Modelling IRF8 Deficient Human Hematopoiesis and Dendritic Cell Development with Engineered iPS Cells

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ABSTRACT

Human induced pluripotent stem (iPS) cells can differentiate into cells of all three germ layers, including hematopoietic stem cells and their progeny. Interferon regulatory factor 8 (IRF8) is a transcription factor, which acts in hematopoiesis as lineage determining factor for myeloid cells, including dendritic cells (DC). Autosomal recessive or dominant IRF8 mutations occurring in patients cause severe monocytic and DC immunodeficiency. To study IRF8 in human hematopoiesis we generated human IRF8−/− iPS cells and IRF8−/− embryonic stem (ES) cells using RNA guided CRISPR/Cas9n genome editing. Upon induction of hematopoietic differentiation, we demonstrate that IRF8 is dispensable for iPS cell and ES cell differentiation into homogentic endothelium and for endothelial-to-hematopoietic transition, and thus development of hematopoietic progenitors. We differentiated iPS cell and ES cell derived progenitors into CD141+ cross-presenting cDC1 and CD1c+ classical cDC2 and CD303+ plasmacytoid DC (pDC). We found that IRF8 deficiency compromised cDC1 and pDC development, while cDC2 development was largely unaffected. Additionally, in an unrestricted differentiation regimen, IRF8−/− iPS cells and ES cells exhibited a clear bias toward granulocytes at the expense of monocytes. IRF8−/− DC showed reduced MHC class II expression and were impaired in cytokine responses, migration, and antigen presentation. Taken together, we engineered a human IRF8 knockout model that allows studying molecular mechanisms of human immunodeficiencies in vitro, including the pathophysiology of IRF8 deficient DC. STEM CELLS 2017;35:898–908

SIGNIFICANCE STATEMENT

Pluripotent stem cells and CRISPR/Cas9n technology are particularly well suited for engineering cells to study the impact of specific factors on cell development, including antigen presenting dendritic cells (DC). So far, DC research was limited to primary cell samples obtained for example, from mice or men. In the mouse system, genetically modified DC are readily obtained by using transgenic, knockout, and knockin mice. In the human system studies with mutated DC relied on patients harboring specific mutations and there was a paucity of techniques for genetic engineering directly in human cells. Induced pluripotent stem (iPS) cell and CRISPR/Cas9n technology now allow to overcome these limitations. Here, we generated interferon regulatory factor 8 (IRF8) knockout human iPS cells and ES cells, and IRF8−/− DC derived thereof. We show that IRF8−/− cells recapitulate the phenotype of individuals with an IRF8 loss of function mutation. Our IRF8−/− iPS cells and ES cells provide a platform to study IRF8 deficient DC subset specification and DC function independent of donor variation or availability. In summary, our IRF8−/− iPS cells and ES cells represent a valid and powerful model to elucidate mechanisms of human DC development and functional diversity.

INTRODUCTION

Induced pluripotent stem (iPS) cells and embryonic stem (ES) cells provide excellent opportunities for modeling human diseases [1–4]. Loss of function mutations in the interferon regulatory factor 8 (IRF8) gene cause life-threatening monocytic and dendritic cell (DC) immunodeficiency [5]. IRF8, also known as interferon consensus sequence binding protein (ICSBP), belongs to a family of helix-turn-helix transcription factors that are...
induced by interferons (IFNα/β and IFNγ) in response to viral infections [6, 7]. Patients suffering from immunodeficiency due to autosomal recessive or dominant IRF8 mutations present a lack of circulating monocytes, DC and basophils but a severe neutrophilia and eosinophilia [5, 6, 8]. Consequently, these patients are particularly susceptible to mycobacterial, viral, and fungal infections. To ensure long-term survival these patients require hematopoietic stem cell transplantation shortly after birth. While these patients demonstrate the impact of IRF8 on myeloid cell development, molecular and functional follow-up studies are difficult due to the limited number of primary cell samples and patients.

Frequently, human hematopoiesis and DC development are studied from cord blood (CB) or bone marrow (BM) derived stem and progenitor cells [9]. However these studies are subject to donor variations and do not capture the very early and embryonic events of hematopoiesis, including the formation of hemogenic endothelium and the endothelial-to-hematopoietic transition (EHT) [10]. Additionally, genetic modifications of CB and BM stem/progenitor cells, for example, by CRISPR/Cas, and the clonal analysis of such cells are challenging due to their limited life span. Here we used iPS cells and ES cells to model hematopoiesis, as they (i) provide an unlimited clonal cell source, (ii) readily differentiate into cell derivatives of all three germ layers, including hematopoietic stem cells and their progeny, and (iii) are efficiently modified by CRISPR/Cas technology, and thus offer a particularly appealing approach for studying gene function during human development, including DC differentiation.

DC are professional antigen presenting cells with a central function in connecting the innate and adaptive immune system [11, 12]. DC are a heterogeneous cell population and comprise several subsets, which are classified according to their anatomical location and specialized function [11, 13]. The two major populations are classical DC (cDC) and plasmacytoid DC (pDC), exhibiting a classical DC or plasma cell morphology, respectively. cDC capture a large plethora of antigens in the periphery and migrate to lymphoid organs for antigen presentation, while pDC are specialized in recognizing viral and bacterial nucleic acids [11, 14]. In humans, CDC and pDC are classified as CD141+ cross-presenting cDC and CD1c+cDC (referred to as cDC1 and cDC2, respectively), and CD303+ pDC [14–16]. cDC1, cDC2, and pDC subsets develop from BM derived stem cells through successive steps of lineage commitment and differentiation [11, 12, 17].

DC have mostly been studied in mice and knockout models have shed light on DC development and function [11, 12, 18–20]. However, translating these findings to human DC is difficult due to phenotypical and ontological differences between species and the limited access to human lymphoid tissues [21–24]. Thus, studying human DC development, identity, and function has remained challenging.

Here we generated human IRF8 knockout iPS cells and ES cells to study the impact of IRF8 on human hematopoiesis, particularly on DC development. iPS cells and ES cells developed into hematopoietic progenitors independent of IRF8 and showed multilineage differentiation potential. IRF8−/− hematopoietic progenitors exhibited a bias toward granulocytes, whereas monocytes were reduced. Development of cDC2 from IRF8−/− progenitors was normal, but development of cDC1 and pDC was impaired.

Materials and Methods
Maintenance and Genome Engineering of Human iPS Cells
iPS cells were obtained by reprogramming of KIT+ progenitors from BM with OCT4, SOX2, c-MYC, and KLF4 Sendai virus vectors (Supporting Information). Human HES-3 ES cells (ES03) were from WiCell Research Institute. iPS cells and ES cells were maintained on irradiated mouse embryonic fibroblasts (MEF) in KnockOut Dulbecco’s modified Eagle medium (KO-DMEM) supplemented with 20% KnockOut serum replacement, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (all Thermo Fisher Scientific), and 10 ng/ml human basic fibroblast growth factor (bFGF, Peprotech).

IRF8−/− iPS cells and ES cells were generated with CRISPR/Cas9n double nicking approach as described in [25]. In brief, two pairs of guide RNA (gRNA) were designed targeting the intron 2-exon 3 boundary of the IRF8 gene (Fig. 1A). gRNA oligonucleotides were cloned individually into a variant of vector pX335 (Addgene 42335) carrying a Puromycin-GFP selection cassette [26, 27]. gRNA plasmids (4 μg each) were transfected into iPS cells and ES cells with the NEON transfection system (1,500 V, 20 ms pulse width, 1 pulse, Thermo Fisher Scientific). Transfected cells were enriched by puromycin treatment (0.4 μg/ml) for 24 hours. Two weeks later individual colonies were picked and screened for deletions in the IRF8 target region by PCR (Supporting Information). Further experiments were performed with three independent founding IRF8+/− iPS cell clones and the respective IRF8−/− iPS cell clones and a pair of IRF8+/+ and IRF8−/− ES cells.

Hematopoietic Progenitor and DC Differentiation
iPS cells and ES cells were subjected to embryoid body (EB) formation and differentiated into hematopoietic cells with a protocol modified from [28]. Briefly, iPS cell and ES cell colonies were treated with collagenase IV and mechanically disrupted to form small cell clusters. Clusters smaller than 70
cytokines were from Peprotech. On day 8 cultures were transfected with CD43 (derived DC) referred to as dd4, dd8, dd10, and dd14, respectively. FSG4 and FSG DC cultures were stimulated with lipopolysaccharid (LPS, Sigma Aldrich, 1 mg/ml) or CpG oligonucleotide (ODN2216, Invivogen, 5 μg/ml) and used for further differentiation or analysis between day 4 and 8.

Flow Cytometry and Cell Sorting
Hematopoietic cell development and DC differentiation were monitored by flow cytometry and with the following antibodies: CD31-PE (clone WM59), CD34-APC (clone S81), CD43-FITC (clone 1G10), CD45-biotin (H530), CD14-PE (clone MOP9), CD66b-PE (clone G10F5), CD123-PE (clone 9F5) (all BD Bioscience), CD45-APC-Cy7 (clone 2D1), CD117-PE-Cy7 (clone 104D2), CD11c-PE-Cy7 (clone 3.9), HLA-DR-FITC (clone LN3), HLA-DR-PE-Cy7 (clone LN3), CD86-PE (clone IT2.2) (all eBioscience), CD31-biotin (clone AC128), CD43-biotin (clone DFT1), CD1c-biotin (clone AD5-8E7), CD1c-PE (clone AD5-8E7), CD303-biotin (clone AC144), CD304-APC (clone AD5-17F6), CD141-VioBlue (clone AD5-14H12), Clec9a-PE (clone 8F9) (all Miltenyi Biotech). CCR7 was detected with a chimeric CCL19-VioBlue (Stemcell Technologies) for 10-15 minutes prior to staining with antibodies. Single cells were incubated with 1% human IgG solution (Privigen, CSL Behring) for 30 minutes at 4°C to block unspecific binding. Biotinylated primary antibodies or IgG fusion proteins were labeled with anti-Biotin-VioBlue (Miltenyi Biotech), Streptavidin-FITC (eBioscience), Streptavidin-APC or goat anti-human IgG FITC (both Thermo Fisher Scientific). Stained cells were analyzed on a FACS Canto II or sorted on a FACS Aria II 3L (both BD Bioscience). Data analysis was performed with FlowJo software (Tree Star).

Reverse Transcription-quantitative PCR (RT-qPCR)
DNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific). RNA was reverse transcribed with random primers and MultiScribe reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific). Quantitative PCR was performed on a StepOnePlus Real-Time cycler with FAST SYBR Green master mix (Thermo Fisher Scientific). Human specific primers (Eurofins) are listed in Supporting Information Table 1. Threshold cycle (Ct) values were represented relative to GAPDH expression (2^(-ΔCt) values). Heatmaps were generated with MultiExperiment Viewer v4.9 (TM4 Software Suite). Expression values were normalized per gene and subjected to bidirectional hierarchical clustering using Euclidian distance and average linkage clustering.

Chemotaxis Assay
To assess migratory potential toward a chemokine gradient DC were cultured in Transwell inserts (5 μm pore size, Costar) as described in [33]. Briefly, Transwells were preincubated with medium to block unspecific binding and 2 x 10^5 cells were stimulated with LPS and seeded into the upper chamber. ELC chemokine (100 ng/ml, Peprotech) was added to the lower chamber and cells were incubated at 37°C for 2 hours. Prior to cell collection, 1 x 10^5 Dynabeads (15 μm diameter, Dynal Polymers) were added to the lower chamber to allow normalization for variations in the experimental procedure. Cells and beads were recovered and analyzed by flow cytometry. Bead/cell ratio was determined and allowed a precise quantification of transmigrated cells. Peripheral blood mononuclear cell (PBMC) derived DC (Supporting Information) were used as control.

Mixed Lymphocyte Reaction
DC and allogenic T cells (Supporting Information) were cultured in a ratio of 1:10 in RPMI medium supplemented with 5% serum of T cell donor, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin for 2 days. Bromdeoxyuridine (BrdU, 10 μM, BD Bioscience) was added and cells were further incubated for 16 hours. Incorporated BrdU was measured by flow cytometry using the APC BrdU Flow Kit (BD Bioscience) according to the manufacturer’s instructions. Concanaavalin A (ConA, 10 μg/ml, Sigma Aldrich) stimulated T cells and unstimulated T cells were used as controls.

Statistical Analyses
Data are presented as mean or median ± standard deviation (SD). Statistical significance was analyzed using two-tailed, unequal Student ‘t’ test (GraphPad Prism version 6) and differences were considered significant (*) when p < .05, very significant (**) when p < .005, and extremely significant (***) when p < .001.

Results
IRF8 is Dispensable for Hematopoietic Progenitor Development from iPS Cells
iPS cells were generated from BM hematopoietic progenitors with OCT4, SOX2, c-MYC, and KLF4 in Sendai virus vectors. iPS
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Figure 2. Hematopoietic progenitor cell development is IRF8 independent. (A): Differentiation protocol of iPS cells and ES cells into hematopoietic progenitors. Undifferentiated IRF8−/− and IRF8+/+ iPS2 cell colonies on day 0 and small embryoid bodies on day 6. Scale bar 500 μm. Hemato-endothelial patches during endothelial-to-hematopoietic transition on day 8 and hematopoietic progenitors on day 10. Scale bar 200 μm. (B): Boxplot of flow cytometry data of hematopoietic differentiation for IRF8−/− and IRF8+/+ iPS cells and ES cells. Frequencies of CD34/31+ (day 6 for iPS cells and day 21 for ES cells), CD43/31+ and CD45+ (both day 10 for iPS cells and day 28 for ES cells) populations in percent of living single cells. Median of four independent experiments (iPS1, iPS2, iPS3, and HES-3) is shown. Whiskers indicate lowest and highest values measured (see (D) and Supporting Information Fig. 3B, 3C). (C): RT-qPCR data during hematopoietic progenitor development (day 0, 4, and 14) for IRF8−/− and IRF8+/+ iPS2 cells depicted in heatmap format. RT-qPCR data (2^−ΔΔCt values) of two independent experiments were normalized per gene and subjected to bidirectional clustering. Gradation bar represents scale of expression levels: red, high expression; blue, low expression. Early mesoendodermal genes (e.g., T and SOX17) are expressed on day 4 and master hematopoietic regulators (e.g., GATA1 and GATA2) and hemoglobins (HBE, HBZ, and HBB) are expressed on day 14 (see also Supporting Information Fig. 3D). (F): Frequency of erythroid (CFU-E), granulocyte-macrophage (CFU-GM), macrophage (CFU-M), and granulocyte (CFU-G) colonies for IRF8−/− and IRF8+/+ iPS cell derived hematopoietic progenitors. Colony numbers represent the mean ± SD of three independent experiments (iPS1, iPS2, and iPS3). Representative phase contrast images of CFU-GM colonies of IRF8−/− and IRF8+/+ iPS2 cell derived hematopoietic progenitors. Scale bar 200 μm. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem; IRF8, interferon regulatory factor 8.
cells were pluripotent by Epi-Pluri-Score test [34], expression of pluripotency markers and three germ layer differentiation potential (Supporting Information Fig. 1A-1C). IRF8 in iPS cells (iPS1, iPS2, and iPS3) was inactivated by CRISPR/Cas9n genome editing (Fig. 1A, Supporting Information Fig. 2A). HES-3 ES cells were used as control for iPS cells. We chose gRNA targeting the splice acceptor of exon 3 (E3), since E3 encodes the DNA binding domain of IRF8. Deletion of sequences around splice acceptor E3 caused a spliced IRF8 mRNA devoid of E3 with a premature stop codon (Supporting Information Fig. 2A, 2B) and abolished IRF8 protein expression (Fig. 1B). Karyotype analysis showed normal GTG banding in IRF8−/− and IRF8+/+ iPS cells (Supporting Information Fig. 2C). Taken together, we have generated IRF8−/− iPS cells and IRF8−/− ES cells by CRISPR guided Cas9n technology, which lack IRF8 protein expression.

IRF8−/− iPS cells and ES cells were differentiated into hematopoietic progenitors as EB with stepwise application of cytokines (Fig. 2A). IRF8+/+ iPS cells and ES cells were used as control. EB of iPS cells became adherent at day 6-8 of differentiation and formed patches of hemogenic endothelium (Fig. 2B), while this was delayed for ES cells (day 21, Supporting Information Fig. 3A). Hemogenic endothelium is a specialized type of endothelium from which hematopoietic progenitors emerge during embryonic development. Both IRF8−/− and IRF8+/+ cells gave rise to a CD34+ CD31+ hemogenic endothelium population, demonstrating that IRF8 deletion did not impact on formation of hemogenic endothelium (Fig. 2C, Supporting Information Fig. 3B).

Emerging hematopoietic progenitors were observed at day 8 in a process referred to as EHT in both IRF8+/− and IRF8+/+ iPS cells (Fig. 2B) and from day 21 onward for IRF8−/− and IRF8+/+ ES cells (Supporting Information Fig. 3A). IRF8−/− and IRF8+/+ hematopoietic progenitors coexpressed the early hematopoietic markers CD43 and CD31 (Fig. 2D, Supporting Information Fig. 3C). This hematopoietic progenitor population expanded overtime and the frequencies of CD43+ CD31+ and CD45+ cells were the same for IRF8−/− and IRF8+/+ iPS cell and ES cell derived progenitors (Fig. 2C). Gene expression profiling and bidirectional clustering showed that hematopoietic progenitors expressed key hematopoietic regulators, including GATA1, GATA2, RUNX1, SCL, and PU.1 irrespective of the presence or absence of IRF8 (Fig. 2E, Supporting Information Fig. 3D).

Importantly, IRF8−/− progenitors formed more and larger granulocyte-macrophage (CFU-GM), macrophage (CFU-M), and granulocyte (CFU-G) colonies compared to IRF8+/+ progenitors (Fig. 2F). In particular, there was a prominent increase in the frequency of CFU-G colonies for IRF8−/− cells, which is in line with the granulocyte bias in IRF8−/− cells (see below). All this is consistent with the role of IRF8 as regulator of myeloid differentiation and cell cycle control in mice and men [7]. Taken together, our data suggests that IRF8 is dispensable for the development of hematopoietic progenitors, but impacts on differentiation propensity into the myeloid lineage.

Loss of IRF8 Enhances Hematopoietic Differentiation Toward Granulocytes

To study IRF8 function in myelopoiesis IRF8−/− and IRF8+/+ progenitors of iPS cells and ES cells were further cultured (Fig. 2A) and analyzed in regular time intervals for mature blood cell markers. One week after the initial emergence of hematopoietic progenitors IRF8+/+ cultures consisted of multiple myeloid cell types, including macrophages, monocytes, eosinophils, and neutrophils (Fig. 3A, Supporting Information Fig. 4A). Strikingly, IRF8−/− cultures were comprised almost exclusively of eosinophils and neutrophils (Fig. 3A, Supporting Information Fig. 4A). Flow cytometry analysis confirmed the reduction of CD14+ monocytes and a threefold increase in CD66b+ granulocytes in IRF8−/− cells (Fig. 3B, 3C, Supporting Information Fig. 4B, 4C). We also found distinct populations of cDC1, cDC2, and pDC in IRF8+/+ cultures, but all DC subsets were reduced in IRF8−/− cells (Fig. 3B, 3C, Supporting Information Fig. 4B, 4C). Moreover, IRF8−/− cultures showed reduced numbers of FcεR+ Kit+ mast cells, which at later time points made up the majority of IRF8+/+ cultures (Fig. 3B, 3C).

Further to this, cells in IRF8+/+ cultures expressed high levels of monocyte/macrophage specific markers (CSF1R, CD163), whereas IRF8−/− cultures were enriched in granulocyte receptors (CSF3R, CSF2RA), as demonstrated with gene expression profiling and bidirectional clustering (Fig. 3D, Supporting Information Fig. 4D). In addition, IRF8−/− cells showed low MHC class II HLA-DR expression compared to IRF8+/+ control. Taken together, in human myelopoiesis IRF8 represents a crucial transcription factor and loss of IRF8 results in a differentiation bias toward granulocytes at the expense of mast cells, monocytes, and DC.

Human DC Subsets are Generated from iPS Cells

Human DC subsets comprise cDC1, cDC2, and pDC. To generate these subsets from iPS cells (in the following referred to as iPS-DC), we adapted the CB based DC differentiation protocols [30, 31] to iPS cell and ES cell derived hematopoietic progenitors. CD43+ progenitors were seeded onto OP9 feeder cells with FLT3L, SCF, GM-CSF, and IL-4 (FSG4) or with FLT3L, SCF, and GM-CSF (FSG) to generate cDC and pDC, respectively (Fig. 4A). On day 4 of DC differentiation (dd4) progenitors had acquired an irregular shape with multiple dendrites and veils extending from the cell body, thus the characteristics of DC (Fig. 4B).

To determine iPS-DC subsets in FSG4 and FSG cultures, differentiated cells were further analyzed for specific and common DC markers (Fig. 4C-4E). cDC1 were identified by CD141 and Clec9a expression and found to occur with similar frequencies in both FSG4 and FSG cultures (Fig. 4C-4E). cDC2 were only found in FSG4 (Fig. 4C, 4E) but not in FSG cultures (Fig. 4D, 4E) because IL-4 suppresses generation of CD14+ monocytes thereby favoring the generation of cDC. Additionally, cDC1 and cDC2 express HLA-DR and the DC marker CD11c, and thus show the same surface marker profile as their in vivo counterparts (Supporting Information Fig. 5). pDC were CD303+ CD304+ HLA-DR+ CD123+ and CD11c+ in FSG4 (Fig. 4C) but CD11c low in FSG cultures (Fig. 4D). pDC in vivo are CD11c− (Supporting Information Fig. 5) and thus we used FSG derived pDC for all further experiments.

Gene expression analyses of iPS-DC subset specific transcription factors further extend the flow cytometry data. FSG cultures showed prominent expression of the pDC gene TCF4 and also expressed the pDC genes SPIB and RUNX2. FSG4 cultures expressed the cDC1 transcription factor BATF3 (Fig. 4F), IRF8 was highly expressed in FSG cultures and to a much lower extent in FSG4 cultures (Fig. 4F).
We then proceeded to stimulate iPS-DC with LPS or CpG, isolated DC subsets by FACS sorting and measured expression of proinflammatory cytokines (Fig. 4G, upper panel). IL-6 and TNFα expression was strongly upregulated in cDC2 and in CD14+ monocytes. IL-12 expression was weakly induced in all DC subsets and CD14+ monocytes. Additionally, HLA-DR was upregulated in cDC1 and pDC (Fig. 4G, lower panel). The costimulatory molecule CD80 and the chemokine receptor CCR7 were efficiently induced in cDC2 and CD14+ monocytes and to a lesser extent in cDC1 and pDC (Fig. 4G, lower panel).

Figure 3. IRF8−/− progenitor cells show a differentiation bias toward granulocytes. (A): Phase contrast images (day 21) and Diff Quik stainings (day 28) of IRF8−/− and IRF8+/+ iPS1 cells during differentiation. Diff Quik staining identifies monocytes (1), macrophages (2), neutrophils (3), and eosinophils (4). Scale bar left, center and right 100 µm, 25 µm, and 10 µm, respectively. (B): Flow cytometry analysis of cells in panel (A). cDC1, cDC2, monocytes (mono), and pDC on day 14; granulocytes (granulo) and mast cells (mast) on day 28. (C): Frequencies of cDC2, monocytes, pDC, granulocytes, and mast cells during differentiation (day 14, 21, 28, and 42) of IRF8−/− and IRF8+/+ iPS cells in percent of living single CD45+ cells. Frequencies represent the mean ± SD of three independent experiments (iPS1, iPS2, and iPS3). Statistical significance was assessed by Student t test (*p < .05, **p < .005, ***p < .001). (D): RT-qPCR data (2−ΔΔCt values) of three independent experiments (iPS1, iPS2, and iPS3) of IRF8−/− and IRF8+/+ iPS cells on day 21 of differentiation are depicted in heatmap format as in Fig. 2E. Gene expression pattern shows bias toward granulocyte genes (CSF3R and CSF2RA) for IRF8−/− cells at the expense of macrophage associated genes (CD163 and CSFR1). Abbreviations: cDC, classical dendritic cells; iPS, induced pluripotent stem; IRF8, interferon regulatory factor 8; pDC, plasmacytoid dendritic cells.
Figure 4. Generation of DC subsets from IRF8+/+ iPSCs. (A): Schematic representation of DC differentiation protocol. CD43+ progenitors are subjected to OP9 co-culture with FSG4 or FSG cytokines to generate cDC or pDC, respectively. (B): Phase contrast images of FSG4 and FSG cultures on DC differentiation day (dd) 4. Scale bar 100 μm. (C-D): Flow cytometry analysis of FSG4 (C) and FSG (D) cultures for DC subsets on dd6. DC subsets were pre-gated on CD45. Top row shows gating for cDC1. Middle row shows gating for cDC2. cDC coexpress HLA-DR and CD11c. Bottom row shows gating for pDC, which coexpress HLA-DR, CD123 and intermediate (C) or low (D) levels of CD11c (blue line in histograms). (E): Frequencies of DC subsets and monocytes from FSG4 and FSG cultures in percent of living single CD45+ cells. Data represent mean ± SD of three independent experiments (iPS1, iPS2, and iPS3). (F): RT-qPCR analysis of FSG4 and FSG cultures for cDC (IRF8 and BATF3) and pDC (IRF8, SPIB, TCF4, and RUNX2) specific genes. Data represent mean ± SD of three independent experiments as in (E). (G): RT-qPCR analysis of stimulated and sorted DC subsets on dd4. Data represent mean ± SD of two independent experiments (iPS1 and iPS3). cDC1 and pDC were from FSG cultures stimulated with CpG and IL-3. cDC2 and monocytes were from FSG4 cultures stimulated with LPS. Gene expression is depicted as fold change compared to unstimulated samples. Abbreviations: cDC, classical dendritic cells; iPS, induced pluripotent stem; IRF8, interferon regulatory factor 8; pDC, plasmacytoid dendritic cells.
Thus, iPS-DC subsets responded to inflammatory stimuli by upregulation of cytokines, costimulatory markers, and HLA-DR.

To validate our differentiation protocol, we used CB derived CD34+ hematopoietic progenitors. iPS-DC subsets showed the same surface marker profiles (Supporting Figure 5).

Figure 5. DC development and function is impaired in IRF8−/− cells. (A): Flow cytometry data of IRF8−/− and IRF8+/+ iPS-DC subsets on dd4. DC subsets were pre-gated on CD45. (B): Frequencies of DC subsets and monocytes from IRF8−/− and IRF8+/+ iPS cells and IRF8−/− and IRF8+/+ ES cells in percent of living single CD45+ cells. Frequencies represent the mean ± SD of four independent experiments (iPS1, iPS2, iPS3, and HES-3). Statistical significance was assessed by Student t test (*p < .05). (C): RT-qPCR data (2^−ΔΔCT values) of stimulated and sorted IRF8−/− and IRF8+/+ DC subsets on dd4 of two independent experiments (iPS1 and iPS3) depicted in heatmap format as in Fig. 2E. (D): Chemotactic responses of IRF8−/− and IRF8+/+ iPS-DC subsets on dd4. Migration is depicted as fold change compared to samples without ELC (see Supporting Information Fig. 8). Data represent the mean ± SD of three independent experiments (iPS1, iPS2, and iPS3). Statistical significance was assessed by Student t test (*p < .05). PBMNC derived DC were used as control (con). (E): Frequencies of BrdU+ T cells following coculture with IRF8−/− and IRF8+/+ stimulated (LPS) and unstimulated FSG4 and FSG derived DC on dd4. Percent of cells in the population of living single cells is shown. Frequencies represent the mean ± SD of two independent experiments (iPS2 and iPS3). Statistical significance was assessed by Student t test (*p < .05). Concanavalin A (ConA) treated and unstimulated T cells were used as control. Abbreviations: DC, dendritic cells; ES, embryonic stem; iPS, induced pluripotent stem; IRF8, interferon regulatory factor 8; PBMNC, peripheral blood mononuclear cells.

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of IRF8 on human hematopoiesis, DC lineage commitment, and subset specification. To this end, we engineered human iPS cells, which match their in vivo equivalents found in blood.

**DC Development and Function is Impaired in IRF8<sup>−/−</sup> Cells**

Next, we investigated the impact of IRF8 on DC development and subset specification (Fig. 4A). Surprisingly, cDC2 were generated from IRF8<sup>−/−</sup> cells in similar numbers as for IRF8<sup>+/+</sup> cells (Fig. 5A, center row). In contrast, cDC1 and pDC were strongly reduced in IRF8<sup>−/−</sup> cultures (Fig. 5A, top and bottom row). Moreover, IRF8<sup>−/−</sup> cDC1 and pDC but not cDC2 presented a severe defect in HLA-DR expression (Fig. 5A). This result was obtained for all three IRF8<sup>−/−</sup> iPS cell clones (iPS1, iPS2, and iPS3) analyzed and for IRF8<sup>−/−</sup> ES cells (Fig. 5B).

Additionally, the result is also consistent with the low HLA-DR expression in IRF8<sup>−/−</sup> cultures without lineage restrictive cytokines (Fig. 3D, Supporting Information Fig. 4D). Time course experiments showed that DC and CD14<sup>+</sup> monocyte numbers were reduced in IRF8<sup>−/−</sup> cells at all time points analyzed (Supporting Information Fig. 7).

IRF8<sup>−/−</sup> DC were stimulated and analyzed for cytokine production (IL-6, IL-12, and TNFα) and CD80, CCR7, and HLA-DR expression. IL-12 production was diminished in cDC1, pDC, and CD14<sup>+</sup> monocytes compared to IRF8<sup>+/+</sup> control (Fig. 5C). IRF8<sup>−/−</sup> cDC2 and CD14<sup>+</sup> monocytes showed a dampened IL-6 and TNFα response and a moderate reduction of CD80 and CCR7. HLA-DR expression was reduced in all IRF8<sup>−/−</sup> DC subsets, although the degree of reduction was higher in cDC1 and pDC than in cDC2 and CD14<sup>+</sup> monocytes (Fig. 5C). Furthermore, the capacity of IRF8<sup>−/−</sup> DC subsets to migrate toward the chemokine ELC, which is the ligand of CCR7, was compromised compared to IRF8<sup>+/+</sup> DC subsets (Fig. 5D, Supporting Information Fig. 8). Finally, we investigated the potential of IRF8<sup>−/−</sup> DC to present antigens to allogenic T cells in a mixed lymphocyte reaction (MLR). IRF8<sup>+/+</sup> FSG4 and FSG derived iPS-DC stimulated T cell proliferation as measured by BrdU incorporation, while the corresponding IRF8<sup>−/−</sup> iPS-DC were impaired in induction of T cell proliferation (Fig. 5E). Taken together, we demonstrated that IRF8<sup>−/−</sup> iPS-DC are impaired in development and functionally compromised.

**DISCUSSION**

Hematopoietic cell development is orchestrated by a network of master transcription factors that regulate lineage commitment and specification [35, 36]. In this study, we established an iPS/ES cell based IRF8 knockout model to study the impact of IRF8 on human hematopoiesis, DC lineage commitment, and subset specification. To this end, we engineered human IRF8<sup>−/−</sup> iPS cells and ES cells by genome editing with CRISPR/Cas. We describe a differentiation protocol to generate specific DC subsets from iPS cells and ES cells. We demonstrate that specification of hematogenic endothelial cells from iPS cells and ES cells, and the subsequent EHT are IRF8 independent. In contrast, IRF8<sup>−/−</sup> hematopoietic progenitors showed a granulocyte differentiation bias and a severe deficiency in monocyte development. Further to this, we report on the selective loss of cDC1 and of pDC in IRF8<sup>−/−</sup> DC cultures, whereas cDC2 were not affected. Finally, all IRF8<sup>−/−</sup> DC subsets were defective in HLA-DR and cytokine expression and presented an impaired migration and antigen presentation capacity and thus are functionally compromised.

Studying development and function of human DC subsets has been challenging due to the limited access to human lymphoid tissues and the low abundance of DC in human tissues [15, 22]. In recent years progress has been made in identifying human DC subsets [21, 22, 24], yet most of our knowledge on DC development stems from mouse knockout models and overexpression studies [11, 12, 37, 38]. These approaches provided valuable information on, for example, the role of transcription factors during DC fate decisions, however there are species specific differences between mouse and men in hematopoietic cell development [23]. As a result, there is still a paucity of knowledge on how human DC develop and on which transcription factors are involved.

Several groups generated DC from pluripotent stem cells as a potentially unlimited source of autologous DC [39–45]. Yet, these iPS/ES cell derived DC exhibited a phenotype similar to inflammatory DC and were not subset specific. However, to apply DC, for example, as anti-tumor therapeutics, specific DC subsets need to be generated in vitro. Silk et al. reported on the differentiation of functional cDC1 from iPS cells under feeder-free conditions [46]. However, recent genome wide transcriptional profiling studies highlighted the impact of microenvironmental cues during DC development [47], indicating that feeder support is important. We tested conventional feeder-free GM-CSF/IL-4 based protocols, which are frequently used for DC differentiation from monocytes, for studying DC development from iPS cells and ES cells [12]. But the DC generated coexpressed subset specific markers, which hampered their assignment to specific subsets (data not shown). Distinct populations of cDC1, cDC2, and pDC from iPS cells and ES cells were generated when specific cytokines were used in combination with OP9 stroma cells, as demonstrated here. These DC subsets showed the characteristic DC surface marker profile and when stimulated responded with an increased expression of HLA-DR and costimulatory molecules. Furthermore, such iPS-DC were able to actively migrate toward a chemokine gradient and induce T cell proliferation following antigen presentation.

Our iPS cell and ES cell based human IRF8<sup>−/−</sup> model recapitulates the phenotype of IRF8<sup>−/−</sup> mice, demonstrating the conserved central role of IRF8 during myelopoiesis between mice and men. IRF8<sup>−/−</sup> mice lack cDC1 and pDC [48–51]. cDC2 were not affected in IRF8<sup>−/−</sup> mice and in our human IRF8<sup>−/−</sup> in vitro model. In this context, it is interesting to note that a patient with a homozygous IRF8 loss of function mutation showed a depletion of all DC subsets, including cDC2 [5]. In contrast, patients with a dominant negative IRF8 mutation selectively lacked cDC2, but had normal numbers of other DC subsets [5]. Based on the IRF8 loss of function phenotype in humans, we speculated that in our IRF8<sup>−/−</sup> model cDC2 were only generated under selective cytokine pressure. In fact, in an unrestricted differentiation regimen without lineage specifying cytokines cDC2 were absent in IRF8<sup>−/−</sup> cultures, indicating that cDC2
development requires IRF8. This contrasts results in IRF8−/− mice. It seems possible that other IRF family members might substitute for IRF8 and from mouse studies IRF4 stands out as strong candidate [52, 53].

Recently, Sichien et al. [54] reported on a detailed mapping of IRF8 function during myelopoiesis in mice, which appears to be more complicated than previously anticipated. They demonstrated that IRF8 is dispensable for the development of monocyte progenitors, but required for terminal differentiation into macrophages and monocytes. Similarly, IRF8 seems to influence the development of different DC subsets at different progenitor stages. In line with these findings the remaining CD14+ population or DC in our IRF8−/− differentiation cultures might represent monocyte/macrophage or DC progenitors, respectively, that are blocked in their development.

In summary, here we present an iPS cell and ES cell based human IRF8−/− model that reflects the clinical phenotype of IRF8 deficient individuals. Patients with autosomal recessive or dominant IRF8 deficiency are very rare, which limits the opportunity to investigate the impact of IRF8 in the human system. iPS cells and ES cells are immortal and exhibit an essentially unlimited lifespan and thus represent an inexhaustible source of IRF8−/− hematopoietic progenitors to study molecular mechanisms of DC subset specification and functional diversity. IRF8 is also important in granulocyte, mast cell and osteoclast development [7] and IRF8−/− mice develop a chronic myelogenous leukemia (CML) like syndrome [51] and thus our model provides further opportunities on analyzing IRF8 function on the development of other hematopoietic lineages and their pathologies.

**CONCLUSION**

In summary, we have engineered human IRF8−/− iPS cells and IRF8−/− ES cells that upon differentiation recapitulate human immunodeficiency associated with IRF8 mutations. Patient samples express IRF8 mutant proteins, yet with impaired function, while our IRF8 knockout cells are completely devoid of IRF8 protein. Thus, our study provides a clear-cut model to investigate molecular mechanisms, transcriptional networks, and developmental pathways underlying DC subset specification and function orchestrated by IRF8. A deeper understanding of human DC and their specialization will help to advance DC based therapies. On a broader level, our IRF8−/− iPS cells and ES cells can also be used to study the delineation of other myeloid cells, for example, granulocytes, monocytes, mast cells, and osteoclasts. Ultimately, our IRF8−/− in vitro model can provide novel insights into the early events of human hematopoiesis and thereby might identify new therapeutic targets for human hematopoietic diseases.

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**AUTHOR CONTRIBUTIONS**

S.S.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; M.F.: Collection of data; J.Q.: Data analysis and interpretation; P.W.: Collection of data; S.M.: Collection of data; H.M.S.: Data analysis and interpretation; S.K.: Provision of study material and patients, data analysis and interpretation; S.R.-J.: Provision of study material; K.S.: Conception and design, data analysis and interpretation; M.Z.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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