Mesenchymal stromal cells in renal transplantation: opportunities and challenges

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Abstract | Lifelong immunosuppressive therapy is essential to prevent allograft rejection in transplant recipients. Long-term, nonspecific immunosuppression can, however, result in life-threatening complications and fail to prevent chronic graft rejection. Bone marrow (BM)-derived multipotent mesenchymal stromal cells (MSCs) have emerged as a potential candidate for cell-based therapy to modulate the immune response in organ transplantation. These cells can repair tissue after injury and downregulate many of the effector functions of immune cells that participate in the alloimmune response, converting them into regulatory cells. The findings of preclinical and initial clinical studies support the potential tolerance-inducing effects of MSCs and highlight the unanticipated complexity of MSC therapy in kidney transplantation. In animal models of transplantation MSCs promote donor-specific tolerance through the generation of regulatory T cells and antigen-presenting cells. In some settings, however, MSCs can acquire proinflammatory properties and contribute to allograft dysfunction. The available data from small clinical studies suggest that cell infusion is safe and well tolerated by kidney transplant recipients. Ongoing and future trials will provide evidence regarding the long-term safety of MSC therapy and determine the optimum cell source (either autologous or allogeneic) and infusion protocol to achieve operational tolerance in kidney transplant recipients. These studies will also provide additional evidence regarding the risks and benefits of MSC infusion and will hopefully offer definitive answers to the important questions of when, where, how many and which types of MSCs should be infused to fully exploit their immunomodulatory, pro-tolerogenic and tissue-repairing properties.

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doi:10.1038/nrneph.2016.7
Published online 8 Feb 2016

Kidney transplantation is now the therapy of choice for end-stage renal disease (ESRD). However, the lifelong global immunosuppression that is required to circumvent graft rejection in transplant recipients imposes substantial risks of morbidity and mortality, impairs protective responses against pathogens[1] and hinders tumour immunosurveillance[2]. Immunosuppressive drugs have important adverse effects including nephrotoxicity and an increased risk of cardiovascular diseases and diabetes[3–5]. Moreover, these drugs have failed to substantially prolong long-term graft survival in the past two decades, despite a dramatic improvement in short-term graft survival[6]. The attention of transplant immunologists has, therefore, turned to identifying novel strategies to achieve allograft tolerance and avoid the need for long-term immunosuppression[7].

Cell-based therapies have been proposed as innovative approaches to induce immune tolerance in organ transplantation[8–10]. The hope is that administration of cells with immunoregulatory properties to transplant recipients could tip the balance between effector and regulatory pathways, ultimately promoting the potential of the host immune system to control the immune response to the allograft[11]. In particular, mesenchymal stem or stromal cells are emerging as a promising cell therapy in clinical transplantation. In this Review we provide an overview of preclinical data that support the potential tolerance-inducing effects of bone marrow (BM)-derived multipotent mesenchymal stromal cells (MSCs) in transplant models, and the results of initial clinical studies of BM-derived MSC therapy in kidney transplant recipients. We also discuss lessons learned so far regarding the safety, efficacy, and mechanisms of action of MSC-based therapy in the setting of kidney transplantation.

Discovery and definition of MSCs

Mesenchymal stem cells are non-haematopoietic progenitors that can differentiate into several mesenchymal tissues. They were identified 50 years ago by

Key points

- The unique immunomodulatory properties of multipotent mesenchymal stromal cells (MSCs) make MSC-based therapy one of the most promising tolerance-promoting cell therapies in solid organ transplantation
- MSCs can down-modulate the effector functions of cells that are involved in the alloimmune response, including those of dendritic cells, T cells, B cells and macrophages, converting them into regulatory cells
- In experimental models of solid organ transplantation, MSCs can induce long-term graft acceptance when given alone or in combination with short-term treatment with immunosuppressive drugs
- In the setting of kidney transplantation MSCs can also acquire proinflammatory function and worsen allograft outcomes
- Initial clinical experience with bone-marrow-derived MSCs in kidney transplantation indicates the safety and feasibility of the procedure and suggests that MSCs can promote donor-specific immunomodulation and possibly a pro-tolerogenic environment
- Future studies should provide evidence for the long-term safety of MSC therapy as well as their efficacy in inducing operational tolerance in kidney transplant recipients

Friedenstein *et al.*, who described the existence of non-haematopoietic stem cells in rodent BM[12]. These cells adhered to plastic, differentiated *in vitro* into skeletal tissue cells (osteoblasts, adipocytes and chondrocytes) and, when seeded at clonal density, gave rise to colony-forming unit (CFU) fibroblasts[12,13]. When implanted in ectopic locations in semi-allogeneic animals, the clonal progeny of a single CFU fibroblast led to the production of fibrous tissue and ectopic bone that contained bone marrow[13,14]. This self-renewal capability and skeletogenic potential was subsequently traced to perivascular cells that could be prospectively isolated on the basis of phenotypic markers (nestin, CD105, vascular cell adhesion protein and CD90 in mice; Stro-1, CD146, alkaline phosphatase, CD49a and CD271 in humans)[15–17].

Since the first description of non-haematopoietic stem cells in BM, the properties of adherence to plastic and *in vitro* proliferation have been used as the main criteria for isolating mesenchymal stem cells from BM. Although BM culture-expanded mesenchymal stem cells appear to be morphologically homogeneous, they are actually heterogeneous and not all plastic-adherent stromal cells adequately fulfil stringent stemness criteria[18]. This finding led the International Society for Cellular Therapy (ISCT) to propose the term multipotent mesenchymal stromal cells (MSCs) and suggest minimum criteria required to phenotypically define these cells: adherence to plastic under standard culture conditions; expression of CD105, CD73 and CD90; no expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface antigens; and ability to differentiate into the cells of three skeletal tissues under appropriate *in vitro* conditions[19]. All of the studies described below adhere to these criteria.

MSCs have not yet been unequivocally identified *in vivo*, but increasing evidence suggests that they might reside in a perivascular niche[20,21]. Perivascular cells that envelop microvessels and adventitial cells surrounding larger arteries and veins have been described as possible *in vivo* counterparts of MSCs or MSC precursors[20]. Such localization might account for the ability to isolate MSCs not only from BM, but also from different tissues and organs throughout the body, including adipose tissue and dental pulp[22]. Nevertheless, MSCs from non-haematopoietic tissues are not necessarily identical to those derived from the BM[18]. BM-derived MSCs obtained using plastic adherence and *in vitro* expansion exhibit powerful immunomodulatory properties, thereby emerging as attractive candidates for therapeutic applications in autoimmune diseases and transplantation.

The alloimmune response

In organ transplantation, recognition of donor graft alloantigens, mainly MHC molecules, by recipient T cells is the central mechanism that underlies the process of acute cellular rejection (FIG. 1). Recipient T cells recognize donor antigens either as intact MHC–peptide complexes on the surface of donor antigen-presenting cells (APCs) migrating outside the graft — a direct allorecognition pathway that is unique to the transplant setting — or presented as a processed peptide in the context of self MHC molecules by recipient APCs — an indirect pathway that resembles the recognition of a foreign peptide[23] (FIG. 1).

The direct allorecognition pathway is dependent on short-lived donor APCs[24], has a dominant role in the initiation of the adaptive immune response, and leads to the activation of a very high number of T cells. Estimates suggest that up to 10% of human T cells can directly recognize donor MHC molecules; this frequency is 100-fold higher than the frequency of T cells that can be activated by the indirect pathway[25,26]. The magnitude of the alloresponse is reflected by the strong activation of T cells when added to allogeneic stimulator cells in a mixed lymphocyte reaction (MLR). Once activated in transplant recipients, $CD4^+$ and $CD8^+$ T cells undergo clonal expansion, differentiate into effector cells and migrate into the graft where they participate in destroying the transplanted organ[27].

In response to alloantigens, effector $CD4^+$ T cells are induced to differentiate mainly into type 1 T helper (T_H1) cells[28], which produce and release high levels of interferon-γ (IFNγ) and IL-2 (FIG. 1). IFNγ increases the expression of graft MHC and adhesion molecules, promoting graft infiltration of T cells, which in turn activate macrophages to mediate a destructive delayed type hypersensitivity reaction[29,30]. IL-2 acts as growth factor to sustain the proliferation and survival of T cells and B cells, converting the latter into antibody-producing plasma cells[27,30]. Antibody-mediated graft damage then takes place by complement activation or through the recruitment of antibody-dependent cell-mediated cytotoxic effector cells[31]. Cytotoxic $CD8^+$ T cells destroy the graft by inducing cell death either by releasing lytic enzymes or by promoting apoptosis of the target tissue cells via Fas–FasL interactions[32]. Antigen-specific effector T cells include naive T cells, which are primed in the secondary lymphoid organs, and memory T cells, which can be reactivated and undergo clonal expansion at nonlymphoid sites[33].

In the context of clinical transplantation, memory T cells are a major problem[33]. The memory T cell pool generated in response to previous infections or vaccinations contains memory T cells that can recognize and respond to MHC molecules on donor cells, a process termed heterologous immunity. Memory T cells are

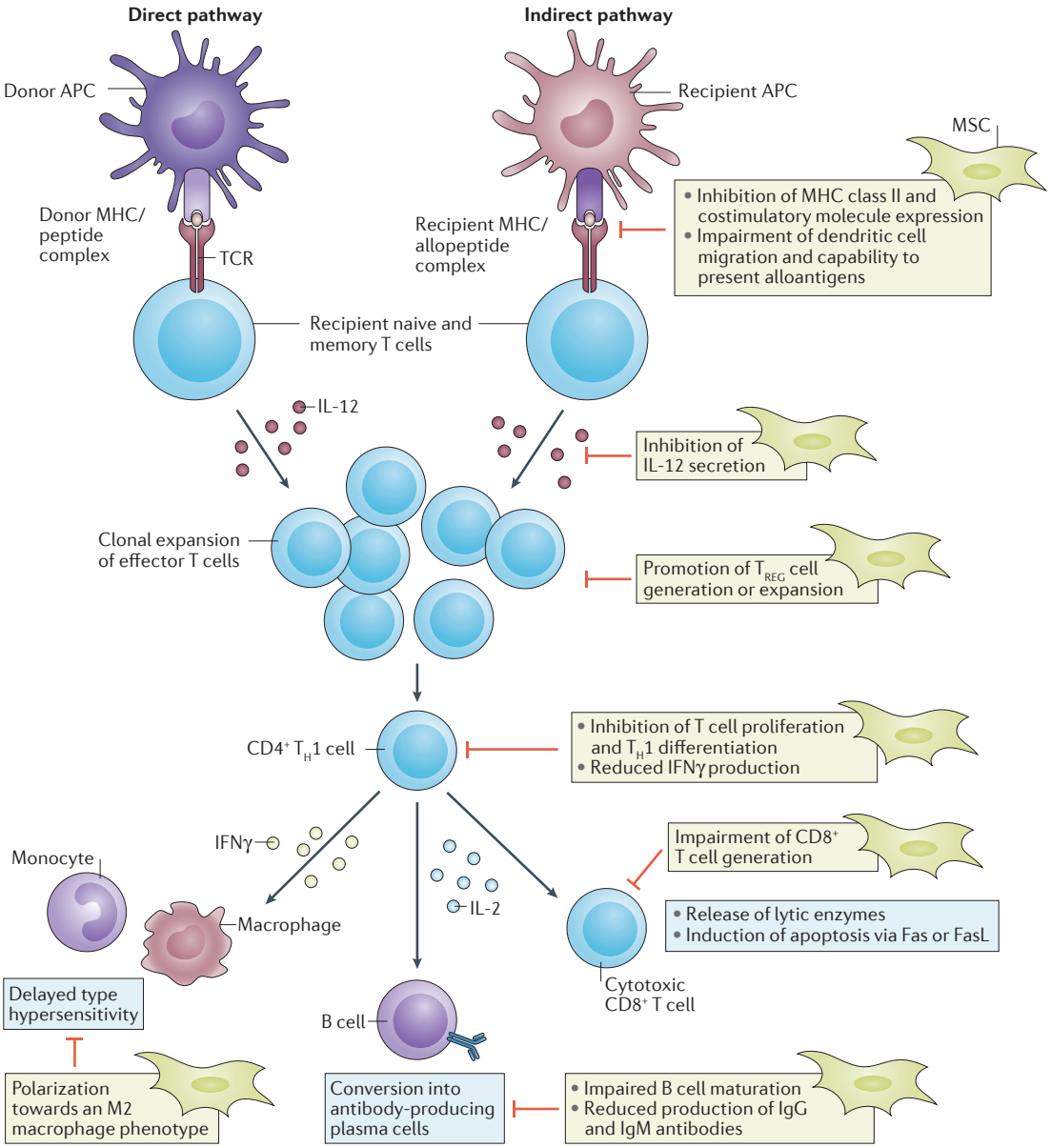

Figure 1 | **The immunomodulatory effects of mesenchymal stromal cells (MSCs) during the alloimmune response.** In response to recognition of donor alloantigens via the direct or indirect pathways, $CD4^+$ effector T cells differentiate into type 1 T helper (T_H1) cells, which produce interferon-γ (IFNγ) and IL-2. IFNγ promotes graft infiltration of T cells, which activate macrophages resulting in a destructive delayed type hypersensitivity reaction. Antibody-mediated graft damage occurs as a result of complement activation or through the recruitment of cytotoxic $CD8^+$ T cells. MSCs interfere with the effector functions of dendritic cells, naive and memory $CD4^+$ and $CD8^+$ T cells, macrophages and B cells through various mechanisms, and might also promote the generation of regulatory T (T_{REG}) cells, B cells and M2 macrophages. APC, antigen-presenting cells.

more difficult to deplete than naive T cells and survive treatment with alemtuzumab or polyclonal rabbit anti-thymocyte globulin (rATG)[34,35]. In lymphopenic conditions, depletion-resistant memory T cells undergo fast homeostatic proliferation driven by the homeostatic cytokines IL-7 and IL-15, which are present at supra-physiological concentrations because of diminished use owing to reduced T cell numbers or increased production by stromal tissue[36]. Moreover, donor-specific memory-T-cell-mediated rejection is extremely difficult to prevent or inhibit. Current immunosuppressive drugs that inhibit naive T cells have minimal effects on preventing memory-T-cell-mediated rejection[37]. Thus, evidence exists of a strong correlation between the presence of pre-transplant alloreactive memory T cells and acute rejection episodes that occur despite tacrolimus-based or sirolimus-based immunosuppressive therapies[33].

Activated T cells might also differentiate into regulatory T (T_{REG}) cells, a distinct subset of $CD4^+CD25^+$ T cells that express high levels of the transcription

factor forkhead box protein P3 (FOXP3)[38]. T_{REG} cells have a major role in the control of autoimmunity, and experimental evidence indicates that they also actively downregulate the alloimmune T-cell response, promoting transplantation tolerance[39]. Without intervention, however, graft inflammation fosters the generation of donor-reactive T_H1 subsets, leading to a disproportionally higher number of graft-destructive effector T cells than graft-protective T_{REG} cells.

Immunosuppressive properties of MSCs

In the past two decades, a large amount of research has provided insight into the immunomodulatory properties of MSCs. The available data provide compelling evidence that these cells can downregulate the function of immune effector cells that drive the host anti-graft immune response and potentially promote the development of tolerance (FIG. 1).

Dendritic cells

Murine studies have shown that MSCs impair the maturation and allostimulatory function of dendritic cells (DCs)[40,41]. In addition to preventing tumour-necrosis-factor (TNF)-induced[40] or lipopolysaccharide-induced[41] upregulation of MHC and co-stimulatory molecules, MSCs interfere with indirect antigen presentation by DCs. When pulsed with ovalbumin (OVA), MSC-conditioned DCs had an impaired ability to induce proliferation of OVA-specific T-cell receptor (TCR)-transgenic $CD4^+$ T cells[40,41] and $CD8^+$ T cells[41], and showed a defective indirect presentation of MHC-derived allopeptides[40]. In addition, by downregulating the expression of C-C chemokine receptor type 7, MSCs inhibited DC migration in response to C-C motif chemokine 19 *in vitro*[40] and *in vivo*[41]. Subcutaneous injection of OVA-pulsed DCs into naive mice injected with OVA-specific transgenic TCR $CD4^+$ T cells and MSCs resulted in impaired priming of T cells owing to the failure of MSC-conditioned DCs to migrate to the draining lymph nodes[41].

Human monocytes induced to differentiate into DCs by granulocyte-macrophage colony-stimulating factor and IL-4, and cultured with BM-derived MSCs, failed to upregulate co-stimulatory molecules and MHC class II expression, as well as to secrete IL-12 (REFS 42–44). Moreover, DCs conditioned by MSC exposure had a reduced allostimulatory effect, as evidenced by an impaired ability to stimulate the proliferation of allogeneic T cells[42–44]. Hence these DCs might promote the generation of alloantigen-specific $FOXP3^+$ T_{REG} cells[45]. Modulation of human DC functions by MSCs might be mediated by cell-contact-dependent mechanisms[43,46] and by the release of soluble factors, such as prostaglandin E2 (PGE2)[44,47]. MSCs might, however, lose their capacity to maintain human DCs in a semi-mature phenotype when incubated with these cells at a later differentiation phase. Indeed MSCs that were added to differentiated DCs[46] or to immature DCs that had been primed with lipopolysaccharide[47,48] failed to reduce DC surface expression of co-stimulatory molecules or to inhibit allogeneic T-cell stimulatory pathways, suggesting that MSCs could be more effective immunomodulators if they are present at the early stages of DC activation. Together, these studies clearly indicate that MSCs are capable of affecting DC phenotype and function by inhibiting their maturation and migration to lymph nodes, and eventually converting them into APCs with defective allostimulatory properties.

T cells

MSCs can also interfere directly with T-cell function. As initially demonstrated using baboon cells[49], addition of BM–MSCs to a MLR inhibits T-cell proliferation. MSCs are effective inhibitors of T cells in MLRs irrespective of MHC matching with the responder or stimulator lymphocytes in the assay. Moreover, murine BM–MSCs inhibited the proliferation[50,51], IFNγ production and cytotoxic function of naive and memory T cells that were stimulated by indirect presentation of the male HY peptide[51]. In this setting, T cells were arrested in the G1 phase of the cell cycle; their proliferation was, therefore, irreversibly abrogated[50].

Consistent with the findings of murine studies, human MSCs inhibited T-cell proliferation in response to anti-CD3 and anti-CD28 antibodies, suggesting a direct, APC-independent effect of MSCs on T-cell proliferation[52]. Moreover, human MSCs inhibited the proliferation of naive $CD45RA^+$ T cells and memory $CD45RO^+$ T cells in response to allogeneic DCs, but had little effect on the specific memory T cell response to viral antigens[53]. MSCs also inhibited the proliferation of human memory T cells in response to the homeostatic cytokines IL-7 and IL-15 (REF. 54); this finding is relevant to the transplant setting in which homeostatic cell proliferation occurs after T-cell depletion resulting from use of induction therapies. Human MSCs were also shown to interfere with the generation of cytotoxic $CD8^+$ T cells *in vitro,* but they did not seem to inhibit the activity of existing cytotoxic $CD8^+$ T cells[55].

Various mediators might have a role in MSC-induced inhibition of T-cell proliferation. Human MSCs have been suggested to inhibit T-cell activation via indoleamine 2,3-dioxygenase (IDO)[54,56,57], PGE2 (REF. 58), TGF-β[59], galectins[60,61] and HLA-G[62], whereas nitric oxide (NO) has been implicated as a mediator of inhibition induced by murine MSCs[63–65]. In inflammatory conditions, especially in the presence of high levels of IFNγ, MSCs release large amounts of these mediators[56,57,64], thereby increasing their capability to exert immunoregulation. Moreover, proinflammatory cytokines stimulate MSCs to express T-cell-specific cytokines, such as C-X-C motif chemokine (CXCL) 9 and CXCL10, which attract T cells into close proximity with the MSCs where they are exposed to high levels of soluble factors[64].

The intriguing ability of MSCs to promote T_{REG} cell generation while inhibiting the activity of effector T cells makes them unique immunomodulators. In the murine setting, MSCs polarized T cells towards a regulatory phenotype, an effect that was increased when they were added to T_H0 cells rather than to mature T_H1 cells[66]. In MLRs or purified T-cell cultures, the addition of human BM-derived MSCs promoted the differentiation of T cells toward $CD25^+FOXP3^+$ T_{REG} cells, which

were able to suppress the syngeneic T-cell alloresponse, a process that was mediated by PGE2 and TGF-β1 (REF. 67) or HLA-G5 (REF. 68). The ability of MSCs to generate T_{REG} cells might also involve immune cells other than T cells or DCs. TGF-β produced by human MSCs polarized monocytes towards type 2 macrophages that expressed high levels of IL-10 and CCL18, and expanded T_{REG} cells *in vitro*[69]. In mice MSCs promoted T-cell apoptosis and uptake of the resulting debris induced macrophages to express high levels of TGF-β, which stimulated the generation of T_{REG} cells[70].

B cells

BM-derived MSCs have been shown to arrest B cells in the G0/G1 phase of the cell cycle[71], inhibit their proliferation and impair their maturation into antibody-secreting cells[71,72]. When injected into mice that had been immunized with T-cell-dependent or T-cell-independent antigens, BM-derived MSCs prevented antigen-specific IgM and IgG production[73]. Notably, MSCs isolated from human adipose tissue can promote the differentiation of B cells into IL-10-producing $CD19^+CD24^{high}CD38^{high}$ regulatory B (B_{REG}) cells *in vitro*[74], suggesting that MSCs might induce B cells with regulatory phenotype and function.

Together the studies described above provide a complex picture of the potential immunomodulatory effects of MSCs during the alloimmune response (FIG. 1). The findings indicate that MSCs can interfere with the effector functions of DCs, naive and memory $CD4^+$ and $CD8^+$ T cells, macrophages and B cells via more than one mechanism and pathway, and might also promote the generation of T_{REG} cells, B_{REG} cells and regulatory macrophages.

MSCs in animal models of transplantation

Heart transplantation

A large number of rodent models have been used to evaluate the immunoregulatory properties of MSCs related to alloreactive responses in the setting of heart transplantation (TABLE 1). An initial study showed that MSCs isolated from the BM of Wistar rats before heart donation and given via multiple infusions to fully MHC-mismatched Fisher344 rat recipients before and after transplantation prolonged graft survival in the absence of immunosuppressive drugs (mean 12.4 days versus 6.4 days in controls)[75]. By contrast, peritransplant administration of donor or recipient-derived BM-derived MSCs into ACI rat recipients of fully-MHC-mismatched hearts from Lewis rats had no effect on graft survival[76]. Rather than improving the immunosuppressive effect of donor-derived or recipient-derived MSCs, the introduction of ciclosporin A (CsA) treatment from day 5 to day 9 post-transplantation seemed to accelerate rejection in this model[76]. A later study reported that administration of donor MSCs at high doses 7 days before transplantation and on the day of transplantation, but not at just one of these time points, prolonged the mean survival of fully-MHC mismatched LEW.1W hearts in LEW.1A recipients from 6 days to 23 days[77]. The researchers found that haem oxygenase-1 and NO expressed by MSCs were the main factors responsible for this beneficial effect. Interestingly, the expression of these immunomodulatory molecules decreased during *in vitro* expansion of MSCs[77], suggesting that differences in expansion protocols (that is number of passages) might explain the contrasting results of previous studies[72,73].

A subsequent study showed that pretransplant intravenous administration of donor MSCs in ACI rat recipients that received mycophenolate mofetil (MMF) immunosuppression during the first week post-transplantation resulted in the induction of long-term acceptance of LEW heart grafts[78]. The donor MSCs promoted graft acceptance when given 4 days before transplantation, but not when given at day 0 or at 3 days post-transplantation; these data provided the first evidence that the timing of MSC infusion has a crucial role in their tolerogenic effect. In the same model, BM-derived MSCs isolated from third-party rats were ineffective, whereas infusion of syngeneic MSCs resulted in long-term graft acceptance in three of eight recipients. Long-term graft survival was associated with a significant increase in the frequency of peripheral $CD4^+CD25^+Foxp3^+$ T cells, although mRNA expression of *Foxp3* in the graft and lymphoid organs 10 days after transplantation was comparable in rats that received MSCs and MMF and those that received MMF alone. Based on these data, the researchers suggest that in their model T_{REG} cells could have a role in the maintenance phase of MSC-induced immune tolerance. By contrast, the induction phase of tolerance was associated with the development in lymphoid organs of immature DCs expressing low levels of MHC class II and the co-stimulatory molecule CD86 (REF. 78).

Further studies in the setting of fully allogeneic heart transplantation showed that tolerance induction could be achieved using donor or third-party-derived multipotent adult progenitor cells (MAPCs). These cells are a class of *in vitro* expanded, adherent, BM progenitors that are isolated from adult BM precursors. In contrast to MSCs, MAPCs are isolated using hypoxic conditions and maintained at subconfluent density in media supplemented with epidermal growth factor and platelet-derived growth factor[79]. Although MAPCs and MSCs can be considered to be distinct cell populations based on different culture conditions, cell-surface phenotype and proliferative capacity[79], they have comparable suppressive effects on T-cell alloreactivity *in vitro*[54,80]. MAPC infusion in combination with MMF therapy was associated with the development of a population of myeloid-derived suppressor cells (MDSCs) that expressed CD11b/c and inducible NO synthase in the spleen and graft early after transplantation[81]. Donor MAPCs were most effective at prolonging allograft survival when given into the portal circulation by intrasplenic injection rather than administered intravenously[81]. Intriguingly, in a murine heart transplant model that used the same MMF treatment protocol, a complex mechanism was identified through which MDSCs generated by donor MSCs induced a strong, short-term T_H17 cell response, which was able to mediate acute graft rejection[82]. In the presence of MMF therapy, however, these T_H17 cells were converted to T_{REG} cells that were able to sustain long-term graft acceptance.

Table 1 | **Graft survival with BM-derived MSC infusion in animal models of transplantation**

Model (donor/ recipient)	BM-MSCs			Immuno-suppression (dose; timing)	Mean graft survival (days)				Refs
	Source	Timing of infusion* (injection site)	Dose		Untreated	MSC	Immuno-suppression	MSC plus immuno-suppression	
Heart transplantation									
Rat (Wistar/ Fisher 344)	Donor	Day −7 and 0–3 (IV)	2×10^6	None	6.4	12.4	NA	NA	75
Rat (Lewis/ACI)	Donor or recipient	Day 0 (vena cava or portal vein) and 3 (IV)	2×10^6	CsA (0.5 mg/kg; day 5–9)	9	9 (donor or recipient MSCs)	>32	10 (donor or recipient MSCs)	76
Rat (LEW.1W/ LEW.1A)	Donor	Day −7 and 0 (IV)	$5\text{-}7\times10^6$	None	6	23	NA	NA	77
Rat (Lewis/ACI)	Donor, recipient or third-party	Day −4 (IV)	2×10^6	MMF (20 mg/kg; day 0–7)	8	6 (donor, recipient or third party MSCs)	15	>100 (donor MSCs); 20 (recipient or third-party MSCs)	78
Rat (Lewis/ACI)	Donor‡ or third-party‡	Day −4 (spleen) and 0 (IV)	5×10^6	MMF (20 mg/kg; day 0–15)	7–12	15 (donor MSCs)§	15	>100 in 80% recipients (donor or third-party MSCs)	81
Mouse (C57/Balb/c	Donor	Day −4 (IV)	1×10^6	MMF (160 mg/kg; day 0–7)	11	9	17	34	82
Mouse (C57/Balb/c)	Donor	Day 1 (IV)	1×10^6	Rapamycin (2 mg/kg; day 0–13)	7.5	14	17	>100	83
Mouse (Balb/c/C57)	Recipient	Day −7 and −1 (IV)	0.5×10^6	None	9	8	NA	NA	84
Mouse (B6C3/C57)	Recipient or donor	Day −7 (portal vein) and −1 (IV)	0.5×10^6	None	10	>60 (recipient MSCs); >30 (donor MSCs)	NA	NA	84
Kidney transplantation									
Mouse (C57/Balb/c)	Donor	Day 1 (IV)	1×10^6	None	31	>100	NA	NA	89
Rabbit (New Zealand/ Japanese white)	Donor IDO transfected	Day 0 (IV)	2×10^6	None	7	63	NA	NA	90
Rat (Fischer 344/Lewis)	Third-party	Week 11 (IV)	2×10^6	CsA (5 mg/kg; day 0–15)	Sacrificed at 24 weeks	Sacrificed at 24 weeks	NA	NA	91
Rat (LEW/LEW.1U)	Recipient	Day 0 (IV or IA)	1.5×10^6	None	28	9–12	NA	NA	94
Mouse (Balb/c/C57)	Recipient	Day −1 or 2 (IV)	0.5×10^6	None	7	>39 (MSCs infused on day −1); 7.5 (MSCs infused on day 2)	NA	NA	95

BM, bone marrow; CsA, ciclosporin A; IA, intra-arterial; IDO, indoleamine 2,3-dioxygenase; IV, intravenous; MSC, mesenchymal stromal cell. *0 = day of transplantation. ‡Multipotent adult progenitor cells. §Effect of third-party MSCs on graft survival was not determined.

BM-derived MSCs have also been reported to synergize with rapamycin in inducing tolerance to murine cardiac transplants. Infusion of donor MSCs only slightly prolonged the survival of fully MHC-mismatched C57 hearts in Balb/c recipients (from a median of 7.5 days to 14 days), whereas the combination of donor MSCs with short-term rapamycin therapy prevented acute and antibody-mediated rejection and induced indefinite allograft survival[83]. Tolerance was donor-specific and characterized by the generation of immature DCs and T_{REG} cells[83]. In mice we found that donor or recipient BM-derived MSCs injected at various time points before heart transplantation were unable to induce long-term graft survival of fully MHC-mismatched heart transplants, but did promote tolerance to semi-allogeneic B6C3 heart transplants in B6 recipients[84]. Both the induction and maintenance phases of MSC-induced donor-specific tolerance were associated with T_{REG} cell expansion in the periphery and in the graft[84].

In non-immunosuppressed murine recipients, donor MSCs were ineffective in inducing cardiac graft tolerance when injected into the tail vein, but were highly effective when administered via the portal vein[84]. By contrast, syngeneic MSCs were able to induce long-term graft acceptance when injected into either the portal or tail vein[84]. These findings suggest that in mice, injection into immunoprivileged sites, such as the liver, is required for the *in vivo* survival of allogeneic MSCs. Nevertheless, the best source of MSCs to be applied in organ transplantation (whether syngeneic, donor or third-party) is still a matter of debate. Although it is possible that the donor MHC component has a role in the effect of MSCs in transplantation, no definitive conclusions can be drawn based on the available evidence. Studies in experimental models of solid organ or cell transplantation comparing MSCs from different sources found that in some cases donor MSCs were more effective in prolonging graft survival than syngeneic MSCs[78] and vice versa[85,86]. In other studies, MSCs from syngeneic, donor or third-party sources showed comparable effects in prolonging graft survival[83,87]. These reports highlight the need for further research in this area.

The available data indicate that post-transplant administration of either allogeneic (donor or third-party) or syngeneic MSCs in fully MHC-mismatched heart transplant models had no effect or induced only a mild prolongation of graft survival. Better results can be achieved by injecting the cells before transplantation[77,78,81,84,88] and by increasing the cell dose[77,88]. Moreover, allogeneic MSCs are effective when administered together with immunosuppressive drugs, especially MMF and rapamycin[78,81,83]. More promising results in terms of long-term graft acceptance can be achieved in the milder alloreactive environment of semi-allogeneic heart transplants than in the fully allogeneic setting, particularly when syngeneic MSCs alone are given before transplantation. Thus, robust evidence suggests that in heart transplant models, MSCs combined with a short course of immunosuppressive drugs in the fully MHC-mismatched setting or alone in the semi-allogeneic environment, are able to exert their potent immunomodulatory function, thereby promoting donor-specific tolerance characterized by the generation of regulatory APCs (that is DCs and MDSCs) as well as T_{REG} cells.

Kidney transplantation

Few studies have assessed the immunomodulatory properties of MSCs in kidney transplant models. Although MSC treatment protocols similar to those applied in heart transplant models have been adopted, more complex features have emerged in the kidney transplant setting. In mice, post-transplant infusion of donor BM-derived MSCs induced donor-specific tolerance toward life-supporting, fully allogeneic kidney transplants, by generating immature DCs and T_{REG} cells[89]. Administration of MSCs together with the IDO inhibitor 1-methyl-tryptophan or infusion of MSCs that lacked the *IDO* gene completely prevented the development of tolerance, indicating a key role of IDO in mediating the immunomodulatory effect of these cells[89]. Consistent with this finding, transfected MSCs that overexpressed IDO were more effective than wild-type MSCs in promoting long-term graft acceptance in a rabbit model of kidney transplantation[90]. Tolerance induction was associated with a dramatic expansion in the population of circulating $CD4^+CD25^+Foxp3^+$ T cells, starting from day 7 post-transplantation. The development of donor-specific tolerance was further evidenced by the long-term acceptance of subsequent skin allografts from the kidney donor, but not of third-party skin allografts in the recipient rabbits.

MSCs from a third-party source have also been shown to confer long-term protection of kidney allografts. In Lewis rat recipients of Fisher344 rat kidneys, infusion of third-party MSCs at 11 weeks after transplantation inhibited intragraft T-cell and macrophage infiltration and prevented the development of interstitial fibrosis and tubular atrophy[91]. Notably, in this model MSCs also limited the activation of the humoral immune response, as indicated by lower levels of anti-donor MHC antibodies in MSC-treated rats than in untreated controls.

In contrast to these positive studies, multiple peri transplant infusions of syngeneic MSCs have failed to prolong rat kidney graft survival, although they significantly inhibited intragraft macrophage and DC infiltration in response to ischaemia–reperfusion injury[92]. Rat kidney recipients that received syngeneic MSCs pre-transplantation in combination with low-dose MMF developed graft dysfunction 7 days post-transplantation, which was associated with histological evidence of tissue damage, increased expression of proinflammatory cytokines and increased B-cell infiltration and C4d deposition in the graft[93]. Similarly, other studies in rats have shown that peritransplant injection of syngeneic BM-derived MSCs results in severe renal insufficiency within 15 days after allogeneic kidney transplantation[94]. Histologic analysis of kidney grafts from MSC-treated rats showed granulocyte infiltration and signs of thrombotic microangiopathy, despite decreased graft T-cell infiltration[94]. The conflicting findings with donor and syngeneic BM-derived MSCs with or without MMF therapy are difficult to interpret and reconcile; however, they indicate that MSCs could acquire *in vivo* pro-tolerogenic or proinflammatory function, at least in experimental kidney transplant models.

Several factors might contribute to dictating the tolerogenic or inflammatory properties of MSCs. As comparable doses of cells have been used in different experimental settings, we hypothesized that the timing of cell infusion in relation to kidney transplantation could have a major role. In a murine kidney transplant model, we found that intravenous infusion of syngeneic MSCs 1 day before transplantation induced indefinite graft survival[95]. This effect occurred even when MSCs were administered to recipient mice that had previously been sensitized with an infusion of donor spleen cells, and thus carried donor-specific memory T cells. Tolerance induction was associated with

T_{REG} cell expansion within the recipient lymphoid organs in which the syngeneic MSCs localized. By contrast, MSCs that were given 2 days after transplantation migrated into the transplanted kidney. Similarly other investigators have shown that in rodents the majority of intravenously infused MSCs localize immediately to the lungs, with a small proportion gradually migrating to other tissues such as the liver, spleen and kidney, and to the site of injuries and tumours within hours of the infusion[96]. Notably, the level of engraftment of MSCs into the kidney increases following ischaemia–reperfusion injury caused by kidney transplantation[97] or renal pedicle clamping[98]. We found that engrafted MSCs in the injured kidney promote neutrophil infiltration, complement activation and expression of proinflammatory cytokines, leading to impairment of graft function[95]. The mechanism by which intragraft MSCs can be activated to a proinflammatory phenotype remains ill-defined. The possibility exists that MSCs might be exposed to several inflammatory mediators induced by renal ischemia–reperfusion injury that ultimately direct them to acquire proinflammatory functions. Further studies are needed to identify and characterize the molecular mechanisms that underlie the protolerogenic and proinflammatory phenotypes of MSCs, in order to optimize the conditions of MSC administration and fully exploit the tolerogenic potential of this cell therapy in kidney transplantation.

Clinical studies of MSCs in kidney transplantation

Autologous BM-derived MSCs

Based on some encouraging data from experimental models of solid organ transplantation, clinical studies of MSC-based therapy in kidney transplant recipients are currently underway[99–104] (TABLE 2). In these vulnerable patients safety and prevention of adverse reactions are essential. Moreover pilot clinical trials of MSC therapy in the setting of kidney transplantation have to be designed on top of the current pharmacologic immunosuppressive therapy.

Our first clinical study of autologous BM-derived MSC infusion in two living-donor kidney transplant recipients had the primary aim of establishing the safety and feasibility of the procedure, and the secondary aim of dissecting the mechanisms by which MSCs could modulate the host alloimmune response toward a protolerogenic environment. In our centre an immunosuppression minimization protocol is in place that includes induction therapy with low-dose rATG and basiliximab, and maintenance immunosuppression with low-dose CsA and MMF[105]. We chose to infuse the MSCs 7 days post-transplantation for safety reasons (at this time point patients are well recovered from surgery) and because of the inclusion of T-cell depleting rATG in the induction protocol[99]. Our *in vitro* studies showed that antibodies in rATG preparations recognized and bound to human MSCs[99], and so potentially

Table 2 | **Clinical studies of BM-derived MSCs in kidney transplantation**

Study	Induction therapy (dose)	Maintenance immunosuppression	No. of patients	MSC			Main finding
				Source	Dose (cells per kg ×10^6)	Timing	
Perico *et al.* (2011)[99]	rATG (0.5 mg, day 0–6); basiliximab (20 mg days 0 and 4); steroids (day 0 to 7)	CsA, MMF	2	Autologous	1.7–2.0	Day 7	Increased T_{REG} cell:memory CD8 T cell ratio from baseline; engraftment syndrome in two patients
Perico *et al.* (2013)[100]	rATG (0.5 mg, day 0–6); steroids (day 0–7)	CsA, MMF	2	Autologous	2.0	Day -1	Increased T_{REG} cell:memory CD8 T cell ratio from baseline; acute cellular rejection in one patient
Tan *et al.* (2012)[101]	Basiliximab in control group only (20 mg, days 0 and 4)	CNI, MMF, steroids	105 (53 on standard CNI dose; 52 on 80% CNI dose)	Autologous	1.0–2.0	Day 0 and 14	Reduced incidence of acute rejection at 6 months and lower incidence of viral infections in the MSC group than in the control group
Reinders *et al.* (2013)[102]	Basiliximab (20 mg, day 0 and 4)	CNI, MMF, steroids	6	Autologous	1.0–2.0 (two doses 7 days apart)	Week 4 or month 6	MSC infusion enabled resolution of tubulitis and IFTA in two patients with subclinical rejection; opportunistic viral infection in three patients
Mudrabettu *et al.* (2015)[103]	rATG (1mg/kg, day -1 to +1)	Tacrolimus, MMF, steroids	4	Autologous	0.2–0.8	Day -1 and 30	No early or late kidney graft dysfunction and no viral infections in the KTRs
Peng *et al.* (2013)[104]	Cytoxan (200 mg)	Tacrolimus, MMF, steroids	6	Donor	5.0 (renal artery at day 0) and 0.2 (IV day 30)	Day 0 and 30	50% reduction of tacrolimus dose in the MSC group

BM, bone marrow; CNI, calcineurin inhibitor (CsA or tacrolimus); CsA, ciclosporin A; IFTA, interstitial fibrosis and tubular atrophy; IV, intravenous; KTR, kidney transplant recipient; MMF, mycophenolate mofetil; MSC, mesenchymal stromal cell; rATG, rabbit anti-thymocyte globulin; T_{REG}, T regulatory.

could lead to lysis of these cells and impairment of their immunosuppressive functions *in vivo*. By contrast, serum samples from a kidney transplant recipient who had received six low-dose rATG infusions did not have any cytotoxic effect on MSCs *in vitro*[99]. We also found that ciclosporin and steroids did not hamper the ability of MSCs to inhibit T-cell proliferation *in vitro*, and confirmed that MMF synergizes with the immunosuppressive effect of MSCs[99].

Another potential advantage of MSC administration 7 days post-transplantation was that the cells were infused during a period in which T cells were undergoing lymphopenia-induced homeostatic proliferation — a setting in which the MSCs can exert their unique T_{REG}-cell promoting and effector and memory T-cell dampening effects. In both patients MSC infusion promoted a pro-tolerogenic environment in the long-term that was characterized by an enrichment of $CD4^+CD25^{high}CD127^-FOXP3^+$ T cells and reductions in the frequency of $CD45RO^+RA^-$ memory $CD8^+$ T cells in the peripheral blood and the *ex vivo* cytotoxic function of donor-specific $CD8^+$ T cells, compared with cells from kidney transplant recipients who received induction therapy without MSC infusion[99]. Both patients, however, developed transient acute renal insufficiency 7–14 days after MSC infusion. A graft kidney biopsy performed in one patient showed a focal inflammatory infiltrate — mostly of granulocytes with very few T cells and B cells — in the renal interstitium, but no evidence of acute cellular or humoral rejection[99].

As no specific marker for MSCs is available, we evaluated the presence of $CD44^+CD105^+$ cells in an attempt to localize MSCs in the graft. These markers are highly co-expressed by MSCs but are only singly expressed by most other local or circulating cells. Notably $CD44^+CD105^+$ cells were undetectable or the levels were negligible in renal biopsy samples from patients with acute graft rejection who did not receive MSCs, in protocol biopsy samples taken 1-year post-transplantation from patients who were given MSCs peritransplant and in normal renal tissue from a patient undergoing nephrectomy for renal carcinoma, but were present in the graft biopsy sample obtained from a MSC-treated patient a few days after MSC infusion. Based on this evidence, we confidently assumed that the intragraft $CD44^+CD105^+$ cells were *bona fide*, previously infused, autologous MSCs. Immunohistochemical analysis also showed intragraft complement C3 deposition, mainly close to granulocytes[99]. We interpreted these findings to indicate that the subclinical inflammatory environment of the graft in the few days post-surgery could have favoured intragraft recruitment and activation of the infused MSCs, promoting a proinflammatory milieu with eventual acute renal dysfunction (engraftment syndrome), as reported in other studies of combined kidney and BM transplantation[106]. This hypothesis was supported by data from a murine kidney transplant model showing that MSC administration 1 day before, but not a few days after transplantation avoided deterioration of graft function, while maintaining the immunomodulatory effect of MSCs[95]. Despite the initial acute renal insufficiency, both patients who received MSCs at 7 days post-transplantation are healthy with stable graft function after more than 6 years of follow-up.

Based on our observation of acute renal insufficiency after MSC infusion and the similar findings in mice[95], we revised our clinical protocol and gave two subsequent living-related kidney transplant recipients pretransplant (1 day) intravenous infusions of BM-derived autologous MSCs before T-cell-depleting induction therapy[100]. To avoid any potential negative effect of the anti-CD25 antibody basiliximab[107] on the MSC-induced generation of T_{REG} cells, we removed this agent from the induction protocol. In the first patient who received the new protocol MSC treatment was uneventful and graft function remained normal during 5-year follow-up, whereas the second patient experienced acute cellular rejection 2 weeks after transplantation[100]. A higher number of HLA mismatches ($n = 3$ versus $n = 2$) in the latter patient in the presence of an induction therapy regimen of low-rATG without basiliximab might explain these differing outcomes. Nevertheless, a few months after kidney transplantation both patients showed an increased ratio of T_{REG} cells to memory $CD8^+$ T cells in the peripheral blood as well as a profound and persistent reduction in *ex vivo* anti-donor $CD8^+$ T-cell cytotoxicity from baseline. Although based on findings in only two patients, these observations underscore the possibility that autologous MSCs might have a low capacity to control the host immune response early post-transplantation in the context of a highly alloreactive environment. As MSC immunomodulatory function develops slowly in the early post-transplantation period, adequate induction therapy, including basiliximab, could be of value to limit the risk of acute graft rejection.

Notably we did not find any significant difference in the frequencies of $CD4^+CD25^{high}CD127^-FOXP3^+$ T cells and $CD4^+FOXP3^+$ T cells between the patients who received post-transplantation MSC infusion and those who received pre-transplantation MSC infusion[100]. We concluded, therefore, that basiliximab does not exert a major negative effect on T_{REG} cell expansion. Given this finding we further modified our protocol; in our ongoing study living-donor kidney transplant recipients are treated with autologous MSCs the day before transplantation and also receive induction therapy that includes basiliximab and low-dose rATG[108].

A large randomized controlled study performed in China examined the efficacy of autologous BM-derived MSC infusion (at the time of kidney reperfusion and 2 weeks later) as an alternative to basiliximab induction therapy in 156 living-donor kidney transplant recipients[101]. At 6 months post-transplantation, the researchers found a lower frequency of acute rejection episodes in the MSC-treated patients than in those who had received basiliximab induction therapy. However, the rate of acute rejection at 6 months in patients given MSCs was similar to that reported in other trials in which participants received induction regimens that included basiliximab and maintenance immunosuppression with low-dose calcineurin inhibitor, MMF and steroids[109]. This observation raises concerns about

the unexpectedly high rate of acute rejection in the basiliximab group in the Chinese trial, which might have led to a biased conclusion. Furthermore, 1 year after transplantation, the rate of acute rejection and the difference in renal function were not significantly different between the study groups[110], suggesting that MSC infusion does not improve these outcomes, even though transient significant differences might occur during the year.

This study in a large cohort provides the best evidence to date of the safety and efficacy of autologous MSC infusion as an alternative induction therapy in low-risk organ transplant recipients, but ultimately raises the question of whether a costly MSC-based therapy should be used to prevent acute rejection, an event that is well controlled by conventional immunosuppressive drugs. The study also showed that MSC-treated patients had a lower incidence of opportunistic infection at 1 year follow-up than did patients who did not receive cell therapy[101]. This result contrasts with data from the Leiden safety and feasibility study in six living-donor kidney transplant recipients that showed an increased incidence of opportunistic viral infections in patients who received MSC infusions[102]. In that study the participants were given two intravenous infusions of autologous BM-derived MSCs when a protocol biopsy showed signs of subclinical rejection and/or increased interstitial fibrosis and tubular atrophy 4 weeks or 6 months post-transplantation. They also received induction therapy with basiliximab and maintenance immunosuppression with a calcineurin inhibitor, MMF and prednisone. In two patients with subclinical rejection, MSC treatment enabled the resolution of tubulitis. Five patients displayed a donor-specific downregulation of MLR proliferation, indicating an immunomodulatory effect of MSCs, but three patients developed opportunistic viral infections, raising some concerns about possible MSC-induced over-immunosuppression.

A subsequent safety and feasibility study was conducted in four Indian patients undergoing living kidney transplantation[103]. They were given autologous BM-derived MSCs 1 day before and 1 month after transplantation, and also received induction therapy with rATG followed by standard immunosuppression with tacrolimus, MMF and prednisolone. The MSC infusion was safe and was not associated with early or late graft dysfunction even though three of the four patients received fully HLA-mismatched kidneys. MSC infusion was also associated with T_{REG} cell expansion and reduced *ex vivo* T-cell proliferation in response to polyclonal stimuli, compared to pre-transplantation values. Despite this evidence of nonspecific MSC-induced immunosuppression, none of the patients developed cytomegalovirus infection or BK virus nephropathy[103].

With the exception of the Chinese trial, the available clinical data on the effects of autologous MSCs are from very small studies, and data on the risk of opportunistic infections associated with the procedure are conflicting. Robust conclusions regarding the safety and immunomodulatory effect of autologous MSC-based therapy in kidney transplantation can, therefore, not yet be made.

Allogeneic BM-derived MSCs

The decision to treat kidney transplant recipients with autologous or allogeneic MSCs remains a matter of debate. Studies in experimental transplant models have shown that both allogeneic and syngeneic MSCs exert potent immunomodulatory properties when given with additional immunosuppression. However, despite their low expression of HLA molecules and inherent immunosuppressive properties, allogeneic MSCs also have the potential to induce anti-donor immune responses *in vivo*[111]. Animal studies in which alloantibody monitoring and donor antigen re-challenge assays were performed to test immune responses against donor MSC antigens reported the development of donor-specific antibodies following systemic or local injection of MSCs[112–114]. Although the presence of alloantibodies or memory T cells against allogeneic MSCs does not cause any specific disease, and MSC immunogenicity does not necessarily mean lack of efficacy or safety, concerns about recipient sensitization are of special relevance when allogeneic MSCs are used in organ transplantation. Memory T cell generation and the development of antibodies specific to the donor cell HLA in sensitized recipients would be an important barrier to a subsequent organ transplantation.

For these reasons, initial clinical studies in kidney transplantation have used autologous BM-derived MSCs, which are a safer option than allogeneic MSCs in terms of the risks of recipient sensitization and rejection of the infused cells. Only one study has investigated the effects of allogeneic donor MSCs in kidney transplant recipients[104]. In this study, living-related donor kidney transplant recipients received two doses of BM-derived MSCs (directly into the renal artery at the time of transplantation and intravenously 1 month later) in combination with cytoxan induction therapy and maintenance immunosuppression with MMF, steroids and a sparing dose of tacrolimus. During 12 months of follow-up none of the MSC-treated patients experienced acute rejection, whereas one acute rejection episode occurred in a control group of patients who received conventional tacrolimus dosing without MSC infusion. Based on these findings, the authors concluded that allogenic MSCs enable sparing long-term tacrolimus treatment. Peripheral lymphocytes from the MSC-treated patients showed a tendency to higher anti-donor proliferative responses than those from patients in the control group[104], but these *ex vivo* findings do not permit any sound conclusions about the safety and feasibility of donor-derived allogenic BM-derived MSCs in kidney transplantation. Moreover, donor-derived MSCs are not technically applicable to deceased-donor transplant programmes.

Third-party BM-derived MSCs

Unfortunately the autologous MSC approach is feasible only in limited living-donor transplant programs, given the several weeks to months required to manufacture these cells. Making autologous MSC-based therapy available to the larger cohort of deceased-donor transplant recipients would not be economically viable.

Use of third-party allogeneic MSCs from large-scale clinical manufacturing might, therefore, represent the ideal strategy for future application of MSC therapy in kidney transplantation. A major advantage of this approach is the possibility of expanding MSCs in strict, controlled, Good Manufacturing Practice conditions, starting with healthy third-party allogeneic BM from selected young donors. This approach would avoid variations in the quality and efficacy of BM-derived MSCs according to the age[115,116] and gender[117] of the donors from whom BM is collected, and potentially reduce discrepancies between the results of clinical studies of MSC-based therapy. Moreover, 'off-the-shelf' allogeneic third-party BM-derived MSCs would provide an immediate source of cells ready for clinical use, bypassing the need for the difficult, expensive and time-consuming production of personalized MSCs. The risk of sensitization could be reduced by selecting allogeneic third-party MSCs matched for HLA with the kidney recipient, preventing the possible development of antibodies against donor cell HLA. Clinical trials using third-party MSCs in organ transplantation are underway (see Supplementary information S1 (table)).

Conclusions

MSC therapy undoubtedly has great potential in kidney transplantation. Preclinical and early clinical results seem promising, but moving the concept of MSC-based therapy forward to large-scale clinical application should be assessed critically. Knowledge about MSCs in organ transplantation is still too limited to embark on large randomized clinical trials, and many questions remain regarding the risks, the mechanisms of action in humans, and the benefits of these cells[118]. The available data in human kidney transplantation suggest that infusion of BM-derived MSCs at a dose of $1–2 \times 10^6$ cells per kg body weight is well tolerated with no toxic effects in the short-term. However, trial participants require careful long-term monitoring for possible adverse effects of MSC infusion, such as an increased risk of infections and malignancies[119,120], which are a particular concern in chronically immunosuppressed patients. Moreover, all trials of MSC-based therapy should include mechanistic studies with long-term immune monitoring using standardized and possibly shared methods, to enable more reliable comparisons of their results. Despite data supporting a degree of MSC-induced donor-specific immunomodulation in kidney transplant recipients, no study has provided or attempted to provide evidence that MSC-based therapy is capable of promoting operational tolerance. Such an effect has been reported with other cell-based strategies, including infusions of BM cells, $CD34^+$ cells and facilitating cells, which require the use of potentially toxic conditioning regimens[9,10,121].

In addition to the evaluation of their tolerogenic potential, autologous or allogeneic MSCs are currently being tested in kidney transplantation as a strategy for immunosuppressive drug minimization, calcineurin inhibitor withdrawal, prevention of rejection and delayed graft function in recipients of kidneys donated after cardiac death and reduction of chronic transplant inflammation and fibrosis. Results from some of these studies are expected to be available by the end of 2016. They will provide additional evidence regarding the risks and benefits of MSC infusion and will hopefully offer definitive answers to the important questions of when, where, how many and which types of MSCs should be infused to fully exploit their immunomodulatory, pro-tolerogenic and tissue-repairing properties.

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Acknowledgements
The authors' experimental studies in organ transplantation are supported by the Fondazione ART (Fondazione per la Ricerca sui Trapianti, Milan, Italy).

Author contributions
F.C., N.P. and M.C. researched the data and wrote the article. All authors made substantial contributions to discussions of the content and reviewed or edited the manuscript before submission.

Competing interests statement
The authors declare no competing interests.

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Subject ontology terms

Health sciences / Health care / Therapeutics / Surgery / Transplantation / Transplantation tolerance
[URI /692/700/565/545/576/2033]
Biological sciences / Stem cells / Mesenchymal stem cells
[URI /631/532/2074]
Health sciences / Health care / Therapeutics / Stem-cell therapies
[URI /692/700/565/2319]
Health sciences / Nephrology / Kidney diseases / Chronic kidney disease / End-stage renal disease
[URI /692/4022/1585/104/1586]

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