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Short communication

Heterozygous missense *CSF1R* variants hamper *in vitro* CD34+-derived dendritic cell generation but not *in vivo* dendritic cell development

Shanice Beerepoot [a,b,c], Nicole I. Wolf [a], Marjo S. van der Knaap [a,d], Stefan Nierkens [b,c,*,1], Maud Plantinga [b,1]

[a] Department of Child Neurology, Amsterdam Leukodystrophy Center, Emma Children's Hospital, Amsterdam University Medical Center, Amsterdam, The Netherlands
[b] Center for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands
[c] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
[d] Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, VU University, Amsterdam, The Netherlands

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ABSTRACT

Colony stimulating factor 1 receptor (CSF1R) is an essential receptor for both colony stimulating factor 1 (CSF1) and interleukin (IL) 34 signaling expressed on monocyte precursors and myeloid cells, including monocytes, dendritic cells (DC), and microglia. In humans, dominant heterozygous pathogenic variants in *CSF1R* cause a neurological condition known as CSF1R-related disorder (CSF1R-RD), typically with late onset, previously referred to as adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP). CSF1R-RD is characterized by microglia reduction and altered monocyte function; however, the impact of pathogenic *CSF1R* variants on the human DC lineage remains largely unknown.

We previously reported that cord blood CD34+ stem cell-derived DCs generated *in vitro* originate specifically from CSF1R expressing precursors. In this study, we examined the DC lineage of four unrelated patients with late-onset CSF1R-RD who carried heterozygous missense *CSF1R* variants (c.2330G>A, c.2375C>A, c.2329C>T, and c.2381T>C) affecting different amino acids in the protein tyrosine kinase domain of CSF1R. CD34+ stem cells and CD14+ monocytes were isolated from peripheral blood and subjected to an *in vitro* culture protocol to differentiate towards conventional DCs and monocyte-derived DCs, respectively. Flow cytometric analysis revealed that monocytes from patients with late-onset CSF1R-RD were still able to differentiate into monocyte-derived DCs *in vitro*, whereas the ability of CD34+ stem cells to differentiate into conventional DCs was impaired. Strikingly, the peripheral blood of patients contained all naturally occurring DC subsets. We conclude that the *in vitro* abrogation of DC-development in patients with heterozygous pathogenic missense *CSF1R* variants does not translate to an impairment in DC development *in vivo* and speculate that CSF1R signalling *in vivo* is compensated, which needs further study.

1. Introduction

Colony stimulating factor 1 (CSF1) signals via a high-affinity receptor tyrosine kinase (CSF1R) encoded by the c-*fms* proto-oncogene *CSF1R* (Sherr et al., 1985). CSF1 regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells and is the primary regulator of mononuclear phagocyte production *in vivo* (Stanley and Chitu, 2014). Mononuclear phagocytes include monocytes, macrophages, and dendritic cells (DCs), which share common features, but are still heterogeneous regarding origin, phenotype, and function. The understanding of myeloid cell development has increased with the recognition of a common monocyte DC progenitor (MDP) to generate both monocytes and DCs without the potential to develop into granulocytes (Fogg et al., 2006). MDPs give rise to common monocyte progenitors and common DC progenitors (Kawamura et al., 2017), under the influence of CSF1 in addition to CSF2 and FMS-like tyrosine kinase 3

Abbreviations: ADC, apparent diffusion coefficient; ALSP, Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; CSF1R-RD, CSF1R-related disorder CSF1R-RD; DWI, Diffusion-weighted images; GNDS, Guy's Neurological Disability Scale.

* Correspondence to: Center for Translational Immunology, University Medical Center Utrecht, Heidelberglaan 100, Utrecht 3584 CX, the Netherlands.
E-mail address: snierken@umcutrecht.nl (S. Nierkens).

[1] Shared last authorship.

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ligand (Flt3l) (MacDonald et al., 2005; Sallusto and Lanzavecchia, 1994; Caux et al., 1997; Karsunky et al., 2003). Although expression of the CSF1 receptor (CSF1R), also known as Cluster of Differentiation 115 (CD115), on MDPs and common DC progenitors has been confirmed (Fogg et al., 2006; Plantinga et al., 2019a), and expression of CSF1R on common DC progenitors has been associated with the rise of precursor cells committed to either conventional (c)DC or plasmacytoid (p)DC fates (Musumeci et al., 2019), the exact role of CSF1R in myeloid differentiation remains unclear.

Several mouse models have been described to study the role of CSF1 and CSF1R in myeloid development. A spontaneous autosomal recessively inherited inactivating mutation of the *Csf1* gene resulted in *Csf1*$^{op/op}$ mice with reduced numbers of osteoclasts and macrophages (Marks and Lane, 1976). Targeted ablation of the *Csf1r* gene in mice also caused severely reduced macrophage populations and showed an even more severe phenotype with poorer viability than *Csf1*$^{op/op}$mice (Dai et al., 2002). Interestingly, in *Csf1r* $^{-/-}$ mice, DCs were normally differentiated in peripheral lymphoid organs, but were impaired in specific non-lymphoid tissue (Bogunovic et al., 2009; Ginhoux et al., 2006). In addition, *Csf1r*$^{-/-}$ mice manifested defects in the differentiation of monocytes into monocyte-derived DCs, indicating involvement of CSF1R-mediated signals in monocyte-derived DC differentiation (Greter et al., 2012). The involvement of CSF1R in DC development is supported by the findings that inactivation of the *Flt3l* gene leads to increased CSF1R signalling with partial rescue of the DC deficiency in mice, and that additional deletion of *Csf1r* in *Flt3*$^{-/-}$ mice further reduces DC development (Durai et al., 2018).

In humans, dominant heterozygous pathogenic *CSF1R* variants, most often located in the protein tyrosine kinase (PTK) domain of CSF1R (Rademakers et al., 2011), have been linked to a neurological condition known as CSF1R-related disorder (CSF1R-RD), typically with late onset, previously referred to as adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) (MIM221820) (Dulski et al., 2024). The disease is characterized by severe microglia reduction, resulting in neurological decline and premature death in adults (Chitu et al., 2015). *In vitro* evidence suggests that auto-phosphorylation of the PTK domain of the CSF1R in microglia is impaired by a dominant-negative mechanism, although a haploinsufficiency genetic mechanism has also been proposed (Chitu et al., 2015; Stables et al., 2022). In addition to microglia reduction, altered features of peripheral monocytes, including altered expression of antigen presentation molecules, an inflammatory shift in cytokine production and impaired phagocytic function, and a reduction of slan-positive CD14+CD16++ non-classical monocytes have been reported (Hamatani et al., 2020; Hofer et al., 2015), although patients do not suffer from systemic immunological disease. The impact of dominant heterozygous pathogenic *CSF1R* variants on the DC lineage in human patients remains largely unexplored. Hofer *et al.* (2015) mentioned in their study that the numbers of cDCs and pDCs in four patients with late-onset CSF1R-RD were similar to those in healthy individuals, but data were not shown (Hofer et al., 2015).

Peripheral blood mononuclear cells (PBMCs) from patients with CSF1R-RD provide an opportunity to study the role of CSF1R in the development of DCs. To investigate if CSF1R plays an essential role in DC differentiation, in addition to differentiation of monocytes into inflammatory monocytes, we cultured CD14+ monocytes and CD34+ stem cells of four unrelated patients with late-onset CSF1R-RD resulting from different heterozygous dominant pathogenic *CSF1R* variants, using a previously developed *in vitro* culture protocol (Plantinga et al., 2019a). Furthermore, we investigated the PBMCs of these patients for the presence of naturally occurring DC subsets. Whereas CD34+ stem cells from healthy donors differentiated into cDC, CD34+ stem cells from patients failed to do so. Nevertheless, we confirmed the statement by Hofer et al. (2015) that all peripheral blood DC subsets, including cDC1 and cDC2, are present in blood from patients with late-onset CSF1R-RD.

2. Patients and methods

2.1. Patients

In this study, approved by the institutional review board of Amsterdam University Medical Center (protocol number 2018.300) and with obtained informed consent, we included four patients (CSF1R-RD1, CSF1R-RD2, CSF1R-RD3, and CSF1R-RD4) with a clinically and genetically confirmed diagnosis of late-onset CSF1R-RD. All patients were compound heterozygous for a previously reported pathogenic *CSF1R* missense variant localized in the intracellular PTK domain, including: c.2330G>A, p.(Arg777Gln); c.2375C>A, p.(Ala792Asp); c.2329C>T, p.(Arg777Trp); and c.2381T>C, p.(Ile794Thr). The patients, one female (CSF1R-RD1) and three males (CSF1R-RD2, CSF1R-RD3, and CSF1R-RD4) aged between 38 and 56 years, were all classified as having an early-to-intermediate-stage late-onset CSF1R-RD at the moment of blood sampling, considering their disease duration (between 1 and 3 years after symptom onset) in combination with a relatively low Guy's Neurological Disability Scale (GNDS) score (Sharrack and Hughes, 1999) (between 9 and 21 out of 60, 60 indicating maximum disability) and mild to moderately impaired cognitive function. The two youngest patients (CSF1R-RD2 and CSF1R-RD3) presented primarily with motor impairment, while the predominant initial symptoms of the two oldest patients (CSF1R-RD1and CSF1R-RD4) consisted of memory deficits and slow processing speed (supplementary table 1). None of the patients received any medical treatment at time of the study. Brain magnetic resonance imaging at 3 T showed bilateral focal and confluent white matter abnormalities, thinning of the corpus callosum, cerebral atrophy as indicated by slightly to mildly enlarged lateral ventricles and subarachnoid spaces in all patients (supplementary figure 1A and 1B). No evidence of calcifications was observed in any of the patients. CSF1R-RD1 and CSF1R-RD2 additionally showed involvement of the corticospinal tracts (supplementary figure 1 C) and areas of restricted diffusion in the white matter (supplementary figure 1D). Except for asthma (CSF1R-RD3), none of the patients had a medical history suggesting systemic immunological disease involvement.

2.2. Sample collection

Blood samples of patients with late-onset CSF1R-RD consisted of fresh leftover material from venous puncture performed in the context of routine clinical care. For CD34+-derived dendritic cell cultures, fresh cord blood served as a control, and was obtained after informed consent and with approval of the institutional review board of University Medical Center Utrecht (protocol number TC-bio 15–345). For monocyte-derived dendritic cell cultures and analysis of monocytes and DC subsets *in vivo*, blood samples of adult healthy controls (HCs) were recruited from our in-house healthy donor service. All samples were collected in ethylenediaminetetraacetic acid tubes. None of the patients and HCs had fever or other signs or symptoms of acute illness at time of collection.

2.3. Dendritic cell cultures

Mononuclear cells were isolated from peripheral blood by density centrifugation over Ficoll-Paque solution (GE Healthcare Bio-Sciences AB). Cells were diluted in magnetic-activated cell sorting buffer, containing phosphate-buffered saline, 2 % 0.1 M ethylenediaminetetraacetic acid, and 2 % fetal calf serum. Cells were incubated for 30 minutes at 4°C and stained with anti-CD3 and anti-CD19 magnetic beads according to the manufacturer protocol. CD3+ and CD19+ cells were removed by magnetic separation (Miltenyi Biotec). The leftover cells were incubated for 30 minutes on 4°C and stained with anti-CD3, CD19, CD56, CD11c, Human Leukocyte Antigen - DR isotype (HLA-DR), CD14, CD141, CD1c, CD123, CD304 and CD34. Progenitors and DCs were sorted using FACS ARIA II (BD) flow cytometer. The protocol for CD34+ stem cell-derived DCs have been described previously (de Haar et al.,

2015; Poulin et al., 2010). In short, in the expansion phase 5×10^4 $CD34^+$ cells/ml were cultured in X-VIVO 15 medium supplemented with Flt3l (50 ng/ml), stem cell factor (50 ng/ml), IL-3 (20 ng/ml) and IL-6 (20 ng/ml) for 14 days. Medium was replenished after 7 days. Thereafter, the cells were differentiated at 2×10^5 cells/ml in differentiation medium, X-VIVO 15 containing 5 % human serum and supplemented with Flt3l (100 ng/ml), stem cell factor (20 ng/ml), granulocyte-macrophage colony-stimulating factor (20 ng/ml) and IL-4 (20 ng/ml) for another week (de Haar et al., 2015; Poulin et al., 2010). Recombinant cytokines were all obtained from Miltenyi Biotec. For generation of monocyte-derived DC, CD14+ cells were cultured for 7 days in X-VIVO 15 containing 5 % human serum and supplemented with IL-4 (25 ng/ml) and granulocyte-macrophage colony-stimulating factor (100 ng/ml). After 3–4 days, medium was replenished.

2.4. Flow cytometry analysis

CD34+-derived DCs in suspension were collected and stained after 1 and 2 weeks of expansion with the expansion mix, after 1 week of differentiation with the DC mix and after 24 h maturation with the maturation mix (supplementary table 2). Monocyte-derived DC were stained after 7 days culture. To obtain monocyte-derived DCs, suspension cells were pooled with cells detached from the surface with an incubation using ice cold PBS for 1 h on a rocking table. Cells were collected, washed, and incubated on 4°C with appropriate antibody combinations. After 30-minute incubation cells were washed and diluted in fluorescence-activated single cell sorting (FACS) buffer (phosphate-buffered saline containing 2 % fetal calf serum and 0.1 % sodium azide). Multiparameter analysis was performed on a FACS LSR Fortessa (BD) flow cytometer. Dead cells were excluded by scatter gating. For Fig. 1A, cells were pre-gated on a live cell population from FSC-A/SSC-A. Cells larger than 50k were considered alive, but were limited in number. For Figs. 1C and 1D, cells were gated after additional expansion and differentiation as live, singlets using FSC-A/FSC-W, as described previously (Plantinga et al., 2020). Thereafter, a graph with either HLA-DR/CD11c (Fig. 1C) or HLA-DR/CD83 (Fig. 1D) was plotted. For Fig. 2, the cells

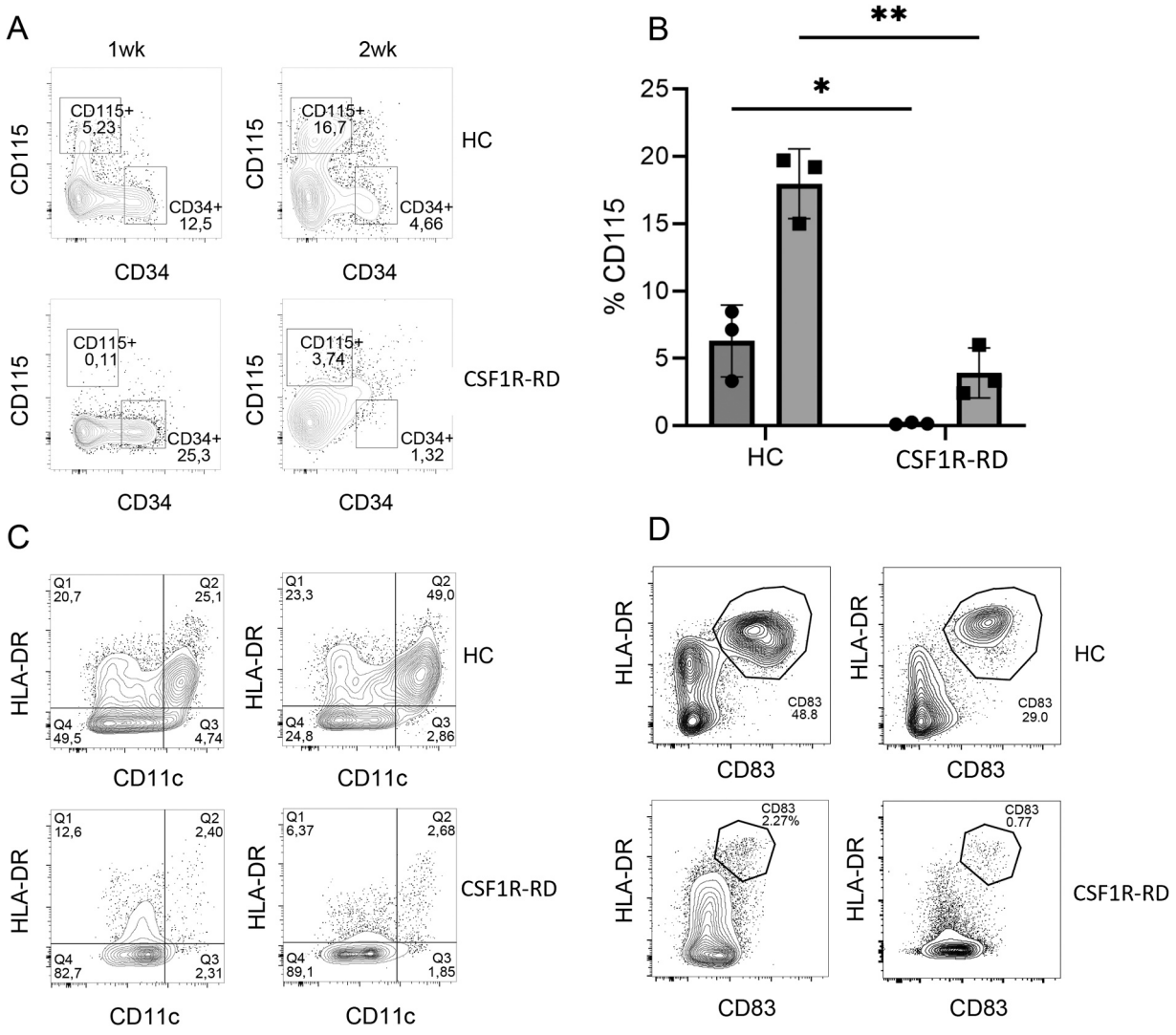

Fig. 1. CD34+-derived dendritic cell culture. (A) CSF1R (CD115) expression after 1 and 2 weeks of expansion of control and CSF1R-RD CD34+ stem cells. Data of one representative healthy control (HC) and one representative patient with late-onset CSF1R-RD are shown. (B) Percentage of CD115 expression on 1 or 2 week expanded CD34+ stem cells from HCs and patients with late-onset CSF1R-RD. Error bar represents SD. Three HCs and three patients were analyzed (number of CD34+ stem cells in PBMCs from CSF1R-RD4 was too low to culture). CD34+ stem cells of patients with late-onset CSF1R-RD developed significantly lower CSF1R expression after 1 week ($p = 0.0461$) and 2 weeks ($p = 0.0091$) than HCs, as analyzed with one-way ANOVA with Bonferroni's multiple comparisons test. (C) HLA-DR and CD11c expression in culture after 2 weeks expansion and 1 week differentiation towards DCs. Data of two HCs and two patients with late-onset CSF1R-RD are shown. (D) HLA-DR and CD83 expression after 24 h maturation of the DC culture of two HCs and two patients with late-onset CSF1R-RD. CD34+-derived dendritic cell data of all patients with late-onset CSF1R-RD is available in supplementary figure 3.

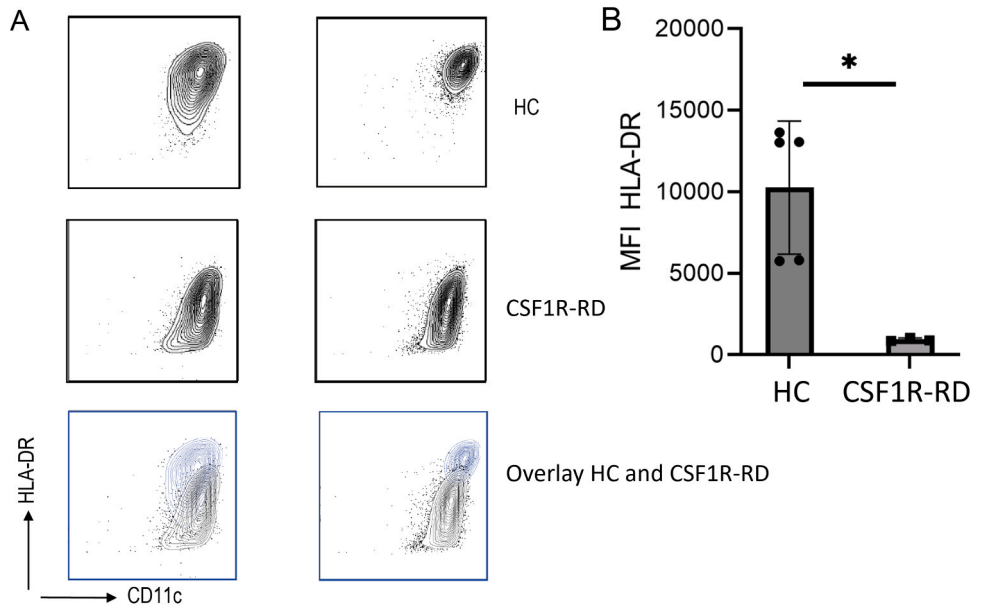

Fig. 2. Differentiation of monocyte-derived dendritic cells. (A) HLA-DR and CD11c expression in culture after 1 week differentiation of FACS sorted CD14+ monocytes. Two representatives are shown out of five HCs and three patients with late-onset CSF1R-RD analyzed (experiment with CSF1R-RD4 failed due to technical issues) in the two upper panels and an overlay with HC data in blue is presented in the lower panel. (B) Mean fluorescent intensity of HLA-DR expression was significantly lower on monocyte-derived DCs of patients with late-onset CSF1R-RD than on monocyte-derived DCs of HCs ($p = 0.036$), as analyzed with the Mann-Whitney U test.

were pre-gated on a live cell population from FSC-A/SSC-A. Cells larger than 50k were considered alive. Analysis was performed using FlowJo software (Tree Star, Inc.). *Ex vivo* analysis of PBMCs was performed on lineage negative (Lin-) cells, encompassing a mix of cells that express mature cell lineage markers and are negatively selected by CD3, CD19 and CD56. cDC1 were gated as CD141+CD123-, and, subsequently, pDCs were gated as CD123+CD304+. All used gating protocols were developed using a fluorescence minus one (FMO) as negative staining control and have been published previously (Plantinga et al., 2019b). Thereafter, the remaining DC population was selected for CD11c+HLA-DR+, followed by cDC2 (CD1c+CD14-) and monocytes (CD14+CD1c-). The small numbers of DCs in the patient samples did not allow functional analyses of antigen presentation or T-cell activation.

2.5. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 8 software (GraphPad, La Jolla, CA). Data are presented as means ± standard deviation. Data were analyzed for Gaussian distribution by Shapiro–Wilk normality test. Differences between patients and HCs were determined using one-way ANOVA with Bonferroni's multiple comparisons test (Fig. 1B) and Mann-Whitney U test (Fig. 2B). The level of significance was set at $p < .05$. Sample sizes for each experiment are indicated in the figure legends.

3. Results

3.1. CD34+-derived dendritic cell cultures

We previously reported that cord blood CD34+ stem cell-derived DCs generated *in vitro* originate specifically from CSF1R expressing precursors (Plantinga et al., 2019a). These CD34+-derived DCs displayed a cDC phenotype, while CSF1R is mainly linked to the monocyte lineage and, more specifically, to microglia maintenance (Hume et al., 2020; Oosterhof et al., 2018). To confirm that CSF1R plays a role in cDC development, we differentiated cDCs from isolated CD34+ stem cells from peripheral blood of patients with late-onset CSF1R-RD. We used CD34+ stem cells from cord blood, peripheral blood and bone marrow of healthy donors as controls, which showed comparable results (supplementary figure 2). Differences in CD34+ stem cell number between peripheral blood and cord blood were controlled by titration. After 2 weeks expanding CSF1R-RD CD34+ stem cells, only very limited CSF1R+ cells were observed *in vitro* (Figs. 1A and 1B and supplementary figure 3) in contrast to CD34+ cells from healthy controls. Strikingly, we could hardly detect any cDCs after an additional week of differentiation (Fig. 1C and supplementary figure 3) nor after 24 h maturation (Fig. 1D and supplementary figure 3) of CSF1R-RD cells. These findings indicate that CSF1R is necessary for the generation of cDCs from stem cells *in vitro*.

3.2. Monocyte-derived dendritic cell cultures

Next, we questioned whether we could generate monocyte-derived DCs in contrast to cDCs. CSF1R expression on CD14+ monocytes from peripheral blood of patients with late-onset CSF1R-RD was reduced compared to CD14+ monocytes from peripheral blood of adult HCs, but not as low as the negative control (supplementary figure 4). Isolated CD14+ cells were subsequently cultured in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor. After one week differentiation, the cells expressed the classical hallmarks for monocyte-derived DCs, including CD11c and HLA-DR (Fig. 2A). However, the mean fluorescence intensity of HLA-DR was significantly lower on monocyte-derived DCs of patients with late-onset CSF1R-RD than on monocyte-derived DCs of HCs ($p = 0.036$, Fig. 2B). This might suggest that monocyte-derived DCs of patients with late-onset CSF1R-RD have a lower differentiation status than monocyte-derived DCs of healthy individuals, in concordance with the previously reported altered behavior of monocytes in patients with late-onset CSF1R-RD (Hamatani et al., 2020).

3.3. Percentages of monocytes and dendritic cells in vivo

Since no cDCs were observed after *in vitro* differentiation of CD34+ stem cells, we evaluated PBMCs of patients with late-onset CSF1R-RD for

monocytes and DC subsets *in vivo*. Complete blood counts, analyzed as part of standard clinical practice, were normal. Further analysis of the composition of PBMCs using flow cytometry, validated the presence of monocytes (Lin- DC1-pDC-CD14+; Fig. 3A and supplementary figure 5). Although patients showed the highest values, no statistically significant difference in the percentage of monocytes between patients with late-onset CSF1R-RD and HCs was observed (Fig. 3B). These results are in line with previously reported findings in *Csf1r*$^{+/-}$ heterozygous mice and rats, which show normal percentages of PBMCs and monocytes in contrast to the decreased percentages found in *Csf1r*$^{-/-}$ mice and rats (Hume et al., 2020; Pridans et al., 2018). Focusing on DCs, we also did not find any deficiency in HLA-DR expression nor in the presence of cDC1 (CD141+), cDC2 (CD1c+CD14-) and pDC (CD304+CD123+) *in vivo*, confirming the findings of Hofer *et al.* (2015), although a higher variability was seen in the percentages of cDC2 and pDCs from patients with late-onset CSF1R-RD compared to HCs (Fig. 3C).

3.4. Conclusion

Altogether, these data contribute to the understanding of the role of CSF1R in DC development in humans. Isolated CD34+ stem cells from patients with late-onset CSF1R-RD did not differentiate towards cDCs using our robust *in vitro* CD34+-derived DC culture protocol. *In vitro* differentiation of isolated CD14+ monocytes towards monocyte-derived DCs was however unaffected, although CD14+ monocytes of patients did limitedly express CSF1R. These findings suggest that *in vitro* differentiation of monocyte-derived DCs in contrast to CD34+-derived DCs occurs through a CSF1R-independent pathway. Strikingly, our data prove that peripheral blood of patients with late-onset CSF1R-RD contains all naturally occurring DC subsets, including cDC1 and cDC2, in line with the observation of Hofer et al. (2015). We speculate that CSF1R signaling is necessary for *in vitro* stem cell-derived DC development, but deficiency of CSF1R in DC development has less impact or can be compensated for by other cytokine pathways *in vivo*, as is observed in *Flt3*$^{-/-}$ *and Flt3l*$^{-/-}$ mice that exhibited an increased sensitivity of other cytokine receptors on DC progenitors (Durai et al., 2018). This hypothesis is supported by the absence of systemic immunological involvement in patients with CSF1R-RD, though we could not perform functional assays with the DCs due to the study design and small cell numbers obtained from leftover peripheral blood. For example, increased signaling through Flt3 might compensate for a deficiency in CSF1R during DC development, while enhanced signaling through the triggering receptor expressed on myeloid cells 2 (TREM-2) could similarly compensate for CSF1R deficiency during DC maturation. However, further investigation is required to validate these hypotheses (Cueto and Sancho, 2021; Bouchon et al., 2001).

CRediT authorship contribution statement

Maud Plantinga: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Stefan Nierkens:** Writing – review & editing, Supervision, Conceptualization. **Marjo S. van der Knaap:** Writing – review & editing, Supervision, Data curation. **Nicole I. Wolf:** Writing – review & editing, Supervision, Data curation. **Shanice Beerepoot:** Writing – original draft, Data curation, Conceptualization.

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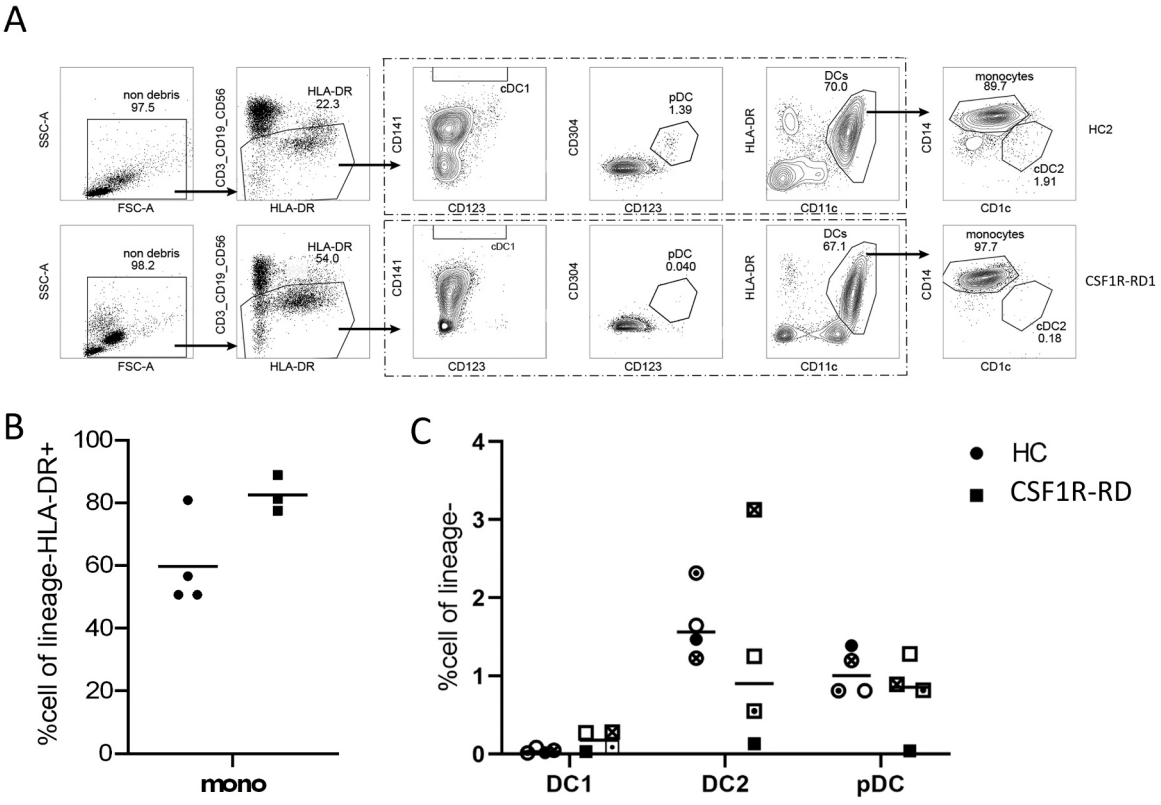

Fig. 3. Identification of myeloid lineage in PBMCs. (A) Gating strategies of CD3/19 depleted PBMCs using flow cytometry for one representative HC and one representative patient with late-onset CSF1R-RD are shown. Gating strategies for all HCs and patients late-onset CSF1R-RD are shown in supplementary figure 5. (B) Percentage of monocytes of the Lin- fraction in three patients with late-onset CSF1R-RD compared to four HCs. (C) Percentage of DC subsets (cDC1, cDC2 and pDC) of the Lin- fraction in four patients with late-onset CSF1R-RD compared to four HCs. Symbols represent the same individual across subsets.

Declaration of Competing Interest

None

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2024.08.002.

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