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Anti-Donor Antibody Induction Following Intramuscular Injections of Allogeneic Mesenchymal Stromal Cells.

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**Running Title:** Anti-donor Antibody Induction by Allogeneic MSCs.

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**Key words:** mesenchymal stromal cells, immunogenicity, immunosuppression, antibodies, cell therapy, vascular disease.
Allogeneic mesenchymal stromal cells (allo-MSC) are a promising “off-the-shelf” therapy with anti-inflammatory and pro-repair properties. This study investigated humoral immune responses to intramuscular (IM) injections of allo-MSC. Total and isotype-specific anti-donor IgG and donor-specific complement-mediated lysis were determined in sera from healthy mice two weeks after single or repeated IM injections of fully-MHC-mismatched allo-MSC with comparison to mice receiving syngeneic MSC, allogeneic splenocytes or saline. In mice subjected to hind limb ischemia (HLI), anti-donor IgG was analysed following IM allo-MSC injection with and without administration of the T-cell immunosuppressant tacrolimus. Recipients of single and repeated IM allo-MSC developed readily-detectable anti-donor IgG. Serum anti-donor IgG levels were similar to those of allo-splenocyte recipients but had higher IgG1/IgG2a ratio and variable capacity for complement-mediated lysis of donor cells. The induced anti-donor IgG bound readily to allo-MSC and this binding was increased following allo-MSC pre-treatment with interferon gamma. In mouse with HLI, IM injection of allo-MSC into the ischemic limb was also associated with induction of anti-donor IgG but this was abrogated by tacrolimus. The results indicate that allo-MSC are inherently immunogenic when delivered intramuscularly to healthy and ischemic mouse hind limb but induce an IgG1-skewed humoral response that is suppressed by tacrolimus.
INTRODUCTION

Mesenchymal stromal cells (MSC) are known to possess potent immunomodulatory and anti-inflammatory properties, as well as pro-angiogenic and tissue repair activities. Encouraging results from pre-clinical safety and efficacy studies have contributed to an increase in early-phase clinical trials of systemic or localized MSC for a range of vascular diseases. The most common routes for MSC administration are intravenous (IV) or intra-arterial (IA) infusion and direct injection into affected tissue. The latter has the potential advantage of precise localization of cells to the site at which their biological actions are required. For diseases of heart and limbs, therefore, intramuscular (IM) is an important delivery route.

Another critical aspect of MSC therapy is the choice between autologous and allogeneic cell source. For many clinical applications, the possibility of having a ready-to-use “off the shelf” product makes allogeneic MSC (allo-MSC) the preferred option. Furthermore, allo-MSC therapies may allow for selection of optimal donors or for matching of cell potency to the disease indication. It has been cited that allo-MSC are not subject to the types of anti-donor immune responses that typically result in adverse immune reaction to allogeneic organ and tissue transplants in the absence of immunosuppressive therapy. However, it is becoming more evident from pre-clinical experiments, as well as early-phase human studies, that allo-MSC are not inherently immune privileged and may also induce immunological responses against allogeneic antigens.

In addition to T-cell mediated allograft rejection, B-cells and their secreted product (antibodies) also contribute significantly to the failure of allogeneic organ and tissue transplants. High levels of donor-specific IgG antibodies (commonly directed against donor major histocompatibility (MHC)-I and MHC-II proteins) have been associated with complement fixation and/or antibody-dependent cellular cytotoxicity via macrophages and
natural killer cells, a known mediator of acute transplant injury \textsuperscript{20,21}. In addition, donor antigen-specific memory B cells and plasma cells serve as a long-lived source of anti-MHC antibodies as well as producing stronger secondary response upon re-exposure to allo-antigens \textsuperscript{21}. The prolonged persistence of anti-donor antibodies following an initial sensitisation event represents one of the most challenging barriers to successful organ and tissue transplantation \textsuperscript{20}. Although the induction of anti-donor IgG antibodies and the mechanisms underlying antibody-mediated rejection of organs transplants have been extensively studied in recent years \textsuperscript{22}, much less is known about the generation and potential clinical consequences of anti-donor antibodies following allo-MSC administration for specific disease indications \textsuperscript{23}.

In this study, we have investigated the donor-specific humoral response to IM delivered allo-MSCs in healthy mice as well as in mice with hind limb ischemia (HLI). In addition, we have experimentally addressed the potential for a short-course of anti-T-cell immunosuppressive drug therapy to prevent allo-MSC induced anti-donor antibodies.

**RESULTS**

**Single and repeated intra-muscular allo-MSC injections result in an IgG1-skewed anti-donor humoral immune response.** For semi-quantitative analysis of anti-B6 IgG antibodies in sera from BALB/C recipients of IM allo-MSC and other experimental groups, a flow cytometry-based assay was developed using B6 splenocytes as “targets” and graded concentrations of a purified preparation of anti-H-2K\textsuperscript{b} detected with anti-mouse IgG Fc-FITC to generate a standard curve of mean fluorescence values (Figure 1). Because of the varying expression levels of MHC proteins by T-cells and B-cells, the numerical readout for the assay (mean fluorescence intensity (MFI)) was restricted to TCR\textsuperscript{β} cells.
This assay was applied to serum samples collected from 6 groups of mice two weeks after the second of two sequential IM injections of cells and/or saline vehicle (n = 4 per group). These groups (first injection/second injection) were as follows: Group 1 - Saline/Saline. Group 2 - Syn-MSC/Syn-MSC. Group 3 – Allo-splenocytes/Saline. Group 4 – Allo-MSC/Saline. Group 5 – Allo-splenocytes/Allo-splenocytes. Group 6 – Allo-MSC/Allo-MSC. Results for semi-quantitative analysis total anti-donor IgG are shown in Figure 2a. As expected, animals injected with saline alone or with Syn-MSC had no detectable anti-B6 IgG. In contrast, animals injected once or twice with allo-splenocytes or allo-MSC had readily detectable anti-donor IgG. Although there was a trend toward lower levels in those that received two injections of allo-MSC, this difference did not reach statistical significance and the trend was not observed in subsequent experiments. It was concluded that even a single IM injection of allo-MSC was associated with the induction of anti-donor IgG to levels comparable to those induced by IM injection of immunogenic primary cells (allo-splenocytes). It was also noteworthy that neither allo-MSC nor allo-splenocytes resulted in further increases in serum IgG level following a second IM injection.

To determine the relative contributions of individual IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) to the overall amount of anti-B6 IgG present in the sera of the different experimental groups, the flow cytometric assay was repeated using FITC-coupled secondary antibodies with specificities for mouse IgG isotypes. These results were expressed in terms of fold-change over the average fluorescence value for the samples from the Saline/Saline group (Figures 2b-e).

As shown, both single and double injections of allo-splenocytes resulted in comparable increases in detectable anti-B6 IgG1, IgG2a and IgG3. In contrast, recipients of single injections of allo-MSCs developed readily detectable anti-B6 IgG1 and IgG2a with low or absent IgG3. For the recipients of two allo-MSC injections, the trends were similar but with lower values in
of 4 animals. Nevertheless, the allo-splenocyte and allo-MSC recipients appeared to develop distinct IgG isotype patterns, despite similar titres of total anti-donor IgG.

To further clarify, a repeat experiment was carried in which total, IgG1 and IgG2a anti-donor antibodies were analysed in serum samples collected from three groups of mice two weeks after the second of two sequential IM injections (n = 10 per group): Group 1 - Saline/Saline. Group 2 – Allo-splenocytes/Allo-splenocytes. Group 3 – Allo-MSC/Allo-MSC. As shown in Figure 3a, anti-B6 IgG was absent in saline injected mice but was readily detectable in sera from both cell-injected groups with no difference in mean level between recipients of allo-splenocytes and allo-MSC. However, the IgG isotype analysis confirmed higher average IgG1 levels among the allo-MSC recipients with no difference in IgG2a levels. Thus, it was concluded that: (a) Allo-MSC are as potent as primary splenocytes from the same allogeneic strain in inducing anti-donor IgG following single or repeated IM injection in healthy mice. (b) The isotype profile of anti-donor IgG induced by IM allo-MSC is characterized by a relatively higher amount of IgG1 compared to IgG2a and other isotypes.

**Anti-donor IgG induced by IM injection of allo-MSC binds to interferon gamma-regulated allo-antigens on the surface of allo-MSC.** We next adapted the flow cytometry assay to determine whether the anti-donor IgG induced by allo-MSC bound specifically to MSC from the same donor strain. Serum samples from the 3-group experiment described above were used for this analysis. As shown in Figure 4b, IgG in sera from mice that had received two IM injections of allo-splenocytes or allo-MSC bound to donor strain (B6) splenocytes and MSC but not to syngeneic (BALB/c) MSC. Notably, the average binding intensity of allo-MSC induced IgG to allo-MSCs was significantly greater than that of allo-splenocyte-induced IgG suggesting that the humoral immune response was directed against allo-antigens that are highly
expressed by culture-expanded allo-MSC. To further investigate, the assay was repeated using untreated and IFNγ-primed allo-MSC as targets for anti-donor IgG binding. As shown in Figure 4c and d, the binding intensity of allo-MSC-induced but not allo-splenocyte-induced IgG was further increased following IFNγ priming. The results indicated that, in healthy, immunocompetent mice, IM injection of allo-MSC potently induces the production of IgG with specificity for allo-antigens that are expressed on the surface of MSC and are regulated by IFNγ.

**Anti-donor IgG induced by IM injection of allo-MSC has variable capacity to support complement-mediated lysis of donor strain cells.** An *in vitro* assay, developed as described in Materials and Methods, was next used to determine the potential for anti-donor antibodies induced by single and repeated IM injections of allo-splenocytes and allo-MSC to promote complement-mediated lysis of cells from the same mouse strain. Serum dilutions of 1:10 were used for this analysis based on serial dilution experiments (data not shown). Frozen serum samples retained from multiple, similarly designed *in vivo* experiments were thawed and used for this analysis. Donor-specific lysis was calculated as fold change in B6 cell death compared to incubation with stored serum from saline-injected mice (Figure 5). The results indicated >2-fold increase in complement-mediated lysis of B6 cells for 7/9 (78%) serum samples from mice that had received single or repeated IM injections of allo-splenocytes compared to 10/30 (33%) samples from animals that had received single or repeated injections of allo-MSC. Thus, IM injection of allo-MSC has the potential to induce complement fixing anti-donor antibodies but this may occur less frequently than following IM injection of primary immune cells. We also performed multiple experiments in which allogeneic B6-MSC and a B6-derived stromal cell line were incubated with sera from various experimental groups in the complement mediated assay. However, in these experiments we observed a relatively high level of
background lysis with no evidence of a specific increase in lysis following incubation with
experimental sera (data not shown). Thus, we could not conclude that the anti-donor IgG
induced by IM injections of either allo-splenocytes or allo-MSC donor-specific complement-
mediated lysis of MSC in vitro.

Intramuscular injection of allo-MSC into ischemic limbs of mice is associated with induction
of anti-donor IgG which is abrogated by a short course of tacrolimus. To examine anti-donor
antibody induction by IM allo-MSC in a disease-relevant scenario, five groups of 8 BALB/C mice
were subjected to hind limb ischemia by surgical ligation of the femoral artery followed by
injection of saline, syngeneic MSC or allo-MSC into the muscles of the ischemic limb. One
saline-injected and one allo-MSC-injected group also received daily intra-peritoneal injections
of tacrolimus from 2 days before to 14 days after the surgery. The remaining three groups
(saline-, syngeneic MSC- and allo-MSC-injected) received intra-peritoneal injections of vehicle
during the same time-period. Twenty-one days after the procedure, serum samples were
collected and analysed by flow cytometric assay for anti-donor IgG. As shown in Figure 6, 4/8
vehicle-treated allo-MSC-injected mice with hind limb ischemia had detectable anti-donor IgG
(\(P< 0.05\) compared to saline-injected group) while anti-donor IgG was absent in tacrolimus-
treated allo-MSC-injected mice with hind limb ischemia. This result indicated that allo-MSC
retain the capacity to induce anti-donor antibody following injection in ischemic muscle and
that this immune response can be inhibited by a short course of a clinically-relevant T-cell
immunosuppressant.
The range and types of immune response associated with administration of allo-MSC remains a significant area of research interest in the field of cellular therapy. In this study, we report that IM injection of allo-MSC into the hind limb of healthy mice, as well as mice with HLI, is associated with the induction of anti-donor IgG. Of interest, when compared to circulating antibodies induced by allo-splenocytes, the anti-donor IgG response to IM allo-MSC was characterized by relatively higher levels of IgG1 antibodies and more variable capacity for complement-mediated donor cell lysis. Suppression of the response by co-administration of tacrolimus in mice with HLI indicated that generation of anti-donor IgG was dependent on T-cell help.

The development of anti-donor antibody by allo-MSC has been reported in a range of animal models but, in many of these studies, the intensity and functional potential of allo-MSC-induced IgG antibody responses have not been characterized in detail or compared to those induced by other allogeneic cell types. Some in vitro studies of the direct interactions of MSC with activated B-cells have suggested that they mediate context-dependent suppressive effects. However, our results for IM-delivered allo-MSCs, along with previously reported observations in other animal models, indicate that such suppressive interactions either do not occur in the in vivo setting or are insufficient to prevent allo-antigen-specific B-cell activation. Importantly, the clinical implications of anti-donor IgG antibodies are influenced not only by the antibody titre but also by the heavy chain isotype and by the capacity for complement fixation. In our study, despite comparable serum levels, the anti-donor IgG response to IM injections of allo-MSCs in mice was characterised by a lack of IgG3 and by a relative predominance of IgG1 antibodies - which has been reported to be associated with an
underlying Th2-type cellular immune response\textsuperscript{31}. Skewing of the humoral response toward IgG1 isotype may also indicate suppressive effects of allo-MSCs on Th1- and Th17-type anti-donor T-cell responses, the influence of induced regulatory T-cell populations, promotion of anti-inflammatory monocytes or myeloid-derived suppressor cells or direct effects modulatory upon activated B-cells\textsuperscript{27,32-34}. Our initial results comparing anti-donor IgG response following single and double injections of allo-MSC suggested a trend toward lower total anti-donor IgG titres following the second injection (Figure 2a) – raising the interesting possibility of a tolerizing effect of multiple IM injections on B cell responses. However, in the subsequent experiments, the serum total IgG levels following double-injection of B6-MSCs were quite comparable to those detected following double-injection of B6-splenocytes and even trended toward being higher (Figure 3). These diverse trends highlight the fact that inter-individual variability in total IgG titers and IgG isotype fold-changes was a notable feature of all of the \textit{in vivo} experiments we performed. We attribute this to the inherent complexity and variability in the \textit{in vivo} generation of IgG immune responses (akin to variable vaccination responses among human recipients). However, we cannot rule out the possibility that the second dose of allo-MSC bound sufficient anti-donor IgG to modulate the circulating level. Overall, we believe that the weight of experimental evidence from our study suggests that multiple doses of allo-splenocytes and allo-MSC induce comparable ranges of anti-donor IgG titers but with differentially skewed IgG isotype repertoires reflecting modulation of anti-donor humoral immune response by the administered MSC either through direct or indirect mechanisms. Confirmation of whether these observations in mice also apply to humans will require further immune monitoring studies of subjects taking part in clinical trials of recipients of IM-administered allo-MSC for critical limb ischemia (CLI) or other diseases\textsuperscript{7,8}. 
Recently, there has been a growing interest in understanding the consequences of antibody binding to MSC in the *in vivo* setting and in the potential clinical significance of MSC interactions with antibodies and other blood components. While it has been reported that MSC express factors that render them resistant to complement-mediated lysis, others have observed them to be susceptible to complement *in vitro*. It has also been reported by Moll *et al.*, that thawed, cryopreserved human MSC are more susceptible to complement-mediated lysis than their freshly cultured counterparts – a possibility that we did not address in the current study. Furthermore, in a limited number of animal model studies involving single or multiple allo-MSC injections, the capacity of the induced anti-donor antibodies to mediate donor cell lysis has been demonstrated. Our results confirm such findings. Less clear, however, is whether allo-MSC-induced anti-donor antibody and complement-mediated lysis may be responsible for “rejection” of subsequent doses of allo-MSC. Of interest, *in vivo* antibody-mediated complement fixation on the surface of allo-MSC may also indirectly mediate MSC damage and elimination by other elements of the immune system. For example, Lin *et al.* have reported that release of C5a following complement fixation by MSC results in activation of neutrophils and cell damage via the resulting oxidative burst. Furthermore, the complement cleavage product iC3b may serve as an activating ligand to enhance NK cell-mediated lysis of MSC *in vivo*. The results we report here demonstrate that anti-donor IgG antibodies induced by single or repeated injections of allo-MSC – in this case by the IM route – bind specifically to MSC and are directed against IFN-γ-regulated cell surface allo-antigens. Although pre-activation ("licensing") of MSC with IFN-γ has been shown in *in vitro* and *in vivo* studies to be associated with enhanced immunomodulatory properties, IFN-γ is also known to induce expression of
surface proteins such as MHC I, MHC II, B7-H1 and ICAM-1 that could serve as allo-antigens and/or enhance immunogenicity \(^{46-49}\). Although we did not formally identify the IFN-\(\gamma\)-inducible proteins that served as targets for anti-donor IgG in the serum of BALB/C mice following IM injection of B6 MSC, it is likely that these predominantly represent strain-specific MHC I and MHC II. Whether binding of pre-existing or induced anti-MHC IgG to implanted allo-MSC is sufficient to cause loss of therapeutic efficacy or increased inflammatory response \textit{in vivo} remains unclear. Of interest, however, Huang \textit{et al.} observed that up-regulation of MHC proteins on allo-MSC undergoing spontaneous differentiation toward myogenic, endothelial, or smooth muscle lineages following intra-cardiac injection was associated with anti-donor T-cell and IgG responses and with late loss of therapeutic efficacy in a rat model of post-infarct cardiac remodelling \(^{43}\). Also of interest, Owens \textit{et al.} recently demonstrated that sera from horses that received multiple injections of allo-MSC via intravenous, intraarticular and intra-lesional routes contained anti-donor antibody that bound at high levels to donor MSC \(^{18}\). Once bound to complement-susceptible donor cells (splenocytes), however, the capacity for the allo-MSC induced IgG to trigger complement-mediated lysis in our study was variable and less consistent that that of anti-donor IgG induced by allo-splenocytes. These observations are in keeping with the higher levels of anti-donor IgG1 and absence of IgG3 induced by allo-MSC as well with a high potential for inter-individual variability in the \textit{in vivo} B-cell responses to allo-MSC administration – a finding that is consistent with the limited amount of data currently available from human clinical trials \(^{9-11,50,51}\).

The donor-specific immune response to allo-MSC has been largely studied in healthy animals \(^{16,17,44}\). However, understanding the influence of specific disease states on the immunogenicity of allo-MSC is important for effective clinical translation. In this study, we observed anti-donor IgG production in 50% of a group of mice with HLI following a single IM
injection of allo-MSC. Results of immune monitoring from early-phase human trials using allo-
MSC injection for limb ischemia and heart disease have provided variable evidence of
induction of donor-specific responses \(^7\)\(^-\)\(^11\). In studies of direct intra-myocardial injection of
allo-MSC, some subjects were found to have pre-sensitization to HLA while a minority
developed low-level donor-specific antibody responses with no reported cases of acute
immunologic rejection \(^9\)\(^-\)\(^11\). In the case of IM injection of allo-MSC into ischemic limbs, Gupta
et al. did not find evidence of adverse response based on serum concentrations of IFN-\(\gamma\), IL-1
and TNF-\(\alpha\) and on the CD4, CD8 and CD25 profile of circulating lymphocytes \(^7\). However, the
authors did not determine whether there was induction of anti-donor antibody among their
study subjects. Thus, it will be important for future clinical trials of IM-injected allo-MSC to
incorporate an immune monitoring strategy that includes sensitive assays for donor-specific
 cellular and humoral immune responses in addition to non-specific monitoring of innate
immune activity.

We also investigated the effectiveness of a plausible strategy for controlling the production of
anti-donor antibodies in the setting of limb ischemia by co-administering allo-MSC with a short
course of the FDA-approved and frequently used immuno-suppressive drug tacrolimus in the
mouse HLI model. Interestingly, this resulted in a diminished anti-donor IgG antibody
response to allo-MSC, suggesting that the activation of recipient B-cells to MSC-delivered allo-
antigens is preceded by anti-donor CD4\(^+\) T-cell activation. This implies robust delivery of allo-
MSC-derived protein/peptide antigens into the recipient antigen presentation system – most
likely via dendritic cell uptake through indirect (uptake of material from dead cells) or semi-
direct (uptake of shed vesicles) pathways – as well as contact of intact allo-antigens with naïve
B-cells \(^20\). Our demonstration that this process can be overcome by co-administration of
tacrolimus is consistent with a limited number of studies in other models \(^52\),\(^53\). These
observations are of particular relevance to the use of allo-MSC therapies in patients with potential future need for an organ transplant, such as those with chronic heart, lung, liver or kidney disease, in whom high level sensitization against HLA proteins can represent a significant barrier to successful transplantation.

Translating the results of rodent model-based research findings such as those reported here, into insights about the immunogenicity and therapeutic efficacy of allo-MSC in human subjects, is not straightforward. In particular, differences in the properties of mouse and human MSC, in the nature of the allo-immune response that occur in the two species and in the technical and logistical details of animal model experiments compared to human clinical trials/therapeutic protocols must be taken into consideration. As a general rule, pre-clinical studies should resemble the intended clinical situation as closely as possible and, in this regard, regulatory agencies recommend that the ultimate cell product should be tested in pre-clinical studies. Frequently, however, researchers must decide whether to use the ultimate human cell product in a xenogenic (e.g. human to mouse) setting or to use an “equivalent” animal cell product to allow for more accurate assessment of pre-clinical efficacy and safety in an autologous or allogeneic treatment setting. In studies such as ours, that aim to evaluate the immune responses to allogeneic cell therapies, allogeneic mouse cells and immunocompetent animals must be used as xenogeneic cells induce immune responses that are of no relevance to the clinical setting. Nonetheless, an important caveat to our results is the possibility that the immune responses that occur in mice, the phenotype of the mouse allo-MSC administered and the technical details of the experimental protocol cannot fully replicate a clinical cell therapy scenario in human subjects. Although mouse and human bone marrow-derived MSC share the expression of a number of commonly-used stromal cell markers (e.g. CD90, CD105, CD73, CD44, MHC I), their typical surface levels of some of these
markers may differ and they are also known to express species-specific proteins such as Sca-1 in mouse. Furthermore, typical methods for the in vitro isolation and expansion of murine MSCs differ from those of their human counterpart. For example, cultures of bone marrow-derived mouse MSC tend remain contaminated with haematopoietic (CD45+) cells up to 7-9 passages whereas human bone marrow-derived MSC are usually sufficiently pure to be clinically administered following 2-4 passages. Of interest, we have observed that healthy, immunocompetent BALB/C mice also generate readily detectable anti-human IgG following IM administration of human bone marrow derived MSC (Alagesan S, Sanz-Nogués C and Griffin MD unpublished data). Furthermore, MSC from both species respond to IFN-γ by up-regulating both MHC I and MHC II.

Pre-clinical and clinical studies of MSC also tend to differ widely in regard to weight-adjusted dose of the administered cell product. In our study, the average weight per mouse was 0.024 kg and the dose administered was $1 \times 10^6$ cells giving an average dose per unit weight of $41.7 \times 10^6$ cells kg$^{-1}$. Three clinical trials have been reported in which autologous or allogeneic bone marrow-derived MSC were administered intramuscularly to patients with critical limb ischemia (CLI). Lu et al. reported results of a Phase I study that compared the administration of MSC with that of bone marrow mononuclear cells for the treatment of CLI and foot ulcers. The average weight of each patient was not provided but the authors stated that an average cell number of $9.3 \times 10^8$ MSCs was administered to CLI patients suggesting a dose per unit weight of $13-16 \times 10^6$ cells kg$^{-1}$. In contrast, in phase I/II and phase II trials of allo MSC, Gupta et al. administered doses $1 \times 10^6$ or $2 \times 10^6$ cells kg$^{-1}$. Thus, the induction of anti-donor IgG observed in mouse recipients of allo-MSC in our study represents a response to cell doses several-fold in excess of those that have been used to date in early phase clinical trials.
Overall, while it is essential to emphasize the significant differences in the typical resting
surface phenotype, passage number at administration and weight-adjusted cell dose involved
in mouse experiments and human clinical trials, the expression of MHC I and cytokine-
inducible upregulation of both MHC I and II that is common to cells from both species suggests
that the results we report here in the mouse have relevance to the potential \textit{in vivo}
immunogenicity of allo-MSC in human subjects.

\textbf{SUMMARY AND CONCLUSIONS}

The results we report here add to the weight of evidence, from both animal and human
studies, that allo-MSC administered by various routes have the capacity to induce anti-donor
IgG antibodies that readily bind to allo-antigens on the MSC surface. Prevention of this
response by co-administration of tacrolimus indicates that this facet of their immunogenicity
represents a T-cell dependent B-cell response. For the IM route of administration, which is of
particular relevance to MSC therapy for lower limb ischemia and other diseases of the
cardiovascular system, we show that allo-MSC-induced anti-donor IgG has a characteristic
isotype profile compared to other immunogenic cell types. This isotype profile may be less
potent for complement fixation and likely represents the outcome of a complex, short-lived
\textit{in vivo} interaction between administered MSC and localised cells of the innate and adaptive
immune system \textsuperscript{34}. Along with the existing literature discussed above, our findings help to
better understand the nature and mechanisms of allo-MSC immunogenicity as well as its
potential clinical relevance. Further studies of multiple-dosing of allo-MSCs with co-
administration of immuno-suppressive drugs will clarify whether lasting therapeutic benefits
can be enhanced by preventing the induction of anti-donor antibodies. The alternative
possibility, that anti-donor antibody response indeed do not influence the efficacy of the cell product, or those allo-antigen-driven interactions between MSC and immune effectors are necessary for full therapeutic effect in some disease settings, must also be considered.

METHODS

Animals. Male 8-12 weeks-old BALB/c (recipient) and C57BL/6 mice (donor) were purchased from Charles River Laboratories, United Kingdom and were housed in a licensed bio-resource with monitoring and support from qualified animal technicians and a veterinary surgeon. All animal experiments were carried out with ethical approval from the Animal Care Research Ethics Committee (ACREC) of the National University of Ireland, Galway and under appropriate individual and project authorisations from Health Products Regulatory Authority (HPRA) of Ireland.

Mouse bone marrow MSC isolation and culture. Donor-strain MSC were obtained from bone marrow (BM) of C57BL/6 and recipient autologous MSC from BALB/c. Briefly, femurs and tibiae from 6-8-week-old mice were removed and excess tissue cut away. Marrow was flushed from the bones using a 27-gauge needle filled with complete isolation medium (CIM, RPMI1640, 9% equine serum, 9% fetal bovine serum (FBS), 1% penicillin/streptomycin and 2mM L-glutamine). After two passages (p), the cells were transferred to T25 or T75 tissue culture flasks at $1 \times 10^6$ per flask in complete expansion medium (CEM, IMDM, 9% equine serum, 9% FBS, 1% penicillin/streptomycin and 2mM L-glutamine.) for further expansion. At passage 5, the cells were cryopreserved in freezing medium and then were subsequently thawed and expanded for 1-4 further passages in culture for use in experiments.
experiments were carried out with p6-p9 MSCs grown to 80% confluence in supplemented Iscove’s modified Dulbecco’s medium (IMDM). The surface phenotypes and tri-lineage differentiation characteristics of the MSCs were confirmed by flow cytometry and in vitro adipogenic, osteogenic and chondrogenic assays as previously described.

**Intramuscular injection of MSC in healthy mice.** Recipient (BALB/c) mice were anaesthetized with intra-peritoneal ketamine (75-100 mg kg\(^{-1}\)) and xylazine (10mg kg\(^{-1}\)) following which intramuscular injections were carried out at five individual sites [40 \(\mu\)L \((2\times10^5\) cells in sterile saline) each] in the left thigh muscle. Cells or sterile saline injections were given at two time-points two weeks apart. After an additional week (21 days following the first injection), the mice were humanely euthanized and blood was collected by cardiac puncture for serum preparation.

**Flow cytometric assay for detection of anti-donor IgG and IgG Isotypes.** Blood samples collected at the time of euthanasia were allowed to clot at room temperature for 4 hours then separated by centrifugation for 10 minutes at 500 x g following which aliquots of serum were frozen at -80°C. For flow cytometric detection of anti-donor (B6) IgG antibody in serum, freshly prepared, RBC-free B6 splenocytes were suspended at 8 \(\times\) 10^6 cells mL\(^{-1}\) in FACS buffer (1x PBS, 2% FCS, 0.05% NaN\(_3\)) and were incubated for 30 minutes at 4°C with either FACS buffer alone (negative control), serial dilutions of purified anti-H-2K\(^b\) (clone AF6-88.5, BD Biosciences, Franklin Lakes, NJ) or serum samples diluted 1:100 in FACS buffer. Next, splenocytes were washed twice in FACS buffer then incubated for 30 min at 4°C with goat anti-mouse IgG Fc F(ab)_2-FITC (Beckman Coulter, Brea, CA) at an optimised dilution of 1:400 in addition to anti-mouse T-cell Receptor (TCR)-\(\beta\)- PE (BD Biosciences) for total IgG or incubated with anti-mouse
IgG1-FITC, anti-mouse IgG2a-FITC, anti-mouse IgG2b-FITC or anti-mouse IgG3-APC (BD Pharmingen, UK) for IgG isotyping. In some experiments, freshly-cultured B6 MSC (untreated) or B6 MSC that had been cultured for 24 hours in the presence of 100ng mL⁻¹ of IFNγ for 24 hours (IFNγ-primed) were incubated for 30 min at 4°C with either FACS buffer alone (negative control), serial dilutions of purified anti-H-2Kb or serum samples diluted 1:100 in FACS buffer. The MSC were washed twice in FACS buffer then incubated for 30 min at 4°C with 1:400 goat anti-mouse IgG Fc F(ab)²-FITC. Stained splenocytes and MSC were washed twice with FACS buffer, re-suspended in 0.5 mL of FACS buffer and analysed immediately on a Becton Dickinson FACSCanto A™ flow cytometer. Analysis was carried out using FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR) with fluorescence intensity of TCRβ-negative cells (B-cells) compared between negative control sample and individual serum-incubated samples.

**Complement-mediated lysis assay.** Red blood cell-free B6 splenocytes were prepared as described above for use as “target cells”. The splenocytes were re-suspended at 5 × 10⁵ mL⁻¹ in 1X PBS, 2% FCS, 0.05% NaN₃ and were incubated either with mouse sera diluted 1:10 in PBS or with PBS alone (negative control). Next, cells were washed to remove unbound antibodies and incubated in 20% baby rabbit complement (AbD Serotec, UK) in PBS at a final volume of 100 µl for 1 hour at 27°C following which 2 mL of ice cold PBS were added. The cells were pelleted by centrifugation, re-suspended in 50 µL of PBS, stained with 0.5 µg propidium iodide/sample and analysed immediately on a Becton Dickinson FACSCanto™ flow cytometer. Analysis was carried out using FlowJo® v7.0 software (TreeStar® Inc, Ashland, OR).

**Hind limb ischemia with MSC and tacrolimus administration.** Hind limb ischemia was induced in eight-week-old male BALB/c mice. Animals were anesthetized with 75-100 mg kg⁻¹ ketamine.
and 10 mg kg\(^{-1}\) xylazine injected intra-peritoneally. The femoral triangle was exposed through an incision in the inguinal region and the femoral artery was separated from the femoral vein and nerve by blunt dissection distal to the inguinal ligament. The femoral artery was occluded by placement of sutures at the proximal and distal regions above the proximal caudal femoral artery branch and a cut was made between the ligation sites. Finally, the incision was closed with interrupted sutures followed by IM injection of cell suspensions or saline alone in the thigh muscle as described above. The mice received analgesia (0.05-0.1 mg kg\(^{-1}\) of buprenorphine) 30 min prior to surgery and every 12 hours for the next 3 days, and thereafter as required. Prophylactic antibiotic (0.1mg kg\(^{-1}\) of Enrofloxacin/Baytril) was also given once post-operatively. For administration of tacrolimus or vehicle, daily intra-peritoneal injections were performed from pre-operative day 2 until post-operative day 14. Tacrolimus was administered at a dose of 1 mg kg\(^{-1}\) day\(^{-1}\) in accordance with published studies\(^{25}\). A working solution of tacrolimus (0.2 mg mL\(^{-1}\)) was prepared each day by mixing stock solution (40 mg mL\(^{-1}\)) with a vehicle solution containing 10% ethanol and 1% Tween80 in sterile physiological saline\(^{26}\). Control-treated mice received injections of equal volumes of vehicle alone. At 21 days after surgery, animals were humanely euthanized, and blood was collected for preparation of serum samples.

**Statistical analysis.** Results were expressed throughout as mean ± standard deviations (SD) and differences among multiple groups were tested statistically by ANOVA with post-hoc testing. All statistical analyses were performed with GraphPad Prism\(^{\circledR}\) 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was assigned at \( P < 0.05 \).
CONFLICT OF INTEREST STATEMENT.

Prof. Timothy O’ Brien is a founder, director, and equity holder in Orbsen Therapeutics Ltd., Ireland. The other authors have no conflicts of interest to declare.
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**Figure 1.** Semi-quantitative flow cytometry assay for anti-donor IgG. (a) Representative dot plots illustrating the gating strategy used for analysis of fluorescence shift among TCR-β B6 mouse splenocytes stained with anti-TCRβ. (b) Representative histograms illustrating the fluorescence shifts on the FITC channel of B6 TCR-β cells incubated with no primary antibody (shaded histogram) or with concentrations of anti-H-2Kb between 0.02 to 14 ng mL⁻¹ followed by anti-mouse IgG-FITC. (c) Graph of the mean fluorescence intensities (MFI) versus anti-H-2Kb concentrations for the histogram shown in b. Examples of the MFIs of target cells incubated with 1:64 diluted sera from a BALB/c recipient of saline (black cross) or from a BALB/c recipient of B6 allo-splenocytes (red cross) are overlaid on the plot. The data shown in b and c are representative of results from 6 independent optimization experiments.
Figure 2. Induction of anti-donor IgG following single and double allo-MSC injections with IgG isotyping – compared with Syn-MSCs and allo-splenocytes. (a) Graph of anti-B6 IgG titres in sera from 6 experimental groups (n = 4 each). Titres were calculated based on a standard curve for monoclonal anti-H-2Kb and expressed as "ng mL\(^{-1}\) equivalent". Filled and open symbols represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. Results are shown for semi-quantitative analysis of anti-B6 IgG isotype titres in 5 groups of mice: IgG1 (b), IgG2a (c), IgG2b (d), IgG3 (e). Titres were calculated as fold change over the average fluorescence intensity of the saline control group. Data-points represent the results for individual animals in each group. Statistical analyses were performed by One-Way ANOVA, **P<0.01 and ***P<0.001 compared to saline control group †P < 0.05, ††P < 0.01, ††† P<0.001 compared to the Syn-MSC group.
Figure 3

Differential IgG isotype patterns in allo-MSC and allo-splenocyte double-injected mice. (a) Results are shown for semi-quantitative flow cytometric analysis of anti-B6 total serum IgG in BALB/C recipients of two sequential IM injections of saline, allo-splenocytes and allo-MSCs (n = 10 each). (b and c) Results are shown for semi-quantitative analysis of anti-B6 IgG1 and IgG2a in two groups of cell-injected mice expressed as fold change over the average fluorescence intensity of the saline control group. Data-points represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. Statistical analysis was performed by One-Way ANOVA, ***P < 0.001 compared to the Saline group ††P < 0.01 compared to the allo-splenocyte group.
Figure 4. Binding of induced IgG to allo-MSC with and without IFN-γ priming. (a) Overlay histograms illustrating the standard curve generated by incubation of serial dilutions of purified anti-B6 Class I (H-2Kb) antibody with B6 splenocytes followed by anti-IgG-FITC. (b) Graph of equivalent concentrations of anti-B6 total IgG in sera from three groups of mice (n = 10 each) against three different target cell populations: allo-(B6)-splenocytes, allo (B6)-MSC and syngeneic (BALB/C)-MSC. Levels of IgG were calculated based on the standard curve and expressed as “ng mL⁻¹ equivalent”. (c) Representative histogram overlays of the anti-IgG staining levels on untreated (left) and IFNγ-pre-treated (right) B6 MSC following incubation without serum (no serum control) or with sera from mice that received double injection of saline, allo-splenocytes or allo-MSC as indicated in the legend. (d) Graph of IgG titres in sera from three groups of mice (n = 10 each) against untreated or IFNγ-pre-treated B6 MSC. Levels of IgG were calculated based on the standard curve and expressed as “ng mL⁻¹ equivalent”. Data represent the group means ± SD. Statistical analysis was performed by One-Way ANOVA, ***P < 0.001 compared to the appropriate results for the saline and allo-splenocyte group, ††P < 0.01 compared to the appropriate result for untreated MSCs.
Figure 5. Variable complement-mediated lysis of donor-specific targets by allo-MSC-induced anti-donor IgG. Results of complement-mediated lysis assays performed with 1:10 dilutions of stored sera from groups of mice that had received 1 of 5 different IM cell injection regimens. Results are expressed as the fold-increase in % lysis of target B6 splenocytes over the mean result for sera from saline-injected animals. Data-points represent the results for individual animals in each group. Horizontal bars and error bars represent the group means ± SD. Statistical analysis was performed by One-Way ANOVA, ***P < 0.001 compared to the syngeneic MSC group.
Figure 6. Effect of tacrolimus administration on anti-donor antibody response induced by IM injection of allo-MSC at the time of hind limb ischemia induction. Results of semi-quantitative flow cytometry assays for anti-B6 IgG in 1:100 diluted sera from five groups of BALB/C mice (n = 8 each) that had undergone hind limb ischemia 21 days previously with single IM injections on the day of surgery. Antibody levels were calculated based on a standard curve generated with serial dilutions of purified anti-H-2Kb and were expressed as “ng mL⁻¹ equivalent”. Data represent the group means ± SD. Statistical analysis was performed by One-Way ANOVA, *P < 0.05 compared to Saline group; †P < 0.05 compared to allo-MSC group. Abbreviations: Tac = tacrolimus.