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MESENCHYMAL STEM CELLS IN TRANSPLANTATION AND TISSUE REGENERATION

Topic Editors
Martin J. Hoogduijn and Frank J. Dor

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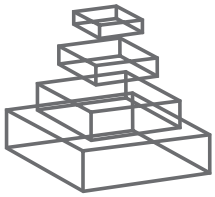

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MESENCHYMAL STEM CELLS IN TRANSPLANTATION AND TISSUE REGENERATION

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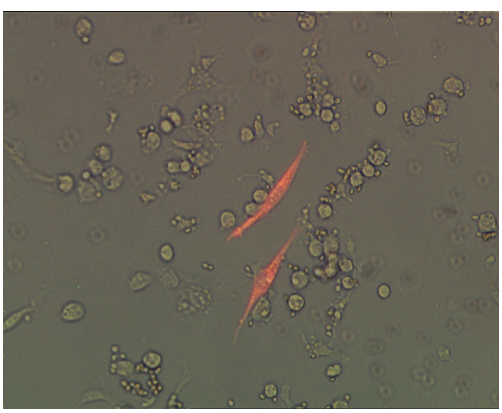

Re-culture of intravenously infused red-fluorescent MSC from lung tissue. Figure taken from Eggenhofer E, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, Baan CC, Dahlke MH and Hoogduijn MJ (2012) Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front. Immun.* 3:297. doi: 10.3389/fimmu.2012.00297.

Mesenchymal stem cells (MSC) have induced expansive interest from both scientific and clinical points of view over the last decennium. This interest stems from the capacity of these cells to differentiate into multiple lineages and from their secretion of growth factors that can activate progenitor cells, which make MSC potentially applicable for tissue regenerative purposes. When exposed to inflammatory conditions, MSC furthermore exhibit immunosuppressive properties. The reduction of chronic or acute inflammatory responses by MSC may halt the development of injury and allow regenerative processes to take place. This prospective has initiated attempts to use MSC as an immunosuppressive and regenerative agent in transplantation and regeneration of tissues like kidney, liver, heart, bone and multiple others. Nevertheless, the conditions under which MSC therapy is effective and via what mechanisms MSC operate are, in particular in in vivo settings, not clear. The route of administration and the reigning immunological conditions are likely to be key for the efficacy of MSC. Studies have demonstrated for instance that MSC may be immunostimulatory under immunological quiescent conditions. Whereas some reports indicate that homing of MSC to

inflamed tissues and their secretion of growth and anti-inflammatory factors are responsible for their effect, others show that MSC rapidly disappear after administration and may not have time to actively contribute to immunosuppression and tissue repair. Many aspects of MSC have to be clarified in order to develop efficient therapies.

In this special topic, different views on the mechanisms of immunomodulation and regeneration by MSC and on the possible applications of MSC in transplantation and tissue regeneration will be put together.

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Mesenchymal stem cells in transplantation and tissue regeneration

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Mesenchymal stem cells (MSC) were identified in the 1960s as bone marrow cells capable of osteogenic differentiation (Friedenstein and Petrakova, 1966). In the following decennia, these cells were further attributed with the capacity to differentiate into adipogenic, chondrogenic, and myogenic lineages (Pittenger et al., 1999), to secrete trophic factors that stimulate other cell types (Caplan and Dennis, 2006), and to possess immunomodulatory properties (Di Nicola et al., 2002). Cells with these properties were found not to be restricted to the bone marrow, but also to reside at other locations including adipose tissue (Zuk et al., 2002), skin (Toma et al., 2001), and in organs like liver, kidney, and brain (da Silva Meirelles et al., 2006). In particular adipose tissue has proven to be a valuable source of MSC due to its accessibility and its abundance.

The characteristics of MSC initiated interest in their potential clinical use for tissue regenerative and immunomodulatory purposes. The first clinical applications were in the treatment of osteogenesis imperfecta (Horwitz et al., 1999) and graft versus host disease (Le Blanc et al., 2004). Since then, the use of MSC for the treatment of a variety of diseases has been investigated in clinical trials, including in Crohn's disease (Duijvestein et al., 2010), myocardial infarction (Hare et al., 2009), rheumatoid arthritis (Liang et al., 2011), multiple sclerosis (Freedman et al., 2010), and organ transplantation (Dahlke et al., 2009).

In organ transplantation, the use of MSC is aimed at the prolongation of allograft survival. Thus, MSC therapy may be used for the treatment of acute rejection, but also to prevent currently untreatable chronic rejection. Furthermore, there is evidence that MSC therapy has a tissue regenerative component that repairs organ injury caused by immunological or ischemic events and thereby prevents the loss of organ transplants in animal models (Morigi et al., 2008; Popp et al., 2008). This may offer another window of opportunity for MSC therapy in particular shortly after transplantation, when organ loss peaks partly due to ischemia–reperfusion injury of the graft.

As with all therapies in development, the reasons for the use of MSC as an immunomodulatory and regenerative agent should be taken into careful consideration. Open questions are whether MSC therapy is effective and, if so, whether it is more efficient than existing drugs? Are there safety issues involved? Is MSC therapy cost-effective? Whether MSC can replace existing drugs is not clear at the moment as the efficacy of MSC therapy is difficult to determine. A one to one comparison of efficacy between MSC and conventional drugs is not easy to make. While pharmacological drugs target specific molecular pathways, MSC have a wide range of effects. Furthermore, while pharmaceuticals can be given to patients at a daily frequency, for safety, practical, and financial reasons there is a limit to the frequency at which cell therapy can be applied. However, at these early stages, a head on comparison with standard therapy may not be required as the use of MSC will primarily be aimed at applications where conventional therapies fail. As such, MSC will be applied as an adjuvant for current therapies. In the more distant future, MSC may be used to replace medication that has significant side effects, as may be the case with calcineurin inhibitors in organ transplantation. Although very effective in preventing organ rejection, calcineurin inhibitors are nephrotoxic, thereby limiting the life span of kidney transplants. Side effects of MSC therapy have not been reported yet, but certainly some will occur when MSC are used more widely. A high incidence of infections after MSC therapy in graft versus host disease patients was recently reported (von Bahr et al., 2011). Whether the risk for infection was significantly elevated in MSC treated patients compared to controls was however not demonstrated. To be able to map the side effects of MSC therapy, these effects should be investigated parallel to their clinical effects in placebo-controlled studies. Nevertheless, from where we stand today, we can conclude with considerable certainty that the infusion of MSC does not harbor serious health threats.

While the *in vitro* properties of MSC suggest a beneficial effect of MSC in immunological and degenerative diseases and early clinical trials are triumphant about the feasibility and safety of MSC therapy, there is thus far little evidence that MSC are effective in curing disease. The effectiveness of MSC therapy needs to be established in follow up trials and knowledge of the mechanisms of action of MSC may help optimizing the therapy. The mechanisms of action of MSC after infusion may be very different to those observed *in vitro*. There is for instance accumulating evidence that MSC are short-lived after infusion (Popp et al., 2008). Even though, long-term effects are observed after infusion of MSC. These effects may be mediated by other cell types to which the effect of MSC is transferred. It has been demonstrated that MSC induce immunoregulatory capacity of T cells (Prevosto et al., 2007) and macrophages (Maggini et al., 2010). More knowledge on how MSC interact with these cell types could provide tools for optimizing MSC therapy.

An alternative approach for MSC therapy is to design drugs or therapies that target tissue resident MSC. MSC respond to cytokines and growth factors by changing their immunoregulatory function and/or their differentiation status. Therefore, these factors could be the basis of such drugs, which should induce a specific response in MSC that reside in transplanted organs. In

this way, MSC therapy can be more localized and more specific compared to the infusion of MSC.

In order for a therapy to be successful, it needs to be cost-effective. MSC treatment is a costly therapy as MSC need to be cultured under GMP conditions. In particular the generation of custom-made, i.e., autologous, MSC of clinical grade is an expensive process. Centralization of MSC production at specialized laboratories can reduce costs. Furthermore, for some applications allogeneic MSC may be suitable and these can be generated in large batches, which further brings down costs. A real cost–effect analysis can however only be made once the efficacy of MSC therapy has become clear.

Summarizing, MSC have the potential to be used as an immunomodulatory and regenerative therapy in organ transplantation and immune and degenerative diseases. Basic and clinical research will have to point toward the right directions on the effective use of MSC. In this special feature of Frontiers in Immunology, the most recent findings on the immunomodulatory capacity of MSC, such as their interaction with regulatory T cells, and on their potential to induce regeneration of liver, kidney, and heart after ischemia–reperfusion injury and of bone and cartilage damage in rheumatoid and osteoarthritis, will be presented. Furthermore, challenges on how to generate a high quality and effective cell product will receive attention. Finally, the use of MSC in transplantation and regenerative medicine and ideas on how to drive this field forward will be reviewed and discussed by leaders in the field. We are confident that this special topic will generate new directions to be followed in translational research and clinical trials.

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Application of MultiStem® allogeneic cells for immunomodulatory therapy: clinical progress and pre-clinical challenges in prophylaxis for graft versus host disease

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The last decade has seen much progress in adjunctive cell therapy for immune disorders. Both corporate and institutional Phase III studies have been run using mesenchymal stromal cells (MSC) for treatment of Graft versus Host Disease (GvHD), and product approval has been achieved for treatment of pediatric GvHD in Canada and New Zealand (Prochymal®; Osiris Therapeutics). This effectiveness has prompted the prophylactic use of adherent stem cells at the time of allogeneic hematopoietic stem cell transplantation (HSCT) to prevent occurrence of GvHD and possibly provide stromal support for hematopoietic recovery. The MultiStem® product is an adult adherent stem cell product derived from bone marrow which has significant clinical exposure. MultiStem cells are currently in phase II clinical studies for treatment of ischemic stroke and ulcerative colitis, with Phase I studies completed in acute myocardial infarction and for GvHD prophylaxis in allogeneic HSCT, demonstrating that MultiStem administration was well tolerated while the incidence and severity of GvHD was reduced. In advancing this clinical approach, it is important to recognize that alternate models exist based on clinical manufacturing strategies. Corporate sponsors exploit the universal donor properties of adherent stem cells and manufacture at large scale, with many products obtained from one or limited donors and used across many patients. In Europe, institutional sponsors often produce allogeneic product in a patient designated context. For this approach, disposable bioreactors producing <10 products/donor in a closed system manner are very well suited. In this review, the use of adherent stem cells for GvHD prophylaxis is summarized and the suitability of disposable bioreactors for MultiStem production is presented, with an emphasis on quality control parameters, which are critical with a multiple donor approach for manufacturing.

Keywords: MultiStem cells, GvHD prophylaxis, regenerative medicine, adherent stem cells, bioreactor

RATIONALE FOR ADHERENT STEM CELLS IN PROPHYLAXIS

Graft versus Host Disease (GVHD) is a potential life-threatening complication and one of the major limitations of allogeneic hematopoietic stem cell transplantation (HSCT). The complication is thought to be initiated by activation of mature donor T-cells, which are co-infused with the hematopoietic stem cell (HSC) transplant, through recognition of target antigens presented on MHC molecules expressed on antigen presenting cells that reside within host tissue. Upon alloantigen recognition, the co-infused donor T-cells become activated, expand, and induce cytolytic effects that target several organs including skin, gut, and liver.

Current therapies to prevent acute GvHD (aGvHD) make use of pharmacological suppression of T-cell activation, however, such immunomodulatory therapy appears not sufficient to treat a GvHD and it may increase the risk of opportunistic infections (Perales et al., 2007) and disease relapse (Lee et al., 2004). Additional strategies are thus required to improve the response rate to immunosuppression.

The last decades have seen major improvements in stem cell research and the translational application of adult stem cells (Armstrong et al., 2012). This has led to numerous clinical trials to investigate the efficacy of various types of stem cells to treat immune disorders, neurodegenerative and cardiovascular disease, bone and cartilage repair, and type I diabetes (Busch et al., 2011b; Trounson et al., 2011; Penn et al., 2012).

Adherent non-hematopoietic bone marrow-derived stem cells have been demonstrated to reduce proliferation of GvHD patient-derived T-cells (Le Blanc et al., 2004), inhibit alloreactive T-cell responses and support HSC engraftment (Auletta et al., 2010). Their use has therefore gained particular interest to treat and prevent GvHD in patients with hematopoietic malignancies such as acute myeloid or lymphoid leukemia (AML, ALL), chronic myeloid leukemia (CML), or myelodysplasia (MDS).

MULTISTEM CELLS

Multipotent adult progenitor cells (MAPC) are bone-marrow-derived non-hematopoietic adherent cells that were first described

by Jiang et al. (2002). The MultiStem clinical product is based on MAPC isolation and expansion protocols (Boozer et al., 2009). Pre-clinical animal studies have clearly shown therapeutic benefits of MAPC/MultiStem cells by preventing GvHD (Kovacsovics-Bankowski et al., 2009), and improving tissue regeneration and function in cardiovascular and neurological disorders, including acute myocardial infarct, traumatic brain injury, spinal cord injury, and ischemic limb injury (van't Hof et al., 2007; Aranguren et al., 2008, 2011; Mays et al., 2010; Walker et al., 2010, 2012; Busch et al., 2011a).

MultiStem cells are in Phase II clinical testing for use in treatment of inflammatory bowel diseases (ulcerative colitis), acute myocardial infarct, and ischemic stroke. Safety studies (Phase I clinical trials) using MultiStem as an adjunct in allogeneic bone marrow transplantation for prevention of GvHD (Maziarz et al., 2012) and acute myocardial infarction (AMI) have been completed (Penn et al., 2012). **Table 1** summarizes the current status of the MultiStem (pre)clinical trials.

The therapeutic benefits of MAPC are multimodulatory and have been shown to be caused, at least in part, by their pro-angiogenic effect through trophic support (Aranguren et al., 2007, 2011; Lehman et al., 2012) and their ability to modulate the immune system (Kovacsovics-Bankowski et al., 2009; Walker et al., 2010). In particular the immune-regulatory properties are of paramount importance for GvHD treatment. Human and rodent MAPC are non-immunogenic. The cells lack MHC II expression, and therefore do not induce a proliferative response when co-cultured with allogeneic T-cells (Kovacsovics-Bankowski et al., 2009; Jacobs et al., 2012). These studies showed that MAPC significantly reduce T-cell proliferation when responder T-cells are stimulated with allogeneic irradiated stimulator cells. The study by Kovacsovics–Bankowski furthermore demonstrated the absence of MAPC *in vivo* immunogenicity, since injection of allogeneic Lewis rat MAPC into Buffalo rats failed to prime an anti-Lewis T-cell response as was observed with allogeneic splenocytes. This immuno-privileged nature and capacity of human and rodent MAPC to inhibit T-cell proliferation is of importance for their use in GvHD prophylaxis. A study by Highfill et al. (2009) demonstrated that MAPC had a prophylactic effect on GvHD after intrasplenic injection. Improved animal survival was seen in MAPC treated mice, while reduced CD4+ and CD8+ T-cells in the spleen were observed.

The effect of MAPC on the inhibition of T-cell proliferation in the study by Highfill and coworkers was shown to be dependent on the ability of MAPC to express PGE synthase and the production of prostaglandin E2 (PGE2). Other studies have also shown that MAPC immunosuppression is partially mediated by soluble factors. A role for indoleamine 2,3-dioxygenase has been demonstrated for human and rat MAPC (Kovacsovics-Bankowski et al., 2009; Jacobs et al., 2012), but this was not found in murine MAPC (Highfill et al., 2009). On the contrary, blocking PGE2 activity had no effect on the suppressive effect of human MAPC (Jacobs et al., 2012) indicating that the molecular mechanisms of immunosuppression occur in a species specific manner.

MULTISTEM CELLS VERSUS MSC

Mesenchymal stromal cells (MSC) may use similar immunosuppression mechanisms (Gebler et al., 2012) and although MAPC and MSC exert comparable activity in an *in vitro* T-cell suppression assay (Jacobs et al., 2012), it is evident that they are distinct cell types. Both cells are adherent bone marrow-derived stem cells, but due to different culture conditions they adopt different phenotypes (Roobrouck et al., 2011b). The cells express distinct cytokine profiles which may explain the observations that MAPC can induce tube formation by HUVEC cells in *in vitro* assays while MSC lack this pro-angiogenic effect (Lehman et al., 2012). Moreover, MAPC are able to induce functional blood vessels *in vivo* when the cells are implanted in a Matrigel plug with VEGF and bFGF under the skin of nude mice, where vessels induced by MSC appeared leaky (Roobrouck et al., 2011a). This latter study showed by means of transcriptome analysis that MAPC and MSC are clearly distinguishable cells types. In a recent study, intracranial injection of human MAPC and human MSC 2 days after induction of stroke revealed that MAPC had a stronger effect on the attenuation of the inflammatory response and had more potency to promote endogenous tissue regeneration than MSC (Mora-Lee et al., 2012). Thus differences in *in vivo* activity between MAPC and MSC have been described, but it is not clear yet how this relates for instance to the therapeutic activity of these cells in GvHD prophylaxis.

MAPC and MultiStem cells are thus immune-privileged in the currently tested settings, and for MSC low-immunogenicity and an anatomical site-specific immuno-privileged nature have been demonstrated (reviewed by Griffin et al., 2010). Before safe application as an allogeneic cell product to patients, cells need to be isolated, expanded, and quality tested in order to reach sufficient cells with therapeutic activity. This hampers the use of autologous cells for applications where cells are needed immediately, as is the case for stroke for instance. Particularly MAPC and MultiStem cells have the capacity to undergo extensive expansion doublings *in vitro*, which, combined with the immuno-privileged properties, enable an off-the-shelf use for MultiStem cells, with therapeutic product available at the time of need and usable without patient matching.

Pre-clinical animal studies using multipotent MSC in HSCT have shown positive effects on survival benefit and the prevention of GvHD, although contradictory effects are observed depending on the origin of adherent stem cells, timing, and dose of infusion (see reviews by Auletta et al., 2010; Baron and Storb, 2012). Pre-clinical studies have shown safety for intravenous infusion of MultiStem cells and demonstrated that the survival rate in a haploidentical aGvHD rat model increased from less than 20 to 50% in rats that received two MultiStem doses in a prophylactic manner (Kovacsovics-Bankowski et al., 2008, 2009).

Human clinical studies have been conducted to evaluate the safety and efficacy of MSC to treat GvHD. Review of current data show that the application of MSC is safe, but that inconsistent benefit is seen in the treatment of acute and steroid-refractory GvHD (Ball et al., 2008; Auletta et al., 2010; Kebriaei and Robinson, 2011a,b; Baron and Storb, 2012). While these first studies are encouraging and prompt evaluation of optimal dosing strategies for MSC treatment of active clinical GvHD, an equally important

Table 1 | Summary of (pre)clinical studies using MultiStem cells.

Program	Developmental stage clinical trials.gov identifier	Cell administration	Observations	Reference
INFLAMMATORY AND IMMUNE				
Ulcerative Colitis	Phase II (in progress) NCT01240915	Single or multiple dose IV	In progress	
HSC transplant/GvHD prevention	Phase I (completed) NCT00677859	1, 5, or 10 million/kg IV, single or repeated dose, adjunctive to HSCT	Doses were safe and tolerated at all doses. Low incidence (11%) of day 100 acute GvHD at 10 million/kg	Maziarz et al. (2012)
Solid organ transplant	Phase I (approved)	2 × 150–600 million cells, first dose transplanted into portal vein at day 1 after transplantation, second dose IV on day 3	In progress	Popp et al. (2011)
	Pre-clinical	2, 4, or 10 million on day-4, or 5 million on days 4 and 0 of allo heart transplant	All doses were pre-clinically safe and well tolerated. Increased long-term pre-clinical allograft protection	Eggenhofer et al. (2011)
CARDIOVASCULAR				
Acute myocardial infarction	Phase I (completed) NCT00677222	20 or 100 million via transarterial catheter, 2–5 days after AMI	All doses were safe and well tolerated. Improvement of Ejection Fraction.	Penn et al. (2012)
PVD/PAD/CLI	Pre-clinical	1 million cells intramuscular, 1 day after iliac artery ligation	MAPC induced a more rapid and complete recovery of blood flow than control	Ryu et al. (2011)
NEUROLOGICAL				
Ischemic stroke	Phase II (In progress) NCT01436487	IV dose (low/high), 1–2 days after ischemic stroke	All doses were safe and well tolerated	Athersys Press Release, 2 October 2012
Traumatic brain injury	Pre-clinical	2, 10 million cells/kg, IV, 2 + 24 h after injury	MAPC injection has a neuroprotective effect by preserving splenic mass, blood brain barrier integrity, and increasing the brain M2/M1 macrophage ratio	Walker et al. (2010, 2012)
Multiple sclerosis	Pre-clinical	1, 3, or 9 million cells IV after EAE symptom onset	Decreased lesion burden in spinal cord and improved remyelination	Hamilton et al. (2011)
Spinal cord injury	Pre-clinical	200,000 cells transplanted immediately after dorsal column crush injury	Transplantation of MAPC 500 μm caudal to lesion results in prevention of axonal dieback and regeneration of injured axons	Busch et al. (2011a)
Hurler's syndrome	Pre-clinical	Transplantation into cerebral lateral ventricles	Injection of MultiStem cells in neonatal MPS-I mice reduces the accumulation of GAGs in the brain	Nan et al. (2012)

Adapted from company website (www.Athersys.com). IV, Intravenous; PVD, peripheral vascular disease; PAD, peripheral arterial disease; CLI, critical limb ischemia; EAE, experimental allergic encephalomyelitis; MPS-I, mucopolysaccharidosis type I; GAG, glycosaminoglycan.

strategy is the use of adherent stem cells as adjunct to HSC for prophylaxis of GvHD. To date a limited number of clinical studies have been conducted to investigate co-transplantation of MSC with HSC and the prevention of GvHD. Recently a phase I clinical dose escalation study was finished in which MultiStem cells were administered to adult patients undergoing allogeneic HSCT for the treatment of leukemia and related conditions (Maziarz et al., 2012).

CLINICAL EXPERIENCE USING ADHERENT STEM CELLS AS HSCT CO-TRANSPLANT

Several studies have evaluated the effect of MSC co-transplantation with HSC on engraftment, safety, and GvHD in pediatric (Ball et al., 2007; Macmillan et al., 2009; Bernardo et al., 2011) and adult patients (Lazarus et al., 2005; Ning et al., 2008; Baron et al., 2010; **Table 2**). Ball et al. (2007) reported a Phase I/II trial in which 14 children received 1–5 million donor MSC/kg body weight 4 h before peripheral blood stem cell (PBSC) transplantation of HLA-disparate relative donors. No infusion-related toxicity was observed. Graft rejection did not occur in the 14 patients receiving MSC, while seven graft failures were observed in a historic control group of 47 children.

A decreased incidence of aGvHD was observed in a group of 13 pediatric hematological disorder patients who received paternal HLA-disparate MSC co-transplantation with umbilical cord blood cells (Bernardo et al., 2011). Single dose injections of 1–3.9 million MSC/kg body weight were safe and revealed no significant difference of cumulative graft rejection when compared with a group of 39 historical controls. Grade II–IV aGvHD showed no significant difference between MSC-receiving patients and controls (31 versus 41%, $p = \text{NS}$). However, patients in the MSC group did not develop grade III or IV aGvHD, while the incidence of these severe forms in the control groups was 26% ($p = 0.05$). None of the patients developed cGvHD, while 11% were observed in the control ($p = \text{NS}$).

Macmillan et al. (2009) reported another Phase I/II clinical trial in pediatric patients receiving MSC co-transplanted with umbilical cord blood transplantation. Eight patients received a dose of 0.9–5 million MSC/kg body weight MCS of haploidentical parental donors, 4 h prior to transplantation of unrelated donor blood cell and three of them were given a second dose at day 21. Three patients developed grade II GvHD, and no patient developed cGvHD. No statistical difference with a historical cohort was observed, but the authors mention a

Table 2 | Summary of clinical studies using adherent stem cells for GvHD prophylaxis.

Study	HSCT specifics	Stromal cell therapy	Stromal cell dosing	Observations
Maziarz et al. (2012)	URD, MRD, BM/PB, Adults CSA + MTX, Tac + MTX	Third party, universal donor, GHVD prophylaxis	1, 5, or 10 million/kg, single dose day 2 after HSCT, or 1 or 5 million/kg repeat dose on day 2, 9, and 16, or days 2, 9, 16, 23, and 30 after HSCT	Grade II–IV and III–IV GVHD at Day 100 was 37 and 14%, resp. ($n = 36$). 11% II–IV GVHD and no grade III–IV GVHD and in 10 million/kg group single dose ($n = 9$). Anticipated rates in this population; 47% II–IV and 15% III–IV
Kuzmina et al. (2012)	RD, HSCT, adults CSA, MTX, prednisolone	HSC donor-derived MSC, GVHD treatment	0.9–1.3 million/kg, 19–54 days after HSCT	Grade II–IV aGVHD in 33.3% of control patients and 5.3% in MSC prophylaxis group
Bernardo et al. (2011)	URD, RD, UCB, pediatric CSA + steroids, CSA + MTX	Paternal derived MSC, GVHD prophylaxis	1–3.9 million/kg, single dose at day of HSCT	Reduced grade III–IV GVHD (0%, compared to historic controls 18/8%)
Baron et al. (2010)	URD, PB, adults MMF + Tac	Unrelated MSC, safety of MSC co-transplantation	1–2 million/kg at day of HSCT	Day 100 incidence of grade II–IV was 35%. Cumulative incidence of grade II–IV GVHD was 45%, compared with 56% in historic group
Macmillan et al. (2009)	URD, UCB, pediatric CSA + steroids	Parental MSC, promote engraftment	0.9–5 million/kg at day of HSCT; three patients second dose at day 21	At day 100, cumulative incidence of grade II–IV similar between MSC and historic control (38 versus 22%, $p = 0.44$)
Ning et al. (2008)	RD, BM/PB, adult CSA + MTX	Sibling derived MSC, MSC prophylaxis	0.03–1.53 million/kg at day of HSCT	Grade II–IV was 11.1% in MSC group and 53.3% in non-MSC group. Overall aGVHD incidence was 44.4% in MSC and 73.3% in non-MSC group
Ball et al. (2007)	MRD, PB, pediatric	HSC donor-derived MSC, graft failure	1–5 million/kg single dose at day of HSCT	No graft rejection in patients receiving MSC, 14.8% failure in control group ($p = 0.14$)
Lazarus et al. (2005)	RD, PB/BM, adults CSA + MTX	HSC donor-derived MSC, GVHD prophylaxis	1, 2.5, or 5 million/kg single dose at day of HSCT	Overall, 50% of patients developed aGVHD, at least grade II in 28% of patients. 11 and 4% developed grade III and IV respectively

URD, unrelated donor; MRD, mismatched related donor; RD, related donor; BM, bone marrow; PB, peripheral blood; UCB, umbilical cord blood; CSA, cyclosporine; MTX, methotrexate; Tac, tacrolimus.

non-significant trend toward improved 3-year survival in the MSC group.

A study by Lazarus et al. (2005) was done on 46 adult patients receiving bone marrow ($n = 19$) or PBSCs ($n = 27$) co-transplanted 1–5 × million/kg MSC from HLA-identical sibling donors. A total of 28% of the patients developed at least grade II aGvHD, while grade III and IV were observed by 11 and 4% respectively. The authors indicate a literature-based percentage of 44–64% for grade III and 12–26% for grade IV, suggesting a benefit of MSC infusion.

Ning et al. (2008) compared patients receiving HLA-identical sibling HSCs from blood or bone marrow without ($n = 15$) or with ($n = 10$) co-transplantation of MSC ($0.3–15.3 \times 10^5$/kg body weight). Grade II–IV GvHD was developed in 11% of the MSC group and 53% of the non-MSC group. None of the patients in both groups showed grade III–IV aGvHD.

Baron et al. (2010) performed a safety study in which patients were transplanted with PBSCs from HLA-mismatched donors in combination with MSC from third party unrelated donors. Twenty patients were co-infused with PBSC and 1–2 million MSC/kg body weight and compared with historical group of 16 patients treated with unrelated donor PBSC without MSC. In the MSC group, 45% experienced grade II–IV aGvHD and 56% in the control group. Grade IV aGvHD developed in 10% of the MSC group and 19% in the historic group.

The studies by Bernardo, Ning and Baron show that development of aGvHD after HSC transplantation may be reduced after co-injection of MSC. Efficacy of MSC as a therapy for aGvHD has recently also been reported by Kuzmina et al. (2012). In this study the MSC were administered after HSCT at the time of graft activation and GvHD manifestation and the authors showed a significant reduction of the incidence of grade II–IV aGvHD in the group of patients having received MSC. The combined results provide a promising base for adherent stem cells as an adjunct therapy for graft support and GvHD prophylaxis. Still, the number of studies and evaluated patients remain limited, and additional evaluations are essential to determine optimal cell dose, timing, and frequency of administration in achieving maximum clinical benefit.

MULTISTEM THERAPY FOR PROPHYLAXIS OF ACUTE GvHD

The primary objective of the clinical Phase I study was to evaluate the safety of MultiStem administration in single dose or as repeat doses to patients receiving allogeneic HSCT (Maziarz et al., 2012). A total of 36 patients was treated with MultiStem, 18 each in the single dose arm (1, 5, and 10 million cells/kg on day 2 after transplant) or the repeated dose arm (1 or 5 million/kg on days 2, 9, and 16 (3 weekly doses), or 5 million/kg on days 2, 9, 16, 23, and 30 (5 weekly doses).

The study demonstrated that MultiStem therapy was well tolerated in both the single infusion and repeat infusion arms and also suggested that the therapy may provide benefit to recipients of allogeneic HSCT, such as reducing the incidence and severity of GvHD, as compared to historical clinical experience (Ratanatharathorn et al., 1998; Nash et al., 2000; Anasetti et al., 2011). The majority of patients participating in the study received transplants from unrelated donors (19 of 36), and nearly all of the patients received PBSC transplants (34 of 36), both of which are associated with a higher risk of GvHD. Importantly, all patients experienced successful neutrophil engraftment (median time of engraftment 15 days), and 86% of patients experienced successful platelet engraftment (median time of engraftment 16 days) which compares favorably to historical clinical experience for this patient population supporting a positive impact on blood and immune system recovery. Relative to the published experience for this specific patient population (Ratanatharathorn et al., 1998; Nash et al., 2000; Anasetti et al., 2011), there was a substantial reduction in aGvHD incidence after administration of the highest single dose of 10 million MultiStem cells/kg, i.e., 11% grade II–IV GvHD, and 0% grade III–IV GvHD, versus 45–70 and 15–20%, respectively. There appeared to be a trend in dose response relationship, with patients receiving the highest single dose of MultiStem cells having a 33% lower absolute incidence of aGvHD relative to patients who received a single low or medium dose, and patients receiving once weekly dosing of the medium dose through the first 30 days having reduced GvHD incidence relative to single or weekly dosing over the first 2 weeks post-transplant. Finally, relapse-free survival rate at 100 days and infection-related complications over the first 100 days were favorable relative to historical clinical experience, consistent with the positive effect on engraftment rates.

CHALLENGES IN STEM CELL THERAPEUTIC PRODUCT DEVELOPMENT

Review of the stromal cell co-transplant and GvHD prophylaxis studies summarized above reveal an important limitation to the complete and optimal use of MSC as an effective therapy. Three of the studies could not be performed as planned because of insufficient availability of MSC at the time of transplantation (Lazarus et al., 2005; Ning et al., 2008; Macmillan et al., 2009). As a result, patients were not injected, not given a repeat dose, or given lower doses of MSC. This illustrates that the use of donor-related MSC is hampered by the limited proliferative capacity of these cells and/or sub-optimal cell expansion protocols or procedures. For efficient therapeutic application in the clinic, most of these limitations would be overcome by use of an allogeneic of-the-shelf stem cells product that is expanded to large scale with consistency in yields and quality.

To illustrate how the cell dose requirements for clinical studies impact the associated expansion and quality control needs we will detail the MultiStem study as a paradigm. For the completion of the entire MultiStem GvHD study, a total of 35 billion cells were injected, all of which were derived from expansions of seed-stock obtained from a single donor. Current MultiStem production units contain a surplus of cells, and consequently, over 50 billion cells were required for this trial. Of course, a multiplicity of cells will be needed for future trials and new manufacture procedures are required to produce the cells in a safe and cost-effective manner. Current process development efforts focus on the optimization of stem cell manufacturing in order to achieve a consistent and safe product for off-the-shelf use.

MULTISTEM MANUFACTURING

One of the most advantageous features of MultiStem cells is the proliferative capacity, and cells can undergo more than 60 population doublings (PD) before senescence. The extensive

proliferation capacity allows creation of a master and working cell bank as production intermediates. The current manufacturing strategy is based on clinical doses generated at about PD28 (master cell bank campaign) or PD38 (working cell bank campaign) that allows for the production of >100,000 clinical doses from a single donor.

MultiStem clinical production is currently performed by a contract manufacturing organization (Lonza) for creation of master cell banks and for production campaigns starting from those banks. A production run typically generates 40–50 clinical doses. The clinical dose varies according to indication, but current production units contain 180 million cells. The cells are cryopreserved and stored in a mixture of PlasmaLyte, i.e., an isotonic solution that mimics human plasma electrolytes, pH and osmolality (Baxter), DMSO, and human serum albumin. Each production run is tested for adventitious agents such as sterility, mycoplasma, and endotoxin. The product is also tested to show a normal karyotype. Only after completion and validation of all tests, the product is released from the contract manufacturer and stored for sites that take part in MultiStem clinical trials. Currently, the product has a validated shelf-life of 5 years.

MULTISTEM CELL EXPANSION IN A HOLLOW-FIBER BIOREACTOR

The Quantum Cell Expansion System (TerumoBCT) is being explored as an alternate platform for larger scale cell culture. This instrument was developed for *ex vivo* expansion of stem cells using a hollow-fiber bioreactor (Antwiler et al., 2009). The functionally closed automated culture system is comprised of a disposable synthetic hollow-fiber bioreactor of 2.1 m^2 surface area connected to a sterile closed-loop, computer-controlled media perfusion platform and gas exchangers. In addition, the system contains sterile closed sample ports by which fluid samples can be taken during expansion in order to monitor expansion and estimate the appropriate moment of harvest.

The Quantum system has been tested to optimize the complete workflow of MultiStem culture in a two-step procedure of stem cell isolation from bone marrow and subsequent expansion up to the scale of clinical dose. During the first step, whole bone marrow is loaded onto a bioreactor and maintained for 10 days, yielding 1×10^7 MultiStem cells. These cells are loaded onto a new bioreactor and expanded to 1×10^9 cells within a period of 6 days. Thus five doses of 180 million cells are obtained by using two consecutive runs on this bioreactor. This encourages the further exploration of this system to upscale MultiStem batches that are sufficient for clinical studies.

A crucial aspect of the research is to confirm by means of *in vitro* cell equivalency testing that the expanded cells are of consistent high quality and that cellular features that relate to *in vivo* function are maintained after manufacture adjustments (**Figure 1**).

MULTISTEM QUALITY CONTROL AND EQUIVALENCY TESTING

In advancing toward a final optimized manufacturing process, modifications of the MultiStem manufacturing process are extensively controlled in order to keep a consistent quality of the product. A panel of cell assays has been developed that allows for MultiStem QC testing in a tiered testing strategy (**Figure 1**). First, MultiStem identity is measured by marker gene and protein expression analysis by means of QPCR, ELISA, and flow cytometry. For MultiStem batches that pass these criteria, multilineage differentiation assays that are typically associated with stem cells from mesenchymal origin are performed. Osteogenic, adipogenic, and chondrogenic differentiation are measured by means of *in situ* cytochemistry and specific gene induction profiles associated with the corresponding cell types. As indicated above, one of the MultiStem product's mode of action *in vivo* has been shown to be based on pro-angiogenic activity, which is supported by an *in vitro* tube formation assay correlated with *in vivo* angiogenic activity and cytokine expression (Lehman et al., 2012).

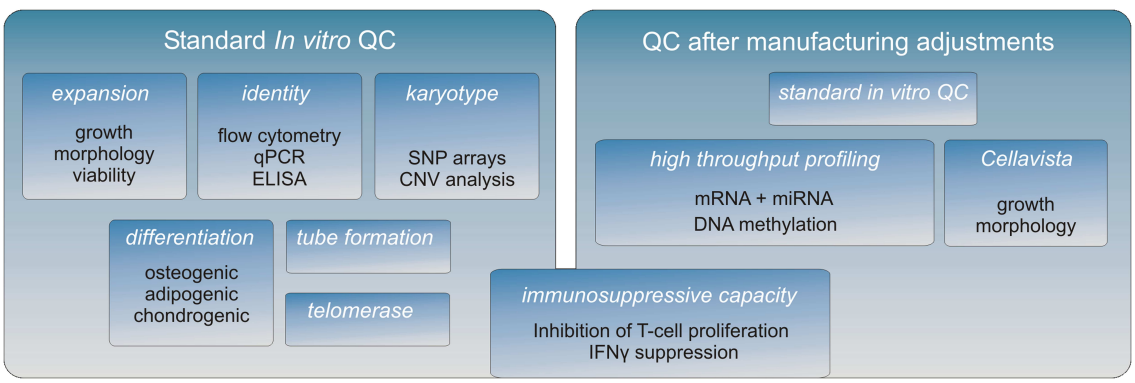

FIGURE 1 | The MultiStem QC pipeline. A full characterization of the MultiStem product is being conducted after each important adjustment of the manufacturing procedure. First, a standard QC is performed to establish MultiStem growth and typical stem cell properties (left panel). Subsequently, high throughput screens are performed to investigate the molecular phenotype of MultiStem (right panel). The Cellavista image-based platform (Roche) is used to study various morphological aspects of different cell cultures. Genome-wide molecular phenotype analyses are carried out on different platforms including array technology, PCR-based screening, and next-generation sequencing (NGS). Combining these "omics" data facilitates on the one hand the identification of unique MultiStem features, while on the other hand the retention of the molecular identity after applying alternative culturing methodologies can be validated. For MultiStem equivalency testing, the immunosuppressive capacity is evaluated by two assays: one is based on inhibition of T-cell proliferation and the other is based on the corresponding reduction of IFNγ secretion by T-cells.

Given the indications that inhibition of T-cell proliferation is a major contributor to immune suppression by stromal cells, we consider *in vitro* immune suppression and potency assays as highly important in our QC in order to guarantee a consistently safe product for treatment of GvHD and other disorders. With the purpose of application in GvHD and other disorders in which immunosuppression by MultiStem cells is critical, our QC emphasizes on *in vitro* assays that reflect such suppression (Kovacsovics-Bankowski et al., 2009). An *in vitro* criterion that is often used to assess the immunosuppressive capacity of stem cells is their inhibitory effect on the proliferation of activated T-cells. In our QC we use two different standardized assays to quantify MultiStem immunosuppression. One of the assays directly measures the inhibition of T-cell proliferation in a co-culture model of MultiStem cells and responder T-cells that are activated by CD3/CD28 or PBMC (Jacobs et al., 2012) while the other assay quantifies interferon gamma (IFNγ) which is secreted by activated T-cells.

In the context of biosafety, a normal karyotype is demonstrated by means of copy number variation (CNV) analysis of MultiStem cells and donor-derived non-expanded mononuclear cells on SNP arrays and the data is analyzed for genomic insertions or deletions at a resolution of 50 kb.

EPIGENETIC SCREENS FOR CELL EQUIVALENCY TESTING

Our current QC pipeline is sufficient to determine MultiStem identity and lot release assays for early to mid-stage clinical studies. However, it is anticipated that for late stage clinical trials (Phase III) and product release, more stringent quality controls are required by the regulatory organizations FDA and EMA, particularly in terms of potency and comparability following process improvements. The QC pipeline is being extended with various genome-wide screening methods to comprehensively characterize the molecular phenotype of our product. Transcriptome analysis is already implemented as a powerful tool in cell comparability testing, and we currently explore emerging epigenetic analysis tools that on the one hand identify robust MultiStem markers and on the other hand provide insight in the mechanisms underlying MultiStem function.

One of the epigenetic tools to investigate MultiStem identity and comparability is miRNA screening, since miRNA profiles determine the identity of stem cells (Chen et al., 2007) and distinguish between embryonic or adult stem cells, as well as between the adult stem cells MAPC and MSC (Aranda et al., 2009). Epigenetic modifications such as DNA methylation or histone modification can influence the function of the associated genes. As a consequence, stem cell identity is related to the epigenetic profile and differentiation capacity is determined by epigenetic components, including DNA methylation and histone modification (Bloushtain-Qimron et al., 2009; Weishaupt et al., 2010). Epigenetic processes can become altered by cell culture processes, since methylation of genes related to differentiation can change during *in vitro* passaging (Bork et al., 2010), while the maintenance of unmethylated regions appears serum-dependent (Dahl et al., 2008). This underscores that monitoring of epigenetic processes may lead to a breakthrough in therapeutic stem cell manufacturing development. Recently, a next-generation sequencing methodology was started to map cytosine methylated regions in MultiStem cells and to explore the possibility of identifying DNA methylation markers. An additional epigenetic assay that is under development to distinguish MultiStem cells from other adherent stromal cells such as MSC is based on telomere biology, an important predictor for proliferative capacity, and it was recently shown that MultiStem telomerase activity is much higher than that of MSC (Boozer et al., 2009).

Altogether, these assays will serve as controls for epigenetic stability, and the product uniqueness and consistency, in particular after modification of the MultiStem manufacture procedure. Application of these QC assays confirmed that characteristics of MultiStem cells harvested from the Quantum Cell Expansion System were maintained compared to those under standard cell culture conditions (data not shown). All QC assays performed thus far indicate successful MultiStem expansion in this bioreactor format, with significant advantages in air-handling requirements and reductions in labor.

PERSPECTIVE

Currently for the stromal cell therapy field as a whole, and for MultiStem in particular, the development is still mainly in the pre-clinical and early and mid clinical stages, during which safety and dose effects are being evaluated. The optimal dosing strategy for stromal cells is considered to be the composite of optimal individual dose level/administration and minimal number of administrations required to fully cover therapeutic opportunity windows. E.g., in the case of GvHD prophylaxis, the therapeutic window covers 30–45 days after allo HSCT. Ideally, clear efficacy is observed after infusion of a single dose level of cell product, but this has not consistently been the experience in pre-clinical or clinical evaluations (**Table 2**). As a consequence, current manufacture strategies are based on the anticipated need to repeat infuse medium to high dose levels (5–10 million cells/kg of bodyweight) in order to observe efficacy. This equates roughly to 400 million to 1 billion cells/infusion, or >1 billion cells/patient for repeat administration, which levels are outside of the range for MSC production from individual donors on a consistent scale. This is especially the case in context of anticipated late stage Phase III clinical studies with large numbers of subjects (>100). However, for the MultiStem product these cell requirements can feasibly be covered with material from individual donors by using a staged expansion and banking approach based on the extensive expansion capacity of the MultiStem platform.

In all, the early clinical observations indicate that the class of stromal stem cells can be safely infused via single or repeat dose regimens in humans without long-term complications. There are no apparent disadvantages of MultiStem *per se*, compared to MSC. Still, continued clinical evaluation and scrutiny will be required to address the still fairly limited experience with immune sensitization as a consequence of repeat dosing of allogeneic product, or long-term risk of ectopic tissue formation, especially in immune-compromised subjects. One remaining shared disadvantage in current use of MultiStem and MSC is the use of FBS for product manufacture and this will be a major area of need in the development of next-generation cell therapy products. Immune responses have been detected against serum components on the stromal cells, but no significant

alloantibody production has been reported (Spees et al., 2004; Sundin et al., 2007). Completion of a serum-free workflow will be beneficial because of limited serum availability, batch-to-batch differences, the possibility of adventitious pathogens and ethical considerations. It is anticipated that Phase III studies using MultiStem in several clinical indications will have integrated serum-free media formulation and production in a closed bioreactor format.

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Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative *in vitro* study

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Background: In diseased joints, the catabolic environment results in progressive joint damage. Mesenchymal stem cells (MSCs) can have immunomodulatory effects by secreting anti-inflammatory factors. To exert these effects, MSCs need to be triggered by pro-inflammatory cytokines. To explore the potential of MSCs as a treatment for diseased joints, we studied the effect of synovial fluid (SF) from donors with different joint diseases and donors without joint pathology on the immunomodulatory capacities of human MSCs *in vitro*. We hypothesized that SF of diseased joints influences the immunomodulatory effects of MSCs. **Materials and Methods:** MSCs were cultured in medium with SF of six osteoarthritis (OA) or six rheumatoid arthritis (RA) donors and three donors without joint pathology were used as control. Gene expressions of IL-6, HGF, TNFa, TGFb1, and indoleamine 2,3-dioxygenase (IDO) were analyzed. L-kynurenine concentration in conditioned medium (CM) by MSCs with SF was determined as a measure of IDO activity by MSCs. Furthermore, the effect of CM with SF on proliferation of activated lymphocytes was analyzed. **Results:** Addition of SF significantly up-regulated the mRNA expression of IL-6 and IDO in MSCs. SF(OA) induced significantly higher expression of IDO than SF(control), although no difference in IDO activity of the MSCs could be shown with a L-kynurenine assay. Medium conditioned by MSCs with SF(OA or RA) suppressed activated lymphocyte proliferation *in vitro* more than medium conditioned by MSCs without SF or with SF(control). **Discussion:** SF can influence the expression of genes involved in immunomodulation by MSCs and the effect on lymphocyte proliferation. We found indications for disease-specific differences between SFs but the variation between donors, even within one disease group was high. These data warrant further research to examine the potential application of MSC therapy in arthritic joints.

Keywords: MSC, osteoarthritis, rheumatoid arthritis, synovial fluid, immunomodulation

INTRODUCTION

Osteoarthritis (OA) and rheumatoid arthritis (RA) are high prevalent forms of arthritis. OA is mainly characterized by progressive functional loss and cartilage degeneration. Main factors involved in cartilage degeneration are a variety of matrix degrading enzymes and pro-inflammatory cytokines (Goldring, 2000; Goldring and Marcu, 2009). It is possible to treat the symptoms of OA with lifestyle changes, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), or intra-articular injections with corticosteroids or hyaluronic acid and the ultimate treatment for end stage OA is joint replacement. A treatment to cure OA, however, is still not available. RA is an auto-immune disease initiated by immune complexes that together with cytokines, complement, and metalloproteinases (Weissmann, 2006) cause an inflammatory and catabolic environment in the joint (Goldring and Marcu, 2009). It is a systemic disease characterized by persistent synovitis, systemic inflammation, and auto-antibodies which eventually cause joint damage with progressive cartilage degeneration and bone alterations. There is a wide range of therapeutic options for RA like analgesics, NSAIDs, disease-modifying anti rheumatic drugs (DMARDs), and biologicals (Lee and Weinblatt, 2001; Scott et al., 2010). However, to date there is no treatment available to cure RA.

Human mesenchymal stem cells (MSCs), the progenitors of connective tissue cells, are able to differentiate into different cell types including chondrocytes (Caplan, 1991, 1994; Solchaga et al., 2004; Caplan and Dennis, 2006). This has attracted the interest of many people working in the area of cartilage repair. Besides the ability to reconstruct tissues, MSCs also have the ability to modulate the environment by secreting many immunomodulating

and trophic factors like cytokines, chemokines, and growth factors (Deans and Moseley, 2000; Minguell et al., 2001; Kim et al., 2005; Caplan and Dennis, 2006; Chen et al., 2006; Schinkothe et al., 2008; Hoogduijn et al., 2010; Meisel et al., 2011). These factors have potent immunomodulatory capacity as demonstrated *in vitro* by inhibition of T-lymphocyte proliferation after adding MSCs in mixed lymphocyte reactions (Hoogduijn et al., 2010; Landgraf et al., 2011). MSCs also inhibit the antibody production of B lymphocytes and inhibit the generation and function of antigen presenting cells (Sze et al., 2007; Chen et al., 2008; Hoogduijn et al., 2010). The stimulation of MSC by pro-inflammatory cytokines like TNFa and IFNg strongly enhances the immunosuppressive function of MSCs (Klyushnenkova et al., 2005; Schinkothe et al., 2008; Siegel et al., 2009; Eggenhofer et al., 2010; Hoogduijn et al., 2010).

In a healthy joint environment, a balance exists between an anabolic and catabolic state. In a situation of inflammation or chronic damage, i.e., OA or RA, the environment becomes more catabolic (Findlay and Haynes, 2005; Goldring and Marcu, 2009). All joint tissues are exposed to synovial fluid (SF) and in OA and RA inflammatory factors are secreted into the SF. The aim of the present study was to investigate whether SF of donors with OA, RA, or no joint pathology triggers MSCs to become immunomodulatory. Since inflammation plays a large role in RA and OA, we hypothesized that MSCs will be triggered to become immunomodulatory. We explored this by studying the effect of SF of OA and RA patients as well as SF of non-pathological(control) donors on MSCs. Our hypothesis was that MSCs conditioned in SF(RA) will express a large anti-inflammatory effect compared to SF(control) due to the high inflammation state of RA patients and MSCs conditioned with SF(OA) will express a mild anti-inflammatory effect compared to SF(control) as a reaction to a less inflamed environment in joints of OA patients.

We evaluated the effect of SF on expression of genes of MSCs for immunomodulatory factors. Furthermore, we performed a functional assay to study the capacity of factors secreted by MSCs in response of SF to inhibit proliferation of activated lymphocytes.

MATERIALS AND METHODS

SYNOVIAL FLUIDS

Fifteen SF samples were obtained from six OA patients, six RA patients, and three donors without any joint pathology. SFs(OA) were obtained from patients undergoing total knee replacement surgery. All patients implicitly consented to the use of these fluids for scientific research (with approval by Erasmus MC medical ethical committee protocol # MEC-2004-322). SFs(RA) were obtained from RA patients with active inflammation of the knee during consultation at the rheumatology outpatient clinic (with approval by Erasmus MS medical ethical committee protocol # MEC-236.904-2003-255). SFs(control) were purchased from SF donors without joint diseases, post mortem within 24 h of death (Articular Engineering, Northbrook, IL, USA). After aspiration, all SF samples from the joints of all donors were centrifuged to remove debris. Supernatant was stored at −80°C.

To evaluate the inflammatory aspects of the different SFs we did amplified enzyme linked immunosorbent assays (ELISA) to quantify cytokines IL-6, TNFa (R&D Systems, Minneapolis, MN, USA), and IFNg (Invitrogen, Carlsbad, CA, USA). Measurements of IL-6, TNFa, and IFNg were performed in duplicate. All SFs were treated with 1:3 hyaluronidase (1000 U/ml PBS, 10 min at 37°C) prior to ELISA measurements. ELISAs were carried out according to the manufacturer's instructions by means of a multilabel plate reader (VersaMax™, Molecular Devices, Sunnyvale, CA, USA).

MSC ISOLATION

Mesenchymal stem cells were isolated from heparinized femoral-shaft marrow aspirate of patients undergoing total hip arthroplasty (with informed consent after approval by Erasmus MC medical ethical committee protocol # MEC-2004-142). About 5–10 ml marrow was harvested with a sterile Jamshidi needle into sterile 10 ml syringes containing 0.5 ml of heparin (1000 U/ml). About $30–100 \times 10^6$ mononuclear cells were plated in a T175 flask in 25 ml expansion medium (Dulbecco's Modified Eagle Medium (DMEM) low glucose (Invitrogen, Carlsbad, CA, USA) containing 15% heat inactivated fetal calf serum (Lonza, Verviers, Belgium, selected batch), 1.5 μg/ml fungizone (All Invitrogen, Carlsbad, CA, USA), 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA, USA), 1 ng/ml fibroblast growth factor-2 (Instruchemie B.V., Delfzijl, The Netherlands), and 0.1 mM of L-ascorbic acid 2-phosphate (vitamin C; Sigma, St. Louis, MO, USA). After 24 h, non-adherent cells and erythrocytes were removed by washing three times with 2% FCS in 1× PBS (Invitrogen, Carlsbad, CA, USA). Remaining adherent cells were cultured in expansion medium at 37°C and 5% carbon dioxide (CO_2). Expansion media were renewed twice a week. At subconfluent cells were trypsinized with a 0.25% trypsin solution containing 0.01% EDTA (Invitrogen, Carlsbad, CA, USA) and plated at a density of 2300 cells/cm^2.

MSC CULTURE WITH SF

Cryopreserved MSCs of passage two were used for the experiments. After thawing, MSCs were seeded in a T175 flask at a density of 2300 cells/cm^2, expanded for one passage and subsequently plated in six well plates at a density of 4000 cells/cm^2 for the experimental conditions. At 70% confluence the existing medium was discarded and the cells were washed three times using PBS (Invitrogen, Carlsbad, CA, USA). Subsequently 0.8 ml of DMEM low glucose containing 9 μg/ml fungizone and 50 μg/ml gentamicin, was applied per well. The different SFs(OA, RA, and control) were added in triplicates to the media in a concentration of 20%. In preliminary tests MSCs were cultured in 0, 10, or 25% SF of four OA donors, Gene expression was not significantly different in 10 and 25% SF. Based on this and taking into account the availability of the SF (from SF(control) we obtained maximal 1 ml per donor) we decided to use 20% SF for all further experiments. All conditions contained a total concentration of 1% ITS (BD Bioscience, Bedford, MA, USA). Nine wells with only medium plus 1% ITS were used as negative controls for unstimulated MSCs. After 48 h of incubation, MSCs were harvested for gene expression analyses and the conditioned medium (CM) was harvested and stored at −80°C.

GENE EXPRESSION ANALYSIS

After 48 h of incubation total RNA from MSCs was isolated using RNeasy® microkit (Qiagen, Hilden, Germany) with RNeasy

MinElute spin columns. After quantification of nucleic acids by spectrophotometry (NanoDrop 2000, Thermo Scientific, Isogen Life Science, IJsselstein, The Netherlands) the RNA was reverse transcribed using a First Strand cDNA Synthesis kit (RevertAid™; MBI Fermentas, St. Leon-Rot, Germany). Amplifications were performed as 20 μl reactions with real-time PCR. Thermocycler conditions comprised an initial holding at 95°C for 10 min, followed by one step at 95°C for 15 s and 60°C for 60 s for 40 cycles. A dissociation stage was added at the end using 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. For UBC, IL-6, HGF, TNF-α, qPCR™Mastermix Plus for SYBR® Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) was used. For GAPDH, HPRT, IDO, and TGF-β1 TaqMan Master Mix (ABI, Branchburg, NJ, USA) was used. Sets of primers and probes used in this study: GAPDH (NM_002046.3) Fw: ATGGGGAAGGTGAAGGTCG Rv: TAAAAGCAGCCCTGGTGACC Probe: Fam-CGCCCAATACGACCAAATCCGTTGAC; HPRT (NM_000194.2) Fw: TATGGACAGGACTGAACGTCTTG Rv: CACACAGAGGGCTACAATGTG Probe: Fam-AGATGTGATG AAGGAGATGGGAGGCCA; UBC (NM_021009.5) Fw: ATTTGG GTCGCGGTTCTTG Rv: TGCCTTGACATTCTCGATGGT; IL-6 (NM_000600.3) Fw: TCGAGCCCACCGGGAACGAA Rv: GCAGGGAAGGCAGCAGGCAA; HGF (NM_000601.4) Fw: GGCTGGGGCTACACTGGATTG Rv: CCACCATAATCCCCCTCACAT; TNF-aplha (NM_000594.2) Fw: GCCGCATCGCCGTCTCCTAC Rv: AGCGCTGAGTCGGTCACCCT; TGF-beta1 (NM_000660.4) Fw: GTGACAGCAGGGATAACACACTG Rv: CATGAATGGTGGCCAGGTC Probe: Fam-ACATCAACGGGTTC ACTACCGGC. IDO was detected using a taqman assay on demand (Applied Biosystems, Capelle a/d IJssel, The Netherlands) of which the primer sequence is not known to us. Data were collected and quantitatively analyzed on an ABI Prism 7000 Sequence Detection System (SDS) with SDS software, version 1.2.3 (Applied Biosystems, Capelle a/d IJssel, The Netherlands). Gene expressions of the cytokines and IDO in MSCs were calculated by cycle threshold (CT) values. CT values of 36 and higher were considered as non-expressed and set to 100 for further calculations. The CT values of the housekeeper genes GAPDH, HPRT, and UBC were averaged by using geometric averaging of every sample. This average is the best keeper index (BKI) for every single sample. All separate CT values were corrected to the BKI by using the $2^{-\Delta CT}$ formula.

L-KYNURENIN ASSAY

In order to evaluate whether SF influenced IDO activity in MSCs, we measured the concentration of L-kynurenine in the CM and SFs. To correct for possible L-kynurenine in SF, the SFs were diluted in the same concentration and the same media as the CM and values were subtracted from the CM values. Values of one of the OA donors could not be used since no remaining SF was available for correction. Thirty percent trichloroacetic acid was added to the samples in a 1:3 ratio and after 30 min incubation at 50°C the samples were centrifuged at 12000 rpm for 5 min. Supernatant of all conditions were diluted 1:1 in Ehrlich reagent (200 μg 4-dimethylaminobenzaldehyde (Sigma, St. Louis, MO, USA) in 10 ml of glacial acetic acid) in duplicate in a 96-wells flat bottom plate and absorbance was determined at 490 nm in a multilabel plate reader (VersaMax™, Molecular Devices, Sunnyvale, CA, USA). L-kynurenine (Sigma, St. Louis, MO, USA) was used as standard.

PBMC PROLIFERATION ASSAY

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Rotterdam, The Netherlands) of healthy volunteers using Ficoll-Paque™Plus (GE Healthcare, Uppsala, Sweden) separation and stored at −135°C until use. PBMCs were thawed and centrifuged at 2000 rpm for 5 min. Viable cells were counted using trypan blue exclusion test. PBMCs were seeded in alpha-modified Minimum Essential Medium (aMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat inactivated FCS (Lonza, Verviers, Belgium, selected batch), 2% pen-strep (Penicillin 10,000 UI/ml, Streptomycin 10,000 UI/ml, Lonza, Verviers, Belgium), and 2% L-glutamine (200 mM, Lonza, Verviers, Belgium) and activated with anti-CD3 and anti-CD28 linked with linker goat-anti-mouse antibody (BD Pharmingen, San Diego, CA, USA). 5×10^4 PBMCs in 100 μl expansion medium were seeded per well in round-bottom 96-well plates (Nunc, Roskilde, Denmark) and incubated for 5 days. The immunosuppressive capacity of factors secreted by MSCs was evaluated by using the CM from the MSC culture conditions described earlier. One hundred microliters of CM of MSCs incubated with each of the SFs, except one of the OA donors were no SF was left, was added in triplicate to the PBMCs for 5 days. CM of MSCs without SF and unconditioned medium, identical to the medium used in the CM except for the fact that it had not been in contact with MSCs, were added in triplicate as a control. To correct for direct effects of the SF present in the CM on the PBMCs we added controls of medium not conditioned by MSCs with similar concentration of SF of each of the donors. At day four of incubation, ^{3}H-thymidine (0.5 μCi/well; Perkin Elmer, Inc., San Jose, CA, USA) was added. At day five, after 16 h of incorporation of ^{3}H-thymidine, PBMCs were harvested, and ^{3}H-thymidine incorporation measured using a β-plate reader (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter and Luminometer, Perkin Elmer, Inc., San Jose, CA, USA).

DATA ANALYSIS

Statistical difference in gene expression by MSCs conditioned with SF(OA), SF(RA), and SF(control) was analyzed by using a mixed linear model in which condition(SF of OA, RA, or no joint pathology donors) was considered a fixed factor and the different SF donors for all conditions a random factor. Values for the genes IDO, TNFa, and TGFb were log-transformed to approach a normal distribution. Statistical differences of inhibitory capacity of the different CM was analyzed by using a mixed linear model in which condition (CM by MSCs incubated with OA, RA, or control SF) was considered a fixed factor, different donors a random factor and Sidak was used as adjustment for multiple comparisons. Inhibitory effects of CM with SF compared to SF only were explored by statistical analyses with the Wilcoxon signed ranks test. Data are presented as the mean ± standard deviation and 2.5–97.5 percentile. P-value of ≤0.05 was considered statistical significant; $^*P < 0.05$, $^{**}P < 0.001$. Analyses were performed using SPSS 17.0 Statistics (SPSS, Inc., Chicago, IL, USA).

RESULTS

EFFECT OF SF ON GENE EXPRESSION OF MSCs

To evaluate the effect of SF on mRNA expression of IL-6, HGF, IDO, TNFa, and TGFb1 by MSCs, MSCs were cultured in medium containing 20% SF of each of the 15 different donors. Medium with 1% ITS was used as SF free culture control and represented as a dotted line in **Figure 1**. Addition of SF significantly up-regulated IL-6 (2.43 ± 0.22-fold; $P < 0.001$) and IDO (1.72 ± 0.17-fold; $P = 0.007$) expression. There is a trend of down-regulation of TNFa albeit not significant. Gene expressions of HGF, TNFa, and TGFb1 were not significantly affected by SF compared to the SF free control (**Figure 1**).

Next, we explored the effect of three different types of SF separately. MSCs cultured in SF(OA) expressed IDO 1.69-fold ($P = 0.048$) higher than MSCs cultured in SF(control). For SF(RA) we also found an up-regulation in gene expression for IDO, albeit

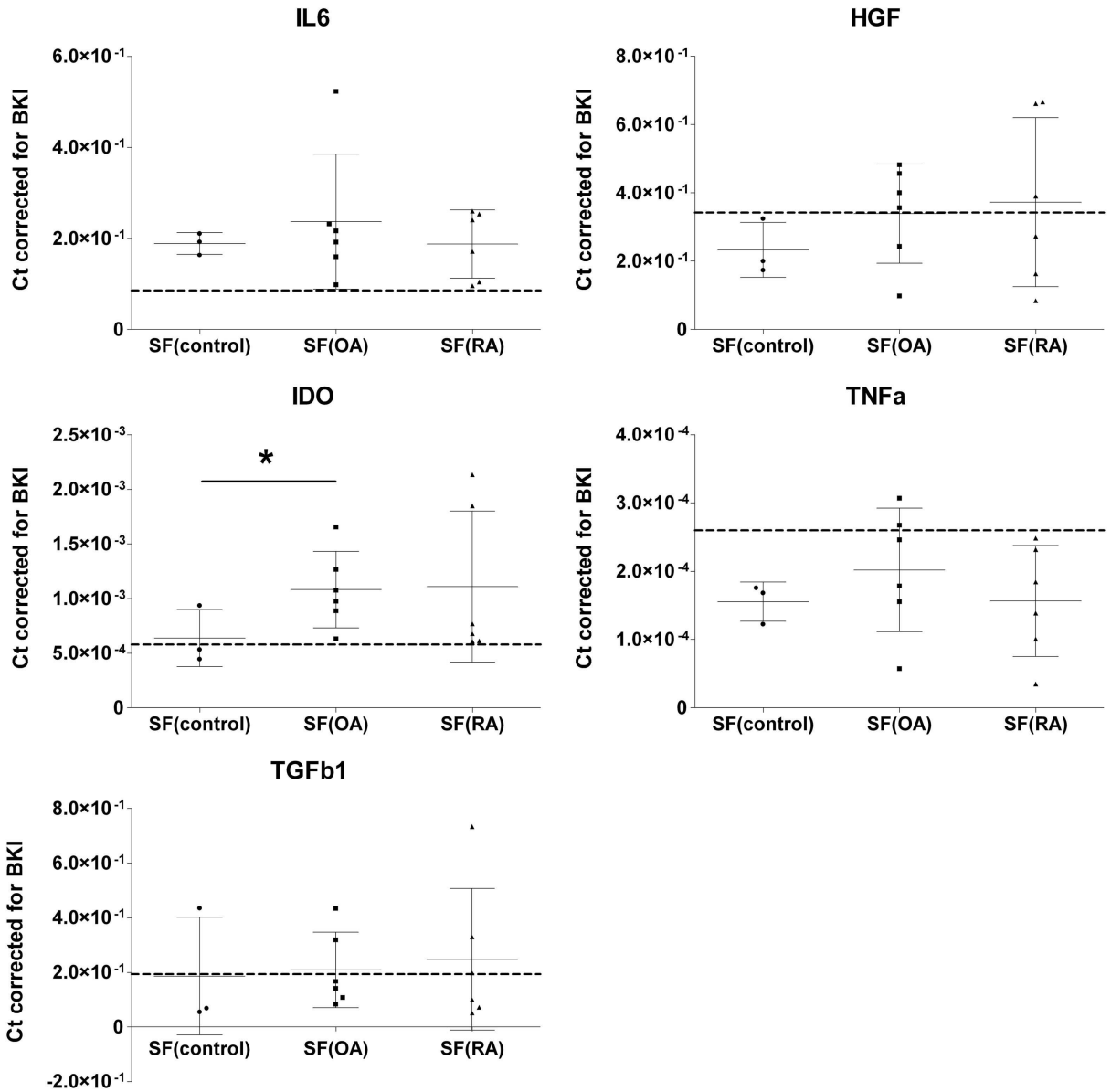

FIGURE 1 | Effect of different synovial fluids on gene expression of immunomodulatory factors by MSCs. Gene expressions expressed in cycle thresholds (ct) normalized to BKI in every sample. MSCs were cultured in 20% SF of six OA and six RA donors and three donors without joint pathology. Dotted lines indicate the average gene expression in MSCs cultured in medium without SF. The data are presented as median scatterplots, each point represents an average of three measurements per donor (Mean ± SD). SF, synovial fluid; SF(control, OA, and RA) culture media of the MSCs supplemented with respectively non-pathological SF, OA SF, and RA SF. IDO, indoleamine 2,3-dioxygenase; IL-6, interleukin-6; TNFa, tumor necrosis factor-alpha; TGFb1, transforming growth factor-beta 1; HGF, hepatocyte growth factor; BKI, BestKeeper Index consisting of: GAPDH, UBC, and HPRT. *Expression in MSCs after culture in SF(OA) different from SF(control) by mixed linear test of these two conditions, $P < 0.05$.

not significant which is probably caused by the large variation between the six different RA donors. No further significant differences in gene expression of IL-6, TNFa, TGFb1, and HGF were found between MSCs cultured in the three different SFs (**Figure 1**). IDO activity of MSCs was analyzed by an L-kynurenine assay on all different CM with SF corrected for L-kynurenine content in SF of that donor. No significant differences of IDO activity by MSCs cultured in SF of different donors were found (data not shown).

EFFECT OF CONDITIONED MEDIUM ON LYMPHOCYTE PROLIFERATION

Conditioned medium harvested after culturing MSCs in 20% SF was used to analyze the effect of secreted factors of MSCs on the proliferation of CD3/CD28 activated PBMCs (**Figure 2**). The CM was mixed 1:1 with fresh medium and added to PBMCs. CM of MSCs without SF (CM control) did not influence PBMC proliferation. There was no difference in PBMC proliferation between CM of MSCs without SF and CM of MSCs incubated in SF(control). There was significantly more inhibition of PBMC proliferation by CM with SF(OA) compared to CM with SF(control; $P < 0.001$) and by CM with SF(RA) compared to CM with SF(control; $P < 0.001$).

To correct for direct effects of SF on PBMCs we added controls with unconditioned medium with SF. The SF appeared to inhibit PBMC proliferation, independent of disease state. A preliminary experiment with different concentrations of SF(OA) indicated that the effect of SF on lymphocyte proliferation is dose dependent (data not shown).

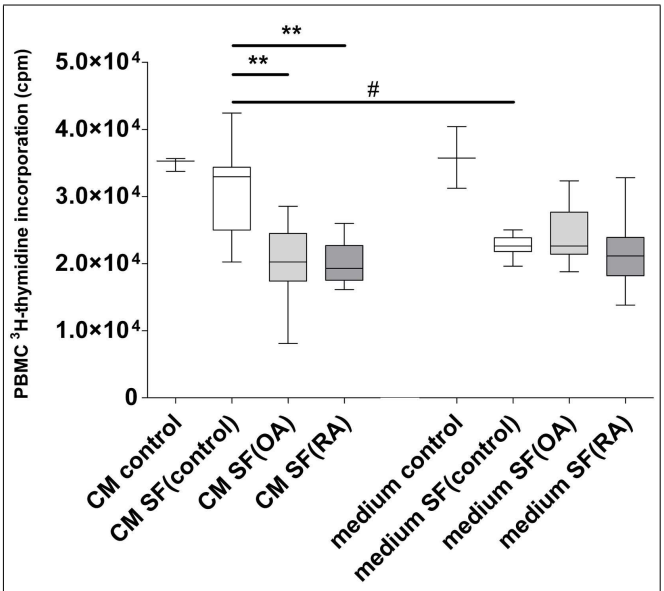

FIGURE 2 | Effect of conditioned medium of MSCs with different types of synovial fluid (SF) on proliferation of CD3/28 activated PBMCs. Box-and-Whisker plot 2.5–97.5 percentile; *$P < 0.05$; **$P < 0.001$; #$P < 0.05$ between CM and SF effect. CM, conditioned medium; SF, synovial fluid; (medium) culture medium with ITS; (CM control) MSC conditioned medium consisting of only culture medium with ITS; (Medium control) Only culture medium with ITS; SF (control) synovial fluid of donors without joint pathology; SF(OA) synovial fluid of patients with osteoarthritis; SF(RA) synovial fluid of patients with rheumatoid arthritis.

Conditioned medium of MSCs incubated in SF(control) caused significantly less inhibition of PBMC proliferation than unconditioned medium [medium that was not in contact with MSCs but contained SF(control)]. No significant differences in proliferation inhibition were found between OA and RA CM (**Figure 2**).

DISCUSSION

The aim of the study was to evaluate to what extent SF influences the immunomodulation of MSCs. This study indicates that SF can influence the expression of genes in MSCs that are involved in immunomodulation. Moreover, factors secreted by MSCs incubated with SF(OA and RA) inhibited the proliferation of activated lymphocytes significantly more than factors secreted by MSCs incubated without SF or with SF(control). This indicates that factors in diseased SF stimulate MSCs to secrete anti-inflammatory factors.

To our knowledge this is the first report on the effect of SF on the expression and secretion of immunomodulatory factors in MSCs. This information is important for the application of MSCs in joints of patients with joint diseases. Upon injection or implantation in the joint the MSCs will be exposed to SF. SF is known to contain a mix of factors secreted by the tissues of the joint. In the current study we investigated whether SF from non-diseased and OA and RA donors triggers MSCs to have an immunomodulatory effect. We demonstrated that SF(OA) can up-regulate MSC gene expression of IDO. In addition, MSCs treated with SF up-regulated expression of IL-6, a pleiotropic cytokine with pro-inflammatory functions, but also involved in regenerative processes and regulation of metabolism (Scheller et al., 2011). Which factor(s) secreted by MSCs cause the immunomodulatory effects cannot be concluded from our study and deserves further investigation.

We hypothesized that MSCs will be triggered by a catabolic environment in the joint to become immunomodulatory and that SF(RA) will induce large anti-inflammatory and SF(OA) will induce mild anti-inflammatory effects compared to SF(control). Our data could partly confirm this hypothesis. Diseased SF triggered MSCs to become immunomodulatory but we did not find any differences between the effects of SF(OA and RA) on gene expression of MSCs and PBMC proliferation. Whereas we assumed SF(control) would be immunological quiescent and diseased SF inflammatory, surprisingly we found inhibited lymphocyte proliferation by all SFs. This inhibition further increased by secreted factors of MSCs cultured with addition of SF(OA or RA), albeit non-significant. Surprisingly in the presence of SF(control), the inhibition of lymphocyte proliferation by SF was significantly reduced. This unexpected outcome suggests different effects of non-pathologic SF on excretion of factors by MSCs. Since the composition of healthy or diseased SF is not precisely known, it is difficult to explain the effects of SFs on MSCs and on PBMCs.

To provide a relatively clean way to study the effect of factors secreted by MSCs on lymphocyte proliferation, we used CM of MSCs exposed to SF. Different durations of exposure to SF and direct interactions between lymphocytes and MSCs in the presence of SF can play a role as well and this should be investigated in the future.

We here demonstrate that MSCs can be differently influenced by exposure to SF from diseased and non-pathological joints, but the effects were small compared to commonly used stimulation with TNFa and IFNg (Crisostomo et al., 2008; Hemeda et al., 2010; Hoogduijn et al., 2010). This might explain why resident MSCs in joints cannot prevent disease development; they might not be properly activated by the environment. It can also be regarded as somewhat disappointing in respect to the application of MSCs in the diseased joint since exposure to SF might not be sufficient to stimulate the healing activity of the MSCs.

Although it is unknown which factor in SF does stimulate MSCs, we performed ELISA on SFs. SF(RA) has a higher concentration of IL-6 compared to SF(OA; 7380 vs. 525.4 pg/ml; $P = 0.009$) and SF(control; 7380 vs. 22.4 pg/ml; $P = 0.024$), confirming previous reports (Kokebie et al., 2011). TNFa was measurable in only one OA donor and two RA donors and IFNg was measurable in only one OA and one RA SF donor (data not shown). Neither of these cytokines correlated with the effects of SF on MSCs or PBMCs but we can not exclude that other factors evoke an effect on MSCs. Moreover, *in vivo*, direct contact with MSCs and inflamed synovial tissue, immune cells in the synovium, or degenerated cartilage might, however, activate the MSCs. Finally, it should be noted that we have selected a limited number of immunomodulatory factors to evaluate the effect on MSCs and we cannot exclude that SF stimulates other processes in MSCs that can effect healing of the diseased joint.

Since the SFs were considered as redundant materials, ethical regulations preclude the availability of patient-specific information. It is very likely that the OA and RA patients used medication that might have influenced the compositions of the SFs. It has been demonstrated that analgesic drugs, NSAIDs, and DMARDs can change concentrations of immunomodulatory factors in SF (Bianchi et al., 2003, 2007; Alvarez-Soria et al., 2006). Use of different types of medication within donor groups could be a cause for the high variations within the groups.

Moreover, this explorative study was performed with SF of six OA donors, six RA donors, and three donors without joint pathology. To gain sufficient power the study should be repeated with larger numbers of pathological and non-pathological SFs. SF was used in a concentration of 20% for 48 h in analyses on MSCs. It remains unknown how MSCs will react on 100% SF over a longer period of time, which eventually will be the environment for MSCs when they are injected in a joint.

Although MSCs appear a promising therapy for degenerative joint diseases, the working mechanisms are not entirely clear. In animal studies it is possible to track MSCs injected in the joint. It was demonstrated that some of the injected MSCs stayed in the joint and adhered to the synovium or affected areas (Qi et al., 2011; Sato et al., 2012) from where they could exert a modulating effect and decrease the inflammatory or catabolic environment in diseased joints. The immunomodulatory capacity of MSCs can be useful for patients with OA and RA. Good therapeutic options for RA are already available, such as DMARDs and biologicals. However MSCs are capable of secreting many different factors, possibly for a prolonged time, which can influence many different mechanisms and are not restricted to one single target, unlike for example anti-TNFα. This explorative study shows that (1) SF can influence the expression of genes by MSCs involved in immunomodulation and (2) factors in CM by MSCs cultured with arthritic SF inhibit lymphocyte proliferation more than factors in CM by MSCs cultured without SF or with SF(control). These results warrant further research to examine the potential application of MSC therapy in arthritic joints.

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Toll-like receptors as modulators of mesenchymal stem cells

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Mesenchymal stem cells (MSCs) have differentiation and immunomodulatory properties that make them interesting tools for the treatment of degenerative disorders, allograft rejection, or inflammatory and autoimmune diseases. Biological properties of MSCs can be modulated by the inflammatory microenvironment they face at the sites of injury or inflammation. Indeed, MSCs do not constitutively exert their immunomodulating properties but have to be primed by inflammatory mediators released from immune cells and inflamed tissue. A polarization process, mediated by Toll-like receptors (TLRs), toward either an anti-inflammatory or a pro-inflammatory phenotype has been described for MSCs. TLRs have been linked to allograft rejection and the perpetuation of chronic inflammatory diseases (e.g., Crohn's disease, rheumatoid arthritis) through the recognition of conserved pathogen-derived components or endogenous ligands (danger signals) produced upon injury. Interest in understanding the effects of TLR activation on MSCs has greatly increased in the last few years since MSCs will likely encounter TLR ligands at sites of injury, and it has been proven that the activation of TLRs in MSCs can modulate their function and therapeutic effect.

Keywords: toll-like receptor, mesenchymal stem cells, cell therapy

ADULT MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) have emerged in recent years as therapeutic tools based on three important features: (i) differentiation potential, (ii) capacity to modulate immune responses, and (iii) low immunogenicity, which would may allow allogeneic treatments.

Mesenchymal stem cells have been isolated from multiple tissues of mesodermal origin, such as bone marrow (Friedenstein et al., 1976), adipose tissue (Zuk et al., 2002), umbilical cord blood (Romanov et al., 2003), placenta (Fukuchi et al., 2004), synovium (De Bari et al., 2001), or dental pulp (Gronthos et al., 2000), among others. Despite significant efforts, no exclusive surface markers have been identified for MSCs. To date, MSCs are defined according to the three criteria of the International Society for Cellular Therapy (Dominici et al., 2006): (a) *Adhesion to plastic*: MSCs can be isolated by adhesion to plastic and expanded *in vitro* in serum containing media with no additional requirements for growth factors or cytokines; (b) *Expression of a specific combination of surface markers*: MSCs are negative for CD45, CD34, CD14, or CD11b, CD79α, or CD19 and HLA-DR, and positive for a variety of other markers, including CD73, CD90, and CD105; (c) *Differentiation potential*: MSCs can be identified *in vitro* by their ability to differentiate into mesenchymal-type cells (trilineage differentiation into adipocytes, osteoblasts, and chondrocytes; Pittenger et al., 1999). Although sharing these main characteristics, differences between MSCs from different sources can be found. The secretome differs between cell types, and bone marrow-derived MSCs (BM-MSCs) and adipose-derived MSCs (AD-MSCs), for instance, show specific RNA and protein expression profiles (De Ugarte et al., 2003; Noël et al., 2008; Skalnikova et al., 2011).

In homeostatic conditions, allogeneic cells are rejected by the immune system upon recognition of their foreign human leukocyte antigen (HLA). Allogeneic cells can also activate T cells through an indirect pathway where their HLA antigens are presented by professional antigen-presenting cells (APC). MSCs express low levels of cell surface HLA class I molecules whereas HLA class II, CD40, CD80, and CD86 are not detectable on the cell surface which theoretically opens the possibility of allogeneic treatments without the requirement of suppression of host immunity. Stimulation with interferon (IFN)γ has been shown to increase both class I and class II molecules. However, MSCs do not express classic co-stimulatory molecules such as CD40, CD80, CD86, even after stimulation in an inflammatory milieu. These features may allow MSCs to avoid or delay immune recognition (Le Blanc et al., 2003a,b; Majumdar et al., 2003; Rasmusson et al., 2003; McIntosh et al., 2006; Chamberlain et al., 2007), although this is a question that needs to be further investigated in both experimental animal models and clinical trials (Griffin et al., 2010).

Mesenchymal stem cells have immunomodulating properties and inhibit function of immune cells (Bartholomew et al., 2002; Krampera et al., 2003; Zhang et al., 2004; Beyth et al., 2005; Glennie et al., 2005; Puissant et al., 2005; Nauta et al., 2006; Yañez et al., 2006; Cui et al., 2007; Chiesa et al., 2011; DelaRosa et al., 2012). The specific molecular and cellular mechanisms involved in the immunoregulatory activity of MSCs are still under investigation and remain poorly understood. There is evidence that the capability to modulate immune responses rely on both cell

contact-dependent mechanisms (i.e., through Jagged1–Notch1 interactions; Liotta et al., 2008) and paracrine effects through the release of soluble factors (reviewed by Doorn et al., 2012). A broad panel of soluble factors have been involved including hepatocyte growth factor (HGF), prostanglandin-E2 (PGE2), transforming growth factor (TGF)-β1, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), interleukin (IL)-10, heme oxygenase-1 (HO-1), and HLA-G5 (Krampera et al., 2003; Beyth et al., 2005; Puissant et al., 2005; Yañez et al., 2006; Chabannes et al., 2007; Cui et al., 2007; Oh et al., 2007; Selmani et al., 2008; DelaRosa et al., 2009). Differences in the mechanisms of immunomodulation employed by MSCs from different species have been reported. Whereas IDO activity appears to be a key player in human MSC-mediated immunomodulation, mouse MSCs do not express IDO and seem to use NO as the main mediator (DelaRosa et al., 2009; Ren et al., 2009; Meisel et al., 2011). Interestingly, MSCs may also modulate immune responses through the generation of regulatory T cells (Tregs; Krampera et al., 2003; Zhang et al., 2004; Maccario et al., 2005; Nauta et al., 2006; Gonzalez-Rey et al., 2010). Whether this MSC-mediated Treg induction is due to an expansion of pre-existing Tregs, to a *de novo* induction or to a combination of both needs to be further explored.

Importantly, MSCs do not constitutively exert their immunomodulating properties but have to be "primed" by inflammatory mediators released from activated immune cells, such as IFNγ, IL1β, and TNFα (Krampera et al., 2006; Prasanna et al., 2010). Also, the functionality of MSCs can be modulated by other inflammatory mediators such as APRIL and BAFF (Zonca et al., 2012). The thinking that MSCs are only anti-proliferative and immune-inhibitory on immune cells has been recently challenged by Waterman et al. (2010) who reported a "licensing" process of MSCs toward either anti-inflammatory or pro-inflammatory phenotypes, depending on the toll-like receptor (TLR) ligand used for activation. For extensive review on the concept of MSC "licensing" see the excellent review by Krampera (2011).

The biological characteristics mentioned above make MSCs an interesting tool for cellular therapy. This is supported by a number of studies in experimental models of inflammatory diseases demonstrating an efficient protection against allograft rejection, graft-versus-host disease, experimental autoimmune encephalomyelitis, collagen-induced arthritis, sepsis, and autoimmune myocarditis (Le Blanc et al., 2004; Zappia et al., 2005; Ohnishi et al., 2007; González et al., 2009a,b; Gonzalez-Rey et al., 2009; Németh et al., 2009). As indicated previously, TLRs have been implicated in the pathology of graft transplantation and inflammatory diseases (Ishihara et al., 2006; Yamamoto-Furusho and Podolsky, 2007) and therefore may modulate MSC function *in vivo* (DelaRosa and Lombardo, 2010; Krampera, 2011).

TOLL-LIKE RECEPTORS

Innate immunity relies on the existence of a mechanism of recognition that identifies conserved molecular structures, known as pathogen associated molecular patterns (PAMPs), broadly expressed by different groups of microorganisms. These PAMPs include lipids, lipoproteins, carbohydrates, and nucleic acids (Akira et al., 2006). The recognition of these PAMPs is mediated by a set of germ line-encoded receptors known as pattern recognition receptors (PRRs). This recognition enables eukaryotic hosts to reliably detect a microbial infection, activating a number of signaling pathways that culminate in the induction of pro-inflammatory cytokines, chemokines, and inflammatory mediators. PRRs include TLRs, Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and NOD-like receptors (NLRs). PRRs, through their modulation of innate and adaptive immune responses, are essential players in the battle for tolerance or rejection of transplanted organs (Methe et al., 2004; Penack et al., 2010). The molecular and cellular mechanisms involved remain poorly understood and represent an emerging field of research with potential therapeutic implications.

Toll-like receptors are type I membrane proteins expressed by immune and non-immune cells (i.e., monocytes, macrophages, endothelial cells) either in the plasma membrane or intracellularly (endosomes). To date, 11 human and 13 mouse TLRs have been identified that recognize distinct microbial products from bacteria, viruses, protozoa, and fungi (Moresco et al., 2011). In addition, the recognition of endogenous ligands by TLRs is thought to have an important role in the regulation of inflammation, both in infectious and non-infectious diseases. A number of endogenous ligands have been identified, including heat shock protein (HSP) 60, HSP 70 (Asea et al., 2000; Oashi et al., 2000), heparan sulfate (Johnson et al., 2002), hyaluronan (Termeer et al., 2002), fibronectin extra domain A (Okamura et al., 2001), uric acid (Liu-Bryan et al., 2005), oxidized LDL (Miller et al., 2003), intracellular components of fragmented cells (Boule et al., 2004; Barrat et al., 2005), myeloid-related proteins-8 and 14 (Vogl et al., 2007), eosinophil-derived neurotoxin (Yang et al., 2008), and human defensin-3 (Funderburg et al., 2007). As these ligands are accessible to TLRs in the setting of injury or non-infectious threat, they have been called "danger signals."

Toll-like receptor activation triggers intracellular signaling pathways that lead to the induction of inflammatory cytokines, type I IFNs, and upregulation of co-stimulatory molecules leading to the activation of the adaptive immune response. Ligand recognition results in the recruitment of intracellular adaptor proteins, including myeloid-differentiation primary-response protein 88 (MyD88), shared by all TLRs except TLR3, and Toll/IL-1R domain-containing adaptor-inducing IFNβ (Trif), employed by TLR3 and TLR4 (O'Neill and Bowie, 2007). Recruitment of MyD88 leads to the activation of the mitogen-activated protein (MAP)-kinases (MAPKs) and nuclear translocation of the transcription factor nuclear factor-κB (NF-κB; *MyD88-dependent pathway*; Hoebe et al., 2006; Meylan et al., 2006). The activation of these signaling pathways is absent in MyD88-deficient mice in response to all TLRs, except TLR4 and TLR3. This is due to the activation of an alternative pathway triggered by Trif (*MyD88-independent pathway*) that culminates in the activation of NF-κB, MAPKs, and the transcription factors interferon-responsive factors (IRFs), whose are responsible for induction of type I IFNs, in particular IFNβ (Honda et al., 2006; Stetson and Medzhitov, 2006). Besides MyD88 and Trif, two other adaptor proteins have been described: TIR-domain-containing adaptor protein (TIRAP, required for MyD88-dependent signaling by TLR2 and TLR4), and Trif-related adaptor molecule (TRAM, required for Trif-dependent signaling through TLR4, but not TLR3; Takeda and

Akira, 2005; O'Neill and Bowie, 2007). Specific adaptors used by different TLRs combined with cell type-specific signaling pathways determine differential responses: inflammatory response, cell differentiation, proliferation, or apoptosis.

MODULATION OF MSCs THROUGH TLRs

Expression of TLR 1, 2, 3, 4, 5, and 6 has been reported in human and mice AD-MSCs and BM-MSCs, human umbilical cord blood MSCs (UCB-MSCs), human Wharton jelly's MSCs (WJ-MSCs), human dental pulp (DP), and dental follicle (DF)-MSCs (van den Berk et al., 2009; DelaRosa and Lombardo, 2010; Kim et al., 2010; Raicevic et al., 2011; Tomic et al., 2011). Expression and function of TLRs can be modulated in different ways in MSCs. Hypoxia significantly increased mRNA of TLR1, 2, 5, 9, and 10 (Hwa Cho et al., 2010). Infection of MSCs with baculoviral vectors upregulated expression of TLR3 and activated TLR3 signaling pathway (Chen et al., 2009). Interestingly, the inflammatory environment may also modulate the pattern and function of TLRs expressed by MSCs. When cultured in the presence of an "inflammatory cocktail" (made with IFNα, IFNγ, TNFα, and IL1β) expression of TLR2, 3, and 4 was increased, while TLR6 was downregulated (Raicevic et al., 2010). This modulatory effect seems to depend on the origin of MSCs as differences between BM, AD, and WJ-MSCs was found recently (Raicevic et al., 2011). Fatty acids may also modulate TLR signaling in ob/ob mouse AD-MSCs. Stearidonic and eicosapentainoic acids inhibited LPS-mediated upregulation of TLR2 through a mechanism that involves NF-κB but not ERK signaling pathway (Hsueh et al., 2011).

EFFECT OF TLRs ON DIFFERENTIATION OF MSCs

Adipogenic differentiation of human MSCs does not seem to be affected by TLRs (Hwa Cho et al., 2006; Liotta et al., 2008; Lombardo et al., 2009; Kim et al., 2010; Raicevic et al., 2010). Chondrogenic differentiation of human BM-MSCs has not been reported to be altered by activation through LPS, PolyIC, or R848 (Liotta et al., 2008), but was increased by TLR2 activation on human UCB-MSCs (Kim et al., 2010). The osteogenic differentiation seems to be enhanced in human BM-MSCs, AD-MSCs, and UCB-MSCs after LPS, PGN, or Poly IC activation (Hwa Cho et al., 2006; Mo et al., 2008; Lombardo et al., 2009; Kim et al., 2010), while CpG oligodeoxynucleotides (CpG ODN), have been reported to inhibit it on human AD-MSCs and BM-MSCs (Hwa Cho et al., 2006; Pevsner-Fischer et al., 2007; Liotta et al., 2008; Lombardo et al., 2009; Nørgaard et al., 2010). It has been reported recently that TNFα and TLRs activate osteogenic differentiation of AD-MSC via upregulation of transcriptional coactivator with PDZ-binding motif (TAZ; Hwa Cho et al., 2010).

On the other hand in mouse BM-MSCs, TLR2 was found to reduce differentiation into the three mesodermal lineages (Pevsner-Fischer et al., 2007). Interestingly, some reports link TLR signaling pathways with MSC multipotency. MyD88-deficient mouse BM-MSCs, when cultured in the appropriate differentiation media without additional stimulation with TLR ligands, effectively differentiated into adipocytes but failed to differentiate into osteocytes and chondrocytes (Pevsner-Fischer et al., 2007). However, TLR4-deficient mouse BM-MSCs showed higher differentiation rates compared to wild-type BM-MSCs (Wang et al., 2010). Nevertheless, TLR2-deficient mouse BM-MSCs failed to accumulate vacuoles in differentiated adipocytes, suggesting some impairment in the terminal differentiation process (Abarbanell et al., 2010). Therefore, the role of TLR signaling pathways in MSC multipotency needs to be further clarified.

EFFECT OF TLRs ON PROLIFERATION AND MIGRATION OF MSCs

So far, most of the studies have not found effects of TLR activation on human MSC proliferation. Only Hwa Cho et al. (2006) reported that TLR9 activation of AD-MSCs inhibited their proliferation. Interestingly, the use of TLR-deficient mouse BM-MSCs provided some insight on the role of TLRs on proliferation as TLR4-deficient BM-MSCs showed higher proliferation rates and TLR2-deficient showed reduced proliferation compared to wild-type MSCs (Abarbanell et al., 2010; Wang et al., 2010). In addition, TLR2 and TLR4 activation promoted proliferation of mouse BM-MSCs (Pevsner-Fischer et al., 2007; Wang et al., 2009).

Migration to the appropriate site of injury is believed to play a key role in the therapeutic efficacy of MSCs. Tomchuck et al. (2008) demonstrated that TLR3 activation drives the migration of human BM-MSCs *in vitro*. However, other reports found that TLR activation either impaired or had no effect on mouse BM-MSC migration (Pevsner-Fischer et al., 2007; Lei et al., 2011). In addition, TLR9 activation enhanced human BM-MSC invasion through a mechanism mediated, at least in part, by increased expression of MMP-13 (Nurmenniemi et al., 2010).

EFFECT OF TLRs ON INTERACTION OF MSCs WITH IMMUNE CELLS

Mesenchymal stem cells have been shown to possess the capacity to inhibit proliferation of immune cells upon mitogenic or allogeneic activation. In recent years, inconsistent results have been reported regarding the role of TLR ligands on MSCs capacity to modulate immune responses. We and others found no significant effect of TLR activation on human AD-MSC or mouse BM-MSC-mediated immunosuppression (Pevsner-Fischer et al., 2007; Lombardo et al., 2009). However, other groups have reported that TLR activation may modulate the immunosuppressive properties of human BM-MSCs, although in very different ways. Liotta et al. (2008) found that TLR3 and TLR4 activation reduce the inhibitory activity of human BM-MSCs on T cell proliferation without influencing IDO activity or PGE2 levels, but downregulated expression of Jagged1, suggesting that the Notch signaling pathway mediates cell contact-mediated immunosuppression by MSCs. In contrast, Opitz et al. (2009) reported that TLR3 and TLR4 engagement enhances the immunosuppressive properties of human BM-MSCs through the indirect induction of IDO1. Induction of IDO1 involved an autocrine IFNβ signaling loop, which was dependent on protein kinase R (PKR) and independent of IFNγ. The role of IDO seems to be species dependent as Lanz et al. (2010) reported recently that IDO activity is not required for mouse BM-MSC immunosuppressive capacity both *in vitro* and *in vivo*, using IDO-deficient MSCs. Interestingly, TLR2 activation has been reported to impair the capacity of mouse BM-MSCs to induce the generation of regulatory T cells (Lei et al., 2011). Adding more uncertainty, Raicevic et al. (2010) reported that preactivation of human BM-MSCs with TLR3 or TLR4 ligands reduced production of HGF and PGE2 which impaired their capacity to

inhibit lymphocyte proliferation. However, these authors found in a later report, that triggering of TLR3 or TLR4 on human MSCs from BM, AD, and Wharton jelly´s did not affect their immunosuppressive capacity (Raicevic et al., 2011). Dental pulp (DP) and Dental follicle (DF)-MSCs can also modulate lymphocyte proliferation *in vitro*, which is potentiated by TLR3 activation in both cell types, whereas TLR4 activation increased the suppressive role of DF-MSCs and reduced it in DP-MSCs (Tomic et al., 2011). Immunomodulating properties of human umbilical cord blood (UCB-MSCs) were not affected by prestimulation with TLR4 or TLR5 ligands (van den Berk et al., 2009).

Toll-like receptors may polarize MSCs toward pro-inflammatory and antigen-presenting-like phenotypes leading to release of pro-inflammatory cytokines and chemokines capable of enhancing recruitment of inflammatory immune cells (Romieu-Mourez et al., 2009). In line with this, a "licensing" process of MSCs toward either pro-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotypes, which depends on the ligand concentration, timing, and kinetics of activation, has been proposed (Waterman et al., 2010). TLR4 priming results in upregulation of mostly pro-inflammatory cytokines such as IL6 or IL8 (MSC1 phenotype), while TLR3 priming results in production of anti-inflammatory molecules such as IL4, IDO, or PGE2 (MSC2 phenotype). TLR3-activated MSCs maintained the capacity to inhibit lymphocyte proliferation *in vitro*, while TLR4-primed MSCs activated T lymphocytes. As suggested by the authors, the polarizing effects of TLR priming may also explain the contradictory results obtained so far on the effects of TLRs on immunomodulation by MSCs.

There are other immune functions mediated by MSCs which have been found to be modulated by TLRs. BM-MSCs and parotid-derived MSCs have been shown to support neutrophil survival and chemotaxis in a ratio dependent manner through the release of soluble factors (Raffaghello et al., 2008; Brandau et al., 2010). Recently, Cassatella and colleagues found that TLR3 and TLR4 ligands enhanced the capacity of MSCs to delay neutrophil apoptosis through the induction of IL6, IFNγ, and GM-CSF. Moreover, TLR activation of BM-MSCs strongly increased respiratory burst of neutrophils. This supportive role on neutrophil function was confirmed using MSCs from thymus, spleen, or adipose tissue (Cassatella et al., 2011).

TLR2 and TLR4 mediate the capacity of human BM-MSCs to support short-term expansion of umbilical cord CD34+ cells, promoting myeloid-differentiation through the induction of hematopoietic growth factors (Wang et al., 2012). Moreover, it has been recently reported that resident mouse BM-MSCs, by producing MCP-1 in response to LPS, induce monocyte emigration from bone marrow into circulation to confront potential infections (Shi et al., 2011). These findings suggest an important role for TLRs in the modulation of the immune system by resident MSCs since BM-MSCs could function as sensors of circulating TLR ligands and determine, by expressing MCP-1, the frequency of circulating inflammatory Ly6Chigh, CCR2+ monocytes.

EFFECT OF TLRs ON THERAPEUTIC EFFECTS OF MSCs *IN VIVO*

Several studies have reported beneficial effects of MSC treatment in animal models of sepsis or LPS-induced lung injury (in which MSCs were administered within 1 h following LPS challenge; Mei et al., 2007, 2010; Xu et al., 2007; Gonzalez-Rey et al., 2009; Németh et al., 2009, 2010). Based on the therapeutic benefit observed in these experimental models, it can be interpreted that high concentrations of LPS did not polarize MSCs toward a pro-inflammatory phenotype, in apparent contradiction to the reported polarizing process observed *in vitro* (Waterman et al., 2010). However, Waterman et al. (2010) reported that MSC1 and MSC2 cells were used in mouse models of lung injury and MSC1 aggravated the inflammatory injury, whereas MSC2 improved it, when compared to unstimulated BM-MSCs.

Conflicting results have been reported regarding the modulation of MSC-mediated cardiac protection by TLRs. LPS preconditioning of mouse BM-MSCs can, when compared to unconditioned MSCs, improve their survival and engraftment and increases the release of vascular endothelial growth factor (VEGF) in a model of rat acute myocardial infarction leading to enhanced therapeutic effects (Yao et al., 2009). These effects can be mediated through a TLR4-mediated protection of MSCs from apoptosis induced by oxidative stress (Wang et al., 2009). In contrast, TLR4-deficient mouse BM-MSCs had increased cardiac protection which was mediated by activated STAT3 signaling, leading to expression of higher levels of angiogenic factors such as VEGF and HGF (Wang et al., 2010). TLR2 activity also seems to be involved in cardioprotective effects by mouse BM-MSCs after ischemia/reperfusion injury. TLR2-deficient mouse BM-MSC showed impaired capacity to recover heart function, which correlates with reduced production of VEGF in hearts treated with TLR2-deficient MSCs compared to wild-type controls (Abarbanell et al., 2010). Therefore, further investigation in experimental animal models is required to clarify the role of TLRs in the licensing process as well as in the therapeutic potential of MSCs *in vivo*.

CONCLUDING REMARKS

Despite discrepancies and inconsistencies reported by authors, some general conclusions can be made: (a) TLR expression: MSCs from different sources express TLRs at the mRNA level, although expression at a protein level seems to be low (i.e., compared to monocytes), and often makes difficult detection by flow cytometry, (b) MSC differentiation: in human MSCs, adipogenic differentiation does not seem to be affected by TLRs but osteogenic differentiation seems to be enhanced by TLR2, TLR3, or TLR4, while inhibited by TLR9. In mouse MSCs, TLR signaling might be linked to multipotency of MSCs as MyD88-deficient BM-MSCs failed to efficiently differentiate into chodrogenic and osteogenic lineage, (c) MSC proliferation: in human MSCs, only TLR9 activation has been reported to affect AD-MSC proliferation, (d) immunomodulatory capacity of MSCs: contradictory results have been reported that can be explained, at least in part, by the experimental conditions and the source of MSCs. The fact that differences in the experimental settings may lead MSCs to behave differently, suggests that MSCs can adjust their response in a dynamic way to the specific environmental conditions they face. In this regard, Waterman et al. (2010) challenged the concept of MSCs being always immunosuppressive and suggested that a polarizing process toward a pro-inflammatory or anti-inflammatory phenotype may occur depending on the TLR activated. However, the anti-inflammatory and therapeutic effects reported in mouse

models of sepsis and lung injury, where MSCs were exposed to high levels of LPS, seems to be in apparent contradiction to the polarizing process described *in vitro*. Therefore, the *in vivo* modulation of MSC biology by TLR ligands deserves to be further investigated and clarified.

The inflammatory conditions MSCs face when administered *in vivo* is now believed to play a fundamental role in their successful therapeutic use. Research on modulation of MSCs by TLRs can strongly contribute to better understand the immunomodulating properties of MSCs under different inflammatory environments and to characterize the features an inflammatory milieu should have for MSCs to best modulate immune reactions (i.e., composition, ratio or activity of immune cells, cytokines or other inflammatory mediators such as TLR ligands).

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Rat mesenchymal stromal cells inhibit T cell proliferation but not cytokine production through inducible nitric oxide synthase

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Mesenchymal stromal cells (MSC) have important immunomodulatory properties, they inhibit T lymphocyte allo-activation and have been used to treat graft-versus-host disease. How MSC exert their immunosuppressive functions is not completely understood but species specific mechanisms have been implicated. In this study we have investigated the mechanisms for rat MSC mediated inhibition of T lymphocyte proliferation and secretion of inflammatory cytokines in response to allogeneic and mitogenic stimuli *in vitro*. MSC inhibited the proliferation of T cells in allogeneic mixed lymphocyte reactions and in response to mitogen with similar efficacy. The anti-proliferative effect was mediated by the induced expression of nitric oxide (NO) synthase and production of NO by MSC. This pathway was required and sufficient to fully suppress lymphocyte proliferation and depended on proximity of MSC and target cells. Expression of inducible NO synthase by MSC was induced through synergistic stimulation with tumor necrosis factor α and interferon γ secreted by activated lymphocytes. Conversely, MSC had a pronounced inhibitory effect on the secretion of these cytokines by T cells which did not depend on NO synthase activity or cell contact, but was partially reversed by addition of the cyclooxygenase (COX) inhibitor indomethacin. In conclusion, rat MSC use different mechanisms to inhibit proliferative and inflammatory responses of activated T cells. While proliferation is suppressed by production of NO, cytokine secretion appears to be impaired at least in part by COX-dependent production of prostaglandin E_2.

Keywords: mesenchymal stem cells, immunosuppression, T lymphocyte activation, mixed lymphocyte reaction, nitric oxide synthase 2, cytokines, rodent, prostaglandin E2

INTRODUCTION

Mesenchymal stem cells have self-renewing capacity and differentiation potential for all mesodermal cell lineages (Pittenger et al., 1999). They are present within a heterogeneous cell population referred to as mesenchymal stromal cells (MSC) which are presently defined by a set of criteria based on their morphology, phenotype, and multipotency (Dominici et al., 2006). To date, MSC have been studied most thoroughly in humans and mice. They can be isolated from the bone marrow (BM) and a variety of other adult and fetal tissues (Pittenger et al., 1999; Zuk et al., 2001; in 't Anker et al., 2003, 2004; da Silva Meirelles et al., 2006; Yoshimura et al., 2007). MSC have potent modulatory effects on immune cells including T cells, B cells, natural killer cells, and dendritic cells as well as regulatory T (T_{reg}) cells (Uccelli et al., 2006; Nauta and Fibbe, 2007; Tolar et al., 2011). A range of distinct mediator molecules have been implicated (Uccelli et al., 2006; Nasef et al., 2008) but the molecular mechanisms by which MSC exert these effects are not entirely understood and the influences of tissue source, species origin, and cell culture conditions have yet to be firmly established.

Nitric oxide (NO) is a short-lived bioactive compound which is catalyzed by different tissue-specific NO synthases, of which inducible NO synthase (iNOS) encoded by the *NOS2* gene is active in macrophages, fibroblasts, and endothelial cells (Bogdan, 2001; Lukacs-Kornek et al., 2011). iNOS expression can be induced by synergistic signals of interferon (IFN) γ and tumor necrosis factor (TNF) α or Toll-like receptor (TLR) ligands (Liew et al., 1991; Muñoz-Fernández et al., 1992; Deng et al., 1993; Lorsbach et al., 1993; Lukacs-Kornek et al., 2011). NO acts as a regulator of cellular and immune functions (Bogdan, 2001) such as inhibition of T cell responses (Lejeune et al., 1994; Medot-Pirenne et al., 1999; Niedbala et al., 2006) and induction of T_{reg} cells (Niedbala et al., 2007). The iNOS pathway also has a role in the immunosuppressive potential of MSC (Sato et al., 2007). A combination of pro-inflammatory cytokines, namely IFNγ together with TNFα, interleukin (IL)1α, or IL1β, has been shown to trigger the expression of iNOS in murine BM-derived MSC (Ren et al., 2008). Mouse MSC (mMSC) utilize NO to arrest T cell proliferation and activation *in vitro* and *in vivo* (Oh et al., 2007; Sato et al., 2007; Ren et al., 2008).

The capacity of MSC to suppress the activation of T lymphocytes has become of interest for clinical prevention and treatment of both autoimmune diseases and graft-versus-host disease (GVHD; Dazzi and Krampera, 2011; Tolar et al., 2011). GVHD has been treated successfully with MSC infusions clinically (Le Blanc et al., 2004, 2008; Ringdén et al., 2006; Martin et al., 2010; Tolar et al., 2011) and experimentally in animal models (Yanez et al., 2006; Min et al., 2007; Tisato et al., 2007; Polchert et al., 2008; Tian et al., 2008; Joo et al., 2010). Ren et al. (2008) reported that amelioration of experimental GVHD by mMSC depended on NO production. Human MSC (hMSC), on the other hand, do not utilize NO conversion, but rather employ alternative signaling pathways such as indoleamine-2,3-dioxygenase (IDO), cyclooxygenase (COX)-2 required for synthesis of prostaglandin E_2 (PGE_2), and heme oxygenase-1 expression to inhibit T cell activation and induce expansion of T_{reg} cells (Meisel et al., 2004; Aggarwal and Pittenger, 2005; Ren et al., 2009; Mougiakakos et al., 2011).

It has been suggested that MSC are "licensed" by certain effector molecules to exert immunomodulatory functions (Dazzi and Krampera, 2011). When exposed to an inflammatory milieu, hMSC upregulated the expression of IDO and COX-2 genes and showed increased inhibitory potential in mixed lymphocyte reactions (MLR; Crop et al., 2010). In another recent paper, the immunomodulatory properties of rat MSC (rMSC) were primed by the addition of different cytokines resulting in either enhanced inhibition of proliferation or the opposite effect depending on the type of stimulatory signal (Renner et al., 2009).

In this report, we generated rMSC lines from the BM and evaluated their potential to inhibit T cell proliferation and cytokine secretion *in vitro*. We show that the regulation of immunosuppression by rMSC was more similar to mouse than to hMSC. rMSC depended on cell-to-cell contact, iNOS expression, and NO production to mediate potent anti-proliferative effects. The putative mechanism that inhibits secretion of inflammatory cytokines is distinct and does not depend on cellular contact but on a soluble factor, likely PGE_2 produced by COX-2.

MATERIALS AND METHODS

ETHICS STATEMENT

Approval for the use of organs of rats euthanized with CO_2 (license number: VIT09.1512) was obtained from our institutional veterinarian with delegated authority from the Norwegian Animal Research Authority under the Ministry of Agriculture of Norway. All experiments were conducted in compliance with institutional guidelines. All animals were sacrificed with CO_2 and every effort was made to minimize their suffering.

ANIMAL CARE

PVG strain rats express the *c* haplotype of the rat MHC (*RT1*^{c}, i.e., *RT1-A*^{c}*-B/D*^{c}*-CE/N/M*^{c}; for short, *c-c-c*). PVG-*RT7*^{b} strain (abbreviated PVG.7B) rats express the RT7.2 allotype of CD45, but are used interchangeably with the standard PVG strain (encoding the RT7.1 allotype) as both strains carry the *RT1*^{c} haplotype. The MHC-congenic PVG-*RT1*^{u} strain (PVG.1U) expresses the *u-u-u* MHC haplotype, the PVG-*RT1*^{n} strain (PVG.1N) the *n-n-n* haplotype and the intra-MHC recombinant PVG-*RT1*^{r23} strain (PVG.R23) the *u-a-av1* haplotype on the PVG background.

PVG.R23, PVG.1N, PVG.1U, and PVG.7B rats were bred at the Institute of Basic Medical Sciences, University of Oslo. PVG and BN/RijHsd (BN; *RT1*^{n}, *n-n-n*) rats were purchased from Harlan, The Netherlands[1]. The animals were housed under a 12:12 h light/dark cycle with access to food and filtered drinking water *ad libitum* and were routinely screened for common pathogens following recommendations by the Federation of European Laboratory Animal Science Associations (Nicklas et al., 2002).

MATERIALS

Nylon cell strainers (70 μm mesh size) were purchased from BD Falcon, MA, USA[2]; GIBCO® RPMI medium 1640, OPTI-MEM® I, α-modified minimal essential medium, fetal bovine serum (FBS), penicillin and streptomycin, sodium pyruvate, 2-mercaptoethanol, trypsin and EDTA, lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (poly-I:C) from Invitrogen, UK[3]; L-glutamine, Immobilon®-P transfer membrane from Millipore, MA, USA[4]; biotin, Brefeldin A, Concanavalin A (ConA), sodium nitrate, sodium dodecyl sulfate, 2-mercaptoethanol, glycerol, sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), fluorescein isothiocyanate (FITC), propidium iodide, paraformaldehyde, saponin from Quillaja bark, 1-methyl-DL-tryptophan (1-MT), N^G-monomethyl-L-arginine acetate (L-NMMA) from Sigma-Aldrich, MO, USA[5]; indomethacin (IMC; Confortid®) from Dumex-Alpharma A/S, Denmark[6]; Criterion™ Precast gels from Bio-Rad Laboratories, CA, USA[7]; SuperSignal® West Pico chemiluminescent substrate from Thermo Scientific, IL, USA[8]; Amersham Hyperfilm™ ECL from GE Healthcare Ltd., UK[9]; culture flasks from Nunc, Denmark[10]; 96-well cell culture clusters, HTS Transwell® 96-well system with 0.4 μm polycarbonate membrane insert plates from Corning, NY, USA[11]; recombinant rat IFNγ from Biomedical Primate Research Centre, The Netherlands[12]; recombinant rat TNFα from PeproTech, UK[13]; [methyl-^{3}H]-thymidine (^{3}H-TTP) from Hartmann-Analytic, Germany[14]; MicroScint™ O solution from PerkinElmer, MA, USA[15].

ANTIBODIES

Monoclonal mouse anti-rat CD25 (OX39) and anti-CD3 (G4.18) antibodies were conjugated with biotin and anti-CD4 (W3/25) antibody with FITC in our laboratory using standard methods. Supernatants of monoclonal mouse anti-rat

[1] www.harlan.com
[2] www.bdbiosciences.com
[3] www.invitrogen.com
[4] www.millipore.com
[5] www.sigma-aldrich.com
[6] www.alpharma.com
[7] www.bio-rad.com
[8] www.piercenet.com
[9] www.gelifesciences.com
[10] www.nuncbrand.com
[11] www.corning.com
[12] www.bprc.nl
[13] www.peprotech.com
[14] www.hartmann-analytic.de
[15] www.perkinelmer.com

CD45 (OX1), anti-RT1-B/D (pan-MHC class II; OX6), anti-CD71 (OX26), anti-CD11b (OX42), anti-CD86 (OX48), anti-CD44 (OX49), anti-RT1-A (pan-MHC class I; OX18, purified) antibodies, as well as phycoerythrin-conjugated mouse anti-rat IFNγ (DB-1), anti-CD31 (TLD-3A12), anti-CD90 (OX7), anti-CD3 (G4.18), FITC-conjugated anti-CD59 (TH9) from BD Biosciences and allophycocyanin-conjugated rat anti-mouse/rat FoxP3 (FJK-16s) from eBioscience, CA, USA[16] were used for immunostaining. Phycoerythrin-conjugated donkey anti-mouse immunoglobulin (Ig) G or peridinin chlorophyll protein-conjugated Streptavidin from BD Biosciences were used as secondary antibodies for flow cytometric analysis.

Polyclonal rabbit anti-rat TNFα, goat anti-rat IFNγ, and rabbit anti-rat IL6 antibodies were from PeproTech; polyclonal rabbit anti-rat NOS2 (M-19) from Santa Cruz Biotechnology, CA, USA[17]; monoclonal mouse anti-GAPDH (6C5) from Millipore; horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG from Jackson ImmunoResearch, PA, USA[18].

CELL LINES

The rat macrophage cell line R2 is derived from pleural macrophages induced by a silica injection in the pleural cavity of Wistar rats (Damoiseaux et al., 1994). R2 cells were maintained in complete medium comprising RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO_2.

MSC lines were obtained from 7- to 8-week-old female PVG.7B and PVG.1U rats as described elsewhere (Lennon and Caplan, 2006). In short, BM cells were aspirated from femurs and tibias, filtered through nylon cell strainers, and cultured in α-modified minimal essential medium supplemented with 20% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2 mM L-glutamine in 175 cm^2 culture flasks at 37°C in a humidified atmosphere of 5% CO_2. Non-adherent cells were removed after 24 h by replacement of culture medium with complete, antibiotics-free MSC medium comprising α-modified minimal essential medium supplemented with 15% FBS and 2 mM L-glutamine. Adherent cells were allowed to expand to near confluence, detached using 500 μg mL^{-1} trypsin and 200 μg mL^{-1} EDTA·4Na and reseeded at a density of approximately 400–600 cells cm^{-2}. MSC were used in experiments after the third passage. Supernatants from confluent cultures were frequently controlled for mycoplasma contamination by PCR as previously described (Zinöcker et al., 2011b).

CELL CULTURE

Mesenteric and cervical lymph nodes from 7- to 14-week-old male or female PVG, PVG.7B, PVG.1N, PVG.1U, PVG.R23, and BN rats were removed and filtered through nylon cell strainers. The lymphocyte population was purified by density gradient centrifugation using Lymphoprep™ 1.077 (Medinor AS, Norway)[19]. Stimulator cells were irradiated (2000 cGy) using a ^{137}Cs source (Gammacell® 3000; MDS Nordion, ON, Canada)[20] to inhibit mitosis. MLR were performed in a total volume of 200 μL complete MLR medium comprising RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol using round bottom 96-well plates. Equal numbers (2×10^5) of responder and stimulator cells were mixed and incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2. ConA was used at 5 μg mL^{-1} final concentration for mitogenic stimulation of 2×10^5 responder cells unless specified otherwise. MSC were harvested from culture, washed twice (500 *g* for 6 min) in phosphate-buffered saline (PBS), resuspended in MLR medium and seeded at least 2 h before lymphocytes were added to allow attachment.

For stimulation experiments, cell-free supernatants were centrifuged at 400 × *g* for 10 min before transfer of equal volumes to MSC culture. For transwell experiments, MSC were seeded either in 0.4 μm polycarbonate membrane inserts or in the reservoirs of 96-well flat-bottom receiver plates. Responder cells were added to the bottom reservoirs and co-incubated for 3 days.

RADIONUCLIDE INCORPORATION ASSAY

DNA synthesis during mitogen stimulation or mixed lymphocyte culture was assessed after 20 h pulsing with 1 μCi ^{3}H-TTP before termination of the culture. Cells were harvested on glass fiber filters using a Filtermate 196 cell harvester (Packard Bioscience Co., CT, USA)[21] and radioactivity was measured using a Wallac 1450 MicroBeta® TriLux (PerkinElmer) microplate scintillation counter. Relative inhibition of the culture was calculated by the following equation:

$$inhibition = 1 - (proliferation\ count\ of\ the\ sample / mean\ proliferation\ count\ of\ the\ positive\ control)$$

CFSE DILUTION ASSAY

Responder cells were stained with CFSE prior to *in vitro* culture as previously described (Zinöcker et al., 2011b). Briefly, cells were resuspended in OPTI-MEM at 2×10^6 mL^{-1} and incubated with 0.5 μM CFSE for 10 min at 37°C. Stained cells were then washed (400 *g* for 8 min) in MLR medium, incubated once more for 5 min at 37°C, washed twice and resuspended in MLR medium.

At the termination of MLR and ConA cultures, cells were harvested and washed in PBS before immunostaining and flow cytometric analysis. Fifty micromolar propidium iodide was added before flow cytometric analysis to exclude non-viable cells. The percentage of dividing cells was determined as the fraction of cells that had undergone one or several cell divisions (CFSE^{lo}) relative to the total number of CFSE$^+$ cells including cells that had not undergone cell divisions (CFSE^{hi}).

IMMUNOSTAINING AND FLOW CYTOMETRIC ANALYSIS

All MSC lines were tested for surface marker expression by flow cytometry. Cells were labeled with anti-CD11b, -CD31, -CD44, -CD45, -CD59, -CD71, -CD86, -CD90, anti-class I, and anti-class II

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MHC monoclonal antibodies (cf. *Antibodies*). Monoclonal mouse IgG_1 and IgG_{2a} were used as isotype controls.

For intracellular IFNγ staining, Brefeldin A (10 μg mL^{-1}) was added 4 h prior to termination of ConA-stimulated LNC cultures to inhibit protein secretion. Cells from triplicate or quadruplicate wells were pooled, stained with anti-CD3 antibody, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% saponin in water and stained with anti-IFNγ antibody.

For intracellular FoxP3 staining, ConA-stimulated LNC were harvested after 3 days and stained with anti-CD3, -CD4, -CD25 antibodies. Immunostained cells were subsequently treated with fixation/permeabilization buffer (eBioscience) and stained with anti-FoxP3 antibody following the manufacturer's protocol. Cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences) using CellQuest™ software (BD Biosciences). FACS data were further analyzed using FlowJo™ software (Treestar, OR, USA)[22].

WESTERN BLOT

Cells were pooled from triplicates and lysed for 30 min on ice with buffer consisting of 25 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton® X-100, 1 mM phenylmethanesulfonyl fluoride in isopropanol, 1 mM Na_3VO_4 and proteinase inhibitor cocktail (all from Sigma-Aldrich). Lysates were centrifuged at 10,000 × *g* for 10 min to remove debris, and supernatants were resuspended in standard sample buffer containing 2% (w/v) sodium dodecyl sulfate and 2.5% (v/v) 2-mercaptoethanol. Samples were then heated at 95°C for 5 min, allowed to cool at ambient temperature and loaded onto polyacrylamide gels. Electrophoresis was run at 80–200 V. Proteins were transferred onto a polyvinylidene fluoride membrane in a TE 70 semi-dry transfer unit (GE Healthcare) using a current of 100 mA for 65 min. Western blotting was performed using anti-rat NOS2 (iNOS) and anti-rat GAPDH antibodies followed by secondary staining with horseradish peroxidase-conjugated IgG. Chemiluminescent substrate was used to visualize the immunoreactive proteins by horseradish peroxidase detection.

NO QUANTIFICATION

The nitrite concentration in the medium was measured as an indicator of NO production by virtue of the Griess reaction (Beda and Nedospasov, 2005). Fifty microliters of cell-free supernatant from MLR or ConA cultures was mixed with equal volumes of 1% (w/v) sulfanilamide in 5% phosphoric acid and 0.1% (w/v) naphthylethylenediamine dihydrochloride in water. Absorbance of the reaction at 540 nm was measured using a Labsystems Multiskan® bichromatic plate reader (Titertek Instruments, AL, USA)[23] and concentrations were calculated based on a standard curve of twofold dilutions of sodium nitrate which was assayed in parallel.

CYTOKINE ASSAYS

For cytokine measurements, supernatants from ConA cultures were collected after 2–3 days, pooled from triplicate or quadruplicate wells, centrifuged at 1,500 × *g* for 4 min to remove cellular debris and stored at −80°C or −20°C until analysis. Samples were thawed at 37°C in a water bath and analyzed using the Bio-Plex™ Rat Cytokine 9-Plex A Panel (Bio-Rad) according to the manufacturer's protocol. Concentrations were determined based on a standard curve using defined reference samples which were assayed (Luminex xMAP® Technology; Bio-Rad) in parallel.

STATISTICAL ANALYSIS

Normal distribution of data was assumed and tested by Shapiro–Wilk's test. The paired Student's *t* test (two-tailed) was used to evaluate the probability of differences between group means. Statistical analysis was performed using SPSS® software version 18.0.1 (SPSS, IL, USA)[24].

RESULTS

GENERATION OF MSC LINES FROM RAT BM

We produced MSC lines from the BM of PVG.7B and PVG.1U MHC-congenic rats. The cells showed adherence to plastic and spindle-shaped fibroblast-like morphology in culture (data not shown). They expressed MHC class I, CD44, CD59, CD71, and CD90 surface markers, but lacked CD11b, CD31, CD45, CD86, and MHC class II expression (**Figure 1**). Their potential to develop into adipocytes and osteocytes was confirmed by *in vitro* differentiation assays (Zinöcker et al., manuscript submitted). Together, this data was in accordance with the current definition of the MSC phenotype (Dominici et al., 2006; Harting et al., 2008).

RAT BM–MSC INHIBIT ALLO-ANTIGEN AND MITOGEN-INDUCED T CELL PROLIFERATION *IN VITRO* INDEPENDENT OF MHC HAPLOTYPE

We tested the ability of MSC to inhibit lymphocyte proliferation induced by ConA or in allogeneic mixed lymphocyte cultures. ConA stimulation induced proliferation of LNC from PVG.7B (*RT1*^{c}) rats as assessed by CFSE dilution and radiolabeled nucleotide incorporation. Proliferation was fully inhibited in the presence of PVG.7B MSC at a cell ratio of 1 MSC per 10 LNC (1:10); significant suppression was also observed at a ratio of 1:100 (**Figure 2A**). The inhibitory effect was not dependent on the MHC combination of MSC and responder cells as allogeneic PVG.1U (*RT1*^{u}) and syngeneic PVG.7B MSC suppressed ConA-induced proliferation of PVG.7B responder cells equally well (**Figure 3A**). Similarly, both PVG.1U and PVG.7B MSC inhibited antigen-induced proliferation of both PVG.1U and PVG.7B responder cells in allogeneic MLR with equal efficiency (**Figure 3B** and data not shown). This suggested that the inhibitory capacity of MSC did not depend on the MHC-allotype, in line with previous findings (Krampera et al., 2003; Le Blanc et al., 2003). Proliferation of MSC alone was negligible and below the negative control (LNC proliferation in the absence of a stimulus), which was consistently less than 10% of the positive control (data not shown). Irradiation (2000 cGy) of MSC did not reduce their inhibitory capacity significantly in ConA- and MLR-induced lymphocyte cultures (data not shown), suggesting that this property is not dependent on their ability to proliferate. Cell-free supernatant from MSC culture had no suppressive effect (data not shown).

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[24] www.spss.com

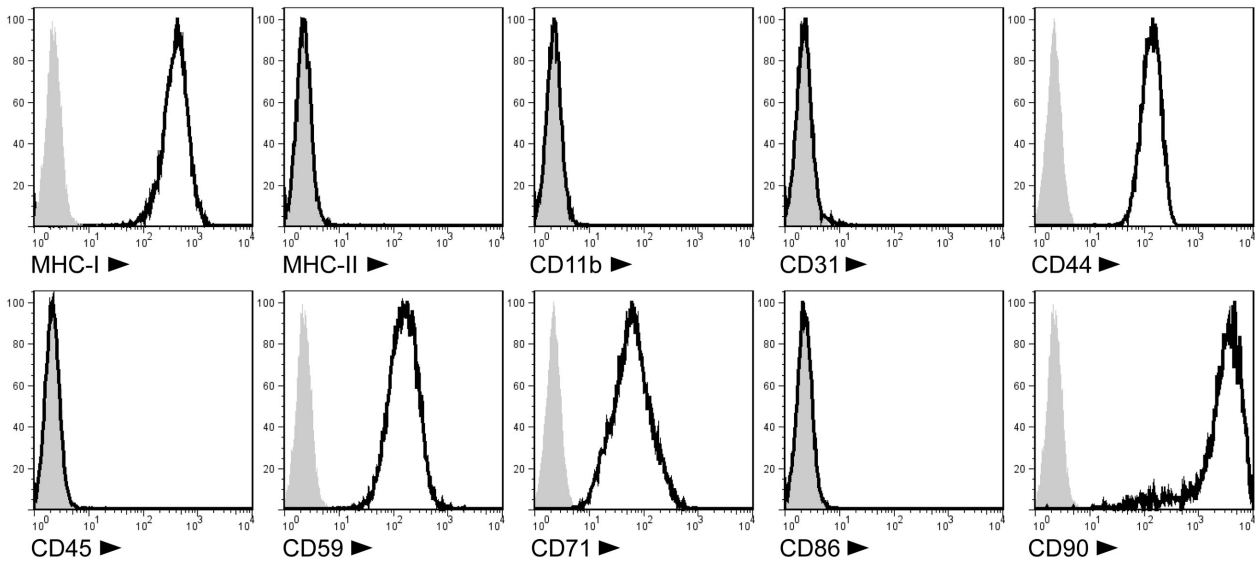

FIGURE 1 | Cell surface phenotype of PVG.7B MSC isolated from rat BM. *Ex vivo*-expanded MSC express MHC class I (RT1-A), CD44H (H-CAM), CD59 (MAC inhibitor), CD71 (transferrin receptor), and CD90 (Thy-1), but not MHC class II (RT1-B/D), CD11b (MAC-1), CD31 (PECAM-1), CD45 (LCA), or CD86 (B7-2) as detected by single-color flow cytometry. Representative stainings of MSC derived from PVG.7B BM are displayed as histograms [*x*-axis, signal intensity (log); *y*-axis, relative cell count (percent of max)] of surface antigens (solid line) and negative controls (gray histograms).

T CELL PROLIFERATION IN THE PRESENCE OF MSC IS RESTORED BY ADDITION OF THE iNOS INHIBITOR L-NMMA

We tested inhibitors of different signaling pathways which have been implicated in immunosuppression by MSC (Meisel et al., 2004; Aggarwal and Pittenger, 2005; Sato et al., 2007) and found that the addition of L-NMMA, an inhibitor of iNOS, at the start of MLR or ConA stimulation (**Figure 2** and data not shown) restored proliferation to normal levels. Conversely, addition of 1-MT, an IDO inhibitor, or IMC, a COX inhibitor, had no effect. The presence of all three inhibitors in lymphocyte culture had no additional effect compared to L-NMMA alone (**Figure 2B**). L-NMMA fully reversed the proliferative arrest of both $CD4^+$ and $CD8^+$ T cells which were equally affected by MSC inhibition (data not shown). Together, these data indicated that rMSC use iNOS to suppress T cell proliferation *in vitro*.

MSC EXPRESS iNOS AND PRODUCE NO IN RESPONSE TO LYMPHOCYTE ACTIVATION

Our findings suggested that the production of NO through iNOS was responsible for the suppression of T cell proliferation by rMSC. To test this hypothesis further, we measured the expression of iNOS by Western blot. iNOS was only detected in ConA cultures upon addition of MSC (**Figure 4A**) and correlated with the number of MSC present. iNOS was not detected in cultures of MSC alone, which suggested that its expression was induced in co-cultures with activated T lymphocytes. We also measured the concentration of nitrate as a proxy of NO (Beda and Nedospasov, 2005) and found that NO levels correlated with the observed iNOS levels. Neither MSC nor ConA-activated LNC cultures produced significant levels of NO (**Figure 4B** and data not shown). Notably, addition of L-NMMA did not affect iNOS protein levels (data not shown) but completely abrogated NO production (**Figure 4B**). We obtained identical results from MLR:MSC co-cultures (unpublished observations). The upregulation of iNOS in MSC co-cultures with stimulated lymphocytes correlated with the observed dose-dependent inhibition of proliferation (as shown in **Figures 2** and **3**) and thus provided further evidence that inducible NO production represents a key mechanism for the suppressive capacity of rMSC.

IMMUNOSTIMULATORY CYTOKINES INDUCE iNOS EXPRESSION IN MSC

It has been proposed that MSC depend on "licensing" in order to assume immunosuppressive functions, e.g., by stimulation through IFNγ or a TLR3 ligand (Krampera et al., 2006; Waterman et al., 2010; Dazzi and Krampera, 2011). Furthermore, it has been shown that iNOS expression is induced in response to synergistic stimulation by pro-inflammatory cytokines in mMSC (IFNγ together with either TNF or IL1 signals; Ren et al., 2008; Ren et al., 2009), and we therefore tested whether this was the case also for rMSC. Different combinations of IFNγ, TNFα, and TLR agonists induced iNOS expression in rMSC as judged by Western blot analysis. Incubation with TNFα for 24 h was sufficient to induce iNOS expression and increase NO concentrations in fresh MSC cultures (**Figure 5A**). Conversely, addition of IFNγ did not activate iNOS at the concentrations tested (titrations of 15 up to 5000 U mL^{-1}, data not shown) but potentiated the effect of TNFα resulting in significantly higher levels of both iNOS expression and NO compared with addition of TNFα alone (**Figure 5A**). LPS, a TLR4 agonist, or poly-I:C, a TLR3 agonist, resulted neither in iNOS expression nor NO production by MSC, in contrast to rat macrophages used as positive control (**Figure 5A**; **Figure A1** in

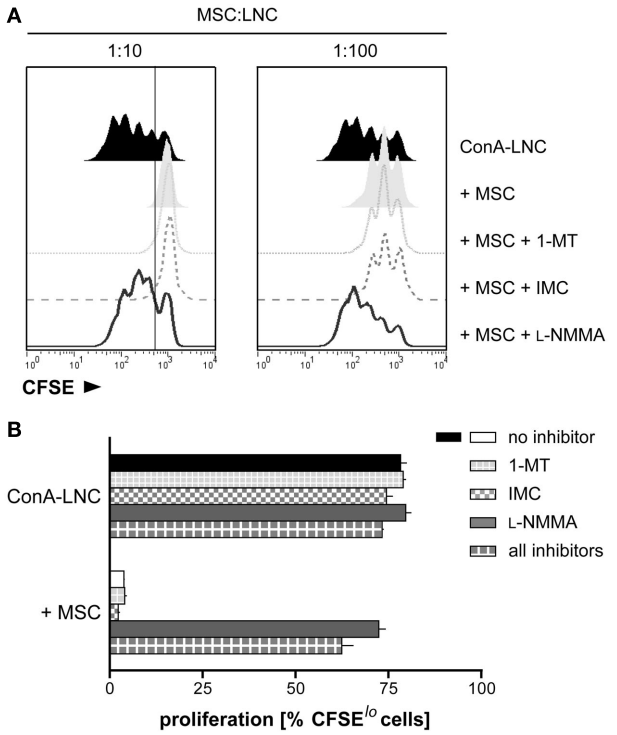

FIGURE 2 | MSC mediate suppression of mitogen-induced T cell proliferation through iNOS. (A) LNC from PVG.7B rats were labeled with CFSE and cultured in the presence of ConA for 3 days. Dilution of CFSE intensity was measured by flow cytometry to quantify lymphocyte proliferation. PVG.7B MSC were added at the start of culture at MSC:LNC ratios of 1:10 (left panel) or 1:100 (right panel), either alone (shaded off-set histograms) or together with inhibitors of IDO (1-MT, 1 mM; dotted line), COX (IMC, 5 μg mL^{-1}; dashed line), or iNOS (L-NMMA, 1 mM; solid line). Proliferation in the absence of MSC is also shown (ConA–LNC, black histograms). MSC addition resulted in a complete or partial inhibition of ConA-induced proliferation at 1:10 and 1:100 MSC:LNC ratios, respectively. The proliferative response was fully restored by addition of L-NMMA while 1-MT or IMC had no effect. **(B)** In the same experimental set-up as shown in **(A)**, inhibitors were added to ConA-stimulated LNC cultures in the absence or presence (MSC:LNC ratio 1:10) of MSC. The percentage of CFSE^{lo} cells was used as a measure of cells that had undergone one or several cell divisions. The gate shown in **(A)** marks the distinction between CFSE^{lo} and non-dividing CFSE^{hi} cells. Data are representative of three independent experiments and are shown as mean and SD of triplicates.

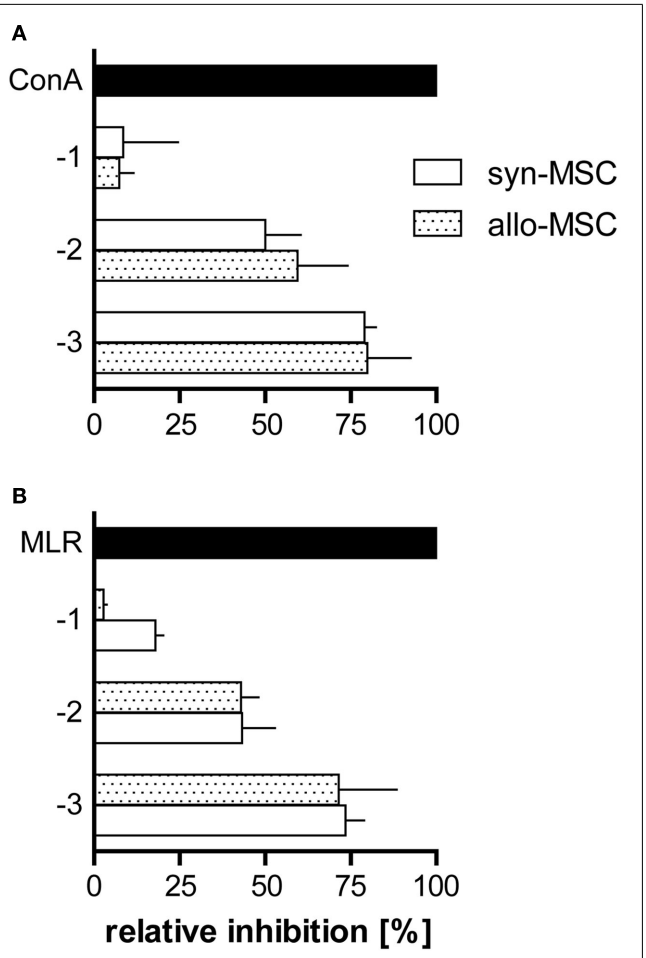

FIGURE 3 | Syngeneic and allogeneic MSC block T cell proliferation in MLR and ConA-stimulated cultures with similar potency. PVG.7B and PVG.1U MSC were added at the start of LNC cultures [values on *y*-axis signify the common logarithm (log) of MSC:LNC cell ratios, i.e., 1:10 (−1), 1:100 (−2), 1:1000 (−3)] and proliferation was assessed by radio-labeled thymidine incorporation for the last 20 h of co-incubation *in vitro*. Inhibition was calculated relative to the positive control (normal proliferation in the absence of MSC; black bars) as described in Section "Materials and Methods." **(A)** PVG.7B LNC responder cells were cultured in the presence of syngeneic (PVG.7B; white bars) or allogeneic (PVG.1U; dotted bars) MSC and ConA for 3 days. **(B)** PVG.1U LNC responder cells were cultured in the presence of irradiated PVG.R23 stimulator cells and syngeneic (PVG.1U; white bars) or allogeneic (PVG.7B; dotted bars) MSC for 4 days. Proliferation was efficiently suppressed by both syngeneic and allogeneic MSC in a cell dose-dependent manner. Data from representative experiments are shown as mean and SD of quadruplicates **(A)** or triplicates **(B)**.

Appendix). LPS synergized with IFNγ in inducing iNOS expression in MSC, albeit with variable potency, while simultaneous stimulation with poly-I:C and IFNγ had no effect (**Figure 5A**; **Figure A1** in Appendix).

Supernatants from stimulated LNC cultures (allogeneic MLR or ConA culture) also led to a potent induction of iNOS (**Figure 5B** and data not shown) in MSC. The concentrations of NO in the culture medium were concordant with the observed protein expression levels. Induction of iNOS and NO production was inhibited by addition of anti-IFNγ antibody and, more potently, anti-TNFα antibody either alone or in combination (**Figure 5B**). These data indicate that the inflammatory cytokine TNFα has a key role in priming MSC for their immunosuppressive function, and that IFNγ potentiates this effect.

MSC-MEDIATED INHIBITION OF CYTOKINE SECRETION BY ACTIVATED T CELLS IS NOT DEPENDENT ON iNOS ACTIVITY BUT ON COX-MEDIATED PRODUCTION OF PROSTAGLANDIN

Next, we tested the influence of MSC on the cytokine secretion patterns of ConA-stimulated lymphocytes. Although iNOS was important for MSC-mediated inhibition of T cell proliferation, this factor could not explain the inhibitory effect on IFNγ production as evaluated by intracellular flow cytometry following addition of L-NMMA to the LNC–MSC co-cultures

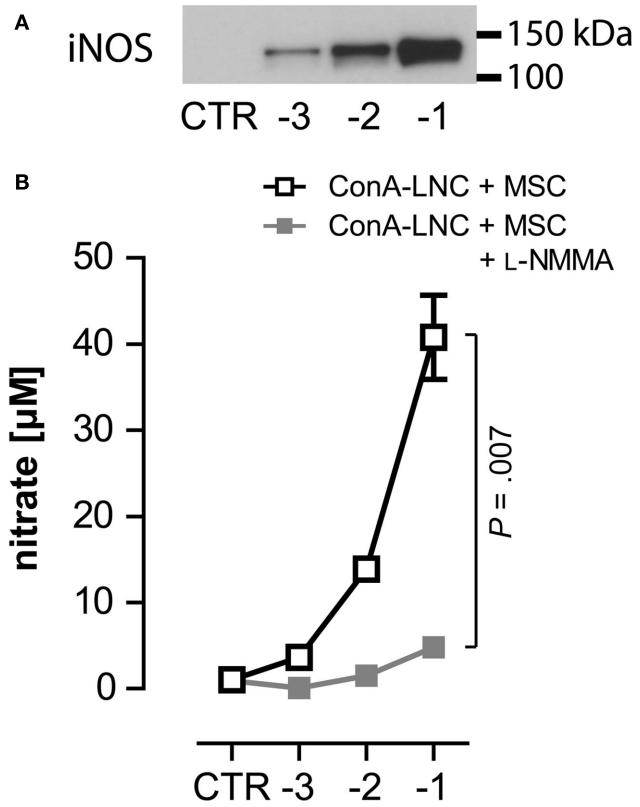

FIGURE 4 | MSC express iNOS and produce NO in response to lymphocyte stimulation. (A) 1×10^5 ConA-stimulated PVG LNC were cultured in the absence (CTR) or presence of syngeneic PVG.7B MSC [log (MSC:LNC)] and expression of iNOS was analyzed after 24 h. iNOS was detected in co-cultures of ConA–LNC with MSC and correlated with the numbers of MSC present. **(B)** In the same set-up as shown in **(A)** the amount of nitrate (shown as the mean and the SD of triplicates) as a proxy for NO concentration was determined in the absence (□) or presence (■) of L-NMMA. Addition of L-NMMA abolished NO levels produced in MSC:LNC co-cultures. NO was not detected in the absence of MSC (CTR). Data are representative of three independent experiments.

(**Figures 6A,B**). We further measured cytokine profiles by multiplex analysis and found that MSC constitutively secreted IL6 and vascular endothelial growth factor (VEGF) but not other cytokines analyzed (data not shown). The addition of MSC resulted in an accumulation of IL6 and VEGF also in ConA-stimulated LNC–MSC co-cultures (**Figure 6C**) as reported previously (Djouad et al., 2007). Addition of neutralizing concentrations ($2\,\mu g\,mL^{-1}$) of anti-rat IL6 antibody to the co-culture failed to reverse inhibition (data not shown), in contrast to previous studies of human and mouse MSC (Di Nicola et al., 2002; Djouad et al., 2007; Najar et al., 2009). LNC secreted significant amounts of the cytokines IL18, TNFα, and in particular IFNγ in response to ConA, which were markedly reduced when MSC were present. The inhibitory effect was proportionate to the numbers of MSC added (**Figure 6C** and data not shown). Addition of recombinant rat IFNγ ($1000\,U\,mL^{-1}$) to the co-culture failed to reverse the suppressive effect (data not shown). Expression levels of IL4 and IL10, cytokines which are typically considered as anti-inflammatory, were not increased in co-culture supernatants (data not shown).

Addition of L-NMMA had no effect on MSC-mediated modulation of cytokine secretion in co-culture experiments, and the same was true for the IDO antagonist 1-MT. By contrast, addition of the COX inhibitor indomethacin resulted in a striking reversal of inhibition of IFNγ and TNFα secretion at a MSC:LNC ratio of 1:100, but not at the highest ratio of 1:10 (**Figure 6C**). This result suggested that MSC can inhibit T cell mediated secretion of inflammatory cytokines by COX-dependent synthesis of PGE_2.

INHIBITION OF PROLIFERATION BUT NOT CYTOKINE PRODUCTION IS DEPENDENT ON CO-LOCALIZATION OF MSC AND T CELLS

The immunosuppressive effect of hMSC depends on soluble factors such as IDO, PGE_2, hepatocyte growth factor and transforming growth factor β1 (Di Nicola et al., 2002; Meisel et al., 2004; Aggarwal and Pittenger, 2005) and does not require cell contact (Hoogduijn et al., 2010) although there have been reports that co-localization of MSC with lymphocytes augmented inhibition (Krampera et al., 2003). mMSC require proximity for the effective inhibition of T cells by short-range activity of NO (Ren et al., 2008). Therefore, we examined whether inhibition of T cell effector function by MSC in the rat depended on cell-to-cell contact or if other soluble factors might be important. Physical separation of MSC from LNC using transwell membrane inserts restored ConA-stimulated proliferation which was inhibited in co-cultures where LNC and MSC were in contact (**Figure 7A**), indicating that suppression of proliferation required close proximity of MSC and target cells. In marked contrast, the cytokine secretion profiles in these cultures were altered irrespective of cell-to-cell contact (**Figure 7B**). IL6 and VEGF were increased to similar levels when MSC and LNC were either co-localized or separated (data not shown). Inflammatory cytokines were significantly reduced in the presence of MSC in either experimental set-up (**Figure 7B**). These latter data showed that inhibition of cytokine expression required a soluble factor, likely PGE_2, and provided further evidence that this property of rMSC is not dependent on close cellular contact.

T_{REG} CELL NUMBERS ARE REDUCED IN STIMULATED LYMPHOCYTE CO-CULTURES WITH MSC

We also analyzed the effect of MSC on the $CD4^+CD25^{hi}FoxP3^+$ T_{reg} cell population using mitogen-induced lymphocyte cultures. The proportion of T_{reg} cells was significantly diminished in the $CD4^+$ T cell population by addition of MSC at the start of the culture (**Figure 8**). This finding is in contrast to a number of previous studies which have demonstrated the induction of T_{reg} cells by MSC in the human system (Aggarwal and Pittenger, 2005; Di Ianni et al., 2008; English et al., 2009; Ghannam et al., 2010; Mougiakakos et al., 2011). It should be noted that the functional potential of these T_{reg}-like cells has not been tested in the present study.

DISCUSSION

Herein we show that rat BM-derived MSC up-regulate iNOS in response to TNFα and IFNγ secreted by activated lymphocytes and produce NO, which exerts a potent inhibitory effect on the proliferative T cell response to mitogen or allogeneic stimuli *in vitro*.

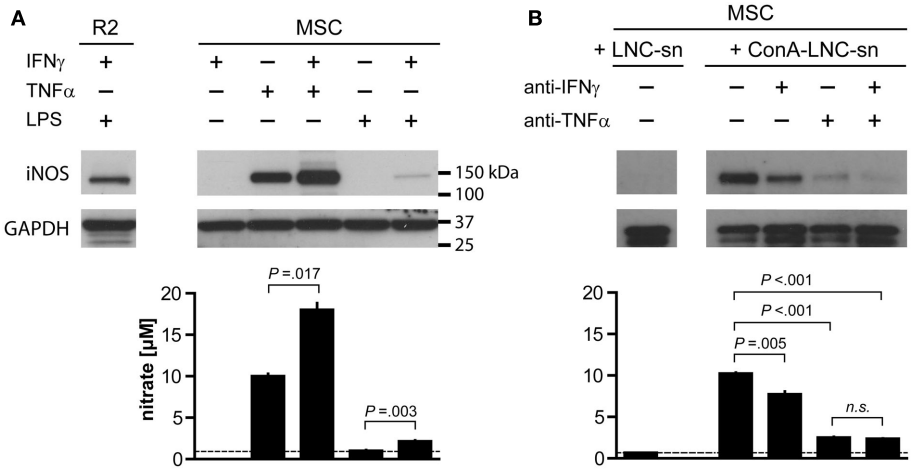

FIGURE 5 | Tumor necrosis factor α and IFNγ synergistically induce iNOS expression and NO production by MSC. 1×10^4 MSC (PVG.7B) were stimulated for 24 h with combinations of IFNγ, TNFα, and a TLR agonist **(A)** or by medium supernatants from ConA-activated LNC (PVG.7B or PVG.1U) cultures **(B)**. Pooled triplicates and quadruplicates, respectively, were analyzed for iNOS expression by Western blot with GAPDH assayed as a loading control. Nitrate concentrations were determined in the culture supernatants of the same wells (bottom panels). **(A)** MSC produced iNOS (130 kDa) in response to TNFα (25 ng mL^{-1}) alone or together with IFNγ (100 U mL^{-1}) or by a combination of IFNγ and LPS (100 ng mL^{-1}). Nitrate concentration levels correlated with the observed levels of iNOS protein expression. The dotted line indicates baseline NO production without cytokines added. The R2 macrophage cell line was used as positive control for iNOS expression. **(B)** Supernatants from ConA-stimulated (ConA–LNC-sn) and unstimulated LNC cultures as a control (LNC-sn; baseline) were added to MSC cultures. Neutralizing antibodies (each 10 μg mL^{-1} final concentration) against either IFNγ or TNFα or both (indicated) were added at the start of culture and incubated for 24 h. iNOS was induced by addition of supernatant from stimulated LNC cultures and was blocked by IFNγ-specific antibody and, more potently, by TNFα-specific antibody alone or in combination. Nitrate concentrations correlated with the observed iNOS expression levels. Data are representative of four independent experiments. Nitrate concentration data are shown as the mean and the SD of triplicates or quadruplicates.

The present study supports an important but not exclusive role of iNOS in the immunosuppressive function of MSC in rodents (Oh et al., 2007; Sato et al., 2007; Ren et al., 2008). COX-dependent PGE_2 is apparently also involved, in the inhibition of IFNγ and TNFα secretion by activated T cells.

To our knowledge, the only study that has investigated the immunosuppressive function of rMSC via iNOS to date (Chabannes et al., 2007) showed an effect in combination with heme oxygenase-1, but not a critical role for iNOS *per se*. Furthermore, that study showed that stimulation with recombinant IFNγ alone resulted in detectable expression of iNOS, which is in contrast to our findings showing that TNFα is sufficient and IFNγ is not required. This difference could be related to variations in cell isolation protocols, culture conditions, or between different rat strains. The MSC line generated from LEW.1A BM by Chabannes et al. (2007) were applied before the fourth passage and a minority of myeloid cells present in this population (3.2% of the cells expressed CD45) could account for the reported detection of iNOS expression in response to IFNγ.

Tumor necrosis factor α, a potent mediator of immune stimulation, induced iNOS expression in rMSC *in vitro* without a requirement for auxiliary signals, and neutralizing the TNFα signal in the supernatant from stimulated lymphocyte cultures abolished it, underlining the importance of this cytokine in MSC modulation of T cell activation. The pro-inflammatory cytokine IFNγ has also been ascribed an important role in the inhibition of T cell responses mediated by MSC (Krampera et al., 2006; Ryan et al., 2007). IFNγ levels were markedly reduced after addition of MSC to LNC cultures and attempts to restore T cell proliferation by addition of exogenous IFNγ were unsuccessful. Although IFNγ did not by itself induce iNOS expression in MSC it did show a synergistic effect in combination with TNFα or certain TLR ligands (LPS, but not poly-I:C) in support of studies performed in the mouse (Oh et al., 2007; Ren et al., 2008, 2009). This could explain why blocking the IFNγ signal by addition of anti-IFNγ antibody resulted in a significant reduction of iNOS expression in co-culture experiments as others also have shown (Oh et al., 2007; Ren et al., 2008).

Our data are in agreement with reports that have argued for "licensing" of MSC to acquire suppressor functionality (Waterman et al., 2010; Dazzi and Krampera, 2011). Collectively, our results support a model for the regulation of suppressor functions by rat BM stromal cells where MSC are primed by inflammatory signals, e.g., immunostimulatory cytokines, primarily TNFα, to induce iNOS expression leading to the accumulation of NO and in turn the inhibition of proliferation of activated T cells in the immediate proximity. In addition, MSC effectively shut off the generation of inflammatory cytokines by activated T cells (Ren et al., 2008) via a soluble factor dependent on COX activity, likely PGE_2.

The mechanisms employed to achieve immunosuppression are not identical in different species. In mMSC (Ren et al., 2008), as in rMSC, iNOS expression seems critically important for the inhibition of T cell proliferation. Our data imply that the cytokine requirement of MSC to activate this inhibitory pathway is not identical in rats (TNFα alone is sufficient) and mice (IFNγ and

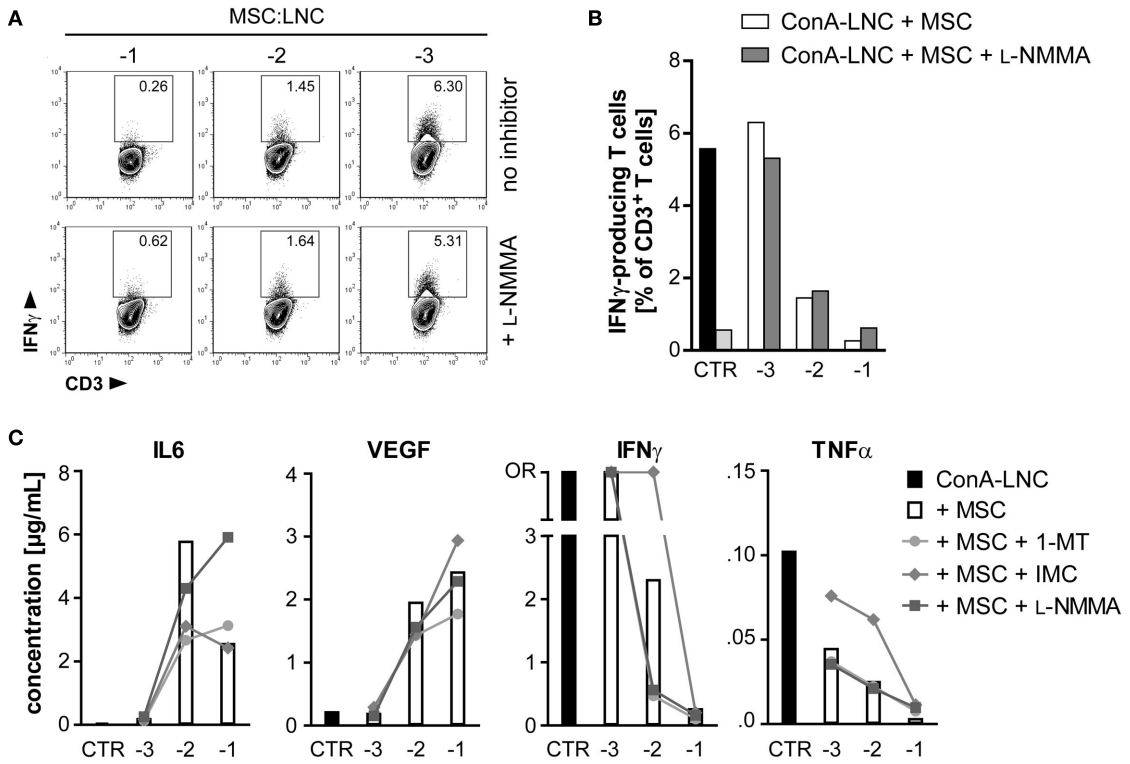

FIGURE 6 | MSC inhibition of IFNγ and TNFα secretion is not dependent on iNOS activity, but on COX-dependent production of prostaglandin. **(A,B)** MSC (PVG.7B) were added at the indicated dilutions [log (MSC:LNC)] at the start of ConA–LNC (PVG.7B) cultures. Cells from triplicate wells were pooled after 24 h and the number of IFNγ-positive T cells ($IFN\gamma^+CD3^+$ lymphocyte gate, values indicate relative frequencies) determined by flow cytometry. Inhibition of iNOS by L-NMMA (bottom row and shaded bars) did not influence the MSC-mediated suppression of IFNγ expression (top row and unshaded bars). Controls in **(B)** show IFNγ production by T cells alone in the presence (black) or absence (light gray) of ConA. **(C)** MSC (PVG.1U) were added at the indicated cell ratios [log] at the start of ConA–LNC (PVG.7B) cultures in the absence (white bars) or presence of IDO (1-MT, 1 mM; circles), PGE_2 (IMC, 5 μg mL^{-1}; diamonds), or iNOS (L-NMMA, 1 mM; squares) inhibitors. Cytokine concentrations in the culture supernatants were determined after 3 days of incubation. As a control (CTR) was used supernatant from ConA–LNC culture without MSC. Levels of IL6 and VEGF were significantly increased while IFNγ and TNFα were significantly reduced in a MSC dose-dependent manner. Addition of L-NMMA or 1-MT had no effect on the cytokine expression profiles. Addition of IMC reversed in part the suppression of IFNγ and TNFα by MSC. Data are representative of two independent experiments and are shown as the average of duplicates; OR, data point out of detection range.

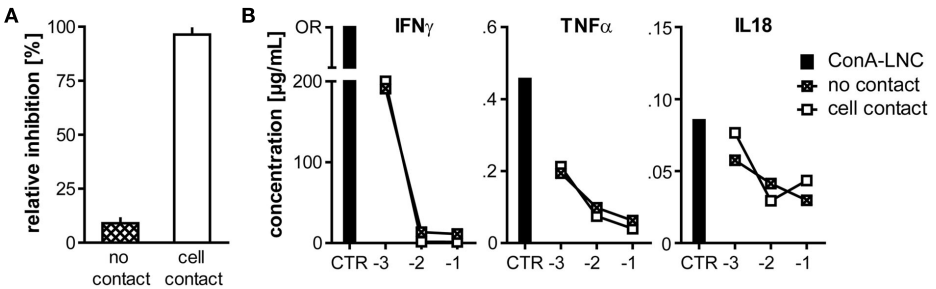

FIGURE 7 | MSC-mediated inhibition of proliferation, but not cytokine secretion requires close cellular contact. **(A)** 5×10^4 MSC (PVG.7B) were cultured together with ConA (3 μg mL^{-1})-stimulated LNC (5×10^5, i.e., a cell ratio of 1:10) either separate from LNC (PVG.7B) in transwell membrane inserts (no contact) or co-localized (cell contact) for 3 days. Relative inhibition of proliferation measured as ^{3}H-TTP incorporation is shown as the mean and the SD of triplicates. Cell proliferation was inhibited by MSC only when in proximity to target cells ($P = 0.001$). **(B)** In the same set-up as shown in **(A)** increasing numbers of MSC [log] were co-cultured with ConA–LNC in transwell plates either separately (crossed squares) or co-localized (unfilled squares). Cytokine concentrations were assayed in the culture supernatants after 3 days (shown as the average of duplicates). IL18, IFNγ, and TNFα were significantly reduced in a MSC dose-dependent, contact-independent manner. OR, data point out of range.

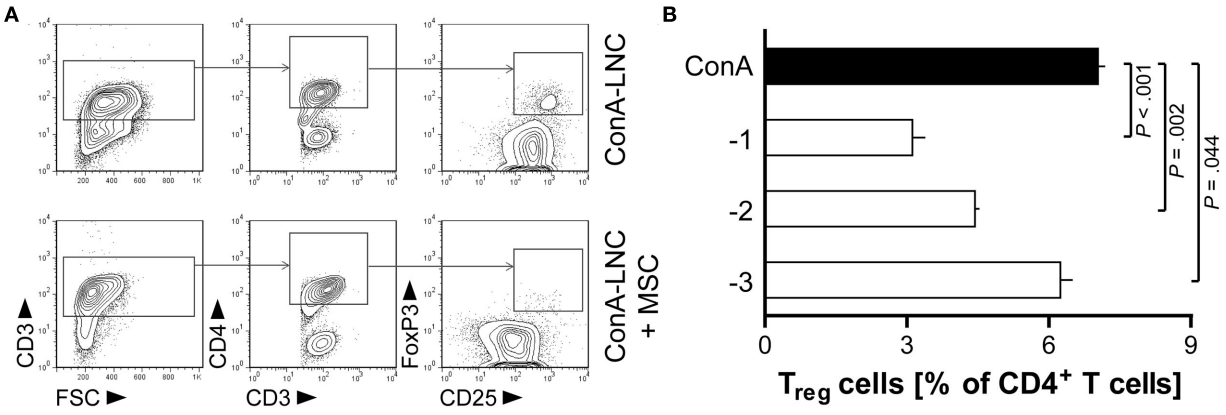

FIGURE 8 | MSC diminish the proportion of CD4$^+$CD25^{hi}FoxP3$^+$ T$_{reg}$-like cells in ConA-stimulated lymphocyte cultures. MSC (PVG.7B) were added at the start of ConA-stimulated LNC cultures *in vitro*. The frequencies of T$_{reg}$ cells were analyzed after 3 days by flow cytometry. Representative plots depict in **(A)** the gating of lymphocytes (lymphocyte gate not shown) for CD3$^+$CD4$^+$CD25^{hi}FoxP3$^+$cells in the absence or presence of MSC (MSC:LNC ratio of 1:10). **(B)** The relative fraction of T$_{reg}$ cells among CD4$^+$ T cells (means and SD of triplicates) was reduced compared to control (black bar) in a MSC dose-dependent manner. Data are representative of three independent experiments.

any of the cytokines TNFα, IL1α, or IL1β are required). Ren et al. (2009) demonstrated that other inhibitory mechanisms, namely the secretion of IDO, but not iNOS, were employed by monkey and human BM-derived MSC in this context.

MSC-mediated inhibition of secretion of IFNγ and TNFα by activated T cells was dissociated from iNOS activity and NO, as demonstrated by transwell cultures and biochemical inhibition of iNOS. Our data are in contrast with a study of mMSC, which suggested that iNOS is involved in inhibiting IFNγ production by T cells stimulated with anti-CD3/CD28 beads in the presence of IL12 and anti-IL4 antibody (Oh et al., 2007). Our study identifies PGE_2 as a candidate soluble factor responsible for the dampening of inflammatory cytokine production by activated lymphocytes, in agreement with a previous study of hMSC (Aggarwal and Pittenger, 2005). Taken together, it can be concluded that rMSC utilize different pathways to regulate proliferation and cytokine production by T cells. The species-specific mechanisms regulating the suppression of immune cell activation and effector functions through BM stromal progenitor cells should be investigated further, because this research might have important implications for the use of MSC or related cell types in the clinical treatment of GVHD, autoimmune diseases or other syndromes of undesired T cell activity.

In a separate series of experiments, we have tested the therapeutic potential of the MSC lines presented here in a model of experimental GVHD induced by donor lymphocyte infusions after MHC-mismatched allogeneic stem cell transplantation (Zinöcker et al., 2011a). Despite their marked inhibitory potential *in vitro*, repeated systemic injections of MSC did not improve GVHD in these experiments even after prestimulaton with IFNγ and TNFα to boost the iNOS pathway in MSC (Zinöcker et al., manuscript submitted). This suggests that their immunosuppressive efficacy is limited with respect to GVHD-related morbidity and mortality.

We show here that rMSC suppress proliferation *in vitro* only when in close proximity to stimulated T cells through short-range activity of NO. The route of administration may determine the effectiveness of treatment if co-localization of MSC is required for the efficient suppression of alloreactive immune cells *in vivo*. We have previously observed by *in vivo* imaging that MSC which expressed enhanced green fluorescence protein transgenically accumulated in the lungs shortly (within minutes) after intravenous injection but did not detect these cells at that site or in other organs after several days *in vivo* and *post mortem* (unpublished observations). Failure to migrate to sites of allopriming and alloreactivity in GVHD is a plausible explanation for the observed lack of efficiency of MSC therapy.

Besides NO synthases, arginase 1 is an important enzyme that regulates L-arginine metabolism and production of NO and has the ability to inhibit T cell proliferation (Grohmann and Bronte, 2010). We did not address a potential role of arginase 1 in the suppression of activated T cells by rMSC in this study. If these cells make use of other, potentially redundant molecular pathways to inhibit T cells or other immune cell types, the strength of NO-dependent immunosuppression observed in this study would suggest that such alternative mechanisms are of minor importance regarding MSC-mediated inhibition of T cell proliferation in the rat species.

MSC have been shown to induce different subtypes of T$_{reg}$ cells (Aggarwal and Pittenger, 2005; Di Ianni et al., 2008; English et al., 2009; Ghannam et al., 2010; Mougiakakos et al., 2011) as potential immunomodulatory mechanisms operating *in vivo* (Hoogduijn et al., 2010). Our finding that CD4$^+$CD25^{hi}FOXP3$^+$ T$_{reg}$ cells were reduced after ConA-stimulation of T cells in the presence of rMSC was in marked contrast to the studies with hMSC and makes it less likely that T$_{reg}$ cells are involved in the observed inhibition of proliferation or cytokine secretion of activated rat T cells *in vitro*. Further experiments could clarify the functional characteristics of this putative T$_{reg}$ cell type.

Recently, Turley and co-workers presented findings which showed that iNOS represents a central pathway employed by both fibroblastic reticular and lymphoid endothelial cells to regulate

T cell activation in the lymph nodes of mice (Lukacs-Kornek et al., 2011). The observed inhibitory effects depended on IFNγ, TNF and direct cell contact (Lukacs-Kornek et al., 2011) and correlate well with our observations for rMSC, which displayed very similar cytokine requirements and inhibitory effects. We therefore speculate that the enzymatic conversion of NO is a common feature of stromal cells in rodents (Jones et al., 2007), which, in combination with PGE_2, control T cell expansion at sites of priming of the adaptive immune system (Lukacs-Kornek et al., 2011) and subsequent inflammation (Nombela-Arrieta et al., 2011).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX

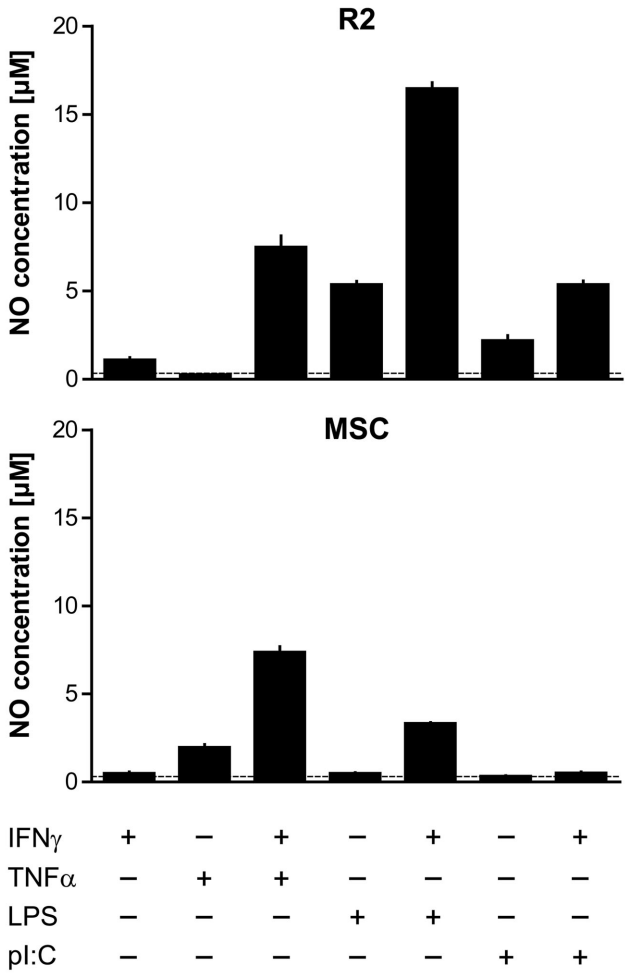

FIGURE A1 | Comparison of cytokine and TLR ligand requirements for NO production by macrophages and MSC. R2 macrophages (top panel) and MSC (PVG.7B; bottom panel) were stimulated for 24 h with IFNγ (100 U mL^{-1}), TNFα (25 ng mL^{-1}), LPS (1 μg mL^{-1}), and poly-I:C (1 μg mL^{-1}), either alone or in combination with IFNγ as indicated. R2 macrophages produced significant levels of NO in response to the TLR agonists LPS and poly-I:C as well as IFNγ. In MSC, NO was only generated in response to TNFα but not to the other stimulatory agents. Addition of IFNγ had a synergistic effect in combination with TNFα or LPS in both macrophages and MSC. The dotted lines indicate baseline NO production of the respective cell type without cytokines added.

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Immunomodulatory effect of mesenchymal stem cells on B cells

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The research on T cell immunosuppression therapies has attracted most of the attention in clinical transplantation. However, B cells and humoral immune responses are increasingly acknowledged as crucial mediators of chronic allograft rejection. Indeed, humoral immune responses can lead to renal allograft rejection even in patients whose cell-mediated immune responses are well controlled. On the other hand, newly studied B cell subsets with regulatory effects have been linked to tolerance achievement in transplantation. Better understanding of the regulatory and effector B cell responses may therefore lead to new therapeutic approaches. Mesenchymal stem cells (MSC) are arising as a potent therapeutic tool in transplantation due to their regenerative and immunomodulatory properties. The research on MSCs has mainly focused on their effects on T cells and although data regarding the modulatory effects of MSCs on alloantigen-specific humoral response in humans is scarce, it has been demonstrated that MSCs significantly affect B cell functioning. In the present review we will analyze and discuss the results in this field.

Keywords: MSC, B cells, humoral rejection, chronic allograft rejection, immunomodulation

INTRODUCTION

B cells are a major cell type involved in adaptive immune responses, specialized in antigen presentation and antibody production. The balance between the different B cell subsets has been identified as an important factor for graft outcome. On one hand, effector B cells generate humoral rejection and pre-formed donor-specific antibodies (DSA) against human leukocyte antigen (HLA)-I or HLA-II that have been correlated to worst graft outcome. On the other hand, pro-tolerogenic B cell subsets have been identified. An increase in immature transitional and naïve B cells has been related to tolerance (Liu et al., 2007) and increased B cell numbers and a differential expression of B cell-related genes were observed in the peripheral blood of a small cohort of tolerant kidney and liver transplant patients compared to stable patients under immunosuppression or to healthy controls (Newell et al., 2010; Sagoo et al., 2010).

Mesenchymal stem cells are multipotent stromal cells localized in virtually every tissue. They are characterized by their adherence to plastic, the expression of surface markers as CD73, CD90, and CD105 among others and the lack of expression of typical hematopoietic markers as CD45 and CD11b (Roemeling-van Rhijn et al., 2012). They also show differentiation potential into different cell lineages under controlled culture conditions. MSCs have been considered as naturally immunoprivileged cells due to low expression of HLA and co-stimulatory molecules in unstimulated conditions and although it is now well-known that under inflammatory stimulation they can express both HLA-I and HLA-II it is also known that under this condition they exert more potent immunosuppressive actions (Crop et al., 2010).

The effect of MSCs on effector and regulatory T cells has been widely studied (Duffy et al., 2011a) and there is also evidence for a suppressive role of MSCs on natural killer (NK) cells (Spaggiari et al., 2008), inhibition of dendritic cells (DCs) maturation (Spaggiari et al., 2009), and alternative activation of macrophages leading to an anti-inflammatory phenotype (Francois et al., 2012). The interaction between MSCs and B cells is gaining interest but data is still scarce and controversial. Here we review the available data on the immunomodulatory actions of MSCs on B cells.

B CELLS IN TRANSPLANTATION

T cell-mediated rejection is together with antibody-mediated rejection the main cause of graft loss. Although research on T cell immunosuppressive therapies has efficiently improved the incidence of acute cellular rejection, long-term allograft survival remains challenged by chronic rejection. Activated B cells have been found to play a significant role on long-term allograft function. Their ability to present antigen to T cells via the indirect pathway and the generation of DSAs are emerging as the major mediators of allograft rejection. Pre-existing DSAs in the allograft recipient mediate hyperacute and acute-antibody-mediated rejection while the presence of *de novo* DSAs (specific for HLA and non-HLA) in recipients compromises long-term allograft survival (Redfield et al., 2011). Furthermore, it has been observed that CD8 and CD4 T cell memory is impaired when the antigen presenting function of B cells is absent (Ng et al., 2010). This finding would support the idea of a beneficial effect of B cell depletion at the time of transplantation to impair T cell mediated alloresponses.

However, there is increasing evidence for a tolerogenic role of specific B cell subsets. Naïve B cells have been shown to stimulate the development of regulatory T cells by antigen presentation to naïve T cells (Reichardt et al., 2007). And more recently, increased expression levels of B cell genes were found

in peripheral blood of kidney transplant patients that spontaneously became tolerant (Newell et al., 2010; Sagoo et al., 2010). The three main genes with predictive value for discerning tolerant from non-tolerant (*IGKV4-1*, *IGLL1*, and *IGKV1D-13*) are expressed by transitional B cells, which are considered to be tolerogenic. Moreover, there is evidence of a subset of B cells with anti-inflammatory properties and the ability to secrete IL10, which down-regulation is known to be involved in the development of autoimmune diseases (Mauri and Bosma, 2011) and in solid organ transplantation there is preliminary evidence for their presence in immunosuppressive free kidney transplant patients (Le Texier et al., 2011).

The use of B cell directed monoclonal antibodies (anti-CD20, Rituximab), antibody depleting strategies (plasmapheresis), plasma cell depleting agents (anti-proteasome, Bortezomib), or complement-inhibitor agents (Eculizumab) have been reported to be efficient in promoting graft survival (Rocha et al., 2003; Tyden et al., 2009; Walsh et al., 2012). It is however still controversial which approach is the best to avoid humoral rejection without compromising regulatory mechanisms.

IMMUNOMODULATORY EFFECT OF MSCs IN TRANSPLANTATION

T cells, as key initiators and mediators of transplant rejection, have been the main target to prove the immunomodulatory potential of MSCs. Multiple studies have demonstrated that MSCs inhibit effector T cell proliferation and cytokine release through mechanisms that are cell-contact-dependent (PD-L1, Augello et al., 2005; B7-H4, Xue et al., 2010; ICAM1; VCAM1, Ren et al., 2010) and contact-independent (IDO, Ge et al., 2010; PGE_2, Najar et al., 2010; Duffy et al., 2011b; HLA-G, Selmani et al., 2009; TGFβ, Liu et al., 2012; galectins, Sioud, 2011). *In vivo* models have shown that MSCs have an indirect effect on T cell activation by inhibition of maturation of DCs. Injected MSCs prevent DCs maturation (Spaggiari et al., 2009) and their migration to lymph nodes by down-regulating CCR7 expression, thus inhibiting T cell priming (Chiesa et al., 2011). MSC-exposed DCs have also the ability to promote Tregs induction (Ge et al., 2009). MSCs posses also the ability to induce Tregs directly via the production of TGFβ, PGE_2 together with cell-contact as key factors. *In vivo*, $FoxP3^+$ Treg generation has been associated with IDO expression by MSC (English et al., 2009). This factor is produced by MSCs under IFNγ conditioning (Croitoru-Lamoury et al., 2011) and is essential to achieve allograft tolerance in an experimental kidney transplantation model (Ge et al., 2010). It appears that MSCs under inflammatory conditions act as super-regulators on T cells inhibiting the effector responses and enhancing the regulation inducing Tregs.

Of note, these actions are not only relegated to the experimental and *in vitro* setting as the applicability of injected MSCs as induction therapy in human kidney transplantation has been recently proved. Injection of autologous MSCs at the moment of transplantation and 2 weeks post-transplantation resulted in lower incidence of acute rejection, decreased risk of opportunistic infection and better estimated renal function at 1 year compared to anti-IL2 receptor antibody (Basiliximab) induction therapy (Tan et al., 2012).

EFFECT OF MSCs ON B CELLS *IN VITRO*

To the moment, the few published papers studying the effect of MSCs on B cells proliferation, differentiation, and function show disparity in their approaches and results. The different results among the groups might be explained by the different starting B cell population (purity and isolation method) and the stimuli used to trigger B cell differentiation and proliferation. MSC: B cell ratio is also an important point, as the most effective ratios used are very high and it is hardly observed a dose dilution effect, contrarily to what happens with the immunosuppressive effect of MSCs on T cells (Hoogduijn et al., 2008).

CELL SOURCE AND ISOLATION METHOD

If we refer to *in vitro* data (**Table 1**), the main starting difference of those studies is the B cell isolation method. On one hand, some authors decided for a more "physiological" model by using a B cell enriched system in which we can still find T helper cells (in different proportion depending on the depleting technique and the source used) and other mononuclear cells found in peripheral blood or spleen (Rasmusson et al., 2007; Comoli et al., 2008). On the other hand, some authors use CD19 positive selection to start with a pure B cell population (Corcione et al., 2006; Tabera et al., 2008; Traggiai et al., 2008), or a CD43 depleted population to have an isolated "untouched" non-activated B cell population to start with (Asari et al., 2009; Schena et al., 2010). The purity of the starting population and the stimuli used to trigger B cell proliferation and differentiation are key factors in determining the effect of MSCs on B cells.

Of note, the source of MCS used in the various studies is bone marrow and the use of allogeneic or autologous MSCs does not seem to affect the interaction between MSC and B cells (Comoli et al., 2008).

The first key study to understand the role of MSC on B cells, on a non-purified starting population, was performed by Comoli et al. (2008). The exposure of enriched B cell populations to irradiated third party PBMCs led to an increase in immunoglobulin (Ig) production that was abrogated by the addition of MSCs. Interestingly the effect exerted by MSCs was abolished by the addition of anti-CD40 and IL10 indicating that MSCs suppression of Ig production was produced by T help suppression rather than by a direct effect on B cells. This is in tune with Rasmusson et al. (2007) who showed that under strong stimulation of mononuclear cell fraction (non-purified B cells), MSCs inhibited the Ig secretion. However, the same cells without or under mild polyclonal stimulation increased their IgG production in the presence of MSCs (Rasmusson et al., 2007).

However, when the effect of MSCs is studied on purified B cells (or B cell subsets) the effect is diverse depending on the stimuli used to induce proliferation and/or differentiation.

CELL STIMULATION

The activation of naïve B cells requires three signals: B cell receptor (BCR) activation (via anti-Ig), T cell co-stimulatory help (via CD40/CD40L), and appropriate cytokines or toll-like receptor (TLR) activation (microbial products, CpG, dsRNA), while memory B cells can be activated in the absence of BCR stimulation and triggered via stimulation of TLR or bystander T cell help only

Table 1 | Effect of MSCs on B cells *in vitro*.

Species and model	B cell isolation	B: MSC ratio	B cell stimuli	Effect of MSCs	Reference
MICE					
Mice C57Bl/10 and Balb/C	Spleen B cell isolation kit	1:1	PWM	Inhibition of B cell proliferation. PD-1/PD-1L/PD-2L pathway is involved.	Augello et al. (2005)
Mouse B6 and CCR2−	Spleen sorted CD19−CD138$^+$ plasma cells	1:1	rOVA	Inhibition of Ig production by a cleaved form of CCL2 secreted by MSCs.	Rafei et al. (2008)
Mouse C57Bl/g	Spleen CD43 depletion	2:1 5:1 10:1	LPS T cell dependent/ independent *in vivo*	Inhibition of Plasma cells (Blimp-1+) induced by LPS. Suppression of B cell proliferation but do not induce plasma cell apoptosis. B cell differentiation inhibition is cell-contact-independent (also not CCL2, IL10, TGFβ, or IDO).	Asari et al. (2009)
Mouse NZBxNZW F1	Spleen CD43 depletion and sorted marginal vs. follicular zone	1:1 3:1 9:1	CpG + CD40L + anti-IgM + IL2	Inhibition of proliferation and differentiation in the presence of IFNγ of BCR stimulated naive B cells. This effect is IDO independent and cell-contact-dependent and not related to apoptosis. Inhibition of phosphorylation of 3 main pathways downstream de BCR and PD-1/PD-L1 upstream the BCR.	Schena et al. (2010)
Mouse NZBxNZW F1	Spleen, BM, kidney CD138$^+$ plasma cells isolation	1:1 1:5	OVA	Coculture MSCs increase survival and function of plasma cells leading to increased IgG production.	Youd et al. (2010)
HUMANS					
Human healthy volunteer	PB T cell depleted + CD19$^+$ positive selection MACS	1:1 1:2	CpG + rCD40L + anti-Ig + IL2 + IL4 ± IL10	Inhibition of proliferation (not apoptosis) by arrest of cell cycle G0/G1. Mediated by soluble factors. Inhibition of IgG, IgA, IgM secretion. Inhibition of homig molecules CXCR4, CXCR5, CCR7, and chemotaxis to CXCL12, CXCL13.	Corcione et al. (2006)
Human healthy volunteer	Spleen or PB enriched B cell population MACS	10:1	LPS/CMV/VZV	Increase IgG producing cells in coculture with MNCs or B cells. The effect on enriched B cells is cell-contact-dependent while is mediated by soluble factors in MNCs. Under strong stimulation MSC reduce Ig production, under low stimulation increases Ig production.	Rasmusson et al. (2007)
Human healthy volunteer and highly sensitized patients	PB partial depletion CD4 and full depletion CD8 MACS	4:1 20:1	MLC ± CD40 agonist + IL10	MSCs inhibit IgG, IgA, IgM production induced in MLC (different ratios and allogeneic or syngeneic MSCs have same effect). Sensitized patients allo-sera induce ADCC but supernatant of MLC + MSC do not induce ADCC. In the presence of agonist CD40 + IL10, MSCs have no effect on Ig reduction and in transwell the effect is not lost.	Comoli et al. (2008)
Human healthy volunteer	Buffy Coat CD19$^+$ and CD3$^+$ selection MACS	5:1 10:1	CpG + anti-Ig ± CD40L + IL4	Promotion of B cell proliferation and viability but under highly proliferative conditions, MSCs arrest B cell cycle in G0/G1. Inhibit Plasma cells induced by pDCs mediated by ERK 1/2 and p38 phosphorylation.	Tabera et al. (2008)

(Continued)

Table 1 | Continued

Species and model	B cell isolation	B: MSC ratio	B cell stimuli	Effect of MSCs	Reference
Human healthy and SLE	PB $CD19^+$ selection MACS + subsets sorting	1:1	CpG + IL2 ± CD40L + anti-Ig	Induction of survival and proliferation of transitional, naïve, IgM memory, and switch memory subsets with/out stimulation. Up-regulation of CD38 and IGM but naïve B cells do not increase IgA and IgG. Cell-contact-dependent effect. Enhancement of survival of SLE patient B cell subsets, increase CD38 expression and IgM and IgG secretion.	Traggiai et al. (2008)

Summary of the published works on the effect of MSCs on B cells in vitro. In all cases the source of MSCs is bone marrow. PWM, Pokeweed mitogen; OVA, ovalbumin; LPS, lipopolysaccharide; BM, bone marrow; PB, peripheral blood; MACS, magnetic cell sorting technology; CMV, cytomegalovirus; VZV, varicella zoster virus; MNCs, mononuclear cells; MLC, mixed lymphocyte culture.

(Lanzavecchia et al., 2006). MSC may have a role in modulating some of these B cell activating pathways.

Among the studies that isolated pure B cells and exposed them to different stimuli to analyze the effect of MSCs we find diverse results. Although MSC were shown to increase the viability of B cells (Tabera et al., 2008), they arrest them in G_0/G_1 (Corcione et al., 2006; Tabera et al., 2008) and inhibit their differentiation into plasma cells and subsequent Ig formation. This effect has been shown to be cell-contact-independent (Asari et al., 2009) or indirect through inhibition of pDCs induced B cell maturation (Tabera et al., 2008). Contrarily, some authors (Augello et al., 2005; Schena et al., 2010) found PD-1/PD-L1 interaction and the inhibition of pathways downstream the BCR (Schena et al., 2010) to be responsible for B cell inhibition by MSC. However, Schena et al. (2010) observed that pre-exposure of MSCs to IFNγ was mandatory for their suppressive effect on B cells, similar to their effect on T cells (Crop et al., 2010). These studies on isolated B cells were performed in the presence of stimuli targeting the three signals for B cell activation, suggesting a role of MSCs directly on B cells besides their effect on T helper cells (contrarily to what was observed in studies using mixed starting population).

In contrast to activated B cells, isolated naïve, transitional, and memory B cell subsets exposed to MSCs increased their survival and proliferation (Traggiai et al., 2008). MSCs synergize with TLR stimuli and IL2, with or without T cell help (CD40L or anti-CD40) and BCR mediated stimulation by inducing proliferation and differentiation into plasma cells. This effect was shown to be contact-dependent although some of the factors released by MSCs are important to modulate this effect.

In this setting, the effect of the stimuli on MSCs should be also taken into account. It has been proved that MSCs express TLRs (DelaRosa and Lombardo, 2010), and their activation promote mainly a different cytokine secretion. MSC stimulated with CpG (one of the main stimuli used in B cell activation that acts through TLR9) produce IL6. This cytokine stimulates B cell proliferation (Friederichs et al., 2001) and could be an explanation for the MSC induction of naïve B cell proliferation under TLR9 stimulation in the absence of BCR triggering (Traggiai et al., 2008).

All these studies give a hint on a potential dual effect of MSCs on B cells. While in the enriched system the effect of MSCs on B cells appear to be by-passed by their immunosuppressive action on T cells, in an activated pure B cell population MSCs efficiently arrest or increase the proliferation depending on the potency of the stimuli on B cells but also on MSCs. Both cell-contact-dependent and independent factors are involved.

EFFECT OF MSCs ON PLASMA CELLS

Mesenchymal stem cells inhibit plasma cell formation induced by allostimulation (Comoli et al., 2008), by LPS (Asari et al., 2009) or by plasmatic DCs (Tabera et al., 2008) and subsequent Ig production (Corcione et al., 2006; Rasmusson et al., 2007). The mechanisms of action described to be involved are cell-contact-independent (alternatively cleaved CCL2 Rafei et al., 2008) or dependent (PD-1/PD-L1 interaction, Schena et al., 2010).

However, we also find some disparity in the results obtained *in vitro*, as some authors observed and increased differentiation into plasma cells with increased Ig production (Traggiai et al., 2008) along with a better survival and function (Youd et al., 2010). This observation is reflected in *in vivo* systemic lupus erythomatosus (SLE) models treated with MSCs.

EFFECT OF MSCs ON B CELLS *IN VIVO*

Similar to the controversial *in vitro* effects of MSC on B cells, there are contradictory reports on the effects of MSC on B cells in animal models.

Different groups have approached the treatment of a SLE model with MSCs. A single injection of human BM-MSCs combined with cyclophosphamide (CTX) increased survival, decreased proteinuria, and reduced the levels of circulating anti-dsDNA IgG in a MRL/Lpr mice model (Zhou et al., 2008), and similar results were obtained in NZBxNZW F1 mice injected preventively with adipose tissue MSCs every 2 weeks for 54 weeks although this protective effect was lost when the animals were treated after the onset of the disease (Choi et al., 2012). This late treatment does not prevent from developing anti-dsDNA IgG or proteinuria, neither increases the survival of the treated animals but it decreases lymphocytic infiltration, glomerular proliferation, and immune complex deposition (Schena et al., 2010). Contrarily, the use of mouse allogeneic MSCs in this model, increases serum anti-dsDNA antibodies and the glomerular deposition of IgG, along with higher interstitial fibrosis and inflammation and protein casts in the kidney when compared to CTX treatment (Youd et al., 2010). Of note the

numbers of IgG^+ plasma cells in the bone marrow of MSC treated mice are also increased (**Table 2**).

In the transplantation setting, we (Franquesa et al., 2012) and others (Ge et al., 2009) have demonstrated that MSCs injection can significantly reduce levels of allospecific circulating antibodies and intragraft allospecific IgG deposits (Ge et al., 2009) leading to long-term graft acceptance.

INDUCTION OF B CELL RESPONSES BY ALLO-MSC

Despite the low immunogenicity that MSCs are supposed to exert (low HLA class I and negative for HLA class II in unstimulated conditions), there is evidence that MSC may be capable of inducing an adaptive immune response (Nauta et al., 2006; Sbano et al., 2008). Therefore it is still a matter of debate whether allogeneic MSCs exert a humoral response in the recipient.

In rats a single injection of allogeneic MSC (1×10^6 cells/animals) induced substantial alloantibody production (IgG1, IgG2) in contrast to syngeneic cells injection in an immunocompetent host (Schu et al., 2011). Also immunocompetent non-human primates (baboons) injected with two doses of allogeneic MSCs (5×10^6 cells/kg body weight) developed alloantibodies (Beggs et al., 2006). Contrarily, in a clinical study with 12 patients which were treated with MSCs ($0.8–2.0 \times 10^6$ cells/kg body weight) after hematopoietic stem cell (HSC) transplantation, none of them developed anti-MSC antibodies (Sundin et al., 2007). And our own experience with a single injection of third party MSCs (0.5×10^6/300 g body weight) in a rat kidney transplantation model is that the injected animals do not develop specific anti-MSCs antibodies while they do increase antibody levels against the third party when they are injected with the full fraction of bone marrow mononuclear cells (Franquesa et al., 2012).

Those studies reflect some disparity of humoral response directed against the injected MSCs that could be explained by the source of MSCs (allo- vs. syngeneic), the number of injected cells, the number of injections, the route of administration or concurrent immunosuppression used. More *in vivo* studies need to be done to develop safe long-term protocols for the clinical setting.

CONCLUSION

The role of B cells in transplantation is multifaceted due to the opposed roles of different B cell subsets in tolerance and rejection. This enlightens the need for more refined immunosuppressive regimens to treat humoral rejection without compromising the effect of the pro-tolerogenic B cell subsets, namely transitional and regulatory B cells.

Mesenchymal stem cells have proven immunomodulatory properties, suppressing inflammatory cell (effector T cells, DCs, inflammatory macrophages) functions, and differentiation and increasing or synergizing with regulatory cells such as Tregs.

Their effect on B cells has been scarcely studied and although the results obtained are contradictory so far, it seems clear there

Table 2 | Effect of MSCs on B cells *in vivo*.

Model	Species	MSC source	MSC dose	Effect of MSCs	Reference
SLE	Mouse female MRL/Lpr	BM human	1×10^6/mice	MSCs alone or combined with Cyclophosphamide (CTX) reduce serum creatinine levels and C3 deposition compared to CTX alone. CTX + MSC reduce circulating dsDNA antibodies.	Zhou et al. (2008)
Heart allograft	Mouse C57BL/6 BALB/c C3H	BM	1×10^6/mice	Inhibition of intragraft and circulating alloreactive antibody levels. In combination with rapamycin induce tolerance.	Ge et al. (2009)
SLE	Mouse C57Bl/g	BM conditioned medium	Conditioned medium	Suppression of antigen specific IgM and IgG1 secretion in immunized mice with T cell-dependent and -independent effect	Asari et al. (2009)
SLE	Mouse NZBxNZW F1	BM C57BL/6J mice	3 Injections 1.25×10^6	Injections of MSC in SLE mice has no effect on IgG dsDNA, proteinuria and survival, but improves glomerular proliferation, lymphocytic infiltration, and IgG immune complex deposition.	Schena et al. (2010)
SLE	Mouse NZBxNZW F1	BM Allogeneic Balb/C	1×10^6 Bi-weekly for 18 or 7 weeks	MSC enhance autoantibody production, pathology and proteinuria.	Youd et al. (2010)
SLE	Mouse NZBxNZW F1	AT human	28 Injections 5×10^5	Higher survival, improvement of histologic, and serologic abnormalities and immunologic function and decreased proteinuria. Anti-dsDNA antibodies and BUN decreased. GM-CSF, IL4, and IL10 increase. Increase of Tregs proportion. Early injections have best results than late treatment.	Choi et al. (2012)

Summary of the published works on the effect of MSCs on B cells in vivo. BM, bone marrow; SLE, systemic lupus erythematosus; AT, adipose tissue.

is a close interaction between MSC and B cells. It appears that this interaction occurs partly through the modulation of T cell help by MSCs, but also in the absence of helper cells MSCs can inhibit activated B cells. The study of this threesome relation is of special interest in the transplantation setting. Another interesting point that remains to be studied is the potential of MSCs to induce pro-tolerogenic B cell subsets that have themselves proved immunomodulatory properties.

The potential of MSCs in B cell immunomodulation appears to be promising and not fully understood. The advent of new and well designed studies can give important insights to fully picture the therapeutic role of MSCs in B cell mediated rejection.

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On the interactions between mesenchymal stem cells and regulatory T cells for immunomodulation in transplantation

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Experimental studies have established the use of mesenchymal stem cells (MSC) as a candidate immunosuppressive therapy. MSC exert their immunomodulatory function through the inhibition of $CD4^+$ and $CD8^+$ T cell proliferation. It is unknown whether MSC impair the immunosuppressive function of regulatory T cells (Treg). *In vitro* and *in vivo* studies suggest that MSC mediate their immunomodulatory effects through the induction of Treg. In this review we will focus on the interactions between MSC and Treg, and evaluate the consequences of these cellular interplays for prospective MSC immunotherapy in organ transplantation.

Keywords: mesenchymal stem cell, regulatory T cell, transplantation

INTRODUCTION TO MSC AND TREG

Since the discovery of mesenchymal stem cells (MSC) in bone marrow by Friedenstein et al. (1970), researchers were able to isolate these adherent cells from various postnatal and adult tissue sources (Pittenger et al., 1999; Zuk et al., 2002; Hoogduijn et al., 2007; Antonucci et al., 2011). MSC are self-renewing and capable of forming colonies while retaining their multilineage differentiation potential. They are able to differentiate into adipocytes, chondrocytes, osteoblasts, and myocytes (Pittenger et al., 1999; de la Garza-Rodea et al., 2012). These features represent part of the criteria that define MSC (Dominici et al., 2006). MSC are immunophenotypically characterized by the expression of the cell surface markers CD73, CD90, CD105, and $HLA\text{-}DR^{low}$, and the absence of CD11b, CD14, CD19, CD34, CD45, and CD79α. Interest in MSC for their use in transplantation was fostered when it was first discovered that MSC possess T cell suppressive properties (Bartholomew et al., 2002). Intensive research was undertaken to unravel the mechanisms by which MSC exert their immunomodulatory functions. Besides $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, activated B cells and natural killer (NK) cells are also susceptible to the suppressive activity of MSC (Corcione et al., 2006; Sotiropoulou et al., 2006). Further, MSC hamper the maturation of dendritic cells (DC) through the downregulation of MHC class II molecules and co-stimulatory molecules (Aggarwal and Pittenger, 2005; Jiang et al., 2005; Nauta et al., 2006). In addition to these immunosuppressive effects, MSC also have immunosupportive properties; they delay the apoptosis of neutrophils, preserving them to be readily available to counter infections (Raffaghello et al., 2008). Immunomodulation by MSC is mediated by cell–cell contact and the release of soluble factors. Important mediators are indoleamine 2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β), interleukin-10 (IL-10), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), human leukocyte antigen-G5 (HLA-G5), and nitric oxide (NO; Di Nicola et al., 2002; Meisel et al., 2004; Groh et al., 2005; Batten et al., 2006; Nasef et al., 2007; Sato et al., 2007; Hoogduijn et al., 2010a; Deuse et al., 2011). The ability of MSC to exert their immunosuppressive function requires MSC activation in a pro-inflammatory microenvironment through the presence of cytokines like interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-1α, and IL-1β (Crop et al., 2010). Collecting *in vivo* data suggest that MSC could be used as immunotherapy (Bartholomew et al., 2002; Popp et al., 2008). In clinical studies MSC have successfully attenuated graft-versus-host-disease (GvHD) after hematopoietic stem cell transplantation (Le Blanc et al., 2004, 2008). Currently, research groups strive to confirm the safety and feasibility of MSC therapy in solid organ transplantation (Hoogduijn et al., 2010b; Perico et al., 2011).

Primarily T cells are targeted by the immunosuppressive effect of MSC which raises the question, to what extent MSC affect T cells with immunomodulatory properties. Regulatory T cells (Treg) were first characterized by Sakaguchi et al. (1995) as activated $CD4^+$ T cells expressing CD25, the IL-2 receptor alpha-chain, which are involved in the maintenance of tolerance to self-antigens. Human Treg are commonly characterized by their expression of the transcription factor forkhead box P3 (FOXP3) and the FOXP3 reciprocal expression of CD127, the IL-7 receptor alpha-chain (Fontenot et al., 2003; Liu et al., 2006; Seddiki et al., 2006); additional markers are the co-stimulatory

molecules cytotoxic T lymphocyte antigen-4 (CTLA-4) and the glucocorticoid-induced TNF receptor-related protein (GITR; Read et al., 2000; Salomon et al., 2000; Takahashi et al., 2000; McHugh et al., 2002; Shimizu et al., 2002). While a unique marker for human Treg is yet to be identified, FOXP3 presents a reliable marker for Treg in mice (Ziegler, 2006).

The importance of Treg in the maintenance of tolerance is highlighted in humans suffering from immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX; Bennett et al., 2001; Kobayashi et al., 2001). IPEX patients lack functional Treg as a result of a point mutation in the *Foxp3* gene (Bacchetta et al., 2006). In other autoimmune diseases, the role of Treg remains controversial (Buckner, 2010). The immunomodulating nature of Treg made them interesting candidates for the induction of transplantation tolerance. Indeed, Treg have been reported to control alloreactivity by inhibiting the functionality of cell types similar to those suppressed by MSC; Treg hamper the proliferation of $CD4^+$ T cells, $CD8^+$ T cells, and DC (Velthuis et al., 2006; Bestard et al., 2007; Tang and Bluestone, 2008; Hendrikx et al., 2009a). Further, molecules associated with the tolerogenic and suppressive function of Treg partially overlap with those involved in MSC-mediated immunomodulation and include IL-10, TGF-β, and heme oxygenase-1 (HO-1; Tang and Bluestone, 2008). In addition, it has been hypothesized that Treg are able to influence their target cells in a granzyme B-dependent and perforin-dependent manner (Grossman et al., 2004; Gondek et al., 2005). Results from different humanized mouse models and clinical studies in patients with GvHD encourage the use of freshly isolated or *ex vivo* expanded Treg (Trzonkowski et al., 2009; Issa et al., 2010; Nadig et al., 2010; Brunstein et al., 2011; Di Ianni et al., 2011). Despite the indication that Treg therapy is promising and well tolerated in patients receiving stem cell treatment, clinical information about the application of Treg in solid organ transplantation is still lacking. However, Treg represent one of the immunomodulatory cell types whose clinical safety is currently under investigation (McMurchy et al., 2011; Schliesser et al., 2012).

Both MSC and Treg are able to influence the adaptive immune system by utilizing similar and distinct mechanisms. Therefore, it is of interest whether the overlapping mechanisms cause interference of the immunomodulatory properties of both cell types.

IN VITRO INTERACTION BETWEEN MSC AND TREG

Recently we investigated the interaction between MSC and Treg in a transplantation setting (Engela et al., 2012). We found that MSC derived from healthy kidney donors permitted the function of Treg from healthy donors as well as Treg from renal transplant patients. Vice versa, Treg did not hamper MSC function.

One of the first indications that T cells with a regulatory phenotype are unaffected by the suppressive capacities of MSC was provided by Maccario et al. (2005). This group reported that although autologous MSC and third-party MSC were able to suppress the proliferation of $CD4^+$ T cells and $CD8^+$ T cells in primary mixed lymphocyte cultures (MLC), after restimulation in secondary MLC the numbers of $CD4^+$ T cells co-expressing CD25 and/or CTLA-4 increased. This indicates an MSC-dependent preferential differentiation into T cells with a regulatory phenotype. Similar observations were made by Prevosto et al. (2007) after PBMC–MSC co-culture. In addition, IL-2 stimulation of PBMC in the presence of MSC also led to elevated proportions of $CD4^+CD25^+$ cells (Aggarwal and Pittenger, 2005). It has to be considered that due to lack of appropriate markers no distinction could be made between activated effector cells and Treg in these early research studies. Employing newly discovered markers, Di Ianni et al. (2008) performed an extensive study to shed light on the T cell population that is most responsive to the MSC-stimulus for Treg generation. The largest numbers of $CD4^+CD25^+FOXP3^+CD127^-$ Treg were found when MSC were cultured with immunoselected $CD3^+CD45RA^+$ or $CD3^+CD45RO^+$ fractions. Co-culture of MSC with different Treg populations such as $CD4^+CD25^+$ cells, $CD4^+CD25^+CD45RA^+$ cells, and $CD4^+CD25^+CD45RO^+$ cells maintained FOXP3 expression, CD127 downregulation, and the immunosuppressive activities of Treg for about 2 weeks. In the absence of MSC the Treg populations lost their suppressive capacities during this period.

The apparent interplay between MSC and Treg in the allosuppression of T cell proliferation triggered researchers' interest in the factors and mediators involved. Although conflicting data exist, it is the current opinion that key factors involved in Treg induction by MSC are MSC-derived TGF-β and PGE2 (Prevosto et al., 2007; English et al., 2009). TGF-β is a key regulator of the initiation and maintenance of FOXP3 expression, and the suppressive function of Treg (Chen et al., 2003; Fu et al., 2004). PGE2 is an immunosuppressant that inhibits T cell mitogenesis and the production of IL-2. TGF-β and PGE2 are constitutively produced by MSC. Secretion of these two immunomodulatory molecules can be increased through "MSC licensing," the activation of MSC with TNF-α and IFN-γ which improves their immunosuppressive capacity (English et al., 2007; Ryan et al., 2007). MSC-derived PGE2 also exerts suppressive functions by increasing the IL-10 production of macrophages and by limiting monocyte differentiation into DC (Nemeth et al., 2009; Spaggiari et al., 2009). It has also been reported that PGE2 induces a regulatory phenotype in $CD4^+CD25^-$ T cells by modulating the expression of FOXP3 and therefore contributes to Treg function (Baratelli et al., 2005). In addition, cell–cell contact seems to play a non-redundant role in the induction of Treg (English et al., 2009). After culture of $CD4^+$ cells with MSC, increases in mRNA levels of CD25 and FOXP3 mRNA were only detected when cells were in close proximity; when cells were separated by a tissue culture insert, this effect was not observed. For enhancement of FOXP3 expression in PBMC, however, direct MSC–PBMC contact was not required. This suggests that cell–cell contact between certain PBMC subpopulations contributes to the FOXP3 expression and substitutes for MSC–$CD4^+$ cell contact interactions. Another factor influencing the immunomodulatory effect of MSC on alloactivated T cells as well as the expansion of $CD4^+CD25^+FOXP3^+$ Treg is the soluble protein HLA-G5 (Selmani et al., 2008). In the presence of anti-HLA-G5 antibody, MSC-mediated immunosuppression was hampered. Neutralization of the HLA-G5 protein led to a decrease in the generation of $CD4^+CD25^+FOXP3^+$ T cells. In addition, the

tryptophan-catabolizing enzyme IDO was identified as a crucial modulator of the immunosuppressive effect of MSC (Meisel et al., 2004; Jurgens et al., 2009). Using a renal allograft model, Ge et al. (2010) investigated whether MSC-driven T cell suppression is also a consequence of the induction of Treg. Graft survival in untreated kidney recipients was significantly lower than graft survival in mice after MSC treatment. Increased serum levels of kynurenine in MSC-treated allograft recipients indicated increased IDO enzymatic activity and correlated with higher frequencies of $CD4^+CD25^+FOXP3^+$ Treg in recipient spleen and in the allograft. In this model recipient treatment with IDO-knockout MSC or the IDO inhibitor 1-methyl-tryptophan did not achieve graft tolerance. Therefore, the expression of functional IDO seems to be a prerequisite for MSC-mediated graft acceptance via direct T cell suppression as well as indirect modulation of the graft recipient's immune system through Treg induction. Skewing of $CD4^+$ T cell differentiation toward a more regulatory phenotype leads to the inhibition of T helper 17 (Th17) cell differentiation, an effect which is partly caused by MSC-derived IDO and PGE2 (Ghannam et al., 2010; Tatara et al., 2011). The role of IL-17, produced by Th17 cells, in the onset of GvHD is still controversial. Disease-ameliorating effects and an inductive role of *ex vivo* differentiated Th17 cells have been reported (Carlson et al., 2009; Kappel et al., 2009).

In summary, the mechanisms employed by MSC to inhibit effector T cell proliferation overlap with the mechanisms involved in Treg induction, yet, they do not interfere with Treg function.

IN VIVO INTERACTION BETWEEN MSC AND TREG

Whether MSC-mediated *in vitro* induction of Treg can be translated into the *in vivo* setting or even into the clinical setting remains to be thoroughly investigated. To date little evidence from animal models exists. One of the first *in vivo* studies, reporting the induction of Treg after MSC administration, was conducted by Gonzalez et al. (2009). Mice suffering from induced colitis were treated with a systemic infusion of human MSC. MSC ameliorated the severity of colitis through the reduction of inflammatory cytokines and chemokines and an increase of IL-10 concentrations; an overall downregulation of Th1-driven autoimmune responses and inflammatory responses was observed. Although MSC infusion impaired the expansion of Th1 cells, functional $CD4^+CD25^+FOXP3^+$ Treg were induced, confirming the *in vitro* MSC–Treg interplay.

A similar observation was recently made in a mouse model of allergen-driven airway inflammation (Kavanagh and Mahon, 2011). Systemic administration of allogeneic mouse MSC reduced the classical pathologies in this model as airway-mucus secretion, allergen-driven lung eosinophilia, and IgE induction were diminished. The improved pathological outcome coincided with a higher percentage of $CD4^+FOXP3^+$ cells in both lungs and spleens of MSC-treated mice when compared to control mice. Kavanagh and Mahon (2011) demonstrated the importance of Treg induction by MSC in this model; after *in vivo* depletion of Treg with cyclophosphamide an amelioration of the disease pathologies was observed, which serves as further evidence that the capability of MSC therapy to induce Treg is essential for this therapeutic modality.

In transplantation, one of the first models demonstrating the *in vivo* induction of Treg after MSC administration was described by Casiraghi et al. (2008). In a semi-allogeneic heart transplant mouse model, pre-transplant infusion of donor-derived MSC into the portal vein led to T cell hyporesponsiveness, prolonged cardiac allograft survival and expanded donor-specific Treg expressing CD4, CD25, and FOXP3. Similar observations were made after administration of recipient-derived MSC. Of relevance for the translation into a clinical setting, this group noticed that double pre-transplant infusions were more tolerogenically effective than a single MSC infusion. In contrast, post-transplant infusion of MSC was not effective. This suggests that pre-exposure to donor-MSC is required or that pre-activation of MSC might be necessary for MSC to successfully exert their tolerogenic action when graft alloantigens challenge the recipient's immune system.

Further evidence that the generation of Treg by MSC contributes to graft survival is provided by a kidney allograft mouse model (Ge et al., 2010). In contrast to the findings by Casiraghi et al. (2008), in this model intravenous administration of MSC 24 h after renal transplantation inhibited T cell proliferation. In tolerant recipients a Th2-dominant cytokine shift was observed as a significant decrease of IFN-γ production was detected while IL-4 levels were significantly increased. Further, in recipient spleens, frequencies of $CD4^+CD25^+FOXP3^+$ T cells were higher in MSC-treated mice. Ge et al. (2010) also found a significant increase of intragraft $FOXP3^+$ cells after MSC treatment suggesting Treg recruitment to the renal allograft. In this model, depletion of Treg using an anti-CD25 monoclonal antibody also reversed the beneficial effect of MSC therapy. $CD25^+$ T cell depletion caused graft rejection despite MSC treatment. This study again emphasized the importance of IDO as a mediator of MSC function. MSC derived from IDO-knockout mice were not able to induce graft tolerance. In a similar fashion, *in vivo* inhibition of IDO also abrogated the tolerogenic effect of MSC revealing a significant role of IDO in MSC immunosuppressivity.

INTERACTION BETWEEN MSC AND TREG IN THE CLINICAL SETTING

Results from preclinical studies support the plethora of *in vitro* data and confirm that MSC-mediated induction of Treg has functional relevance *in vivo*. Nevertheless, to date only one clinical study looked into this aspect of MSC therapy in a transplantation setting (Perico et al., 2011). In this safety and clinical feasibility study, autologous MSC were administered intravenously to two patients 7 days after they received living-related kidney grafts. Despite a concerning increase in serum-creatine levels after MSC infusion, stable graft function was reported for both patients 1 year after transplantation. Both patients received induction therapy in combination with standard maintenance immunosuppression. The induction regimen consisting of basiliximab and low-dose rabbit anti-thymocyte globulin (rATG) caused a profound depletion of $CD4^+$ and $CD8^+$ T cells in the peripheral blood in MSC-treated and non-MSC treated patients during the first 30 days. In patients with MSC therapy the number of $CD4^+$ T cells remained lower during the entire follow-up time when compared to the number of $CD4^+$ T cells in non-MSC patients.

In contrast, $CD8^+$ T cell repopulation to pre-transplant levels was achieved in both patient groups. During T cell depletion, the percentage of $CD4^+CD25^+FOXP3^+CD127^-$ Treg within the total $CD4^+$ T cell population was reduced. However, after 30 days the percentage of Treg increased in both MSC-treated patients. The memory $CD8^+CD45RO^+RA^-$ T cells in both MSC-treated patients remained lower than pre-transplant levels after 30 days post-transplant despite the full recovery of total $CD8^+$ T cell counts around this time-point. This effect was less prominent in patients who did not receive MSC. These findings indicate that in comparison to the immunosuppressive medication MSC might have an additional inhibitory effect on memory $CD8^+$ T cell proliferation. The combination of reduced memory $CD8^+$ T cells and increased percentages of Treg suggests that MSC treatment leads to a more pro-tolerogenic environment. Yet, it cannot entirely be ruled out that the observed effects are due to the MSC treatment and not evoked by the rATG induction therapy. More clinical experience with MSC therapy is required to obtain confident data.

INTERACTION OF MSC AND TREG WITH IMMUNOSUPPRESSIVE MEDICATION

Achieving graft tolerance, the long-term goal in transplantation immunology, is a major challenge. Controlling the immune response to donor-antigen in the graft recipient is currently accomplished by the administration of immunosuppressive drugs. Upon the introduction of novel cellular immunotherapies they will be applied in combination with standard immunosuppressive regimens. It therefore has to be considered that these drugs may not be permissive for MSC and/or Treg function. Conversely, MSC and Treg may interfere with the efficacy of the agents.

In vitro studies show that MSC reduce the efficacy of rapamycin and tacrolimus and, conversely, that these immunomodulatory agents negatively affect MSC function (Hoogduijn et al., 2008; Buron et al., 2009). However, cumulative inhibition of effector cell proliferation has been reported for combination therapy of MSC with mycophenolic acid (MPA) while dexamethasone did not influence MSC functionality. A synergistic effect of MSC and mycophenolate mofetil (MMF), the prodrug of MPA, on prolonged graft survival was observed in a fully allogeneic heart transplant mouse model (Eggenhofer et al., 2011); treatment with MSC and cyclosporine A failed to prolong allograft survival. In contrast to *in vitro* results, combination therapy of MSC and rapamycin achieved long-term heart allograft tolerance in mice and increased the frequency of splenic $CD4^+CD25^+FOXP3^+$ T cells (Ge et al., 2009). This finding is important as rapamycin is currently used for the *ex vivo* expansion of Treg (Battaglia et al., 2012). In renal transplant patients rapamycin led to an increase of $CD4^+CD25^+FOXP3^+$ Treg (Hendrikx et al., 2009b). A recent study by Ma et al. (2011) indicated that *in vitro* rapamycin had better synergistic effects on Treg function than cyclosporine A and tacrolimus. Subsequent adoptive infusion of donor-alloantigen-specific Treg in combination with low-dose of rapamycin delayed the acute rejection of kidney allografts in *Cynomolgus* monkeys. In addition, selective expansion of donor-type $CD4^+CD25^+FOXP3^+$ Treg after *in vivo* administration of rapamycin in combination with IL-2 suppressed acute GvHD in mice (Shin et al., 2011).

The interactions between both immunomodulatory cell types and immunosuppressive drugs demonstrate that the choice of immunosuppressive regimen will affect the outcome of cellular therapies.

HETEROGENEITY OF THE TREG POPULATION

Recent developments in Treg research revealed a heterogeneity of the Treg population. Apart from the well-described thymic-derived naturally occurring $CD4^+CD25^+CD127$-$FOXP3^+$ Treg (nTreg), other Treg subsets have been identified. Of these the most studied are induced Treg (iTreg). iTreg develop from naïve T cells in the periphery and their induction occurs upon T cell receptor stimulation, CD28 co-stimulatory signaling, and in the presence of IL-2 and TGF-β. Phenotypically iTreg resemble nTreg, yet, both populations can be distinguished by the methylation status of a special locus within the Foxp3 gene (Baron et al., 2007; Wieczorek et al., 2009). In nTreg this locus, the Treg-specific-demethylated-region (TSDR), is fully demethylated allowing for easy transcription while the TSDR in iTreg is methylated. Further discrimination between nTreg and iTreg may be provided by Helios, an Icaros family transcription factor. It has been reported that nTreg express Helios while iTreg do not (Thornton et al., 2010). Opinion on this topic is divided, some groups claim that Helios only presents an additional activation marker and is mutually expressed by natural Treg and induced Treg (Akimova et al., 2011; Gottschalk et al., 2012).

Apart from these two Treg subsets, non-FOXP3 expressing $CD4^+$ Treg have been described such as the IL-10 expressing T regulatory (Tr) 1 cells and TGF-β expressing Th3 cells (Weiner, 2001; Roncarolo et al., 2006). Other T cells with regulatory functions have been studied, but less extensively (Hayday and Tigelaar, 2003; Reibke et al., 2006; Ford McIntyre et al., 2008; Monteiro et al., 2010).

These new findings have to be considered when previous Treg work is evaluated. Further research will be required to investigate and to distinguish the influence of MSC on Treg expansion and Treg induction.

THE IMPORTANCE OF TREG INDUCTION BY MSC

The fate of MSC in the body after administration has been revealed by multiple distribution studies (Barbash et al., 2003; Kraitchman et al., 2005; Fischer et al., 2009; Assis et al., 2010; Zonta et al., 2010). Using various types of tracking techniques it was determined that MSC accumulate in the lung after intravenous infusion. The size of cultured MSC is significantly larger than the size of other immune cells in the circulation. This might cause MSC to be trapped in the capillaries of the lung. When MSC were administered via alternative routes, they were also found in other organs such as liver and spleen (Shi et al., 2010). Despite the consistency of the data, the drawback of these studies is that researchers cannot be certain that label detection confirms the presence of living MSC. Retrieved label can originate from viable MSC, deceased MSC or possibly phagocytosed debris of MSC.

To gain more clarity on this issue, different approaches have been examined (Hoogduijn et al., 2011). After intravenous

administration of labeled MSC to mice, organs were harvested, and MSC were isolated and re-cultured. Interestingly, labeled MSC were found in the lung up to 24 h after infusion, but in none of the other observed organs at any time after administration.

The apparent shortevity of MSC after infusion fortifies the importance of rapid Treg induction by MSC. While MSC are retained in different tissues shortly after administration and subsequently cleared, they "transfer" their immunomodulatory effect to other immunosuppressive mediators. Hence, when infused MSC are not present any longer to execute their suppressive functions, increased numbers of Treg are available to enforce graft acceptance.

CONCLUSION

Although currently prescribed drug-based immunosuppressive regimen are effective in preventing graft rejection in transplant patients, their main shortcoming is that their long-term application causes malignancies, infections, and nephrotoxicity. Due to their immunomodulatory capabilities, MSC have a high potential to function as alternative immunosuppressive therapy possibly with less side effects. While drugs mainly target a specific molecular pathway to achieve immunomodulation, MSC appear to have a broader effect on the patient's immune system. Yet, further characterization of this more global intervention by MSC is required. Despite inflicting their immunosuppressive effect on most lymphocyte subsets including T cells, MSC spare Treg. In fact, preclinical and clinical studies indicate that MSC mediate the expansion of natural Treg and/or the induction of novel Treg. This becomes an important feature of MSC immunomodulation as MSC appear to be cleared by the recipient's immune system shortly after infusion and hence will not be able to perform their suppressive capacities via direct cell–cell contact or soluble factors. Because of the multifaceted mechanisms by which MSC apply their immunosuppressivity combined with the fact that they do not impair the functionality of host Treg, MSC are very interesting candidates for cellular therapy in transplantation. First clinical results in the field of transplantation should encourage investigators to continue their research to bring MSC therapy to the patient.

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Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion

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Mesenchymal stem cells (MSC) are under investigation as a therapy for a variety of disorders. Although animal models show long term regenerative and immunomodulatory effects of MSC, the fate of MSC after infusion remains to be elucidated. In the present study the localization and viability of MSC was examined by isolation and re-culture of intravenously infused MSC. C57BL/6 MSC (500,000) constitutively expressing DsRed-fluorescent protein and radioactively labeled with Cr-51 were infused via the tail vein in wild-type C57BL/6 mice. After 5 min, 1, 24, or 72 h, mice were sacrificed and blood, lungs, liver, spleen, kidneys, and bone marrow removed. One hour after MSC infusion the majority of Cr-51 was found in the lungs, whereas after 24 h Cr-51 was mainly found in the liver. Tissue cultures demonstrated that viable donor MSC were present in the lungs up to 24 h after infusion, after which they disappeared. No viable MSC were found in the other organs examined at any time. The induction of ischemia-reperfusion injury in the liver did not trigger the migration of viable MSC to the liver. These results demonstrate that MSC are short-lived after i.v. infusion and that viable MSC do not pass the lungs. Cell debris may be transported to the liver. Long term immunomodulatory and regenerative effects of infused MSC must therefore be mediated via other cell types.

Keywords: mesenchymal stem cell, infusion, localization, survival, lung, liver

INTRODUCTION

Mesenchymal stem cells (MSC) are considered as a potential therapy for a wide variety of degenerating and immunological disorders (Giordano et al., 2007; Reinders et al., 2010; Salem and Thiemermann, 2010). Animal models demonstrate that MSC induce the repair of injured organs and ameliorate inflammatory processes (Morigi et al., 2008; Aurich et al., 2009; Gonzalez-Rey et al., 2009; Fisher-Shoval et al., 2012). The encouraging results in such models have initiated the translation of MSC therapy in clinical trials in a range of disorders, including graft versus host disease, inflammatory bowel disease, and cardiac infarct (Le Blanc et al., 2008; Hare et al., 2009; Duijvestein et al., 2010). Trials in multiple sclerosis (Freedman et al., 2010), systemic lupus erythematosus (Liang et al., 2010), and in organ transplantation (Perico et al., 2011; Popp et al., 2011; Tan et al., 2012) are currently ongoing or in preparation.

A problem with the interpretation of results and optimization of trials with MSC is a lack of understanding of the mechanism of action of MSC. While MSC have the capacity to differentiate into multiple cell types (Pittenger et al., 1999; Long et al., 2005), secrete growth factors that stimulate the proliferation and differentiation of other cells (Lee et al., 2011), and inhibit the proliferation of immune cells *in vitro* via the secretion of anti-inflammatory factors (Di Nicola et al., 2002), it is unknown whether these mechanisms are operational after administration of MSC. Moreover, there is controversy about the localization and persistence of MSC in the body after administration. The route of administration is an important factor determining the fate of MSC. The favorite route of administration in human is intravenously, as this has proven to be safe and allows the administration of large amounts of MSC. Tracking studies have shown that the majority of MSC localize to the lungs after intravenous infusion (Barbash et al., 2003; Kraitchman et al., 2005; Fischer et al., 2009; Assis et al., 2010). The detainment of MSC in the lungs is due to space restriction, as cultured MSC are more than 20 μm in diameter (Crop et al., 2010) and therefore much larger than circulating immune cells and larger than the width of the micro-capillaries of the lungs. Administration of MSC via alternative routes leads to detainment of MSC in other filtering organs. For instance, MSC administered via the portal vein are found in the liver (Shi et al., 2010), while MSC administered in tissues like muscle, spine, and fat pads remain present locally up to several weeks (Boulland et al., 2012; Hu et al., 2012; Nam et al., 2012).

After intravenous administration, MSC tend to disappear from the lungs within hours and migrate to other tissues such as the spleen and liver (Devine et al., 2003; Kraitchman et al., 2005) and preferentially to sites of injury (Chapel et al., 2003; Assis et al., 2010; Jackson et al., 2010; Jin et al., 2012). However, care should be taken with interpreting these results. Studies examining the distribution of MSC after intravenous infusion rely on PCR techniques, immunofluorescence, or bioluminescence to detect DNA, fluorescence label, or luciferase enzyme activity from infused MSC, but do not encompass the detection of living MSC. It is not unlikely that label is detected in dead MSC or in macrophages that

have phagocytosed MSC. Detection of label provides therefore no information on the localization and persistence of living MSC. Many studies examining the distribution of MSC use (severely) immuno-compromised recipient animals (Pereira et al., 1995; Liechty et al., 2000; Devine et al., 2003; Boulland et al., 2012). In most human studies, MSC recipients will have a more functional immune system and this is likely to affect the survival of MSC. The idea that MSC may not survive long after administration is supported by evidence demonstrating that the majority of MSC become apoptotic after administration (Liu et al., 2012).

In the present study we examined the localization of living MSC after intravenous infusion in immunocompetent mice by re-establishing cultures of administered MSC. Bone marrow-derived MSC of C57BL/6 mice that constitutively express DsRed were infused via the tail vein of wild-type C57BL/6 mice. After 5 min, 1, 24, or 72 h blood was taken and lung, spleen, liver, kidney, and bone marrow removed, MSC isolated and brought into culture. After 1 week of culture, the presence of adherent DsRed-MSC was analyzed by microscopy and flow cytometry. The outcome was compared to the results obtained from distribution experiments with radioactive labeled MSC in the same model.

MATERIALS AND METHODS

ETHICS STATEMENT

All animal experiments were carried out in accordance with European communities council directive (86/609/EEC) and institutional guidelines for animal care after local ethics committee approval (Ethics committee for animal laboratories, Medical Faculty, University of Regensburg, 93042, Regensburg, Germany). The MSC tracking studies were conducted after approval by the local authorities governing health care (Regierung der Oberpfalz, Emmeransplatz 8, 93047, Regensburg, Germany, www.ropf.de; AZ: 54-2532.1-33/08).

EXPERIMENTAL ANIMALS

We used DsRed C57BL/6 mice (Jax, stock number 006051; http://www.jaxmice.jax.org) as MSC donors. These transgenic mice, which carry an Actb-DsRed.T3 transgene, express the red fluorescent protein variant DsRed.MST under the control of the chicken beta actin promoter coupled with the cytomegalovirus (CMV) immediate early enhancer. All tissues of homozygotes are red fluorescent. 6–8-weeks-old wild-type C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were used as MSC recipients. For some experiments, $Rag2^{-/-}$ × common cytokine receptor γ-$chain^{-/-}$ double knock out ($Rag2^{-/-}$ × γ-$chain^{-/-}$) mice lacking T, B, and NK cells were used as MSC recipients. Laboratory animals were housed with access to food and water provided *ad libitum*. Water was provided via standard lab water bottles which were replenished daily. Cages were cleaned weekly. All veterinary procedures were performed only with sedated animals. All efforts were taken to ameliorate any suffering.

Liver ischemia-reperfusion injury was induced according to Abe et al. (2009). In brief, ischemia-reperfusion injury was induced by placing an atraumatic clip across the portal vein, hepatic artery, and bile duct just above the branching to the right lateral lobe. The median and lateral lobe (approximately 70% of the liver) showed significant blanching. After 45 min of ischemia, the clamp was removed and the liver reperfused.

ISOLATION AND CULTURE OF MSC

MSC were isolated from tibias and femurs of DsRed C57BL/6 mice by flushing. The obtained cell suspension was washed and plated in tissue culture flasks in MEM alpha supplemented with 10% heat-inactivated fetal calf serum (FCS), and 100 U/mL penicillin and 100 mg/mL streptomycin (1% p/s) (all Invitrogen, Karlsruhe, Germany). After 2–3 days non-adherent cells were removed. Plastic adherent cells were removed by trypsinization after reaching 70–80% confluency. After the first passage, $CD11b^{+}$ cells were depleted from the cultures by MACS (Miltenyi, Bergisch Gladbach, Germany). Cells were maintained at 37°C, 5% CO_2, and 95% humidity, culture medium refreshed twice weekly and used for experiments between passage 2 and 5.

CHARACTERIZATION OF DsRed-MSC

DsRed-MSC were examined for DsRed expression by fluorescence microscopy and flow cytometry using a FACS Calibur (BD Biosciences, San Jose, USA). For surface marker characterization, MSC were harvested and washed twice in ice-cold phosphate-buffered saline (PBS). Stainings were performed in 50 µl of pre-diluted FITC-conjugated anti-mouse CD34, CD11b, Sca-1 or MHC-I, and APC-conjugated anti-mouse CD44, CD11c, or CD117 antibody (all from BD Biosciences, Heidelberg, Germany). After 20 min incubation at 4°C in the dark, 400 µl of PBS was added, and the cells analyzed with the FACS Calibur flow cytometer.

Differentiation of MSC into adipocytes was achieved by plating MSC into six-well plates in expansion medium without FCS for 2 days. Differentiation was induced by culturing the cells in expansion medium without FCS supplemented with insulin (15 U/ml; Sanofi-aventis, Paris, France), dexamethasone (10^{-6} M; Sigma-Aldrich, St. Louis, USA), goat serum (5 ml/100 ml; PromoCell, Heidelberg, Germany), and 3-isobutyl-1-methylxanthin (0.1 mg/ml; Sigma-Aldrich) for 3 days. The cells were then cultured in expansion medium without FCS supplemented with insulin (15 U/ml; Sanovi-aventis) for 5 days. Cell differentiation into adipocytes was confirmed by oil red O staining. Cells were washed in cold PBS, fixed with 10% formaldehyde at 4°C for 10 min, and then incubated with 5 mg/ml oil red O solution (Sigma-Aldrich, Munchen, Germany) for 2 h at room temperature.

To induce osteogenic differentiation, MSC were seeded in six-well plates at a density of 15,000 cells per cm^2 in DMEM without FCS for 1 day. Cells were then treated with osteogenic medium for two weeks, changing the medium twice a week. Osteogenic medium consisted of DMEM supplemented with 0.1 µM dexamethasone, 0.3 mM ascorbic acid, and 10 mM α-glycerolphosphate (all Sigma-Aldrich). Osteogenic differentiation was assessed by von Kossa staining. Cells were covered with 5% silver nitrate solution for 40 min in bright light followed by an incubation step in UV light for 2 min. After rinsing with distilled water, cells were incubated for 5 min in 1% pyrogallol (Sigma-Aldrich) and rinsed again. Non-specific staining was removed by washing the cells in 5% sodium thiosulfate (Sigma-Aldrich) for 5 min.

For examination of the immunomodulatory capacity of DsRed-MSC, splenocytes of C57Bl/6, or Balb/c mice were labeled with CFSE (Vybrant Cell Tracer Kit, Molecular Probes, Eugene, Oregon, USA) and stimulated with 2 ng/ml Concanavalin A (ConA) in MEM alpha with 10% FCS and 1% p/s. DsRed-MSC were added at a 1:5 ratio. After 3 days, splenocytes were collected, stained for CD3 and CD4 (BD Biosciences), and analyzed on the flow cytometer.

INFUSION OF DsRed-MSC

DsRed-MSC were trypsinized and washed twice with PBS. A suspension of 500,000 DsRed-MSC in PBS was infused via the tail vein of C57BL/6 mice or Rag2$^{-/-}$ × γ-chain$^{-/-}$ mice. Control animals received PBS only. Mice were sacrificed after 5 min, 1, 24, or 72 h, blood collected, and lungs, liver, spleen, kidneys, and bone marrow removed.

ORGAN HARVEST AND RE-ISOLATION OF DsRed-MSC

Blood

Approximately 1 ml blood was collected and red blood cells lysed in red cell removal buffer (Roche, Germany). The cells were then washed and plated out in tissue culture flasks in MEM alpha with 10% FCS and 1% p/s.

Lungs, kidneys, spleen

Organs were minced with a scalpel knife and incubated in 0.5 mg/ml collagenase in PBS for 30 min in a shaker at 37°C. The tissue was then put several times through a 19 G needle with a syringe, washed, taken up in MEM alpha with 10% FCS and 1% p/s, filtered through a 100 μm cell strainer, and plated out in tissue culture flasks.

Liver

Livers were harvested and prepared with the Gentle MACS dissociator according to the protocol (Miltenyi, Bergisch Gladbach, Germany) without the final centrifugation step to obtain a single cell suspension including all liver cells. Cells were washed, taken up in MEM alpha with 10% FCS and 1% p/s, and plated out in tissue culture flasks.

Bone marrow

Tibias and femurs were flushed with PBS and the cell suspension was washed and plated out in tissue culture flasks in MEM alpha with 10% FCS and 1% p/s.

After 2 and 7 days of culture, non-adherent cells were removed by washing and the adherent cells examined for the presence of DsRed-MSC by fluorescence microscopy.

INFUSION OF RADIO-LABELED DsRed-MSC

DsRed-MSC were radio-labeled using a protocol described by Sprent (1976). Briefly, MSC were diluted to a concentration of 20 × 10^6 /ml and incubated with 20 μCi/ml Cr-51 Sodium (PerkinElmer, Waltham, MA, USA) for 1 h followed by two washes with medium. Subsequently, 500,000 radio-labeled cells were infused via the tail vein of C57BL/6 mice. The animals were sacrificed and blood and organs removed after 1 or 24 h. Organs were place in vials and radioactivity measured as counts/min with a 1470 Wallac Wizard gamma counter (PerkinElmer).

RESULTS

CHARACTERIZATION OF DsRed-MSC

DsRed-MSC isolated from the bone marrow had a typical fibroblast-like appearance in culture, similar to wild-type MSC (**Figure 1A**). They expressed the MSC markers Sca-1 and CD44, and were negative for CD34, CD11b, CD11c, and CD117. They had only a weak expression of MHC class I, and constitutively expressed high levels of DsRed (**Figure 1B**).

To show that DsRed-MSC are able to differentiate like wild-type MSC, we cultivated them under adipogenic and osteogenic conditions. After 2 weeks in culture, DsRed-MSC started to differentiate into adipocytes, as demonstrated by positive oil red O staining of lipid-filled vesicles (**Figure 1C**, left). Importantly, these differentiated cells still remained red fluorescent (**Figure 1C**, right). After 2 weeks in osteogenic differentiation medium, DsRed-MSC started to deposit calcified nodules, which stained black with silver nitrate (**Figure 1D**, left). Also, the differentiated osteoblasts remained red fluorescent (**Figure 1D**, right).

DsRed-MSC furthermore shared the property of wild-type MSC of inhibiting the proliferation of ConA stimulated splenocyte proliferation. The proliferation of CD3$^+$CD4$^+$ T cells and CD3$^+$CD4$^-$ T cell subsets was significantly inhibited on day 3 by DsRed-MSC added at a 1:5 ratio. DsRed-MSC inhibited both syngeneic (C57BL/6 Responders cells) (**Figure 1E**) as well as allogeneic responder cells (Balb/c Responder cells) (**Figure 1F**).

DISTRIBUTION OF RADIOACTIVE LABELED SYNGENEIC MSC

We radio-labeled DsRed-MSC and followed their distribution after intravenous infusion by tracking the radioactive signal. One hour after administration of MSC, the majority (60%) of radioactivity was found in the lungs, while a smaller proportion was found in the liver (**Figure 2**). After 24 h, the amount of radioactivity in the lungs was strongly reduced, while the amount of radioactivity in the liver was increased. A small amount of radioactivity was found in the spleen. Radioactivity in other organs was around background levels.

PRESENCE OF LIVING DsRed-MSC IN TISSUE CULTURES

To examine whether infused MSC home to lungs, liver and perhaps other tissues as living cells, DsRed-MSC (500,000) were infused via the tail vein in syngeneic C57BL/6 mice and tissues isolated at various time points after infusion and brought into culture to detect the presence of viable DsRed-MSC. Thus, after 5 min, 1, 24, or 72 h mice were sacrificed and blood, lungs, liver, spleen, kidneys, and bone marrow collected and cell cultures established in MSC-supporting culture medium. Adherent cell cultures reaching confluency after 7–10 days were obtained from lung, spleen, kidney, and bone marrow tissues. The cultures consisted of multiple cell types, including macrophage-like cells, endothelial cells, and fibroblastic cells. Single colonies of fibroblastic cells were obtained from blood and liver tissue.

Analysis by fluorescence microscopy revealed that cultures obtained from blood collected 5 min after MSC infusion contained no DsRed-MSC. However, lung tissue cultures contained red fluorescent cells, indicating that living donor MSC were present in the lungs 5 min after infusion of DsRed-MSC. Interestingly, after 2 days of culturing multiple DsRed-MSC were

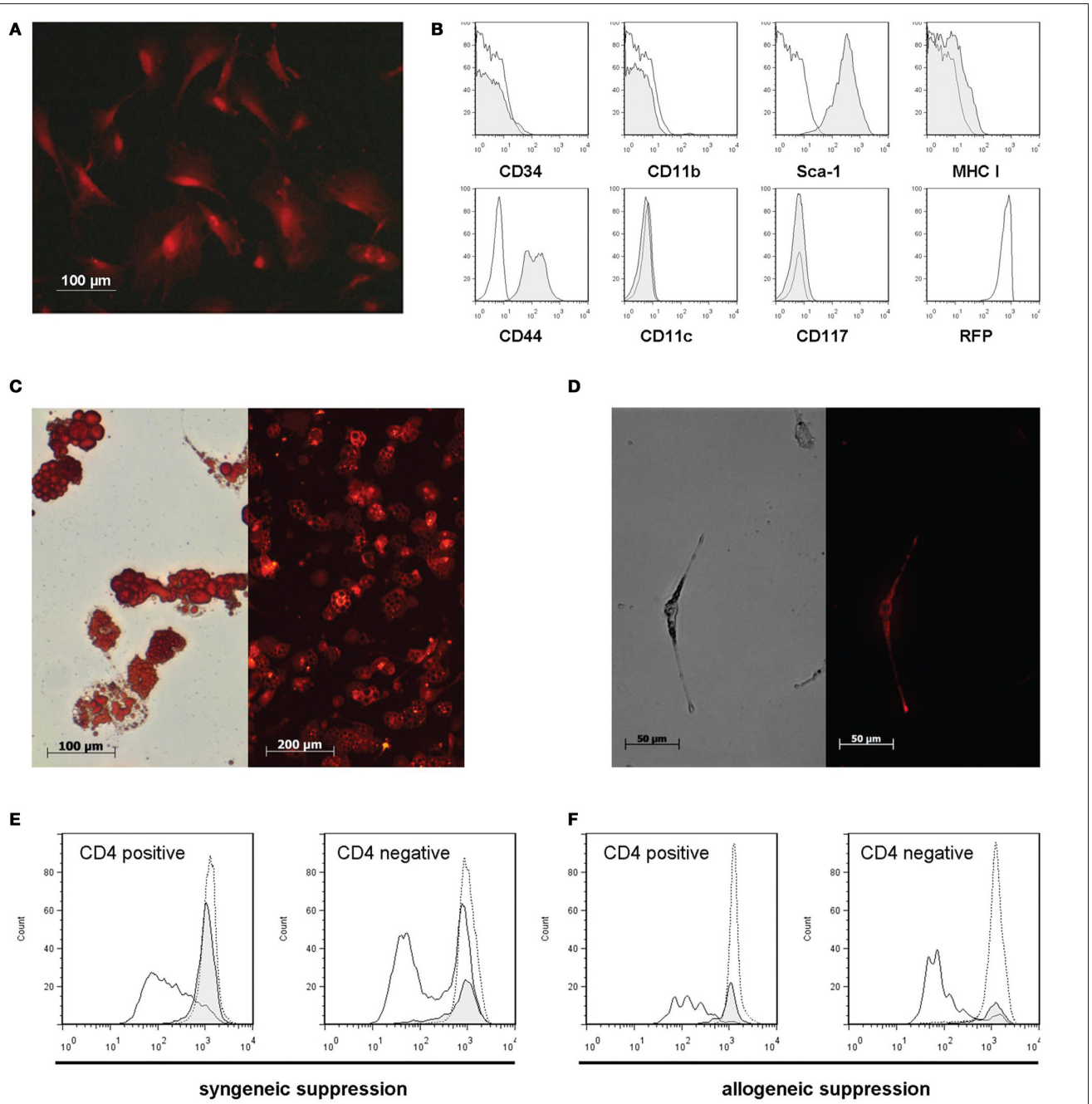

FIGURE 1 | DsRed-MSC are phenotypically and functionally comparable to wild-type MSC. (A) Fluorescence microscopy of plastic adherent DsRed-MSC in culture shows bright red fluorescence. **(B)** Flow cytometric analysis demonstrates that DsRed-MSC are negative for the expression of CD34, CD11b, CD11c, and CD117, show weak expression of MHC class I, and are positive for Sca-1, CD44, and RFP (DsRed). **(C)** DsRed-MSC are capable of differentiating into adipocytes, as demonstrated by staining of lipid vesicles by Oil-Red-O (left), while remaining red fluorescent (right). **(D)** DsRed-MSC are capable of differentiating into osteoblasts, indicated by positive silver nitrate staining for calcium deposits (left), while remaining red fluorescent (right). (**E** and **F**) DsRed-MSC suppress ConA induced proliferation of CD4 positive and negative T cells efficiently, determined by CFSE dilution on day 3 (solid line: ConA stimulated T cells, gray shaded curve: ConA stimulated T cells + MSC, dotted line: non-stimulated T cells). DsRed-MSC suppressed the proliferation of syngeneic C57BL/6 responder T cells **(E)** as well as allogeneic Balb/c responder T cells **(F)**. Representative data of 3 experiments shown.

frequently shown in close proximity, suggesting that the cells were proliferating (**Figure 3A**). After 7 days of culture, DsRed-MSC were mostly found in colonies rather than equally distributed throughout the cultures (**Figure 3B**). Flow cytometric analysis of lung tissue after 7 days of culture confirmed the presence of DsRed-MSC expressing the MSC marker $CD44^+$ (**Figure 3C**). Cultures from spleen, kidney, liver, and bone marrow established 5 min after MSC infusion contained no DsRed-MSC.

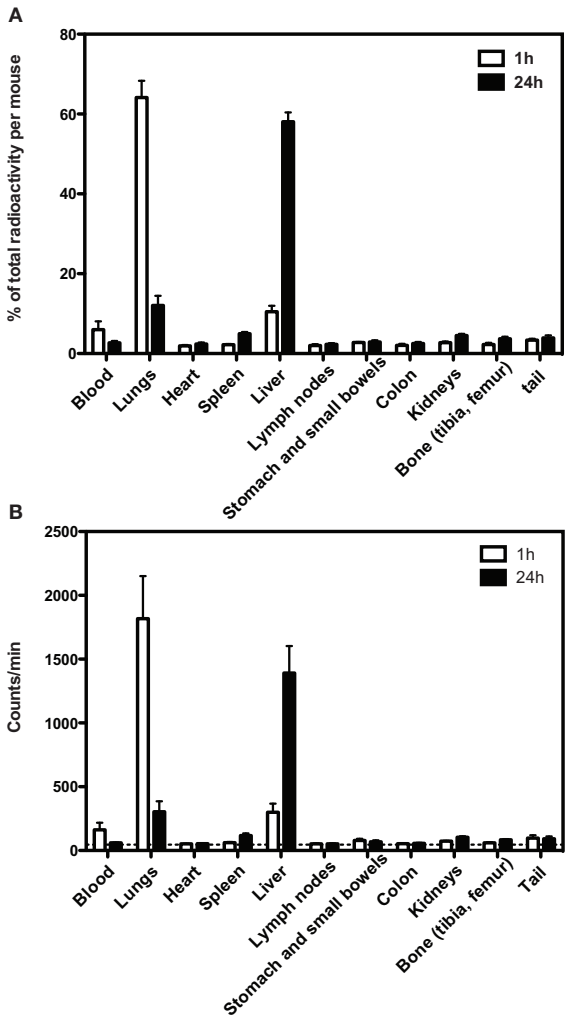

FIGURE 2 | Distribution of Cr-51 radioactivity after infusion of Cr-51 labeled DsRed-MSC. (A) One hour after intravenous injection, more than 60% of total radioactivity is located in the lungs and approximately 10% in the liver. Other tissues contain only residual radioactivity. After 24 h, the majority of radioactivity is located in the liver. **(B)** As in **(A)**, but absolute counts shown. Dashed line indicates background radioactivity. Cervical, axillary, inguinal, hepatic, para-aortal, and mesenteric lymph nodes were collected. Average data of 2 experiments is shown.

DsRed-MSC were also present in lung cultures established 1 h after MSC infusion, but their numbers were strongly reduced in cultures obtained at 24 h. No DsRed-MSC were detected in lung cultures established 72 h after MSC infusion, suggesting that the infused MSC were either no longer viable or had migrated to other tissues. Surprisingly, living DsRed-MSC were not detected in cultures of any of the other tissues established at 1, 24, or 72 h after MSC infusion (**Table 1**).

PRESENCE OF LIVING DsRed-MSC IN ISCHEMIA-REPERFUSION INJURY LIVER TISSUE

To examine whether organ injury would provide a trigger for MSC to migrate to the organ, ischemia-reperfusion injury was induced in the liver of mice by clamping the hepatic artery, portal vein, and bile duct for 45 min in order to prevent about 70% of the liver lobes from blood supply. One hour before ischemia-reperfusion injury, 500,000 DsRed-MSC were infused via the tail vein. At this time point we know from the previously described experiments that living MSC are present in the lung. Twenty-four hours after reperfusion, the organs were removed and MSC brought into culture. No living DsRed-MSC were detected in liver tissue after ischemia-reperfusion injury (**Table 1**). DsRed-MSC were present in lung tissue, like in control animals.

PRESENCE OF LIVING DsRed-MSC IN $Rag2^{-/-} \times \gamma$-$chain^{-/-}$ RECIPIENTS

To determine whether NK, T, and B cells were responsible for the rapid disappearance of living DsRed-MSC after infusion, DsRed-MSC were infused in $Rag2^{-/-} \times \gamma$-$chain^{-/-}$ double knock-out mice that are deficient for these cells, and organs removed and cultures established. Like in wild-type mice, living DsRed-MSC were found in lung tissue up to 24 h after infusion, but not in any other tissue at any time point (results not shown).

DISCUSSION

MSC therapy has shown to be effective as an immunomodulatory and regenerative therapy in a number of animal models, including transplant models (Popp et al., 2008), experimental colitis (Gonzalez et al., 2009), pancreatitis (Jung et al., 2011), experimental multiple sclerosis (Fisher-Shoval et al., 2012), and several others. The mechanisms that mediate the effects of MSC in these models are not clear. The old dogma that administered MSC engraft and differentiate in specialized cell types has been abandoned, whereas the proposition that the effects of MSC are mediated via the secretion of trophic and immunoregulatory factors has gained in popularity.

In the present study we demonstrated that MSC accumulate in the lungs within the first few hours after intravenous infusion. This is in agreement with earlier findings (Barbash et al., 2003; Kraitchman et al., 2005; Assis et al., 2010). Importantly, we were able to demonstrate that at least some of the exogenous MSC remained viable in the lungs up to 24 h after infusion. Re-culturing of these MSC demonstrated that they maintained their proliferation capacity. During the first 24 h after infusion, living MSC were not found in blood, liver, spleen, kidney, or bone marrow. After 24 h, living MSC disappeared from the lungs, but did not reappear in the other tissues examined, suggesting they did not survive long term in the recipient animals.

As it has been suggested that MSC migrate to sites of injury, we induced ischemia-reperfusion injury in the liver and examined whether viable administered MSC would appear in the injured liver. We found, however, no living donor MSC in the injured liver, indicating there is no difference in the migration of viable MSC to injured and non-injured organs.

The identification of viable MSC after infusion has not been demonstrated earlier and shines a new light on the fate of MSC after administration. Other studies reported the migration of MSC to various sites, particularly liver and spleen, and to sites of injury. In our experiments, radioactivity was found in the liver and spleen 24 h after injection of radio-labeled MSC but we

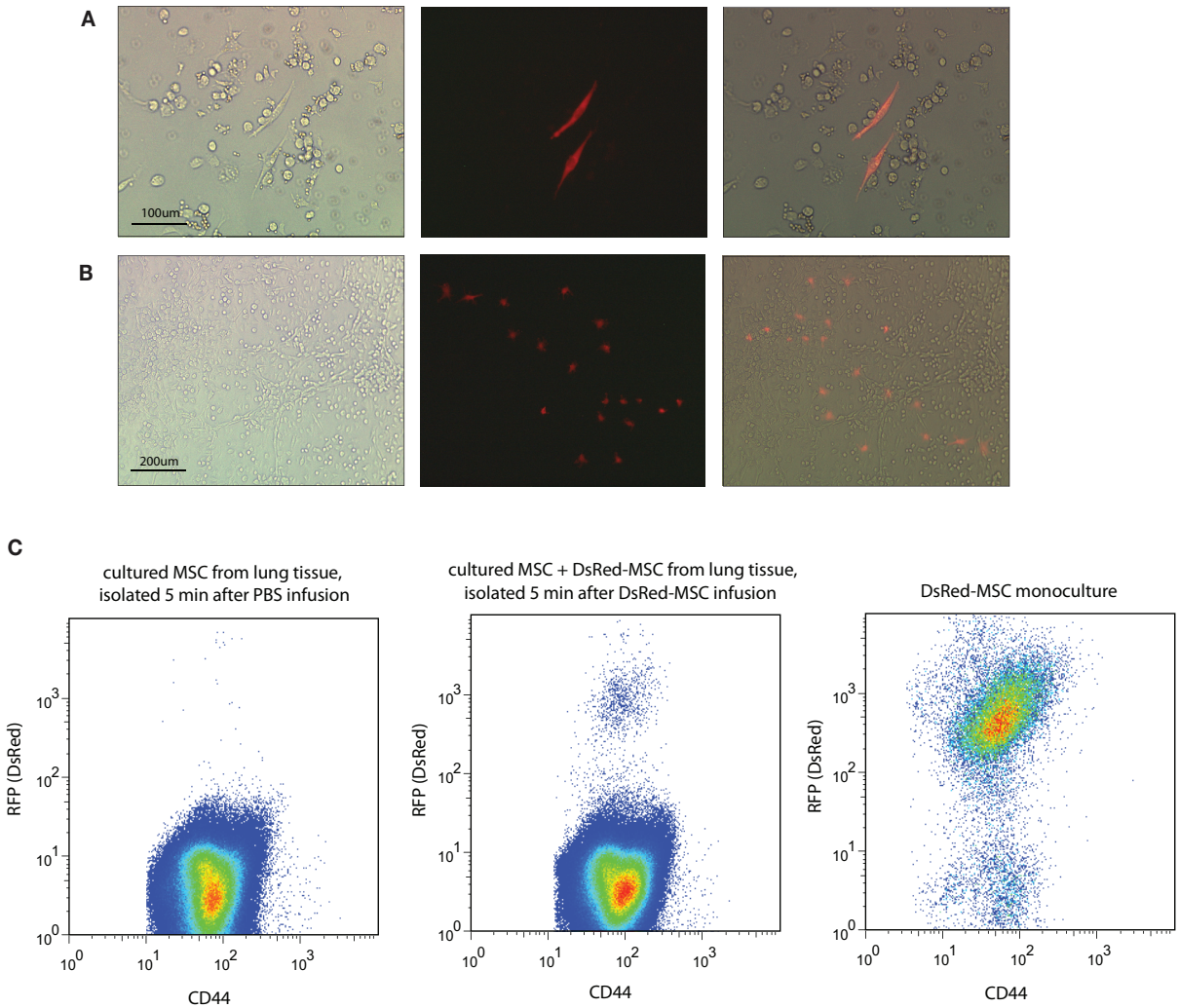

FIGURE 3 | Viable MSC are detected in lung tissue only after intravenous infusion. (A) Brightfield, immunofluorescent, and merged microscopic images show two DsRed-MSC in a two days-old culture of lung tissue established 5 min after MSC infusion. **(B)** After 7 days of culture clusters of DsRed-MSC are seen. **(C)** Flow cytometric analysis of cultures established from lung tissue 5 min after DsRed-MSC infusion cultured for 7 days revealed a distinct population of $CD44^+$ DsRed-MSC (middle plot), while in cultures of PBS treated animals no DsRed-MSC were present (left plot). Mono-cultures of DsRed-MSC served as gating control (right plot). Five animals were included per group. Representative data shown.

Table 1 | The presence of living MSC in tissues 5 min, 1, 24, and 72 h after infusion of 500,000 DsRed-MSC via the tail vein at $T = 0$.

	$T = 0$	$T = 5$ min	$T = 1$ h	$T = 24$ h	$T = 24$ h liver IRI*	$T = 72$ h
Blood	+	–	–	–	–	–
Lung	–	+	+	+	+	–
Liver	–	–	–	–	–	–
Spleen	–	–	–	–	–	–
Kidney	–	–	–	–	–	–
Bone marrow	–	–	–	–	–	–

Five animals were included per group.

**Liver IRI: 45 min of ischemia-reperfusion injury of the liver, MSC infusion 1 h before ischemia-reperfusion injury.*

failed to isolate viable MSC from these organs. Our data indicate that living MSC do not pass the capillary bed of the lungs after intravenous infusion. It is therefore likely that previous studies describing MSC in other tissues detected MSC-label (e.g., radioactivity, fluorescence) from MSC debris or from phagocytosed MSC rather than living MSC. Our studies suggest that other routes of administration have to be investigated if MSC need to be delivered to tissues other than the lungs. For instance, administration via the portal vein could be used for delivery of MSC to the liver, while arterial administration may deliver MSC to specific organs. The survival of MSC administered via such alternative routes could be examined by the methods used in the present paper.

How intravenously administered MSC disappear from the lungs is not clear at this stage. One possibility could be that MSC

are damaged by shear forces after infusion. However, the survival of MSC for up to 24 h in the lungs suggests that their removal is caused by other mechanisms. Immune cells may well be involved in this process. In the present study, the administered MSC were of syngeneic origin, which would suggest a role for cells of the innate immune system in the removal of MSC. Activated NK cells have been shown to be capable of lysing autologous MSC *in vitro* (Spaggiari et al., 2006; Crop et al., 2011). We, however, demonstrated that infused MSC do not have an increased life span in mice that lack NK cells. Other cells of the innate immune system, in particular macrophages, may play a more important role. If the innate immune system is responsible for the loss of administered MSC, it is questionable whether conventional immunosuppressive drugs would be capable of preventing the removal of MSC, as these drugs mainly target the adaptive immune system.

Our data clearly demonstrate the short-term survival of infused MSC and a lack of distribution of viable MSC beyond the lungs. Nevertheless, several studies have demonstrated beneficial effects of MSC in a variety of disease models (Gonzalez et al., 2009; Semedo et al., 2009; Kanazawa et al., 2011) even when MSC were no longer around (Yang et al., 2012). The question now arises how these effects are mediated. It seems clear that delivery of MSC to a site of injury is not required for a therapeutic effect. It has been hypothesized that apoptosis of infused cells can trigger an immunomodulatory response (Thum et al., 2005) and recently it was demonstrated that macrophages adapt an immunoregulatory function after phagocytosis of dead (MSC) (Lu et al., 2012). Our results suggest this process may happen in the lungs and from there develop into a response that eventually targets the immune response at sites of inflammation and injury. Future research will have to reveal which signals conduct this response through the body.

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Therapeutic potential of bone marrow-derived mesenchymal stem cells for cutaneous wound healing

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Despite advances in wound care, many wounds never heal and become chronic problems that result in significant morbidity and mortality to the patient. Cellular therapy for cutaneous wounds has recently come under investigation as a potential treatment modality for impaired wound healing. Bone marrow-derived mesenchymal stem cells (MSCs) are a promising source of adult progenitor cells for cytotherapy as they are easy to isolate and expand and have been shown to differentiate into various cell lineages. Early studies have demonstrated that MSCs may enhance epithelialization, granulation tissue formation, and neovascularization resulting in accelerated wound closure. It is currently unclear if these effects are mediated through cellular differentiation or by secretion of cytokines and growth factors. This review discusses the proposed biological contributions of MSCs to cutaneous repair and their clinical potential in cell-based therapies.

Keywords: mesenchymal stem cells, wound healing, differentiation, paracrine signaling, tissue engineering

INTRODUCTION

Chronic wounds are a cause of significant morbidity and mortality and pose a large financial burden on the healthcare system. Proper cutaneous wound repair requires a well-coordinated response of inflammation, neovascularization, extracellular matrix formation, and epithelialization. Failure of any of these processes due to ischemia, reperfusion injury, bacterial infection, or aging can result in chronic inflammation and a non-healing wound (Mustoe et al., 2006).

Traditional therapies for the treatment of chronic wounds include debridement, minimization of bacterial load, pressure offloading, negative-pressure therapy, biological dressings, skin grafting, and reconstructive tissue flaps. Despite the most recent advances in wound management, up to 50% of chronic wounds still fail to heal (Cha and Falanga, 2007). One hypothesis for this problem is that resident cells in non-healing wounds are intrinsically impaired and demonstrate increased senescence and decreased response to growth factors (Hasan et al., 1997; Vande Berg et al., 1998).

Bone marrow-derived mesenchymal stem cells (MSCs) were originally described as plastic-adherent fibroblast-like cells and can differentiate into osteoblasts, adipocytes, and chondrocytes (Friedenstein et al., 1976; Pittenger et al., 1999). Their plasticity has since been expanded to include contribution to cell lineages in brain (Brazelton et al., 2000), muscle (Ferrari et al., 1998), liver (Alison et al., 2000), and kidney tissue (Poulsom et al., 2001). MSCs are easy to isolate and expand in culture and studies have suggested minimal immunogenic response when allogeneic or syngeneic cells are used (Ryan et al., 2005; Chen et al., 2009). Given these qualities and the current barriers limiting embryonic stem cell research, MSCs have become a recent focus of interest for cellular therapy in tissue regeneration. The application of MSCs for tissue repair has ranged from intravenous infusion to reduce the size of brain infarcts in a rat stroke model (Li et al., 2005) to implantation of cells in the myocardium to reduce left ventricular dysfunction in a swine model of myocardial ischemia (Amado et al., 2005). Here we review the existing evidence for MSC-based therapies for cutaneous wound healing and future directions to bring their potential to the clinical setting.

BONE MARROW-DERIVED CELLS IN NORMAL POST-NATAL SKIN DEVELOPMENT

Early literature demonstrating the contribution of bone-marrow derived cells to the epidermis formed the basis for investigating the role for BM-MSCs specifically in cutaneous repair. The development of transgenic mice strains expressing green fluorescent protein (GFP) has been essential in allowing investigators to understand the behavior of cells *in vivo*. When coupled with bone marrow transplantation or parabiosis models, GFP+ donor cells can be identified in wild-type mice using basic immunohistochemistry techniques to track cellular fate and differentiation. The ability for precursor cells to mobilize from the bone marrow niche to peripheral tissue remains controversial, however, several investigators have demonstrated the existence of circulating bone marrow precursor cell (Roufosse et al., 2004). Several early animal studies have reported that in normal skin homeostasis, bone marrow-derived cells may contribute to keratinocytes in the epidermis and sebaceous glands as well as dendritic cells within the dermis. The aggregate contribution to the epidermis and dermis by cells of bone marrow origin has been described as 11–14% of the total cell population (Fathke et al., 2004; Deng et al., 2005). Cell fusion between bone marrow-derived cells and mature resident cells has been observed in *in vitro* co-culture systems by several groups resulting in cells that adapt a "differentiated"

phenotype but fail to undergo true differentiation (Terada et al., 2002; Spees et al., 2003). Several investigators have attempted to address this phenomenon and have demonstrated a lack of cell fusion in these models by using sex-mismatched donor cells and performing FISH analysis (Brittan et al., 2005; Wu et al., 2007; Sasaki et al., 2008). In a similar model, Badiavas et al. utilized a total bone marrow transplantation model and discovered bone marrow-derived CD34+ (a hematopoietic stem cell marker) keratinocytes in the hair bulge region which is thought to be the stem cell niche for epidermal stem cells (Badiavas et al., 2003; Trempus et al., 2003). This suggests that potentially circulating bone marrow-derived cells may serve to replenish the epithelial stem cell compartment throughout life. While still early, these studies highlight the potential role of bone marrow-derived stem cells in differentiating into various lineages to maintain skin homeostasis.

MSC DELIVERY ENHANCES CUTANEOUS WOUND REPAIR

Wound healing studies have subsequently focused on MSCs as the potential cell population within bone marrow that can contribute to cutaneous regeneration. Studies in both mice and humans have consistently demonstrated enhanced wound repair following treatment with bone marrow-derived MSCs (**Table 1**).

The use of murine models has been crucial for advancing the understating of wound healing, however, fundamental differences exist between the mouse and human skin. Murine skin lacks apocrine sweat glands and rete ridges/dermal papillae, which are both found in human skin. However, rete ridge-like structures may become apparent during mouse wound healing and are often described as "pseudoepitheliomatous" or "pseudocarcinomatous hyperplasia" (Sundberg, 2004). Mouse skin also has a panniculosus carnosus layer, a thin subcutaneous muscle layer only found in the human neck (platysma). This muscle layer produces rapid wound contraction following injury which is the primary method of wound healing in the mouse as opposed to granulation tissue formation and re-epithelialization in humans. Mouse skin has also been shown to be thinner and more compliant than human skin (Aarabi et al., 2007). A more complete summary of murine wound healing models is reviewed here (Wong et al., 2011b).

Experiments with diabetic murine models have been particularly useful in assessing the clinical utility of MSCs in wound repair. Many non-healing ulcers are caused by diabetic pathology which has been shown to attenuate the recruitment of inflammatory cells and down-regulate expression of various growth factors (Falanga, 2005). Local delivery of MSCs significantly increased granulation tissue formation and decreased wound healing time in leptin receptor-deficient db/db diabetic mice compared to those treated with either PBS or non-cell type-specific bone marrow aspirate (Javazon et al., 2007). Analysis of the mechanical properties of treated wounds revealed that administration of MSCs not only accelerated wound closure but also enhanced wound repair quality, resulting in healed tissue with increased tensile strength. This effect is thought to be secondary to increased collagen composition within the healed tissue (McFarlin et al., 2006; Kwon et al., 2008). The mechanism for this observed increase in collagen secretion is currently under investigation.

Promising findings in animal models have led to a very limited number of small-scale human trials examining the effects of autologous MSCs on chronic wounds. Injection of primary bone marrow cells into the wound edge followed by topical application of cultured MSCs resulted in the complete closure of three chronic wounds which had failed traditional therapy including autologous skin grafting (Badiavas and Falanga, 2003). Hallmarks of the healing wounds were a massive influx of mature and immature inflammatory cells, increased vascularity, and increased dermal thickness. It must be noted that this study utilized the injection of whole bone marrow aspirate which includes a large and mixed population of hematopoietic stem cells and inflammatory cells.

Dash et al. conducted the only randomized controlled trial investigating the use of MSCs in 24 patients with non-healing lower extremity ulcers secondary to diabetes or vasculitis. Autologous MSCs expanded in culture were injected intramuscularly into the wound edges of the treatment group. Twelve weeks after implantation, ulcer size in the MSC-treated group decreased 73% while those receiving standard wound care only decreased 23%. In addition, subjects receiving MSC injections increased their pain-free walking distance 7.5-fold compared to 2.2-fold in the control group with no reported adverse effects (Dash et al., 2009). Increased numbers of mature immune cells in the dermis of wound biopsies in the treatment group suggest an augmented inflammatory response as a possible mechanism for enhanced repair.

MSCs ENHANCE WOUND HEALING BY DIFFERENTIATION INTO EPIDERMAL CELLS

There is data to suggest that MSCs mobilize from the bone marrow niche and traffic to ischemic tissue via the peripheral circulation in response to cytokine signaling (Hamou et al., 2009). Once at the site of injury, it is hypothesized that they contribute to wound healing by differentiating into various cells of the epidermis and dermis (**Figure 1**).

In culture, MSCs have been observed to differentiate into K14+ keratinocytes when grown in the presence of the growth factor BMP-4 (Sasaki et al., 2008). Histological examination of murine wounds treated with GFP + MSCs has demonstrated various levels of direct engraftment of donor cells into the epidermis as mature keratinocytes (Badiavas et al., 2003; Fathke et al., 2004; Harris et al., 2004). There is also evidence that transplanted MSCs are capable of ongoing differentiation as the percentage of GFP+ keratinocytes increases when wounds are analyzed over time. However, long-term engraftment has not been observed (Wu et al., 2007). Furthermore, differentiated cells have been observed to maintain active proliferation *in vivo* (Borue et al., 2004).

MSCs ENHANCE NEOVASCULARIZATION DURING WOUND REPAIR

New vessel formation, or neovascularization, is a critical component of wound healing as it is necessary to supply oxygen and nutrients to and carry waste away from the damaged tissue. Neovascularization can occur by two mechanisms: angiogenesis and vasculogenesis. During angiogenesis, tissue ischemia

Table 1 | Study design and results for treatment of cutaneous wounds with mesenchymal stem cell therapy.

Species	Wound type	Therapy type	Delivery method	Control(s)	Findings	Reference
Mouse	Excisional wounds	Concentrated conditioned medium from allogeneic P3 MSCs	Single subcutaneous injection of 80 ul and topical application of 20 ul conditioned medium	Concentrated conditioned medium from dermal fibroblasts	Accelerated wound closure. Increased recruitment of macrophages and endothelial progenitor cells	Chen et al., 2008
Mouse	Excisional wounds	Allogeneic P3 MSCs	Single systemic injection of 1×10^6 cells	PBS	Accelerated wound healing	Sasaki et al., 2008
Mouse	Excisional wounds	Allogeneic P3-6 MSCs	Topical application of 2.5×10^5 cells seeded on hydrogel scaffold	No treatment, hydrogen alone, and intradermal injection	Accelerated wound healing. Increased angiogenesis and restoration of hair follicles and sebaceous glands.	Rustad et al., 2012
Mouse (wild type and diabetic)	Excisional wounds	Allogeneic P3-5 MSCs	Single intradermal injection of 1×10^6 cells	Dermal fibroblasts	Accelerated wound closure. Increased granulation tissue, angiogenesis, and restoration of hair follicles.	Wu et al., 2007
Mouse (diabetic)	Excisional wounds	Allogeneic P33 MSCs	Single topical application of 7.5×10^5 cells	PBS	Accelerated wound closure. Increased granulation tissue and angiogenesis.	Javazon et al., 2007
Rat	Incisional fascial wounds	Allogeneic P3-5 MSCs	Four systemic injections of 2×10^6 cells OR. Single intradermal injection of 6×10^6 cells	PBS	Increased wound burst strength. Increased collagen composition	McFarlin et al., 2006
Rat (diabetic)	Incisional fascial wounds	Allogeneic P2-6 MSCs	Four systemic injections of 1.5×10^6 cells OR. Single intradermal injection of 6×10^6 cells	PBS	Increased wound burst strength. Increased collagen composition	Kwon et al., 2008
Human	Chronic non-healing wounds ($n = 3$)	Autologous bone marrow aspirate and cultured MSCs	Subcutaneous injection of bone marrow aspirate and 1–3 topical applications of MSCs	None	Complete closure of wounds. Increased inflammatory response and angiogenesis.	Badiavas and Falanga, 2003

(Continued)

Table 1 | Continued

Species	Wound type	Therapy type	Delivery method	Control(s)	Findings	Reference
Human	Chronic non-healing wounds ($n = 24$)	Autologous P0 MSCs	Intramuscular and subcutaneous injection of $> 1 \times 10^6$ cells/cm^2 ulcer area and topical application	Standard wound care	Decreased wound size. Increased pain-free walking distance	Dash et al., 2009
Human	Acute ($n = 4$) and chronic ($n = 6$) non-healing wounds	Autologous P2-10 MSCs	1–3 topical applications by fibrin spray	None	Complete healing of acute wounds. Reduction or complete closure of chronic wounds. Dose dependent effect.	Falanga et al., 2007
Human	Chronic non-healing wounds ($n = 20$)	Autologous P0 MSCs	Topical application of MSC seeded collagen sponge	None	Complete closure of 13 wounds. Partial closure of five wounds.	Yoshikawa et al., 2008

MSC, mesenchymal stem cell; P, passage.

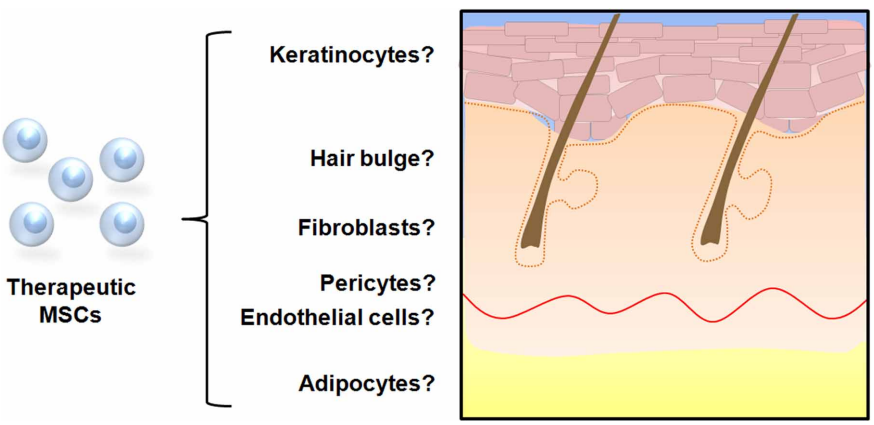

FIGURE 1 | Possible cell fate of mesenchymal stem cells in cutaneous repair. *In vitro* studies have demonstrated that MSCs are capable of differentiating into cells with keratinocytic, fibroblastic, endothelial, and adipocytic phenotypes when cultured under specific conditions. Several studies utilizing wound healing models and transplanted labeled MSCs have provided evidence that cellular differentiation occurs *in vivo* to contribute to cutaneous repair.

initiates signals that stimulate mature resident endothelial cells to proliferate and sprout new vessels (Folkman, 1995). In contrast, vasculogenesis involves the formation of de novo blood vessels from circulating vascular progenitor cells that home to the ischemic site (Tepper et al., 2005). *In vitro* experiments have demonstrated that MSCs are capable of differentiating into vessel forming endothelial cells suggesting that they may contribute to postnatal vasculogenesis during the wound healing process. When cultured in medium supplemented with VEGF, MSCs exhibit an endothelial-like phenotype such as expression of the vascular markers von Willebrand Factor (vWF), kinase insert domain receptor (KDR), and vascular cell adhesion molecule (VCAM). Furthermore, these MSCs form tube-like structures when cultured on Matrigel, an established *in vitro* model for neovessel formation (Oswald et al., 2004).

Similar evidence for endothelial differentiation has been extended to animal models. In a parabiosis model, GFP+ MSCs were found to traffic from the bone marrow to ischemic wounds and engraft into neovessels. Approximately 12% of all endothelial cells within the wound bed were determined to originate from donor MSCs (Hamou et al., 2009). Similar wound healing studies report the incidence of MSC-derived endothelial cells ranging from 0.1% to 13% (Badiavas et al., 2003; Fathke et al., 2004; Sasaki et al., 2008). MSC-treated excisional wounds in BALB/c mice demonstrated nearly two times the capillary density as quantified by CD31 staining than vehicle and fibroblast-treated wounds. In this study, engrafted MSCs were located in the

perivascular space as opposed to the endothelium suggesting differentiation into pericytes. These cells do not directly incorporate into neovessels but are proposed to participate in angiogenesis by guiding endothelial sprouts (Nehls et al., 1992). Current research is elucidating how pericytes continue to support and regulate mature vessels through local secretion of soluble growth factors and mechanical signaling (Hirschi and D'Amore, 1997; Gerhardt and Betsholtz, 2003; Wu et al., 2007; Rustad et al., 2012).

MSC-MEDIATED PARACRINE SIGNALING ENHANCES WOUND REPAIR

There is growing evidence to suggest that MSCs may elicit the majority of their wound healing properties via paracrine mechanisms. When compared to dermal fibroblasts, which are normally the main source of growth factors during cutaneous wound healing, MSCs express significantly greater amounts of VEGF-A, epidermal growth factor (EGF), erythropoietin, and stromal cell-derived factor -1α (SDF-1α) (Chen et al., 2008). The same group demonstrated that soluble proteins secreted by MSCs are potent mitogens. For example, keratinocytes and endothelial cells exhibit significantly greater proliferation rates when cultured in conditioned medium from MSCs compared to medium from fibroblast cultures. These proteins are also powerful chemoattractants and promote the migration of inflammatory cells, endothelial cells, and keratinocytes (Chen et al., 2008). Paracrine factors from MSCs have also been shown to stimulate collagen secretion from dermal fibroblasts *in vitro* (Kim et al., 2007). Excisional wounds treated with conditioned medium alone from MSC cultures demonstrate accelerated closure in wild-type mice, corroborating the importance of MSC-secreted factors in wound healing. These wounds also exhibited increased recruitment of macrophages, key cells in the acute healing process, and CD34+ and c-kit+ cells which have been described as the putative endothelial progenitor cell (Chen et al., 2008).

OPTIMIZING DELIVERY OF MSCs TO CUTANEOUS WOUNDS

As evidence for the wound healing capacity of MSCs continues to grow, research has now shifted toward modalities to optimize cell delivery as studies have shown that the clinical effectiveness of MSC-therapy is dependent on the number of cells delivered (Falanga et al., 2007). Most studies have utilized the technically simple method of injecting a cell suspension intradermally into or around the wound defect. As described, this method has demonstrated enhanced wound healing, however, the true therapeutic potential of MSCs appears to be limited due to poor engraftment efficiency and cell retention at the wound site (Freyman et al., 2006). Causes of this phenomenon are still under investigation with evidence suggesting that the hostile wound environment may impede high MSC engraftment in acute wounds. Elevated levels of reactive oxygen species such as those found in ischemic wounds are thought to impede cell engraftment in tissue (Angelos et al., 2006; Yao et al., 2006). In addition, the shear forces generated by the injection process itself may lead to anoikis (Rustad et al., 2012). Alternative delivery systems are therefore being investigated to enhance MSC function within non-healing wounds (**Figure 2**).

Falanga et al. utilized a fibrin spray system to topically administer autologous MSCs to non-healing lower extremity wounds in human subjects. Stem cells were found to survive within the fibrin layer and migrate into the wound tissue. One subject demonstrated no improvement, four had an average 40% reduction in wound size, and one subject had complete closure of a wound that had previously existed for over 10 years (Falanga et al., 2007).

Building on the idea of providing a scaffolding and external niche from the ischemic tissue, Yoshikawa et al. developed a composite graft combining a commercially available collagen matrix with cultured autologous MSCs. Grafts were applied to 20 patients with intractable dermatopathies due to thermal burns, traumatic wounds, and decubitus ulcers. Thirteen of the 20 wounds demonstrated regeneration of fibrous and fat tissue and underwent re-epithelialization resulting in complete wound closure. Treatment with the composite graft led to sufficient granulation tissue and dermal regeneration to allow for successful skin grafting in an additional five wounds (Yoshikawa et al., 2008).

Hydrogels are synthetic biomaterials that emulate the hygroscopic nature of extracellular matrix making them an ideal vehicle for MSC delivery (Lutolf and Hubbell, 2005). A novel collagen-pullulan hydrogel that is non-cytotoxic and provides protection from oxidative stress was recently described (Wong et al., 2011a). MSCs seeded and cultured in this hydrogel demonstrate significantly greater expression of the stemness genes Oct-4, SOX2, and KLF4 compared to cells plated on standard two-dimensional culture dishes. Secretion of the wound healing and angiogenic cytokines MCP1 and VEGF-A are also found to be increased. Topical hydrogel delivery of MSCs demonstrated significantly accelerated wound closure and improved quality of cutaneous regeneration with greater return of hair follicles and sebaceous glands when compared to intradermal injection strategies. The number of MSCs found within the wound tissue was nearly three times greater at day 7 post-wounding and nearly 10 times greater at day 10 in animals receiving the seeded hydrogen compared to local injection. Co-localization analysis of healed wounds revealed a small percentage (12.5%) of MSCs expressing the endothelial cell marker CD31 representing cells directed toward angiogenesis. The vast majority of engrafted cells differentiated into dermal fibroblasts and pericytes suggesting the wound healing effects were largely secondary to enhanced secretion of paracrine factors. Indeed, levels of VEGF, FGF1, MMP8, and MMP9 were all found to be significantly higher in tissue from wounds treated with MSCs delivered by hydrogel versus intradermal injection (Rustad et al., 2012).

HETEROGENEITY IN MSC PREPARATIONS

Despite the data supporting the potential of MSC-based therapy for wound repair, controversy remains. The reported contribution of MSC engraftment to wound repair varies widely in the literature with some authors reporting little to no evidence of cellular engraftment (Duffield et al., 2005; Rustad et al., 2012). The reason for these discrepancies is likely multi-factorial with cell population heterogeneity being a possible contributor. Clonal

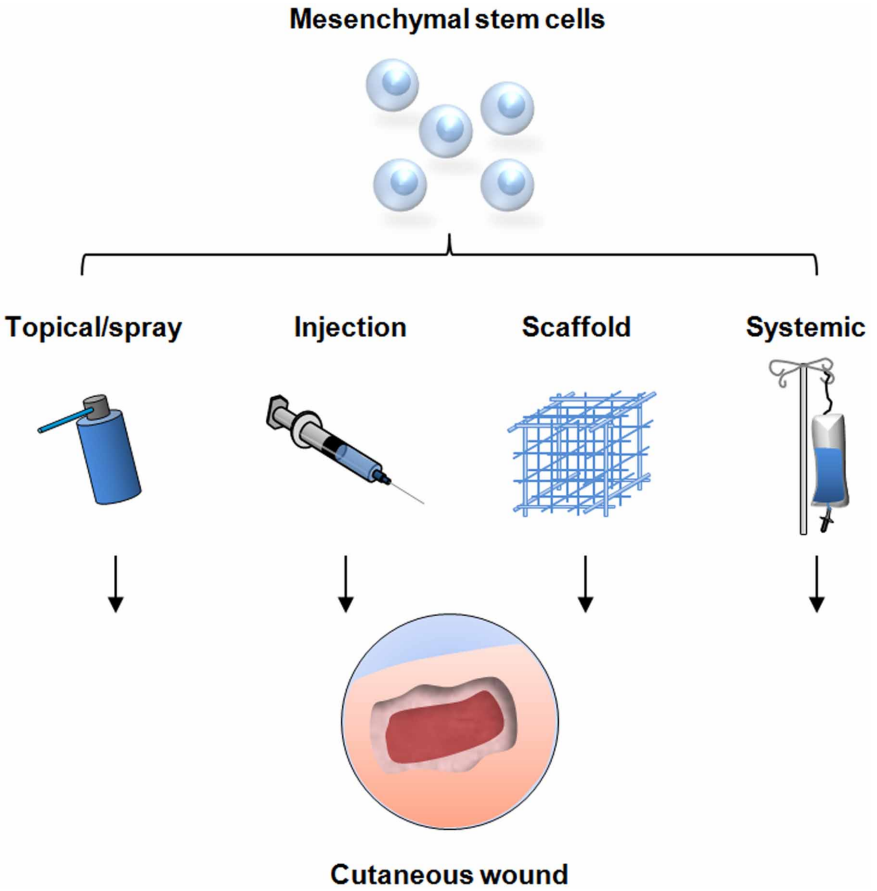

FIGURE 2 | Strategies for mesenchymal stem cell delivery to cutaneous wounds. Traditional techniques include local injection of cells into the soft tissue, direct topical application, and systemic delivery via injection into the peripheral circulation. These methods have resulted in improved wound healing but are limited by sub-optimal cell survival and engraftment. Novel delivery methods are being developed utilizing tissue scaffolds to optimize stem cell function and maximize the therapeutic potential for cellular therapy.

studies have demonstrated that even with identical isolation and expansion methods, MSC isolation by the traditional plastic adherence technique results in cells that are functionally heterogenous with varying capacities of differentiation (Phinney et al., 1999). Previous studies have also used MSCs from various culture passages, however, investigators have shown that MSCs exhibit different gene expression profiles as they undergo serial passage (Gregory et al., 2005). In addition, MSC function is highly dependent on cues from the culture condition therefore different seeding densities and growth media utilized by investigators add to the heterogeneity of cell preparations used in these studies. These issues speak to the importance of establishing a standardized language when isolating and defining MSCs in the literature and the need for a method of prospective isolation by specific cell surface markers.

IMMUNOMODULATORY PROPERTIES OF MSCs

An important property of MSCs which has been demonstrated both *in vitro* and *in vivo* is the immunosuppressive effect elicited by allogeneic cells. Human MSCs have been shown to suppress CD4+ and CD8+ T-cell proliferation through the secretion of soluble factors including hepatocyte growth factor (Di Nicola et al., 2002) and alter the cytokine secretion profiles of dendritic cells, effector T-cells, and natural killer cells to more anti-inflammatory phenotypes (Aggarwal and Pittenger, 2005). This phenomenon has been exploited to use MSC-therapy to help treat skin graft rejection and graft-versus-host disease (Bartholomew et al., 2002; Le Blanc et al., 2004). How this property may affect the role of MSCs in wound healing has yet to be fully investigated. Some authors have anecdotally theorized that the beneficial effects of MSC therapy on cutaneous repair may in part be due to the prevention or reversal of chronic inflammation. The possible negative side effects of immunosuppression also raise questions that continue to be investigated concerning increased tumorigenicity of cancer cells in animals receiving MSC injections (Djouad et al., 2003). Further work in understanding the systemic effects of MSC are certainly required especially if the use of allogeneic cells is to be considered a clinical possibility.

CONCLUSIONS AND FUTURE WORK

The possible benefits of MSC-based therapy in the clinically important area of chronic wounds have been demonstrated in

numerous studies. Administration of MSCs has been shown to augment the acute inflammatory response, enhances angiogenesis, accelerates re-epithelialization, and increases wound strength. More importantly, these effects have been observed in clinically relevant conditions of impaired healing such as diabetes.

Although these preliminary findings are promising, several areas require further investigation before large-scale randomized human studies can become feasible. Although the International Society for Cellular Therapy has published minimum criteria to define human MSCs, significant heterogeneity certainly exists within this population (Dominici et al., 2006). Researchers have typically utilized plastic adherence to isolate MSCs in culture, however, macrophages, lymphocytes, endothelial cells, and smooth muscle cells also adhere to plastic and may contaminate early passage populations (Deans and Moseley, 2000). Even in late passage MSCs, cells display morphological and functional heterogeneity (Javazon et al., 2004). Identifying these various subpopulations, understanding their phenotypic properties, and developing methods for prospective isolation by surface marker profiles will be a crucial step in optimizing directed therapy. Also, it remains to be elucidated if the primary contribution of MSCs to cutaneous regeneration is by cellular differentiation or indirectly through paracrine activity. A better understanding of the mechanism of action is needed to develop more efficient treatment strategies. Long-term systemic effects of MSC-therapy have yet to be fully established. Limited data has suggested that the immunosuppressive properties of MSCs could increase susceptibility to malignancies and opportunistic infections (Djouad et al., 2003; Sundin et al., 2006). Additionally, although no instances have been reported in humans, BM-MSCs have been shown to be able to differentiate into carcinoma-associated fibroblasts and sarcomas (Tolar et al., 2007; Mishra et al., 2008). Finally, further investigation into delivery methods specifically designed for the delivery progenitor cells to chronic wounds is necessary to maximize the regenerative properties of MSC-based cell therapy.

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Mesenchymal stromal cells in renal ischemia/reperfusion injury

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Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival in kidney transplantation. Renal I/R injury can lead to fibrosis and graft failure. Although the exact sequence of events in the pathophysiology of I/R injury remains unknown, the role of inflammation has become increasingly clear. In this perspective, mesenchymal stromal cells (MSCs) are under extensive investigation as potential therapy for I/R injury, since MSCs are able to exert immune regulatory and reparative effects. Various preclinical studies indicate the beneficial effects of MSCs in ameliorating renal injury and accelerating tissue repair. These versatile cells have been shown to migrate to sites of injury and to enhance repair by paracrine mechanisms instead of by differentiating and replacing the injured cells. The first phase I studies of MSCs in human renal I/R injury and kidney transplantation have been started, and results are awaited soon. In this review, preliminary results and opportunities of MSCs in human renal I/R injury are summarized. We might be heading towards a cell-based paradigm shift in the treatment of renal I/R injury.

Keywords: mesenchymal stromal cells, stem cells, ischemia/reperfusion injury, kidney transplantation

INTRODUCTION

Ischemia/reperfusion (I/R) injury is the exacerbation of tissue damage upon reestablishment of circulation after a period of ischemia. I/R injury is considered a major contributor to tissue damage in multiple clinical situations such as myocardial infarction, stroke, and organ transplantation. In many clinical settings, the duration of ischemia is beyond control, and preventive and therapeutical measures are required to reduce the extent of I/R injury. Unfortunately, current treatment is primarily supportive. The pathophysiology of I/R injury is multifactorial and only partially understood. However, the general local reaction to reperfusion is thought to involve an inflammatory response that leads to tissue damage. In the quest for new therapeutical options for renal I/R injury, stem cells have come into play. With their multipotent immune modulating properties they hold promise to lead to improvement in the treatment of renal I/R injury.

PATHOPHYSIOLOGY OF ISCHEMIA/REPERFUSION INJURY

Although there may be differences in the exact pathophysiological mechanisms of I/R injury between different organs, some processes appear to play a universal role (Eltzschig and Eckle, 2011). The endothelium and microvasculature are very sensitive to hypoxia and easily affected in I/R injury. Upon reperfusion, the vascular endothelial cell lining can undergo swelling which may lead to narrowing of the vascular lumen (Summers and Jamison, 1971; Leaf, 1973). Moreover, vasorelaxation can be impaired, together contributing to the no-reflow phenomenon (Lieberthal et al., 1989). Endothelial injury can increase microvascular permeability which may lead to inflammatory cell recruitment into the diseased organ. There have been many reports of invading granulocytes, monocytes, dendritic cells (DCs), and lymphocytes after reperfusion (Shigematsu et al., 2002; Burne-Taney et al., 2003; Day et al., 2005, 2006; de Vries et al., 2011).

Together with leukocytes, platelets can be activated by injured endothelium. In myocardial infarction, platelets mediate thrombotic occlusion and increase damage by contributing to the no-reflow phenomenon (Gawaz, 2004). However, platelets are also able to invade the tissue (Weissmuller et al., 2008). This is essential since platelets can contribute to the inflammatory response through release of cytokines, chemokines, and growth factors from their granules (Reed, 2004; Lisman and Porte, 2010; Thornton et al., 2010). In fact, platelets have been suggested to be involved in the inflammatory response of I/R injury in various organs. They are able to roll and adhere to post-reperfusion endothelium in a P-selectin-dependent mechanism (Massberg et al., 1998; Sindram et al., 2000; Khandoga et al., 2002; am Esch et al., 2005). In mouse myocardial tissue, the first activated platelets are present within minutes after reperfusion (Xu et al., 2006), and then accumulate in the infarcted myocardium (Liu et al., 2011).

The ensuing inflammatory response is considered to exacerbate damage. Both the innate and the adaptive immune system can be activated after reperfusion. Activation of the innate immune system is probably mediated via pattern-recognition receptors such as toll-like receptors that recognize their endogenous ligands that are released upon tissue damage (Chen and Nunez, 2010). Besides, the complement system is part of the humoral immune response and can play a role both as first line innate defense, but may also contribute to the adaptive immune response (Dunkelberger and Song, 2010). In many animal experiments a role for (terminal)

complement activation in I/R injury has been suggested (Zhou et al., 2000; Park et al., 2001; de Vries et al., 2003; Zheng et al., 2008; Zhang et al., 2011), although recent experiments doubt the involvement of the complement system itself in the initiation of injury (van der Pol et al., 2012). The role of complement activation in human I/R injury is even more complex. While in human myocardial I/R injury a role of complement activation was suggested (Yasojima et al., 1998; Baldwin et al., 1999), the diverse intervention studies using anti-complement therapy did not lead to major improvements (Granger et al., 2003; Mahaffey et al., 2003; Lazar et al., 2004; Verrier et al., 2004; Testa et al., 2008).

Ischemia-related metabolic adaptations and dysregulated mitochondrial homeostasis are thought to result in substantial release of reactive oxygen and nitrogen species (RONS) upon reintroduction of oxygen. The RONS overload can overwhelm the endogenous antioxidant system, resulting in oxidative damage. This may trigger secondary processes and contribute to the pro-inflammatory response upon reperfusion (Crimi et al., 2006; Valko et al., 2007; Gourdin et al., 2009; Misra et al., 2009). Numerous animal studies clearly demonstrated that antioxidant therapy ameliorates I/R injury (Ambrosio et al., 1991; Yellon and Hausenloy, 2007; Lakhan et al., 2009). Despite these findings, studies in humans consistently fail to show any clinically relevant effect (Land and Zweler, 1997; Bath et al., 2001; El-Hamamsy et al., 2007; Yellon and Hausenloy, 2007; Suzuki, 2009). The basis for this discrepancy between human and animal studies is still unclear, yet it may suggest that the contribution of RONS to I/R injury in humans is less than commonly thought.

Ultimately, when I/R injury to the cell is severe, various programs of cell death can be activated. There are three major forms of cell death: necrosis, apoptosis, and autophagy. Besides acute cell death during and directly after the ischemic period, cell death continues for several days following reperfusion. All three types of cell death can contribute to the continued loss of cells for days and even weeks in the reperfused tissue (Zhao et al., 2000, 2001). Autophagy during the ischemic episode appears to keep cells viable and might play a protective role. However, it is suggested that activation of autophagy after reperfusion is detrimental (Matsui et al., 2007; Hariharan et al., 2011).

ISCHEMIA/REPERFUSION INJURY IN KIDNEY TRANSPLANTATION

Ischemia/reperfusion injury is an inevitable consequence of kidney transplantation. Graft survival for living unrelated donation is superior compared to grafts from brain dead and cardiac dead donors, although the average human leukocyte antigen (HLA) matching is worse (Terasaki et al., 1995). Therefore, the poor graft survival of deceased donor kidneys cannot be exclusively attributed to differences in immunogenicity. I/R injury can induce delayed graft function and has a major influence on graft function and survival (Yarlagadda et al., 2009).

Inflammation is regarded the crucial event in the development of tissue injury and graft dysfunction in renal I/R injury. Many individual factors, such as cytokines and complement have been identified to be involved in the inflammatory response. However, intervention studies aiming at specific inhibition of a single factor have generally shown disappointing results (Park et al., 2001; de Vries et al., 2009). Cooperation, redundancy, and interactions play a role and mechanisms appear to be more complex than previously thought. Pharmacological inhibition of the entire inflammatory cascade would appear a logical intervention, however, the negative side effects appear larger than the anticipated beneficial effects (Morariu et al., 2005).

ISCHEMIA/REPERFUSION INJURY: LONG-TERM IMPACT

Although short-term results of kidney transplantation are excellent, 5 year graft loss can be up to 30% in older recipients (Keith et al., 2006). Protocol biopsies obtained in the first years after transplantation have shown interstitial fibrosis/tubular atrophy (IF/TA). This finding has been correlated with later allograft dysfunction and loss (Nankivell et al., 2003; Park et al., 2010). Both allogen dependent and independent factors determine IF/TA. I/R injury is an important non-allogeneic factor and the duration of the cold ischemic period is directly correlated to delayed graft function and even allograft failure (Ojo et al., 1997; Salahudeen et al., 2004). I/R injury itself, without allogeneic transplantation, has been shown to cause interstitial fibrosis and glomerulosclerosis in experimental models (Tullius et al., 1994; Herrero-Fresneda et al., 2000; Basile et al., 2001; **Figure 1**).

RENAL REPAIR

In recent years, it has become clear that in response to kidney injury not only fibrotic repair but also restoration of damaged kidney tissue can occur. This has been best established for acute kidney injury, where surviving resident tubular epithelial cells dedifferentiate and subsequently re-enter the cell cycle to replace the necrotic tubular epithelium. Dedifferentiated cells outside the injured kidney may also migrate to the site of injury within the kidney. Kidney biopsies in male recipients of a female donor kidney with acute tubular necrosis showed presence of the male Y chromosome in renal tubular cells. No Y chromosome staining was seen in patients without acute tubular necrosis. This provides evidence that extra-renal cells participate in renal regeneration (Poulsom et al., 2001; Gupta et al., 2002).

The call for better treatment strategies for I/R injury has directed research toward more encompassing cellular-based therapies, particularly aimed at the use of stem cells. The multi-factorial pathophysiology of I/R injury makes a pharmacological agent that has a single mechanistic target less likely to be therapeutically effective. In contrast, stem cells are versatile, and able to target a whole cascade of repair mechanisms simultaneously and successively, thereby improving organ protection and repair.

MESENCHYMAL STROMAL CELLS

Of all bone marrow (bm)-derived cells, mesenchymal stromal cells (MSCs) hold special promise in attenuating kidney injury, since nephrons are largely of mesenchymal origin and stromal cells are of crucial importance for signaling leading to differentiation of both nephrons and collecting ducts. MSCs are characterized by three main criteria: (1) The ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*, (2) the expression of surface makers CD73, CD90, and CD105, and lack of expression of haematopoietic markers including CD34 and CD45, and (3) plastic adherence in culture (Dominici et al., 2006).

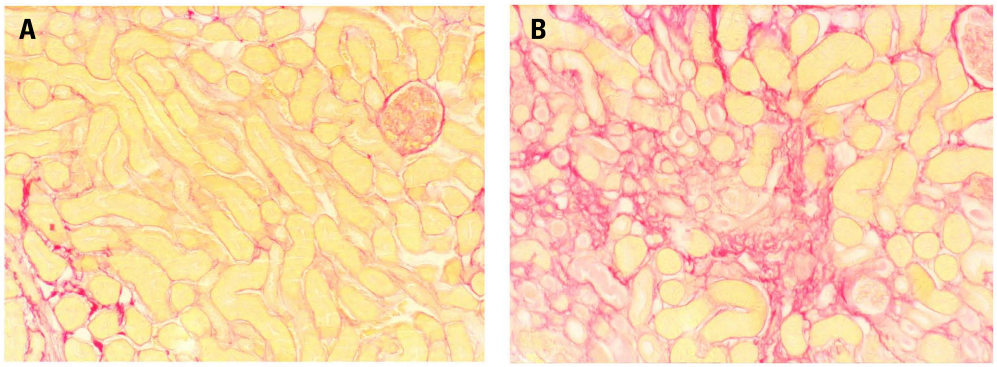

FIGURE 1 | Male, 8-week-old Bl6 mice underwent bilateral (warm) renal ischemia for 25 min by clamping of the renal artery and vein. Long-term structural damage and fibrosis were assessed. I/R injury induced severe patchy renal fibrosis three weeks after ischemia, although kidney function partially recovered. Sirius red staining shows **(A)** normal mouse kidney and **(B)** severe fibrosis 3 weeks after the mouse underwent 25 min of warm renal I/R injury (Representative images, unpublished data).

Mesenchymal stromal cells have the ability to secrete numerous growth factors and cytokines that collectively stimulate mitogenesis, inhibit apoptosis, and modulate immune responses. They can alter cytokine secretion profiles of T cells (Krampera et al., 2003), DCs, and natural killer cells to induce a more anti-inflammatory or tolerant phenotype (Aggarwal and Pittenger, 2005; Stagg, 2007). These immune modulating effects could be achieved both with autologous and allogeneic MSCs.

An important aspect of the effect of MSCs is their ability to home to areas of injury or inflammation. Exogenously administered MSCs can engraft into various injured structures in the kidney (Ninichuk et al., 2006; Herrera et al., 2007; Wong et al., 2008). Recently, studies have shed light on the exact factors that facilitate homing of MSCs. Amid them, CD44 and hyaluronic acid interactions, and stromal-derived factor-1 (SDF-1) and CXCR4 interactions may be crucial in recruiting exogenous MSCs to injured renal (Togel et al., 2005b; Herrera et al., 2007).

SOURCES OF MSCs

While initially isolated from the bm, MSCs have now been identified within most tissues and are thought to represent a perivascular cell population involved in normal tissue homeostasis (Crisan et al., 2008). Indeed, MSCs have been isolated from adipose tissue, umbilical cord (uc) blood, placenta, and various organs (Zuk et al., 2002; Morigi et al., 2004; Toma et al., 2005; da Silva et al., 2006; Hoogduijn et al., 2006). Recently, MSCs have also been isolated from the human and mouse kidney. In mice these cells were extensively compared to bmMSCs (Pelekanos et al., 2012). Transcriptome and immunophenotype analysis of the renal MSC-like populations supported strong congruence with bmMSCs. Future studies need to elucidate whether regeneration and functional repair can be enhanced via the resident renal stem cells. In the meantime, bmMSCs are the best characterized population and currently more than 200 clinical trials are ongoing using bmMSCs[1].

[1]www.clinicaltrials.gov

MSCs AMELIORATE RENAL ISCHEMIA/REPERFUSION INJURY *IN VIVO*

Although MSCs most probably do not replace damaged cells, evidence on beneficial effects of MSCs in renal I/R injury is accumulating in animal experiments. Intravenous injection of bm-derived lineage-negative pluripotent cells after experimental renal I/R significantly attenuated the creatinine rise (Duffield et al., 2005). Peripherally administered purified MSCs significantly attenuated functional and histological damage (Furuichi et al., 2012). Even when administered 24 h or later after I/R injury, MSCs still were able to ameliorate damage and fibrosis (Lange et al., 2005; Togel et al., 2005a; Donizetti-Oliveira et al., 2012). In experimental renal allograft transplantation MSCs decreased inflammation (Hara et al., 2011).

Different studies have reported beneficial effects of human MSCs on acute repair in the kidney (Morigi et al., 2006). The therapeutic potential of human bmMSCs was studied in immunodeficient NOD-SCID mice. Infused human bmMSCs reduced renal cell apoptosis and increased proliferation after cisplatin-induced acute renal failure. bmMSCs also preserved the integrity of the tubular epithelium and peritubular vessels, and prolonged survival (Morigi et al., 2008). In search for new sources of MSCs for renal repair, human ucMSCs were shown to ameliorate both renal dysfunction and tubular cell injury, and prolong survival in cisplatin-induced acute kidney injury (Morigi et al., 2010).

The mechanism of MSC-induced kidney repair has been the subject of numerous studies. There is growing evidence that the process of transdifferentiation is probably not relevant to renal repair *in vivo*. The primary means of these cells most likely involve paracrine and endocrine effects; including mitogenic, anti-apoptotic, anti-inflammatory, antifibrotic, and angiogenic influences (**Figure 2;** Ninichuk et al., 2006). The factors that mediate the paracrine effects are obviously of great interest. Several factors that are abundant in MSC-conditioned medium have been mentioned (Togel et al., 2007). Recently, it was suggested that microvesicles released from MSCs may account for this paracrine mechanism. Administration of isolated microvesicles from human MSCs indeed protected

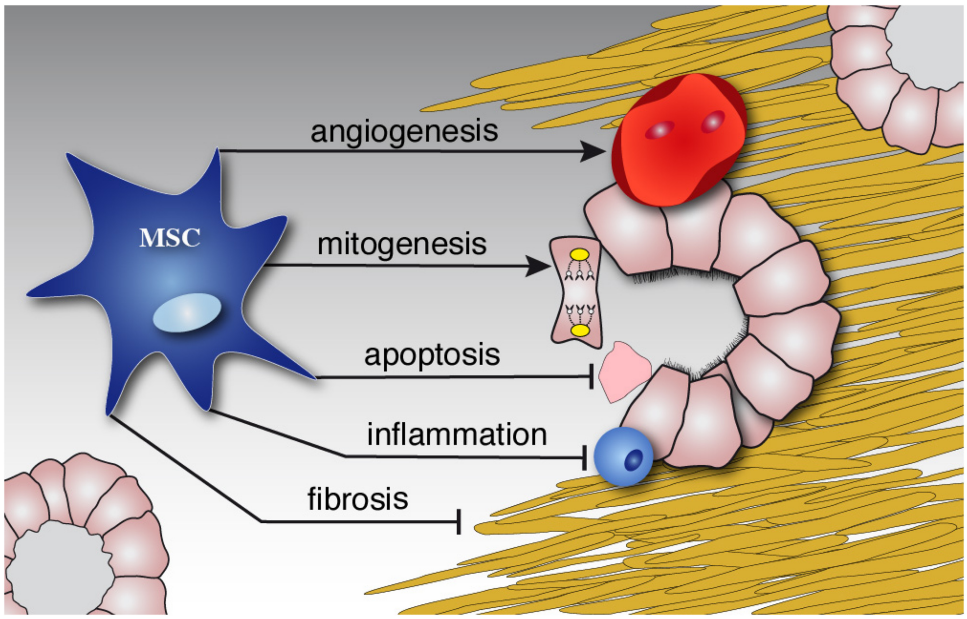

FIGURE 2 | MSCs diminish damage and induce repair. Schematic illustration of the paracrine effects of MSCs on the kidney. While stimulating repair by mitogenic and angiogenic effects, MSCs inhibit ongoing inflammation, apoptosis and later fibrosis of injured tissue.

rats from acute ischemic kidney injury (Bruno et al., 2009; Gatti et al., 2011).

CLINICAL APPLICATIONS OF MSCs IN RENAL DISEASE

There are only limited clinical data about MSC therapy in renal disease. The first safety and feasibility data of autologous MSC administration in the week after kidney transplantation were published in 2011 (Perico et al., 2011). Although data are limited to two patients, MSC infusion appeared feasible and restricted memory T cell expansion while enlarging Treg population. However, both patients showed transient increase in serum creatinine levels within 2 weeks after cell infusion that might be related to intragraft recruitment of granulocytes, suggesting that timing of infusion is of particular importance (Ortiz et al., 2003; Fang et al., 2004; Lange et al., 2005). This is probably related to the necessity for the appropriate micro-environment to allow MSCs to acquire their anti-inflammatory properties. In addition, in a recent study the use of autologous MSCs resulted in lower incidence of acute rejection, decreased risk of opportunistic infection and better estimated renal function at 1 year compared with anti-IL-2 receptor antibody as induction therapy (Tan et al., 2012). In our clinical trial we investigate safety and feasibility of autologous bmMSC treatment in patients with subclinical rejection and/or IF/TA in the renal biopsy at 4 weeks or 6 months after renal transplantation (Clinical trials NCT00734396). Hereby we expect to provide additional information about the importance of timing in the transplant setting.

AUTOLOGOUS VERSUS ALLOGENEIC MSCs

Until now, most studies have focused on the use of autologous cells since allogeneic cell transplantation may promote allograft rejection and possibly sensitization (Nauta et al., 2006; Stagg et al., 2006). However, autologous MSCs also have disadvantages. The cells need weeks of culture and a concern for the use of autologous MSCs includes their potential dysfunction due to the underlying disease. Few studies have reported influence of renal failure on MSC behavior. In mice, functional incompetence of MSCs was reported under uremic conditions (Noh et al., 2012). In addition, in human MSCs it was shown that uremic serum induced an osteoblast-like phenotype in MSCs accompanied by matrix remodeling and calcification (Kramann et al., 2011). In contrast, it was recently shown that human adipose tissue-derived MSCs are not affected by renal disease (Roemeling-van Rhijn et al., 2012).

MSC NUMBER, ROUTE OF ADMINISTRATION, AND INTERACTION WITH IMMUNOSUPPRESSIVES

Alongside the cell source, the number of MSCs and the timing of administration are critical. In most clinical trials doses of 0.4 to 10×10^6/kg body weight were used (Lazarus et al., 2005; Le Blanc et al., 2008; Macmillan et al., 2009). However, no clear correlations have been made between cell dose and clinical effect. Dose escalation studies to monitor safety and efficacy are one of the major objectives for future studies of MSCs.

Mesenchymal stromal cells have been administered intravenously in most human trials. Other possible successful routes of administration include intra-arterial or intra-renal infusion (Kunter et al., 2006, 2007; Ding et al., 2009). An advantage of these routes may be the direct administration at the place of injury, whereas disadvantages include the complexity and possible side effects such as obstruction of capillaries. To date, there are no reports of these treatment modalities in humans.

Current immunosuppressive drugs cannot be withheld from patients receiving MSC treatment after renal transplantation. Therefore, it is of importance that an optimal concurrent immunosuppressive regimen is chosen in which drugs have no negative impact on MSC function and vice versa. So far, this interaction has mainly been assessed by *in vitro* studies (Maccario et al., 2005; Prevosto et al., 2007) and future studies are needed to elucidate their interaction with concurrent immunosuppression *in vivo* in order to facilitate successful translation to the clinic.

POSSIBLE HURDLES OF MSC TREATMENT

Although cell therapy with MSCs holds enormous promise for the treatment of many diseases, unwanted side effects of MSC infusions must be assessed with the greatest care. Experimental studies have demonstrated maldifferentiation after injecting MSCs directly into damaged tissue (Breitbach et al., 2007; Kunter et al., 2007). In addition, MSCs may adopt and unwanted, myofibroblast-like phenotype after administration (Wu et al., 2003; di Bonzo et al., 2008). Another important concern is that MSCs may differentiate into neoplastic cells or may cause promotion of tumor cell growth (Djouad et al., 2003; Karnoub et al., 2007; Tolar et al., 2007), although an increased risk of tumor formation has never been confirmed in humans (Centeno et al., 2010). Currently, more than 2000 patients have been treated with allogeneic or autologous MSCs worldwide for a variety of diseases and so far no major side effects have been reported. However, still little is known about long-term side effects.

SUMMARY

The pathophysiology of I/R injury is complex and characterized by inflammation, leading to tissue injury and graft dysfunction. Given current shortage of donor organs and usage of marginal donor kidneys for transplantation, novel treatment options to minimize renal I/R injury are urgently needed. Recent developments in stem cell research and derived clinical stem cell therapies have given reason to believe that such cell-based treatments will become generally available in the near future. Although substantial additional time for the maturation of these therapies for routine clinical use is needed, the first steps of MSC-based therapeutic strategies in the treatment of I/R injury have been taken.

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Detrimental effects of rat mesenchymal stromal cell pre-treatment in a model of acute kidney rejection

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Mesenchymal stromal cells (MSC) have shown immunomodulatory and tissue repair potential including partial tolerance induction by pre-treatment of donor-specific cells in a rat heart transplantation model. Very recently, we could show that autologous MSC attenuated ischemia reperfusion injury in a highly mismatched donor–recipient rat kidney transplant model. Therefore, we investigated donor-specific MSC pre-treatment in this rat kidney transplantation model to study whether graft function could be improved, or if tolerance could be induced. Donor- and recipient-type MSC or phosphate buffered saline (PBS) as a control was injected i.v. 4 days before kidney transplantation. Mycophenolate mofetil immunosuppression (20 mg/kg body weight) was applied for 7 days. Kidney grafts and spleens were harvested between days 8 and 10 and analyzed by quantitative RT-PCR and immunohistology. In addition, creatinine levels in the blood were measured and serum was screened for the presence of donor-specific antibodies. Surprisingly, application of both donor- and recipient-specific MSC resulted in enhanced humoral immune responses verified by intragraft B cell infiltration and complement factor C4d deposits. Moreover, signs of inflammation and rejection were generally enhanced in both MSC-treated groups relative to PBS control group. Additionally, pre-treatment with donor-specific MSC significantly enhanced the level of donor-specific antibody formation when compared with PBS- or recipient MSC-treated groups. Pre-treatment with both MSC types resulted in a higher degree of kidney cortex tissue damage and elevated creatinine levels at the time point of rejection. Thus, MSC pre-sensitization in this model impairs the allograft outcome. Our data from this pre-clinical kidney transplantation model indicate that pre-operative MSC administration may not be optimal in kidney transplantation and caution must be exerted before moving forward with clinical studies in order to avoid adverse effects.

Keywords: mesenchymal stromal cell, inflammation, kidney transplantation, acute rejection, humoral response

INTRODUCTION

Kidney transplantation outcomes have been greatly improved over the last few years by better immunosuppression regimens and post-operative care. However, due to organ shortages, often the donor kidneys available are sub-optimal or so-called "marginal organs," which has been shown to lead to greater problems with immunogenicity and worse long-term function (Audard et al., 2008; Stallone et al., 2010). Several attempts have been developed to help reduce damage to the graft that may occur before the transplant (van der Woude et al., 2004; Kotsch et al., 2007; Caumartin et al., 2011), however, many treatment regimes are not suited for use with human patients. More recent strategies have focused on using cell therapies from different sources to help stimulate the regeneration of cells inside the transplanted organ (Bussolati and Camussi, 2006; Morigi et al., 2006; Choi et al., 2010; Harari-Steinberg et al., 2011; Little, 2011; Bussolati et al., 2012). In particular, the reduction of ischemia reperfusion injury by use of protective cells or their products has been an area of intense research in the hopes of increasing long-term survival and kidney function (Donizetti-Oliveira et al., 2012; Furuichi et al., 2012).

Recently, the potential therapeutic use of mesenchymal stromal cells (MSC) has been investigated in many model systems. Based on the discovery of various properties of MSC to help in the repair of damaged tissues and to promote immunomodulatory functions, a great deal of promise has been invested in this cell type (Yagi et al., 2010; Hoogduijn et al., 2011; Shi et al., 2011; Singer and Caplan, 2011; Tögel and Westenfelder, 2011). In animal experiment models of graft versus host disease (GvHD), skin transplantation, and in particular heart transplantation, MSC have been described as promoting protective effects (Bartholomew et al., 2002; Le Blanc et al., 2004; Maitra et al., 2004; Zhou et al., 2006; Eggenhofer et al., 2011). In a rat heart transplantation model, bone marrow derived donor- and recipient-type MSC administered concurrent to the time point of grafting were not able to prolong heart allograft survival or even led to accelerated rejection with concurrent low-dose Cyclosporin A treatment (Inoue et al., 2006). In contrast, pre-treatment with allogeneic MSC

under mycophenolate mofetil (MMF) immunosuppression in the same rat transplantation model induced partial tolerance toward the transplanted organ, whereas syngeneic cells were less effective (Popp et al., 2009).

Beneficial effects of MSC on renal function were mostly described in models of acute kidney injury induced by temporary vessel ligation. In this experimental setup, MSC administration has been clearly shown to reduce kidney damage as measured by reduced serum creatinine and urea levels (Tögel et al., 2005; Semedo et al., 2007; Cao et al., 2010; De Martino et al., 2010; Morigi et al., 2010). In addition, we could very recently show in a rat renal transplantation model that repeated recipient MSC application was able to ameliorate damage following prolonged cold ischemia at early time points by reducing the expression of pro-inflammatory cytokines and infiltration by antigen-presenting cells (APC) in the grafted kidney (Hara et al., 2011). However, in this acute pre-clinical model the allograft survival was not improved. As these results fell short of our expectations, we have focused on reports in the heart model that indicated that allogeneic MSC under MMF immunosuppression might be more effective (Popp et al., 2009).

Here, we describe that the protocol which was successful in a heart transplant model cannot simply be transferred to kidney transplantation. Allogeneic MSC do not induce tolerance to the graft, but they actually worsen the outcome. We have found that the deleterious effects of both donor- and recipient-type MSC are related to the induction of humoral immune responses, associated infiltration of B cells, and increased C4d deposits attributed to complement activation in the allograft. We also found indications that the allogeneic MSC could lead to a pre-sensitization of the recipient to donor antigens as shown by the enhancement of donor-specific antibodies that could accelerate the pace of organ rejection instead of hindering it.

MATERIALS AND METHODS

ANIMALS

Adult male Dark Agouti (DA; MHC haplotype $RT1^{av}$; Harlan-Winkelmann, Borchen, Germany) and Lewis (LEW; MHC haplotype $RT1^{l}$) inbred rats (Charles River, Sulzfeld, Germany) weighing approximately 250–300 g were maintained in the animal facility of the Charité Virchow clinic. All animal procedures were performed in accordance with the approval of the local authority for animal research procedures, the Landesamt für Gesundheit und Soziales, Berlin, Germany, and conformed to all relevant regulatory standards for animal research. The rats were anesthetized with inhaled isoflurane.

MSC ISOLATION AND CULTURE

Mesenchymal stromal cells were harvested from bone marrow of femurs and tibias from adult male LEW or DA rats by centrifugation of the bone shaft as described elsewhere (Hara et al., 2011). MSC at passages 3–5 and a content of $<5\%$ $CD45^+$ cells as confirmed by flow cytometry were used for all experiments described. MSC displayed a typical phenotype pattern: $CD90^+$, $CD73^+$, major histocompatibility complex (MHC) I^+, intercellular adhesion molecule $(ICAM)^+$, $VCAM^-$, $MHCII^-$, $CD86^-$, and weak $CD80^+$ as described elsewhere (Hara et al., 2011; see also Appendix **Figure A1**).

KIDNEY TRANSPLANTATION

Donor kidneys were removed from DA rats and then perfused with and stored in University of Wisconsin (UW) perfusion solution (Charité, Berlin, Germany) at 4°C while the recipient animal was prepared. The total cold ischemic time was 35 ± 5 min. Following cross-clamping of the abdominal aorta and the inferior vena cava, the left kidney of the LEW recipient rat was removed. The DA kidney was transplanted orthotopically with an end-to-side aortic patch and performing an end-to-end venous anastomosis using 10-0 Prolene® (Ethicon; Johnson & Johnson Medical GmbH, Norderstedt, Germany) running sutures. The ureter was reconstructed by using an end-to-end anastomosis, performed by four discontinuous stitches with 10-0 Ethilon® (Ethicon). The total warm ischemic time of the graft during the attachment of the new kidney was approximately 15 min.

EXPERIMENTAL DESIGN

As outlined in **Figure 1**, 4 days prior to kidney transplantation, two million bone marrow-derived MSCs from DA or LEW rats, or phosphate buffered saline (PBS) as control was injected intravenously. The immunosuppressant mycophenolate mofetil (MMF/Cell Cept; Roche, Basel, Switzerland) was injected i.p. at a dosage of 20 mg/kg body weight as described in a study of MSC in heart transplantation (Popp et al., 2009) under mild isoflurane anesthesia daily for 7 days from the day of transplant (day 0). Transplanted rats were monitored daily for signs of illness due to rejection or side effects of the MMF treatment. The contralateral (right side) kidney was removed at day 7 after transplant. Signs of rejection appeared beginning at day 8 for all groups. Data were collected from five to six individual animals in each treatment group that were transplanted and treated independently with two to three transplantations performed per week ($n = 5$–6). Please note that the creatinine measurement is only shown for $n = 4$–6 animals per group as this data was not measured from one animal.

The rats were anesthetized with isoflurane and blood was collected from the aorta using a catheter (Venflon™ Pro 22GA; BD Biosciences, Heidelberg, Germany) into a serum collection tube (Vacutainer® SST II, 8.5 ml; BD Biosciences) with an additional blood drop placed onto a CREA Reflotron strip to measure creatinine levels using a Reflotron® Plus Clinical Chemistry analyser (Roche Diagnostics, Mannheim, Germany). After perfusing the transplanted kidney with cold saline, the grafted kidney and recipient spleen were collected for further analysis by PCR or immunohistochemistry.

QUANTITATIVE REAL-TIME RT-PCR

Harvested organs were carefully cut into smaller pieces, immediately snap frozen in liquid nitrogen and stored at −80°C. Kidneys and spleens were thawed and homogenized before total RNA was extracted using the Nucleospin II RNA kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany) and quantified using the Nanodrop 1000 device and v3.7.1 software (Peqlab, Erlangen, Germany). A reverse transcription reaction was performed using 3 μg total RNA in a total volume of 30 μl using the high capacity cDNA

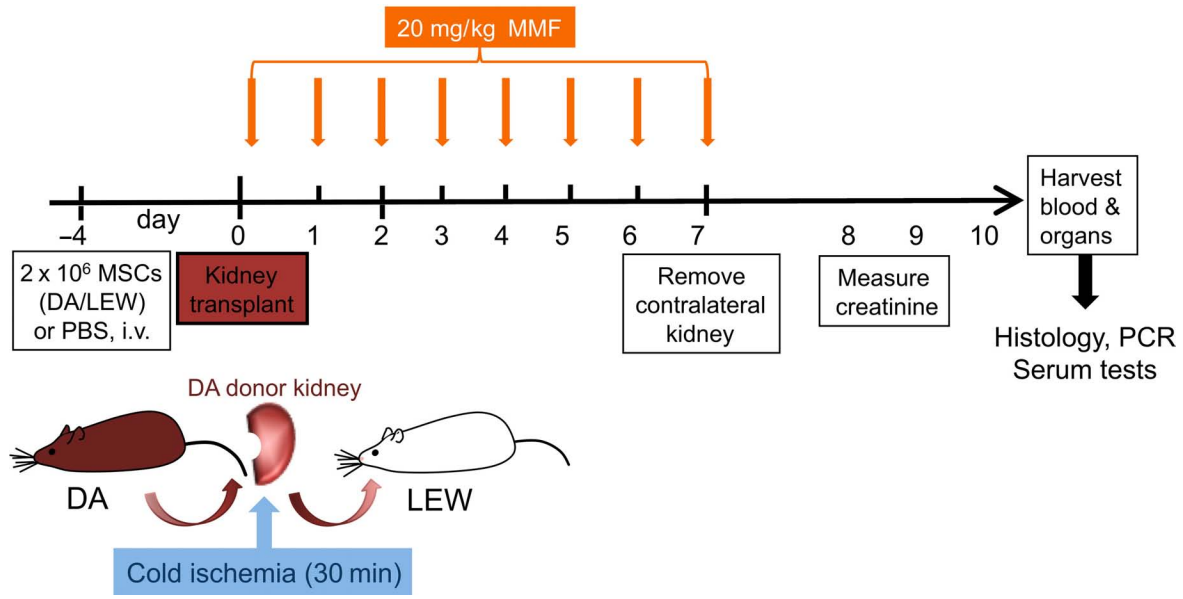

FIGURE 1 | Experimental flow chart of *in vivo* study design. DA (DA, RT1^{av}) kidneys were harvested, perfused with UW solution and kept at 4°C before being transplanted orthotopically into Lewis (LEW, RT1A^{l}) recipients. MMF treatment was given daily from day 0 to 7 at a dosage of 20 mg/kg body weight. Two million donor-type DA-MSC or recipient-type LEW-MSC or PBS controls were intravenously injected 4 days before transplantation with five to six animals in each group. Eight to ten days after transplantation, rats were humanely euthanized and tissues were harvested for further analysis by real-time PCR and immunohistology.

reverse transcription kit (Applied Biosystems/Life Technologies GmbH, Darmstadt, Germany) in an Eppendorf Mastercycler personal thermal cycler (Eppendorf, Hamburg, Germany) using the conditions 10 min at 25°C, 2 h at 37°C, and 5 s at 85°C as recommended by the manufacturer. Quantitative real-time PCR was performed using the Eppendorf realplex^2 Mastercycler machine with a total reaction volume of 20 µl in 0.2 ml MicroAmp® Optical Tubes and strip lids (Applied Biosystems) for a total of 40 cycles. The PCRs for tumor necrosis factor α (TNFα), interferon γ (IFNγ), interleukin-6 (IL-6), CD25, and MHC class II were performed using TaqMan chemistry (TaqMan® Universal PCR Mastermix; Applied Biosystems), and for chemokine ligand (CCL) 21, IL-1β, and β-actin using SYBR® Green qPCR MasterMix Plus dTTP for SYBR® Assay ROX (Eurogentec, Seraing, Belgium). Primers and probes were synthesized by Metabion (Martinsried, Germany) with sequences given in **Table 1**. For ICAM-1, an assay on demand was used (Applied Biosystems). The specificity of the desired gene products was determined by melting-curve analysis. Expression of the housekeeping gene β-actin was used to normalize expression of the target gene within the test sample and the mean fold increase of the target gene in the test samples compared to the values in the kidneys or spleens of three naïve rats was calculated using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

IMMUNOHISTOCHEMISTRY FOR INTRAGRAFT CELLULAR INFILTRATION

Harvested organs were prepared for immunohistochemistry by first fixing the tissues with 2% paraformaldehyde (PFA; Sigma-Aldrich, Taufkirchen, Germany) for 2 h and were then transferred to 30% filter-sterilized sucrose (Calbiochem/Merck, Darmstadt, Germany) for 1 or 2 days before being embedded in Jung Tissue Freezing Medium (Leica, Nussloch, Germany) and stored at −80°C. Sections of kidney or spleen tissues 5–8 µm thick were prepared using a Leica CM3050S cryostat and mounted onto Superfrost Plus slides (R. Langenbrinck, Emmendingen, Germany). Slides were blocked with Dual Enzyme blocking reagent (Dako Deutschland GmbH, Hamburg, Germany) for 10 min and washed, followed by 1 h with Tris buffered saline (TBS)/Tween/1% Bovine serum albumin (BSA)/10% horse serum before the addition of specific monoclonal mouse anti-rat antibodies to a B cell marker (clone KiB1R; BMA Biomedicals, Augst, Switzerland), MHC class II (MHCII/RT1B; clone OX-6; BD Pharmingen, San Diego, USA), CD45 (clone OX-1; AbD-Serotec, Düsseldorf, Germany), CD68 (clone ED1; AbDSerotec), T cell receptor (TCR; clone R73; Biolegend, San Diego, USA) or with IgG isotype-identical control antibody (clone MOPC; Biolegend) overnight at 4°C. The primary antibody was thoroughly washed before incubation with the Secondary Antibody (anti-mouse IgG (H + L)-biotin, rat absorbed (Vector, Burlingame, CA, USA) for 1 h at room temperature, followed by incubation with streptavidin/horseradish peroxidase (Streptavidin/HRP; Dako Deutschland GmbH) and then visualized using substrate (3-amino-9-ethylcarbazole; AEC)-solution (Dako Deutschland GmbH). Samples were counterstained with Harris's hematoxylin to detect cell nuclei and embedded in Aquatex (Merck, Darmstadt, Germany). Images were obtained by light microscopy using a Zeiss Axioskop 40 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with three images captured from each slide and then analyzed in a blinded approach by three different independent investigators. Signal intensities were graded

Table 1 | Primer and probe sequences used for real-time RT-PCR analysis.

Gene	Forward primer	Reverse primer	Probe
TNFα	5′-tcg agt gac aag ccc gta gc-3′	5′-ctc agc cac tcc agc tgc tc-3′	5′-cgt cgt agc aaa cca cca agc aga-3′
IFNγ	5′-aac agt aaa gca aaa aag gat gca tt-3′	5′-ttc att gac agc ttt gtg ctg g-3′	5′-cgc caa gtt cga ggt gaa caa ccc-3′
IL-1β	5′-acc aaa aat gcc tcg tgc tgt ct-3′	5′-tgt tgg ctt atg ttc tgt cca ttg-3′	5′-acc cat gtg agc tga aag ctc tcc acc-3′
IL-6	5′-aac tcc atc tgc cct tca gga-3′	5′-ggc agt ggc tgt caa caa cat-3′	5′-ttt ctc tcc gca aga gac ttc cag cca-3′
CCL21	5′-cca tcc cag caa tcc tgt tc-3′	5′-cct cag ggt ttg cgc ata-3′	–
MHC class II	5′-ggt tga gaa cag caa gcc agt c-3′	5′-ggt gag gta agc cat ctt gtg g-3′	5′-tga gac cag ctt cct ttc caa ccc tga-3′
CD25	5′-cac agt ctg tgt acc aggaga acc t-3′	5′-cca cga agt ggt aga ttc tct tgg-3′	5′-cag gtc act gca ggg agc ccc c-3′
β-actin	5′-gta caa cct cct tgc agc tcc t-3′	5′-ttg tcg acg acg agc gc-3′	5′-cgc cac cag ttc gcc atg gat-3′

as scores between 0 and 3 (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). The scores obtained were graphed and analyzed in GraphPad Prism v5. Additional slides were stained for 4 min with Harris's hematoxylin, washed twice with water, counterstained for 2 min with Eosin (both from Sigma-Aldrich, Taufkirchen, Germany), washed again and embedded with Entellan® (Merck) to evaluate tissue integrity.

C4d STAINING

Immunofluorescence techniques were used to evaluate complement staining using a polyclonal antibody to rat C4d (Hycult Biotech, Uden, The Netherlands) which was incubated overnight at 4°C followed by an Alexa Fluor® 488 donkey anti-rabbit IgG (H + L) antibody (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) for 90 min and covered with a DAPI mounting medium (Dianova, Hamburg, Germany). Images were obtained by fluorescence microscopy using a Zeiss Axis Observer Z1 microscope. The total area of positive C4d staining in square pixels was quantified using the Columbus™ Image Data Storage and Analysis System v2.3.0 (Perkin Elmer, Waltham, USA).

DETECTION OF DONOR-SPECIFIC ANTIBODIES

Thymocytes were isolated from naïve male DA rats (200–250 g body weight) and made into a single cell suspension in Dulbecco's PBS (PAA, Pasching, Austria) by homogenization through a 40μm cell strainer (Falcon, Oxnard, USA), and frozen in 90% fetal calf serum (FCS; Biochrom AG, Berlin, Germany), 10% dimethylsulfoxide (DMSO; Sigma, Taufkirchen, Germany) until use. The cells were thawed, washed twice in RPMI (PAA) with 2 mM L-glutamine (Life Technologies, Darmstadt, Germany) 100 units/ml penicillin and 100 μg/ml streptomycin (both from Life Technologies), containing 10% FCS (Biochrom AG) and incubated in a 37°C humidified incubator with 5% CO_2 for 2 h. Thymocytes were washed again in cold PBS containing 1% FCS, strained through a 40μm cell sieve to remove clumps, and counted using a Fuchs Rosenthal cell chamber before 0.5 million cells were distributed into each 1.4 ml flow cytometry tube (Micronic, Lelystad, The Netherlands) and incubated with the serum collected from the test rats (or a naïve LEW rat as control) diluted 1:10 with PBS and incubated for 45 min at 4°C with occasional vortexing. Cells were washed thoroughly before incubation with Goat-anti-Rat-Fab2-FITC secondary antibodies for anti-IgG or anti-IgM (STAR 69 and STAR 116F; both from AbDSerotec, Düsseldorf, Germany) for 30 min at 4°C, washed again and fixed with 1% PFA (Sigma) until FACS analysis. Flow cytometry was performed using the BD FACS Canto II (BD Biosciences, Heidelberg, Germany) and further analysis with FlowJo 8.8.5 Software (TreeStar Inc., Ashland, USA) was used to determine the geometric mean fluorescence intensity (MFI) of FITC labeling. Background staining was calculated for a naïve Lew serum sample and subtracted from the test rat values.

STATISTICAL ANALYSIS

All data are presented as means ± SEM. Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) with the Bonferroni post-test for differences between groups using GraphPad Prism v5 software. P values of <0.05 were considered significant.

RESULTS

NEGATIVE IMPACT OF MSC ON KIDNEY GRAFT FUNCTION

When serum creatinine levels were measured after removing the contralateral kidney, we found highest values in the DA-MSC-treated group which were significantly different to the PBS control group. Moreover, the creatinine values of the LEW-MSC-treated group were also significantly elevated, indicating reduced kidney function after injection of either type of MSC (**Figure 2A**). Hematoxylin-Eosin (HE) staining of cryosections corroborated these kidney function findings by indicating extreme destruction of the architecture of glomeruli and tubuli within the DA-MSC and LEW-MSC groups (**Figures 2B,C**) in comparison to the PBS-treated animals (**Figure 2D**).

ENHANCED INFLAMMATION BY MSC IN RENAL ALLOGRAFTS

Kidney grafts were analyzed by quantitative PCR for their expression levels of inflammatory cytokines, chemokines, and cellular markers compared to naïve rats as shown in **Figure 3**. Although we could not find any significant differences between the expression level for all tested markers due to individual variations, it was obvious that PBS-injected animals in general showed lower values, especially for TNFα (**Figure 3A**), CCL21 (**Figure 3E**), and ICAM-1 (**Figure 3F**) when compared to both MSC-treated groups (DA-

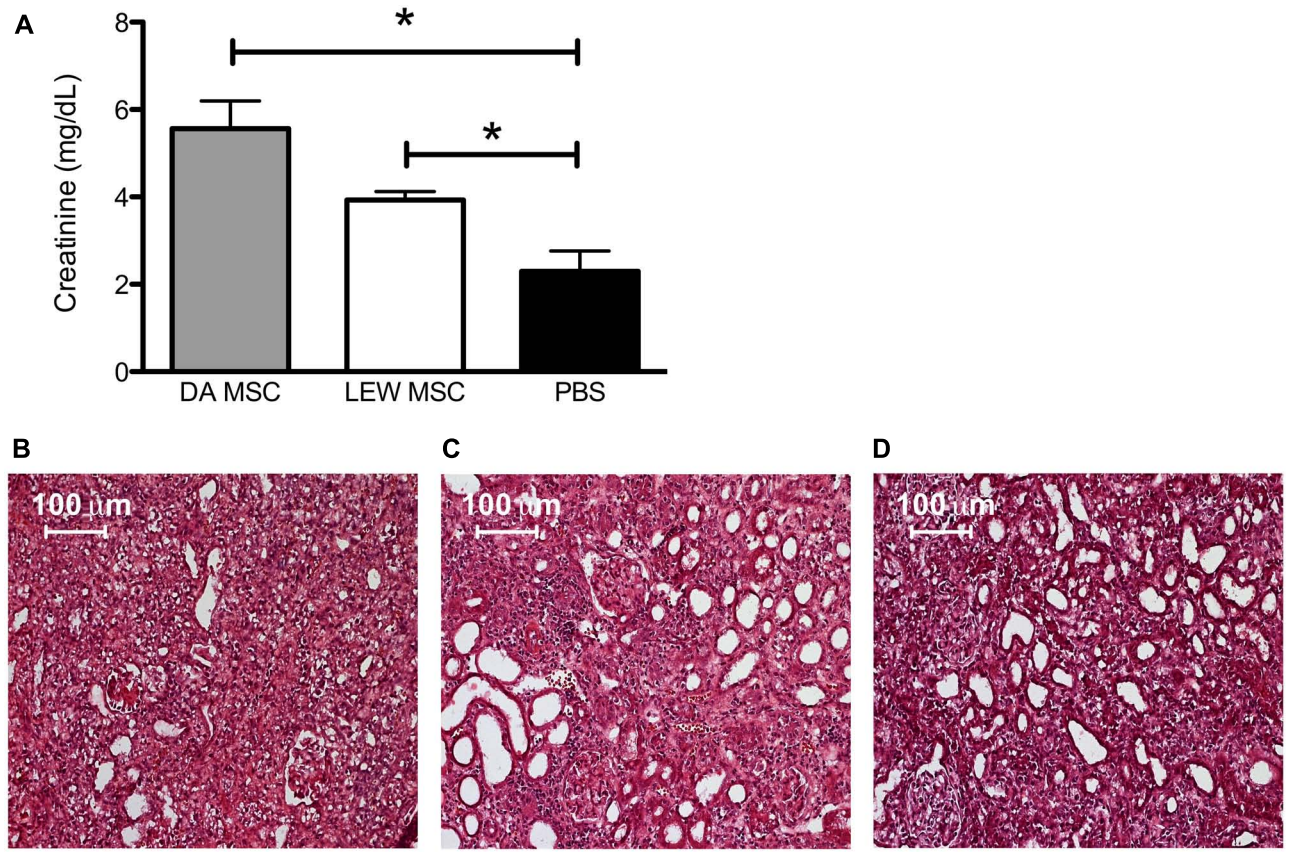

FIGURE 2 | MSC pre-treatment induces enhanced creatinine levels and impairs kidney cortex architecture. Blood creatinine levels were measured 8–10 days after kidney transplantation and data are presented as mean ± SEM with 4–6 rats in each group **(A)**; $*P < 0.05$. Transplanted kidneys were then harvested, fixed with 2% PFA for 2 h, incubated in sterile 30% sucrose and embedded. Five to eight micron thick sections were stained with Harris's hematoxylin followed by Eosin to evaluate tissue integrity. Representative images are shown for rats injected 4 days prior to transplant with **(B)** DA-MSC, **(C)** LEW-MSC, or **(D)** PBS. Scale bars represent 100 µm.

and LEW-MSC). IFNγ, IL-1β, IL-6, and CD25 mRNA expression was rather comparable between all groups (**Figures 3B–D,G**).

Analyzing the mRNA expression levels in recipient spleens, we generally detected low expression levels for nearly all tested markers, except for CD25 (**Figure 4**). In addition, both MSC-treated groups displayed higher CD25 mRNA expression levels relative to the PBS control group (**Figure 4H**). Notably, values within the DA-MSC-injected group were more variable between the single recipients. Although not significant, more animals per group with higher expression levels were detected for the DA-MSC group and especially for the markers IL-1β (**Figure 4C**), ICAM-1 (**Figure 4F**), and MHCII (**Figure 4G**).

IMPACT OF MSC ON CELLULAR INTRAGRAFT INFILTRATION

Kidney grafts were analyzed by immunohistological staining for their cellular infiltration pattern at post-operative days 8–10 by staining with antibodies to the major subsets of immune cells. In **Figure 5**, the summarized data of staining scores for B cells, T cells, $CD68^+$ macrophages, $CD45^+$ leucocytes, and $MHCII^+$ APC are shown. Significant differences between the experimental groups treated with LEW-MSC compared to DA-MSC-treated and PBS control animals were detected regarding the scores for TCR-positive cells (**Figure 5A**) and B cell marker-positive cells (**Figure 5B**). Surprisingly, higher values were detected for the LEW-MSC-treated group relative to the DA-MSC- and also the PBS-treated group as illustrated in representative images (**Figures 5F–H**). For all the other markers tested; CD68, CD45, and MHCII, the scores were comparable (**Figures 5C–E**).

DEPOSITION OF COMPLEMENT FACTOR C4d IN THE KIDNEY CORTEX

To evaluate whether humoral mediated responses might contribute to the poorer graft function of MSC-treated rats, we performed immunofluorescence staining for C4d deposits. The staining intensities of the fluorescence signal on microscopic images of all samples were quantified by a specific algorithm of the Columbus™ Image Data Storage and Analysis System and the total area of positive staining ($pixels^2$) was calculated for all treatment groups (**Figure 6A**). Representative images of the C4d staining for all treatment groups are shown (**Figures 6B–D**) as well for the quantification method (**Figure 6E**). A control staining performed using a transplanted syngeneic kidney demonstrated the absence of C4d deposits when rejection was not induced (please refer to Appendix **Figure A2**). It is apparent that LEW-MSC-treated animals express higher levels of C4d in the kidney cortex

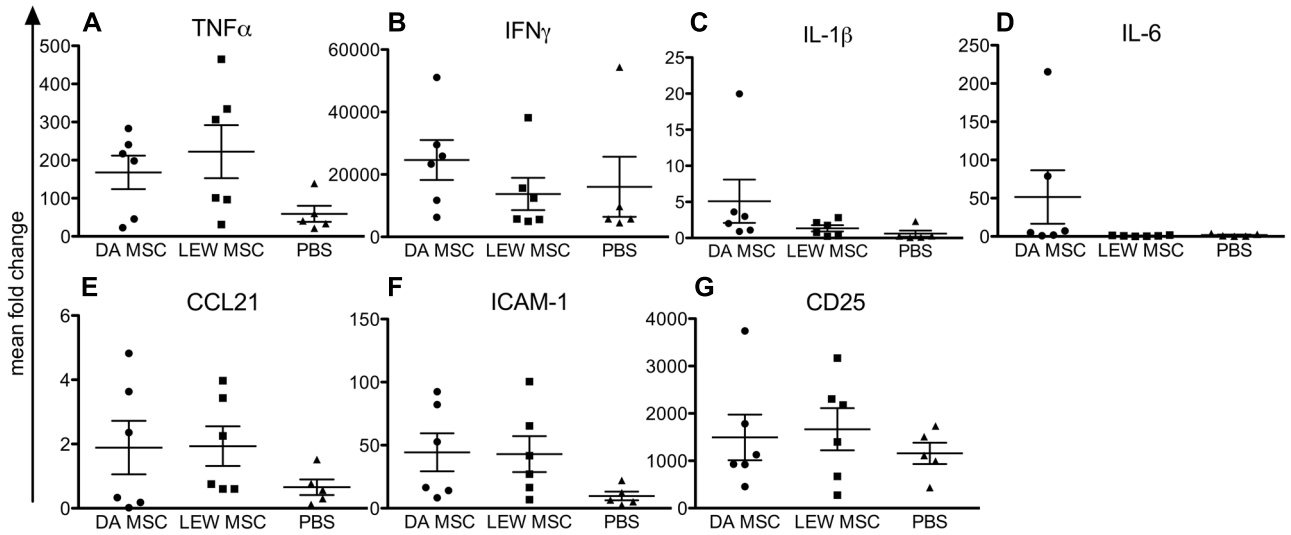

FIGURE 3 | Inflammation within the kidney grafts following MSC pre-treatment measured by intragraft gene expression analysis. Quantitative real-time PCR was performed on kidney grafts harvested 8–10 days after transplant. After expression of the target gene was normalized to the housekeeping gene β-actin, the mean fold increase of the target gene in the test samples compared to the values in the kidneys of three naïve rats was calculated using the formula $2^{-\Delta\Delta CT}$ for **(A)** TNFα, **(B)** IFNγ, **(C)** IL-1β, **(D)** IL-6, **(E)** CCL21, **(F)** ICAM-1, and **(G)** CD25. Data are presented as the mean ± SEM of the mean fold change from five to six transplanted rats per group from PCR analyses performed in duplicate.

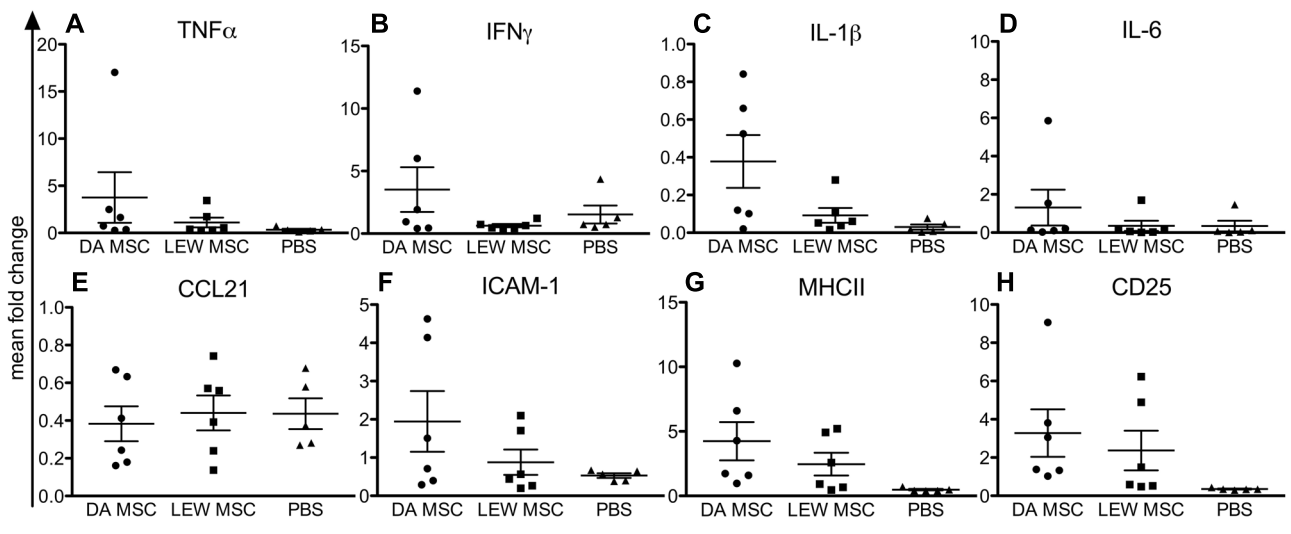

FIGURE 4 | Elevated immune cell activation in rat spleens following MSC pre-treatment measured by gene expression analysis. Quantitative real-time PCR was performed on spleens harvested 8–10 days after kidney transplantation. After expression of the target gene was normalized to the housekeeping gene β-actin, the mean fold increase of the target gene in the test samples compared to the values in the spleens of three naïve rats was calculated using the formula $2^{-\Delta\Delta CT}$ for **(A)** TNFα, **(B)** IFNγ, **(C)** IL-1β, **(D)** IL-6, **(E)** CCL21, **(F)** ICAM-1, **(G)** MHCII, and **(H)** CD25. Data are presented as the mean ± SEM of the mean fold change from five to six transplanted rats per group from PCR analyses performed in duplicate.

area then DA-MSC-treated rats but without significant differences between both treatment groups and the PBS-injected group.

INDUCTION OF DONOR-SPECIFIC ANTIBODIES BY MSC APPLICATION

Sera of all LEW recipient rats were screened for the presence of donor (DA)-specific antibodies at the time point of graft harvest using a flow cytometry based assay with isolated thymocytes. The geometric MFIs were calculated by subtracting the value of a naïve rat from the value of all kidney transplant recipient rats and a representative histogram of the fluorescence staining is shown in **Figure 7A**. As shown in the summarized data a distinct and significantly higher MFI for donor-specific IgG antibodies was measured for the DA-MSC-treated group in comparison to the LEW-MSC and PBS group (**Figure 7B**). IgM antibody values were only marginally enhanced in the DA-MSC-treated group (**Figure 7C**).

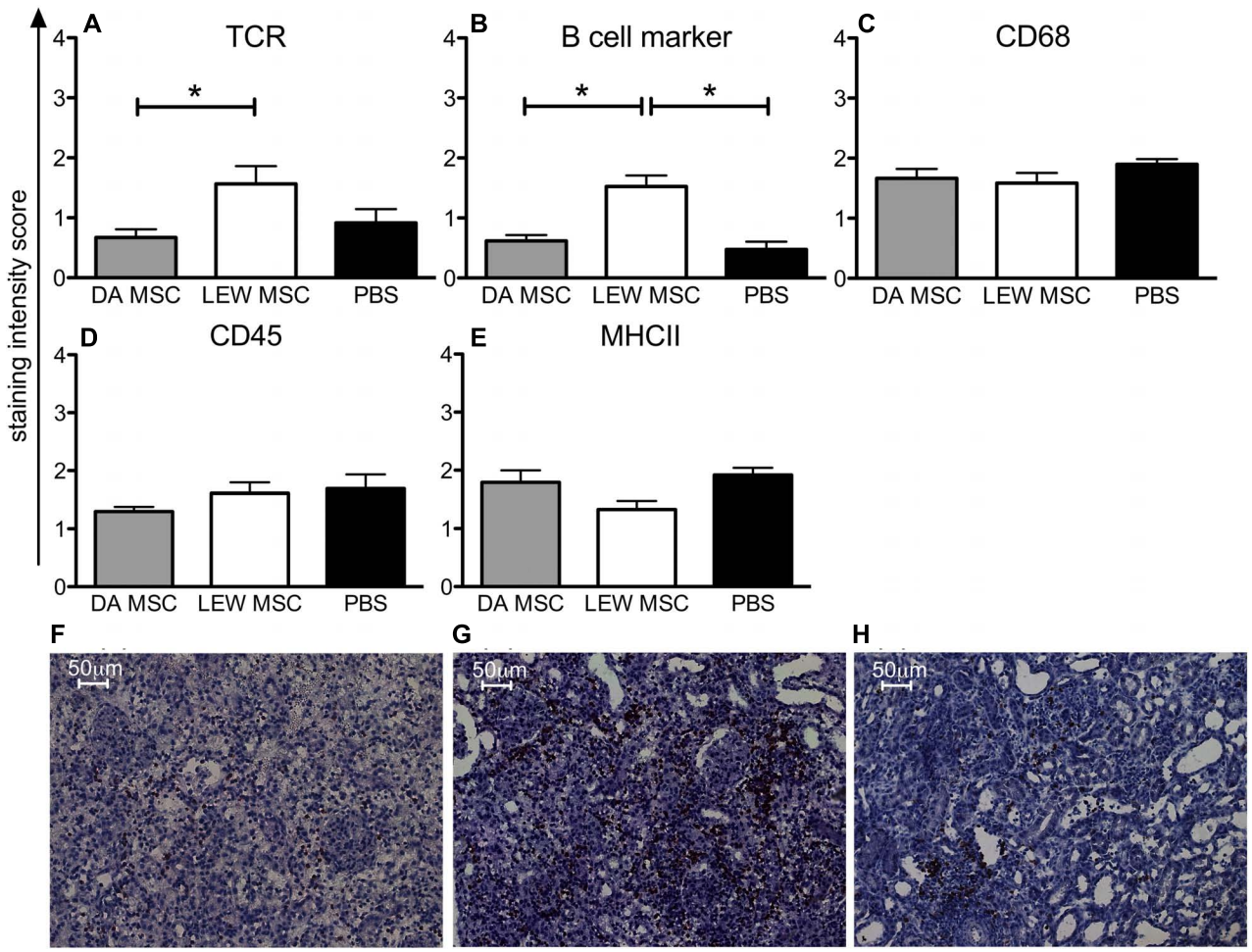

FIGURE 5 | Enhanced intragraft accumulation of T and B cells following MSC pre-treatment measured by immunohistology. Five to eight micron thick sections of transplanted kidneys were labeled with specific monoclonal mouse anti-rat antibodies to **(A)** TCR, **(B)** B cell marker, **(C)** CD68, **(D)** CD45, and **(E)** MHCII overnight at 4°C. A biotin-conjugated secondary antibody and streptavidin/HRP were applied before visualization with the substrate solution and counterstaining with Harris's hematoxylin. Staining scores are given as mean ± SEM for triplicate slides with five to six rats in each group; *P<0.05. Representative images of B cell staining are shown for rats injected 4 days prior to transplant with **(F)** DA-MSC, **(G)** LEW-MSC, or **(H)** PBS. Scale bars represent 50μm.

Whether the higher levels of IgG donor-specific antibodies within the DA-MSC-treated group correlated with higher B cell activity in the spleen was analyzed by immunohistological staining of tissue sections with B cell- and MHCII-specific antibodies (**Figure 8**). The staining intensity score for MHCII was significantly enhanced in the DA-MSC transplant group compared to the PBS-treated control (**Figure 8A**). A trend toward an increase in MHCII was also observed for the LEW-MSC group. In contrast, B cell staining scores were nearly equal for all treatment groups (**Figure 8B**).

DISCUSSION

In the present study, we investigated the effect of the donor-type MSC pre-treatment on the modulation of inflammation and rejection responses in an acute rat renal transplantation model of high MHC disparity with concomitant immunosuppression.

In our clinically relevant transplant model, we found that in contrast to our expectations, the application of either donor- or recipient-type MSC 4 days before kidney grafting resulted in the induction of increased signs of inflammation and higher levels of cellular infiltration, especially of B cells, at the time point of rejection. This was combined with C4d deposits within the glomeruli and the peritubular capillaries. In addition, when donor-type MSC were applied, significantly higher donor-specific IgG-antibody levels were induced, in contrast to the application of recipient-type MSC. These data lead us to the conclusion that donor-type MSC administration before kidney transplantation causes enhanced humoral rejection processes.

Our data in a renal transplant model are in contrast to the clear beneficial effects of the day-4 MSC application in a rat model of heterotopic heart transplantation with the same MMF immunosuppressive regimen (Popp et al., 2009). We neither see a prolonged graft survival, nor the development of partial tolerance.

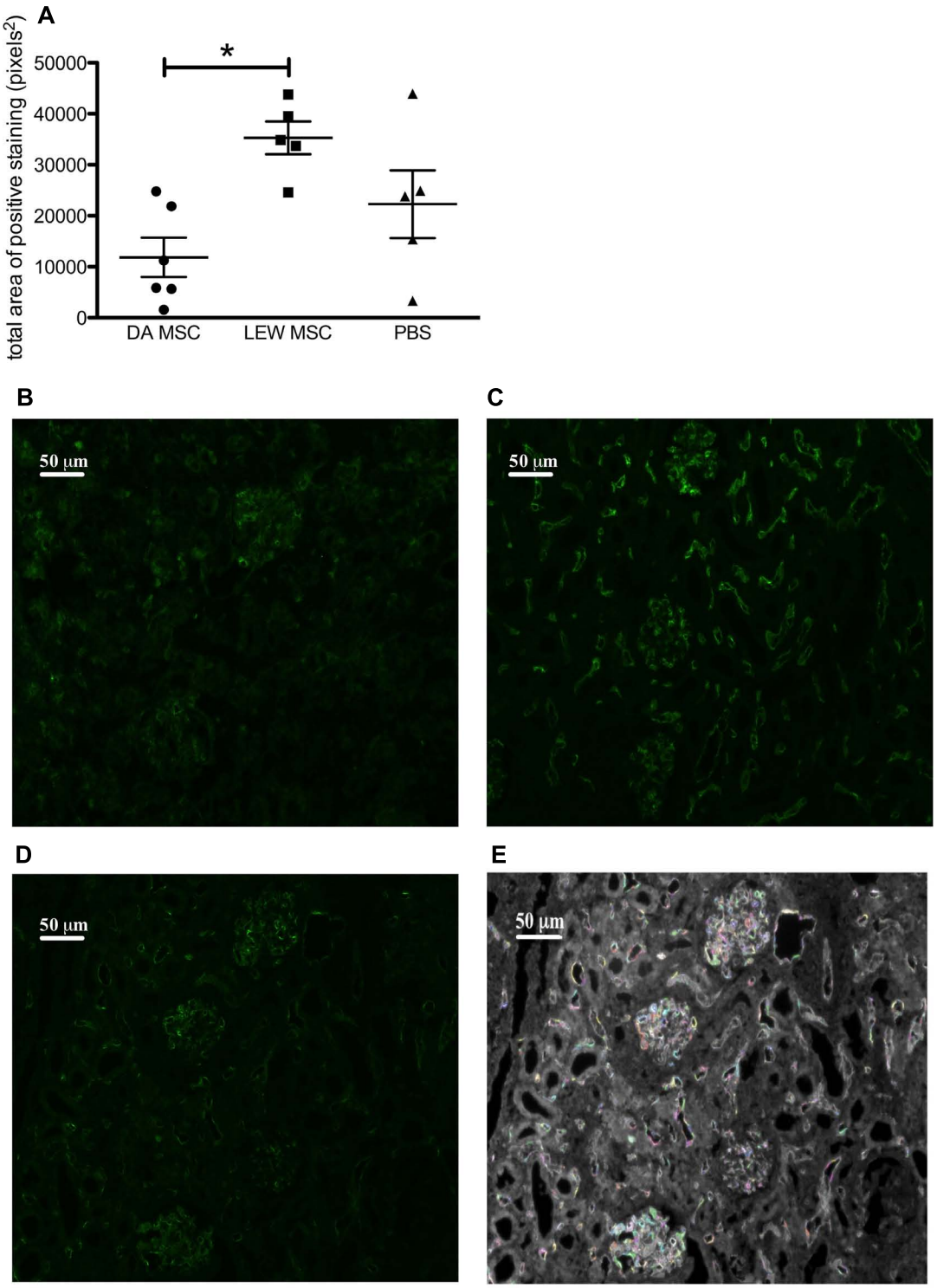

FIGURE 6 | Amplified C4d deposits in transplanted kidneys following MSC pre-treatment. Immunofluorescence labeling was performed on sections prepared from transplanted kidneys using a polyclonal antibody to rat C4d incubated overnight followed by detection with an Alexa Fluor® 488 donkey anti-rabbit IgG (H + L) secondary antibody. **(A)** Images obtained by fluorescence microscopy were evaluated using the Columbus™ Image Data Storage and Analysis System to quantify C4d (green fluorescence) labeling. Data are given as mean ± SEM for total area of positive "spots" in pixels^2 from duplicate images from five to six rats per group; $*P < 0.05$. Representative images of C4d labeling are shown for rats injected 4 days prior to transplant with **(B)** DA-MSC, **(C)** LEW-MSC, or **(D)** PBS. An example of the spot identification using Columbus™ software is shown **(E)** with various colors indicting quantified spots for the same PBS-injected animal image. Scale bars represent 50µm.

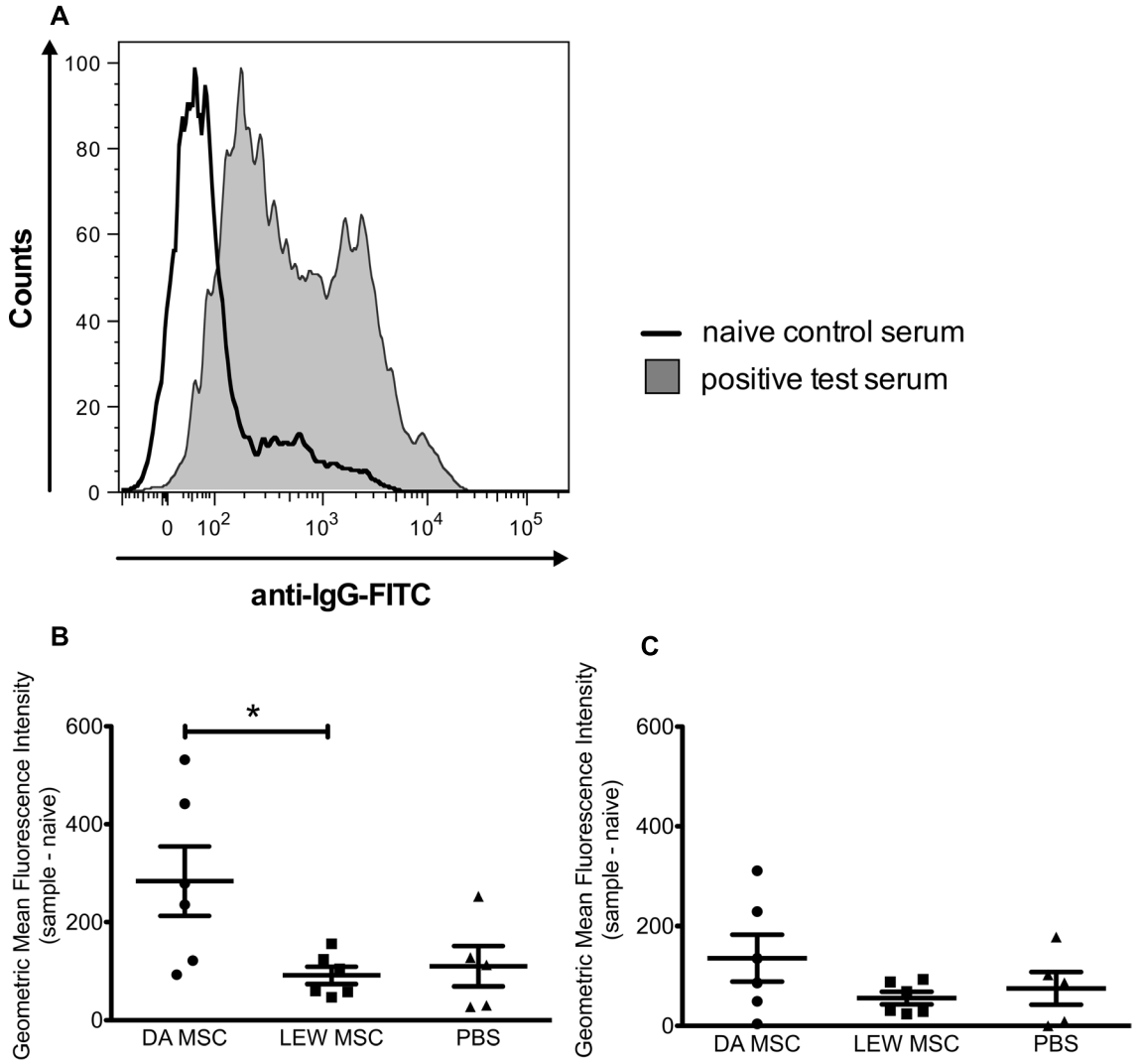

FIGURE 7 | Enhanced levels of donor-specific antibodies in serum following donor-specific MSC pre-treatment. Serum collected from transplanted rats at the time of harvest was evaluated for the presence of IgG and IgM donor-specific antibodies. **(A)** A representative staining histogram is shown for a naïve control serum (black solid line) and a positive test serum (filled gray curve). Data were collected using a BD FACS Canto II flow cytometer and the geometric MFI was determined. Background values for a naïve animal were subtracted from test rat serum values. Data are presented for **(B)** IgG and **(C)** IgM donor-specific antibodies as the mean ± SEM of the geometric MFI from five to six transplanted rats per group; $*P < 0.05$.

Discrepancies might be caused by differences in the experimental parameters including the selected rat strain combination, and the fact that while the heterotopic heart is not required for survival, our model requires the transplanted kidney to function.

Renal grafts were rejected between days 8 and 10 regardless of whether the groups were pre-treated with MSC or a PBS control. The overall condition of the animals was extremely poor in the donor-type MSC-treated group, and higher creatinine levels were measured at the time point of rejection. This was also reflected by the observation of histological signs of destruction which damaged the typical renal cortex architecture of glomeruli and tubuli and visible interstitial cellular infiltration in HE staining.

Analyzing the degree of cytokine and cellular marker expression within the grafted kidneys, we found not significantly changed gene expression levels between the MSC-treated and the PBS-treated control group. However, most animals in both MSC-treated groups tended toward higher mRNA expression levels for the pro-inflammatory cytokine TNFα and the chemokine CCL21 as well as cellular markers of immune cell activation (e.g. ICAM-1, CD25). Therefore, the protective effect of MSC by reducing signs of inflammation at early time points after kidney transplantation we recently described (Hara et al., 2011) seems to be undetectable in a later phase of the rejection process. In this former *in vivo* study, we saw significant effects using a higher number of cells which were injected at multiple time points both before and after transplantation. In addition, the type of immunosuppressive treatment

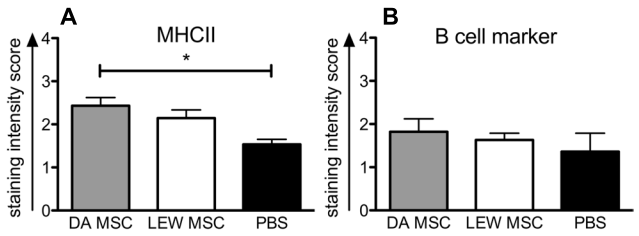

FIGURE 8 | Enhanced detection of MHCII-positive cells in rat spleens following MSC pre-treatment measured by immunohistology. Spleens were harvested at 8–10 days after kidney transplant and cryosections were labeled with specific monoclonal mouse anti-rat antibodies to B cell marker, MHCII, or with the isotype control antibody overnight at 4°C. A biotin-conjugated secondary antibody and streptavidin/horseradish peroxidase were applied before visualization with the substrate solution and counterstaining with Harris's hematoxylin. Triplicate images were captured from each slide and the intensity of positive brown staining for **(A)** MHC II or **(B)** B cell marker was analyzed in a blinded approach on a scale of 0–3. Scores are given as mean ± SEM for triplicate slides with five to six rats in each group; $*P < 0.05$.

(Cyclosporine A instead of MMF) might influence the difference in MSC effectiveness observed.

Splenic mRNA levels for IL-1β and for ICAM-1 and MHCII also trended toward an increase in the donor-type MSC-treated group, indicating signs of sensitization against the donor type cells and induced immunogenicity. Whether the observed marginally higher CD25 expression in both MSC-treated groups is related to expansion of regulatory T cells or activation of conventional T cells remains unclear.

Our analysis of graft infiltrating cell subsets gave rise to interesting and surprising findings. We observed that B cells were more abundant within the grafts pre-treated with recipient-type MSC compared to the PBS control group. T cell and macrophage infiltration were not significantly different between the PBS and both MSC groups. Why the donor MSC-treated animals do not display the same high B cell staining is unclear. One explanation might be that the process of rejection and organ damage is even more accelerated over the same time frame in this group and cells had already disappeared from the allograft at days 8–10. Hints for stronger organ damage in the donor MSC-treated group were clearly seen in the HE histology and additionally resulted in higher blood creatinine levels. Whether the higher B cell infiltration within the kidneys and the higher degree of graft destruction is caused by enhanced IL-6, as described in rat kidney transplantation models with low weight grafts (Gong et al., 2009) was examined. However, we detected neither significantly elevated mRNA expression levels for IL-6 in the grafted kidneys, nor higher levels of circulating IL-6 in the serum of MSC-treated animals at the time point of rejection (data not shown). Future studies could clarify if MSC cause a rise in systemic IL-6 levels soon after they are injected which decreases over time.

It is known that B cells are an important immune cell subset with antigen-presenting capacity in renal graft rejection. B cell involvement is in general characterized by intragraft B cell infiltration, C4d deposition and circulating donor-specific antibodies (Barnett et al., 2011). In humans, about 5–7% of the kidney transplant patients develop acute humoral rejection (Takemoto et al., 2004). Therefore, we had a closer look into the detection of complement factor deposits and the circulation of donor-specific antibodies. Animals that were treated with recipient-type MSC have significantly higher levels of C4d deposits and more infiltrated B and T cells, when compared to the donor-type MSC group.

The analysis of the donor-specific antibody levels demonstrated a detectable IgG response in all animals which we attribute to the transplant of a strongly mismatched kidney. However, IgG levels were significantly higher in the donor MSC-treated group. These results clearly demonstrate the sensitization in recipients to the donor-type antigen resulting in a more accelerated rejection process. Our observations are in agreement with evidence from other groups demonstrating recognition of MSC by the adaptive immune system (Crop et al., 2011) or even sensitization of the recipient (Nauta et al., 2006; Sbano et al., 2008). Another group has recently published that i.v. injection of allogeneic MSC provoked the generation of allo-antibodies and that repeated injections reduce the survival of injected allogeneic MSC (Schu et al., 2011). However, it still remains unclear how this may interfere with their potential immunomodulatory effects in pre-clinical or clinical trials (Griffin et al., 2010; Hoogduijn et al., 2011).

The first clinical study in kidney transplantation with autologous MSC treatment was reported by Perico et al. (2011) as a safety and feasibility study, but with limited success. Other groups are preparing to set up clinical trials using autologous or even allogeneic MSC as described in a recent review (Roemeling-van Rhijn et al., 2012). Although in solid organ transplantation new treatment strategies are essential, our results from the pre-clinical rat kidney transplantation model advise that MSC administration may not be optimal in all types of solid organ transplants and also that the specific treatment regimen might be crucial for graft success. Contrary data have also been published for the rat heart transplantation model, with either accelerated rejection (Inoue et al., 2006) or prolonged graft survival (Popp et al., 2008) obtained depending on the experimental approach. Therefore, the time point of injection, number of cells applied and the type of immunosuppressive treatments used seem to be important parameters influencing the success of the MSC treatment. A recent study using autologous MSC as a replacement for induction therapy in living, related kidney transplants (Tan et al., 2012) demonstrated reduced acute rejection, faster recovery of renal function and reduced opportunistic infections. Whereas, another group observed prolonged graft survival by a Treg-dependent mechanism in a mouse model of kidney transplantation where they applied a pre-treatment of animals with syngeneic MSC (Casiraghi et al., 2012), indicating that the proper time point for MSC administration is still up for debate. Nevertheless, based on conflicting results in pre-clinical studies caution must be exerted in order to avoid adverse effects in future clinical studies.

Though the administration of whole MSC may lead to adverse effects, it is possible that many of the positive effects published in earlier studies could be due to paracrine modes of MSC action. As many researchers do not believe that MSC act to improve regeneration by differentiating into cells of the target organ to exert their effects (Tögel et al., 2007; Wise and Ricardo, 2012), rather that they might work by secreting paracrine factors or

microvesicles (Bruno et al., 2009; Gatti et al., 2011; Ratajczak, 2011; Tetta et al., 2011), we would suggest that further studies focus on investigating the positive protective effects of MSC in organ regeneration without the risks of injecting whole cells. This approach would also alleviate concerns related to the possible malignant outgrowth of MSC injected into a patient subjected to long-term immunosuppression.

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APPENDIX

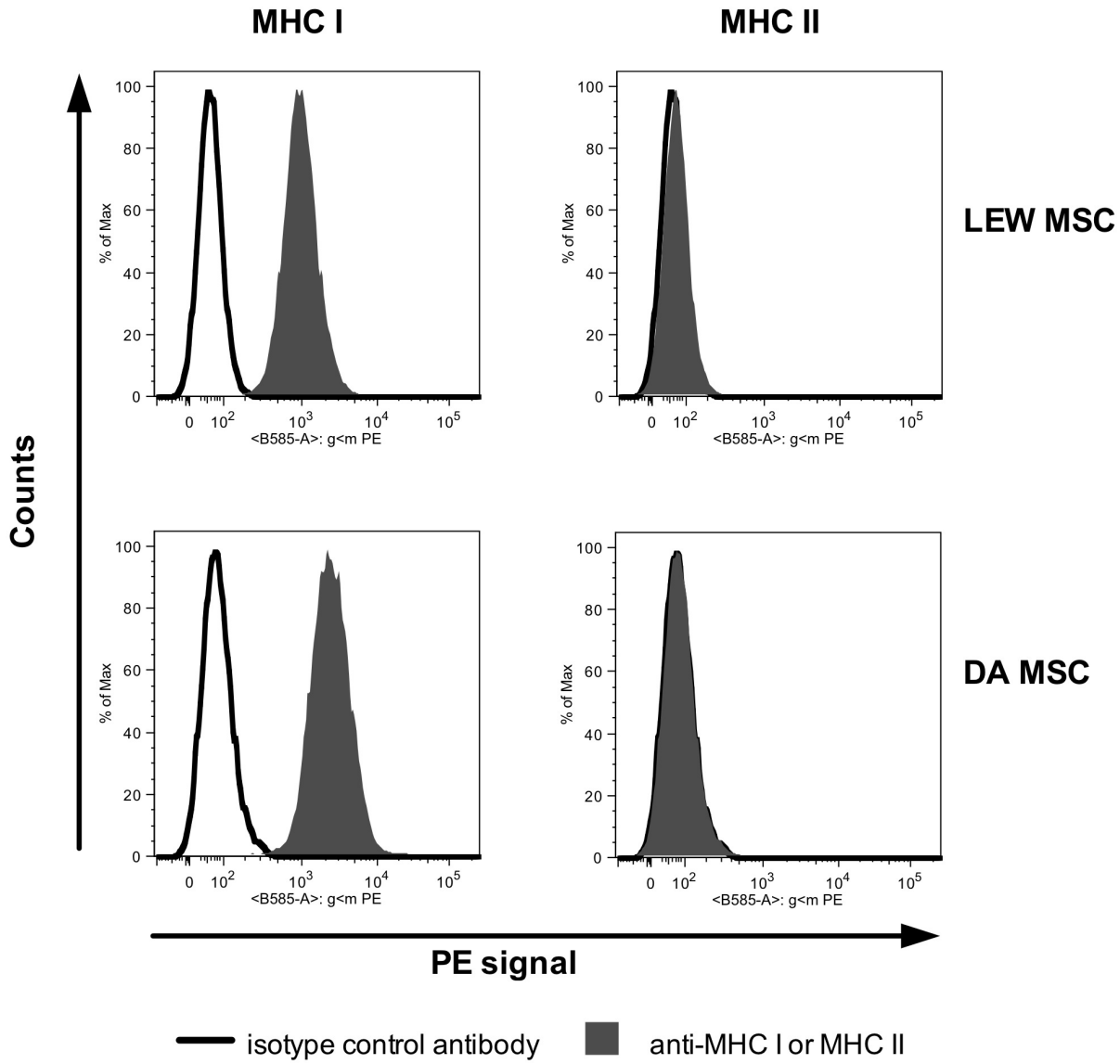

FIGURE A1 | FACS histograms of a representative labeling of LEW MSC (recipient-type) and DA MSC (donor-type) with antibodies against rat MHC I and MHC II are shown (gray filled curve) in comparison to labeling with the isotype control antibody (black bold line). Both MSC types constitutively express MHC I, but not MHC II. Data were collected using a BD FACS Canto II flow cytometer analyzed using FlowJo software.

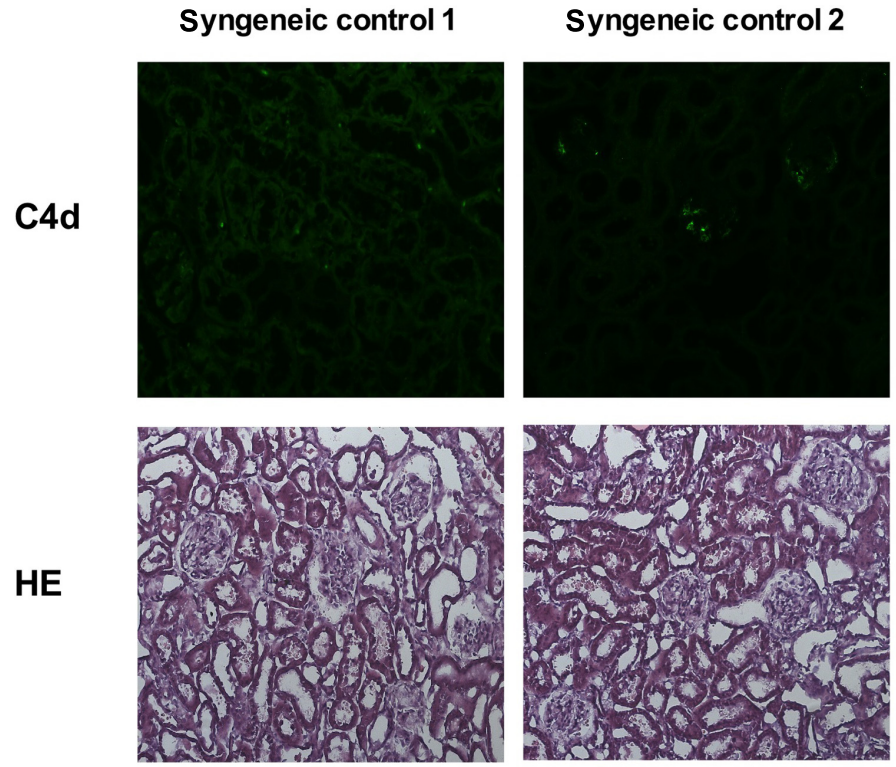

FIGURE A2 | Representative images of kidney cortex tissue of two animals 3 days after syngeneic kidney transplantation are shown. Cryostat sections of the kidney tissue were stained with an anti-C4d antibody (upper row) or with HE (lower row); original magnification × 20. Both rats show a normal kidney cortex architecture and lack specific C4d signal.

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Mesenchymal stem cell-derived hepatocytes for functional liver replacement

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Mesenchymal stem cells represent an alternate cell source to substitute for primary hepatocytes in hepatocyte transplantation because of their multiple differentiation potential and nearly unlimited availability. They may differentiate into hepatocyte-like cells *in vitro* and maintain specific hepatocyte functions also after transplantation into the regenerating livers of mice or rats both under injury and non-injury conditions. Depending on the underlying liver disease their mode of action is either to replace the diseased liver tissue or to support liver regeneration through their anti-inflammatory and anti-apoptotic as well as their pro-proliferative action.

Keywords: cell transplantation, mesenchymal stem cells, liver, hepatocyte

WHY TALK ABOUT ALTERNATIVES?

It could have been a good idea to replace diseased liver tissue by healthy hepatocytes in order to provide the metabolic power, which gets lost during liver damage from any kind of challenge – viral, genetic, chemical intoxication, etc., (Muraca, 2011; Puppi et al., 2011; Hughes et al., 2012). This concept is based on the assumption that the hepatocyte represents the smallest functional unit of the liver executing all the single metabolic services as the liver does as a whole. Indeed, hepatocyte transplantation has been proven feasible in animal trials and turned out to promise an alternative to liver transplantation in clinical settings. Usually, in rodents cells are administered to the liver either via the splenic vein after injection into the spleen or via the portal vein. Cells then spread with the blood stream over the entire organ and enter the parenchyma after endothelial penetration. They integrate and proliferate and ideally take over the hepatocytes' metabolic functions in the long-term range. There is huge experience in hepatocyte transplantation available from animal trials comprising acute and chronic liver disease models. Provided that a mitotic challenge and a regenerative advantage is presented to the donor hepatocytes then significant or even nearly complete repopulation of the host liver might be achieved. Yet, without this the rate of repopulation is rather low ranging at about 1% (Santoni-Rugiu et al., 2005; Christ, 2006; Weber et al., 2009; Shafritz and Oertel, 2011). There is doubt whether this is sufficient to supply the metabolic capacity needed to overcome the malfunction of the damaged host liver in clinical applications. An estimate of 1–5% of repopulating hepatocytes has been considered to suffice for the correction of a genetic metabolic defect of the liver (Fox and Roy-Chowdhury, 2004a; Lee et al., 2004b). Patients suffering from the defect of UDP-glucoronosyltransferase (Crigler–Najjar-Syndrome; Fox et al., 1998) or of glucose-6-phosphatase (glycogen storage disease type Ia; Muraca et al., 2002) improved after receiving human hepatocyte transplants at least for a transient period of time. Thus, hepatocyte transplantation has also gained proof-of-concept in clinical trials, which is documented by more than 30 ongoing or published studies (Muraca, 2011; Christ and Brückner, 2012; Hughes et al., 2012). Yet, one problem seriously hampers clinical breakthrough of hepatocyte transplantation. There are 30% more patients on the waiting list for liver transplantation than actually receive the life-saving organ both in the United States[1] and in Europe[2] indicating the scarcity of donor livers. It is self-evident that in this situation also livers to isolate primary hepatocytes for purposes of cell transplantation are scarce and, they are often marginal yielding hepatocytes of minor quality and insufficient quantity. Hence, even if hepatocyte transplantation turned out a versatile alternative to liver transplantation the shortage of donor livers prompted the search for novel cell resources to generate hepatocytes or hepatocyte-like cells. It might be assumed that the principles of hepatocyte transplantation are also valid for these "artificial hepatocytes" in terms of cell transplant quantity, site of application, mode of action, principles of tissue integration, and finally therapeutic support in the short- and long-term range. This approach sounds rather straightforward and therefore it is worthy to talk about alternatives, which would aid to provide surgical potential in order to manage the bottleneck of donor liver availability both for organ and hepatocyte transplantation.

[1] www.unos.org/
[2] http://www.eurotransplant.nl/

HEPATIC STEM CELLS ARE DOING THE JOB OF LIVER REGENERATION

Tissue turnover is not the liver's most prominent quality under resting, i.e., healthy conditions. With only 0.01% hepatocytes undergoing mitosis the organ seems rather indolent without any provocation (Steiner et al., 1966; Koniaris et al., 2003). Yet, in case of liver damage accompanied by massive hepatocyte loss the organ displays a remarkable regenerative potential. After two third partial hepatectomy the liver mass is restored after only about 1–2 weeks in rodents. The regenerative process is tightly regulated by a plethora of cytokines, hormones, factors, and their interactions. The initial response is triggered by the liver-resident macrophages, the Kupffer cells, which engages the activation by tumor necrosis factor α (TNF-α), components of the complement system (C3a/5a), lymphotoxin, just to mention some of those best known until today. The Kupffer cells then secrete the pro-inflammatory cytokine IL-6, which targets the hepatocytes and activates the canonical IL-6-signaling pathway involving gp130-mediated dimerization of STAT3 and the downstream activation of IL-6 target genes. This so-called priming phase initiates hepatocyte proliferation involving a second set of factors comprising hepatocyte growth factor (HGF) and epidermal growth factor (EGF) receptor ligand family such as transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor and amphiregulin. Hepatocyte proliferation continues until the original mass of the liver is restored (Fausto and Campbell, 2003; Michalopoulos, 2007, 2010; Riehle et al., 2011). Thus, liver regeneration after partial hepatectomy obviously does not involve liver stem cells. Yet, there is evidence that hepatocytes may not only generate hepatocytes but may also differentiate into other liver cell types such as biliary epithelial cells (Michalopoulos et al., 2005) or pancreatic cells (Horb et al., 2003; Burke et al., 2006). Hence, hepatocytes themselves fulfill the basic criteria of stem cells, the self-renewal and multiple differentiation potential giving rise to progeny of at least two different lineages.

The liver contains a parenchymal back-up compartment, which is activated under injury conditions preventing mature hepatocyte proliferation and/or causing hepatocyte replicative senescence. Experimentally, such situations may be provoked in rodents by feeding a choline-deficient diet in combination with the administration of acetylaminofluorene (AAF) or ethionine, by galactosamine or dipin combined with partial hepatectomy to mention a few (Koniaris et al., 2003; Santoni-Rugiu et al., 2005; Shafritz and Oertel, 2011). Liver progenitor cells – called oval cells in rodents – emerge in the periportal areas of the liver lobule comprising the Canals of Hering, structural links between the terminal biliary branches and the periportal hepatocytes surrounding the proximal parts of the sinusoids. Under healthy conditions oval cells are rare and hardly detectable. It is widely agreed upon that the oval cells are the bipotent progeny of hepatic stem cells, of which their real nature and existence in the adult liver has still to be substantiated (Sell, 2001; Fausto, 2004; Kofman et al., 2005; Santoni-Rugiu et al., 2005; Oertel and Shafritz, 2008). But, due to similar marker gene expression patterns it has been proposed that there might exist a precursor/product relationship between the embryonic hepatoblasts and the oval cells (Fausto and Campbell, 2003). In humans hepatic progenitor cells, perhaps equivalent to the oval cells in rodents, appear in the pathophysiological situations of viral hepatitis, liver cancer and massive drug intoxication (Roskams et al., 2004, 2010). Injuries occurring under these conditions provoke so-called ductular reactions, of which the hallmark is the appearance of transit amplifying cells, the progeny of hepatic progenitor cells residing in the liver stem cell niche, the Canals of Hering (Roskams et al., 2004; Gouw et al., 2011). Hepatic progenitor cells may be identified based on the expression of cytokeratin 7 (CK7), epithelial cell adhesion molecule (EpCAM), neural adhesion molecule, and CD133 (Alison et al., 2009; Gouw et al., 2011; Rountree et al., 2012). The cells of the ductular reactions display an intermediate immunophenotype featuring both biliary and hepatocyte marker expression. There is emerging evidence that the etiology of the liver disease may imprint the phenotype of the cells of the ductular reaction indicating their bipotent differentiation capacity, but which may also be the result of stimulation of different hepatic stem cell niches or the differential activation of one and the same niche under different hepatic injury conditions (Van Den Heuvel et al., 2001; Spee et al., 2010; Turner et al., 2011). Indeed, active NOTCH signaling seems to specify cholangiocyte differentiation whereas this pathway must be shut off for hepatocyte differentiation, which, however, requires in addition active Wnt signaling (Spee et al., 2010; Nejak-Bowen and Monga, 2011; Boulter et al., 2012).

In recent times it became obvious that hepatic stem cells might also derive from extrahepatic sources such as the bone marrow. In the animal model of fumarylacetoacetate hydrolase (FAH) deficiency featuring human Tyrosinemia type I transplantation of hematopoietic stem cells (HSC) resulted in the rescue of the disease phenotype in the mouse liver due to the generation of HSC-derived functional hepatocytes (Lagasse et al., 2000; Grompe, 2003; Wang et al., 2003a). Oval cells were also attributed to be of bone marrow origin (Petersen et al., 1999; Alison et al., 2000; Theise et al., 2000). Yet, not differentiation of the HSC into hepatocytes but rather fusion with host hepatocytes was the product of donor cell-derived hepatocytes (Petersen et al., 1999; Alison et al., 2000; Theise et al., 2000; Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003; Wang et al., 2003a,b; Camargo et al., 2004).

Besides HSC the bone marrow harbors mesenchymal stem cells (MSC), which are CD34- and CD45-negative indicating their non-hematopoietic nature. They feature multiple differentiation potential including lineage commitment into cells of all three germ layers (Pereira et al., 1995; Pittenger et al., 1999; Jiang et al., 2002). Their hepatocyte differentiation capacity *in vitro* and *in vivo* has been demonstrated and because of their easy availability and low ethical risks MSC have become an attractive cell source for clinical cell therapy approaches including cell therapy of liver diseases (see below).

There is great hope in induced pluripotent stem cells (iPS), which were generated first in mice from somatic cells complemented with the so-called pluripotency factors, Oct4, Sox2, Klf4, and c-Myc re-programming the cells into an embryonic stem cell-like genotype (Takahashi and Yamanaka, 2006). One prominent feature of these cells is their pluripotent differentiation capability, which comprises differentiation into cells from the three germ layers such as cardiomyocytes, adipocytes, neurons, hematopoietic precursors, osteoclasts, pancreatic cells (for recent reviews cf.; Hanna et al., 2010; Okita and Yamanaka, 2011; Bilic and Belmonte,

2012). Endodermal differentiation includes also hepatocyte differentiation (Yagi et al., 2009; Ghodsizadeh et al., 2010; Takayama et al., 2012). Introduction of the factors is achieved by viral, chemical, and DNA-mediated delivery. All of these methods raise safety concerns, which in addition to the tendency of the iPS to form teratoma, restrict the clinical use of these cells so far. However, first liver repopulation experiments in mice demonstrated the high regenerative potential of iPS (Espejel et al., 2010), which certainly opens a clinical perspective. This is highly relevant since application of cells of autologous origin back to the patient avoids the long-term risks associated with immunosuppression.

Thus, in summary liver regeneration might be accomplished by liver stem cells either of intrinsic origin or from extrahepatic sources like bone marrow (MSC) or any somatic cell. This clearly opens the perspective to generate "artificial hepatocytes" from stem cells for clinical hepatocyte transplantation (Fox and Roy-Chowdhury, 2004b).

MESENCHYMAL STEM CELLS – THE PREMIUM LIVER CELLS?

One feasible alternative to human adult hepatocytes is the use of hepatocytes derived from human MSC. Experiments in rats (Wang et al., 2004; Lange et al., 2005), mice (Jiang et al., 2002), and humans (Schwartz et al., 2002; Lee et al., 2004a; Hong et al., 2005; Seo et al., 2005; Taléns-Visconti et al., 2006; Aurich et al., 2007; Banas et al., 2007) confirmed the *in vitro* differentiation potential of MSC from prominent sources like bone marrow or adipose tissue. In the following we will reference some of the studies using MSC in order to indicate their versatile application in animal models of different liver diseases.

Cultured bone marrow-derived MSC from male albino rats were infused into the tail vein of female rats treated with carbontetrachloride (CCl_4) to induce liver fibrosis. Y chromosome-positive donor cells were found in the female host liver exhibiting reduced collagen depositions and improved liver functions (Abdel Aziz et al., 2007). Attenuation of CCl_4-induced liver fibrosis was also demonstrated using hepatocyte-like cells differentiated from bone marrow-derived MSC in the rat (Oyagi et al., 2006). Undifferentiated human bone marrow-derived MSC attenuated acute liver injury induced by allyl alcohol in Sprague Dawley rats (Sato et al., 2005). Hepatic integration and function of human adipose tissue-derived MSC pre-differentiated into hepatocyte-like cells prior to transplantation was shown both in CCl_4-treated mice (Seo et al., 2005; Banas et al., 2007) and rats after partial hepatectomy (Sgodda et al., 2007). Not surprisingly, hepatocyte pre-differentiated MSC were more effective as compared to their undifferentiated precursors. In the hepatectomized SCID mouse model bone marrow-derived MSC pre-differentiated into hepatocyte-like cells *in vitro* xenografted to the mouse livers and expressed hepatocyte markers such as albumin and CK18 (Lysy et al., 2008; Aurich et al., 2009). MSCs engrafted predominantly in the periportal portion of the liver lobule displaying hepatocyte-specific features like glycogen storage and expression of phosphoenolpyruvate carboxykinase, connexin32, albumin, and the human hepatocyte-specific antigen HepPar1 (Aurich et al., 2007).

In summary, irrespective of the site of application, i.e., systemic infusion, intrahepatic injection, intrasplenic delivery, or portal vein infusion MSC were found in the liver of the host animal forming clusters of donor cells. These cells were functional in terms of expression of specific markers and secretion of albumin. In the case of acute and chronic liver architecture deterioration MSC improved the disease. Both undifferentiated and hepatocyte-differentiated MSC integrate functionally into the host liver but at significant higher rates using differentiated cells. Facing the fact that functional characterization of MSC-derived hepatocyte-like cells after transplantation is fragmentary at best, is it then reasonable to use MSC in clinical applications?

MSC FOR HEPATIC REPAIR – SAFE OR NOT SAFE?

In the following section animal studies will be exemplified to delineate critical aspects of potential safety concerns before translation of MSC-based hepatocyte transplantation into the clinics. These include site of administration, distribution, bioavailability, elimination, and tumorigenicity.

MSC display migratory competence. After systemic application they migrate to inflammatory sites attracted by chemokines liberated from the regions of tissue injury. Intrasplenic and hepatic injection have been chosen as the sites primarily used for transplantation of adult hepatocytes. It may be anticipated that a portion of injected cells resides in the spleen, which provides an acceptable tissue environment for adult hepatocytes to survive, proliferate, and execute hepatocyte-specific functions without systemic side effects (Kusano and Mito, 1982). Very likely the mechansims of hepatic integration of stem cell-derived hepatocytes is similar or even equal to that of adult hepatocytes as discussed above. Transplanted hepatocytes mainly engraft in the periportal regions of the liver lobule and acquire the gene expression pattern of periportal hepatocytes (Aurich et al., 2005). However, shifting transplanted hepatocytes into the perivenous areas by treatment with carbontetrachloride resulted in the change from a periportal to a perivenous hepatocyte expression pattern in these cells. This indicates that the hepatic microenvironment governs the differentiation state of transplanted cells directing position-specific gene expression (Gupta et al., 1999; Koenig et al., 2007). Intraportal infusion of hepatocytes resulted also in entrapment of cells passaged through the liver into the lung parenchyma of New Zealand rabbits (Schneider et al., 2003). Yet, virtually all hepatocytes were cleared from the pulmonary capillaries within 24 h (Rajvanshi et al., 1999; Schneider et al., 2003). Hepatic engraftment after transplantation of human bone marrow-derived MSC into the spleen or the liver was similar in SCID mice (Lysy et al., 2008). As mentioned above MSC-derived hepatocyte-like cells both after intrasplenic and portal administration were found in the periportal areas of the liver lobule where they featured typical characteristics of periportal hepatocytes 10 weeks post-transplant (Aurich et al., 2007, 2009).

Principally, undifferentiated MSC may contribute to the formation of most if not all somatic cell types. This has been confirmed by injecting mouse multipotent adult progenitor cells into mouse blastocysts. Donor cells were found in hematopoietic organs and in the epithelia of the lung, liver, and gut (Jiang et al., 2002). Injection of undifferentiated murine bone marrow-derived MSC into the tail vein of NOD/SCID mice resulted in engraftment of donor cells into gastrointestinal organs but also in the lung and skin (Anjos-Afonso et al., 2004), a result, which was also demonstrated after

intravenous application of undifferentiated bone marrow MSC into baboons (Devine et al., 2003). Taking advantage of the permissive milieu of tissues and organs during organogenesis, fetal sheep were transplanted i.p. with human bone marrow-derived MSC, which integrated and differentiated into blood, liver, and skin cells (Almeida-Porada and Zanjani, 2004). Intrahepatic versus intraperitoneal injection of human MSC improved the percentage amount of human hepatocytes in sheep livers by fivefold (Chamberlain et al., 2007). It may be concluded that under minimal injury conditions as in the models described here MSC may give rise to cell types of different tissues and organs but that hepatic injury leads primarily to engraftment in the liver. Hence, it is very likely that MSC, both native and hepatocyte-differentiated home to the liver without significant extrahepatic tissue colonization.

There is evidence that MSC might contribute to extrahepatic manifestation of cancer or even liver cancer. It is a generally accepted concept that mature differentiated cells in a tissue originate from multipotent stem cells via tissue-specific stem and/or progenitor cell differentiation. Tumorigenic transformation at each step of this one-way lineage leads to loss of the differentiated phenotype and may give rise to a putative cancer (stem) cell developing into tumors of the respective tissues (cf.; Martínez-Climent et al., 2006; Polyak and Hahn, 2006; Ailles and Weissman, 2007; Wu, 2008; for recent reviews). Common properties of both tissue-specific stem cells and tumor (stem) cells are their potential of self-renewal, differentiation, and gene expression signatures supporting the abovementioned concept. Thus, any mutational event given will cause expansion of stem/progenitor cells normally quiescent in the healthy tissue thereby increasing their propensity to tumor development. The tumor stroma contains mesenchymal cells (MTC) with an invasive phenotype contributing to neoangiogenesis, which they share with MSC. Thus, similar morphological and immunological features, as well as the expression of a common set of stemness signature genes might indicate the risk of the therapeutic use of MSCs under tumor-promoting conditions (Studeny et al., 2004; Galie et al., 2008). There is controversy whether or not somatic stem cells are involved in hepatocarcinogenesis (Wu and Yu, 2007). It is known that MSC tend to malignant transformation in culture after extended expansion. However, in a transgenic mouse model of hepatocellular cancer (HCC) induced by diethylnitrosamine and phenobarbital bone marrow cells did not progress to HCC (Ishikawa et al., 2004). Similar results were found in non-transgenic Balb/c mice after chemical induction of HCC by diethylnitrosamine (Zheng and Liang, 2008). In the Lewis rat, bone marrow-derived stem cells were recruited to the liver after feeding a choline-deficient diet, fused with hepatic oval cells but did not contribute to pre-neoplastic nodule formation (Kubota et al., 2008). Numerous studies have since shown hepatogenic differentiation from both hematopoietic and MSC without cellular fusion in a variety of different animal disease models (Ishikawa et al., 2003; Newsome et al., 2003; Jang et al., 2004; Sato et al., 2005). Thus, even under conditions favoring tumorigenesis in the liver, no contribution of MSC to tumor formation in the liver has been reported so far whatever site of application or carcinogen was being used. Most of the studies described above applied MSC not pre-differentiated into hepatocyte-like cells prior to hepatic transplantation. In a murine melanoma model the impact of differentiated and undifferentiated MSC on tumor growth and metastasis was investigated. Ectopic administration of allogeneic MSC showed that MSC after chondrogenic differentiation did not display migratory activity and reduced the promotion of tumor growth while undifferentiated MSC migrated to the site of the tumor and favored tumor growth and metastasis (Akay et al., 2010). It seems to be a general feature of undifferentiated MSC to be recruited to the tumor stroma as shown previously in a culture model of human glioblastoma (Birnbaum et al., 2007). Nevertheless it cannot be excluded that MSC promote tumor growth indirectly due to their propensity to form progenitor cells of tumor vessels exemplifying the pro-angiogenic properties of MSC (Kinnaird et al., 2004) and/or stromal-fibroblast like cells thus impacting the tumor stroma and supporting tumor growth (Huss et al., 2004; Feng and Chen, 2009; Mishra et al., 2009; Zischek et al., 2009). There is also evidence that MSC by producing anti-inflammatory molecules reduce pancreatic tumor growth (Zischek et al., 2009). On the other hand the immunosuppressive features of undifferentiated MSC might favor tumor growth and metastasis as shown in rodent animal models (Djouad et al., 2003; Zhu et al., 2006; Krampera et al., 2007).

Thus, the current knowledge does not allow for the safe use of MSC in clinical settings at least in terms of tumorigenicity. Therefore, investigations in large animal models of liver diseases like in the pig are appreciated to study the behavior of MSC under the given environment produced by the specific disease. In recent times pig models for isolation and transplantation of MSC became available (Casado et al., 2012), which now allow for the evaluation of both the therapeutic and the potential side effects of MSC as close as possible to the human situation (Shi et al., 2010; Groth et al., 2012; Li et al., 2012).

MSC FOR HEPATIC REPAIR –WHICH MODE OF ACTION DO WE NEED?

Due to their specific properties like low immunogenicity and promotion of anti-inflammatory responses MSC act immunomodulatory (Djouad et al., 2003; Krampera et al., 2007; Newman et al., 2009). The application of allogeneic MSC does not provoke an immune response *in vitro* or *in vivo*. This might be partially due to the expression of intermediate levels of HLA class I antigens and lack of expression of HLA class II antigens on the cell surface (Di Nicola et al., 2002; Le Blanc et al., 2003; Klyushnenkova et al., 2005; Sotiropoulou et al., 2006). The MSC-mediated immune modulation mechanistically varies depending on the immune cell type affected (Meisel et al., 2004; Aggarwal and Pittenger, 2005; Nasef et al., 2007; Feng and Chen, 2009; Siegel et al., 2009). MSC interact with dendritic cells (DC) as well as with T-cells, B-lymphocytes, and with NK cells (Aggarwal and Pittenger, 2005; Nauta and Fibbe, 2007; Noel et al., 2007; Stagg and Galipeau, 2007). They modulate generation, activation as well as function of DC at different levels of differentiation (Jiang et al., 2005; Nauta et al., 2006; Hematti, 2008).They inhibit the maturation and migration of DC to the lymph nodes and the secretion of TNF-α by DC (Krampera et al., 2006; Spaggiari et al., 2006; Nasef et al., 2007; Ramasamy et al., 2007).

Recent studies identified two different functional types of MSC. Depending on the prevailing conditions immunosuppressive MSC

or immunogenic MSC may be distinguished. In the presence of pro-inflammatory cytokines like TNF-α and interferon-gamma (IFN-γ) the immunosuppressive phenotype of MSC is favored. If anti-inflammatory cytokines like IL-10 are predominant the suppressive effect of MSC is abrogated (Renner et al., 2009). Depending on the level of IFN-γ MSC furthermore exhibit antigen-presenting properties (Chan et al., 2006). After chondrogenic differentiation the immunological properties of xenogeneic MSC changed. Differentiated MSC promoted human DC maturation by stimulation of CD38 expression on the DC and upregulation of B7 expression on MSC. Yet, osteogenic, chondrogenic and adipogenic differentiation did not alter the immunosuppressive properties of MSC (Chen et al., 2007), which supported the conclusion that MSC, undifferentiated, or differentiated, may be accepted even by HLA-incompatible patients.

MSC attenuate secretion of major pro-inflammatory cytokines like TNF-α and IFN-γ and thus reverse tissue inflammation, which is supported by an increased expression of the immunosuppressive cytokines IL-10 and TGF-β secreted by the MSC (Krampera et al., 2006; Ryan et al., 2007; Zheng et al., 2008). Taken together this would explain the anti-inflammatory features of MSC (Di Nicola et al., 2002; Aggarwal and Pittenger, 2005; Chabannes et al., 2007; Feng and Chen, 2009; Kode et al., 2009; Newman et al., 2009; Mao et al., 2010). Immunosuppressive and anti-inflammatory effects of MSC may be mediated on the molecular level by heme oxygenase (HO-1) and iNOS (Munn et al., 1998), indoleamine 2,3-dioxygenase (IDO) preventing the T-cell response through tryptophan depletion (Aggarwal and Pittenger, 2005) or prostaglandin E2 (PGE_2; Bartholomew et al., 2002; Aggarwal and Pittenger, 2005; Beyth et al., 2005; Le Blanc and Ringden, 2005; Yanez et al., 2006; Nasef et al., 2007; Hematti, 2008). So far major attempts are under way to apply MSC for the prevention of Graft versus Host Disease (GVHD), rejection of organ transplants and for modulation of inflammation in general.

It may be concluded that MSC may play a pleiotropic role impacting a given disease by a specified mode of action, which is triggered by the diseased tissue environment. This includes tissue regeneration through substitution of the tissue lesion by functional cells differentiated from the MSC but also modulation of an inflammatory tissue environment thus improving or stimulating self-regeneration of the affected tissue. These pleiotropic mode of action is highly appreciated to treat liver diseases of different etiology. Acute or chronic liver injuries require the down-regulation of inflammatory processes in order to prevent progressing tissue damage whereas ample liver resection due to liver cancer might require substitution of functional loss. Hence, in the one case undifferentiated MSC might represent the cell source of choice while in the latter hepatocyte-differentiated MSC might be appreciated.

WHAT TO DO NEXT?

It must be anticipated that nearly all tissues harbor MSC, which upon tissue injury proliferate and differentiate into the cells of the tissue of origin to replace and functionally regenerate the injured tissue regions. Recently, MSC-like cells have even been isolated from adult human liver (Najimi et al., 2007; Covas et al., 2008) and liver grafts (Pan et al., 2011) suggesting that these cells might contribute to tissue repair after hepatic injury. So why not use MSC for allogeneic stem cell transplantation in liver diseases? As outlined above this concept has widely been proven in animal models of a great variety of different liver diseases, and indeed, finds increasing interest to progress into clinical translation. Liver cirrhosis is characterized by the irreversible deterioration of the liver's architecture resulting in the formation of regenerative nodules, which are separated by fibrotic septae. It may progress to liver cancer and/or liver failure with a very high incidence of mortality. MSC have been shown to ameliorate liver fibrosis in mice and rats, which was likely due to the reduction of collagen synthesis and the induction of expression of metalloproteinases, the major players in matrix degradation and remodeling (Parekkadan et al., 2007; Banas et al., 2008; Tsai et al., 2009). Acute liver failure is a highly inflammatory response of the liver to exogenous toxic insults, which is characterized by parenchymal dysfunction leading to systemic organ failures due to the lack of metabolic homeostasis normally provided by the healthy liver. The disease requires intensive care and like liver failure due to chronic dysfunction bears a high risk of mortality (Ostapowicz and Lee, 2000; Gill and Sterling, 2001; Rahman and Hodgson, 2001; O'Grady, 2005). Taking advantage of the anti-inflammatory, anti-apoptotic, and pro-proliferative features of MSC it has been shown in animal models that the cells attenuated acute liver failure by inhibition of inflammatory infiltration, reducing the rate of cell death, by increasing tissue recovery through stimulation of hepatocyte proliferation, and finally by augmenting survival rate (Parekkadan et al., 2007; van Poll et al., 2008; Zagoura et al., 2012). These encouraging results from animal studies prompted clinical application of MSC in chronic and acute liver failure[3]. However, so far there is only limited information available on the clinical outcome. In patients suffering from decompensated liver cirrhosis treatment with umbilical cord-derived MSC reduced ascites volume and improved liver function in the short-term range (Kharaziha et al., 2009; Zhang et al., 2012) and patients with end-stage liver failure improved in terms of ascites volume reduction and improvement in Child score after autologous bone marrow-derived MSC transplantation (Amer et al., 2011). These phase I/II clinical trials demonstrated safety of hepatic MSC transplantation at least under these indications but efficacy still awaits confirmation. Even if some clinical parameters might improve, the fate and long-term survival of the transplanted cells in the host liver, their mode of action, and finally safety in the long-term range have to be demonstrated.

CONCLUSION

It is likely that depending on the etiology and pathophysiology of the liver disease to be treated MSC act differently according to their pleiotropic spectrum of action. Thus, the anti-inflammatory, anti-apoptotic, and pro-proliferative features of MSC might be favorable in cases of chronic inflammatory liver diseases but in addition a functional tissue replacement is warranted in cases where massive tissue loss has to be substituted to provide sufficient metabolic capacity like in acute liver failure and huge liver resections. Therefore, it is necessary to understand the impact of

[3]http://clinicaltrials.gov

MSC both on the molecular and cellular level and their interactions with the host liver tissue under a given microenvironment as created by the diseased liver. It might also be thought to use MSC in combination with primary human hepatocytes to either support hepatocyte function and moreover to minimize immunological rejection of the transplant in the short-term range taking advantage of the immunosuppressive features of MSC (Stutchfield et al., 2010). This could help to bridge the patient to liver transplantation and even through the critical phase of acute liver failure until the host liver recovers from the acute insult. This is of high interest because this setting would enable allogeneic hepatocyte transplantation avoiding long-term immunosuppression with all the known undesired adverse effects.

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Composite tissue allotransplantation and dysregulation in tissue repair and regeneration: a role for mesenchymal stem cells

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Vascularized composite tissue allotransplantation is a rapidly evolving area that has brought technological advances to the forefront of plastic surgery, hand surgery, and transplant biology. Composite tissue allografts (CTAs) may have profound functional, esthetic, and psychological benefits, but carry with them the risks of life-long immunosuppression and the inadequate abilities to monitor and prevent rejection. Allografts may suffer from additional insults further weakening their overall benefits. Changes in local blood flow, lack of fully restored neurologic function, infection, inflammation with subsequent dysregulated regenerative activity, and paucity of appropriate growth factors may all be involved in reducing the potential of CTAs and therefore serve as new therapeutic targets to improve outcomes. Strategies involving minimized immunosuppression and pro-regenerative therapy may provide a greater path to optimizing long-term CTA function. One such strategy may include mesenchymal stem cells (MSCs), which can provide unique anti-inflammatory and pro-regenerative effects. Insights gained from new studies with MSCs on composite allografts, advances in tissue regeneration reported in other MSC-based clinical studies, as well as consideration of newly described capacities of MSCs, may provide new regenerative based strategies for the care of CTAs.

Keywords: composite tissue allotransplantation, tissue regeneration, mesenchymal stem cells, vascularized composite tissue allotransplantation, cell therapy

OBSTACLES FACING VASCULARIZED COMPOSITE TISSUE ALLOTRANSPLANTATION

Patients receiving life-saving solid organ transplants directly contrast with those considered for vascularized composite tissue allotransplantation (VCTA). In general, VCTA recipients are physically healthy individuals except for the tissue defect; tissue transplantation is not considered life-saving or life-prolonging (1). The benefits of hand or face transplantation can include limb function as well as improvements in psychological and social well-being. A chronic immunosuppressive regimen may be needed to prevent rejection of highly antigenic tissues of multiple embryonic origins, including skin, muscles, and nerve is required (2). Rejection may not adequately present all the potential obstacles, which serve to weaken the potential of these allografts; the regenerative and repair capacity of the allograft must also be considered. Factors that impair tissue repair and regeneration in chronic wounds and therapies that modulate these responses may also be considered for composite tissue allograft (CTA) survival. Endothelial injury and corresponding changes in local blood flow, neuropathy, infection, inappropriate inflammation, and paucity of appropriate growth factors may all be involved in reducing the overall benefit of CTAs and as a consequence, serve as new therapeutic targets to improve composite allograft outcomes. While the risks of life-long immunosuppressive therapy serve as the impetus for finding alternative modulators of host immune responses to these transplanted allografts (3), focus will be directed toward regenerative and reparative strategies, including those based on mesenchymal stem cells (MSCs).

HAND COMPOSITE TISSUE ALLOTRANSPLANTATION

Since the first successful hand transplantation by Dubernard in 1998, over 70 hand composite tissue allotransplantations (HCTAs) ranging from wrist level to shoulder level have been performed around the world (4). The devastating loss of function after the amputation of the upper extremity makes HCTA an appealing method of restoration, as prostheses provide limited functionality. Plasticity of the human brain allows for cortical organization and adaptation after HCTA, with reversal of the cortical organization shift that occurs after sensory and motor deprivation in amputees (5). Functionality post-hand transplant can be evaluated using a 100-point functional score system, evaluating appearance (15 points), sensibility (20 points), motility (20 points), psychological and social acceptance (15 points), daily activities and work status (15 points), and patient satisfaction

and general well-being (15 points) (6). In a study of 38 HCTA, all patients developed protective sensibility, 90% of patients had tactile sensibility, and approximately 80% of patients had discriminative sensation (7). Motor recovery, however, was not immediate; intrinsic muscle function was observed between 9 and 15 months post-transplantation. All patients were able to perform grasp and pinch grip, hold small objects, turn door knobs, write, and work (7). More than 85% were treated for acute rejection the first year. Correspondingly, 63.6% were diagnosed with opportunistic infections and 50% experienced metabolic complications of immunosuppression including hyperglycemia, renal dysfunction, hypertension, Cushing's syndrome, and aseptic necrosis of the hip with bilateral replacements. It is possible that, due to the number of events occurring during the earlier period post-transplant, the mean functional scores tended to be lower in the first 4 years (range 65.5–69, single hand transplant; range 60.5–82.5 bilateral hand transplant) than the sixth and seventh years (88 points, single hand; range 84.5–94 points, bilateral hand). These findings suggest that while hand transplant provides a significant advance in regained function, its early time course is plagued with delayed function, potential tissue injury due to acute rejection, and significant complications due to chronic immunosuppression.

In terms of long-term success, there are other, less well-characterized variables which play a role. While cerebral plasticity allows adaptation to use of the hands, it does not predict psychological acceptance (8). Such acceptance may impact compliance with post-transplant medication and the success of life-long immunosuppressive therapy. Candidacy screening also presents a recognized but less discussed variable in long-term success. In the past year, in contrast to prior perception that hand transplant recipients did not experience chronic rejection (7), new evidence demonstrated, with devastating results, the effect of chronic rejection, originating with the endothelium. The amputation of the Louisville hand patient ensued after the patient had received substantive immunosuppression; Campath 1H induction (Alemtuzumab) was followed by tacrolimus and mycophenolate mofetil maintenance therapy. The patient had three episodes of acute rejection in the first 8 months characterized as a rash or slight swelling and was treated with topical tacrolimus and/or steroids. At 9 months, unmanageable ischemia secondary to severe intimal hyperplasia, confluent in the donor arteries of the allograft, resulted in amputation (9). Chronic rejection manifested as severe obliterative intimal hyperplasia of the deep vessels of the arterial tree, with the endothelium identified as a target of chronic rejection (9). The series of acute rejection preceding chronic rejection and graft loss suggests that intervention during the early course of the transplant mitigating or eliminating repetitive endothelial injury may improve long-term results. Additionally, it is plausible targeted therapeutics which provide pro-regenerative strategies for the endothelium may offset or reduce manifesting cardinal events that precipitate chronic rejection.

FACE COMPOSITE TISSUE ALLOTRANSPLANTATION

The first partial face allotransplantation was achieved in 2005 by Dubernard in Amiens, France, and the first full face allotransplantation, containing soft tissue and bony structures, was performed in 2010 by Barret in Barcelona, Spain (10, 11). To date, over 20 facial allotransplantations with increasing comprehensiveness have been performed thus far in France, USA, Spain, and China. Face composite tissue allotransplantation (FCTA) furnishes the perfect match in facial texture, pliability, and color, as well as mimetic of function (12). While the lack of a suitable autologous substitute serves as a driving force behind FCTA, significant challenges include control of infection, prevention of rejection, psychological adaption, rehabilitation, cortical integration, and ethical practice (13).

The face plays a major role in an individual's interaction with the outside environment. The face represents sense-of-self and identity. In addition to the senses (smelling, hearing, etc.), it conveys emotion (smiling, kissing) and plays a major role in basic physical functions (swallowing, breathing) (14). Facial disfigurement or loss of motor and sensory function has devastating psychological and social impact on an individual and FCTA manifests as a utopia for restoration (15). However, the high visibility of the face and its intimate relationship with the individual serves as a source of controversy, with ethical considerations in face donation and the donor's family. Concerns that face transplant represents an identity exchange or that the face of a loved one would be recognizable in a stranger impart an emotive barrier to FCTA (16, 17). Related challenges arise in the recipient with psychological adaption, rehabilitation, and cortical integration.

Like hand transplant, neurologic sensory function precedes motor function. Reestablishing sensation and motion for speech, swallow, and mimicry through coaptation of the sensory and motor nerves (trigeminal and facial nerves, respectively) remain challenging. Near normal sensory recovery of the early cases has been demonstrated between 3 and 8 months postoperatively by quantitative sensory tests (18). Motor recovery has been slower with limited published objective data on motor recovery; though the first four patients were able to eat, drink, and speak within 7–10 days after transplantation (14). Functional MRI and electromyographic studies have been suggested as an objective tool to determine motor recovery in FCTA (19).

Unlike hand transplantation, facial transplantation must overcome the added hurdle of host responses directed against the transplanted mucosal barrier and associated microbiome (2, 20, 21). Much like intestinal and lung transplants which establish donor derived cellular barriers and associated microbiomes in the transplanted host, the balance between diagnosis of infection and rejection is likely to be equally problematic. So far, rates of infection compared to solid organ transplantation have been less. This observation may reflect the overall good health of the recipient prior to transplant (17), the low numbers transplanted, or an incomplete ability to differentiate infection from rejection. Alternatively, a sub-population of face transplant recipients, severely burned patients, may experience higher risk of both infection and rejection due to their proclivity for developing sepsis (17). Infection and tissue damage has been noted to associate with poorer graft outcomes (22).

Even with an ever-increasing number of FCTAs being performed, consternation still remains over the unexpected death of the world's second face transplant recipient (China) and the death of the world's first concomitant hand and FCTA patient (France). In 2009, Lantieri transplanted the upper 2/3 of face and bilateral

hands to a 37-year-old recipient with significant burns. No acute rejection episodes occurred but the patient suffered multiresistant *Pseudomonas aeruginosa* infection on post-operative day 15 with destructive soft tissue infection, and subsequent death 65 days after transplant due to anoxic cardiac arrest from tracheostomy obstruction in the context of septic complications (23). This most recent death has spurred new questions regarding the appropriateness of concomitant face and hand tissue (CFHT) allotransplantation, stemming from length of procedure, cortical integration, antigenic load, and safety in burn patients who often undergo presensitizing events such as temporary cadaveric skin allograft coverage or blood transfusions (24, 25) and retain indolent, resistant bacteria which can reemerge in a clinically significant manner during systemic immunosuppression (26).

CURRENT IMMUNOSUPPRESSIVE STRATEGIES

Original work involving cyclosporine A and successful rat hindlimb allotransplantation have paved the way for modern immunosuppressive therapy for composite tissue allotransplantation (27). Immunosuppressive protocols applied in VCTA are derived from those used in solid organ transplantation using triple-drug regimens (7). Following the guidelines established by Petruzzo et al. (7), the majority of VCTA patients began immunosuppressive induction therapy for T-cell depletion using either the polyclonal anti-thymocyte globulin (ATG) antibody or the monoclonal antibodies directed against CD25 (basiliximab) or CD52 (Campath1/alemtuzumab), followed with immunosuppressive maintenance therapy accomplished using a triple-drug cocktail of tacrolimus, mycophenolate mofetil, and steroids. Acute rejection episodes are treated with adjustment in steroid or a short course of steroid or induction agent, and use of topical tacrolimus and steroid (7). More recently, steroid reduction/avoidance and conversion of tacrolimus to sirolimus for long-term therapy (improved renal function) has been applied (28).

In addition to triple therapy, some centers have attempted novel methods to reduce immunosuppression requirements. Devauchelle and Dubernard included bone marrow donor infusion in the first case, anticipating improved survival in recipients of solid organ transplant and donor hematopoietic stem cells (29, 30). However, no benefit was seen. Hivelin et al. (31) and Lantieri et al. (23) later introduced extracorporeal photopheresis to face transplantation to reverse rejection crises by viral infection.

Decreasing the risk profile of CTA requires eliminating or reducing the obligatory life-long immunosuppression component. Induction of donor-specific immunologic tolerance has been proposed in various clinical and animal models. The Pittsburg Hand Transplant Program has found early benefit with donor bone marrow transfusion at day 15, with reduction in immunosuppressive burden (maintenance with oral tacrolimus versus triple therapy) (21) though long-term results are pending. Immunotolerance is a goal for organ and composite tissue transplantation, though particularly relevant for CTA, where procedures are not considered life-saving. Kawai demonstrated donor-specific immunotolerance across major histocompatibility complex barriers using a conditioning regimen of cyclophosphamide, anti-CD2 monoclonal antibody, thymic irradiation, and cyclosporine A before a combined bone marrow and kidney transplant in HLA haplotype mismatched living-related donor. Cyclosporine A immunosuppression was tapered over the next several months (in four of five patients) with maintenance of stable renal function without immunosuppression to date. Transient lymphohematopoietic mixed chimerism without chronic rejection was observed (32).

Progressive steps in achieving stable mixed chimerism with a non-myeloproliferative conditioning regimen and donor hematopoietic stem cell infusion (33) have been achieved in laboratory models, but clinical application is limited by the need for donor preconditioning (26). Tolerance induction through the establishment of mixed chimerism seems to require engraftment of donor hematopoietic stem cells in the recipient bone marrow compartment (26). Engrafted stem cells facilitate central and peripheral tolerance, by providing a persisting source of donor cells. Recent studies have identified novel approaches with cotransplantation of MSCs in addition to bone marrow transplantation (34) or cotransplantation of polyclonal T-regulatory cells with fully mismatched allogeneic donor bone marrow (35) to reduce the toxicity of the conditioning regimen while enhancing CTA survival. More recently, treatment with MSCs combined with preoperative irradiation and short term cyclosporine A, but without bone marrow transplantation, has contributed to prolonged composite tissue allotransplantation survival in a heterotopic hindlimb swine model (36). Current clinical translation is impeded by the lack of feasible protocols devoid of cytotoxic conditioning (e.g., irradiation and cytotoxic cells/mAbs). These treatment algorithms offer potential realization of long-term multilineage chimerism with graft tolerance.

MESENCHYMAL STEM CELLS IN SOLID ORGAN TRANSPLANTATION

There is already strong preclinical and clinical indication for the use of MSCs in solid organ transplantation suggesting this approach could be beneficial in VCTA. In preclinical models, MSCs have not only been shown to limit the extent of injury following renal ischemia-reperfusion (37) but also demonstrated the ability to prevent rejection in a mouse model of semiallogeneic heart transplantation and a in a model of fully allogeneic islet cell transplantation (38). Additionally, MSC are capable of promoting a state of tolerance after cardiac allograft transplantation and kidney transplantation (39, 40). While there is some preclinical data suggesting that pretreatment with allogeneic MSCs may actually be detrimental to solid organ transplant by accelerating graft rejection (41), graft rejection in this study occurred at the same time when pretreated with MSCs as compared to their non-MSC-treated controls. While MSC-treated animals showed increased cellular and molecular markers for acute rejection as well a decline in functional markers, overall rejection levels, and timing were not affected by MSC pretreatment. It is unclear if this study's findings represent unique findings or are a result of differences in immunosuppression and technical approaches as the majority or preclinical work shows great promise for MSC as a therapeutic agent. Currently, Phase I/II clinical studies are underway to determine the efficacy of MSC therapy in solid organ transplantation (42–44).

Another benefit to using MSCs is the opportunity to capitalize on the growing body of literature supporting the

non-immunogenic properties of these cell populations. One key obstacle in solid organ transplantation is the need for a donor-recipient crossmatch. There is currently a large set of data suggesting that MSCs are non-immunogenic (45–47) thereby making the need for a crossmatch unnecessary. However, while these studies demonstrate the apparent immune-privileged nature of MSCs, some recent studies (48, 49) have shown the presence of anti-donor immune responses (T-cell and B-cell/antibody) following *in vivo* transplantation of allogeneic MSCs. These more recent data question both the concept that MSCs are truly immune-privileged and the reality of non-crossmatched allogeneic MSC transplantation. While it is clear that further investigation is necessary to fully understand the immunogenicity of MSCs, the potential to utilize MSCs as an "off the shelf" therapeutic agent cannot be overlooked and may offer a significant advantage in the setting of VCTA.

Because of increasing evidence to support beneficial effects of MSC, extension of MSC therapy from solid organ transplantation to VCTA is an appropriate next step for therapy in improving outcomes in CTAs. Beyond potential for facilitating immunotolerance, MSC application may potentiate therapeutic effects of repair and regeneration to those reported in acute and chronic wound models as VCTA contain skin elements, in contrast to solid organ allografts, which would benefit from accelerated closure, granulation, and angiogenesis.

VCTA AND SKIN MODELS (CHRONIC WOUNDS, FETAL WOUNDS)

In VCTAs that include skin components, an effective progression through the phases of inflammation, tissue formation, and remodeling must occur since these are the overlapping phases of skin regeneration (50). Neutrophil and macrophage infiltration are necessary prerequisites to regeneration since their absence leads to deranged healing, chronic wounds with persistent inflammatory responses and associated collateral tissue destruction.

Tissue injury typically results in the secretion of several mediators of wound healing, such as platelet-derived growth factor, which attract and activate macrophages and fibroblasts (51). MSC-based therapies modulating neutrophil and macrophage responses hold potential for targeted therapies in VCTA. To date, MSC treatment of acute and chronic wounds results in more rapid epithelialization, granulation tissue formation, and angiogenesis. MSC differentiation to endothelial, keratinocyte, and pericyte cellular types in cutaneous wounds has been observed despite low engraftment efficiency (52).

The perspective of the VCTA as a chronic wound is based on similarities seen in tissue dysregulation and potential pro-regenerative synergistic targets that may act as counteragents to retarded repair mechanisms in the setting of: (1) inflammation, (2) macrophage-mediated inflammatory processes, (3) impaired epithelialization and attenuated matrix deposition, and (4) endothelial injury and intimal hyperplasia. All of these processes serve as potential targets for MSC-based therapy, due to the effect of MSCs on cytokine signaling pathways regulating immune responses and inflammation.

Mesenchymal stem cell-potentiated tissue regeneration and repair responses in chronic wound healing that may be paralleled and exploited in VCTA include: (1) MSC signaling with enhancement of cellular responses including cell survival, proliferation, migration, and gene expression; (2) MSC-conditioned media exhibiting paracrine activity as a chemoattractant recruiting macrophages and endothelial cells to the wound, including epidermal keratinocytes and dermal fibroblasts (53); (3) downstream effects of MSC signaling with reduced duration of inflammation with promotion of phagocytosis and macrophage modulation from pro-inflammatory to pro-regenerative; and (4) appropriate matrix deposition with enhanced repair and regeneration of endothelium. In this vein, MSC-based repair and regeneration in adult injury can be compared and contrasted with fetal wounding models which exhibit rapid re-epithelialization and "scarless" healing.

Fetal healing serves as an ideal model for wound repair. Pluripotent MSCs have been touted as the adult cellular proxy to reenact the tissue regenerative capacity seen early in development. Despite improved wound regeneration orchestrated by MSCs, there is no substantial evidence that MSCs promote "scarless" healing seen in fetal tissues mechanism due to MSC differentiation to replace damaged skin (52). Fetal wounds are rich in metalloproteinases and display reduced levels of transforming growth factor β1 (TGF-β1), which may serve as the basis behind scarless healing (50). Thus, many distinct signaling pathways act in concert to achieve scarless healing. MSC-based alteration of cellular activity via paracrine signaling may produce analogous effects and bear an important role in potentiating wound repair and regeneration and enhanced tissue survival. The remainder of this review will focus on aspects of MSC which can be exploited to promote long-term survival of VCTAs.

PROSPECTS OF MESENCHYMAL STEM CELLS IN VCTA

While a great deal of research has focused on immunosuppressive strategies following VCTA, very little has focused on promoting regeneration of the allograft components. Such a strategy, in combination with immunosuppressive regimens, may improve early function and reduce immunosuppressive requirements. Induction of MSC differentiation into many of the components of VCTAs, has been reported to induce significant regenerative effects on tissues (54). However, low survival and proliferation rates of MSCs at tissue injury sites have been observed, indicating that the regenerative effects of MSCs are not derived mainly from engraftment and differentiation, but rather paracrine signaling mechanisms (52).

Since MSC-based regenerative properties are likely not due to terminal differentiation, with several studies have demonstrated that the number of MSCs administered could not numerically account for all the components of regenerating tissue (55–58) MSC-based signaling pathway modulation offers the greatest potential for enhanced regeneration and repair in VCTA (52). MSCs appear to provide their greatest regenerative effects through paracrine regulation of multiple cell types. Secreted molecules by MSCs attract macrophages, endothelial cells, keratinocytes, and dermal fibroblasts to wounds in addition to stimulating increased cellular function of these cells. This serves to not only reduce the duration of inflammation and promote phagocytosis of tissue debris, but it also induces appropriate matrix deposition, promotes the repair and regeneration of endothelium, switches macrophages from pro-inflammatory to pro-regenerative, and

provide anti-microbial effects. On close evaluation of wound histology, MSCs accelerate granulation tissue formation, epithelialization, and angiogenesis (52). This enhanced tissue repair can also be seen with application of MSC preconditioned medium alone (37, 53, 59), demonstrating significant benefit from MSC-secreted signaling factors without the additive advantage of MSC pluripotency as the basis for regeneration.

Multiple strategies have been investigated to increase MSC presence at wounds, including the recruitment of endogenous MSCs as well as the direct application of MSCs to the wound. The total benefit derived from direct application of MSCs is additive with the potential for regeneration coming through both cellular engraftment and differentiation – exploiting MSC "stemness" – in addition to repair via paracrine signaling. Another strategy involves signaling endogenous MSCs to mobilize from the bone marrow and preferentially deposit in injured tissue over the surrounding, uninjured tissue (60–62). While the signaling mechanism promoting the trafficking of MSCs to skin/wounds are not fully understood at this time, chemokines SLC/CCL2 and substance P have been implicated in recruitment and circulation (52, 63, 64). These modalities of increasing MSC-potentiated effects can be paralleled into VCTA for enhanced regenerative capacity and cellular survival through improved engraftment efficiency in addition to observed paracrine-mediated responses in wound models. Overall increased potency and resilience of transplanted allografts may result from improved early graft function with MSC-enacted reduction of macrophage-derived inflammation, stimulated pro-regenerative responses with enhanced epithelialization and wound healing, and improvement in endothelium regeneration.

CONCLUSION

While the risks of life-long immunosuppressive therapy serve as the impetus for finding alternative modulators of host immune responses in VCTA, MSCs may offer novel reparative and regenerative based strategies with application in these transplanted allografts. Components of tissue dysregulation involving the endothelial injury, delayed epithelialization, inappropriate inflammation, and paucity of appropriate growth factors may all be involved in reducing the overall benefit of CTAs and as a consequence, serve as novel therapeutic targets for MSCs to improve allograft outcomes.

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Cell therapy medicinal product regulatory framework in Europe and its application for MSC-based therapy development

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Advanced therapy medicinal products (ATMPs), including cell therapy products, form a new class of medicines in the European Union. Since the ATMPs are at the forefront of scientific innovation in medicine, specific regulatory framework has been developed for these medicines and implemented from 2009. The Committee for Advanced Therapies (CAT) has been established at the European Medicines Agency (EMA) for centralized classification, certification and evaluation procedures, and other ATMP-related tasks. Guidance documents, initiatives, and interaction platforms are available to make the new framework more accessible for small- and medium-sized enterprises, academia, hospitals, and foundations. Good understanding of the centralized and national components of the regulatory system is required to plan product development. It is in the best interests of the cell therapy developers to utilize the resources provided starting with the pre-clinical stage. Whilst there have been no mesenchymal stem cell (MSC)-based medicine authorizations in the EU, three MSC products have received marketing approval in other regions since 2011. The information provided on the regulatory requirements, procedures, and initiatives is aimed at facilitating MSC-based medicinal product development and authorization in the EU.

Keywords: advanced therapy medicinal product, cell therapy medicinal product, mesenchymal stem/progenitor cell, Committee for Advanced Therapies, Hospital Exemption, national competent authority

INTRODUCTION

The scientific progress and advances in the biotechnology sector have led to the development of therapies which are based on the use of living cells, recombinant genetic material, and *in vitro* engineered tissue. A number of cell therapy and tissue engineered products have been introduced into the national markets of several Member States during the last decade. Due to the novelty, complexity, and technical specificity of such products, specially tailored and harmonized rules were necessary to ensure free movement of those products within the EU. Consequently, the Regulation (EC) N° 1394/2007 on advanced therapy medicinal products (ATMPs) was drafted and came into force on December 30, 2008. The Regulation laid down specific rules concerning centralized authorization, supervision, and pharmacovigilance of the ATMPs (Committee for Advanced Therapies and CAT Scientific Secretariat, 2010).

The term "advanced therapy medicinal product" covers the following medicinal products for human use: somatic cell therapy medicinal products (CTMPs), gene therapy medicinal products, and tissue engineered products. Combined ATMPs incorporate one or more medical devices as an integral part of the product. The scope of this article is primarily CTMP. For cells to be classified as medicinal products they have to fulfill at least one of the following conditions: the cells have been subject to substantial manipulation and/or these cells are not intended for use for the same essential function (the term "non-homologous use" is also used). By "substantial manipulation" it is understood that the biological characteristics, functions, or properties relevant for the therapeutic effect have been altered. Taking into account the methodological complexity of the cell therapy products, and in order to reduce the possible interpretations, it has been defined that certain manipulations with the cells and tissues are not to be considered as substantial. These include (tissue) cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, freezing, cryopreservation, and vitrification (all listed in the Annex I of the Regulation (EC) N° 1394/2007). From the scientific or clinical perspective it can be argued that cell irradiation, for instance, can have a substantial effect on the biological characteristics and physiological functions that may be relevant also for the intended therapeutic application. Since interpretations by product developer and the regulator may differ, the exact legal definition for somatic CTMP is provided in **Table 1**.

The requirements for the cell therapy product marketing authorization dossier are prescribed in the Directive 2001/83/EC. It has to be verified whether the text of the directive includes the amendments introduced until at least 2011. A link to the consolidated version is provided in the references. Thus, cell therapy requirements were introduced in 2010 by the Directive 2009/120/EC amending the Directive 2001/83/EC. In brief, the particulars and documents of a cell therapy dossier are presented as five modules: Module 1 provides the European Community specific administrative data; Module 2 provides the quality, non-clinical, and

Table 1 | Glossary of key terms for advanced therapy developers in the EU.

Advisory procedures available for ATMP development process:

(a) *Classification* of product by the EMA: optional incentive for applicants, fast procedure, applied preferably during the early development stage;

(b) EMA *Innovation Task Force (ITF) briefing meeting*: early dialog with product developers with confidential and legally non-binding advice; the NCAs of some Member States may also provide similar "introductory" meetings with regulatory experts;

(c) *Certification* of quality and non-clinical data by the EMA: preferably applied before the clinical development stage, but the presence of clinical data does not preclude this procedure;

(d) *Scientific Advice* procedure: can address any quality, non-clinical, and clinical question at any time point of the product development (post-marketing advice is also available); provided by the EMA, but similar procedures can be offered in some countries by the national competent authority (NCA).

Advanced therapy medicinal product (ATMP)

Any of the following medicinal products for human use:

(a) gene therapy medicinal product,

(b) somatic cell therapy medicinal product,

(c) tissue engineered product.

Depending on the product characteristics, ATMP can be placed on the market in accordance with centralized marketing authorization procedure according to Regulation (EC) N° 1394/2007 or, if applicable to product, via Hospital Exemption clause in individual Member State.

Somatic cell therapy medicinal product (CTMP)

ATMP which has the following characteristics:

(a) contains or consists of somatic cells or tissues that have been subject to substantial manipulation so that the biological characteristics, physiological functions, or structural properties relevant for the intended clinical use have been altered; or of cells or tissues that are not intended for the same essential function(s) in the recipient and the donor;

(b) is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the action of its cells or tissues.

Centralized marketing authorization (MA)

The centralized MA procedure is required for certain categories of medicines in Europe, including the ATMPs. This procedure results in a single marketing authorization that is valid in all EU countries, as well as in Iceland, Liechtenstein, and Norway. The European pharmaceutical regulatory framework is applied, in particular the requirements of the Directive 2001/83/EC and the Regulation (EC) N° 726/2004, also the Regulation (EC) N° 1394/2007 with regard to the ATMPs. The EMA is responsible for the centralized procedure for medicines and the Committee for Advanced Therapies (CAT) evaluates ATMP submissions.

In order to address serious unmet medical needs of patients, it may be possible to obtain MA on the basis of less complete data than normally. Besides a standardized marketing authorization some medicinal product indications may present a case for a conditional approval. *Conditional marketing authorization* is subject to specific post-marketing obligations as set out in the Regulation (EC) N° 507/2006. However, such an authorization is not supposed to remain conditional indefinitely. Once the missing data are provided, it should be possible to replace it with a marketing authorization which is not conditional.

Hospital Exemption (HE)

Centralized MA is not required for such ATMPs which are prepared on a non-routine basis according to specific quality standards and are used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner in order to comply with an individual medical prescription for a custom-made product for an individual patient.

Manufacturing of such ATMP products is authorized by the NCA of the Member State. The Member States have to ensure compliance with the adequate quality standards, as well as the traceability and pharmacovigilance requirements. The HE clause is implemented through the national legislative acts and there are differences among the HE regulations of individual Member States.

Transitional period

Advanced therapy medicinal products, excluding tissue engineered products, which were legally on the markets of the Member States in accordance with the or the EU legislation on December 30, 2008, had to comply with the Regulation (EC) N° 1394/2007 no later than by December 30, 2011. Tissue engineered products have to comply with the regulation no later than by December 30, 2012.

clinical summaries, Module 3 provides chemical, pharmaceutical, and biological information, Module 4 provides the non-clinical reports, and Module 5 provides the clinical study reports. The Regulation (EC) N° 1394/2007 also introduced amendments to the Directive 2001/83/EC, for instance Article 28 added provisions for the Hospital Exemption (HE, Article 3.7. of the consolidated Directive 2001/83/EC).

OVERVIEW OF THE EUROPEAN REGULATORY FRAMEWORK

Directives and regulations are the two types of the EU legislative acts that form the regulatory framework for all medicines, including cell-based products. This legal framework provides the basis for centralized and national competencies. The directives set the general requirements for the Member States which implement these requirements by adopting national legislative acts. Certain variability of these implementation measures exists amongst the Member States. The regulations have to be implemented directly and uniformly, without the national legislative acts. The Regulation (EC) N° 1394/2007 provides the legal basis for a centralized authorization procedure of the ATMPs – it involves a single scientific evaluation of the quality, safety, and efficacy of the product carried out to the highest possible standard by the European Medicines Agency (EMA). National (NCA) and centralized [EMA/Committee for Advanced Therapies (CAT)] competences at the different stages of cell therapy product development are summarized in **Table 2**.

Centralized procedures are provided by the EMA which is an interface for the cooperation and coordination of the activities of all 27 Member States with respect to the medicinal products. It is responsible for coordinating the existing scientific resources for the evaluation, supervision, and pharmacovigilance of the medicinal products. The EMA has seven committees including the CAT as well as a number of working parties which are expert groups with a specific scope and mandate. In addition to the evaluation of product marketing applications, the EMA mandates include scientific and procedure advice, the Innovation Task Force (ITF) meetings with product developers, coordination of the inspections of the Member States (GMP, GCP, GLP), and other. The mandates of the EMA do not cover the following ATMP development-related issues: pre-clinical development, clinical trial authorization, products legally on the market during the transitional period, HE authorization, functions of the ethics committee (but the EMA has the expertise to evaluate ethical issues), pricing and reimbursement of medicinal products. These issues are regulated at the Member State level by the national competent authority (NCA). Some states have one regulatory office whilst others have several NCAs that cover different regulatory tasks. The respective EU directives set the scene for the national regulatory frameworks. The Directive 2004/23/EC (with the implementing Directives 2006/17/EC and 2006/86/EC) defines the quality and safety standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells. In the case of blood cells or blood components for the ATMP manufacture, the requirements of the Directive 2002/98/EC apply. For ATMPs that contain human cells or tissues, Directive 2004/23/EC and 2002/98/EC derived national provisions will apply as far as donation, procurement and testing are concerned. The requirements of these directives do not apply to research projects, their scope is only the tissues and cells intended for human use. Clinical trials with ATMPs should be conducted in accordance with the overarching principles and the requirements laid down in the Directive 2001/20/EC on approximation of the national laws, regulations and the administrative provisions for the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Compared with the regulatory system in the US, the EMA is not "FDA of Europe" since the FDA has a direct mandate to regulate the above mentioned issues and perform other tasks, including research. The current developments indicate that the EMA is likely to acquire more mandates in the future which will reduce the historical fragmentation of the European regulatory framework for medicinal product development.

The CAT provides a centralized cell therapy product evaluation procedure. The CAT formulates a draft opinion on the quality, safety, and efficacy of a product for the final approval by the Committee for the Medicinal Products for Human Use (CHMP). The EU marketing authorization which is based on a centralized evaluation procedure takes 210 days excluding clock-stops, it is defined in the Regulation (EC) N° 726/2004. The evaluation is done by two independent (Reporter and Co-Reporter) assessor

Table 2 | Centralized and national components of the regulatory framework for ATMP development in the EU.

Type of activity	Legislation	NCA	EMA/CAT
Cell and tissue donation, procurement, processing	National	Inspection, authorization	n/a
Pre-clinical development	National	GLP inspection, consultation	Certification procedure (optional)
Clinical development	National	GCP, GMP inspections, authorization	n/a
ATMP classification	EU	Consultation	Procedure/opinion
ATMP certification	EU	n/a	Procedure/opinion/certificate
ATMP evaluation	EU	n/a	Procedure/opinion
Transition period	EU	Consultation	ATMP evaluation
Hospital exemption	National	Consultation, GMP inspection, production license	n/a

GLP, good laboratory practice; GCP, good clinical practice; GMP, good manufacturing practice.

teams with the CAT and the CHMP representatives. The fee for marketing authorization is reduced by 50% if the applicant is a hospital or a small- or medium-sized enterprise and can prove that there is particular public health interest in the ATMP concerned. This is prescribed in the Article 19 of the Regulation (EC) N° 2007/1394. However, even if the marketing authorization for a cell therapy product is granted, the Regulation does not interfere with the decisions of the Member State on whether to allow the use of any specific type of human cells such as embryonic stem cells or xenogeneic cells. It does not affect the application of the national legislation prohibiting or restricting the sale, supply or use of medicinal products containing, consisting of or derived from particular cells. Several cell therapy products were legally on the Member State markets before December 30, 2008. Such products have been granted a transition period defined in the Article 29 of the Regulation (EC) N° 1394/2007 during which products have to comply with the Regulation. The transition period for CTMPs has already expired at the end of 2011, and for tissue engineered products the transition period expires in 2012.

The evaluation-related tasks of the CAT include the classification of the advanced therapy products and certification of their pre-clinical data quality. Product developers have access to the classification procedure in order to determine whether a given product based on cells, genes or tissues meets the scientific criteria which define it as an ATMP. It is an incentive but not a legal requirement for the applicants and an opinion is delivered within 60 days after the receipt of the request. More than 50 classifications have been completed by 2012 and non-confidential summaries are available on the EMA website. ATMP classification procedure does not determine whether product dossier will be evaluated by the centralized procedure in or it can be submitted for the HE in Member State. However, the CAT classification procedure opinion is not legally binding for the NCAs in case product is submitted for the HE. Finally, certification of the quality and non-clinical data is a new and unique procedure available only for the medicinal products of advanced therapy classification and it is based on the Regulation (EC) N° 668/2009. Micro businesses and SMEs developing an ATMP can submit all the relevant quality and where available non-clinical data to the EMA for scientific evaluation by the CAT. It is a 90-day procedure and in the case of a favorable CAT opinion the EMA will issue a corresponding certificate. The certification is not legally binding but it will facilitate the development and improve the clinical trial and marketing authorization applications based on the same data. Only one cell therapy certification has been completed since the launching of this procedure. This is possibly due to the optional nature of the procedure and the interpretation that the resulting opinion is not legally binding for the EMA. It is also possible that SMEs developing cell therapy products may not be yet fully aware of this procedure and the related potential benefits. It has to be emphasized that a positive outcome of the certification procedure indicates that the regulatory agency has evaluated and recognized the quality of non-clinical data. Certification of the data quality also minimizes the possibility of major objections at the marketing application evaluation stage and may serve as an incentive for investments in the development of cell therapy.

Upon request of the Executive Director of the EMA or the European Commission the CAT provides advice and scientific support to drafting documents related to the fulfillment of the objectives of the ATMP regulation. On request of the European Commission the committee provides scientific expertise and advice for initiatives related to the development of innovative medicines and therapies which require the expertise in advanced therapy-related scientific areas. The activities proposed in the CAT Work programme 2010–2015 may be of interest for the ATMP stakeholders. The document is available at the EMA website and link is provided in the references. Considering the potential of the ATMPs and due to the lack of product progress to the market, the CAT has adopted proactive approach in providing the guidance tools and ensuring a dialog with the relevant parties. A number of program activities are targeted at the needs of the ATMP developers and the stakeholders are welcome to communicate their opinion via the EMA website. The CAT Work programme for the period of 2010–2015 is aimed at providing positive long term impact on the advanced therapy sector in Europe.

CONSIDERATIONS FOR DEVELOPMENT OF MSC-BASED MEDICINES

The mesenchymal stem cells (MSCs) have produced beneficial effects in a wide range of pre-clinical development disease models, even though there are as yet no adequate explanations for many of the effects observed (Prockop and Oh, 2012). During the last decade the MSC-based therapy clinical trials have been conducted for at least a dozen of different medical conditions (Wang et al., 2012). The results of clinical studies have led to the conclusion that MSC applications have been safe and feasible. However, the efficacy often could not be convincingly demonstrated as the therapies advanced along with the clinical development. This is also illustrated by the absence of MSC-based products in the European market. Only a few MSC-based cell therapy products have been approved in other markets worldwide. South Korea is leading with two MSC products registered and the first authorization granted in 2011. It might be linked to the procedure of a conditional marketing approval in the regulatory framework of South Korea that allows commercial sale in certain instances whilst pivotal trials are underway. There is also a procedure of conditional marketing authorization in the EU prescribed by the Regulation (EC) N° 507/2006 but certain differences exist in the regulatory systems. With the approval from the Korean FDA in January 2012, Cartistem has become the world's first allogenic, off-the-shelf MSC-based product. The product contains the umbilical cord blood (UCB)-derived MSCs and it is indicated for the treatment of traumatic and degenerative osteoarthritis. In 2011 the Korean company FCB PharmiCell received Korean FDA approval for commercial sale of HeartiCellgram indicated for post-acute myocardial infarction treatment. It is autologous bone marrow-derived MSC therapy product. The company provides 50–90 million cells (depending on the weight of the patient) which are administered by infusion into the coronary arteries. The regulatory approval for HeartiCellgram was granted after 6 years of clinical trials. The company has announced that the patients displayed a 6% improvement in the left ventricular ejection fraction 6 months after one dose of HeartiCellgram. However, the company has not published the results in a peer-reviewed journal (Wohn, 2012). It seems that a similar regulatory decision has

been adopted for Osiris Therapeutics Inc. product Prochymal which consists of allogenic MSCs. The company was granted an authorization for the treatment of acute graft-vs-host disease (GvHD) in children under Health Canada's Notice of Compliance with conditions (NOC/c) in May 2012. This is an authorization to market on condition that the manufacturer undertakes additional studies to verify the clinical benefit. Such a regulatory pathway provides access to treatments for unmet medical conditions and has demonstrated the benefits outweigh the risks in the clinical trials. Overall this may represent a regulatory trend to consider the evaluation procedures that could address medical needs more efficiently. Adaptive licensing, e.g., conditional approvals, would be based on stepwise learning in circumstances of acknowledged uncertainty, with iterative phases of data gathering and regulatory re-evaluation (Eichler et al., 2012). Adaptive licensing requires a different approach from the standardized dichotomous unapproved/approved product paradigm.

Better understanding of the regulatory framework should improve the development strategy and create opportunities for MSC-based therapy product authorization also in Europe. From the regulatory perspective all MSC-based products in the EU will be classified as ATMPs unless the developer claims that the MSCs have been obtained without *in vitro* culture step. According to the CAT opinion, the cell culture process corresponds to a "substantial manipulation" and the derived cells qualify as an active substance of a medicinal product. The MSCs containing medicinal product can be classified as cell therapy or tissue engineered product depending on the intended use and the claimed mode of action. The addition of recombinant proteins, chemicals and biologically active molecules *in vitro* to the MSCs will not change the regulatory status of the product. Introduction of gene expression vector into MSCs does not change the ATMP status but will result in reclassification from the cell to gene therapy product, since the product which may fall within the definition of the CTMP and the gene therapy medicinal product should be considered as a latter (as defined in Part IV of Annex I to Directive 2001/83/EC). The studies that report MSC clinical application often present the development strategy decisions which could be re-evaluated in case the overall aim was to develop a cell therapy product. It is often reported that unmodified primary MSC cultures have been used. Correspondingly, pre-clinical screening has been absent since there was only one active substance candidate. The rationale of the use of a particular MSC culture should be evaluated if mesenchymal cell clinical application does not require the same essential function as in the tissue of origin. Instead, the MSC trials often aim to facilitate the tissue regeneration or to achieve the immunomodulatory effect. Pre-clinical screening and selection is an integral part of conventional medicinal product development and there is no reason to assume that it should not be introduced also for the cell therapy developments. With the technology available there are several strategies that might be considered in order to introduce the MSC therapy candidate selection step. For instance, modification of a primary MSC population with small chemical compounds and subsequent screening for expression or secretome profiles could be considered to improve the study design and the efficacy (Ranganath et al., 2012). Genetic modification to express factor(s) that are expected to mediate or enhance the therapeutic effect and characterization of transfected clones also represents rational development (Olson et al., 2012). The comparison of subpopulations or primary cultures of different origin will increase the number of candidates for the screening and has been applied for the cell-based medicinal product development (Li et al., 2012). These examples illustrate the feasibility of the pre-clinical screening step also for the cell-based therapies. Whilst the screening will increase the costs and may not be attractive or necessary for the academic research activities, it may present a cost efficient improvement for the discovery of new MSC therapy candidates. It may provide benefits also from novel intellectual property acquisition perspective.

The MSC product developers are advised to start with the EMA scientific guidelines that provide a detailed description of the quality, safety, efficacy, and pharmacovigilance issues for CTMPs. These guidance documents include the "Guideline on human cell-based medicinal products," "Guideline on the safety and efficacy follow-up – risk management of advanced therapy medicinal products," "Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products," "Guideline on the quality, preclinical and clinical aspects of medicinal products containing genetically modified cells," and "Reflection paper on stem cell-based medicinal products." "Guideline on the risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products" is an important ATMP guidance document which has been published for public discussion and will be finalized by the end of 2012 (the e-links for all guidance documents are provided in the references). The above-mentioned guidance documents should be examined in detail but it is neither the scope of these documents nor is it technically feasible to describe the whole variety of cell therapy products. Instead, the risk analysis may cover the entire development process and an adequate risk-based approach strategy has to be applied. According to the Directive 2001/83/EC, due to the specific nature of the ATMPs, the application of a risk-based approach is encouraged to determine the extent of quality, the non-clinical and clinical data to be included in the marketing authorization application. The risk analysis methodology followed, the nature of the identified risks and the implications of the risk-based approach for the development and evaluation program has to be discussed and the risk analysis has to be described in the product application. For instance, if conventional pharmacological and toxicological tests may not be considered as appropriate for a cell therapy product, the relevant biological parameters such as the cell viability, biodistribution, ectopic growth, and the expression patterns can be investigated. It is acknowledged that relevant animal models for cell therapy product and indication might not exist. Immunocompromised animals may have a limited value, the structural and functional dissimilarities between the animal and human target organs/tissues may not produce relevant data. It would be reasonable to assume that the marketing authorization and certification application evaluation will be carried out in accordance with the risk-based approach until a certain number of products reach the market.

The whole range of assistance procedures and initiatives available from the EMA and the NCAs should be considered (summary

in **Table 1**). The product classification procedure is highly recommended at an early stage of development. This will confirm the legal framework and the relevant guidance documents can be applied for the development. Certification of non-clinical and quality data should be considered since a positive outcome would facilitate and streamline the product development for authorization. Since there are no MSC products authorized in the EU, certification by the regulatory agency may encourage funding agencies and the potential investors. Application for EMA ITF briefing meeting definitely should be considered. The ITF provides a multidisciplinary group that contains scientific, regulatory, and legal competences. It is a forum for an early dialog with the applicants. The ITF briefing meetings are meant to complement other regulatory procedures, such as the classification, certification, and scientific advice (SA). The ITF meetings are free of charge; the application forms and information about the procedure are available at the EMA website. The SA procedure provides considerably more detailed information to the applicant and can be used at any stage of the MSC product development. The procedure helps the applicant to make sure that appropriate tests and studies are performed. Consequently no major objections are likely to be raised during the evaluation of the marketing authorization application. Such major objections could significantly delay the marketing of a product and may result in a refusal of the authorization. Adherence to the SA recommendations can substantially increase the probability of a positive marketing outcome (Regnstrom et al., 2010). Within the current initiative the EMA provides SA to the SMEs for a fee reduced by 90%, and with a 65% reduction for other applicants that develop the ATMPs. Several NCAs also have the capacity and the expertise to provide similar advice but this has to be confirmed with the particular agency. The protocol assistance procedure should be considered for orphan or rare disease products, i.e., a condition affecting no more than 5 in 10,000 people in the EU. This procedure is available at the EMA and presents a special form of SA. As a result the "orphan designation" may be applied for medicinal products that meet the criteria and the incentives for "orphan designation" include the fee reduction and 10 years of market exclusivity once authorized. Products developed for ultra rare diseases may qualify for a marketing authorization under exceptional circumstances which requires a less complete data set. The following guidance document has been published for further information: "Guideline on procedures for the granting of a marketing authorization under exceptional circumstances, pursuant to Article 14 (8) of Regulation (EC) N° 726/2004."

Alternatively, an MSC therapy product may be considered for national authorization procedure under the HE clause in a particular Member State. In order to qualify for HE authorization, a product has to be prepared on a non-routine basis according to specific quality standards, and has to be used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner (legal definition provided in **Table 1**). The HE regulatory instrument could be in a way perceived as adaptive licensing at the national level for advanced therapy medicinal products. It has to be acknowledged that inherent and unavoidable autologous cell material differences can make certain cell therapy applications more similar to the development of medical technology than to classical medicinal product development process with standard clinical trials (Webster et al., 2011). This does not mean that autologous cell products by definition would qualify for HE approval since regulatory decision is not based on the origin of cell material. However, autologous products combined with complex medical procedures are more likely to qualify for the HE due to the inherent product characteristics. In either case the HE is still a very new regulatory procedure which, if considered, should be discussed in advance with the experts of an NCA. The implementation of the HE clause has been accomplished in the majority of EU states by 2012, but the terms and conditions of the authorization vary and each Member State decides on the implementation tools. For instance, a recent publication illustrates the differences between France and the UK regarding the HE authorizations and also provides information on several other ATMP development-related issues that are regulated differently at the national level (Mahalatchimy et al., 2012). This reference should be examined in case the application for the HE authorization is planned in the UK or France. For instance, the UK agency provides options for either the HE authorization or the "Specials" exemption status according to Article 5.1. of Directive 2001/83/EC. The website of the UK NCA – Medicines and Healthcare products Regulatory Agency (MHRA) – provides user friendly information on the ATMP-related questions and relevant flowcharts in the section "How we regulate advanced therapy medicinal products." Information on the HE clause in Germany is available on the website of Paul-Ehrlich-Institute (PEI) which is the German NCA. The PEI Innovation Office website includes summaries in English and flow-chart on how the ATMPs are regulated in Germany (links are provided in references). German ATMP framework is reviewed and analyzed in detail in a recent publication (Buchholz et al., 2012).

CONCLUSION

Regulatory centralization has been introduced for cell therapy product marketing in the EU since 2009 but the remaining national procedures can be quite heterogeneous. Still, general provisions in the national legal acts are based on the EU directives and therefore will be common for all Member States. The EMA and NCA guidance documents, initiatives, and interaction platforms are available to make the regulatory framework more understandable and accessible for investigators both in the public and private sectors. Good understanding of centralized and national components of the framework will form an essential part of a product development plan and initial shortfalls will be difficult to compensate at the marketing authorization application stage. It is in the best interests of the investigators and investors to communicate with the NCAs and the EMA already during early phase of MSC product formulation. Resulting strategy improvements may facilitate MSC-based medicine development and authorization in the European Union.

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APPENDIX

LEGISLATIVE ACTS

Regulation (EC) N° 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:136:0001:0033:en:PDF

Regulation (EC) N° 507/2006 on the conditional marketing authorisation for medicinal products for human use falling within the scope of Regulation (EC) No 726/2004 of the European Parliament and of the Council. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:092:0006:0009:EN:PDF

Regulation (EC) N° 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) N° 726/2004. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF

Commission Regulation (EC) N° 668/2009 of 24 July 2009 implementing Regulation (EC) No 1394/2007 of the European Parliament and of the Council with regard to the evaluation and certification of quality and non-clinical data relating to advanced therapy medicinal products developed by micro, small and medium-sized enterprises. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:194:0007:0010:EN:PDF

Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:121:0034:0044:EN:PDF

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Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:en:PDF

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GUIDANCE DOCUMENTS

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Mesenchymal stem cells: are we ready for clinical application in transplantation and tissue regeneration?

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Mesenchymal stem cells (MSC) are emerging as a therapeutic option for a plethora of immunological and degenerative diseases. Preclinical research is focusing on the mechanisms of MSC-mediated immunomodulation and regeneration. This Research Topic shines light on mechanistic and regulatory aspects that are of importance for understanding the full potential of MSC for clinical use in transplantation and tissue regeneration.

A first consideration to make when studying MSC that is addressed in this Research Topic is the source of the cells. MSC are present in bone marrow and adipose tissue, amongst other tissues, and can be used in an autologous or allogeneic way. A commercially available allogeneic "off the shelf" cell product as described by Vaes et al. (2012) has the advantage that it is thoroughly characterized and can be produced at a relatively low cost, although for particular applications in transplantation autologous cell usage may be preferable because of the expression of HLA class I on MSC and the potential risk of sensitization upon the use of allogeneic MSC.

Functionally, MSC are influenced by environmental factors. Leijs et al. (2012) demonstrated that synovial fluid of arthritic patients modulates the expression of genes with immunomodulatory function in MSC. Also, toll-like receptor activation by pathogen-derived components or danger signals produced upon tissue injury can modulate MSC toward either an anti-inflammatory or a pro-inflammatory phenotype and alter their function (Delarosa et al., 2012). The local milieu appears therefore crucial in the therapeutic effect of MSC.

It becomes more and more clear that multiple mechanisms are responsible for the immunomodulatory effect of MSC. Different mechanisms target a variety of facets of immune cell functioning. For instance, as shown by Zinocker and Vaage (2012). The inhibition of T cell proliferation by rat MSC is dependent on nitric oxide, whereas cytokine production is modulated by the production of prostaglandin E2 by MSC. Interest is rising in the effect of MSC on B cells, as B cells are increasingly held responsible for transplant rejection. MSC can inhibit immunoglobulin production by B cells and may induce regulatory B cells, although the conditions under which this takes place are not clear yet, as lined out by Franquesa et al. (2012). More is known about the effect of MSC on regulatory T cells. Engela et al. (2012) reviewed studies that demonstrate the induction of regulatory T cells by MSC *in vitro* and *in vivo* after infusion. Although MSC and regulatory T cells target the same cell types, there is no evidence that they impede each other's function. The induction of regulatory T cells by MSC may be crucial for the long-term effects of MSC after infusion. Eggenhofer et al. (2012) demonstrated that intravenous infusion of MSC leads to accumulation of the cells in the lungs and that they disappear within 24 h, after which they cannot be detected in other tissues. This suggests that MSC are short-lived after infusion and that their effects are transferred to other cell types.

Chen et al. (2012) reviewed the clinical potential of MSC-based regenerative therapy for chronic wounds. Studies have shown that MSC administration augments the acute inflammatory response, enhances angiogenesis, accelerates re-epithelialization, and increases wound healing, even in conditions of impaired healing such as diabetes. However, whether MSC induced cutaneous regeneration by cellular differentiation or indirectly through paracrine activity is still unknown. A better understanding of the mechanism of action is needed to develop more efficient treatment strategies. Also, further investigation into delivery methods specifically designed for the delivery of progenitor cells to chronic wounds is necessary to maximize the regenerative properties of MSC-based cell therapy.

Different studies have reported beneficial effects of human MSC on repair of ischemia-reperfusion and other acute kidney injury, as discussed by de Vries et al. (2012). The therapeutic potential of human MSC was studied in immunodeficient NOD-SCID mice after cisplatin-induced acute renal failure; MSC reduced renal cell apoptosis and increased proliferation. MSC also preserved the integrity of the tubular epithelium and peritubular vessels, and prolonged survival.

Mechanistically, there is growing evidence that the process of transdifferentiation is unlikely to be relevant to renal repair *in vivo*. The primary means of these cells most likely involve paracrine and endocrine effects, including mitogenic, anti-apoptotic, anti-inflammatory, antifibrotic, and angiogenic influences. The factors that mediate the paracrine effects are obviously of great interest. Several factors that are abundant in MSC-conditioned medium have been mentioned, including microvesicles released from MSC may account for this paracrine mechanism.

Seifert et al. (2012) warn that treatment with both donor- and recipient-specific MSC in a preclinical kidney transplantation model surprisingly resulted in enhanced humoral immune responses. Signs of inflammation and rejection were generally enhanced in both MSC-treated groups compared to PBS control groups. Additionally, pre-treatment with donor-specific MSC significantly enhanced the level of donor-specific antibody formation when compared with PBS- or recipient MSC-treated groups. Pre-treatment with both MSC types resulted in a higher degree of kidney cortex tissue damage and elevated creatinine levels at the

time point of rejection. Thus, MSC pre-sensitization in this model impaired the renal allograft outcome. In liver disease, it is thought that MSC act differently according to their pleiotropic spectrum of action, depending on the etiology, and pathophysiology of the specific liver disease. Thus, the anti-inflammatory, anti-apoptotic, and pro-proliferative features of MSC might be favorable in cases of chronic inflammatory liver diseases. Additionally, functional tissue replacement is warranted in cases of massive tissue loss in order to provide sufficient metabolic capacity, such as in acute liver failure and extended liver resections, as discussed by Christ and Stock (2012). Therefore, understanding the impact of MSC both on the molecular and cellular level and their interactions with the host liver tissue in a microenvironment created by the diseased liver, is important for the development of effective MSC therapy. Another interesting option is to administer MSC in combination with human hepatocytes; to support hepatocyte function, but moreover to minimize short-term rejection of the hepatocyte transplant. This would facilitate bridging the patient to liver transplantation, or help the patient through the critical phase of acute liver failure until the native liver recovers. This setting would enable allogeneic hepatocyte transplantation avoiding long-term immunosuppression and its adverse effects.

The translation of a cell-based therapy from bench to bedside is challenging under a regulatory framework involving multiple responsible authorities, EU members, and continents. Regulatory centralization has been introduced in the EU since 2009 but the remaining national procedures can be quite heterogeneous. As pointed out by Ancans, European Medical Agency (EMA), and National Competent Authorities (NCA) guidance documents, initiatives, and interaction platforms are available to make the regulatory framework more understandable and accessible for investigators both in the public and private sectors (Ancans, 2012). Good understanding of the regulatory framework is essential for product development; initial steps will determine the marketing authorization application stage. Therefore, early communications between researchers/clinicians and the NCAs and the EMA are advised. Resulting strategy improvements may facilitate MSC-based medicine development and authorization in the European Union.

The tremendous therapeutic potential of MSC has once again been highlighted in this Research Topic. MSC can alleviate alloreactivity, but also reduce inflammatory responses involved in ischemia/reperfusion injury. We are encouraged by the results of the first clinical trials, however, as we have seen in this topic, there are still several questions to be resolved before MSC can be broadly applied in transplantation. The knowledge obtained from this Research Topic may aid to further development of MSC-based therapies in transplantation and tissue regeneration.

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