


# Monocyte-derived dendritic cells display a highly activated phenotype and altered function in patients with familial Mediterranean fever<sup>1</sup>

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## Summary

Dendritic cells (DCs) are sentinels of the immune system that bridge innate and adaptive immunity. By capturing antigens in peripheral tissue, processing and presenting them with concurrent expression of co-stimulatory molecules and cytokine secretion they control and modulate immune reactions. Through pattern recognition receptors, DCs sense molecules that are associated with infection or tissue damage, frequently resulting in the formation of inflammasomes upon intracellular stimulation. The inherited autoinflammatory familial Mediterranean fever (FMF) is associated with deregulated activity of the pyrin inflammasome leading to acute inflammatory episodes. However, differentiation and function of DCs in this disease are as yet unclear. Therefore, we first determined DC subpopulation frequency in peripheral blood of a cohort of FMF patients. Joint evaluation without classification according to specific patient characteristics, such as mutational status, did not disclose significant differences compared to healthy controls. For the further examination of phenotype and function, we used immature and mature monocyte-derived DCs (imMo-DCs, mMo-DCs) that were generated *in vitro* from FMF patients. Immunophenotypical analysis of imMo-DCs revealed a significantly elevated expression of CD83, CD86 and human leukocyte antigen D-related (HLA-DR) as well as a significant down-regulation of CD206, CD209 and glycoprotein NMB (GP-NMB) in our FMF patient group. Furthermore, FMF imMo-DCs presented a significantly higher capacity to migrate and to stimulate the proliferation of unmatched allogeneic T cells. Finally, the transition towards a more mature, and therefore activated, phenotype was additionally reinforced by the fact that peripheral blood DC populations in FMF patients exhibited significantly increased expression of the co-stimulatory molecule CD86.

**Keywords:** dendritic cells, familial Mediterranean fever, inflammation

## Introduction

As the most potent professional antigen-presenting cells, dendritic cells (DCs) have the unique capacity to initiate and maintain primary immune responses [1,2]. In their immature state, they sample their environment continuously. After recognition of conserved danger or pathogen-associated molecular patterns (DAMPs, PAMPs) via pattern recognition receptors (PRRs) a maturation process is initiated, resulting in a multitude of immunophenotypical and

functional changes. Most importantly, an enhanced capacity to prime naive T cells but also increased mobility and migratory capacity, as well as the secretion of proinflammatory cytokines, can be observed [1–3]. Upon intracellular activation, DCs are able to form cytoplasmic multi-protein complexes called inflammasomes, involving different intracellular receptors and sensors that can bind the adaptor protein ASC. This central molecule of inflammasome complexes recruits caspase-1, which activates the proforms

of the potent pyrogens and inflammatory cytokines interleukin (IL)-1 $\beta$  and IL-18 [4–6].

Hereditary disorders classified as autoinflammatory diseases are characterized by unprovoked inflammatory episodes in the absence of autoantibodies or autoreactive T cells [7]. Familial Mediterranean fever (FMF) is the most common monogenetic autoinflammatory disease. The majority of patients hold a mutation in the *MEFV* gene (Mediterranean fever; alias Marenosttrin, pyrin innate immunity regulator) coding for the intracellular pattern recognition receptor (PRR) pyrin, which can form its own pyrin inflammasome in response to bacterial modifications of the Rho GTPase or if mutated [8–12]. A total of 342 mutations have been identified so far, but it is unclear whether all are disease causal. Pyrin is expressed mainly in neutrophils, monocytes and DCs [13]. Therefore, this study aims to evaluate potential numerical, phenotypical and functional changes in DCs of FMF patients without grouping them into categories based on disease characteristics. However, due to its low frequencies it is somewhat difficult to analyse the activation states of blood DCs. To avoid this problem, we used monocyte-derived DCs (Mo-DCs) as a well-established *in-vitro* model that ensures sufficient cell numbers as well as stable and homogeneous cellular conditions for our extensive analyses.

The results obtained by this study could significantly contribute to a better understanding of the pathophysiology and pathogenesis of FMF and other autoinflammatory diseases, and could open up new diagnostic and treatment approaches. The aims of this study were as follows.

1. To determine DC subpopulation frequency in peripheral blood of a cohort of FMF patients compared to healthy controls.
2. To assess phenotype and function of Mo-DCs that were generated from healthy donors and a cohort of FMF patients irrespective of disease characteristics such as mutational status.
3. To verify CD83 and CD86 up-regulation in peripheral blood DCs of FMF patients.

## Materials and methods

### Study subjects

After written informed consent, peripheral blood samples of 25 FMF patients and age- and gender-matched healthy volunteers were obtained at the University of Tübingen. Detailed patient characteristics are presented in Table 1. FMF was classified according to the Tel Hashomer criteria [14]. The local Institutional Review Board (Ethics

committee at the Medical Faculty and at the University Hospital Tübingen) approved the study (111/2017BO2) to be in accordance with ethical standards and with the Helsinki Declaration.

### Flow cytometry and reagents

For characterization of peripheral blood DCs, surface expression was analysed using fluorescence conjugated mouse anti-human monoclonal antibodies against CD14 fluorescein isothiocyanate (FITC) (BD Bioscience, Heidelberg, Germany), CD1c phycoerythrin (PE)-Dazzle, CD3 BV510, CD11c PE-cyanin 7 (Cy7), CD16 AF700, CD19 BV510, CD20 BV510, CD56 BV510, CD141 allophycocyanin (APC), CD303 peridinin chlorophyll (PerCP)-Cy5-5, human leukocyte antigen D-related (HLA-DR) BV650 and Zombie Aqua (BioLegend/Biozol Diagnostica, Eching, Germany). For the evaluation of CD83 and CD86 expression, peripheral blood leukocytes were stained with CD14 FITC, CD86 BV421 (BD Bioscience), CD83 APC (eBioscience, Frankfurt, Germany), CD3 BV510, CD16 AF700, CD19 BV510, CD20 BV510, CD56 BV510, HLA-DR BV650 and Zombie Aqua (BioLegend/Biozol Diagnostica). Samples were analysed on an LSR II Fortessa with DIVA software (BD Bioscience). Data analysis was performed using FlowJo software version 10 (TreeStar, FlowJo LCC, Ashland, OR, USA) identifying DC subpopulations with a hierarchical gating strategy. A singlet gate excluded cell doublets before leukocytes were gated based on forward (FSC) and side-scatter (SSC). Next, lineage marker-positive (CD19, CD20, CD3, CD56) and dead cells were excluded (lin<sup>-</sup>/live population). After exclusion of monocytes by gating CD14<sup>-</sup>/CD16<sup>-</sup> cells, HLA-DR<sup>+</sup> cells were defined as total peripheral blood DCs (PBDCs). DC subgroups were identified as CD11c<sup>-</sup>/CD303<sup>+</sup> PDC, CD11c<sup>+</sup>/CD1c<sup>+</sup> myeloid type 1 DC (MDC)1 and CD11c<sup>+</sup>/CD141<sup>+</sup> MDC2. CD83 and CD86 were analysed in PBDCs without discriminating between DC subpopulations. The cut-off between negative and positive populations for CD83 and CD86 was defined with unstained samples [fluorescence minus one (FMO) controls] and results were expressed as median fluorescence intensity (MFI) of the positive population.

For phenotype analysis of Mo-DCs, Fc receptors were blocked using FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Mo-DCs were stained with the following monoclonal mouse anti-human antibodies: FITC-labeled immunoglobulin (Ig)G1 isotype, anti-CD1a, anti-CD206 (BD Bioscience) and anti-CD80 (eBioscience), PE-labeled IgG1 isotype, anti-CD83 (eBioscience), anti-glycoprotein NMB (GPNMB) and anti-CCR7 (R&D Systems/Bio-Techne, Wiesbaden-Nordenstadt, Germany), PerCP-labeled IgG2b isotype, anti-CD209 (R&D Systems) and anti-CD14 (BD Bioscience), APC-labeled IgG1 isotype (R&D Systems), anti-HLA-DR (eBioscience), anti-CD40 and anti-CD86 (BD Bioscience). Cells were analysed on

**Table 1.** Clinical characteristics of familial Mediterranean fever (FMF) patients

UPN	Age	Sex	Age at diagnosis	M694V mutation	Other mutation	WBC (1/pl)	LY abs (×1000/pl)	MO abs (×1000/pl)	ESR (mm/h)	CR (mg/dl)	CRP (mg/dl)	Iron (ng/dl)	SAA (mg/l)
1	38	M	35	Heterozygous	E1480/-	9160	3.21	0.45	5	0.7	1.06	39	15
2	31	NI	30	Heterozygous		5720	1.39	0.48	2	0.9	0.20	67	3
3	38	M	32	Homozygous		3800	0.95	0.42	16	6.5	0.05	60	2
4	26	M	24	None	R202Q/-	5880	1.59	0.64	1	0.7	0.17	135	2
5	35	F	28	None	E1480I <sup>-</sup> S339E/-	8500	2.56	0.62	6	0.4	0.64	85	8
6	42	F	31	None		5860	1.64	0.37	19	0.6	0.69	47	12
7	47	M	38	n.a.	n.a.	7580	2.27	0.51	2	0.9	0.12	113	3
5	39	F	3	n.a.	n.a.	7490	2.44	0.38	7	0.5	0.01	45	3
9	24	F	8	Heterozygous	V726A/-	7920	3.14	0.46	13	0.9	1.45	67	12
10	26	F	23	Heterozygous	E148QI <sup>-</sup>	6890	2.26	0.39	4	0.6	0.06	149	
11	29	NI	21	None	M680I <sup>-</sup> V726A1 <sup>-</sup>	4620	1.71	0.31	1	0.9	0.01	161	2
12	20	F	3	Homozygous		10 260	2.7	0.37	23	0.7	6.83	62	459
13	22	M	2	Homozygous		5190	1.67	0.42	2	0.6	0.81	112	
14	35	F	34	None	E148QI <sup>-</sup> R2020 <sup>-</sup> P369S/P369S R408Q/R408Q	6510	1.89	0.38	16	0.5	0.61	59	
15	33	NI	23	n.a.	n.a.	9100	2.79	0.64	2	1.0	0.69	41	14
16	41	F	34	None	M680I/M680I	7590	1.41	0.52	6	0.6	0.03	53	
17	41	M	28	Heterozygous		5000	1.91	0.36	4	0.9	0.02	85	2
18	23	F	11	Homozygous		5990	2.56	0.34	19	0.6	1.54	31	5
19	22	F	4	Heterozygous	R761A1 <sup>-</sup>	5760	2.05	0.47	41	0.6	3.15	32	121
20	21	F	8	Heterozygous	V726A/-	7190	1.92	0.38	32	0.6	2.01	75	7
21	24	M	12	Heterozygous	M680I/-	5860	1.66	0.39	4	1.0	0.35	112	16
22	42	M	13	Heterozygous	V726A/-	6570	2.58	0.51	4	0.8	0.35	82	4
23	19	M	12	None	E148QI <sup>-</sup>	6640	1.82	0.67	29	0.7	2.49	53	140
24	36	F	32	Heterozygous		4970	1.59	0.3	11	0.6	1.3	32	16
25	28	F	18	n.a.	n.a.	7510	2.21	0.56	17	0.7	0.65	67	12

M = male; F = female; n.a. = not assessed; WBC = white blood count; LY = lymphocytes; abs = absolute; MO = monocytes; ESR = erythrocyte sedimentation rate; CR, creatinine; CRP = C-reactive protein; SAA = serum amyloid A; mm/h = millimeter in 1 h; mg/dl = milligrams per deciliter; pg/dl = micrograms per deciliter.

a FACSCalibur flow cytometer with Cell Quest Pro software (BD Biosciences). Data analysis was conducted using FlowJo software version 10 (TreeStar). Specific fluorescence indices (SFI) were calculated by dividing median fluorescence obtained with specific monoclonal antibodies by median fluorescence obtained with isotypes.

### Cell isolation and generation of Mo-DCs

Mo-DC generation was performed as described previously [15]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. PBMCs were washed with phosphate-buffered saline (PBS) (Sigma Aldrich, Taufkirchen, Germany) and resuspended in serum-free X-VIVO 20 medium (Lonza/Biozym Scientific, Hessisch Oldendorf, Germany) in cell culture flasks (Falcon/BD Bioscience) for 2 h at 37°C and 5% CO<sub>2</sub>. After removal of non-adherent cells PBMCs were cultured for 1 week in RP10 medium (RPMI-1640 with GlutaMAX and HEPES; Gibco/Life Technologies, Darmstadt, Germany), supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Sargramostim, Leukine; Bayer HealthCare Pharmaceuticals, Leverkusen, Germany) and 20 ng/ml IL-4 (R&D Systems/Bio-Techne, Wiesbaden-Nordenstadt, Germany) for generation of imMo-DCs. Cytokines were replenished every second day. For maturation and generation of mMo-DCs, lipopolysaccharide (LPS) (100 ng/ml; Sigma-Aldrich, St Louis, MO, USA) was added on day 6 for 24 h.

### CFSE proliferation assay

Mo-DCs (stimulator cells) were inactivated by  $\gamma$ -radiation at 30 GY, 100% and seeded into 96-well flat-bottomed microplates (Greiner Bio-One, Frickenhausen, Germany) at concentrations of  $2 \times 10^4$  cells/well. A total of  $2 \times 10^5$  responding cells from unmatched allogenic 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE)-labeled PBMCs from healthy volunteers were added. Phytohemagglutinin (PHA) stimulation (3  $\mu$ g/ml; Sigma-Aldrich) of CFSE-labeled PBMCs and native CFSE-labeled PBMCs served as positive and negative proliferation controls, respectively. Distinct generations of proliferating cells were monitored by CFSE dye (Life Technologies/Thermo Fisher Scientific, Darmstadt, Germany) dilution. After coculture for 6 days, fluorescence activated cell sorter (FACS) analysis of recovered lymphocytes was performed and CD4-PE antibody (BD Bioscience) served to identify CD4<sup>+</sup> T cells. All cell counts were scaled to the 0–100% range using GraphPad Prism's 'normalize' function; 0% was defined as the value of PBMC without PHA and 100% as the value of PHA-stimulated PBMC. GraphPad Prism version

8.1.0 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

### *In-vitro* migration assay

After 1 week, Mo-DCs ( $2 \times 10^5$ /well) were seeded into Transwell chambers (8  $\mu$ m; Falcon/BD Bioscience) in a 24-well plate. After 16 h of incubation at 37°C and 5% CO<sub>2</sub>, migration to the CC chemokine 19 (CCL19) (100  $\mu$ g/ml; R&D Systems) was analysed by counting gated Mo-DCs for 60 s on a FACSCalibur cytometer. Migrated cells were normalized to control imMo-DCs without CCL19.

### Statistical analysis

All experiments were performed at least three times. If not indicated otherwise, values depict medians with interquartile range. The Mann-Whitney *U*-test for unpaired non-parametric values was applied using GraphPad Prism version 8.1.0 for Windows software. *P*-values < 0.05 were considered as indicating statistical significance.

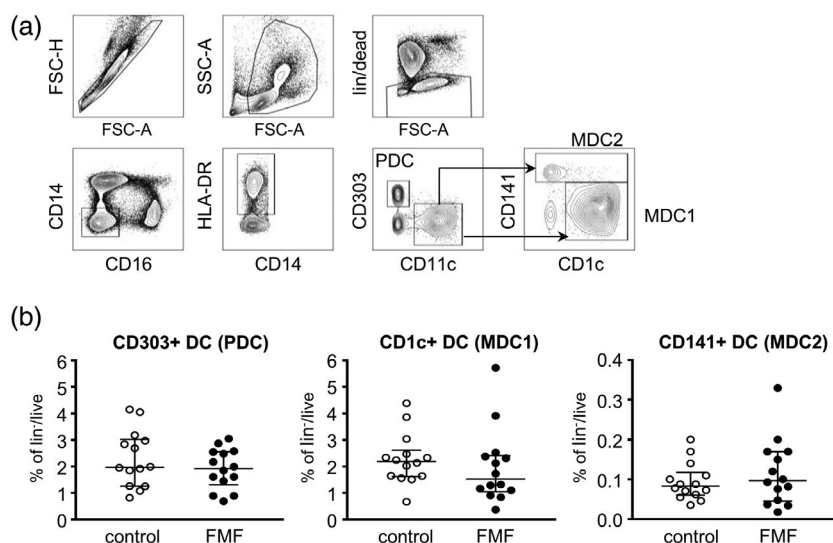
## Results

### Frequencies of peripheral blood DCs in FMF patients are comparable to healthy controls

Blood contains immature precursors of tissue and lymphoid organ DCs that can be divided into three subsets: CD1c<sup>+</sup>MDC1, CD141<sup>+</sup>MDC2 and CD303<sup>+</sup>plasmacytoid DCs (PDC) [16]. DC subset frequencies are altered in a multitude of different diseases, but no data are available regarding FMF [3,17]. Therefore, DC subpopulations were identified by flow cytometry using a hierarchical gating strategy (Fig. 1a). Peripheral blood samples from 14 FMF patients were assessed and compared to matched healthy controls. During the evaluation patients were not grouped according to specific characteristics such as mutational status. As depicted in Fig. 1b and Table 2, there were no significant changes in the frequencies of both MDC and PDC subsets among FMF patients compared to matched healthy controls.

### Mo-DCs can be efficiently generated from FMF patients

To assess phenotype and function of Mo-DCs from FMF patients, we used a well-established *in-vitro* model system. Therefore, monocytes obtained by plastic adherence were differentiated into imMo-DCs via GM-CSF and IL-4 supplementation. Morphologically, large, round, loosely adherent cells showing the typical dendritic cytoplasmic extensions could be observed. There were no obvious differences in morphology between FMF patients and healthy controls (data not shown). Phenotypical analysis after



**Fig. 1.** Dendritic cell (DC) subpopulations in peripheral blood of familial Mediterranean fever (FMF) patients compared to matched healthy controls. (a) Subpopulations were identified by fluorescence activated cell sorter (FACS) analysis using a hierarchical gating strategy. After a singlet gate to exclude cell doublets, leukocytes were gated based on forward- (FSC) and side-scatter (SSC), followed by lineage (CD19, CD20, CD3, CD56) and dead cell exclusion (lin<sup>-</sup>/live). Lin<sup>-</sup> cells were gated on CD14<sup>-</sup>/CD16<sup>-</sup> cells to exclude monocytes. Subsequently, human leukocyte antigen D-related (HLA-DR)<sup>+</sup> cells were defined as total peripheral blood DCs, subgroups were identified as CD11c<sup>-</sup>/CD303<sup>+</sup> plasmacytoid DC (PDC), CD11c<sup>+</sup>/CD1c<sup>+</sup> MDC1 and CD11c<sup>+</sup>/CD141<sup>+</sup> myeloid type 2 DCs (MDC2). One representative example is shown. (b) Combined analyses of peripheral blood DC (PBDC) subpopulation frequencies are presented as percentage of the lin<sup>-</sup>/live population. No significant changes in the frequencies of both MDC and PDC subsets between FMF patients ( $n = 14$ ) and matched healthy controls ( $n = 14$ ) could be observed. Median with interquartile range. \*Statistically significant difference,  $P < 0.05$ .

**Table 2.** Frequencies of peripheral blood DCs subpopulations in FMF patients and healthy controls

Cell type		PBDC	MDC1	MDC2	PDC
FMF	Frequency (% of lin <sup>-</sup> /live)	5.70	1.87	0.12	1.91
	s.d.	1.71	1.12	0.08	0.75
Control	Frequency (% of lin <sup>-</sup> /live)	5.62	2.28	0.09	2.29
	s.d.	1.96	0.97	0.05	1.06

MO = monocytes; PBDC = peripheral blood dendritic cells; MDC1 = myeloid dendritic cells type 1; MDC2 = myeloid dendritic cells type 2; PDC = plasmacytoid dendritic cells; FMF = familial Mediterranean fever; lin<sup>-</sup>/live, population of lineage negative and live cells; s.d. = standard deviation.

1 week of cell culture demonstrated acquisition of a typical imMo-DC phenotype characterized by low expression of CD14 and expression of CD1a and HLA-DR.

Mo-DC yield based on total numbers of seeded cells was comparable between FMF patients and healthy controls.

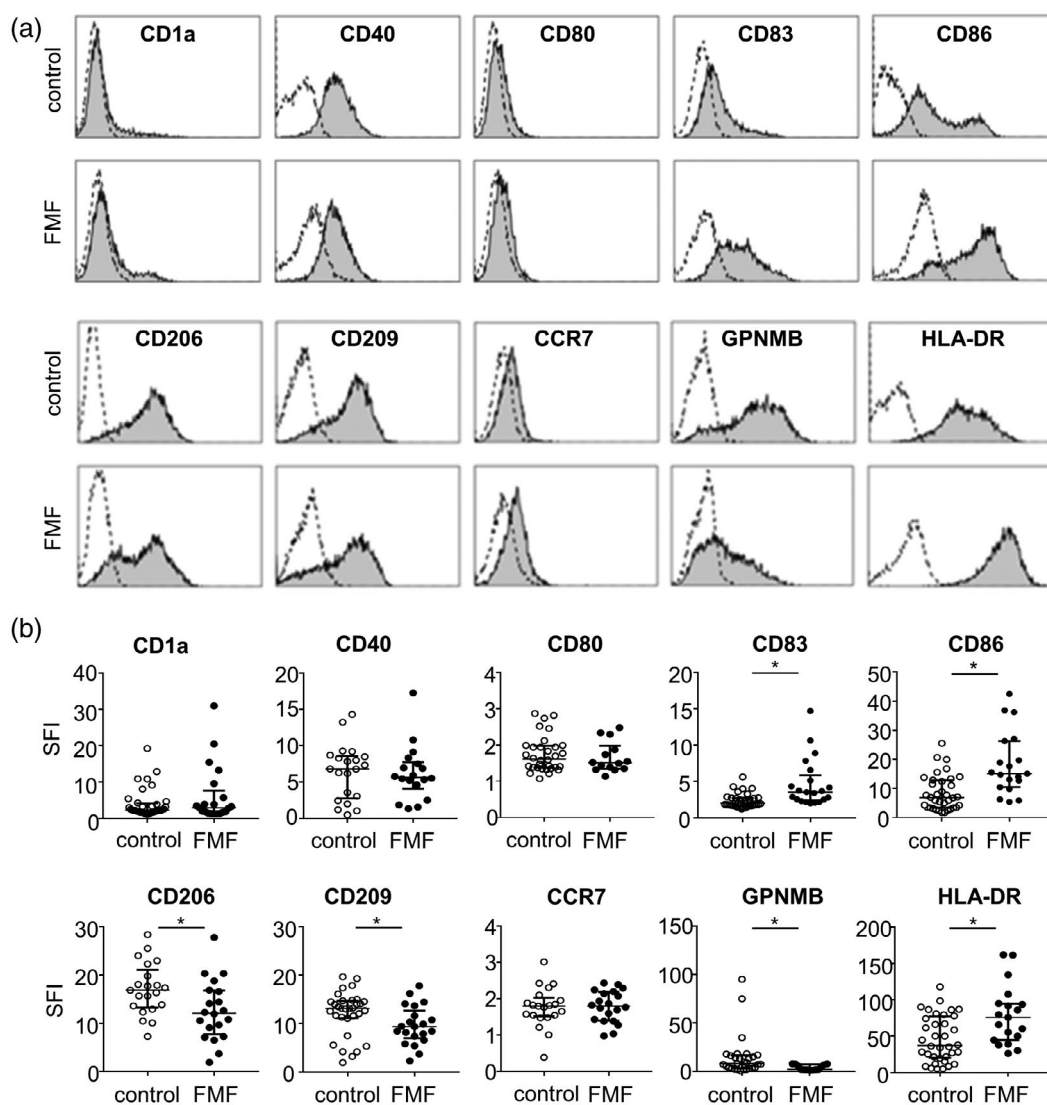
These data show that Mo-DCs can be efficiently generated from FMF patients.

#### imMo-DCs from patients with FMF show increased expression of maturation markers

To evaluate possible phenotypical alterations in FMF imMo-DCs, the expression of typical surface markers

was analysed in our patient cohort without taking account of specific patients' characteristics, such as mutational status. Matched healthy subjects served as controls. Representative histograms are depicted in Fig. 2a. Immunophenotypical characterization after 1 week of cell culture revealed that *in-vitro*-generated imMo-DCs from FMF patients exhibited a significantly increased expression of the maturation marker CD83, the costimulatory molecule CD86 and HLA-DR. In contrast, the mannose receptor CD206 and CD209, also known as DC-specific C-type lectin (DC-SIGN), were significantly down-regulated (Fig. 2b; asterisks indicate significance). GPNMB (glycoprotein NMB, Osteoactivin, DC-HIL), a transmembrane protein known to be expressed in Mo-DCs, can interact with syndecan-4 to inhibit T cell activation. We recently demonstrated significant down-regulation of GPNMB expression in Mo-DCs upon Toll-like receptor (TLR) ligand stimulation [18–20]. Interestingly, in our analysis a significantly decreased GPNMB expression could also be observed in FMF imMo-DCs. No significant changes in surface expression of CD1a, CD40, CD80 and CCR7 could be detected.

Taken together, these analyses revealed that imMo-DCs from FMF patients display a transition towards a more mature phenotype.



**Fig. 2.** Expression of different monocyte-derived dendritic cell (Mo-DC) surface markers was analysed by flow cytometry. (a,b) Immunophenotypical characterization of familial Mediterranean fever (FMF) immature Mo-DCs (imMo-DCs). (a) Representative histogram of healthy controls (upper panel) and FMF patients (lower panel) are shown. Dotted lines represent the respective isotype control. (b) FMF imMo-DCs revealed a significantly increased expression of the maturation marker CD83, the co-stimulatory molecule CD86 and human leukocyte antigen D-related (HLA-DR). Glycoprotein NMB (GPNMB), CD206 and CD209 (DC-SIGN) were significantly down-regulated. Surface expression of CD1a, CD40, CD80 and C-C chemokine receptor type 7 (CCR7) showed no significant change between FMF patients ( $n \geq 13$ ) and healthy controls ( $n \geq 21$ ). (c,d) Mo-DCs have been subjected to lipopolysaccharide (LPS) as a maturation stimulus 24 h before harvest for the generation of mature Mo-DCs (mMo-DCs). (c) Representative example of healthy controls (upper panel) and FMF patients (lower panel) are shown. Dotted lines represent the respective isotype control. (d) Analysis of the immunophenotype of FMF mMo-DCs and healthy controls demonstrated no significant difference in the expression of surface markers ( $n \geq 10$ ). Specific fluorescence indices (SFI) were calculated by dividing median fluorescence obtained with specific monoclonal antibodies by median fluorescence obtained with isotypes. Median with interquartile range. \*Statistically significant difference,  $P < 0.05$ .

### Mature mMo-DCs from patients with FMF do not show increased expression of maturation markers

To evaluate the responsiveness of imMo-DCs generated from FMF patients to a maturation signal, we added the TLR-4 ligand LPS 24 h before cell harvest to generate mature mMo-DCs. Representative histograms are depicted in Fig. 2c. Comparing FMF mMo-DCs and mMo-DCs

of healthy controls, expression of the analysed DC surface molecules showed no significant difference (Fig. 2d).

### FMF imMo-DCs are susceptible to a maturation stimulus

In comparison to GM-CSF/IL-4-generated imMo-DCs, LPS treatment resulted in the typical significant

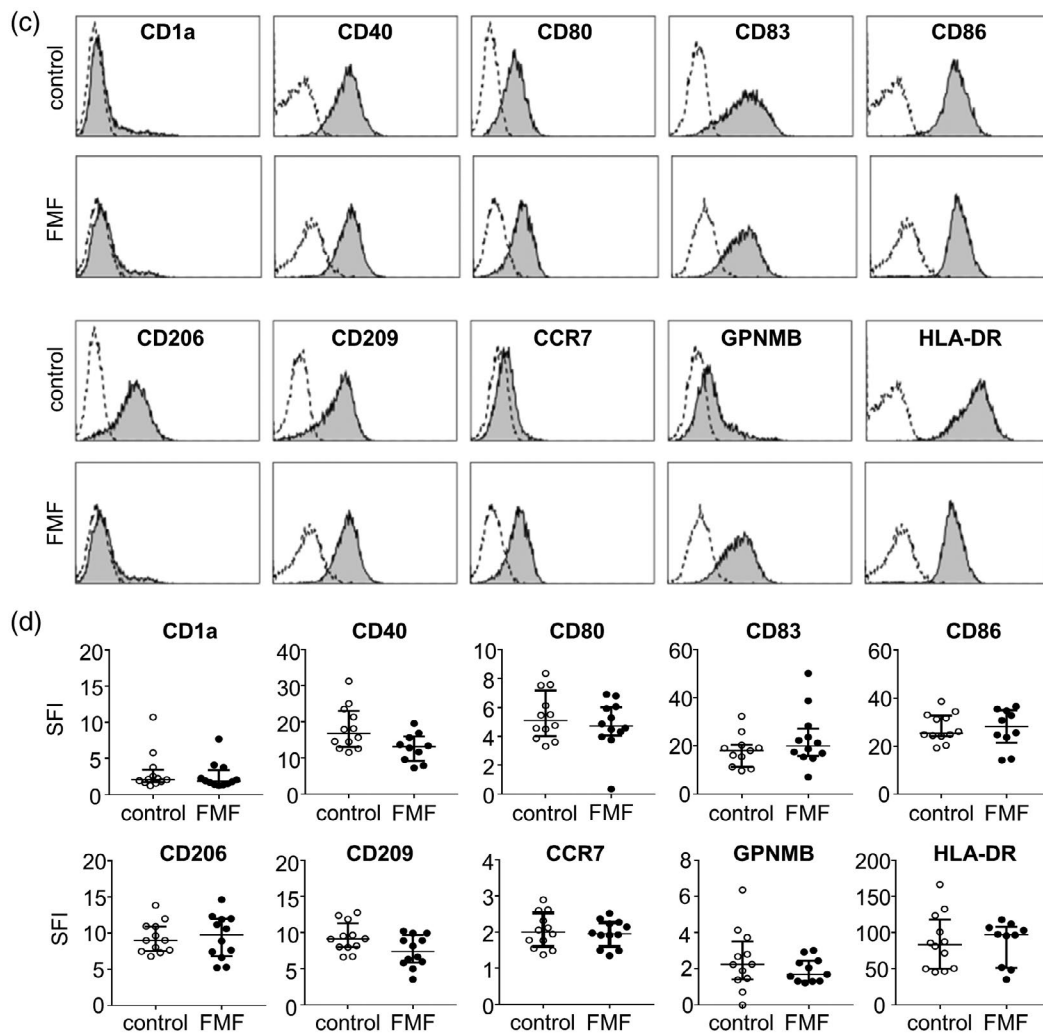


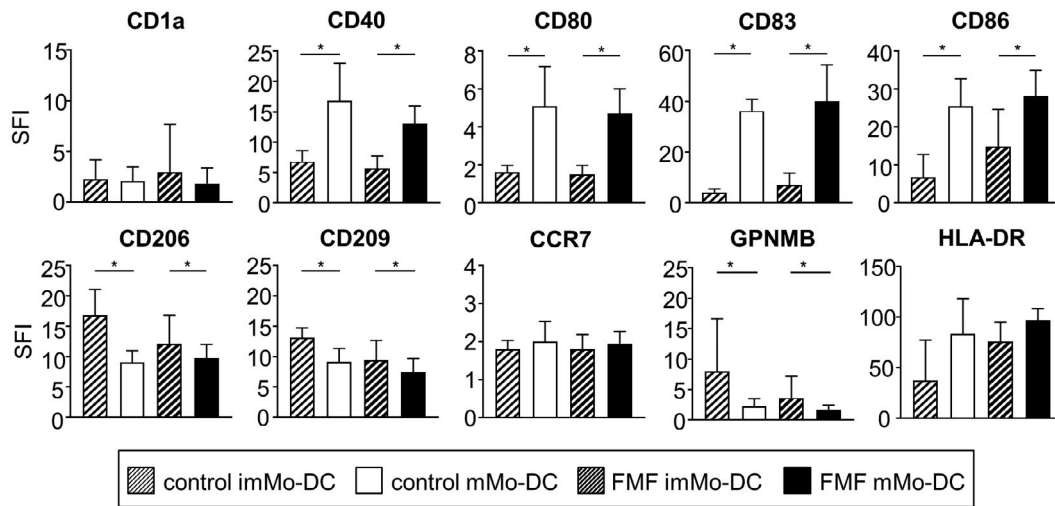
Fig. 2. Continued

up-regulation of co-stimulatory molecules CD80, CD86, and CD40 as well as the maturation marker CD83 and the down-regulation of PRRs such as CD206 and CD209 and the co-inhibitory transmembrane protein GPNMB (Fig. 3; asterisks indicate significance). These findings indicate that FMF imMo-DCs, although intrinsically activated, are still susceptible to TLR-4 triggering. Interestingly, no significant further increase could be detected for HLA-DR, indicating that expression of this molecule has already reached maximum expression in FMF imMo-DCs comparable to mMo-DCs. No changes in expression of CCR7 could be detected between FMF imMo-DCs and FMF mMo-DCs.

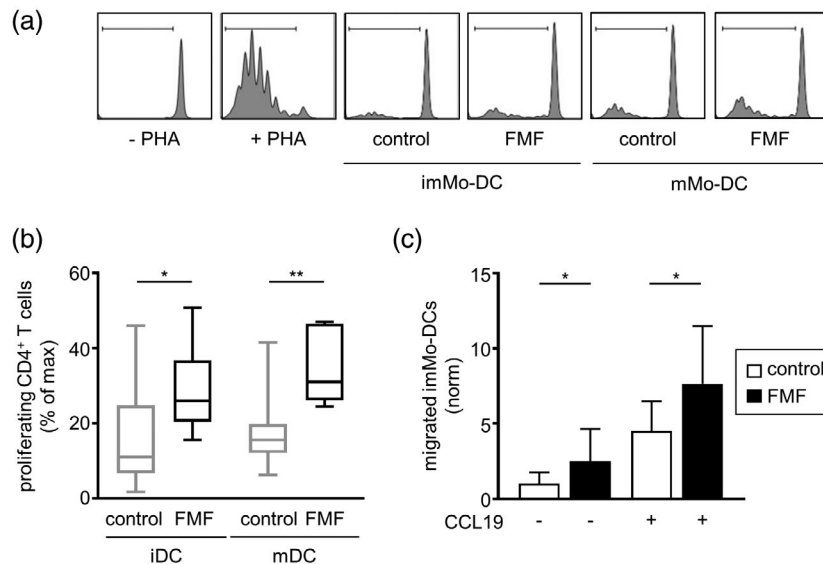
These data demonstrate that FMF imMo-DCs generally are not anergic to signaling through TLR-4, which plays a key role as PRR.

#### FMF imMo-DCs have a significantly enhanced T cell stimulatory capacity

imMo-DCs are weak stimulators of T cells. Upon maturation, these cells acquire the capability to potently induce T cell responses. To evaluate this characteristic property a CFSE-based assay was employed. Exemplary histogram data are shown in Fig. 4a. We could observe an excellent allogeneic T cell stimulatory function of imMo-DCs obtained from FMF patients: FMF imMo-DCs compared to imMo-DCs from healthy controls showed a significantly higher capacity to stimulate proliferation of unmatched allogeneic T cells exceeding even the potential of the mMo-DC control (Fig. 4b). This significantly increased stimulatory capacity is maintained in FMF mMo-DCs compared to mMo-DC controls.

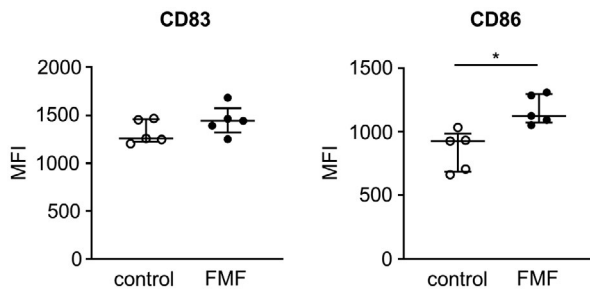


**Fig. 3.** Familial Mediterranean fever (FMF) immature monocyte-derived dendritic cells (imMo-DCs) are susceptible to a maturation stimulus. Comparison of imMo-DCs and mature Mo-DCs (mMo-DCs) from FMF patients and healthy controls showed that CD40, CD80, CD83 and CD86 are up-regulated in both cohorts upon maturation due to lipopolysaccharide (LPS) stimulation. Conversely, CD206, CD209 and glycoprotein NMB (GPNMB) exhibited a decreased expression in mMo-DCs of FMF patients and healthy controls concerning their immature counterparts. Specific fluorescence indices (SFI) were calculated by dividing median fluorescence obtained with specific monoclonal antibodies by median fluorescence obtained with isotypes. Median with interquartile range. \*Statistically significant difference,  $P < 0.05$ .



**Fig. 4.** Increased functional activity of familial Mediterranean fever (FMF) immature monocyte-derived dendritic cells (imMo-DCs). (a,b) T cell stimulatory capacity of FMF imMo-DCs or FMF mature Mo-DCs (mMo-DCs) in comparison to healthy control imMo-DCs or mMo-DCs was analysed by 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) proliferation assays with unmatched allogeneic peripheral blood mononuclear cells (PBMCs). Phytohemagglutinin (PHA) stimulation of CFSE-labeled PBMCs and native CFSE-labeled PBMCs served as positive and negative reference, respectively. Distinct generations of proliferating cells were monitored by CFSE dye dilution. After co-culture for 6 days, fluorescence activated cell sorter (FACS) analysis of recovered lymphocytes was performed. Representative FACS histograms of CFSE dilution are shown in (a). Box-and-whisker plots of normalized (% of maximum) pooled data of  $\geq 6$  FMF patients and  $\geq 12$  healthy controls are shown in (b). This analysis revealed that imMo-DCs from FMF patients are more potent stimulators of CD4<sup>+</sup> T cells than imMo-DCs from healthy controls. Moreover, this effect was sustained in FMF mMo-DCs. (c) FMF imMo-DCs display both a significantly higher spontaneous and C-C motif chemokine ligand 19 (CCL19)-mediated migratory capacity compared to healthy controls ( $n \geq 9$ ). Cell migration was determined by Transwell-migration assays with or without the chemoattractant CCL19. Median with interquartile range. \*Statistically significant difference,  $P < 0.05$ .





**Fig. 5.** Increased expression of maturation markers in peripheral blood dendritic cells (PBDCs) of familial Mediterranean fever (FMF) patients. Expression of CD83 and CD86 was analysed by flow cytometry. The analysis of five FMF patients revealed a significantly increased expression of the co-stimulatory molecule CD86 and increase in the expression of maturation marker CD83 that was not statistically significant. Results are expressed as median fluorescence intensity (MFI) of CD83<sup>+</sup> and CD86<sup>+</sup> PBDC populations, respectively. Median with interquartile range. \*Statistically significant difference,  $P < 0.05$ .

These experiments revealed that imMo-DCs from our FMF patient cohort are very potent stimulators of T cells and confirmed their shift towards a mature phenotype.

#### imMo-DCs from FMF patients show increased chemotaxis to CCL19 signals

*In-vivo* homing of DCs to secondary lymphoid organs is essential to induce immune responses. Upon maturation, DCs acquire the capability to migrate in response to the chemokine CCL19. In line with our previous results, FMF imMo-DCs showed a significantly higher spontaneous as well as chemokine-mediated migratory capacity compared to healthy controls in Transwell migration assays (Fig. 4c). No relevant differences were found in mature mMo-DCs (data not shown).

These findings further support the concept that FMF imMo-DCs exhibit the functional properties of more mature cells.

#### PBDCs in FMF patients show increased expression of maturation markers

As we have observed significantly increased expression of maturation markers in imMo-DCs from patients with FMF, we finally examined the expression of CD83 and CD86 in total PBDCs of five patients without discriminating between DC subpopulations and compared it to matched healthy controls. All patients were under treatment with colchicine and free of clinical attacks.

PBDC populations in FMF patients exhibited a significantly increased expression of the co-stimulatory molecule CD86 compared to matched healthy controls. Furthermore, Fig. 5 illustrates that there was an obvious

increase in the expression of maturation marker CD83. However, this increase was not yet statistically significant.

In line with our previous findings, these analyses revealed that blood DC in FMF patients are intrinsically activated and display a transition towards a more mature phenotype.

#### Discussion

Autoinflammatory diseases are caused by defects or dysregulation of the innate immune system. They are characterized by inflammation and lack of a primary pathogenic role of the adaptive immune system, such as autoreactive T cells or autoantibody production [7]. DCs as professional antigen-presenting cells are key players in the initiation and regulation of immune responses that link innate and adaptive immunity. Thus, it is not surprising that numerous studies show that altered DC function can promote breach of tolerance to self-antigens and development of autoimmunity and inflammation [21,22].

FMF has long been considered an autosomal recessive disease caused by mutations in the *MEFV* gene, which encodes for a protein called pyrin. This immune sensor has a unique structure different from nucleotide-binding oligomerization domain-like receptors (NLRs) and is mainly expressed in neutrophils, monocytes and DCs [23,24]. Recently, pyrin was shown to trigger 'pyrin inflammasome' activation in response to Rho-GTPase-modifying bacterial toxins [8,25]. Accordingly, *MEFV* gene mutations, and thus modified pyrin, may modulate DC function. However, to our knowledge, so far nothing is known regarding the role of DCs in the pathogenesis and maintenance of auto-inflammatory diseases. Here, we evaluated phenotypical and functional changes in Mo-DCs of FMF patients without taking their mutation status or individual characteristics into account.

When comparing peripheral blood FMF DCs to healthy controls, we found no significant differences in the frequencies of CD1c<sup>+</sup>MDC1, CD141<sup>+</sup>MDC2 and CD303<sup>+</sup>PDC. While PDC are thought to play a special role in responding to viral infections through interferon (IFN) and cytokine secretion, MDC2 have a higher capacity to cross-present antigens than other DCs, and MDC1 have a wide range of PRRs [16,17]. However, this is only a glimpse at the wide and still not fully understood variety of functions of different DC subsets.

As peripheral blood contains very low DC frequencies, we subsequently used *in-vitro*-generated monocyte-derived Mo-DCs for further experiments. Phenotypical analysis revealed that FMF imMo-DCs are in a

transitional stage towards a more mature phenotype, which is of interest as the T cell system reportedly is abnormally activated in FMF patients [26]. It appears possible that the observed up-regulation of B7 (CD80/CD86) and CD83 molecules enhances T cell stimulatory capacity of Mo-DCs and mediates an efficient amplification of signaling in naive T cells [27]. Moreover, high levels of HLA molecules, as observed in our analyses, ensure higher capacity for antigen presentation and triggering of TCRs. In fact, when compared to imMo-DCs from healthy controls, FMF imMo-DCs exhibited a significantly higher stimulatory capacity of unmatched allogeneic T cells, exceeding even the potential of mMo-DCs from healthy controls. Physiologically, DCs only mature upon encounter with PAMP signals and subsequently promote T cell immunity mainly by marked up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules. Our results suggest that even in the absence of PAMP signaling, imMo-DCs from FMF patients are very potent intrinsic stimulators of T cell proliferation.

When DCs mature, they down-regulate their responsiveness to inflammatory stimuli and traffic to the draining lymph nodes. This is mediated by up-regulating chemokine receptors, which respond to chemokine ligands such as CCL19 and CCL21 [28]. Interestingly, we found increased chemotaxis to CCL19 signals in FMF imMo-DCs, further supporting our concept that FMF imMo-DCs exhibit the phenotypical and functional properties of mature cells.

TLRs trigger intracellular signaling cascades resulting in the induction of genes coding for inflammatory cytokines such as type I IFN and chemokines [29]. TLR-signaling induces DC maturation by up-regulation of co-stimulatory and HLA-molecules. This is crucial for the induction of pathogen-specific immune responses, thereby linking innate and adaptive immunity [30]. We could show that FMF imMo-DCs further mature, and thus are not anergic to signaling through TLR-4, which plays a key role as PRR for bacterial lipopolysaccharide.

In our experimental setting, monocytes obtained from FMF patients were differentiated *ex vivo* to obtain imMo-DCs. Humoral activation of the cells by proinflammatory cytokines in cell culture can thus be virtually ruled out. However, it cannot be excluded that the monocytes were pre-activated *in vivo*, before differentiation into Mo-DCs. Taken together, our study provides evidence that imMo-DCs of FMF patients are pathologically activated.

Based on these findings, up-regulation of maturation markers was checked in PBDCs of five patients who were treated with colchicine and free of clinical attacks. Here, we found that CD86 was significantly higher than in healthy controls. Furthermore, FACS analyses suggested that surface expression

of CD83 also seemed to be increased. However, this increase was not statistically significant. Considering that, in contrast to Mo-DCs, PBDCs of patients have been constantly exposed to the therapeutic drug colchicine, these analyses provide valuable data strongly confirming the hypothesis that DCs of FMF patients are activated intrinsically. Rare samples of newly diagnosed, so far untreated, patients would be very valuable to further verify these findings.

As DCs play a central role in T cell activation, it is likely that the latter are stimulated accordingly in this autoinflammatory disease. However, only few studies reported an involvement of T cells in FMF: Musabak *et al.* found that both absolute counts and percentages of CD4<sup>+</sup>CD69<sup>+</sup> T cells in peripheral blood of FMF patients were significantly higher than in healthy controls. The authors concluded that FMF patients have an abnormal activation of the T cell system [26]. Kholoussi and co-workers detected an increased percentage of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared to controls [31]. Rimar *et al.* evaluated the role of regulatory T cells (T<sub>regs</sub>) in FMF and proposed that T<sub>regs</sub> may have a role in terminating FMF attacks [32]. Our results suggest conducting further studies in this context; for example, co-cultures of T cells and FMF Mo-DCs to comprehensively examine T cell activation or inhibition. Moreover, further analyses of T cells in peripheral blood of patients with FMF appear warranted based on our findings to further improve the understanding of the pathophysiology of autoinflammatory diseases in general and FMF in particular. Finally, assessment and targeted treatment of DC in FMF could open up new diagnostic and therapeutic options.

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### Disclosures

The authors declare no competing financial interests.

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