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## The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy

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### ABSTRACT

There is growing interest in the use of mesenchymal stem cells (MSC) for immune therapy. Clinical trials that use MSC for treatment of therapy resistant graft versus host disease, Crohn's disease and organ transplantation have initiated. Nevertheless, the immunomodulatory effects of MSC are only partly understood. Clinical trials that are supported by basic research will lead to better understanding of the potential of MSC for immunomodulatory applications and to optimization of such therapies. In this manuscript we review some recent literature on the mechanisms of immunomodulation by MSC *in vitro* and animal models, present new data on the secretion of pro-inflammatory and anti-inflammatory cytokines, chemokines and prostaglandins by MSC under resting and inflammatory conditions and discuss the hopes and expectations of MSC-based immune therapy.

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### 1. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are the current focus of a growing number of research laboratories for their potential use for immune therapy. They are found in the bone marrow and at multiple other sites including adipose tissue [1], skin [2], spleen, and heart [3]. *In vitro*, MSC are characterized by plastic adherence, colony forming capacity and rapid proliferation. The immunophenotype of MSC, CD45<sup>-</sup>, CD34<sup>-</sup>, CD13<sup>+</sup>, CD44<sup>+</sup>, CD73<sup>+</sup>, CD90<sup>+</sup>, CD166<sup>+</sup>, CD80<sup>-</sup>, CD86<sup>-</sup>, HLA class I<sup>low</sup>, HLA class II<sup>-</sup>, distinguishes them from hematopoietic stem cells, which are CD34<sup>+</sup>, CD45<sup>+</sup> and CD13<sup>-</sup>, and positions them close to fibroblasts. This phenotype also suggests MSC are low immunogenic [4]. A key property of MSC is their multi-lineage differentiation potential [5]. MSC can be induced to differentiate into osteogenic, adipogenic and chondrogenic lineages, while they may have additional potential to differentiate into myogenic, hepatogenic and other lineages. The differentiation potential of MSC has raised interest in their use for repair of injured tissue and to combat ageing. Nevertheless, recent data suggests that the tissue regenerative effects of MSC do not solely stem from their differentiation, but depend for a major part on the secretion

of growth factors that stimulate local progenitor cells [6]. Furthermore, some of the apparent regenerative effects of MSC may in fact originate from their immunomodulatory properties, which are the focus of this manuscript.

### 2. The immunomodulatory properties of MSC

MSC have potent immunosuppressive capacity. This is demonstrated *in vitro* by the inhibition of T lymphocyte proliferation and pro-inflammatory cytokine production after mitogen or cellular stimulation by MSC [7,8]. MSC furthermore inhibit the antibody production of B cells [9] and inhibit the generation and function of antigen presenting cells [10]. The immunosuppressive effects of MSC have been further evidenced in a number of *in vivo* models, where MSC were demonstrated to alleviate experimental colitis [11], were effective in reducing immune activity in autoimmune enteropathy [12] and were capable of prolonging heart and skin allograft survival [13,14]. Via their immunosuppressive properties MSC may be able to prevent immune inflicted damage of tissues and organs and allow repair after injury. Immunosuppression is, however, not the only aspect of the immunomodulatory capacity of MSC. Under immunological quiescent conditions, MSC promote T lymphocyte survival [15] and can stimulate the activation and proliferation of CD4<sup>+</sup> T cells [16]. The activation of T cells by MSC is independent of cell contact. Although T cells that are activated by MSC

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are responsive to mitogen and allo-antigen stimulation, they contain an increased number of CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>-</sup> regulatory T cells that exhibit suppressive activity. To fully understand the possibilities of the use of MSC for immune therapy, it is important to study the mechanisms of immunomodulation by MSC in more detail.

### 3. Mechanisms of immunomodulation by MSC

The immunosuppressive effect of MSC is for a large extent mediated via soluble factors. Separation of MSC by a transwell membrane does not prevent inhibition of activated immune cell proliferation. Several factors have been proposed to play a role in the immunosuppressive effect of MSC, including TGF- $\beta$ , hepatocyte growth factor (HGF) [7], nitric oxide [17] and HLA-G [18]. Furthermore, the tryptophan depleting enzyme indoleamine 2,3-dioxygenase (IDO) plays a crucial role in the immunosuppressive effect of MSC [19]. There is strong evidence that the immunosuppressive capacity of MSC is induced under inflammatory conditions [20]. Pre-stimulation of MSC with the pro-inflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL6 not only increased the potency of MSC to inhibit lymphocyte proliferation, it also boosts the speed of lymphocyte inhibition (Crop et al. submitted). The enhanced immunosuppressive capacity of MSC after treatment with pro-inflammatory cytokines is in particular associated with increased IDO expression, while also the expression of HGF and HLA-G is augmented. The response of MSC to inflammatory conditions is, however, not a one-way road leading to enhanced immunosuppression. Recently it was reported that MSC possess antigen-presenting properties [21]. They express antigen-processing and transporter proteins and can cross-present antigen to CD8<sup>+</sup> T cells via MHC class I. In response to IFN- $\gamma$ , MHC class I expression on MSC is up regulated and their antigen-presenting capacity improved [21]. MSC express low levels of MHC class II, but prolonged treatment with IFN- $\gamma$  up regulates MHC class II levels on MSC [22]. This suggests that under inflammatory conditions MSC can act as professional antigen-presenting cells.

### 4. The secretion of cytokines and chemokines by MSC

In addition to anti-inflammatory factors, MSC produce and secrete pro-inflammatory cytokines and chemokines. We analyzed the secretion of 40 soluble factors by MSC after 48 h of culture in serum-free MEM- $\alpha$  using Raybio® arrays (Raybiotech, Norcross, GA). MSC constitutively secreted large amounts of the cytokines IL6 and IL8, the chemokine CCL2, and of TIMP-2, known for long to have tumor cell invasion inhibitory capacity [23] (Fig. 1A). When IFN- $\gamma$  (50 ng/ml) was added to the MSC for 48 h, there was a trend for increased secretion of soluble ICAM-1, CXCL10, and CCL8 by MSC, whereas the secretion of IL8 was decreased (Fig. 1B). The trend for decreased IL8 levels, high levels of CCL2 and tendencies for increased levels of CCL8 and CXCL10 after IFN- $\gamma$  treatment would suggest that MSC-mediated chemotaxis targets neutrophils and monocytes under non-inflammatory conditions, while under inflammatory conditions MSC attract monocytes, dendritic cells and T and NK lymphocytes. The function of chemotaxis of immune cells for the immunomodulatory effects of MSC is not clear. Chemotaxis may bring specific immune cell subsets in close proximity to MSC, which makes them more susceptible for the immunomodulatory effects of MSC. Chemotaxis can be followed by binding of immune cells to MSC [24]. We have recently demonstrated that in particular activated lymphocytes bind to MSC [25]. The low levels of secreted TGF $\beta$ 1 that were observed and the failure of IFN- $\gamma$  to increase these levels are remarkable, as a number of studies have identified TGF $\beta$ 1 as one of the key factors of immunomodulation by MSC [7,26]. There is a possibility that the adipose tissue-derived MSC used in our studies show different TGF $\beta$ 1 secretion patterns than bone marrow-derived MSC, which are used in most other studies. However, it is reported earlier that TGF- $\beta$ 1 cannot be detected in conditioned medium of both adipose tissue and in bone marrow-derived MSC [27]. It has also been reported that TGF- $\beta$ 1

does not support the immunomodulatory capacity of MSC in the presence of IFN- $\gamma$ , but that other factors such as COX-2 and PGE<sub>2</sub> are responsible [28]. This suggests that environmental factors may be crucial in determining which immunomodulatory pathways are operational in MSC.

### 5. The secretion of prostaglandins by MSC

In addition to cytokines and chemokines, MSC secrete prostaglandins, of which PGE<sub>2</sub> has earlier been associated with the immunosuppressive effect of MSC [29]. We recently analyzed the secretion of prostaglandins by human heart-derived MSC in more detail. MSC were isolated from atrial tissue that became available after heart transplantation, as described before [30]. After expansion for 3 up to 5 passages, MSC were kept in serum-free medium for 48 h. Levels of prostaglandins were measured in the conditioned medium by liquid chromatography coupled to tandem mass spectrometry (LC/ESI-MS/MS) as previously described [31]. MSC secreted prostaglandins PGE<sub>1</sub>, PGE<sub>2</sub>, PGE<sub>3</sub>, PGI<sub>2</sub> (measured as its stable metabolite 6-keto PGF<sub>1</sub> $\alpha$ ), PGF<sub>2</sub> $\alpha$  and PGJ<sub>2</sub>. When MSC were cultured in the presence of allo-activated PBMC separated by a transwell membrane for 7 days prior to 48 h incubation in serum-free medium, the secretion of these prostaglandins was enhanced (Fig. 1C). Although PGE<sub>2</sub> has been linked to the immunosuppressive effects of MSC [29], prostaglandins are best known for their role in the process of inflammation. Prostaglandins mediate vasodilatation that allows immune cells to invade inflamed tissue. Recent evidence also suggests that PGE<sub>2</sub> may in fact have an immunostimulatory role by facilitating Th1 differentiation and expanding the Th17 T cell population [32]. As prostaglandins have a short half life, they act as paracrine and autocrine factors in the local environment in which they were produced. MSC themselves also express receptors for prostaglandins, of which prostaglandin subtypes receptors EP1, EP2, EP4, FP and IP show the highest expression. The effect of stimulation of these receptors on MSC function is not known. However the profile of prostaglandins produced by MSC is in agreement with the profile of receptors (prostaglandins type E and F, and prostacyclin).

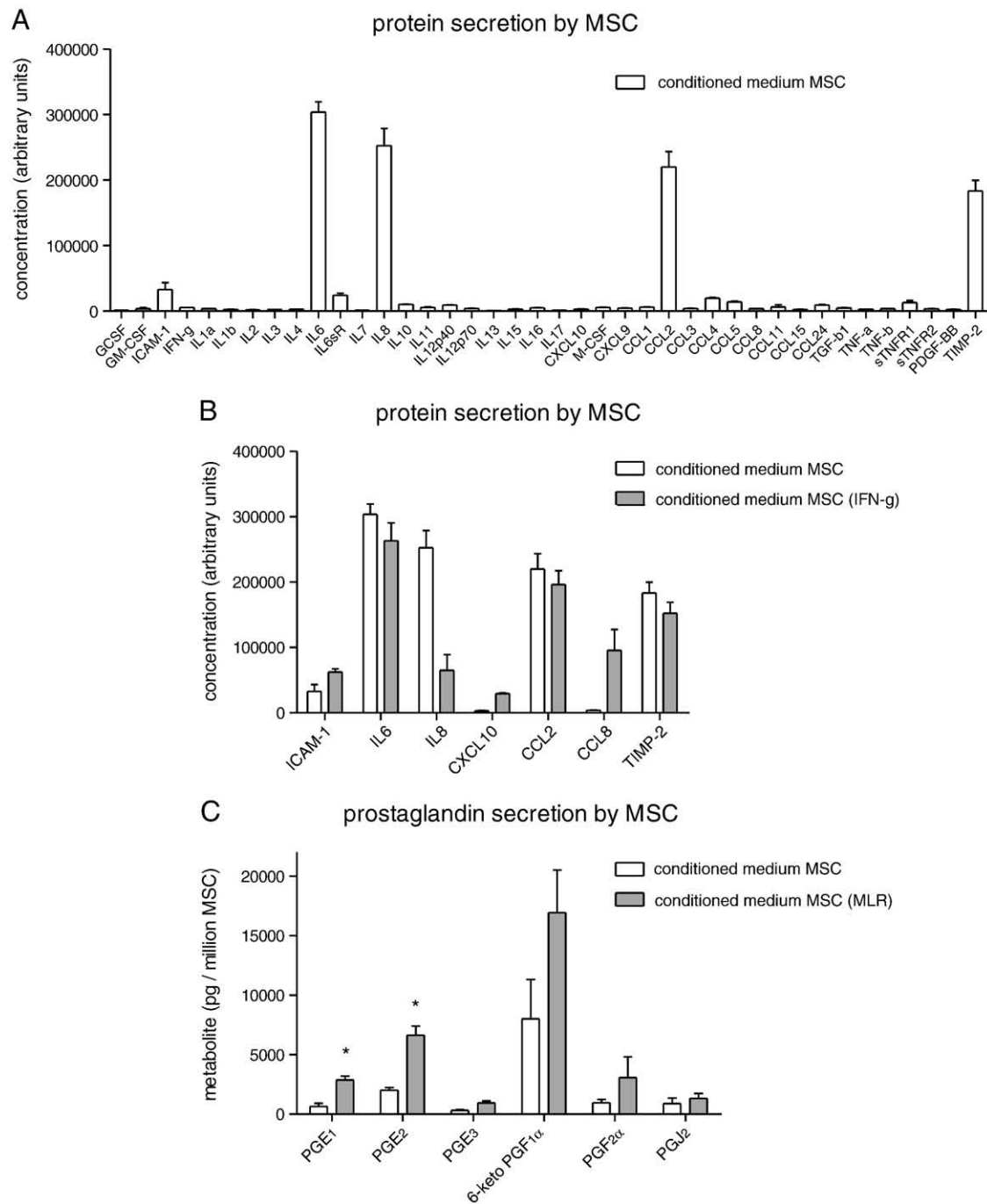
Thus, MSC appear to have dual immunomodulatory capacity; they have the ability to inhibit immune cell activation and proliferation via IDO, PGE<sub>2</sub> and other factors, but also enhance immune responses via the secretion of pro-inflammatory factors and chemokines. The nature of the immunomodulatory effect of MSC depends on local immunological conditions, where in particular IFN- $\gamma$  and TNF- $\alpha$  play a crucial role in inducing the immunosuppressive function of MSC [28,33].

### 6. Immunological response to MSC infusion

While the proactive effects of MSC on immune cells, such as the secretion of anti-inflammatory factors, are subject of intense research, there is little attention for potential passive immunomodulatory effects of MSC. It is for instance possible that infusion of MSC elicits an immune response, as cultured MSC have a phenotype that is different to the phenotype of MSC in tissues. MSC may therefore be recognized by the immune system and induce an immunomodulatory effect that is independent on active cytokine production of MSC. Speculative, such a response could eventually be down regulated, leading to a relieving side-effect on chronic inflammations. Whether immune activation is one of the mechanisms that mediate the beneficial immunomodulatory effects of MSC needs further investigation.

### 7. Immunogenicity of MSC

MSC have a low immunogenic phenotype as they express low levels of HLA and co-stimulatory molecules and they do not elicit alloreactive T lymphocyte responses *in vitro* [4]. Nevertheless, there is now convincing evidence that MSC are immunogenic after *in vivo* infusion. Nauta et al. demonstrated some years ago that infusion of



**Fig. 1.** Effect of IFN- $\gamma$  and MLR on cytokine, chemokine and prostaglandin secretion by MSC. A) Secretion of cytokines and chemokines by MSC cultured in serum-free MEM- $\alpha$  for 48 h measured by Raybio® arrays according to the manufacturer's instructions ( $n = 2 \pm SD$ ). B) Effect of culturing of MSC for 48 h in the presence of 50 ng/ml IFN- $\gamma$  on the secretion of cytokines and chemokines. Secreted factors present in the highest levels and with the largest changes in concentration are depicted ( $n = 2 \pm SD$ ). C) Prostaglandin levels in MSC conditioned medium. MSC were cultured in the absence or presence of allo-activated PBMC separated by a transwell membrane for 7 days. MSC were then cultured in serum free medium for 48 h and prostaglandin levels determined by LC/ESI-MS/MS ( $n = 3 \pm SD$ ). \* indicates  $p < 0.05$  determined by Student's t-test.

donor, but not host-derived, MSC can promote rejection of donor bone marrow transplants in sublethally irradiated mice [34]. A similar finding was observed in a skin transplant model in the rat, in which infusion of donor-derived MSC accelerated donor skin graft rejection [35]. This suggests that MSC can sensitize for HLA directed immune responses *in vivo*. Under inflammatory conditions, HLA expression on MSC is upregulated and this could lead to increased recognition of the antigen and thereby increased sensitization against MSC. On the contrary, HLA recognition is a pre-requisite for desensitization and there is evidence suggesting that the application of donor MSC in

combination with immunosuppressive drugs can lead to desensitization against donor grafts [35].

## 8. Interaction between MSC and immunosuppressive drugs

There is hope that MSC may be capable of complementing or even replacing immunosuppressive drugs in the future. As this therapy emerges, there is no doubt that MSC will be administered in combination with immunosuppressive drugs. As these drugs and MSC have common targets (the proliferating T cell pool), there is a possibility

that MSC and immunosuppressive drugs have a reciprocal effect on each other's efficacy. *In vitro* data demonstrates that the immunosuppressive capacity of MSC is reduced in the presence of the calcineurin inhibitor tacrolimus and of the mTOR inhibitor rapamycin, which are commonly used drugs to prevent organ rejection after transplantation [36,37]. In contrast, the cell cycle inhibitor mycophenolic acid (MPA) and MSC have cumulative immunosuppressive effects. These findings are supported by animal studies that show that MSC in combination with MPA have a stronger effect on the survival of transplanted hearts than MSC and MPA alone [13]. A different model, however, demonstrated that MSC also synergize with rapamycin to prolong allograft survival [38]. However, in these latter experiments additional growth factors were supplied to the culture, presumably producing a different cell type compared to a classic MSC. In summary, more work is needed to work out under what conditions, which immunosuppressive drug is most appropriate to use in combination with MSC.

## 9. MSC immunotherapy in animal studies

Data from animal models of immunotherapy with MSC must still be considered heterogenous. In the first promising studies, MSC were able to control lethal GvHD after allogeneic bone marrow transplantation [39] whereas in a different mouse model MSC failed to prevent GvHD [40]. These findings clearly reflect that MSC biology is complex in living organisms and that results depend on a multitude of factors, one of which are non-standardized culture conditions used in different laboratories. More and more experience with MSC also shows that distinct MSC preparations can have different effects *in vivo*, even when cultured under the same conditions. It is thus hard to prospectively predict the *in vivo* effects of a given MSC culture. The opposing results observed in the above studies suggest that MSC might act differently *in vivo*, depending on their state of activation. Here again, INF- $\gamma$  seems to play a pivotal role since INF- $\gamma$  pre-stimulated MSC were more efficient in preventing and treating GvHD compared to naïve MSC [41].

MSC were also applied to prolong graft survival in several preclinical models of solid organ allo-transplantation. The immunological mechanism of action remains controversial, although a variety of these transplantation models outlined a clear benefit of MSC on graft survival. Among these positive studies are the initial description of prolonged skin graft survival in the baboon, and several rodent models of vascularized grafts [13,14,38,42]. Tolerogenic dendritic cells, regulatory T cells as well as IDO expression have been described as immunomodulatory mechanisms operating *in vivo* [13,31]. Interestingly, different immunomodulatory mechanisms of action have been demonstrated for human and mouse MSC *in vitro* [43]. If this also applies to the *in vivo* application of MSC, the diverse mechanisms of action might be related to a fixed species variation in MSC.

## 10. MSC as immune therapy in the clinic

MSC have been successfully applied for the treatment of steroid-resistant GvHD in a multicenter, clinical phase II study that was recently published [44]. This study represents the most successful clinical application of MSC so far reported and was considered a breakthrough within this field. However, grade IV GvHD must be considered a unique clinical situation, as overall the treatment options are very limited, which makes the decision to commit to experimental therapies relatively easy. Furthermore, grade IV GvHD is accompanied by severe systemic inflammation, whereas this is not the case in many other immunological disorders. The immunomodulatory properties of MSC are, however, evaluated to treat other diseases. Phase III studies are underway that are enrolling patients with Crohn's disease refractory to conventional immunosuppressants. The first results confirm that MSC are well tolerated, non-toxic and reduce disease severity [45,46]. More patients need to be treated to elucidate whether MSC can emerge as a

true alternative to established clinical practice. The same is true for additional studies applying MSC in kidney failure, ischemic stroke and multiple sclerosis.

In the field of solid organ transplantation, there are very effective treatment regimes available for immunosuppression and good arguments are needed to justify an additional application of MSC. On the other hand, patients still have to take immunosuppressants for life and thus suffer from side effects like infections and increased risk of cancer development. Together with the expected regenerative effect on ischemia reperfusion injury of organ transplants, the application of MSC can therefore be considered feasible and is currently explored by our groups and others [47,48]. First studies are recruiting patients undergoing kidney transplantation. Here patients are treated with calcineurin inhibitors and MPA concurrently with MSC, and we hope that the first results will give further insight into the interactions of MSC with conventional immunosuppressants in patients.

## 11. Conclusions

MSC are a promising cell population that is currently investigated on the preclinical and clinical level by a growing number of laboratories around the globe. In the recent years this has led to a significant increase in the knowledge about MSC immunobiology and first clinical therapies are evolving.

However, failure will follow the first "MSC-hype" and further thorough analysis and discussion needs to be undertaken by dedicated MSC immunobiologists to achieve a real future clinical benefit. Collaborative work groups like the MISOT consortium (Mesenchymal stem cells in Solid Organ Transplantation), founded by the authors of this article, will hopefully play a crucial role in this process [47].

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