Supplementary Information

Using peripheral blood immune signatures to stratify patients with adult

and juvenile inflammatory myopathies

Meredyth G Ll Wilkinson BSc, PhD [1,2,3], Anna Radziszewska BSc[1], Christopher Wincup MBBS[1,4], Yiannis Ioannou MBBS, FRCP, PhD [1,2], David A Isenberg, MBBS, FRCP, FAMS, MD [1,2,4]*, Jessica J Manson MB ChB FRCP, PhD [4]*, Elizabeth C Jury MSc, PhD [1,2]*

* Share senior authorship

1. Division of Medicine, University College London, The Rayne Building, 4th Floor, 5 University Street, London, WC1E 6JF, UK.
2. Arthritis Research UK Centre for Adolescent Rheumatology, University College London Hospital and Great Ormond Street Hospital, London, United Kingdom.
3. Infection Inflammation and Rheumatology, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH.
4. Department of Rheumatology, 3rd Floor Central, University College London Hospital, 250 Euston Road, London, NW1 2PG

Supplementary Tables:

Supplementary Table 1:   Conjugated antibodies and reagents used for flow cytometry

Marker	Fluorochrome	Clone	Isotype	Manufacturer
Live/dead fixable blue	Live/dead blue	N/A	N/A	Life technologies
CD3	BUV396	UCHT1	Mouse BALB/cIgG1, κ	BD
CD185	BV605	J252D4	Mouse IgG1, κ	Biolegend
CD4	BUV737	SK3	Mouse BALB/c IgG1, κ	BD
CD4	BV711	OKT4	Mouse IgG2b, κ	Biolegend
CD45RA	BV421	HI100	Mouse IgG2b, κ	Biolegend
CD27	BV785	O323	Mouse IgG1, κ	Biolegend
CD127	BV711	A019D5	Mouse IgG1, κ	Biolegend
CD25	PE-Cy7	M-A251	Mouse IgG1, κ	Biolegend
TCR V a24-Ja18	APC	6B11	Mouse IgG1, κ	Biolegend
CD8	APC-cy7	RPA-T8	Mouse IgG1, κ	Biolegend
CD8	APC	SK1	Mouse IgG1, κ	eBioscience
CD69	BV510	FN50	Mouse IgG1, κ	Biolegend
PD-1	PE	NAT105	Mouse IgG1, κ	Biolegend
CD278	AF488	C398.4A	Armenian Hamster IgG	Biolegend
CD19	BV786	HIB19	Mouse IgG1, κ	Biolegend
CD19	Pe-Cy7	SJ25C1	Mouse IgG1, κ	eBioscience
CD38	BUV737	HB7	Mouse IgG1, κ	BD
CD24	BV421	ML5	Mouse IgG2a, κ	Biolegend
CD27	BV711	O323	Mouse IgG1, κ	Biolegend
CD14	BV605	M5E2	Mouse IgG2a, κ	Biolegend
CD14	FITC	61D3	Mouse IgG1, κ	eBioscience
CD16	APC-cy7	B73.1	Mouse IgG1, κ	Biolegend
IgD	FITC	IA6-2	Mouse IgG2a, κ	Biolegend
CD69	PE-cy7	FN50	Mouse IgG1, κ	Biolegend
HLA-DR	BV510	L243	Mouse IgG2a, κ	Biolegend
CD24	APC	SN2 A5-2H10	Mouse IgG1, κ	eBioscience
CD27	PECy7	O323	Mouse IgG1, κ	eBioscience
CD38	BV605	HIT2	Mouse IgG1, κ	Biolegend
IgM	Pacific Blue	MHM-88	Mouse IgG1, κ	eBioscience
IL-6	PE	MQ2-13A5	Rat IgG1,k	eBioscience
IL-17A	AF 488	N49-653	Mouse IgG1, k	BD

Supplementary Table 2: Immune cell subsets with defined sub-population by surface cell markers

Cell type	Markers
T cell subpopulations	
T cell	CD3+
Invariant killer T cells (inKT)	CD3+$\gamma\delta$TCR+
CD8+ T cell	CD3+CD8+
CD8+ Effector memory (EM)	CD3+CD8+CD45RA-CD27-
CD8+ Central memory (CM)	CD3+CD8+CD45RA-CD27+
CD8+ CD45RA+ Effector memory (EMRA)	CD3+CD8+CD45RA+CD27-
CD8+ naïve	CD3+CD8+CD45RA+CD27+
CD4+ T cell	CD3+CD8+
CD4+ Effector memory (EM)	CD3+CD4+CD45RA-CD27-
CD4+ Central memory (CM)	CD3+CD4+CD45RA-CD27+
CD4+ CD45RA+ Effector memory (EMRA)	CD3+CD4+CD45RA+CD27-
CD4+ naïve	CD3+CD4+CD45RA+CD27+
Tregs	CD3+CD4+CD127^{low}CD25+
CXCR5+ T cell	CD3+CD4+CD185+
Tfregs	CD3+CD4+CD185+CD25+CD127^{low}
TfH	CD3+CD4+CD185+ CD25^{low}CD127+
Activated T cells	CD3+CD4+CD185+ CD25^{low}CD127+PD1+ICOS+
B cell subpopulations	
Total B cells	CD3-CD19+
CD27+ B cells	CD3-CD19+CD27+
CD27- B cells	CD3-CD19+CD27-
BM1	CD3-CD19+IgD+CD38-
BM2	CD3-CD19+IgD+CD38+
BM2 transitional	CD3-CD19+IgD^{hi}CD38^{hi}
BM3-4	CD3-CD19+IgD-CD38^{hi}
BM5 early	CD3-CD19+IgD-CD38+
BM5 late	CD3-CD19+IgD+CD38-
Monocyte subpopulations	
Total Monocytes	CD3-HLA-DR+
Natural killer cells (NK)	CD3-HLA-DR-CD16+
Non-classical monocytes	CD3-HLA-DR+CD14-CD16+
Intermediate monocytes	CD3-HLA-DR+CD14+CD16+
Classical monocytes	CD3-HLA-DR+CD14+CD16-

Supplementary Table 3: Heterogeneous immune cell profiles in IIM patient subgroups the significantly different cell populations

Significant cell population	AM mean	AHC mean	Fold change	p value
CD27+ B cell	40.12	33.01	0.22	0.0008
CD27- B cells	31.14	65.83	-0.53	0.0007
CD8+CM	20.84	33.96	-0.39	0.0002
Intermediate monocytes	25.82	18.6	0.39	0.0147
CD4+ CM	34.71	44.73	-0.22	0.0043
BM5 early	8.66	11.23	-0.23	0.0802
CD4+ naïve	50.59	40.99	0.23	0.0515
CD8+ EMRA	22.52	15.55	0.45	0.0877
BM3-4	3.28	1.845	0.78	0.0696
Monocytes	19.01	14.23	0.34	0.0658
Activated T cells	0.29	2.693	-0.89	0.0084
NK cells	7.37	9.806	-0.25	0.1207

Significant cell population	ADM mean	AHC mean	Fold change	p value
BM5 early	6.37	11.23	-0.43	0.0223
CD8+ CM	23.55	33.96	-0.31	0.0578
Tregs	6.02	4.211	0.43	0.0044
Th17	2.33	0.8383	1.78	0.0093
CD4+ naïve	55.28	40.99	0.35	0.0648
BM3-4	4.74	1.845	1.57	0.0976

Significant cell population	APM mean	AHC mean	Fold change	p value
CD27+ B cell	15.23	33.01	-0.54	0.0141
CD27- B cells	84.10	65.83	0.28	0.0117
CD8+CM	17.03	33.96	-0.50	0.0093

Significant cell population	JDM mean	THC mean	Fold change	p value
CD8+ T cells	23.69	32.62	-0.27	0.0168
CD8+ CM	19.31	28.93	-0.33	0.0202
NK cells	6.763	10.03	-0.33	0.0403
CD4+ T cells	62.69	52.01	0.21	0.0184
CXCR5+	7.794	5.807	0.34	0.0691
BM1	9.623	5.452	0.77	0.0646

Significant cell population	ADM mean	JDM mean	Fold change	p value
CD27+ B cell	23.78	33.77	-0.30	0.1047

CD27- B cells	74.88	63.75	0.17	0.0807
CD8+naïve	46.53	62.07	-0.25	0.0464
Intermediate monocytes	24.08	36.17	-0.33	0.0348
Th17	2.249	0.6753	2.33	0.0032
BM5 early	6.726	15.35	-0.56	0.0071
Tregs	6.019	4.697	0.28	0.0488
CD8+ EM	11.13	5.444	1.04	0.0621

Significant cell population	AHC mean	THC mean	Fold change	p value
Intermediate monocytes	18.60	44.18	-0.58	0.0001
CD4+ naïve	40.99	57.73	-0.29	0.0001
CD8+ naïve	38.04	56.81	-0.33	0.0030
BM2	53.34	64.38	-0.17	0.0051
Classical monocytes	58.12	43.69	0.33	0.0066

Significant cell population	Anti-synthetase AAB mean	AHC mean	Fold change	p value
CD27+ B cell	13.27	33.01	-0.60	0.0149
CD27- B cells	85.43	65.83	0.30	0.0166
BM1	4.83	13.75	-0.65	0.0761

Significant cell population	ARD AAB overlap mean	AHC mean	Fold change	p value
Th17	2.90	0.8383	2.46	0.0135
CD8+ CM	18.97	33.96	-0.44	0.0274
CD8+ EMRA	28.96	15.55	0.86	0.0958

Significant cell population	Cancer AAB mean	AHC mean	Fold change	p value
Monocytes	42.05	14.23	1.96	0.0001
T cells	26.50	47.07	-0.44	0.0150
BM3-4	9.49	1.844	4.15	0.0057
Intermediate monocytes	34.55	18.6	0.86	0.0406

Significant cell population	Remission off-treatment mean	AHC mean	Fold change	p value
BM2	77.08	53.34	0.45	0.0469
BM5 early	3.575	11.23	-0.68	0.0739
CD27+ B cell	11.02	33.01	-0.67	0.0520
CD27- B cells	88.48	65.83	0.34	0.0434
Non-classical monocytes	20.38	8.286	1.46	0.0019

Significant cell population	Remission on-treatment mean	AHC mean	Fold change	p value
Th17	2.677	0.8383	2.19	0.0381

Significant cell population	Active mean	AHC mean	Fold change	p value
CD8+ CM	12.05	33.96	-0.65	0.0542

Supplementary Table 3: Heterogeneous immune cell profiles in IIM patient subgroups the significantly different cell populations. Table showing the significantly different cells populations when disease groups and healthy control groups were compared. Comparisons include: adult myositis (AM) Vs. adult healthy control (AHC); adult dermatomyositis (ADM) Vs. AHC; adult polymyositis (APM) Vs. AHC; juvenile dermatomyositis (JDM) Vs. teenage healthy control (THC); ADM Vs. JDM; AHC Vs. THC; Anti-synthetase group (anti-Jo-1 and anti-Pl-12) Vs. AHC; Autoimmune rheumatic disease overlap (ARD) group (anti-Ro, anti-PmScl and anti-RNP) Vs. AHC; Cancer associated group (anti-NXP2 and anti-TIF1γ) Vs. AHC;; remission off-treatment Vs. AHC; remission on-treatment Vs. AHC;; active Vs. AHC. Mean average was recorded for each group and fold change difference. All p-values represent adjusted p-values calculated by Tukey's multiple comparison.

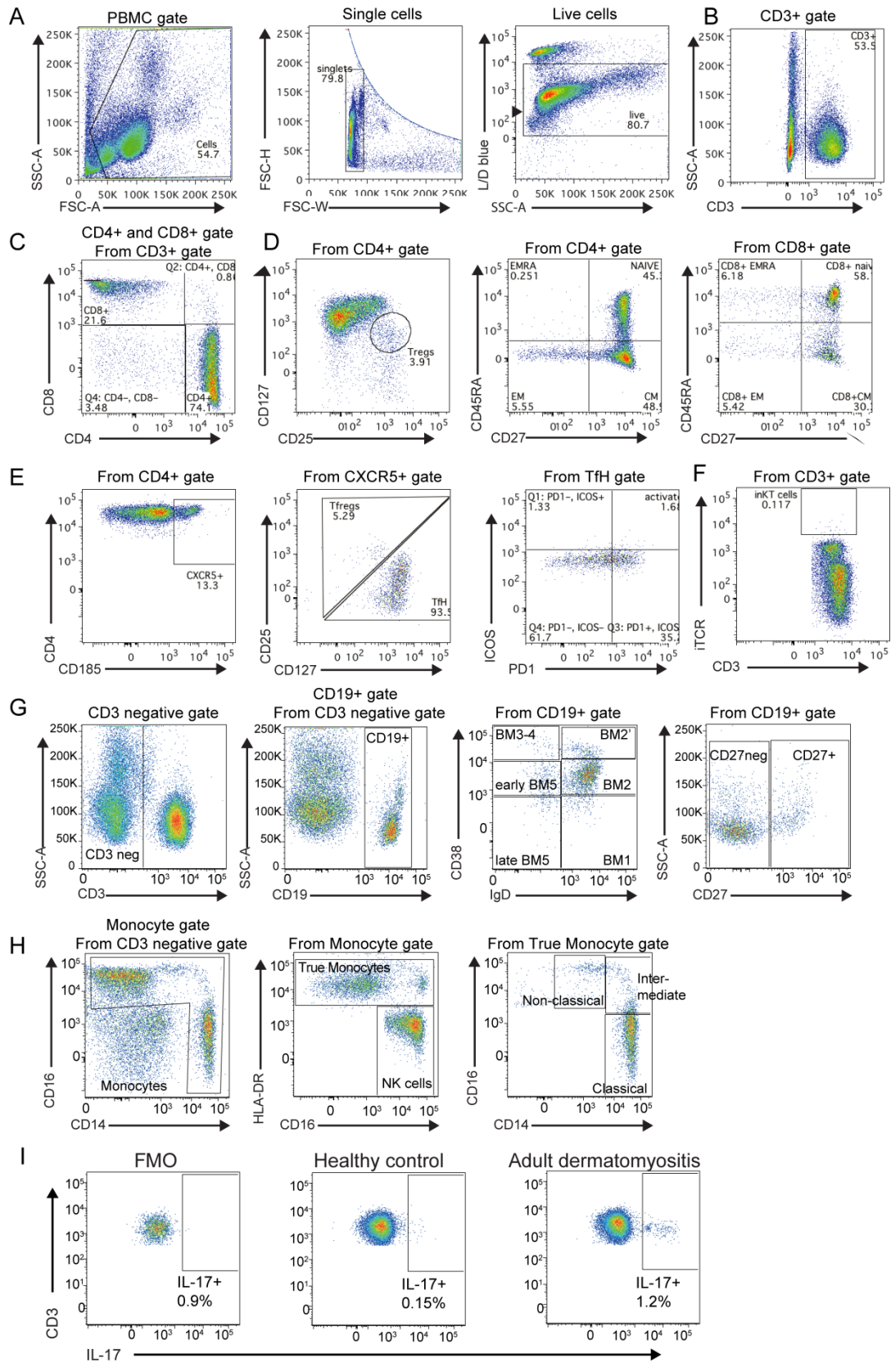**Supplementary Figures**
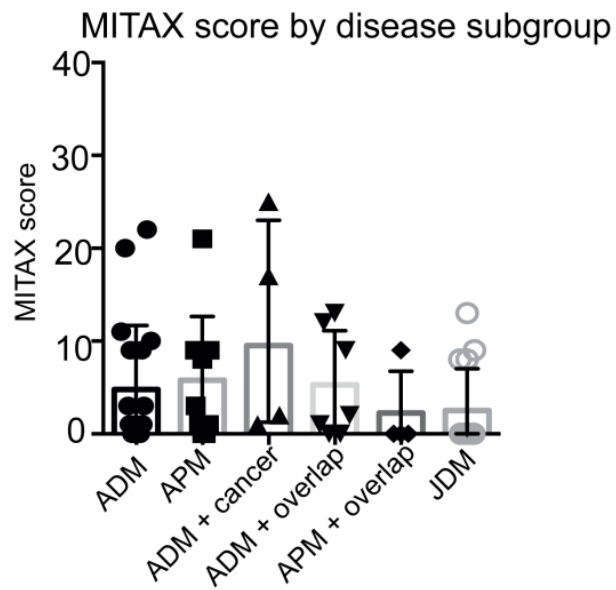
Supplementary Figure 1: no difference in disease activity MITAX score between IIM subgroups. Scatter plot showing MITAX scores for each IIM sample split into subgroups; ADM, APM, ADM +cancer, ADM + overlap, APM + overlap and JDM. Represented mean average with standard deviation.

Viability of PBMC by sample group
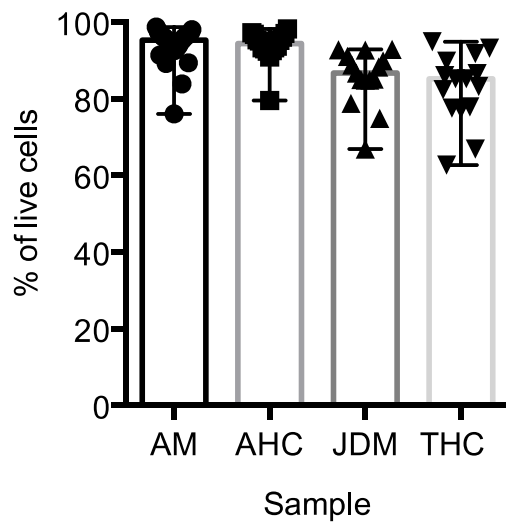
Supplementary Figure 2: no difference in cell viability between sample groups.
PBMC were stained with viability dye to measure the cell viability. Scatter plot showing % of live cells for each sample group; adult myositis (AM), adult healthy control (AHC), adolescent-onset juvenile dermatomyositis (a-JDM). Represented median average with range.

A PBMC gate — Single cells — Live cells

B CD3+ gate — CD3+ 53.5

C CD4+ and CD8+ gate From CD3+ gate — Q2: CD4+, CD8 0.8; CD8+ 21.6; Q4: CD4−, CD8− 3.48; CD4+ 74.1

D From CD4+ gate — Tregs 3.91
From CD4+ gate — EMRA 0.251; NAIVE 45.; EM 5.55; CM 48.
From CD8+ gate — CD8+ EMRA 6.18; CD8+ naïv 58.; CD8+ EM 5.42; CD8+CM 30.

E From CD4+ gate — CXCR5+ 13.3
From CXCR5+ gate — Tfregs 5.29; TfH 93.5
From TfH gate — Q1: PD1−, ICOS+ 1.33; activat 1.6; Q4: PD1−, ICOS− 61.7; Q3: PD1+, ICOS 35.

F From CD3+ gate — iNKT cells 0.117

G CD19+ gate
CD3 negative gate — CD3 neg
From CD3 negative gate — CD19+
From CD19+ gate — BM3-4; BM2'; early BM5; BM2; late BM5; BM1
From CD19+ gate — CD27neg; CD27+

H Monocyte gate
From CD3 negative gate — Monocytes
From Monocyte gate — True Monocytes; NK cells
From True Monocyte gate — Non-classical; Intermediate; Classical

I FMO — IL-17+ 0.9%
Healthy control — IL-17+ 0.15%
Adult dermatomyositis — IL-17+ 1.2%

Supplementary Figure 3: Flow cytometry gating strategy for T cells and subsets

PBMCs were taken from all sample groups and stained ex-vivo for **A** live cells, **B** total T cells (CD3+) and **C-F** 16 T cell sub-populations, **G** total B cell (CD19+), 8 B cell sub-populations, **H** total monocytes, 3 monocyte sub-populations and natural killer(NK) cells (see Supplementary Table 2). These populations were categorised and measured ex-vivo by flow cytometry. **I** Flow cytometry gating strategy for Th17 cells. Total PBMC were taken from all sample groups and incubated for 4hrs in the presence of PMA, ionomycin and golgi plug. Surface and intracellular markers were stained for. The T cell populations expression of IL-17 were categorised and measured by flow cytometry. The plots show positive gates for IL-17 staining on CD3+ T cells for a healthy control fluorescence minus one (FMO), a healthy control and adult dermatomyositis PBMC sample. Application settings were created; Cytometer Setup and Tracking (CS&T) beads were run on the flow cytometer before each session. Application settings were applied to panel templates each time prior to compensation to ensure that all immunophenotyping data is comparable over time. Data were analysed with Flowjo (Tree Star).
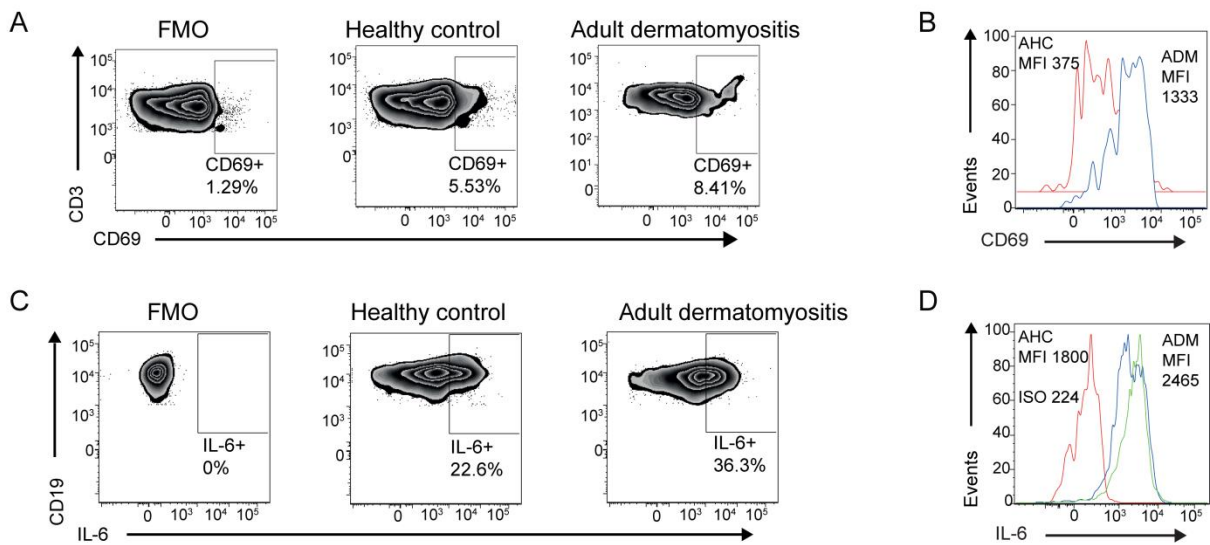
Supplementary Figure 4: Flow cytometry gating strategy for CD69 and IL-6 expression on lymphocyte and monocytes populations.

A Flow cytometry gating strategy for CD69 expression. Total PBMC were taken from all sample groups and stained ex-vivo for 28 lymphocyte and 5 monocyte populations and their expression of the activation marker CD69. These populations were categorised and measured ex-vivo by flow cytometry. The plots show positive gates for CD69 staining on T cells for a healthy control fluorescence minus one (FMO), a healthy control and adult dermatomyositis PBMC sample. **B** Flow cytometry histograms of CD69. **C** Flow cytometry gating strategy for IL-6 expression. Total PBMC were taken from all sample groups and incubated for 4hrs in the presence of PMA, ionomycin and golgi plug. Surface and intracellular markers were stained. The monocyte and lymphocyte populations expressing IL-6 were categorised and measured by flow cytometry. The plots show positive gates for IL-6 staining on CD19+ B cells for a healthy control fluorescence minus one (FMO), a healthy control and adult dermatomyositis PBMC sample. **D** Flow cytometry histograms of IL-6 median fluorescence intensity (MFI).
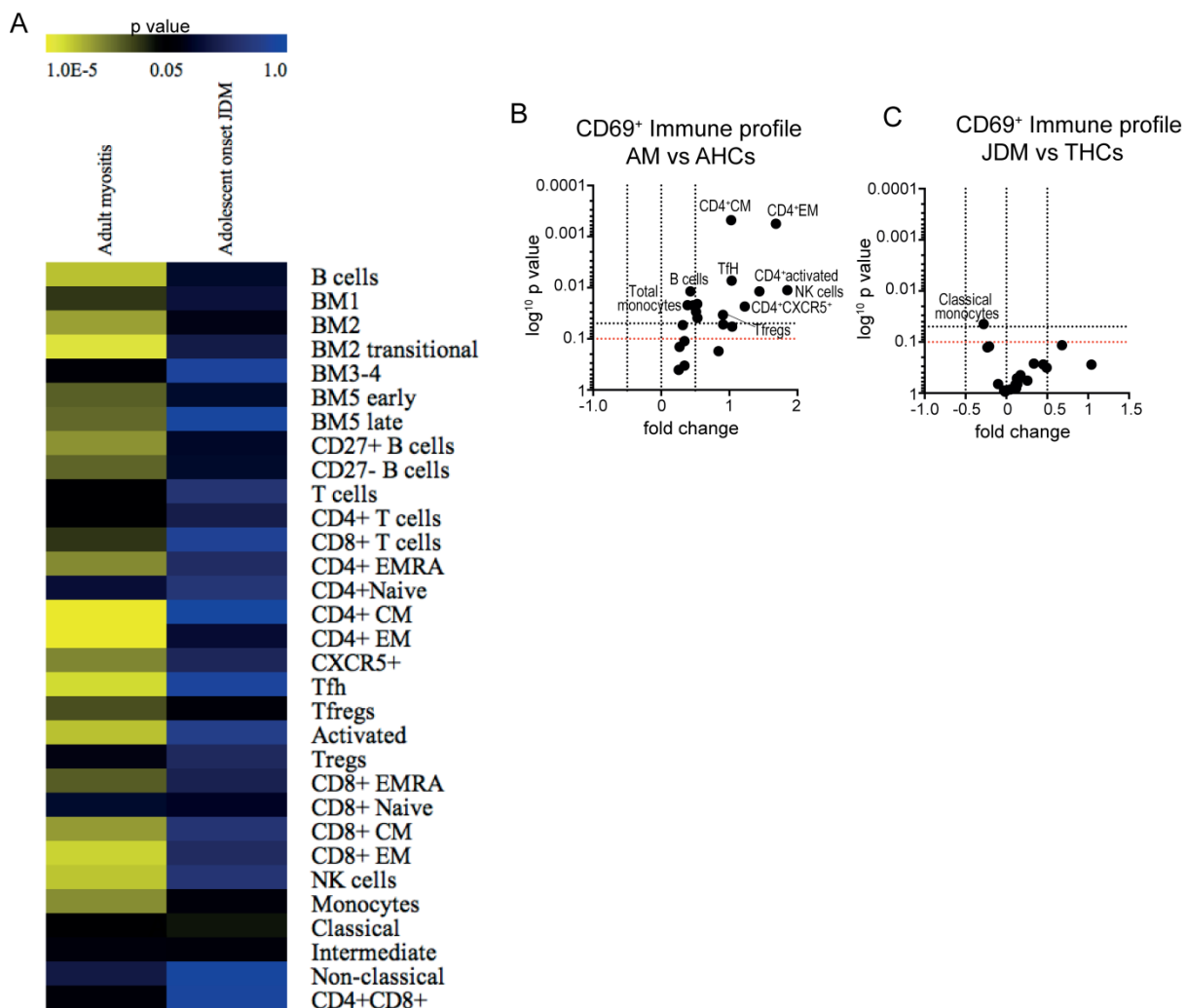
Supplementary Figure 5: Activation status of immune cell populations in patients with IMMs. The expression of CD69 from PBMC populations were measured by flow cytometry from 44 adult myositis (AM) patients, 25 adult healthy controls (AHC), 15 adolescent-onset juvenile dermatomyositis (JDM) patients and 15 teenage healthy controls (THC). **A** Heat map identifying significant differences in cell populations expression of CD69, a comparison of IIM to healthy samples. This heat map demonstrates the statistical difference between each cell population by comparing the mean of each group of samples; AM vs. AHC, and JDM vs. THC. A

student t-test calculated the p-values. Black to yellow – p<0.05. Blue to black – p>0.05. Volcano plots representing the fold change and p value for each cell population expressing of IL-6 CD69 when sample groups were compared. **B** AM vs. AHC; and **C** JDM vs. THC. The p values were calculated by one-way ANOVA. All p-values represent adjusted p-value calculated by Tukey's multiple comparison. Stringent significance p<0.05 (black dotted line). Adjusted significance by false discovery rate (FDR) for small sample size p≤0.1 (red dotted line).
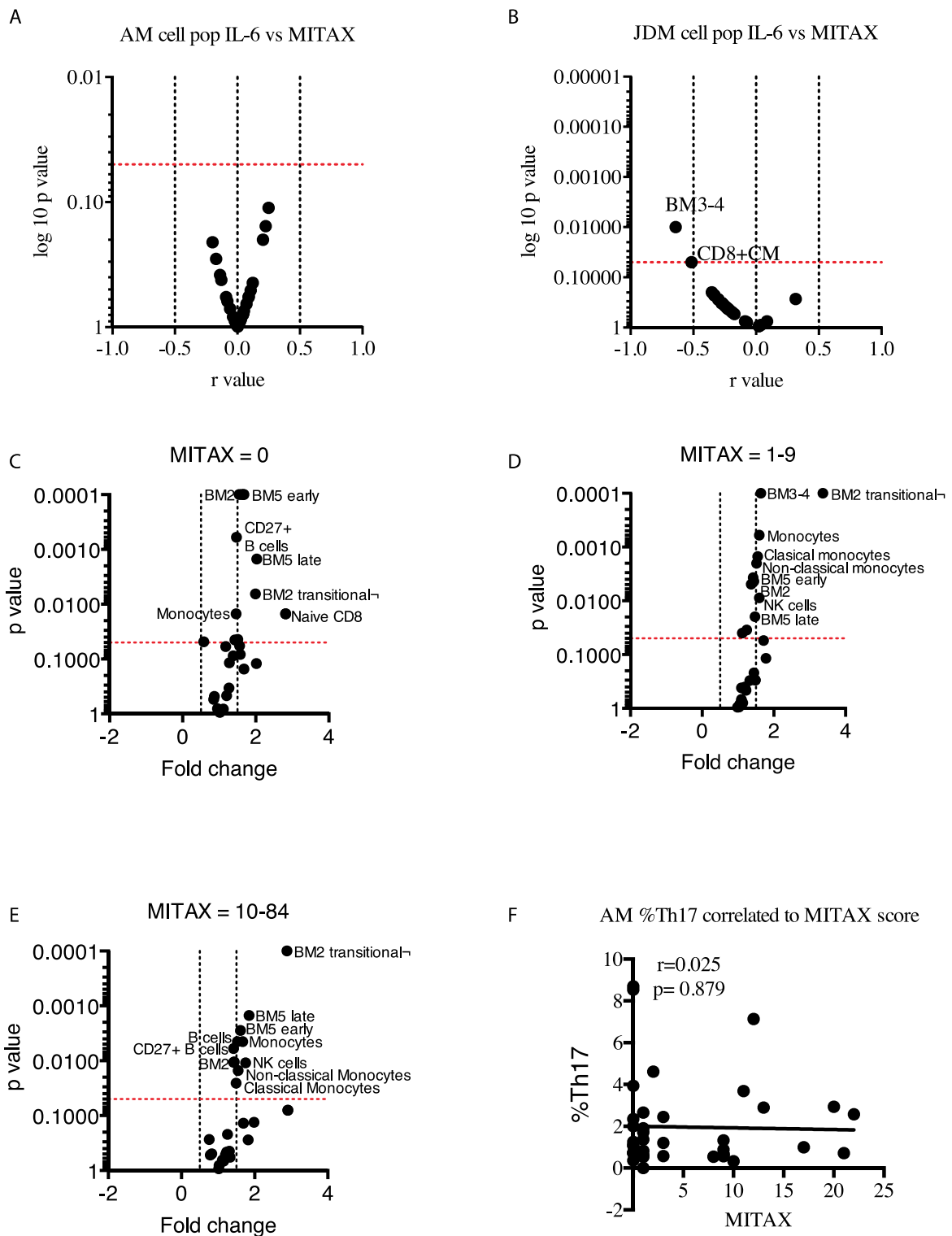
Supplementary figure 6: IL-6 and Th17 do not increase with higher MITAX score. Volcano plots showing all PBMC populations, from the total **A)** AM group and **B)** JDM group, expression of IL-6 correlation to MITAX score. Pearson's correlation was conducted, r-value (x-axis) and log10 p value (y-axis). Significance reached p≤0.05 (horizontal dotted red line). The fold change significance is ≤-0.5 and ≥0.5

(vertical dotted black lines). Volcano plots showing all cell populations expression of IL-6 comparing AM (n=44) to AHC (n=25) [calculated fold change] grouped by MITAX score (C) =0), (D) = 1-9), (E) = 10-84). P values were calculated by one-way ANOVA. All p-values represent adjusted p-values calculated by Tukey's multiple comparison. Stringent significance p$\leq$0.05 (horizontal red dotted line). **F)** Scatter plot showing linear regression correlation between %Th17 cells from AM samples and MITAX score.