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ARTICLE

Evaluation of gene delivery strategies to efficiently overexpress functional HLA-G on human bone marrow stromal cells

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Mesenchymal stromal cells (MSC) constitutively express low levels of human leukocyte antigen-G (HLA-G), which has been shown to contribute to their immunomodulatory and anti-inflammatory properties. Here, we hypothesized that overexpression of HLA-G on bone marrow-derived MSC would improve their immunomodulatory function, thus increasing their therapeutic potential. Therefore, we investigated which gene transfer system is best suited for delivering this molecule while maintaining its immunomodulatory effects. We performed a side-by-side comparison between three nonviral plasmid-based platforms (pmax-HLA-G1; MC-HLA-G1; pEP-HLA-G1) and a viral system (Lv-HLA-G1) using gene transfer parameters that yielded similar levels of HLA-G1-expressing MSC. Natural killer (NK) cell–mediated lysis assays and T cell proliferation assays showed that MSC modified with the HLA-G1 expressing viral vector had significantly lower susceptibility to NK-lysis and significantly reduced T cell proliferation when compared to nonmodified cells or MSC modified with plasmid. We also show that, in plasmid-modified MSC, an increase in Toll-like receptor (TLR)9 expression is the mechanism responsible for the abrogation of HLA-G1's immunomodulatory effect. Although MSC can be efficiently modified to overexpress HLA-G1 using viral and nonviral strategies, only viral-based delivery of HLA-G1 is suitable for improvement of MSC's immunomodulatory properties.

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INTRODUCTION

Human mesenchymal stromal cells (MSC) have been recognized for their trophic, anti-inflammatory, and immunomodulatory properties, and have been used in the treatment of a wide range of diseases, including those that involve degenerative or aberrant immune/inflammatory responses.[1] MSC preferentially home to sites of injury and/or inflammation, whereupon they promote tissue repair through mechanisms that involve both secretion of bioactive molecules and cell-to-cell interactions, which regulate and/or modulate local innate and adaptive immune responses, and promote tissue-specific cell proliferation and repair.[2,3] Although MSC's therapeutic benefit has been reported in numerous studies,[4,5] some of the larger-scale clinical trials to-date either produced conflicting results or shown only modest benefits.[6] Failure to achieve a therapeutic effect is likely due in large part to inadequate engraftment, poor tissue survival, or insufficient trophic and/or immunomodulatory effects of the transplanted MSC. Therefore, strategies that will enable MSC therapies to consistently achieve robust and reliable efficacy are urgently needed.

Human leukocyte antigen-G (HLA-G), a nonclassical HLA class I molecule (HLA-1b), known for its tolerogenic and powerful immune inhibitory function,[7] exists in seven different isoforms, of which the full-length transmembrane HLA-G1, and its soluble counterpart HLA-G5, are the most extensively studied.[8,9] Both HLA-G1 and HLA-G5 are potent suppressors of allogeneic T-cell response through induction of $CD8^+$ T-cell apoptosis and arrest of T- and B-cell proliferation, inhibitors of natural killer (NK) cell cytotoxicity, inducers of regulatory T cells, and are known to modify maturation of antigen-presenting cells.[9–11] In addition, it has also been reported that higher levels of HLA-G expression are associated with a reduction of acute and chronic transplant rejection, while low levels of this molecule have a positive correlation with graft versus host disease incidence. Additionally, a genetic association between recipients homozygous for an HLA-G 14 bp polymorphism (an insertion of 14 bp that decreases the stability of HLA-G mRNA, leading to lower protein synthesis) and graft versus host disease incidence and relapse has also been described.[12] Furthermore, low HLA-G levels have a positive correlation with the incidence of inflammatory processes that often are responsible for the etiology of autoimmune diseases. This was reported in patients with multiple sclerosis, rheumatoid arthritis, and Crohn's disease, among others, and suggests that the restoration of HLA-G expression can induce the re-establishment of a favorable tolerogenic environment in the affected tissues through protection against NK and T-cell lysis and prevalence of inflammatory processes that are often responsible for autoimmune disease etiology.[9,12,13]

Since MSC have been shown to constitutively express HLA-G at low levels,[10,13] and this molecule is known to be involved in MSC-mediated immunomodulatory function, we hypothesized that

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genetically engineering MSC to overexpress HLA-G1 (MSC-HLA-G1) could be used as an approach to improve upon MSC's immunomodulatory properties and thereby enhance the efficacy of existing MSC-based therapies. In addition, since MSC, despite their immunomodulatory properties, can still be a target of activated NK[14–16] and cytotoxic T cells,[15] it is possible that overexpression of HLA-G1 could lead to increased survival of MSC after infusion.[17] Therefore, here we investigated which gene transfer system is best suited for delivering this molecule while maintaining its immunomodulatory effect by performing a side-by-side comparison between a lentiviral vector (Lv-HLA-G1), a murine retroviral vector (Rv-HLA-G1), and three nonviral plasmid constructs including a conventional plasmid

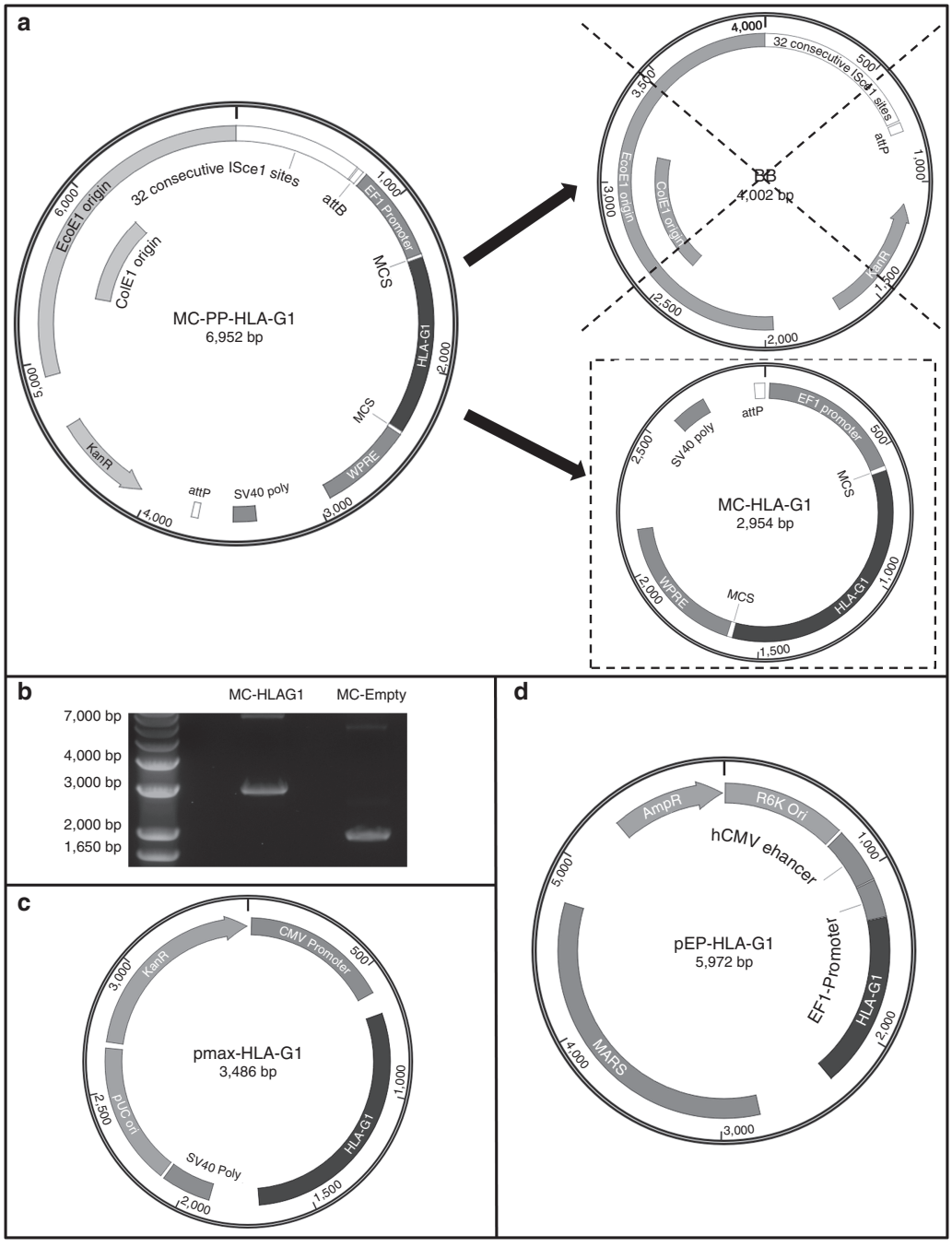

Figure 1 Schematic representation of minicircle (MC) production and map of pDNA constructs used for nonviral gene delivery. The MC-PP-HLA-G1 contains the ColE1 and EcoE1 origin of replication sequences required for bacterial propagation, the kanamycin-resistance gene, the φC31 integrase recognition sites attB and attP, and a block of 32 tandem repeats of the recognition sequence for the I-*Sce*I homing endonuclease. Following MC-PP-HLA-G1 propagation, arabinose-mediated induction of φC31 integrase activity generates a minicircle containing the HLA-G1 expression cassette (MC-HLA-G1) and a circular plasmid backbone (BB); the latter is subsequently degraded by host exonucleases (also induced by arabinose) (**a**). Agarose gel electrophoresis analysis of linearized MC-HLA-G1 and MC-Empty with no visible PP contaminants (**b**). pmax-HLA-G1 is a conventional plasmid that harbors the pUC origin of replication, a kanamycin-resistance gene, a hCMV promoter driving the HLA-G1 gene, and an SV40 poly-adenylation sequence (**c**). The episomal plasmid pEP-HLA-G1 includes the R6K origin of replication, an ampicillin-resistance gene, a S/MAR sequence, a hCMV enhancer, an EF1 promoter driving the HLA-G1 gene, and an IRES module (**d**).

(pmax-HLA-G1), a minicircle (MC-HLA-G1), and an episomal plasmid (pEP-HLAG1). The results obtained indicate that MSC can be efficiently modified to overexpress HLA-G1 using both viral and nonviral strategies; however, only lentiviral transduction of the HLA-G1 transgene resulted in an enhancement of MSC's immunomodulatory properties.

RESULTS

Generation of HLA-G1-engineered MSC, and efficiency of nucleofection-mediated HLA-G1 gene transfer

In order to establish a nonviral platform to successfully and efficiently overexpress HLA-G1 in MSC, Nucleofector technology was used as a delivery system, and three different DNA constructs were explored: (i) a conventional plasmid (pmax-HLA-G1); (ii) a minicircle (MC-HLA-G1; plasmid vectors that lack bacterial elements); and (iii) an episomal plasmid (pEP-HLA-G1; replicates autonomously within the cell nucleus) (Figure 1). Nucleofection-mediated transfection was performed as described in detail in Materials and Methods. Nontransfected cells and cells subjected to the nucleofection procedure without addition of exogenous DNA served as negative controls. HLA-G1 expression was assessed by flow cytometry at 24 hours, 48 hours, 4 days, and 9 days after transfection, and results demonstrated that, with all delivery platforms, the maximum levels of gene expression were obtained at day 2 ($n = 4$). While MC-HLA-G1 and pmax-HLA-G1 induced 56 ± 2.7% and 53 ± 2.9% of the transfected cells, respectively, to express HLA-G1, by contrast, only 13 ± 0.9% of the cells transfected with pEP-HLA-G1 overexpressed HLA-G1 (Figure 2). Duration and level of transgene expression profile was also determined 12 days post-nucleofection, at which time HLA-G expression mediated by pEP-HLA-G1 was lost, while MC-HLA-G1 and pmax, still drove levels of HLA-G1 expression of 31 ± 4.6% and 31 ± 5.5%, respectively ($n = 4$). Importantly, for all DNA constructs, cell viability was always found to be greater than 85%.

To further evaluate gene modification efficacy, both transfected cell recovery and yield of transfection were also calculated at 48 hours post-nucleofection (Figure 3). Transfected cell recovery corresponds to the ratio of the number of live nucleofection-modified cells to the number of cells before transfection, while the yield of transfection is given by the percentage of viable HLA-G1-positive cells relative to nontransfected cells. Together, these two parameters evaluate the number of MSC that maintained viability and successfully overexpressed the transgene, and therefore provide a more accurate assessment of the transfection performance. MSC modified with MC-HLA-G1 displayed values of cell recovery (53 ± 4.3%) that were significantly higher than MSC transfected with pEP-HLA-G1 (31 ± 2.9%; $P < 0.05$) ($n = 4$). Additionally, no significant differences in cell recovery were seen between MC-HLA-G1-modified cells (53 ± 4.3%) and control cells that were nucleofected without DNA (58 ± 5.2%) (Figure 3a). However, we found that the transfection rate (Figure 3b) obtained for MC-HLA-G1 (46 ± 3.6%) was significantly higher than that attained with pmax-HLA-G1 (37 ± 1.8%; $P < 0.05$) or pEP-HLA-G1 (6.3 ± 0.8%; $P < 0.05$). Since the MC-HLA-G1 construct provided the best overall rate of transfection, MSC were nucleofected with an MC-empty vector (no HLA-G1) to exclude any possible effect of the MC backbone on endogenous HLA-G1 expression. Importantly, while the percentages of cell viability and cell recovery were similar to that observed for MC-HLA-G1, no HLA-G1 upregulation was found in MSC nucleofected with the empty MC vector (data not shown).

Among viral vectors, retroviruses and lentiviruses (a subfamily of retrovirus) are the most commonly used for long-term transgene expression, as they are able to efficiently and stably integrate into the host genome.[18] However, while murine-retroviruses were the first class of viral vectors to be developed, and one of the most widely applied gene transfer carriers in the clinic, lentiviruses have the important advantage of modifying both nondividing and dividing cells.[6,19] Therefore, stably transduced MSC expressing HLA-G1 were generated using lentivirus (Lv-HLA-G1). In order to perform a side-by-side comparison between nonviral and viral systems, MSC were transduced at a multiplicity of infection (MOI) known to modify ~50–60% of MSC, and thus generate MSC with HLA-G expression levels similar to day 2 nonvirally transfected cells (Figure 4).

MSC modified with a lentiviral vector expressing HLA-G, but not with the nonviral plasmids, have significantly lower susceptibility to NK-lysis when compared to unmodified cells

Since HLA-G has been shown to inhibit NK cytotoxicity,[8,9] we investigated whether MSC modified to overexpress HLA-G1 (MSC-HLA-G1) would have an increased resistance to NK-mediated lysis in comparison with nonmodified MSC (MSC) ($n = 4$). MSC nucleofected with MC-HLA-G1, pmax-HLA-G1, or pEP-HLA-G1 were examined at day 2 post-transfection, as were MSC that had been stably transduced with Lv-HLA-G1. A broad range of NK:MSC ratios (effector:target) was tested. Results show that only the viral delivery system render MSC more resistant to activated NK cells, indicating that NK-mediated lysis is differentially affected by the system used to deliver the HLA-G1 gene (Figure 5). This effect is particularly evident at the highest NK:MSC ratio (20:1), where a reduction of ~30% was observed for virally-transduced MSC when compared to nonmodified MSC ($P < 0.05$). In contrast, when MSC were transfected with MC-HLA-G1 (64 ± 3.3%) and pmax-HLA-G1 (68 ± 5.6%) vectors, NK-mediated lysis was not decreased, compared to nonmodified cells (62 ± 5.5%). Even more remarkably transfection of MSC with pEP-HLA-G1 actually caused a statistically significant increase in their susceptibility to NK lysis (89 ± 6.6%; $P < 0.05$). Similar results were also obtained for 10:1 ratio. Although at the 5:1; 1:1, 0.2:1, and 0.1:1 NK:MSC ratios, a statistically significant difference between Lv-HLA-G1 engineered MSC and unmodified MSC was no longer present, the cytotoxic

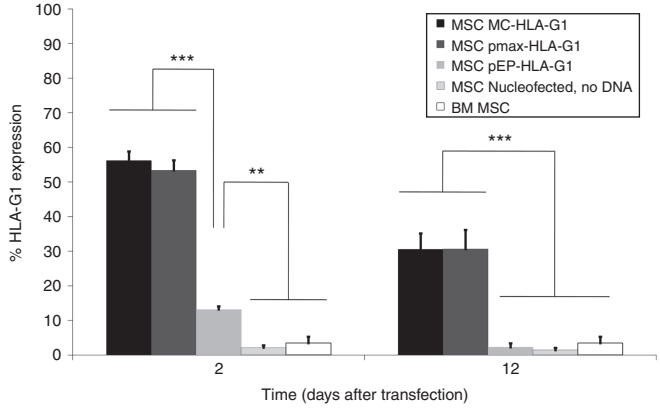

Figure 2 Evaluation of HLA-G1 transfection efficiency by flow cytometric analysis. Bone marrow-derived mesenchymal/stromal cells (MSC) were nucleofected with three distinct DNA constructs: MC-HLA-G1 (black), pmax-HLA-G1 (dark grey), and pEP-HLA-G1 (light grey). Controls consisted of untransfected cells (white) and cells that were subjected to the identical transfection protocol but without addition of DNA (dashed). Transgene expression measured at day 2 and day 12 postnucleofection. Each bar represents the mean ± standard error of mean, $n = 4$; $^{**}P < 0.01$; $^{***}P < 0.001$.

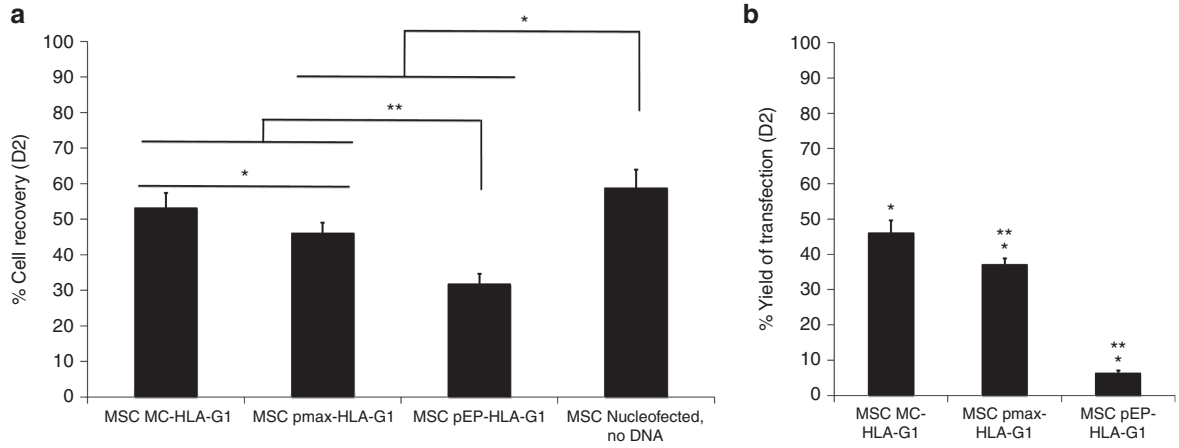

Figure 3 Cell recovery and transfection yield at day 2 after nucleofection. Comparison of the cell recovery (**a**) and yields of transfection (**b**) obtained for all DNA constructs. Each bar represents the mean ± standard error of mean, $n = 4$; $^{*}P < 0.05$; $^{***}P < 0.001$.

inhibitory effect for Lv-HLA-G1 remained significantly lower when compared to nonvirally transfected cells ($P < 0.05$).

Only HLA-G virally-modified MSC are able to significantly reduce T-cell proliferation

In order to determine whether MSC modified to overexpress HLA-G1 (MSC-HLA-G1) would further reduce peripheral blood mononuclear cells (PBMNC) proliferation when compared to nonmodified MSC, a one-way ($n = 4$) or two-way mixed lymphocyte reaction (MLR) was performed ($n = 4$). The one-way proliferation assay measures the ability of PBMNC to respond and proliferate to allogeneic MSC in culture, while the two-way assay determines the ability of MSC to alter the allogeneic immune response (proliferation) when PBMNC of two unrelated individuals are cultured together. In similarity to our observations with susceptibility to NK lysis, results with MLR show that PBMNC proliferation was also differentially affected by the HLA-G1 gene delivery system used (Figure 6). MLR results shown in Figure 6a (one-way) and Figure 6b (two-way) clearly demonstrate that only HLA-G overexpressed by the lentiviral vector was able to significantly decrease PBMNC proliferation when compared with unmodified MSC ($P < 0.05$). Results from one-way assays show that, when compared to unmodified MSC, cells transfected with MC-HLA-G1 (1.02 ± 0.05) and pmax-HLA-G1 (1.04 ± 0.07) were unable to decrease the levels of PBMNC proliferation, while modifying the MSC with pEP-HLA-G1 (1.12 ± 0.05) actually enhanced significantly PBMNC proliferation ($P < 0.05$; Figure 6a). Furthermore, as shown in (Figure 6b) which depicts representative results obtained by the two-way MLR, HLA-G1 overexpression, via pmax-HLA-G1 (1.23 ± 0.11) and pEP-HLA-G1 (1.29 ± 0.09), significantly decreased the ability of MSC to inhibit allogeneic PBMNC proliferation, when compared with unmodified MSC ($P < 0.05$). Nucleofection of MSC with MC-empty (1.26 ± 0.10) also resulted in an increased PBMNC proliferation in two-way assays ($P < 0.05$), whereas no major differences were observed between cells transfected with MC-HLA-G1, unmodified cells, and MSC nucleofected without the addition of plasmid DNA.

Expression levels of different immunoreceptors on MSC overexpressing HLA-G1

In order to determine why HLA-G1 was unable to alter T and NK cell responses, when delivered through nonviral systems, we investigated whether MSC's expression repertoire of inhibitory or

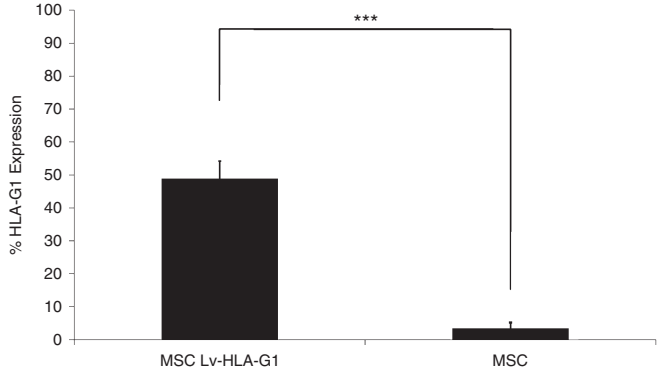

Figure 4 Stable transduction of MSC overexpressing HLA-G1. MSC were modified using a lentiviral delivery system (Lentiv-HLA-G1). Stable transduction was achieved 3 days after viral infection and transgene expression levels were determined by flow cytometric analysis. Each bar represents the mean ± standard error of mean; $n = 4$; $^{***}P < 0.001$.

stimulatory immunoreceptors/ligands had changed as a result of transfection.

NK lytic activity is determined by the balance between activating and inhibitory signals, and the activating NK cell receptors NKp30, NKG2D, and DNAM-1 are the main receptors responsible for the induction of NK-mediated cytotoxicity against MSC.[14,20] Therefore, we analyzed the presence/levels of NK cells' NKG2D activating ligands, including ULBP 1, 2/5/6 and 3, and MICA/B, as well as DNAM-1 activating ligands, CD122 (nectin-2) and CD155 (PVR),[16] on the surface of genetically engineered and unmodified MSC. As shown in Figure 7a, all MSC, independent of the treatment, expressed moderate and low levels of ULBP-2/5/6 and ULBP-3, respectively, and negligible levels of MICA/B and ULBP-1. Furthermore, CD122 was barely detectable regardless of whether MSC were modified to upregulate HLA-G1 or not. CD58, a surface molecule that has been associated with NK-cytotoxic activity,[21] was absent on all MSC populations (Figure 7a), and CD155 was highly expressed in both MSC-HLA-G1 and MSC.

Since the interaction between HLA class I (HLA-I) molecules and specific inhibitory/activating receptors on the NK-cell surface, such as KIRs, CD94, and NKG2A, engages signaling pathways that prevent target cell lysis,[22] the expression of the classical HLA-I molecules HLA-ABC and the nonclassical HLA-Ib molecule HLA-E was also investigated. HLA-ABC was present on more than 80% of all

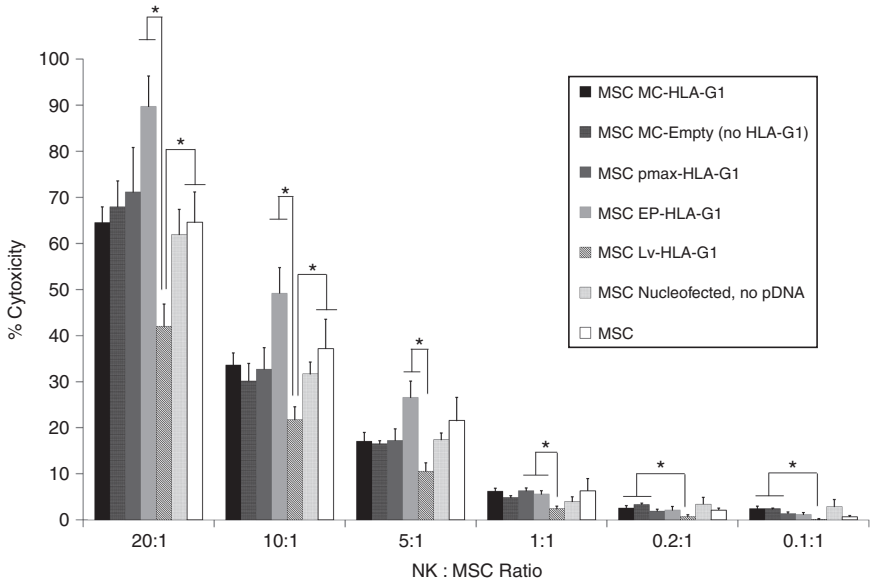

Figure 5 Susceptibility to NK-mediated lysis is altered by the gene delivery system used to overexpress HLA-G. MSC nucleofected with MC-HLA-G1 ($n = 4$), pmax-HLA-G1 ($n = 4$), or pEP-HLA-G1 ($n = 4$) at day 2 post-transfection, as well as cells stably transduced with Lv-HLA-G1 ($n = 4$) were cocultured independently with different concentrations of NK-92MI cells at 20:1, 10:1, 5:1, 1:1, 0.2:1, 0.1:1 NK:MSC (effector:target) ratios. Unmodified MSC ($n = 4$) and MSC nucleofected without pDNA ($n = 4$) were included as control. After 4 hours of incubation, cell lysis of each MSC population was assessed through the measurement of lactate dehydrogenase release, and the specific percentage of cell lysis was calculated. Each bar represents the mean ± standard error of mean; *$P < 0.05$.

modified and unmodified MSC, whereas HLA-E exhibited low levels of expression (Figure 7b). Additionally, the expression of HLA-DR, a class II HLA molecule that plays a pivotal role in eliciting immune responses was negligible (Figure 7b).

Because no significant differences were observed in the expression of any of the molecules described above, the Toll-like receptor (TLR) pathway was investigated (Figure 7c). Importantly, TLR9, a receptor that specifically recognizes nonmethylated CpG dinucleotides present in bacterial sequences,[23] was found to be significantly upregulated in MSC transfected with any of the nonviral delivery systems ($P < 0.05$). Nevertheless, it is important to note that, among nonviral constructs, MC-HLA-G1 induced the least TLR9 expression (17 ± 3.4%) when compared to MSC modified with MC-empty (25.7 ± 3.4%), pmax-HLA-G (135 ± 11%), or pEP-HLA-G1 (36 ± 11%). By contrast, when a lentiviral vector was used to deliver HLA-G1, the levels of TLR9 expression remained similar to those present in unmodified cells. The levels of TLR4 (a receptor for bacterial lipopolysaccharides)[23] were found to be almost negligible for all conditions, while the percentage of cells expressing TLR3 (a receptor for viral double-stranded RNA (dsRNA), was only increased ($P < 0.05$) in Lv-HLA-G1 when compared to unmodified cells.

DISCUSSION

Given MSC's intrinsic anti-inflammatory and immunomodulatory properties, these cells have been explored as promising therapeutic tools to treat several immune-based disorders.[2,24] Therefore, engineering MSC to overexpress proteins that exert strong immune-inhibitory functions, such as HLA-G[10] could be used to enhance the beneficial effect of MSC and, consequently, improve the clinical outcome of current MSC-based therapies. To achieve this goal, it is thus pivotal to identify the gene delivery strategy that provides the optimal combination of gene transfer performance, safety, and therapeutic efficacy of the transgene. Current options for delivering therapeutic genes include both viral and nonviral approaches, each with advantages and shortcomings.[19]

In the present study, we evaluated the ability of three different nonviral vectors and a lentiviral vector to serve as effective gene delivery platforms to overexpress HLA-G1 in MSC, and used NK lysis, MLR assay, and immunophenotyping to define the effects each of these systems exerts on known MSC immunomodulatory functions.

Although nonviral approaches have been reported to be less efficient at introducing genetic material into cells when compared to viral systems, they could potentially provide superior safety to viral vectors.[19,25] Therefore, we first focused on establishing transfection conditions that maximized HLA-G1 overexpression. Because, among nonviral gene transfer systems available, nucleofection has emerged as a powerful technique that consistently provides MSC transfection levels of 50–80%,[26–28] our strategy consisted of combining this technology not only with a standard plasmid DNA molecule (pMax),[29] but also with newer generation DNA constructs, such as minicircles (MC), which have the advantage of a greatly reduced size,[30] and a self-replicating episomal plasmid encoding for scaffold/matrix attachment region (S/MAR; pEP).[31,32] In addition, both MC and pEP constructs have reduced numbers of unmethylated CpG sequences (169 and 170 respectively), which have been proposed as one of the mediators for triggering the immune response that leads to loss of gene expression following plasmid-mediated delivery.[33]

Here we show that nucleofection using either MC-HLA-G1 or pmax-HLA-G1 plasmid constructs constitutes an effective gene delivery approach to overexpress HLA-G1 on MSC and, although similar levels of HLA-G1-positive cells were obtained with both constructs, MC-HLA-G produced significantly higher cell recovery and yield of transfection. In addition, the percentages of cell recovery and yield of transfection obtained for MC-HLA-G1 were higher than what has previously been reported using the same or alternative electrotransfer methods such as microporation.[27,34] We also showed that although pEP-HLA-G1 induced HLA-G1 overexpression, the overall transfection efficiency was much lower when compared with MC-HLA-G1 or pmax-HLA-G1 constructs. This difference between

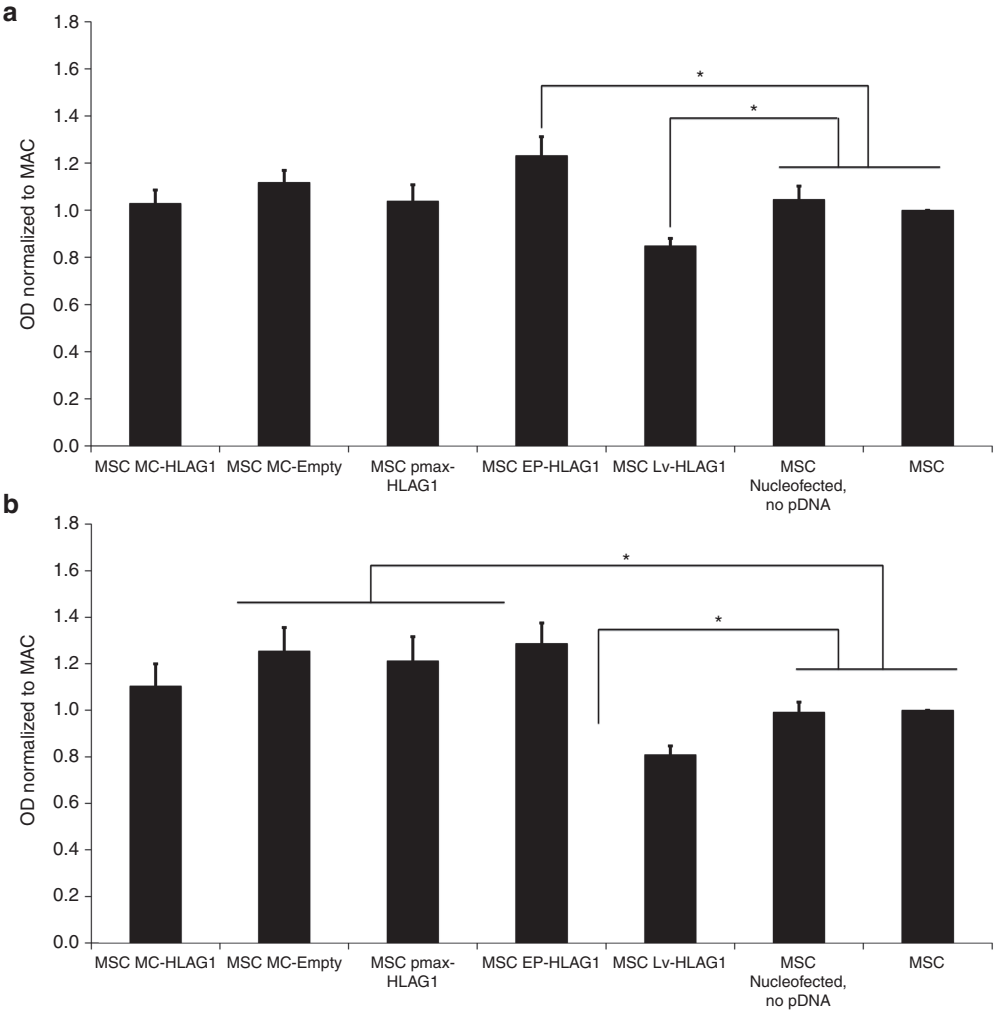

Figure 6 PBMNC proliferative response toward MSC-HLA-G1 engineered using different gene delivery systems. Each of the HLA-G1 modified and unmodified MSC (stimulator cells) were cocultured with allogeneic PBMNC (responder cells) in a one-way ($n = 8$), (**a**) or two-way ($n = 4$), (**b**) assay. After 5 days, the proliferation of alloreactive cells was measured using a colorimetric BrdU cell proliferation ELISA. For all conditions, the arbitrary values of PBMNC proliferation were normalized to unmodified MSC (MSC). Each bar represents the mean ± standard error of mean; *$P < 0.05$.

MC-HLA-G1 (2954 bp), pmax-HLA-G1 (3486 bp), and pEP-HLA-G1 (5972 bp) is consistent with the previously described inverse correlation between transfection efficiency and the size of the DNA molecule introduced.[31] Surprisingly, transgene expression induced by pEP-HLA-G1 was rapidly lost with time in culture, despite the presence of the S/MAR element.

Since viral systems are still the most routinely used gene transfer platforms and are able to offer robust efficiency and sustained gene expression,[19] we performed a side-by-side comparison between nonviral and a lentiviral system in order to investigate which gene transfer system is best suited for delivering HLA-G1, while maintaining its immunomodulatory effects. We found that only virally-transduced MSC were capable of significantly decreasing the susceptibility of MSC to NK-mediated lysis and reducing PBMNC alloproliferation in comparison with unmodified MSC. In addition, preliminary studies using murine retroviral vectors to deliver HLA-G1 resulted in similar levels of immunomodulation to those seen with lentiviral vectors (data not shown). Although the use of nonviral constructs, resulted in a similar percentage of HLA-G1 expressing cells as the viral-based delivery system, NK-mediated lysis and PBMNC alloproliferation were both increased following nonviral transfer of HLA-G1. These results led us to investigate the expression of molecules known to have an active role in eliciting immune responses on MSC. Therefore, we evaluated and compared the expression of the NK killing activation ligands ULBP 1, 2, and 3, MICA/B, CD122, and CD155, as well as two of the main NK inhibitory ligands, HLA-ABC and HLA-E, on genetically engineered and unmodified MSC. Since we were unable to detect a correlation between the levels of expression of these molecules and the effects observed in the functional assays, the expression of TLRs were also investigated. TLRs represent the most well studied family of immune sensors of invading pathogens and play an important role in activation of the adaptive immune response.[19] These receptors are broadly distributed throughout the cells of the immune system, and their activation is crucial for the engagement of immune response and enhancing adaptive immunity against microbes.[23] More recently, it has also been shown that MSC constitutively express TLRs at different levels,[35,36] with TLR3 and TLR4[37] being expressed at higher levels than TLR9.[35] Importantly, it has also been shown that TLRs participate in MSC's immunological function and that although the mechanisms involved are still not completely understood, TLR activation differentially affects MSC immunomodulatory response.[37] Some studies indicate that TLR3 and TLR4 activation reduce the inhibitory activity of human BM-MSCs on T-cell proliferation and induce

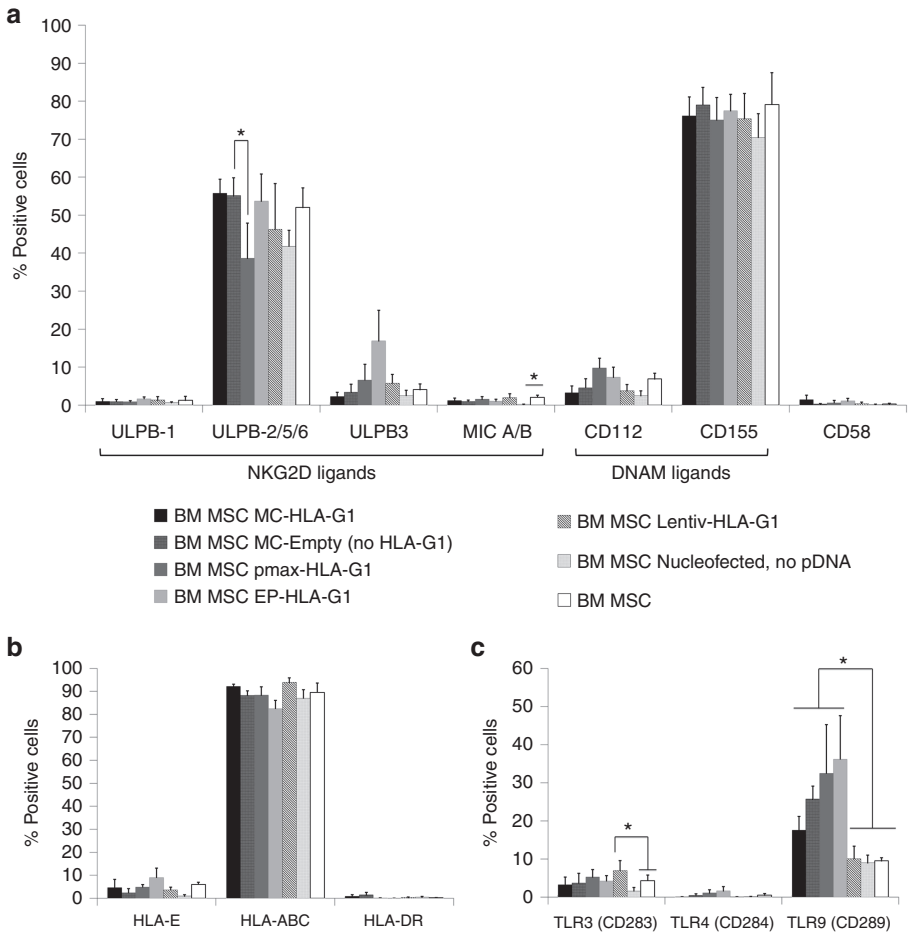

Figure 7 Characterization of immunoreceptors on MSC-HLA-G1 and unmodified MSC. Cells were analyzed by flow cytometry for expression of NK-activating ligands, including ULPP 1, 2/5/6 and 3 and MICA/B (NKG2D ligands), and CD122 and CD155 (DNAM-1 ligands) (**a**), for HLA-ABC, HLA-E, and HLA-DR (**b**), as well as for TLR3, TLR4, and TLR9 (**c**). Each bar represents the mean ± standard error of mean; $n = 4$; *$P < 0.05$.

the secretion of proinflammatory molecules capable of promoting the recruitment of inflammatory immune cells,[36,38,39] whereas others report that TLR3 and/or TLR4 engagement enhances the immunosuppressive properties of human BM-MSCs.[39,40]

Waterman *et al*.[41] suggested a model in which TLR ligand concentration, timing, and kinetics of activation are responsible for polarizing MSC toward either proinflammatory (MSC1) or anti-inflammatory (MSC2) phenotypes. More specifically, in TLR4-primed MSC, upregulation of proinflammatory cytokines, such as interleukin (IL)-6 and IL-8, and activation of T-cell proliferation (MSC1 phenotype) are observed, whereas TLR3 engagement results in secretion of anti-inflammatory molecules, such as IL4, IDO, and PGE2, and inhibition of lymphocyte proliferation (MSC2 phenotype).

Here, we did not find changes on TLR3 or TLR4 expression, but rather, a significant increase in TLR9 was found for all nonvirally transfected MSC, indicating that TLR9 function in MSCs can be modulated by artificially introducing pDNA into these cells. In fact, despite HLA-G1 overexpression and efforts to minimize CpG content by using newer generation DNA constructs (MC-HLA-G1 and pEP-HLA-G1), it is important to note that the small number of residual CpG sequences may still result in activation of the TLR9 signaling following introduction of these bacterial-based platforms into MSC's cytoplasm, thereby negating the functional effect of HLA-G1 overexpression. Although TLR9 has thus far only been implicated in MSC mobilization toward inflammation sites,[42] we showed a potential correlation between TLR9 activation in transfected MSC, and impaired MSC immunomodulatory potential. This effect was not observed for virally-transduced MSC, most likely due to the fact that viruses have evolutionary characteristics that naturally allow them to bypass recognition by some DNA sensors.[18]

Further studies on MSC-HLA-G1 secretome for pro- and anti-inflammatory cytokines and chemokines should be performed in order to better understand the role and physiological implications of MSC's innate DNA sensing on gene delivery of immunomodulatory molecules. Additionally, downregulating TLRs could potentially be used as a strategy to minimize the effects of the presence of naked DNA and therefore prevent CpG recognition. Our current results with MSC are in agreement with recent studies by Wang and colleagues, in which the authors report that nucleofected B cells over-expressing factor IX (FIX) antigen fused with IgG heavy chain induced an increased anti-FIX response and a marked upregulation of proinflammatory cytokines, when compared to B cells modified with retroviral vectors to express this same cassette. In further agreement with our results, these authors also demonstrated that this effect was due to TLR9 activation by the plasmid DNA.[43]

In conclusion, we demonstrated that HLA-G1 expression on human MSC can be efficiently boosted using both nonviral and viral approaches. Nevertheless, despite the encouraging results obtained with nonviral systems, we show for the first time, to our knowledge, that only viral-based delivery of HLA-G1 improves MSC's immunomodulatory properties, indicating that the gene transfer approach can influence the immune behavior of MSC. These differences

observed between non-viral and viral systems could be attributed to TLR9 upregulation caused by the CpG sequences present in non-viral plasmids; however, further investigation into the processes involved in DNA sensing in MSC can clarify how signals delivered by TLR can influence genetically-engineered MSC's biological function.

MATERIALS AND METHODS

Human BM-MSC isolation and culture

Bone marrow-derived MSC (BM-MSC) were isolated using anti-Stro-1 antibody (R&D Systems, Minneapolis, MN) and magnetic cell sorting (Miltenyi Biotec, Auburn, CA) as previously described.[44] Cell cultures were maintained in gelatin (Sigma-Aldrich, St Louis, MO)-coated flasks and MSCGM (Mesenchymal Stem Cell Growth Medium BulletKit, Lonza, Atlanta, GA) supplemented with penicillin/streptomycin (100 U/ml) (Gibco-Life Technologies, Carlsbad, CA) at 37 °C and 5% CO_2 in a humidified atmosphere. MSC were passaged when they reached 70% confluence using trypsin solution (Lonza) for 7 minutes at 37 °C. Cell number and viability were determined using the Trypan Blue (Gibco-Life Technologies) exclusion method, and cells were replated at 3,000 cells/cm^2. Phenotypic and differentiative characterization of Stro-1+ BM-MSC has been previously reported.[44] MSC were found to be positive (>95%) for CD29, CD73, CD90, and CD105; and negative (<0.05%) for CD34, CD45, CD14, CD19, and HLA-DR. Furthermore, upon culture in appropriate media, these cells differentiated into bone, cartilage, and adipocytes.

All experiments were performed using cells from four different donors (n = 4), at passages 6–9. After genetic modification with HLA-G1, independently of the method used, cells are designated MSC-HLA-G1.

Vector preparation and purification

The coding region of the human HLA-G1 gene (h*hlag*, 1017 bp) was amplified by polymerase chain reaction (PCR) using, as template, a cDNA clone (GenBank NM_002127.3) obtained from OriGene (pCMV6-XL5-HLAG, Rockville, MD). The primers were specifically designed to amplify the full coding sequence and to introduce additional restriction sites for further cloning purposes (primer sequences are listed in Table 1). All PCR reactions were performed using High Fidelity Platinum Taq DNA Polymerase (Invitrogen-Life Technologies, Carlsbad, CA) under the following conditions: 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 1 minute; and 72 °C for 10 minutes. Each PCR product was then cloned into the pCR4-TOPO vector (Invitrogen) following manufacturer's instructions. The HLA-G1 sequence was confirmed by DNA sequencing of all five pCR4-TOPO-HLAG1 vectors. Subsequently, the HLA-G1 gene was isolated from the pCR4-TOPO-HLAG1 vectors by restriction digestion and gel purification. Digestion was performed using standard methodology with *Bam*HI/*Eco*RI, *Nhe*I/*Bsr*GI, *Nhe*I/*Xho*I, *Spe*I/*Eco*RI, or *Eco*RI/*Xho*I (all from New England Biolabs, Ipswich, MA). Minicircle parental plasmid-HLA-G1 (MC-PP-HLA-G1, 6952 bp) was derived by inserting HLA-G1 in the multiple cloning site of MC-PP MN502A1 (5956 bp, System Biosciences, Mountain View, CA), using *Eco*RI and *Bam*HI restriction sites.

Table 1 List of polymerase chain reaction primers used for plasmid construction

Primer name	*F/R*	*Sequence (5′ to 3′)*
HLAG1_F_EcoRI	F	AAA ***GAATTC*** ACCACCATGGTGGT CATGGCGC
HLAG1_F_NheI	F	AAA ***GCTAGC*** ACCACCATGGTGGT CATGGCGC
HLAG1_F_SpeI	F	AAA ***ACTAGT*** ACCACCATGGTGG TCATGGCGC
HLAG1_R_BamHI	R	AAA ***GGATCC*** TCAATCTGAGCTC TTCTTTCTCCAC
HLAG1_R_BsrGI	R	AAA ***ACATGT*** TCAATCTGAGCTC TTCTTTCTCCAC
HLAG1_R_XhoI	R	AAA ***GAGCTC*** TCAATCTGAGCTCTTCTTTCTCCAC

The MC-PP-HLA-G1 (Figure 1a) was transformed into *Escherichia coli* ZYCY10P3S2T (System Biosciences), a strain that has been specifically engineered to allow both propagation of the MC-PP and the production of the MC DNA.[30] MC-PP-HLA-G1 propagation and MC-HLA-G1 production (Figure 1a) was performed according to System Biosciences' instructions.[30] After MC-HLA-G1 purification, a restriction digest-mediated linearization step allowed the evaluation of the residual amount of MC-PP in the MC preparations (Figure 1b). MC-empty (no HLA-G1) was also produced. The pmax-HLA-G1 plasmid (3486 bp, Figure 1c) was constructed by replacing the GFP from pmaxGFP (3486 bp, Lonza, Amaxa GmbH) with HLA-G1 using *Nhe*I-*Xho*I sites. The pEP-HLA-G1 plasmid (5972 bp, Figure 1d) was generated by replacing the GFP from pEPito-EGFP-IB (5680 bp, PlasmidFactory, Bielfield, Germany) with HLA-G1 using *Nhe*I and *Bsr*GI restriction sites. pmax-HLA-G1 and pEP-HLA-G1 were constructed and propagated in *E. coli* strain DB3.1λpir (kindly provided by Dr Michael Kahn, Washington State University) and *E. coli* strain DH5α (ZYMO Research Corporation, Irvine, CA), respectively. The HLA-G1 gene was also cloned into the multiple cloning site of the pMSCV-Neo retroviral vector backbone (Clontech, Mountain View, CA) using the restriction sites *Eco*RI and *Xho*I. The pSIN-EF2-HLA-G1 was obtained by replacing the Nanog gene in pSIN-EF2-Nanog-Pur, obtained from Addgene (Addgene, http://www.addgene.org), with HLA-G1 using the *Spe*I and *Eco*RI restriction sites. All cloning products were confirmed by restriction digest-mediated linearization followed by agarose gel electrophoresis and DNA sequencing. All plasmid constructs were purified using an endotoxin-free plasmid purification kit (Macherey-Nagel, Bethlehem, PA). The concentration of purified pDNA solutions was assayed by Nanodrop (Thermo Scientific, Wilmington, DE), and DNA integrity was confirmed using DNA agarose gel stained with ethidium bromide.

Viral particle production

Lentiviral particles were produced by cotransfecting 293T cells with pSIN-EF2-HLA-G1, pMD2.G, and psPAX2 plasmids using FuGENE 6 (Promega, Madison, WI). Vector-containing supernatants were collected 48 hours after transfection.The collected supernatants, for both retrovirus and lentivirus, were filtered with 0.2 µm low protein-binding syringe filters (Pall Corporation, Ann Arbor, MI), concentrated using 100K Amicon Ultra centrifugal filters (Millipore, Billerica, MA), and stored in aliquots at −80 °C until further use.

Nucleofection-mediated transfection

BM-MSC nucleofection was performed according to Human MSC Nucleofector Kit (Lonza) protocol. Briefly, 4×10^5 MSC were resuspended in 100 µl of nucleofection buffer with 2 µg pDNA (MC-HLA-G1, pmax-HLA-G1, or pEP-HLA-G1) and pulsed with the U-23 program of the Amaxa Nucleofection device (Lonza). After nucleofection, cells were carefully transferred to MSCGM, and the cell number and percent viability estimated using Trypan Blue. Cells were then replated at 3,000 cells/cm^2 and kept in culture at 37 °C and 5% CO_2 in a humidified atmosphere. HLA-G1 expression levels were measured by flow cytometric analysis 2 and 10 days after nucleofection. Non-nucleofected cells were used as a control, as were MSC only pulsed with U-23 (no pDNA). Cell recovery and rate of nucleofection were determined using equations previously described by Madeira *et al.*[34] MSC nucleofected with plasmids encoding HLA-G1 were designated nvMSC-HLA-G1.

MSC viral transduction

MSC cultures at 60% confluence were incubated overnight with supernatants containing lentiviral particles encoding HLA-G1 diluted in serum-free QBSF-60 medium (Quality Biological, Gaithersburg, MD) and 8 µg/ml protamine sulfate (Calbiochem, San Diego, CA). Transduction was performed at an MOI that yielded similar levels of HLA-G1-expressing MSC for all gene delivery systems. After transduction, cells were washed, and media was changed to MSCGM (Lonza). Stably transduced MSC with HLA-G1 (LvMSC-HLA-G1) were analyzed for transgene expression using flow cytometry.

NK cytotoxicity assay

NK cytotoxicity assays were performed as previously described by Soland *et al.*[16] Briefly, MSC, nvMSC-HLA-G1 48 hours after transfection, and vMSC-HLA-G1 were plated in a flat-bottomed 96-well microplate (BD Falcon, Franklin Lakes, NJ) (1×10^5 cells/ml; 50 µl/well), and incubated in triplicate

with different concentrations of NK-92 MI cells (ATCC, Rockville, MD) (20×10^5 cells/ml; 10×10^5 cells/ml; 5×10^5 cells/ml; 1×10^5 cells/ml; 2×10^4 cells/ml; 1×10^4 cells/ml; 50 µl/well) in α-MEM complete medium without phenol red (Gibco). NK-92MI cell line was used due to its robust proliferation rate in cell culture, and strong cytolytic activity towards several cells lines and primary cells without IL-2 supplementation. Furthermore, the NK-92 MI are also similar to activated NK cells with respect to their surface receptor expression.[45] The cytotoxicity tests were run at 20:1, 10:1, 5:1, 1:1, 0.2:1, and 0.1:1 E:T ratios following the guidelines of the CytoTox 96H Non-Radioactive Cytotoxicity Assay (Promega).

MLR

Human PBMNC (hPBMNC) were prepared from freshly collected, heparinized whole blood samples from healthy donors, after informed consent, according to guidelines from the Office of Human Research Protection at Wake Forest University Health Sciences.

MSC, nvMSC-HLA-G1 48 hours after transfection, and vMSC-HLA-G1 were used as stimulator cells, and allogeneic hPBMNC were used as responder cells. Firstly, 1×10^4 of each stimulator cell type was plated in triplicate into a 96-well flat-bottom plate (BD Falcon) containing MSCGM and incubated overnight. Cells were treated with 5 µg/ml mitomycin C (Roche Applied Science, Indianapolis, IN) at 37 °C for 2.5 hours in a humidified incubator with 5% CO_2 to impair further proliferation. After washing three times with Iscove's modified Dulbecco's medium, cocultures were established as recommended by the manufacturer of the 5-bromo-2′-deoxyuridine (BrdU) cell proliferation colorimetric ELISA kit (Roche, Mannheim, Germany). In brief, 1×105 hPBMNC in Dulbecco's modified Eagle's medium 10% fetal bovine serum were added to each well (100 µl/well) to obtain a final MSC/hPBMNC ratio of 1/10. Controls included: media control and stimulator and responder controls, which contain either stimulator or responder cells alone. Cultures were incubated for 5 days at 37 °C in 5% CO_2 and humidified atmosphere. On the fifth day, BrdU (Roche) was added to each well to a final concentration of 10 µmol/l. After overnight incubation at 37 °C in 5% CO_2 and 100% humidified atmosphere, DNA synthesis was assayed with the BrdU cell proliferation colorimetric enzyme-linked immunosorbent assay (Roche) according to the manufacturer's instructions.

Flow cytometric immunophenotypic analysis

The expression of surface markers on MSC, nvMSC-HLA-G1, and vMSC-HLA-G1 was evaluated by flow cytometry using the following monoclonal anti-human antibodies: HLA-G FITC (ABD Serotec, Oxford, UK), HLA DR, DP, DQ FITC (BD Bioscience, San Jose, CA), HLA-ABC PE (BD Pharmingen, San Jose, CA), MICA/MICB FITC, CD155 FITC (both from ABD Serotec), ULBP-1 PE, ULBP-2 PE (both from R&D Systems), CD58 PE (BD Biosciences), TLR9 FITC, TLR4 PE (both from Abcam, Cambridge, UK) and TLR3 Alexa 647 (Imagenex, Port Coquitlam, BC, Canada). Cells were also stained for HLA E (Abcam), ULBP-3 (R&D Systems), and CD112 (ABD Serotec) using Alexa Fluor 488 Goat Anti-Mouse IgG (Molecular Probes-Life Technologies) as secondary antibody. Briefly, cells were incubated for 15 minutes in the dark with saturating concentrations of each antibody. Stained cells were then washed with phosphate-buffered saline 0.1% azide, fixed with 1% formaldehyde, and analyzed by flow cytometry using the CellQuest software (Becton Dickinson Biosciences, San Jose, CA). A total of 10,000 events was acquired for each sample. Appropriate isotype (negative) controls were performed for each antibody.

Data analysis

All data are presented as mean ± standard error of the mean, and were analyzed with PASW Statistics software (IBM, Armonk, NY) one-way analysis of variance followed by a *post hoc* Tukey's honestly significant difference test. When both distribution and equality of variances of the data were not verified, a nonparametric Mann–Whitney test was performed. For all analyses, a P value <0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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