Molecular Medicine

Functional Analysis and Transcriptomic Profiling of iPSC-Derived Macrophages and Their Application in Modeling Mendelian Disease

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**Rationale:** An efficient and reproducible source of genotype-specific human macrophages is essential for study of human macrophage biology and related diseases.

**Objective:** To perform integrated functional and transcriptome analyses of human induced pluripotent stem cell–derived macrophages (IPSDMs) and their isogenic human peripheral blood mononuclear cell–derived macrophage (HMDM) counterparts and assess the application of IPSDM in modeling macrophage polarization and Mendelian disease.

**Methods and Results:** We developed an efficient protocol for differentiation of IPSDM, which expressed macrophage-specific markers and took up modified lipoproteins in a similar manner to HMDM. Like HMDM, IPSDM revealed reduction in phagocytosis, increase in cholesterol efflux capacity and characteristic secretion of inflammatory cytokines in response to M1 (lipopolysaccharide+interferon-γ) activation. RNA-Seq revealed that nonpolarized (M0) as well as M1 or M2 (interleukin-4) polarized IPSDM shared transcriptomic profiles with their isogenic HMDM counterparts while also revealing novel markers of macrophage polarization. Relative to IPSDM and HMDM of control individuals, patterns of defective cholesterol efflux to apolipoprotein A-I and high-density lipoprotein-3 were qualitatively and quantitatively similar in IPSDM and HMDM of patients with Tangier disease, an autosomal recessive disorder because of mutations in ATP-binding cassette transporter AI. Tangier disease-IPSDM also revealed novel defects of enhanced proinflammatory response to lipopolysaccharide stimulus.

**Conclusions:** Our protocol-derived IPSDM are comparable with HMDM at phenotypic, functional, and transcriptomic levels. Tangier disease-IPSDM recapitulated hallmark features observed in HMDM and revealed novel inflammatory phenotypes. IPSDMs provide a powerful tool for study of macrophage-specific function in human genetic disorders as well as molecular studies of human macrophage activation and polarization.

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**Key Words:** cholesterol ■ genomics ■ induced pluripotent stem cells ■ inflammation ■ macrophages

Macrophages are the most plastic cells of the hematopoietic system and represent a critical cell type at the intersection of metabolism, immunity, and cardiovascular diseases. An imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall is the hallmark of atherosclerosis. Experimental human macrophages have been mainly derived from 2 sources: tumor-derived cell lines (eg, U937, THP-1 cells) and primary cells (eg, human peripheral blood mononuclear cells [PBMC]–derived macrophage [HMDM]). The former are endowed with unlimited replicative potential but are karyotypically abnormal and phenotypically immature and do not provide the opportunity for genotype-specific studies. HMDMs are reflective of tissue macrophages and provide genotype-specific tools, but do not self-renew, and are refractory to genetic manipulation.

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Induced pluripotent stem cells (iPSCs) provide an unlimited source of subject genotype-specific cells and are a powerful platform for disease modeling, drug screening, and cell therapeutics. The differentiation of human iPSCs to macrophages (iPSC-derived macrophages [IPSDMs]) offers a powerful alternative system for using a bank of previously created genotype-specific iPSCs to derive terminally differentiated, karyotypically normal, and genetically consistent human macrophages. This approach provides a potential tool for study of macrophage-specific functions of genomic loci for human disease and novel mechanisms of macrophage biology in homeostasis, disease states, and in therapeutic translation. However, there is some uncertainty as to the functional and molecular fidelity of iPSC-differentiated macrophages from iPSC. We show that IPSDMs, like HMDM, can be polarized in vitro to functionally and molecularly distinct M0 (nonpolarized macrophages), M1, and M2 subtypes, and we demonstrate that IPSDMs are comparable with their primary isogenic counterparts by phenotypic, functional, secretome, and transcriptional profiling. Finally, we use IPSDM from subjects with Tangier disease (TD) to demonstrate the fidelity of IPSDM, relative to primary HMDM, for gene and disease-specific macrophage functional studies in humans.

Methods

Supplemental information includes Online Methods, Online Figures I to IV, and Tables I to XXXIX.

PBMC to Macrophage Differentiation (HMDM) and Polarization

PBMCs collected using BD VACUTAINER Mononuclear Cell Preparation Tube were cultured in macrophage culture media, 20% fetal bovine serum in RPMI 1640 media with 100 ng/mL human macrophage colony-stimulating factor (M-CSF), for 7 days on BD Primaria tissue culture plate to induce macrophage differentiation. Polarization was obtained in the presence of M-CSF by 18- to 20-hour incubation with 20 ng/mL interferon-γ and 100 ng/mL lipopoly saccharide for M1-like polarization or 20 ng/mL interleukin (IL)-4 for M2-like polarization as we described.

Subject-Specific iPSCs Derivation, Culture, and Maintenance

All human protocols for this work were approved by the University of Pennsylvania Human Subjects Research Institutional Review Board. Generation and characterization of subject-specific PBMC-derived iPSCs using Sendai viral vectors were performed by the iPSC Core Facility at Penn’s Institute of Regenerative Medicine as described.

Differentiation of iPSCs to Macrophages (IPSDM)

Embryoid bodies were generated by culturing small aggregates of feeder-depleted iPSCs in Corning ultralow attachment multwell plate in StemPro-34 media supplemented with different cytokine cocktails as summarized in Online Table I. Since D8, macrophage culture media was used to enrich for myeloid precursors. At D15, single cells were transferred to BD Primaria tissue culture plate for expansion and maturation.

Cholesterol Efflux

HMDM and IPSDM were labeled by 6 μCi [3H]-cholesterol for 24 hours, followed by 14- to 16-hour equilibration with or without treatment of the liver X receptor agonists, 10 μmol/L 9-cis-retinoic acid (9cisRA), and 5 μmol/L 22-hydroxycholesterol (22OH), that up-regulate ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1). In some experiments, polarization was obtained during equilibration to determine the effects on cholesterol efflux capacity. Efflux to apolipoprotein A-I (apoA-I; 10 μg/mL) or high-density lipoprotein-3 (HDL3; 25 μg/mL) was performed for 4 hours. Efflux was presented as the percentage of counts recovered from the medium in relation to the total counts (sum of medium and cells), with subtraction of efflux to medium with no acceptors, which was comparable among groups.

RNA-Seq Library Preparation and Sequencing

RNA samples were extracted using All Prep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA). With a minimum of 300 ng input RNA, libraries were prepared using the TrueSeq RNA Sample Preparation Kit (Illumina, San Diego, CA), followed by 100 bp paired-end sequencing on an Illumina’s HiSeq 2000 as we described.

Alignment of RNA-Seq Reads

As we described, RNA-Seq data were aligned to the hg19 reference genome using STAR 2.5.0a with default options. Analyses were based on filtered alignment files. Mapping statistics are summarized in Online Table II. RNA-Seq data are available from the NCBI Gene Expression Omnibus under the accession numbers GSE55536.

RNA-Seq Data Analysis and Bioinformatics

Transcript abundance was measured in fragments per kilobase of transcript per million fragments mapped (FPKM) using Cufflinks 2.1.1. Differential expression (DE) was tested with cuffdiff, using the RefSeq annotation. Genes with a false discovery rate (FDR)-adjusted P value <0.01 and a fold change (FC) >2 were considered differentially expressed. Multidimensional scaling was done with Euclidean distance based on log2 FPKM+0.1 in R programming languages. To visualize the overall sample to sample relationship within the data set, we performed coregulation analysis based on Pearson correlation coefficients using BioLayout Express3D. FPKM values were normalized using method from Anders and Huber separately for M0 (nonpolarized macrophages), M1, and M2 samples in HMDM and IPSDM. Normalized FPKM values were transformed by log2 (FPKM+0.1) on heat map. Heat maps illustrating expression patterns of DE genes were generated using ggplot2 in R.

Statistical Analysis

Data were analyzed with GraphPad Prism 6 (GraphPad Software, San Diego, CA) and were presented as mean±SD. Statistical differences between groups were determined using Mann–Whitney test and
Wilcoxon tests because nonparametric tests are more appropriate for small sample sizes. For analysis of gene ontology (GO) pathways in RNA-Seq data, significant enrichment was declared at FDR-adjusted \( P \) values < 0.05 using Benjamini and Hochberg method.\(^{16}\) Enrichment analysis was performed in BiNGO plugin using Biological Process category and visualized in Cytoscape.\(^{19}\)

**Results**

**Generation of Subject-Specific iPSCs**

Subject demographics of PBMC-derived iPSC lines used in this study are listed in Online Table III. As shown in Online Figure I, iPSCs expressed typical pluripotency markers, maintained a normal karyotype, and exhibited silencing of exogenous transgenes beyond passage 10.

**Efficient Differentiation of Human iPSCs to Macrophages**

We established a stepwise protocol for efficient IPSDM differentiation (Figure 1). With this approach, commitment to hematopoiesis was achieved at stage 2 in serum-free media containing different cytokine cocktails (Figure 1; Online Table I). At D8, single cells emerged from the culture exhibited >90% of CD43\(^+/\)CD34\(^-\) hematopoietic cells. Around 40% of single cells expressed CD41 and CD235 and possessed restricted erythromegakaryocytic potential but CD45\(^+/\)CD18\(^-\) myeloid progenitors remained absent. From D8 to D15 in RPMI+ 20% fetal bovine serum+100 ng/mL M-CSF, a significant amount of CD45\(^+/\)CD18\(^-\) myeloid progenitors emerged from the culture, and the CD41\(^+/\)CD235\(^-\) lineage was lost. Single cells in suspension were harvested at D15 and were plated for adherent culture at \( \approx 0.1 \) to \( 0.15 \times 10^6 \) per well of 6-well plate for macrophage maturation. From D15 to D22, CD45\(^+/\)CD18\(^-\) myeloid cells showed progressive maturation toward mature macrophages assessed by morphological changes (Figure 1). This protocol produces an almost pure (>95% CD45\(^+/\)CD18\(^-\)), high-yield macrophage population (>2x10\(^7\) of CD45\(^+/\)CD18\(^-\) differentiated macrophages per 6-well plate of confluent iPSCs) and is highly consistent across the multiple lines/clones.

IPSDM characteristics (Figure 2A and 2C) recapitulated those of primary HMDM (Figure 2B and 2D). Specifically, IPSDM exhibited macrophage-like morphology (both by phase-contrast imaging and May-Grünwald-Giemsa staining), phagocytosis of Alexa Fluor 594-labeled zymosan particles, uptake of Dil-label acetylated-low density lipoprotein. Immunofluorescence staining also demonstrated expression of CD68 and MCP-1 (monocyte chemoattractant protein-1) in IPSDM (Figure 2A). Flow cytometry revealed that IPSDM acquired expression of myeloid/macrophage
markers CD18, CD11b, CD11c, CD14, CD16, CD115, and other well-defined macrophage markers (CX3CR1 and CCR2). Importantly, neither IPSDMs (Figure 2C) nor HMDMs (Figure 2D) were positive for markers of dendritic cells (CD1a and CD83), T lymphocytes (CD3), or B lymphocytes (CD19), indicating the specificity in myelomonocytic development.

Taken together, these results showed that IPSDM and HMDM shared comparable morphological and phenotypic characteristics and that our protocol for IPSDM differentiation to macrophages is consistent, efficient, and scalable.

**Human IPSDMs Share Functional Characteristics With HMDM**

Macrophages are plastic and efficiently respond to a variety of stimuli, which result in a spectrum of macrophage phenotypic modulation that is observed in vivo during physiological and pathological stresses. Within this spectrum, the classical M1-associated stimuli, lipopolysaccharide, and interferon-γ, as well as M2-associated stimulus, IL-4, represent the stereotypic extremes of M1 and M2 axis in macrophage activation, with the general perception that M1 macrophages are inflammatory and M2 macrophages promote tissue repair and metabolic homeostasis. In this study, HMDMs and IPSDMs were polarized to M1 and M2 states as shown in Figure 3A.

Macrophage phagocytic activity is an essential early function in tissue remodeling and clearance of pathogens and dying cells in infection and inflammation. Using the CytoSelect Phagocytosis Assay kit (Cell Biolabs), we found that IPSDMs were highly phagocytic, showing comparable engulfment of enzyme-labeled zymosan prepared from yeast as that of HMDM.
derived from the same subject (Figure 3B, control-6, white, male. Relative to nonpolarized and M2-IPSDM and M2-HMDM, both M1-IPSDM and M1-HMDM had reduced (=50%) phagocytic capacity (Figure 3B) consistent with reports that lipopolysaccharide treatment inhibits phagocytosis in human macrophages.21

Efflux of cellular cholesterol to lipoprotein acceptors reduces macrophage cholesterol accumulation in arterial neointima limiting atherosclerosis progression and promoting disease regression.2 Efflux pattern of [3H]-labeled cholesterol to both apoA-I and mature HDL3, in vivo acceptors for macrophage free cholesterol via ABCA1 and ABCG1, respectively, was almost identical in IPSDM and HMDM of the same subject (Figure 3C, control-6). Cholesterol efflux capacity has not been reported in human polarized macrophages. During polarization, ABCA1 and ABCG1 mRNA were markedly upregulated in M1-HMDM (=6- and 20-fold, respectively) and significantly reduced in M2-HMDM (Figure 3C). Although not statistically significant using nonparametric Wilcoxon tests in our small sample (n=4 subjects), M1-HMDM showed clear patterns of increased efflux to apoA-I (by ≈2-fold; P=0.12) and HDL3 (by ≈40%; P=0.12), whereas M2 macrophages had small trends toward reduced cholesterol efflux to apoA-I (Figure 3C). IPSDM resembled HMDM in polarization-induced change in ABCA1/ABCG1 mRNA expression and cholesterol efflux (Figure 3C). The mechanism of polarization-related changes in ABCA1 and ABCG1 expression is not fully elucidated, but previous literature has reported that lipopolysaccharide induced ABCA1 expression through liver X receptor–dependent mechanisms in THP-1 monocytes.21 These findings support fidelity of IPSDM, relative to primary HMDM, in important macrophage cholesterol metabolic functions.

Basal and polarization-dependent secretion of cytokines and chemokines was determined in culture media (Figure 3D and 3E) using semiquantitative human cytokine array (R&D, Minneapolis, MN). Isogenic M0-IPSDM and M0-HMDM showed remarkably similar secretome profiles (Figure 3D, left). Polarization to M1-IPSDM resulted in secretion of multiple cytokines, for example, IL-6, IP-10 (interferon gamma-induced protein 10), RANTES, and tumor necrosis factor-α, in a pattern almost identical to that in M1-HMDM (Figure 3D, middle). In contrast to M1, secretome profile of M2-IPSDM, which mirrored that in M2-HMDM, differed little from their nonpolarized macrophage counterparts (Figure 3D, left versus right). These secretome findings were generalizable across HMDM and IPSDM of 5 age/sex-matched subjects shown by heat map in Figure 3E.

Overall, these studies suggest secretome, phagocytic, and efflux similarities between IPSDM and HMDM in both nonpolarized and polarized states and support a novel finding of loss of phagocytosis potential but increase in ABCA1/ABCG1 expression and cholesterol efflux capacity during M1 polarization (Figure 3B and 3C).

**Human IPSDMs Share Transcriptome Characteristics With HMDM**

An important issue in the field is to what extent somatic cells differentiated from iPSCs lose hallmarks of pluripotent iPSCs and adopt the transcriptomic characteristics of the somatic cells. To address this question in our IPSDM, we performed RNA-Seq (≈130 million reads per sample, 95% mapping rate to the reference genome, ≈70% reads uniquely mapped and filtered; see Online Table II) and compared transcriptome profiles of IPSM, M0-IPSDM, M1-IPSDM, and M2-IPSDM (including duplicate clones for individual iPSC lines) as well as M0-HMDM and M1-HMDM and M2-HMDM of 3 healthy individuals control-1, control-3, and control-4 (Figure 4A for schematic experimental design and Online Table III for subject demographics).

**Marked Transcriptome Changes During iPSC Transition to IPSDM**

Multidimensional scaling (Figure 4B) and coregulation analysis (Figure 4C) revealed that IPSDM had different transcriptome profiles relative to their precursor iPSC but displayed similar profiles to that of isogeneic HMDM. Differentiation to IPSDM had profound effects on the global transcriptome. DE analysis ( cuffdiff) revealed 6305 DE genes (3470 upregulated, 2835 downregulated, FDR-adjusted P<0.01, FC >2; Figure 4E). IPSM to IPSDM transition resulted in 100 to >4000-fold decreased expression of key pluripotency genes including LEFTY1, DNMT3B, and LIN28a (Online Table V), with a concomitant increase in the expression of macrophage genes including CD14, CD33, CD68, and CCL2, which were induced at levels comparable with that observed in HMDM (Figure 4D; Online Table IX). Analysis of upregulated genes showed an over-representation of expected GO terms using BiNGO plugin22 associated with immune response, defense response, response to wounding, and inflammatory response (Figure 4E; Online Table VIII).

**Transcriptome Similarities Between HMDM Versus IPSDM**

At >1 percentile FPKM expression level, we detected 13418 expressed genes in HMDM and 13471 genes in IPSDM (Figure 4F) with significant overlap (=98%) and strong correlation (Pearson r=0.85). DE analysis of HMDM versus IPSDM revealed 89% genes to have similar expression (11948 non-DE genes out of a total number of 13331 genes expressed in both HMDM and IPSDM; Online Table IX) with ≈12% of genes (1610) to be differentially expressed (FDR-adjusted P<0.01, FC >2). Of the 1610 DE genes, 1019 genes had higher expression in IPSDM, whereas 591 genes had higher expression in HMDM (Figure 4F; Online Tables X and XI). Genes expressed at higher levels in IPSDM include typical fibroblast markers (PDGFRα, PDGFRβ, LOX, THY1, FGFI, TIMP1, TIMP3, ZEB1, and CDH2), and several genes that encode collagen and extracellular matrix (COL1A1, COL1A1, COL1A1, COL1A2, and DCN), but importantly, no markers of undifferentiated mesenchymal stem cells, for example, GNL3 (Online Table X). Of 591 genes with lower expression in IPSDM, 100 (20.5%) belonged to the immune response GO term (FDR=8.64E−36 for enrichment); Online Table XIII). This group of genes included several members of human leukocyte antigen (HLA) system corresponding to major histocompatibility complex (MHC) protein complex, as well as a few of established M1 polarization markers. Importantly, although expressed at lower levels in M0-IPSDM versus M0-HMDM, most of these chemokines/cytokines (eg, CCL5, CXCL9, and CXCL10) and HLA genes (eg, MHC class I, HLA-A, HLA-B, HLA-C, etc) were markedly upregulated during M1-polarization of IPSDM to levels comparable with that in HMDM-derived M1 lines (Online Table IV).
Figure 3. Functional phenotypes in response to M1 (lipopolysaccharide [LPS]+interferon [IFN]-γ) and M2 (interleukin [IL]-4) polarization are comparable between induced pluripotent stem cell–derived macrophages (IPSDMs) and human peripheral blood mononuclear cell–derived macrophages (HMDMs). A, Schematic protocol for HMDM and IPSDM polarization. B, M1-HMDM and M1-IPSDM showed lower phagocytosis capacities (n=7 age/sex-matched subjects). HMDM and IPSDM derived from the same subject (control-6) have comparable phagocytosis capacities (data represent mean of triplicate experiments). C, ABCA1 and ABCG1 mRNA were upregulated in M1-HMDM and suppressed in M2-HMDM. M1-HMDM showed a clear pattern of increased efflux to apolipoprotein (apo) A-I and high-density lipoprotein-3 (HDL3). IPSDM resembled HMDM in polarization-induced change in ABCA1/ABCG1 mRNA expression and cholesterol efflux. Each dot in the plots represents 1 subject. In addition, HMDM and IPSDM derived from control-6 showed comparable efflux levels. D, Representative cytokine array determining the relative level of 36 human cytokines and chemokines in culture media. HMDM and IPSDM derived from 5 age/sex-matched subjects are quantified by Image J and visualized. IPSD indicates induced pluripotent stem cell; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; and TNF, tumor necrosis factor.
Figure 4. RNA-Seq transcriptome analysis of induced pluripotent stem cells (iPSCs), iPSC-derived macrophages (IPSDMs) and human peripheral blood mononuclear cell-derived macrophages (HMDMs). A, Schematic figure of RNA-Seq design and data analysis (n=3 subjects, control-1, control-3, and control-4, with 2 iPSC clones per subject). B, Multidimensional scaling (MDS) and (C) coregulation analysis (CRA) confirmed the distinct transcriptome profile of iPSC from HMDM and IPSDM. M1 polarization profoundly affects the transcriptional profile, whereas M2 polarization results in more subtle expression changes. D, IPSDM showed expression of macrophage markers and the absence of markers of pluripotency and other hepatopoietic cells. E and F, The total number of genes (fragments per kilobase of transcript per million fragments mapped [FPKM] > 1% expression of all genes) and differential expression (DE) genes in iPSC vs IPSDM, and IPSDM vs HMDM. The top 10 gene ontology (GO) terms ranked by false discovery rate (FDR)-adjusted P values for DE genes are illustrated. The size of the circle is inversely correlated to FDR-adjusted P values of enrichment in the respective GO term. The thickness of the lines corresponds to similarity between 2 GO terms. Scatterplot (F) suggests the strong Pearson correlation (r=0.85) of transcriptome between HMDM and IPSDM. Green, DE genes with higher expression in M0-HMDM; red, DE genes with higher expression in M0-IPSDM; and blue, non-DE genes. CEACAM8 indicates carcinoembryonic antigen-related cell adhesion molecule 8; DNMT3B, DNA (cytosine-5)-methyltransferase 3 beta; EPCAM, epithelial cell adhesion molecule; LEFTY1, left-right determination factor 1; MS4A1, membrane-spanning 4-domains, subfamily A, member 1; NCAM1, neural cell adhesion molecule 1; NK cells, natural killer cells; and POU5F1, POU domain, class 5, transcription factor 1.
Transcriptome Changes During M1 or M2 Polarization in HMDM and IPSDM

Multidimensional scaling and coregulation analysis (Figure 4B and 4C) demonstrated that polarization to M1-type macrophages markedly altered the transcriptional profile of both IPSDM and HMDM precursors. DE analysis in M0-HMDM versus M1-HMDM and M0-IPSDM versus M1-IPSDM revealed 1931 and 1643 upregulated DE genes, respectively (FDR-adjusted P<0.01, FC >2; Online Tables XIV and XV), with marked overlap between HMDM and IPSDM during M1 polarization (1114 genes; Online Table XVI). Among these overlapped DE genes were key prototypic markers of M1 polarization, such as surface molecules CD86, the cytokine/chemokine genes, CXCL9, CXCL10, TNF, CCL5, IL6, and IL8, and genes encoding intracellular protein GBP1 to GBP5, which were inducible by interferon-γ (Online Table XVI). Consistent with our cytokine and chemokine secretion data (Figure 3D and 3E) and earlier microarray data sets showing modest difference between IL-4-derived M2-HMDM and their M-CSF differentiated nonpolarized HMDM precursors,23 RNA-Seq of M2-HMDM and M2-IPSDM revealed subtle transcriptomic changes during M2 polarization of HMDM and IPSDM (Figure 4B and 4C; Online Tables XXII and XXIII), suggesting that M-CSF-differentiated macrophages are already shifted toward the M2 transcriptome and phenotype. Yet, the overlapping DE genes (n=85) during M2-HMDM and M2-IPSDM polarization did include several classic M2 polarization markers, such as the Fj3A1, Clec4a4, and Mrc1 (Online Table XXIV). Scatterplots of Log2 FC of DE genes revealed a slightly stronger correlation between HMDM and IPSDM in M1 polarization (r=0.80; Figure 5A), than that in M2 polarization (r=0.71; Figure 5B). To eliminate noise from genes expressed at low levels and genes with small or nonsignificant FC, only genes with FPKM >1% expression and also defined as DE genes in either HMDM or IPSDM were included in the analysis (refer to Online Tables XXX–XXXV for the list of genes).

Known and Novel Macrophage Polarization Markers

We found higher expression of 1790 and 1623 genes (Figure 5C; HMDM and IPSDM, respectively; FDR-adjusted P<0.01, FC >2) in M1 versus M2 macrophages with 1082 overlapped genes (Online Table XXXVI). GO analysis of these DE genes (Online Table XXXVIII) showed enrichment for genes involved in immune response, defense response, inflammatory response, and response to wounding, suggesting the expected upregulation of host defense and inflammatory response programs in M1 polarization (Figure 5C). In contrast, 2072 and 1552 genes (Figure 5D) were identified as being elevated in M2 versus M1 macrophages with 1221 overlapped genes (Online Table XXXVII). Representative GO terms (Online Table XXXIX) include oxidation reduction, cellular respiration, and small molecule metabolic process, suggesting distinct metabolic phenotypes in M2-polarized versus M1-polarized macrophages (Figure 5D). Heat map visualization of well-established M1- and M2-like macrophage markers showed similar expression profile in both HMDM and IPSDM (Figure 5E). We also identified potential novel markers for M1 and M2 polarization by ranking the absolute value of Log2 FC of genes with FPKM > 5% expression in M1 versus M2-HMDM and M1 versus M2-IPSDM, and the common top DE genes were classified by known functional categories and visualized by heat map (Online Figure II).

Overall, RNA-Seq revealed distinct transcriptome profile of iPSC from HMDM and IPSDM, suggested that M1 polarization profoundly affects the transcriptional profile, whereas M2 polarization results in more subtle expression changes, confirmed known markers for M1 and M2 macrophage polarization, and identified novel genes related to specific polarization programs that were common to both HMDM and IPSDM.

IPSDMs Reproduce Macrophage Cholesterol Defects in TD

Loss of function mutations in the ABCA1 transporter underlies the rare Mendelian disorder TD.24 Generation of iPSC from patients with TD provides an opportunity to examine the reproducibility of the TD phenotypic defects in IPSDM and to probe additional functional impact of ABCA1 deficiency in the IPSDM system. We recruited 2 TD individuals, TD-1, compound heterozygote at S2046R/K531N, and TD-2, homozygous for the E1005X/E1005X truncation mutation. The sibling of TD-1, heterozygous at K531N (hetero-1), was recruited as a family comparator. Age, race, and sex-matched healthy subjects, control-1 for TD-1, and control-2 for TD-2 were also recruited.

IPSDM and HMDM of patients with TD had similarly abolished cholesterol efflux to apoA-I and equally impaired efflux to HDL₃ (Figure 6A). The heterozygote ABCA1 mutation carrier had an intermediate defect in cholesterol efflux consistent with the presence of 1 functional allele (Figure 6A). Despite small sample sizes (n=3 replicates for TD-1/hetero-1/control-1 HMDM), it is apparent that efflux to apoA-I in TD-HMDM was completely abolished (Figure 6A, upper). Further, liver X receptor agonists upregulated efflux in both hetero-1 and control-1 HMDM but failed to induce any cholesterol efflux of TD-1 HMDM (Figure 6A, upper). Relative to control-2 IPSDM, the cholesterol efflux defect to apoA-I and HDL₃ in TD-2 IPSDM (Figure 6B) was almost identical to that observed in TD-1 studies and was consistent in longer efflux studies (20 hours) and with higher concentration of HDL₃ (50 μg/mL; Online Figure III). Thus, IPSDMs recapitulate the key cellular defect in TD macrophages in a highly reproducible manner.

ABCA1 Deficiency in Human IPSDMs Leads to Increased Inflammatory Response

In addition to its canonical role in mediating cholesterol efflux and reverse cholesterol transport, ABCA1 has been implicated in the interface of inflammation and cholesterol metabolism. Indeed, peritoneal macrophages isolated from macrophage-specific Abca1⁻/⁻ mice are hypersensitive to lipopolysaccharide stimulus and showed higher expression of inflammatory genes, including Il1b, Il6, and Tnf.25 Macrophage-specific Abca1⁻/⁻ Abcg1⁻/⁻ bone marrow–transplanted Ldlr⁻/⁻ mice after 10 weeks of Paigen diet showed increased inflammatory gene expression in macrophages in atherosclerotic lesions.26 However, data on inflammatory response in human ABCA1 deficiency are lacking. Because of the limited availability of blood samples from subjects with rare TD, TD-IPSDMs provide a unique opportunity to study novel phenotypes in human ABCA1-deficiency. In lipopolysaccharide-primed (100 ng/mL for 4 hours) macrophages, inflammatory gene expression (IL1B, IL8, TNF, and...
CCL5) was increased in both HMDM and IPSDM of control and TD subjects, but with a markedly greater response in TD-HMDM and TD-IPSDM relative to their controls (Figure 6C). In contrast, baseline expression of IL1B, IL8, and TNF, but not CCL5, in TD-HMDM was only marginally increased compared with control-HMDM and IPSDM. Only genes with fragments per kilobase of transcript per million fragments mapped >1% expression and also defined as DE genes in either HMDM or IPSDM were plotted. Genes with the same direction of change were highlighted in red for upregulation and green for downregulation. Genes with opposite direction of change were highlighted in purple. The DE genes of M1 vs M2 are separated into those expressed at higher levels in M1 (C), or in M2 (D). Venn diagrams of the number of common DE genes in HMDM and IPSDM, and gene ontology (GO) enrichment analysis of these common DE genes are performed and the top 10 GO terms ranked by false discovery rate–adjusted P values are visualized. E, The heat maps depict expression profiles of subsets of well-established macrophage polarization markers for selected M1-enriched (top) and M2-enriched (bottom) genes in HMDM and IPSDM. TNF indicates tumor necrosis factor.

Discussion

Despite a substantial appreciation for the dual function of macrophages in innate immunity and lipid metabolism, understanding of human macrophage biology has been hampered by the lack of reliable and scalable models for cellular and genetic studies. IPSDM, as an unlimited source of subject genotype-specific cells, not only can shed light on the molecular physiology of macrophages in complex cardiometabolic disorders but also permit deeper insights into consequences of rare genetic variants associated with monogenic Mendelian disorders, where primary samples are often not readily accessible. In the current work, we combined human iPSC technology, RNA-Seq, and a Mendelian genetic disorder to gain new insights into the application of IPSDM in cell-specific functional disease modeling of human genetic disorders and in human macrophage biology. Using human iPSCs derived by our Sendai virus reprogramming protocol, we present (1) a relatively simple protocol that uses fewer cytokines for rapid, high-throughput generation of...
IPSMD of high purity and functional homogeneity, (2) comparable morphological, functional, and transcriptome profiles in IPSMD and HMDM, (3) remarkably similar in vitro functional plasticity and polarization in phagocytosis and cholesterol efflux capacity and inflammatory cytokine secretion in IPSMD and HMDM, (4) almost identical hallmark cholesterol metabolism phenotypes in IPSMD and HMDM of patients with TD, and (5) novel insights via IPSMD into macrophage inflammatory response in human ABCA1 deficiency. Thus, our IPSMD system paves the way toward large-scale applications in functional, disease, and therapeutic modeling of macrophages in innate immunity and cellular cholesterol homeostasis in human.

We present the first high-resolution transcriptome map of isogenic HMDM and IPSMD during differentiation and polarization. These data highlight the fidelity of our IPSMD but also reveal challenges as well as opportunity for application to complex mechanistic studies. First, IPSMD lost expression of pluripotency markers, had remarkably distinct gene expression profiles relative to precursor iPSCs, and had similar gene expression as HMDM. Second, although our protocol yields \( \approx 95\% \) pure CD45+/CD18+ myeloid cells, a CD45-negative population accounting for \( \approx 5\% \) of the differentiated cells may have contributed to a minor difference in gene expression between HMDM and IPSMD. This might impact the use of IPSMD in macrophage modeling of certain genes.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Tangier disease (TD)-induced pluripotent stem cell–derived macrophages (IPSDMs) recapitulate hallmark phenotypes of impaired cholesterol efflux and reveal novel inflammatory phenotype in TD-IPSDM. **A**, In human peripheral blood mononuclear cell–derived macrophages (HMDMs), efflux of \([\text{H}]\)-labeled cholesterol to apolipoprotein (apo) A-I was completely abolished, and efflux to high-density lipoprotein-3 (HDL) was impaired in TD-1 and partially impaired in the heterozygote sibling. Liver X receptor agonists enhanced efflux in the hetero-1 and age/sex/race-matched healthy control, but not in the TD patient. The TD-1 IPSDM faithfully reproduced the impaired cholesterol efflux function. **B**, IPSDM of TD-2 and age/sex/race-matched control-2 also showed abolished efflux to apoA-I and impaired efflux to HDL. **C**, TD-HMDMs showed marginal increase in baseline expression of interleukin (IL)-1B, IL8, and tumor necrosis factor (TNF), and are hypersensitive to lipopolysaccharide (LPS) stimulus as evidenced by greater increases in inflammatory gene expression. IPSDMs reproduce the enhanced inflammatory response in TD-HMDM. The \( \Delta \text{Cts} \) represent the mean cycle threshold for target genes relative to \( \text{ACTB} \) as the internal reference. **n=4** replicates of control-2 and TD-2.
associated with diseases, such as for PDGFD in coronary heart disease, FGFI in cardiac hypertrophy, DCN in sudden cardiac arrest, and TIMP3 in age-related macular degeneration. Thus, specific customization of the IPSDM protocol may be required to suit each question addressed. Our study represents a starting point and provides a resource to assist investigators in determining the appropriateness of the current IPSDM protocol and whether modifications are required. We note also the potential to eliminate the ≤5% CD45-negative cells by fluorescence-activated cell sorting or magnetic beads separation when a highly purified macrophage population is needed. Third, and of some concern, there were 591 genes expressed at lower levels in IPSDM versus HMDM, and these were enriched in immune response and defense response genes. Many of these, however, were upregulated during polarization to levels comparable with M1- or M2-HMDM (Online Table IV) suggesting that polarization leads to more complete convergence of ISPD M and HMDM transcriptomes. Nevertheless, a few members of MHC protein complex class II (DOA, DP, DQ, and DR) and chemokines (CCL1, CCL18, and CCL22, etc) were expressed at generally lower levels in IPSDM (Online Table IV), suggesting that optimized protocols are required for specific applications. Fourth, consistent with functional and secretome similarities, expression profiles of IPSDM- and HMDM-derived M1 lines were highly correlated with each other and both were dramatically different from their respective IPSDM and HMDM precursors. This suggests particular value of the IPSDM system in modeling inflammatory and M1-type macrophage functions. Similarly, there was substantial transcriptomic overlap between IPSDM- and HMDM-derived M2 lines that included classical M2 polarization markers. Fifth, through RNA-Seq we identified many new genes modulated during polarization in both HMDM and IPSDM, thus revealing novel, and potentially regulatory, polarization markers that warrant further study. Finally, the pairwise Pearson correlation of mRNA expression in all M0-HMDM and M0-IPSDM pairs (Online Figure IV) reveals that the correlation between isogenic HMDM and IPSDM was high (ranging from 0.83 to 0.92). Yet, the correlation across HMDMs of the three subjects in our study was also high (0.90–0.96). This sample is too small to draw inference of within and across subject correlations but larger samples and diverse populations will result in larger variation between subjects. We emphasize, however, that the high expression correlation between isogenic HMDM and IPSDM is in support of the fidelity of IPSDM for human macrophage functional genomics studies.

We demonstrate the specific use of IPSDM for interrogation of functional impact of human genetic variation in macrophages. IPSDM faithfully recapitulate the hallmark macrophage cholesterol homeostasis defects in TD, both in a qualitative and quantitative manner. Deficiency of ABCA1 in IPSDM also revealed a novel phenotype, of hypersensitivity to inflammatory stress, in human TD-IPSDM. Whether this relates to FC toxicity, as proposed in rodent cells, or distinct signaling actions in human TD macrophages is uncertain but our IPSDM system provides a renewable tool for molecular studies to address the underlying mechanism(s). These observations provide support for the use of IPSDM in defining subtle macrophage phenotypes in Mendelian disorders and for ongoing studies of common genetic variation for complex diseases such as atherosclerosis. This work lays the groundwork for large-scale use of genome editing technologies, in conjunction with iPSC-macrophage differentiation in cells of isogenic background, for modeling of genetic effects on human macrophage functions in Mendelian disorders (eg, gene editing in isogenic wild-type IPSDMs to study novel inflammatory phenotypes in TDs) as well as in complex-disease macrophage genomics at the intersection of metabolism, immunity, and cardiovascular diseases.

In summary, we describe an efficient protocol for generation of human IPSDM, establish functional and transcriptomic fidelity of IPSDM relative to HMDM, demonstrate equivalent plasticity in IPSDM and HMDM polarization in vitro, and recapitulate hallmark macrophage lipid phenotypes while revealing novel inflammatory defects in IPSDM of patients with TD, a Mendelian disorder of ABCA1 deficiency. Through high-resolution RNA-Seq of HMDM and IPSDM under nonpolarized, M1-like and M2-like conditions, we also provide novel insights into the human macrophage function and transcriptome and identify gene clusters for further study in macrophage polarization and biology. This work suggests genome-to-phenome fidelity at the individual level during IPSDM differentiation and polarization. The IPSDM protocol provides a unique tool to study the macrophage-specific functions of novel genomic loci for human disease, to execute gene-editing strategies to prove causality and to advance clinical and therapeutic translation of human genomic discoveries.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Macrophages represent a critical cell type at the intersection of metabolism, immunity, and cardiovascular diseases.
- The differentiation of human induced pluripotent stem cells to macrophages (induced pluripotent stem cell-derived macrophages [IPSM]) provides a ready source of subject genotype-specific cells with potential applications in disease modeling, drug screening, and cell therapies.

What New Information Does This Article Contribute?

- We present the first high-resolution transcriptome map of isogenic human peripheral blood mononuclear cell-derived macrophages (HMDM) and IPSDM during differentiation and polarization.
- M1 activation of HMDM and IPSDM is associated with an apparent coordinate reduction in phagocytosis and increase in cholesterol efflux capacity as well as characteristic secretion of inflammatory cytokines, whereas M2 activation of HMDM and IPSDM is characterized by a preservation of phagocytosis and trend toward reduced cholesterol efflux capacity with a distinct pattern of cytokine secretion.
- Tangier disease subject–specific IPSDMs recapitulate hallmark macrophage lipid phenotypes of impaired cholesterol efflux, while revealing novel inflammatory phenotypes of enhanced inflammatory responses to lipopolysaccharide stimulus.

Induced pluripotent stem cell technology offers a promising approach to in vitro disease modeling and drug screening; however, it is unclear to what extent induced pluripotent stem cell–differentiated cells adopt the functional and transcriptomic characteristics of their primary somatic cells. We developed a protocol for rapid, high-throughput generation of IPSDM of high purity and functional homogeneity. IPSDM, like their primary isogenic HMDM, can be polarized in vitro to functionally and molecularly distinct M1 and M2 subtypes, with in vitro functional plasticity and polarization in phagocytosis and cholesterol efflux capacity, secretome, and transcriptome profiles similar to native cells. Tangier disease–IPSDM showed identical hallmark phenotypes of impaired cholesterol efflux in HMDM of patients with Tangier disease, while revealing novel insights into the enhanced inflammatory response to lipopolysaccharide stimulus because of human ATP-binding cassette transporter A1 deficiency. This work represents a significant step forward for the utilization of IPSDM in defining subtle macrophage phenotype differences in Mendelian disorders, lays the groundwork for large-scale use of genome editing technologies in conjunction with IPSDM to model genetic effects of rare Mendelian mutations as well as novel genomic loci for complex diseases in human macrophages, and to advance clinical and therapeutic translation of human genomic discoveries.
Functional Analysis and Transcriptomic Profiling of iPSC-Derived Macrophages and Their Application in Modeling Mendelian Disease

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SUPPLEMENTAL MATERIAL

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This PDF file includes:

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Supplemental Figure II. Potential novel macrophage polarization markers
Supplemental Figure III. Cholesterol efflux of TD-2 and Control-2 IPSDM to 10 μg/ml apoA-I and 50 μg/ml HDL3 for 20-hour
Supplemental Figure IV. Heat map of Pearson’s correlation coefficient (r) for pairwise comparisons of M0-HMDM and M0-IPSDM samples
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Other Supplemental Materials for this manuscript includes the following:
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Supplemental Methods

Human blood collection and PBMC isolation

The BD VACUTAINER® Mononuclear Cell Preparation Tube (CPT™) with Sodium Citrate (BD, Franklin Lakes, NJ) for the separation of mononuclear cells from whole blood was used for blood collection using the standard technique for BD Vacutainer™. After collection, the tube was stored upright at room temperature (RT) and was processed within 2 h. Tubes were centrifuged at 1,800 RCF for 30 minutes at RT. After centrifugation, lymphocyte and monocyte band (PBMC layer) was collected using a serological pipette, and was washed with ample amount of Dulbecco’s Phosphate Buffered Saline (PBS, modified, without calcium chloride and magnesium chloride) followed by centrifuging at 330 RCF for 10 minutes at RT. PBMC cryopreserved in freezing media (90% FBS + 10% DMSO) at 2-3 x 10^6 cells/ml freezing media was stored in liquid nitrogen vapor phase for subsequent recovery and iPSC generation.

Subject-specific iPSC derivation and culture

All of the protocols for this study were approved by the University of Pennsylvania Human Subjects Research Institutional Review Board. Tangier patients and healthy controls were recruited. Generation of subject-specific iPSC lines derived from PBMC was performed by iPSC Core Facility at Penn’s Institute of Regenerative Medicine as previously described. Briefly, freshly collected or thawed PBMC were cultured in QBSF-60 hematopoietic stem cell media (Quality Biological, Gaithersburg, MD) to expand the erythroblast population for transduction using four recombinant Sendai viral vectors (Cytotune™ – iPS Sendai Reprogramming Kit, Life Technologies, Grand Island, NY), expressing the four reprogramming factors Oct4, Sox2, Klf4 and c-Myc. Initial iPSC colony selection was based on morphologic resemblance to human embryonic stem cell colonies. Undifferentiated iPSC lines were maintained in hES media consisting of Dulbecco modified Eagle medium/F12 (50:50; Life Technologies, Grand Island, NY) supplemented with 20% knockout serum replacement, 100 μM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (Life Technologies), 10^{-4} M β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 10 ng/ml human basic fibroblast growth factor (bFGF) on irradiated mouse embryonic feeder cells (MEF, GlobalStem, Rockville, MD) as described previously.

Characterization of iPSC lines

Immunofluorescence staining

iPSCs were grown to confluence in 48-well tissue culture plates on mouse feeders. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Indirect immunofluorescence staining was then performed with antibodies against Oct3/4 (Abcam, Cambridge, MA), Sox2 (R&D systems, Minneapolis, MN) SSEA3 (Millipore, Billerica, MA) or Nanog (Abcam, Cambridge, MA) followed by Alexa Fluor-conjugated secondary antibodies (Life Technologies, Grand Island, NY). Results were observed using Nikon Eclipse Ti-U inverted microscope with DS-Qi1 monochrome digital camera (Nikon Instruments, Melville, NY).
Flow cytometric analysis

iPSCs were dissociated into single cells with Accutase Enzyme Cell Detachment Medium (Innovative Cell Technologies). Flow cytometric analyses were performed using SSEA4-PE, and Tra1-60-FITC fluorophore-conjugated antibodies (Millipore, Billerica, MA). Samples were analyzed using BD FACSCalibur (BD Biosciences, San Jose, CA). Cells were plotted according to forward scatter and side scatter profiles and gated to exclude cell doublets and debris. Data were analyzed by using FlowJo software (Tree Star, Ashland, OR).

RT-PCR confirmation of loss of Sendai viral transgenes

Total RNA was isolated using an RNeasy kit (Qiagen) according to manufacturer’s instructions. cDNA synthesis was primed via random primers, and RT–PCR was performed with primer sets against the Sendai viral genome (forward primer: 5’-GGATCACTAGGTGATATCGAGC-3’ and reverse primer: 5’ACCAGACAAGAGTTAAGAGATATGTATC-3’) and GAPDH as a normalization control (forward primer: 5’-GTGGACCTGACCTGCCGTCT-3’ and reverse primer” 5’-GGAGGAGTGGGTGTCGCTGT-3’). PCR products were analyzed using 2% agarose gel electrophoresis.

Karyotypic analysis

iPSCs were cultured in T25 flasks on mouse feeders and live cultures were sent to Cell Line Genetics (Madison, WI) for cytogenetic analysis using G-banded Karyotyping. An average of 20 cells per cell line was analyzed for chromosome integrity.

Differentiation of human PBMC to macrophages and polarization

Human macrophage differentiation was performed as described. Freshly isolated PBMC were plated (6-well plates, 3x10^6 cells per well, or 24-well plates, 1x10^6 cells per well) (BD Primaria™ cell culture plates, BD Biosciences, Bedford, MA), allowed to adhere (overnight), washed with RPMI-1640 and cultured in macrophage culture media (20% FBS in RPMI 1640 media with 100 ng/ml human M-CSF) (Peprotech, Rocky Hill, NJ) for 7 days. Macrophage polarization was obtained by removing culture medium at Day 6 and culturing cells for an additional 18-20 hours in 10% FBS in RPMI 1640 media with 50 ng/ml M-CSF, and supplemented with 20 ng/ml IFN-γ (R&D, Minneapolis, MN) and 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) for M1 polarization, or 20 ng/ml IL-4 (R&D, Minneapolis, MN) for M2 polarization.

Differentiation of iPSC to macrophages

iPSC lines were passaged to beyond passage 20 for differentiation to macrophage. Before differentiation, the iPSC populations (80% to 90% confluent on MEF feeder) were depleted of feeder cells by culturing on plates coated with Matrigel (1:2 dilution of Growth Factor Reduced BD matrigel with IMDM medium) and cultured for 48 hours in the presence of 5 μM ROCK inhibitor for the first 24 hours. Cells were fed daily with hES media. At Day 0, iPSCs were harvested by incubation with collagenase B (0.2% collagenase B in 20% FBS in IMDM media + 10 μg/ml DNase I) for 25 min at 37 °C. To induce differentiation, embryoid bodies (EBs) were generated by culturing small aggregates of feeder-depleted iPSCs in COSTAR ultra-low attachment surface multiwell plate (CORNING, Corning, NY) in StemPro-34 media (Life Technologies) supplemented with penicillin/streptomycin, 2 mM glutamine, 4x10^-4 M monothioglycerol, 50 μg/ml ascorbic acid, 150 μg/ml human transferrin, and 25 ng/ml human bone morphogenetic protein-4 (BMP-4), and 50 ng/ml VEGF at 37°C in an environment of 5%
CO2, 5% O2, and 90% N2 for 2 days. At Day 2, EBs were harvested, washed, and cultured in the aforementioned media supplemented with 50 ng/ml human stem cell factor (SCF), 50 ng/ml thrombopoietin (TPO), 50 ng/ml Flt-3, and 20 ng/ml bFGF for an additional 2 days to induce primitive streak/mesoderm formation. At Day 4, BMP-4 was omitted from the medium and media change was performed every two days until D8. At Day 8, EBs and singles cells were collected and were resuspended in macrophage culture media composed with 20% FBS/RPMI with 100 ng/ml human M-CSF in the same ultra-low attachment surface plates for the enrichment of myeloid precursors, and cells were cultured in normoxic conditions at 37°C, and 95% air and 5% CO2 in a humidified incubator. At Day 15, single cells were transferred to BD Primaria™ tissue culture plate (BD Biosciences, Bedford, MA) with fresh macrophage culture media for expansion and macrophage maturation for 7 days (Day 22) in macrophage culture media. Please refer to Supplemental Table I for sources of recombinant cytokines used in differentiation protocol.

Assessment of hematopoietic and macrophage differentiation by FACS analysis

Analysis of a number of hematopoietic markers was performed at different time points during differentiation using flow cytometric analysis by collecting single cells emerged from cultured EBs. Cells were prepared in PBS + 1% BSA containing 0.05% sodium azide, and were blocked with 20 μl Fc-receptor antibodies for 10 min at RT. Next, the cells were labeled with multicolor monoclonal antibody combinations for 20 min at RT. The cells were then fixed with 200 μl of 2% paraformaldehyde. The fluorophore-labeled monoclonal antibodies used for identification and characterization of cell subsets included CD41-PE, CD235-APC, CD34-PECy7, CD18-APC, CD83-FITC, CD3-APC, CD11c-PECy7 (BD Biosciences, San Jose, CA) and CD43-FITC, CD45-Pacific Blue, CD11b-PECy7, CD16-PECy7, CD115-PE, CCR2-APC, CD1a-APC (Biolegend, San Diego, CA). Samples were analyzed using BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Cells were plotted according to forward scatter and side scatter profiles and gated to exclude cell doublets and debris. Data were analyzed by using FlowJo software (Tree Star, Ashland, OR).

IPSDM polarization

IPSDM polarization was performed at Day 21 using the same protocol for HMDM polarization.

May-Grünwald-Giemsa (MGG) staining

MGG staining was performed using a standard eosin-methylene blue solution and Giemsa solution. Images were acquired on Nikon Eclipse 80i upright microscope with DS-Qi1Mc digital camera (Nikon Instruments, Melville, NY).

Dil-Ac-LDL uptake

IPSDM or HMDM were incubated in 10 μg/ml Dil-labeled Ac-LDL (Biomedical Technologies, Stoughton, MA) for 4 h, counterstained with DAPI and observed by Nikon Eclipse Ti-U inverted microscope with DS-Qi1 monochrome digital camera (Nikon Instruments, Melville, NY).

Phagocytosis of Alexa Fluor-594 conjugated zymosan particle

Non-opsonized Zymosan A (S. cerevisiae) BioParticles®, Alexa Fluor® 594 Conjugate (Life Technologies, Grand Island, NY) were resuspended at approximately 4x10⁸ particles/ml PBS. One microliter suspension was added to one well of 24-well plate and incubated for 1 hour at 37
ºC. Results were observed using Nikon Eclipse Ti-U inverted microscope with DS-Qi1 monochrome digital camera (Nikon Instruments, Melville, NY).

**Quantitative assay of phagocytosis**

CytoSelect 24-Well Phagocytosis Assay kit (zymosan, colorimetric format, Cell Biolabs, San Diego, CA) was used for quantitative assessment of phagocytosis. Zymosan particles were prepared from yeast cell wall and resuspended in PBS at 5x10⁸ particles/ml. 20 μl suspension of non-opsonized zymosan particles were added to one well of 24-well plate and treated for one hour at 37 ºC. External zymosan particles were blocked, and engulfed particles were detected by colorimetric methods. Absorbance measurements were performed on a Bio-Tek PowerWave XS2 Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT) with a 405 filter.

**Immunofluorescence staining**

IPSDM or HMDM were fixed and stained with primary antibodies for macrophage markers, including CD68 (ab955, Abcam, Cambridge, MA) and MCP-1 (500-M71, PeproTech, Rocky Hill, NJ), followed by fluorescence-conjugated secondary antibodies. Cells were counterstained with DAPI and observed by Nikon Eclipse Ti-U inverted microscope with DS-Qi1 monochrome digital camera (Nikon Instruments, Melville, NY).

**Semi-quantitative real-time reverse transcriptase PCR (qRT-PCR)**

Total RNA was isolated using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Germantown, MD). Reverse transcription was performed in a total volume of 20 μl including 200 ng total RNA with the High Capacity RNA to cDNA Master Mix (Applied Biosystems, Life Technologies). Real time PCR was performed in a total volume of 10 μl on a 7900HT Real-Time PCR System (Applied Biosystems, Life Technologies) using TaqMan Gene Expression assays (Applied Biosystems, Life Technologies). ACTB expression levels were used to normalize Ct values obtained for each gene.

**Proteome profiler human cytokine array**

Culture media were collected from non-polarized, or M1/M2 polarized HMDM and IPSDM. The Proteome Profiler Human Cytokine Array (Panel A, R&D, Minneapolis, MN) was used to detect the presence of 36 different cytokines in culture media. This uses a membrane antibody array, with biotinylated detection antibodies followed by streptavidin conjugated to horseradish peroxidase and then developed with a chemiluminescent substrate (Thermo Scientific, Rockford, IL). Data from 5 age/sex matched subjects were quantified by Image J and visualized by heat map. The original blots for array data derived from the same subject (Control-6) were also presented.

**Measurement of cholesterol efflux capacity**

Preparation of samples and measurement of efflux capacity were performed as previously described. Briefly, IPSDM and HMDM were plated as described above in 24-well Primaria cell culture plate. At Day 20 (for IPSDM) or Day 5 (for HMDM), cells were radiolabelled with 6 μCi/ml of [³H]-cholesterol (Perkin-Elmer, Waltham, MA). Twenty-four hours later, cells were washed and equilibrated overnight in RPMI1640 with 0.2% bovine serum albumin (BSA) and 0.1% FBS with or without the treatment liver X receptor (LXR) agonists 10 μM 9-cis-Retinoic acid and 5 μg/ml 22-hydroxycholesterol to up-regulate ATP-binding cassette transporter A1 (ABCA1) and
G1 (ABCG1). Efflux to apoA-I (10 μg/ml), HDL3 (25 μg/ml), or no acceptor control (RPMI 1640 media) was assessed over 4 hours as described previously for macrophages. After 4 hours, aliquots of the medium were removed, and the [3H]-cholesterol released was measured by Liquid scintillation counting. The [3H]-cholesterol present in the cells was determined by extracting the cell lipids in isopropanol and measured by Multipurpose Scintillation Counter LS 6500 (Beckman Coulter, Indianapolis IN). Efflux was presented as the percentage of counts recovered from the medium in relation to the total counts (sum of medium and cells), with subtraction of efflux to medium with no acceptors, which was comparable among groups. In some experiments, polarization was obtained during equilibration to determine the effects on cholesterol efflux capacity. Assays were performed in triplicate or quadruplicate.

**RNA-Seq library preparation and sequencing**

RNA samples were extracted using All Prep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA). Extracted RNA samples underwent quality control (QC) assessment using the Agilent Bioanalyzer (Agilent, Santa Clara, CA) and all RNA samples submitted for sequencing had an RNA Integrity Number (RIN) in the range of 7 to 10, with a minimum of 300 ng input RNA. We generated first-strand cDNA using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis using RNase H and DNA polymerase, and ligation of sequencing adapters using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). Fragments of ~350 bp were selected by gel electrophoresis. To minimize lane and batch effects in RNA-Seq experiments, barcoded libraries were mixed and pooled in equimolar (2 nmol/L) amounts and diluted for cluster formation on a single flow cell lane, followed by 100 bp paired-end sequencing on Illumina’s HiSeq 2000. On average, we obtained ~130 million (M)/reads per sample with a high mapping rate of ~95% to the reference genome and ~70% reads were uniquely mapped and properly filtered.

**Alignment of RNA-Seq reads**

The RNA-Seq data were aligned to the hg19 reference genome using STAR 2.3.0e with default options. We applied several filtering steps; (1) the mapping quality score of each read is ≥ 30, (2) reads from the same pair were mapped to the same chromosome with expected orientations and mapping distance between the read pair was < 500,000 bp, and (3) each read was uniquely mapped. All subsequent analyses were based on filtered alignment files. Mapping statistics are summarized in Supplemental Table II. RNA-Seq data are available from the NCBI Gene Expression Omnibus (GEO) under the accession number GSE55536.

**Transcriptional profile analysis**

Transcript abundances were measured in FPKM (fragments per kilobase of exon per million reads mapped) using Cufflinks 2.1.1. Differential expression (DE) was tested with Cuffdiff, using the RefSeq annotation. Genes with a false discovery rate (FDR)-adjusted P value<0.01 and a fold change (FC)>2 were considered differentially expressed. Multidimensional scaling (MDS) was done with Euclidean distance based on log10 (FPKM + 0.1) in R programming languages. To visualize the overall sample to sample relationship within the data set, we performed co-regulation analysis (CRA) based on Pearson’s correlation coefficients by using BioLayout Express 3D. FPKM values were normalized using method from Anders and Huber separately for M0 (non-polarized macrophages), M1, M2 samples in HMDM and IPSDM. Normalized FPKM values were transformed by log10 (FPKM + 0.1) on heat map. Heat maps illustrating expression patterns of DE genes were generated by using ggplot2 in R.
Statistical analysis

Data were analyzed with GraphPad Prism 6 (GraphPad Software, San Diego, CA) and were shown as mean±SD. Statistical differences between groups were determined using Mann-Whitney test and Wilcoxon tests because non-parametric tests are more appropriate for small sample sizes. For analysis of gene ontology (GO) pathways in RNA-Seq data, significant enrichment was declared at FDR adjusted P values <0.05 using Benjamini and Hochberg method. Enrichment analysis was performed in BiNGO plugin using Biological Process category and visualized in Cytoscape. Given a background set of N genes expressed and X DE genes (non-DE genes for Table IX), the P-value is the probability that at least x of X genes belong to a GO Biological Process category shared by n of the N genes in the background set.
Supplemental Tables

Supplemental Table I: Cytokines and chemicals used in differentiation and polarization of IPSDM and HMDM.

<table>
<thead>
<tr>
<th>Cytokines/Chemicals</th>
<th>Company</th>
<th>Catalog #</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Recombinant Human BMP-4</td>
<td>R&amp;D</td>
<td>314-BP-050/CF</td>
<td>25 ng/ml</td>
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<tr>
<td>Recombinant Human VEGF 165</td>
<td>R&amp;D</td>
<td>293-VE-001mg/CF</td>
<td>50 ng/ml</td>
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<td>Recombinant Human Flt-3 Ligand (hTPO)</td>
<td>R&amp;D</td>
<td>308-FK-025/CF</td>
<td>50 ng/ml</td>
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<tr>
<td>Recombinant Human Thrombopoietin (hTPO)</td>
<td>R&amp;D</td>
<td>288-TP-500/CF</td>
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<tr>
<td>Recombinant Human SCF</td>
<td>R&amp;D</td>
<td>255-SC-001MG/CF</td>
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<td>Recombinant Human FGF-Basic (bFGF)</td>
<td>Life Technologies</td>
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<td>Recombinant Human M-CSF</td>
<td>PeproTech</td>
<td>300-25</td>
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<tr>
<td>Recombinant Human IFN-γ</td>
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<td>Recombinant Human IL-4</td>
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<td>Lipopolysaccharides (LPS)</td>
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Supplemental Table II: QC of RNA-Seq.

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<th>Phenotype</th>
<th>HMDM</th>
<th>M0-HMDM</th>
<th>M1-HMDM</th>
<th>M2-HMDM</th>
<th>IPSC</th>
<th>M0-IPSC</th>
<th>M1-IPSC</th>
<th>M2-IPSC</th>
<th>IPSDM</th>
<th>M0-IPSMD</th>
<th>M1-IPSMD</th>
<th>M2-IPSMD</th>
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<td>Aligned Reads (X10^6)</td>
<td>123.7±7.4</td>
<td>134.8±16.6</td>
<td>139.5±3.3</td>
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<td>140.7±22.5</td>
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<td>Exonic Reads (%)</td>
<td>92.3±0.7</td>
<td>89.8±0.5</td>
<td>91.6±0.9</td>
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<td>89.8±2.0</td>
<td>90.9±1.1</td>
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<td>Intronic Reads (%)</td>
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<td>Intergenic Reads (%)</td>
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Supplemental Table III: Subject demographics of iPSC lines.

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<tr>
<th>Phenotype</th>
<th>TD-1</th>
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<th>Hetero-1 (Sibling of TD-1)</th>
<th>Control</th>
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<td>No. of clones per iPSC line</td>
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<td>Age</td>
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TD, Tangier disease; C, Caucasian; M, male; F, female
Supplemental Table IV. Selected DE genes enriched in GO term - immune response that were expressed at lower levels in IPSDM vs. HMDM.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>FPKM M0-HMDM</th>
<th>FPKM M1-HMDM</th>
<th>Log2 FC</th>
<th>FDR</th>
<th>FPKM M0-IPSDM</th>
<th>FPKM M1-IPSDM</th>
<th>Log2 FC</th>
<th>FDR</th>
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<td>MHC protein complex</td>
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<tr>
<td>HLA-A</td>
<td>1311.48</td>
<td>4848.62</td>
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<td>HLA-E</td>
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</tbody>
</table>

* Indicate genes not identified as DE genes with threshold criteria (fold-change >2, FDR adjusted P <0.01).
Supplemental Figure I. Subject-specific PBMC-derived iPSC generation and characterization.

(A) Schematic of iPSC generation. PBMCs were isolated from freshly collected blood and were cultured for 9-12 days to enrich for erythroblasts. At Day 0, erythroblasts were transduced with Sendai viral vectors expressing the 4 reprogramming factors. Colonies were picked 2-3 weeks later. Stable iPSC lines were established within 2-3 months. (B) and (C) The iPSC lines showed expression of various pluripotency markers including Oct4, Sox2, Nanog, SSEA3, SSEA4 and Tra1-60 as analyzed by indirect immunofluorescence staining or FACS. (D) Loss of Sendai viral
transgenes was confirmed by RT-PCR beyond passage 10. (E) iPSCs generated by the method described in (A) have normal karyotype as shown by a representative G-banded karyotyping analysis.

Supplemental Figure II. Potential novel macrophage polarization markers.

To identify potential novel markers for M1 and M2 macrophages, we first excluded the genes listed in Figure 5E, then ranked the absolute value of log₂ FC of genes with FPKM > 5% of expression in M1 vs. M2-HMDM and M1 vs. M2-IPSDM. The top 150 genes with the highest fold change for HMDM and IPSDM were derived separately and the common 65 genes between HMDM and IPSDM were classified by known functional categories and were visualized by heat map. Cell surface molecules and membrane receptors IL2RA and IL31RA showed preferential expression in M1 macrophages. Other surface molecules, including CD1B, CD1C, CD93, ADORA3 etc., and important lipid metabolism enzyme ALOX15, were markedly increased in M2 macrophages.
Supplemental Figure III. Cholesterol efflux of TD-2 and Control-2 IPSDM to 10 μg/ml apoA-I and 50 μg/ml HDL₃ for 20-hour.

IPSDM were radiolabelled with 6 μCi/ml of [³H]-cholesterol. Twenty-four hours later, cells were washed and equilibrated for 4 hours in RPMI1640 with 0.2% bovine serum albumin (BSA) and 0.1% FBS. Efflux to apoA-I (10 μg/ml), HDL₃ (50 μg/ml), or no acceptor control (RPMI 1640 media) was assessed over 20 hours. Efflux was presented as the percentage of counts recovered from the medium in relation to the total counts (sum of medium and cells), with subtraction of efflux to medium with no acceptors. Data represent mean±SD of 4-6 replicates. * P < 0.05.
Supplemental Figure IV. Heat map of Pearson’s correlation coefficient (r) for pairwise comparisons of M0-HMDM and M0-IPSDM samples.

Genes expressed at FPKM > 1% expression of all genes were included for each sample. FPKM values were then normalized across samples. Only genes expressed in all M0-HMDM and M0-IPSDM were included in calculation. Pearson’s r was calculated for each pair of samples and visualized by heat map.
Supplemental References


