Characterization of Human Mesenchymal Stromal Cells Derived from Extraembryonic Gestational Tissue

- A Study *In Vitro* and *In Vivo* -

DISSERTATION

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SUMMARY

Mesenchymal stromal cell (MSC) therapy is being investigated for the treatment of various degenerative and immunological disorders. The placenta and umbilical cord are rich sources of MSC populations, but in-depth studies testing their suitability for cell-based therapies are lacking. Therefore, the properties of four post-natal extraembryonic gestational tissue-derived MSCs isolated from the umbilical cord lining (CL-MSC), umbilical cord blood (CB-MSC), placenta (P-MSC) and Wharton's jelly (WJ-MSC) were examined. MSCs used in clinical applications apart from supporting tissue regeneration, have to meet several criteria: (i) fast expansion* to large cell numbers; (ii) a high migration rate; (iii) prolonged survival *in vivo* after transplantation; and (iv) lack of immune rejection making allogeneic applications possible. Ideally they should also modulate immune responses. For this, the proliferation rate, survival, migration, immunogenicity and immunomodulatory capabilities of extraembryonic tissue-derived MSCs were explored. Fulfillment of the criteria suggested for human multipotent MSCs was also examined.

The extraembryonic tissue-derived cells differed in their expression of typical MSC markers. More importantly, only CL-MSCs showed tri-lineage developmental potential and could be differentiated into adipocytes, chondrocytes and osteocytes. Further differences were noted on the level of cell proliferation and migration, with CL-MSCs showing the highest proliferation and migration rates. CL-MSCs enhanced proliferation translated to a prolongation in survival in immunodeficient mice. Moreover, CL-MSCs showed a prolongation in survival in immune competent mice which was attributed to their ability to dampen xenogeneic T helper (T_H)1 and T_H2 cell responses. Weaker human cellular immune responses were detected against CL-MSCs and P-MSCs, which correlated with their low human leukocyte antigen (HLA) class I expression. Furthermore, HLA class II was up-regulated less substantially by CL-MSCs and CB-MSCs after interferon-γ (IFN-γ) stimulation.

*The expression "expansion" refers to the *in vitro* proliferation of cell populations.

SUMMARY

CB-MSCs expressed the highest levels of immunomodulatory human leukocyte antigen G (HLA-G) and HLA-E. They secreted the highest amount of the toleragenic cytokine transforming growth factor-β1 (TGF-β1), while the MSC types did not differ in indolamine 2,3-dioxygenase (IDO) expression after IFN-γ stimulation. Despite having a lower IDO, HLA-G and TGF-β1 expression, only CL-MSCs were able to reduce the release of IFN-γ by lymphocytes in a mixed-lymphocyte reaction.

Next, the migration pattern of CL-MSCs was examined *in vivo* as data describing the behaviour of CL-MSCs *in vivo* is lacking. After systemic infusion in immunodeficient mice, CL-MSCs were trapped in the lungs and no migration to other organs was observed. Therefore, a more suitable method for CL-MSC administration remains to be determined.

The results of this study demonstrate that, CL-MSCs show the best characteristics for cell-based strategies, as they are hypo-immunogenic and show high proliferation, and migration rates. In addition, this work shows for the first time that although immunomodulatory molecules HLA-G, HLA-E, and TGF-β play an important role in MSC immune evasion, HLA expression is decisive in determining the immunogenicity of MSCs.

Keywords: Mesenchymal stromal cells, Cell transplantation, Immunogenicity, Migration.

ZUSAMMENFASSUNG

Zur Behandlung verschiedener degenerativer und immunologischer Erkrankungen werden Therapien mit Mesenchymalen Stromazellen (MSC) untersucht. Verschiedene MSC Populationen können aus der Plazenta und Nabelschnur isoliert werden. Studien, in denen die Eignung dieser Populationen für zell-basierte Therapien analysiert werden, fehlen jedoch bis heute. In der vorliegenden Arbeit wurden vier MSC Populationen aus extraembryonalen Gewebe isoliert und in ihren Eigenschaften verglichen. Dabei handelt es sich um aus der Nabelschnur isolierte MSCs (CL-MSC), aus Nabelschnurblut isolierte MSCs (CB-MSC), aus der Plazenta isolierte MSCs (P-MSC) und aus Wharton-Jelly isolierte MSCs (WJ-MSC). MSCs, die für klinische Anwendungen vorgesehen sind, müssen - abgesehen von ihrer unterstützenden Funktion während der Geweberegeneration - mehrere Kriterien erfüllen: (i) schnelle Expansion* zu großen Zellzahlen, (ii) hohe Migrationsraten, (iii) verlängerte Überlebensraten nach Transplantation und iv) fehlende Immunabwehr, die eine allogene Anwendung ermöglicht und idealerweise die Immunreaktion moduliert. Hierzu wurden in dieser Arbeit der Phänotyp, die Proliferationsrate, Migration, Immunogenität und immunmodulatorische Kapazitäten der vier MSC Populationen erforscht. Weiterhin wurde für alle MSC Populationen analysiert ob diese die Kriterien für humane multipotente MSCs erfüllen.

Alle MSC Populationen unterschieden sich in der Expression typischer MSC Marker. Nur CL-MSCs waren in der Lage zu multipotenter Differenzierung hin zu Adipozyten, Chondrozyten und Osteozyten. Weitere Unterschiede wurden auf Ebene der Zellproliferation und Migration festgestellt. Hier wiesen CL-MSCs die höchste Proliferations- und Migrationsraten auf, was zu einer Verlängerung der Überlebenszeit in immundefizienten Mäusen führte. Auch in immunkompetenten Mäusen zeigten CL-MSCs eine verlängerte Überlebensrate, was ihrer Fähigkeit xenogene T-Helferzellen (T_H) 1 und T_H2-Zell-Reaktionen zu dämpfen, zugeschrieben wurde.

* Der Begriff „Expansion“ bezieht sich auf die *in vitro* Proliferation der Zellpopulationen.

CL-MSCs und P-MSCs stimmulierten schwächere humane zelluläre Immunantworten, was mit ihrer niedrigen Expression von HLA Klasse I-Molekülen korreliert. Ebenso waren nach einer IFN-γ Stimmulation von CL-MSCs und CB-MSCs die HLA-Klasse II-Moleküle weniger stark hochreguliert. CB-MSCs zeigten das höchste Expressionsniveau an immunmodulatorischen HLA-G und HLA-E, und sezernierten die größte Menge des tolerogenen Zytokins TGF-β1. Die Expression von Indolamine 2,3-dioxygenase (IDO) in den vier MSC-Gruppen zeigte keine Unterschiede nach IFN-γ Stimmulation. Trotz einer geringen Ausprägung der IDO, HLA-G und TGF-β1Expression konnten lediglich CL-MSCs die Freisetzung von IFN-γ durch Lymphozyten in einer gemischten Lymphozyten-Reaktion reduzieren.

Die Ergebnisse dieser Arbeit verdeutlichen, dass CL-MSCs die besten Eigenschaften für zell-basierte Strategien aufweisen. CL-MSCs sind hypo-immunogen und besitzen hohe Proliferations- und Migrationraten. Darüber hinaus zeigt die vorliegende Arbeit zum ersten Mal, dass obwohl die immunmodulatorischen Moleküle HLA-G, HLA-E, und TGF-β eine wichtige Rolle bei der MSC Immunevasion spielen, die HLA-Expression entscheidend für die Immunogenität von MSCs ist.

Daten, die das Verhalten von CL-MSCs *in vivo* veranschaulichen, fehlen und Studien über das Migrationsverhalten von CL-MSC sind notwendig, bevor die Zellen in einem klinischen Ansatz angewendet werden können. Intravenös (IV) injizierte CL-MSCs wurden in der Lunge abgefangen, und eine weitere Migration in andere Organe konnte nicht beobachtet werden.

Schlagwörter: Mesenchymale Stromazellen, Zelltransplantation, Immunogenität, Migration.

1. INTRODUCTION

1.1. Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) have gained a lot of attention in the last decade as candidates for tissue repair (1,2), as modulators of immune responses in autoimmune diseases (3,4), in prolonging solid organ survival after transplantation (5,6), and in combating graft-versus-host-disease after bone marrow transplantation (7). The existence of a non-hematopoietic stem cell population resident in the bone marrow was postulated nearly 140 years ago by Cohneim (8). Friedenstein and co-workers were the first to identify and isolate spindle-shaped cells with clonogenic potential from rodent bone marrow (9). These cells, initially named osteogenic stem cells or bone marrow stromal stem cells were able to form bone when injected subcutaneously into animals and supported the reconstitution of the haematopoietic system (10,11). In 1991 osteogenic stem cells were renamed by Caplan as mesenchymal stem cells, as they can also differentiate into adipocytes and chondrocytes which are of mesenchymal lineage (12).

Presently the term mesenchymal stem cell is being replaced by the term multipotent mesenchymal stromal cell (MSC) (13), due to uncertainties with respect to stem cell defining characteristics (14). For a cell to be defined as a stem cell various criteria have to be met including, clonogenicity, multi-lineage developmental and self-renewal capacity. MSCs show clonogenic potential and after plating *in vitro* form so called fibroblast colony-forming units (CFU-F). Pittenger *et al.* and Liechty *et al.* demonstrated the multi-lineage developmental capacity of MSCs *in vitro* (15) and *in vivo* (16). Colter *et al*. demonstrated that human BM-MSCs seeded *in vitro* at low density are able to self-renew (17), while others have found that MSCs fail to show an unlimited self-renewal capacity in culture (18). The most stringent test for stem cells was developed for haematopoietic stem cells and involves the transplantation of the candidate cell into a primary recipient, next harvesting the candidate cells from reconstituted tissues and finally transplanting them into a second recipient (19). So far MSCs

have been unable to pass this process successfully and therefore cannot be called stem cells.

The identity and role of MSCs *in vivo* remains poorly understood. Da Silva Meirelles *et al.,* have suggested that MSCs are associated with the perivascular niche and play a role in endogenous tissue repair by supporting other cell types, including adult stem cells (20). Bone marrow-derived MSCs (BM-MSCs) are proposed to play a role in haematopoietic stem cell (HSC) survival and regulate HSC migration into the blood-stream from their niche in the bone marrow ((21) and reviewed in (22)). The association of MSCs with the vasculature is interesting and could explain the fact that so far MSCs have been isolated from a wide range of organs including; heart, liver, bone marrow (23), peripheral blood (24), placenta (25), umbilical cord tissue and blood (26–28), amniotic fluid (29), spleen, thymus (30) and adipose tissue (31,32).

Table 1: Minimal criteria to identify human multipotent mesenchymal stromal cells (13). *CD, cluster of differentiation; HLA, human leukocyte antigen.*

1. Adherence to plastic in standard culture conditions *in vitro.*
2. Phenotype:

Positive (≥ 95%):	Negative (≤ 2%):
CD105, CD90, CD73.	HLA-DR, CD45, CD34, CD79α or CD19, CD14 or CD11b.

3. *In vitro* differentiation into osteoblasts, adipocytes and chondroblasts as demonstrated by staining of *in vitro* cell cultures.

Results from MSC studies are difficult to compare due to a lack of standardized MSC isolation methods and a lack of a unique cell surface marker that can be utilized to distinguish the cells *in vitro*. In 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) tried to tackle this problem

by issuing a position statement defining the minimal criteria to identify human multipotent mesenchymal stromal cells *in vitro* (Table 1) (13).

Presently, it is commonly accepted that MSCs are a heterogeneous population, differing in respect to cell size, morphology, proliferative capacity and potential for differentiation (33,34). Differences in differentiation potential and epitope expression have even been reported among MSCs from different strains of inbred mice (35). These observed differences among MSCs may also translate into differences in therapeutic outcome (36) and therefore highlight the importance of choosing the correct cell type for cellular therapy.

1.2. Therapeutic Potential of Mesenchymal Stromal Cells

MSCs are under evaluation as a novel treatment strategy for a wide range of degenerative and immunological disorders. The immunomodulatory capabilities of MSCs have been used to treat steroid-resistant graft versus host disease (GvHD), which is evoked after the transplantation of bone marrow and is caused by donor-derived immune cells targeting the skin and gastrointestinal tract of the recipient (7). Crohn's disease is another inflammatory disease of the gastrointestinal tract where the immunomodulatory capabilities of MSCs are of use (reviewed in (22)). Studies have shown that the systemic administration of MSCs can also prolong solid organ and graft survival after transplantation by suppressing host immune responses (6,37,38). Wound victims could also benefit from MSC-based therapies as factor secreted by MSCs have shown to enhance wound healing and inhibit scar formation (39). MSCs show also neuroprotective effects and so are under investigation for the treatment of neurological disorders, such as stroke and multiple sclerosis (4,40). The potential of MSCs to regenerate bone has also been explored to treat children with osteogenesis imperfecta (2). Most clinical trials evaluating the benefits of MSC therapies have concentrated on myocardial regeneration, therefore here I provide an overview of the therapeutic mechanisms of MSCs, concentrating on the repair of injured myocardium after infarction.

1.2.1. MSC-Mediated Cardiac Repair

There is an urgent need for new therapeutic strategies for cardiac repair. After coronary occlusion, ischemia leads to cardiomyocyte necrosis and since the regenerative potential of the adult heart is modest (41), the process of infarct healing results in scar formation, leading ultimately to so called "ventricular remodelling" and worsened cardiac function (42). Many studies have suggested that bone marrow cells (43–45) and more specifically bone marrow-derived MSCs (BM-MSCs) (46,47) can differentiate directly into cardiomyocytes and so support heart regeneration after myocardial infarction (MI). In contrast, other studies have questioned this (48,49) and limited the

potential of MSC to differentiate into cells of the mesenchymal lineage namely; adipocytes, chondrocytes and osteocytes (15,50). These discrepancies in the multipotency of MSC could be reconciled with the reported phenomena among bone marrow cells of fusion with other cell types *in vivo* (51,52) or the selection of rare cell populations during prolonged cell culture (33). More importantly, the detection of cardiac proteins, such as atrial natriuretic factor or α-cardiac actin alone is often interpreted as transdifferentiation. Evidence in the form of a cardiomyocyte functional assay would be a more valid proof of true differentiation (49).

Table 2: Mechanisms of MSC-mediated heart regeneration. *CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; FGF, fibroblast growth factor; G-CSF, growth colony-stimulating factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MMP, metalloproteinase; PDGF, platelet-derived growth factor; SDF, stromal cell-derived factor; TNF, tumour necrosis factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.*

Mechanism	Comment / Description
Transdifferentiation into cardiomyocytes.	Supported by: Toma *et al.* (47), Kawada *et al.* (46), Rota *et al.* (43). Contested by: Bianco *et al.* (14), Rose *et al*. (49).
Secretion of factors supporting (53,54):	
1. Angiogenesis,	FGF-2, FGF-7, MCP-1, PDGF, TGF-β, VEGF.
2. Extracellular matrix remodelling,	MMP1, MMP2, MMP9, TNF-α.
3. Stem cell proliferation and recruitment,	bFGF, G-CSF, IGF-1, M-CSF, SDF.
4. Immunomodulation,	See Table 4.
5. Anti-scarring (anti-fibrosis),	HGF, FGF.
6. Cell migration (chemoattractive).	CCL2, CCL3, CCL4, CCL5, CCL6, CCL20, CCL26, CX3CL1, CXCL5, CXCL11, CXCL1, CXCL2, CXCL8, CXCL10, CXCL12.
Inhibiting cardiomyocyte apoptosis.	Li *et al.* (55).

Studies have suggested that the direct conversion of MSCs into cardiomyocytes is not needed for cardiac regeneration, that MSC therapy has been observed to be beneficial without even long-term engraftment of MSCs into the target organ (56). Presently a widely accepted mode of action states that MSCs support heart regeneration by secreting a range of cytokines and growth factors which are able to decrease inflammation and fibrosis, limit negative “ventricular remodelling“ and enhance endogenous cardiomyogenesis and angiogenesis (57–62) (Table 2).

More importantly, factors secreted by MSCs play a cardioprotective role by reducing cardiomyocyte apoptosis and so limiting cardiomyocyte loss after an ischaemic insult (55). This fact is further supported by the recent discovery that MSCs stimulate the proliferation and differentiation of resident cardiac stem cells (63). It is important to point out that improvement in cardiac function after the injection of MSC-conditioned medium alone has been observed by some groups (64), although not by others (63). In summary, the potential of MSCs to exert trophic effects on tissue regeneration, combined with their immunomodulatory properties and low immunogenicity make them a promising option for cell-based repair strategies.

1.2.2. Mesenchymal Stromal Cells in Clinical Trials to Treat Heart Failure

The first clinical trial using bone marrow-derived cells to treat myocardial infarction (65) was conducted just months after the publication of two studies demonstrating bone marrow cell cardiomyocyte transdifferentiation in rodent models of MI (45,66), a process later disproved by Murry *et al*. (67) and others (14,49). Since 2001 numerous have confirmed that bone marrow-derived cells can improve heart function in animal models, although through indirect non-myogenic pathways (68,69). Disappointingly, clinical trial results with bone marrow-derived cells and MSCs are inconsistent (Table 3).

Table 3: Results of clinical trials investigating the use of cell therapy in the treatment of acute myocardial infarction. *ASTAMI, Autologous Stem cell Transplantation in Acute Myocardial Infarction; BOOST, BOne marrOw transfer to enhance ST-elevation infarct regeneration; EF, ejection fraction; REPAIR-AMI, Reinfusion of Enriched Progenitor cells and Infarct Remodelling in Acute Myocardial Infarction.*

Trial	Findings	References
BOOST	Transient functional benefit at 6 month post-infusion.	(70)
REPAIR-AMI	Improvement of EF at 4 months and 2 years.	(71,72)
ASTAMI	No significant improvement in EF.	(73)
Janssens *et al.*	No significant improvement of EF at 4 months, decrease in scar size.	(74)
Prochymal®	Improvement of EF at 6 months post-infusion (only anterior MI patients), reduction in ventricular arrhythmias, improved pulmonary function.	(75)
Chen *et al.*	Improvement of EF at 3 and 6 months post-infusion.	(76)
Katritsis *et al.*	Improvement in left ventricular EF and myocardial perfusion, cellular re-population of previously non-viable infarct scars.	(77)
Yang *et al.*	Improvement of EF and myocardial perfusion at 6 months post-infusion.	(78)

Meta-analysis of clinical trials has revealed an average gain in heart left ventricle ejection fraction (LVEF) of 4 - 5% (79). Unfortunately, due to study heterogeneity and small group sizes in certain trials it is difficult to draw any definitive conclusions. Conclusive evaluation of MSC therapy will only be possible after the completion of large-scale, multicenter, placebo controlled trials. Nevertheless clinicians have learned a number of lessons from phase I and II clinical trials: (i) efficacy is inconsistent and modest, but meaningful benefits have been reported, especially among patients with the greatest myocardial damage; (ii) cell engraftment correlates well with functional benefit, however cardiac engraftment and retention of cells is low; (iii) bone marrow cells show a very limited cardiomyogenic potential; and (iv) autologous cell therapy has severe limitations due to timing constraints and inter-patient variability in cell potency (80). So far none of the heart targeting cell therapies investigated in rodents or humans have been able to demonstrate complete *restitutio ad integrum* of the heart.

Low cell engraftment and retention after transplantation remains a significant obstacle for cardiac cell therapy. Regardless of cell type or delivery route, animal studies have shown that 24 hours after cell delivery less than 10% of administered cells are found in the myocardium (80). One week after delivery the number of cells detected falls below 1% (81). Similarly, clinical studies have confirmed that only 1.3 to 2.6% of infused cells are retained in the heart (82). Reasons for this low cell number in the myocardium are manifold (reviewed in (80)). Firstly, the recently transplanted cells may not be able to survive in a hostile inflammatory or ischemic environment. There could be a lack of stimulatory or pro-survival signals needed to support the exogenous cells. Furthermore, transplanted cells undergo severe sheer stress during injection, which could lead to their apoptosis. Additionally, there could be a lack of cell retention in the tissue as transplanted cells may be washed out by the coronary venous system or from the injection site (80). Finally, if the cells are intravenously administered, there is a problem of limited cell migration to the infarct, due to the lack of appropriate recruitment signals and the trapping of cells in small capillaries of organs such as the lung (83–86).

1.3. Mesenchymal Stromal Cell Immunobiology

Knowledge of how transplanted cells interact with the recipient‘s immune system has great importance for cell-based therapies. Autologous MSC therapy has been explored to treat several diseases, including multiple sclerosis (4), stroke (87), and heart failure (88). Due to a limited access to Good Manufacturing Practice (GMP) grade facilities needed to expand and culture MSCs, economical contraints, inter-patient differences in cell potency, patient age and comorbidities and a narrow time window for cell administration, allogeneic MSC banks could be a more valuable off-the-shelf option for MSC-based therapies. Moreover, a recent study by Wang *et al.*, limits the autologous use of MSCs as the health of the MSC donor has a significant effect on the therapeutic potency of the MSCs (89).

As allogeneic MSCs could be targeted by the recipient‘s immune system, MSC immunogenicity has been extensively studied. The discovery that MSCs express moderate levels of HLA I suggests that they could be recognized by alloreactive host T cells and rejected after transplantation (90). On the contrary, studies have shown that transplantated HLA mismatched MSCs have been well tolerated in animals (37,91). Moreover, *in vitro* studies with human MSCs have shown that they are resistant to cytotoxic T-cell-mediated lysis (92). Furthermore, human MSCs engraft and persist in multiple tissues, when transplanted into fetal lambs, even if injected after the development of immunocompetence by the fetuses (16). The micro-environment of damaged tissue is rich in IFN-γ, which increases HLA class I and II expression on MSCs and could potentially increase their immunogenicity. Interestingly, IFN-γ-treated MSCs fail to elicit alloreactive lymphocyte responses (90). The bulk of research data to date suggest that, MSCs are able to actively influence an immune response (immunomodulation) and thus induce a state which facilitates their survival *in vivo* after transplantation (93). MSCs have been shown to inhibit T-cell activation (94,95), proliferation (96,97) and function (98). Similarly, some studies have shown that MSCs inhibit Natural Killer (NK)-cell proliferation (99–101). Whether MSCs inhibit NK cytotoxicity (99,102) or not (95) is still widely disputed. Conflicting data has come from studies on the effect of MSCs on B-cells. One study suggests that MSCs promote B-cell prolifer-

ation and antibody production (103), while another suggests an inhibitory effect on B-cell terminal differentiation (104). MSCs also influence the formation of T_H17 cells (105,106) and effect the T_H1/ T_H2 cell balance by altering the cytokine milieu during T_H cell differentiation (93). Moreover, MSCs can activate various immunosuppressive mechanisms by inducing regulatory T cells (105,107), directing dendritic cells to an immune suppressive phenotype and compromising their migration and function (108,109). An elegant set of experiments carried out by Potian *et al.,* showed that MSCs are able to blunt allogeneic immune responses in a mixed-lymphocyte reaction (MLR) (110). This described “veto-like” activity of MSCs does not, however, induce a state of general immune suppression as T-cell responses to viral antigens remain intact (111).

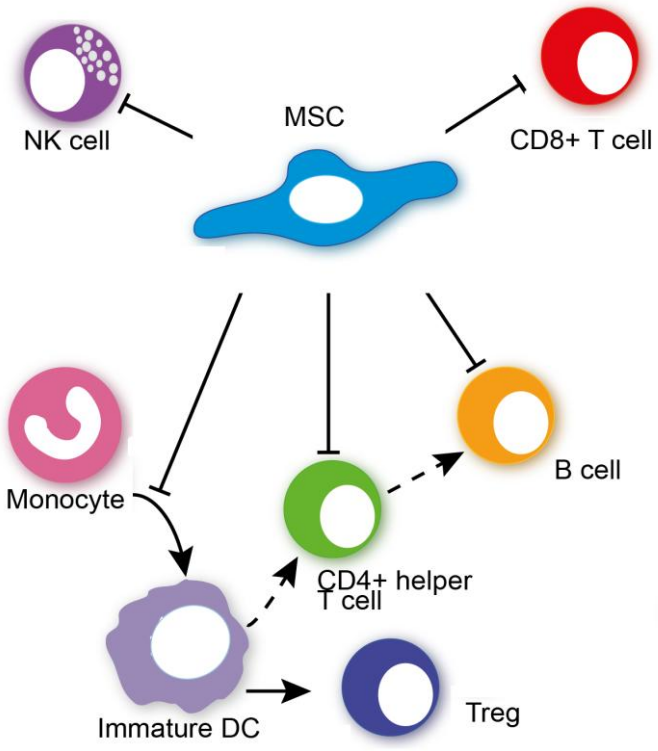

Figure 1: Immunoregulatory properties of MSCs (modified from (58)). *MSCs are able to affect the humoral as well as the cellular arm of the immune system by secreting a range of factors or directly interacting with immune cells via receptors on their cell surface (listed in Table 4). DC, dendritic cells; MSC, mesenchymal stromal cell; NK, natural killer; Treg, regulatory T cell.*

Table 4: Immunomodulatory factors expressed by MSCs. *COX, cyclic oxide synthase; DC, dendritic cells; Gal-1, galectin-1; HLA, human leukocyte antigen; HGF, hepatocyte growth factor; HO-1, hemoxygenase-1; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; LIF, leukaemia inhibitory factor; MLR, mixed-lymphocyte reaction; NK, natural killer; NOS, nitric oxide synthase; PGE_2, prostaglangin E_2; TGF-β1, transforming growth factor-1; TSG-6, TNF-α-stimulated gene-6; TNF-α, tumour necrosis factor-alpha; Tregs, regulatory T cells.*

Factor	Function	References
Gal-1	Suppression of T-cell proliferation. Inhibition of IFN-γ, TNF-α and IL-2-secretion.	(112)
HLA-G	Suppression of T- and NK-cell function, induction of Tregs. Inhibition of IFN-γ-secretion.	(113,114)
HLA-E	Suppression of NK-cell responses.	(115,116)
HGF	Suppression of T-cell proliferation.	(94)
HO-1	Production of biliverdin and carbon monoxide, which inhibit T-cell responses in a MLR and promote Treg induction.	(117)
IDO	Depletion of the essential aminoacid L-tryptophan and production of kynurenine, which inhibits allogeneic T-cell responses and NK-cell proliferation and function.	(99,118)
IL-10	Inhibition of T-cell proliferation. Inhibition of IFN-γ and TNF-α-secretion.	(119)
IL-1ra	Inhibition of TNF-α-secretion by IL-1α-activated macrophages.	(120)
LIF	Generation of Tregs and inhibition of T-cell proliferation.	(121)
NOS	Production of nitric oxide, which inhibits T-cell proliferation.	(122)
PGE_2	Inhibition of DC maturation and function. Inhibition of NK-cell proliferation and effector function. Reprogramming macrophages to an anti-inflammatory M2 phenotype. Produced by COX.	(93,99,101,109,123)
TGF-β1	Induction of Tregs. Inhibition of T- and NK-cell function. Inhibition of IFN-γ and TNF-α-secretion.	(101,119,124)
TSG-6	Inhibition of pro-inflammatory cytokine-release by macrophages.	(125)

The notion that MSCs could be used in therapy without additional immunosuppression is highly attractive and long-term engraftment of allogeneic MSCs without immune rejection has been reported (126). On the other hand some concerns remain, as a few studies have suggested that allogeneic MSC transplantation can lead to the induction of immune responses in the recipient and lead to the rejection of implanted cells (127–131). Donor-specific allo-antibodies have also been detected in animals after multiple, high-dose administrations of MSCs (132). Further arguing against the use of allogeneic MSCs are studies by Huang *et al.* (130) and Poncelet *et al.* (131) demonstrating that MSCs that had shown low immunogenicity in *in vitro* assays, differentiate and induce recipient immune responses after injection into injured myocardium. Surprisingly, MSCs that are differentiated *in vitro* before transplantation do not elicit immune responses *in vivo* (90).

In summary, the observed discrepancies in results from past studies emphasize the importance of continuing studies on MSC immunobiology.

1.4. Mesenchymal Stromal Cell Migration

MSCs seem to employ mechanisms similar to leukocytes transmigrating into inflamed tissue (133). The leukocyte adhesion cascade, which describes the exit of leukocytes from the circulation and entry into tissue involves four steps; cell rolling, integrin activation, firm cell adhesion and finally transmigration (134). Each step is mediated by a distinct set of selectins, integrins, chemokine receptors and ligands.

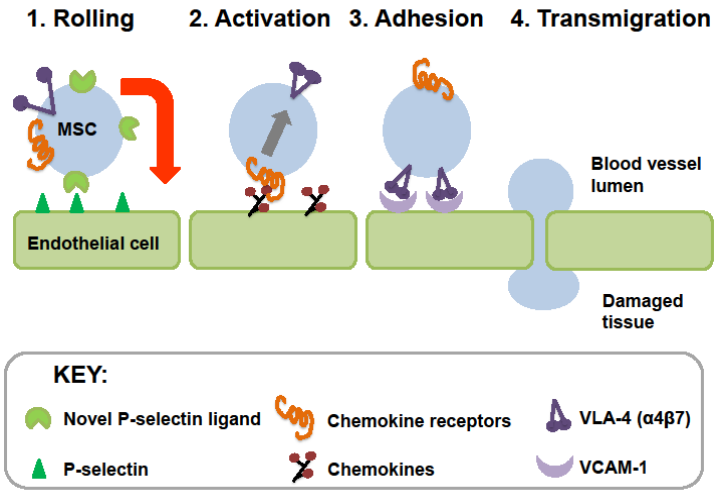

Figure 2: MSC adhesion and transendothelial migration is a multi-step process (modified from (135)). *P-selectin, platelet selectin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.*

The first step of MSC transmigration involves the rolling of MSCs on endothelial cells (Figure 2). P-selectin expression on endothelial cells has been suggested by Ruster *et al.* to mediate MSC rolling (136). MSCs bind to P-selectin with an unidentified ligand, as they do not express any previously described P-selectin ligands. During rolling MSCs encounter chemokines that lead to an increase in integrin ligand-binding capability (so called “integrin activation”). The reported chemokine receptor repertoire for MSCs has been inconsistent (135). The most often reported receptors include: chemokine (C-C motif) receptor-1 (CCR1), CCR7, CCR9, CCR10, chemokine (C-X-C motif) receptor-4 (CXCR4), CXCR5, CXCR6 and CX3CR1 (136–141). Long-term cul-

ture, culture conditions and passage number are proposed to induce chemokine receptor expression heterogeneity (137,142). MSC arrest under sheer flow conditions is mediated by integrins. Studies of the integrin profile of MSCs show that MSCs are positive for α1, α2, α3, aα, αv, β3, β4 (15) and the α4β1 (VLA-4) integrin (15,136), which binds to VCAM-1. Neutralising antibody experiments have proven that the VLA-4/VCAM-1 axis in indispensable for MSC adherence (136), however they also express other molecules; intracellular adhesion molecule-1 (ICAM-1, CD54), ICAM-3 (CD50) and activated-leukocyte cell adhesion molecule (ALCAM, CD166) (15). Although, MSC transmigration over the endothelial barrier has been observed (143), the molecular mechanism is not understood as MSCs do not express the most commonly used adhesion molecule by leukocytes, CD34.

MSCs are released from their niche after injury as suggested by studies documenting elevated levels of MSCs in the peripheral blood of acute burn patients (144). A decrease in circulating MSC pool size one week after myocardial infarction has also been observed, which was attributed to increased recruitment to the injured myocardium (145). Similarly, injected MSCs are able to migrate specifically to injured heart and brain tissue (86,146). Studies by Abbott *et al.* have shown that CXCR4/CXCL12 interactions are needed for MSC migration to the heart (147). This has been supported by observations that CXCL12 (stromal cell-derived factor-1, SDF-1) expression increases in the ischemic heart. However, the concept that the CXCR4/CXCL12 axis plays an important role in MSC migration to myocardium is controversial because, an increase in CXCL12 expression was not detected in the injured heart by Vandervelde *et al*. (148). Furthermore, the surface expression of CXCR4 on MSCs is low and can only be significantly increased after incubating MSCs with cytokines (139). The CXCR4/CXCL12 axis has been proposed to play an important role in MSC migration to the bone marrow (139). Several groups have reported the migration of BM-MSCs to bone marrow after their systemic infusion into immunodeficient mice (149,150) and non-human primates (91). Other organ-specific migration has also been reported including, liver, spleen (150,151) and muscle (149). Lung entrapment within 24 hours of MSC infusion is widely observed and is considered a barrier to administering MSCs via the intravenous route (83,84,149).

1.5. Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

Most of the research on MSCs has concentrated on bone marrow-derived cells (BM-MSCs). They are not an ideal source for cellular therapy, as their isolation is associated with donor morbidity, they may carry a risk of viral and bacterial contamination and more importantly the number of MSCs found in the bone marrow and their regenerative potential significantly declines with donor age (152,153). Hence, MSCs from other tissue sources could be an interesting alternative for BM-MSC-based therapies.

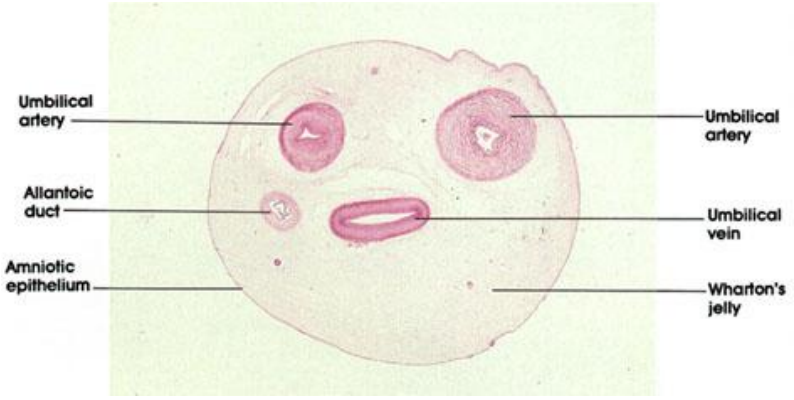

Figure 3: Transverse section of a human umbilical cord (funiculus umbilicalis) (154). *Haematoxylin and eosin staining. Magnification 8.5X.*

Human post-natal gestational tissues are a rich source of MSCs (reviewed in (155)). MSCs have been isolated from the placenta (so called P-MSCs) (25), umbilical cord lining (CL-MSCs) (26,116), cord blood (CB-MSCs) (28) and Wharton's jelly (WJ-MSCs) (27). Their future therapeutic use shows great promise due to their high replicative potential and the fact that their isolation is non-invasive and thus safe for both the new born and mother (155). Extraembryonic tissue-derived MSCs have a lower level of mutations and can be kept in culture for longer periods of time due to the young chronological age of the cells (155). In contrast to BM-MSCs, the procurement of MSCs from post-natal gestational tissue is associated with minimal ethical and legal issues. The premise that post-natal gestational tissue-derived MSCs are less immunogenic to BM-MSCs (116), makes them particularly interesting for allogeneic cell

therapies. Furthermore, they demonstrate immunomodulatory capabilities superior to BM-MSCs (116).

Like BM-MSCs, extraembryonic tissue-derived MSCs are spindle-shaped and plastic-adherent (155). They also show a similarity to BM-MSCs immunophenotype (25,27,116,156). *In vitro* studies have provided evidence for extraembryonic tissue-derived MSC multipotency (25,27,28,116,156). There is also evidence for extraembryonic tissue MSC differentiation into cells outside the mesodermal lineage, including hepatocyte-like, neuronal and glial cells (155,157). Interestingly, some researchers have also reported that MSCs from the extraembryonic tissue compartment express markers associated with pluripotent stem cells; Nanog and Oct-4 (26). MSCs from gestational tissue clearly differ from BM-MSC in terms of origin. MSCs from the umbilical blood and cord are of fetal origin (155), whereas placental MSCs can be fetal or maternal (25). Presently, it is not known whether MSCs from this compartment originate from a common progenitor cell or how they relate to each other.

2. AIM OF STUDY

Mesenchymal stromal cells (MSCs) are presently being investigated as candidates for cellular therapies. To date most of the research on MSCs has concentrated on bone marrow-derived cells (BM-MSCs) which are not an ideal source for cellular therapy. On the other hand, MSCs have also been isolated from post-natal gestational extraembryonic tissues such as, the placenta (P-MSC), umbilical cord lining (CL-MSC), umbilical cord blood (CB-MSC) and Wharton's jelly (WJ-MSC). Therefore, the aim of this work was to investigate the suitability of these cells for cell-based therapies. For this the following properties were explored:

- fulfillment of criteria accepted for human multipotent MSCs,
- proliferation and migration rates,
- survival potential *in vivo*,
- immunogenicity *in vitro* and *in vivo*,
- immunomodulatory properties *in vitro* and *in vivo*.

CL-MSC migration studies are warranted before the cells can be applied in a clinical setting. Therefore, the second aim of this study was to examine the migration of intravenously-injected human CL-MSCs in immunodeficient, NOD SCID mice.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Consumables

Table 5: Consumable materials

Consumable Material	Manufacturer
96-Well Delta Surface Flat-Bottom Plates	Nunc, Thermo Fischer Scientific, GER
96-Well MAXISORP Plates	Nunc, Thermo Fischer Scientific, GER
96-Well Round-Bottom Plates	SARSTEDT AG & Co, GER
48-Well Flat-Bottom Plates	Cellstar®, Greiner Bio-One, GER
24-, 12- and 6-Well Flat-Bottom Plates	Falcon™, BD Biosciences, GER
Micro slides Super Frost / Plus (76 x 26 mm)	Assistant® Glaswarenfabrik Karl Hecht, GER
Cover Slips (24 x 50 mm)	Carl Roth GmbH & Co. KG, GER
50- and 15-ml Polypropylene Tubes	Cellstar®, Greiner Bio-One, GER
0.2-, 1.5- and 2.0-ml Micro-tubes	SARSTEDT AG & Co, GER
5 ml Round-Bottom FACS Tubes	Falcon™, BD Biosciences, GER
40 µm Cell Strainers	Falcon™, BD Biosciences, GER
1 ml Syringes	B. Braun Melsungen AG, GER
26 G Needles (0.45 x 25 mm)	B. Braun Melsungen AG, GER
0.2 µm Syringe Filter	VWR International GmbH, GER
1 ml Cryotubes	Nunc, Thermo Fischer Scientific, GER
1000, 100 and 10 µl Pipette Tips	SARSTEDT AG & Co, GER
ART® 1000, 200, 100 and 10 µl Pipette Filter Tips	Molecular BioProducts, Inc., GER
25, 10, 5 and 1 ml Pipettes	Falcon™, BD Biosciences, GER
175 and 75 cm^2 Cell Culture Flasks	SARSTEDT AG & Co, GER
50 and 25 cm^2 Cell Culture Flasks	Falcon™, BD Biosciences, GER

100 x 15 mm Petri-Dishes	Falcon™, BD Biosciences, GER
60 x 15 mm cell Culture Dishes	Falcon™, BD Biosciences, GER
18 cm Cell Scraper	Falcon™, BD Biosciences, GER
Plate Sealing Film	Axygen, GER
100 ml Disposable Reagent Reservoir	VWR International GmbH, GER
Disposable Scalpels	B. Braun Aesculap AG, GER
Disposable, Powder Free Gloves	HARTMANN, GER
Polypropylene Sutures	Ethicon, Inc., GER
Neubauer Chamber	Assistant® Glaswarenfabrik Karl Hecht, GER

3.1.2. Reagents, Kits and Buffers

Table 6: Reagents

Reagent	Manufacturer
Acetic Acid (CH_3COOH), 100%	Sigma-Aldrich® Co. GER
Alizarin Red S	Sigma-Aldrich® Co., GER
Bovine Serum Albumin (BSA)	Sigma-Aldrich® Co., GER
D-Luciferin Firefly Potassium Salt	BIOSYNTH®, Switzerland
Dimethyl Sulfoxide (DMSO) Hybri-Max™	Sigma-Aldrich® Co., GER
EmbryoMAX® UltraPure Water with 0.1% Gelatin	Millipore™, Merck KGaA, GER
Ethanol (C_2H_5OH), absolute 100%	Merck KGaA, GER
Foetal Calf Serum (FCS)	Gibco®, Invitrogen, USA
Ficoll-Paque™ PLUS Solution	GE Healthcare, GER
Formaldehyde (CH_2OH), 37%	Carl Roth GmbH & Co. KG, GER
Haematoxylin	Waldeck GmbH & Co., GER
Hydrogen Chloride (HCl), 1N (1 Mol/l)	Merck KGaA, GER

Isoflurane	Abbot GmbH & Co KG, GER
Isopropanol (C_3H_7OH), 99%	Sigma-Aldrich® Co., GER
Methanol (CH_3OH)	Merck KGaA, GER
Mitomycin C	Sigma-Aldrich® Co., GER
Oil Red O	Merck KGaA, GER
Paraformaldehyde ($H[CH_2O]_{8-100}OH$), 20%	EMS Science Services GmbH, GER
Polybrene®	Sigma-Aldrich® Co., GER
Puromycin	Santa Cruz, USA
Protease Inhibitor Cocktail	Sigma-Aldrich® Co., GER
Sodium Chloride (NaCl), 1N (1 Mol/l)	Merck KGaA, GER
Sodium Nitroprusside (SNP)	Sigma-Aldrich® Co., GER
Triton® X-100	Sigma-Aldrich® Co., GER
Trypan Blue Stain, 0.4%	Gibco®, Invitrogen, USA
Toluidine Blue	Fluka, Sigma-Aldrich® Co., GER
TWEEN® 20	Sigma-Aldrich® Co., GER
UltraPure™ DNase and RNase Free Distilled Water	Gibco®, Invitrogen, USA

Table 7: Cytokines

Cytokine	Concentrations	Manufacturer
Human Basic Fibroblast Growth Factor (bFGF)	10.0 ng/ml	Peprotech, Inc., USA
Human Recombinant Interferon-γ (IFN-γ)	25 ng/ml, 500 ng/ml	Peprotech, Inc., USA

Table 8: Kits

Kit	Manufacturer
AEC Detection System for ELISPOT	BD Biosciences, GER
BCA Protein Assay Kit	Thermo Scientific, USA
CellTiter 96® Aqueous One Solution Cell Proliferation Assay	Promega GmbH, GER
Human IFN-γ ELISPOT Set	BD Biosciences, GER
Human IL-4 ELISPOT Set	BD Biosciences, GER
Mouse IFN-γ ELISPOT Set	BD Biosciences, GER
Mouse IL-4 ELISPOT Set	BD Biosciences, GER
Mouse IL-17 ELISPOT Set	BD Biosciences, GER
OptEIA™ ELISA Set Human IL-2	BD Biosciences, GER
OptEIA™ ELISA Set Human IL-10	BD Biosciences, GER
OptEIA™ ELISA Set Human TGF-β1	BD Biosciences, GER
OptEIA™ Reagent Set B	BD Biosciences, GER

Table 9: Ready-to-use buffers

Buffer	Manufacturer
D-PBS (Dulbecco's PBS) (+) $MgCl_2$, (+) $CaCl_2$	Gibco®, Invitrogen, USA
PBS pH 7.2 (-) $MgCl_2$, (-) $CaCl_2$	Gibco®, Invitrogen, USA
PBS pH 7.4 (-) $MgCl_2$, (-) $CaCl_2$	Gibco®, Invitrogen, USA
Restore Western Blot Stripping Buffer	Pierce, Thermo Scientific, USA
RIPA Buffer	Sigma-Aldrich® Co., GER
Saline 0.9% NaCl	B. Braun Melsungen AG, GER

Table 10: Self-made buffers

Buffer	Content	Manufacturer
FACS Buffer:	10 ml FCS (heat inactivated)	Gibco®, Invitrogen, USA
	500 ml PBS pH 7.4	Gibco®, Invitrogen, USA
Permeabilization Buffer:	500 ml TBS	See recipe below
	5 ml Triton® X-100	Sigma-Aldrich® Co., GER
	5 g BSA	Sigma-Aldrich® Co., GER
TBS:	1 pack BupH Tris Buffered Saline	Thermo Scientific, USA
	500 ml UltraPure™ DNase and RNase Free Distilled Water	Gibco®, Invitrogen, USA

3.1.3. Cells and Media

Table 11: Cells

Cell Type	Description	Reference
CL-MSC	Isolated from the umbilical cord (*funiculus umbilicalis*) lining of full-term human neonatals.	(26)
CB-MSC	Isolated from the umbilical cord (*funiculus umbilicalis*) blood of full-term human neonatals.	(28)
P-MSC	Isolated from the placenta (*placenta*) of human neonatals.	(25)
WJ-MSC	Isolated from the umbilical cord (*funiculus umbilicalis*) matrix of full-term human neonatals.	(27)

Table 12: Cell culture media

Cell Type	Medium	Manufacturer
CL-MSC	PTT4	Cell Research Corporation, Singapore
CB-MSC	500 ml DMEM + GlutaMAX-1 Low Glucose (1000 mg/ml), phenol red,110 mg/l sodium pyruvate	Gibco®, Invitrogen, USA
	MSCGM Supplement Mix:	Pioetics®, Lonza, GER
	50 ml MCGS	
	0.5 ml Gentamicin Sulfate	
	500 µl Amphotericin – B	
P-MSC	500 ml DMEM + GlutaMAX-1 Low Glucose (1000 mg/ml), phenol red,110 mg/l sodium pyruvate	Gibco®, Invitrogen, USA
	100 ml Heat Inactivated FCS	Gibco®, Invitrogen, USA
	5 ml Penicillin – Streptomycin 100X	Gibco®, Invitrogen, USA
WJ-MSC	450 ml Human MSC Expansion Medium	CET, USA
	50 ml Heat Inactivated FCS	Gibco®, Invitrogen, USA
	5 ml Penicillin – Streptomycin 100X	Gibco®, Invitrogen, USA

Table 13: Cell culture supplements and differentiation media

Medium	Manufacturer
Foetal Calf Serum (FCS) (heat inactivated)	Gibco®, Invitrogen, USA
MSC Adipogenic Differentiation Medium	PromoCell GmbH, GER
MSC Chondrogenic Differentiation Medium	PromoCell GmbH, GER
MSC Osteogenic Differentiation Medium	PromoCell GmbH, GER
Trypsin TrpLE Express	Gibco®, Invitrogen, USA

3.1.4. Antibodies

All primary antibodies used in this work were targeted against human epitopes.

Table 14: Primary antibodies used for flow cytometry

Antibody	Host species	Clone	Manufacturer	Conjugate	Dilution
HLA-ABC	Mouse	DX17	BD Pharmingen™	PE	1:10
HLA-DR+DP+DQ	Mouse	WR18	Abcam	PE	1:20
HLA-E	Mouse	MEM-E/06	Santa Cruz	-	1:20
HLA-G	Mouse	MEM-G/9	Santa Cruz	-	1:20
CD31	Mouse	WM59	BD Pharmingen™	PE	1:10
CD34	Mouse	563	BD Pharmingen™	PE	1:10
CD40	Mouse	5C3	BD Pharmingen™	PE	1:10
CD44	Mouse	G44-26C26	BD Pharmingen™	PE	1:10
CD45	Mouse	H130	BD Pharmingen™	PE	1:10
CD54	Mouse	HA58	BD Pharmingen™	PE	1:10
CD80	Mouse	L307:4	BD Pharmingen™	PE	1:10
CD86	Mouse	2331, FUN-1	BD Pharmingen™	PE	1:10
CD90	Mouse	5E10	BD Pharmingen™	PE	1:20

CD105	Mouse	166707	R&D Systems	PE	1:20
CD117	Mouse	104D2	BD Pharmingen™	PE	1:10
SSEA-1	Mouse	MC-480	R&D Systems	PE	1:20
SSEA-4	Mouse	MCB13-70	R&D Systems	PE	1:20
TRA-1-60	Mouse	TRA-1-60	BD Pharmingen™	PE	1:10

Table 15: Secondary antibodies used for flow cytometry

Antibody	Clone	Manufacturer	Conjugate	Dilution
Anti-Mouse IgG1	Polyclonal	Santa Cruz	PE	1:20
Anti-Mouse IgG2a	Polyclonal	Santa Cruz	PE	1:20

Table 16: Isotype controls used for flow cytometry

Antibody	Clone	Manufacturer	Conjugate
Mouse IgG1, κ	MOPC-21	BD Pharmingen™	PE
Mouse IgG2a, κ	MOPC-173	BD Pharmingen™	PE
Mouse IgG2b, κ	27-35	BD Pharmingen™	PE
Mouse IgG3	133316	R&D Systems	PE
Mouse IgM, κ	G155-228	BD Pharmingen™	PE

Table 17: Antibodies used for western blotting

Antibody	Host species	Clone	Manufacturer	Conjugate	Dilution
IDO	Rabbit	H-110	Santa Cruz	-	1:500
GAPDH	Rabbit	14C10	Cell Signalling	-	1:500
Anti-Rabbit IgG	Donkey	Polyclonal	Amersham	HRP	1:5000

3.1.5. Instruments

Table 18: Instruments

Instruments	Model	Manufacturer
Block Heater	Digital dry block heater	VWR International, Belgium
Blotting System	Xcell II™ Blot Module	Invitrogen, USA
Centrifuge	Heraeus Biofuge PRIMO	Thermo Scientific, GER
Centrifuge	ESPRESSO	Thermo Scientific, GER
Class II Biological Safety Cabinet	MSC – Advantage	Thermo Scientific, GER
CO_2 Incubator	MCO-20AIC	Sanyo Electronic Co., Ltd Japan
Freezing Container	Cryo 1°C „Mr Frosty“	Nalgene®, Thermo Scientific, GER
Dehydrating Machine	TP 1020	Leica Microsystems, GmbH GER
Electrophoresis System	Xcell SureLock™ Mini-Cell	Invitrogen, USA
ELISPOT Plate Reader		Cellular Technology Limited, USA
Flow Cytometer	FACSCalibur	BD Biosciences, USA
Gas Anesthesia System	XGI-8 (Xenogen) with Matrx (MidMark) Xenogen	Caliper Lifesystems, USA
IVIS Imaging System	200 Series. Xenogen Vivo Vision	Caliper Lifesystems, USA
Light Microscope	IT400	VWR International, Belgium
Light Microscope	DMIRE2 / CTRMIC	Leica Microsystems, GmbH GER
Light Microscope		Carl Zeiss MicroImaging GmbH, GER
Microscope Workstation	Q5501W	Leica Microsystems, GmbH GER
Microtome	RM 2145	Leica Microsystems, GmbH GER
Paraffin-Embedding Module	EG 1150H	Leica Microsystems, GmbH GER
“Pipette Boy”		Eppendorf AG, GER

Pipettes	5000-, 1000-, 200-, 10 µl	Eppendorf AG, GER
Plate Reader	Sunrise Magellan v4.0	Tecan Group Ltd., GER
Rocking Rlatform	PMR-30	Grant-Bio, GER
Water Bath	WNB 22	Memmert, GER
Vacuum System	VACUSAFE Comfort IBS	INTEGRA Biosciences, GER
Vortex	REAX top	Heidolph, GER

3.1.6. Software

Table 19: Software programs

Program	Manufacturer
Adobe Ilustrator CS5 version 1	Adobe Systems Inc., USA
Adobe Photoshop CS3 extended version 10	Adobe Systems Inc., USA
CellQuest Pro	BD Biosciences, USA
Corel PHOTO-PAINT X3 version 13	Corel Corporation, USA
EndNote version X2	Thomson Reuters Corporation, USA
Flow Jo version 7.2.5	Tree Star, Inc., USA
Graphpad PRISM versions 4.0 and 5.0	Graphpad Software Inc., USA
Image J version 1.44p	National Institute of Health, USA
Living Image 3.0	MediaCybernetics, USA
Mendeley version 1.3.1	Mendeley, USA
MRX Revelation version 4.22	Dynex Technologies GmbH, GER
QWin acquisition	Leica Microsystems, GmbH GER
Statistical Package for the Social Sciences (SPSS) version 17.0	SPSS Inc., USA

3.2. Methods

3.2.1. Mesenchymal Stromal Cell Isolation and Culture

All MSCs were cultured in the media listed in Table 12. CL-MSCs (kindly provided by CellResearch Corporation) were isolated as previously described (26). CB-MSCs (kindly provided by Dr. Bieback) were isolated and cultured according to a previous protocol (28). P-MSCs (a kind gift of Prof. Atkinson) were isolated as described by Barlow *et al.* (25). WJ-MSCs were obtained from Thermo Scientific and cultured in medium supplemented with or without 10 ng/ml recombinant basic fibroblast growth factor (bFGF). WJ-MSCs and P-MSCs were cultured in 0.1% gelatin coated culture flasks.

All MSCs were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until 70 - 80% confluent before trypsinization or being removed by scraping with a cell scraper (for flow cytometry, proliferation and western blot analysis) for further analysis. Cells used in this study were passages 2 to 15.

3.2.2. Animals

Six to 8-week old male BALB/c and SCID Beige (CB17.Cg-*Prkdc*^{scid} Lyst^{bg}/Crl) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under specific pathogen-free conditions in the animal care facilities of the University Heart Center Hamburg, Germany. Severe-combined immunodeficient mice (SCID) carry a mutation in the protein kinase, DNA activated catalytic polypeptide (*Prkdc*) gene, which plays a pivotal role in T- and B-cell receptor recombination. Therefore, SCID mice do not have any mature B and T cells. The additional presence of a mutation at the *beige* loci is responsible for the lack of a functional NK-cell response in these mice (158).

To study CL-MSC migration *in vivo* immunodeficient NOD SCID (NOD/NCrCrl-Prkdc^{scid}) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Non-obese diabetic (NOD) have a deficient NK-cell response, lack complement activation and have defective myeloid cell development and function (158). All animals

received humane care in compliance with University of Hamburg Guidelines (Germany).

3.2.3. Lentiviral-Mediated Gene Transfer

To study MSC survival and rejection *in vivo*, cells were made to express luciferase (FLuc) from the firefly (*Photinus pyralis)* under control of the cytomegalovirus promoter (pCMV) by transducing with the lentiviral (LV) vector LV-pCMV-FLuc (Figure 4A) (Addgene plasmid 17477) (159). To study CL-MSC migration *in vivo*, CL-MSCs were made to express FLuc by transducing with the LV-pEF-1α-FLuc (SN1) vector (Addgene plasmid 22524) (Figure 4B) (159).

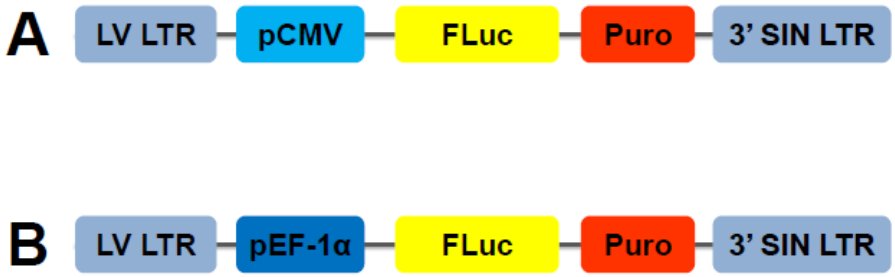

Figure 4: Schema of the lentiviral constructs, which allow for the stable expression of FLuc. *(A): All extraembryonic tissue-derived MSCs were made to express constitutively FLuc. (B): In addition, to study CL-MSC migration in vivo, CL-MSCs were made to express FLuc under control of the EF-1α promoter. CMV, cytomegalovirus; EF-1α, elongation factor-1 alpha; FLuc, firefly luciferase; LV, lentivirus; p, promoter; Puro, puromycin; SIN, self inactivating; LTR, long terminal repeats.*

Transduction efficiency of low passage number MSCs (maximum passage 5) was enhanced by adding 8 µg/ml polybrene into cell culture medium with the lentiviral supernatant. Stable transfectants were enriched by puromycin selection at 0.08 µg/ml (CL-MSC, P-MSC, WJ-MSC) or 0.5 µg/ml (CB-MSC) before cryo-preservation. Before injection into mice, each thawed MSC batch was tested for FLuc expression.

To estimate transduction efficiency, the cells were tested for FLuc activity in a cell number titration assay. For this, MSCs were seeded in a 24-well plate. After 2 hours the medium was replaced with 1 ml of D-luciferin (0.9 mg/ml) dissolved in PBS. The cells were considered positive if a distinct signal above background level was observed.

3.2.4. ELISPOT

To test the immunogenicity of MSCs *in vivo* 1.0 x 10^6 MSCs in 60 µl PBS were injected into the thigh muscle of BALB/c mice (*n* = 6 - 8 *per* cell type). Spleens were harvested after 5 days and 1.0 x 10^7 splenocytes were used as responder cells with 1.0 x 10^6 mitomycin C treated-MSCs as stimulators. After 24 hours mouse IL-4, IL-17 or IFN-γ-secreting cells were detected in an enzyme-linked immunosorbent spot (ELISPOT) assay according to the manufacturer's instructions (BD Biosciences).

To test the potential of MSCs to elicit an immune response from human PBMCs, 5.0 x 10^6 Ficoll-isolated PBMCs were used as responder cells with 0.1 x 10^6 MSCs. Human IL-4 and IFN-γ responses were detected after a 4-day incubation period with an ELISPOT assay according to the manufacturer's instructions (BD Biosciences).

To test the potential of MSCs to inhibit a one-way mixed-lymphocyte reaction (MLR), 5.0 x 10^6 allogeneic Ficoll-isolated human PBMCs were used as responder cells with 0.5 x 10^6 mitomycin C-treated PBMCs and 0.1 x 10^6 MSCs. Human IL-4 and IFN-γ cytokines were detected after 4 days in an ELISPOT assay according to the manufacturer's protocol (BD Biosciences). All ELISPOT spots were counted using an ELISPOT plate reader (CTL, USA).

3.2.5. ELISA

Human IL-2, IL-10 and TGF-β1 were detected in cell culture supernatants using the BD OptEIA™ ELISA sets and BD OptEIA™ Reagent SET B according to manufacturer’s instructions (BD Biosciences). The cells were cultured at a density of 2.7 x 10^4 cells / cm^2 for 48 hours before the cell culture supernatant was collected and stored

at -20°C. Where specified recombinant IFN-γ was added to the medium at a concentration of 25 ng/ml. The detection limit of the enzyme-linked immunosorbent assay (ELISA) is 125 pg/ml for TGF-β1 and 7.8 pg/ml for IL-2 and IL-10.

3.2.6. MSC Lineage Differentiation

For the adipocyte and osteocyte differentiation the cells were plated in 24-well plates (6×10^4 cells / well in 2 ml) in MSC culture medium and incubated at 37°C in 5% humidified CO_2. After 24 hours the culture medium was changed to the appropriate differentiation medium (PromoCell). For the chondrocyte differentiation the cells were plated in 96-well suspension plates (1×10^5 cells / well in 200 µl). For all cultures the differentiation medium was changed three times *per* week for 21 days or 14 days (adipocytes). Next, the cells were stained for adipocyte (oil red O and haematoxylin counter-staining), chondrocyte (toluidine blue) and osteocyte (alizarin red S) differentiation as previously described (116).

MSC differentiated into adipocytes were fixed with 10% PFA for 10 minutes. After washing with deionized water, 60% isopropanol was added for 10 minutes. After another washing step, the cells were stained with oil red O solution (3 mg/ml) dissolved in isopropanol for 5 minutes. The cells were counter-stained with haematoxylin (30 seconds), before a short wash step with 1% acetic acid. Lastly, the cells were washed with tap water before acquiring images with a phase contrast microscope (Leica Microsysteme).

Paraffin-embedded chondrospheres were sectioned (5 µm) and stained with toluidine blue staining solution dissolved 5-fold in 1% NaCl for 3 minutes. The toluidine stock solution was created by dissolving 1 mg/ml toluidine blue in 70% ethanol. Chondrocyte images were acquired with a Zeiss microscope.

Calcium deposits were detected with an alizarin red S staining. MSC differentiated into osteocytes were fixed with 10% PFA for 10 minutes. After washing with distilled water, the alizarin red S staining solution (20 mg/ml) diluted in distilled water was

added for 2 minutes. Next, the cells were washed with tap water and viewed under a phase contrast microscope (Leica Microsysteme).

3.2.7. Antibody Staining and Flow Cytometry

MSCs were incubated with antibody in 100 µl fluorescence-activated cell sorting (FACS) buffer for 45 minutes at 4°C in the dark. Next the cells were washed with 600 µl FACS buffer and centrifuged for 3 minutes (1200 revolutions *per* minute). After the supernatant was removed, the cells were re-suspended in 200 µl FACS buffer for data acquisition. Data was acquired on a FACSCalibur flow cytometer using CellQuest Pro software (all BD Biosciences) and analysed using FlowJo software (Tree Star, Inc.). The fluorescence of 10 000 gated live cells was measured per sample. The samples were positive when the mean fluorescent intensity (MFI) was at least one fold higher than the MFI of the matched isotype control. The data are shown as the mean from at least three independent experiments. Where specified, 25 ng/ml or 500 ng/ml recombinant IFN-γ was added to the cell culture medium for 48 hours before flow cytometry.

3.2.8. MTS Proliferation Assay

To compare the proliferation rate of MSCs, cells were seeded in a 96-well flat-bottom plate (4000 cells *per* well in 100 µl culture medium) and incubated at 37°C in 5% humidified CO_2. Medium was changed every second day. Cell counts were quantified every day for 4 consecutive days using a methyl-tetrazolium salt (MTS)-based assay according to manufacturer's instruction's (Promega). Absorbance at 490 nm was measured with the Magellan ELISA Reader and Software (Tecan Systems Inc.).

3.2.9. Scratch Migration Assay

MSCs were plated in 0.1% gelatin coated 6-well plates (1×10^6 cells *per* well in 2 ml medium). A scratch in the confluent cell mono-layer was made with a pipette tip

(P200) and after a 6-hour migration period the cells were fixed with 10% PFA for 10 minutes and then washed with PBS. Phase contrast images were acquired with a Leica Microscope and QWin acquisition software (Leica Microsysteme). Migrated cells in 3 - 4 segments of the scratch area (300 µm x 500 µm each) were counted. Each analysis was performed 10 times (30 – 40 *per* cell type).

3.2.10. IDO and GAPDH Detection

To induce indoleamine 2,3-dioxygenase (IDO) expression in MSCs, 500 ng/ml recombinant human IFN-γ was added to the cell culture medium 48 hours prior to cell harvesting. Next, cellular proteins were extracted from native and IFN-γ-treated MSCs using RIPA Buffer supplemented with Protease Inhibitor Cocktail according to manufacturer's instructions (both Sigma). Protein concentrations were measured using the Pierce BCA Protein Assay Kit. Ten µg protein *per* well was loaded on a NuPAGE Novex 4 - 12% Bis-Tris Gel and separated using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen). Proteins were next transferred onto a 0.2 µm PVDF membrane using the NuPAGE Transfer System where IDO or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected using unconjugated antibodies. After an overnight incubation step horse radish peroxidase (HRP) conjugated antibodies were used to detect rabbit monoclonal antibodies. The antigens were next identified with the ECL Plus Western Blotting Detection Kit according to the manufacturer's protocol (Amersham Biosciences) and imaged with the IVIS 200 system (Xenogen, Caliper Lifesystems). Blots were stripped with Restore Western Blot Stripping Buffer before being re-probed.

3.2.11. MSC *In Vivo* Survival and Rejection Assays

To test the survival of MSCs *in vivo* 1.0 x 10^6 firefly luciferase (FLuc)-positive MSCs in 60 µl PBS were injected into the thigh muscle of immunodeficient, SCID Beige mice n = 4 - 6 *per* cell type). To test for rejection FLuc-positive MSCs were injected into the thigh muscle of immune competent, BALB/c mice (n = 4 - 6 *per* cell type).

FLuc expression was examined just after cell injection, one day later and thereafter every second day until the Fluc signal dropped to levels before injecting labeled cells.

3.2.12. Bioluminescence Imaging (BLI)

For BLI, D-luciferin Firefly, potassium salt (375 mg/kg) dissolved in PBS pH 7.4 was injected intraperitonealy (200 µl *per* mouse) into mice anesthetized with 2% isoflorane. Next the animals were imaged using the IVIS 200 system (Xenogen, Caliper Lifesystems). Region of interest (ROI) bioluminescence was quantified in units of maximum photons *per* second *per* square centimeter *per* steridian (p/s/cm^2/sr). The maximum signal from a ROI was measured using Living Image 3.1 software (Media-Cybernetics).

3.2.13. CL-MSC *In Vivo* Migration Assay

Confluent mono-layers of FLuc expressing CL-MSCs were trypsinated and 0.5×10^6 cells were re-suspended in 100 µl PBS *per* animal (immunodeficient NOD SCID mice). Mice underwent mid-line laparotomy for injection of MSCs into the *inferior vena cava* (IVC). Five minutes prior to MSC injections 25 µl of sodium nitroprusside (SNP) (1 mg/ml) diluted in 200 µl saline was administered. One hour, 24, 48 hours, 7 and 14 days after the injection of MSCs the animals were imaged (BLI) and then sacrificed (n = 2 – 3 animals *per* time point). Removed organs (brain, femur, lungs, heart, liver, kidneys, spleen) and peripheral blood were imaged to trace CL-MSCs.

3.2.14. Statistics

Data are presented as the arithmetic mean ± standard deviation (SD). Comparisons were done by analysis of variance between groups (ANOVA) with least-significant difference (LSD) *post-hoc* tests. Probability values (*p*) of less than 0.05 were considered significant. Statistical analysis was performed using the SPSS statistical software package for Windows (SPSS Inc.).

4. RESULTS

4.1. Characterization of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

It is difficult to define MSCs as they are a heterogeneous group of cells and to date there is no unique marker to easily distinguish them. There is also no standard protocol for their isolation and the available protocols vary depending on the tissue source. For these reasons, in this work criteria suggested by the International Society for Cellular Therapy (ISCT) (Table 1 on page 6) as well as markers known from the literature, were used to support the notion that the cells studied are MSCs (13,160). Furthermore, by following the criteria it was possible to exclude a contamination from other cell types, such as endothelial or haematopoietic stem cells that may have been included in the isolation process.

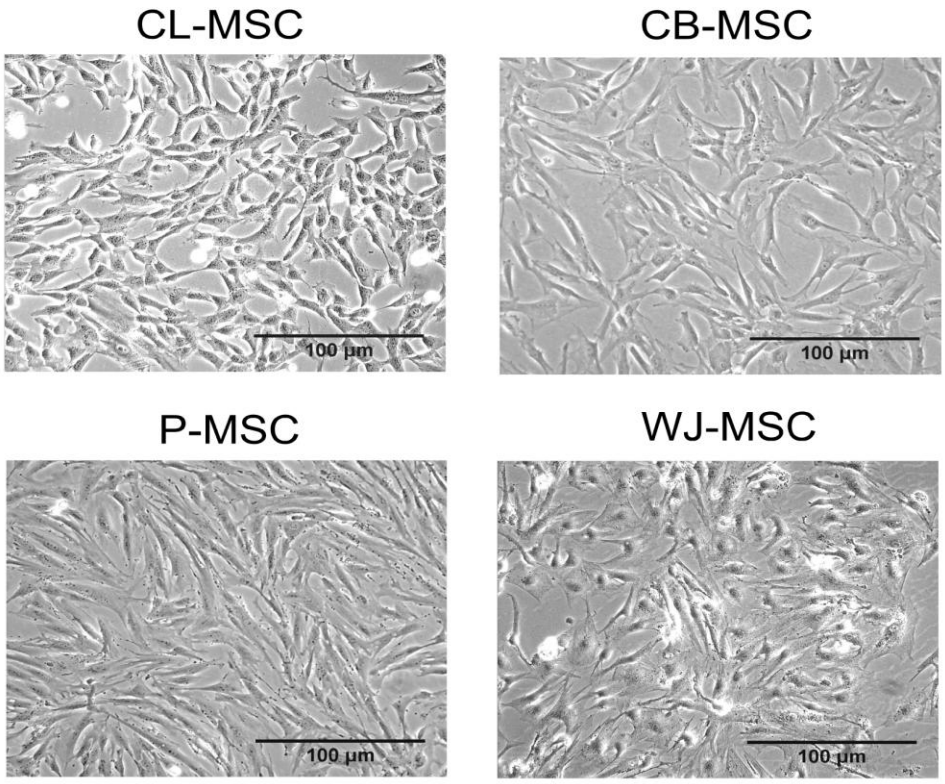

Figure 5: Morphology of human extraembryonic tissue-derived MSCs plated at low density. *Representative phase contrast images show that all the cell types were spindle-shaped and plastic-adherent, so fulfilling the first criterion for MSCs. Scale bar: 100 µm.*

Firstly, all four cell types adhered to plastic and were spindle-shaped (Figure 5). All cell types were highly positive for CD44, CD90 and CD105 (> 95%), which are typically expressed by MSCs (Figures 6 and 7). The cells were negative for endothelial (CD31), haematopoietic lineage (CD34 and CD45) (< 2%) and pluripotent stem cell markers (CD117, SSEA-1, TRA-1-60).

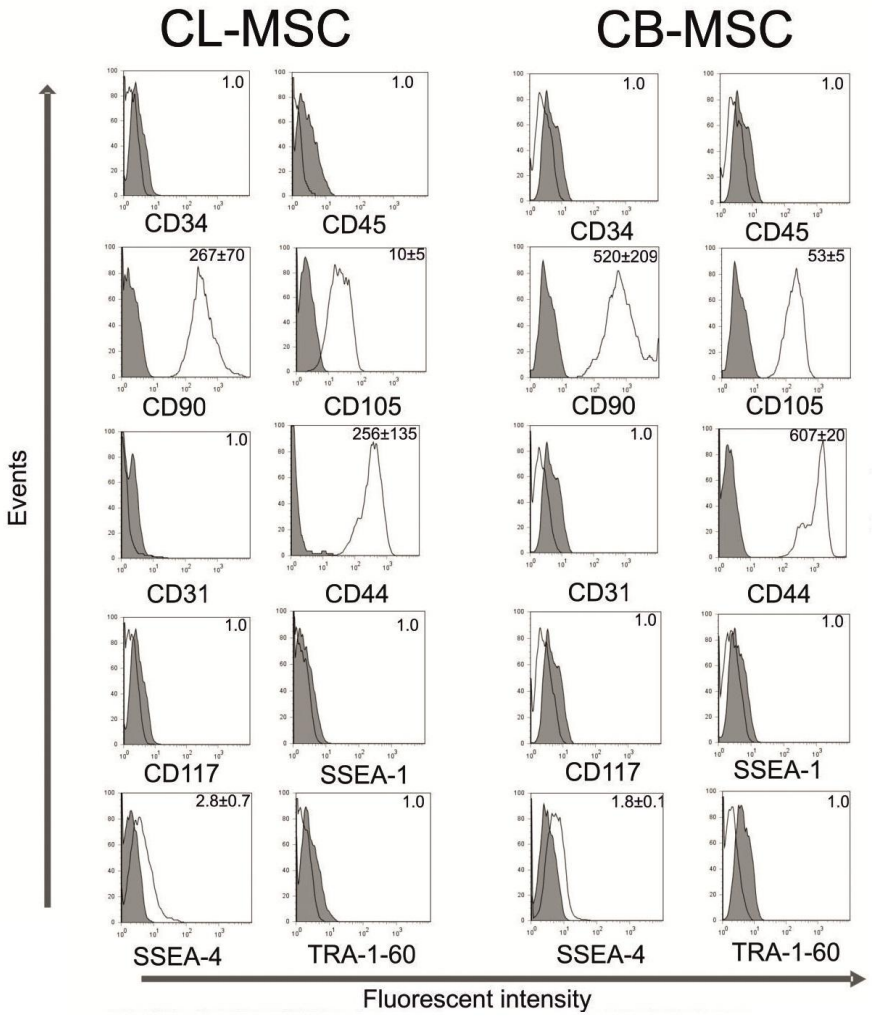

Figure 6: MSC marker expression by human CL-MSCs and CB-MSCs. *Flow cytometry shows that the MSCs were negative for CD34, CD45, CD31, CD117, SSEA-1 and TRA-1-60, but positive for CD44, CD90, CD105 and SSEA-4. The marker expression in each experiment was calculated by dividing the MFI of the marker (empty histograms) with the MFI of the isotype control (filled grey histograms). One representative histogram from 3 independent experiments is shown (10 000 gated cells per cell type in each experiment) with the standard deviations (where applicable) noted in the top right corner. MFI, mean fluorescent intensity.*

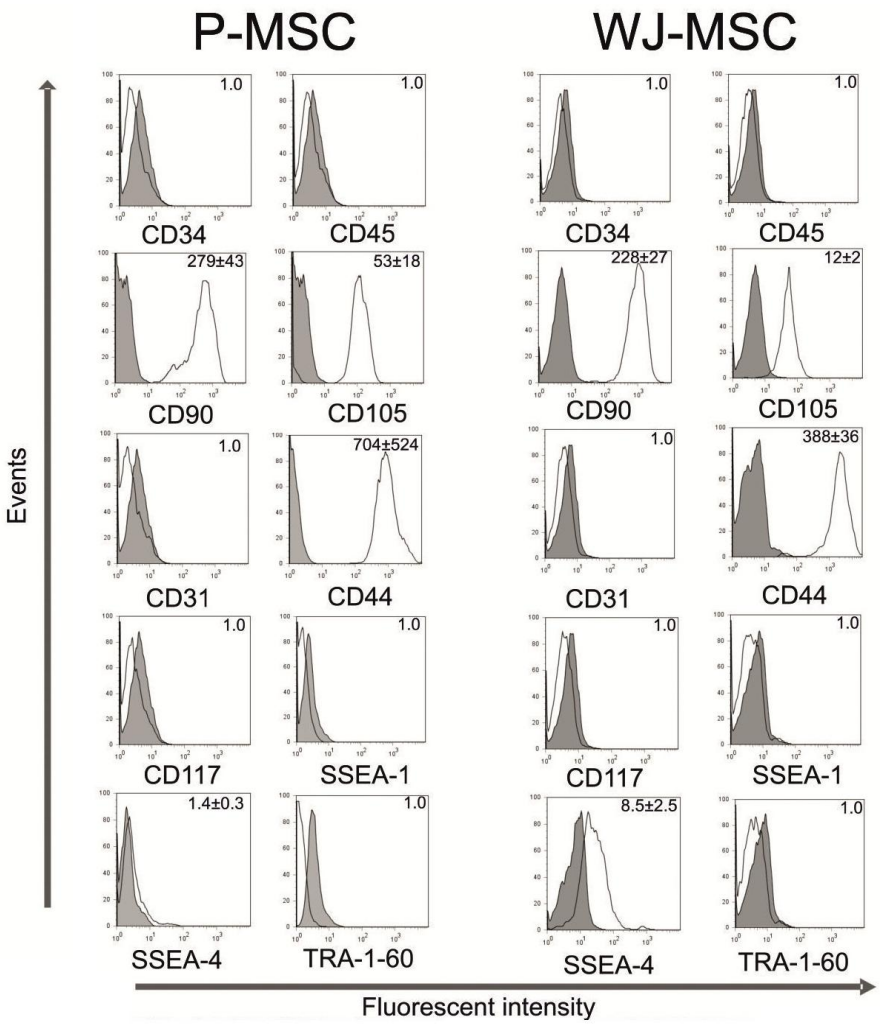

Figure 7: MSC marker expression by human P-MSCs and WJ-MSCs. *Flow cytometry shows that the MSCs were negative for CD34, CD45, CD31, CD117, SSEA-1 and TRA-1-60, but positive for CD44, CD90, CD105 and SSEA-4. The marker expression in each experiment was calculated by dividing the MFI of the marker (empty histograms) with the MFI of the isotype control (filled grey histograms). One representative histogram from 3 independent experiments is shown (10 000 gated cells per cell type in each experiment) with the standard deviations (where applicable) noted in the top right corner. MFI, mean fluorescent intensity.*

The extraembryonic tissue-derived cells were only mildly positive for stage-specific embryonic antigen-4 (SSEA-4). WJ-MSCs expressed SSEA-4 significantly higher than the other extraembryonic tissue-derived cells ($p < 0.001$). SSEA-4 was previous-

ly reported to be present on bone marrow-derived MSCs, however it is expressed at a much higher level (161).

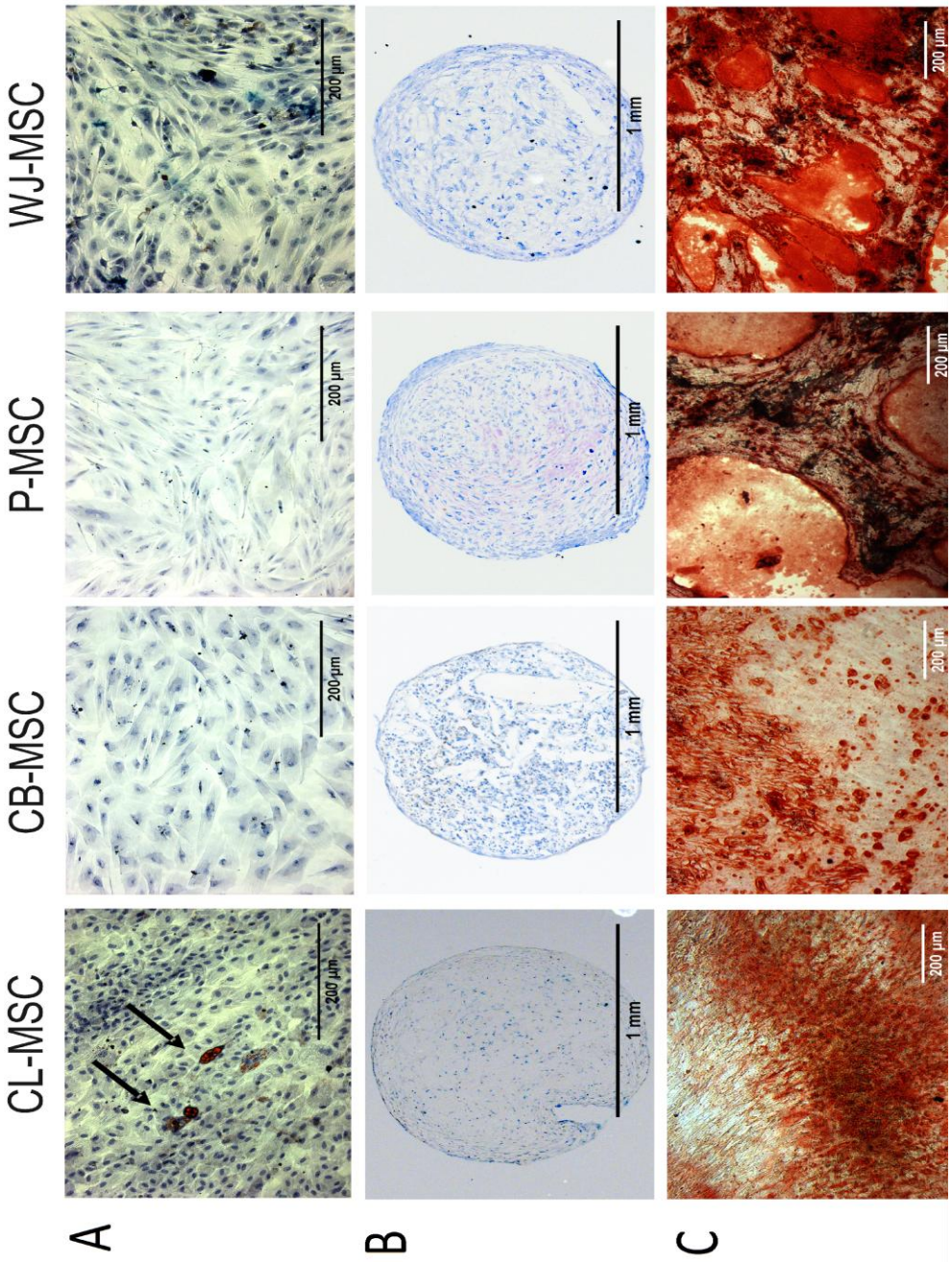

Figure 8: Extraembryonic tissue-derived MSC differentiation. *(A): Only CL-MSCs stained positive for lipid vacuoles with oil red O (indicated by arrows) verified a successful adipocyte differentiation. Counter-staining with haematoxylin. (B): Extraembryonic tissue-derived MSCs are able to differentiate into the chondrogenic lineage as shown with a toluidine blue staining. (C): A positive alizarin red S staining of calcium deposits shows that extraembryonic tissue-derived MSCs are able to differentiate into osteocytes. Scale bar: 200 µm (adipocytes and osteocytes) and 1 mm (chondrocytes).*

To confirm multipotency the cells were differentiated into osteocytes, chondrocytes and adipocytes in culture (described in Material and Methods on page 35). Chondrocyte and osteocyte differentiation was confirmed by detecting proteoglycan or calcium deposits with toluidine blue or alizarin red S, respectively (Figure 8). Oil red O positive-lipid vacuoles, demonstrating a positive adipocyte differentiation were only detected in the CL-MSCs cultures. In agreement with previous studies, CB-MSCs and P-MSCs did not readily differentiate into cells of the adipogenic lineage (25,28,156). In this study, WJ-MSCs also did not show any adipogenic differentiation potential within the 3 week time frame.

In summary, this data supports the notion that only CL-MSCs fulfill the criteria for human multipotent mesenchymal stromal cells according to the International Society for Cellular Therapy (13). As the other cell types were not multipotent, in this work, the abbreviation “MSCs” refers to mesenchymal stromal cells.

WJ-MSCs were the most difficult MSC type to keep in culture. WJ-MSCs rarely survived passaging and were also difficult to expand due to their low proliferation rate. Furthermore, large, pleiomorphic cells similar to senescent fibroblasts dominated the cell culture (162). This state was however reversible as the addition of basic fibroblast growth factor (bFGF) (suggested by Mr. Anant Kamath, Chief Operating Officer Cellular Engineering Technologies, WJ-MSCs supplier) to the cell culture medium induced a dramatic change in WJ-MSC morphology and proliferation rate. The cells became much smaller and spindle-shaped.

4.2. Proliferative and Migratory Properties of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells *In Vivo*

To investigate whether the extraembryonic tissue-derived MSCs differed in their proliferation potential, they were compared in a methyl-tetrazolium salt (MTS)-based assay. In this assay, colourless MTS is converted by cells to a coloured formazan product. Formazan changes the optical density of the culture medium and is correlated with the number of cells in the culture well.

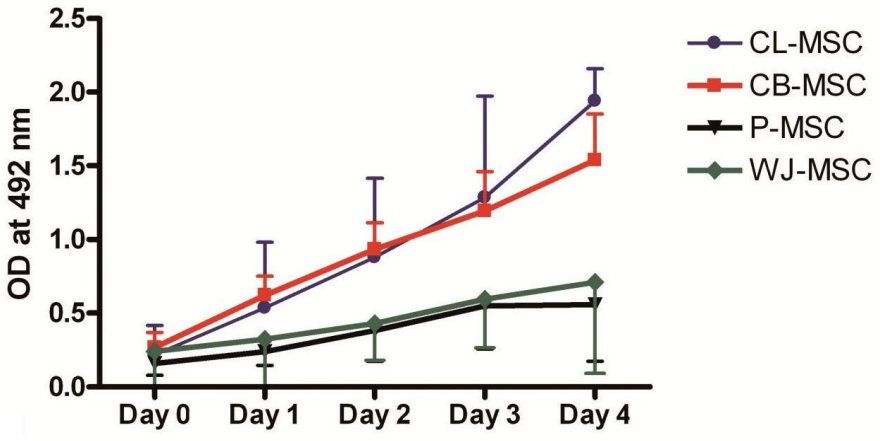

Figure 9: Extraembryonic tissue-derived MSC proliferation. *CL-MSCs showed the highest proliferation capabilities in the MTS assay. Presented are the mean OD +/- SD at 492 nm from 4 independent experiments per cell type (each assay was performed in quadruples). OD, optical density; SD, standard deviation; MTS, methyl-tetrazolium salt.*

The results of the four-day MTS assay showed that CL-MSCs have a significantly higher proliferation rate than P-MSCs (p = 0.003) and WJ-MSCs (p = 0.009), whereas there was no significant difference in the observed proliferation between CB-MSCs and CL-MSCs (Figure 9). CB-MSC showed a significantly higher proliferation rate than P-MSCs (p = 0.015) and this difference was already significant by day 2 (p = 0.047).

The migration capabilities of extraembryonic tissue-derived MSCs were evaluated in a scratch assay, in which the number of cells entering an empty scratch area after 6 hours was counted. CL-MSCs were able to migrate into the scratch area the fastest

(p < 0.001 compared to P-MSC and WJ-MSC) (Figure 10). CB-MSCs showed a slightly lower level of migratory potential, however were still faster than P-MSCs (p < 0.001) and WJ-MSCs (p < 0.001). P-MSCs and WJ-MSCs demonstrated the lowest migratory potential, with the least cells entering the scratch area after 6 hours.

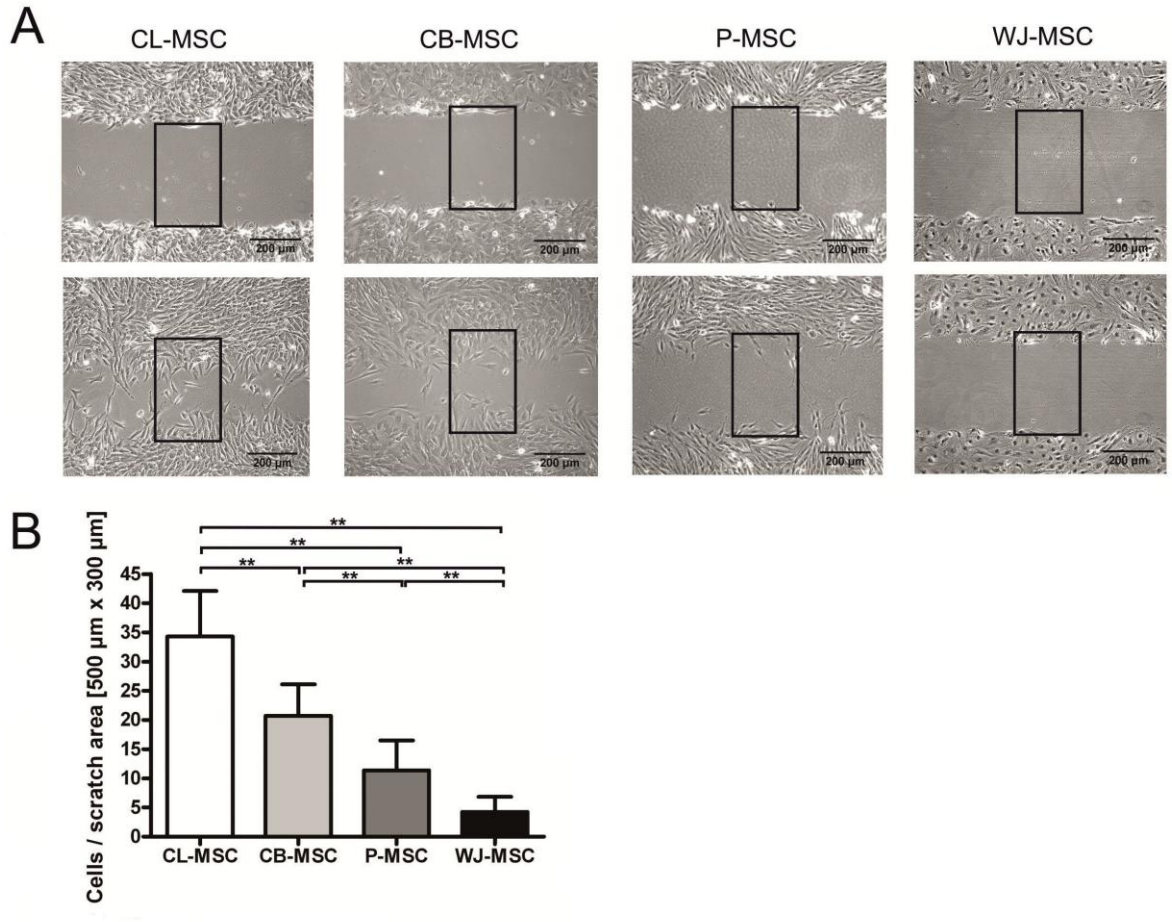

Figure 10: Extraembryonic tissue-derived MSC migration. *(A): Phase contrast images of MSCs show the scratch area at 0 (top panel) and 6 hours thereafter (lower panel). The rectangle represents the scratch area (300 x 500 µm). Scale bar: 200 µm. (B): Quantified scratch assay data showing the mean number of migrated cells into the scratch area + SD after 6 hours. Each analysis was performed 10 times (4 scratch areas were counted per scratch with the exception of WJ-MSCs, were 3 areas were counted per scratch). CL-MSCs migrated into the scratch area significantly faster than the other MSC types. While WJ-MSCs were the slowest migrators. **p < 0.001 (ANOVA) with LSD post-hoc tests. SD, standard deviation.*

4.3. Survival of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells *In Vivo*

To follow the survival of MSCs *in vivo*, the cells were made to constitutively express the ATP-dependent enzyme, firefly luciferase (FLuc). FLuc converts luciferin to oxiluciferin with the emission of light. The intensity of this light signal can be measured and correlates with the number of live FLuc-positive cells.

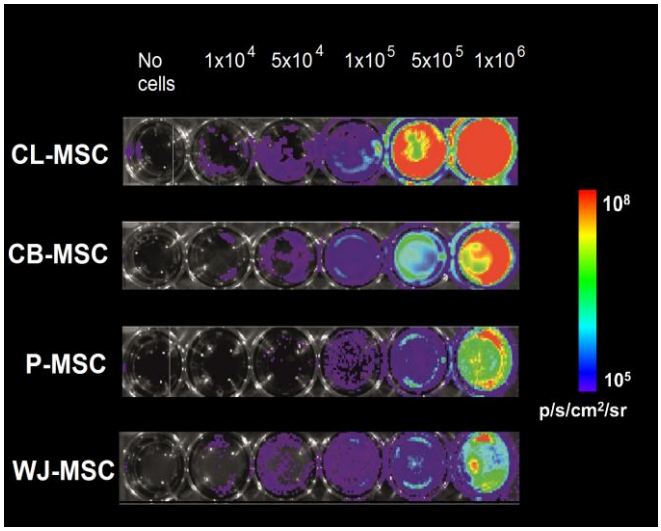

Figure 11: Extraembryonic tissue-derived MSCs were made to constitutively express FLuc under the control of the cytomegalovirus promoter. *To confirm FLuc expression, the cells were plated at densities ranging from 1 x 10^4 to 1 x 10^6 cells per well in cell culture medium. After two hours the medium was replaced with the FLuc enzyme substrate, luciferin and the cells were imaged. Bioluminescence was quantified in units of maximum photons per second per centimeter square per steridian (p/s/cm^2/sr). FLuc, firefly luciferase.*

The FLuc signal measured *in vitro* correlated with live extraembryonic tissue-derived MSC cell numbers after lentiviral transduction ($R^2 \geq 0.94$) (Figure 11). The FLuc signal intensity differed between the extraembryonic tissue-derived MSC types with a stronger FLuc signal detected from CL-MSCs and CB-MSCs. Although an identical number of MSCs were plated in this assay, the difference in the FLuc signal intensity might be due to differences in cell numbers after the two-hour incubation period, just prior to imaging. As demonstrated before in the MTS assay, CL-MSCs and CB-MSCs proliferate faster than P-MSCs and WJ-MSCs and therefore it is likely that a larger

number of CL-MSCs and CB-MSCs were imaged. It is important to note that, FLuc signal intensities just after cell injection into mice and one day later were similar.

To study the survival of MSCs *in vivo*, FLuc-positive MSCs were injected into the thigh muscle of immunodeficient SCID Beige mice. Bioluminescent images were acquired just after cell injection, one day later and thereafter every second day until the signal reached background levels. Extraembryonic tissue-derived MSC proliferation rates measured in the MTS assay correlated with their survival capabilities in SCID Beige mice (Figure 12). FLuc-positive CL-MSC survived 21 days in SCID Beige mice compared to 15 days survival for CB-MSCs ($p < 0.05$), while P-MSC and WJ-MSC showed the shortest survival capacity as FLuc signals reached background levels already by day 11 ($p = 0.01$ and $p = 0.007$ for CL-MSCs versus P-MSCs and WJ-MSCs, respectively).

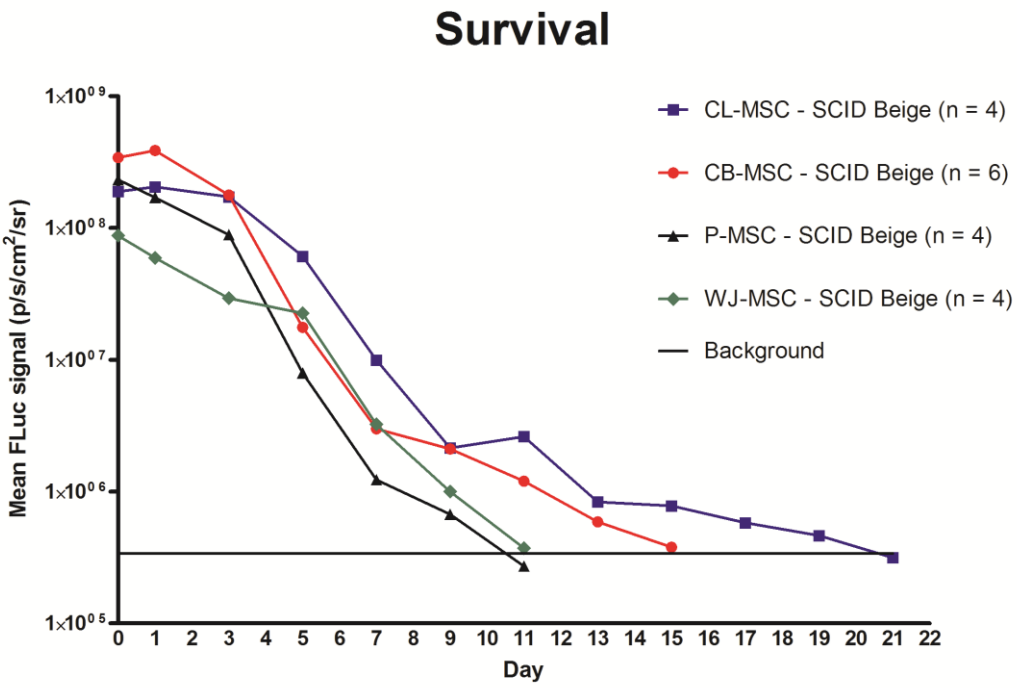

Figure 12: Survival of extraembryonic tissue-derived MSCs in immune deficient mice. *Mean FLuc maximum values are depicted per time point (n = 4 - 6 mice per cell type). Standard deviations are not shown for clarity. FLuc signals from CL-MSCs were detected until day 21, while P-MSC and WJ-MSC signals were detected only until day 11 post-injection. CB-MSCs were able to survive for 15 days in SCID Beige mice. Fluc, firefly luciferase; p/s/cm²/sr, photons per second per square centimeter per steridian; SCID, severe-combined immunodeficient.*

4.4. Immunogenicity of Extraembryonic Tissue-Derived Mesenchaml Stromal Cells

Next the ability of human extraembryonic tissue-derived MSCs to evade a xenogeneic immune response *in vivo* was elucidated in BALB/c mice (Figure 13). For this FLuc-positive MSCs were injected into the thigh muscle of immune competent BALB/c mice and bioluminescent images were acquired on the day of injection (day 0), one day later and thereafter every second day until the signal reached background levels.

In BALB/c mice, FLuc-positive CL-MSCs were detected up to 11 days after injection. The FLuc signal of CB-MSCs dropped substantially between days 5 and 7, and reached background levels on day 9 in BALB/c mice. Both P-MSCs and WJ-MSCs were rejected by day 7 which differs significantly from CL-MSCs (p = 0.008 and p = 0.007 for CL-MSCs versus P-MSCs and WJ-MSCs, respectively).

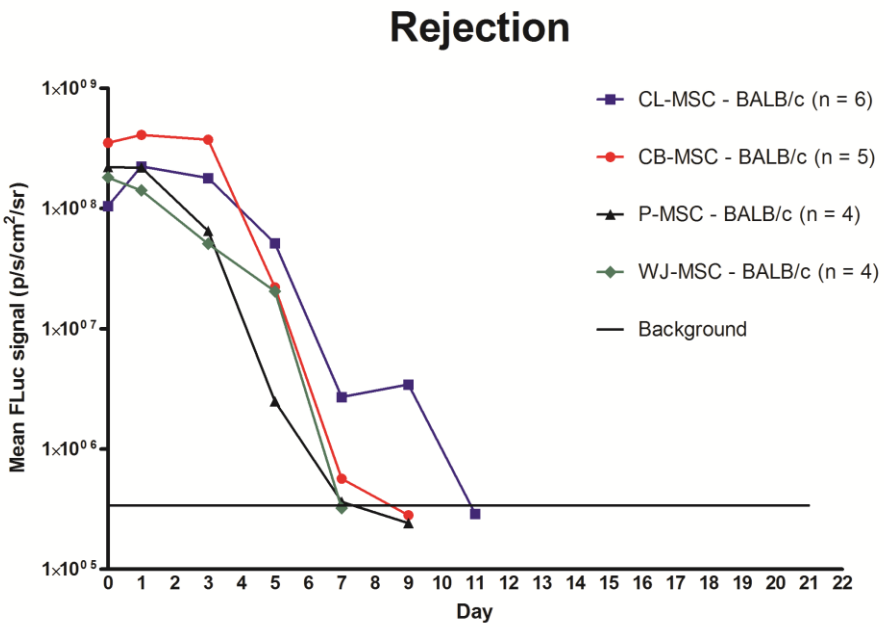

Figure 13: Rejection of extraembryonic tissue-derived MSCs by immune competent mice. *The rejection profile of FLuc-positive MSCs injected into BALB/c mice was monitored by bioluminescence imaging. Mean FLuc maximum values are depicted per time point (n = 4 - 6 mice per cell type). CL-MSCs and CB-MSC were detected the longest while; P-MSC and WJ-MSCs were rejected by day 7. FLuc, firefly luciferase; p/s/cm^2/sr, photons per second per square centimeter per steridian.*

Combining the results from both experiments (“Survival” Figure 12 and “Rejection” Figure 13) we observed that FLuc-positive extraembryonic tissue-derived MSCs were detected significantly longer in immunodeficient SCID Beige mice (P-MSCs and WJ-MSCs, 4 days; CB-MSC, 9 days and CL-MSCs 10 days) than in immune competent BALB/c mice ($p < 0.001$ for all cell types).

To test whether the differences in MSC rejection in immune competent mice are due to their ability to evoke T helper (T_H)1, T_H2 or T_H17 cell responses, ELISPOT assays were performed (Figure 14). In this assay extraembryonic tissue-derived MSCs were injected into BALB/c mice. Five days later spleens were removed and the isolated splenocytes used as responder cells in either an IFN-γ (T_H1), IL-4 (T_H2) or IL-17 (T_H17) spot assay.

CL-MSCs evoked the weakest immune response from T_H1 (IFN-γ assays: $p < 0.001$, $p = 0.001$, $p = 0.004$ compared to CB-MSCs, P-MSCs, and WJ-MSCs, respectively) and T_H2 cells (IL-4 assays: all $p < 0.001$ compared to CB-MSC, P-MSC, and WJ-MSCs). CB-MSC, P-MSC, and WJ-MSC evoked a similar T_H1 and T_H2 immune response. The T_H17 immune response was mildly evoked in BALB/c mice by the injection of extraembryonic tissue-derived MSCs. T_H17 immune responses against CL-MSCs and P-MSCs were weaker than against CB-MSCs and WJ-MSCs (IL-17 assays: $p < 0.05$ compared to CB-MSC and WJ-MSCs, for both CL-MSC and P-MSC).

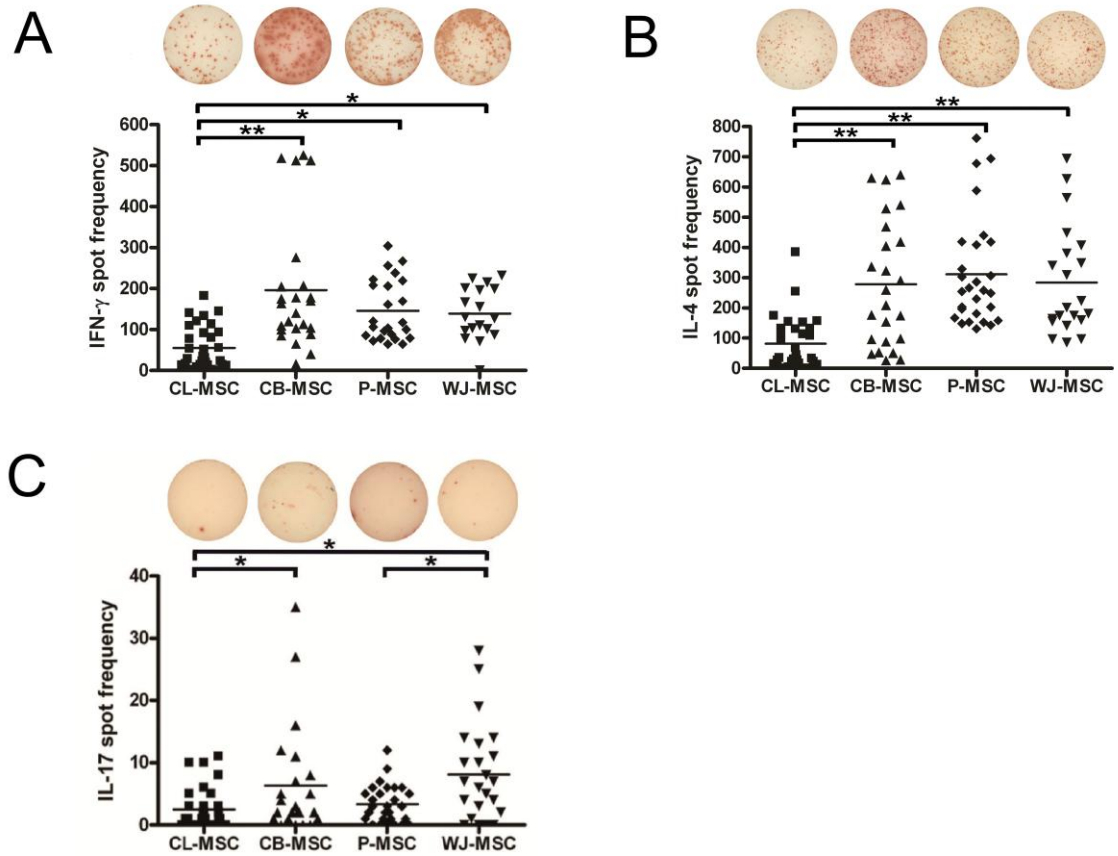

Figure 14: Xenogeneic immune responses against extraembryonic tissue-derived MSCs. *(A): To detect murine IFN-γ-secreting cells five days after injecting 1 x 10^6 MSCs into BALB/c mice (5 – 8 mice per cell type), spleens were removed and used as responders in an ELISPOT assay (4 wells per mouse). Mice showed the lowest spot frequency against CL-MSCs. (B): Similarly, mice showed the lowest IL-4 spot frequencies against CL-MSCs, corresponding to a very mild immune response. (C): CL-MSC and P-MSCs evoked the weakest T_H17 response. Each data point represents one ELISPOT plate well (18 – 32 wells per cell type). The number ration of responder splenocytes to MSCs was 10:1. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. The horizontal lines represent the mean. IFN-γ, interferon-gamma; IL, interleukin; PBMC A, peripheral blood mono-nuclear cells from responder A.*

To further investigate the immunogenicity of extraembryonic tissue-derived MSCs, ELISPOT assays using human allogeneic peripheral blood mono-nuclear cells (PBMCs) as responders were carried-out.

Strong human T_H1 and T_H2 immune responses were not induced against any of the MSCs examined. However, CB-MSCs and WJ-MSCs provoked stronger cellular responses by T_H1 cells in comparison to CL-MSCs and P-MSCs (Figure 15A) (both $p < 0.001$). The number of IL-4 secreting PBMCs was comparable among cell types and only differed between CB-MSCs and P-MSCs ($p = 0.043$) (Figure 15B).

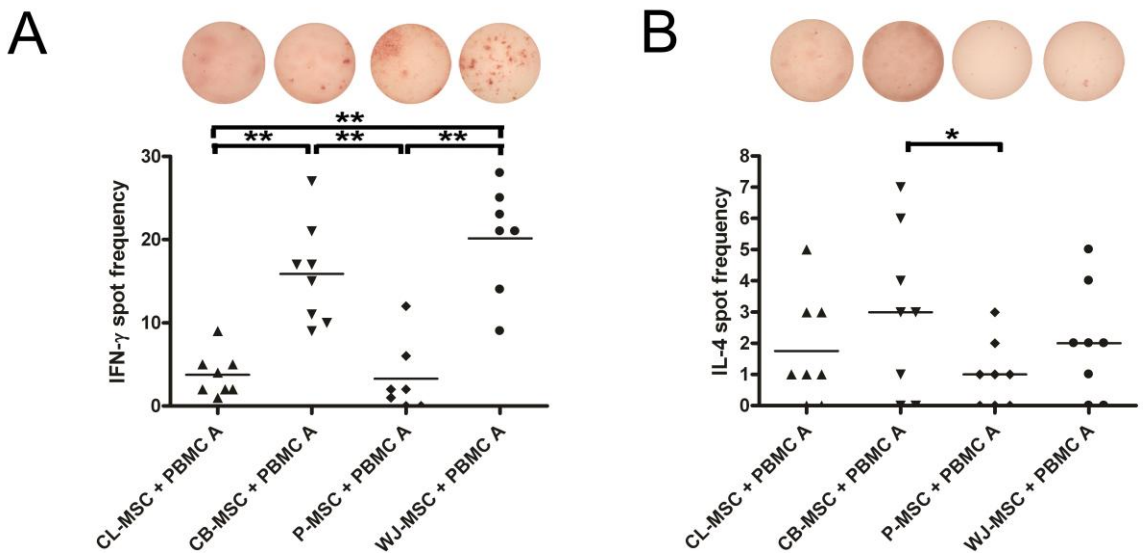

Figure 15: Immunogenicity of extraembryonic tissue-derived MSCs. *(A): ELISPOT using human allogeneic PBMCs as responders showed that CB-MSCs and WJ-MSCs induced a stronger IFN-γ response compared to CL-MSCs and P-MSCs. (B): Only CB-MSCs and P-MSCs differed in the IL-4 response among the cell types. Each data point represents one ELISPOT plate well (7 – 8 wells per cell type). *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. The number ration of responder PBMCs to MSCs was 50:1. The horizontal lines represent the mean. IFN-γ, interferon-gamma; IL-4, interleukin-4; PBMC A, peripheral blood mono-nuclear cells from responder A.*

The observed differences in the induction of T_H1 and T_H2 responses evoked by MSCs could be potentially attributed to the difference in HLA-type between the MSCs and the responder PBMCs, therefore the HLA-type of the cells was determined (Table 20).

Interestingly, WJ-MSCs induced a significant human T_H1 immune response, despite having the least HLA mismatches with responder PBMCs (Table 20). Similarly, CB-

MSCs induced a strong immune response from human responder PBMCs despite similarities in the HLA-type.

Table 20: The HLA-typing results of extraembryonic tissue-derived MSCs and responder PBMCs. *Differences and similarities in haplotype were observed between MSCs and responder PBMCs. Identical HLA alleles are in bold. HLA, human leukocyte antigen; PBMCs, peripheral blood mono-nuclear cells.*

	HLA-A	HLA-B	HLA-DR
Responder PBMCs	0301	0702	1501
	2402	-	-
CL-MSCs	0201	4801	1201
	2402	-	**1501**
CB-MSCs	**0301**	**0702**	**1501**
	2402	4402	1104
P-MSCs	0201	**0702**	**1501**
(donor #1)	1101	**-**	**-**
P-MSCs	**0301**	5101	1102
(donor #2)	6801	1803	1104
WJ-MSCs	**0301**	**0702**	**1501**
	2402	**-**	1701

Differences in MSC immunogenicity might be attributed to differences in human leukocyte antigen (HLA) class I or II expression, therefore surface HLA molecule expression was examined via flow cytometry. As the pro-inflammatory cytokine interferon-γ (IFN-γ) has been reported to increase surface HLA molecule expression on MSCs (163), To determine the extent to which MSCs up-regulate HLA expression in

an inflammatory environment, HLA expression after a 48-hour incubation period with IFN-γ was also examined.

Native CL-MSCs and CB-MSCs express a very low level of HLA class I, while P-MSCs and WJ-MSC express a moderate level (Figure 16). A 48-hour stimulation period with a moderate (25 ng/ml) concentration of IFN-γ lead to a significant increase in surface expression of HLA class I by all MSC types, except P-MSCs ($p < 0.001$, $p = 0.003$ and $p = 0.017$ for CL-MSCs, CB-MSCs, and WJ-MSCs, respectively). The increase in HLA class I expression after IFN-γ-stimulation by P-MSCs was not statistically significant ($p = 0.053$).

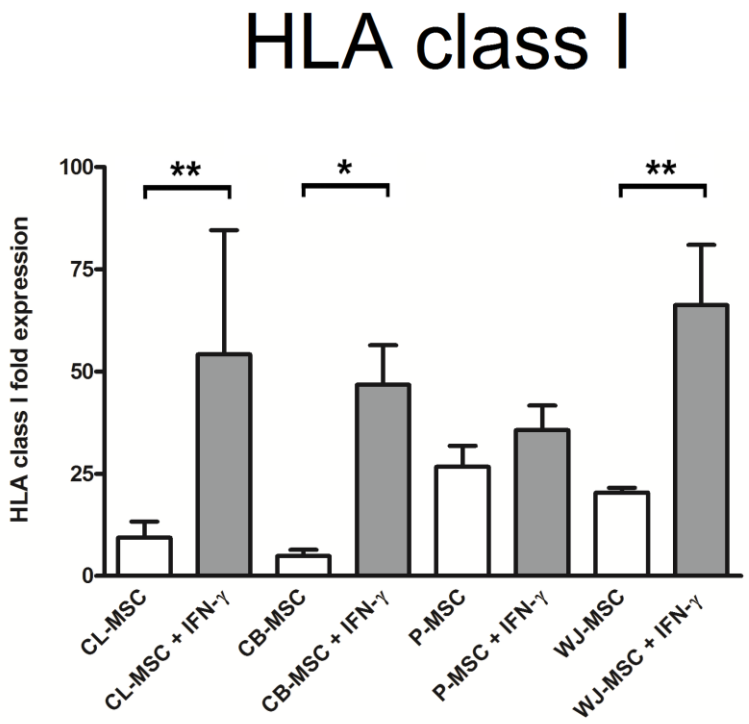

Figure 16: Human extraembryonic tissue-derived MSCs increase HLA class I expression after IFN-γ stimulation. *Flow cytometric analysis revealed that native MSCs express a low to moderate level of HLA class I, which can be increased after IFN-γ is added to the cell culture for 48 hours prior to cell harvesting. HLA expression in each experiment was calculated by dividing the MFI of the anti-HLA antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± standard deviation from independent experiments (3 – 8 per cell type). *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. (+) 25 ng/ml IFN-γ. HLA, human leukocyte antigen; IFN-γ, interferon-gamma; MFI, mean fluorescent intensity.*

All native MSCs showed a very low HLA class II expression prior to the addition of IFN-γ (Figure 17). IFN-γ induced the up-regulation in HLA class II expression in all extraembryonic tissue-derived MSCs (p = 0.001 for CL-MSCs, p < 0.01 for CB-MSCs, p < 0.001 for both P-MSCs and WJ-MSCs). WJ-MSCs showed the highest expression level after stimulation with IFN-γ (p < 0.001 for all MSC types versus WJ-MSCs). HLA class II expression by P-MSCs was also higher after IFN-γ stimulation than in CL-MSCs and CB-MSCs (p = 0.03 and p = 0.003, respectively).

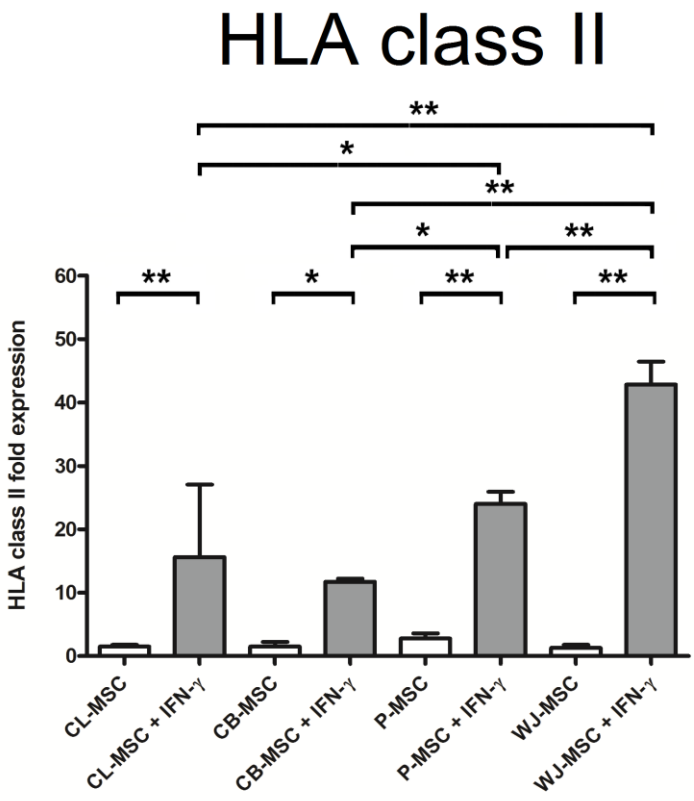

Figure 17: Human extraembryonic tissue-derived MSCs increase HLA class II expression after IFN-γ stimulation. *Similarly, to HLA class I, class II expression can be increased significantly on extraembryonic tissue-derived MSCs after incubation with IFN-γ. HLA expression in each experiment was calculated by dividing the MFI of the anti-HLA antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± standard deviation from 3 independent experiments. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. (+) 25 ng/ml IFN-γ. HLA, human leukocyte antigen; IFN-γ, interferon-gamma; MFI, mean fluorescent intensity.*

As IFN-γ-treated MSCs up-regulated HLA II expression, it was important to investigate the potential of MSCs to express co-stimulatory molecules (CD40, CD80 and CD86) (Figure 18). The presence of both co-stimulatory molecules and HLA class II could imply that MSCs can function as non-professional antigen-presenting cells (APCs) and stimulate T-cell responses *in vivo* (164). In this study, only native WJ-MSCs expressed CD86, although at a very low level. CD86 expression by WJ-MSCs was down-regulated in the presence of IFN-γ (p = 0.001). Co-stimulatory molecule expression (CD40, CD80 and CD86) by other MSCs was not detected and remained unaffected by IFN-γ. It is therefore, possible to conclude, that CL-MSCs, CB-MSCs and P-MSCs do not have the potential to function as APCs even after IFN-γ stimulation.

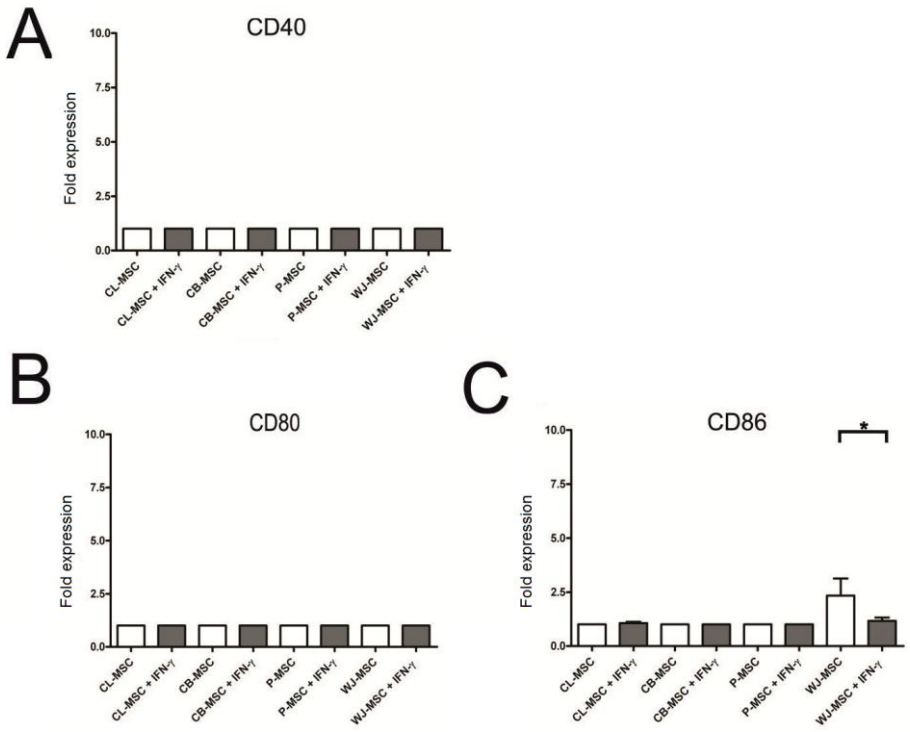

Figure 18: Human extraembryonic tissue-derived MSCs do not express co-stimulatory molecules after IFN-γ stimulation. *Native MSCs were CD40-, (A) CD80- (B) and with the exception of WJ-MSCs CD86- (C). Co-stimulatory marker expression in each experiment was calculated by dividing the MFI of the antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± standard deviation from 3 independent experiments. *p < 0.05 (ANOVA) with LSD post-hoc tests. (+) 25 ng/ml IFN-γ. CD, cluster of differentiation; IFN-γ, interferon-gamma; MFI, mean fluorescent intensity.*

4.5. Immunomodulatory Properties of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

HLA-G expression is associated with the immunosuppressive phenotype of MSCs (113,165). All extraembryonic tissue-derived MSCs expressed HLA-G on their surface as detected by flow cytometry. The up-regulation of surface HLA-G in the presence of IFN-γ with the exception of CB-MSCs ($p < 0.001$) was determined to be not significant (Figure 19). Differences in the level of HLA-G expression, were however, observed among the MSC types studied. CB-MSCs expressed a significantly higher amount of surface HLA-G compared to CL-MSCs ($p = 0.015$) and P-MSCs ($p = 0.033$). The addition of IFN-γ to CB-MSC cultures induced a higher level of HLA-G expression compared to the other MSCs ($p < 0.001$ for all MSCs versus CB-MSCs).

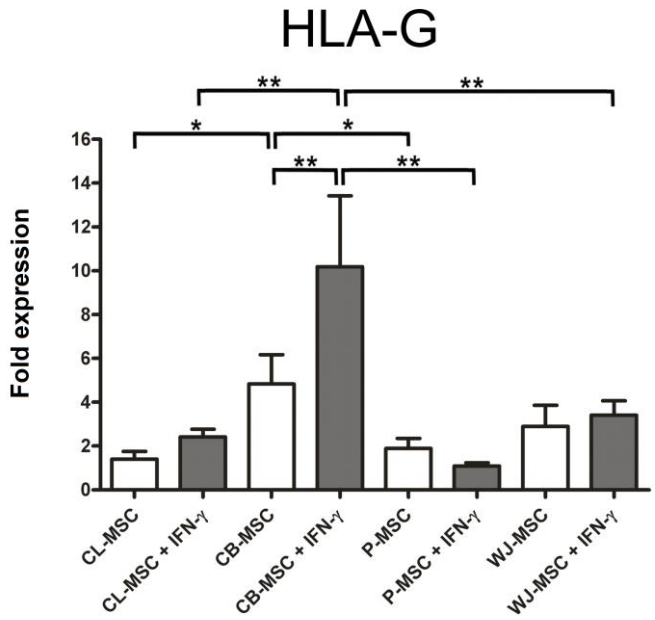

Figure 19: Human extraembryonic tissue-derived MSCs express immunomodulatory HLA-G. *CB-MSCs express the highest level of surface HLA-G. The addition of IFN-γ to CB-MSC cultures 24 hours prior to flow cytometry further enhances HLA-G expression. Expression in each experiment was calculated by dividing the MFI of the antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± SD from 3 independent experiments. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. (+) 25 ng/ml IFN-γ. IFN-γ, interferon-gamma; MFI, mean fluorescent intensity; SD, standard deviation.*

In addition, extraembryonic tissue-derived MSCs express the NK-cell ligand, HLA-E (Figure 20). Only IFN-γ-stimulated CB-MSCs were able to significantly up-regulate HLA-E expression ($p < 0.001$). Furthermore, IFN-γ-stimulated CB-MSCs expressed the highest level of surface HLA-E, compared to other cytokine-stimulated MSCs ($p < 0.001$ for all MSCs versus CB-MSCs).

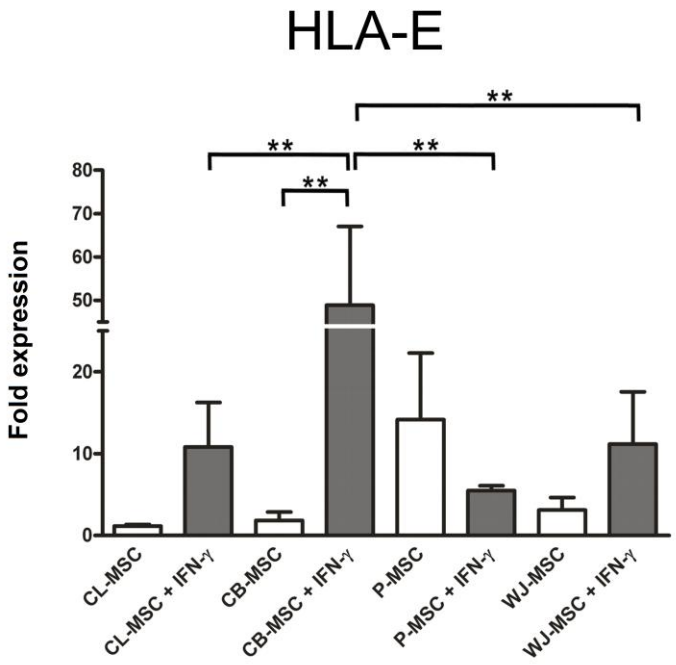

Figure 20: Human extraembryonic tissue-derived MSCs express immunomodulatory HLA-E. *Native MSCs express a low level of surface HLA-E. The effect of IFN-γ on HLA-E expression was most pronounced in CB-MSCs. Expression in each experiment was calculated by dividing the MFI of the antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± SD from 3 independent experiments. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. (+) 25 ng/ml IFN-γ. IFN-γ, interferon-gamma; MFI, mean fluorescent intensity; SD, standard deviation.*

Others have demonstrated that the enzyme, indolamine 2,3-dioxygenase (IDO) plays an important role in mediating the suppressive effect of MSCs on T and NK cells by producing kynurenine and catabolising the essential for immune cell-activation aminoacid, L-tryptophan (99,118). In the present work, IDO was not detected in native (cytokine unstimulated) extraembryonic tissue-derived MSCs (Figure 21). All

MSC types showed a similar degree of IDO up-regulation after a 48-hour stimulation period with high-dose IFN-γ which, in this experiment, mimicked an inflammatory milieu.

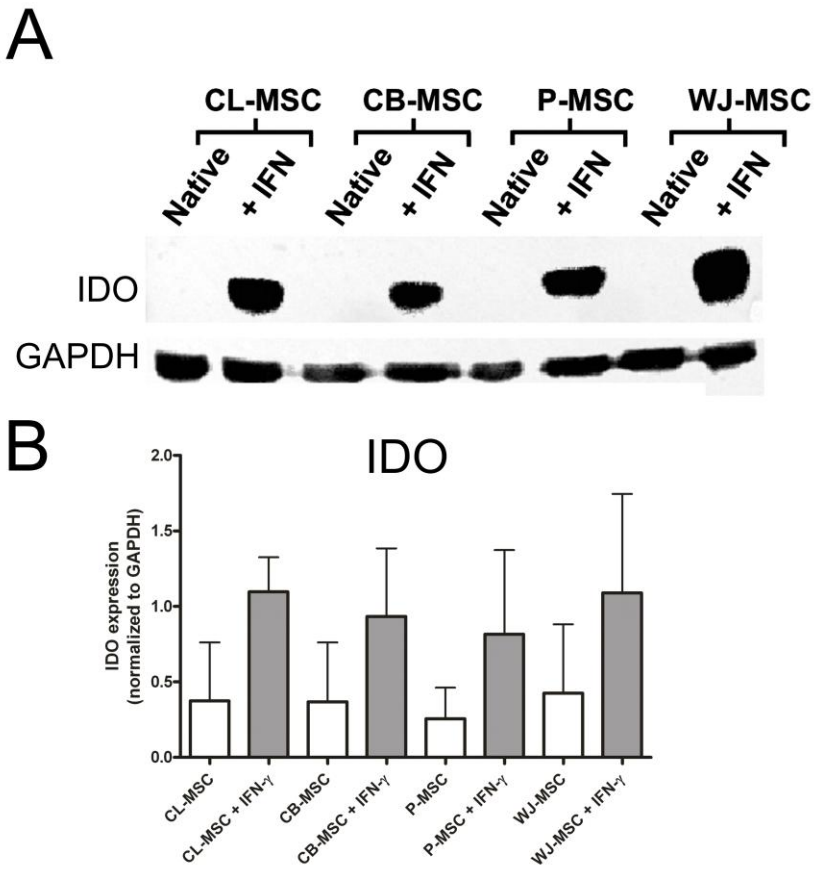

Figure 21: Human extraembryonic tissue-derived MSCs express IDO after IFN-γ stimulation. *(A): IDO protein was not detected in unstimulated MSCs (native), but could be induced after stimulation with 500 ng/ml IFN-γ. One representative immunoblot from three independent experiments is shown. (B): Quantification of immunoblot data after IDO (42 kDa) expression was normalized to GAPDH (37 kDa) ± standard deviation (n = 3). IDO, indolamine 2,3-dioxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.*

In a previous study, resting CL-MSCs were shown to release interleukin-10 (IL-10) and transforming growth factor-beta1 (TGF-β1), which could be increased with IFN-γ stimulation (116). In the present study, the amount of cytokines released by MSCs was quantified using a sensitive ELISA assay.

IL-10 and IL-2 were not detected in the 48-hour conditioned supernatants from the MSCs (assay detection limit 7.8 pg/ml) (data not shown). The addition of IFN-γ 48 hours before supernatant collection did not induce an increase in the amount of IL-2 or IL-10 released by extraembryonic tissue-derived MSCs. On the other hand, TGF-β1 was secreted by all extraembryonic tissue-derived MSCs (detection limit 125 pg/ml) (Figure 22).

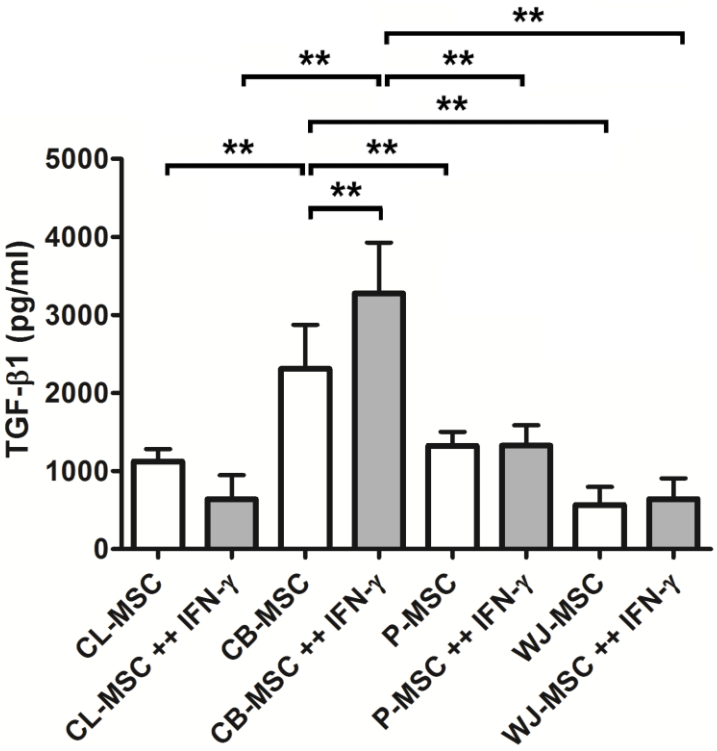

Figure 22: Human extraembryonic tissue-derived MSCs secrete the toleragenic cytokine, TGF-β1. *Supernatants from 48-hour MSC cultures were examined for the presence of TGF-β1. Displayed are means ± standard deviation from 3 independent experiments per cell type. Each analysis was performed in triplicates. ELISA data show that the addition of IFN-γ to CB-MSCs cultures, but not other MSCs induced the release of TGF-β1. **p < 0.001 (ANOVA) with LSD post-hoc tests. IFN-γ, interferon-gamma; TGF-β1, transforming growth factor-beta1. (++) 500 ng/ml IFN-γ.*

Native CB-MSC secreted over twice the amount of TGF-β1 than CL-MSCs (p < 0.001), P-MSCs (p < 0.001) and WJ-MSCs (p < 0.001), which was further significantly increased in the presence of IFN-γ. Surprisingly, the addition of IFN-γ to the CL-

MSC culture 48 hours before supernatant collection slightly reduced TGF-β1-secretion (not significant), while secretion of TGF-β1 by P-MSC and WJ-MSC remained unchanged.

Recently CD54 (intracellular adhesion molecule-1, ICAM-1) expression has been proposed to be critical for the immunosuppressive capabilities of MSCs (166). CD54, which binds to lymphocytes via lymphocyte function-associated antigen-1 (LFA-1) was expressed by all the MSCs types examined and was further up-regulated in the presence of IFN-γ (Figure 23).

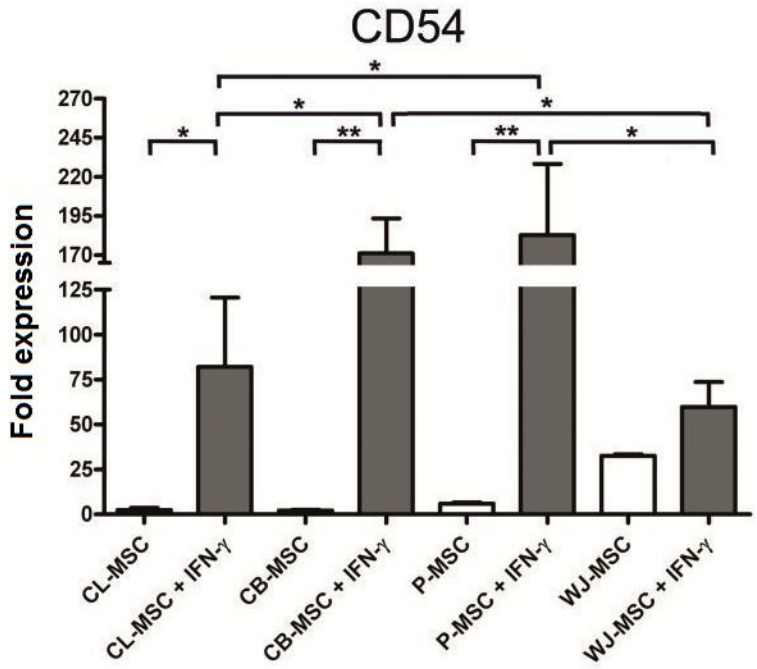

Figure 23: Human extraembryonic tissue-derived MSCs express CD54 (ICAM-1). *The addition of IFN-γ to the cell cultures 24 hours prior to flow cytometry induced the up-regulation of surface CD54 (ICAM-1) expression by all MSCs. CD54 expression in each experiment was calculated by dividing the MFI of the anti-CD54 antibody-stained sample with the MFI of the isotype-matched control. Ten thousand live cells where acquired per sample. Presented are means ± SD from 3 independent experiments. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. ICAM-1, intracellular adhesion molecule-1; IFN-γ, interferon-γ; MFI, mean fluorescent intensity. (+) 25 ng/ml IFN-γ.*

Although WJ-MSCs up-regulated the surface expression of ICAM-1 after IFN-γ-stimulation, CL-MSCs, CB-MSCs and P-MSCs expressed ICAM-1 at levels signifi-

cantly higher after IFN-γ-stimulation (p = 0.014 for native CL-MSCs versus stimulated CL-MSCs, $p < 0.001$ for native CB-MSCs versus stimulated CB-MSCs and $p < 0.001$ for native P-MSCs versus stimulated P-MSCs, respectively). IFN-γ-stimulated CB-MSCs and P-MSCs expressed higher levels of CD54 than stimulated CL-MSCs ($p < 0.05$ for both cell types) and WJ-MSCs ($p < 0.05$ for both cell types).

The immunomodulatory effect of MSCs on T cells can be demonstrated in a one-way mixed-lymphocyte reaction (MLR) (110,116). For this assay, mismatched responder peripheral blood mono-nuclear cells (A PBMCs) and stimulator PBMCs (B PBMCs) were included (Table 21).

Table 21: The HLA-typing results of PBMCs used in the mixed-lymphocyte reaction. *Stimulator and responder PBMCs shared the HLA-A2402 and HLA-DR1501 epitopes. Identical alleles are in bold. HLA, human leukocyte antigen; PBMC, peripheral blood mono-nuclear cells.*

	HLA-A	HLA-B	HLA-DR
Responder A PBMCs	0301	0702	**1501**
	2402	-	-
Stimulator B PBMCs	0101	1501	**1501**
	2402	4405	1601

In MLRs, PBMCs respond to HLA-mismatched stimulator PBMCs by proliferating and producing cytokines. Extraembryonic tissue-derived MSCs were added as a third-party cell, at the beginning of the co-culture period. Stimulator PBMCs were treated with mitomycin C before the MLRs to inhibit cytokine-release. After four days, cytokines released by the responder PBMCs were detected with an ELISPOT assay.

The number of IFN-γ-releasing human responder PBMCs in response to allogeneic PBMCs was significantly higher when CB-MSCs and WJ-MSCs were added (p = 0.02 and $p < 0.001$ compared to PBMC A+B, respectively) (Figure 23). CB-MSC and WJ-MSC induced a significant stronger IFN-γ response compared to CL-MSC (p =

0.003 and $p < 0.001$, respectively). T_H1 responses of WJ-MSC MLRs were also significantly higher than in those of P-MSCs ($p = 0.001$).

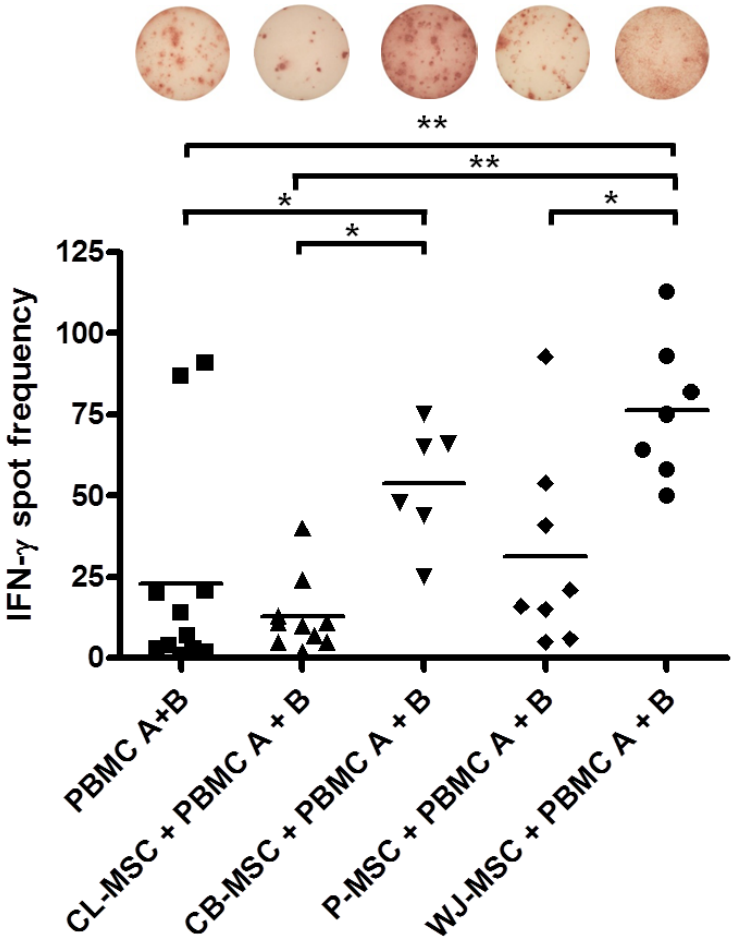

Figure 23: Human extraembryonic tissue-derived MSCs did not show inhibition of T_H1 responses in MLRs. *Skewing of the cytokine profile by MSCs as a third-party cell in a one-way MLR was quantified with the ELISPOT assay. Responder PBMCs released IFN-γ in response to allogeneic PBMCs, which could only be moderately inhibited by CL-MSCs. Each data point represents one ELISPOT plate well (6 - 10 wells per MSC type, 11 wells for PBMC A + B). The number ratio of responder PBMCs to recipient PBMCs was 10:1. The number ratio of responder PBMC to MSCs was 50:1. *p < 0.05, **p < 0.001 (ANOVA) with LSD post-hoc tests. The horizontal lines represent the mean. IFN-γ, interferon-gamma; MLR, mixed-lymphocyte reaction; PBMCs, peripheral blood mononuclear cells. A – responder, B – stimulator.*

Similarly, the number of IL-4-releasing human PBMCs in response to allogeneic PBMCs was significantly increased when CB-MSCs and WJ-MSCs were added ($p <$

0.05 compared to PBMC A+B) (Figure 24). T_H2 responses in the MLR were not significantly reduced by the addition of neither P-MSCs nor CL-MSCs.

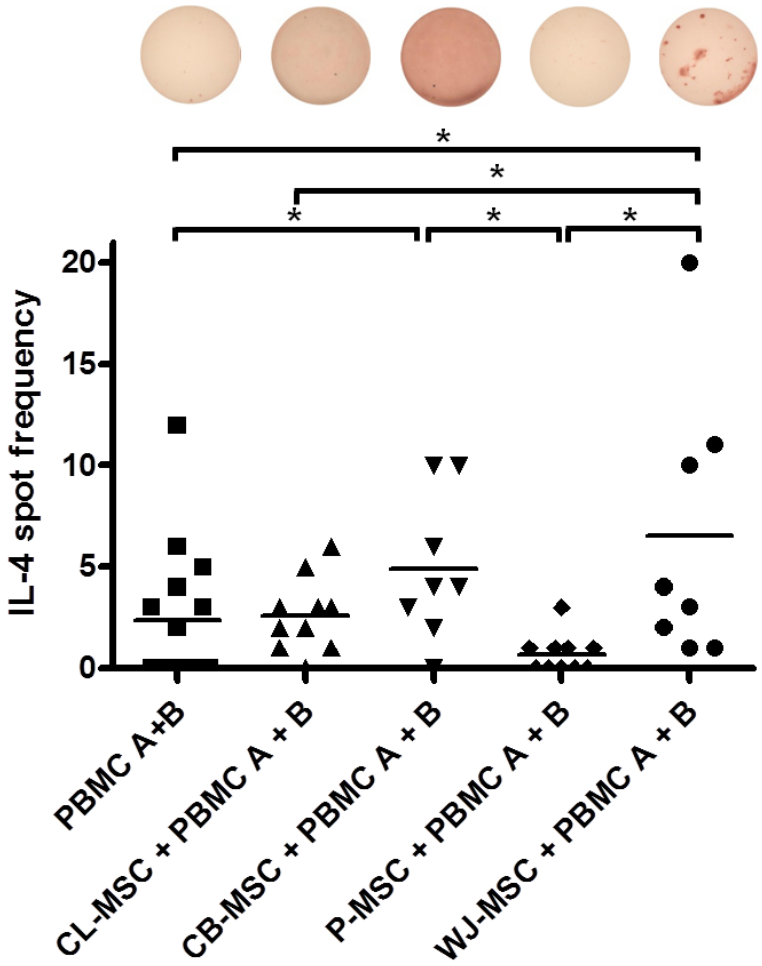

Figure 24: Human extraembryonic tissue-derived MSCs did not show inhibition of T_H2 responses in MLRs. *Skewing of the cytokine profile by MSCs as a third-party cell in a one-way MLR was quantified with the ELISPOT assay. PBMCs released IL-4 in response to allogeneic PBMCs, which could not be inhibited by MSCs. Each data point represents one ELISPOT plate well (8 - 10 wells per cell type, 16 wells for PBMC A + B). The number ratio of responder PBMCs to recipient PBMCs was 10:1. The number ratio of responder PBMC to MSCs was 50:1. *p < 0.05 (ANOVA) with LSD post-hoc tests. The horizontal lines represent the mean. IL-4, interleukin-4; MLR, mixed-lymphocyte reaction; PBMCs, peripheral blood mono-nuclear cells. A – responder, B – stimulator.*

4.6. Cord Lining Mesenchymal Stromal Cell Migration *In Vivo*

4.6.1. CL-MSC Migration in Immunodeficient NOD SCID Mice

The migration pattern of CL-MSCs *in vivo* has not been examined before; therefore CL-MSCs were injected directly into the IVC (inferior vena cava) of immunodeficient NOD SCID mice. To follow the migration of CL-MSCs *in vivo*, CL-MSCs were made to constitutively express firefly luciferase (FLuc) before infusion (Appendix Figure 2).

Next, 0.5 x 10^6 FLuc-positive CL-MSCs were infused intravenously (IV) into immunodeficient NOD SCID mice.

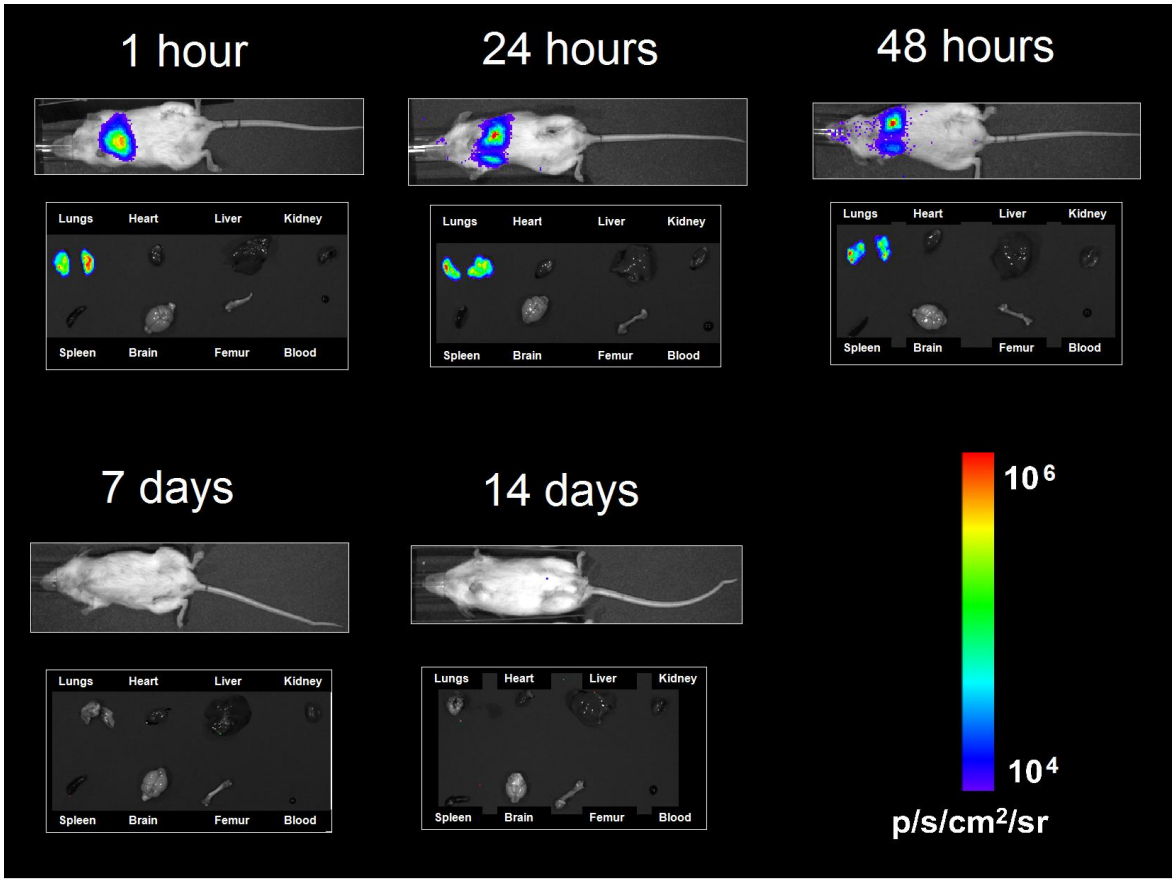

Figure 25: Intravenously-injected CL-MSCs were found in the lungs. *0.5 x 10^6 FLuc-positive CL-MSCs were infused intravenously into NOD SCID mice. Mice were imaged 1, 24, 48 hours, 7 and 14 days after infusion. After the mice were sacrificed bioluminescent images were taken of peripheral blood, long bones and organs (lungs, heart, liver, kidney, spleen, and brain) ex vivo. At the 1, 24 and 48 hour time points, CL-MSCs were only detected in the lungs. Data are shown as peak bioluminescence signal. A single representative image is shown (2 – 3 animals per time point). FLuc, firefly luciferase; p/s/cm^2/sr, photons per second per square centimeter per steridian.*

Each mouse received a vasodilator prior to cell injection to minimize the trapping of cells in the small capillaries of the lung. Despite the vasodilator pre-treatment at the one hour, 24 and 48 hour time points a strong FLuc signal could only be detected in the lungs (Figure 25). On day 7 and 14 post-injection no FLuc signal was detected in immunodeficient mice.

4.6.2. CL-MSC Size

Large-cell size could inhibit the circulation of intravenously-injected cells through small lung capillaries. Therefore, the diameter of CL-MSCs was measured. The mean diameter of trypsinated CL-MSCs was measured with light microscopy to be 15.7 µm (Figure 26).

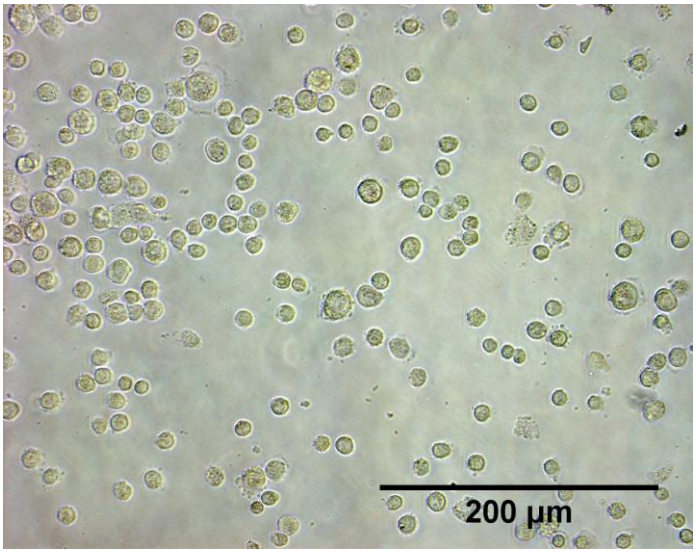

Figure 26: Trypsinated CL-MSCs have a mean diameter of 15.7 µm. *Scale bar: 200 µm. One representative bright field image is shown (n = 30).*

5. DISCUSSION

Mesenchymal stromal cell-based therapies have shown to be of benefit for patients suffering from a wide range of conditions, including heart failure (75), osteogenesis imperfecta (2), multiple sclerosis (4) and graft-versus-host disease (7). Many questions concerning MSC-based therapies remain unanswered, including; (i) what is the range of diseases in which MSCs could be of therapeutic use; (ii) what is the best route of application; (iii) how can MSC therapy be further enhanced; (iv) how can MSC potency *in vivo* be predicted; and finally (v) what is the best tissue source of MSCs (54).

To date MSCs have been isolated from organs such as bone marrow, liver, spleen and adipose tissue (23,31). MSCs derived from post-natal extraembryonic gestational tissues show great promise because, their isolation is simple, and an invasive procedure is not necessary since it involves the isolation of cells from tissues discarded after birth. Furthermore, gestational tissue MSCs have a higher replicative potential and younger chronological age compared to bone marrow (BM)-MSCs (reviewed in (155)). Lower immunogenicity and superior to BM-MSCs immunomodulatory capabilities have also been reported for extraembryonic tissue-derived MSCs (116).

It was therefore, the aim of this work to answer the relevant question of whether post-natal extraembryonic gestational tissue-derived MSCs differ in their suitability for cell-based therapies and if so, which extraembryonic tissue-derived MSC is a better source of cells for therapy. For this, immunogenicity, immunomodulatory and survival capabilities, proliferative as well as migratory potential of four MSCs derived from post-natal extraembryonic gestational tissues (umbilical cord lining, umbilical cord blood, placenta and Wharton’s jelly), where examined. In addition, the cells were tested whether they fulfill the accepted criteria for human multipotent mesenchymal stromal cells.

5.1. Characterization of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

MSC heterogeneity is a well described phenomenon, reflected in MSC morphology, marker expression and multi-lineage potential (33,34). Heterogeneity has been observed between BM-MSC donors, mouse strains, within an individual isolate and even within one fibroblast colony-forming unit (CFU-F) colony (34). The four extraembryonic tissue-derived cells compared in this study, differed in their cell culture requirements. These differences in cell culture requirements could be explained by the fact that the cells were isolated using different techniques, from different extraembryonic tissue compartments and from different donors. Heterogeneity was also observed in cell morphology and size. It is presently unclear, how these differences in cell morphology and size relate to cell function (34).

Some researchers have reported that WJ-MSCs can be easily cultured (167), while others have not (personal communication Mr. Kamath, Chief Operating Officer Cellular Engineering Technologies). In our experience, WJ-MSCs proved to be the most difficult MSC type to expand and preserve in culture. WJ-MSCs were sensitive to passaging and rarely attached to the new flask bottom after trypsinization. They also displayed a morphology characteristic of senescent cells (162). The reasons for this are not known, however the addition of the cytokine basic FGF to the cell culture medium induced a dramatic change in WJ-MSC morphology and proliferation rate. To date FGF has shown to decrease human adult BM-MSC cell size and increase cell proliferation (168).

Although the cell types examined in this study fulfilled most of the criteria accepted for human multipotent MSCs (13), only CL-MSCs were truly multipotent as they were able to differentiate into adipocytes, osteocytes and chondrocytes. Therefore, in this work the abbreviation “MSCs” refers to mesenchymal stromal cells. MSC-type and donor-specific differences in adipocyte differentiation have been observed in the past by other groups (25,35,156,169). Pittenger *et al.* were the first to test the differentiation potential of six human MSC colonies (15). All six colonies differentiated into osteocytes, five into adipocytes and only two into chondrocytes. Colonies which dis-

played a limited differentiation potential could have lost it *in vitro* or they could represent another cell type. In contrast to chondrogenic and osteogenic lineage differentiation, the ability of MSCs to differentiate into adipocytes seems to be more sensitive to cell age as this ability rapidly declines with passage number (170). In the present study, only low passage number MSCs were used for the differentiation assays. It is not known how often the MSCs had undergone mitosis before harvesting. A hierarchical organization of the differentiation cascade has been used to explain heterogeneity in MSC multipotency (171). Within this hierarchy, adipogenic potency is the first to be eliminated. MSCs also demonstrate a vast inter-donor variability in respect to differentiation into other cell types (172). Differences in MSC adipocyte differentiation capabilities have even been reported among different mouse strains (35). Interestingly, contrary findings have been reported for BM-MSCs isolated via different methods, which did not differ in their differentiation potential (173). It is conceivable that MSCs are not only a heterogeneous population due to different isolation methods and culture conditions (33) but, also that their origin distinguishes them and directs their potential therapeutic use (30,36). The observed heterogeneity in MSC populations highlight the importance of clarification of whether extraembryonic tissue-derived MSCs differ in their suitability for cell-based therapies and if so, which cell type is more suitable for cell-based strategies.

5.2. Proliferation and Migration of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells *In Vitro*

Large numbers of MSCs are needed for regenerative therapies, which prompted us to examine whether the MSCs differed in their proliferative potential *in vitro*. In this assay, CL-MSCs and CB-MSCs showed the highest proliferation rates, making their expansion *in vitro* to large cell numbers much easier. MSC proliferation rates were compared using the MTS-based assay, in which the colourless substrate is converted into a coloured formazan product in a NADPH-dependent reaction. As the conversion of MTS is dependent on mitochondrial dehydrogenase enzymes, the assay could be influenced by cell metabolic activity. Although it cannot be excluded that, a difference in metabolic rates between the examined MSCs exists, the results of the MTS assay are supported by the observation that CL-MSCs, were passaged more often than other cell types. On the other hand, WJ-MSCs not only displayed the lowest proliferation in the MTS assay but, were also split into new culture flasks less often.

Cell migration plays a pivotal role in tissue healing processes (39). Significant differences in migration rates were observed between extraembryonic tissue-derived MSC types, with CL-MSCs showing the most promise by migrating into an empty scratch area the fastest.

5.3. Survival and Immunogenicity of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

The proliferation capabilities of extraembryonic tissue-derived MSCs were correlated with their ability to survive *in vivo* in immunodeficient mice. Bioluminescence imaging showed that, CL-MSCs were able to survive longer in SCID Beige mice than the other MSC types. Despite the fact that SCID Beige mice lack mature T, B, and functional NK cells (158), MSC survival was limited. This could be explained by; stress during cell injection, combined with the lack of appropriate MSC survival signals *in vivo*, and different MSC proliferation rates.

There is a growing body of evidence suggesting that MSCs do not induce immune reactivity, enabling them to survive *in vivo* for prolonged periods of time in allogeneic (93) and xenogeneic (16) settings. On the other hand, previous studies have also suggested that allogeneic MSC transplantation can lead to the induction of immune responses in the recipient and to the rejection of the implanted cells (127). Similalry, Grinnemo *et al.* reported xenoreactivity after human BM-MSCs were transplanted into rats (174). The present study demonstrated the low immunogenicity of extraembryonic tissue-derived MSCs and especially CL-MSCs. The MSC-targeted xenogeneic immune responses *in vivo* were investigated using immune competent BALB/c mice. CL-MSCs, which showed the latest rejection, also demonstrated the lowest T_H1 and T_H2 cell activation in the ELISPOT assay using BALB/c sensitized splenocytes as responders. Similarly, when human PBMCs were used as responders, the spot frequency was low, which suggests that human CL-MSCs have developed a strategy to limit murine as well as human immune responses. Interestingly, although P-MSCs induced a high xenogeneic response, they were able to limit human T_H1 and T_H2 cell activation. Although allo-responses against MSCs have been reported by Nauta *et al.* (127), in the present study it was not observed against HLA mismatched CL-MSCs and P-MSCs. On the contrary, CB-MSCs and WJ-MSCs induced the strongest human T_H1 cell activation, despite being identical in the HLA-A loci with the responder PBMCs. These findings suggest that allogeneic extraembryonic tissue-derived MSC banks could be of extreme value as sources of

cells for therapy. It should be noted that, previous studies showed no correlation between the BM-MSC's HLA-type and it's effect on lymphocyte responses (175).

IFN-γ is a pro-inflammatory cytokine released by activated T cells and NK cells (164). Studies have demonstrated that IFN-γ plays a pivotal role in so called MSC-activation or licensing (176–178), as MSCs that lack the IFN-γ receptor cannot exert any immunosuppressive effects on T cells (179). On the other hand, IFN-γ increases the level of HLA class I expression on MSCs, which could lead to an increase in MSC immunogenicity and ultimately make transplanted cells more susceptible to immune rejection by T cells. As IFN-γ has such a profound effect on MSC immunogenicity and biology, cytokine-stimulated MSCs as well as native MSCs were compared.

In concordance to previous studies (90,163,176), all MSCs showed a low to moderate HLA class I, low HLA class II and, with the exception of WJ-MSCs, lack of co-stimulatory molecule expression. As reported previously for BM-MSCs (163), extraembryonic tissue-derived MSCs increased their HLA class I expression in the presence of IFN-γ. The reason for WJ-MSCs inducing a very high IFN-γ and IL-4 response in the MLR assay, despite only one HLA loci mismatch with the responder PBMCs, could be their high HLA class I and II expression.

Previous studies, have demonstrated that IFN-γ-stimulated BM-MSCs can behave like non-professional antigen-presenting cells (APCs) by up-regulating HLA molecules and presenting antigens to both CD4- and CD8-positive T cells, inducing their activation (163,180,181). This process is dependent on the amount of IFN-γ in the MSC's micro-environment (163). T_H-cell activation would facilitate MSC rejection after transplantation into immune competent hosts. However, for T-cell activation to occur, the non-professional APC must also co-express co-stimulatory molecules such as CD80 or CD86 (164). Therefore, the co-expression of CD86 and HLA class II by WJ-MSCs could potentially have lead to the induction of T_H immune responses. IFN-γ-induced up-regulation of HLA class II by CL-MSCs, CB-MSCs and P-MSCs was not accompanied with the co-expression of co-stimulatory molecules and therefore would not lead to T-cell activation, but rather induce a state of T-cell anergy (164).

In the present work, the immunogenicity of extraembryonic tissue-derived MSC was evaluated in both *in vivo* and *in vitro* assays. The results of studies by Poncelet *et al*., in which allogeneic MSCs were found not be immunogenic *in vitro*, but after intracardiac infusion elicited an immune response, further emphasizes the importance of both *in vitro* and *in vivo* studies when testing the immunogenicity of MSCs for cell-based therapies (131).

5.4. Immunomodulatory Properties of Extraembryonic Tissue-Derived Mesenchymal Stromal Cells

The increase in MSC immunogenicity due to HLA class I or II up-regulation after IFN-γ stimulation could be potentially counter-balanced by the up-regulation of immunomodulatory molecules, such as HLA-G, HLA-E or IDO that can influence the recipient´s immune responses. The role of IFN-γ in enhancing the immunomodulatory properties of MSCs is well described in the literature (116,177). Non-classical major histocompatibility (MHC) expression (HLA-G) has been implemented in playing a role in MSCs evading immune responses (165). More importantly, it has been shown that both HLA-E and HLA-G expression on the target cell are needed for the inhibition of immune cell-mediated lysis (182). Thus, in this study HLA-G and HLA-E expression was compared among the MSCs as well as in an environment rich in IFN-γ. Flow cytometry revealed that only CB-MSCs showed a significant increase in both HLA-G and HLA-E surface expression.

Campioni *et al*, suggested that as HLA-G and IL-10 expression by MSCs is correlated with their surface CD90 expression, CD90 could be a new predictive marker for MSC inhibitory capabilities (183). In this study, a correlation between CD90 and HLA-G expression was detected; however the observed increase in HLA-G expression by CB-MSCs did not translate to an increase in their immunosuppressive abilities. This could be due to the inability of extraembryonic MSCs to produce a significant amount of IL-10.

In contrary to mouse MSCs which mediate their immunosuppressive effects mainly via nitric oxide, human MSCs rely on the enzyme IDO (184). In this study, IDO ex-

pression was determined after a 48-hour stimulation period with high-dose IFN-γ. Interestingly the MSCs did not differ in their capacity to express IDO. Therefore, it can be concluded that the observed differences in extraembryonic tissue-derived MSC survival *in vivo* and immunogenicity are not due to a difference in IDO expression.

Interestingly, IFN-γ had a significant effect on the expression of ICAM-1 (CD54) which, recently has been shown to be critical for the immunosuppressive capabilities of MSCs (166). Although ICAM-1 up-regulation after cytokine exposure has been demonstrated by Ren *et al.*, in this study it is shown for the first time that a very high concentration of IFN-γ (500 ng/ml) over a prolonged period of time alone is enough to robustly up-regulate ICAM-1 significantly. Clearly the expression of ICAM-1 alone is not sufficient to protect MSCs from immune attack as native WJ-MSCs expressed the highest levels of ICAM-1, however induced the highest level of IFN-γ-secretion by responder human PBMCs. This decreased immune evasion response could possibly be linked with their lower TGF-β1 and higher HLA expression.

Since MSCs exert immunomodulatory effects not only via direct cell-cell contact but also by releasing soluble factors such as IL-10 and TGF-β1 (119,185), a varied expression of these factors will lead to differences in exerting immunosuppressive effects. IL-10 secreting CL-MSCs have been previously detected in ELISPOT assays (116). Interestingly, in this study extraembryonic MSCs did not secrete a detectable amount of IL-10. CB-MSC released significant amounts of TGF-β1, which could be further increased with the supplementation of IFN-γ during cell culture. In a previous study, IFN-γ was shown to increase the number of TGF-β-secreting CL-MSCs (116). In this study an increase in the total amount of TGF-β1 released by CL-MSCs after IFN-γ stimulation was not observed.

There are reports of MSCs exerting a so called “veto-like” activity in which, bone marrow MSCs decrease peripheral blood lymphocyte proliferation in a one-way mixed-lymphocyte reaction (MLR) (110,175). A previous study demonstrated a superior inhibition of IL-2 release by lymphocytes in a MLR by CL-MSCs compared to BM-MSCs (116). In this study, the effect of CL-MSCs on IFN-γ-secretion during a MLR was demonstrated. Other extraembryonic tissue-derived MSCs were unable to weaken

the immune reaction. On the contrary, CB-MSCs and WJ-MSCs significantly enhanced the amount of responding lymphocytes secreting IFN-γ and IL-4. Similarly, human first trimester liver-derived MSCs are unable to suppress a MLR, which has been attributed to their immunological immaturity (170). Contrary findings have been reported for second trimester MSCs from gestational tissues, which suppress MLR responses in an IL-10 dependent manner (186). In this study, post-natal gestational tissue MSCs from full-term births were investigated, which clearly differ from second trimester-derived MSCs in terms of MLR suppressive potential, which could be due to their lack of IL-10 production. Second trimester MSCs from the fetal-maternal interface could be better equipped to suppress a MLR, however could possibly lose this capability around the time of birth, when immune rejection no longer poses such a danger to the developing fetus. Moreover, differences in extraembryonic tissue-derived MSC origin could be reflected in their potential to suppress immune reactions. Roelen *et al.* reported that gestational tissue-derived MSCs of fetal origin (amnion and amniotic fluid) show a stronger inhibition of MLRs compared to MSCs of maternal (decidua) origin (186). In the present study, MSCs from the umbilical blood and cord were of fetal (155), whereas placental MSCs were of maternal origin (25). Origin cannot explain the observed differences between MSC types in our immunological assays.

Studies by Le Blanc *et al.* have concluded that using fewer MSCs (10 – 1000 MSCs) in the MLR results in inconsistent results and that both suppression as well as activation of lymphocyte responses is observed (175). Additions of a larger number of MSCs to the MLR (over 1 x 10^3 MSCs) consistently lead to the suppression of the MLR. In the present study, 1 x 10^5 MSCs were used as the third-party cell in the MLR and the ratio between responder PBMC and MSC cell numbers was calculated to be 50:1. Therefore, low MSC cell number cannot explain the lack of MLR suppression.

It would be possible to envision that in this study these immunomodulatory defense mechanisms played a pivotal role in evading immune responses. However, this was not confirmed. CL-MSCs expressed moderate levels of HLA-G, HLA-E and TGF-β1 and also did not differ in IDO production from other MSCs. They nevertheless showed the longest *in vivo* survival rates and the lowest immune stimulation in mu-

rine and human ELISPOT assays. More importantly, this finding is further supported by the result of the MLR as only CL-MSCs slightly reduced the production of IFN-γ by PBMCs in the allo-response.

The present work sought to clarify, which extraembryonic tissue-derived MSC is better suited for MSC-based therapy. Presently, BM-MSCs are the most often examined MSC type. In a previous study conducted in our laboratory, CL-MSCs showed superior to BM-MSCs immunomodulatory properties (116). Whether other extraembryonic tissue-derived MSCs are immunomodulatory, was not known. To address this question the expression of various immunomodulatory molecules were compared between the different MSC types. As MSCs express a broad range of immunomodulatory molecules, only the most often described in the literature for human MSCs were compared. However, although unlikely, it cannot be excluded that other non-examined or unidentified molecules exert a very strong immunomodulatory function and are responsible for the observed differences between extraembryonic tissue-derived MSCs.

Safety is always of concern when developing new therapeutic strategies. Although the safety of MSC therapy was not closely monitored in this study; it is important to point out that no adverse effects were noted in mice in the MSC survival, immunogenicity or migration assays. So far clinical trials have shown MSC therapy to be safe (75), however there is some evidence suggesting that the injection of undifferentiated MSCs can lead to ossifications (187). Whether the observed calcifications are a result of MSC differentiation into osteocytes or the host's response to the injection of foreign cells remains unclear (reviewed in (188)). Furthermore, arrhythmias were reported in a swine model of myocardial infarction after systemic infusions of MSCs (69), which was not found in human studies. Another possible side-effect of MSC therapy is associated with MSC tropism towards tumours, where their presence is correlated with tumour growth and metastasis (189). The strong immunosuppressive properties exerted by MSCs could potentially lead to an increase in leukaemia-relapse rates in patients after haematopoietic stem cell (HSC) transplantation. A recent study challenged this notion and confirmed that the infusion of MSCs with HSCs is safe and moreover, leads to a decrease in graft versus host disease (GvHD) rates

in recipients (190). On the other hand, van Bahr *et al.* reported an increase in cytomegalovirus infection-related deaths in patients with GvHD after BM-MSC therapy (191). This observation further highlights the advantage of extraembryonic tissue-derived MSCs as gestational tissues carry a lower risk of viral contamination (155).

5.5. Cord Lining Mesenchymal Stromal Cell Migration *In Vivo*

5.5.1. CL-MSC Migration in Immunodeficient NOD SCID Mice

CL-MSCs are a newly described population of MSCs and data describing their behaviour *in vivo* is lacking. CL-MSC migration studies are warranted before the cells can be administered in a clinical setting. For this a NOD SCID migration model, which is often used to study human bone marrow-derived MSC (BM-MSC) migration *in vivo* was used (192). In this model, BM-MSC are found in the bone marrow 24 hours post-infusion and human DNA was detected up to 13 months (193).

CL-MSCs were detected up to 21 days after intramuscular injection into immunodeficient mice. By IV-injection, CL-MSCs were trapped within the fine capillaries of the lungs, which probably also decreased the number of MSCs available to migrate to other organs. After 7 days, no FLuc signal could be detected in the lungs therefore, no live cells were present past this time point in the mice. It could also be envisioned that, CL-MSCs in contrast to human BM-MSCs (83,194) do not migrate to the bone marrow. CL-MSCs thus may not express the same adhesion molecules as BM-MSCs needed for bone marrow migration. The migration profile of CL-MSCs should be elucidated in further studies.

It is also important to point out, that in many studies, only human DNA is detected within mouse tissues and interpreted as the presence of MSCs (193). In this study, CL-MSCs were made to express firefly luciferase (Fluc) to enable cell tracking. FLuc-labelling was chosen instead of human DNA detection because; nucleic acid detection is not dependent on cell survival *in vivo.* On the other hand, the FLuc signal can only be detected in living cells, as this enzyme is ATP-dependent. DNA from apoptotic cells, phagocytosed cells, cellular debris, as well as from live cells can be detected and misinterpreted as cell survival. Bioluminescent imaging has, however, two disadvantages: the detection limit is 400 - 1000 FLuc-positive cells and the FLuc signal intensity is dependent on tissue depth and structure (reviewed in (195)). Therefore in this study, organs were also examined *ex vivo* for FLuc-expressing cells.

Finally, a disadvantage of the labeling technique is that CL-MSCs were culture-expanded after lentiviral-mediated gene transfer of FLuc. Although the CL-MSCs

were passages 11 to 15 before infusion into mice, it cannot be excluded that this *in vitro* culture step could have influenced the migration capacity of the cells (142).

5.5.2. CL-MSC Size and Pulmonary Embolism

Large IV-injected cells can get trapped in the lung vasculature due to the small diameter of lung capillaries (83). Intravenous-administration of MSCs without vasodilator pre-treatment leads to episodes of tachypnea, apnea and hemodynamic alterations in mice (83). The use of a vasodilator prior to cell administration results in a marked reduction of these symptoms (83). Pulmonary embolism can influence the results of *in vivo* migration studies as well as impede the usage of the intravenous route for MSC administration. Interestingly, human BM-MSCs have been successfully administered *per* IV in patients after myocardial infarction, however the number of cells reaching the myocardium was low (75). The smallest human lung capillaries are 5.5 µm in diameter and leukocytes which are 6 to 8 µm have to deform to pass through the capillary bed (196). It could be that human MSCs are not only trapped in the lung capillaries due to their larger cell size but also are unable to deform as readily as leukocytes and so cannot transit through the capillary bed. Although, the diameter of trypsinated CL-MSCs was measured to be smaller than that reported for human BM-MSCs, they were still larger than the diameter of murine lung capillaries (83). Therefore, I conclude that despite vasodilator pre-treatment, most CL-MSCs get physically trapped in lung capillaries and pulmonary embolism remains a barrier for the IV-administration of MSCs.

Another possible explanation for MSC trapping in the lung micro-vasculature is the binding of CD44-expressing MSCs to hyaluronan, which is expressed within mouse and human lung vasculature. Hyaluronan-CD44 interactions have been proposed to play a role in tumour cell metastasis to the lung (197). Therefore, migration studies with CD44-negative MSCs would help in elucidating the role of CD44 in MSC migration and perhaps decrease lung embolism. The result of this study further highlights the importance of increasing MSC organ-specific migration and inhibiting pulmonary embolism after IV-administration of MSCs.

6. CONCLUSIONS AND OUTLOOK

In the present work, different immunomodulatory molecules were examined. However, a correlation between the expression of these molecules and MSC immunomodulatory capacity was not found. It should be noted that presently there is no marker or *in vitro* assay, which could be used to successfully predict MSC potency *in vivo*. Thus, it remains desirable for research on MSC markers and immunomodulatory capacity to continue.

This study also highlights that although immunomodulatory molecules play an important role in MSC immune evasion, HLA expression is decisive in determining the immunogenicity of MSCs, and therefore should be examined when choosing suitable cell types for cell-based therapies. In order to avoid MSC rejection by the host's immune system two strategies can be envisioned.

Allogeneic MSC banks would allow for the possibility of matching donor and recipient HLA-type in the same manner as solid organ transplants are matched (198). The number of donor cell lines needed for such a human bank has been estimated for embryonic stem cells to be at least 150 cell lines (199). To establish an MSC bank covering most HLA-types found in the human population would be a challenging task and it is probably not feasible without the support of the international community. More importantly, the results of this dissertation are interesting as they support the possibility of MSC transplantation in an allogeneic setting even without a complete HLA matching.

Another option would be to modify MSCs immunogenicity *in vitro* before transplantation. A study using HLA I-targeting intrabodies (intracellular antibodies) and small interfering RNA showed a pro-longation in human embryonic stem cell survival in a xenogeneic setting (200). A similar strategy could be used to make other therapeutic cell types less immunogenic.

In this work the migration pattern of CL-MSCs was examined for the first time. In contrast to what has been reported for human CB-MSCs and BM-MSCs (139,192,194), CL-MSCs were not detected in murine bone marrow after infusion. It was also found

that, a major obstacle for the IV-administration of MSCs remains lung embolism. It is important to point out that, CL-MSCs migration was studied in healthy NOD SCID mice. This model has also been previously used to examine human BM-MSC migration to the bone marrow (150). The specific migration of BM-MSCs to injured organs has been demonstrated (86,146), and depends on sufficient chemotactic signals directing the injected MSCs to the site of injury (147). Future studies could concentrate on determining whether CL-MSCs also specifically migrate to injured organs. Finally, when designing MSC-based therapies, the appropriate MSC-type, their therapeutic efficacy and also their *in vivo* behaviour should be taken into account.

In conclusion, in this dissertation extraembryonic tissue-derived MSCs showed a varied potential to evade immune responses as well as exert immunomodulatory effects. CL-MSCs showed the best potential for a cell-based therapy, as the cells were not only hypo-immunogenic, but they also showed enhanced proliferative and migratory potential. Future research should concentrate on the best disease models in which CL-MSCs could be administered. Furthermore, after systemic infusion into mice, no organ-specific migration was demonstrated; a more suitable method for CL-MSC administration remains to be determined.

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8. APPENDIX

8.1. ADDITIONAL FIGURES

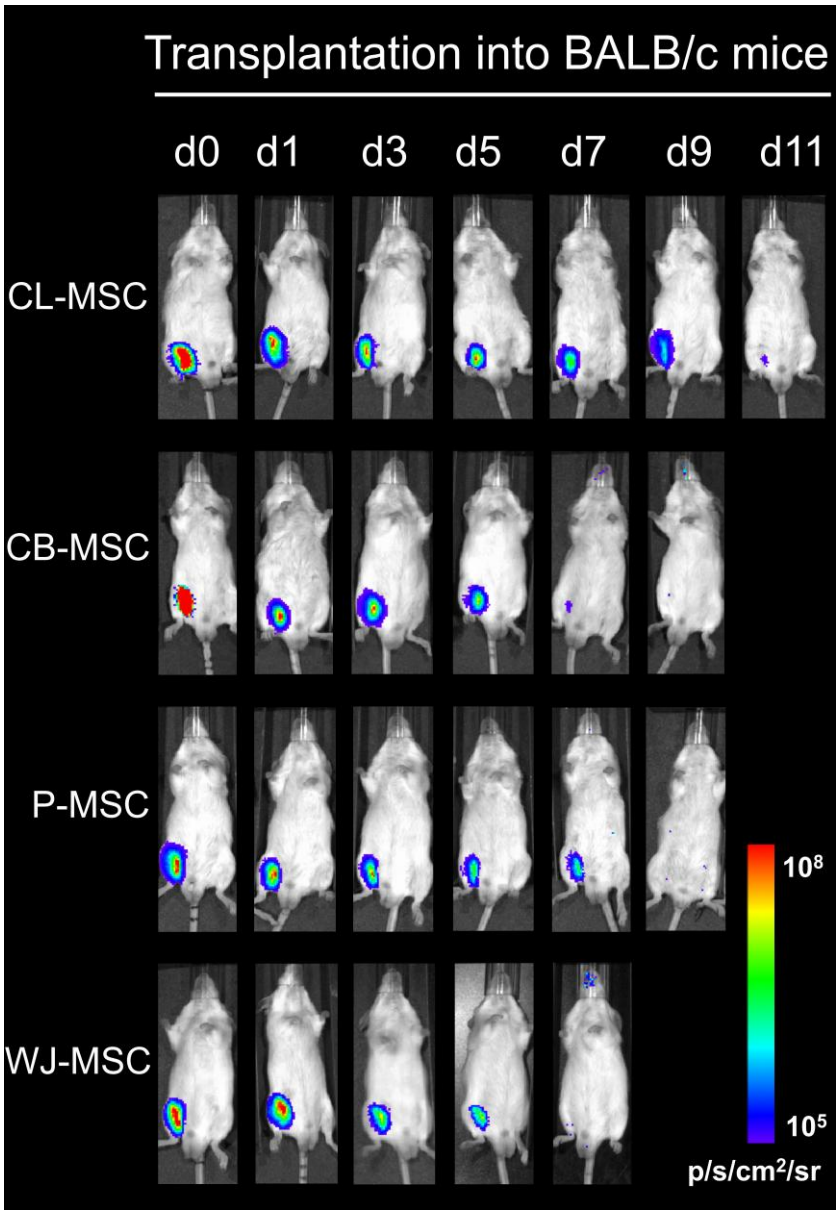

Figure 1: Rejection of FLuc-expressing extraembryonic tissue-derived MSCs in BALB/c mice. *1 x 10^6 FLuc-positive MSCs were injected into the hind leg muscle of BALB/c mice. The mice were imaged on the day of the injection (day 0), one day later and then every second day until the maximal FLuc signal reached background levels (3.4 x 10^5 p/s/cm^2/sr). Representative mice are shown for each time point (n = 4 – 6 mice per cell type). Bioluminescence was quantified in units of maximum photons per second per square centimeter per steridian (p/s/cm^2/sr). d, day; FLuc, firefly luciferase.The quantified data are shown in Figure 13.*

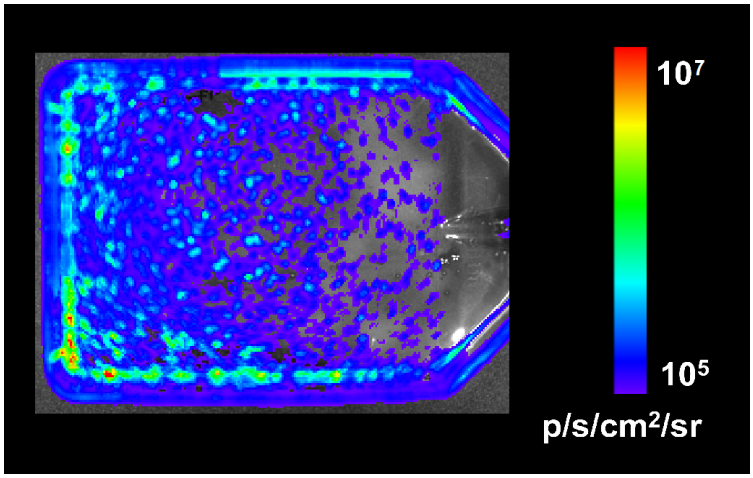

Figure 2: To monitor live CL-MSC migration, the cells were made to express FLuc. *To confirm FLuc expression, luciferin was added to 60% confluent CL-MSC cultures, before being imaged using the IVIS 200 system. Bioluminescence was quantified in units of maximum photons per second per square centimeter per steridian (p/s/cm^2/sr). FLuc, firefly luciferase.*

8.2. ABBREVIATIONS

ALCAM	Activated-leukocyte cell adhesion molecule
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BALB/c	Bagg-albino/c
BLI	Bioluminescence imaging
BM-MSC	Bone marrow mesenchymal stromal cells
BSA	Bovine serum albumin
CB-MSC	Cord blood mesenchymal stromal cells
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CL-MSC	Cord lining mesenchymal stromal cells
COX	Cyclic oxide synthase
CXCL	Chemokine (C-X-C motif) ligand
°C	Degrees Celsius
Da	Dalton
DC	Dendritic cell
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
et al.	*Et alli* (Latin for “and others”)
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FLuc	Firefly luciferase
Gal-1	Galectin-1

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Growth colony-stimulating factor
GER	Germany
G/M-CSF	Granulocyte/macrophage colony stimulating factor
GMP	Good manufacturing practice
GvHD	Graft versus host disease
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HO-1	Hemoxygenase-1
HSC	Haematopoietic stem cell
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
ISCT	International Society for Cellular Therapy
IV	Intravenous
LIF	Leukaemia inhibitory factor
LSD	Least-significant difference
LVEF	Left ventricular ejection fraction
MFI	Mean fluorescent intensity
MHC	Major histocompatibility antigen
MLR	Mixed-lymphocyte reaction
MMP	Metalloproteinase
MI	Myocardial infarction
MSC	Mesenchymal stromal cell
MTS	(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt, methyl-tetrazolium salt

NK	Natural killer cell
NOD	Non-obese diabetic
NOS	Nitric oxide synthase
Post-hoc	Latin for “after this”
P-MSC	Placental mesenchymal stromal cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
Sca-1	Stem cell antigen-1 (Ly-6A/E)
SCID	Severe-combined immune deficiency
SDF-1	Stromal cell-derived factor-1 (CXCL12)
SNP	Sodium nitroprusside
SSEA	Stage-specific embryonic antigen
TBS	Tris-buffered saline
T_H	T helper cell
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
TSG-6	TNF-α-stimulated gene/protein-6
USA	United States of America
WJ-MSC	Wharton‘s jelly mesenchymal stromal cells
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

APPENDIX

International system of units (SI):

m	Metre
kg	Kilogram
l	Litre

Prefixes for SI units:

k	kilo (10^{3})
c	centi (10^{-2})
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})

8.3. MANUSCRIPT

J. Kawalkowska*, M. Stubbendorff*, T. Deuse, T. T. Phan, K. Bieback, K. Atkinson, X. Hua, J. Velden, T. H. Eiermann, H. D. Volk, R. C. Robbins, S. Schrepfer. Immunological properties of extraembryonic tissue-derived mesenchymal stromal cells derived from gestational tissue. Submitted for publication June 2012. *co-shared authorship. The author of the present dissertation was responsible for: writing the manuscript, preparing figures, data analysis and interpretation, and acquiring parts of the data. For other contributions please refer to Contributions on page 103.

8.4. PRESENTATIONS

J. Kawalkowska, T. Deuse, H. Reichenspurner, R. C. Robbins, S. Schrepfer. Talk title: No significant homing of IV injected mesenchymal stem cells in a murine model of myocardial infarction. 40th Annual Meeting of the German Society for Thoracic and Cardiovascular Surgery, Stuttgart, Germany, 2011 (abstract A-296-0005-00282).

Data from the present work was presented at:

M. Stubbendorff, **J. Kawalkowska**, T. Deuse, T. T. Phan, K. Bieback, K. Atkinson, Velden, T. H. Eiermann, H. Reichenspurner, R. C. Robbins, S. Schrepfer. Talk title: Immunological and immunomodulatory properties of extraembryonic tissue-derived mesenchymal stromal cells. 41st Annual Meeting of the German Society for Thoracic and Cardiovascular Surgery, Freiburg, Germany, 2012 (abstract V243).

8.5. CONTRIBUTIONS

I thank Dr. med. vet. Stubbendorff (TSI lab) for contributing to this dissertation by gathering the following data: differentiation and histology of P-MSCs and WJ-MSCs, part of the FACS data characterizing WJ-MSCs and P-MSCs, MTS, and ELISPOT assays. Furthermore, she contributed to this work by injecting MSCs into mice and organ harvesting. She was also responsible for the paper work associated with the animal studies.

I thank Christiane Pahrmann (TSI lab) for gathering most of the BLI data. I would like to thank Xiaoqin Hua (TSI lab) and Dr. med. Velden (Department of Pathology, University Hospital Hamburg-Eppendorf) for taking the chondrocyte photos and Prof. Eiermann (Institute for Transfusion Medicine, University Hospital Hamburg-Eppendorf) for the HLA-typing.

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10. STATEMENT

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, April 2012

Joanna Kawalkowska