Chapter 4

Mesenchymal stem cells, derived from a local source, accumulate in lymph nodes upon acute inflammation

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Abstract

Mesenchymal stem cells have evolved as a biological treatment option for patients with inflammatory bowel disease since they possess the capacity to dampen inflammatory responses and contribute to tissue repair upon inflammation. There is however no data on the behavior of endogenous mesenchymal stem cells during inflammatory bowel disease. In the present study we used two mouse strains, C57BL/6 and BALB/c mice, to study the consequences of both acute and chronic colitis on the presence of mesenchymal stem cells in mesenteric lymph nodes and bone marrow. We showed that during the acute phase, mesenchymal stem cells accumulate in intestinal draining lymph nodes of mice that exhibit the most severe form of colitis while no concurrent changes in the bone marrow were observed at that time. Additional experiments suggest that perinodal adipose tissue surrounding the lymph nodes, rather than the bone marrow, served as a reservoir for mesenchymal stem cells that mobilized into the inflamed lymph node upon induction of local peripheral immune responses.
Introduction

Crohn’s disease and Ulcerative Colitis, the two major forms of inflammatory bowel disease (IBD), are caused by an aberrant mucosal immune response against normal intestinal flora as a consequence of defects in the barrier of the intestinal epithelium. Available treatments mainly focus on dampening the inflammatory immune response, whereas restoring the epithelial barrier is left untreated. Over the past years, mesenchymal stem cells (MSC) have gained particular interest as a possible biological treatment for a variety of inflammatory diseases, including IBD. MSC are multipotent progenitor cells and are able to differentiate into a number of cell types, encompassing adipocytes, chondrocytes, osteoblasts, and stromal cells. Their relative abundance is highest in bone marrow but they reside in virtually all organs.

MSC have the capacity to modulate effector functions of both innate and adaptive immune cells such as NK cells, dendritic cells, T and B lymphocytes. Several factors produced by MSC contribute to these immunomodulatory effects including PG2, IDO, TGF-β, IL-10 and Nitric Oxide (NO). Strikingly, the pro-inflammatory cytokines IFN-γ and TNF-α produced by activated T cells, appeared to be instructive for MSC to produce these immunosuppressive mediators. Additionally, MSC are able to increase the number of regulatory T cells by inducing the production of IL-10 by DCs, although data on this is limited. However, some discrepancy on the immunomodulatory effects of MSC exists, especially since the infusion of allogeneic MSC in an experimental model of allogeneic bone marrow transplantation, elicited a T cell mediated immune response resulting in increased graft rejection. Dextran sodium sulfate (DSS) induced colitis is one of the most commonly used animal models of IBD, in which DSS is orally administered. Upon administration the intestinal epithelial lining is damaged, leading to increased permeability of the epithelial barrier and consequently, an immune response against the intestinal microflora. Neither T cells nor B cells are required for the induction of acute colitis since severe combined immunodeficient mice are still able to develop DSS induced acute colitis. However, it is believed that T cells are the main cause of tissue damage observed in chronic induced colitis.

So far, experimental studies with chemically induced colitis have shown that infusion of MSC derived from various organs including bone marrow, gingiva, or adipose tissue, shortly after disease induction, ameliorates clinical disease symptoms and histopathological signs of chronic colitis. In these studies, MSC exerted their positive effect mainly through inhibition of the ongoing immune response. In addition, recent reports also showed that MSC support regeneration of the epithelial barrier. It has been shown that bone marrow derived non hematopoietic cells were incorporated into inflamed areas of the colon upon chemical induction of colitis and thus could contribute to tissue regeneration.

In the present study, we used two mouse strains, C57BL/6 and BALB/c mice, to study the consequences of both the acute and chronic phase of DSS induced colitis on the presence of MSC in mesenteric lymph nodes and bone marrow during. Several research groups,
including our own, have shown that BALB/c mice are more resistant to DSS induced colitis compared to C57BL/6 mice (R. Molenaar; submitted). Our findings show that in the acute phase of colitis in C57BL/6 mice, which exhibit the most severe form of colitis, the number of MSC increased in the intestine draining mesenteric lymph nodes, while the number of MSC in the bone marrow were only affected at later time points of the disease. Similarly, subcutaneous administration of antigen together with adjuvant in the skin resulted in an increase of MSC in the draining peripheral lymph nodes, with a concomitant decrease of MSC numbers in the surrounding fat tissue. Our results suggest a model in which perinodal fat tissue may serve as a local source of MSC upon immune activation within lymph nodes.
Results

Different kinetics of mesenchymal stem cells in mesenteric lymph nodes of C57BL/6 vs BALB/c during the acute phase of colitis

Over the past years, mesenchymal stem cells (MSC) have gained much interest as a potential biological treatment of several inflammatory diseases, including inflammatory bowel disease (IBD)\textsuperscript{3}. Animal models resembling IBD have shown promising results but so far, there is no data on the behavior of endogenous MSC during experimentally induced IBD. Therefore, we induced colitis in two mouse strains that differ in their susceptibility for DSS colitis and monitored the change of MSC numbers at various stages of the disease. Hereeto, various organs including gut draining mesenteric and non draining peripheral lymph nodes, as well as the bone marrow were analyzed for the absolute number of MSC. BALB/c mice are more resistant to DSS induced colitis compared to C57BL/6 mice\textsuperscript{28,37}. To induce comparable level of disease in both strains, BALB/c mice were given 5\% DSS in drinking water while C57BL/6 received 2\% DSS during the same period and body weight was scored daily. Whereas BALB/c mice started to lose weight around day 6 with a maximum weight loss around day 8, C57BL/6 mice began to lose weight around day 6 with a maximum weight loss around day 8 (Fig. 1A). Despite these different kinetics in body weight changes, we and others have shown that using this DSS administration protocol, disease severity was comparable between the two mouse strains, based on reduction in colon length, inflammatory score and diarrhea scores\textsuperscript{28,37}. Mesenteric lymph nodes are part of the intestinal mucosal immune system and play, as draining lymph node for the intestine, a major role in the induction of mucosal tolerance and immunity\textsuperscript{38-40}. During DSS induced colitis, intestinal antigens are presented in mesenteric lymph nodes resulting in priming and activation of naïve T cells that subsequently migrate to mucosal tissue and contribute to the immune response\textsuperscript{39}. Since MSC have anti-inflammatory capacities, we investigated whether the numbers of MSC in mesenteric lymph nodes of both C57BL/6 mice and BALB/c mice changed during the acute and chronic phase of colitis by means of the Colony Forming Unit (CFU) assay. Before onset of the disease, both strains showed comparable amount of MSC in mesenteric lymph nodes. However, due to significantly higher cell numbers in mesenteric lymph nodes of BALB/c when compared to mesenteric lymph nodes of C57BL/6, MSC were present at lower frequency in BALB/c mesenteric lymph nodes (Fig. 1C). Interestingly, during the acute phase of colitis, we observed a dramatic increase in the absolute number of MSC in mesenteric lymph nodes of C57BL/6 mice. This increase was not observed in BALB/c mice where numbers of MSC remained comparable to control mice. The increase of MSC in mesenteric lymph nodes of C57BL/6 mice appeared to be transient, as we observed no significant increase in numbers of MSC at the chronic phase in either mouse strains, when compared to untreated control mice. The increase in MSC in C57BL/6 is likely to be restricted to mesenteric lymph nodes, since we observed no significant increase in the number of MSC in lymph nodes that did not drain the intestines (Fig 1D). It is interesting to observe that in both mouse strains,
the relative abundance of MSC in peripheral lymph nodes is substantially lower when compared to mesenteric lymph nodes.

**Bone marrow MSC increase upon progression to chronic colitis in C57BL/6 mice**

Since the bone marrow is the major location where MSC reside, we addressed whether the induction of acute colitis would result in the mobilization of MSC from the bone marrow and thus could account for the increased number of MSC observed in the intestine draining mesenteric lymph nodes. Therefore, we analyzed the number of MSC in the bone marrow both during the acute as well as the chronic phase of DSS induced colitis in both mouse strains. Again, both strains showed comparable number of MSC in the bone marrow before disease onset. Strikingly, we did not observe any changes in MSC numbers at the
Figure 1 Different kinetics of MSCs in mesenteric lymph nodes of C57BL/6 vs BALB/c upon acute colitis

Daily changes in body weight (± SEM) of control mice that received normal drinking water versus either (A) C57BL/6 mice that received 2% DSS or (B) BALB/c mice that received 5% DSS in their drinking water for 5 days, followed by 30 days normal drinking water. Body weight changes presented as percentage change compared to day 0 (starting body weight) (C + D). Absolute number of MSCs, determined by colony forming unit assay, together with total cell count in mesenteric (C) and peripheral (D) lymph nodes (MLN and PLN respectively), in control mice and in mice during the acute or chronic phase of DSS induced colitis in both C57BL/6 (black bars) and BALB/c mice (open bars). Data represent average of either absolute number of MSCs or total cell count of 3 animals per group (± SEM). **p<0.01. PLN = peripheral lymph nodes, MLN = mesenteric lymph nodes.

Concomitantly, bone marrow cellularity did not change as cell counts of both C57BL/6 and BALB/c mice remained constant at the indicated timepoints (data not shown). These data suggest that during acute colitis there is no systemic recruitment of MSC from the bone marrow to the site of inflammation.

To gain more insight in the behavior of bone marrow MSC during progression to chronic colitis, we monitored the number of bone marrow MSC over time in C57BL/6 mice. Interestingly, upon progression to chronicity, we observed a transient increase in the number of MSC in the bone marrow compared to control animals. This increase was first observed at day 15 and reached a maximum at day 18 after start of DSS administration. At this time point a threefold increase of MSC numbers was seen. At day 22, MSC numbers returned to levels that were comparable to control mice (Fig. 2B).
MSC accumulate in peripheral lymph nodes upon activation of CD4 or CD8 T cells

Although the initial phase of colitis induction depends on the innate immune system, the adaptive immune response seems to be the driving force of tissue damage that is observed during the chronic phase of the disease.

To address whether activation of adaptive immune cells can indeed induce an increase in absolute numbers of MSC in the draining lymph nodes, we mimicked the continuous exposure to antigen, as seen in mesenteric lymph nodes during colitis. Hereto, we transferred either OT-I or OT-II T cells i.v. into C57BL/6 naïve mice and administered 400µg OVA subcutaneously 24 hrs later. To maintain T cell activation, mice received an additional subcutaneous OVA stimulation in IFA at day 6 after initial antigen administration. Subsequent analysis of the number of MSC in peripheral lymph nodes was performed at day 8 and 12 after the first antigen administration.

Antigenic stimulation of transferred OVA specific CD4 or CD8 T cells resulted in the increase in number of MSC in draining peripheral lymph nodes compared to untreated control animals. At day 8 we observed a significant increase in number of MSC in peripheral lymph nodes of mice that received either OVA specific CD4 or CD8 T cells. At day 12, when the total number of cells within the lymph nodes is decreasing again and antigenic driven T cell expansion wanes, the absolute number of MSC also decreased and was not significantly elevated above control levels anymore (Fig. 3A). We did not observe any changes in MSC numbers in the spleen, indicating that only locally, in the lymph nodes that drain the site of infection, T cell activation could affect MSC numbers (Fig 3B).

In addition to the lymph nodes and spleen, we also analyzed bone marrow and peripheral blood for the presence of MSC. At the timepoint that increased numbers of MSC were...
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measured in peripheral lymph nodes, no significant changes in CFUs from the bone marrow were observed (Fig. 3C). These data suggest that peripheral T cell activation does not result in mobilization of MSC from the bone marrow. Furthermore, we were not able to detect reasonable numbers of MSC in peripheral blood (data not shown). These data suggest that the increase in MSC in the skin draining lymph nodes did not arrive via the blood and could imply that MSC may be mobilized from a local source.

**Perinodal fat tissue contains MSC that decrease upon activation while they increase in the draining lymph node**

Since mammalian lymph nodes are surrounded by fat tissue and MSC can be isolated from fat tissue, we hypothesized that lymph node activation could result in recruitment of MSC from the surrounding fat tissue into the lymph nodes. To address whether perinodal
adipose tissue could serve as a local source of MSC, we stimulated popliteal lymph nodes by subcutaneous injection of OVA and LPS in the hind paw of C57BL/6 mice and analyzed the number of MSC in both the popliteal lymph nodes and the surrounding fat from the popliteal cavity at various timepoints after stimulation.

Although the numbers of MSC were very low in popliteal lymph nodes, we observed an increase in MSC within the popliteal lymph nodes, 48 and 72 hrs after stimulation with OVA + LPS (Fig. 4A). Interestingly, analysis of the surrounding fat tissue revealed a concomitant decrease in the number of MSC. This decrease already started at 24 hrs after antigen administration and continued to decrease at 48 and 72 hrs (Fig 4B). We were unable to detect MSC in peripheral blood (data not shown) at any given timepoint. Furthermore, upon intravenous transfer of $5 \times 10^6$ GFP expressing MSC, followed by subcutaneous injection of OVA and LPS in the hind paw 24 hrs later, no GFP expressing MSC could be observed within the popliteal lymph nodes that drained the administration site, based on FACS analysis as well as immunofluorescence (data not shown). Together these data indicate indeed that upon LN activation, the surrounding fat tissue also participates in the ongoing immune response by a decrease of MSC and concomitant increase in the lymph nodes at the time that T cell activation is initiated.
Discussion

Mesenchymal stem cells (MSC) have emerged as potential therapeutic mediators of several inflammatory diseases including inflammatory bowel disease (IBD)\(^5\). Their therapeutic efficiency not only involves immunomodulatory actions\(^{13,41}\), but studies in animal models suggest that MSC may also play a role in restoring and maintaining epithelial barrier integrity\(^{34,35}\). Here we show that during the acute phase of colitis, MSC accumulate in the intestine draining mesenteric lymph nodes, while at this time point no alterations in absolute number of MSC could be observed in the bone marrow. In addition, we showed that local immune activation of peripheral lymph nodes also resulted in an increase of MSC in these peripheral lymph nodes and a concomitant decrease of MSC from the surrounding perinodal adipose tissue. We therefore propose that during an inflammatory response, immediate recruitment of MSC is from local sources rather than from the bone marrow. Only upon prolonged immune activation, as during the chronic phase of DSS induced colitis, we observed transient alterations of MSC numbers within the bone marrow.

To our knowledge, there is no information regarding the behavior of endogenous MSC during experimentally induced colitis. In the present study we made use of two mouse strains, C57BL/6 and BALB/c, to study the effect of DSS induced colitis on the presence of MSC in mesenteric lymph nodes and bone marrow. C57BL/6 and BALB/c mice exhibit different susceptibility to DSS induced colitis and different doses of DSS are required to induce comparable severity of disease in these strains\(^{28,37}\). Immune cells from both the innate and adaptive immune system are involved in DSS mediated pathology, however, the observed tissue damage involves activated effector CD4 T cells\(^2\). Priming and activation of intestinal CD4 T cells occurs in secondary lymphoid organs and in the case of colitis, this will be mainly the mesenteric lymph nodes\(^{42}\). Notably, only C57BL/6 mice showed an increase in total cell count in mesenteric lymph nodes while cell numbers of mesenteric lymph nodes in BALB/c mice did not change, indicating that immune activation was more evident in C57BL/6 than in BALB/c mice. We and others have seen that BALB/c mice possess more organized lymphoid structures in the colon which contain more T and B cells as well as regulatory T cells when compared to C57BL/6 mice\(^{43}\). It could be that the immune response in Balb/c mice, induced upon DSS administration, can take place in the lymphoid structures rather than the mesenteric lymph nodes, while in C57BL/6 mice, these structures may not be sufficient and thus immune activation will also occur in the mesenteric lymph nodes. Concurrently, MSC accumulated in mesenteric lymph nodes of C57BL/6, but not BALB/c mice, which we propose to be a consequence of the immune activation caused by DSS induced damage within the intestine. Indeed, immune activation induced in the hind leg also resulted in an accumulation of MSC in the draining lymph nodes, in a similar time frame as we observed during DSS induced colitis. Although the major reservoir for MSC is the bone marrow, analysis at the acute timepoint for the number of MSC in the bone marrow revealed no change compared to control animals. Strikingly, bone marrow MSC showed completely different kinetics compared to...
MSC present in mesenteric lymph nodes, as we observed a transient, threefold increase of bone marrow MSC in the progressive phase of the disease. These observed differences support our hypothesis that during acute inflammation a local source may serve as a MSC reservoir for lymph nodes that drain inflammatory sites, rather than the bone marrow. This was further supported by our inability to detect substantial numbers of MSC in peripheral blood, through which MSC, derived from the bone marrow, would have to travel to be recruited to a certain organ. MSC would, upon arrival at the lymph nodes, leave the bloodstream through tethering, rolling, and subsequent firm adhesion to the endothelial cells, as MSC were shown to harbor the same properties as has been shown for leukocytes to cross blood endothelial cells\textsuperscript{44,45}.

Patients with inflammatory bowel disease suffer from bone loss and osteoporosis\textsuperscript{46,47}. It has been shown in various other diseases, including rheumatoid arthritis and estrogen deficiency, that the manifestation of bone loss is mediated via T cells and appears to involve pro-inflammatory mediators like TNF-α and IFN-γ\textsuperscript{48}. Inflammatory infiltrates of T cells within the bone marrow have been shown to occur in experimentally induced colitis in mice during the chronic phase of the disease\textsuperscript{49}. And thus, the increase in MSC numbers that we observed in the bone marrow in the progressive phase of the disease may be caused by such an influx of activated leukocytes. In addition, also a systemic increase of cytokines or growth factors as a result of the ongoing intestinal inflammation, may affect the MSC population within the bone marrow. The trigger for recruitment of MSC from the local perinodal fat tissue as well as their influence on the ongoing immune response within the draining lymph nodes remains unknown. Although activated T cells may be instrumental for recruitment of MSC from perinodal adipose tissue, it cannot be excluded that direct activation of MSC through TLRs allows for their migration to the draining lymph node. Upon recruitment, MSC could contribute to the ongoing immune response both by curtailing the activation of immune cells and by the provision of precursor cells needed for lymph node remodeling\textsuperscript{50-53}. In addition, the observed increase in bone marrow MSC during the chronic phase of inflammation in C57BL/6 mice might indicate that MSC are mobilized from the bone marrow for regenerative purposes as it has been shown that MSC support endothelial regeneration\textsuperscript{15}.

Perinodal adipose tissue surrounding lymph nodes serves specialized functions that support local immune responses\textsuperscript{54,55}. Perinodal adipose tissue can be distinguished from normal fat tissue based on their fatty acid content. It was shown that adipose tissue closely associated with lymph nodes contains more unsaturated fatty acids compared to fat tissue located elsewhere\textsuperscript{56}. These fatty acids have been shown to be instrumental to lymphocytes with respect to cell mobility, signaling and tolerance for oxidative stress\textsuperscript{55,57}. Whereas normal fat tissue mass increases in Crohn’s Disease (CD) patients, perinodal adipose tissue appeared to be absent in CD patients\textsuperscript{56,59}. Although in our study we did not discriminate specifically between perinodal adipose tissue and remote adipose tissue, it is tempting to speculate that MSC reside in perinodal adipose tissue and are recruited to the draining lymph node upon activation to modulate immune responses. Loss of perinodal adipose tissue as a consequence of chronic inflammation may therefore ultimately lead
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to reduced immune suppression by MSC in the activated lymph node and a diminished capacity to support remodeling of the draining lymph nodes. All together, we have shown that, upon acute inflammation, MSC increase in number in draining lymph nodes. This increase is not paralleled by mobilization of MSC from the bone marrow, the major reservoir for MSC. Only upon chronic inflammation, MSC numbers increase in the bone marrow transiently. We have shown that fat tissue surrounding lymph nodes serves as a local source for MSC that are recruited upon acute inflammation.
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Experimental Procedures

Mice
Specific pathogen free (SPF) C57BL/6, C57BL/6 Ly5.1, and BALB/c female mice were purchased from Charles River Laboratories (France). OT-I and OT-II mice were bred at our own facility. All animals were maintained under standard animal housing conditions in the animal facility at the Vrije Universiteit (Amsterdam, The Netherlands). The Animal Experiments Committee of the VU (Vrije Universiteit) University Medical Center approved all of the experiments described in this study.

DSS Colitis induction
DSS Colitis was induced as previously described. BALB/c mice are more resistant to DSS induced colitis compared to C57BL/6 mice. In accordance with earlier publications, BALB/c mice were given 5% DSS and C57BL/6 mice 2% DSS in drinking water, which also in our facility resulted in comparable severity of disease in both strains. DSS in drinking water was provided ad libitum and water was changed on a daily basis. For analysis of DSS colitis in the acute phase, animals were given DSS for 7 days. For analysis of the chronic phase of the disease, animals were given DSS for 5 days followed by 30 days normal drinking water. Acute colitis animals were euthanized at day 7 after start of DSS treatment while chronic colitis animals were euthanized at day 35 after the start of DSS treatment.

Single cell suspension
Bone marrow single cell suspensions were obtained according to the following protocol. Freshly isolated femur and tibia were flushed once with 1ml icecold DMEM (Gibco) supplemented with 2% FCS, 2% antibiotics and glutamine (Flush Fraction). To release additional MSC from the flushed bones, femur and tibia were subsequently incubated with DMEM containing Blendzyme 2 (150µg/ml), DNAse I (200ug/ml, both Roche Applied Sciences, The Netherlands), 2% FCS and 2% antibiotics for 20 minutes at 37°C while continuous stirring. After incubation, enzymatic activity was stopped and femur and tibia were flushed once more with 1ml icecold DMEM with 2% FCS and 2% antibiotics (Blendzyme 2 fraction).

Single cell suspensions from lymph nodes and perinodal fat tissues were made as follows. Isolated lymph nodes and surrounding fat tissue were collected separately and cut extensively with small scissors, and subsequently enzymatically digested in DMEM containing Blendzyme 2 (150µg/ml), DNAse I (200ug/ml), 2% FCS and 2% antibiotics for 15 minutes at 37°C while continuously stirring. After incubation, enzymatic activity was stopped with icecold DMEM, 10% FCS, 2% antibiotics.

All single cell suspensions were washed once with excess medium to remove any residual Blendzyme 2 and resuspended in DMEM (Gibco) supplemented with 2% FCS, 2% antibiotics and glutamine, and cells were counted.
Colony Forming Unit assay
Colony Forming Unit (CFU) assays were performed according to the following protocol. Bone marrow single cell suspensions and lymph node single cell suspensions were seeded at 3 or 2 different concentrations respectively, in 2 ml Mesencult + stimulatory supplements (Mesencult Proliferation Kit, Stem Cell Technologies, France) and 2% antibiotics/well in a 6-wells plate. CFU assays were always performed in duplo. Fat single cell suspensions were seeded in quadruplo in a 6 wells plate. After 2 weeks of culture, plates with colonies were washed twice with PBS, fixed in methanol and stained with Giemsa. CFUs were counted using a stereo microscope. A cluster of cells containing ≥10 cells was considered a colony.

OT-I and OT-II cell transfer and antigenic stimulation
Spleens and lymph nodes from OT-I or OT-II mice were collected and minced through a 100µm nylon mesh to obtain single cell suspensions. Red blood cells present in the splenic single cell suspensions were lysed with lysis buffer (150mM NH₄, 1mM NaHCO₃, pH 7.4). Enriched OT-I or OT-II populations were obtained by negative selection using the CD8 or CD4 negative selection kit (Dynal, Oslo, Norway), respectively. Purity was assessed by flow cytometry. Subsequently, at day -1, 5 x 10⁶ OVA specific Ly5.2⁺ T cells were i.v. transferred into C57BL/6 Ly5.1⁺ congenic mice. At day 0, mice received OVA (400µg in 20 µl saline) s.c. in their hind leg followed by restimulation with OVA and incomplete Freund's adjuvant (400µg OVA in 20 µl) s.c in the tail base another 6 days later. Mice were euthanized at day 8 and 12 after the first OVA injection.
For popliteal lymph node stimulation, mice were injected subcutaneously in each hindpaw with OVA (0.5mg) and LPS (0.5mg). Mice were euthanized 24, 48 and 72 hrs after stimulation. Non-immunized mice served as controls.
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