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New Paradigms in Cell Therapy: Repeated Dosing, Intravenous Delivery, Immunomodulatory Actions, and New Cell Types

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Abstract

Perhaps the most important advance in the field of cell therapy for heart disease has been the recognition that all stem/progenitor cells (both adult and embryonic) fail to engraft in the heart to a significant extent, and thus work via paracrine mechanisms. This fundamental advance has led to four new paradigms that are discussed in this review and that may importantly shape, or even revolutionize, the future of the field: i) repeated cell therapy, ii) intravenous cell therapy, iii) immunomodulatory actions of cell therapy, and iv) new cell types. Since virtually all of our current knowledge of cell therapy is predicated upon the effects of a single cell dose, the idea that the full therapeutic effects of a cell product require repeated doses is disruptive, and has far-reaching implications. For example, inadequate dosing (single-dose protocols) may be responsible, at least in part, for the borderline or disappointing results obtained to date in clinical trials; furthermore, future studies (both preclinical and clinical) may need to incorporate repeated cell administrations. Another disruptive idea, supported by emerging preclinical and clinical evidence, is that intravenously injected cells can produce beneficial effects on the heart, presumably via release of paracrine factors in extracardiac organs or endocrine factors into the systemic circulation. Intravenous administration would obviate the need for direct delivery of cells to the heart, making cell therapy simpler, cheaper, safer, more scalable, and more broadly available, even on an outpatient basis. While the mechanism of action of cell therapy remains elusive, there is compelling *in vitro* evidence that transplanted cells modulate the function of various immune cell types via release of paracrine factors such as extracellular vesicles, although *in vivo* evidence is still limited. Investigation of the new paradigms reviewed herein should be a top priority, as it may profoundly transform cell therapy and finally make it a reality.

Keywords

cell therapy; heart failure; repeated administration; intravenous; immune system

Cell therapy is a promising new approach that may transform the management of heart failure (HF), refractory angina, and peripheral artery disease. Many reviews have been published in this burgeoning field. The purpose of the present article is not to repeat what is already in the literature, but rather to discuss novel paradigms that have emerged in recent

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years and that may importantly shape, or even revolutionize, the future of the field. These include 1) repeated cell therapy, 2) intravenous cell therapy, 3) immunomodulatory actions of cell therapy, and 4) use of new cell types.

Repeated Cell Therapy

The concept of repeated cell therapy was proposed recently[1] on the basis of studies in rodents.[2–4] It is a major paradigm shift that may fundamentally change the entire field of cell therapy.[1] Here, we will briefly recapitulate the salient points.

Intuitively, and as a general consideration, expecting one dose of cells to be enough to bring about a therapeutic benefit seems unrealistic. Is it reasonable to expect that a single administration of cells would be sufficient to correct a chronic process, such as HF, that has taken years or decades to develop? How many therapies are there that can produce a sustained improvement in LV function with a single treatment? It would appear more sensible to assume that just like pharmacologic therapies need to be repeated, so do cell-based therapies. This becomes even more logical when one considers that a major factor limiting the benefits of cell therapy is the poor engraftment of the cells, which disappear rapidly after transplantation, regardless of the type of cell used. In fact, poor engraftment is a universal phenomenon that has been observed with virtually all types of cells studied heretofore[5], including embryonic stem cells[6]. In order for this problem to be fully appreciated, it is critical that survival and engraftment of transplanted cells be carefully quantitated (as opposed to showing anecdotal microphotographs). When such a quantitative analysis is performed, there is no convincing evidence in the literature that any cell type engrafts in significant numbers beyond the first 1–2 months after transplantation. A few cells can persist for 1 year, but their numbers are vanishingly low[7]. We described this phenomenon a few years ago. Using a highly sensitive assay to measure cell retention[8], we found that after either intramyocardial or intracoronary administration of 100,000 c-kit$^+$ cardiac progenitor cells (CPCs) in mice, the number of cells remaining in the heart declined precipitously, so that 35 days later there were only ~1,000 cells left in the entire heart[9].

Because of this poor engraftment, it seems self-evident that administering one dose of cells cannot be considered an adequate test of the efficacy of that cell product. However, almost all preclinical and virtually all clinical studies of cell therapy performed heretofore have used one cell administration. There are several reasons for this insistence on single-dose treatments. First, the old paradigm assumed that transplanted cells would engraft, proliferate, and differentiate into new mature myocytes; therefore, it was thought that adequate regeneration could be attained simply by administering one (sufficiently large) dose of cells. However, as mentioned above, we now know that there is minimal, if any, long-term engraftment, regardless of the cell type used. Secondly, in rodents repeated injections of cells (either by the intramyocardial or intracoronary route) are technically difficult and associated with prohibitive mortality. In patients, the use of repeated intramyocardial or intracoronary cell injections is hindered by logistic, safety, financial, and regulatory issues. It is difficult enough to obtain regulatory approval for a single treatment; the hurdles are even greater for multiple treatments.

Several strategies have been attempted to overcome poor engraftment, but these approaches have not been particularly successful in preventing cell loss; although they may prolong survival of transplanted cells, they do not result in significant *long-term* engraftment. We put forth the idea that the problem of poor engraftment can be overcome, in part, by administering repeated cell doses[1]. We argued that just as most pharmacologic agents are ineffective when given once but can be highly effective when given repeatedly, so a cell product may be ineffective, or modestly effective, when given as a single treatment, but may turn out to be quite efficacious if given repeatedly[1].

To determine whether repeated treatments are superior to a single treatment, we conducted a study in a rat model of chronic ischemic cardiomyopathy in which c-kit$^+$ CPCs were given either once or three times at 35-day intervals[2]. We found that each administration of c-kit$^+$ CPCs resulted in an increase in LVEF, such that the total cumulative increase after the 3rd dose was approximately triple that observed after a single dose (Figure 1). We obtained similar results in a mouse model of chronic ischemic cardiomyopathy using a different cell type (cardiac mesenchymal cells)[3]. In either study, there were no significant differences between three doses and one dose with respect to scar size and amount of viable myocardium. In addition, even after three doses, the number of transplanted cells remaining in the heart at the end of the study was vanishingly low, as was the number of new myocytes derived from transplanted cells (assessed with FISH) and the number of new myocytes derived from endogenous cells (assessed with BrdU/EdU labeling). Thus, the improvement in LV function effected by cell therapy cannot be explained by differentiation of exogenous cells into myocytes or formation of new myocytes from endogenous cells. One possible mechanism whereby cell therapy ameliorated LV function is a reduction in fibrosis (collagen content), particularly in the noninfarcted region[2, 3] (the region that is mainly responsible for LV performance). An additional possibility is that cell therapy reduced the content of inflammatory cells in the myocardium and/or inhibited the detrimental actions of these cells[3]. Much work needs to be done to elucidate the mechanism of action of cell therapy (whether with single or repeated doses).

The studies reviewed above[2,3] employed two different species and two different cell types and arrived at similar conclusions, which can be summarized as follows (Figure 2). When only one dose of cells is given, the benefits of cell therapy are significantly underestimated. Repeated cell administrations have cumulative beneficial effects and, as a result, are markedly more effective than a single administration. The beneficial effects of repeated cell doses cannot be explained by engraftment and differentiation of transplanted cells, and thus must reflect paracrine mechanisms. Importantly, even repeated doses do not appear to stimulate the formation of new myocytes from endogenous sources, which calls into question the concept that cell therapy promotes true myocardial regeneration. Potential mechanisms of action of transplanted cells include anti-fibrotic and anti-inflammatory effects.

In both of the aforementioned studies[2, 3], the total number of transplanted cells was three times greater in the multiple-dose group than in the single-dose group. Thus, the superiority of three doses could be caused by the greater total number of cells given rather than by repeated treatments. If this is the case, then the effects of three repeated doses should be

recapitulated by one large dose that contains the same total number of cells as the three doses. We tested this concept in our rat model of chronic ischemic cardiomyopathy[4]. We found that rats given one large dose of CPCs exhibited an improvement in LV function, but the magnitude of that improvement was less than that of rats receiving multiple doses. In addition, the multiple dose-treated animals had less collagen and less infiltration of $CD45^+$ cells in the noninfarcted region, suggesting greater anti-fibrotic and anti-inflammatory actions of repeated doses compared with one large dose. These results support the concept that splitting a dose of CPCs into three smaller doses given a few weeks apart is more effective than giving the same number of cells as a single dose. The fact that the cumulative effects of repeated cell doses are not recapitulated by a single combined dose, even though the total number of cells infused is the same, suggests that the duration of myocardial exposure to the transplanted cells is more important than the intensity of such exposure[4].

The idea that the full therapeutic effects of cells cannot be properly evaluated after a single dose and require repeated doses is simple but potentially disruptive. Its implications are far-reaching. For example, the conclusions of previous “negative” studies could be questioned because the benefits of the treatment may have been underestimated or even completely overlooked, due to the use of inadequate treatment protocols. Is it possible that, with repeated dosing, many of these studies would have been “positive”? Is it possible that the traditional single-dose paradigm may be responsible for at least some of the borderline or disappointing results obtained to date in clinical trials of cell therapy? Perhaps the most important implication of this new paradigm is that the protocols of future preclinical and clinical trials of cell therapy need to be changed to incorporate repeated treatments.

Looking ahead, we believe it is important that the concept of repeated dosing be carefully tested in large animal models and in humans. Investigation of these issues should be a top priority in cell therapy, because it may change the way research studies are conducted.

Intravenous route of delivery

The concept of repeated treatments inevitably leads to the question as to whether cells can be delivered intravenously (i.v.), because this route would be by far the most practical for administering multiple doses. Therefore, it is important to discuss i.v. cell delivery in depth.

Until now, every effort has been made to deliver cells (as many as possible) directly to the heart, in the hope that the transplanted cells would engraft and improve LV function. Since i.v. injection results in only a minuscule percentage of cells engrafting in the heart (*vide infra*),[10–14] direct delivery to the myocardium via intracoronary (i.c.), transendocardial, or transepicardial approaches has been used. However, these approaches entail a small but significant risk of adverse events related to the nature of the invasive procedures. For example, i.c. infusion can cause coronary dissection and transendocardial injection can be complicated by cardiac perforation and hemopericardium[15]. Transendocardial injection cannot be performed in certain patients with prosthetic mechanical valves and devices. Furthermore, i.c., transendocardial, and epicardial delivery of cells necessitates special facilities, expensive equipment, and specialized personnel, making widespread use of cell therapy difficult. On the other hand, the recent recognition that the overwhelming majority

of cells die soon after transplantation[1, 9, 16, 17,8] and that their beneficial effects are mediated by the release of various factors that act in a paracrine manner to reduce collagen deposition, inhibit apoptosis, augment angiogenesis, inhibit inflammation,[18–20] and suppress innate and adaptive immunity[18, 21] raises the question as to whether similar beneficial effects can be accrued by i.v. administration of cells and release of paracrine factors into the systemic circulation; if so, this approach would obviate the need for direct delivery of cells to the heart, making cell therapy simpler, cheaper, safer, and more broadly available.

The preclinical and clinical studies that have evaluated i.v. delivery of cells to date are summarized in Table 1.

Efficacy in Preclinical Studies

Intravenous delivery of stem or progenitor cells has been investigated in small[11–14, 20, 22–27] and large[28–32] animal models (Table 1). The earliest report was from Nagaya *et al.*[11] in 2004. These investigators injected mesenchymal stem cells (MSCs) i.v. in isogenic rats 3 h after coronary ligation and found a reduction in infarct size and an increase in capillary density and LV function in treated hearts four weeks later. Shortly thereafter, other studies in rodents also found an improvement in LV function and reported that some MSCs homed to the infarcted myocardium[22] and that SDF-1 may play a role in this process.[12] This concept is consistent with the finding in mice, also reported in 2004, that SDF-1/CXCR-4 interactions play a crucial role in the recruitment of i.v. injected BM unfractionated cells to the infarcted heart.[33] Over the ensuing decade, evidence supporting the utility of i.v. cell therapy has grown considerably. With rare exceptions,[14] beneficial effects of i.v. cell therapy on LV function have been observed in a variety of experimental settings (acute MI, [11–13, 20, 22, 23, 26–32, 34, 35] chronic MI,[14, 20] and nonischemic cardiomyopathy[24, 25, 35, 36]) and with various cells types, including naïve BM MSCs,[11–14, 20, 22, 24–31] stromal vascular fraction from adipose tissue combined with cultured adipose-derived cells,[23] and cells pretreated with azacytidine[24, 27] or atorvastatin[26] (Table 1).

Given the promising results of the studies in rodents, the i.v. route of cell delivery has also been evaluated in large animal models. All studies published so far were conducted in pigs subjected to acute MI (Table 1).[28–32] Price *et al.*[28] reported that administration of allogeneic MSCs 30 min after reperfusion resulted in improved LV EF and reduced LV hypertrophy 3 months later. Improved LV function was also observed by Halkos *et al.*[30] 12 weeks after infusion of allogeneic MSCs, given 15 min after occlusion/reperfusion, concomitant with augmented vasculogenesis and perfusion in the infarcted region; interestingly, escalating doses of MSCs (1, 3, and 10 million/kg) did not produce dose-dependent effects.[30] Consistent with these reports, improved LV function and smaller infarct size were observed 1 month after treatment with autologous MSCs[29] and autologous, allogeneic porcine, or human MSCs[31], given 48 h after MI. At 4 weeks after MI, MSCs could still be found in the peri-infarct region, although they were more abundant in the lungs and liver[29], a distribution that resembles that reported 24 h after MSC injection in mice.[20] In non-immunosuppressed pigs, both a single i.v. dose (150 million) and repeated doses (50 million/day for three consecutive days) of human adipose-derived stem cells (hASCs), given immediately after MI, have been reported to improve LV function and coronary flow reserve, promote

angiogenesis, and decrease the myocardial perfusion defect 28 days later.[32] The hASCs also had systemic immunomodulatory effects, e.g., they prevented the decrease in circulating CD8+ T cells at 7 days after MI. At 2 h after injection, most (>85%) hASCs were trapped in the lungs and only few could be found in the heart (in the peri-infarct area); by 28 days, hASCs disappeared in both tissues.[32]

Distribution of Cells After i.v. Injection

Do cells home to the heart after i.v. injection? Several studies have examined the distribution and retention of MSCs delivered by the i.v. route. In rat models of acute MI, it has been reported that only ~3% of the transplanted cells are detectable in the myocardium 24 h after delivery,[11] and that this number becomes negligible (<200 cells/mm^2) by 3 days.[12] In this latter study,[12] cell retention in the heart at 3 days after injection was greater if MSCs were given at 1 day after MI compared with earlier or later time points, but in all cases retention was minimal.[12] Similarly, when MSCs were injected in rats 10–14 days after MI, most were trapped in the lung and <1% could be found in the heart 4 h later (mainly in the infarcted region).[37]

Lee *et al.*[13] delivered 2×10^6 human MSCs (hMSCs) i.v. to immunodeficient NOD/SCID mice after acute MI; 99% of the infused cells were cleared from the circulation within 5 min and almost all of them were entrapped in the lungs (from which they disappeared with a half-life of ~24 h). Only a minuscule number (0.04% of injected cells, averaging ~400 cells) were found in the heart 15 min after infusion; this number increased at 1 day to an average of 1,480 cells (0.15% of injected cells) (likely reflecting redistribution from the lungs), but declined to zero by 3 days.[13] Despite such low retention in the heart, however, infarct size was reduced and LV function was improved 21 days later. Based on additional studies, the authors attributed this effect to secretion of the TSG-6 - a protein with strong anti-inflammatory actions[13, 38] - by hMSCs trapped in the lungs.[13] Specifically, transcriptomic analysis of hMSCs recovered from the lungs 10 h after injection showed marked upregulation of the message for TSG-6. The reparative effets of hMSCs were recapitulated in part by recombinanat TSG-6 but not by i.v. injection of hMSCs with TSG-6 knockdown[13]. Similarly, using a rat model of chronic MI, Wang and colleagues[14] found that 1 day after infusion of 5×10^6 MSCs, most (52%) injected cells were trapped in the lungs and only very few (<1%) were retained in the heart; at 30 days, no MSCs were detected in either the lungs or the heart.

As expected, when i.v. infusion was compared with other delivery routes, myocardial cell retention was found to be much lower in the former. In rats with acute or recent MI, the number of BM MSCS retained in the heart was much less 4 h after i.v. delivery than 4 h after infusion into the LV cavity (in both cases it was <1% of the injected dose); again, most cells infused by the i.v. route were found in the lungs.[37] Similarly, in rats with acute MI, no BM MSCs could be detected in the heart 1 week after i.v. injection, whereas 15% of the injected cells were still present after epicardial injection.[39] Freyman *et al.*[10] compared i.v., i.c., and transendocardial delivery of allogeneic BM MSCs in pigs immediately after MI; two weeks after cell delivery, 6% and 3% of the injected cells were present in the heart when the MSCs were given via the i.c. and transendocardial route, respectively, but none could be detected

after i.v. injection. More than 20% of injected MSCs were in the lung at 14 days after i.v. delivery; interestingly, significant MSC retention in the lung was observed also after i.c. delivery (similar to that seen after i.v. injection) and after transendocardial delivery (approximately one-third of that after i.v. injection), indicating that the lung is a major site of homing for transplanted cells no matter what route is used.[10] In a pig model of acute MI, i.c delivery resulted in a 10-fold higher number of cells in the reperfused myocardium compared with i.v. delivery.[40]

In summary, all available studies indicate that after i.v. administration, the fraction of MSCs that home to the heart within 24 h is minuscule (≤3% of the total number injected) and that it becomes essentially nil by 7 days. All studies have consistently documented significant MSC entrapment in the lungs and, in some cases, [20] in other extracardiac organs (spleen, liver, kidney) (*vide infra*) (Figure 3A and B).

Mechanism of Action of Cells After i.v. Delivery: Are Cells a New Endocrine Organ?

The mechanism(s) responsible for the salubrious effects of i.v. therapy remains to be elucidated. As detailed above, all studies have demonstrated that cells injected i.v. accumulate preferentially in the lungs,[13, 14, 28, 37, 4129, 32] and that homing to the heart is minimal.[13, 14, 29, 37, 41,32] This implies a systemic mechanism of action (Figure 3A and B).

It is possible that transplanted cells trapped in the lungs (and other organs) release immunomodulatory signals that produce systemic anti-inflammatory effects leading to reduced inflammation in the myocardium (Figures 3A and 4A). This hypothesis is supported by multiple lines of evidence. Previous studies have documented the ability of i.v. injected cells (e.g., MSCs) that are trapped in the lungs to affect distant organs [13, 32, 42–44]. For example, Noort *et al.*[42] showed that when hMSCs are infused i.v. into mice, they reside in the lungs and promote the engraftment of CD34+ cells into the bone marrow and spleen by mechanisms that do not require hMSC homing in these tissues. Similarly, ASCs delivered by the i.v. route have systemic activity including effects on BM progenitors and adipose tissue mass.[43] As mentioned above, Lee *et al.* reported that the beneficial effects of i.v. hMSCs in mice were mediated in part by the secretion of the anti-inflammatory protein, TSG-6, by hMSCs trapped in the lungs[13] (Figure 4A).

Immune cells are known to travel via the blood and lymph between the hematopoietic organs (BM and spleen), lymphoid tissues, and various organs including the heart.[45–47] Therefore, a constant turnover of immune cells is part of tissue and organ homeostasis. Since i.v. injected cells, such as BM MSCs, can home to the spleen[13, 20] and various lymphoid and non-lymphoid organs,[13, 14, 28, 37, 4129, 32] it is conceivable that they can regulate the locally residing host immune cells, which in turn may affect systemic and local myocardial inflammation (Figure 4B). There is some evidence in support of this concept. In the study by Lee et al. mentioned above,[13] i.v. hMSCs, but not hMSCs with TGS-6 knockdown, reduced myocardial infiltration of inflammatory cells, assessed by fluorescent microscopy with Ly6G and Ly6C staining. However, a detailed analysis to identify the specific cell populations in the myocardium was not performed. Dayan *et al.*[48] administered hMSCs or human umbilical cord perivascular cells i.v. 48 h after coronary artery ligation in immunodeficient NOD/SCID mice. They found improved LV function 2 and 4 weeks, but not 16 weeks, later,

suggesting that the infused cells had only a short-term effect on myocardial function. Intravenous administration of cells reduced the number of macrophages in the myocardium, and the remaining macrophages had a pro-reparative/anti-inflammatory phenotype; this was accompanied by a decrease in myocardial mRNA expression of pro-inflammatory cytokines (IL-1β and IL-6) and an increase in that of the anti-inflammatory cytokine, IL-10. There was also a reduction in cardiomyocyte apoptosis, suggesting a cardioprotective effect of cell therapy[48]. Decreased myocardial infiltration by macrophages was also observed when adipose-derived MSCs were injected i.v. at 7 days (but not at 1 day) after a reperfused MI in rats, concomitant with a reduction in scar size[49]. Hong *et al.* found that the therapeutic actions of i.v. hASCs in pigs were associated with preservation of circulating CD8+ T cells.[32]

Further mechanistic insights were recently provided by Luger *et al.*,[20] who examined the effects of i.v. delivery of hMSCs in immunocompetent mice in two models, acute MI and old MI (chronic ischemic cardiomyopathy), in which cells were given 24 h or 4 weeks after coronary artery occlusion/reperfusion, respectively. hMSCs were grown at physiological oxygen levels (5%). When hMSCs were injected 24 h after MI, hMSC concentration 1 day later was much higher (20–40 fold higher) in the kidney, liver, and spleen than in the heart, where hMSCs were found mostly in the infarcted region. (Interestingly, in this model hMSC content in the lung was 25–50% of that in the kidney, liver, and spleen, but still greater than in the heart, which was very low.) Despite minimal myocardial homing, in the acute MI setting hMSCs reduced adverse LV remodeling and dysfunction at 21 days. Similar salubrious effects were observed in the setting of an old MI, in which hMSC-treated mice exhibited a significant improvement in LV end-systolic volume and LV EF. Evidence was provided that these beneficial effects of hMSCs were associated with immunomodulatory actions. Flow cytometric analysis showed that administration of hMSCs 24 h after MI resulted, 7 days later, in a significant reduction in the splenic and myocardial content of NK cells and in the myocardial content of neutrophils; *in vitro* studies showed that MSCs suppressed NK cell proliferation via secreted factors. In the model of old MI, injection of MSCs lowered the content of myeloid cells in the spleen. A causal role of NK cell depletion in the salubrious effects of hMSCs was supported by the finding that these effects were mimicked when NK cells were depleted systemically with antibodies given prior to MI (interpretation of these data, however, is complicated by the fact that NK cell-depleted mice also exhibited a reduction in infarct size at 21 days; if this reduction occurred early as a result of acute cardioprotection during ischemia/reperfusion, it could explain the improvement in LV function and volumes 21 days later).

Taken together, the observations of Luger *et al.*[20] suggest that i.v. administration of hMSCs improves cardiac function in both the acute MI and the old MI setting, and that these effects are mediated in part by systemic anti-inflammatory actions, specifically, a reduction in the myocardial infiltration of NK cells.[20] However, the results must be interpreted with caution because the setting of xenogenic hMSC transplantation in immunocompetent mice differs from the clinical setting of allogeneic MSC transplantation (hMSCs in humans). Further studies need to be performed in allogeneic and syngeneic models to confirm these findings. In this regard, a reduction in circulating NK cells after i.v. injection of MSCs in humans has recently been reported by Butler *et al.*[36] (*vide infra*).

In summary, the mechanism(s) of i.v. cell therapy remains unclear, but the available evidence suggests that trapping of cells in the lung, spleen, and other extracardiac tissues may result in anti-inflammatory effects, either via paracrine actions of the cells that are lodged in extracardiac tissues or via endocrine actions of these cells, i.e., systemic release of anti-inflammatory factors (Figure 3A and B). In addition, since MSCs and other stem/progenitor cells secrete a panoply of extracellular vesicles, cytokines, non-coding RNAs, etc.,[50] it is possible that endocrine release of these factors into the circulation from extracardiac sites may exert other actions that are beneficial to the infarcted heart, e.g., anti-apoptotic, angiogenic, and anti-fibrotic actions. In this scenario, transplanted cells may act as a new (albeit transient) endocrine organ. This is a major area for future investigation, one that may unveil novel cell-free approaches to the treatment of HF.

It is important to note that the systemic and extracardiac actions of cells delivered i.v. could also be operative when cells are delivered via other routes, because many cells home to the lungs even after i.c., epicardial, or transendocardial delivery.[51] Using a highly sensitive and accurate PCR-based method to quantify transplanted cells, we have shown that after i.c. infusion of c-kit+ cardiac progenitor cells (CPCs) in mice with acute MI, the number of CPCs present in the lungs 24 h later was more than double that in the heart, and that at 7 days and 35 days, the number of cells found in the lungs and in the heart was similar.[9] Even after intramyocardial (epicardial) injection, the number of CPCs present in the lungs is similar to that present in the heart at 5 min and 1 day.[8] Therefore, it is conceivable that when cells are delivered locally to the heart, the salubrious effects of cell therapy could be underlain, at least in part, by systemic actions (anti-inflammatory or otherwise) elicited by cells lodged in the lungs, spleen, and other extracardiac tissues. If proven correct, this scenario would be a major shift in our understanding of the mechanism of action of cell therapy in general.

Summary of Preclinical Studies

In summary, a considerable body of preclinical evidence (>15 studies) suggests that i.v. cell therapy exerts beneficial effects on LV function after MI. As shown in Table 1, the data reported in rodents[11–14, 20, 22–27] suggest that i.v. infusion of MSCs is effective in alleviating MI-induced LV dysfunction both in the setting of an acute and an old MI. Similarly, the five studies reported in pigs[28–32] indicate that i.v. administration of MSCs (autologous, allogeneic, or even xenogeneic) soon (within 48 h) after MI is effective in alleviating subsequent LV dysfunction. One notable gap is the lack of data regarding the effects of i.v. cell therapy in large animal models of chronic ischemic cardiomyopathy (old MI); such data are necessary to translate i.v. cell therapy to the large population of patients with chronic ischemic HF. Filling this gap should be a priority for future investigations. Since almost all studies have used MSCs, further work with other cell types will be important to fully evaluate the utility of i.v. cell therapy. The i.v. route of cell delivery appears to be safe, as no adverse effects have been reported in animals despite infusion of relatively large numbers of cells.

The mechanism of action of i.v. cell therapy remains speculative but clearly does not involve engraftment of exogenous cells in the heart. Rather, the transplanted cells must act by

releasing factors that act at a distance (endocrine action) or by modulating cells that circulate in the blood (such as immune cells) (Figure 3A, 3B, 4A, 4B). A few animal studies[13, 20, 48] and one small clinical trial[36] suggest that, in HF, the improvement in LV function elicited by i.v. administration of MSCs is associated with systemic anti-inflammatory actions *in vivo*. Whether the functional benefits are caused by the anti-inflammatory actions is unclear. It also remains unclear whether other cells types exhibit similar effects when given by the i.v. route. These are important areas for future investigation. The recognition that the beneficial effects of i.v. cell therapy in HF are mediated by systemic anti-inflammatory actions would usher in a novel strategy for the management of this syndrome based on inhibition of delerious immune responses. Such a concept would be consistent with clinical trials showing systemic anti-inflammatory and immunomodulatory actions of i.v. cells therapy in various conditions, including graft-versus-host disease, multiple sclerosis, systemic lupus erythematosous, chronic obstructive pulmonaty disease, and Crohn's disease[52–58].

Clinical Studies

The encouraging results of the preclinical studies summarized above have motivated investigation of i.v. cell delivery in humans. Three randomized clinical trials have been reported thus far (Table 2).[34–36] The first was by Hare *et al.*,[34] who performed a double-blind, placebo-controlled phase I study in which 53 patients with acute MI (either ST-elevation or non-ST-elevation MI occurring 1–10 days prior to randomization) and LV EF ≥ 30% and ≤ 60% received different doses (0.5, 1.6, and 5 million cells/kg) of allogeneic MSCs and were followed for 6 months. The i.v. delivery of MSCs appeared to be safe, with similar adverse event rates between the MSC- and placebo-treated groups. A functional beneficial effect of MSCs was supported by the observation that, in patients who sustained an anterior MI, LV EF (measured by echocardiography) improved significantly at 6 months vs. baseline values in MSC-treated patients but not in in the placebo group. In a subset of patients who underwent cardiac MRI, MSC administration was associated with a significant increase in LV EF at 12 months vs. baseline; furthermore, when plotted relative to EF, MSC-treated patients exhibited evidence of reverse LV remodeling with no increase in LV end-diastolic volume and a decline in LV end-systolic volume, whereas placebo patients demonstrated evidence of LV chamber enlargement. As in the porcine study by Halkos *et al.*,[30] no dose-dependent effects of MSCs were observed. This trial provided evidence of safety, and provisional evidence of efficacy, of allogeneic MSCs in patients with acute MI.[34]

In 2017 Butler *et al.*[36] reported the results of a single-blind, placebo-controlled, crossover, randomized, phase IIa trial of i.v. allogeneic MSCs (grown under hypoxic conditions [5% O_2]) in patients with nonischemic cardiomyopathy (LV EF<40%). Subjects were randomized to i.v. MSCs (n=10, 1.5×10^6 cells/kg) or placebo (n=12) followed by crossover at 90 days, when each group received the alternative treatment. Although LV EF and LV volumes (measured by MRI) did not differ between the two groups, a small, but statistically significant, decrease in LV volumes and increase in LV EF were observed 90 days after MSC infusion in the treated cohort but not in placebo-treated patients. MSC-treated subjects exhibited a significant increase in the 6-min walk distance and in the Kansas City Cardiomyopathy Questionnaire (KCCQ) clinical summary score and functional status score compared with placebo-treated patients. Administration of MSCs, but not placebo, was

associated with a significant decrease in circulating NK cells and a significant increase in CD3 and CD4 cells; the magnitude of the reduction in NK cells was inversely related to the magnitude of the increase on LV EF. There were no discernible differences in serious adverse events between the two groups. This study further supports the concept that i.v. administration of MSCs is safe; in addition, it provides evidence of systemic immunomodulatory effects (as evidenced by the reduction in NK cells, which is consistent with the observations of Luger *et al.* in mice[20]) and it suggests that i.v. MSC delivery may improve clinical end-points and functional status in nonischemic cardiomyopathy.[36]

The RIMECARD trial,[35] also published in 2017, was a phase I, randomized, double-blind, and placebo-controlled study in which patients with either ischemic or nonischemic cardiomyopathy were assigned to i.v. infusion of allogeneic umbilical cord (UC)-derived MSCs (1×10^6 cells/kg) or placebo (n = 15 per group) and were followed for 1 year. No adverse events were reported, suggesting again that i.v. delivery of cells was safe. None of the patients tested developed alloantibodies to the injected cells. Compared with baseline values, patients given UC-derived MSCs, but not those receiving placebo, exhibited a significant improvement in LV EF (assessed by echocardiography as well as MRI) vs. baseline that became apparent at 3 months and was sustained at 6 and 12 months, although LV EF did not differ significantly between the two groups. Throughout the follow-up, the NYHA class, Minnesota Living with Heart Failure questionnaire [MLHFQ] score, and KCCQ score improved in the MSC-treated group, but not in the control group. *In vitro* studies demonstrated that UC-derived MSCs and BM-derived MSCs produced similar degrees of inhibition of T cell proliferation, suggesting similar immunosuppressive properties. Although the secretomes of the two cell types were similar, UC-MSCs exhibited higher expression of hepatocyte growth factor. This study adds to the evidence that i.v. cell therapy is safe and suggests that UC-derived MSCs may improve LV function, functional status, and quality of life in patients with HFrEF of ischemic or nonischemic origin.[35]

Taken together, the results of these three trials, [34–36] conducted in three different patient populations (acute MI, ischemic cardiomyopathy, and nonischemic cardiomyopathy) (Table 2), suggest that i.v. infusion of MSCs (allogeneic or autologous) is well-tolerated, and that no humoral immune reaction develops to allogeneic MSCs. The initial results with respect to efficacy are encouraging and warrant further investigation. Since these studies were small and none of the them was designed to assess efficacy, larger phase II trials are in order to determine the impact of i.v. MSC delivery on LV function, LV remodeling, functional status, exercise capacity, and other relevant clinical outcomes as well as to further establish the safety of i.v. cell delivery. It will also be important to compare the i.v. route with other routes of delivery in direct head-to-head comparisons. The i.v. route offers many major potential advantages, including the fact that it is simple, it does not require expensive equipment or specialized training, it could be implemented in almost every medical facility, and perhaps most importantly, it lends itself to repeated cell administrations, which could be performed easily and inexpensively, even as outpatient procedures. Future studies will be necessary to elucidate the optimal protocols for repeated i.v. infusions of cells (e.g., number, frequency, dose, etc.). If i.v. administration of cell products proves to be safe and effective, its use would be a major revolution in the field of cell-based therapies.

Immunomodulatory Actions of Cell Therapy

The recognition that cells do not engraft in the heart to a significant extent[1, 9, 16, 17,8] has stimulated a search for new mechanisms underlying their beneficial effects. One emerging mechanism whereby cell therapy (given i.v or by other routes) may improve LV function is via anti-inflammatory actions. In this section we review current knowledge of the effects of cell therapy on the immune system.

Immune response after MI

Cell death resulting from myocardial ischemia induces activation of the complement system, production of ROS, and release of danger-associated molecular patterns [59–64], all of which trigger intense sterile inflammation and infiltration of neutrophils and pro-inflammatory monocytes that digest and clear the dead tissue[62, 63, 65–68]. This short-lived pro-inflammatory phase, usually lasting a few days, is necessary for a subsequent reparative phase characterized by resolution of inflammation, accumulation of reparative macrophages, removal of apoptotic neutrophils, neovascularization, proliferation of stromal cells, and deposition of extracellular matrix[63–65, 69–71]. At the end of the reparative phase, immune cells leave the infarcted region and the scar undergoes maturation, with extracellular matrix crosslinking and deactivation and apoptosis of reparative cells[63, 72–76]. That this inflammatory response plays a crucial role in cardiac repair is demonstrated by the fact that both nonselective inhibition of inflammation after MI with corticosteroids or non-steroidal anti-inflammatory drugs and systemic depletion of neutrophils or macrophages prior to MI have detrimental effects on myocardial repair, leading to rupture and exacerbation of adverse LV remodeling[45, 67, 77–79]. However, although the early immune response is essential for myocardial repair, infarcted wall stabilization, and preservation of LV function, incomplete resolution of inflammation after the reparative phase contributes to HF progression. Indeed, chronic ischemic HF is associated with accumulation of monocytes/macrophages, dendritic cells, and T cells in the myocardium (including the noninfarcted region) and with systemic expansion of immune cells resulting in their increase in the spleen and in the peripheral blood[47, 78, 80, 81]. This chronic, non-resolving inflammation contributes to the progression of adverse remodeling and worsens LV function[47, 63, 80, 82–84].

Immunomodulatory actions of cell therapy

The discovery that BM MSCs suppress T cell proliferation led to further studies investigating their immunomodulatory properties.[85, 86] An extensive body of work has now demonstrated that MSCs can modulate both innate and adaptive immunity, *in vitro* as well as *in vivo*[87, 88] (Figure 5). Indeed, numerous phase I, II, and III clinical trials are currently testing the immunomodulatory actions of MSCs in the treatment of inflammatory diseases, including graft-versus-host disease (GVHD), Crohn’s disease, ulcerative colitis, multiple sclerosis, and systemic lupus erythematosus (see ClinicalTrials.gov).[87, 88] Because the immune system is involved not only in tissue healing acutely after MI, but also in the progression of HF in the chronic phase after MI, the immunomodulatory actions of cell therapy in the treatment of heart disease constitute a new, important avenue for basic and clinical research.[63, 73, 77, 89, 90] Cell therapy appears to be a useful tool to identify and

harness the salutary components of the immune system that lead to improved outcome in HF.

Effects on neutrophils

Neutrophils infiltrate infarcted myocardium within minutes after reperfusion in response to DAMPs, cytokines, chemokines, endogenous lipid mediators (e.g. leukotriene B4), histamine, and complement 3 and 5 cleavage components. Extravasated neutrophils secrete pro-inflammatory mediators (IL-1, IL-6, TNF-α), granules containing proteolytic enzymes (e.g., acid hydrolases, elastase, neutral serine proteases, lysozyme, cathepsin G, proteinase 3), neutrophil extracellular traps (NETs), and ROS[59, 61, 62, 64, 91–95], which not only perpetuate the inflammatory response, but also contribute to the clearance of dead cells and extracellular matrix debris. Neutrophil depletion prior to MI dysregulates monocyte/macrophage function, promotes adverse remodeling, and worsens LV function[74, 86, 87, 91]. On the other hand, in the chronic phase of HF, an elevated neutrophil-to-lymphocyte ratio correlates with the severity of the disease and is an indicator of poor prognosis.[96–99] Both *in vivo* and *in vitro* experiments indicate that neutrophils may have cytotoxic effects and contribute further injury to the myocardium[100] Therefore, neutrophils are double-edged swords in myocardial repair.

Little is known regarding the impact of cell therapy on neutrophil function after MI. *In vitro* studies show that co-culture of neutrophils with BM MSCs extends the lifespan of neutrophils by inhibiting apoptosis, reduces production of ROS and pro-inflammatory cytokines (IL-1, IL-6, TNF-α), inhibits degranulation, NET formation, and expression of NADPH oxidase-1, and increases expression of catalase and cyclooxygenase-2.[89, 100–103] Neutralizing experiments have revealed that these effects of BM MSCs on neutrophils are accounted for by production of IL-6, interferon-β (IFN-β), and granulocyte macrophage colony-stimulating factor.[89, 100–103] Administration of cord blood mononuclear cells, which contain MSCs, at the time of coronary occlusion reduces neutrophil infiltration and expression of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β) and chemokines (MCP-1, MIP, fractalkine);[104] however, the mechanisms responsible for these phenomena are not well defined. For example, it is unknown whether the injected cells regulate neutrophil function directly or indirectly through changes in immune cytokine expression. To date, no systematic studies are available regarding the effects of cell therapy on neutrophil function *in vivo*, particularly in the setting of myocardial repair.

Effects on monocytes/macrophages

Monocytes and macrophages are part of the innate immunity and it is well established that they play a key role both in the initial insult to the myocardium and in the chronic phase of cardiac injury. They remove dead tissue and apoptotic cells but also produce cytokines and bioactive lipids that regulate fibroblast and endothelial function, immune cells, and resolution of inflammation[105–108]. Systemic monocyte and macrophage depletion with clodronate liposomes or splenectomy prior to MI impairs myocardial repair and results in LV rupture[45, 67]. In the chronic phase after MI, macrophages accumulate in the noninfarcted region of the heart, where they contribute to adverse remodeling and progression of

contractile dysfunction[47, 80]. Therefore, macrophages are important players in the pathophysiology of ischemic heart disease.

The immunomodulatory role of BM MSCs in the regulation of macrophage function has been well established in *in vitro* co-culture systems, where the MSC secretome induces a pro-reparative and anti-inflammatory phenotype in macrophages but also enhances their phagocytic activity. Within this secretome, a number of MSC-derived factors have been identified that modulate macrophage reparative functions, including PGE2, IDO, TGF-β, and IL-10[48, 103, 109–111]. In addition, the secretome of cardiosphere-derived cells (CDCs) polarizes macrophages to a phenotype distinct from that of classically or alternatively polarized macrophages, enhances their phagocytosis, and decreases expression of CD80 and CD86, two obligatory T cell co-stimulatory receptors[112]. Data are also available *in vivo* that support modulation of macrophages by cell therapy delivered shortly after MI. Infusion of BM MSCs 48 h after MI in mice induces a macrophage switch toward a reparative phenotype[111]. In rat and pig models of MI, injection of allogeneic CDCs soon after reperfusion results not only in improved LV functional recovery, but also in a decrease in the number of myocardial $CD68^+$ macrophages[112–115]. Adoptive transfer of macrophages preconditioned with CDCs reproduces the reparative effects without CDC administration[112, 114, 115]. Further mechanistic studies suggested that CDC-derived exosomes transfer miR-181b, which then mediates the reparative macrophage phenotype through a reduction of PKCδ transcript levels[113]. Taken together, these data[111–115] suggest that the reparative effects of CDCs are mediated through modulation of the resident cardiac macrophage population.

In summary, the actions of cell therapy on macrophage function are well recognized *in vitro* and *in vivo*. However, whether these actions are important to the beneficial effects of exogenous cells, particularly in the chronic phase of HF, remains unclear; furthermore, our understanding of the mechanism(s) whereby transplanted cells modulate macrophage reparative functions is poor. Elucidating these issues is a top priority for future studies and may bring about major advances in the field of cell therapy.

Effects on dendritic cells (DCs)

DCs are the most potent antigen-presenting cells in the immune system. They are capable of uptake of myocardial peptides generated by MI and presentation, resulting in T cell activation[116]. This process modulates myocardial healing in the acute phase after infarction by controlling monocyte/macrophage homeostasis and by activating regulatory and helper T cells[117–120]. However, infiltration of mature DCs in the chronic phase after MI contributes to adverse remodeling, fibrosis, and deterioration of LV function[80]. Therefore, similar to neutrophils and macrophages, DCs can be viewed as double-edged swords in the pathogenesis of HF.[80, 117, 118]

Observations *in vitro* suggest that MSCs have an inhibitory effect on DCs and activate the regulatory T cell response, favoring myocardial repair and resolution of inflammation.[103, 121, 122] Co-culture of MSCs with DCs or their precursors inhibits DC maturation and leads to impaired effector function by decreasing CD80 and CD86 expression, which is necessary for T cell activation[121, 123–125]. This process is at least partially regulated by

MSC-derived IL-6. MSCs also suppress expression of pro-inflammatory cytokines (IL-12, TNF-α, IFN-γ) and promote expression of the anti-inflammatory cytokine IL-10 in DCs. However, virtually nothing is known regarding the effect of cell therapy on DC function in the setting of HF *in vivo*.

Effect on B cells

B cells originate from the BM where hematopoietic progenitor cells differentiate into pro-B cells, which undergo a series of conformational changes to be finally released into the circulation as immature B cells. Next, they home to the secondary lymphoid tissues and/or spleen, where they become transitional B cells. B cell activation and maturation take place once they come in contact with an antigenic stimulus. That causes the cells to either switch isotype and produce antibodies, or to present the foreign molecule to T cells via the major histocompatibility complex class II (MHCII)[126–129]. B cells may contribute to the auto-antibody production against cardiac specific antigens that has been implicated in the progression of HF, and also regulate T cell subpopulation balance[130, 131].

There is considerable evidence that MSCs can regulate B cell function *in vitro*. B cells have a strong chemotactic activity towards the MSC secretome that is PGE2-, CXCL8-, and CXCL10-independent[132]. The specific chemotactic factor(s) secreted by MSCs has not been identified. The effects of MSCs on B cell proliferation are more controversial[133]. Some studies demonstrated that MSCs inhibit B cell proliferation[134, 135] while other show an opposite pro-proliferative effect[136, 137]. Direct contact with MSCs can inhibit caspase 3-mediated apoptosis of peripheral blood B cells through MSC-induced upregulation of VEGF and p-PKBA activation[138]. MSCs can also regulate the effector function of B cells; that is, they can inhibit IgM, IgG, and IgA production[135]. Taken together, these studies indicate that locally injected MSCs can affect B cell homeostasis by promoting their recruitment[132], survival [138] and proliferation[134–137], and also by regulating their effector function by switching the classes of antibodies produced or facilitating their maturation[135].

To date, no studies have been reported examining the effect of cell therapy on B cell function *in vivo* in the context of myocardial repair. Nevertheless, as pointed out above, there is considerable evidence that MSCs can regulate B cell function. This may be another fertile area for future investigation.

Effect on T cells

T lymphocytes are the primary effector cells of the adaptive immune system. Antigen-specific activation and differentiation of naïve T cells lead to the generation of a range of T cell phenotypes that may be defined by the surface markers expression and by the profile of secreted cytokines[139–142]. T cells expressing CD8, or cytotoxic T cells (Tc), have a direct cytotoxic effect on the targeted cells expressing foreign antigens presented by MHC class I. [143, 144] A wide range of CD4-expressing helper T cells (Th) can be identified by cytokine expression profiling. Type one helper T cells (Th1) express the pro-inflammatory cytokines IFNγ, TNF-α, and IL-2, which activate macrophages and perpetuate the immune response. [145, 146] Similarly, Th17 lymphocytes (a subpopulation of CD4-expressing Th cells that produce IL-17) are primarily proinflammatory and regulate neutrophil and macrophage

recruitment.[147] Excessive activation of the Th1 response can lead to uncontrolled tissue damage. Therefore, the proinflammatory response is counterbalanced by Th2 lymphocytes that produce IL-4, IL-13, and IL10, which promote a reparative and anti-inflammatory phenotype in macrophages.[148] Regulatory T cells (T regs) expressing CD4, FOXP3, and CD25 are immunosuppressive and inhibit effector T cell function.[149]

Because T cells are activated in sterile inflammation, including MI, they have been implicated in the processes related to cardioprotection, infarct healing, and progression of HF[150]. The first evidence for a role of T cells in heart disease came from observations in Rag1 KO mice, which produce no mature T or B cells. In these mice, infarct size was reduced after ischemia-reperfusion[151]. Adoptive transfer studies indicated that $CD4^+$ but not $CD8^+$ T cells contributed to the cardiotoxic effects. More detailed analyses showed that IFNγ mediates cardiac injury, suggesting that the Th1 subset is responsible for that effect[151]. This line of evidence is consistent with another study showing that adoptive transfer of regulatory $CD4^+$ T cells induced protection of ischemic myocardium, which was related to ectonuclease (CD39) expression and formation of adenosine[151, 152].

In mouse models of MI, T cells become activated and proliferate in the draining lymph nodes; in addition, both $CD4^+$ and $CD8^+$ T cells are elevated in the myocardium within days after MI[119]. Further analysis indicated that $CD8^+$ T cell deficiency has no effect on infarct healing but lack of $CD4^+$ T cells leads to impaired myocardial repair and worsens myocardial contractile function[119, 150]. Mechanistically, Th1 $CD4^+$ T cells contribute to a reduction of $Ly6C^{HIGH}$ monocyte recruitment, as deficiency of $CD4^+$ Th1 cells increased recruitment of monocytes and was associated with worsening of LV function[119, 150]. The absence of $CD4^+$ T reg lymphocytes led to a proinflammatory skewing of the macrophage phenotype, perpetuated proinflammatory response, and impaired LV function[153]. Therefore, a proper balance between T helper cell subpopulations is necessary for proper myocardial repair.

It is conceivable that stem/progenitor cells can directly interact with T cells to modulate the immune response after MI. Numerous *in vitro* studies have shown that BM MSCs inhibit T cell proliferation and differentiation.[103] Co-culture with MSCs suppresses both $CD4^+$ Th and $CD8^+$ Tc lymphocyte proliferation through cell cycle arrest in the G0/G1 phase.[154] Some MSC-expressed factors have been identified that have an antiproliferative effect on T cells; the most studied are IDO, PGE2, and PD-L1/PD-L2.[103] Activated MSCs can also express MHC class II antigens and present the antigen to naïve T cells; however, because of the lack of co-stimulatory signals (CD80, CD86, CD40), T cells undergo anergy instead of full activation and proliferation.[155, 156] MSCs can also induce an anti-inflammatory response by inhibiting Th1/Th17 cells and activating Th2 polarization of $CD4^+$ Th cells.[157] Moreover, MSCs induce T reg differentiation of naïve T cells, which produces immunostasis of T cells and has further anti-inflammatory effects.[157, 158] However, no data are currently available *in vivo* regarding the effects of MSCs or other cell types on T cell function in models of HF. This is another important area for future investigation.

In summary, it is conceivable that the beneficial effects of cell therapy may be mediated by anti-inflammatory actions in the viable (noninfarcted) myocardium. There is compelling *in*

vitro evidence that cell therapy regulates the function of various immune cell types, and some of the paracrine factors involved in these actions have been identified. In particular, exosomes have been identified as a novel immunomodulatory paracrine mechanism for horizontal transfer of RNAs affecting the targeted immune cell transcriptome and function. Further studies will be necessary to identify the specific components of the exosomes that are responsible for their immunomodulatory function and the downstream pathways in the immune cells that are involved in these effects. In the *in vivo* setting, evidence supporting immunomodulatory actions of cell therapy is still limited; importantly, it is unclear whether these immunomodulatory actions are causally involved in the improvement in LV function and structure after cell therapy. Future research should aim to identify not only the paracrine factors produced by injected cells but also the specific immune cells and signaling pathways responsible for the salutary effects.

New Cell Types

Over the past two decades, several cell types have been studied for the treatment of HF in animal models and in clinical trials[16]. Although various degrees of therapeutic benefit have been reported with almost all cell types tested, the generally modest improvement in LV function[17], the uniformly limited survival of transplanted cells (regardless of cell type[17]), and their failure to differentiate into the cardiac lineage[17] have motivated the search for new, more effective cell types with superior reparative actions on the failing heart. In recent years, three new cell types have been tested in experimental animal models of MI and HF: cortical bone-derived stem cells[159–163], multilineage-differentiating stress enduring cells[164], and cardiac mesenchymal cells[3, 165].

Cortical Bone-Derived Stem Cells

Mesenchymal cells isolated from cortical bone (CB), or cortical bone-derived stem cells (CBSCs), have properties similar to the MSCs isolated from the bone marrow (BM); however, they are more clonogenic and have superior osteogenic potency; thus, they can be considered a more primitive subset of BM MSCs [163, 166–168]. CBSCs express classical mesenchymal markers (CD90, CD105, CD29, CD73, and CD29), lack expression of hematopoietic and endothelial markers (CD45 and CD31, respectively), and possess multilineage differentiation potential (adipocytes, osteoblasts, and chondrocytes) and immunomodulatory properties, both of which are typical MSC characteristics[160, 161, 163]. Compared with BM MSCs and CDCs, CBSCs exhibit greater expression of CD61 and integrin β4, have enhanced proliferative capacity, and are more resistant to apoptotic stimuli[161]. CBSCs have been reported to exert more potent reparative actions than c-kit$^+$ CPCs in murine models of HF[160]. However, as is the case with virtually all other cell types, transplanted CBSCs contribute minimally to cardiomyocytes or endothelium, indicating that their superior reparative potential is accounted for by paracrine actions[160]. This body of evidence suggests that CBSCs represent a distinct and more primitive population of MSCs with a potent paracrine profile. Studies in a porcine model of ischemic cardiomyopathy have shown that CBSCs are safe and exert reparative actions that are similar to those observed in rodents[162]. Thus, these cells appear to be promising candidates for translation in clinical trials.

Multilineage-differentiating, stress enduring (Muse) cells

Muse cells are stage-specific embryonic antigen-3 (SSEA-3) expressing cells that reside in the BM[169–171], adipose tissue[172–174], dermis[175], and connective tissue of various organs[176], but also circulate in the peripheral blood[177]. They express pluripotency markers such as Sox2, Oct3/4, and Nanog, are stress tolerant, and secrete pro-survival factors that are protective against cells death. Even though Muse cells are a small fraction of the tissues of origin, they are easily expandable *in vitro* and thus therapeutically relevant[170]. After i.v. injection, they have been shown to home to the site of organ or tissue injury and contribute to repair[176]. Muse cells have been reported to repair diabetic skin ulcers[178] and brain[179–181], liver[171, 182], kidney[183], and skin injury[184].

Recently, these cells have also been tested for their reparative potential in a rabbit model of MI[164]. Yamada *et al.*[164] reported that BM-derived rabbit and human Muse cells, expanded *in vitro* and injected i.v. in immunocompetent rabbits, home to infarcted hearts at 3 days and 2 weeks. The migration and homing of i.v. injected Muse cells were toward a sphingosine-1 phosphate (S1P) gradient in the infarcted myocardium via S1P receptor 2 (S1PR2) expressed on the Muse cells. Co-injection of JTE-013 (an S1PR2 antagonist) or knockdown of the receptor with siRNA resulted in reduced recruitment of Muse cell to the heart[164]. Some of the cells that homed and engrafted in the infarcted heart expressed cardiac-specific markers, such as cardiac troponin I, sarcomeric α-actinin, and connexin 43, as well as vascular markers (CD31 and α-SMA). Rabbits given Muse cells exhibited a reduction in infarct size (~52%) and an increase in LV EF (~17 units) compared with vehicle-treated animals at 2 months. Interestingly, both allogeneic and xenogeneic Muse cells engrafted and induced recovery of LV function in immunocompetent rabbits for up to 6 months without immunosuppression,[164] suggesting that they have efficient mechanisms protecting them from immunorejection. This study[164] suggests that Muse cells have potential clinical utility, but these results need to be reproduced in other models; furthermore, the mechanism whereby these cells repair the heart remains unclear. Although some of the Muse cells that engrafted after injection expressed cardiac and vascular specific markers, the number of these cells was too small to explain the recovery of LV function. Thus, like all other cell types, Muse cells most likely repair the heart via paracrine or endocrine mechanisms.

Cardiac Mesenchymal Cells

Cardiac mesenchymal cells (CMCs) are the myocardial equivalents of BM MSCs. A population of mesenchymal (or stromal) cells residing in the human heart was described in 2011 by Rossini *et al.*[185], who reported that these cells (termed cardiac mesenchymal or stromal cells) have a phenotype similar to BM MSCs, express mesenchymal markers (CD105, CD73, CD29, CD44, and CD13), and are negative for CD34, CD45, CD14, CD117 (c-kit), CD31, VEGFR2, CD144, and HLA-DR. Expression of CD90 was heterogeneous but lower than in BM MSCs. Transcriptome analysis showed no difference in the expression of pluripotent markers (Oct-4, Nanog, Sox-2, Klf4, and MDR-1) between CMCs and BM MSCs; the expression of cardiac markers (Nkx2.5, alpha-MHC, TnI) was low or absent in both cell populations. However, CMCs had a distinct intracellular miRNA profile compared with BM MSCs. In immunosuppressed rats that received human cells 3 weeks after coronary artery ligation, CMCs, but not BM MSCs, reduced LV end-diastolic pressure and increased

dP/dt, although both cells reduced LV weight compared with vehicle-injected rats. Similar to many other cell types, CMC survival after transplantation was negligible; therefore, the functional improvement was most likely related to secretion of paracrine factors[185].

We have recently performed a series of studies to evaluate the therapeutic potential of CMCs and to identify their most effective subsets. We have stratified CMCs into two subpopulations based on their adherence to plastic tissue culture dishes. Rapidly adhering cells (RA-CMCs) adhere within hours from the initial plating, whereas slowly adhering cells (SA-CMCs) requires days to attach[165, 186]. Both cell populations have a similar fibroblast-like morphology, express typical mesenchymal markers (CD90, CD105, CD29, CD73, CD44), and are negative for CD45, CD31, CD34, and c-kit[165]. Despite their indistinguishable morphology and phenotype, transcriptomic analysis showed striking differences between these two cell types. CMCs were studied *in vivo* using appropriate protocols and data were analyzed as previously described [187–191]. When injected intramyocardially in mice 2 days after MI, SA-CMCs, but not RA-CMCs, improved LV function 1 month later[165]. However, a negligible number of transplanted cells (either RA-CMCs or SA-CMCs) survived at 1 month after injection. In parallel to the changes in LV function, SA-CMCs, but not RA-CMCs, reduced fibrosis in the noninfarcted region and lowered the number of immune cells detected with CD45 staining at 1 month after cell injection. *In vitro* experiments showed that compared with RA-CMCs, SA-CMCs exert stronger anti-inflammatory effects on M1 polarized BM macrophages[165]. Administration of repetitive doses of SA-CMCs in a murine model of chronic ischemic cardiomyopathy resulted in cumulative beneficial effects[3, 165, 186] similar to those of c-kit+ CPCs in rats.[2]

Taken together, these data[3, 165, 186] demonstrate that slow adherence to plastic identifies a population of cells with robust therapeutic potential and anti-fibrotic and anti-inflammatory properties. This simple method of CMC stratification has potential clinical application, as SA-CMCs can be isolated and expanded from a small tissue sample, for example, endomyocardial biopsies (EMBs). Indeed, we have recently found that large quantities of CMCs, sufficient for therapeutic administration, can be produced from EMBs harvested in pigs. Compared with c-kit positive CPCs, c-kit negative CMCs offer several advantages. Their expansion from EMBs to generate therapeutically relevant quantities (several million) is simpler (not requiring immunosorting) and thus more consistent and less prone to controversies; it is also faster (because of much higher starting numbers in myocardial tissue), thereby enabling administration of younger cells at low passage numbers. For example, the time required to generate 5 million or more cells would be ~2 weeks for CMCs vs. ~ 2 months for CMCs. For these reasons, the use of CMCs may be cheaper and more widely applicable than c-kit positive CPCs. CMCs are therefore an attractive new option for clinical use. Preclinical studies in pigs are currently ongoing in our lab to confirm the above results in rodents, with a goal toward clinical translation.

Concluding remarks

The field of cell therapy is evolving rapidly. The fundamental shift has been the recognition that all cell types, both adult and embryonic, fail to engraft in the heart, and instead work via paracrine mechanisms[1–3, 5–7, 9, 165]. This has led to new paradigms and therapeutic

approaches, which may revolutionize cell therapy. Since transplanted cells do not persist in the heart more than a few weeks, it seems logical to give repeated doses. Since cells work by releasing factors in the environment, i.v. therapy may also be effective via systemic release of these factors. Since cells do not differentiate into new myocytes, alternative mechanisms are being explored, such as anti-inflammatory and anti-fibrotic actions. Since the cells studied heretofore exhibit limited survival and efficacy, new cell types have been investigated. Future preclinical and clinical research needs to focus on these new strategies, which may finally make cell therapy a reality.

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Non standard abbreviations and acronyms

BM	bone marrow
CBSCs	cortical bone-derived stem cells
CDCs	cardiosphere-derived cells
CMCs	cardiac mesenchymal cells
CPCs	cardiac progenitor cells
EF	ejection fraction
FISH	fluorescence in situ hybridization
GVHD	graft-versus-host disease
hASCs	human adipose-derived stem cells
IL	interleukin
HF	heart failure
HFrEF	heart failure with reduced ejection fraction
KCCQ	Kansas City Cardiomyopathy questionnaire
LV	left ventricle/left ventricular
MI	myocardial infarction
MLHFQ	Minnesota Living with Heart Failure questionnaire
MRI	magnetic resonance imaging
MSCs	mesenchymal stromal cells
MUSE	multilineage, stress enduring
NETs	neutrophil extracellular traps

NK	natural killer
NYHA	New Your Heart Association
RA-CMCs	rapidly-adhering CMCs
ROS	reactive oxygen species
SA-CMCs	slowly-adhering CMCs
SDF-1	stromal derived factor 1
CXCR-4	C-X-C chemokine receptor type 4
UC	umbilical cord
TGS-6	tumor necrosis factor – stimulated gene 6
TNF-α	tumor necrosis alpha

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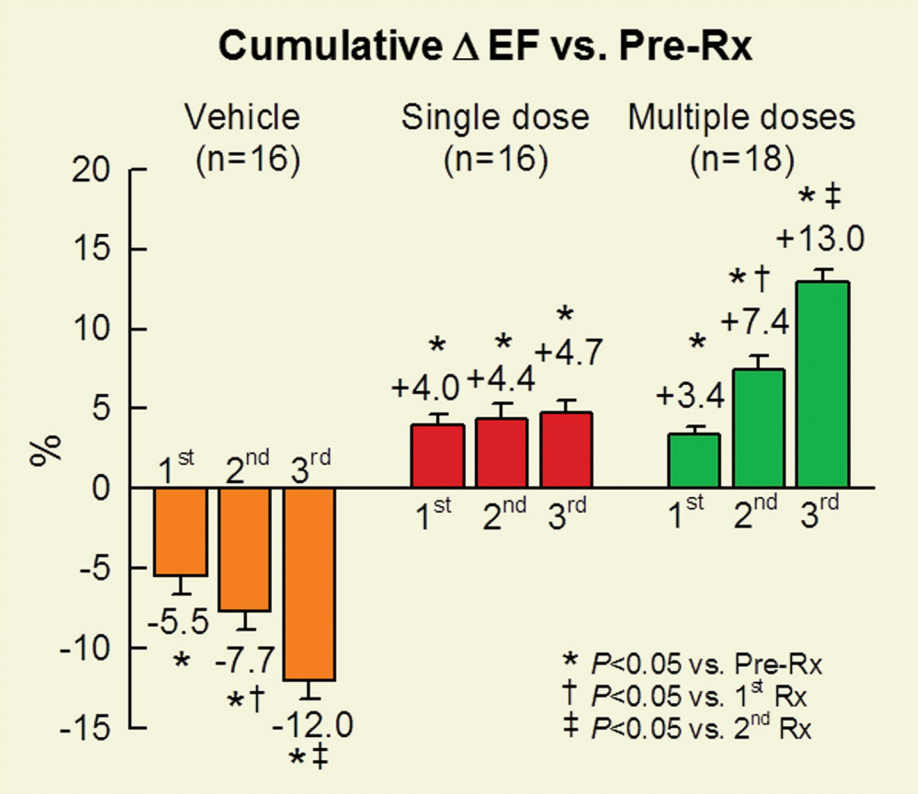

Figure 1. Cumulative improvement in LV EF after repeated cell doses

Rats with a 1 month-old MI were given, at 35 day intervals, three injections of vehicle (orange), one injection of c-kit+ CPCs and two of vehicle (red), or three injections of c-kit+ CPCs (green) into the LV cavity. Depicted here are the changes in EF (absolute units) from pretreatment (Pre-Rx), i.e., from before the 1st injection. Data are means±SEM. Reproduced with permission from ref. 1.

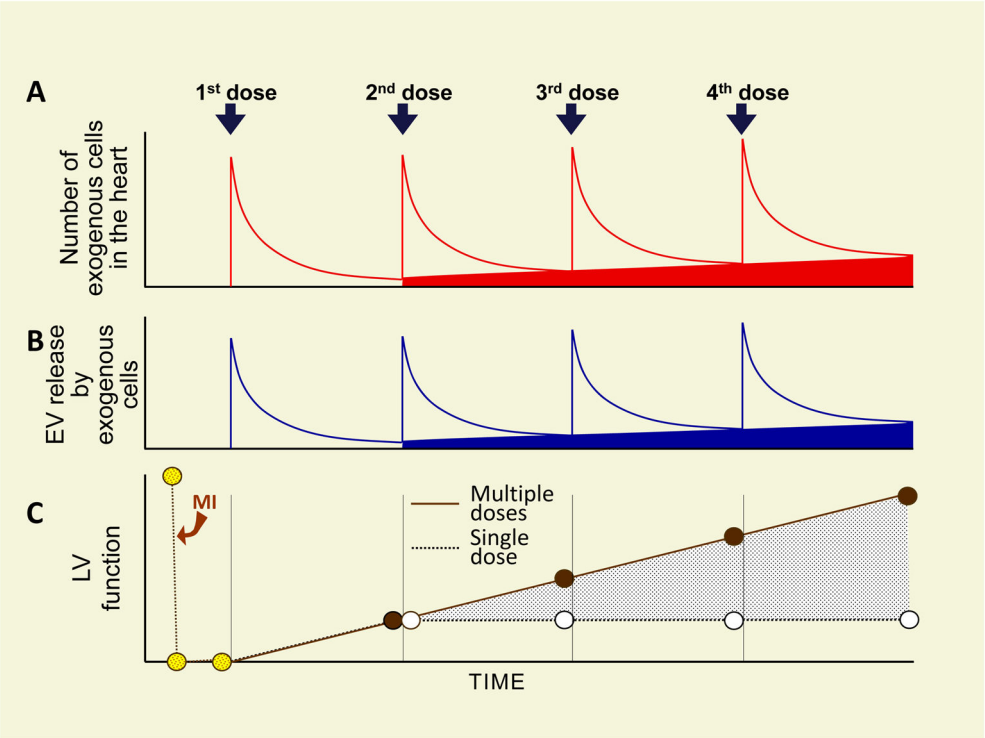

Figure 2. Conceptual paradigm of repeated cell therapy

(A), After cell administration, the number of transplanted cells in the myocardium falls rapidly, but a small number persists for many weeks (at least 1 year). Repeated cell doses produce a cumulative increase in the number of transplanted cells that persist long term in the myocardium (red area)[7]. Each cell dose produces a burst of extracellular vesicles (EV) release **(B)**, which is responsible for the cumulative improvement in LV function **(C)**. The cumulative increase in long-lasting exogenous cells (red area) may be associated with a cumulative increase in sustained EV release by these cells (blue area). **A** and **C** are based on experimental data whereas **B** is speculative. Reproduced with permission from ref. 4

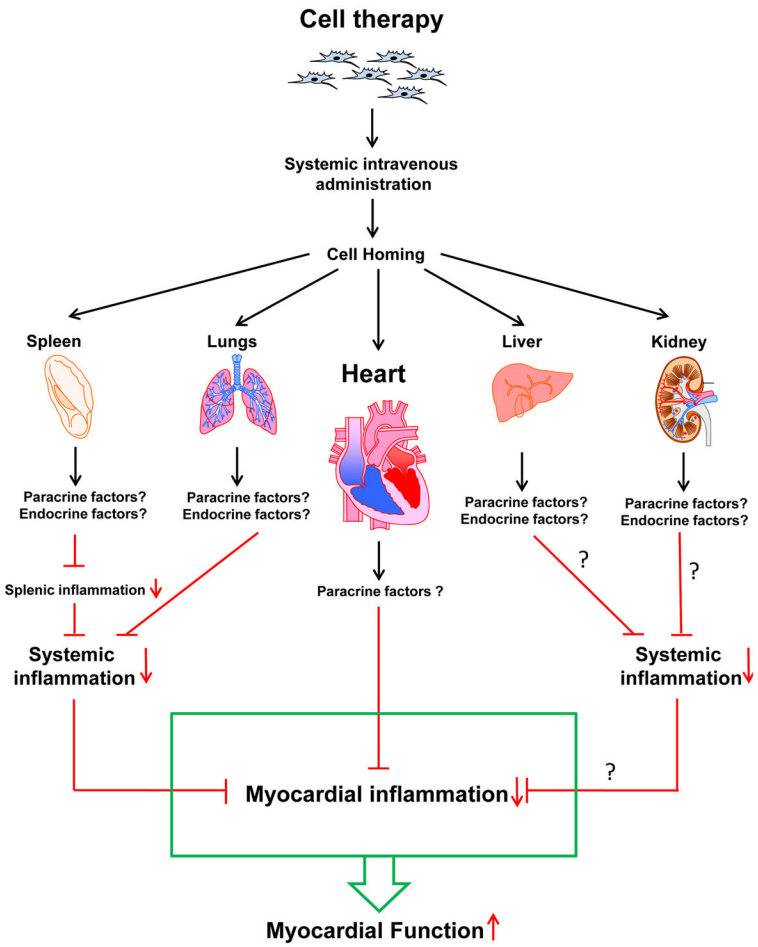

Figure 3A

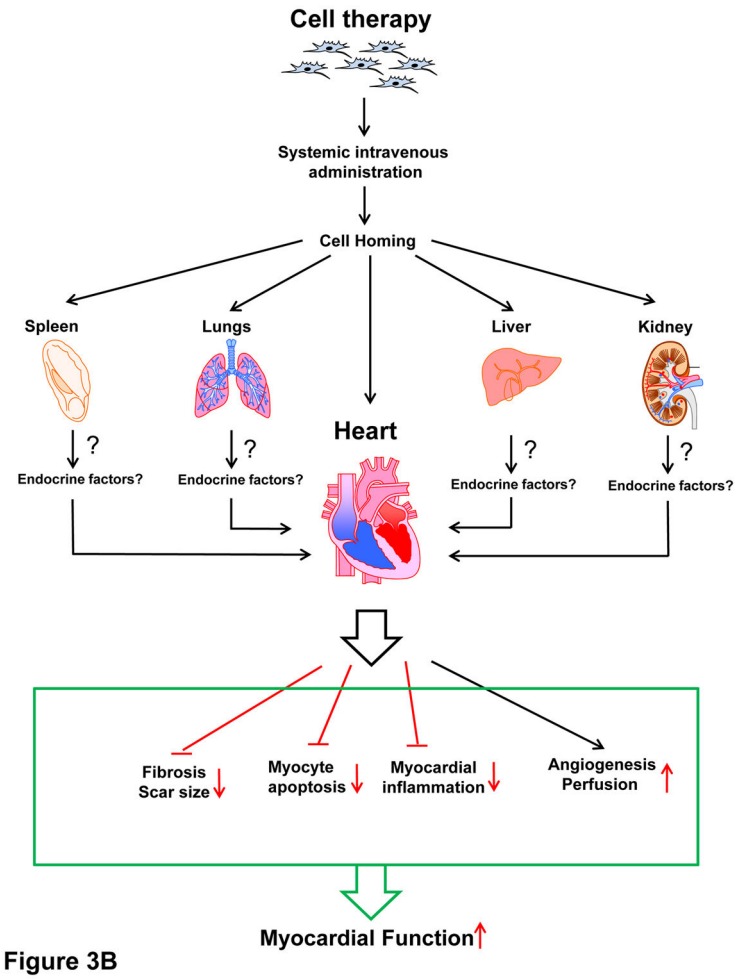

Figure 3. Potential mechanisms of intravenous cell therapy

Intravenously injected cells home to various organs and affect the heart in a paracrine and/or endocrine fashion. **(A) Paracrine and/or endocrine anti-inflammatory actions.** Transplanted cells trapped in extracardiac organs may affect local inflammatory cells (paracrine action) that are released into the circulation and/or they may affect systemic inflammatory cells (endocrine action via release of immunomodulatory factors), ultimately leading to a reduction in myocardial inflammation. For example, cells trapped in the lungs may modulate immune cells circulating through the lungs (paracrine mechanism) and/or release factors (cytokines, miRs, EVs, etc.) that modulate immune cells systemically (endocrine effect). **(B). Other endocrine actions.** In addition, cells trapped in the lungs may release factors (cytokines, miRs, EVs, etc.) that affect the myocardial milieu at a distance (endocrine effects), resulting in a reduction of scar size, fibrosis in viable tissue, and apoptosis, and in an increase in angiogenesis and myocardial perfusion, thereby leading to improved myocardial function. In theory, transplanted cells that home to the heart may also exert paracrine effects, but the number of such cells is very low.

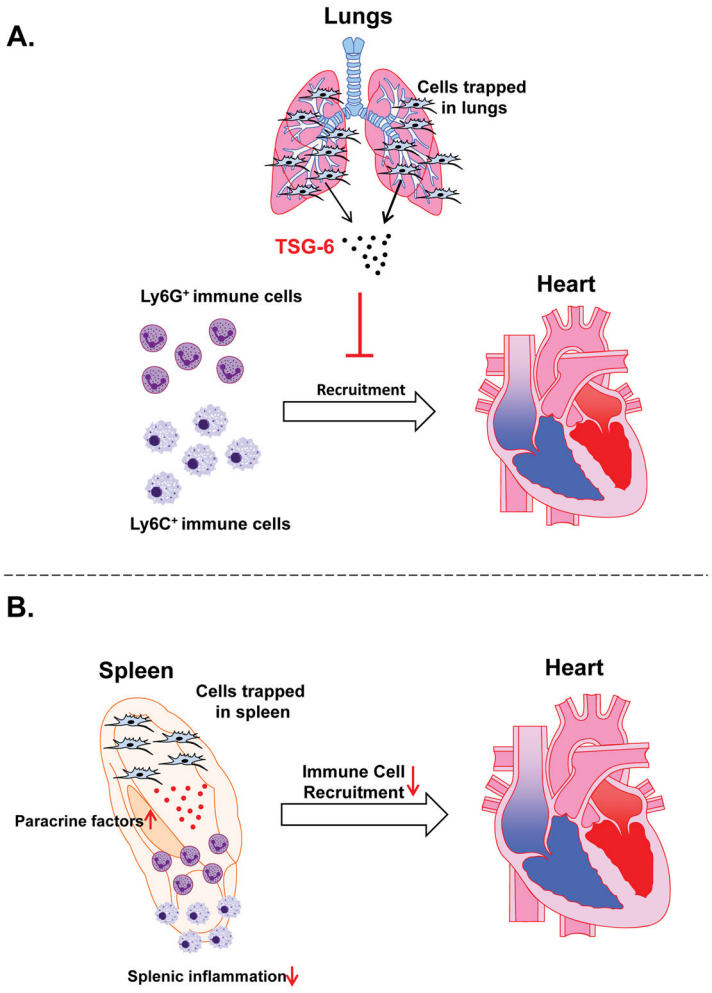

Figure 4. Examples of paracrine/endocrine actions of intravenously delivered cells

(A) Intravenously injected cells trapped in the lungs produce TSG-6, which has anti-inflammatory actions and inhibits myocardial recruitment of $Ly6G^+$ and $Ly6C^+$ immune cells. This is an example of endocrine actions of transplanted cells. **(B)** Intravenously injected cells home to the spleen and modulate splenic inflammatory cells by secreting paracrine factors; this reduces immune cells recruitment in the myocardium.

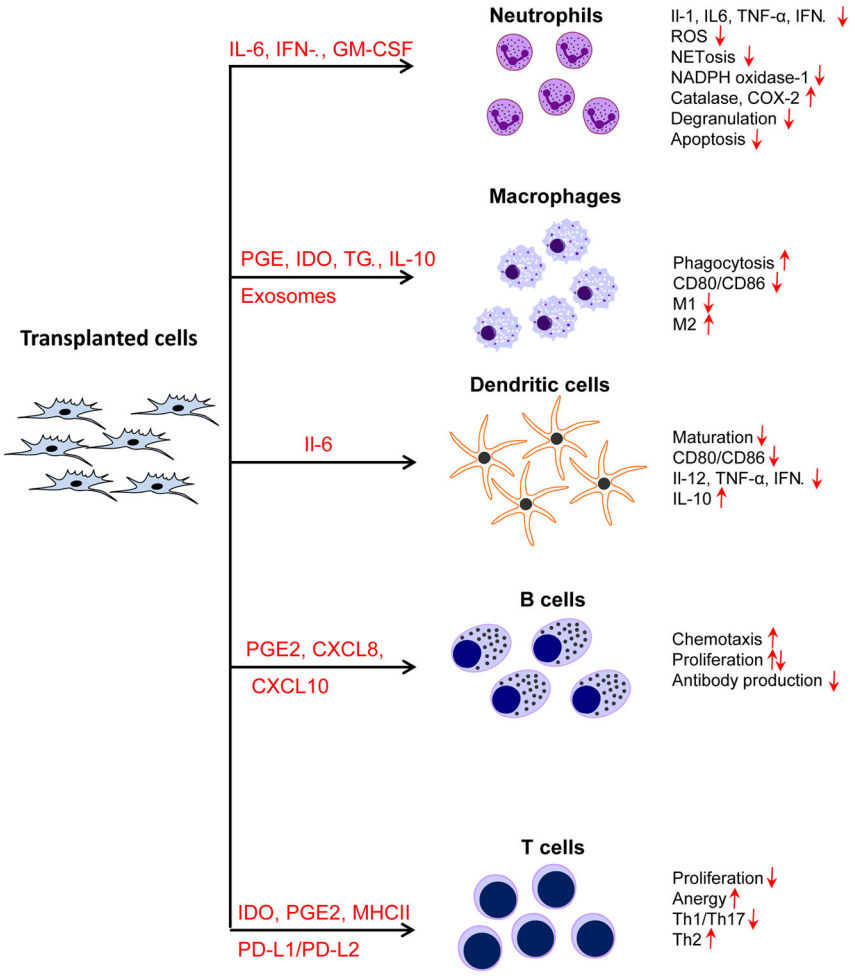

Figure 5. Potential mechanisms whereby transplanted cells modulate the various components of the immune system

Cells injected into the host secrete a panoply of factors that affect the function of various immune cells, locally (paracrine actions) and potentially at a distance (endocrine actions), as detailed in the text.

Table 1

Preclinical Studies of Intravenous Cell Therapy

Study	Species	Setting	Cell Type	Cell dose	Follow-up	Outcomes
Nagaya,[11] 2004	Rats *	Acute MI	BM MSCs, allogeneic	5×10^6	4 weeks	↓ IS, ↑ LV EF, ↑ capillary density
Ma,[12] 2005	Rats *	Acute MI	BM MSCs, allogeneic	5×10^6	28 days	↑ cardiac function, ↑ angiogenesis
Boomsma,[22] 2007	Mice *	Acute MI	BM MSCs, allogeneic	0.5×10^6	2 weeks	↔ infarct morphology, ↑ systolic function
Guo[49], 2007	Rats *	Acute MI	BM MSCs, allogeneic	1×10^7	4 weeks	↑ LV EF ↑ fractional shortening
Lee,[13] 2009	NOD-SCID mice (immunodeficient)	Acute MI	BM MSCs, human	2×10^6	21 days	↑ LV EF ↑ fractional shortening
Dijk,[23] 2011	Rats *	Acute MI	AD SCs, allogeneic	5×10^6 SVF 1×10^6 AD	35 days	↓ IS No adverse events
Wang,[14] 2011	Rats *	Chronic MI	BM MSCs allogeneic	5×10^6	4 weeks	↔ LV function
Dayan[48], 2011	NOD-SCID mice (immunodeficient)	Acute MI	BM MSCs, HUCPVC allogeneic	2×10^6	4 weeks	↑ fractional shortening at 4 weeks then ↔
Yang,[24] 2013	Rats *	Nonischemic	BM MSCs allogeneic	5×10^6 5×10^6 +AZA	4 weeks	↑ cardiac function with double infusion
Yu,[25] 2014	Rats *	Nonischemic	BM MSCs, allogeneic	5×10^6	20 weeks	↑ LV function with repeated infusion
Li,[26] 2015	Rats *	Acute MI	BM MSCs, allogeneic	2×10^6 2×10^6 + atorvastatin	30 days	↑ LV EF Atorvastatin-pretreated cells homed to the myocardium
Mykhaylichenko,[27] 2016	Rats *	Acute MI	BM MSCs, allogeneic	$1–5 \times 10^6$ $1–5 \times 10^6$ +AZA	4 weeks	↑ LV function and vascularization
Luger,[20] 2017	Mice *	Acute MI, chronic ischemic cardiomyopathy	BM MSCs, human	2×10^6	6 weeks	↓ IS ↑ LVEF ↓ Adverse remodeling
Price,[28] 2006	Swine, *	Acute MI	BM MSCs, allogeneic	3.2×10^8	3 months	↑ EF, ↔ IS ↓ eccentric hypertrophy
Krause,[29] 2007	Swine	Acute MI	BM MSCs, autologous	1×10^6	1 month	↓ IS ↑ hemodynamic
Halkos,[30] 2008	Swine *	Acute MI	BM MSCs, allogeneic	1×10^6 3×10^6 10×10^6	12 weeks	↑ LV function
Wolf,[31] 2009	Swine *	Acute MI	BM MSCs, autologous, allogeneic, and human	1×10^6	4 weeks	↑ FAS ↓ IS

Study	Species	Setting	Cell Type	Cell dose	Follow-up	Outcomes
Hong,[32] 2015	Swine *	Acute MI	AD SCs, human	15×10^7 5×10^7 (3 doses)	28 days	↑ LV EF ↑ CFR ↓ Perfusion defect area
Yamada,[164] 2018	Rabbit *	Acute MI	BM MSCs, autologous, allogeneic, and human	3×10^5	2 and 4 weeks	↑ LV EF ↑ fractional shortening ↑ cardiac remodeling

Abbreviations: AZA, azacytidine; AD SCs, adipose-derived stem cells; SVF, stromal vascular fraction; BM MSCs, bone marrow mesenchymal stromal cells; UC MSCs, umbilical cord-derived mesenchymal stromal cells; EF, ejection fraction; HFrEF, heart failure with reduced ejection fraction; MI, myocardial infarction; LV, Left ventricular; IS, infarct size; FAS, fractional area shortening; HUCPVC, Human umbilical cord perivascular cell

* non-immunosuppressed

Table 2

Clinical Studies of Intravenous Cell Therapy

Study	Trial Characteristics	Clinical Setting	Cell Type	Cell dose	Follow-up	Outcomes
Hare,[34] 2009	Randomized (i.v. MSCs = 34, control = 19)	Acute MI, RWMA EF ≥ 30% to ≤ 60%	BM MSC, allogeneic	0.5×10^6 1.6×10^6 5×10^6	6 months	↓ arrhythmia burden ↑ EF (more in AWMI) ↔ wall thickness ↑ global assessment scale
Butler,[36] 2017	Randomized (i.v. MSCs = 10, control = 12)	Nonischemic EF ≤ 40%	BM MSC, allogeneic	1×10^6	90 d	↑ 6MWD ↑ Kansas City Questionnaire
Bartolucci,[35] 2017	Randomized (i.v. MSCs = 15, control = 15)	HFrEF (ischemic and nonischemic)	UC MSC, allogeneic	1×10^6	12 months	↑ EF, ↑ NYHA class ↑ MLHFQ

Abbreviations: AZA, azacytidine; AD SC, adipose-derived stem cells; SVF, stromal vascular fraction; BM MSCs, bone marrow mesenchymal stromal cells; UC MSCs, umbilical cord-derived mesenchymal stromal cells; EF, ejection fraction; HFrEF, heart failure with reduced ejection fraction; MI, myocardial infarction; NYHA, New York Heart Association class; 6MWD, six-minute walk distance; MLHFQ, Minnesota living with heart failure questionnaire; LV, Left ventricular; IS, infarct size; FAS, fractional area shortening.