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Supplemental Information

Impaired IFNγ-Signaling and Mycobacterial Clearance in IFNγR1-Deficient Human iPSC-Derived Macrophages

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Figure S1

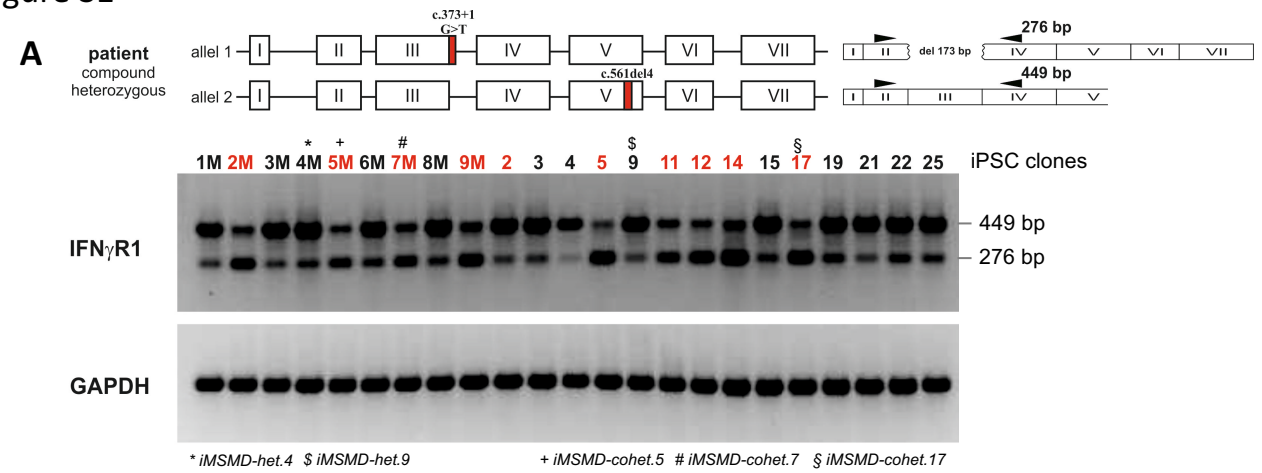

A
patient
compound
heterozygous
allel 1
allel 2
c.373+1
G>T
c.561del4
I
II
III
IV
V
VI
VII
del 173 bp
276 bp
449 bp
1M 2M 3M 4M 5M 6M 7M 8M 9M 2 3 4 5 9 11 12 14 15 17 19 21 22 25
iPSC clones
IFNγR1
GAPDH
449 bp
276 bp
* iMSMD-het.4 $ iMSMD-het.9
+ iMSMD-cohet.5 # iMSMD-cohet.7 § iMSMD-cohet.17

Figure S2

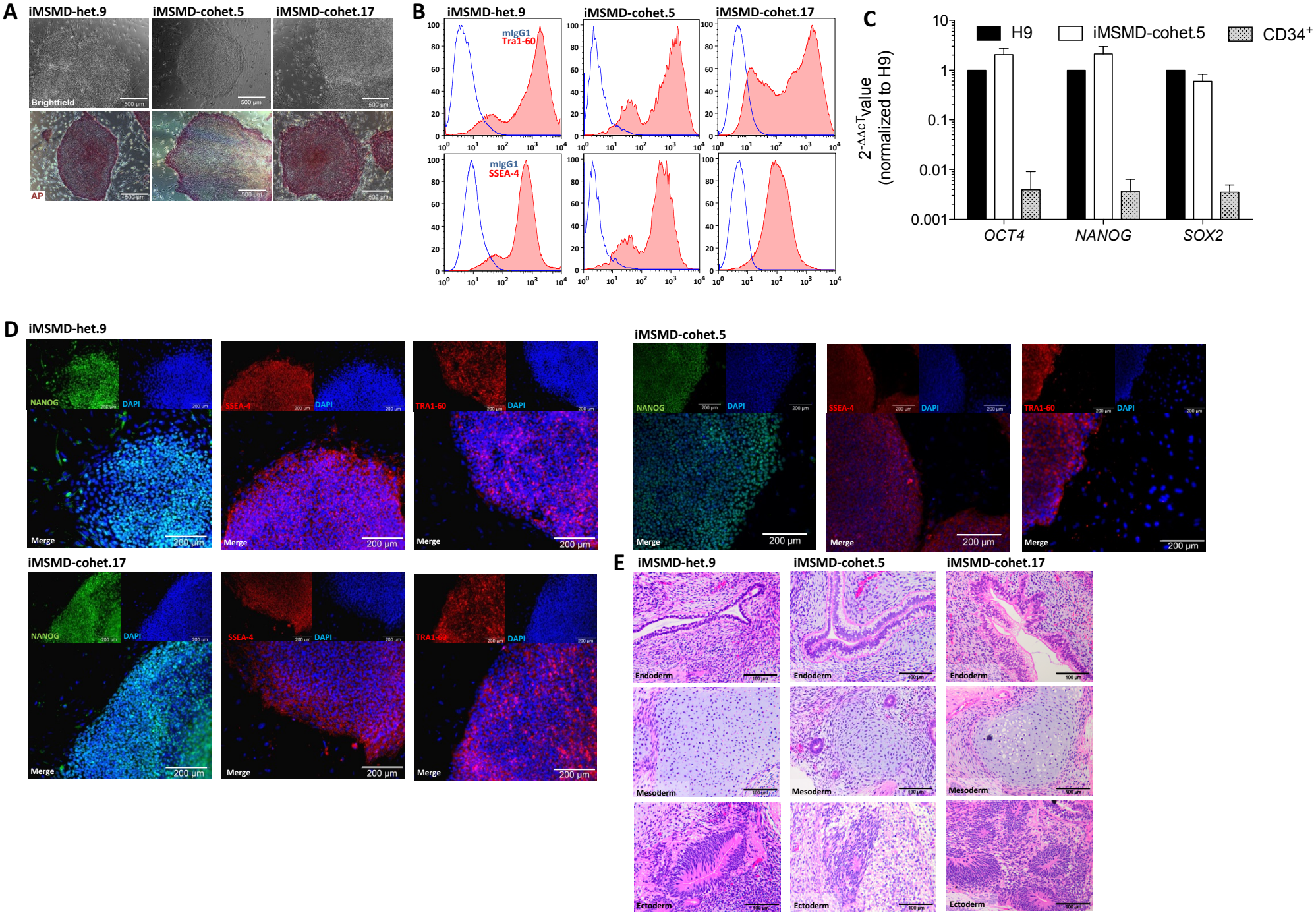

A
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
Brightfield
AP
B
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
mIgG1
Tra1-60
mIgG1
SSEA-4
C
H9
iMSMD-cohet.5
CD34+
2-ΔΔcTvalue
(normalized to H9)
OCT4
NANOG
SOX2
D
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
NANOG
SSEA-4
TRA1-60
DAPI
Merge
200 μm
E
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
Endoderm
Mesoderm
Ectoderm

Figure S3

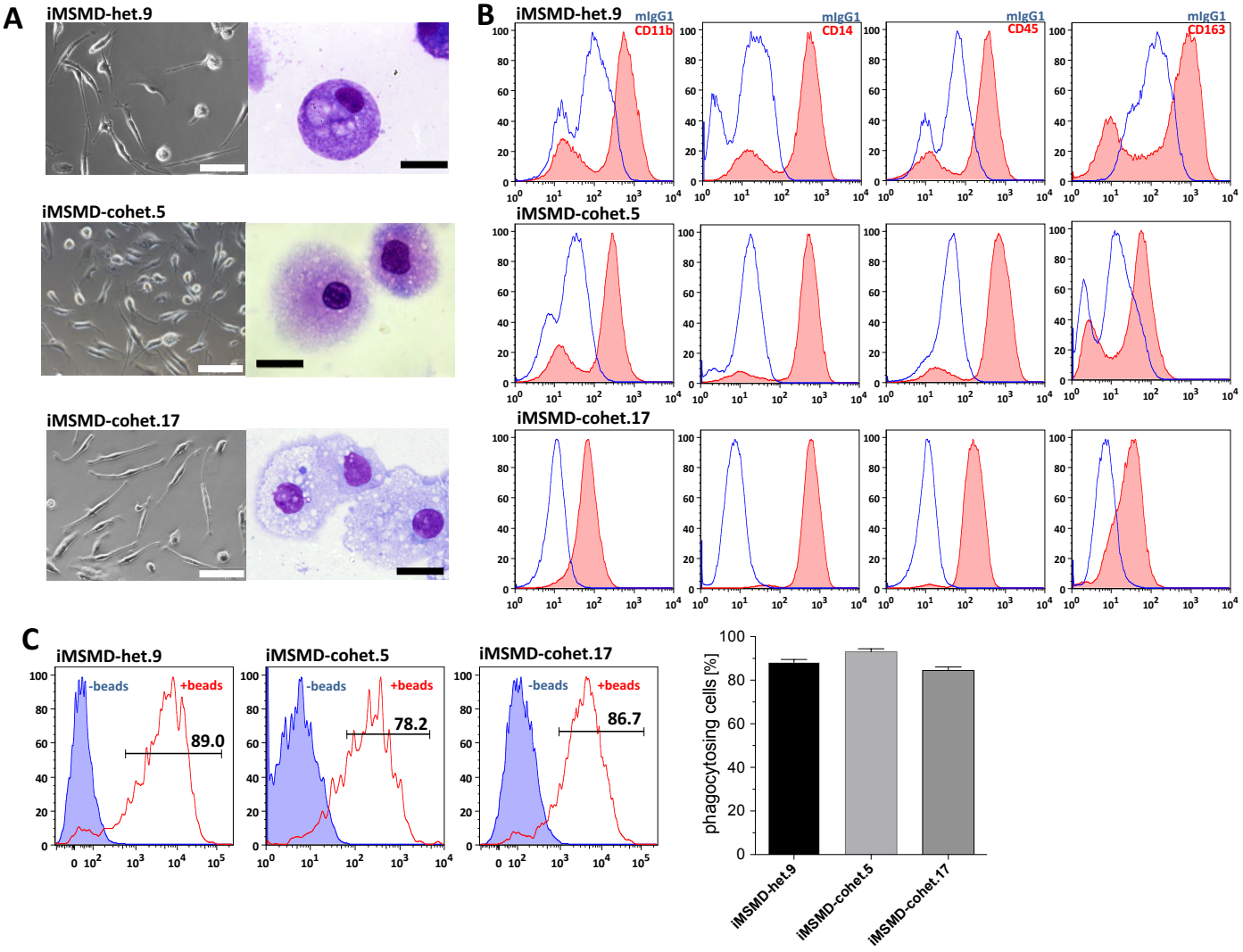
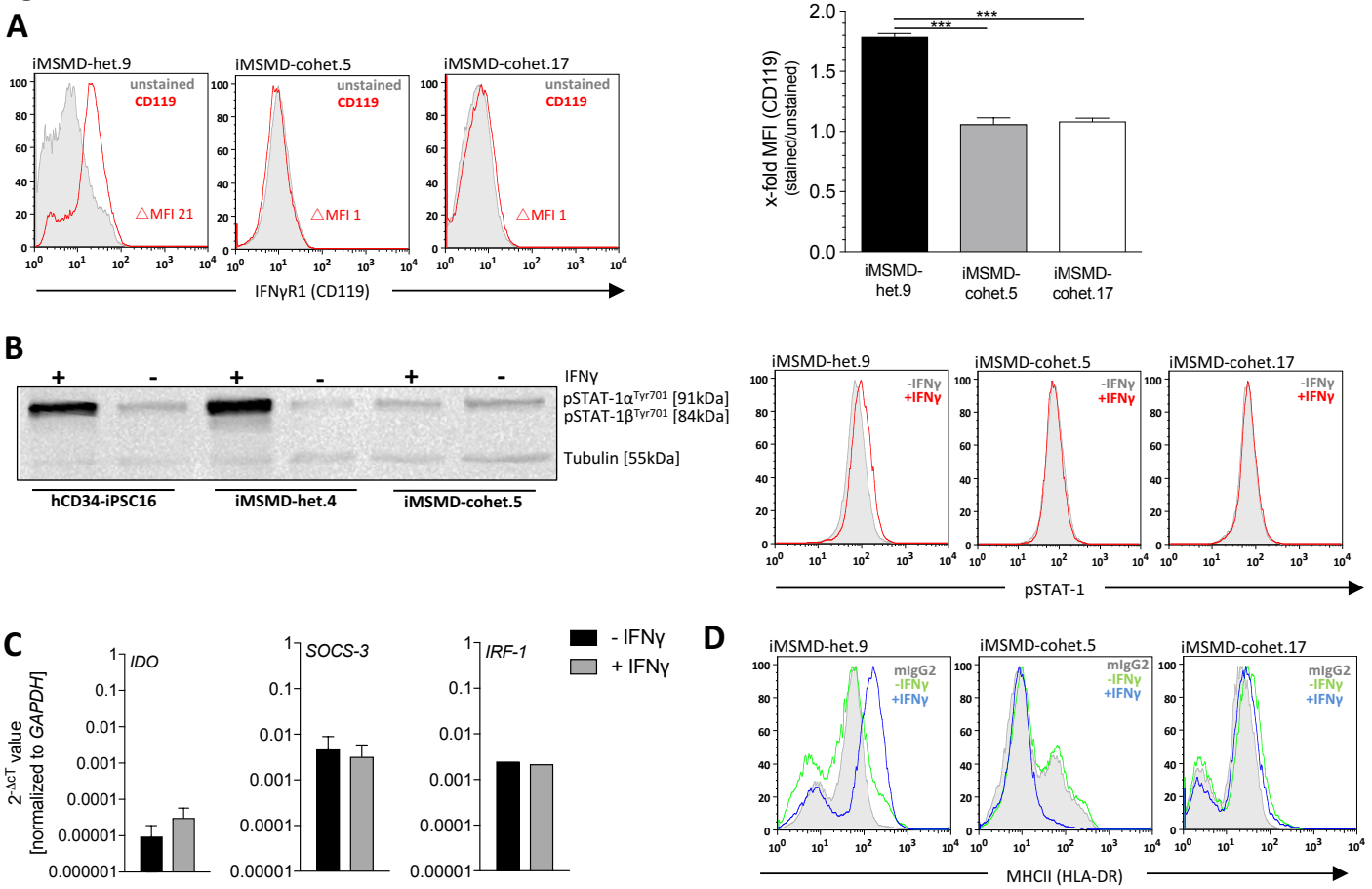

A
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
B
iMSMD-het.9
mIgG1
CD11b
mIgG1
CD14
mIgG1
CD45
mIgG1
CD163
iMSMD-cohet.5
iMSMD-cohet.17
C
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
-beads
+beads
89.0
78.2
86.7
phagocytosing cells [%]
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17

Figure S4


A
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
unstained
CD119
△MFI 21
△MFI 1
△MFI 1
IFNγR1 (CD119)
x-fold MFI (CD119)
(stained/unstained)
***
***
iMSMD-het.9
iMSMD-cohet.5
iMSMD-cohet.17
B
IFNγ
pSTAT-1αTyr701 [91kDa]
pSTAT-1βTyr701 [84kDa]
Tubulin [55kDa]
hCD34-iPSC16
iMSMD-het.4
iMSMD-cohet.5
-IFNγ
+IFNγ
pSTAT-1
C
IDO
SOCS-3
IRF-1
- IFNγ
+ IFNγ
2-ΔCT value
[normalized to GAPDH]
D
mIgG2
-IFNγ
+IFNγ
MHCII (HLA-DR)

Supplemental Information

Online Materials and Methods

Ethical statement

Human peripheral blood samples were collected after written informed consent of the donor at Universitätsklinikum Dresden (Germany). Ethical vote No. 2127-2014 was approved by the Institutional Ethical Committee at Hannover Medical School (Hannover, Germany).

Generation and cultivation of human iPS cells

Peripheral blood was collected from a previously described patient (Reuter et al., 2002; Roesler et al., 1999) $CD34^+$ cells were isolated using magnetic activated cell sorting (MACS) (Miltenyi, Bergisch-Gladbach, Germany), and cultured in StemSpan (StemCells, Cologne, Germany) supplemented with 2 mM L-glutamine, 1 % penicillin-streptomycin, 100 ng/ml human stem cell factor, 100 ng/ml hFlt3-L and 50 ng/ml human thrombopoietin (Peprotech, Hamburg, Germany) for 24 hours. For iPSC generation $CD34^+$ cells were transduced with the "4 in 1" all in one third-generation self-inactivating lentiviral reprogramming vector (Warlich et al., 2011) on Retronectin (Takahara, Otsu, Japan)-coated dishes with a multiplicity of infections of 5, 10, or 20 in complete StemSpan medium. 24 hours after transduction 0.5 µM valproic acid was added and medium change was performed daily. After 4 days, 20 ng/ml basic fibroblast growth factor (bFGF) (Preprotech) was added and 5 days after transduction, cells were transferred to irradiated mouse embryonic fibroblasts from CF1 mouse strain and grown in half-half medium consisting of medium I (complete StemSpan) and human embryonic stem cell (ESC) medium II (knock-out Dulbecco's modified Eagle medium, 20% knock-out serum replacement, 0.1 mM beta-mercaptoethanol, 1 mM L-glutamine, 1% nonessential amino acids, and 20 ng/ml bFGF; Invitrogen, Darmstadt, Germany). When initial colonies were observed, medium was completely changed to human ESC medium. iPSC cultivation was performed by splitting every 5-7 days depending on colony size by 2 mg/ml collagenase (Invitrogen).

Characterization of iPSCs

Immunfluorescence staining was carried out according to standard protocols. Primary antibodies used were anti-OCT4 (Santa-Cruz, Heidelberg, Germany (sc-5279); 1:200), anti-NANOG (Abcam, Cambridge, UK; (ab80892) 1:50), anti-TRA1-60 (Millipore, Billerica, MA (MAB4360); 1:250) and anti-SSEA-4 (Millipore; (MAB4304) 1:250). Secondary antibodies used include goat anti-mouse IgG Alexa-546 (A-11003) and goat anti-mouse IgM Alexa-488 (A-10680) (Invitrogen). Nuclei were costained with 4,6-diamidino-2-phenylindole DAPI (1:2000).
TRA1-60 expression was characterized using the same protocol with the following antibodies: 1 hour TRA1-60 Biotin (1:250; eBioscience, Frankfurt am Main, Germany (13-8863-82)) followed by 45 minutes HRP-Avidine (1:500; eBioscience (18-4100-51)) and 5 minutes diaminobenzidine (Invitrogen). Alkaline Phosphatase staining was performed using the Alkaline Phosphatase Staining Kit (Stemgent, Cambridge, MA, USA) according to the manufacturer's instructions.
For analysis of mRNA expression levels total RNA was isolated using the miRNAeasy Kit (Qiagen, Hilden, Germany) and additional DNase I treatment (DNase I, RNase-free [1 U/µl]; Thermo Fisher Scientific, Waltham, MA, USA). For reverse transcription RevertAid reverse transcriptase and random hexamer primers (Thermo Fisher Scientific) were used. The following predesigned assays were obtained from Applied Biosystems (Darmstadt, Germany) for TaqMan-based qRT-PCR: *GAPDH* (Hs_99999905), *NANOG* (Hs_02387400), *SOX2* (Hs_01053049) and *OCT4* (Hs_3005111).

Undirected in vitro differentiation

hiPSCs were detached from the feeder layer by collagenase IV, dispersed into small clumps and cultured in differentiation medium (80% IMDM supplemented with 20% fetal calf serum, 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 1% nonessential amino acid stock) in ultra-low attachment plates (Corning Inc., NY, USA) for 7 days. Subsequently, EBs were plated onto 0.1% gelatine coated tissue culture dishes and cultured for a further 13 days before fixation and immunostaining. Cells were fixed with 4% paraformaldehyde (w/v) and stained by standard protocols using primary antibodies anti-beta3-Tubulin (Upstate, NY, USA (05-559); IgG2a, 1:400), anti-AFP (R&D Systems, Minneapolis, USA (189502); IgG1, 1:800) and anti-Desmin (Progen, Heidelberg, Germany (10519); IgG1, 1:20) and secondary antibody DyLight®549-donkey-anti-mouse-IgG (1:200, Jackson Immunoresearch Laboratories (715-165-150). Corresponding isotype antibodies were used for negative control staining. Cells were counterstained with DAPI (Sigma) and analysed with an AxioObserver A1 fluorescence microscope and Axiovision software 4.71 (Zeiss).

Teratoma induction

hiPSC were expanded in co-culture with C3H murine embryonic fibroblasts. Cells were detached with Trypsin/EDTA (Pan/Biotech). Harvested cells ($3*10^6$ hiPSC/ flank) were resuspended in medium containing 20µm p160ROCK inhibitor (Biotechne). Final cell suspension volume of 100-150µl (one injection) was mixed

with 150µl Matrigel Matrix (Corning) and kept at 4°C until subcutaneous injection. Teratoma assay was performed in adult NSGS mice ((NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ). All animal experiments have been approved by the animal welfare committee of Lower Saxony and have been performed in accordance to the R3 regulations. NSGS mice have been housed in pathogen-free environment in the animal facility of Hannover Medical School with access to food and water *ad libitum*.

HE staining
Isolated tissue was fixed in 4% buffered formaldehyde (Carl Roth GmbH+ Co. KG). Then, samples were embedded in paraffin and sectioned into 3µm slices. Hematoxylin and Eosin staining was performed using standard histological procedures. Analysis was done with microscope BX51, camera XC50 and software Cell^F version 3.4 (all Olympus).

Genotyping
For genotyping, genomic DNA was prepared using QIAamp Blood Mini Kit (QIAGEN) according to manufacturer's instructions and 100 ng of gDNA was amplified by PCR with Phusion® High-Fidelity DNA polymerase (NEB) according to manufacturer's instructions and following primers: forward 5'- ACA CCT CCA TTG CTC CAC TG -3' and reverse 5'- TCA GTT GTA ACA CCC CAC ACA -3'. The PCR products were sequenced using the reverse primer. For the validation of the exon III skipping, RT-PCR on coding DNA was applied. Therefore, cDNA was amplified by PCR with GoTaq®HotStart Polymerase (Promega) according to manufacturer's instruction and following primers: forward 5'- CTT GTC ATG CAG GGT GTG AG -3' and reverse 5'- TCC TGC TCG TCT CCA TTT ACA -3'.

Flow Cytometric Analysis
Hematopoietic and non-hematopoietic surface marker expression was performed as described before (Lachmann et al., 2015). Antibodies were used as follows: CD11b-APC (12-0118-42), CD14-PE(12-0149-41), CD45-APC (17-0459-42), CD163-PE (12-1639-41), CD119-PE (12-1199), HLA-DR-APC (17-9956), SSEA4-FITC (560126, Biolegend), TRA1-60-PE (12-8863-80), isotype controls; mouse IgG1k-PE (12-4714-81) or APC (17-4714-81), mouse IgG2bk-APC (17-4732), mouse IgMk-PE (401611, Biolegend) and mouse IgG3 (114742) (if not otherwise noted all antibodies are from eBioscience), minus one control represents the staining without the respective specific antibody.
For measurement of HLA-DR upregulation after IFNγ stimulation, iPSC-derived macrophages were either left unstimulated or stimulated with 25 ng/ml IFNγ for 24 hours.

Cytospins
For cytospins $3x10^4$ cells were centrifuged on object slides at 600 x g for 7 minutes. Subsequently, slides were stained for 5 minutes in May-Grünwald staining solution (0,25% (w/v) in methanol**),** followed by 20 minutes in 5% of Giemsa azur-eosin-methylene blue solution (0,4% (w/v) in Methanol, working solution was 0.02%) and washed extensively in aqua dest.

Phagocytosis
For assessment of phagocytosis, $1x10^5$ cells were seeded in 96-well plates in standard medium. After >12 hours of settling, cells were incubated for 2 hours with carboxylate fluorescent latex-beads (1 µm in diameter; Polysciences, Warrington, PA, USA) at a concentration of 1:200. Subsequently, cells were washed extensively, and the amount of incorporated beads was assessed by flow cytometry on a FACSCanto II flow cytometer (BD) and further analyzed using FlowJo V10 (Tree Star).

qRT-PCR
For measurement of mRNA expression levels macrophages were stimulated with 25 ng/ml IFNγ for 24 hours and qRT-PCR was performed as described above.
SYBR Green qRT-PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems) with the following predesigned Quantitect Primer Assay (Qiagen): *GAPDH* (QT01192646), *IDO* (QT00000504), *SOCS-3* (QT00244580), *IRF-1* (QT00494536). qRT-PCR was performed on a StepOnePlus thermocycler (Applied Biosystems) under the following conditions: 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

STAT1 Phosphorylation Assay
Macrophages were stimulated with 25 ng/ml IFNγ for 24 hours. Proteins were isolated by utilizing radioimmunoprecipitation assay buffer and protease as well as phosphatase inhibitor according to the manufacturer's instructions. Phosphorylated STAT1 was detected by Western blot analysis using phospho-STAT1 (Tyr701; 9167) and α-tubulin (2144) antibodies (Cell Signaling Technology, Frankfurt am Main, Germany and Sigma Aldrich). Membranes were incubated with the horseradish-peroxidase-coupled secondary

antibody (111-035-144; Jackson ImmunoResearch, Suffolk, UK) at room temperature for 1 hour, followed by Western blot run with 15 µg of protein in a 12% sodium dodecyl sulfate gel. pSTAT1/α-tubulin intensity ratios were determined by densitometry using Bio-Rad Quantity One analyzing software at an exposure time of 90 seconds.
Flow cytometric analysis of phosphorylated STAT1 was performed using pSTAT1-Ser727 (BioLegend; 686403) according to the manufacturer's instructions. iPSC-derived macrophages were either left unstimulated of were stimulated with 25 ng/ml IFNγ for 24 hours before analysis.

Mycobacterial Killing Assay
Mycobacterial killing by macrophages was determined as previously described (Kuehnel et al., 2001). Briefly, Mycobacterium bovis (BCG) cultures in exponential growth phase were pelleted, washed twice in PBS pH 7.4 and re-suspended in DMEM medium to a final OD_{600}: 0.1. Clumps of bacteria were removed by ultrasonic treatment of bacteria suspensions in an ultrasonic water bath for 5 minutes followed by a low speed centrifugation (120 x *g)* for 2 minutes. Single cell suspension was verified by light microscopy.
iPS cell generated macrophages were seeded onto 12-well tissue culture plates at a density of 0.5×10^5 cells per well. 24 hours before infection cells were stimulated with 25 ng/ml IFNγ. The infection was carried out using a multiplicity of infection (MOI) of 100:1 for 1 h (OD_{600}~0.1). In each experiment, after 1 hour infection gentamicin (10 µg/ml) was added. At 1 and 24 p.i. cells were washed twice with PBS and scraped off the plates in 1 ml of 1% Nonidet P40 in PBS. Macrophages were disrupted by 10 passages through a 24-gauge needle. Tenfold serial dilutions of the homogenates were prepared with PBS, and 100 µl of each was plated on Middlebrook 7H10 agar plates. After incubation for up to 2 weeks at 37°C, colony-forming units (cfu) were counted.

Supplemental references

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Supplemental figure legends

(Related to figure 1) Figure S1 Supplementary figure 1 Identification of the genetic background of iMSMD iPSC lines. (A) RT-PCR based screen to identify the heterozygous c373+1 G>T transition in heterozygous and compound heterozygous iMSMD iPSC lines. Sequence specific primers for exon III were designed to amplify either a 276bp fragment describing the heterozygous 173bp deletion and to amplify a 449bp fragment to amplify the second functional allele. Red color indicates compound heterozygous iPSC clones. Primers for *GAPDH* were used as internal loading control.

(Related to figure 2) Figure S2 Supplementary figure 2 Characterization of MSMD iPSC lines (**A**) Microscopy analysis of iPSC lines derived from either heterozygous (iMSMD-het.9) or compound heterozygous (iMSMD-cohet.5 and .17) donor cells. Images are shown for brightfield and alkaline phosphatase (AP) staining (scale bar = 500 µm). (**B**) Flow cytometry analysis of TRA1-60 and SSEA-4 expression on iMSMD iPSCs (blue line: isotype; red filled line: surface marker). (**C**) qRT-PCR to evaluate endogenous expression of *OCT4*,

NANOG and *SOX2* in MSMD-cohet.5 relative to H9 control cells (n=4, independent experiments, mean ±SEM). (**D**) Immunfluorescence staining of pluripotency associated factors for NANOG (green), SSEA-4 (red) and TRA1-60 (red) (scale bar = 200 µm). (**E**) Representative hematoxylin and eosin (HE) staining of teratoma formation after transplantation of iMSMD-het.9 (endoderm: epithelium lining a luminal surface; mesoderm: cartilage; ectoderm: immature neuroepithelium), iMSMD-cohet.5 (endoderm: epithelium lining a luminal surface; mesoderm: cartilage; ectoderm: immature neuroepithelium) or iMSMD-cohet.17 (endoderm: intestinal-like epithelium with goblet cells; mesoderm: cartilage; ectoderm: immature neuroepithelium) cells into immunocompromised NSGS mice (scale bar = 100µm).

(Related to figure 3) Figure S3 Supplementary figure 3 Hematopoietic differentiation of iMSMD lines
Heterozygous (iMSMD-het.9) and compound heterozygous (iMSMD-cohet.5 and .17) iPSC lines has been differentiated towards macrophages. (**A**) Microscopic analysis of macrophages in brightfield images (left, scale bar: 100µm) or cytospin images (right, scale bar: 20µm). (**B**) Surface marker expression by flow cytometry of hCD11b, hCD14, hCD45 and hCD163 on macrophages derived from iMSMD-het.9, iMSMD-cohet.5 and iMSMD-cohet.17 lines (blue line: isotype; red filled line: surface marker). (**C**) Ability of iPSC-derived macrophages to phagocytose latex-beads. Left images show representative flow cytometric analysis of macrophages (blue filled line: cells without beads, red line: cells beads). Right image shows macrophages which are positive for fluorescent latex-beads (n=4, independent experiments, mean ±SEM).

(Related to figure 4) Figure S4 Supplementary figure 4 Disease phenotype of differentiated iMSMD-derived macrophages. (**A**) Left images show representative flow cytometric analysis of IFNγR1 (CD119) surface marker expression by flow cytometry on iMSMD-derived macrophages (grey filled line: unstained, red line: CD119). Right images show delta mean fluorescent intensity (MFI) of CD119 expression (delta MFI was calculated by subtracting unstained (negative) MFI from CD119 (positive) MFI) relative to unstained control (n=3, independent experiments, mean ±SEM; ***significance of $p< 0.01$ by One-Way ANOVA). (**B**) Analysis of STAT1 phosphorylation (pSTAT1) in macrophage subsets stimulated with IFNγ. Left image shows western blot analysis of pSTAT1 (approx. 90kDa) in iMSMD-cohet.5- and hCD34-iPSC16-derived macrophages (Tubulin (50kDa) served as a control). Right images show pSTAT1 expression by flow cytometry of iMSMD-derived macrophages (grey filled line: unstimulated, red line: IFNγ stimulated). (**C**) Quantitative PCR of *IDO*, *SOCS-3* and *IRF-1* in iMSMD-cohet.5- and hCD34-iPSC16-derived macrophages after stimulation with IFNγ. Values are normalized to *GAPDH* as a housekeeping gene (n= 1-2, independent experiments, mean ±SEM). (**D**) Flow cytometric analysis of MHC-II (HLA-DR) surface marker expression by flow cytometry on iMSMD-derived macrophages before and after stimulation with IFNγ (grey filled line: isotype, green line: non-stimulated control, blue line: IFNγ stimulated).