Immune response to stem cells and strategies to induce tolerance

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Although recent progress in cardiovascular tissue engineering has generated great expectations for the exploitation of stem cells to restore cardiac form and function, the prospects of a common mass-produced cell resource for clinically viable engineered tissues and organs remain problematic. The refinement of stem cell culture protocols to increase induction of the cardiomyocyte phenotype and the assembly of transplantable vascularized tissue are areas of intense current research, but the problem of immune rejection of heterologous cell type poses perhaps the most significant hurdle to overcome. This article focuses on the potential advantages and problems encountered with various stem cell sources for reconstruction of the damaged or failing myocardium or heart valves and also discusses the need for integrating advances in developmental and stem cell biology, immunology and tissue engineering to achieve the full potential of cardiac tissue engineering. The ultimate goal is to produce ‘off-the-shelf’ cells and tissues capable of inducing specific immune tolerance.

Keywords: stem cells; tolerance; immune response; regenerative medicine; tissue engineering

1. INTRODUCTION

Tissue engineering, one of the major components of regenerative medicine, encompasses various disciplines such as biology, material science and engineering, with the single aim of developing living substitutes that can restore and maintain normal function. This involves several strategies including the *ex vivo* use of three-dimensional biological scaffolds seeded with appropriate cells and cultivated in bioreactors which allow formation of the desired tissue or organ (Stock & Vacanti 2001; Vaananen 2005). A critical factor in these strategies is the choice and source of cells to be used. The use of autologous cells from the host is actively being explored to avoid rejection. However, for many patients with end-stage organ failure this approach is not feasible, since in most cases a tissue biopsy does not yield enough normal cells for expansion, and transplantation and the procedure could be severely hampered by time constraints involved in generating a tissue engineered organ such as heart valve leaflets or functional myocardium. Regenerative therapy, in particular cardiac regeneration, may offer a viable solution (Sanchez *et al.* 2006; Yacoub *et al.* 2006).

The use of allogeneic or xenogeneic stem cells to create an ‘off-the-shelf’ organ, suitable for all patients is extremely attractive. However, this poses the significant problem of anti-graft immune responses. During the last few years, a significant amount of knowledge regarding the specific immune responses of stem cells, and to some extent strategies to deal with them, has accumulated. In this article, we will discuss the different sources and types of stem cells that are currently used in both

clinical applications and experimental models of cardiac repair *in vivo* and *in vitro*, with particular reference to their immunogenicity. This is followed by reviewing current strategies used to enhance engraftment and to overcome rejection.

2. SOURCES OF STEM CELLS FOR TISSUE ENGINEERING

(a) Embryonic stem cells

Studies carried out on human embryonic stem (ES) cells have demonstrated that they have an enormous potential for generating tissues of therapeutic value, which offers prospects for cell transplantation that may be applicable to a wide range of human diseases. Three main properties make ES cells very attractive for replacement therapies: (i) their ability to grow indefinitely in culture provides a renewable cell source for transplantation and tissue engineering; (ii) ES cells can be genetically manipulated and can theoretically be targeted with transgenes to replace defective genes or to supplement tissues with growth and survival factors; and (iii) the pluripotent capacity of ES cells to generate almost any cell type has been demonstrated through many reported differentiation protocols using established culture conditions (McCloskey *et al.* 2005; Wobus & Boheler 2005). Although the hope is that human ES cells could be used to treat a wide range of human ailments, the proof of principle has mainly come from studies using animal ES cells where many models are being used to validate their potential use.

(i) ES cells in cardiac repair

The use of ES cells in cardiac repair in animal models has yielded some encouraging results. For instance, the utilization of cardiac-restricted promoters using

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selectable markers in the selection of cardiomyocytes from differentiating ES cells has been well documented (Klug *et al.* 1996; Meyer *et al.* 2000; Muller *et al.* 2000; Fijnvandraat *et al.* 2003). Others have demonstrated the therapeutic capacity of mouse ES cell-derived cardiomyocytes by injecting these cells into the ventricular myocardium of adult dystrophic (*mdx*) mice. ES cell incorporation into the hearts was verified by their presence within the grafts seven weeks after implantation, in the absence of tumour formation (Klug *et al.* 1996). This study was further corroborated by reports demonstrating ameliorated left ventricular function in post-infarcted rats following implantation of ES-derived 'beating cells' (Min *et al.* 2002). The engrafted cells not only displayed typical striations and markers of mature cardiomyocytes such as the myofilament proteins sarcomeric α -actin, α -myosin heavy chain and troponin I, but were also able to establish themselves among the endogenous cardiomyocytes. Whether the transplanted cardiomyocytes coupled normally with endogenous cells or produced long-term beneficial effects in this study was unclear. However, in a large animal model of xenotransplantation, recent data from Kehat *et al.* (2004) observed that when they transplanted human ES-derived cardiomyocytes into the left ventricular wall of a pig heart with complete heart block, the grafted cells could electromechanically integrate with the resident cardiomyocytes and functioned to rescue cardiac rhythm, thus acting as pacemaker cells. Recently, progress in developmental biology has identified the involvement of critical factors in cardiomyocyte differentiation, including bone morphogenic protein (Bin *et al.* 2006) and Wnt signalling proteins (Eisenberg & Eisenberg 1999; Pandur *et al.* 2002; Terami *et al.* 2004), and such factors have the potential to improve the efficiency of stem cell induction. Therefore, it remains to be seen whether the full potential of ES-derived cardiomyocytes in the generation of stable cardiac grafts for cardiac therapy will be fulfilled.

Currently, there are no ES cell-based therapies ongoing in humans. Their use in a clinical setting is beset by ethical constraints due to their isolation from *in vitro* fertilized human embryos, and there are additional concerns such as inefficient differentiation (McLaren 2001) and tumorigenicity (Wobus & Boheler 2005) of human ES cells. In a recent report by Mummery *et al.* (2003), the problem of inefficient differentiation of human ES cells into functional cardiomyocytes was addressed using a novel strategy. They show that by co-culturing human ES cells, which have not undergone spontaneous cardiogenesis, with visceral-endoderm (VE)-like cells from the mouse, differentiation to beating cardiomyocytes could be initiated within 10 days. This suggested that paracrine factors derived from the VE cells act as a cardiac differentiation factor. However, the nature of the specific factors involved was not elucidated. Nevertheless, the differentiated ES cell-derived cardiomyocytes were fully characterized and were found to exhibit sarcomeric proteins, atrial natriuretic peptide, cardiac ion channel genes and the generation of gap junctions, demonstrated by connexin 43 expression and dye-coupling. With regards to tumorigenicity, the neoplastic tendency of embryoid bodies has been

proposed to decrease with increased cell differentiation *in vitro* (Zhang *et al.* 2001; Chinzei *et al.* 2002), but the possibility that differentiated ES cell-derived tissues may still harbour undifferentiated ES cells remains a concern. To circumvent this risk, one possibility is to pre-differentiate ES cells *in vitro* (ideally to 100%) to the desired lineage and/or eliminate undifferentiated cells by positive selection using cell surface markers and fluorescent-activated cell sorting strategies. Such a strategy has been successfully tested in an experimental study (Menard *et al.* 2005).

Perhaps most importantly, the immunogenicity and the high potential for rejection of differentiated cells derived from ES cells in allogeneic recipients have been major impediments to the approval of clinical trials (Bradley *et al.* 2002; Drukker & Benvenisty 2004; Swijnenburg *et al.* 2005). In addition to carrying the risk of infection by non-human pathogens, the exposure of human ES cells to non-human proteins and xenogeneic feeder cells during derivation and propagation may also contribute to their immunogenicity. Non-human sialic acid, Neu5Gc, has been identified as a possible cause for human ES cell immunogenicity (Martin *et al.* 2005). This protein is abundant in animal-derived serum substitutes and feeder cells to which a vast majority of humans have circulating antibodies. Since both embryoid bodies and human ES cells can metabolically take in considerable amounts of Neu5Gc, their exposure to human sera containing antibodies to Neu5Gc will inevitably result in complement induced cell death *in vivo*. In an attempt to avoid this possible scenario, human ES cell lines have now been expanded on both human feeder cells (Richards *et al.* 2002; Amit *et al.* 2004) and in the absence of feeder cells under serum-free conditions (Lee *et al.* 2005; Ellerstrom *et al.* 2006). These newly derived human ES cell lines, free from potential retroviral infections, may then be suitable for eventual therapeutic applications.

(ii) *Immunogenicity of ES cells and routes to escape rejection*
Human ES cells have the potential to be rejected following post-transplantation due to the fact that they express low levels of major histocompatibility complex (MHC) class I antigen which can increase after differentiation both *in vitro* and *in vivo* (Drukker *et al.* 2002). In addition, interferon gamma treatment has been shown to markedly increase MHC class I expression (Draper *et al.* 2002; Drukker *et al.* 2002). Recently, it has also been shown that expression of MHC class I on human ES cells is sufficient for rejection by cytotoxic T cells (Drukker *et al.* 2006). On the other hand, the absence of MHC class II molecules and low-level expression of MHC class I may also lead to natural killer cell rejection of the transplanted cells (Drukker *et al.* 2002); however, several studies have reported that this is not always the case (Hori *et al.* 2003; Mammolenti *et al.* 2004; Wei *et al.* 2004). In addition, mismatched minor histocompatibility antigens as well as ABO blood groups should also be considered as potential triggers for rejection. For further reading, the subject of ES cell immunogenicity has been extensively covered in several reviews (Bradley *et al.* 2002; Drukker & Benvenisty 2004; Fairchild *et al.* 2004; Boyd *et al.* 2005).

Figure 1. Routes for evading immune rejection of human ES cells.

A number of approaches have been proposed to tackle or eliminate ES-derived graft rejection (figure 1). Classic immunosuppression, routinely used for organ transplantation, could reduce the host reactivity to allogeneic ES-derived transplants (Gage 1998), but associated complications such as drug-related toxicities, opportunistic infections (Simon & Levin 2001) and a 100-fold increase in the incidence of malignant tumours (Penn 2000; Buell *et al.* 2006) may reduce the quality of life for the patients. Another possible route is through the induction of tolerance by cotransplanting both ES-derived graft and haematopoietic stem cells established from the same parental ES cell line. This could lead to specific inhibition of immune rejection (Odorico *et al.* 2001). Alternatively, genetically modifying ES cells to eliminate MHC class I molecules to generate a 'universal' undifferentiated ES cell line has also been suggested (Boheler & Fiszman 1999; Drukker *et al.* 2002). In addition to the potential consequences of such drastic gene modifications to the graft (Grusby & Glimcher 1995), it is also worth considering that in addition to direct allo-recognition, the absence of MHC molecules may lead to indirect allo-recognition-mediated rejection and/or natural killer cell-mediated cell destruction (McNerney *et al.* 2006). However, it has been reported that the expression of non-polymorphic class I molecule human leucocyte antigen (HLA)-E on the surface of cells confers protection from death by natural killer cells (Moretta *et al.* 2002, 2003). Another approach is the generation of an ES stem cell bank containing HLA-isotyped and/or genetically modified human ES cell lines where known HLA backgrounds could be established. But this would involve a massive amount of work to attain pure populations of HLA-defined ES cells, which ultimately may be unachievable.

Finally, the process of 'therapeutic cloning' has been proposed for generating pluripotent stem cells that carry the nuclear genome of the patient. In this approach, also known as nuclear transfer, the nucleus of a human oocyte is removed and replaced by a nucleus from a somatic cell obtained from the patient. The manipulated oocyte is allowed to proliferate in culture, creating an ES cell population. Inducing these ES cells to differentiate into replacement cells may

eliminate the critical problem of immune incompatibility since they contain (with the exception of the mitochondrial genome) the same genetic information as the patient (Lanza *et al.* 1999). This strategy was successfully extended to an application known as reproductive cloning, used to create Dolly the sheep (Wilmut *et al.* 1997), although reproductive cloning through nuclear transfer of human cells would not be pursued for ethical reasons. Therapeutic cloning on the other hand is legal in many countries including the United Kingdom (Pincock 2004), other European countries including Sweden and Belgium, and also in certain parts of the United States such as California and New Jersey. Irrespective of what the optimal application proves to be, human ES cells offer an enormous potential for generating tissues of therapeutic value, although further research is required to overcome the problems highlighted earlier, especially those regarding their immunological status following differentiation.

(b) *Adult stem/progenitor cells*

(i) *Sources of adult stem/progenitor cells*

Adult stem cells or tissue-derived progenitor cells are a population of cells found within mature tissue that have the capacity to differentiate and expand into mature cells with specific function. However, unlike ES cells which are defined by their origin (the inner cell mass of the blastocyst), adult stem cells cannot be characterized in the same manner, especially since their true origin is still unclear. Adult human stem cells can be isolated from tissues such as periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, deciduous teeth and, more recently, heart valves (Veinot *et al.* 2006; Latif *et al.* submitted) and cardiac tissue (Anversa *et al.* 2006), in particular the islet-1 positive cells which have been termed as the definitive cardiac progenitor cell (CPC; Laugwitz *et al.* 2005). The most extensively studied source is the bone marrow which contains the multipotential mesenchymal stem cells (MSCs) or stromal cells, resident at low density within the bone marrow stroma (Le Blanc & Pittenger 2005; Vaananen 2005) as well as haematopoietic stem cells (Bryder *et al.* 2006) and endothelial progenitor cells (EPCs; Barber & Iruela-Arispe 2006). The plasticity or

transdifferentiation ability of these bone marrow stem cells to differentiate into specific cell types of another tissue has been under intense investigation, in particular the use of bone marrow-derived stem cells in cardiac repair.

(ii) *Mesenchymal stem cells*

The MSC is the most extensively characterized and most potent adult stem cell in terms of differentiation into multiple cell phenotypes including bone, fat, cartilage, muscle, epithelium, neural and cardiomyocytes (Pittenger *et al.* 2000; Pittenger & Martin 2004; Smits *et al.* 2005). At present, there is no definitive marker or markers which exclusively identify MSCs, which suggests that the cell populations derived by the current methods are heterogeneous. Morphologically they mostly resemble fibroblast-like cells and are characterized by their positive surface staining for CD105 (SH2 or transforming growth factor- β (TGF β) receptor endoglin), CD166 (activated leucocyte-cell adhesion molecule, ALCAM), CD73 (SH3 and SH4 or membrane-bound ecto-5'-nucleotidase) and CD44 (hyaluronan receptor), and negative for haematopoietic surface markers CD34 (haematopoietic stem cells), CD45 (leucocyte common antigen), CD14 (monocytes, macrophages), CD31 (endothelial, T lymphocytes, macrophages) and CD133 (endothelial/stem progenitor cells; Majumdar *et al.* 2003; Barry & Murphy 2004). Although these cells are considered to be involved mainly in the support of haemopoiesis, their ease of isolation, culture and capacity to be greatly expanded without losing the ability to differentiate into multiple lineages, has highlighted their clinical potential for repair or replacement of damaged tissues (Le Blanc & Pittenger 2005).

Another plentiful source for MSCs is from adipose tissue, which not only provides a rich source of MSCs which can be easily harvested by a simple minimally invasive method, but also of other stem/progenitor cells such as EPCs, providing a plentiful reservoir of MSCs and EPCs which would otherwise be normally discarded following cosmetic surgery. As with bone marrow-derived MSCs, adipose tissue-derived MSCs can also be similarly characterized using the same panel of antibodies. For EPCs, these cells are typically defined on the basis of expression of cell surface markers such as CD34, Flk-1 (vascular endothelial growth factor receptor (VEGFR) 2) and CD133. Of particular interest regarding the use of MSCs and EPCs derived from adipose tissue is the fact that these cells can be isolated in greater numbers (0.001–0.01% versus 1–2%; M. Hedrick 2003, personal communication) and expanded more rapidly than from bone marrow. This may obviate the need for long-term *in vitro* propagation since clinically relevant stem cell numbers can be extracted from isolates of adipose tissue (De Ugarte *et al.* 2003; Planat-Benard *et al.* 2004b; Martinez-Estrada *et al.* 2005). Recent reports have demonstrated that adipose-derived MSCs can also be induced to differentiate into cardiomyocytes (Planat-Benard *et al.* 2004a). However, the subject of whether adult bone marrow cells have true *in vivo* differentiation capacity into cardiac myocytes remains controversial. It will be important to clarify whether

the surviving cells within the heart arose from differentiation or fusion and to assess their functional capacity in the heart.

(iii) *Cardiac-derived progenitor cells*

The most commonly held view about the regenerative capacity of the heart is that it cannot regenerate as it is a post-mitotic organ consisting of terminally differentiated cardiomyocytes. This view is now being challenged by reports from several groups who suggest that the heart, similar to other organs such as skeletal muscle and brain, may contain a resident population of progenitor cells with cardiomyogenic and even myocardiogenic potential (ability to differentiate into myocytes, vascular smooth muscle cells and endothelial cells). CPCs have been identified and characterized in the hearts of mice (Hierlihy *et al.* 2002; Oh *et al.* 2003; Matsuura *et al.* 2004), rats (Beltrami *et al.* 2003), dogs (Linke *et al.* 2005) and humans (Evans 1993; Messina *et al.* 2004; Laugwitz *et al.* 2005). Some of the first evidence for the presence of these cells came from Hierlihy *et al.* (2002), who demonstrated that the 'side population' cells determined by their ability to take up the stain Hoechst, represented CPC (approx. 1%) in the hearts of post-natal mice. Studies examining phenotypic and functional characteristics indicated that the CPCs were positive for the stem cell marker c-kit (7–10% of the cells expressed early myocyte-related transcription factors Nkx2.5, GATA-4 and MEF2C) and displayed stem cell qualities of self renewal, clonogenicity and multipotency (Beltrami *et al.* 2003). Furthermore, several studies demonstrated that intramyocardial injection of c-kit positive CPCs, or growth factor activation of local CPCs significantly improved cardiac function (Beltrami *et al.* 2003; Dawn *et al.* 2005; Linke *et al.* 2005). Oh *et al.* (2003), using a different approach, isolated stem cell antigen-1 positive (Sca-1⁺, but c-kit negative) cells from mouse hearts and found that after injecting them intravenously, the Sca-1⁺ cells homed to the site of ischaemia/reperfusion injury where they differentiated and fused with the host cells. Matsuura *et al.* (2004) confirmed these findings and went on to show that Sca-1⁺ cells expressed cardiac transcription factors, contractile proteins, sarcomeric structures and spontaneous beating following treatment with oxytocin.

CPCs isolated from human heart (atrial and ventricular biopsies; Messina *et al.* 2004) express the stem cell markers c-kit and Sca-1, and the endothelial marker CD34, when grown as cardiospheres or clonal spherical clusters, but can also express cardiac differentiation markers when grown on collagen-coated dishes. More recently, the expression of islet-1 (Isl-1), a marker of progenitor cells implicated in the morphogenesis of the embryonic heart (Evans 1993), has been used to identify and isolate another population of CPCs from human, mouse and rat post-natal hearts (Laugwitz *et al.* 2005). These cells are negative for Sca-1 and c-kit but express early cardiac differentiation markers Nkx2.5 and GATA-4. However, there is a dispute whether these newly identified cells represent a new definitive CPC type important for reconstituting the damaged heart (Anversa *et al.* 2006). It has been suggested that Isl-1-positive cells have not been

detected in the failing heart and furthermore, during development, Isl-1-positive cells are not implicated in left ventricle formation (Anversa *et al.* 2006). Nevertheless, there appears to be overwhelming evidence that the heart harbours progenitor cells with the capacity to generate cells that exhibit cardiomyocyte characteristics, as well as other non-cardiac cell types, but further studies are required to ascertain their true identities and the relationship between them.

(iv) *Clinical studies using autologous adult stem cells*

MSCs have become an important tool of tissue regeneration studies and are considered as potential candidates for several clinical applications (Prockop *et al.* 2003). Several phase I clinical studies have reported to have used autologous whole bone marrow, consisting of both MSCs and endothelial stem cells, in cardiac therapy. Although these studies do not define the percentage of each subset of cell type used, their overall outcome is an improvement in a range of cardiac clinical measurements. For instance, one clinical study followed 10 patients with acute myocardial infarction after administering autologous bone marrow cells into the infarct region for a period of several minutes under increased pressure using a double-balloon catheter. Three-month post-procedure evaluation revealed the infarct region to have significantly reduced as well as an improved wall motion score (Strauer *et al.* 2002). Another clinical study utilizing autologous mononuclear bone marrow cells, carried out electromechanical mapping to visualize viable myocardium regions (15 sites in total) for optimal placement of the cells (15 million cells) in patients with severe heart failure. Two months after intramyocardial injections of bone marrow-derived stem cells, an enhancement in myocardial blood flow with associated improvement of regional and global left ventricular function was noted (Perin *et al.* 2003). Also of interest is the use of autologous bone marrow-derived EPCs by Stamm *et al.* (2004) who isolated EPCs using magnetic beads coated with the AC133 antibody (CD133) and injected them into areas of infarct in patients undergoing coronary artery bypass. An improvement in left ventricular function, revascularization and contractility of the infarct areas was seen; however, the exact mechanism of improvement in left ventricular function is not known. Recent evidence indicates that transdifferentiation to either cardiomyocytes or endothelial cells is unlikely and that paracrine factors play a major role. Taken together, these studies suggest that autologous grafts of MSCs may be possible, but engrafting allogeneic MSCs would be more therapeutically attractive. However, there are concerns regarding the very small improvement in ejection fraction in reports from phase I clinical trials using bone marrow-derived putative stem cells and the apparent inconsistency, particularly in longer term results (Nadal-Ginard & Fuster 2007).

(v) *Use of allogeneic or xenogeneic mesenchymal stem cells in cardiac repair and regeneration*

Recent studies have shown that the use of allogeneic adult stem cells may be more feasible for clinical applications, as several preclinical and clinical studies

have been performed in the field of bone marrow transplantation and immune diseases (Devine 2002; Serakinci & Keith 2006). Long-term engraftment of allogeneic MSC has been demonstrated in several animal studies using a range of tissues in the absence of immunosuppression (Liechty *et al.* 2000; Bartholomew *et al.* 2001). Pittenger & Martin (2004) reported the ability of allogeneic MSCs to engraft into infarcted myocardium. Using both rat and porcine animal models, they demonstrated both engraftment of allogeneic MSCs in necrotic myocardium and also improvement in ventricular function after infarction. Furthermore, in the absence of immunosuppressive therapy, they found no evidence of immunological rejection or lymphocytic infiltration. The precise mechanism involved in these animal studies requires further research, but fusion with resident cardiomyocytes has been proposed (Alvarez-Dolado *et al.* 2003). However, subsequent studies using male bone marrow cell donors in female recipients have demonstrated that bone marrow cells can differentiate directly into cardiac cell lineages (Kajstura *et al.* 2005). Further evidence of MSC engraftment was observed when xenogeneic human MSCs were injected into experimentally induced ischaemic rat myocardium (Grinnemo *et al.* 2004). Other studies have documented the presence of resident cardiac stem cells (Beltrami *et al.* 2003) and some recent new data from several groups have reported that these cells can be expanded and differentiated *in vitro* and transplanted to improve function (Ott *et al.* 2005; Tateishi *et al.* 2005). Although further studies are necessary to determine whether the immune privileged status of MSCs may allow for enhanced clinical applicability, allogeneic MSCs can be readily available and administered with immunological acceptance by the recipient.

(vi) *Current non-cardiac therapeutic applications of mesenchymal stem cells*

Clinical trials with encouraging results have already been initiated in a variety of other conditions using MSCs. These include the treatment of brittle bone disease or osteogenesis imperfecta (Horwitz *et al.* 2002; Le Blanc *et al.* 2005) through systemic transplantation of allogeneic MSC which resulted in the MSCs homing to the bone and an increase in new bone formation. Also, similar clinical improvement has been observed in patients with metachromatic leukodystrophy or Hurler's syndrome (Koc *et al.* 2002). Furthermore, cotransplantation of MSCs with heterologous bone marrow transplants have been used to enhance engraftment (Koc *et al.* 2000) as well as to successfully treat severe acute graft-versus-host disease (GVHD; Collins *et al.* 2000a; Bartholomew *et al.* 2002; Potian *et al.* 2003; Le Blanc *et al.* 2004). These clinical data suggest that MSCs possess an immune privileged position that allows their persistence in an allogeneic environment. These studies strongly suggest that MSC grafting may not only treat and prevent GVHD, but may be incorporated in and extended to future tolerance generating regimes in both transplantation and tissue engineering.

(vii) *Immunological obstacles in tissue engineering using adult progenitor cells*

In addition to their applications in tissue regeneration, the use of adult stem cells in the field of tissue engineering is also being vigorously pursued. Target organs include liver (Kaiharu *et al.* 2000), skin (Andreadis 2004), bone (Schek *et al.* 2005), cartilage (Pang *et al.* 2005), teeth (Modino & Sharpe 2005), small intestine (Choi & Vacanti 1997) and cardiovascular organs such as heart valves (Hoerstrup *et al.* 2002; Neuenschwander & Hoerstrup 2004). However, very little is known regarding their behaviour and fate following either systemic infusion or local implantation. In our laboratory, we are actively pursuing the tissue engineering of human heart valves. Through our initial studies comparing interstitial cells derived from the native valve leaflet with bone marrow-derived MSCs, we found that these two cell types share many properties. These include similar functional capacity to synthesize collagen when mechanically stretched (Ku *et al.* 2006), which will be important if MSCs are to be used in tissue engineered heart valves. MSCs were also found to highly resemble valve interstitial cells with respect to phenotype (cell surface molecules including cytoplasmic proteins, ECM components and cell communication molecules; N. Latif 2006, personal communication) and perhaps more importantly, our *in vitro* studies have shown that, like valve interstitial cells, MSCs also downmodulate T cell responses, suggesting that MSCs may prove to be a very good cell source for tissue engineering heart valve allografts (Batten *et al.* 2001, 2006).

Unlike ES cells, there is emerging evidence that donor MSCs have profound immunomodulatory function, both *in vivo* and *in vitro*, suggesting that the clinical application of MSCs for tissue regeneration could be achieved (Devine 2002; Ryan *et al.* 2005). As evidenced by both experimental and clinical data, MSCs have unique immunological characteristics that allow their persistence in an allogeneic environment (Liechty *et al.* 2000; Hoerstrup *et al.* 2002; Tse *et al.* 2003). *In vivo* non-human primate studies have elegantly demonstrated that neither allogeneic nor autologous MSCs are rejected but are engrafted into multiple tissues when transplanted in baboons (Devine *et al.* 2003). Clinical studies (Le Blanc *et al.* 2004) have demonstrated that MSCs expanded *ex vivo* can either engraft into tissues or be infused into patients without being rejected, the best examples of which are bone marrow transplantations for the treatment of leukaemia after myeloablative therapies. *In vitro* studies have indicated that MSCs can directly suppress T cell immune responses (Tse *et al.* 2003; Aggarwal & Pittenger 2004), as well as modulate the immune response indirectly through effects on professional antigen presenting cells such as dendritic cells (Beyth *et al.* 2005) and B cells (Corcione *et al.* 2006).

The immunogenicity of undifferentiated MSCs conforms to the commonly agreed consensus that they are hypoimmunogenic; it is generally accepted that MSCs are MHC class I positive and MHC class II negative or be it at very low levels. It is important to note that MSCs express MHC class I because without this expression NK cells may exert mechanisms of

deletion seen against tumour cells that have down-regulated MHC class I molecules (Ruggeri *et al.* 2001). The absence of MHC class II expression by MSCs may also help protect them from recognition by alloreactive CD4⁺T cells (Le Blanc *et al.* 2003; Majumdar *et al.* 2003). In addition, MSCs do not appear to express the costimulatory molecules CD40, CD40L, CD80 or CD86 required for effector T cell activation (Fijnvandraat *et al.* 2003; Majumdar *et al.* 2003; Tse *et al.* 2003), suggesting that any cognate interaction with the T cell receptor on T helper cells in the absence of costimulation would result in anergy.

At the cellular level, we have shown that the immunoregulatory mechanisms involved in MSC ability to downregulate T cell responses require both cell-to-cell contact (through T cell receptor/MHC class II interaction) and the release of soluble factors (Batten *et al.* 2006). We have described the induction of a distinct cytokine profile as a consequence of T cell co-culture with both allogeneic and third party MSCs which included upregulation of anti-inflammatory T helper (Th) 2 cytokines interleukin (IL)-3, IL-5, IL-10, IL-13 and the Th2 chemokine, I-309, a chemoattractant for regulatory T cells. Simultaneously, there was a downregulation of pro-inflammatory Th1 cytokines IL-1 α and IL-1 β , interferon (IFN) γ and tumour necrosis factor (TNF) α (figure 2). The generation of CD4⁺CD25^{low}CD69^{low}FoxP3⁺ regulatory T cells following co-culture with MSCs may also contribute to their ability to downmodulate the T cell response (Batten *et al.* 2006).

Although we and others have started to unravel the interactions and factors involved in MSC-mediated T cell suppression, there is still dispute concerning the actual mechanisms involved. For instance, several reports have implicated IL-10, hepatocyte growth factor, TGF β (Di Nicola *et al.* 2002; Potian *et al.* 2003) and more recently prostaglandin E2 (PGE2; Aggarwal & Pittenger 2004) as the soluble factors responsible for the suppressive effects exerted by MSCs, while others did not find the presence of IL-10 or inhibition with anti-TGF β antibodies (Djouad *et al.* 2003; Krampera *et al.* 2003) or reversal of suppression with indomethacin which inhibits PGE2 biosynthesis (Tse *et al.* 2003). Other disputed mechanisms which have also been suggested to play a role in MSC-mediated immunosuppression include the role of tryptophan catabolism by the enzyme indoleamine 2,3-dioxygenase (IDO; Meisel *et al.* 2004). In this mechanism, MSCs are thought to deplete tryptophan, essential for cell proliferation from the tissue culture medium via IDO, and thus negate proliferation. However, the suppressive activity of MSCs could not be reversed by the addition of an IDO-specific inhibitor or by the addition of supplementary tryptophan (Tse *et al.* 2003). These inconsistencies between reports suggest that other as yet undefined mechanisms may also be involved.

Although studies to date have uncovered a range of soluble factors that may induce MSC-mediated down-regulation of T cell responses, there are others which also require consideration, such as the chemokines, IL-1 receptor antagonist (IL-1ra) and IL-4ra. For instance, IL-1ra was reported to inhibit the effects of IL-1 α and

Figure 2. MSCs induce the release of anti-inflammatory Th2 cytokines and the Th2 chemokine, I-309, a major chemo-attractant for regulatory T(reg) cells, but downregulate Th1 pro-inflammatory cytokines. Supernatants were collected following 48 h co-culture of either (a) primary T cells with allogeneic peripheral blood mononuclear cells (PBMCs), two isolates of IFN γ -treated MSC (1 and 2) or IFN γ -treated allogeneic endothelial cells (ECs) or (b) primed DR11-specific T cells with allo-specific DR11 expressing PBMCs, IFN γ -treated DR11-expressing ECs, IFN γ -treated DR11-expressing MSCs or IFN γ -treated third party MHC mismatched MSCs. Cytokines detected from 48 h supernatants from T cells alone (primary and primed) and untreated or IFN γ -treated MSCs alone are also shown. Secretion of cytokines was measured using the RayBio Human Cytokine Array III system. Sections of the membrane detecting a range of Th1 and Th2 cytokines are as shown and the results are shown as signal spots.

IL-1 β by competing for type I and type II IL-1 receptors, resulting in significantly attenuated inflammation (Granowitz *et al.* 1991) as well as downregulating the inflammatory response following ischaemia-reperfusion injury in the heart (Suzuki *et al.* 2001). More recently, Murtuza *et al.* (2004) demonstrated that skeletal myoblasts overexpressing secretory IL-1ra enhanced the beneficial effects of skeletal muscle transplantation resulting in improved cardiac function.

Although *in vitro* studies investigating the immunogenicity of MSCs demonstrate that they possess immunomodulatory functions, there is some growing evidence from several studies documenting immune-mediated damage to these cells following implantation and differentiation *in vivo*. In one report, it was demonstrated that implantation of allogeneic murine gene-modified erythropoietin (Epo)-secreting MSCs into non-immunosuppressed classes I and II MHC-mismatched immunocompetent recipients, led to a vigorous and specific cellular immune response

(Eliopoulos *et al.* 2005). This suggests that MSCs become immunogenic following *in vivo* differentiation. Other studies have demonstrated that although differentiated or undifferentiated MSCs remain non-immunogenic *in vitro*, they cannot impart their immunomodulatory activities when cotransplanted to protect allogeneic allografts. One such example was demonstrated by Liu *et al.* (2006) where they showed that osteogenic differentiated MSCs displayed normal immunosuppressive activities *in vitro* and *in vivo*, but their immunomodulatory activity in protecting allogeneic skin allografts was lost following transplantation. More recently, the use of MSCs to prolong a rat model of allogeneic heart transplant was shown to be ineffective (Inoue *et al.* 2006). These studies therefore suggest that upon differentiation *in vivo*, local growth factors, cytokines and other environmental cues such as mechanotransduction and spatial cues may render MSCs immunogenic and therefore markedly limit their use as a universal donor cell type for tissue engineering. If this is the case, the actual mechanisms involved in 'switching off' the non-immunogenic status of MSCs *in vivo* need to be determined.

3. STRATEGIES TO INDUCE TOLERANCE TO OVERCOME REJECTION OF ALLOGENEIC TISSUE ENGINEERED ORGANS

To achieve this objective, several strategies are currently being pursued. One potential avenue involves genetic manipulation of MSCs. MSCs can be readily transduced by a variety of vectors and maintain transgene expression after *in vivo* differentiation (Mosca *et al.* 2000; Toma *et al.* 2002). This may be used to augment cell engraftment or the extent of differentiation. Gene transfer of tolerogenic molecules such as Fas ligand (FasL) and haemoxygenase-1 (HO-1) may offer protection against rejection. These cytoprotective molecules have recently been implicated in MSC-mediated downregulation of T cell responses (Liu *et al.* 2004), as well as in enhanced MSC survival following their transplantation in infarcted rat hearts resulting in improved cardiac function (Zhang *et al.* 2005b). FasL is a type II cell surface protein which has multiple roles (Askenasy *et al.* 2005). It can be induced on T cells and is constitutively expressed in cells from immune privileged organs such as the Sertoli cells in the testis, epithelial cells in the eye, and in the thymus where it contributes to peripheral tolerance. FasL provides and maintains immunological privilege of these sites by destroying all activated lymphocytes that enter (Singer & Abbas 1994; Bellgrau *et al.* 1995; Griffith *et al.* 1995). Studies from our laboratory have demonstrated that tolerance can be induced by FasL through overexpression in human endothelial cells (Li *et al.* 2000). However, normal human MSCs neither express FasL nor can they be upregulated following cytokine treatment (P. Batten 2005, unpublished data). Perhaps genetic modification of MSCs to express FasL may induce peripheral allograft tolerance by selectively deleting alloreactive T cells. Recent data from Liu *et al.* (2004) have indicated a role for FasL-mediated immunosuppression of peripheral blood

Figure 3. MSCs constitutively express high levels of HO-1 when compared with endothelial and valve interstitial cells (IC).

lymphocyte proliferation by MSCs. The consequence of FasL expression on MSCs will therefore be an important immunomodulatory pathway to consider for producing an 'off-the-shelf' tissue engineered organ.

Another likely candidate is the HO-1 system, which may confer cytoprotection. HO-1 has been strongly associated with cytoprotection and immune regulatory functions under conditions of cellular stress such as inflammation, ischaemia, hypoxia, hyperoxia, hyperthermia and radiation (Choi & Alam 1996) as well as in conferring protection from graft rejection in various models of organ transplantation (Katori *et al.* 2002; Braudeau *et al.* 2004; Camara & Soares 2005). This cytoprotective molecule has recently been implicated in enhanced MSC survival following transplantation of HO-1-transduced MSC in infarcted rat hearts resulting in improved cardiac function (Zhang *et al.* 2005b). Although there are three isoforms of HO (HO-1, -2 and -3), only the upregulation of inducible HO-1 or heat shock protein 32 produces the most critical cytoprotective mechanisms activated during times of cellular stress. It is through the elimination of haem and the function of its by-products biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}) that cytoprotection is conferred (Katori *et al.* 2002). Of particular interest is the ability of HO-1 to modulate T cell activation and proliferation. This was first suggested by the observation that HO-1-deficient mice have higher numbers of circulating activated CD4^+ T cells when compared with wild-type mice (Poss & Tonegawa 1997a,b). More recently, both upregulation (Pae *et al.* 2004) and overexpression (Braudeau *et al.* 2004; Pae *et al.* 2004) of HO-1 were shown to inhibit CD4^+ T cell proliferation, which could also be mimicked by adding exogenous CO or biliverdin (Braudeau *et al.* 2004; Pae *et al.* 2004). In addition, recent data from Sawle *et al.* (2005, 2006) have demonstrated that anti-inflammatory responses can be suppressed by novel metal carbonyl-based compounds or CO-releasing molecules (CO-RMs). CO released by CO-RMs may therefore have therapeutic potential since several reports have suggested that they may play a role as vasorelaxants in rat aortic and cardiac tissue (Motterlini *et al.* 2002; Johnson *et al.* 2003; Foresti *et al.* 2004) as well as in decreasing myocardial ischaemia/reperfusion damage (Clark *et al.* 2003; Guo *et al.* 2004). Furthermore, in addition to downregulating T cell immune responses directly, expression of HO-1 in other cell types such as immature dendritic cells has been reported to result in indirect inhibition of pro-inflammatory cytokines and allogeneic immune responses (Chauveau *et al.* 2005).

We have preliminary evidence showing MSCs to exhibit high constitutive expression of HO-1 when compared with other cell types such as endothelial cells and valve interstitial cells (figure 3). This novel finding therefore suggests that HO-1 may have a role in MSC immunoregulatory activities.

Another molecule which has recently been of interest is Akt, a serine–threonine kinase involved in diverse cellular responses such as differentiation and survival. Activation of Akt in cardiomyocytes has been shown to protect against apoptosis after ischaemia/reperfusion injury (Matsui *et al.* 2001). Recent data from several groups (Mangi *et al.* 2003; Lim *et al.* 2006) have used this molecule to overcome the poor survival rate of implanted cells, critical for improving the efficiency of stem cell therapy. Mangi *et al.* (2003) demonstrated that transplantation of Akt-transduced MSCs made resistant to apoptosis, enhanced cardiac repair after transplantation. This group used a retroviral vector to overexpress the pro-survival gene *akt* in MSCs before implantation in infarcted rat myocardium. Akt protein overexpression was reported to not only greatly enhance MSC survival but also prevent pathological remodelling after infarction, with striking improvement in cardiac output. Lim *et al.* (2006) also demonstrated similar effects in a porcine model.

Finally, another molecule which could be of interest is relaxin. Functionally, relaxin is classically described to improve blood supply to multiple organs including the uterus, mammary gland, lung and heart (Bani 1997). It is also known to increase matrix metalloproteinase expression, resulting in collagen turnover of reproductive tissues and in models of fibrosis (Bani 1997; Ivell & Einspanier 2002; Bathgate *et al.* 2003). However, it is relaxin's contribution to the regulation of immune homeostasis during pregnancy which suggests it could have a role in future tolerance induction regimes. Evidence comes from relaxin's ability to promote the development of $\text{IFN}\gamma$ -producing Th1 cells to protect the mother against intracellular pathogens as well as inducing the expression of IDO by macrophages at the materno-fetal interface, thereby suppressing T cell activity and preventing the immunological rejection of the foetal allograft (Munn *et al.* 1998; Piccinni *et al.* 2000). In addition, it has also been shown to have a role in protecting the heart from ischaemia/reperfusion-induced myocardial injury, whereby free radical-induced myocardial injury, infiltration of inflammatory cells and myocardial fibrosis were prevented, thus improving cardiac function (Zhang *et al.* 2005a).

Taken together, it seems highly likely that genetic manipulation of MSCs may provide an attractive strategy to overcome rejection of tissue engineered organs; however, the ethics and clinical outcome of using virally transduced cells in humans remains controversial. Perhaps the use of non-viral transfection techniques (Collins *et al.* 2000a,b; Patel *et al.* 2001) which has been successfully used to transfect primary cultures of vascular smooth muscle cells (Li *et al.* 2001) may provide a solution, but alternative strategies involving the use of novel compounds such as CO-RMs, as mentioned earlier, require further investigation. Given that there are many factors that govern MSC immunomodulatory activities both *in vitro* and *in vivo*, it is more than likely that there will not be a single 'magic bullet' solution for MSCs to be completely accepted in an allogeneic recipient, suggesting that a multifaceted approach will probably be required, with the need for greater understanding of the mechanisms involved.

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