

ORIGINAL ARTICLE

Comparative global immune-related gene profiling of somatic cells, human pluripotent stem cells and their derivatives: implication for human lymphocyte proliferation

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Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), represent potentially unlimited cell sources for clinical applications. Previous studies have suggested that hPSCs may benefit from immune privilege and limited immunogenicity, as reflected by the reduced expression of major histocompatibility complex class-related molecules. Here we investigated the global immune-related gene expression profiles of human ESCs, hiPSCs and somatic cells and identified candidate immune-related genes that may alter their immunogenicity. The expression levels of global immune-related genes were determined by comparing undifferentiated and differentiated stem cells and three types of human somatic cells: dermal papilla cells, ovarian granulosa cells and foreskin fibroblast cells. We identified the differentially expressed genes *CD24*, *GATA3*, *PROM1*, *THBS2*, *LY96*, *IFIT3*, *CXCR4*, *IL1R1*, *FGFR3*, *IDO1* and *KDR*, which overlapped with selected immune-related gene lists. In further analyses, mammalian target of rapamycin complex (mTOR) signaling was investigated in the differentiated stem cells following treatment with rapamycin and lentiviral transduction with specific short-hairpin RNAs. We found that the inhibition of mTOR signal pathways significantly downregulated the immunogenicity of differentiated stem cells. We also tested the immune responses induced in differentiated stem cells by mixed lymphocyte reactions. We found that CD24- and GATA3-deficient differentiated stem cells including neural lineage cells had limited abilities to activate human lymphocytes. By analyzing the transcriptome signature of immune-related genes, we observed a tendency of the hPSCs to differentiate toward an immune cell phenotype. Taken together, these data identify candidate immune-related genes that might constitute valuable targets for clinical applications.

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INTRODUCTION

Derivatives of human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced PSCs (iPSCs), are a potentially unlimited source of cells with considerable promise for basic research and organ transplants. However, the immunogenicity of these cells remains a major hurdle that

precludes their clinical application. Over the past decade, several reports have provided *in vivo* and *in vitro* evidence of reduced immunogenicity and immune privilege of ESCs, iPSCs and their derivatives.^{1–5} These phenomena may reflect inherent characteristics of PSCs, including the lower expression of major histocompatibility complex class I (MHC-I), MHC-II

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and natural killer (NK) cell receptor ligands.^{2,5,6} In addition to reduced MHC expression levels, an alteration of immune-related and immune privilege genes in PSCs may also be associated with their distinct immunogenicity.⁵ Accordingly, various strategies have been proposed to take this hypothesis into account, such as the banking of MHC-matched stem cells, establishment of ESCs by nuclear transfer-derived embryos and derivation of patient-specific iPSCs.^{7,8}

Recently, the discovery of hiPSCs that are reprogrammed from somatic cells by transduction of the factors Oct4, Sox2, Klf4 and c-Myc has revolutionized the stem cell field and demonstrated the potential to evade immune rejection after transplantation.⁹ Although the use of autologous hiPSCs has emerged as a new prospect to overcome immunogenicity caused by MHC mismatching, significant immunogenicity of teratomas derived from syngeneic iPSCs, but not ESCs, was reported in a mouse model.¹⁰ Moreover, reprogramming defects and the genetic instability of iPSCs are reported to lead to the expression of genes such as *Zgl16*, *Hormad1*, *Cyp3a11*, *Retn* and *Lcelf* and the induction of immunogenicity.^{10,11} Evidence shows that iPSCs derived from CD34⁺ hematopoietic stem cells maintain greater genomic stability than do terminally differentiated somatic cells, with relatively few somatic mutations compared with other somatic-derived iPSCs.¹² However, the clinical applicability of potentially reduced immune responses in differentiated cells derived from CD34⁺ hematopoietic stem cell-iPSCs on remains questionable. These reports suggest that in spite of the limited immunogenicity of differentiated cells from iPSCs, which might be similar to the differentiated cells from ESCs, the specifically differentiated cells from iPSCs could still induce certain immune reactions.

The mammalian target of rapamycin (mTOR) is a widely expressed serine/threonine protein kinase that has emerged as an important regulator of immune function, including T-cell activation, differentiation and function.¹³ In addition, the Akt/mTOR signaling pathway has been identified as a key mediator of human immunity and may be leveraged as a therapeutic strategy using rapamycin.^{14,15} Although the immunosuppressive effects of this agent during cell transplantation have been well documented, the ensuing transcriptome signatures and biological functions of rapamycin following stem cell transplantation remain incompletely understood. In the present study, we compare global immune-related gene expression patterns among undifferentiated stem cells, stem cell derivatives and their respective parental somatic cells of origin. In addition, we examine the role of the mTOR pathway in regulating the immunogenicity of hPSC-derived cells.

MATERIALS AND METHODS

Pluripotent stem cell culture, differentiation and reagents

Several hPSCs were used in the present study, including two hESCs: NTU1 (karyotype 46, XX)¹⁶ and H9 cells (karyotype 46, XX; WiCell, Madison, WI, USA).¹⁷ The iGra2 hiPSCs were derived from reprogrammed human granulosa cells⁵ and the iCFB hiPSCs were derived from reprogrammed human foreskin fibroblasts by our group.¹⁸ The CBiPSCs (CB: cord blood) were generated using human

cord blood-derived CD34⁺ progenitors with seven episomally expressed factors (catalog number A18945, Life Technologies, Taipei, Taiwan, R. O.C.).¹⁹ Thus, three types of somatic cells were used for hiPSC generation and were used as somatic cell controls, including human primary dermal papilla cells (adult human origin), human primary foreskin fibroblast cells (parental cells of iCFB iPSCs; adult Taiwanese male foreskin) and human primary granulosa cells (parental cells of iGra2 iPSCs; adult Taiwanese female luteinized granulosa cells). Human granulosa cells were obtained from ovarian follicular aspirates during oocyte retrieval in *in vitro* fertilization programs conducted in the National Taiwan University Hospital. Culture protocols of pluripotent stem cells were modified as previously described.^{4,16,20,21} Briefly, early-passage hPSCs were used for all experiments. The cells were continuously maintained on murine embryonic fibroblast feeders using serum-free medium (ReproCELL ES cell medium, Kanagawa, Japan). The cells were split weekly using 30-gauge insulin needles (Terumo Syringe, Tokyo, Japan) as previously described.^{16,20,22} For *in vitro* differentiation, colony pieces were cultured on gelatin-coated dishes without murine embryonic fibroblast and maintained in complete culture medium (DMEM-based medium (Gibco, Waltham, MA, USA and Stembo Bioscience, La Mirada, CA, USA) supplemented with 15% fetal bovine serum (Gibco), 200 mM glutamin (Invitrogen, Waltham, MA, USA), 10 mM non-essential amino acids (Invitrogen), 100 mM sodium pyruvate (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen). The medium was refreshed every 3 days and the cells were harvested on day 15 after differentiation. The complementary (c)-DNA microarray was undertaken in undifferentiated NTU1 hESCs, undifferentiated iCFB hiPSCs, 15-day differentiated NTU1 cells, differentiated iCFB cells and somatic cell controls of primary dermal papilla cells, primary foreskin fibroblast cells and primary granulosa cells. The neuroectodermal sphere formation was performed as previously described.²³ Briefly, embryoid body formation was generated from hESC H9 cells for 4 days in serum-free ReproCELL ES cell medium and then transferred to N₂ medium for 3 days to form neuroectodermal sphere, then cultured in Matrigel for 18 days. The use of human cells was approved by the Institutional Review Board of Academia Sinica and the Ethical Committee of National Taiwan University Hospital. Informed consent was obtained from all subjects.

RNA extraction, cDNA preparation and qPCR

Total RNA was harvested by Trizol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, #L1642, Waltham, MA, USA). Briefly, 1–10 µg of total RNA was treated with 1 µl of 10 × DNase buffer and 1 µl of amplification grade DNaseI in a 20 µl reaction. The reaction was terminated by the addition of 1 µl of Dnase stop buffer (Promega, M6106, Madison, WI, USA). Reverse transcription was performed at 25 °C for 10 min, 50 °C for an hour and the reaction was terminated by incubating at 85 °C for 5 min. cDNA samples were then aliquoted and stored at –20 °C. Quantitative PCR (qPCR) reaction was performed on 1 × EvaGreen reagent (Biotium, Fremont, CA, USA, catalog number 31014); 1 µl of diluted cDNA and 100 µM selected primers (IDT, San Jose, CA, USA, Supplementary Table S1) were used at 2 µl in 20 µl of qPCR reaction. The qPCR procedure was initiated by 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. The expression level of the target genes was calibrated by GAPDH, and the data were analyzed using the ABI 7500 Fast Real-Time PCR system. Quantification of all samples by the software was calculated from the C_T and relative fold changes were calculated using the 2^{–ΔΔCT}.²⁴

Immunoblotting and flow cytometry

Cells were lysed in RIPA buffer (radioimmunoprecipitation assay buffer, Darmstadt, Germany) supplemented with ProBlock Gold phosphatase inhibitor cocktail (Gold Biotechnology, Inc., St Louis, MO, USA). Immunoblot analysis was performed as previously described.²⁵ All primary antibody incubations were performed at 4 °C overnight at the following dilutions: mouse monoclonal anti-CD24 (eBioscience, San Diego, CA, USA), 1:250; mouse monoclonal anti-GATA3 (Thermo, The Thermo Fisher Scientific Inc., Waltham, MA, USA), 1:100; rabbit polyclonal anti-mTOR (ab2732, Abcam, Cambridge, MA, USA), 1:500; mouse monoclonal AKT (ab32505, Abcam), 1:1000; rabbit monoclonal pAKT (ab81283, Abcam), 1:1500; mouse monoclonal pS6 (Thermo), 1:500 and mouse monoclonal anti- β -actin (Thermo), 1:10 000. For CD24 flow cytometric analysis, anti-CD24 was used in a 1:20 dilution.

Teratoma formation, immunohistochemistry and immunofluorescence analysis

Forming teratoma from human PSCs was performed as previously described.²⁶ Immunohistochemistry and immunofluorescence were performed on formalin-fixed, paraffin-embedded teratoma sections and differentiated stem cells as previously described.²⁷ Briefly, all primary antibody incubations were performed overnight at 4 °C. The primary antibodies were used at the following concentrations: mouse monoclonal anti-CD24 (Thermo), 1:10; mouse monoclonal anti-GATA3 (Thermo), 1:50; goat monoclonal anti-SOX17 (R&D System), 1:10; goat monoclonal anti-brachyury (R&D System, Minneapolis, MN, USA), 1:10; rabbit monoclonal anti-beta III tubulin (ab18207, Abcam), 1:500. For teratoma section staining, antigen retrieval was performed by microwave heating for 10 min with 10 mM sodium citrate (pH 6). Differentiated stem cells were fixed in 4% PFA and permeabilized in 0.5% Triton X-100. Six to eight images of the samples were randomly selected and captured at $\times 400$ magnification for quantification by a blinded investigator (C-EW).

Complementary DNA microarray and ingenuity pathway analysis

First, 10 μ g total RNA was used for cDNA synthesis and further biotin-labeled cDNA hybridization probe generation. The transcriptome profiling was analyzed using an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) by the Microarray Core Laboratory of the National Health Research Institutes of Taiwan. All samples used fulfilled the criteria of an OD260/280 ratio > 1.9 and an OD260/230 ratio > 1.8 . The ribosomal RNA of 28S was greater than 18S by electrophoresis analysis. The final image files were detected with an Affymetrix GeneChip Scanner 3000, and the initial DAT files were processed by Affymetrix GeneChip Operating Software to generate the raw signal intensity CEL files. The raw intensity data were normalized using the robust multi-array average algorithm of GeneSpring software GX11 (Agilent, Santa Clara, CA, USA). The parameter of each chip was normalized to the 50th percentile. Based on our previous findings in an immune chip,⁵ the 85 selected immune-related gene probes were adopted from the normalized data. The study groups were compared to each of the three somatic cell controls. The list of genes obtained from each comparison was used for unsupervised hierarchical clustering displayed using Tree View Software. The significant up- or downregulation of candidate genes was examined by performing the significant analysis of microarrays.²⁸ The results of Log₂ fold change (FC > 2) ratios and immune-related gene probe IDs from the

comparison were further submitted to Ingenuity Pathway Analysis (IPA, Ingenuity Systems, <http://www.ingenuity.com/>) to identify the network connections of the genes of interest. The data were also entered into Gene Ontology terms extraction by applying the Fisher's exact test using Babelomics (<http://babelomics3.bioinfo.cipf.es/>). The top molecules were generated by IPA, and the results were collected for integration. Highly up- or downregulated genes in all experimental groups were listed as specific genes that can be attributed to the effects of differentiation and cell type.

Microarray raw data were uploaded to Gene Expression Omnibus (GEO) database (human primary dermal papilla cells referred to GSE28406-GSM702125, human primary granulosa cells referred to GSE28406-GSM702129 and human primary foreskin cells referred to GSE28406-GSM702135). To include more immune-related genes in the analysis, we collected the top 500 probes with high variance across the samples, incorporated the Immunome database²⁹ into the analysis and compared it with the data using the selected immune-related genes.⁵

RNA interference

A set of Mission short-hairpin RNAs (shRNAs) human CD24 and a shRNA control vector targeting Green Fluorescent Protein were purchased from ORIGENE (Rockville, MD, USA). The shRNA targeting human GATA3 (5'-CATCCAGACCAGAAACCGAAA-3') and CD24 (5'-CTTCTGCATCTCTACTCTTAA-3') lentiviral shRNA transfer vectors and the expression vectors encoding viral packaging proteins were provided by the National RNAi Core Facility, Academia Sinica, Taiwan. The vectors were co-transfected into HEK293 cells as described previously.³⁰ Supernatants of HEK293 cells were collected and used to transduce 15-day differentiated stem cells. Puromycin (2 ng ml⁻¹; Calbiochem, San Diego, CA, USA) was added to the medium for 24 h to enrich transduced cells.

Human peripheral blood mononuclear cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were donated from healthy volunteers and purified by centrifugation on Ficoll-Hypaque PLUS (GE Healthcare Life Science, Pittsburgh, PA, USA), as previously described.⁵ Briefly, PBMCs were isolated by Ficoll-Paque and washed three times with DPBS (Gibco). The purified T cells were maintained in complete culture medium supplemented with 10% fetal bovine serum and the mixed lymphocyte reaction (MLR) was undertaken within a week. The 'T cells' described in this section were human PBMCs, and these cells were used as the sources of enriched responder lymphocytes. For positive controls, T cells were treated with PHA (5 μ g ml⁻¹; Sigma, Irvine, CA, MO, USA) and/or allogeneic T cells. All volunteers involved in this study gave informed consent, and the procedures followed were approved by the institutional ethics committee.

MLR, carboxyfluorescein diacetate succinimidyl ester and cell co-cultures

A MLR assay was used to evaluate the stimulating effects of differentiated stem cells on human T-cell proliferation as previously described.⁵ Two hESCs (NTU1 and H9), two hiPSCs (iGra2 and CBiPS) and syngeneic PBMCs were used as stimulator lymphocytes. Briefly, 15-day differentiated stem cells were treated with mTOR inhibitor rapamycin (50 ng ml⁻¹, Millipore, Billerica, MA, USA) for 24 h. Meanwhile, the differentiated stem cells were transduced with lentiviruses encoding shRNA for CD24, GATA3 or the shControl. The differentiated stem cells, including rapamycin-treated and CD24 or GATA3-knockdown (KD) cells, had their further growth arrested by

the addition of 50 µg ml⁻¹ mitomycin C (Sigma) to the medium before incubating with non-stimulated effector T cells. After 5 days, human T-cell proliferation was measured by BrdU incorporation (Millipore) using a chemiluminescence-based T proliferation ELISA kit and CFSE (eBioscience CFSE) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay

Total human IL-10 and TGFβ (eBioscience) concentrations were determined using an ELISA kit according to the manufacturer's instructions. The intensity of color developed was measured using a Beckman Coulter DTX 800 microplate reader at 450 nm optical density (OD) with correction at 570 nm.

Statistics

Except for the microarray data, all statistical analyses were conducted using GraphPad Prism (version 6.01, La Jolla, CA, USA). The results are presented as the mean ± s.e.m. from at least three independent experiments. Statistical significance was determined with the nonparametric Mann–Whitney *U* (two-tailed) and one-way analysis of variance by a blinded investigator (CWY). *P* < 0.05 was considered statistically significant.

RESULTS

Differential immune-related gene expression between PSCs, their derivatives and somatic cells

Immune-related gene expression in hPSC lines NTU1 hESCs and iCFB hiPSCs was assessed by microarray analysis before and after 15 days of differentiation and compared to the hiPSCs' parental somatic cells. In a first round of analysis, we examined the differential expression of the selected immune-related genes previously characterized as being expressed in hPSCs and their derivatives present on the microarray.⁵

We observed a high number of immune gene expression differences across samples (Supplementary Figure S1). We further focused our analysis on 85 immune-related genes of the top 20 up- and downregulated genes that were identified in undifferentiated NTU1 hESCs and iCFB hiPSCs (Supplementary Figure S2A and B) and in 15-day differentiated stem cells (Supplementary Figure S2C and D) compared to the hiPSCs' parental somatic primary dermal papilla cells, primary foreskin fibroblast cells and primary granulosa cells, respectively.

Among all 85 immune genes, *CD24*, *PROM1* (*CD133*) and *TNFSF11* were consistently significantly upregulated, and *IFIT3*, *IL1R1* and *LY96* were consistently significantly downregulated in undifferentiated stem cells compared with somatic cells (Figure 1a). In contrast, in undifferentiated stem cells, the genes *CXCR4*, *GATA3* and *CD133* were consistently upregulated, and only *THBS2* was uniformly downregulated in 15-day differentiated NTU1 hESCs and iCFB hiPSCs when compared with somatic cells (Figure 1b). Similar expression patterns of the immune-related genes were observed when differentiated hPSCs were compared with somatic cells (Supplementary Figure S2 and Supplementary Tables S2A–D). The results therefore show that immune-related genes are extensively regulated in human stem cells and may correlate with differential developmental immunogenicity.

Identification of immune-related genes using the immunome database and IPA analyses

In a second round of analyses, we considered the entire microarray data set and identified the top 500 probes involving

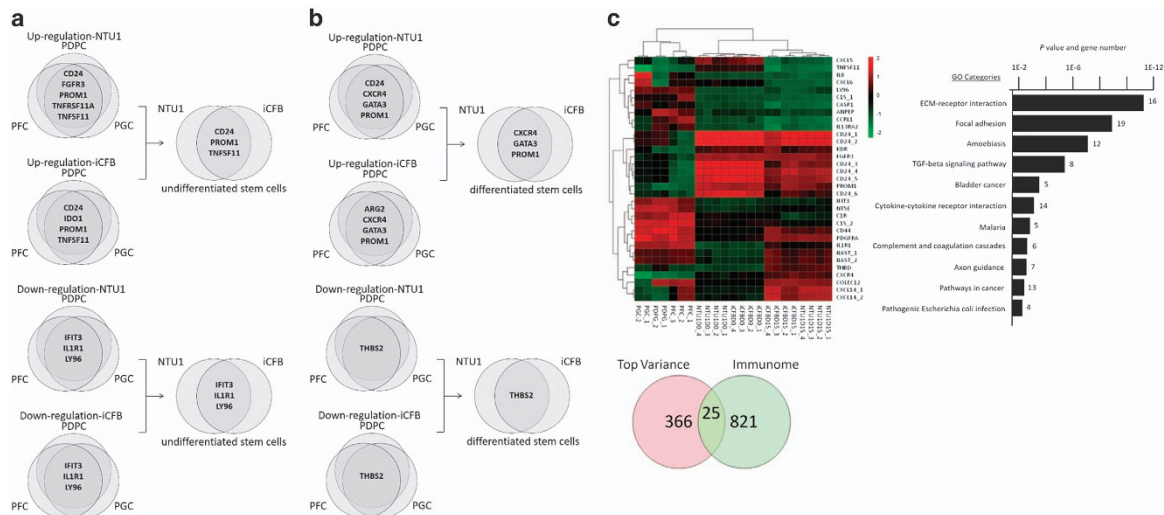


Figure 1 Venn diagrams show overlapping up- or downregulated genes in (a) undifferentiated stem cells and (b) differentiated stem cell compared with somatic cells. (c) The heat map shows that 25 immune-related genes in the Immunome database were expressed with high variance. The enriched GO terms in the 11 resulting clusters are shown. Venn diagram shows overlap with the top variance gene list. GO, Gene Ontology; iCFBD0, undifferentiated iCFB hiPSCs; iCFBD15, 15-day differentiated iCFB hiPSCs; NTU1D0, undifferentiated NTU1 hESCs; NTU1D15, 15 days differentiated NTU1 hESCs; PDPCs, primary dermal papilla cells; PFCs, primary foreskin fibroblast cells; PGCs, primary granulosa cells.

366 genes with high variance between stem cells and somatic cells (Supplementary Figure S3). We selected the top 200 probes with high variance for analysis (>2) using the Immunome database, which is the state-of-the-art reference set of genes for systems biology of the human immune system. Using these criteria, we identified 25 genes from the Immunome database list that also showed high variance between somatic cells and hPSCs (Figure 1c). In agreement with the Immunome database analyses, Gene Ontology analyses identified a significant over-representation of 11 signaling pathways, including ECM–receptor interaction, focal adhesion, amoebiasis and TGF- β signaling pathways, which are all important processes in immune function (Figure 1c and Supplementary Table S3).

To further investigate the differences in immune-related genes between somatic cells and hPSCs, the microarray data were analyzed by IPA to identify significant functional transcriptomic signatures. In experimental groups of hPSCs (NTU1 and iCFB cells), *THBS4*, *IDO1* and *CRIL* (Supplementary Table S4A) were highly upregulated, whereas *TGF β 2* and *TGF β 111* (Supplementary Table S4A) were highly downregulated in undifferentiated stem cells compared with somatic cells. However, these genes were not significantly expressed after differentiation for 15 days (Supplementary Table S4B). In contrast, *GATA3* expression was not significantly expressed in any of the hPSCs compared with somatic cells but was highly upregulated after 15 days of

differentiation (Supplementary Table S4), suggesting the crucial role of *GATA3* in the development of immunogenicity in differentiated hPSCs. Various other genes were also associated with differences between undifferentiated and differentiated stem cells and somatic cells (Supplementary Tables S5A–D). Taken together, we identified a list of 11 genes with immune-related functions that are differentially expressed between hPSCs, their derivatives and various somatic cell types: *CD24*, *GATA3*, *CD133*, *THBS2*, *LY96*, *IFIT3*, *CXCR4*, *IL1R1*, *FGFR3*, *IDO1* and *KDR*. These genes may play important roles in immunological distinctions and were therefore selected for further investigation.

CD24 is a downstream target of mTOR in differentiated stem cells

The Akt/mTOR signaling pathway has been identified as a key mediator of human immunity and has been leveraged as a therapeutic strategy in tissue transplantation by using rapamycin.^{14,15} To investigate the role of mTOR signaling in mediating the immunogenic properties of hPSC-derived cells, we examined the mRNA levels of the 11 immune-related genes identified via microarray analysis after mTOR signaling modulation. To this aim, we measured their expression levels by using quantitative PCR with reverse transcription in rapamycin-treated 15-day differentiated stem cells. CD24 mRNA was significantly reduced by rapamycin in all differentiated stem cells, including in NTU1, H9 hESCs,

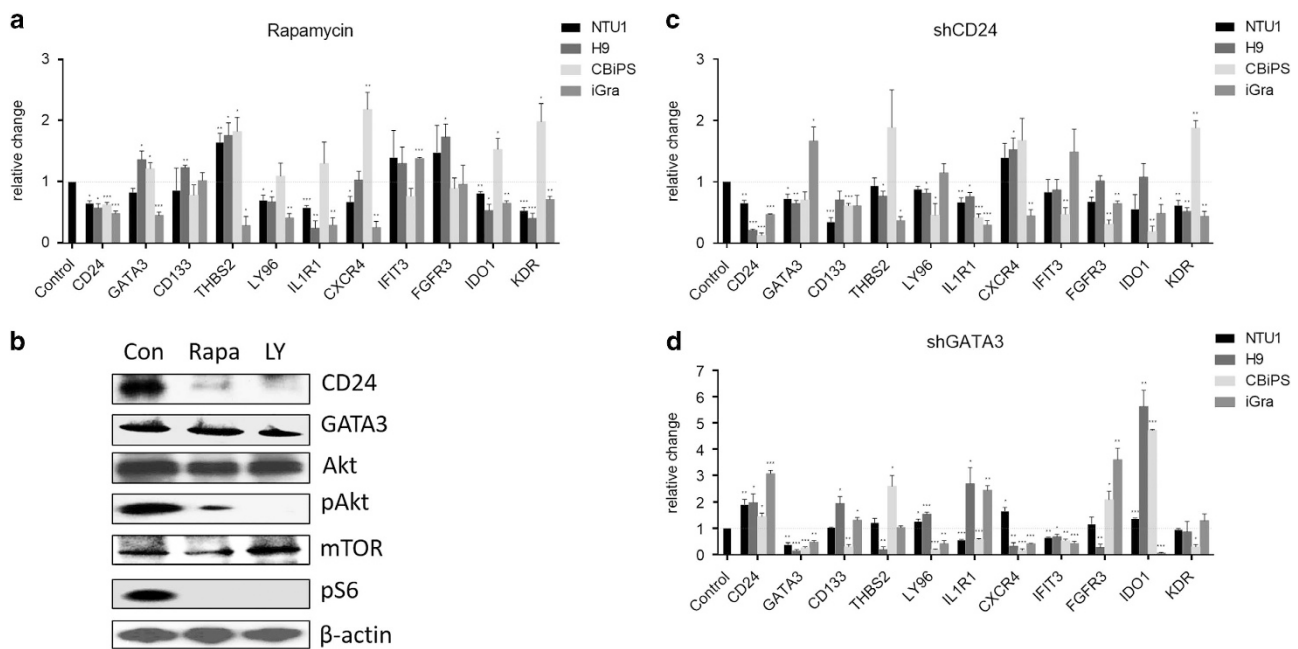


Figure 2 Alterations in mRNA levels of select genes in control and differentiated stem cells. The relative steady-state mRNA levels were determined in (a) rapamycin treatment, (c) shCD24 and (d) shGATA3 cells by real-time qPCR. (b) Differentiated stem cells were treated with rapamycin (50 ng ml⁻¹, Rapa) and LY294002 (50 μ M, LY), and cell lysates were analyzed by immunoblotting using antibodies against total and phosphorylated forms of the signaling molecules CD24, GATA3, AKT, pAKT, mTOR and pS6. Mean \pm s.e.m. is based on three to five replicate experiments using different batches of differential stem cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Human ESCs: NTU1 and H9; human iPSCs: CBiPS and iGra. ESCs, embryonic stem cells; mTOR, mammalian target of rapamycin; PSCs, pluripotent stem cells.

CBiPSCs and iGra2 hiPSCs compared to vehicle-treated controls (Figure 2a). Next, we confirmed the role of Akt/mTOR signaling pathways on CD24 and GATA3 expression at the transcript and protein levels following treatment with two pathway inhibitors. Differentiated stem cells were incubated with rapamycin and LY294002 (a PI3K inhibitor) for 24 h. As expected, phospho-Akt and phospho-S6 levels, but not total Akt and mTOR levels, were significantly diminished in rapamycin- and LY294002-treated differentiated stem cells compared with control cells, which demonstrated the specific inhibition of Akt/mTOR signaling pathways. Interestingly, CD24 protein levels were decreased in differentiated stem cells following treatment with either rapamycin or LY294002 (Figure 2b and Supplementary Figure S4). In contrast, GATA3 expression was not reduced by treatment with rapamycin or the PI3K inhibitor in differentiated stem cells (Figure 2b). Taken together, these results indicate that the Akt/mTOR pathway regulates CD24 in stem cell derivatives.

CD24 and GATA3 regulate immune genes in differentiated hPSCs

We previously showed that derivatives of hPSCs have the ability to activate lymphocytes and that different hPSCs demonstrate distinct MHC expression and immunogenicity.⁵ As shown above, CD24 and GATA3 are consistently upregulated in hPSC derivatives (Figure 1) and may thus play a role in the regulation of their immunogenicity. To test this hypothesis, we generated CD24 and GATA3-KD differentiated stem cells using lentivirus-mediated shRNA transduction and examined the mRNA expression levels of the 11 differentially expressed immune genes by quantitative PCR with reverse transcription. In these experiments, the interleukin receptor IL1R1 mRNA expression was significantly decreased in all CD24-KD differentiated stem cells compared to shRNA control (Figure 2c). The expression of interferon-induced IFIT3, a negative regulator of cell proliferation, was significantly decreased in GATA3-KD differentiated stem cells, whereas CD24 was significantly upregulated (Figure 2d). Other candidate genes were inconsistently regulated by CD24 and GATA3 in hESCs and hiPSC derivatives such as CD133 or KDR (Figure 2c and d). These data suggest that the reduction in the expression of CD24 or GATA3 leads to differential regulation of multiple immune-related genes.

Human T-lymphocyte proliferation is suppressed in CD24 and GATA3-deficient hESC and hiPSC derivatives

To further demonstrate the roles of mTOR, CD24 and GATA3 in the immunogenicity of stem cell derivatives, human T-cell responses were determined with PBMCs by the MLR assay. Colony formation assays of human lymphocyte aggregation is an *ex vivo* cellular immune assay that occurs between two allogeneic lymphocyte populations. Syngeneic lymphocyte reactions used stimulator and responder lymphocytes from the same donor (Figure 3a). In contrast, stimulator and

responder lymphocytes from different sources with unmatched HLA (Figure 3b) or following PHA stimulation (Figure 3c), and differentiated stem cells only (Figure 3d) were used in allogeneic lymphocyte reactions. To determine suppressive effects of the mTOR pathway and deficiencies of CD24 and/or GATA3, rapamycin-treated, CD24-KD and/or GATA3-KD 15-day differentiated stem cells were co-cultured with PBMCs at varying cell numbers (1×10^4 to 1×10^5 cells; Figure 3e–h). MLR analyses showed that 15-day derivatives of both hESCs (NTU1 and H9) and hiPSCs (CBiPSCs and iGra2 stem cells) induced cell number-dependent proliferation of responder lymphocytes and were comparable to stimulation by allogeneic lymphocytes (Figures 3e and 4). These data are in accordance with our previous report,⁵ and suggest potential immunogenicity of differentiated stem cells. In contrast, the activation of responder lymphocytes was reduced by rapamycin, with significant reductions at distinct cell numbers of hESCs and hiPSCs (Figure 4a). In particular, significant decreases were seen with 1×10^5 CD24-KD differentiated cells (Figure 4b) and with similar numbers of GATA3-KD differentiated stem cells (Figure 4c).

To determine whether rapamycin affects the activation or function of CD3⁺CD4⁺CD25⁺ human regulatory T cells (Treg), we screened percentages of Treg cells and expression levels of IL-10 and TGF β in rapamycin-treated 15-day H9 hESC differentiated stem cells co-cultured with human T cells. In these experiments, no significant changes in CD3⁺CD4⁺CD25⁺ Treg cell numbers were observed after the stimulation of PBMCs by differentiated stem cells (Supplementary Figure S5A). However, the production of immunosuppressive cytokines IL-10 and TGF β by PBMCs was significantly increased in response to rapamycin-treated differentiated stem cells when compared with differentiated stem cell controls (Supplementary Figure S5B and C). To further demonstrate whether the rapamycin-mediated immunogenicity suppression of differentiated stem cells is dependent on CD24 expression or on the induction of regulatory cytokines, the immune responses of differentiated stem cells were neutralized with IL-10 or TGF β antibodies in the presence or absence of CD24. Our results indicated that there was no difference in responder lymphocyte proliferation of rapamycin-treated and cytokine-neutralized differentiated stem cells compared to control cells (Supplementary Figure S5D). These results therefore suggest that both immunosuppressive cytokine IL-10 and TGF β -mediated mechanisms are involved in the differential immune responses of hPSC derivatives. Finally, synergistic suppressive effects of double CD24/GATA3-KD in the presence of rapamycin treatment were observed in differentiated H9 hESCs but not in differentiated CBiPSCs or NTU1 or iGra stem cells (Figure 5). Taken together, these data suggest that, consistent with the suppressive effect on rapamycin action, strongly reducing both CD24/GATA3 expression also resulted in a marked suppression in immunogenicity of hPSC derivatives.

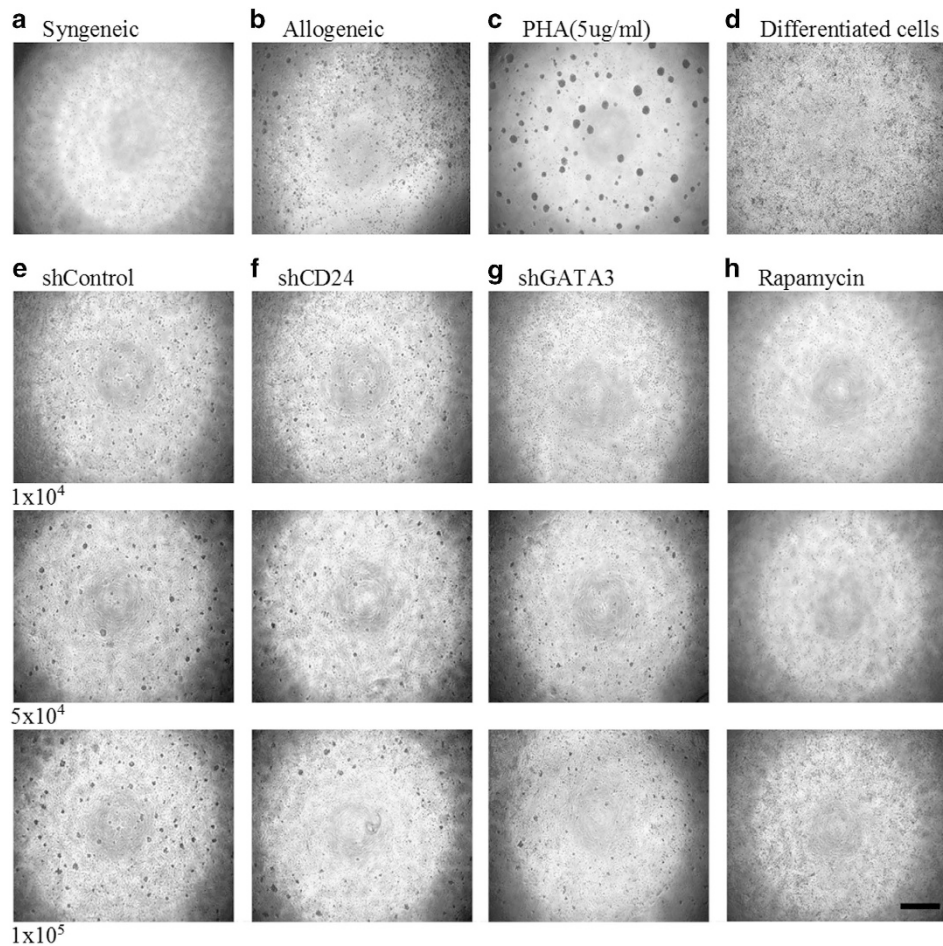


Figure 3 The colony formation shows human lymphocyte proliferation. Mixed lymphocyte reactions evaluate the actions of (a) syngeneic, (b) allogeneic (unmatched HLA) lymphocytes, (c) PHA ($5 \mu\text{g ml}^{-1}$) stimulation, (d) differentiated stem cells only, (e) differentiated stem cells of shControl, (f) shCD24, (g) shGATA3 and (h) rapamycin treatment. Scale bars, $1000 \mu\text{m}$. Human ESCs: NTU1 and H9; human iPSCs: CBiPS and iGra. ESCs, embryonic stem cells; PSCs, pluripotent stem cells.

Human T lymphocyte proliferation is suppressed in CD24 and GATA3-deficient neural lineage cells

To evaluate whether various lineages of cells derived from hPSCs expressed CD24 or GATA3, *in vitro* differentiated stem cells and teratomas were stained with CD24 or GATA3 in three germ layers (endoderm, mesoderm and ectoderm). Abundant CD24 expression was detected in cell surface membrane or cytoplasm co-localized with beta III tubulin in cells that differentiated into ectoderm to form the nervous system. CD24 was also observed in the cells of endoderm both in teratoma and *in vitro* differentiated stem cells (Figure 6a). In contrast, GATA3 was highly expressed in the nucleus and co-localized with SOX17 in the cells that differentiated into endoderm to develop the epithelial lining of multiple systems. GATA3 was detectable in all three germ layers in *in vitro* stem cell differentiation whereas the expression was very weak or not observed in ectoderm of teratoma tissue (Figure 6b). Thus, our data demonstrate that CD24 and GATA3 are expressed upon differentiation and canalization into distinct human cell lineages. To explore the potential clinical cell therapy

applications, rapamycin-treated, CD24-KD and GATA3-KD neural lineage cells were co-cultured with human PBMCs. MLR analyses showed that differentiated neural lineage cells induced cell number-dependent increases in responder lymphocyte proliferation, thus suggesting the potential immunogenicity of differentiated neural cells. However, we observed a significant decrease of responder lymphocyte proliferation in CD24-KD and GATA3-KD neural cells compared to control cells (Figure 6c). Surprisingly, no significant difference was found between the rapamycin-treated and control cells (Figure 6c). Taken together, our results indicate that reducing both CD24 and GATA3 expression resulted in a marked suppression in the immunogenicity of neural lineage cells derived from hPSCs.

DISCUSSION

Previous studies have suggested that ESCs are immune privileged in multiple species.^{1,31–33} However, lineage-specific derivatives from hESCs may remain recognizable by immune systems, and allogeneic immune rejection of hESCs by

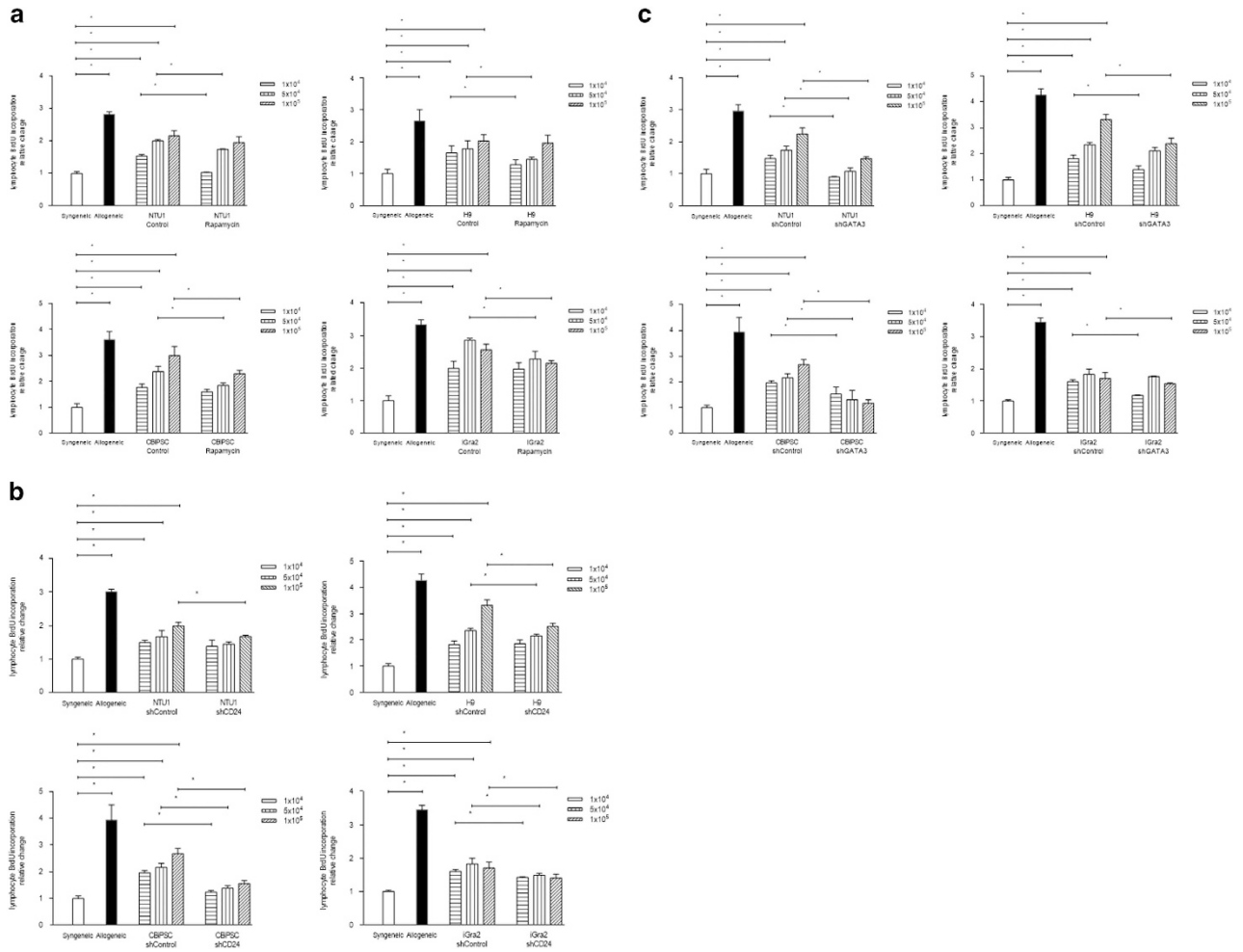


Figure 4 BrdU incorporation of human lymphocytes is measured by co-culture with human pluripotent stem cell derivatives. Proliferation of human PBMCs is suppressed by co-culture with (a) rapamycin treatment, (b) CD24-knockdown and (c) GAT3-knockdown differentiated hESCs/hiPSCs. Bar graph represents mean \pm s.e.m. from four independent experiments. *Significantly different relative proliferation when compared with syngeneic lymphocytes and control ($*P < 0.05$). Human ESCs: NTU1 and H9; human iPSCs: CBiPS and iGra. PBMCs, peripheral blood mononuclear cells

recipients is a major hurdle to clinical application.³⁴ We previously demonstrated that lower transcript levels of MHC-I, $\beta 2$ -microglobulin and non-classical MHC-I in undifferentiated stem cells are involved in the differential immune responses of hESCs compared with those in somatic cells.⁵ Thus, the development of reprogrammed autologous hiPSCs from somatic cells provides an efficient potential strategy for overcoming immune surveillance.⁹ However, various genes that are overexpressed in teratomas were originally attributed to the immunogenicity of syngeneic iPSCs, suggesting that substantial immune responses remain a drawback of syngeneic iPSC derivatives.¹⁰

To address this issue and investigate hPSC-based strategies for regenerative therapy, it is necessary to examine expression profiles of immune-related genes that regulate differential immune functions in hPSC derivatives. Accordingly, differentiated stem cells are much more suitable for clinical application as transplants than undifferentiated stem cells. Hence,

comparisons of the expression of immune genes between differentiated stem cells and somatic cells may facilitate the understanding of the ensuing therapeutic applications. Our data demonstrated that several immune-related genes are differentially expressed among human undifferentiated stem cells, differentiated stem cells, and somatic cells. In particular, several genes were up- or downregulated in somatic cells only (including *ACTB*, *CD59*, *IL6*, *LGALS1*, *CD14*, *CSTA* and *HMOX1*), in undifferentiated stem cells only (*IDO1*, *SERPINE1* and *TGF β 2*), or in differentiated stem cells only (*GATA3*, *OLR1* and *THBS2*). In addition, several immune-related genes were specifically expressed in hESCs and hiPSCs, including *CD24*, *CD133* and *TNFSF11*. Furthermore, these genes were consistently expressed in differentiated stem cells when compared with various somatic cells. In contrast, the genes *IL1R1*, *LY96* and *IFIT3* were specifically and consistently expressed at lower levels after differentiation than those in somatic cells. These results suggest that hPSC derivatives remain distinct from

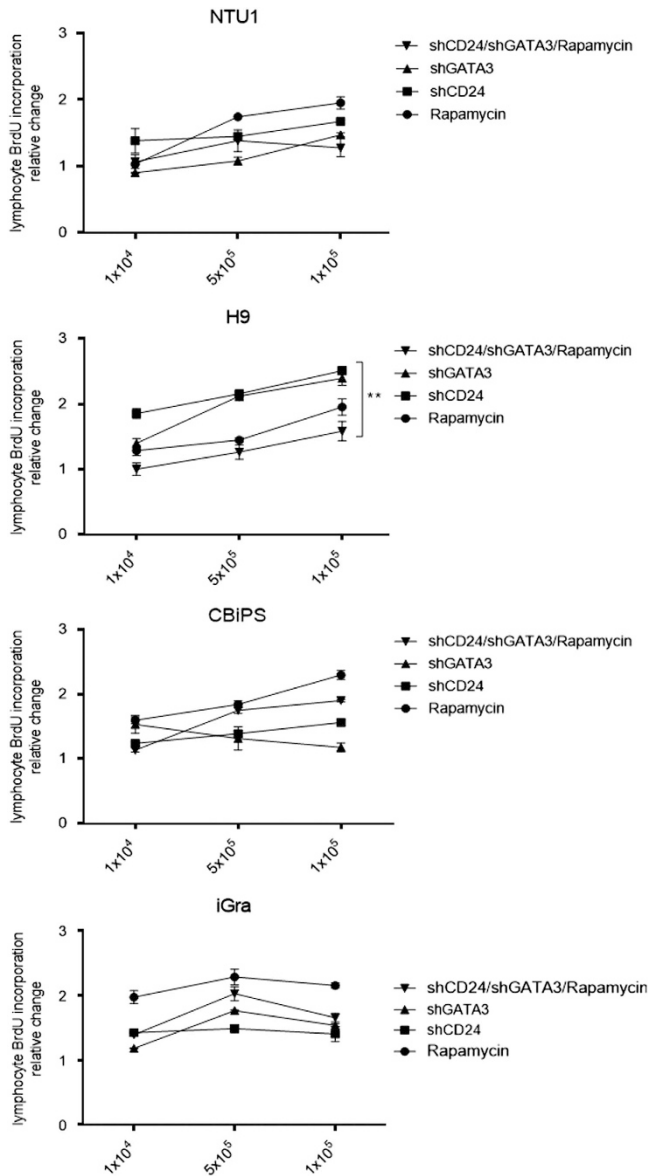


Figure 5 Human PBMCs proliferation compared to co-culture with knockdown of CD24 and/or GATA3 in the presence of rapamycin-treated hESC/hiPSC differentiated cells. The comparative proliferation of human PBMCs was measured by BrdU incorporation to hESCs of NTU1, H9, hiPSCs of CBiPS and iGra. Representative plots and mean results \pm s.e.m. from four independent experiments are shown as comparative lymphocyte proliferation (** $P < 0.01$). Human ESCs: NTU1 and H9; human iPSCs: CBiPS and iGra. ESCs, embryonic stem cells; PBMCs, peripheral blood mononuclear cells; PSCs, pluripotent stem cells.

somatic cells in terms of the gene expression that is important to immune function.^{35–38}

mTOR is a serine/threonine kinase that is present in mammalian cells as two distinct protein complexes, mTORC1 and mTORC2. mTOR consists of mTOR, mLST8 and Raptor, which is preferentially inhibited by rapamycin; this immunosuppressive agent also interferes with mTORC2 activity.³⁹ The mTOR signaling pathway is a key regulator of

cellular metabolism and is specifically involved in anabolic processes that promote cell growth and proliferation.⁴⁰ Rapamycin has also been shown to inhibit and stimulate osteogenesis in mesenchymal stem cells.^{41,42} However, the roles of mTOR in differentiation of hPSCs to other lineages remain unclear. Interestingly, transplant rejection can be selectively inhibited by low-dose rapamycin treatment.⁴³ Previous studies have shown that mTOR may regulate the generation of CD4⁺ Treg cells.^{44,45} Moreover, the mTOR pathway has been implicated in the control of macrophages and the regulation of CD24⁺ dendritic cell development.^{14,46} The glycoprotein CD24 is expressed on the surface of mature granulocytes, B lymphocytes, and differentiating neuroblasts and has been shown to regulate B-lymphocyte activation,⁴⁷ suggesting that CD24 is involved in differential immune responses. In the present study, we demonstrated that the Akt/mTOR pathway regulates CD24 in the tested hPSC derivatives, further indicating by neutralizing immunosuppressive cytokines that the potential immunogenicity of differentiated stem cells can be ameliorated by rapamycin treatment following decreased CD24 expression and/or upregulating the immunosuppressive cytokines IL-10 and TGF β (Figure 7 and Supplementary Figure S5).

We previously demonstrated the potential immunogenicity of differentiated stem cells;⁵ in the present study, an upregulation of GATA3 was consistently observed in both hESC and hiPSC derivatives, suggesting an active role of GATA3 in differentiated stem cells. GATA3 has been characterized as an important transcription factor that binds the T-cell receptor and has been associated with T-cell development, proliferation, maintenance and innate lymphoid cell activities.⁴⁸ Although the functions of GATA3 in hematopoietic lineage commitment are not completely understood, GATA3 reportedly mediates Notch signaling to promote T-cell development and inhibit B-cell development.⁴⁹ In addition to Th cell lineage, GATA3 is essential at multiple stages of CD4⁺ T-cell development and Th2 cytokine responses.⁵⁰ GATA3 deficiency has been reported to cause embryonic lethality⁵¹ and to suppress the expression of IL-4, IL-6, IL-13 and IL-10.^{52,53} Treg cells are critical suppressors of immune responses and maintain self-tolerance,⁵⁴ and previous studies have shown that GATA3 deficiency does not affect the thymic generation of Treg cells as it does in conventional CD4⁺ T cells.^{55,56} In our study, we found that GATA3 is strongly expressed in differentiated stem cells and observed reduced lymphocyte proliferation in GATA3-deficient differentiated stem cells. These findings suggest that the role of GATA3 in embryonic development may be critical in the regulation of differential immune responses, in part by harmonizing different cytokines.

The effectiveness of immune suppressant treatments for preventing immune rejection has been widely observed.⁵⁷ However, the side effects related to the toxicity and potential carcinogenesis are a significant concern in most cell-based therapies, including hPSC derivatives-based therapies. Therefore, genetic manipulation is likely to be an efficient and

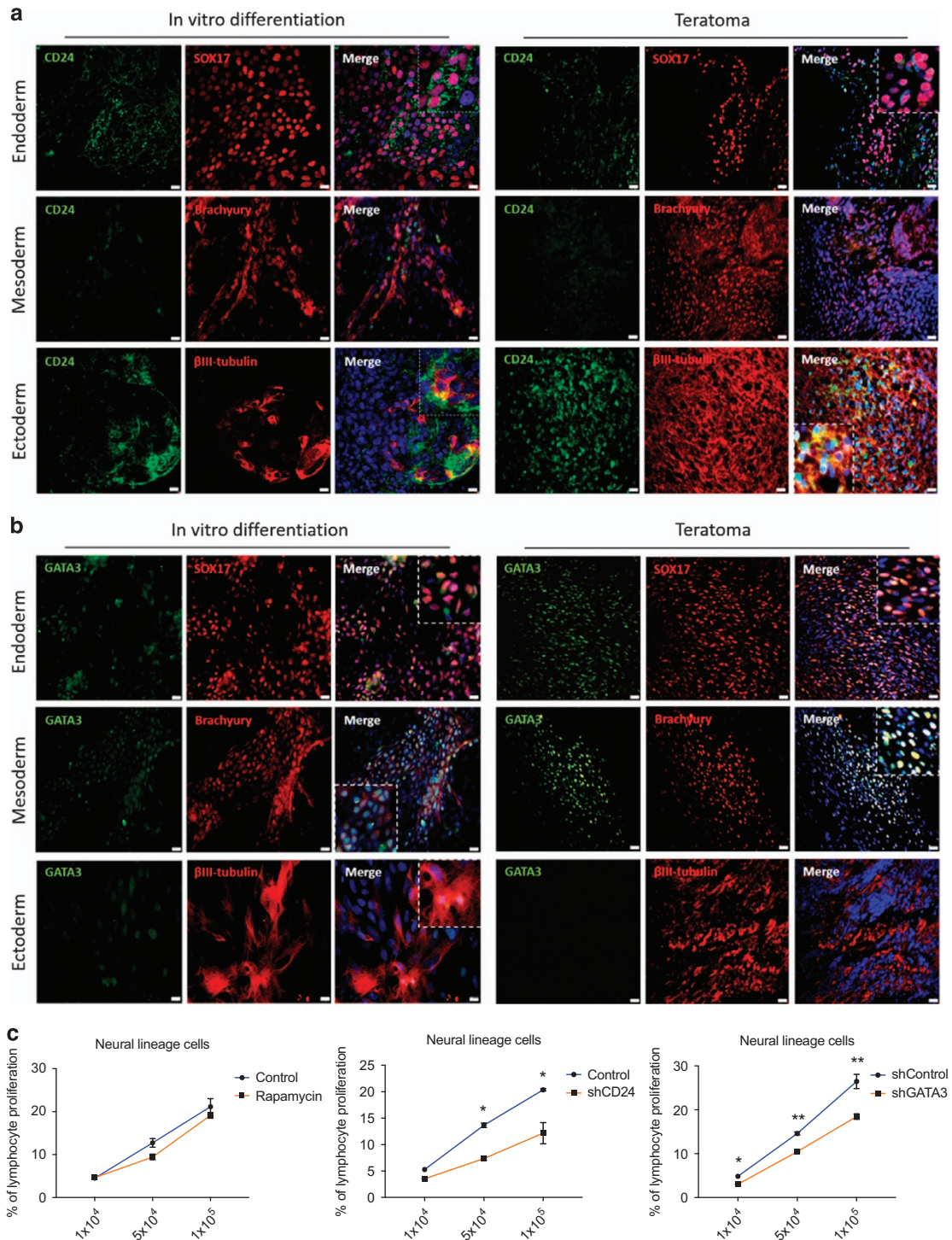


Figure 6 CD24 and GATA3 were expressed in teratoma and the *in vitro* differentiation cells. (a) CD24 was expressed in the cells of endoderm (SOX17) and ectoderm (β III-tubulin). (b) GATA3 was expressed in the cells of endoderm and mesoderm (Brachyury) in teratoma and ectoderm in *in vitro* differentiated cells. (c) The CFSE of human lymphocytes is measured by co-culture with neural lineage cells. Proliferation of human PBMCs is measured by co-culture with rapamycin treatment, CD24-KD and GAT3-KD cells. Bar graph represents mean \pm s.e.m. from three independent experiments. (* $P < 0.05$ and ** $P < 0.01$). Scale bars, 20 μ m. CFSE, carboxyfluorescein diacetate succinimidyl ester; KD, knockdown; PBMC, peripheral blood mononuclear cells.

relatively safe strategy for mitigating these issues by reducing immune responses of hPSC-derived cells without using immune suppressants.⁵⁸ We assessed whether CD24 and/or GATA3 deficiency leads to reduced immunogenicity of hPSC

derivatives using specific shRNAs. We showed that both differentiated CD24-KD and GATA3-KD stem cells resulted in significant reductions in human lymphocyte proliferation. However, our data also showed that CD24 is upregulated in

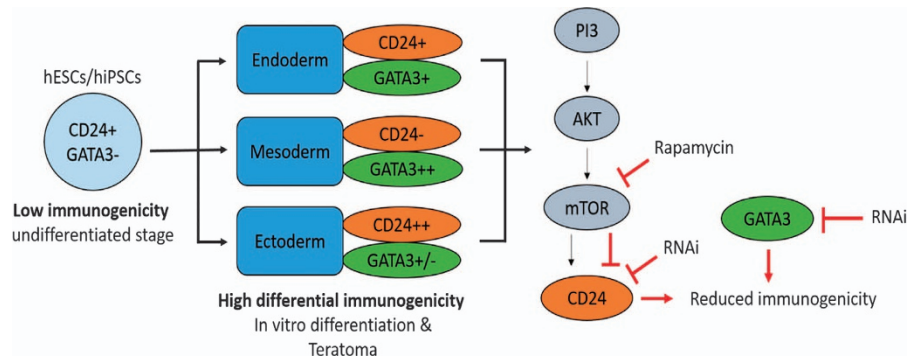


Figure 7 Schematic diagram summarizing immune genetic expression and pathways of hPSC development. Human PSCs are characterized by immune privilege and expressed CD24, but not GATA3, in the undifferentiated stage. Differential immunogenicity was generated and demonstrated by human lymphocyte reactions. The expression levels of CD24 and GATA3 were significantly increased in teratoma and differentiated stem cells of endoderm, mesoderm and ectoderm, respectively. Induction of CD24 by PI3/AKT/mTOR is a critical mechanism that underlies the immunogenic effects. The immunogenicity of differentiated stem cells expressing GATA3 is mediated by other pathways. hPSCs, human pluripotent stem cells; mTOR, mammalian target of rapamycin.

differentiated GATA3-KD stem cells, which suggests that a strategy to protect hPSC-derived cells from immune responses might require double KD of CD24/GATA3. Accordingly, the immune suppressive effects of double CD24-KD/GATA3-KD were synergistic in rapamycin-treated differentiated hESCs but were not observed in hiPSC derivatives. Taken together, these results suggest that the induction of CD24 by mTOR could be a critical mechanism that underlies the immunogenic effects of this pathway. Hence, the immunosuppressive effects of rapamycin are mediated in part by the inhibition of CD24 expression. In contrast, the immunogenicity of differentiated stem cells expressing GATA3 was mediated by other pathways independent of mTOR, thus warranting further studies of GATA3-mediated mechanisms of immunosuppression.

To determine whether various lineages of cells derived from hPSCs expressed CD24 or GATA3, we took advantage of the capability of hPSCs to form teratomas and *in vitro* differentiation, which contain cells derived from the three germ layers. CD24 was observed in cells differentiated into endoderm and ectoderm. By contrast, GATA3 was strongly expressed in cells differentiated into mesoderm and endoderm. Previous studies reported that not all iPSC-derived cells cause immune responses and that the rejection may only be observed in mesodermal cells.^{10,11} Our studies therefore open the door to the further investigation of these genes and their roles in inducing immunogenicity in specific tissues.

We leveraged these findings towards clinical application by using neural lineage cells as a model.²³ Our results showed that CD24 and GATA3 deficiency leads to a significant immunosuppression in human differentiated neural cells derived from hPSCs. Interestingly, in contrast to CD24/GATA3-deficient cells, no significant differences were observed in rapamycin-treated neural lineage cells. Immunosuppressant administration, such as rapamycin, could affect neural lineage cell fate, migration, survival and proliferation, depending on

the treatment time and dosage.⁵⁹ Taken together, our results indicate that reducing both CD24 and GATA3 expression resulted in a marked suppression in immunogenicity of neural lineage cells derived from hPSCs. Our results suggest that genetic manipulation of CD24 and GATA3 should be seriously considered as an immune tolerance strategy to effectively protect hPSC-derived grafts of the cells from allogeneic immune responses in clinical applications.

In summary, the present data demonstrate that several immune-related genes including *CD24*, *GATA3*, *PROM1*, *THBS2*, *LY96*, *IFIT3*, *CXCR4*, *IL1R1*, *FGFR3*, *IDO1* and *KDR*, are associated with differential immunological behaviors of somatic cells and hPSC derivatives. Hence, strategies employing genetic manipulation, such as KD of CD24 and GATA3, and other genes from the lists may effectively suppress immune responses after transplantation of hPSC derivatives. Further studies will be required to fully characterize the biological significance of these specific genes on immune responses using an *in vivo* system of humanized mice.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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