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Increased $CD8^+CD28^-$ circulating T cells and high blood interferon score characterize the systemic inflammation of amyopathic dermatomyositis

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Title: Increased $CD8^+CD28^-$ circulating T cells and high blood interferon score characterize the systemic inflammation of amyopathic dermatomyositis

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ABBREVIATIONS

ACR/EULAR: American college of rheumatology/European league against rheumatism

ADM: amyopathic dermatomyositis

CDM: classic dermatomyositis

DM: dermatomyositis

HC: healthy control

IFN: interferon

PBMC: peripheral blood mononuclear cell

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Body of manuscript:

Amyopathic dermatomyositis (ADM) is a subtype of DM defined by the presence of cutaneous signs of DM with no evidence of muscle weakness or abnormal muscle enzymes for ≥ 6 months.[1] Classical DM (CDM) is characterized by modification of circulating lymphocytes [4], type I interferon (IFN) signature [2], elevation of serum pro-inflammatory cytokines [3]. Pathophysiology of ADM is less studied. We analysed circulating T cells by flowcytometry (using specific monoclonal antibodies), peripheral blood mononuclear cells type I IFN signature by PCR and serum cytokine levels by ELISA in a series of 17 ADM and 15 CDM patients.

CD4+ T cells is the predominant population found in the skin and muscle of DM. Expression of CD45RA was increased in CD4+ T-cells from ADM and CDM patients compared with healthy controls, in accordance with previous findings,[4] which may indicate that they were recruited from the peripheral blood to the inflammation site (Figure 1A). Concerning Tregs, FOXP3 median expression in $CD4^+$ T cells was similar in all groups but CD25 expression was significantly decreased in ADM and CDM patients compared with healthy controls, as described in other auto-immune diseases. CD39+CD26-Tregs have been recently showed as exhausted Tregs.[5] Here, the $CD39^+CD26^-$/$CD39^-CD26^+$ ratio was significantly increased in ADM and CDM patients compared with healthy controls (Figure 1B). The increase of this population and of the $CD25^-$ Treg subset suggests a strong stimulation of Tregs to reach their final maturation stage.

$CD8^+$ cytotoxic T cells play a pivotal role in DM and are found both in the skin and the muscle. $CD8^+CD28^-$ T cells are a subset of long-lived, highly differentiated cells that have been found to be elevated in the PBMCs of CDM and to infiltrate the affected muscles. Here,

ADM patients displayed a significantly higher frequency of circulating $CD8^+CD28^-$ T cells compared with healthy controls (Figure 1C). CD94/NKG2A is an inhibitory C-type lectin receptor, which activation on $CD8^+$ T cells leads to a negative feed-back allowing the return of CD8+ T cells to steady state. CD94/NKG2A expression on CD8+ T cell was downregulated in ADM and CDM patients, potentially leading to a persistent activation state of $CD8^+$ T cells in these patients. (Figure 1C))

Type I IFN have a pivotal role in the pathophysiology of CDM. Among 10 patients, 7 exhibited a high IFN score (3 out of 5 ADM and 4 out of 5 CDM). The IFN score was not significantly different between ADM and CDM patients.

IL-6 and TNF-α have been shown to be elevated in CDM.[3] Here, IL-6, IL-10 and TNF-α levels were higher in ADM patients than in healthy controls. IL-10 and TNF-α levels were correlated with the CDASI (Cutaneous Dermatomyositis Disease Area and Severity Index) (r=0.69, *p*=0.04 and r=0.69, *p*=0.02, respectively) (Figure 1D).

This study argues for a systemic inflammation during ADM including an expansion of CD8+CD28- T cells, a decreased number and altered phenotype of CD4+ Tregs, a type I IFN signature and an elevation of circulating pro-inflammatory cytokines.

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FIGURES LEGENDS

Figure 1. Flow cytometric quantification of T-cell subsets, type I IFN signature and serum cytokine levels found in amyopathic and classical dermatomyositis. Peripheral blood mononuclear cells from healthy controls (n=19, unless specified), amyopathic dermatomyositis (ADM) (n=18, unless specified) and classical dermatomyositis (CDM) (n=14, unless specified) were stained for multi-parameter flow cytometry. **A. $CD4^+$ Tconv from ADM and CDM patients express more CD45RA**. Relative frequency of $CD45RA^+$ and $CD28^-$ $CD4^+$ T cells are showed (ADM=15, CDM=13). Cells were pre-gated on live $CD14^-CD3^+TCR\gamma\delta^-$ $MAIT^-$ cells. **B. $CD4^+$ Treg from ADM and CDM patients have decreased CD25 cell surface expression and are enriched within the $CD39^+/CD26^-$ "exhausted" subset.** Relative FOXP3 expression among $CD4^+$ T cells and relative expression of CD25 in $CD4^+$ $FoxP3^+$ T cells is showed. Cells were pre-gated on live $CD14^-CD3^+TCR\gamma\delta^-MAIT^-CD4^+$ T cells. **C, $CD8^+$ T-cells from ADM patients are frequently CD28null and have decreased $CD94^/NKG2A$ immune checkpoint receptor cell surface expression.** Relative expression of $CD94^+NKG2A^+$ (ADM=10, CDM=12) and frequency of CD28 (ADM=15, CDM=13) among $CD8^+$ T cells is showed. Cells were pre-gated on live $CD14^-CD3^+TCR\gamma\delta^-MAIT^-CD8^+$ cells. **D, Elevated inflammatory cytokines in ADM.** Serum cytokines concentration were measures using Luminex technology in healthy controls (n=8) and ADM patients (n=8), minimum detectable concentration and linear range, pg/mL for IL-6: 0.9, [10.5-2700], IL-10: 1.1 [5.25-1189] and TNF-α: 0.7 [10.72-2511]. Data are presented in scatted dot plots; horizontal bar represent the median and whiskers the interquartile range. P values were calculated using Wilcoxon-Mann-Whitney test. P values < 0.05 were considered significant. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. When not specified, differences are not statistically significant. HC: healthy control; ADM: amyopathic dermatomyositis: CDM: classic dermatomyositis.

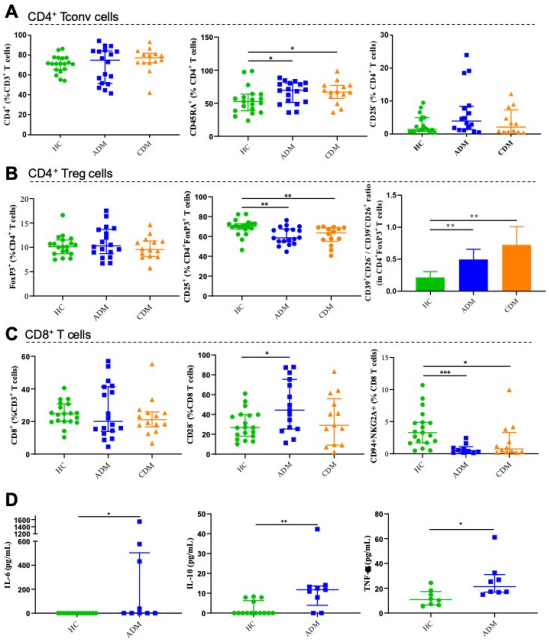
TABLES

Table 1. Clinical characteristics of enrolled patients for flow cytometry analysis

	HC[1] (n=19)	ADM[2] (n=17)	CDM[3] (n=15)	ADM vs. CDM
Age, years (SD[4])	47 (8.5)	54 (14.7)	58 (13.6)	*p*=0.8
Sex female (%)	13 (69)	12 (70)	10 (66)	*p*=1
Serology, MDA5[5]/Mi2/TIF1γ[6]/Jo1		5/3/1/0	2/1/2/1	*p*=0.47
Time of evolution (month, median)		6 (42)	7 (110)	*p*=0.59
Neoplasia (%)		1 (5.6)	1 (6.7)	*p*=1
ILD[7] (%)		5 (26.4)	3 (20)	*p*=0.69
CRP[8], ng/mL [IQR[9]]		4 [0-8]	3.5 [0.75-7.5]	*p=0.8*
NLR[10], no unit [IQR[9]]		2.63 [1.74-3.5]	1.88 [1.42-2.81]	*p=0.3*

[1]HC: healthy controls; [2]ADM: amyopathic dermatomyositis; [3]CDM: classical dermatomyositis; [4]SD: standard deviation; [5]MDA5: melanoma differentiation associated protein 5; [6]TIF1: transcription intermediary factor 1; [7]ILD: interstitial lung disease; [8]CRP: C reactive protein; [9]IQR: inter quartile range; [10]NLR: neutrophil-lymphocyte ratio. Statistical tests were made with Mann-Whitney U test for quantitative data and Fisher's exact test for qualitative data. Data are expressed either in median and standard deviation or absolute number and percentage. Comparison between groups were made either by Wilcoxon-Mann-Whitney test or by Chi square test.

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MATERIAL AND METHODS

Patients

The study population comprised 32 patients (10 men and 22 women), 17 ADM and 15 CDM (Department of Dermatology, Saint-Louis hospital or Tenon hospital, Paris, from 2014 to 2018). ADM and CDM were diagnosed according to ENMC criteria [1] and/or ACR/EULAR criteria.[2,3] Briefly, CDM was defined by a subacute onset of symmetric proximal muscle weakness and typical DM rash confirmed by skin biopsy (purple periorbital oedema, gottron's papules or gottron's sign and/or erythema of chest and neck or upper back). ADM was defined by a typical DM rash confirmed by skin biopsy without muscle weakness or elevation of serum CK for at least 6 months after diagnosis. All patients did not receive any therapy at the time of inclusion, which means that these patients were not treated with antimalarials, corticosteroids, immunosuppressants or plasmapheresis. The control group included 19 healthy controls (HC) who matched the age and sex of the patient group. The demographic features of the patients and healthy donors are shown in ***Table S1***.

Cell preparation

Peripheral venous blood of patients and healthy donors was diluted with Roswell Park Memorial Institute medium (RPMI), and the peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, St Quentin en Yvelines, France) and all were further stored frozen in liquid nitrogen.

Staining and flow cytometric analysis

A multicolor immunophenotyping approach was used for the identification and analysis of different lymphocyte subpopulations. Immunophenotypic studies were performed on frozen

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samples, using 18-colour flow cytometry. Ten common markers and a viability dye were constantly present in all aliquots: CD45RA-BV650 HI100, CD8-BUV395 RPA-T8, CD161-BV605 DX12, CD14-BV510 MPhiP9, CD3-BUV737 UCHT1, CD25-BV785 M-A251, CD94-BV711 HP-3D9, CD4-BB700 Sk3, CD28-APC R700 CD28.2, FOXP3-PECF594 236A/E7 and CTLA4-PC5 BNI3 were obtained from BD (BD Bioscience, Le Pont de Claix, France), TCRγδ-Pc5.5 was obtained from Beckman Coulter (Beckman Coulter France S.A.S, Villepinte, France); NKG2A-PE REA110 was obtained from Miltenyi (Miltenyi Biotec, Bergisch-Gladbach, Germany), CD26-FITC BA5b was obtained from BioLegeng (BioLegend France, Paris, France). The MR1 5-OP-RU and 6-formylpterin (6-FP) BV421-conjugated tetramers were provided by the NIH Tetramer Core Facility. [4]

Cells were stained for membrane markers (at 4°C in the dark for 30 min) using cocktails of Ab diluted in PBS containing BSA/NaN3 (0.5% BSA, 0.01% NaN3) (FACS buffer). FOXP3 intracellular staining was performed according to the manufacturer's instructions. Appropriate isotype control Abs were used for each staining combination. Samples were acquired on a BD LSR-Fortessa flow cytometer using FACSDiva software (Beckton Dickinson). Flow data were analyzed using FlowJo software (FlowJo, LLC).

Polymerase chain reaction mRNA expression analysis

Total RNA was isolated from PBMC conserved in RLT buffer, using RNeasy Plus Mini Kit (Quiagen, Courtaboeuf, France). cDNA was synthesized from total RNA using a reverse transcription kit PrimerScript^{TM} RT reagent Kit with gDNA eraser (Takara Bio Europe S.A.S., Saint-Germain en Laye, France). RNA quantity and quality assessment were performed with a Nanodrop instrument (Thermo Fisher Scientific, Courtaboeur, France).

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The expression levels of 4 genes of the type I IFN signature were tested by real-time quantitative PCR. Real-time quantitative PCR was carried out using the LightCycler® 480 SYBR Green I Master (Roche Applied Science, Meylan, France). A LightCycler 480 II thermocycler (Roche Applied Science, Meylan, France) was used. PCR conditions were 95°C for 5min, followed by 45 cycles of 95°C for 15s and 60°C for 1min. At the end of the amplification reaction, a melting curve analysis was performed to confirm the specificity as well as the integrity of the PCR product by the presence of a single peak. Absence of cross-contamination and primer dimers was verified on a blank water control. The geometric mean of two reference genes (*TOP1* and *ATP5B)* was used for normalization. The relative expression levels of mRNA were determined using the ΔΔCt formula; fold changes were calculated as 2–ΔΔCt. Only means of duplicates with a CV of <15% were analysed. Primers used are the following: IFI27 (IFI27_F: TCCTCCATAGCAGCCAAGA; IFI27_R: TCCAGTTGCTCCCAGTGA), IFI44L (IFI44L_F: TGAGGAAACTGGTGCAATT, IFI44L_R: GCAGGCTGTCAATAGAGATT), MX1 (MX1_F: CCGTTAGCCGTGGTGATT; MX1_R: AAGGTGGAGCGATTCTGAG) and IFIH1 (IFIH1_F: CCTCCTTCAGCCCACTCT; IFIH1_R: CAGCAGCAATCCGGTTTCT).

Calculation of the type I IFN score

The mean (M) and standard deviation (SD) of each 4 IFN inducible genes (*IFI27, IFI44L, MX1 and IFIH1,* chosen from Walsh et al. [5]) for the group of healthy controls (MHC and SDHC) were used to calculate the type I IFN gene's expression score for each study subject, defined as the number of SD-HC above the M-HC, according to a previously published method [6]. Type I IFN score represented by the sum of the scores for each of the 4 genes was calculated for each subject. We considered a type I IFN score to be high if it fulfilled 1 of the 2 following

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criteria: 1) expression of at least 2 of the 3 IFN genes at a level ≤ 2 SD-HC above the M-HC; 2) expression of a single IFN gene at a level ≤ 4 SD-HC above the M-HC.[6]

Cytokines quantification.

Blood samples were collected from 8 patients, and 8 healthy controls and were centrifuged for 15 min at 2500rpm. Serum samples were subdivided into small aliquots to be stored at -20°C until testing. Levels (minimum detectable concentration, pg/mL) of IFN-γ (0.8), IL-1β (0.8), IL-4 (4.5), IL-6 (0.9), IL-10 (1.1), IL-17A (0.7), IL-21 (, IL-23 and TNF-α (0.7) were measured using Luminex Xmap technology (Luminex, Austin, TX, USA) with Human Millipore panels (Milipore S.A.S., Molsheim, France).

Human subject declaration

All studies have been approved by the appropriate institutional review boards and were conducted in accordance with current ethical and legal frameworks of the declaration of Helsinki. Informed written consent was received from participants before inclusion in this study, according to our local ethic rules (CPP Paris 12).

Statistical analysis Data

Data are expressed as median with interquartile range. Data were analysed using a statistical software package (GraphPad Prism), the Mann–Whitney U-test and Spearman's rank correlation. Differences at $P<0.05$ were considered to be statistically significant. In the figures, P values are displayed according to the following representation: * $p< 0.05$, ** $p<0.005$, *** $p<0.001$.

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