University

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ADDENDUM

Chapter 1

p 21, Section 1.5.3: Add at the beginning of line 1:

"To date, several cultured cell populations such as fibroblast and MSC, have been demonstrated to exert immunodulatory effects on immune cells".

Chapter 2

p 52, Section 2.15: Add "Data were tested for normality and" at the beginning of line 2 to read "Data were tested for normality and Krustal-Wallis test were used to compare..."

Chapter 3

p 53, para 1, line 1: Replace "two" with "more than five" to read "Since the discovery of MSC more than five decades ago..."

p 74, para 2, add in line 2 after sentence ending with, "...isolation of BM-MSC."

"To obtain sufficient cell numbers for MSC isolation, 5-FU-treated BM cells (p.0) were cultured for 10 days prior to MSC isolation."

p 81, para 2: Replace first and second sentences with:

"To date, the use of cytotoxic drugs such as 5-FU is largely restricted to the treatment of cancer, and in pre-conditioning in HSCT to ablate immunity. However, the usage of these drugs for *in vivo* cell enrichment has yet been proposed. Therefore, the direct administration of 5-FU to patients prior to MSC isolation may pose a hurdle in effective translation of this methodology into the clinic. Nevertheless, the ability to replicate 5-FU treatment of BM *in vitro* could prove to be useful."

Chapter 4

p 86, Section 4.3.1, line 2: Add "bulk" in front of "BM cells" to read "...5-FU-treated bulk BM cells as described in section 2.4."

p104, FIGURE 4.8, line 1: Replace "differentiation potential" with "growth kinetics".

Chapter 5

p121, para 1: Replace second sentence with:

"As multiple doses of MSC may mask the therapeutic effect of MSC *per se*, mice were given only a single dose of either 1x 10^6 5-FU-MSC or untreated MSC 6 days after MOG immunization to assess the immunosuppressive potential of 5-FU-MSC."

Contents

ABSTRACT

Autoimmune disease is a debilitating disease caused by immune responses towards particular self-antigens whereby inflammation in the target organs leads to tissue damage, which is associated with serious morbidity and disability. An example of autoimmune disease is multiple sclerosis (MS), a chronic inflammatory disorder of the human central nervous system (CNS) in which lymphocytic infiltration into the CNS damages myelin and axons. MS patients generally have poor quality of life with patients suffering from recurrent acute neurological episodes with persistent symptoms or progressive development of the disease. To date, there is no cure for MS, with management of the disease dependent on immunomodulatory interventions to minimize disease relapses and retard disease progression. However, available therapies have poor efficacy and many are associated with serious side-effects. Recently, cell therapy has emerged as a promising treatment regime for various autoimmune diseases including MS. Mesenchymal stem/stromal cells (MSC), plastic adherent fibroblastic-like cells, have emerged as the promising cell therapy for the treatment of MS. In pre-clinical studies, MSC ameliorate disease in experimental autoimmune encephalomyelitis (EAE) with associated decreased in lymphocyte infiltration and demyelination in the CNS. Due to the lack of a consensus method for effective isolation of well-characterized mouse MSC, different MSC preparations have been used across different studies, making comparison of the data difficult. Moreover, the majority of these studies use poorly characterized MSC preparation lacking in tri-lineage differentiation capacity. Therefore, the true therapeutic effect of MSC for the treatment of MS has not been established. In this study, an improved methodology aimed at isolating a well-characterized MSC population from mouse BM has been developed. By preconditioning the donor mice with 5-FU, a cytotoxic drug that kills proliferating cells, to remove hematopoietic cells and combining flushing of the

medullary cavity with scraping of the endosteum to remove additional BM cells, the isolation efficacy was significantly increased. Using MACS® separation column, $CD11b^{-}CD45^{-}$ MSC was efficiently and consistently isolated from multiple mouse strains. Isolated MSC from 5-FU-treated mice (5-FU-MSC) displayed enhanced functional characteristics such as higher proliferation potential, tri-lineage differentiation, immunosuppression capacity and CD49d (α4 integrin) expression compared to untreated MSC. Importantly, the administration of 5-FU-MSC in an EAE model of MS completely remitted disease, associated with the reduction in lymphocytes infiltration into the CNS, demyelination, and reduction in immune response such as lower splenocyte proliferation to MOG and serum anti-MOG antibodies. The complete remission was accompanied by elevated anti-inflammatory Th2 cytokines (IL-4, IL-5 and IL-6) and decreased proinflammatory cytokine, IL-17, in MOG-stimulated splenocytes. Soluble factors, IL-1ra, IL-10 and PGE_2, were found to be the mediators of 5-FU-MSC-induced immunosuppression. Together, these findings suggest that immunosuppression by 5-FU-MSC is mediated by a combination of elevated IL-1ra, IL-10, PGE_2 and anti-inflammatory Th2 cytokines and decreased IL-17. These findings suggest that the improved isolation method developed in this study can firstly, be adopted by other researchers to isolate a well-characterised MSC from multiple mouse strains and secondly, the method can be modified, for example changing 5-FU treatment from *in vivo* to *in vitro*, to isolate a similar population of highly immunosuppressive human MSC for exploitation to remit autoimmune diseases.

STATEMENT OF AUTHENTICITY

I certify that this thesis, except with the Research Graduate School Committee's approval, contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. I affirm that to the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Ding Yuan Oh

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LIST OF ABREVIATIONS

5-FU	5-Fluorouracil
5-FU-MSC	Isolated MSC from 5-FU-treated mice
α-MEM	α-Minimal Essential Media
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
bFGF	Basic-fibroblastic growth factor
CPM	Count per minute
Con A	Concanavalin A
CNS	Central nervous system
CFU-F	Colony forming unit-fibroblasts
DC	Dendritic cells
DMEM	High Glucose Dulbecco's Eagle Medium
dH_2O	Distilled water
EDSS	Expanded Disability Status Score
EAE	Experimental autoimmune encephalomyelitis
ES cells	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FGF	Fibroblastic growth factor
FBS	Fetal bovine serum
GvHD	Graft-versus-Host disease
HSV	Herpes simplex virus
HSC	Hematopoietic stem cells
HO-1	Heme oxygenase-1

HLA-G	Human histocompatibility soluble leukocyte antigen G
HS	Horse serum
HGF	Hepatocyte growth factor
IL7RA	IL-7 alpha receptor chain
IL2RA	IL-2 alpha receptor chain
IDO	Indoleamine 2,3 dioxygenase
IFNγ	Interferon gamma
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
ICAM-1	Intracellular adhesion molecule-1
ICV	Intracerebralventricular
Ig	Immunoglobulin
ISCT	International Society for Cellular Therapy
i.v	Intravenous
i.p	Intraperitoneal
LFB	Luxol fast blue
LN	Lymph node
L-NAME	N-nitro-L-arginine methyl ester hydrochloride
MFI	Mean fluorescence intensity
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MAG	Myelin-associated glycoprotein
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSC	Mesenchymal stem/stromal cells
MPC	Mesenchymal progenitor cell

NO	Nitric oxide
NK	Natural killer
PDGF	Platelet-derived growth factor
PD	Programmed death
PGE_2	Prostaglandin E2
PSG	Penicillin-Streptomycin-Glutamine
PBS	Phosphate buffer solution
PLP	Proteolipid protein
PP-MS	Primary progressive multiple sclerosis
PR-MS	Progressive relapsing multiple sclerosis
RR-MS	Relapsing-remitting multiple sclerosis
RS	Rapidly self-renewing
RT	Room temperature
SnPP	Tin protoporphyrin
SI	Stimulation index
SP-MS	Secondary progressive multiple sclerosis
Tregs	T-regulatory cells
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α

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1 CHAPTER 1 – LITERATURE REVIEW

Several types of stem cells are involved in homeostatic processes in the bone marrow (BM). Among them are mesenchymal stem/stromal cells (MSC) recognized as the putative stem cells for BM stroma via their ability to differentiate into hematopoiesis-supportive cells such as adipocytes, osteoblast, osteoclasts and fibroblasts. The supportive role of MSC to the hematopoietic stem cells (HSC), a well characterized stem cell in the BM, is evident in various co-culture experiments whereby MSC maintain the proliferative and self-renewal capacity of HSC [1-3]. The discovery of MSC not only provides further understanding of the regulation of HSC in the BM niche, but also opens a new avenue for the discovery of some unique therapeutic uses of MSC. In this review, isolation methodologies, therapeutic properties and the application of MSC in autoimmune diseases, in particular, multiple sclerosis (MS) will be discussed.

1.1 Autoimmune disease

Autoimmune diseases are incurable, affecting approximately 5% of the population [4]. They have a significant impact on the community, being associated with serious morbidity and disability. Common examples of autoimmune diseases include MS, Type 1 diabetes and rheumatoid arthritis. These diseases are the consequence of an immune attack directed at particular antigens residing in target organs. Rational strategies to establish life-long immune tolerance to autoantigens, without continual immunosuppression, greatly depend on mechanisms that the immune system uses to establish tolerance to autoantigens throughout life. The entire immune repertoire is generated by HSC residing in the BM. Thymus progenitors arising from

HSC migrate to the thymus, where they develop into mature T cells. Thymus progenitors also develop into the alternative lineage of potent tolerogenic dendritic cells (DC) that present self-antigens to developing T cells, resulting in deletion of autoreactive T cells that bind with high avidity. Not all self-antigens are presented in the thymus; medullary thymic epithelial cells assist further purging of the T cell repertoire by presenting "promiscuous" extra-thymic autoantigens to the developing T cells; these deletion processes are termed central tolerance [5]. However, these deletion processes do not delete all autoreactive T cells. The thymus also generates "natural occurring" Tregs, which suppress peripheral autoreactive T cells that have escaped thymic deletion. Together with peripheral DC (immature) which induce antigen-specific tolerance [6], these processes are termed peripheral tolerance. The emergence of autoimmune diseases in man and animals indicates that these central and peripheral tolerogenic mechanisms are insufficient to remove or suppress all autoreactive T cells. Currently, long-term immunosuppressive therapy is being used to control, but not cure autoimmune diseases. These drugs have severe side effects and therefore a curing strategy is required.

1.2 Multiple sclerosis

MS is a chronic inflammatory disorder of the human central nervous system (CNS) in which lymphocytic infiltration into the CNS leads to the damage of myelin and axons [7]. MS affects more than 2.5 million individuals worldwide, typically in their 20s and 30s [8-11]. Initial symptoms of MS may present as limb weakness, sensory loss, monocular visual loss, double vision, gait instability and ataxia; progressing to include bladder dysfunction, fatigue and heat sensitivity, complications of infections, cognitive deficits and depression [10, 12]. Premature

death occurs in two-thirds of MS cases; the median time to death is approximately 30 years from disease onset, representing a reduction in life expectancy of approximately 5-10 years [13].

Although the different definitions and clinical classifications of MS have been refined over the last 50 years, the etiological agent(s) or event(s) that induces disease still remain unknown. To date, there are four recognised clinical patterns for MS. The majority (~85%) of MS patients experience relapsing-remitting MS (RR-MS) characterised by recurrent acute neurological episodes followed by recovery. Recovery from each episode is incomplete and, over time, persistent symptoms accumulate [7]. After 8 to 20 years, approximately 65% of patients with RR-MS enter secondary progressive MS (SP-MS), characterized by continuous, irreversible neurological deterioration without remission. A smaller proportion (~20%) of MS patients has primary progressive MS (PP-MS), where illness is progressive from disease onset. Both SP-MS and PP-MS occurs at around 40 years of age [14]. The fourth clinical pattern is progressive relapsing MS (PR-MS), which affects approximately 5% of patients [10, 12]. MS patients with PR-MS experience gradual progression of disability from disease onset, accompanied by one or more relapses later in the disease [10, 12].

1.2.1 Causes of MS

A large number of environmental factors have been implicated as potential risks in the development of MS [15, 16], including geographic [17] and socio-economic factors [18], such as solar radiation [19], climate, sanitation, nutritional and dietary factors [20, 21], stress, pollution and viral factors such as measles, rubella, mumps, Epstein Barr virus, Human herpes virus 6,

herpes simplex virus (HSV) 1 and 2 and varicella zoster virus [22-26]. However, no single environmental factor has ever been demonstrated as a definitive trigger for MS. The possible mechanism of the diverse pathology and etiology of MS has been suggested as, "molecular mimicry", in which peptides from pathogens share sequences or structural similarities with CNS self-antigens [27, 28]. Several reports have suggested evidence for infection-induced molecular mimicry by HSV 1 [29], Theiler's murine encephalomyelitis virus [30], Haemophilus influenzae [31] and Murine hepatitis virus [32].

Genetic predisposition is another important consideration in MS, since MS clusters with complex polygenic diseases characterized by modest disease risk heritability for disease susceptibility [10]. There is evidence of inherited risk in familial recurrence of about 20% and twin concordance (clinical concordance rates: monozygotic: 25% vs. dizygotic: 5%) for genetic influences in MS [7, 33]. The strongest genetic factor associated with MS is the *HLA-DRB1* allele [34-38]. Moreover, recent studies have shown the involvement of non-HLA genetic variation in association with disease susceptibility in MS. The interleukin-7 alpha receptor chain (IL7RA) gene was identified as a heritable risk factor in MS [39-41] and more evidence of an association between this gene and MS susceptibility has been shown in several populations [42-44]. Other non-HLA genes have also been identified including KIAA0350 (CLEC16A; C-type lectin domain family 16, member A), IL-2 alpha receptor chain (IL2RA), Ribosomal protein L5 and CD58 (LFA-3; Lymphocyte function associated antigen 3) to be associated with disease susceptibility in MS [42, 45].

1.2.2 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the CNS that is induced in laboratory animals by the generation of an autoimmune response against antigen(s) of the myelin sheath, an insulating covering around nerve fibers [46]. EAE is widely used as an animal model of MS because of the shared resemblance of clinical and pathological features between the animal and human diseases [46]. The origin of EAE goes back to the fundamental work by Rivers *et al* [47] in rhesus macaques, showing that brain extract alone can induce paralysis that is associated with perivascular infiltration and demyelination in brain histology. EAE has been successfully replicated in various species such as guinea pigs, rabbits, goats, rats, hamsters, dogs, sheep, marmosets and chickens [48]. Early methodologies of inducing EAE involved the use of white matter extract homogenate, myelin extracts, or whole myelin protein such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) as immunogens (**Fig. 1.1**). In mouse, it is more common to induce EAE by targeting single major histocompatibility complex (MHC) class II-restricted myelin epitopes [46]. Typically, EAE is induced by vaccination with the immunogenic peptide in Complete Freund's Adjuvant (with *Mycobacterium tuberculosis*), followed by two injections of pertussis toxin 48 hours apart [46]. Similar to EAE in rhesus macaques, EAE in mouse is characterised by inflammation of the CNS, associated with perivascular infiltration and demyelination (generally being more pronounced in the brain stem and spinal cord), leading to progressive hind-limb paralysis and can be scored as shown in **Figure 1.2**. The various forms of MS disease course can be induced in mouse depending on the mouse strain and autoantigens employed (**Table 1.1**). For example, the immunisation of SJL mice with $PLP_{139–151}$ results in a relapsing-remitting course of paralysis mimicking the relapsing-remitting human form of MS. In contrast, C57BL/6 mice

immunised with $MOG_{35–55}$ develop a progressive form of EAE characteristic of primary-progressive human MS.

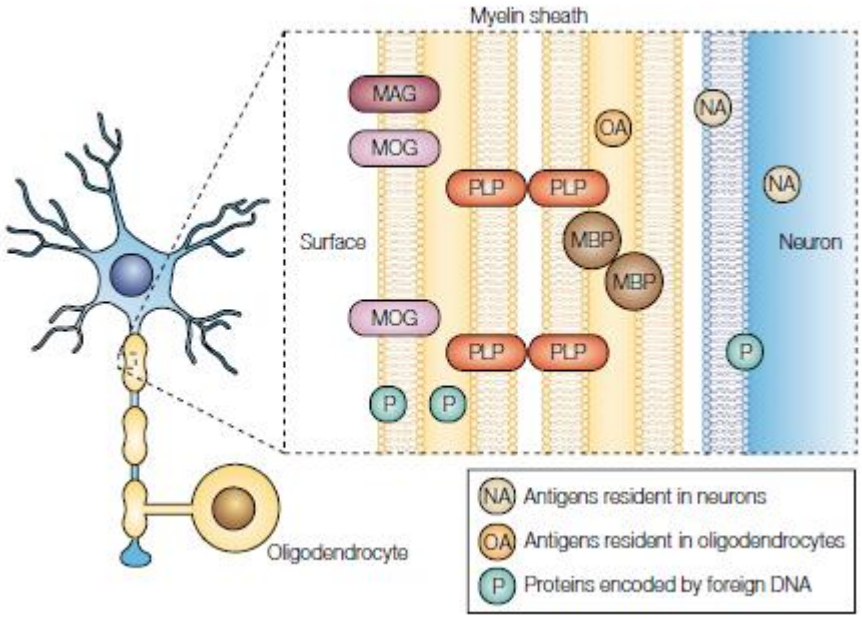

FIGURE 1.1: Possible targets antigens in the white matter. Proteins of the myelin sheath, oligodendrocytes and neurons including myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) are possible targets of the immune response in MS. Adapted from Hemmer *et al* [49].

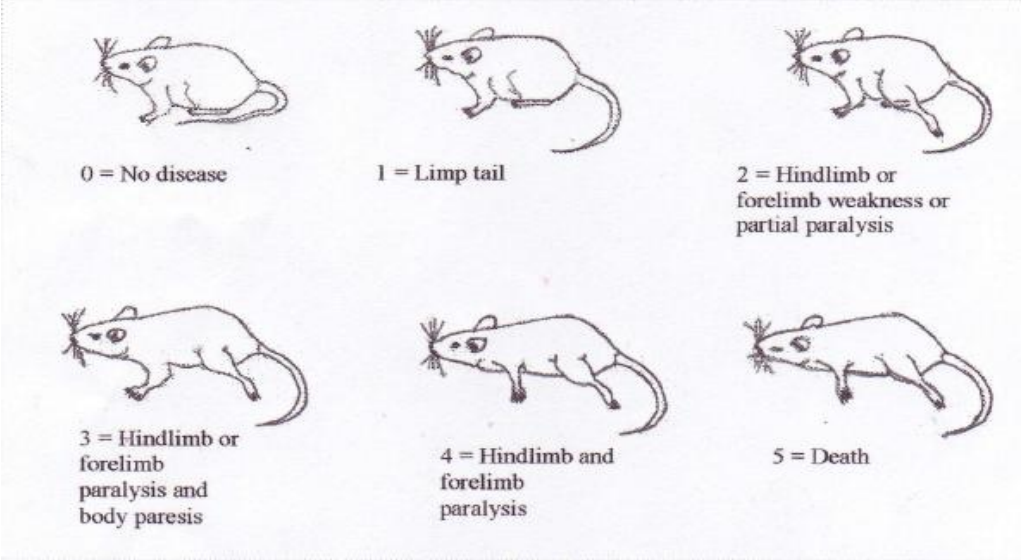

FIGURE 1.2: EAE scoring system in the mouse model. Neurological impairment is scored on an arbitrary clinical score: 0, no clinical sign of disease; 1, limp tail; 2, limp tail and hind limb weakness; 3, severe hind limb paresis; 4, complete hind limb paresis; 5, moribund or death.

TABLE 1.1: Different encephalitogenic peptides induce different forms of MS disease in different mouse stains. Adapted from Miller *et al* [50].

Mouse strain	Antigen specificity	Disease type
C57Bl/6	MOG	PP-MS
	MOG_{35-55}	PP-MS
	MBP	PP-MS
	MBP_{84-104}	PP-MS
SJL	$PLP_{139-151}$	RR-MS
	MBP_{84-104}	RR-MS
	MBP_{89-101}	RR-MS
B10.PL	MBP_{Ac1-11}	PP-MS
B10.S	MBP	PP-MS
	MBP_{87-106}	PP-MS

EAE is a well characterized animal model for human MS. Investigations using EAE over the last 50 years have improved our understanding of the acute inflammatory demyelinating syndromes, advanced the understanding of genetic susceptibility to autoimmunity, mechanisms of lymphocyte trafficking and the role of the blood-brain barrier in CNS inflammation [51]. Indeed, three clinical therapeutics have developed after showing promising results in EAE studies: glatiramer acetate (Copaxone®; Teva Pharmaceutical Industries Ltd – a random mixture of polypeptides containing alanine, glutamic acid, lysine and tyrosine) [52], mitoxantrone (Novantrone®; Amgen Inc. – an anthracenedione cytotoxic and immunosuppressive agent) [53] and natalizumab (Tysabri®; Biogen Idec Inc. and Elan Corporation Plc – a humanized monoclonal antibody specific for $\alpha_4\beta_1$–integrin; also known as VLA4) [54]. However, it has been criticized that dissimilarities that exist between EAE and MS prevented an accurate extrapolation of research data from EAE to human MS [55]. It was noted that although adverse effects were not detected in EAE, they occurred in various clinical trials; for example, progressive multifocal leukoencephalopathy with natalizumab [56], exacerbations of disease with the altered peptide ligand NBI-5788 [57] and worsening of disease with blockade of tumor-necrosis factor [58]. Nevertheless, as the closest model of human MS, EAE still holds great value for screening potential therapeutics and testing the efficacy of stem cell therapy as an immunomodulator in ameliorating disease.

1.2.3 Treatment for MS

The treatment of MS typically involves therapies aimed at indirect immunomodulatory interventions, promoting remyelination to repair the damaged CNS or to minimize disease

relapses and retard disease progression. Their primary mode of action is to suppress the immune pathogenesis of MS. Current approved therapies for RR-MS include interferon β (β-1a and β-1b), glatiramer acetate, natalizumab, and mitroxantrone [59]. Widely used drugs such as interferon β (β-1a and β-1b) and glatiramer acetate have been shown to decrease RR-MS relapse rates by approximately 30% and reduce the development of new gadolinium-enhancing lesions in magnetic resonance imaging (MRI) [60, 61]. Natalizumab, a humanized monoclonal antibody specific for $\alpha_4\beta_1$–integrin on leukocytes, is effective in reducing relapses by 50% with a 92% reduction in gadolinium-enhancing lesions in MRI [59]. However, current therapies have limited efficacy in other forms of MS such as PP-MS and most of them are associated with adverse side-effects. For example, interferon β and glatiramer acetate cause flu-like symptoms, depression and liver enzyme level abnormalities [62]; natalizumab causes progressive multifocal leukoencephalopathy, a rare demyelinating disease caused by the reactivation of John Cunningham virus, and mitroxantrone is associated with cardiomyopathy, leukemia, leucopenia and infection [61]. Besides immunomodulatory drugs, neuroprotective compounds such as Insulin-like Growth Factor-1, chaperonin 10 and sodium channel blockers have been tested in clinical trials, but most have failed to show significant therapeutic efficacy or trials have been prematurely ended due to unexpected adverse effects such as gait, ataxia and increase disability [63]. The employment of current therapies to treat MS are limited by their poor efficacy and associated side-effects, thus new therapies with better therapeutic efficacy and minimal side-effects are urgently needed for the treatment of MS.

Hematopoietic stem cell transplantation (HSCT) has emerged as a promising therapy for treating MS [64]. The rationale of "resetting" the aberrant immune repertoire by HSCT to generate a new

antigen-naïve immune system has shown promising results in several phase I/II clinical trials. Retrospective evaluation of phase I/II HSCT in 85 patients showed disease stabilization in 74% of the treated patients after 3 years, and even neurological improvement [improvement by ≥ 1 point in Expanded Disability Status Score (EDSS)] in 21% of the patients [65]. Similar results were obtained from more recent studies [66-68] involving pre-conditioning MS patients with high-dose immunosuppressive therapy following autologous HSCT. Progression-free survival at 6 years was 72%, with 62% of the treated patients improving at least 0.5 points in EDSS [66]. In the Canadian MS bone marrow transplantation (BMT) study group, progression-free survival was 75% at 3 years without any associated relapse and absence of lesions at 5 years follow up [67, 69]. Although HSCT holds much promise in treating MS, the treatment-related mortality, issues with potential relapses and severe adverse side effects are a major concern [65, 70-72]. Moreover, as reported in clinical studies, the efficacy of HSCT is limited to specific MS cohorts; with young active RR-MS patients having short disease history and who are unresponsive to all available first-line agents, the best candidates for HSCT [73].

Recently, experimental approaches such as antigen-specific BMT have shown favorable outcome in the treatment of autoimmune disease such as MS [74-76]. As demonstrated by Chan *et al* [74], the transplantation of genetically-modified BM with disease causative self-antigen, MOG_{35-55}, can effectively prevent and reverse established disease in BM recipient mice. This treatment regime has proven effective in both myeloablative [74] and non-myeloablative pre-conditioning settings [75, 76]. The antigen-specific tolerance was suggested to be due to clonal deletion of MOG-specific T cells within the thymus and/or peripheral tolerance via programmed death (PD) 1 pathway [74, 76]. Nonetheless, this treatment approach is still in its experimental phase with

much work still needed to address the safety concerns regarding virus delivery of the specific antigen into the BM cells.

1.3 Mesenchymal stem/stromal cells

BM adult stem cells, known as MSC, that can differentiate into non-hematopoietic tissues, were first described by Friedenstein *et al* [77] as a small group of plastic adherent fibroblast-like cells that have the ability to differentiate into osteocytes. Plasticity of MSC was further demonstrated in subsequent studies showing that MSC can differentiate into chondrocytes and adipocytes [78]. Besides BM (the richest source for MSC), these cells are also present in other tissues, including adipose tissue, skeletal muscle, umbilical cord, peripheral blood, dental pulp, amniotic fluid, and fetal tissues such as blood, lung, and liver [79].

MSC populations are comprised of undifferentiated stem cells and lineage-restricted precursors that are morphologically, phenotypically and functionally heterogeneous [80]. The cells differ in size, morphology, proliferative potential and express different levels of alkaline phosphatase [81]. The heterogeneity of MSC populations was clearly demonstrated by Mauraglia *et al [82]*, showing that approximately half of the 185 clones from human MSC culture express bi-lineage osteo-chondrogenic potential and less than one-third of the clones having tri-lineage differentiation potential into adipocytes, osteoblasts and chondrocytes. The primary MSC culture comprised of three different cell morphologies (**Fig. 1.3**) namely small rapidly self-renewing (RS) cells and mature cell types such as spindle-shape cells and large flattened cells [83]. RS cells have been suggested by Colter *et al* [83] to be the earliest MSC progenitors in the primary

BM culture based on their high replication rate and high tri-lineage differentiation potential compared to the other two cell types. Among the three cell types, large flattened cells replicate most slowly and have the lowest tri-lineage differentiation potential [83-85].

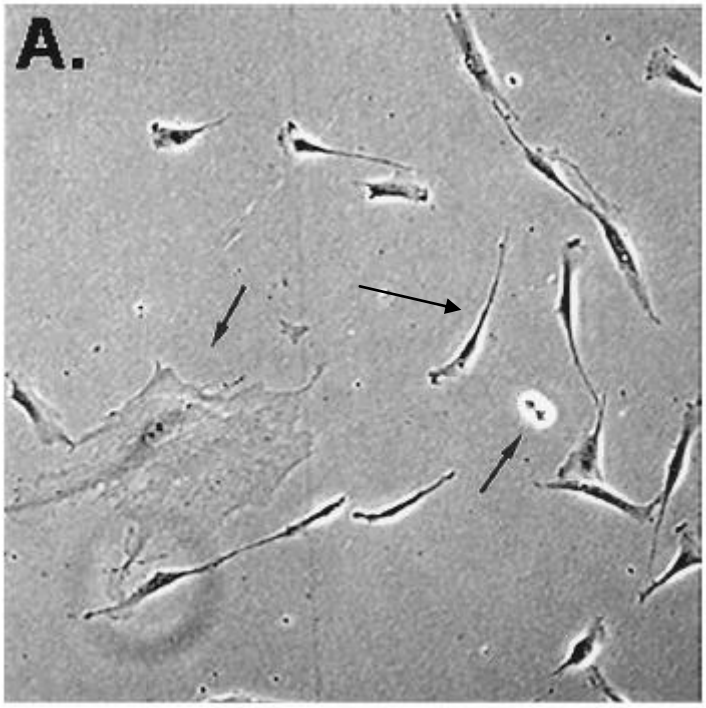

FIGURE 1.3: Phase contrast photomicrograph of different cell types in a MSC culture. Arrows indicate large flattened MSC, spindle-shaped MSC and doublets of recently replicated RS cells (X200). Adapted from Colter *et al* [83].

To date, there is not a specific singular marker, or a set of markers, for the identification of MSC. Rather, a collection of positive and negative markers is generally used to phenotype these cells. The MSC population can express antigens such as CD29, CD44, CD49, CD51, CD73, CD105, CD106, CD166, and Stro-1 [86-88]. The absence of CD45, CD34, CD31, Mac1 (CD11b), CD19 and glycophorin A expression distinguishes MSC from hematopoietic cells, endothelial cells, endothelial progenitors, monocytes, B cells and erythroblasts, respectively. Recent studies have identified potential cell surface marker(s) that can be used to identify putative MSC from different tissues; for example, CD133 expressing MSC in human peripheral and umbilical cord blood, SSEA-4 and SSEA-1 expressing MSC in human/murine BM, p75NTR expressing MSC in murine adipose tissues and W8B2 and frizzled-9 expressing MSC from human BM and placenta [89-91]. Nonetheless, these markers are not exclusive to MSC which makes direct isolation of MSC a challenge.

The conventional method for isolation of BM-MSC involved firstly, the adherent of BM cells onto tissue culture plastic, followed by continuous passaging of the adherent cells to establish MSC culture [92]. This isolation technique has proven effective for both human and rat MSC, as the hematopoietic cell components from these species fail to adhere and thrive upon continuous culture [93]. However, mouse hematopoietic cells readily adhere to tissue culture plastic and persist even after multiple passages thus hindering the effective isolation of MSC from mouse BM [92, 94]. Additionally, MSC from different mouse strains vary widely in terms of their growth kinetics, numbers of MSC within their BM, differentiation capacity and ease of isolation by conventional isolation methods. Therefore, the development of a single isolation methodology for isolation of MSC from different mouse strains poses a significant challenge.

To increase the isolation efficiency of mouse MSC, modifications to conventional isolation procedure such as alterations to cell culturing conditions [95-100] and/or improvement to BM extraction methods to release additional MSC [101, 102], were developed (**Table 1.2**). Although modifications were implemented, these methods still rely heavily on the continuous passaging of BM cells to obtain a pure MSC culture. Prolonged culturing of MSC may potentially introduce characteristic changes such as decreasing multipotency [103] and karyotypic abnormalities [104]. Thus those improved methodologies which include passage-purification steps may be suboptimal for isolating mouse MSC. Other isolation methodologies which reduce the requirement for long-term MSC culturing rely on the expression of non-specific MSC antigens such as CD34, PDGFRα and Sca-1 [97], whilst other methods select for MSC lacking expression of hematopoietic markers such as CD11b, CD34 and CD45 [94, 105]. Although these methodologies have improved isolation efficiency, their applicability as a widely accepted method for isolation of mouse MSC is questionable, as the majority were tested on a limited number of mouse strains and, additionally, important characterisation data such as the ability of the isolated MSC to differentiate into all three classical mesodermal lineages is lacking (**Table 1.2**).

The lack of an efficient method for isolation of mouse MSC has prompted investigators to use human MSC instead of mouse MSC in experiments. Several investigators including Meisel *et al* [106], Aggarwal *et al* [107], Di Nicola *et al* [108], Najar *et al* [109] and Kadri *et al* [110] have used human MSC to investigate the immunomodulatory mechanisms of MSC. Although human MSC are relatively easier to isolate than mouse MSC, their usage has two major limitations. Firstly, the role of specific genes in MSC can only be studied with MSC isolated from

genetically-modified mouse models [95, 100, 102, 111]. Secondly, *in vivo* studies which involved the transplantation of human MSC into xenogenic models such as rat or mouse can potentially provoke the host immune responses such as macrophages infiltration into areas of transplantation [112]. Therefore, the immunogenicity of human MSC even at a weak level [113] can potentially confound the underlying therapeutic effect of MSC. Therefore, the development of a widely accepted methodology to isolate a well-characterised MSC population from mouse BM is urgently needed to facilitate the understanding of MSC in human diseases.

TABLE 1.2: Overview of methodologies that improve isolation of mouse BM-MSC

Study	Modification (s)	Tested mouse strain(s)	Passaged purification	Differentiation		
				Fat	Bone	Cartilage
Piester *et al* [95]	Horse serum in isolation & culture media Low cell seeding density	C57Bl/6; BALB/c; FVB/N; DBA1	Yes	Not all strains	Yes	Not all strains
Eslaminejad *et al* [96]	Seeding density	NMRI	Yes	Yes	Yes	NA
Xu *et al* [102]	Crushing & digestion of bone Immunodepletion	C57Bl/6/KaLwRij	Yes	Yes	Yes	Yes
Guo *et al* [101]	Culturing collagenase-digested bone fragments	C57Bl/6	Yes	Yes	Yes	No
Sun *et al* [98]	BM-conditioned media & bFGF	C57Bl/6	Yes	Yes	Yes	Yes
Tropel *et al* [100]	Immunodepleted cells seeded onto human fibro-nectin coated TC dish with EGF & PDGF-AA	C57Bl/6	Yes	Yes	Yes	Yes
Sung *et al* [99]	Immunodepleted cells cultured with StemXVivo & bFGF	C57Bl/6	Yes	NA	NA	NA
Morikawa *et al* [111]	Selecting for PDGFRα^+, Sca-1^+, $CD45^-$, $TER119^-$ MSC-subset	C57Bl/6; BALB/c; FVB/N; DBA1; 129;ICR	Yes	Not all strains	Not all strains	Yes
Nadri *et al* [114]	Frequent media change after plating BM cells	BALB/c	Yes	Yes	Yes	NA
Baddoo *et al* [105]	Selecting $CD11b^-C45^-CD34^-$ MSC from adherent BM cells	FVB/N	No	Yes	Yes	Yes
Nadri *et al* [97]	Selecting $CD34^+$ MSC from BM cells	C57Bl/6	No	Yes	Yes	NA
Rivkin *et al* [115]	Use fibrin beads to enhance MSC adherence	C57Bl/6	Yes	Yes	Yes	Yes

BM: Bone marrow; TC: Tissue culture; EGF: Epithelial growth factor; bFGF: basic-Fibroblastic growth factor; PDGF-AA: Platelet-derived growth factor-AA; NA: Not available

1.4 Identity of MSC

The discovery of MSC led to a need to increase the understanding of their anatomical distribution and location *in vivo*. To date, the lack of putative marker(s) for MSC impeded such investigations and the true identity of MSC remains elusive. However, MSC have been suggested to be derived from pericytes [116, 117]. Isolated MSC from human BM and dental pulp were found to express pericyte-specific markers such as CD146 and 3G5 [118]. Furthermore, MSC from other human organs such as skeletal muscles, pancreas, adipose tissue and placenta can be positively isolated using pericyte markers such as CD146, NG2 and PDGF-Rbeta, suggesting a similar phenotype to perivascular cells [119]. The ability to isolate MSC from artery and vein, and the correlation of the numbers of MSC to blood vessel density further supports their vascular-origin [120-122]. Although, there is increasing evidence of a relationship between MSC and pericytes, to date, a consensus has yet to be reached for the *in vivo* identity of MSC [123].

1.5 Properties that makes MSC an attractive therapeutic agent

1.5.1 Homing capacity of MSC

The ability of MSC to home to injured tissue or inflammation sites has been an area of interest for therapeutic application. MSC localize in areas of injury, inflammation or BM after systemic infusion into non-human primates [124] or disease models [125-128]. Although MSC were found at the sites of injury or inflammation, their specific-homing efficiency was low, with the majority of the infused cells distributed throughout other tissues and organs such as lungs,

kidney liver and spleen [124, 129, 130]. Besides sites of injury or inflammation, MSC can also home to tumors, suggesting them as a potential delivery vector of gene products to the tumor microenvironment [131]. To date, the mechanisms by which MSC home to injured tissues are still not fully understood. However, the interactions of MSC with integrin[132], chemokine receptors [133], basic-fibroblastic growth factor [134], matrix metalloproteinases [135] and Toll-like receptors [136] have been implicated as important modulators in the MSC-homing process.

1.5.2 In vitro differentiation / transdifferentiation into multiple tissues

The differentiating capacity of MSC into the three classical mesenchymal cell types: adipocytes, osteoblasts and chondrocytes; has been a well-documented phenotype [137]. Over the past decades, it has been shown that MSC may possess a greater plasticity than previously thought. The ability of MSC to differentiate across germinal boundaries, a process known as transdifferentiation, into ectodermal and endodermal cell lineages has generated much interest in their exploitation for therapeutic purposes [78].

MSC have been shown to differentiate into epithelial-like lineages *in vitro* [138, 139] or after systemic administration *in vivo* [126]. The transdifferentiation of MSC into epithelial-like cells was reported in co-culture experiments where MSC cultured with airway epithelial cells assume an epithelial-like cuboidal or columnar cell shape, and expressed epithelial-specific marker genes such as cytokeratin and tight junction protein, occludin [138, 139]. Wang *et al* [138] showed that MSC co-cultured with normal air-way epithelial cells transdifferentiate directly into epithelial-like cells with no evidence of cell-fusion. In contrast, a similar co-culture study by Spees *et al*

[139] reported that only a minority of MSC differentiated directly into epithelial-like cells, and that majority of the MSC had fused with neighboring heat-shock airway epithelial cells.

Similar to *in vitro* studies, the potential for MSC-epithelial transdifferentiation was demonstrated in a mouse model for lung fibrosis. In these studies, MSC administered immediately after bleomycin treatment significantly reduced bleomycin-induced lung fibrosis and the transplanted MSC were detected in areas of lung injury associated with an epithelial-like morphology [126]. However, other studies in post-ischemic kidney [140] or injured cornea models [141] have shown that the transplanted MSC failed to restore the damaged epithelial cells and provided little or no evidence of epithelial transdifferentiation by MSC. Therefore, evidence for the plasticity of MSC to transdifferentiate into epithelial-like lineages is debatable.

The early indication of the differentiation plasticity of MSC to neuro-ectodermal lineages was demonstrated when MSC injected into the lateral ventricle of newborn mice migrated throughout the brain to adopt morphological and phenotypic characteristics of astrocytes and neurons [142]. Subsequently, neuronal differentiation of MSC was demonstrated *in vitro* using a wide range of chemicals including reducing agents, anti-oxidants and chemicals that increase intracellular cyclic AMP [143]. However, the adoption of a neuronal phenotype by MSC upon exposure to these chemicals was dismissed by other research groups which showed that the supposedly ‘neuro-ectoderm plasticity’ was in-fact a chemically-induced artifact rather than a true neuronal differentiation [144-146]. Rather than neurite outgrowth, as seen in neurons, the exposure of MSC to chemical agents such as DMSO or BHA caused cell-shrinkage and loss of focal contact due to the rapid disruption of the actin cytoskeleton of MSC. These neuron-like MSC were also

found to be non-functional, with no exhibition of Na(+) or K(+) currents and the lack of ability to fire action potentials [146]. Other than DMSO or BHA, similar pseudo-neuronal morphology in MSC can also be replicated in fibroblasts or keratinocytes using cellular stressors such as detergents, high-molarity sodium chloride, and extremes of pH [145].

Nevertheless, other protocols which employed the usage of cocktails of cytokines have successfully induced MSC to assume Schwann cell-like morphology and expression of markers such as p75 glial fibrillary acidic protein, S-100, O4 and P0 [147]. Also, co-culturing of MSC in the presence of tumor necrosis factor-α (TNF-α) induced MSC to assume a neuroglial-like morphology with associated up-regulation of genes for neural growth and function [148]. However, to date, there is a lack of data showing electrophysiological features of *bona fide* neurons such as action potentials, presence of voltage-gated channels and presence of Na(+) or K(+) currents in MSC-derived neural cells, thus undermining the evidence for the neuro-ectoderm transdifferentiation of MSC.

1.5.3 Immunomodulation effects of MSC

Discovery of the immunomodulatory effect of MSC has paved the way for the characterisation of their broad immunological effects on various immune cells, and their application as a potential cell therapy for immunological diseases [69, 149]. MSC have been shown to affect both innate and adaptive immunity (**Fig. 1.4**). In innate immunity, MSC inhibit Natural killer (NK) cell activation and target cell killing by down-regulating expression of NKp30, NKp44 and natural-killer group 2 member D activating receptors [150]. Also, MSC inhibit cytotoxicity of NK cells by proliferation inhibition and suppression of interferon gamma (IFN-γ) production by NK cells

via prostaglandin E2 (PGE_2) and indoleamine 2,3 dioxygenase (IDO) [150, 151]. MSC can further influence innate immunity by inhibiting maturation of monocytes into DC and hindering their antigen-presenting functions by decreasing cell surface expression of MHC class II molecules, CD11c, CD83, co-stimulatory molecules as well as decreased interleukin (IL)-12 production [150-152]. Additionally, MSC have been shown to dampen the respiratory burst of neutrophils by suppressing production of hydrogen peroxide in activated neutrophils [153].

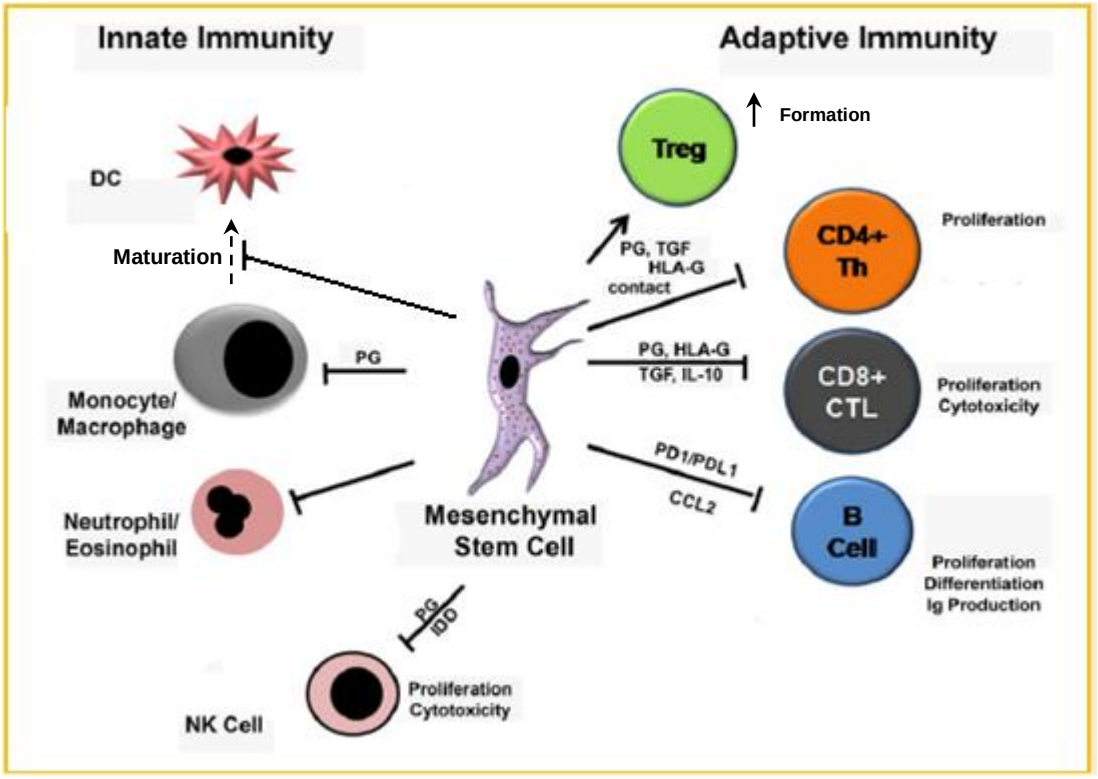

FIGURE 1.4: Overview of the influence of MSC on the different cells of the innate and adaptive immunity. In innate immunity, MSC can inhibit proliferation and cytotoxicity of Natural killer (NK) cells by prostaglandins (PG) and indoleamine 2, 3-dioxygenase (IDO). MSC dampen the respiratory burst of neutrophil by suppressing the production of hydrogen peroxide. Monocytes maturation into dendritic cell (DC) is also inhibited by MSC. In adaptive immunity, MSC inhibit proliferation of $CD4^+$ T cells and decrease the proliferation and cytotoxicity of $CD8^+$ cytotoxic T lymphocytes (CTLs). These effects are mediated by PG, human leukocyte antigen-G (HLA-G), transforming growth factor (TGF) and interleukin-10 (IL-10). MSC promote the generation of T-regulatory cells (Treg) by cell-to-cell contact and soluble factors such as PG, TGF and HLA-G. MSC also inhibit proliferation, differentiation and immunoglobulin (Ig) production of B cells by the programmed death 1 (PD-1)/PD ligand pathway and chemokine ligand 2 (CCL2). Adapted and modified from English *et al* [154].

In adaptive immunity, MSC have been shown to influence both T cells, T-regulatory cells (Tregs) and B cells [149]. MSC are known to inhibit T cell proliferation stimulated by polyclonal mitogen, antigen-specific mitogen and allogeneic cells [106, 108, 155]. Independent of apoptosis, the inhibition of T cell proliferation was proposed by Glennie *et al* [155] to be mediated by division arrest of activated T cells at the G_1 phase of their cell cycle with down-regulation of cyclin D2 expression and up-regulation of p27kip1 by MSC. Additionally, MSC also promote survival of these activated T cells by down-regulating Fas receptors and Fas ligand on T cell surfaces and inhibit endogenous proteases involved in programmed cell death [156]. MSC also influence other T cell functions such as decreased production of IFN-γ, IL-2 and TNF-α and the increased IL-4 production [107]. Experimental data have showed that soluble factors produced by MSC such as IL-1 receptor antagonist (IL-1Ra) [157], IL-6 [158], IL-10 [159], transforming growth factor (TGF) β-1 [108], IDO [106], hepatocyte growth factor (HGF) [108], PGE_2 [107], nitric oxide (NO) [160], soluble human histocompatibility soluble leukocyte antigen G (HLA-G) [161] and galactin [162, 163] also play an important role in inhibiting T cells. Recently, cell-to-cell contact involving adhesion molecules such as ICAM-1 and VCAM-1 have been associated with MSC immunosuppressive capacity, highlighting the requirement for cell-to-cell contact and the importance of adhesion molecules in MSC-mediated immunosuppression [164].

MSC have also been reported to influence other sub-populations of T cells such as $CD8^+$ cytotoxic T cells. The interaction between MSC and cytotoxic T cells has been suggested as a partial explanation for the tolerance of allogeneic MSC across the MHC barrier [165, 166]. Several studies show that MSC are generally resistant to cytotoxic T cell-mediated lysis [165-

167]. Rasmusson *et al* [166] demonstrated that resistance of MSC to cytotoxic T cell-mediated lysis is associated with the failure of MSC to trigger the effector functions of activated cytotoxic T cells. MSC were unable to induce IFN-γ and TNF-α production and resulted in a weaker up-regulation of CD25 and down-regulation of T cell receptor complex [166]. In addition, MSC were also able to significantly inhibit cytotoxic T cell-mediated lysis by inhibiting the formation of cytotoxic T cells in the stimulation phase as demonstrated in mixed lymphocyte assays [167].

MSC have been shown to promote the generation of $CD4^+CD25^+$ or $CD8^+$Tregs with functional properties [107, 168]. The mechanisms involved in the induction of Tregs have been associated with cell-to-cell contact in combination with PGE_2 and TGF-β [169], and also the secretion of HLA-G, IL-10 and leukemia inhibitory factor by MSC to drive Tregs expansion [170]. MSC also inhibit B cell proliferation, down-regulate B cell expression of CXCR4, CXCR5 and CXCR7 chemokine receptors and inhibit B cell maturation and antibody secretion [171, 172]. Their inhibition of B cell effector function, such as immunoglobulin (Ig) production as demonstrated by Rafei *et al* [173], can be mediated by MSC-derived chemokine ligands, CCL2 and CCL7. These ligands inactivate the signal transducer and activator of transcription factor STAT3 and induce transcription factor PAX5, which in turn inhibits Ig production from plasma cells [173]. Additionally, the inhibitory effect of MSC on B cells is also dependent on IFN-γ and is mediated by cell-to-cell interactions involving the PD-1/PD ligand pathway [172].

1.6 Changing paradigm on how MSC improve disease state

The mechanisms by which MSC exert their therapeutic effect in many diseases have been an area of much interest and research. Based on their homing potential and alleged differentiation plasticity, the therapeutic efficacy of MSC was previously thought to be primarily due to their ability to engraft and replace damaged cells. However, increasing experimental data in MSC transplantation have shown otherwise [130, 157, 174-179]. For example, although the infusion of MSC into children with osteogenesis imperfecta improved their growth rate and mobility, the levels of MSC detected in the tissue and the bone were less than 1% [177]. Low engraftment of donor MSC was also repeatedly observed in other animal models of diseases including spinal cord injury [180], stroke [178], myocardial infarction [130, 175], post-ischemic kidney [140], injured cornea [141] and neurological disease such as MS [174, 176]. Therefore, the stem-like ability of MSC to differentiate and replace damaged tissue such as cardiomyocytes in myocardial infarction or neuronal cells in MS, could not be the sole explanation for the functional recovery in these diseases. The paracrine effect of MSC in tissue repair was recently demonstrated by Lee *et al* [130], where MSC-induced reduction of infarct size in mice after myocardial infarction was partly due to their secretion of the anti-inflammatory protein, TSG-6, rather than direct engraftment. In this study, the majority of the intravenous (i.v) infused human MSC were detected as emboli in the lungs instead of the heart, and transcriptome analysis for human-specific protein in the lungs demonstrated an up-regulation of TSG-6. The important role of the secreted TSG-6 by MSC was further demonstrated by functional knockout using TSG-6 siRNA which failed to reduce infarct size and inflammatory responses [130]. Thus, the mechanisms that alter disease outcome are not solely reliant on the ability of MSC to home to the injured area and replace the damaged cells. Rather, they are elicited through a myriad of processes such as

secretion of cytokines and chemokines, and the immunomodulatory effect, which work in concert towards tissue repair and disease amelioration.

1.7 Clinical trials with MSC

The therapeutic efficacy of MSC in pre-clinical models has stimulated the demand to translate MSC as a therapeutic for immune- or inflammatory-related diseases. Over the past decade, a large number of clinical trials involving MSC have occurred world-wide. For example, at the time of writing this review, according to the clinical trials website of the United States of America sponsored by the National Institute of Health (http://clinicaltrials.gov), there are currently 98 recruiting clinical trials exploring the applications of MSC. A diverse range of diseases were registered including inflammatory diseases such as myocardial infarction, liver cirrhosis, renal diseases, wound healing, and immune-related diseases such as Crohn's disease, Sjögren's syndrome, Graft-vesus-Host disease (GvHD), Type I diabetes, amyotrophic lateral sclerosis and MS. MSC applications have also been pursued in other conditions such as osteogenesis imperfecta, Hurler syndrome, metachromatic leukodystrophy and articular cartilage effect in clinical trials. To date, phase I/II clinical trials have established the safety of MSC without any report of cell-related adverse effects in patients [181-186]. Recently, the safety of MSC applications was challenged by reports of the ability of MSC to promote tumour growth [187]. Several studies showed that co-transplantation of MSC together with cancer cells were associated with higher incidence and size of tumour formation *in vivo* [188-191]. Conversely, other studies have shown that MSC inhibit tumour growth resulting in smaller tumour formation. The variation in the observations of the promotion or retardation of tumour by MSC was suggested to be dependent on patient-to-patient variability in MSC isolates, the methods of

isolating and propagation of MSC, the dosage of MSC and the timing at which MSC are introduced into the tumour [187]. Nonetheless, these contradicting reports associated with MSC and tumour formation warrant further studies and suggest caution in the use of MSC in future clinical trials.

1.8 Mesenchymal stem cells in MS

1.8.1 Mesenchymal stem cells in EAE

The ability of MSC to immunomodulate various immune cells makes them an attractive candidate for cell-based therapies in treating immunological diseases such as MS. Therapeutic efficacy of MSC was demonstrated in pre-clinical studies where transplantation of BM-MSC into EAE mice ameliorated disease severity and CNS demyelination [125, 174, 192, 193]. Disease amelioration in EAE by MSC can be reproduced in mice of different genetic backgrounds (C57Bl/6, SJL/J and C3H.SW), or by different routes of cell delivery such as i.v, intraperitoneal (i.p) or intracerebralventricular (ICV) injection. In addition, the therapeutic effect of MSC was not restricted to tissue origin with MSC from both BM and adipose tissue able to ameliorate EAE [193].

MSC are effective in treating different EAE models. In the MOG_{35-55}-non-remitting C57Bl/6 EAE model, early intervention by i.v injection of MSC before [125, 176, 192] or after [192, 194] disease onset significantly reduces clinical score, inflammatory infiltration and CNS demyelination. However, MSC do not exhibit any beneficial effects after disease stabilization in EAE [192]. In the remitting-relapsing SJL EAE model, MSC significantly reduces the relapse

rate by approximately 60% [174] with associated reduction in clinical score, number of inflammatory infiltrates and degree of demyelination [174, 195].

1.8.2 Therapeutic mechanisms of MSC

As demonstrated in EAE, MSC appears useful to ameliorate pathogenic responses as seen in MS, however the mechanisms by which they act are still debatable. Several mechanisms for disease amelioration were proposed; firstly, the limitation of autoreactive immune responses by MSC to reduce pathologic damage to the CNS. Secondly, the secretion of MSC-derived neuroprotective factors to stimulate endogenous repair of damaged tissue in the CNS, and lastly, the transdifferentiation of MSC into brain cells for cell replacement [196].

To date, the mechanism of MSC immunodulating the autoreactive responses in EAE model is best supporting by experimental evidence. Immunosuppressive capacity of MSC in EAE was demonstrated by several studies showing a significantly lower T cell response from MSC-treated EAE mice upon antigen-recall [125, 174, 192-194, 197-199]. Zappia *et al* [192] showed that T cell response to antigen-recall from MSC-treated EAE mice can be restored by IL-2, thus suggesting the induction of T cell anergy by MSC [192]. Chemokine ligands such as CCL2 from MSC, have been shown to influence T cell responses in EAE by paracrine conversion of CCL2 from agonist to antagonist of CD4 T cell function [194]. MSC from $CCL2^{-/-}$ mice lost their ability to ameliorate EAE, resulting in a higher clinical score and a significant increase in pro-inflammatory molecules such as IL-17 and TNF-α in the serum of MSC-treated mice. Besides suppressing T cell functions, MSC can also shift T cell polarization by decreasing

proinflammatory Th1 and Th17 responses and promote anti-inflammatory Th2 responses in EAE mice [192, 194, 198]. Interestingly Tregs, which are potent modulators of T and B cells, are not regulated by MSC in EAE [192]. Besides T cells, B cell responses are also modulated by MSC. In a remitting-relapsing SJL EAE model, MSC treatment significantly reduced the production of serum $PLP_{139-151}$-specific IgG (IgG1, IgG2a, IgG2b and IgG3) in EAE mice [174].

Besides modulating autoreactive responses in EAE, the therapeutic effects of MSC have also been proposed to be mediated by the secretion of MSC-derived neuroprotective factors to support neuroregeneration. In EAE, injected MSC can be detected in the demyelinated regions of the CNS in MSC-treated EAE mice [198, 200]. As demonstrated by several studies [193, 195, 198], migration of MSC to the spinal cord of EAE mice was associated with a higher proportion of $NG2^+$ oligodendrocytes precursor cells in the lesion regions of MSC-treated EAE mice. The exact mechanism of stimulating such repair was not fully elucidated, but the ability of MSC to produce neurotrophic cytokines such as brain-derived neurotrophic factor has been suggested to partially account for these regenerative effects [193, 195, 198]. Indeed, the secretion of MSC-derived neutrophic factors including nerve growth factor, ciliary neurotrophic factor, fibroblastic growth factor (FGF)-2, vascular endothelial growth factor, and polycomb family transcriptional repressor BMI-1; has been associated with the promotion of endogenous neural stem cells to undergo neurogenesis in the mouse hippocampus [201]. Therefore, the secretion of neurotrophic factors by MSC together with their immunomodulatory effects on autoreactive responses may protect and/or promote the regeneration of damage tissues in the CNS of EAE to ameliorate disease.

MSC that migrated into the CNS of MSC-treated EAE mice were found to express markers for oligodendrocyte progenitors (NG2), oligodendrocytes (O4 and galactocerebroside), neurons (β-tubulin type III) and astrocyte (GFAP) [125, 195]. The expression of neural markers suggested the possible transdifferentiation of MSC into neural cells to replace damaged cells in the CNS of EAE mice. However, this remains debatable, as other research groups have found conflicting evident that little or no MSC transdifferentiated in the CNS of MSC-treated EAE mice [174, 176, 194, 198, 200]. In addition, it has been suggested that the supposed transdifferentiation of MSC into a neural phenotype *in vivo* can be the product of cell fusion between donor MSC and host neural cells leading to a false immunophenotype [202, 203]. Other evidence against transdifferentiation includes the expression of a neural phenotype by a low percentage of MSC in CNS of MSC-treated EAE mice [195], the inconsistency between the time required for MSC to transdifferentiate into neural cells to the rapid onset of the therapeutic effect of MSC [196], and a lack of functional neural phenotype in terms of excitability and reaction to neurotransmitter stimuli of transdifferentiated MSC [146]. Hence, the plasticity of MSC to transdifferentiate into neural cells for the repair and/or replacement of damaged cells in the CNS remains to be elucidated.

1.8.3 Limitations in EAE studies

The promising data associated with applications of MSC in EAE models suggest a potential for translation into therapeutic strategies for treatment of MS. However, data from the majority of EAE studies were generated using MSC that lack important characteristics such as the capacity to differentiate into all three classical mesodermal lineages; adipocytes, osteoblasts and

chondrocytes (**Table 1.3**). The majority of these studies rely on immunophenotype assay to verify the identity of their isolated MSC by the expression of positive and absence of negative markers as mentioned in section **1.3**. However, according to the minimal criteria proposed by International Society for Cellular Therapy (ISCT), a prospective MSC population should also have tri-lineage differentiation capacity [204]. Among the EAE studies, only a minority of the studies reported isolation of tri-lineage MSC [174, 200] whilst other studies reported either bi-lineage MSC [125, 194, 199, 205] or provided no differentiation data [192, 193, 197, 198, 206] (**Table 1.3**). The absence of tri-lineage differentiation capacity of those isolated MSC poses significant questions as to whether *bona fide* MSC were used in these EAE studies and hence, the lack of well-characterized MSC in EAE may have confounded and/or undermined the true potential of MSC to treat MS.

TABLE 1.3: Overview of tri-lineage differentiation capacity of the MSC in EAE studies

Study	Source of MSC	Tri-lineage differentiation		
		Adipocytes	**Osteoblasts**	**Chondrocytes**
Zappia *et al* [192]	C57Bl/6 BM-MSC	NA	NA	NA
Matysiak *et al* [197]	SJL BM-MSC	NA	NA	NA
Constantin *et al* [193]	C57Bl/6 adipose-MSC	NA	NA	NA
Rafei *et al* [194]	C57Bl/6 BM-MSC	Yes	Yes	NA
Kassis *et al* [125]	C57Bl/6 BM-MSC	Yes	Yes	NA
Grigoriadis *et al* [205]	C57Bl/6 BM-MSC	Yes	Yes	NA
Gerdoni *et al* [174]	C57Bl/6 BM-MSC	**Yes**	**Yes**	**Yes**
Bai *et al* [198]	Human BM	NA	NA	NA
Zhang *et al* [195]	Human BM	NA	NA	NA
Barhum *et al* [199]	Human BM	Yes	Yes	NA
Gordon *et al* [200]	Human BM	**Yes**	**Yes**	**Yes**

NA: Not available; BM: Bone marrow

1.8.4 Clinical trials in MS

MSC have gone from bench to bedside in a relatively short period of time, despite the lack of a comprehensive understanding of their mechanism(s). However, positive results from pre-clinical studies in EAE have prompted their use as a potential therapeutic in MS. Indeed, several pilot studies administering MSC either intrathecally or intravenously into MS patients have reported the feasibility and safety of MSC therapy [182-184]. The most recent phase I/II open safety clinical trials conducted by Karussis *et al* [181] reported similar findings; no immediate or late adverse effects were observed in MS and amyotrophic lateral sclerosis patients injected intrathecally and/or intravenously with MSC. Additionally, 6 months follow-up of the MS cohort revealed no new gadolinium-enhancing lesions in the brain, and EDSS scores of the MS patients generally remained stable with a declining trend [181]. Immunological analysis showed an increased proportion of $CD4^+$ $CD25^+$ Tregs and, a reduction in the proportion of activated DC and *in vitro* T cell proliferation, 4 and 24 hours post MSC transplantation suggesting a possible immunological down-regulation by MSC. Based on these Phase I/II studies, it appears safe and feasible to inject MSC into MS patients. Further evaluation in subsequent phase III clinical trials and importantly, further investigation of the mechanisms of MSC in EAE model will be required to fully elucidate their efficacy as a potential therapeutic for MS.

1.9 Hypothesis

The hypothesis of this study is that MSC can be effectively isolated from mouse BM using a more efficient methodology, and be employed as a therapy for treatment, and possible cure, of EAE, the animal model for MS.

1.10 Project Aims

The hypothesis was addressed by investigating the following objectives:

Objective 1

To determine the most effective method for isolating of a pure and homogenous MSC population that is well characterized and will be subsequently applied in an animal model of EAE to test their therapeutic potential. The most efficient method of MSC isolation will be investigated by (1) determining the most effective method for collecting BM cells; (2) determining the applicability of *in vivo* 5-FU treatment of the BM as a pre-conditioning step to remove hematopoietic cells from the BM and (3) the optimization of methodology to fractionate prospective fibroblastic MSC from hematopoietic cells from 5-FU-treated BM culture.

Objective 2

To characterize the $CD11b^{-}CD45^{-}$ MSC isolated from 5-FU-treated and untreated C57BL/6 mice by (1) identifying and characterizing BM-MSC-derived CFU-F; (2) investigating the effect of 5-FU on the frequency of CFU-F and differentiation potential of CFU-F; (3) determining their differentiation and proliferation potential; (4) examining cell surface antigen expression and pluripotent marker expression and (5) comparing their *in vitro* immunosuppressive capacity.

Objective 3

To assess the *in vivo* immunosuppressive potential of $CD11b^{-}CD45^{-}$ MSC isolated from 5-FU-treated and untreated C57BL/6 mice in a mouse EAE model. Therapeutic effectiveness of 5-FU-MSC versus untreated MSC in EAE will be investigated by determining (1) the therapeutic effect of untreated MSC and 5-FU-MSC in EAE; (2) the degree of inflammatory infiltrates and demyelination of untreated MSC- and 5-FU-MSC-treated mice; (3) changes to the immune responses of untreated MSC- and 5-FU-MSC-treated mice; (4) Th1-, Th2 and Th17-associated cytokine levels in MOG-stimulated splenocytes from untreated MSC- and 5-FU-MSC-treated mice and (5) the involvement of known immunosuppressive factors in untreated MSC- and 5-FU-MSC-mediated immunosuppression.

2 CHAPTER 2 – MATERIALS AND METHODS

2.1 Reagents and solutions

2.1.1 Complete α-MEM

α-Minimal Essential Media (α-MEM; Sigma, USA) supplemented with non-heat inactivated 10% (v/v) Fetal Bovine Serum (FBS; Gibco^{TM}, USA), 10% (v/v) horse serum (HS; Serum Australis, Australia) and 10% Penicillin-Streptomycin-Glutamine (PSG; Gibco^{TM}, USA).

2.1.2 α-MEM flushing media

α-MEM supplemented with non-heat inactivated 3% (v/v) FBS, 3% (v/v) HS and 10% PSG.

2.1.3 α-MEM buffer

α-MEM supplemented with 0.5% (w/v) bovine serum albumin (BSA), mixed well and filtered through 0.2µm filter.

2.1.4 Complete RPMI media

RPMI 1640 (Gibco^{TM}, USA) supplemented with non-heat inactivated 5% (v/v) FBS, and 10% PSG.

2.1.5 Adipogenic induction media

Complete α-MEM supplemented with 1µM dexamethasone (Sigma, USA), 50µM indomethacin (Sigma, USA) and 0.5µM IBMX (Sigma, USA).

2.1.6 Osteogenic induction media

High Glucose Dulbecco's Eagle Medium (DMEM; Sigma, USA) supplemented with non-heat inactivated 10% (v/v) FBS, 10% (v/v) HS, 50µg/mL L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, USA), 20mM β-Glycerol phosphate disodium salt pentahydrate (Sigma, USA) and 1nM dexamethasone. Prepared freshly for each use.

2.1.7 Chondrogenic media

Serum-free DMEM supplemented with ITS+premix (6.25µg/mL insulin, 6.25µg/mL transferrin, 6.25ng/mL selenious acid, 1.25mg/mL BSA, 5.35µg/mL linoleic acid; BD Biosciences, USA), 50µg/mL L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 40µg/mL L-Proline (Sigma, USA), 100nM dexamethasone, 10ng/mL TGF-β3 (Sigma, USA) and 100nM sodium pyruvate (Gibco^{TM}, USA). Prepared freshly for each use.

2.1.8 Oil red O stain

2.1.8.1 Stock solution

0.5% (w/v) Oil Red O (Sigma, USA) stock solution in isopropanol mixed well and filtered through 0.2µm filter.

2.1.8.2 Working solution

3 parts Oil Red O stock solution and 2 parts phosphate buffer solution (PBS) were mixed and allowed to stand for 10 minutes, then filtered through 0.45µm filter and allowed to stand for 10 minutes before use. Prepared freshly for each use.

2.1.9 Alizarin Red stain

0.01% (w/v) Alizarin Red S (Sigma, USA) was mixed with distilled water (dH_2O) and pH adjusted to 4.2 using 0.1% ammonium hydroxide. Mixture filtered through 0.2µm filter.

2.1.10 Alcian Blue stain

0.01% Alcian blue (Sigma, USA) was mixed with 3% acetic acid (Sigma, USA), then filtered through 0.2µm filter and pH adjusted to 2.5.

2.1.11 Safranin O stain

0.2% (w/v) Safranin O (Sigma, USA) and 1% (v/v) glacial acetic acid mixed with dH_2O, then filtered through 0.2µm filter.

2.1.12 Fast green stain

0.04% (w/v) Fast green FCF (Sigma, USA) and 0.2% (v/v) glacial acetic acid mixed with dH_2O, then filtered through 0.2µm filter.

2.1.13 Cystal violet stain

2.1.13.1 For staining CFU-F

0.1% (w/v) crystal violet (Sigma, USA) mixed with 10% (v/v) ethanol, then filtered through 0.2µm filter.

2.1.13.2 For cell counting

0.1% (w/v) crystal violet (Sigma, USA) and 15% (v/v) glacial acetic acid mixed with 75% (v/v) dH_2O, then filtered through 0.2µm filter.

2.1.14 IDO buffer (2X)

2X IDO buffer were prepared with 100mM PBS (pH 6.5), 40mM ascorbic acid (Sigma, USA), 20µM methylene blue (Sigma, USA), 200µg/mL catalase (Sigma, USA) and 800mM L-Tryptophan (Sigma, USA).

2.2 Animals

8- to 12-week-old female C57BL/6, BALB/c and NOD mice from Monash Animal Services (Monash, Victoria, Australia) were housed in a specific pathogen-free environment (Monash Medical Centre Animal Facilities, Victoria, Australia). All studies had approval of the animal ethics committee under animal ethics permit number, MMCB/2007/38.

2.3 Isolation of MSC from untreated and 5-FU-treated BM

Treated mice were given i.p injection of 300 mg/kg 5-FU 4 days before BM harvest. Untreated and 5-FU-treated mice were culled and tibia and femora removed. BM was flushed out using α-MEM flushing media. BM cells filtered through 70µm nylon mesh were either used for colony forming unit assay or further culturing. Number of extracted BM cells was enumerated with crystal violet stain. For culturing, BM cells were plated at a density of 2 x 10^5 cells/cm^2 onto 75cm^2 tissue culture flasks (BD falcon™, USA). After 24 hours incubation at 37^oC, 10% CO_2, non-adherent cells were removed by washing twice with basal α-MEM. Fresh complete α-MEM media was added and cultured for 10 days before cell sorting. MSC were isolated using MACS® column (Miltenyi Biotec, Germany) according to manufacturer's instructions. Briefly, BM cells

were stained with CD11b-PE and CD45-PE (BD Pharmingen, USA) at 4ºC in the dark for 30 minutes. Unbound antibodies were removed with α-MEM buffer followed by staining with anti-PE MACS® microbeads at 4ºC in the dark for 15 minutes. Stained cells were washed with α-MEM buffer prior to separation using MACS® LS column. The eluted cell fraction (negative for both CD11b and CD45) was collected and homogeneity verified by flow cytometry (MoFlo™, Beckman). Sorted cells were used for proliferation assay, gene and cell surface markers determination, *in vitro* differentiation, splenocyte proliferation assay and *in vivo* EAE study.

2.4 Colony-forming unit assay and criteria for CFU-F sizes

Untreated and 5-FU-treated BM cells were plated at a density of 1 x 10^4 BM cells/cm^2 on 225cm^2 tissue culture flasks and 6-well flat-bottomed tissue culture dish (NUNC™, USA) respectively. A total of 12 x 10^6 cells from untreated BM and 1 x 10^5 cells from 5-FU-treated BM were used. Cells were incubated at 37^{o}C, 10% CO_2 for 24 hours, before non-adherent cells were removed by washing with basal α-MEM. Fresh complete α-MEM media was changed every 3 days over 20 days. Prior to staining with crystal violet for 1 hour, growth media was removed and cells washed twice with PBS. Excess stain was removed by repeated washing with distilled water and colonies air-dried overnight. Stained colonies were enumerated at 4X objective using bright-field microscopy. A colony that contained at least 20 cells was counted as one CFU-F. A grid system made up of 1mm by 1mm squares was used to identify CFU-F sizes according to the following arbitrary criteria. Small CFU-F fell within the 1mm^2 grid while medium or large CFU-F occupied the entire designated grid squares (medium CFU-F $\geq$ 4mm^2 and $\leq$ 9mm^2; large CFU-F $\geq$ 9mm^2).

2.5 In vitro differentiation assay of CFU-F and MACS® isolated MSC

For CFU-F differentiation, 1 x 10^5 BM cells were plated on 6-well flat-bottomed tissue culture dish in duplicate. Cells were incubated at 37°C, 10% CO_2 for 24 hours and non-adherent cells removed by basal α-MEM. Fresh complete α-MEM media was changed every 3 days over 20 days. For MSC differentiation, MACS® isolated MSC were plated in triplicate at 1 x 10^4 MSC/well for adipogenesis and 3 x 10^4 MSC/well for osteogenesis on 48-well flat-bottomed plates (NUNC™, USA) in complete α-MEM media and incubated at 37°C, 10% CO_2 till 70-80% cell confluence.

To induce differentiation, culture media were replaced with adipogenic or osteogenic induction media and changed every 2-3 days for a maximum of 21 days. For chondrocyte differentiation, 2.5 x 10^5 MACS® isolated MSC in a 15mL tube (BD falcon™, USA) were pelleted at 500*g* for 10 minutes followed by replacing the culture media with chondrogenic induction media. The pelleted cells were incubated at 37°C, 10% CO_2 for 21 days with media changed every 2-3 days. Adipocytes were stained with Oil Red O, osteoblasts with Alizarin Red and chondrocytes with Safranin O or Alcian Blue.

2.5.1 Oil Red O staining

The differentiated cells were fixed by carefully replacing half the induction media with 10% (v/v) neutral buffer formalin followed by incubation at room temperature (RT) for 30 minutes. The fixed cells were washed once with PBS followed by staining with Oil Red O for 30 minutes

at RT. Stained cells were washed with PBS until the wash was clear. Cells were counter-stained with hematoxylin for 5 minutes and excess stain was removed by washing with PBS. PBS was added to cover the cell monolayer and adipocytes visualised under bright-field microscopy.

2.5.2 Alizarin red staining

The differentiated cells were washed twice with PBS then fixed with 10% (v/v) neutral buffer formalin at RT for 30 minutes. Fixed cells were washed once with PBS followed by staining with Alizarin red for 30 minutes at RT. Excess stain was removed by repeated washing with dH_2O and the stained cell monolayer air-dried overnight. Calcium accumulation was visualised under bright-field microscopy.

2.5.3 Alcian blue staining

The differentiated cell pellet was incubated in 10% (v/v) neutral buffer formalin for 24 hours at RT. The fixed cell pellet was embedded in paraffin then the histological sections (5µm) deparaffinised, hydrated and stained with Alcian blue stain for 10 minutes. Sections were dehydrated, mounted with a cover slip and proteoglycan accumulation visualised under bright-field microscopy.

2.5.4 Safranin O-fast green staining

The deparaffinised and hydrated sections were stained with Safranin O for 10 minutes followed by rinsing with dH_2O. The sections were counter-stained with fast green stain for 15 seconds,

rinsed with dH_2O and dried with filter paper. The sections were then rinsed with absolute ethanol to remove excess stain and mounted with cover slip after 2 washes in xylene (5 minutes each). The accumulation of proteoglycan was visualised under bright-field microscopy and graded using Bern score [207].

2.6 In situ immunofluoresence staining of colonies

Untreated or 5-FU-treated BM cells (5 x 10^4 cells) were plated on µDish$^{35mm,\ high}$ (Ibidi®, Germany). After 24 hours incubation at 37^{o}C, 10% CO_2, non-adherent cells were removed by washing twice with basal α-MEM and fresh complete α-MEM media added and cultured for 10 days before fixing in fixation/permeabilization solution (BD Biosciences, USA) for 30 minutes. Excess fixative was removed by washing twice with PBS. Cells were stained with CD11b-PE and CD45-PE antibodies at 4ºC in the dark for 30 minutes. Unbound antibodies were removed with PBS and fluorescence visualised under Olympus IX71 fluorescence microscope.

2.7 Flow cytometry

2.7.1 Extraction and preparation of organs from mice

Mice were killed by CO_2 asphyxiation or cervical dislocation. Spleen, thymus, inguinal lymph nodes (LN) were removed and placed in sterile PBS on ice. Single cell suspensions of spleen, thymus and LN were prepared by gently dissociating tissue with the end of a plastic syringe plunger on a sterile plastic dish. Dissociated cells were filtered through 100µm nylon mesh to

remove tissue clumps and fat. Cells were pelleted at 1600rpm and resuspended in sterile PBS for splenocyte proliferation assay or flow activated cell sorting (FACS) staining.

2.7.2 Cell staining and analysis

One million cells were stained with the appropriate antibody cocktail (sections **2.7.3** and **2.7.4**). For primary staining, antibodies were incubated at 4^{o}C for 30 minutes in the dark. Following staining, cells were washed twice with PBS and transferred into FACS tubes. For secondary staining, the stained cells were incubated with secondary antibodies at 4^{o}C for an additional 30 minutes in the dark then washed twice with PBS and transferred into FACS tubes. Samples were analysed using BD FACS Canto Flow II cytometer (at least 10,000 events/sample). Unstained and single stained cells were used as controls.

2.7.3 Antibody cocktail for staining T cells from EAE mice

Cells were stained with CD4-PB (BD Pharmingen, USA) or CD8-APC (BD Pharmingen, USA). Cell were stained with FoxP3-APC (Anti-mouse/rat FoxP3 staining set APC; eBioscience®) according to manufacturer's instructions. Briefly, CD4 stained cells were fixed with fixation/permeabilization buffer at 4^{o}C for 30 minutes prior to FoxP3 staining at 4^{o}C for 30 minutes in the dark. Stained cells were washed twice with permeabilization buffer then resuspended in PBS for FACS analysis.

2.7.4 Antibody cocktail for MSC characterization

Freshly MACS® isolated MSC were stained with CD44-PE, CD49d-PE, CD29-FITC, CD54-biotin, CD73-PE, CD81-PE, CD90-PerCP, CD106-biotin (BD Pharmingen, USA), CD105-PB (BioLegend®), CD140b-APC, CD166-PE, SSEA-4-biotin (eBioscience®) and purified rabbit anti-mouse CD271 (abcam®). Streptavidin-APC-Cy7 (BD Pharmingen, USA) and goat anti-rabbit IgG-PE (Vectashield®) were used as secondary antibodies.

2.8 RT-PCR

Total mRNA from MSC was prepared using the RNeasy RNA isolation kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA was treated with DNase I (QIAGEN, Germany), reverse-transcribed (Superscript™ III First-Strand Synthesis System for RT-PCR; Invitrogen, USA) and amplified by PCR using the primers below (**Table 2.1**). mRNA from murine embryonic stem (ES) cells given as a gift by Dr. Paul Verma was used as positive control.

PCR amplification include a total of 25 cycles of denaturation at 94^{o}C for 45 seconds followed by annealing at the primer-specific temperature outlined in **Table 2.1** for 45 seconds and extension at 72^{o}C for 45 seconds with a first denaturation step at 94^{o}C for 5 minutes and a final extension step at 72^{o}C for 5 minutes. PCR products were analysed by 2% agarose gel electrophoresis containing 0.5 µg/ml of ethidium bromide. Gels were visualised using Bio-Rad

Universal hood II Gel Doc XR system (BioRad Laboratories Inc.) and images taken using Quality One Version 4.6.3 basic software.

TABLE 2.1: PCR primer sequences

Primers		Sequences	Annealing temperature (^{o}C)
Nanog	**Forward**	5'-TCA AGG ACA GGT TTC AGA AGC A-3'	58
	Reverse	5'-GCT GGG ATA CTC CAC TGG TG-3'	
Oct 3/4	**Forward**	5'-CCA ATC AGC TTG GGC TAG AG-3'	60
	Reverse	5'-CCT GGG AAA GGT GTC CCT GTA-3'	
β-actin	**Forward**	5'-TCA CCG AGG CCC CCC TGA ACC CTA-3'	58-60
	Reverse	5'-GGC AGT AAT CTC CTT CTG CAT CCT-3'	

2.9 Induction of experimental autoimmune encephalomyelitis (EAE)

200µg of encephalitogenic peptide, MOG_{35-55} (GL-Biochem, China) was emulsified in Complete Freund's Adjuvant (Sigma-Aldrich, USA) supplemented with 4mg/mL Mycobacterium tuberculosis H37 RA (DIFCO Labs, USA) in a total volume of 200µl, injected subcutaneously

into the flanks. Immunized mice were immediately injected i.v with 350ng pertussis toxin (Sigma-Aldrich, USA) and again 48 hours later. Animals were monitored daily and neurological impairment was scored on an arbitrary clinical score: 0, no clinical sign; 1, limp tail; 2, limp tail and hind limb weakness; 3, severe hind limb paresis; 4, complete hind limb paresis; 5, moribund or death [208]. In accordance with animal ethics requirements, mice were euthanized upon reaching a clinical score of 3. Mice were followed for 40 days following immunization.

2.10 MSC treatment

To test the immunomodulatory effect of MSC in EAE, mice were injected intravenously with 1 x 10^6 MSC in 300µL basal α-MEM, 6 days after immunization as described in section **2.9**. Control mice received an equal volume of basal α-MEM at day 6.

2.11 Histology

Brain and spinal cord were removed and fixed in 10% neutral buffered formalin and the cervical, thoracic and lumbar segments of each spinal cord were embedded in paraffin. Eight to 16 sections, each of at least 20µm apart, were examined. The extent of inflammation and demyelination was evaluated on tissue sections (5µm) with H&E to assess inflammation and Luxol fast blue (LFB) for demyelination. Histological sections were scored blind. For inflammation, evaluation was performed using H&E-stained sections and scored as follows: 0, no inflammation; 1, cellular infiltrate only in the perivascular areas and meninges; 2, mild cellular infiltrate in parenchyma; 3, moderate cellular infiltrate in parenchyma; 4, severe cellular

infiltrate in parenchyma. Myelin breakdown was assessed as pale staining with LFB and scored as follows: 0, no demyelination; 1, mild demyelination; 2, moderate demyelination; 3, severe demyelination [208, 209].

2.12 MOG-stimulated splenocyte proliferation assay

Splenocytes were cultured at 5 x 10^5 cells/well in complete RPMI media. Cells were stimulated with 1-100µg/ml MOG_{35-55} peptide. Concanavalin A (Con A; 5µg/ml final concentration) was used as positive control and cells without peptide as background. After 72 hours incubation at 37°C with 5% CO_2, splenocytes were pulsed with 10 µCi/mL [3H] thymidine (Amersham) for 24 hours. Cells were harvested on glass fiber filters and incorporation of [3H] thymidine determined using a Packard Tri-Carb 1900 TR liquid scintillation analyzer. All cultures were in quintuplicate. Proliferation data are presented as a stimulation index (SI) calculated according to the formula: SI = count per minute (CPM) of splenocytes with Con A / CPM of splenocytes without Con A.

2.13 Sera MOG-specific antibodies detection

MOG-specific antibody was measured by ELISA as described [210]. Sera were collected at the end point of each experiment and tested at dilutions of 1/100, 1/300, and 1/900 in 96-well plates coated with 5µg/ml of $MOG_{35–55}$. Absorbance was measured at 490nm and mean absorbance of triplicate samples calculated. Non-coated wells were used as a blank.

2.14 MSC suppression assay, blocking assay and immunoregulatory molecules detection

In the suppression assay, 20 Gy γ-irradiated MSC (freshly MACS® isolated) were co-cultured with 5 x 10^5 splenocytes in 96-well flat-bottomed plates with complete RPMI media. For antigen non-specific suppression, splenocytes from naïve mice were stimulated with 5 µg/ml Con A, and for antigen-specific suppression, splenocytes from EAE-induced mice were stimulated with 10 µg/ml MOG_{35-55} peptide for 72 hours at 37°C, 10% CO_2 before pulsing with 10 µCi/mL [3H] thymidine for 16 hours. In blocking experiments, suppression assays were performed in the presence of blocking agents: 2 µg/mL anti-IL-1ra, 1.2 µg/mL anti-IL-6, 70 ng/mL anti-IL-10 (R & D Systems, USA), 10mM indomethacin, 12.5 µM tin protoporphyrin (SnPP; Enzo® life sciences), 0.1mM N-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma, USA) or isotype goat or rat IgG antibodies (R & D Systems, USA). Cells were harvested on glass fiber filters and incorporation of [3H] thymidine determined using a Packard Tri-Carb 1900 TR liquid scintillation analyzer. Supernatants from the suppression assay were collected and levels of IL-6, IL-10, TGF-β (Bender MedSystem®, Austria), HGF (abcam®), PGE_2 (Cayman Chemical, USA), NO (Promega®) and IL-1ra (Quantikine®, USA) detected. Heme oxygenase-1 (HO-1) levels in MSC from suppression assay determined with HO-1-FITC antibodies (abcam®) and expressed as mean fluorescence intensity (MFI).

Levels of IDO activity were measured by a colorimetric method as described [211]. Briefly, 5 x 10^5 cells from the suppression assay were pelleted and frozen at -80^{o}C. The pellet was thawed and resuspended in 100µL PBS. An equal amount of 2X IDO buffer was added and the mixture

incubated at 37^{o}C for 30 minutes to permit IDO to convert to L-tryptophan to N-formyl-kynurenine. The reaction was stopped by adding 40µL of 30% (w/v) trichloracetic acid (Sigma, USA) followed by incubation at 52^{o}C for 30 minutes. The mixture was centrifuged to remove cell debris and 100µL supernatant were transferred to 96-well microreader plate and an equal amount of Ehrlich reagent (Sigma, USA) added. The colour was allowed to develop for 10 minutes and absorbance acquired at 490nm. L-Kynurenine (Sigma, USA) was used as standards.

2.15 Statistical analysis

Statistical analyses to compare two groups were performed using the two-tailed unpaired *T* test. Krustal-Wallis test were used to compare more than two groups and significance between the groups analysed by Tukey post-test as appropriate. All statistical analyses were performed using the software GraphPad Prism v5.02. A value of $p<0.05$ was considered significant.

3 CHAPTER 3 – ISOLATION OF MSC FROM BONE MARROW

3.1 Introduction

Since the discovery of MSC two decades ago, the isolation of MSC from BM still relies heavily on the adherence of MSC onto tissue culture plastic [212]. This isolation technique has proven effective for both human and rat MSC, as the hematopoietic cell components from these species fail to adhere and thrive upon continuous culture [93]. However, mouse hematopoietic cells readily adhere to tissue culture plastic and persist even after multiple passages thus hindering the effective isolation of MSC from mouse BM [92, 94]. Several techniques have been developed to increase the isolation efficiency and yield of MSC from mouse BM. For example, alterations to the cell culturing conditions [95] such as reduction in trypsinisation time, supplementation of growth media with horse serum or growth factors, and changing culture medium more frequently, have been found to enrich and increase efficiency of MSC isolation. Methods involving combinations of BM flushing with mechanical crushing and enzymatic digestion of bones have also been reported to increase MSC isolation efficiency by releasing additional MSC localised within the bone matrix [102], which would not be removed by conventional BM flushing alone. To obtain a pure MSC culture, the majority of methods published to date employ a passaging-purification approach, in which the mixture of adherent MSC and hematopoietic contaminants are continuously passaged to remove the hematopoietic contaminants.

To date, no specific-MSC antigen(s) have been identified, so methods that employ antibodies, singly or in combination, select for specific-MSC subsets rather than the MSC population as a whole [89, 91, 97, 105, 111]. An alternative approach developed by Baddoo *et al* [105] is an immunodepletion method that removes hematopoietic contaminants from 8- to 10-day cultured FVB/N mouse BM cells using antibodies against hematopoietic markers such as CD11b, CD34 and CD45 [94, 105]. Anjos-Afonso *et al* [85] also demonstrated the effectiveness of this immunodepletion for isolating MSC from various mouse strains. Nonetheless, no single method has been recognized as the gold standard for isolating mouse BM-MSC.

MSC from different mouse strains vary widely in terms of their growth media requirements, growth kinetics, numbers of MSC within the BM, differentiation capacity and ease of isolation. Phinney *et al* [92] reported significant differences in terms of yield, growth kinetics and differentiation potential of MSC from different mouse strains, including BALB/c, C57Bl/6, DBA/1, FVB/N and 129. Of these, C57Bl/6 and 129 mouse strains were reported to have the lowest numbers of MSC resulting in poor isolation efficacy [92]. Similar observations were reported by Anjos-Afonso *et al* [85] who highlighted the difficulty of isolating BM-MSC from C57Bl/6 mice, a mouse strain that develops a progressive form of EAE after immunization with MOG_{35-55} [46]. Although, several groups have successfully isolated C57Bl/6 MSC for MOG_{35-55}-induced EAE studies [125, 192, 194], a more effective methodology to isolate MSC for studies in EAE model is required to provide further understanding of their therapeutic effect in MS.

5-Fluorouracil (5-FU) is an antimetabolite that kills rapidly proliferating cells by inhibiting thymidylate synthase, thereby limiting thymidine availability for DNA synthesis [213]. By eliminating rapidly proliferating cells in BM, 5-FU enriches for a quiescent and undifferentiated MSC population [214, 215]. Falla *et al* [215] first reported that BM cells from 5-FU-treated mice formed colonies and differentiated into bone, suggesting the presence of a 5-FU-resistant stromal population. Subsequently, Wang *et al* [214] showed that these 5-FU-resistant BM cells obtained from 5-FU-treated mice were enriched for CFU-F, which retained the ability to differentiate into all three classical mesenchymal lineages. Although these studies highlighted the usefulness of 5-FU in enriching for undifferentiated BM-MSC, these MSC populations were contaminated by hematopoietic components that had not been segregated from the 5-FU-treated BM cultures.

3.2 Aims

The aim of this study was to determine the most effective method for the isolation of a pure and homogenous MSC population that is well characterized and can be subsequently applied in an animal model of EAE to test their therapeutic potential. The following investigations were undertaken to determine the most efficient method of MSC isolation:

1. The most effective method of BM cells extraction
2. The applicability of *in vivo* 5-FU treatment of the BM as a pre-conditioning step to remove hematopoietic cells from the BM
3. Optimization of the methodology to fractionate prospective fibroblastic MSC from hematopoietic cells from 5-FU-treated BM culture

3.3 Materials and Methods

3.3.1 Animals

8- to 12-week-old female C57BL/6, BALB/c and NOD mice were used for MSC isolation as described in section **2.2**.

3.3.2 Extraction of BM cells by conventional flushing

The ends of each bone were removed and BM cells were extracted by inserting a syringe needle into the medullary cavity. BM content was flushed out with flushing media. At least two flushes were applied at both ends of the bone. To determine the number of CFU-F from the medullary cavity, CFU-F assay was carried as described in section **2.4**.

3.3.3 Extraction of BM cells by scraping

BM cells within the medullary cavity were removed by flushing as described in section **3.3.2.** The BM cells along the endosteal region were extracted by scraping the endosteum with the tip of the syringe needle along the bone. Extracted BM cells from the endosteum were then flushed out of the bone. To determine the number of CFU-F from the endosteum, CFU-F assay was carried as described in section **2.4**.

3.3.4 Extraction of BM cells by flushing and scraping

BM cells within the medullary cavity and along the endosteal region were extracted as described in sections **3.3.2** and **3.3.3** respectively. Extracted BM cells from both regions were then pooled together.

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3.3.5 Histology of the bone after BM extraction

BM cells from mouse tibia were extracted either by conventional flushing alone or by a combination of flushing and scraping of the endosteum. Tibia bone was decalcified and embedded in paraffin. The bone was sectioned longitudinally (5µm) and stained with H&E to determine the extraction efficiency of BM cells by the different methods under bright-field microscopy.

3.3.6 In situ immunofluoresence staining of BM cells

BM cells from untreated mice were isolated as described in section **2.3**. Different cells in the BM culture were stained with CD11b and CD45 antibodies as described in section **2.6**.

3.3.7 Flow cytometry

Cells were stained with CD45-PE and CD11b-FITC to determine the percentage of double positive and double negative cells in the population. Details described in section **2.7.2.**

3.3.8 Conventional passaged purification method to isolate MSC

Extracted BM cells (section **3.3.4**) from untreated or 5-FU-treated mice were plated at a cell seeding density of 2 x 10^5 cells/cm^2 onto 75cm^2 tissue culture flasks (BD falcon^{TM}, USA). After 24 hours incubation at 37^oC, 10% CO_2, non-adherent cells were removed by washing twice with basal α-MEM. Fresh complete α-MEM media was added and adherent cells were cultured to approximately 60-70% confluency before further passaging.

3.3.9 5-FU treatment at different time points

The 5-FU-treated mice were given intra-peritoneal injection of 300 mg/kg 5-FU 1 day, 2 days, 3 days or 4 days before BM harvest as described in section **2.3**. To determine the number of CFU-F from each time point, CFU-F assay was carried as described in section **2.4**.

3.3.10 Sorting $CD45^-CD11b^-$ MSC from BM culture

$CD45^-CD11b^-$ cells were sorted from 10 days cultured BM cells using MACS® column as described in section **2.3**. For FACS, $CD45^-CD11b^-$ cells were sorted from 10 days cultured BM cells using MoFlo^{TM} XDP cell sorter. CD45 and CD11b stained cells were filtered through 70µm filter prior to FACS.

3.3.11 In vitro 5-FU treatment of BM cells

Freshly isolated BM cells from untreated mice were directly exposed to two different 5-FU concentrations, 0.1mg/mL and 0.6mg/mL *in vitro*. The 5-FU-treated BM cells were incubated at 37^{o}C for five different time duration of 0.5 hour, 1 hour, 2 hours, 3.5 hours and 7 hours. After incubation, the treated cells were washed twice in complete α-MEM media and then plated at 1 x 10^5 cells/cm^2 onto 25cm^2 tissue culture flasks. The treated BM cells were incubated at 37^{o}C, 10% CO_2 for 6 days and cell proliferation visualized under phase-contrast microscopy. Untreated BM cells were used as control.

3.4 Results

3.4.1 Scraping the endosteal surface significantly increases the yield of BM cells and CFU-F

The efficiency of recovery of BM cells is a primary determinant of the effectiveness of MSC isolation. The conventional method of extracting BM involves inserting a syringe needle into the medullary cavity and flushing out the content of the BM with sterile media [77]. To determine the effectiveness of this conventional method in extracting BM cells, BM was flushed out of the medullary cavity, then the flushed bones were sectioned longitudinally and stained with H&E. The histological staining showed an incomplete removal of BM cells from the medullary cavity with residual cells remaining along the endosteal region (**Fig. 3.1**). To maximize the recovery of BM cells, conventional flushing was combined with scraping of the endosteum with the tip of the syringe needle. This combined method successfully removed the majority of the BM cells, including those in the endosteal region (**Fig. 3.1**). Not only does the combined flushing method yield an additional 33% more BM cells compared to regular flushing (**Fig. 3.2*A***), BM cells from the endosteal region also contained a significantly higher numbers of CFU-F compared to those recovered from the medullary cavity (endosteum: 394 ± 103 vs. medullary cavity: 80 ± 30, $p<0.05$) (**Fig. 3.2*B***). Thus, conventional flushing combined with scraping yield more BM cells and permits the extraction of more CFU-F from the endosteal region compared to flushing alone. The combined method of flushing and scraping were employed for BM extraction for the rest of these studies.

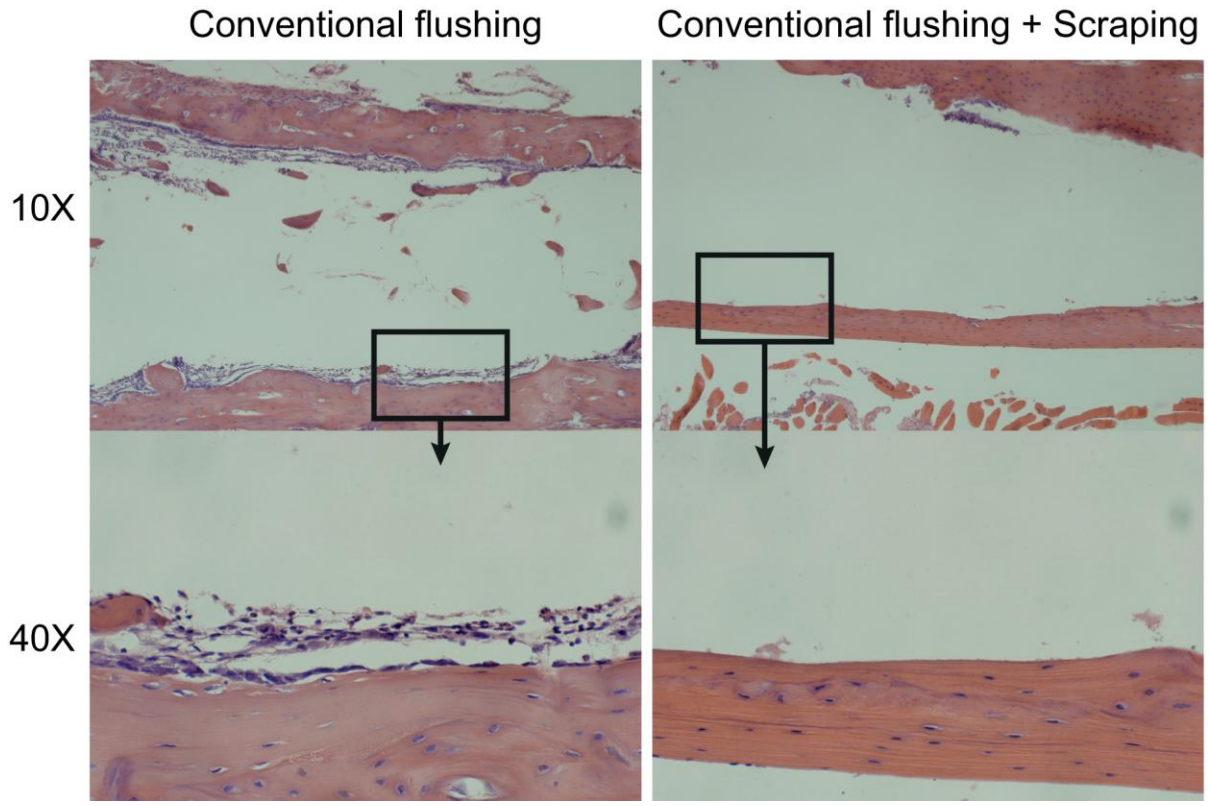


FIGURE 3.1: Representative section of the tibia bone after recovery of BM cells using flushing with or without endosteal scraping. Longitudinal sections were stained with H&E to detect remaining cells in the tibia after BM extraction. BM cells were not completely removed using conventional flushing alone. The combination of flushing and scraping removed the majority of the BM cells especially cells along the endosteal region. Representative photomicrographs taken at X10 and X40 magnification.

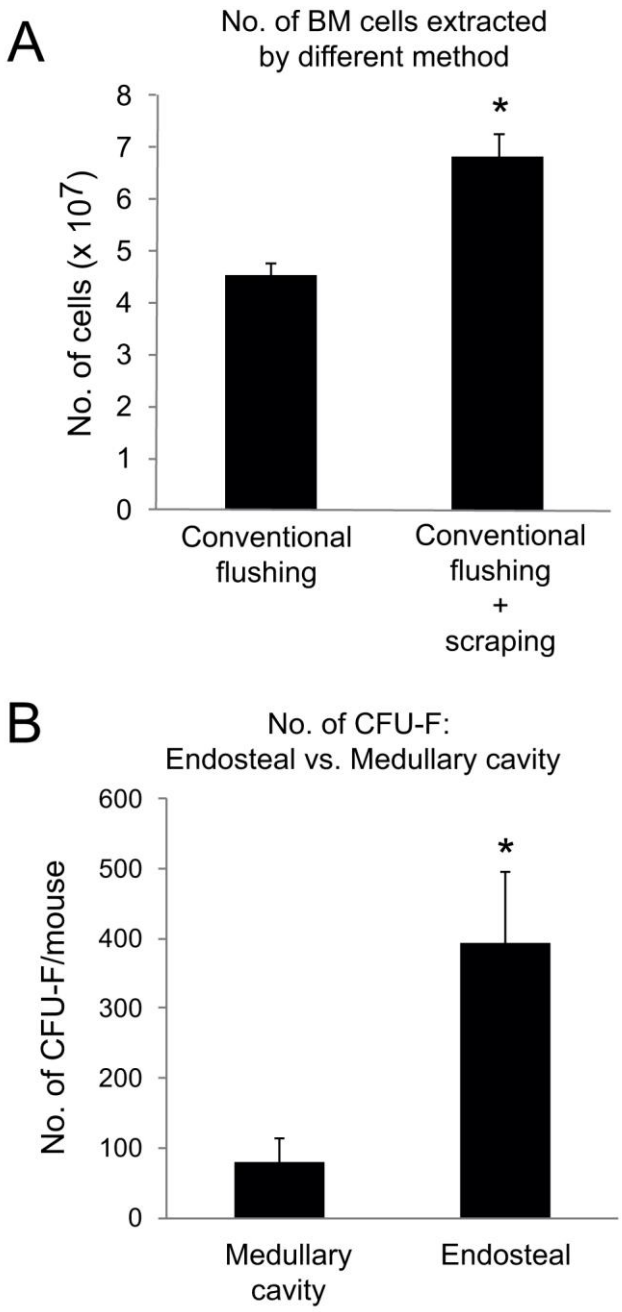
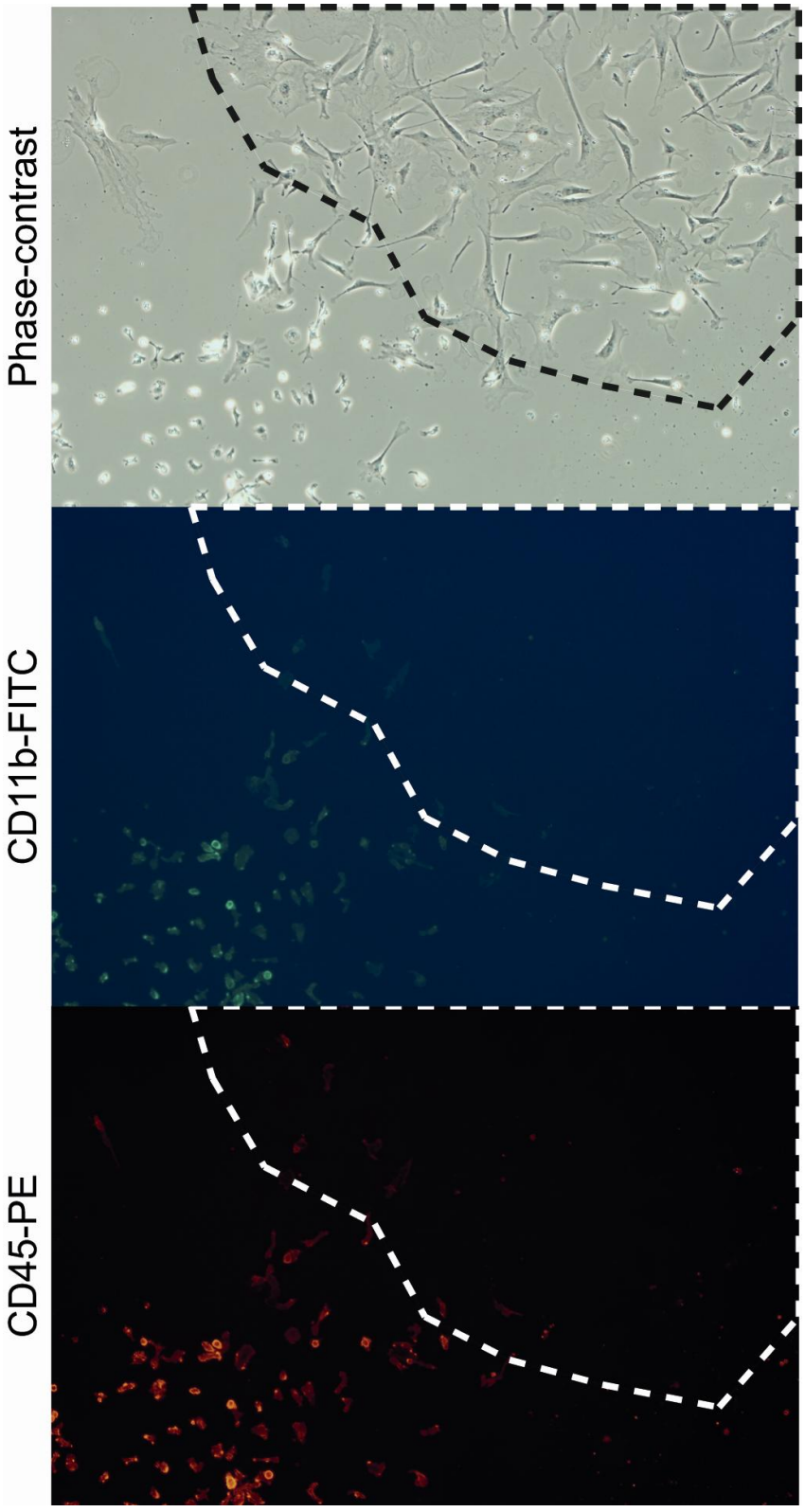


FIGURE 3.2: The efficiency of BM extraction using conventional flushing and combined flushing and scraping methods. (A) The combination of flushing and scraping (n=10) yielded a significant 33% more BM cells compared to flushing alone (n=10). (B) 1 x 10^4 BM cells from the medullary cavity (n=10) and endosteal region (n=10) were plated onto 6-well plates respectively and CFU-F stained with crystal violet after 20 days of culture. The number of CFU-F in the endosteal region is 4-fold higher than in the medullary cavity. Data presented are mean ± SEM. (* $p<0.05$, Two-tailed unpaired *T* test).

3.4.2 CD11b and CD45 antibodies can be used to differentiate prospective MSC from hematopoietic cells within BM culture

Plating of mouse BM cells onto tissue culture plastic resulted in a heterogeneous culture consisted of a mixture of fibroblastic-like cells and non-fibroblastic-like cells as depicted in **Figure 3.3**. Non-fibroblastic-like cells consisted of round cells, cuboidal cells and short rod-like cells characterized by their distinctive glow under phase-contrast microscopy (**Fig. 3.3**). To distinguish hematopoietic cells from the prospective MSC in the culture, cells were stained with the hematopoietic markers, CD11b and CD45, which are not expressed by fibroblastic MSC [87, 92, 95]. As expected, the fibroblastic-like cells stained negative for CD11b and CD45 and the non-fibroblastic-like cells were positive for both CD11b and CD45 (**Fig. 3.3**), confirming that the non-fibroblastic-like cells are the hematopoietic contaminants within the BM culture.

FIGURE 3.3: The expression profile of CD11b and CD45 antigens in the different cell types in BM culture. The isolated BM cells were plated onto tissue culture plastic and cultured for 10 days prior to fixation and staining with CD11b and CD45 antibodies. The fibroblastic cells stained negative (area within the black or white dotted lines) for CD11b and CD45, and the non-fibroblastic cells stained positive for both CD11b and CD45. Representative photomicrographs taken at X10 magnification using Olympus IX71 fluorescence microscope.

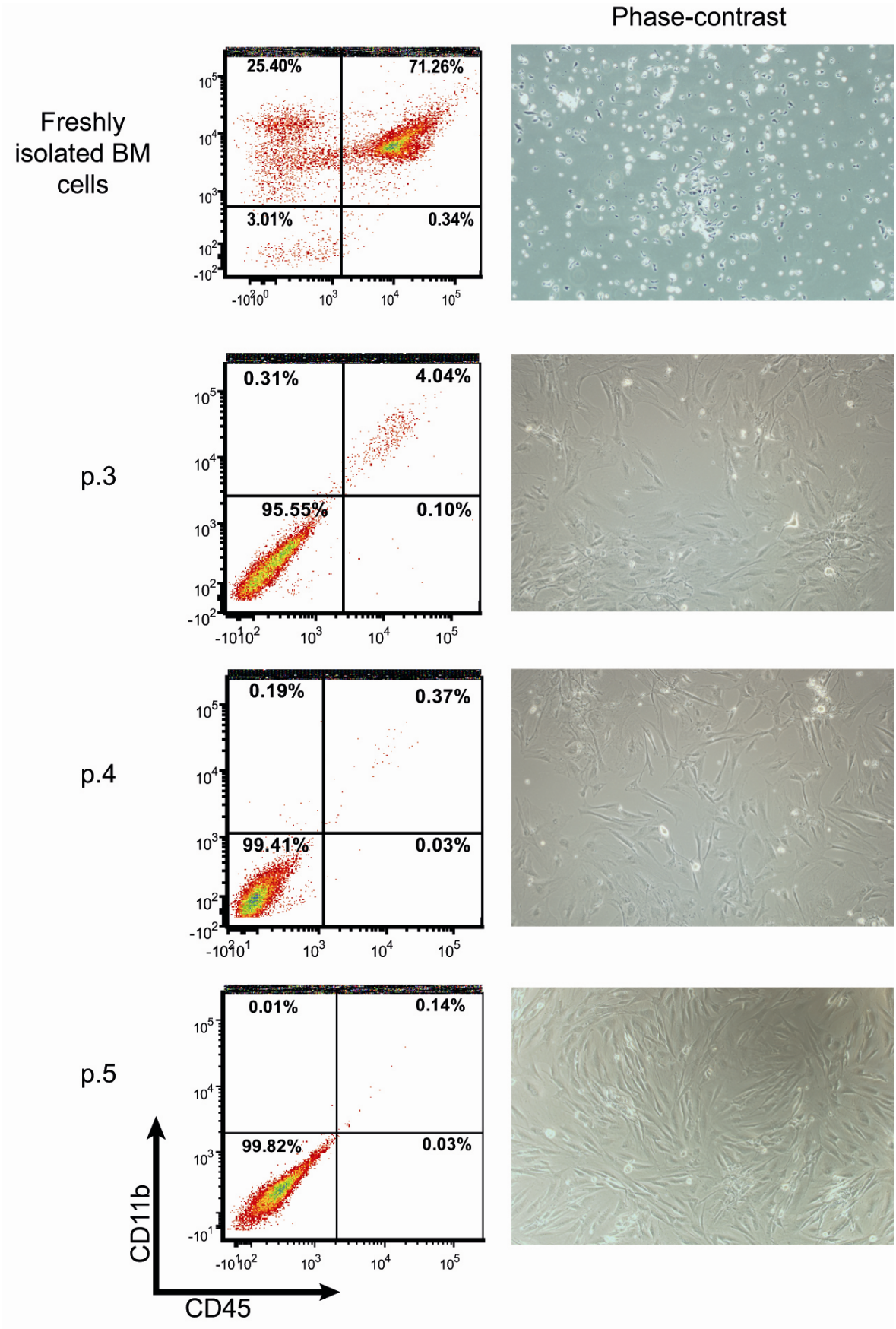

Phase-contrast
CD11b-FITC
CD45-PE

3.4.3 The conventional passage purification method is inefficient in isolating BM-MSC from mice

Although, widely used by many laboratories, the conventional method of passage purification has been suggested to be ineffective in isolating mouse MSC [94, 105]. To determine the suitability of the conventional method in isolating mouse MSC, BM cells were plated onto tissue culture plastic. In some BM cultures from C57Bl/6 mice, discrete colonies of MSC started to form after three passages, accompanied by a reduction in the proportion of $CD11b^{+}CD45^{+}$ hematopoietic contaminants in the culture. Further passaging of the cells resulted in the establishment of a homogenous fibroblastic-like $CD11b^{-}CD45^{-}$ MSC culture with less than 1% of $CD11b^{+}CD45^{+}$ hematopoietic contaminants at passage 5 (**Fig. 3.4**). However, the efficiency of MSC isolation using the conventional method is very low, with 4 out of 5 BM cultures failing to produce MSC cultures (data not shown).

Very poor isolation efficiency of MSC was also observed in BALB/c and NOD mouse strains. BM cells from these mice strains did not increase in confluence after 2-3 weeks of culture (data not shown). The isolation process was repeated with different split ratios and in different culturing media such as DMEM, α-MEM and RPMI, but no MSC cultures were isolated.

FIGURE 3.4: Isolation of MSC from C57BL/6 mice by conventional passage purification methodology. BM cells were plated and cultured to approximately 60-70% confluency before further passaging. At each passage, the relative proportions of $CD11b^{-}CD45^{-}$ and $CD11b^{+}CD45^{+}$ cell populations were determined by flow cytometry. At passage 3 (p.3), fibroblastic-like cells were predominant. A homogenous fibroblastic-like $CD11b^{-}CD45^{-}$ fibroblastic-like MSC with less than 1% of $CD11b^{+}CD45^{+}$ hematopoietic contaminants was established at passage 5. Representative photomicrographs taken at X10 magnification using Olympus IX71 fluorescence microscope.


Phase-contrast
Freshly isolated BM cells
25.40%
71.26%
3.01%
0.34%
p.3
0.31%
4.04%
95.55%
0.10%
p.4
0.19%
0.37%
99.41%
0.03%
p.5
0.01%
0.14%
99.82%
0.03%
CD11b
CD45

3.4.4 In vivo 5-FU treatment increases isolation efficiency of MSC

Hematopoietic cells from mouse BM readily adhere to tissue culture plastic and can persist in culture after multiple passages [92, 94]. *In vivo* 5-FU treatment has been used to eliminate rapidly proliferating hematopoietic precursor cells in the BM as an initial step to increase the purity of hematopoietic stem cells in HSC isolation [216]. To determine if a similar *in vivo* reduction of hematopoietic cells can increase the efficacy of isolating MSC, donor mice were pre-treated with 5-FU prior to BM extraction.

3.4.4.1 4 days of 5-FU pre-conditioning is optimal

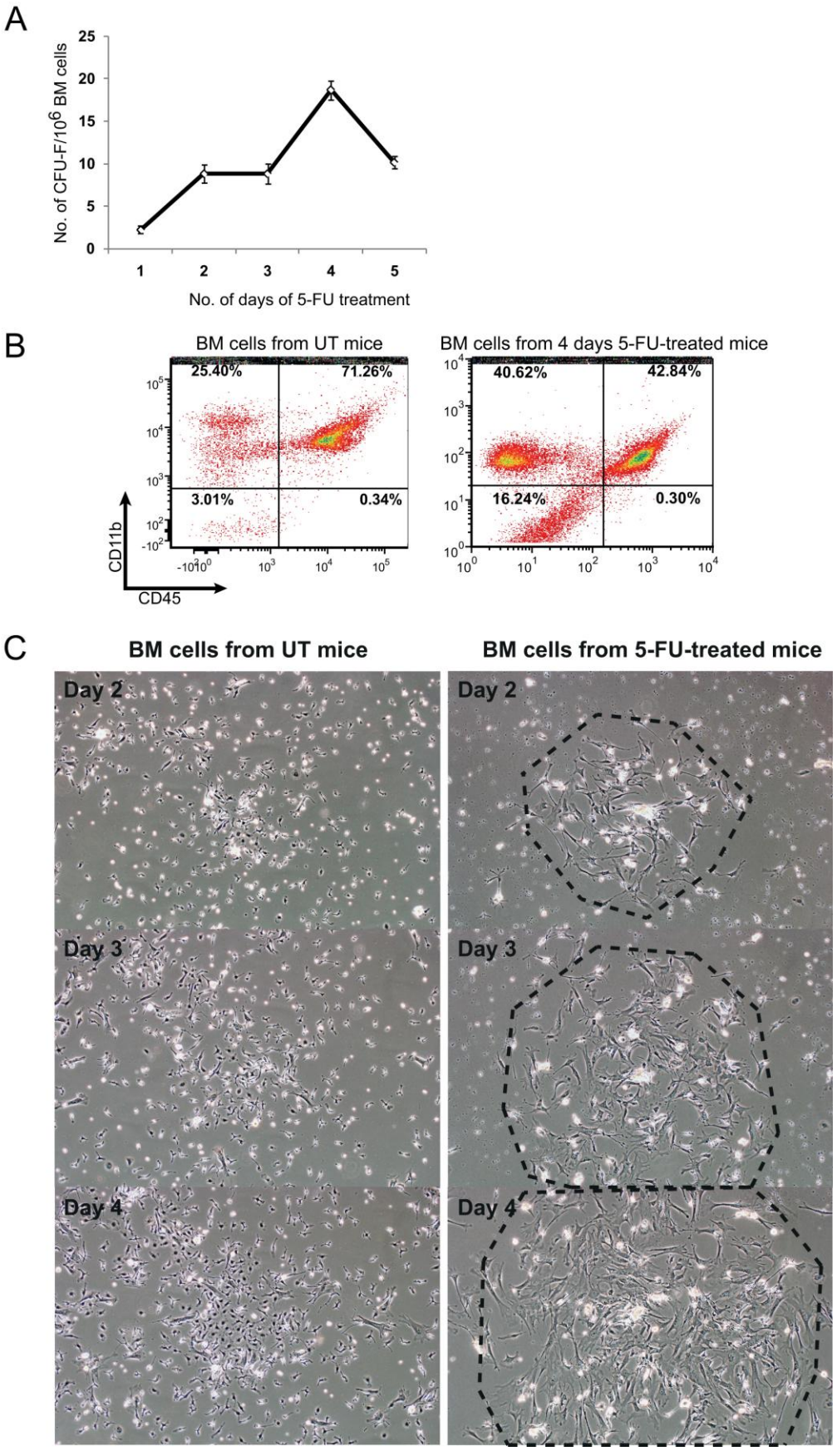
The optimal period from 5-FU administration to BM extraction, which maximizes MSC isolation efficiency was determined. Mice were treated with 5-FU for 1 day, 2 days, 3 days, 4 days and 5 days respectively prior to BM extraction. 1 x 10^5 BM cells were seeded into 6-well plate and incubated at 37^{o}C for 20 days prior to staining CFU-F with crystal violet. All time points were analyzed in sextuplicate. There is a 9-fold increase in the numbers of CFU-F from day 1 to day 4 with the numbers of CFU-F peaking at day 4 and decreased thereafter (**Fig. 3.5*A***). Thus, treating mice with 5-FU for 4 days prior to BM cells isolation was selected as the optimum treatment regime.

To determine whether 5-FU treatment successfully decreased the proportion of hematopoietic component in the BM, freshly isolated BM cells from 5-FU-treated mice were stained with CD11b and CD45 antibodies. In contrast to untreated BM cells, the proportion of $CD11b^+CD45^+$ hematopoietic cells was drastically reduced from ~71% in untreated BM to ~43% after 4 days 5-

FU treatment (**Fig.3.5*B***). In addition, 4 days 5-FU-treatment also increased the proportion of $CD11b^{-}CD45^{-}$ cells in the population by approximately 5-fold from ~3% to ~16% (**Fig.3.5*B***).

The increased proportion of $CD11b^{-}CD45^{-}$ cells in the 5-FU-treated BM was associated with the formation of distinctive fibroblastic-like colonies not seen in untreated BM cultures. When cultured at the same plating density with the same culture area observed from day 2 to day 4, adherent fibroblastic-like cells from 5-FU-treated BM formed distinct colonies but those from untreated BM did not (**Fig. 3.5*C***). The untreated BM culture comprised of a mixture of fibroblastic-like cells and hematopoietic cells (**Fig. 3.5*C***) whereas 5-FU-treated BM culture is comprised mainly of fibroblastic-like cells (**Fig. 3.5*C***).

FIGURE 3.5: The isolation of BM-MSC after treating mice with 5-FU. (A) The optimum time point for isolating MSC after 5-FU treatment. Mice (n=5/time point) were treated with 5-FU for different time periods before BM cells were extracted and plated for CFU-F. Treating mice with 5-FU for 4 days yield the highest numbers of CFU-F. All time points were analyzed in sextuplicate. (B) 4 days 5-FU treatment reduces the proportion of $CD11b^{+}CD45^{+}$ hematopoietic cells and increases the proportion of $CD11b^{-}CD45^{-}$ cells in the BM population. Representative plot of 2 independent experiments. (C) BM cells from untreated mice and 5-FU-treated mice were plated at the same cell density and the same microscopic fields were monitored from day 2 to day 4. BM from 5-FU-treated mice was associated with the formation of distinctive fibroblastic-like colonies (circled with black dotted lines) not seen in untreated BM cultures. Representative photomicrographs taken at X10 magnification using Olympus IX71 fluorescence microscope.


A
No. of CFU-F/10^6 BM cells
25
20
15
10
5
0
1
2
3
4
5
No. of days of 5-FU treatment
B
BM cells from UT mice
25.40%
71.26%
3.01%
0.34%
CD11b
CD45
BM cells from 4 days 5-FU-treated mice
40.62%
42.84%
16.24%
0.30%
C
BM cells from UT mice
BM cells from 5-FU-treated mice
Day 2
Day 3
Day 4
Day 2
Day 3
Day 4

3.4.4.2 Hematopoietic components can be effectively removed from 5-FU-treated BM cultures by MACS® sorting

Similar to untreated BM culture (section **3.4.3**), attempts to isolate 5-FU-MSC by conventional passage purification were unsuccessful. Although 5-FU-treated BM cells formed fibroblastic-like colonies (p.0), these cells did not increase in confluence after sub-culturing (p.1) and the culture failed to survive after 2-3 weeks (data not shown). This failure to isolate MSC using conventional methodology prompted the development of a more direct isolation method.

Negative selection, using hematopoietic markers such as CD11b and CD45, was reported by Phinney *et al* [94] to facilitate the effective isolation of BM-MSC. Therefore, $CD11b^{-}CD45^{-}$ MSC from 10 day cultures of 5-FU-treated BM cells were directly separated from the hematopoietic cells using CD11b and CD45 antibody staining. To optimize isolation efficiency, two different cell sorting methods were investigated, namely using fluorescence activated cell sorting (FACS) or magnetic cell separation using the MACS® column system.

As illustrated in **Table 3.1**, the number of $CD11b^{-}CD45^{-}$ cells/10^6 in cultured BM obtained from MACS® column was 22-fold higher than those obtained using the FACS method. In addition, the isolated $CD11b^{-}CD45^{-}$ cells from MACS® column are generally more than 98% free from hematopoietic contaminants (**Fig. 3.6*A***). By combining *in vivo* 5-FU treatment together with MACS® sorting, MSC were successfully isolated from C57Bl/6, NOD and BALB/c mice even though no MSC were isolated from the latter two mouse strains using conventional passage purifications as described earlier in section **3.4.3** (**Fig. 3.6*B***).

TABLE 3.1: Comparison of the efficiency of MSC separation between FACS and MACS®

Separation method	$CD11b^{-}CD45^{-}$ cells/10^6 stained cultured BM (x 10^6)
FACS	0.0075 ± 0.001
MACS®	0.16 ± 0.02

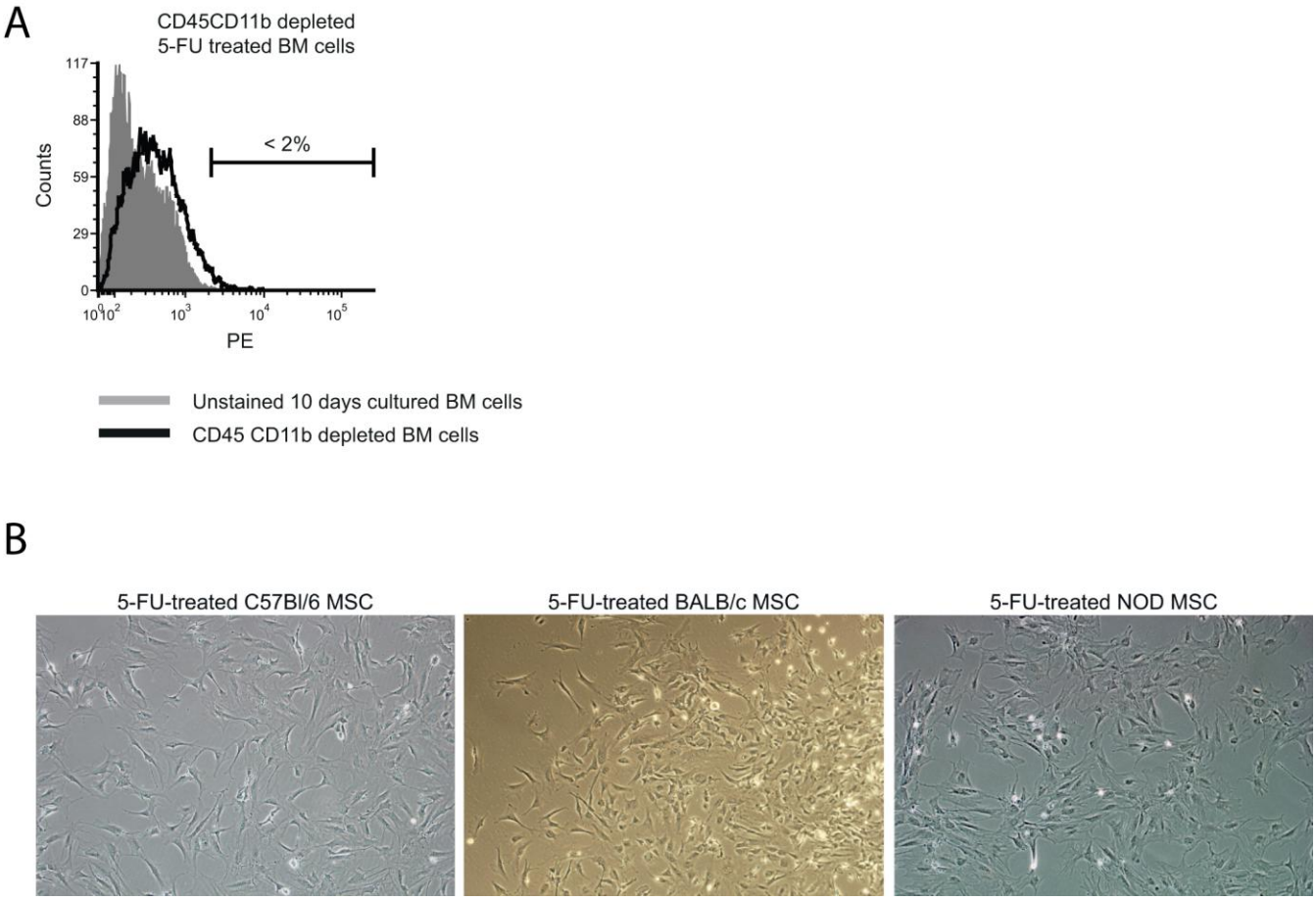


FIGURE 3.6: Cell sorting of 5-FU-treated BM cells with MACS® yielded a homogenous population of $CD11b^{-}CD45^{-}$ MSC and the isolation method is applicable in different mice strains. (A) Representative histogram of the purity of MACS® sorted $CD11b^{-}CD45^{-}$ MSC from 10 days cultured 5-FU-treated C57Bl/6 BM cells. BM cells were stained with CD45-PE and CD11b-PE antibodies prior to sorting for $CD11b^{-}CD45^{-}$ MSC using a MACS® column. Purity of the sorted MSC was determined by the level of PE expressed. More than 98% of the isolated MSC were negative for CD45-PE and CD11b-PE. (B) The MACS® sorted $CD11b^{-}CD45^{-}$ MSC from 5-FU-treated C57Bl/6, NOD and BALB/c mice. The isolated $CD11b^{-}CD45^{-}$ MSC from all three mouse strains displayed fibroblastic morphology. Representative photomicrographs were taken at X10 magnification using Olympus IX71 microscope.

3.4.5 In vitro 5-FU treatment of BM cells is less effective

To determine if similar effect of 5-FU on BM cells can be replicated *ex vivo*, we directly exposed freshly isolated BM cells to 5-FU *in vitro*. Isolated BM cells were exposed *in vitro* to two different 5-FU concentrations, 0.1mg/mL and 0.6mg/mL for five different time durations of 0.5, 1, 2, 3.5 and 7 hours. The untreated BM cells were used as control. After 6 days of culture, fibroblastic-like colonies were detected in the BM culture after 0.5 hr of 5-FU-treatment. The exposure of BM cells to 5-FU for periods of 1 hour or greater did not result in the formation of fibroblastic-like colonies (**Fig. 3.7**). No visible colonies were found in untreated controls.

Treatment with 0.6 mg/mL 5-FU resulted in the formation of larger colonies compared to those seen after treatment with lower concentrations of 5-FU (**Fig. 3.7**). However, the frequencies of fibroblastic-like colonies in 5-FU-treated BM were much lower than seen in BM cultures from 5-FU-treated mice (data not shown).

To further characterize the fibroblastic-like cells in the *in vitro* 5-FU-treated BM (0.6 mg/mL 5-FU, 0.5 hour); the adherent cells were expanded prior to cell sorting as mentioned in section **3.4.4.2**. However, the 5-FU-treated BM did not increase in confluency after sub-culturing (p.1) and the culture failed to survive after 2-3 weeks of culture. The addition of growth factors such as basic-fibroblastic growth factor (bFGF) and platelet-derived growth factor (PDGF) to the culture did not restore proliferation of these cells.

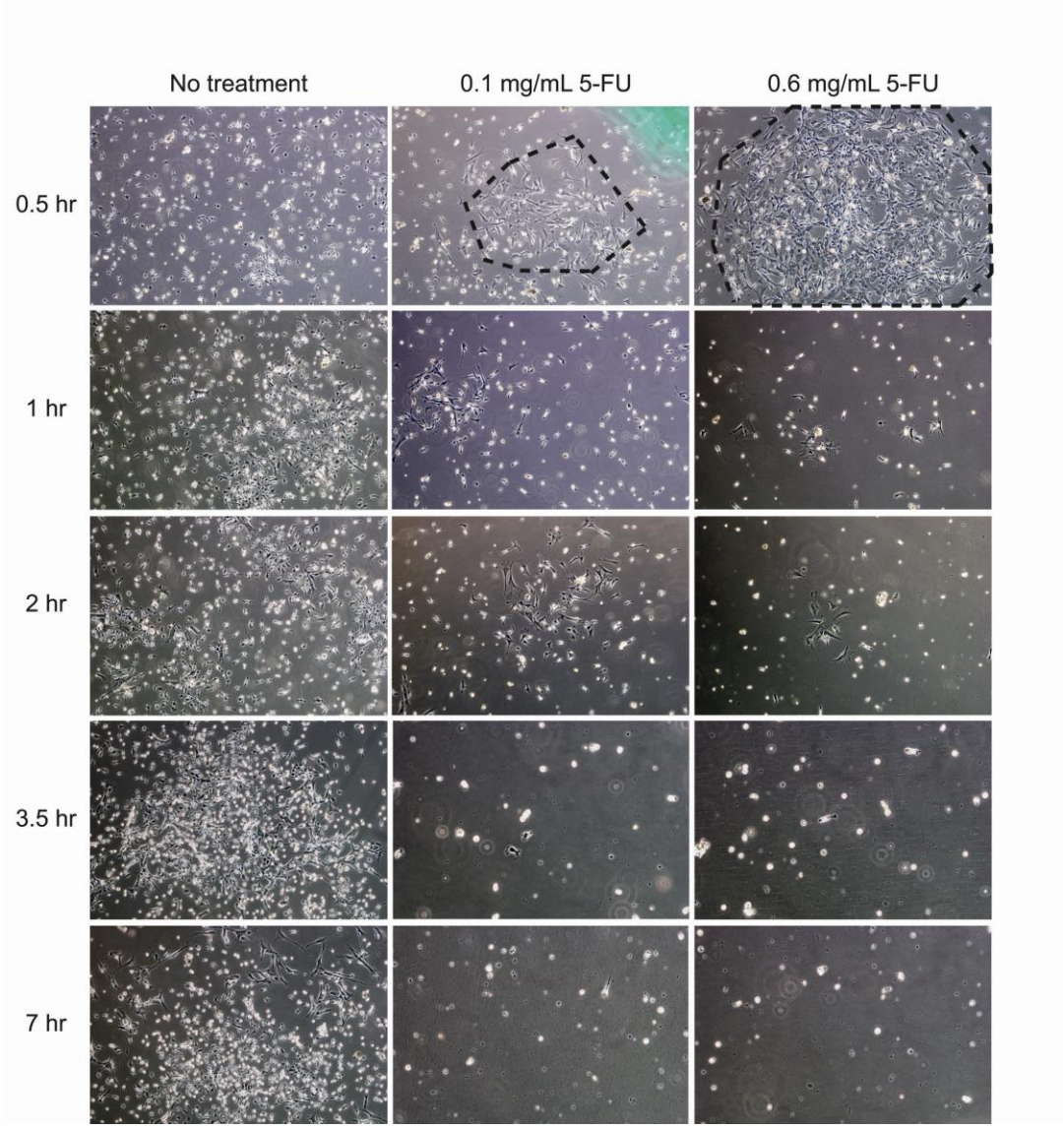


FIGURE 3.7: *In vitro* treatment of BM cells with 5-FU. BM cells from naive C57BL/6 mice (n=5/treatment) were treated with 5-FU at 0.1mg/mL or 0.6mg/mL for 0.5, 1, 2, 3.5 and 7 hours at 37^{o}C. Treated cells were plated at 1 x 10^{5} cells/cm^{2} then incubated at 37^{o}C, 10% CO_2. Colonies of fibroblastic cells (circled with black dotted lines) were observed in BM treated for 0.5 hour with larger colonies observed at 0.6mg/mL 5-FU. No visible colonies were detected in untreated cultures. Representative photomicrographs of 6 day cultures were taken at X10 magnification using Olympus IX71 microscope.

3.5 Discussion

The isolation of mouse MSC from BM has been hampered by problems including the persistence of hematopoietic contaminants and low frequency of MSC. Although several techniques have been developed to address these problems, to date, no single method has been recognized as the gold standard for isolating mouse BM-MSC. In this study, the combination of *in vivo* 5-FU treatment of BM together with immunodepletion using CD11b and CD45 antibodies was shown to permit efficient isolation of MSC from mouse BM.

The frequency of MSC within the BM is extremely low ranging from one to five MSC per million BM cells [217]. Therefore, effective extraction of cells from the BM is crucial in increasing the efficiency of isolating BM-MSC. The conventional method of extracting BM cells involved flushing BM cells out from the medullary cavity of the bone. However, as shown in this study, flushing alone is inefficient, with significant residual BM cells remaining along the endosteal region. Several reports show that crushing and digesting the bones with collagenase can increase the number of BM cells [101, 102]. Using the combination of flushing, and digestion of crushed bones, Xu *et al* reported a 19.4% increase in BM cells compared to the conventional flushing method [102]. In this study, the combination of flushing and scraping produced a higher yield of 33% in harvested BM cells. Most importantly, BM cells from the endosteal region were also found to contain a higher number of CFU-F than those from the medullary cavity, which suggests the presence of a higher number of MSC within endosteal region. Therefore, the method of combining flushing and scraping of BM using the syringe needle can be seen as a more direct and efficient method for harvesting BM and thus MSC than other methods reported to date.

This study confirms that the conventional isolation methodology is inefficient in isolating MSC from mouse strains such as C57Bl/6, BALB/c and NOD. Instead of fibroblastic-like cells, a heterogeneous cell population is obtained after plating the BM cells. As described by Phinney *et al* [92], the adherent cell population of the plated BM cells consist of both $CD11b^{+}CD45^{+}$ hematopoietic cells and $CD11b^{-}CD45^{-}$ fibroblastic-like MSC. Even after several attempts and modifications to culturing conditions, such as media and cell seeding density, MSC within the primary BM cultures of BALB/c and NOD mice failed to propagate. While C57BL/6 MSC were occasionally isolated using the conventional method, outcomes were not reproducible with different batches of BM culture. The low frequency of MSC within the BM culture [92] and persistent hematopoietic contaminants could have explained the unsuccessful isolation of MSC from BALB/c and NOD mice, and also the inconsistency in the isolation of C57BL/6 MSC.

The administration of 5-FU to mice has been reported to eliminate rapidly proliferating cells in the BM and enrich for a quiescent and undifferentiated MSC population [214, 215]. In this study, treating mice with 5-FU for 4 days was shown to greatly reduce the proportion of $CD11b^{+}CD45^{+}$ hematopoietic cells and increase the proportion of $CD11b^{-}CD45^{-}$ cells in the BM population. The increased proportion of $CD11b^{-}CD45^{-}$ cells in 5-FU-treated BM was associated with the formation of distinctive fibroblastic-like colonies not seen in untreated BM culture. This is consistent with several 5-FU studies [214, 215] which demonstrated the presence of a 5-FU-resistant stromal population within the BM. In this study, rather than selecting for specific-cell subsets using single or combination of antibodies [89, 91, 97, 105, 111], the entire MSC population was isolated by CD11b and CD45 antibody staining. This facilitates the isolation of higher numbers of MSC from 5-FU-treated BM and allows for cellular and functional characterization of the effect of 5-FU on the MSC population as a

whole, rather than specific-MSC subsets individually. Significantly more $CD11b^{-}CD45^{-}$ fibroblastic-like cells were isolated using the quicker method of MACS® columns, than the slower FACS method. In addition, other problems such as constant blockage of the FACS machine leading to extensive cell loss render FACS a poor sorting methodology compared to MACS® sorting. Therefore, unlike the conventional isolation method, the combination of *in vivo* 5-FU treatment of BM together with MACS® cell sorting enables the effective isolation of $CD11b^{-}CD45^{-}$ MSC. Importantly, the isolation of MSC by this method is highly reproducible between batches of 5-FU-treated mice and in different mouse strains, such as C57Bl/6, BALB/c and NOD.

The direct administration of 5-FU to patients prior to MSC isolation may pose a hurdle in effective translation of this methodology into the clinic. Therefore, the ability to replicate 5-FU treatment of BM *in vitro* could prove to be useful. Similar to BM culture from 5-FU treated mice, the application of 5-FU directly onto freshly isolated BM cells resulted in formation of distinctive fibroblastic-like colonies. These observations suggested that quiescent MSC within the BM cells were spared from the cytotoxicity of 5-FU. A similar study by Congret *et al* [218] showed that treating expanded mesenchymal progenitor cells with 5-FU resulted in the isolation of a population of quiescent, uncommitted and undifferentiated MPC. However, the *in vitro* methodology used in this study is yet to be optimized for the effective isolation of 5-FU-treated fibroblastic cells. In particular, 5-FU-treated BM could not be expanded in sufficient numbers downstream to allow MACS® column sorting, even in the presence of growth factors such as bFGF and PDGF. These significant obstacles must be overcome before this methodology of *in vitro* treatment can be applied in a therapeutic setting.

3.6 Conclusion

In this study, the isolation of MSC from mouse BM was optimized. The conventional passage-purification method is not effective for isolating mouse MSC. However, the combination of *in vivo* 5-FU treatment together with MACS® cell sorting enables the effective isolation of $CD11b^{-}CD45^{-}$ MSC from multiple mouse strains.

4 CHAPTER 4 – CHARACTERIZATION OF 5-FU-MSC

4.1 Introduction

The widely accepted method for measuring the frequency of MSC relies primarily on detecting colony-forming unit-fibroblast (CFU-F) as it is assumed that each CFU-F is derived from a single MSC [212]. However, conventional methods of detecting CFU-F may not be optimal for the measurement of CFU-F from mouse BM. Different from human and rat BM cells, hematopoietic cells from mouse BM readily adhere to tissue culture plastic and persist even after several days in culture [94]. The adherence of hematopoietic cells and the ability of MSC to support hematopoiesis could potentially interfere with the formation and detection of CFU-F from mouse BM. To date, no studies have reported the characterization of CFU-F from mouse BM cells. Commonly, the criteria for classification of a CFU-F from BM rely on the minimal cell numbers constituting a CFU-F or the size of the colonies [92, 102, 214, 215] without distinguishing the type of colonies being measured. Thus, in a culture of MSC with hematopoietic contaminants, hematopoietic cells mixed with MSC may be counted as a MSC colony. In general, the cells which constitute a CFU-F are not usually characterized. Hence, the discrimination of the 'true' CFU-F from other hematopoietic colonies becomes essential for accurate measurement of the frequency and characterization of CFU-F from mouse BM, so that a detailed and accurate study of these cells becomes possible. For instance, there are differences between CFU-F sizes in human BM cells, where larger CFU-F have been associated with increased potential to differentiate into adipocytes and osteoblasts, and higher proliferation rates [103].

According to the minimal criteria proposed by ISCT, human MSC must firstly be plastic-adherent and secondly, express CD105, CD73, CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD119 and HLA-DR surface molecules. Thirdly, isolated human MSC must have the ability to differentiate into adipocytes, osteoblasts and chondrocytes *in vitro* [204]. In contrast, no such consensus exists for mouse MSC. Mouse MSC isolated by different laboratories vary in characteristics such as antigen expression profile, cellular proliferation, differentiation potential and immunosuppressive properties [95, 97, 98, 101, 111, 192, 219].

Several studies have documented that 5-FU enriches a quiescent and undifferentiated MSC population [214, 215]. Although these studies highlight the usefulness of 5-FU in enriching for undifferentiated BM-MSC, these MSC populations were contaminated by hematopoietic components that had not been segregated from the 5-FU-treated BM cultures. Previous studies did not investigate the effect of 5-FU on CFU-F, nor characterize the MSC from 5-FU-treated BM (5-FU-MSC) [214, 215]. Therefore, the effect of 5-FU on the CFU-F and MSC remained to be determined.

4.2 Aims

The aim of this study was to characterize the CFU-F and $CD11b^{-}CD45^{-}$ MSC isolated from 5-FU-treated C57BL/6 mice prior to their application in EAE to test their therapeutic potential. BM-MSC-derived CFU-F were identified and characterized, and the following attributes of the 5-FU-MSC population were investigated:

1. The effect of 5-FU on the frequency of CFU-F and differentiation potential of CFU-F
2. The differentiation and proliferation potential of 5-FU-MSC
3. Cell surface antigen expression and pluripotent marker expression of 5-FU-MSC
4. The *in vitro* immunosuppressive capacity of 5-FU-MSC

4.3 Materials and Methods

4.3.1 Colony-forming unit assay and criteria for CFU-F sizes

CFU-F assay and criteria for CFU-F sizes were carried out using untreated and 5-FU-treated BM cells as described in section **2.4**.

4.3.2 In vitro differentiation assay of CFU-F and MACS® isolated MSC

Differentiation of CFU-F or MSC into adipocytes, osteoblasts and chondrocytes was carried out as described in section **2.5**. Adipocytes were stained with Oil Red O (section **2.5.1**), osteoblasts with Alizarin Red (section **2.5.2**) and chondrocytes with Alcian Blue (section **2.5.3**) or Safranin O (section **2.5.4**).

4.3.3 In situ immunofluoresence staining of colonies

In situ immunofluoresence staining of CFU-F was carried out as described in section **2.6**.

4.3.4 Flow cytometry

Surface antigen expression profile of MSC was determined by staining with antibodies as described in sections **2.7.2** and **2.7.4**.

4.3.5 RT-PCR analysis for Oct 3/4 and Nanog

Oct 3/4 and Nanog genes were detected from the mRNA of MSC as described in section **2.8**.

4.3.6 MSC suppression assay

MSC suppression of antigen non-specific Con A-stimulated splenocytes and MOG_{35-55}-specific stimulated splenocytes was carried out as described in section **2.14**.

4.4 Results

4.4.1 Identification of CFU-F

To determine the effect of 5-FU on BM-MSC, the CFU-F assay was employed to quantitate MSC. Although each CFU-F is assumed to be derived from a single MSC [212, 220], there was a heterogeneity in the cells that constitute CFU-F. Untreated and 5-FU-treated BM cells formed predominantly two types of CFU-F; Firstly, Fibro-CFU-F comprised of homogenous fibroblastic-like cells resembling the reported MSC phenotype [221] while a second type, named Mixed-CFU-F, comprised a mixture of fibroblastic-, cuboidal- and small spindle-like cells (**Fig. 4.1**). Fibroblastic MSC do not express the hematopoietic markers, CD11b and CD45 [87, 92, 95]. When both types of colonies were stained *in situ* with CD11b and CD45 antibodies, Fibro-CFU-F stained negative for both hematopoietic markers (**Fig. 4.2*A***). Mixed-CFU-F showed mixed staining; fibroblastic-like cells stained negative for both markers, while cuboidal- and spindle-like cells stained positive for both CD11b and CD45 antigens indicating that these are hematopoietic contaminants (**Fig. 4.2*A***).

To confirm the mesenchymal lineage of the fibroblastic-like cells within CFU-F, colonies were differentiated *in situ* into adipocytes and osteoblasts. Most of the fibroblastic-like cells within Fibro-CFU-F differentiated into both lineages while differentiation was limited only to fibroblastic-like cells in Mixed-CFU-F; cuboidal- or spindle-like cells in Mixed-CFU-F did not differentiate (**Fig. 4.2*B***). The chondrogenic potential of these CFU-F at the colony level were examined but differentiation was not observed, probably due to the need for a 3-dimensional pellet for chondrogenesis [85]. To test the chondrogenic potential of Fibro-CFU-F, $CD11b^{-}CD45^{-}$ cells were isolated from untreated and 5-FU-treated BM cultures, and

induced to differentiate in a pellet culture. Positive Alcian blue staining showed that both untreated and 5-FU-treated $CD11b^{-}CD45^{-}$ cells differentiated into chondrocytes efficiently (**Fig. 4.2*C***). Thus, these data clearly showed that the fibroblastic-like cells are MSC and for the rest of these studies only Fibro-CFU-F are considered to be MSC-derived CFU-F.

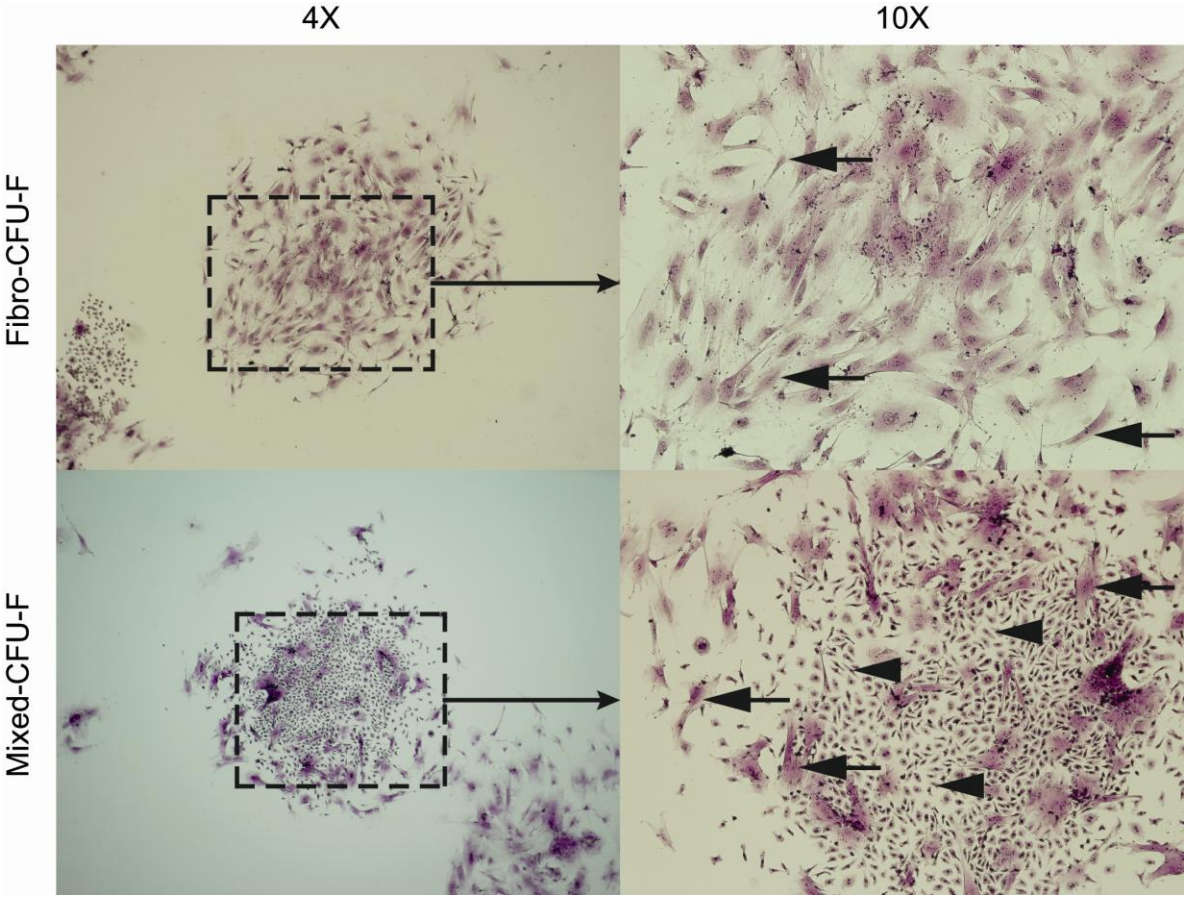
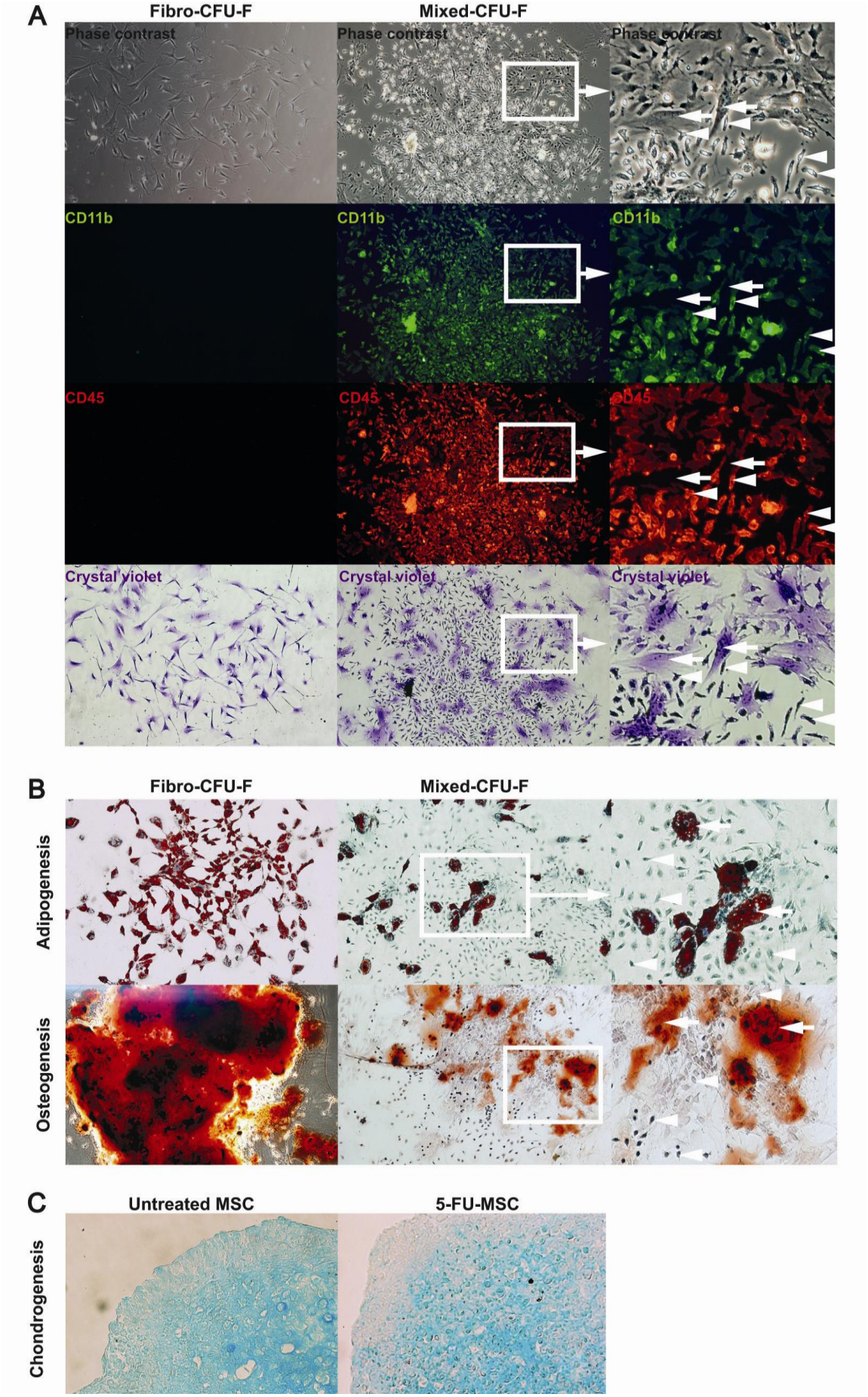


FIGURE 4.1: The two predominant type of colonies in BM culture. BM cells were plated onto 6-well plate and incubated for 20 days prior to fixation and staining with crystal violet. The colony types were identified as Fibro- and Mixed-CFU-F based on different morphology; Fibro-CFU-F contained homogenous fibroblastic-like cells (black arrows) whereas Mixed-CFU-F comprise a mixture of fibroblastic- (black arrows), cuboidal- and small spindle-like cells (black arrow heads). Representative photomicrographs taken at X4 and X10 magnification using Olympus IX71 microscope.

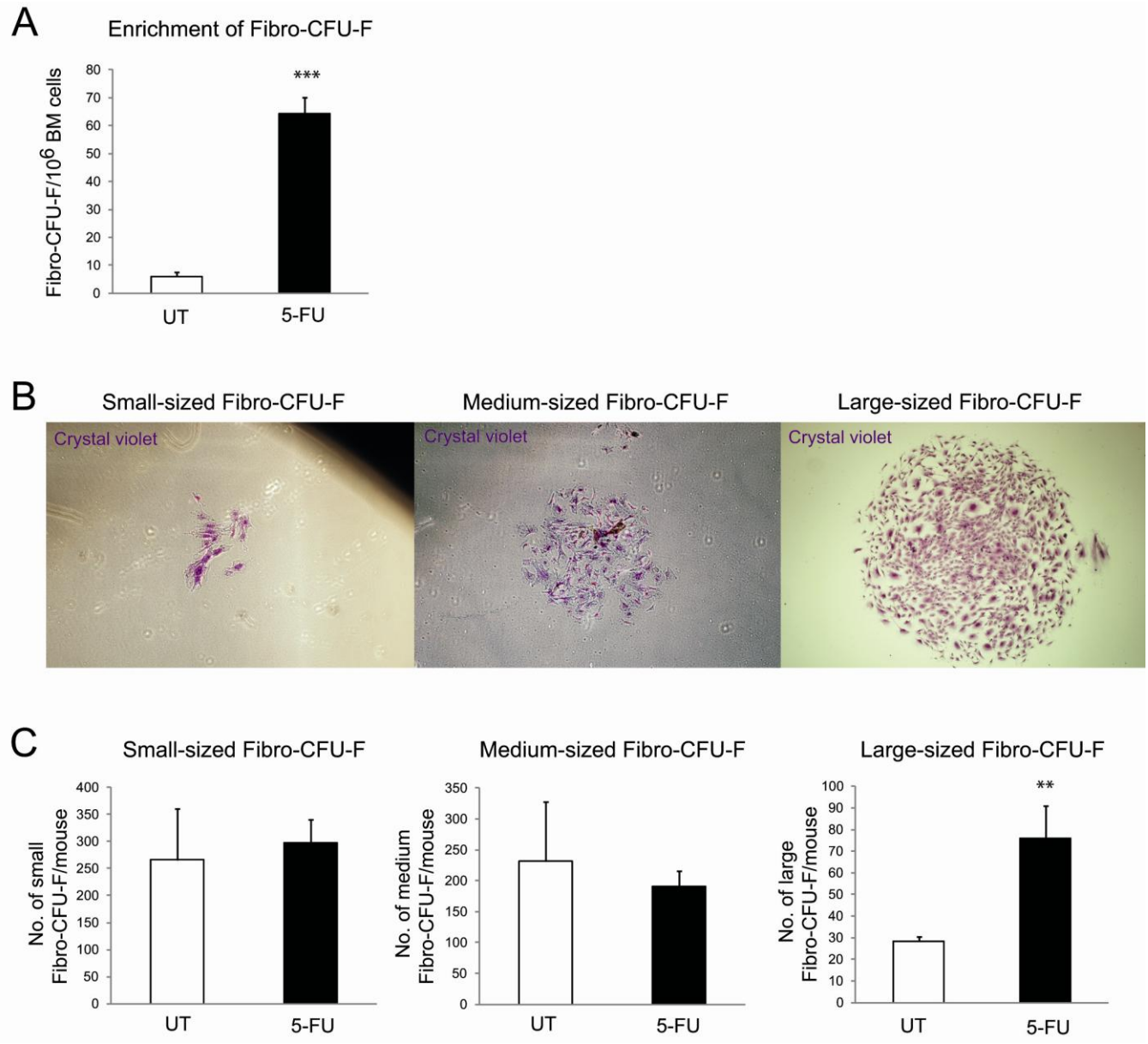
FIGURE 4.2: Identification of MSC-derived CFU-F from BM. (A) Colony types identified as Fibro- and Mixed-CFU-F based on cell morphology were further assessed by phase-contrast microscopy, crystal violet and immunofluorescence staining for CD11b and CD45. Fibro-CFU-F contained homogenous fibroblastic-like cells that do not express CD11b or CD45 antigens. Mixed-CFU-F comprise a mixture of $CD11b^-$ and $CD45^-$ fibroblastic-like cells (white arrows) and $CD11b^+$ and $CD45^+$ hematopoietic cells (white arrow heads). (B) *In situ* differentiation of Fibro- and Mixed-CFU-F into adipocytes (Oil red O) and osteoblasts (Alizarin Red) showing differentiation of Fibro-CFU-F into both lineages. In Mixed-CFU-F, only fibroblastic-like cells (white arrows) differentiated into both lineages; hematopoietic cells (white arrow heads) did not differentiate. (C) Differentiation of $CD11b^-CD45^-$ MSC from untreated and 5-FU-treated mice into chondrocytes. Both untreated and 5-FU-treated $CD11b^-CD45^-$ cells differentiated into chondrocytes which stained positive with Alcian blue. Representative photomicrographs of 3 independent differentiation experiments using 5-FU-treated C57BL/6 BM cells (n=30) taken at X20 magnification using Olympus IX71 fluorescence microscope.


A
Fibro-CFU-F
Mixed-CFU-F
Phase contrast
Phase contrast
Phase contrast
CD11b
CD11b
CD11b
CD45
CD45
CD45
Crystal violet
Crystal violet
Crystal violet
B
Fibro-CFU-F
Mixed-CFU-F
Adipogenesis
Osteogenesis
C
Untreated MSC
5-FU-MSC
Chondrogenesis

4.4.2 5-FU enriched for Fibro-CFU-F and significantly increased the numbers of large-sized (≥ 9mm^2) Fibro-CFU-F

Similar to previous reports [214, 215], there was a significant 11-fold enrichment ($p<0.0001$) for BM Fibro-CFU-F after 5-FU treatment in C57Bl/6 mice (**Fig. 4.3*A***). However, there was no report on the effect of 5-FU on the characteristics of BM-MSC. To determine whether 5-FU affects the numbers of BM-MSC, Fibro-CFU-F were classified according to their sizes (section **2.4**; Chapter 2) and their absolute numbers enumerated. As indicated in **Figure 4.3*B***, the medium- and large-sized Fibro-CFU-F were largely composed of fibroblastic-like cells. In contrast, the small-sized Fibro-CFU-F were composed of broad flattened cells and tended to be less confluent than the larger Fibro-CFU-F (**Fig. 4.3*B***.). Among the three different sized colonies, there was a significant 3-fold increase in large-sized Fibro-CFU-F after 5-FU treatment from 28 ± 2 to 75 ± 15 ($p=0.0037$) (**Fig. 4.3*C***). However, the 5-FU treatment did not have any significant effect on the numbers of small- or medium-sized Fibro-CFU-F (**Fig. 4.3*C***). Similar enrichment for Fibro-CFU-F was also observed in the BALB/c mouse strain after 5-FU treatment (**Fig. 4.4*A***). Interestingly, in BALB/c mice, all three Fibro-CFU-F sizes were significantly increased (small-sized Fibro-CFU-F: 2.5-fold; medium-sized Fibro-CFU-F: 5.5-fold; Large-sized Fibro-CFU-F: 4-fold) after 5-FU treatment (**Fig. 4.4*B***).

FIGURE 4.3: *In vivo* 5-FU treatment enriches for Fibro-CFU-F and increases large-sized (≥ $9mm^2$) Fibro-CFU-F from C57BL/6 mice. Cells from untreated (UT) (n=20) and 5-FU-treated (n=20) BM were plated and numbers and size of Fibro-CFU-F enumerated under phase-contrast microscopy (Magnification, X20) after 20 days culture. (A) Enrichment of Fibro-CFU-F/10^6 BM cells from 5-FU-treated C57BL/6 mice. (B) Morphology of small-, medium- and large-sized Fibro-CFU-F. The small-sized Fibro-CFU-F were composed of broad flattened cells whereas medium- and large-sized Fibro-CFU-F were composed of a majority of fibroblastic-like cells. Representative photomicrographs taken at X4 magnification using Olympus IX71 microscope. (C) Numbers of large-sized (≥ $9mm^2$) Fibro-CFU-F from C57BL/6 mice were significantly increased after *in vivo* 5-FU treatment. No changes to small- (≤$1mm^2$), and medium-sized Fibro-CFU (≥ $4mm^2$ and ≤ $9mm^2$). Data presented are mean ± SEM of 2 independent experiments. (**p<0.01; ***p<0.001, Two-tailed unpaired *T* test).


A
Enrichment of Fibro-CFU-F
Fibro-CFU-F/10^6 BM cells
***
UT
5-FU
B
Small-sized Fibro-CFU-F
Medium-sized Fibro-CFU-F
Large-sized Fibro-CFU-F
Crystal violet
Crystal violet
Crystal violet
C
Small-sized Fibro-CFU-F
Medium-sized Fibro-CFU-F
Large-sized Fibro-CFU-F
No. of small Fibro-CFU-F/mouse
No. of medium Fibro-CFU-F/mouse
No. of large Fibro-CFU-F/mouse
**
UT
5-FU
UT
5-FU
UT
5-FU

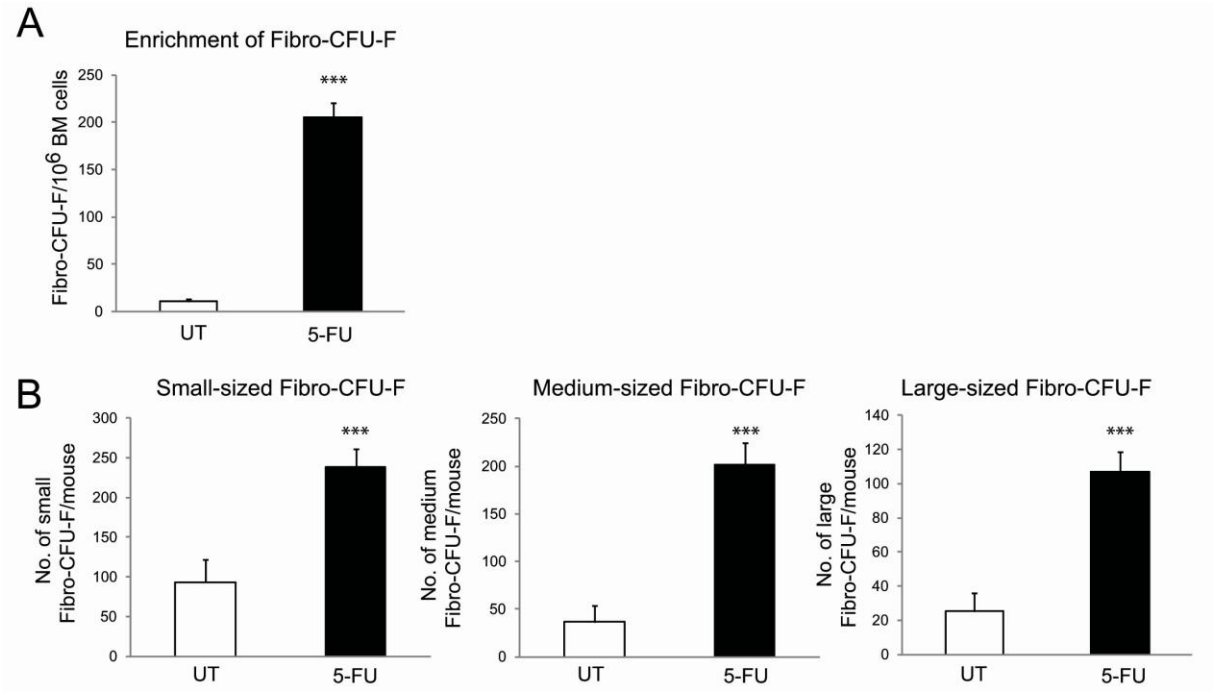


FIGURE 4.4: *In vivo* 5-FU treatment enriches for Fibro-CFU-F and increases large-sized (≥ 9mm^2) Fibro-CFU-F from BALB/c mice. Cells from untreated (UT) (n=20) and 5-FU-treated (n=20) BM were plated and numbers and size of Fibro-CFU-F enumerated under phase-contrast microscopy (Magnification, X20) after 20 days culture. (A) Enrichment of Fibro-CFU-F/10^6 BM cells from 5-FU-treated BALB/c mice. (B) Numbers of small- (≤1mm^2), medium- (≥ 4mm^2 and ≤ 9mm^2) and large-sized (≥ 9mm^2) Fibro-CFU-F from BALB/c mice were significantly increased after *in vivo* 5-FU treatment. Data presented are mean ± SEM of 2 independent experiments. (***p<0.001, Two-tailed unpaired *T* test).

4.4.3 Large-sized Fibro-CFU-F from 5-FU-treated BM have high differentiation potential

The ability of different sizes of Fibro-CFU-F from untreated and 5-FU-treated BM to differentiate into adipocytes and osteoblasts was compared. An association between differentiation capacity and Fibro-CFU-F size from both untreated and 5-FU-treated BM showed that in general, as Fibro-CFU-F size increased, the number of Fibro-CFU-F capable of differentiating into adipocytes and osteoblasts increased (**Fig. 4.5*A*** and ***B***). Even though it appears that the differentiation potentials of CFU-F into adipocytes and osteoblasts are similar across the three colony sizes from both untreated and 5-FU-treated BM, a closer examination revealed that a higher percentage of differentiated cells were detected within Fibro-CFU-F after 5-FU treatment (**Fig. 4.6**). In 5-FU-treated BM, the majority of the large-sized Fibro-CFU-F contained at least 70% of differentiated cells within the CFU-F (**Fig. 4.6*A*** and ***B***). In contrast, none of the untreated large-sized Fibro-CFU-F contained more than 70% of cells capable of adipogenesis and osteogenesis (**Fig. 4.6*A*** and ***B***). Thus, these data clearly show that 5-FU treatment increased the numbers of large-sized Fibro-CFU-F with enhanced differentiation potential.

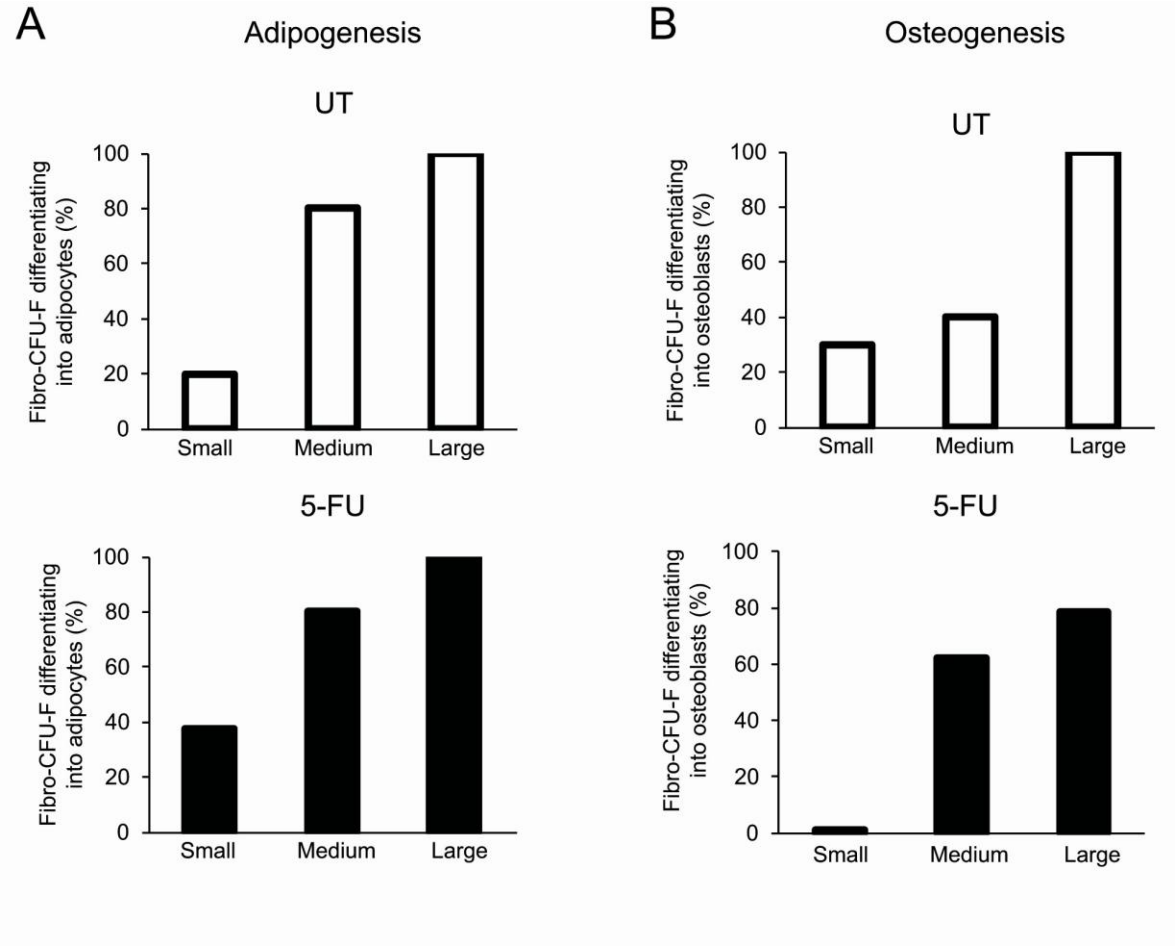


FIGURE 4.5: The differentiation potential of Fibro-CFU-F into adipocytes (A) and osteoblasts (B) increases as colony size increases. Cells from untreated (UT) (n=20) and 5-FU-treated (n=20) BM of C57Bl/6 were plated and induce to differentiate into adipocytes and osteoblasts after 20 days culture. The differentiation potential expressed as the percentage of small, medium or large Fibro-CFU-F differentiating into adipocytes or osteoblasts. 10 – 50 Fibro-CFU-F were enumerated. Positively differentiated CFU-F contained at least 1 differentiated cell.

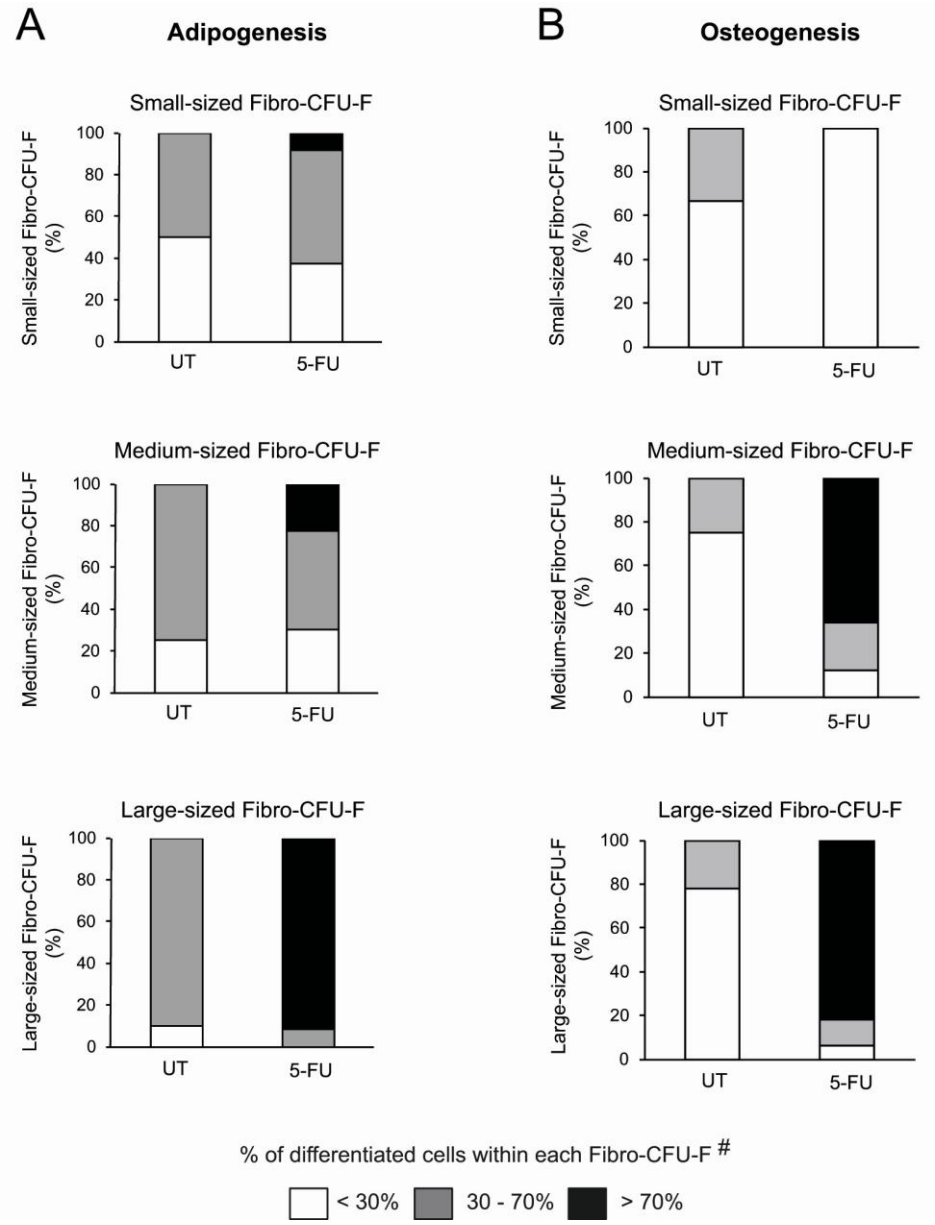


FIGURE 4.6: *In vivo* 5-FU treatment enhanced differentiation capacity of Fibro-CFU-F. Cells from untreated (UT) (n=20) and 5-FU-treated (n=20) BM of C57Bl/6 were plated and induce to differentiate into adipocytes and osteoblasts after 20 days culture. A high percentage of large-sized Fibro-CFU-F from 5-FU-treated BM contained more than 70% of cells capable of adipogenesis (A) and osteogenesis (B). None of the untreated large-sized Fibro-CFU-F contained more than 70% of cells capable of adipogenesis and osteogenesis; 10 – 50 Fibro-CFU-F were enumerated. Representative data of 2 independent experiments. # < 30%: Less than 30% of cells differentiated within each Fibro-CFU-F; 30 – 70%: 30 to 70% of cells differentiated within each Fibro-CFU-F; > 70%: More than 70% of cells differentiated within each Fibro-CFU-F.

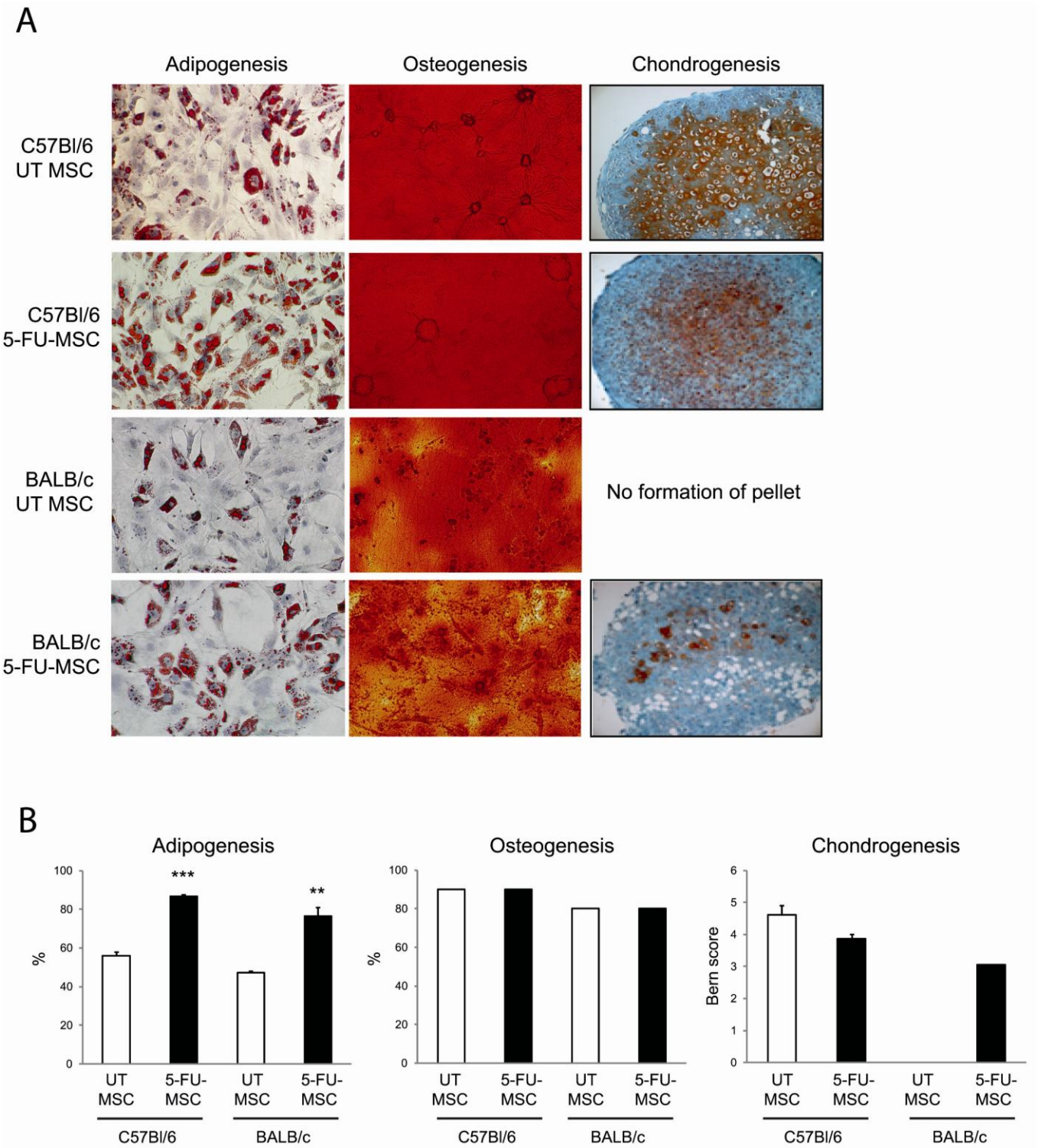
4.4.4 Differentiation potential of untreated and 5-FU-MSC

The ability to differentiate into adipocytes, osteoblasts and chondrocytes is an important criterion that marks the identity of MSC [137]. To determine the tri-lineage potential of the MSC isolated by the improved isolation methodology (Chapter 3), MACS®-sorted $CD11b^-$ $CD45^-$ MSC were plated onto tissue culture plastic and induced to differentiate into adipocytes and osteoblasts upon reaching 70% confluence. For chondrogenesis, MACS®-sorted $CD11b^-CD45^-$ MSC were differentiated in a pellet culture and stained with Safranin O instead of Alcian blue (section **4.4.1**) to measure the extent of chondrocyte differentiation.

The improved isolation methodology developed in this study was shown to isolate tri-lineage MSC with increased adipogenic potential from both C57Bl/6 and BALB/c mice. Both untreated and 5-FU-MSC from C57Bl/6 mice have tri-linage potential, with similar capacity to differentiate into osteoblasts and chondrocytes (**Fig. 4.7*A*** *and* ***B***). However, 5-FU-MSC have a higher capacity for adipogenesis (**Fig. 4.7*A*)**, as indicated by a higher percentage of differentiated cells stained with Oil Red O compared to untreated MSC (5-FU-MSC: 87±1% vs. untreated MSC: 56±2%, $p<0.001$) (**Fig. 4.7*B***).

In contrast, only 5-FU-MSC from BALB/c mice displayed tri-lineage differentiation capacity whereas untreated BALB/c MSC differentiated only into adipocytes and osteoblasts (**Fig. 4.7*A***). Chondrogenesis could not be achieved as untreated BALB/c MSC failed to form stable pellets. As seen in C57Bl/6 mice, both untreated and 5-FU-MSC from BALB/c mice have similar osteogenic capacity with 5-FU-MSC having a higher capacity to differentiate into adipocytes compared to untreated MSC (5-FU-MSC: 77±5% vs. untreated MSC: 47±1%, $p<0.01$) (**Fig. 4.7*B*)**.

FIGURE 4.7: The differentiation potential of untreated (UT) (n=30) and 5-FU-treated MSC (n=30) from C57Bl/6 and BALB/c into adipocytes, osteoblasts and chondrocytes respectively. (A) Representative photomicrographs of the differentiated MSC stained with Oil Red O to detect lipid accumulation (adipogenesis), Alizarin Red for calcium deposit (osteogenesis) and Safranin O for proteoglycan (chondrogenesis). MACS-sorted $CD11b^-$ $CD45^-$ MSC isolated from 10 days BM culture were plated onto tissue culture plastic or as pellet culture prior induced to differentiate into adipocytes, osteoblasts and chondrocytes for a maximum of 21 days. Representative photomicrographs of 2 independent differentiation experiments taken at X20 magnification using Olympus IX71 microscope. (B) The percentage of adipocytes was assayed by examining at least 50 – 100 cells in multiple fields. The percentage of mineralization in osteogenesis was assayed by examining at least 10 fields for the area of mineralization as a percent of the total confluent culture. For chondrogenesis, the amount of accumulated proteoglycan was quantified by Bern Score. In both mouse strains, UT and 5-FU-MSC have similar capacity to differentiate into osteoblasts with a higher percentage of 5-FU-MSC differentiated into adipocytes compared to untreated MSC. For C57Bl/6 mice, UT and 5-FU-MSC have similar capacity to differentiate into chondrocytes. In BALB/c mice, only 5-FU-MSC differentiated into chondrocytes with UT MSC failing to form a cell pellet for differentiation. Data presented are mean ± SEM of 2 independent experiments. (**$p<0.01$; ***$p<0.001$; Two-tailed unpaired *T* test).


A
Adipogenesis
Osteogenesis
Chondrogenesis
C57Bl/6
UT MSC
C57Bl/6
5-FU-MSC
BALB/c
UT MSC
No formation of pellet
BALB/c
5-FU-MSC
B
Adipogenesis
Osteogenesis
Chondrogenesis
%
Bern score
***
**
UT MSC
5-FU-MSC
C57Bl/6
BALB/c

4.4.5 Increased proliferation potential of 5-FU-MSC

The increased numbers of large-sized Fibro-CFU-F suggested the presence of highly proliferative MSC after 5-FU treatment. To determine if the formation of Fibro-CFU-F in 5-FU-treated BM is due to high proliferative capacity of 5-FU-MSC, 5 x 10^6 of BM cells from untreated or 5-FU-treated C57Bl/6 mice were seeded onto tissue culture flasks and the number of $CD11b^-CD45^-$ cells at day 4 or day 10 culture was quantified. In 5-FU-treated BM culture, there was a 4-fold increase in the numbers of $CD11b^-CD45^-$ cell population from ~2.0 x 10^5 on day 4 to ~7.5 x 10^5 MSC on day 10 (**Fig. 4.8*A***). In contrast, the number of $CD11b^-CD45^-$ cells in untreated BM culture remained unchanged (**Fig. 4.8*A***). This is in agreement with the observation described in section **3.4.4.1** (Chapter 3) whereby distinctive fibroblastic-like colonies were detected in 5-FU-treated BM but not in untreated BM culture (**Fig. 3.5*C***).

Consistently, the increased proliferation of 5-FU-MSC was also observed in the homogenous population of MACS®-sorted $CD11b^-CD45^-$ MSC. $CD11b^-CD45^-$ MSC (1 x 10^4) from both untreated and 5-FU-treated BM culture were seeded onto tissue culture flasks and the numbers of proliferating cells enumerated daily for a period of 8 days. The 5-FU-MSC has a higher doubling rate of 2.4 hour with plated cells proliferating from 1 x 10^4 cells on day 0 to 4.9 x 10^4 cells on day 6 (**Fig. 4.8*B***). In contrast, untreated MSC has a longer doubling time of 6.1 hour with their numbers increasing from 1 x 10^4 cells on day 0 to 1.7 x 10^4 cells on day 6 (**Fig. 4.8*B***). Taken together, *in vivo* 5-FU treatment of BM resulted in the isolation of a population of MSC with high proliferative potential.

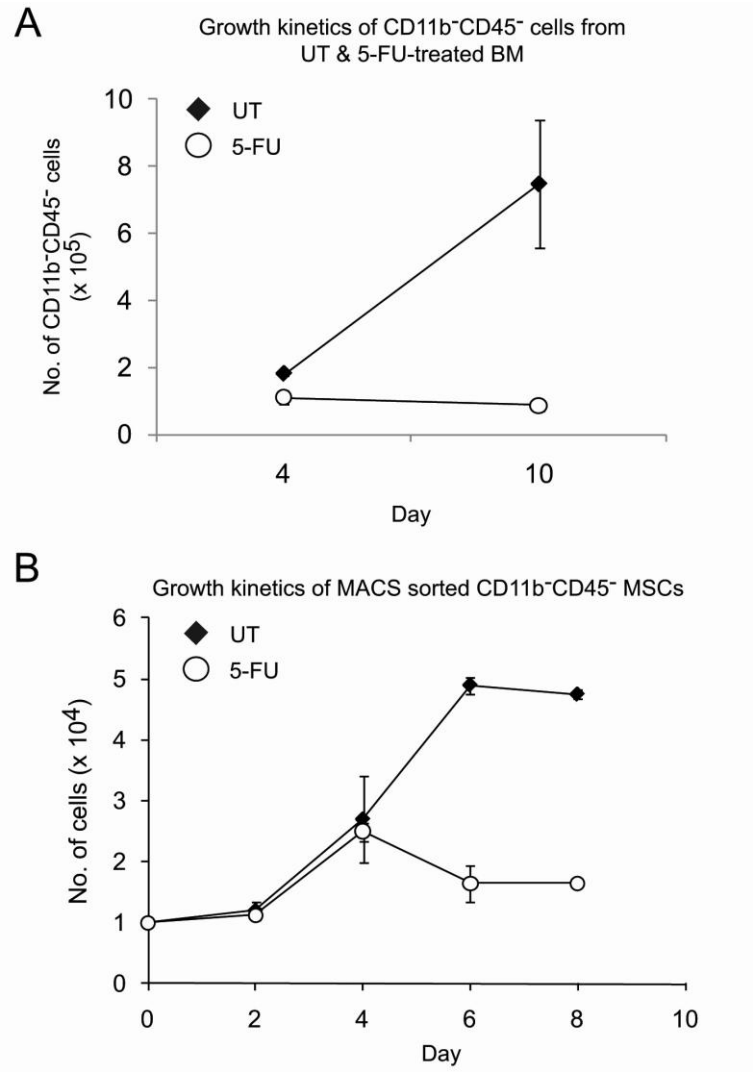


FIGURE 4.8: 5-FU-MSC have increased differentiation potential compared to untreated (UT) MSC. (A) The growth kinetics of $CD11b^{-}CD45^{-}$ cells from untreated (UT) (n=6) and 5-FU-treated BM (n=6) of C57Bl/6. 5 x 10^6 of BM cells from untreated or 5-FU-treated mice were plated and the number of $CD11b^{-}CD45^{-}$ cells at day 4 or day 10 culture determined. There was a 4-fold increase in the numbers of $CD11b^{-}CD45^{-}$ cell population in 5-FU-treated BM culture compared to no change in untreated BM culture. (B) The growth kinetics of MACS-sorted $CD11b^{-}CD45^{-}$ MSC from UT (n=6) and 5-FU-treated BM (n=6) culture of C57Bl/6. 1 x 10^4 of $CD11b^{-}CD45^{-}$ MSC from untreated and 5-FU-treated BM cultures were seeded onto tissue culture flasks and the cell numbers enumerated daily for a period of 8 days. The 5-FU-MSC has a faster (actually lower) doubling rate of 2.4 hour compared to 6.1 hour for UT-MSC.

4.4.6 5-FU-MSC and untreated MSC share similar cell surface antigens and pluripotent marker expression

To further characterize the MSC populations, $CD11b^{-}CD45^{-}$ MSC were isolated from primary BM cultures of untreated and 5-FU-treated C57Bl/6 mice. Flow cytometry characterization of cell surface markers showed that the expression levels of CD49d increased after 5-FU-treatment (5-FU-MSC: 10% vs. untreated MSC: 2%) (**Table 4.1**). Expression levels of CD29, CD44, CD54, CD73, CD81, CD90, CD105, CD106, CD140b, CD166, CD271 and SSEA-4 were similar in both untreated and 5-FU-MSC (**Table 4.1**). In addition, both untreated and 5-FU-MSC expressed Nanog but not Oct 3/4 (**Fig. 4.9**).

TABLE 4.1: Characterization of cell surface markers on untreated (UT) MSC and 5-FU-MSC from C57Bl/6

Marker(s)	UT MSC	5-FU-MSC
CD29	+++	+++
CD44	++	++
CD49d	-	+
CD54	++	++
CD73	+	+
CD81	++	++
CD90	-	-
CD105	+	+
CD106	++	++
CD140b	++	++
CD166	-	-
CD271	-	-
SSEA-4	-	-

Percentage of cells expressing these markers were scored as '–' if less than 5% of the cells were positive, '+' if more than 5% but less than 20% were positive, '++' if more than 20% but less than 75% were positive, and '+++' if more than 75% of the cells were positive.

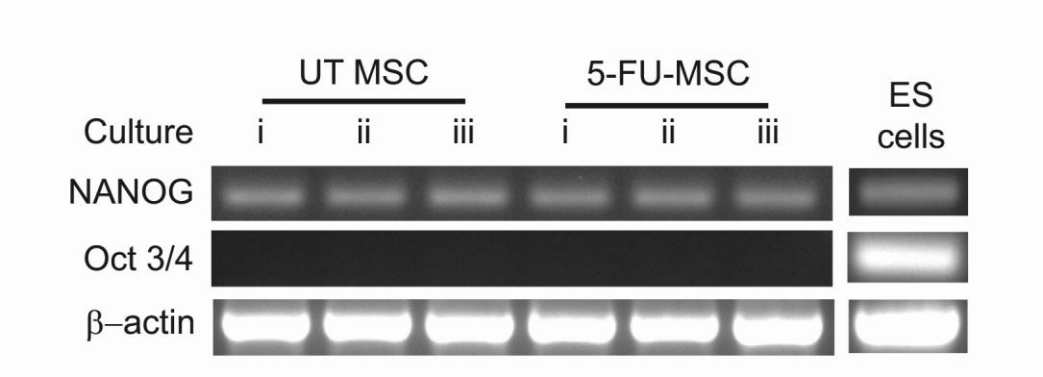


FIGURE 4.9: RT-PCR analysis of Nanog and Oct 3/4 gene expression in untreated (UT) MSC and 5-FU-MSC from C57Bl/6. UT and 5-FU-MSC expressed Nanog but not Oct 3/4. Three independent cultures of UT and 5-FU-MSC were used. ES cells shown as positive control and β-actin gene expressions were used as an internal control.

4.4.7 5-FU-MSC are potently immunosuppressive

The ability of MSC to immunosuppress makes them a potential candidate for application in immune-related diseases. An *in vitro* co-culture assay with Con A-stimulated splenocytes was used to assess the capacity of the MSC to suppress antigen non-specific Con A-stimulated splenocyte proliferation. Both untreated MSC and 5-FU-MSC suppressed Con A-stimulated splenocyte proliferation in a dose-dependent manner (**Fig. 4.10*A***). However, 5-FU-MSC had significantly higher immunosuppressive capacity than untreated MSC, as 5-FU-MSC induced significantly higher levels of suppression up to 1:320 MSC:splenocyte ratios compared to untreated MSC (**Fig. 4.10*A***). In addition, a higher capacity to immunosuppress antigen-specific MOG_{35-55}-stimulated splenocytes was demonstrated by 5-FU-MSC compared to untreated MSC (**Fig. 4.10*B***).

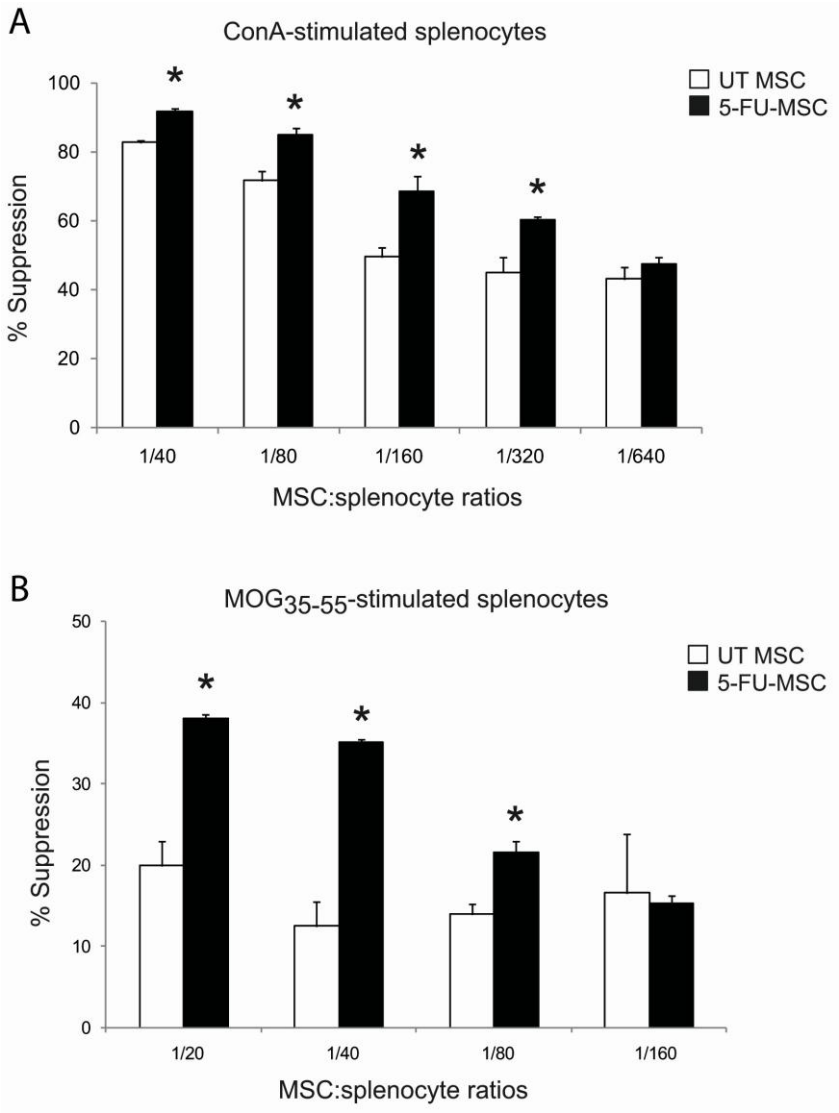


FIGURE 4.10: 5-FU-MSC demonstrate potent immunosuppression *in vitro*. (A) Con A-stimulated splenocytes were co-cultured with untreated (UT) MSC (n=15) or 5-FU-MSC (n=15) from C57BL/6 at different ratios to determine their capacity to suppress antigen non-specific splenocyte proliferation. UT and 5-FU-MSC showed dose-dependent suppression of Con A-stimulated splenocyte proliferation; 5-FU-MSC have significantly higher immunosuppression up to 1:320 MSC:splenocyte ratio. (B) MOG-stimulated splenocytes were co-cultured with UT MSC (n=15) or 5-FU-MSC (n=15) from C57BL/6 at different ratios to determine their capacity to suppress antigen-specific splenocyte proliferation. 5-FU-MSC showed significantly higher capacity to suppress MOG-stimulated splenocyte proliferation compared to UT MSC. Splenocyte proliferation was quantified by [3H] Thymidine incorporation and expressed as percentage of suppression by MSC. Data presented are mean ± SEM of 3 independent experiments. (* $p<0.05$, Two-tailed unpaired *T* test).

4.5 Discussion

The detection of MSC from BM relies primarily on the measurement of CFU-F as it is assumed that each CFU-F is derived from a single MSC [212]. However, the characterization of MSC-derived CFU-F has been confounded by the presence of contaminating hematopoietic elements and the effect of 5-FU on MSC populations free of contaminating hematopoietic elements has not previously been elucidated. In this study, prospective MSC-derived CFU-F termed Fibro-CFU-F from the BM were identified by the absence of cells expressing the hematopoietic markers CD11b and CD45 and by their ability to differentiate into adipocytes and osteoblasts. By excluding Mixed-CFU-F (CFU-F containing hematopoietic contaminants) and considering Fibro-CFU-F alone as MSC-derived, 5-FU treatment was shown to significantly enrich for Fibro-CFU-F and increased the numbers of large-sized Fibro-CFU-F. These effects of 5-FU on Fibro-CFU-F may vary among mouse strains as a more pronounced effect was observed in BALB/c mice, with significant higher 20-fold enrichment of Fibro-CFU-F and a significant increase of all three Fibro-CFU-F sizes after 5-FU treatment, than in C57Bl/6 mice. These effects in BALB/c may be due to the administration of a higher dose of 5-FU (300mg/kg) that could potentially eliminate more hematopoietic cells and enriched for more Fibro-CFU-F. For instance, Falla *et al* [215] reported a lower 12-fold enrichment of CFU-F in BALB/c mice treated with a lower 5-FU dose (150mg/kg). Collectively, our data suggest that the effect of 5-FU on Fibro-CFU-F may be more pronounced in some mouse strains than others, such as BALB/c compared to C57BL/6 mice.

Previous finding in human BM-derived MSC has shown that larger-sized CFU-F have a higher potential for differentiation into adipocytes and osteoblasts compared to smaller CFU-

F [103]. However, in these studies, the differentiation capacity of the CFU-F was determined from expanded cultures of CFU-F rather than directly differentiating the CFU-F *in situ* into adipocytes or osteoblasts [103]. The expansion of CFU-F prior to differentiation will potentially introduce passage-induced changes to the differentiation potential of CFU-F thus undermining the findings reported. In addition, the comparison of the differentiation capacity between the different sized CFU-F was also not reported [103]. In contrast, a more accurate assay involving the *in situ* differentiation of CFU-F into adipocytes or osteoblasts was carried out in this study to assess the differentiation capacity of the different-sized Fibro-CFU-F. Larger-sized Fibro-CFU-F were associated with higher differentiation potential with a higher percentage of cells undergoing differentiation after 5-FU treatment. In particular, 5-FU treatment increased the numbers of large-sized ($\geq$ 9mm^2) Fibro-CFU-F with enhanced differentiation potential. Therefore, *in vivo* 5-FU treatment of BM increases the numbers of large-sized Fibro-CFU-F with enhanced differentiation efficiency, consistent with the isolation of a quiescent and undifferentiated 5-FU-resistant MSC population as suggested [214].

Previously, Wang *et al* [214] reported that the proliferation rate of BM-MSC remained unchanged after 5-FU treatment. However, their study used unfractionated BM cells, which contained other hematopoietic contaminants. In this study, the separation of non-hematopoietic CD11b$^-$CD45$^-$ MSC from unfractionated BM and MACS®-sorted MSC culture, revealed a significantly enhanced proliferative capacity of 5-FU-MSC. The proliferative capacity of 5-FU-MSC is significantly higher (20-fold) compared to untreated MSC isolated by a similar immunodepletion of cultured BM cells [102]. These data indicate that MSC do not just remain in their quiescent state upon 5-FU treatment, but instead show evidence of cellular response by up-regulating their proliferation rate.

The difficulty of inducing mouse MSC, especially those from C57Bl/6 and BALB/c strains, to differentiate into chondrocytes is well-documented [85, 95, 101]. As highlighted by Peister *et al* [95], passage-purified C57BL/6 and BALB/c MSC showed little or no evidence of proteoglycan secretion. In addition, Anjos-Afonso *et al* [85] reported the inability of C57BL/6 MSC to form stable pellets for chondrogenesis. Strikingly, C57Bl/6 and BALB/c MSC isolated by the newly improved method in this study, were able to form cell pellets and differentiated efficiently into chondrocytes. As serial passaging of MSC is associated with the loss of multipotency [103], the usage of multiple-passaged MSC by Peister *et al* [95] (passage 7) and Anjos-Afonso *et al* [85] (passage 3) could have accounted for the low chondrogenic potential observed. In contrast, MSC used in this study were freshly isolated from 10 day BM cultures. Besides having chondrogenic potential, 5-FU-MSC also have a higher adipogenic potential compared to untreated MSC. The capacity to isolate a population of tri-lineage $CD11b^{-}CD45^{-}$ MSC with high proliferative potential from different mouse strains indicates that the isolation methodology developed in this study is more efficient compared to other methods reported to date.

Beside higher differentiation potential, 5-FU-MSC also express a higher level of CD49d (α4 integrin) compared to untreated MSC. CD49d is an important adhesion molecule playing a key role in the pathogenesis of autoimmune disease such as EAE [222]. The transcription factors, Nanog and Oct 3/4 regulate pluripotency in ES cells, however Nanog alone, but not Oct 3/4, is expressed in both untreated MSC and 5-FU-MSC. This is in agreement with two recent studies reporting that MSC express Nanog but not Oct 3/4 [223]. The expression of Nanog in MSC was suggested by Pierantozzi *et al* [224] to be associated with the transition of MSC from *in vivo* quiescence to adaptation to *in vitro* culturing. Therefore, untreated and 5-FU-MSC share similar pluripotent marker expression and antigen expression profiles.

5-FU-MSC had significantly enhanced immunosuppressive capacity *in vitro* compared to untreated MSC. This was demonstrated by the potent 60% suppression of Con A-stimulated splenocyte proliferation at a MSC:splenocyte ratio of 1:320. This immunosuppression is much more potent than the previously reported 30% suppression of Con A-stimulated splenocyte proliferation at a MSC:splenocyte ratio of 1:160 [125] or 1:200 [101]. In a more autoimmune disease relevant setting, where autoantigens play an important role in activating the immune system and subsequent disease manifestation, the enhanced immunosuppressive capacity of 5-FU-MSC in suppressing proliferation of MOG_{35-55}-specific compared to untreated MSC could suggest a better therapeutic outcome when administered to EAE, which will be examined in the next chapter.

4.6 Conclusion

In this study, $CD11b^{-}CD45^{-}$ BM-MSC isolated from 5-FU-treated C57BL/6 mice were characterized. Prospective MSC-derived CFU-F termed Fibro-CFU-F from BM were identified by the absence of cells expressing the hematopoietic markers CD11b and CD45 and by their ability to differentiate into adipocytes, osteoblasts and chondrocytes. The *in vivo* 5-FU treatment of BM significantly enriched for Fibro-CFU-F and in particular the large-sized Fibro-CFU-F. The proliferation potential and immunosuppression capacity of 5-FU-MSC was significantly increased compared to untreated MSC. In addition, 5-FU-MSC have increased tri-lineage differentiation potential, especially for adipogenesis, compared to untreated MSC. Other characteristics such as cell surface antigen and pluripotent marker expression level were found to be similar between untreated and 5-FU-MSC.

5 CHAPTER 5 – 5-FU-MSC IN EAE

5.1 Introduction

MSC are immunosuppressive [69, 149]. *In vitro*, MSC inhibit proliferation of cells stimulated by mitogens and responder cells in mixed lymphocyte reactions [108, 155, 159]. MSC have been shown to affect both innate and adaptive immunity. In adaptive immunity, MSC have the capacity to influence T cells, Tregs and B cells [106, 108, 149, 155]. This inhibition is considered to be mediated by division arrest of activated T cells at the G_1 phase of the cell cycle and is associated with down-regulation of cyclin D2 expression [155]. MSC have also been reported to influence other sub-populations of T cell including down-regulating $CD8^+$ cytotoxic T cells-mediated cytotoxicity and increasing the proportion of Tregs when MSC were co-cultured with stimulated T cells [167]. MSC also inhibit B cell proliferation, down-regulate B cell expression of CXCR4, CXCR5 and CXCR7 chemokine receptors and inhibit B cell maturation and antibody secretion [171].

The influence of MSC in immunity has instigated many studies aiming to elicit the mechanism(s) by which MSC exert their immunomodulatory function. To date, the underlying mechanism(s) of MSC-mediated immunosuppression is still not fully understood. Experimental data have shown that secretion of soluble factors by MSC such as IL-1ra [157], IL-6 [158], IL-10 [159], TGF-β1 [108], IDO [106], HGF [108], PGE_2 [107], NO [160], soluble HLA-G5 [161], HO-1 [225] and galactin [162, 163] play an important role in mediating these effects.

The ability of MSC to immunomodulate various immune cells make them a potential candidate for cell-based therapies to treat immunologically-related diseases such as autoimmune diseases [79]. The therapeutic potential of MSC in autoimmune disease particularly MS, has seen the formation of the 'International MSC Transplantation study group' aiming to develop a consensus on the therapeutic application of MSC [226]. The interest in using MSC to treat MS was initiated by pre-clinical studies demonstrating the efficacy of MSC in ameliorating disease severity and CNS demyelination in EAE [125, 174, 192]. In a MOG_{35-55}-non-remitting C57Bl/6 EAE model, early intervention by i.v injection of MSC before [125, 192] or after [192, 194] disease onset significantly reduced clinical score, inflammatory infiltration and CNS demyelination. However, MSC do not exhibit any beneficial effects after disease stabilization in EAE [192]. In the remitting-relapsing SJL EAE model, MSC significantly reduced the relapse rate of diseased mice by approximately 60% with associated reduction in clinical score, number of inflammatory infiltrates and degree of demyelination [174, 195]. However, to date, no studies have reported a complete remission of EAE by MSC [125, 174, 192].

Several mechanisms for disease amelioration have been proposed including the induction of T cell anergy [192], a reduction in pathogenic B cell responses [174] and paracrine conversion of MSC-derived CCL2 from agonist to antagonist of CD4 Th17 function [194]. The expression of neuronal markers by MSC found in the CNS of MSC-treated EAE mice has also suggested possible transdifferentiation of MSC for neuronal repair or replacement [125, 195]. However, the proposition of neuronal repair or replacement by transdifferentiated MSC remains debatable, as other research groups have found little evidence that MSC transdifferentiated in the CNS of MSC-treated EAE mice [174, 176, 194, 198]. Additionally, there is also a lack of *in vitro* evidence to demonstrate a functional neural phenotype in terms

of excitability and reaction to neurotransmitter stimuli of transdifferentiated MSC [146]. Nonetheless, to date, the therapeutic mechanism(s) of MSC in EAE are primarily recognized to be via immunomodulatory pathways rather than direct neural replacement [226].

5.2 Aims

The aim of this study was to assess the immunosuppressive potential of $CD11b^{-}CD45^{-}$ BM-MSC isolated from 5-FU-treated C57BL/6 mice in a mouse model of MS. This was achieved by investigating the following to determine the therapeutic effectiveness of 5-FU-MSC in EAE:

1. The therapeutic effect of untreated MSC and 5-FU-MSC in EAE
2. The degree of inflammatory infiltrates and demyelination of untreated MSC- and 5-FU-MSC-treated mice
3. The changes to the immune responses of untreated MSC- and 5-FU-MSC-treated mice
4. Th1-, Th2 and Th17-associated cytokine levels in MOG-stimulated splenocytes from untreated MSC- and 5-FU-MSC-treated mice
5. The involvement of known immunosuppressive factors in untreated MSC- and 5-FU-MSC-mediated immunosuppression

5.3 Materials and Methods

5.3.1 Animals

8- to 12-week-old female C57BL/6 mice were used for MSC isolation and EAE as described in section **2.2**.

5.3.2 Isolation of MSC from untreated and 5-FU-treated BM

$CD45^{-}CD11b^{-}$ MSC from untreated and 5-FU-treated mice were isolated as described in section **2.3**.

5.3.3 EAE induction, clinical evaluation and MSC treatment

Mice were induced to develop EAE and monitored daily with neurological impairment scored on an arbitrary clinical score as described in section **2.9**. To test the immunomodulatory effect of MSC, mice were injected i.v with 1 x 10^6 MSC, 6 days after immunization as described in section **2.10**.

5.3.4 MOG-stimulated splenocyte proliferation assay and detection of sera MOG-specific antibodies

The proliferative response of splenocytes to MOG_{35-55} peptide *in vitro* and sera MOG-specific antibodies in disease mice were determined as described in section **2.12** and **2.13** respectively.

5.3.5 Histology

Brain and spinal cord were removed, fixed in 10% formalin, embedded in paraffin, and stained with H&E to assess inflammation and LFB to assess demyelination. Sections were scored for inflammation and demyelination as described in section **2.11**.

5.3.6 MSC suppression assay, blocking assay and immunoregulatory molecules detection

MSC suppression of Con A-stimulated splenocyte proliferation, blocking suppression assay with blocking agents, and the detection of immunoregulatory molecules from the supernatant of suppression assay were carried out as described in section **2.14**.

5.3.7 Cytokine detection

Supernatants of MOG-stimulated splenocytes from MSC-treated and control mice were collected and levels of IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α, GM-CSF and IL-17 detected using FlowCytomix^{TM} (eBioscience$^{®}$).

5.3.8 Flow cytometry

Cells from the spleen and LN of EAE mice were stained with antibodies as described in sections **2.7.2** and **2.7.3**.

5.4 Results

5.4.1 5-FU-MSC induced complete remission of EAE

$CD11b^{-}CD45^{-}$ MSC were isolated from primary BM cultures of 5-FU-treated mice and untreated mice respectively. Using MOG-induced EAE as a model of MS, mice were given either 1 x 10^6 5-FU-MSC or untreated MSC 6 days after MOG immunization. Mice that did not receive MSC served as control (EAE control). All EAE control mice (9 out of 9 mice; **Fig. 5.1*A*** and ***B***) developed severe disease, reaching a mean maximal score of 2.83 ± 0.12 (**Fig. 5.1*C***). Similarly, all the mice (6 out of 6 mice; **Fig. 5.1*A*** and ***B***) injected with untreated MSC developed disease, reaching a similar mean maximal score (untreated MSC: 3.0 ± 0.2 vs. EAE control: 2.8 ± 0.1, $p>0.05$) and cumulative scores (untreated MSC: 76.9 ± 8.4 vs. EAE control: 71.8 ± 7.4, $p>0.05$) as compared to the EAE control group (**Fig. 5.1*C*** and ***D***). In striking contrast, while mice injected with 5-FU-MSC initially developed mild EAE reaching a mean clinical score of 1.2 ± 0.4 (5 out of 6 mice; **Fig. 5.1*A*** and ***B***), they started to regain normal mobility after day 16 to become completely remitted of EAE from day 30 onwards (**Fig. 5.1*B***). Overall, the 5-FU-MSC-treated group had significantly lower mean maximal ($p=0.017$) and cumulative ($p=0.0026$) scores compared to untreated MSC and EAE control groups (**Fig. 5.1*C*** and ***D***).

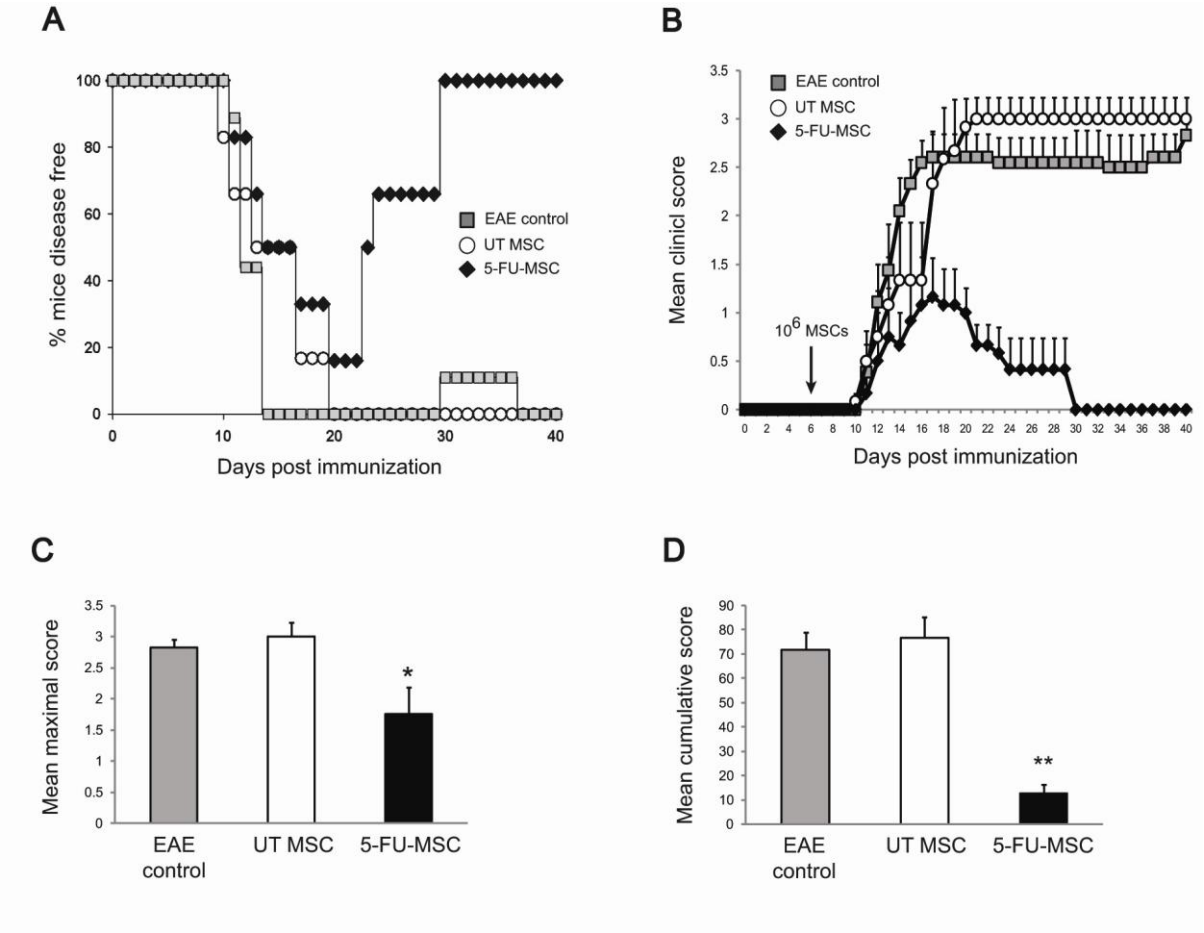


FIGURE 5.1: 5-FU-MSC remit EAE. Each mouse was i.v infused with 1 x 10^6 cells: untreated (UT) MSC (n=6) or 5-FU-MSC (n=6); 6 days after EAE immunization. Mice without MSC were used as EAE control (n=9). (A) Percentage of mice that remained disease-free over time following EAE induction. (B) Mean clinical score of EAE control mice or MSC-treated mice. 5-FU-MSC-treated mice developed mild disease but started regaining normal mobility after day 16 to become completely remitted of EAE from day 30 onwards. (C) Mean maximal score of EAE control mice and MSC-treated mice. (D) Mean cumulative score of EAE control mice and MSC-treated mice. 5-FU-MSC-treated EAE mice showed a significantly lower mean maximal and cumulative score compared to UT MSC-treated group and EAE control group. Data presented as mean ± SEM of 2 independent experiments. (*$p<0.05$; **$p<0.01$, Kruskal-Wallis test).

5.4.2 Complete remission of EAE by 5-FU-MSC is associated with decreased inflammatory infiltrates and demyelination of the CNS

In the EAE control group, severe EAE was associated with extensive inflammatory infiltrates and myelin loss in the spinal cord (**Fig. 5.2*A*** and ***B***). EAE mice given untreated MSC showed similar degrees of inflammatory infiltrates (untreated MSC: 2.17 ± 0.28 vs. EAE control: 2.28 ± 0.18, $p>0.05$) and demyelination (untreated MSC: 1.43 ± 0.19 vs. EAE control: 1.94 ± 0.21, $p>0.05$) as the EAE control mice (**Fig. 5.2*A*** and ***B***). In contrast, histological analysis of spinal cord from 5-FU-MSC-treated mice revealed a significant reduction in inflammatory infiltrates ($p=0.0027$) and demyelination ($p=0.024$) compared to untreated MSC and EAE control groups (**Fig. 5.2*A*** and ***B***).

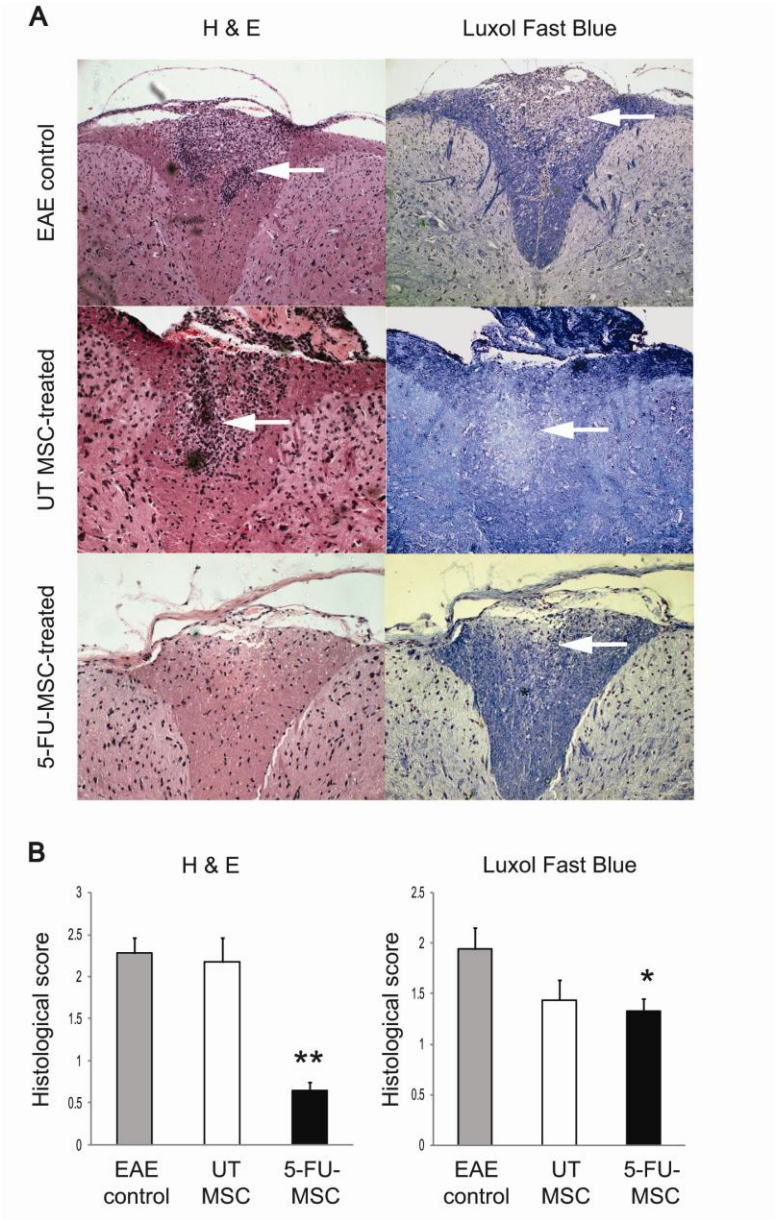


FIGURE 5.2: Histology of spinal cord. (A) Representative sections from EAE control, untreated (UT) MSC- and 5-FU-MSC-treated mice. Spinal cord sections were stained with H&E to detect lymphocyte infiltration and Luxol fast blue to indicate myelin loss. White arrows indicate regions of lymphocytic infiltrates or myelin loss. (B) Histological score of H&E and Luxol Fast Blue staining. Mice from EAE control and UT MSC-treated group showed extensive cellular infiltrates compared to lower infiltrates in mice treated with 5-FU-MSC. Demyelination was evident in mice from all three experimental groups with the 5-FU-MSC-treated group showing significantly lower demyelination. Representative photomicrographs taken at X20 magnification. Data presented are mean ± SEM. (*$p<0.05$; **$p<0.01$, Kruskal-Wallis test).

5.4.3 Complete remission of EAE by 5-FU-MSC is associated with decreased immune responses to MOG

In EAE, the transplantation of MSC has been shown to dampen the immune response of antigen-specific T cells [174, 192]. Splenocytes from EAE control, untreated MSC- and 5-FU-MSC-treated mice displayed dose-dependent proliferative responses upon *in vitro* MOG peptide stimulation (**Fig. 5.3*A***). Decreased disease severity in the 5-FU-MSC-treated group was associated with a significant reduction in the proliferative response of splenocytes to MOG_{35-55} peptide compared to the EAE control group (**Fig. 5.3*A***). Although injection of untreated MSC did not reduce EAE disease severity, the proliferative responses of splenocytes from untreated MSC mice showed significantly lower dose-dependent proliferation to MOG_{35-55} peptide compared to the EAE control group and were similar to the 5-FU-MSC-treated group (**Fig. 5.3*A***). There was no significant difference between all three groups in splenocyte proliferation in response to Con A-stimulation (EAE control: 71.9 ± 5.6; untreated MSC: 45.2 ± 12.4; 5-FU-MSC: 41.7 ± 11.0; $p>0.05$).

Besides T cells, the role of circulating MOG-specific antibody from B cells can contribute to disease pathogenesis [227]. Interestingly, 5-FU-MSC-treated mice which were completely remitted of disease produced significantly less anti-MOG antibody than untreated MSC-treated and EAE control mice (**Fig. 5.3*B***). Untreated MSC-treated mice, whose clinical score did not improve, have a similar level of anti-MOG antibody to EAE control mice (**Fig. 5.3*B***)

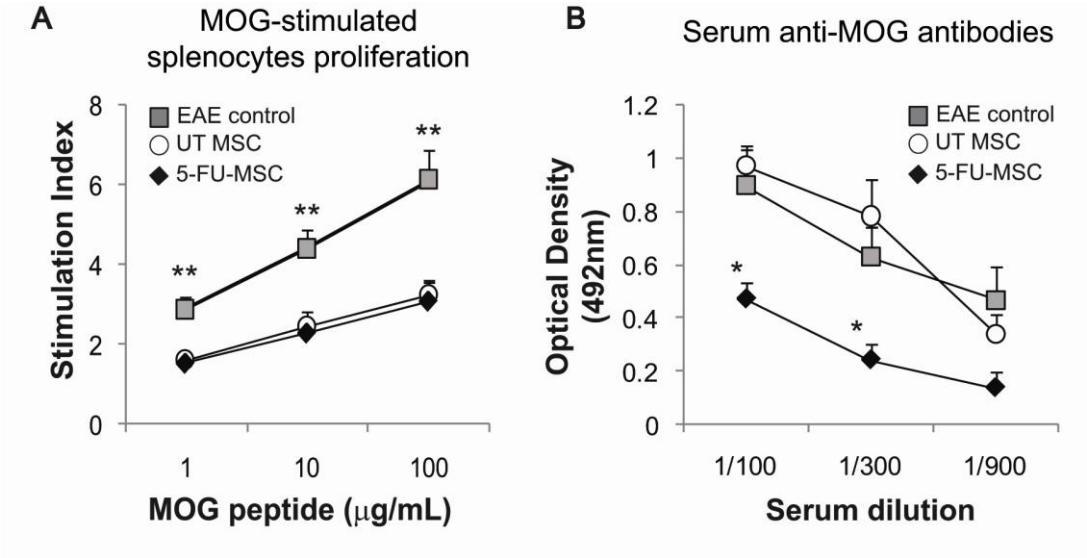


FIGURE 5.3: Splenocyte proliferation and antibody response. (A) Stimulation index of splenocyte proliferation from untreated (UT) MSC- and 5-FU-MSC-treated EAE mice stimulated with 1, 10 and 100μg/mL MOG peptide show significantly lower dose-dependent proliferation to MOG peptide compared to EAE control. (B) Serum MOG-specific antibody response demonstrated reduced anti-MOG reactivity in mice treated with 5-FU-MSC compared to UT MSC-treated and EAE control group. Specific absorbance corrected against background staining. EAE control (n=9), UT MSC (n=6) and 5-FU-MSC (n=6) for all 2 assays. (*$p<0.05$; **$p<0.01$, Kruskal-Wallis test).

5.4.4 Complete remission of EAE by 5-FU-MSC is associated with increased Th2 cytokines and lower IL-17

T cell polarization has been associated with disease pathogenesis in MS [228]. For instance, proinflammatory cytokines such as Th1-cytokines and IL-17 promote disease development whereas anti-inflammatory Th2-cytokines have a protective effect [206]. Cytokine analysis of the supernatant of MOG_{35-55}-stimulated splenocytes revealed similar levels of Th1-associated cytokines; IFN-γ, TNF-α, and GM-CSF in all three groups (**Fig. 5.4**). A higher level of Th2-associated cytokines IL-4, IL-5, IL-6 was produced by splenocytes from 5-FU-MSC-treated mice than by splenocytes from mice treated with untreated MSC or from EAE control mice. IL-10 was detected at comparable levels in both 5-FU-MSC treated and EAE control mice but not in mice treated with untreated MSC (**Fig. 5.4**). Splenocytes from 5-FU-MSC-treated mice produced significantly less IL-17 (**Fig. 5.4**). Further analysis of the T cells subsets in the three experimental groups did not reveal any significant changes in the absolute numbers of spleen and lymph node $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+FoxP3^+$ Tregs cells (**Fig. 5.5**).

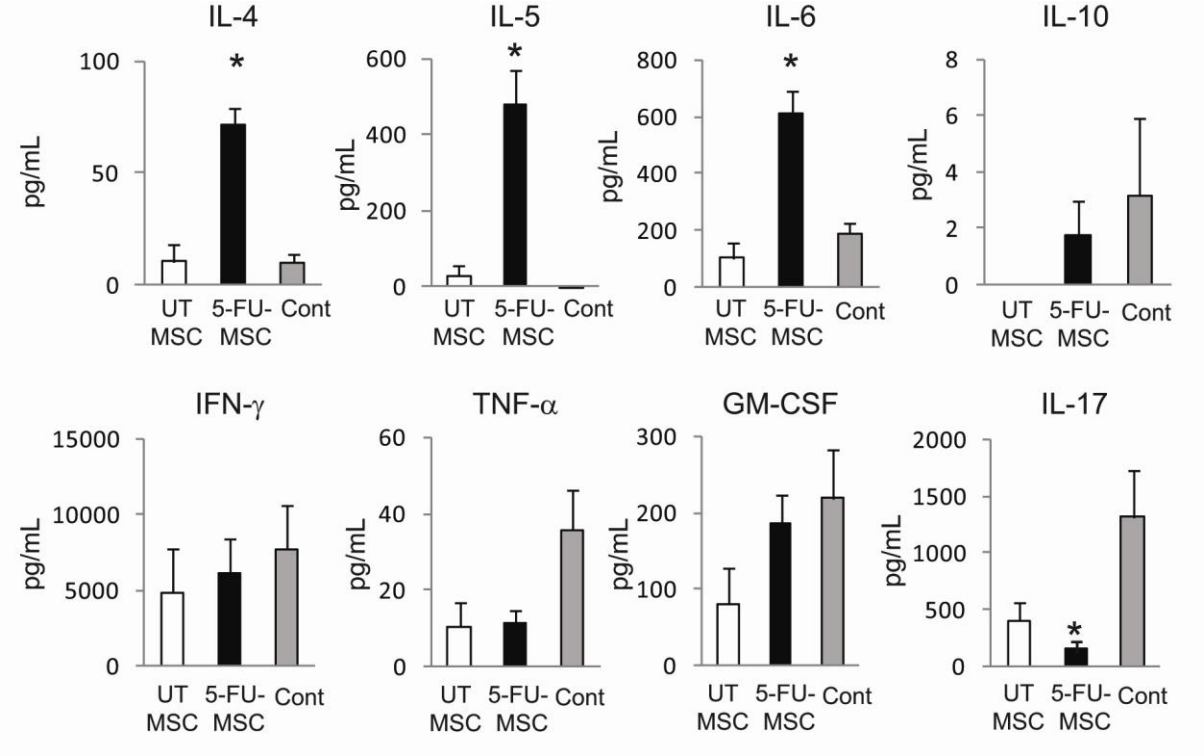


FIGURE 5.4: Splenocyte cytokine production in response to MOG peptide demonstrated significant increases in IL-4, IL-5, IL-6 and reduction in IL-17 of 5-FU-MSC-treated EAE mice (n=6) compared to untreated (UT) MSC-treated (n=6) and EAE control group (Cont) (n=9). IL-10, IFN-γ TNF-α and GM-CSF remained unchanged. Splenocytes were cultured for 72 hours with 10 µg/mL MOG peptide and supernatant collected for cytokine detection. (*$p<0.05$, Kruskal-Wallis test).

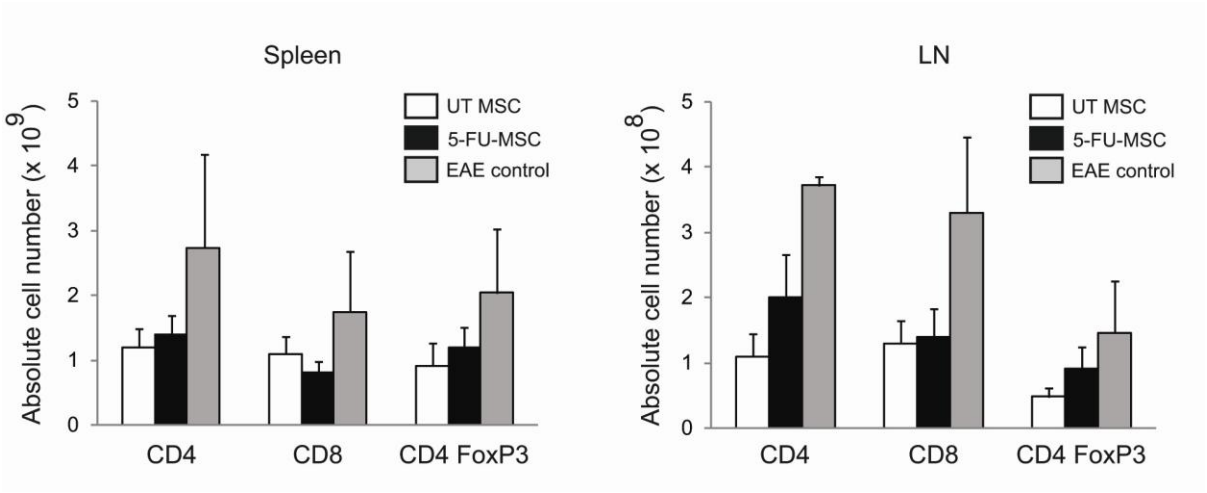


FIGURE 5.5: Absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+$ $FoxP3^+$ Tregs cells from (A) spleen and (B) lymph node (LN) reveal no significance differences between untreated (UT) MSC- (n=6), 5-FU-MSC-treated mice (n=6) and EAE control mice (n=9).

5.4.5 5-FU-MSC are potently immunosuppressive and produce elevated amounts of IL-1ra, IL-10 and PGE_2

The enhanced immunosuppressive capacity of 5-FU-MSC (section **4.4.7**; Chapter 4) associated with complete remission of disease in EAE mice prompted an investigation of the mechanism(s) underlying 5-FU-MSC-induced immunosuppression. Immunosuppression by MSC has largely been attributed to immunoregulatory molecules [106, 108, 219, 225]. Therefore, the supernatants of the *in vitro* immunosuppression assay were screened for anti-inflammatory factors IL-1ra, IL-6, IL-10, HGF, PGE_2, TGF-β and NO. Levels of NO and HO-1 in both co-cultures were similar (**Fig. 5.6**). Levels of IL-10, HGF and PGE_2 were higher in 5-FU-MSC co-culture compared to untreated MSC although these differences did not reach statistical significance. However IL-1ra was significantly increased ($p=0.042$) in the supernatant of 5-FU-MSC compared to untreated MSC (**Fig. 5.6**). TGF-β and IDO were not detected in either co-culture (data not shown).

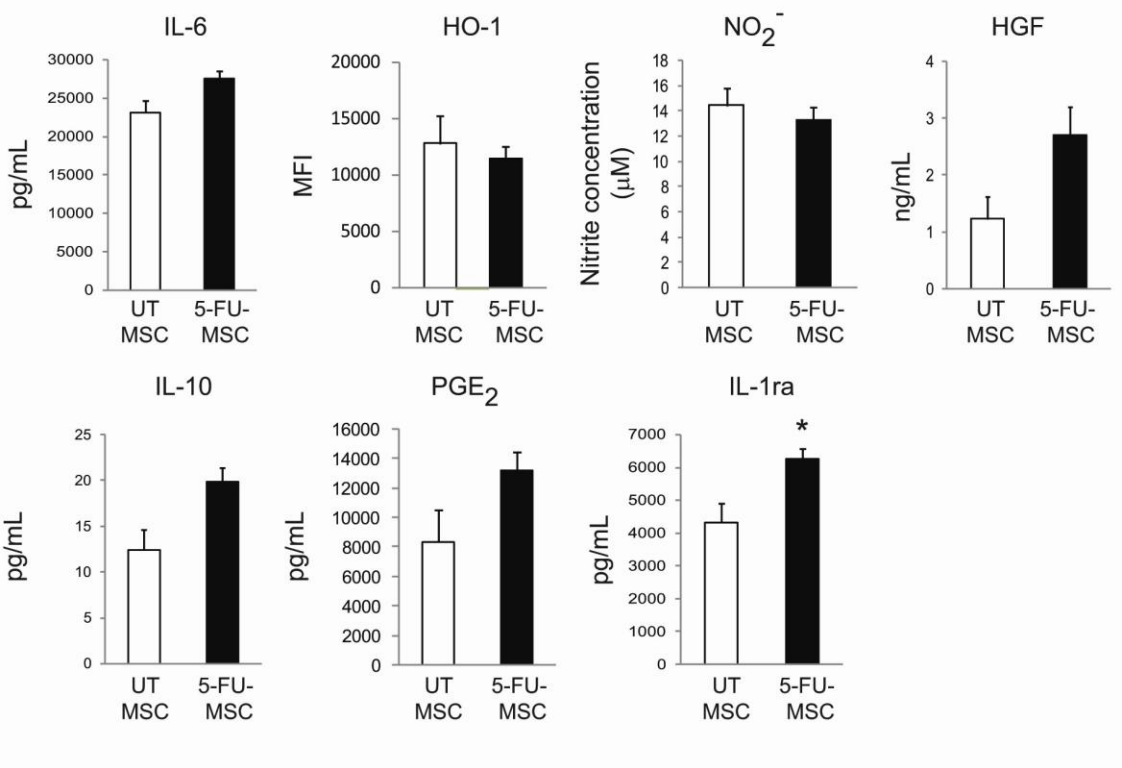


FIGURE 5.6: IL-1ra, IL-6, IL-10, HGF, PGE_2 and NO_2^- were measured in supernatants of MSC co-cultured with Con A-stimulated splenocytes at 1/40 MSC:splenocyte ratio. HO-1 levels (MFI) expressed in MSC from suppression assay were determined by HO-1 antibody staining. 5-FU-MSC co-cultures show significant increase of IL-1ra compared to untreated (UT) MSC co-cultures. Data presented are mean ± SEM of 3 independent experiments. (* $p<0.05$, Two-tailed unpaired *T* test).

5.4.6 The enhanced immunosuppressive capacity of 5-FU-MSC is mediated by IL-1ra, IL-10 and PGE_2

To investigate the role of secreted molecules produced by these MSC and their contribution to immunosuppressive capacity, neutralizing agents were added to 5-FU-MSC co-cultured with Con A-stimulated splenocytes. Blockade of HGF was not performed due to the unavailability of HGF neutralizing monoclonal antibodies. Blockade of IL-1ra, IL-10 and PGE_2 significantly attenuated 5-FU-MSC suppression of Con A-stimulated splenocyte proliferation by 61%, 49% and 62% respectively (**Fig. 5.7**). In contrast, suppression levels of splenocyte proliferation by untreated MSC remained unchanged after IL-1ra, IL-10 or PGE_2 blockade (**Fig. 5.7**). Blockade of IL-6, HO-1 and NO had no significant effect on the immunosuppressive capacity of untreated MSC or 5-FU-MSC (**Fig. 5.7**).

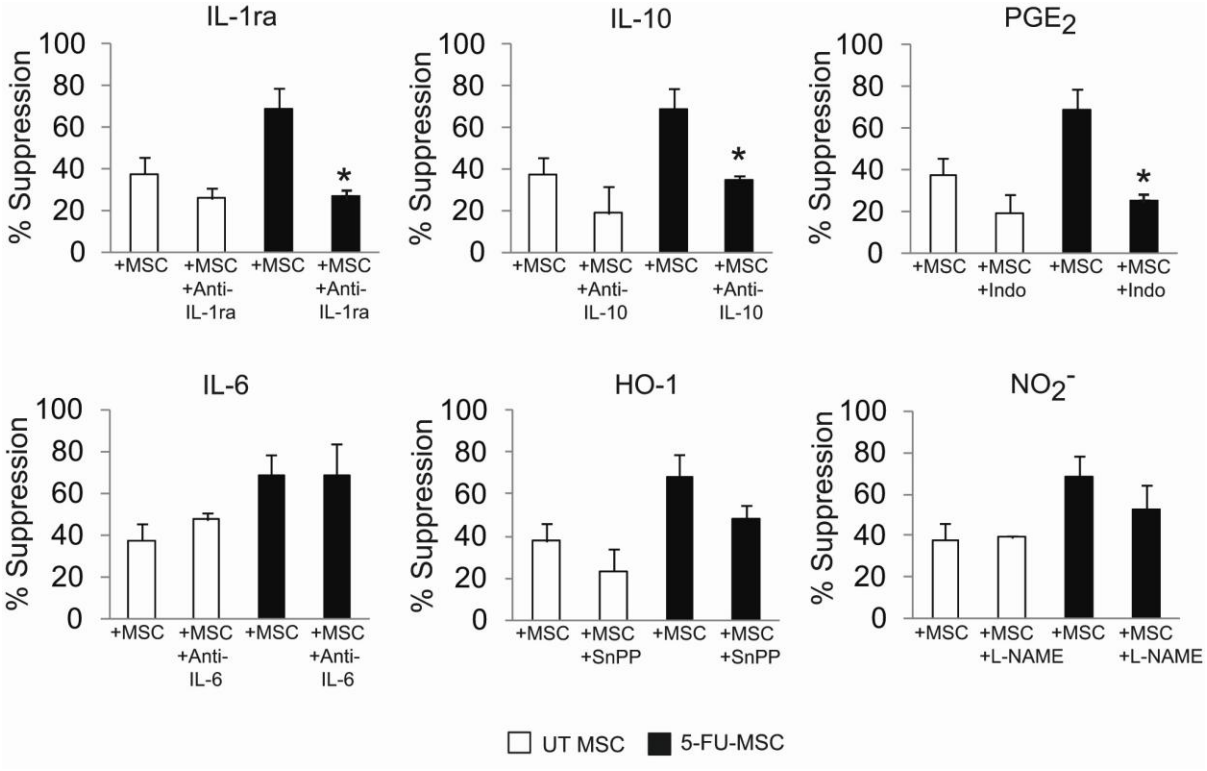


FIGURE 5.7: IL-1ra, IL-10 and PGE_2 blockade attenuated 5-FU-MSC suppression of Con A-stimulated splenocyte proliferation. Blocking agents for IL-1ra, IL-6, IL-10, PGE_2, NO_2^- and the expression of HO-1 were anti-IL-1ra, anti-IL-6, anti-IL-10, indomethacin, L-NAME and SnPP respectively. Blockade of IL-1ra, IL-10 and PGE_2 attenuated 5-FU-MSC-induced suppression by 61%, 49% and 62% respectively; no significant effect was observed with untreated (UT) MSC-induced suppression. Data presented are mean ± SEM of 3 independent experiments. (* $p<0.05$, Two-tailed unpaired *T* test).

5.5 Discussion

The immunosuppressive capacity of 5-FU-MSC *in vivo* was significantly enhanced. The potent *in vivo* immunosuppressive capacity of 5-FU-MSC was demonstrated in the EAE model of MS, where mice receiving 5-FU-MSC on day 6 after MOG-immunization were completely remitted after initial development of a mild disease. Previous studies of MSC transplantation in EAE reported attenuation of disease but not complete disease remission [125, 174, 192]. It appears that this study is the first report of complete remission of EAE after MSC transplantation.

Complete remission of EAE after 5-FU-MSC infusion was associated with a significant reduction in cellular infiltrates in the CNS, significantly reduced MOG-stimulated splenocyte proliferation and much lower levels of serum MOG-specific antibodies. These observations are consistent with reports that transplantation of MSC reduces pathogenic T and/or B cell responses towards encephalogenic antigens such as MOG_{35-55} or $PLP_{139-159}$ [174, 192]. On the other hand, although proliferation of MOG-stimulated splenocytes was reduced by infusion of untreated MSC, the levels of MOG-specific antibody were unaffected, EAE was not ameliorated, and the degree of cellular infiltrates in the CNS was unchanged. In EAE, MOG-specific antibodies have been reported to enhance demyelination and inflammation in the CNS [227]. However, in a study of methylprednisolone-induced remission of EAE in mice, reduced cellular infiltrates in the CNS was accompanied with disease remission even in the presence of demyelination [229]. Thus, a reduction in cellular infiltrates within the CNS of mice treated with 5-FU-MSC may be an important factor leading to the remission observed in this study.

These data also revealed a significant increase in Th2-associated cytokines in the supernatant of MOG-stimulated splenocytes from 5-FU-MSC-treated mice. This is in agreement with the observed complete disease remission in 5-FU-MSC-treated mice, as upregulation of Th2 cytokines have been shown to have a protective effect on EAE [206]. Additionally, IL-17 was significantly lower in the supernatant of MOG-stimulated splenocytes from 5-FU-MSC-treated mice compared to untreated MSC or EAE control mice. IL-17 is a proinflammatory cytokine that is upregulated in various autoimmune diseases including MS [230]. Recently, several reports have shown that MSC are able to inhibit Th17 differentiation [194, 231, 232] and a study by Rafei *et al* [194] showed that MSC ameliorate EAE by inhibiting Th17 T cells through a CCL-2 dependent mechanism. Thus, the significantly lower level of IL-17 in 5-FU-MSC-treated mice may also contribute to the remission observed in this study.

MSC have been shown to influence Tregs differentiation [107]. However, the inhibitory effect of MSC in EAE was suggested to be independent of Tregs whereby administration of MSC in EAE mice did not change the proportion of LN Tregs [192]. Similarly, the untreated MSC-, 5-FU-MSC-treated and EAE mice in this study were associated with no significant changes in the absolute numbers of spleen and LN $CD4^{+}FoxP3^{+}$ Tregs. These findings are in agreement with Zappia *et al* [192] whereby the inhibitory effect of MSC in EAE is not dependent on Tregs.

Unlike 5-FU-MSC, transfer of untreated MSC into EAE mice did not ameliorate disease severity. This result is at variance with other studies where transplantation of BM-MSC attenuated EAE disease [125, 174, 192]. The discrepancy may reflect the different methods employed to isolate BM-MSC. In previously reported EAE studies, MSC were isolated by

repeated-passaging of adherent BM cells whereas, in this study, MSC were negatively selected from a primary culture of BM cells using CD11b and CD45 antibodies. Different isolation methods have been reported to yield different MSC populations varying in their immunosuppressive capacity [98, 101, 192]. The untreated $CD11b^{-}CD45^{-}$ MSC used in this study may represent a population of MSC with a different *in vivo* therapeutic effect compared to passage-purified MSC.

The mechanism(s) by which 5-FU-MSC remit EAE disease were investigated by screening for immunoregulatory molecules produced by 5-FU-MSC. IL-1ra, IL-10 and PGE_2 were identified to be the mediators of this immunosuppression. The levels of IL-1ra, IL-10 and PGE_2 were elevated in 5-FU-MSC-induced suppression of Con A-stimulated splenocytes and blockade of these molecules significantly attenuated the immunosuppression. Secretion of IL-1ra by BM-MSC was first reported to mediate anti-inflammatory and anti-fibrotic effects in bleomycin-induced lung injury by Ortiz *et al* [157]. IL-1 is a pro-inflammatory cytokine with a pivotal role in diseases such as rheumatoid arthritis [233], Type 1 diabetes [234] and MS [235-238]. Binding of IL-1 to its receptor initiates pro-inflammatory events, including production of other highly inflammatory molecules such as NO, and synthesis and release of chemokines that attract other inflammatory cells. Besides inducing pathogenic IL-17 producing T cells, IL-1 also induces expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) [239]. ICAM-$1^{-/-}$ mice develop significantly attenuated EAE with reduced T cell responses to MOG and reduced inflammatory infiltrates in the spinal cord [240]. IL-1ra is a natural inhibitor of IL-1 that competes for the IL-1 receptor. Common drugs for MS treatment including steroids, interferon beta and glatiramer acetate; are thought to act by inducing IL-1ra production [241, 242]. Viral delivery of IL-1ra into autoimmune disease models, including EAE, has produced positive therapeutic outcomes [243, 244]. Furthermore,

direct administration of recombinant IL-1ra into EAE mice has been shown to delay disease onset and reduced disease severity [239, 245].

Both IL-10 and PGE_2 regulate EAE development [246, 247]. IL-10-deficient mice are more susceptible to EAE and disease is more severe. Consistent with these observations, overexpression of IL-10 in transgenic mice renders them resistant to EAE [246]. The enhanced immunosuppressive capacity of 5-FU-MSC observed in this study could, therefore, be in part attributed to an elevation of IL-10 levels. PGE_2 has a variety of immunosuppressive properties including inhibition of T cell proliferation and stimulation of production of Th2 cytokines such as IL-5 and IL-10 [248, 249]. In EAE, the production of PGE_2 from MSC has been reported to ameliorate disease [197], with MSC producing higher levels of PGE_2 having greater therapeutic effect on the disease [197]. In this study, untreated MSC produced lower levels of PGE_2 and did not improve EAE compared to 5-FU-MSC, suggesting that the combined effect of IL-1ra, IL-10 and PGE_2 may have limited infiltration of pathogenic lymphocytes into the CNS, evidenced by a reduced inflammatory infiltrates in the CNS that was correlated with remission of EAE. Although these molecules were also detected at lower levels in untreated MSC, blockade of IL-1ra, IL-10 and PGE_2 failed to restore splenocyte proliferation suggesting that the lower levels of IL-1ra, IL-10 and PGE_2 may not play an important role in suppressing Con A-stimulated splenocyte proliferation.

Besides the involvement of IL-1ra, IL-10 and PGE_2 in 5-FU-MSC-mediated suppression, the expression of a higher level of CD49d by 5-FU-MSC (Chapter 4) may have contributed to their therapeutic effect in EAE. The importance of CD49d in EAE was recently highlighted by Constantin *et al* [193] where expression of CD49d on MSC was found to facilitate their

migration into the inflamed CNS of EAE mice to ameliorate disease. The higher levels of expression of CD49d by 5-FU-MSC may indicate their increased capacity for migration into the CNS to exert their therapeutic effect compared to untreated MSC. However, the ability of 5-FU-MSC to migrate into the CNS of EAE mice is yet to be demonstrated.

5.6 Conclusion

In this study, the immunosuppressive capacity of $CD11b^{-}CD45^{-}$ MSC isolated from 5-FU-treated C57BL/6 mice was assessed in a mouse model of MS. Administration of these $CD11b^{-}CD45^{-}$ 5-FU-MSC, 6 days after MOG immunisation, completely remitted MOG-induced EAE after initial development of mild disease. The remission was accompanied by reduced CNS cellular infiltration and demyelination, and a significant reduction in anti-MOG antibody and splenocyte proliferation to MOG. MOG-stimulated splenocytes from these mice showed elevated levels of Th2 cytokines (IL-4, IL-5 and IL-6) and decreased IL-17. Compared to untreated MSC, 5-FU-MSC induced immunosuppression of Con A-stimulated splenocytes *in vitro* was accompanied by elevated levels of IL-1ra, IL-10 and PGE_2. Blocking IL-1ra, IL-10 and PGE_2 but not IL-6, HO-1 and NO with neutralizing agents attenuated 5-FU-MSC-induced immunosuppression. Together, these findings suggest that immunosuppression by 5-FU-MSC is mediated by a combination of elevated IL-1ra, IL-10, PGE_2 and anti-inflammatory Th2 cytokines and decreased IL-17. These findings suggest that 5-FU treatment identifies a population of potently immunosuppressive 5-FU-resistant MSC that have the potential to be exploited to remit autoimmune diseases.

6 GENERAL DISCUSSION

Autoimmune diseases are caused by an overactive immune response against self-antigens which eventually destroy their targets leading to pathology. They are a major health burden associated with serious morbidity and disability and, to date, remain incurable [4]. Of particular concern is MS, a chronic inflammatory disorder of the human CNS in which lymphocytic infiltration into the CNS leads to the damage of myelin and axons [7]. Progressive damage to the nerves is represented by clinical symptoms such as limb weakness, sensory loss, gait instability and disability. MS affects more than 2.5 million individuals world-wide with reduction in life expectancy of approximately 5-10 years [8-11]. The treatment of MS involves therapies aimed at indirect immunomodulatory interventions to promote remyelination of the damaged CNS or to minimize disease relapses and retard disease progression. Currently, long-term immunosuppressive therapies are being used for the treatment of MS [59]. However, efficacy of these therapies is often restricted to patients with relapsing-remitting form of MS and the usage of these immunosuppressive drugs is associated with severe side effects [61].

The potential role of adult stem cells in cell-based therapies has led to an enormous interest in harnessing their therapeutic effect in debilitating diseases. One of the promising cell types is MSC, an adult stem cell residing in various tissues including BM that can differentiate into non-hematopoietic tissues [80]. Unlike HSC, MSC have been associated with therapeutic properties such as promotion of tissue repair and immunodulatory properties [78]. The therapeutic potential of MSC has been demonstrated in various diseases including lung

fibrosis [126], cardiovascular diseases [130] and autoimmune diseases [86, 250]. Recently, the increasing body of pre-clinical data in EAE, an animal model of MS, have demonstrated the therapeutic potential of MSC for treating MS [125, 174, 192]. However, their therapeutic potential in MS is confounded by the employment of poorly characterised MSC in EAE studies [125, 194, 199, 205]. The lack of a consensus method to effectively isolate mouse MSC has led to the usage of different MSC preparations in different EAE studies, which makes comparison of their findings a difficult task. Thus, the true potential of MSC as a cell therapy for MS may be undermined by the lack of a consensus method to isolate a well-characterised MSC for studies in EAE.

The hypothesis proposed for this study is that a well-characterised MSC can be effectively isolated from mouse BM by a more efficient methodology, and be employed as a therapeutic to treat and possibly cure MS. This study has specifically addressed issues such as (1) the development of a more effective methodology for isolating BM-MSC by using *in vivo* 5-FU-treatment; (2) the phenotypic and functional characterisation of MSC isolated from 5-FU-treated mice (5-FU-MSC) and (3) evaluating the therapeutic efficacy of 5-FU-MSC as a therapy for MS in EAE model.

In this study, the isolation of mouse MSC was optimised by the development of a method that comprised initial enrichment of MSC by *in vivo* 5-FU treatment. Secondly, extraction of BM cells using a combination of flushing and scraping of the endosteum increased the yield of BM cells. Lastly, $CD11b^{-}CD45^{-}$ MSC from 5-FU-treated BM culture were separated by MACS® column. The primary step of treating the donor mice with 5-FU greatly reduced proliferating $CD11b^{+}CD45^{+}$ hematopoietic cells and enriched for $CD11b^{-}CD45^{-}$ Fibro-CFU-

F. Coupled with an improved BM cell extraction method that combines conventional flushing with scraping of the endosteum, this significantly increased the numbers of BM cells and CFU-F thus increasing isolation efficiency. Using CD11b and CD45 antibodies and MACS® column cell separation to fractionate hematopoietic contaminants, the whole $CD11b^{-}CD45^{-}$ MSC population rather than specific-MSC population was isolated.

Conventional isolation methods for mouse BM-MSC are highly dependent on the initial quality and numbers of the isolated BM cells [85], and the isolation efficiency varies in different mouse strains [92]. To date, no single method has been regarded as the 'gold standard' for isolating mouse MSC. The majority of the reported isolation methods for MSC were either not tested on multiple mouse strains [96-102, 105, 114] or are not well-characterized; lacking important phenotype characterization data such as tri-lineage differentiation potential [95-97, 99, 101, 102, 111]. In this study, the improved isolation method can be used to isolate tri-lineage MSC from multiple mouse strains, including C57Bl/6 which has previously demonstrated poor isolation efficiency and chondrogenic potential [85, 92]. Additionally, MSC can be consistently isolated from different batches of BM cells, thus making this isolation methodology simpler and more effective than conventional isolation methods.

The conventional way of determining the frequency of MSC in the BM is by CFU-F assay. However, the classification of a colony as CFU-F in reported studies was based on either minimal cell numbers constituting a CFU-F or the size of the colonies, without distinguishing the type of colonies being measured [92, 102, 214, 215]. As demonstrated in this study, the conventional determination of CFU-F is not optimal as hematopoietic cells from mouse BM

readily adhere to tissue culture plastic [94] resulting in the formation of Mixed-CFU-F (CFU-F containing hematopoietic contaminants). In this study, the prospective MSC-derived CFU-F termed Fibro-CFU-F from BM was identified by their fibroblastic appearance, the absence of cells expressing the hematopoietic markers CD11b and CD45 and importantly, their ability to differentiate into adipocytes and osteoblasts. Therefore, the discrimination of the 'true' CFU-F (Fibro-CFU-F) from other hematopoietic colonies in this study, will facilitate other similar studies to accurately identify and measure the frequency of MSC colonies in the mouse BM.

Besides increasing the isolation efficiency of mouse MSC, the improved isolation method also yielded a population of 5-FU-MSC with superior functional characteristics. The characterisation of 5-FU-MSC revealed significantly higher proliferation potential, tri-lineage differentiation potential, immunosuppression capacity and CD49d (α4 integrin) expression compared to untreated MSC. Of particular significance is the enhanced immunosuppressive potential of 5-FU-MSC, which has not been reported by other methodologies or studies thus far. *In vitro*, 5-FU-MSC exhibited a higher capacity to suppress Con A-stimulated splenocyte proliferation at a higher MSC:splenocyte ratio of 1:320 compared to reported ratio of 1:160 [125] and 1:200 [101]. *In vivo*, the potent immunosuppressive capacity of 5-FU-MSC was demonstrated in EAE, a model of MS, where mice receiving 5-FU-MSC on day 6 after MOG-immunization were completely remitted after initial development of a mild disease. This is likely to be the first report demonstrating a complete EAE disease remission after MSC administration, as the transplantation of MSC into EAE mice in previous studies only attenuated disease progression with no remission (33-35).

With the employment of the mouse model as a fundamental platform for the study of various human diseases, this improved methodology presents itself as a potent tool to facilitate the isolation of a well-characterised mouse BM-MSC population for future studies. For instance, MSC from gene knock-out mice can be isolated using this improved method to determine the association of specific genes with the different functions of MSC which include immunosuppression, differentiation, and their basic biology. Also, the ability to isolate a well-characterized MSC with high immunosuppressive ability will facilitate the further exploration of their therapeutic potential in other diseases such as Type I diabetes and rheumatoid arthritis. Collectively, this improved isolation method appears superior to other reported methods for isolating mouse MSC for the studying of human diseases.

The therapeutic effects of 5-FU-MSC in EAE support the potential use of MSC in autoimmune diseases as outlined by the 'International MSC Transplantation study group' [226]. Importantly, the ability to completely remit disease in EAE by these immunosuppressive 5-FU-MSC warrants exploration to identify a similar population of highly immunosuppressive human MSC for treatment of MS. However, direct translation of this improved methodology for isolating human MSC may be a difficult task as it can involve the administering of 5-FU directly to patients prior to MSC isolation. Even though chemotherapy has been used as a preconditioning regime in HSCT [64] or for treating cancer [213], the requirement for patients to undergo treatment with 5-FU prior to MSC isolation appears impractical when treatment-associated toxicity and/or complications such as immune suppression by 5-FU are considered. An alternative approach involving the i*n vitro* treatment of MSC with 5-FU was previously described by Congret *et al* [218] whereby a population of human MSC that exhibit properties of uncommitted progenitors can be isolated by treating an expanded MSC culture with 5-FU. In this study, *ex vivo* treatment of MSC appear to respond

in a similar fashion as those MSC from *in vivo* 5-FU treated BM culture by the formation of fibroblastic-like colonies (Chapter 3) [251]. However, these fibroblastic-like colonies from *in vitro* 5-FU-treated BM culture were found at a lower frequency compared to their *in vivo* 5-FU treated counterparts. Additionally, they could not be expanded in sufficient numbers even in the presence of growth factors such as bFGF and PDGF, thus hindering the characterisation of these *in vitro* 5-FU-treated MSC.

Although, methods for the adaptation of *in vitro* 5-FU treatment for isolating highly immunosuppressive human MSC remain to be determined, nevertheless, the preliminary findings in mouse BM encouraged the application of this *in vitro* method in freshly isolated human BM samples. Besides overcoming the need to treat patients with 5-FU prior to MSC isolation, *in vitro* 5-FU treatment could be used to isolate MSC from allogeneic BM thus eliminating the reliance on autologous BM. Additionally, the effect of *in vitro* 5-FU treatment on MSC from other sources such as adipose tissues or dental pulp can also be investigated to further understand the effect of 5-FU on these MSC. Collectively, the exploration of *in vitro* 5-FU treatment to isolate a similar population of highly immunosuppressive MSC from human BM should be a priority.

Besides exploration of the therapeutic potential of 5-FU-MSC, it is also essential to carry out further molecular characterization which could elicit molecular signatures and potentially delineate pathways which are unique to 5-FU-MSC. As demonstrated by Phinney *et al* [252] analysis of gene expression profiles of MSC can potentially identify regulatory proteins expressed exclusively by MSC which can relate to their *in vivo* therapeutic efficacy.

The mechanism(s) by which 5-FU-MSC remit EAE disease was investigated by screening for immunoregulatory molecules produced by MSC namely IL-1ra, IL-6, IL-10, HGF, PGE_2, TGF-β, NO, IDO and HO-1 [106, 108, 219, 225]. IL-1ra, IL-10 and PGE_2 were identified to be the mediators of this immunosuppression. The levels of IL-1ra, IL-10 and PGE_2 were elevated in 5-FU-MSC-induced suppression of Con A-stimulated splenocytes and blockade of these molecules significantly attenuated the immunosuppression. Both IL-10 and PGE_2 regulate EAE development [246, 247] whereas IL-1ra is a natural inhibitor of IL-1, a pro-inflammatory cytokine in MS [235-238]. Therefore, elevated production of these molecules by 5-FU-MSC in EAE could have mediated the complete remission of disease. Additional experiments such as investigating the dose-response of 5-FU-MSC on disease progression, therapeutic effectiveness of 5-FU-MSC after disease stabilization, localization of 5-FU-MSC after systemic infusion, possible transdifferentiation of 5-FU-MSC into neural cells and the effect of 5-FU-MSC in a remitting-relapsing EAE model are necessary to better understand their therapeutic mechanism(s) and potential as a cellular therapeutic for MS. Besides MS, further exploration of these highly immunosuppressive 5-FU-MSC in autoimmune diseases such as Type 1 diabetes and rheumatoid arthritis should also be undertaken to provide further understanding on their mode of action in other autoimmune diseases.

In this study, the development of an efficient method to isolate well-characterized mouse MSC population can be seen as a significant advancement to facilitate future studies of MSC. Importantly, this method enables the isolation of a population of highly immunosuppressive 5-FU-MSC that could potentially be exploited in the development of a safer and more effective treatment regime for autoimmune diseases.

7 CONCLUSION

In this study, the therapeutic potential of MSC to treat and cure MS was determined. A new isolation method was developed to effectively isolate MSC from mouse BM. The isolation methodology comprising of *in vivo* 5-FU treatment, an improved BM cell extraction method and separation of $CD11b^{-}CD45^{-}$ MSC using MACS® column was able to consistently isolate MSC from multiple mouse strains. Isolated 5-FU-MSC demonstrated significantly higher proliferation potential, tri-lineages differentiation potential and, *in vitro* and *in vivo* immunosuppression capacity, compared to untreated MSC. In EAE, administration of these 5-FU-MSC, 6 days after MOG immunisation, completely remitted MOG-induced EAE after initial development of mild disease. The remission was accompanied by reduced CNS cellular infiltration and demyelination, and a significant reduction in anti-MOG antibody and splenocyte proliferation to MOG. MOG-stimulated splenocytes from these mice showed elevated levels of Th2 cytokines (IL-4, IL-5 and IL-6) and decreased IL-17. Compared to untreated MSC, 5-FU-MSC demonstrated potent immunosuppression of Con A-stimulated splenocytes *in vitro* even at 1:320 MSC:splenocyte ratio. Immunosuppression was accompanied by elevated IL-1ra, IL-10 and PGE_2. Blocking IL-1ra, IL-10 and PGE_2 but not IL-6, HO-1 and NO with neutralizing agents attenuated 5-FU-MSC-induced immunosuppression. Together, these findings suggest that immunosuppression by 5-FU-MSC is mediated by a combination of elevated IL-1ra, IL-10 and PGE_2, anti-inflammatory Th2 cytokines and decreased IL-17. Therefore, the development of this improved method that enables the isolation of a well-characterized MSC can be adopted by other researcher as the standard isolation protocol for mouse studies. Also, further investigation should be carried out to elicit the additional therapeutic properties of this highly immunosuppressive 5-FU-MSC and their possible isolation from human.

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9 APPENDICES

9.1 Publications

1. **Oh, D.Y**., Cui P, Hosseini, H., Mosse, J., Toh, BH., Chan, J., *Potently immunosuppressive 5-Fluorouracil-resistant mesenchymal stromal cells completely remit an experimental autoimmune disease.* J Immunol, 2012. 188(5): p. 2207-17.

2. Hosseini, H., **Oh, D.Y**., Chan, S.T., Chen, X.T., Nasa, Z., Yagita, H., Alderuccio, F., Toh, BH., Chan, J., *Non-myeloablative transplantation of bone marrow expressing self-antigen establishes peripheral tolerance and completely prevents autoimmunity in mice.* Gene Ther, 2011.