Title:
The plasticity of mesenchymal stem cells in regulating surface HLA-I

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A low surface expression level of human leukocyte antigen class I (HLA-I) ensures the mesenchymal stem cells' (MSCs) escape from the allogeneic recipients' immunological surveillance. Here, we discovered that both transcriptional and synthesis levels of HLA-I in MSCs increased continuously after IFN-\(\gamma\) treatment, while interestingly, their surface HLA-I expression was downregulated after reaching an HLA-I surface expression peak. Microarray data indicated the post-transcriptional process plays an important role in downregulation of surface HLA-I. Further studies identified that IFN-\(\gamma\)-treated MSCs accelerated HLA-I endocytosis through a Clathrin–independent Dynamin-dependent endocytosis pathway. Furthermore, the cells which have self-downregulated surface HLA-I expression elicit a weaker immune response than they previously could. Thus, uncovering the plasticity of
MSCs in the regulation of HLA-I surface expression would reveal insights into the membrane-transportation events leading to the maintenance of low surface HLA-I expression, providing more evidence for selecting and optimizing low immunogenic MSCs to improve the therapeutic efficiency.

Introduction

Polymorphisms in human leukocyte antigen (HLA) class I genes can cause the rejection of stem cells or tissue grafts in allogeneic recipients, which affect the safety and efficiency of therapies. Mesenchymal stem cells (MSCs) have been widely considered and reported to have the therapeutic function in various degenerative and inflammatory diseases (Hosseini et al., 2018; Wang et al., 2016); so it is important to evaluate the HLA-I expression on MSCs when considering about allogeneic MSCs transplantation therapy. Although MSCs can be isolated from multiple tissues including bone marrow, adipose, and umbilical cord, only a very limited number of MSC can be harvested from specific sources, generally requiring several weeks of ex vivo expansion to reach the demanding therapeutic MSC dose. In addition, they have been reported to exhibit large heterogeneity between different tissue sources and complicated donors physical status in cell qualities following differentiation or immunomodulation abilities (Kim et al., 2018; Kunimatsu et al., 2018; Yang et al., 2018). Therefore, pluripotent stem cells, such as induced pluripotent stem cells and embryonic stem cells, were introduced as potential sources for MSCs due to their capacity to differentiate into the MSCs lineage. However, induced pluripotent stem cells have the potential risks of chromosomal instability and oncogenic transformation associated with the application of viral vectors during the reprogramming process (Okita et al., 2007; Yu et al., 2007). In addition, it raised a concern that the reprogramming of iPSCs may be incomplete so that they still carry donor-specific characteristics, resulting in iPSCs with variable gene expression or DNA methylation (Chin et al., 2009; Doi et al., 2009). Thus, although allogeneic embryonic stem cells carry the risk of teratomas formation and face the challenge of maintaining genetic stability during long-term culture (Hentze et al., 2007). These cells have recently been proposed as an efficient source for MSCs generation to provide a high quality “off-the-shelf” human embryonic stem cell-derived mesenchymal stem cell (hESC-MSCs) products (Hematti, 2011). Hence,
hESC-MSCs must abide by a rigorous quality control system, evaluating their safety and immunogenicity during cell transplantation.

The immunogenicity of MSCs remains poorly defined and controversial. The prevailing dogma considers allogeneic MSCs as immune privileged or immune evasive. However, some studies showed the generation of alloantibodies and immune rejection after allogeneic MSCs transplantation.

Culture-expanded MSCs have been confirmed by expressing a low level of surface HLA-I, no HLA-II and costimulatory molecules including CD40, CD80, and CD86(Klyushnenkova et al., 2005). Furthermore, MSCs were reported capable of producing a variety of immunomodulatory cytokines such as prostaglandin E2, interleukin10, transforming growth factor-β, HLA-G, 2,3-dioxygenase, and inducible nitric oxide synthase, increasing the proportion of regulatory T cells, and inhibiting the function of natural killer cells and effector T cells(Aggarwal and Pittenger, 2005). Some studies illustrated that allogeneic MSCs maintained low immunogenicity even after being immune challenged in vitro. Additionally, when compared with the injection of peripheral blood mononuclear cells in vivo, allogeneic MSC injection did not elicit T cell proliferation and inflammatory cytokines secretion(Lee et al., 2014). Further evidence from Zangi et al. showed the mMSCs(20 days) were able to survive longer when compared to fibroblasts(10 days) in the allogeneic host mice(Zangi et al., 2009). These results suggested that MSCs may exhibit lower immunogenicity than other differentiated cells and that MSCs can regulate themselves, as well as the environment, to maintain a hypo-immunogenic condition.

However, there also exist controversial reports regarding the immunogenicity of MSCs. It was reported that MSCs became highly immunogenic after transplanted into the host (Yang et al., 2017); previous results indicated that allogeneic MSCs injection stimulated the hosts’ T-cell response which threatened MSCs survival(Beggs et al., 2006). In addition, the ability of MSCs is often limited by the cell’s poor engraftment rate, hindering their therapeutic efficiency, as well as the unknown route of MSC administration (Gu et al., 2015). Reviews by Ankrum et al. and Berglund et al. provided a thorough discussion on the immunogenicity of MSCs and insisted that it was worthwhile to consider MSCs immunogenicity in order to improve the efficiency and safety of MSCs therapies(Ankrum et al., 2014; Berglund et al.,
The rate of immune detection and elimination of allogeneic MSCs is dictated by the balance between a given cell’s relative expression of immunogenic and immunosuppressive factors. Meanwhile, the cell cycle may also have an effect on the stem cells’ immunogenicity. Agudo et al. have reported that the hair follicle stem cells (HFSCs) within the telogen phase (quiescent state) can downregulate the antigen presentation machinery to evade cellular immunity (Agudo et al., 2018). The cell state of MSCs can also be regulated into a quiescent state by altering the culture medium or plate as previously reported (Moya et al., 2017; Rumman et al., 2018), but a published microarray data reported that quiescent MSCs induced a stronger immune response in contrast (GO:0006954, PTSG2, IL10, IL1A, IL1B, CCR7) (Rumman et al., 2018). Thus, it is still unknown whether it is beneficial to maintain MSCs in the quiescent phase in order to maintain low immunogenicity, especially low HLA-I expression. The alterations of MSCs’ immunogenicity possibly depend on various factors including both the cell microenvironment and cell state. Therefore, more studies need to be conducted in order to understand details related to MSCs’ immunogenicity, which could help improve the efficiency of MSCs transplantation.

The major role of HLA-I is to act as an identifying card for all nucleated cells. In healthy individuals, all of these molecules are autologous to avoid being attacked by CD8$^+$ T cells; however, when cells exhibit abnormal characteristics (e.g., cancer cells, allotransplantation, and viral infections), they express aberrant or non-self HLA-I molecules or antigens on their surface, rendering them being targeted by CD8$^+$ T cells for destruction and elimination from the body to then be destroyed and eliminated from the body (Bjorkman et al., 1987). Meanwhile, cells that have HLA-I defects will be targeted by natural killer (NK) cells (Kollnberger, 2016); therefore, HLA-I molecules is critical when considering allogeneic MSCs transplantation.

The surface HLA-I expression level depends on both the HLA-I presentation and HLA-I endocytosis. HLA-I antigen processing and presenting machinery (APM) is relatively similar among different cell types, and is believed to contain several steps including protein breakdown, peptide transport, peptide trimming, HLA-I assembling, and HLA-I-peptide complex exportation; however, the internalization components of HLA-I vary between different cell types and status. Additionally, because the HLA-I pathway gradually evolves to
improve its detection and elimination of abnormal cells, tumor cells and some virus-infected cells have also coevolved and improved its cloaking mechanisms to avoid such detection. For example, the abnormalities of antigen processing and presentation in HLA-I are discovered in many types of cancers (Campoli et al., 2004), and a large majority of viruses have evolved ways to tamper with the APM or internalize HLA-I from the cell membrane (Hewitt, 2003). Nearly every steps in the assembly and trafficking of HLA-I represent potential targets for ablating HLA-I expression on the cell surface. Nevertheless, it is still unknown how MSCs regulate their HLA-I expression.

The majority of immunogenicity studies on MSCs were carried out in vitro, and it should be noted that culture-expanded hMSCs typically express low levels of HLA-I on the surface, allowing them to evade immune surveillance. MSCs have been verified to have therapeutic functions in many inflammatory diseases such as osteoarthritis and autoimmune encephalitis, however, MSCs exposed to the inflammatory cytokine, IFN-gamma, can significantly express more HLA-I (Barrachina et al., 2016; Martini et al., 2010). Therefore, we investigated the expression level of HLA-I on hESC-MSCs and examined its underlying regulatory mechanism, particularly in an inflamed microenvironment, hoping to better understand the immunogenicity of MSCs and improve the efficiency of MSC therapy.

**Results**

**MSCs can self-downregulate their HLA-I surface expression**

In our previous study, characterization of MSC phenotype in the generated hESC-MSCs have been identified (Wang et al., 2017); here we aimed to study the expression of HLA-I on hESC-MSCs in an inflamed niche. IFN-γ is one of the common inflammatory cytokines, secreted mainly from cytotoxic T lymphocytes. It has been reported that IFN-γ can upregulate HLA-I expression on cancer cells, leading to the activation of tumor-specific immune response (Martini et al., 2010); however, it is still unclear whether IFN-γ treatment will have an effect on hESC-MSCs’ HLA-I expression. In this study, we stimulated hESC-MSCs with 100U/mL IFN-γ for 0, 1, 2, and 3 days; qRT-PCR results demonstrated a continuous upregulation of polymorphic class I molecule (HLA-A, HLA-B, and HLA-C) expressions in hESC-MSCs following IFN-γ stimulation (Figure 1A). Additionally, western blot results also
illustrated a more prominent HLA-I band with longer stimulation time, indicating that hESC-MSCs’ total HLA-I protein level expression was upregulated in the presence of IFN-γ, where the intensity also increases over time (Figures 1B and 1C). In contrast, data analyzed by flow cytometry suggested a different expression outcome. Results illustrated that hESC-MSCs exhibited a degree of plasticity to maintain homeostasis; at day 1, the surface HLA-I expression on hESC-MSCs was first observed to be upregulated after IFN-γ stimulation, then the expression was automatically downregulated (Figures 1D and E). When compared to hESC-MSCs, bone marrow MSCs exhibited weaker HLA-I autoregulation ability as the downregulation of HLA-I initiated at day 2 after IFN-γ stimulation (Figures S1A and S1B); therefore, the surface HLA-I auto-downregulation ability varies between different sources of MSCs. Taken together, the presence of IFN-γ upregulates HLA-I expression level in hESC-MSCs, however, the cells are able to independently reverse the effect leading to the downregulation of HLA-I surface expression in the latter stage.

**Microarray data reveals the transcriptional changes in MSCs under IFN-γ treatment**

To investigate hESC-MSCs’ underlying mechanism in the downregulation of HLA-I surface expression, we performed microarray analysis on IFN-γ-stimulated hESC-MSCs after 0, 1, and 3 days of stimulation (marked as Day0, Day1, Day3); a total of 3054 differential expressed genes were significantly regulated in these three groups (Figure 2A). According to the STEM analysis, the differential genes can be classified into 8 groups (Figure 2B), where we further focused on the group (indicated as green background, N=312 genes) that were continuously upregulated with IFN-γ treatment (Day3>Day1>Day0) (Figure 2C). Gene ontology (GO) enrichment analysis on this specific group was performed using DAVID; results indicated that these genes were not only high related with the immune response but also enriched in terms associated with vesicle transportation and endocytosis pathway (Figure 2D, indicated as the red bar). Taken together, microarray data indicated that the vesicle transportation and endocytosis pathway may play an important role in regulating the hESC-MSCs surface HLA-I expression after IFN-γ treatment.
MSCs accelerate the endocytosis of surface HLA-I

The HLA-I surface expression level is regulated by the balance between the endoplasmic reticulum (ER)-Golgi-plasma membrane transportation rate and endocytosis transportation rate. Immunofluorescence was performed to determine the location of HLA-I in different organelles (ER, Golgi, endosome, and lysosome) on samples of Day0, Day1, and Day3. Results illustrated that the co-localization of HLA-I in ER was low at both before and after IFN-γ stimulation, while HLA-I molecules co-localized in Golgi, endosome, and lysosome were significantly upregulated after IFN-γ treatment (Figure 3A). Consistently, immunofluorescence intensity peaks of HLA-I (green line) were detected to be overlapped with Golgi, endosome, and lysosome intensity peak (red line) separately, but not with the ER intensity peak (red line) (Figure 3B). Quantitative analysis was also executed by measuring the co-localization rate with Mander’s values ranging from 0 to 1, where bigger values indicate higher co-localization rate. Quantitative results suggested the HLA-I co-localized in Golgi, endosome, and lysosome of hESC-MSCs were all upregulated after IFN-γ treatment; furthermore, the HLA-I in endosome was significantly upregulated at Day3 when compared with Day1 (Figure 3C). Furthermore, flow cytometry was utilized to examine HLA-I endocytosis rate in hESC-MSCs; results revealed that the HLA-I surface expression on hESC-MSCs was significantly upregulated after IFN-γ stimulation, however, it also showed faster endocytosis rate to down-regulate surface HLA-I (Figure 3D). On the whole, these data demonstrated that hESC-MSCs upregulate the plasma HLA-I endocytosis rate as a mechanism to reduce HLA-I surface expression.

MSCs regulate HLA-I endocytosis through the clathrin-independent dynamin-dependent pathway

To determine the exact endocytosis pathway responsible for the regulation of HLA-I surface expression, we collected and applied several small molecules known to inhibit different endocytosis pathway (Figure 4A). Selected inhibitors were then separately applied on IFN-γ stimulated hESC-MSCs where inhibitors marked as S8047 (Dynasore, Dynamin inhibitor) and S1342 (Genistein, Clathrin-independent inhibitor) were shown to lead to a significant increase in surface HLA-I expression; Chloroquine, a lysosome inhibitor, also led
to an upregulation of HLA-1 surface expression, but not as significant as S8047 and S1342. Other endocytosis and degeneration inhibitors showed very little or no effect on the cells’ HLA-I surface expression (Figure 4B).

To further validate the influence of S1342 and S8047 on hESC-MSCs’ HLA-I surface expression, cells were treated with a concentration gradient of these inhibitors. Data revealed that the application of S8047 or S1342 at a higher dosage resulted in a stronger level of HLA-I surface expression (Figures 4C and 4D). Furthermore, the combination of S8047 and S1342 was detected to further upregulate the HLA-I surface expression when compared to a single inhibitor (Figure 4E). We also conducted a siRNA experiment to knock down the DNM2 and RHOA respectively, and data showed both of them upregulate the surface HLA-I expression in the inflamed environment (Figures 4F and 4G). In conclusion, we suggest that hESC-MSCs regulate HLA-I endocytosis through the clathrin-independent and dynamin-dependent pathway.

**The HLA-I surface expression is related with MSC immunogenicity**

To investigate whether the downregulation of surface HLA-I in hESC-MSCs is correlated with hESC-MSCs immunogenicity, we applied a one-way mixed lymphocytes reaction culture to evaluate the immunogenicity of Day0, Day1, and Day3 hESC-MSCs. Allogeneic Peripheral Blood Mononuclear Cells (PBMCs) were stained with CFDA-SE as responder cells to reflect the proliferation rate. Results illustrated that hESC-MSCs in all groups were able to induce allogenic PBMCs proliferation to a certain extent; however, PBMCs stimulated with Day1 hESC-MSCs had fewer cells remaining in the un-proliferated stage when compared to Day0 and Day3 (Figure 5A). The corresponding quantitative result indicated that the proliferation rate of PBMCs was the highest in the Day1 stimulation group, while the Day3 stimulation group was lower but still a little stronger than Day0 (Figure 5B).

We further developed a humanized CD34+ mouse model to evaluate the immunogenicity of hESC-MSCs in vivo, imitating the human immune system. Immunodeficient mice (NSG) were first irradiated and then engrafted with human CD34+ hematopoietic stem cells (HSCs) to be reconstituted with human lymphocytes lineages. After 6 weeks post-engraftment, we subcutaneously injected the DiI-labeled hESC-MSCs into the humanized mice;
immunofluorescence staining results of hESC-MSCs revealed that these cells were able to retain in the host mice for two weeks, but the intensity varied between different groups (Figure 5C). Quantification analysis demonstrated that the immunofluorescence intensity of Day1 retained in the humanized mice was especially weaker than Day0 and Day3 groups (Figure 5D). In addition, we adopted the delayed-type hypersensitivity (DTH) test to assess T cell responses stimulated with different hESC-MSCs. The humanized mice were first primed with Day0, Day1, or Day3 hESC-MSCs for two weeks before the host mice were challenged with the same priming cells in footpads; thicker footpads signify a stronger T-cell response reaction. DTH results showed that all hESC-MSCs with different HLA-I expressions were able to elicit allergic responses, where Day1 IFN-γ-stimulated cells induced a stronger immune response as reflected by the mice’s thicker foot pads compared to other groups (Figure 5E). Consistently, histological HE stainings revealed that the Day1-injected footpad exhibited more lymphocytes infiltration (Figure 5F). In summary, we compared the immunogenicity of Day0, Day1, and Day3 IFN-γ-treated hESC-MSCs, and the results suggested that hESC-MSCs immunogenicity is consistent with HLA-I surface expression level.

Discussion

Taken together, these data demonstrated that hESC-MSCs exhibited plasticity in the regulation of surface HLA-I expression under an inflamed environment. HLA-I molecules are expressed on all nucleated cells and play an important role in allogeneic rejection through their presentation of peptide antigens to CD8+ T cells (Braciale, 1992), therefore a higher HLA-I expression on allogeneic cells will increase the risk of rejection by the host. Our results indicated that HLA-I expression levels varied in different cell types, with MSCs possessing a lower HLA-I surface expression when compared to somatic cells (Figures S2A-S2D). Previous studies have reported that MSCs showed low immunogenicity, including low expression of MHC-I (also named HLA-I in human) and no expression of MHC-II and its co-stimulators CD40, CD80, and CD86 (Klyushnenkova et al., 2005). This phenomenon suggests that hESC-MSCs may be a universal off-the-shelf cell for MSC therapeutic applications. However, previous reports showed that the HLA-I expression level was also
influenced by the different cell state and different cellular environment. We compared the HLA-I expression between hESC-MSCs at a late passage (slow cycle) and early passage (fast cycle) (Figures S3), where late passage MSCs showed higher expression of HLA-I. Moreover, we also induced hESC-MSCs into quiescence by serum deprivation (SD) for 48 hours, and then compared their HLA-I surface expression with control hESC-MSCs in a standard culture condition; the result illustrated that there was more HLA-I expressed on cells in the SD group (Figures S4). These results are consistent with the outcome reported by Rumman et al. (Rumman et al., 2018), suggesting that MSCs express a higher surface level of HLA-I in the quiescent state. However, a study completed by Judith Agudo (Agudo et al., 2018) reported that HFSCs downregulate MHC-I in their quiescent state to evade immune surveillance. Thus, MSCs and HFSCs had a different relationship between cell immunogenicity and the quiescent cell state. We suppose that this may be caused by the different types of stem cells, MSCs come from the mesenchyme, while the HFSCs are from the epithelium. To MSCs there were no benefits but disadvantages, to maintain the quiescent state in order to downregulate HLA-I expression. When referring to the cellular microenvironment, IFN-γ plays a major role in multiple scenarios during immune activation; it is one of the most important pro-inflammatory cytokines produced primarily by natural killer cells, CD4⁺T helper type 1 cells, cytotoxic CD8⁺T cells, as well as by dendritic cells, macrophages, and natural killer T cells (Eberl et al., 2015; Schroder et al., 2004), etc. Previous evidence indicated that IFN-γ could induce the immunosuppressive function of human MSCs, such as increasing the enzymatic activity of IDO1 or inhibiting CD4⁺/CD8⁺ T cell proliferation (Liotta et al., 2015; Sivanathan et al., 2014). Moreover, this cytokine has also been reported to upregulate MHC-I transcription level, which increased tumor immunogenicity (Weber and Rosenberg, 1988); this avenue of tumor surveillance was determined to involve recognition and elimination of tumor cells by cytotoxic T lymphocytes, recruited to the tumor mass via IFN-γ-induced chemokine signaling (Kunz et al., 1999). When considering MSCs, Wing Keung Chan et al. also found that IFN-γ markedly and instantly induced the expression of MHC-I in human bone marrow MSCs (Chan et al., 2008). However, it is still unclear whether IFN-γ treatment will have any impact on hESC-MSCs’ immunogenicity.

Consistent with tumor cells and bone marrow MSCs (Martini et al., 2010; Saric et al.,...
2002), our results showed that IFN-γ treatment was able to constantly upregulate both HLA-I transcription and total protein levels. Interestingly, we observed that even though the HLA-I surface expression on hESC-MSCs was first upregulated, the expression level then auto-downregulated in the latter stage; therefore, this suggested that hESC-MSCs have the plasticity to maintain the level of HLA-I expression. However, recent reports have stated that MSCs’ immunogenicity is influenced by multiple factors, including cell passages, donors’ age, physiological status, and tissue sources. Comparing with our data on bone marrow-derived MSCs (Figure S2A), we predicted that the auto-regulating ability of MSCs’ HLA-I surface expression may also be different between different sources of MSCs.

It has been reported that HIV infected cells can downregulate the HLA-I surface expression by accelerating the ARF6 endocytic pathway (Blagoveshchenskaya et al., 2002), as well as blocking the transportation of HLA-I to the cell’s surface (Swann et al., 2001). However, the mechanism of hESC-MSCs in modulating the downregulation of HLA-I surface expression is still unknown. Data obtained from our microarray analysis revealed a hint that the vesicle transport and endocytosis pathway may play a part in mediating the downregulation of HLA-I surface expression. In order to define which particular pathway was involved in the downregulation of HLA-I expression, we determined the location where HLA-I was overexpressed in the different subcellular fraction. We discovered a little amount of HLA-I co-localized within the ER, indicating that hESC-MSCs do not avoid HLA-I translocating onto the cell surface through restricting the molecules to ER, whereas plenty of HLA-I was found located in the Golgi, endosome, and lysosome. Moreover, the percentage of HLA-I located in the endosome and lysosome was found to be higher at Day3 when compared to Day1, indicating that the downregulation of surface HLA-I is mainly due to the endocytosis and degradation of HLA-I. Our flow cytometry results confirmed that HLA-I surface expression on hESC-MSCs was upregulated under IFN-γ treatment, however, these cells exhibited faster surface HLA-I endocytosis rate to maintain a low expression level of HLA-I.

Endocytosis, the process by which cells internalize macromolecules and surface proteins, involves several distinct pathways (Elkin et al., 2016) including clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-independent endocytosis pathway. In order to
identify the endocytosis pathway involved in hESC-MSCs’ HLA-I expression regulation, we applied selective inhibitors of different pathways. When compared to the non-treated group, results illustrated an increase in HLA-I surface expression when inhibitors dynasore and genistin were applied; a higher dosage of these two inhibitors also resulted in higher surface expression of HLA-I. Moreover, the combination of these two inhibitors also resulted in a more efficient upregulation of HLA-I surface expression. The time-series experiment implicated that both dynasore and genistin have the ability to block the downregulation of HLA-I surface expression on hESC-MSCs. These results all indicate that the HLA-I endocytosis in IFN-γ treated hESC-MSCs goes through the clathrin-independent and dynamin-dependent endocytosis pathway.

HLA-I is one of the major molecules reflecting the graft cells’ immunogenicity, however, we were not sure if the downregulation of HLA-I can exactly affect the cells’ immunogenicity. Mixed lymphocytes culture assay has been considered as a traditional and superior method in predicting allograft rejection in vitro. We co-cultured CFDA-SE stained PBMCs with Day0, Day1, and Day3 hESC-MSCs separately then tested the proliferation rate of PBMCs. Flow cytometry results showed that Day1 hESC-MSCs induced the highest stimulation index when compared to other groups (Day0 and Day3). Furthermore, we applied the DTH model in humanized mice to evaluate cell-mediated immune responses associated with IFN-γ-stimulated hESC-MSCs stimulated at different time points. The mice challenged with Day1 hESC-MSCs showed a more robust response in footpad swelling assays compared to Day0 and Day3. Meanwhile, histological staining results revealed that mice footpads challenges with Day1 hESC-MSCs had a significantly higher lymphocytes infiltration compared to Day0 and Day3. All of these results indicated the importance of HLA-I surface expression in inducing an allogeneic immune response, and the hESC-MSCs’ ability in regulating HLA-I expression can exactly downregulate cell immunogenicity, and then reduce the risk of immune rejection.

In previous studies, researchers tried to use the gene-editing method to modify HLA-I expression, either targeted disruption of B2M gene in ESC to minimize the cells’ immunogenicity(2015) or replaced the classical HLA-I with minimally polymorphic HLA-E molecules to escape allogeneic responses(Gornalusse et al., 2017). Here we discovered that
hESC-MSCs obtained the ability to downregulate HLA-I surface expression naturally, even when stressed under pulsed IFN-γ treatment (Figure S5). Refer to a previous study, the MSCs showed enhanced T-cell suppression when they were preconditioned by incubating them with IFN-γ for 48h (Le Blanc et al., 2003). Thus, maybe we can take advantage of this special property by priming hESC-MSCs with an appropriate dosage of IFN-γ for a specific time duration in order to enhance their immune-suppressive capacity, and also to maintain their low surface HLA-I expression to evade immune surveillance. This may push forward the application of MSCs in clinical trials.

In summary, we revealed the ability of hESC-MSCs in auto-downregulating the surface HLA-I expression with IFN-γ treatment. This behavior has been manifested to leave the cell with low immunogenicity. This discovery not only broadens our knowledge of hESC-MSCs but also provides a beneficial strategy to minimize the risk of rejection.

Limitations of the Study

This study focused on the plasticity of MSCs’ immunogenicity, which determines the cells’ survival in the allogeneic host. IFN-γ is an essential inflammatory cytokine, which has been identified to have the ability in strengthening the MSCs’ immunomodulatory. So here we only focused on the IFN-γ simulated inflammatory environment, without considering other inflammatory cytokines or other specific disease microenvironment.

Methods

All methods can be found in the accompanying Transparent Methods supplemental file.

Acknowledgments

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**Author Contributions**

Yafei Wang designed the experiment, performed the most experiment, analyzed the data and wrote the manuscript. Jiayun Huang, Lin Gong, and Dongsheng Yu performed the animal experiment and histological staining. Varitsara Bunpetch contributed to language polishing. Chenrui An helped with the Microarray data analysis. Jun Dai performed some molecular experiment. He Huang provided the donor blood to reconstitute humanized mice. Xiaohui Zou provided materials including human embryonic stem cell lines. Prof. Hongwei Ouyang and Prof. Hua Liu provided the most materials, all facilities, and financial support. Prof. Hua Liu provided the idea and approved the manuscript submission.

**Declaration of Interests**

The authors declare no competing interests.

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Figure Legends

Figure 1. The HLA-I expression of hESC-MSCs under IFN-γ treatment.

(A) qRT-PCR expression analysis of HLA-I related genes (include HLA-A, HLA-B, HLA-C, and B2M) on hESC-MSCs under IFN-γ treatment. RNA expression levels were normalized to the level of GAPDH expression. Data are shown as means±SEM;

(B) HLA-I expression on hESC-MSCs within IFN-γ treatment for 0 day, 1 day, 2 days and 3 days were compared by western blotting;

(C) The western blotting results quantified analysis of HLA-I total expression level on hESC-MSCs within IFN-γ treatment for 0 day, 1 day, 2 days and 3 days. HLA-I expression levels were normalized to the level of GAPDH expression. Data are shown as means±SEM;
(D) HLA-I surface expression on hESC-MSCs (red line) within IFN-γ treatment for 0 day, 1 day, 2 days and 3 days were compared by flow cytometry analysis. Blue line demarcate isotype control;

(E) The flow cytometry results quantified analysis of HLA-I surface expression level on hESC-MSCs with IFN-γ treatment for 0 day, 1 day, 2 days and 3 days by mean fluorescence index. Data are shown as means±SEM.

See also Figure S1, Figure S2, Figure S3, Figure S4, Figure S5 and Figure S6.

**Figure 2. The transcription microarray analysis of hESC-MSCs under IFN-γ treatment.**

(A) The heatmap of differential expressed genes between Day0, Day1 and Day3 hESC-MSCs;

(B) STEM analysis of all transcript levels differential expressed genes between Day0, Day1 and Day3 hESC-MSCs. “n” represents the gene numbers of each profile;

(C) The STEM result of genes expression in the selected profile;

(D) Functional analysis of differential expressed genes between Day0, Day1 and Day3 hESC-MSCs based on Gene Ontology. Blue bars indicate immune activation related GO, red bars indicate transportation-related GO.

**Figure 3. The transportation of HLA-I in hESC-MSCs under IFN-γ treatment.**

(A) HLA-I’ subcellular location in different organelles (include endoplasmic reticulum, Golgi, endosome, and lysosome) of Day0, Day1 and Day3 hESC-MSCs. Red indicates organelles, green indicates HLA-I and blue indicates DAPI. Scale bar,50µm;

(B) The immunofluorescence intensity analysis of HLA-I with different organelles in Day0, Day1 and Day3 hESC-MSCs (focused on the white line drawn on Figure 3A). The red line indicates organelles and the green line indicates HLA-I;

(C) The Mannder’s value analysis of colocalization rate of HLA-I and different organelles in Day1 and Day3 hESC-MSCs with the immunofluorescence results. n=3 technical replicates. Data are shown as means±SEM;

(D) The HLA-I endocytosis analysis on hESC-MSCs within or without IFN-γ treatment by flow cytometry. The red line indicates IFN-γ treated hESC-MSCs, and the blue line indicates
IFN-γ untreated hESC-MSCs, dash line indicates the endocytosis rate of surface HLA-I.

**Figure 4. The identification of the selected endocytosis pathway for HLA-I in hESC-MSCs.**

(A) Summary of the selected inhibitors for different endocytosis pathway;
(B) The mean fluorescence index of HLA-I surface expression on hESC-MSCs with different inhibitor treatment were compared by flow cytometry analysis. The black dash line indicates the compared hESC-MSCs group with only IFN-γ treatment. Data are shown as mean fluorescence index;
(C) The mean fluorescence index of HLA-I surface expression on IFN-γ treated hESC-MSCs with different dosage of S1342 treatment were compared by flow cytometry analysis. Data are shown as means±SEM;
(D) The mean fluorescence index of HLA-I surface expression on IFN-γ treated hESC-MSCs with different dosage of S8047 treatment were compared by flow cytometry analysis. Data are shown as means±SEM;
(E) The mean fluorescence index of HLA-I surface expression on IFN-γ treated hESC-MSCs with the combination of S1342 and S8047 treatment was compared by flow cytometry analysis. Data are shown as means±SEM;
(F) The mean fluorescence index of HLA-I surface expression on hESC-MSCs with different siRNA transfection were compared by flow cytometry analysis. Data are shown as means±SEM;
(G) The mean fluorescence index of HLA-I surface expression on IFN-γ treated hESC-MSCs with different siRNA transfection were compared by flow cytometry analysis. Data are shown as means±SEM.

**Figure 5. The immunogenicity of hESC-MSCs with Day0, Day1 and Day3 hESC-MSCs.**

(A) PBMCs proliferation rates with Day0, Day1 or Day3 hESC-MSCs stimulation were compared by flow cytometry analysis. The FL1 subset indicates the non-proliferated PBMCs; the FL1 subset-1, FL1 subset-2, FL1 subset-3 indicate the proliferated PBMCs, the PBMCs in later subset proliferate more times that the PBMCs in the former subset;
(B) PBMCs proliferation rates with Day0, Day1 or Day3 hESC-MSCs stimulation were quantified based on flow cytometry analysis. Data are shown as means±SEM;

(C) The humanized mice imaging with Day0, Day1 or Day3 hESC-MSCs injection after 2 weeks;

(D) The quantification analysis of Day0, Day1 or Day3 hESC-MSCs immunofluorescence retained in humanized mice. Data are shown as means±SEM;

(E) The footpad thickness of humanized mice with Day0, Day1 or Day3 hESC-MSCs challenge. Data are shown as means±SEM;

(F) The H&E staining of humanized mice’ footpads with Day0, Day1 or Day3 hESC-MSCs challenge. The black arrow indicated the infiltrated lymphocytes in the footpad. Bar=200μm.
Figure A: Images of day 0, day 1, and day 3 stained for organelles, HLA-I, and DAPI.

Figure B: Graphs showing the change in expression levels of organelles, HLA-I, and Endosome Lysosome from day 0 to day 3.

Figure C: Mander's overlap coefficient comparison among day 0, day 1, and day 3.

Figure D: Time-dependent MFI of IFN-γ and CON with a significant decrease in MFI from day 0 to day 1.
A

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B

The HLA-I surface expression on hESC-MSCs

C

The HLA-I surface expression on hESC-MSCs

D

The HLA-I surface expression on hESC-MSCs

E

The HLA-I surface expression on hESC-MSCs

F

The HLA-I surface expression on hESC-MSCs

G

The HLA-I surface expression on hESC-MSCs
Highlights

- hESC-MSCs have the plasticity of maintaining low HLA-I expression on cell surface
- hESC-MSCs downregulate the surface HLA-I expression through endocytosis of HLA-I
- hESC-MSCs with lower HLA-I surface expression induce weaker MLR and slighter DTH