Increase in circulating cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis

Alterations in macrophage polarisation are recognised among the possible immune system abnormalities contributing to systemic sclerosis (SSc) pathogenesis.[1]

Macrophages have been classified as classically (M1) or alternatively (M2) activated, although growing evidence indicates that they may exhibit characteristics shared by more than one of the described phenotypes.[2–5]

An M2 pre-eminent phenotype has been postulated for SSc monocytes/macrophages.[2] The aim of the study was to widen a phenotype characterisation (M1, M2 and mixed M1/M2) of circulating monocytes/macrophages in patients with SSc and healthy subjects (HSs) through flow cytometry analysis. Fifty-eight consecutive patients with SSc (38 limited and 20 diffuse SSc) and 27 age-matched and gender-matched HSs were enrolled after signing informed consent. SSc diagnosis was based on the 2013 American College of Rheumatology/European League Against Rheumatism criteria (online supplementary file 1).[6] For flow cytometry analysis, peripheral blood was collected and anti-CD14 and anti-CD45 antibodies were used to identify the monocyte/macrophage lineage; macrophage scavenger receptors (CD204, CD163) and mannose receptor 1 (CD206) were used as M2 phenotype markers; and co-stimulatory molecules (CD80, CD86) and Toll-like receptors (TLR4, TLR2) were used as M1 phenotype markers. CD66b was used to distinguish granulocytes (Miltenyi Biotech, Germany). Flow cytometry analysis was performed using the Navios flow cytometer and Kaluza analysis software (Beckman Coulter). A total of 5×10^6 cells were evaluated and more than 30 events were detected in the smallest subset investigated, according to the consensus guidelines for minimal residual disease. Results were expressed in percentages over total circulating leucocytes, unless otherwise specified. The non-parametric al Mann-Whitney U test was used for statistical analysis and any p value lower than 0.05 was considered statistically significant. Two initial gating strategies were used to study circulating M2-like monocytes/macrophages, the first gated $CD14^+$cells and the second gated $CD204^+$cells.[1 2 7]

Using $CD14^+$cells for the initial gating strategy, patients with SSc showed a significantly increased percentage of $CD14^+CD163^+CD206^+$cells compared with HSs (6.35%±2.8% vs 5.38±1.98%, p=0.047) (figure 1A). In this subset, cells expressing CD204 were significantly higher in patients with SSc compared with HSs (0.23±0.25 vs 0.14±0.13; p=0.02) (figure 1B). In circulating $CD14^+CD206^+CD163^+CD204^+$M2 cells, a significantly higher percentage of $TLR4^+$cells was observed compared with HSs (0.0091%±0.013% vs 0.003±0.0026%; p=0.003) (figure 1C).

Using $CD204^+$cells for the initial gating strategy, the percentage of $CD204^+CD163^+CD206^+$M2 cells was significantly higher in patients with SSc compared with HSs (5.6%±6.9% vs 1.4±1.3% of $CD204^+$cells; p<0.0001), representing 0.042% of the leucocyte population in patients with SSc compared with 0.01% in HSs (figure 1D). In this cell population, cells expressing TLR4 were significantly higher in patients with SSc than in HSs

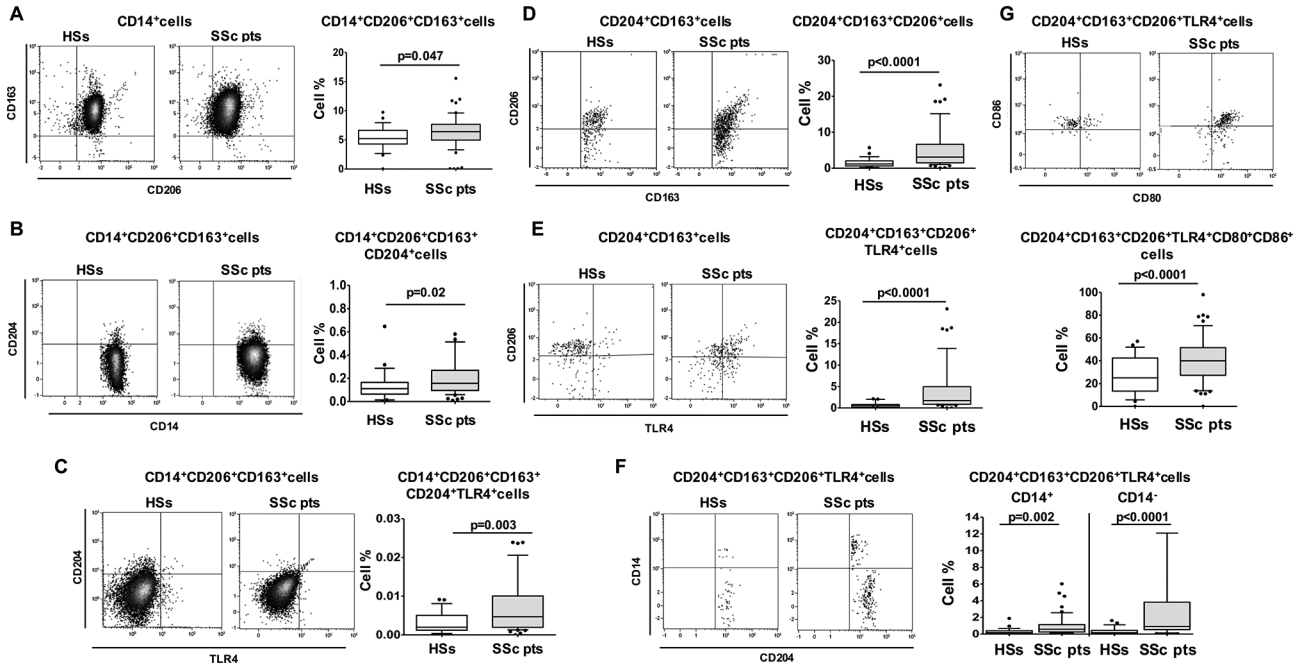

Figure 1 Evaluation of circulating cells expressing M1 and M2 phenotype markers in patients with systemic sclerosis (SSc) and healthy subjects (HSs). Representative flow cytometry panels with quadrant regions and box plot representation of the percentage (%) of circulating (A) $CD14^+CD206^+CD163^+$cells in the $CD14^+$cell population; (B) $CD14^+CD206^+CD163^+CD204^+$cells in the $CD14^+CD206^+CD163^+$cell subset; (C) $CD14^+CD206^+CD163^+CD204^+TLR4^+$cells in the $CD14^+CD206^+CD163^+$cell subset, detecting $CD14^+$cells in the leucocyte population as the initial gating strategy in HSs and patients with SSc. Representative flow cytometry panels with quadrant regions and box plot representation of the % of circulating (D) $CD204^+CD163^+CD206^+$cells in the $CD204^+CD163^+$cell subset; (E) $CD204^+CD163^+CD206^+TLR4^+$cells in the $CD204^+CD163^+$cell subset; (F) $CD14^+$ and CD14cells in the $CD204^+CD163^+CD206^+TLR4^+$cell subset; (G) $CD204^+CD163^+CD206^+TLR4^+CD80^+CD86^+$cells in the $CD204^+CD163^+CD206^+TLR4^+$cell subset, detecting the $CD204^+$cells in the leucocyte population as the initial gating strategy in HSs and SSc pts. The flow cytometry panels are representative of the mean % that was obtained. The box plots show the median and the whiskers (which represent the ninth and the ninety-first centiles). The results were expressed as mean±SD.

(4.65%±6.8% vs 0.66±0.6% of CD204$^+$cells; p<0.0001) (figure 1E). In the CD204$^+$CD163$^+$CD206$^+$TLR4$^+$cell subset, CD14$^+$cells made up 1% of the CD204$^+$cells in patients with SSc compared with 0.31% in HSs (p=0.002), whereas the CD14 cells made up 3.65% of CD204$^+$cells in patients with SSc compared with 0.35% in HSs (p<0.0001) (figure 1F). Moreover, 41.6% of the CD204$^+$CD163$^+$CD206$^+$TLR4^{+-} cells in patients with SSc were characterised by the coexpression of CD80 and CD86 (M1) compared with 26.8% in HSs (0.02%±0.07% vs 0.001±0.002% of the leucocyte population, p<0.0001) (figure 1G).Interestingly the four outliers with higher percentages of M2 and M1/M2 cells calculated over the total CD204$^+$cells in the patient populations with SSc , are always related to the same patients (figure 1D, E, F, G). These patients are all women of different ages, characterised by Scl70 positivity, interstitial lung disease (ILD) at lung CT scan, reduced diffusing capacity of the lung for carbon monoxide (DLCO), presence of oesophageal or heart involvement. Two of them were treated with glucocorticoids.

No difference in circulating M1 cell percentage was observed between patients with SSc and HSs (online supplementary file 2).

The patient subgroup analysis according to the different treatment regimens pointed out that the lowest percentages of M2 and M1/M2 circulating cell populations were observed in HSs, while the highest values were observed in patients taking only glucocorticoids. Intermediate values were observed for circulating M2 and M1/M2 populations in not treated patients or in patients treated with both drugs. Highly significant differences in the percentages of the circulating M2 and M1/M2 studied populations were constant between patients with SSc not treated with any of the considered medications and HSs. No significant correlation was reported with other treatment regimens (ie, ERAs) (figure 2). Moreover, no correlations were observed with either limited or diffused cutaneous disease form.

Based on a wide flow cytometry surface marker analysis of circulating monocytes/macrophages, the study demonstrated that M2 and more significantly cells expressing both M1 and M2 surface markers characterise patients with SSc compared with HSs. No difference was identified when only M1 markers were used. Moreover, the initial gating strategy based on the CD204$^+$cells resulted the most efficient to describe monocyte/macrophage phenotype differences between patients with SSc and HSs.

The result is supported by a recent study demonstrating a remarkable plasticity of circulating monocytes/macrophages, resulting in a 'spectrum' of activation states.[5] Consistently with our data, a recent study from Moreno-Moral *et al* showed a downregulation of interferon-γ response and IL6/JAK/STAT3 pathway in SSc monocyte-derived macrophages, possibly describing a 'SSc specific macrophage'.[8–10]

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A

CELL PHENOTYPE	PTs GCs	PTs IMM	PTs IMM+GCs	PTs NO IMM/GCs	HSs	p
CD204$^+$CD163$^+$ (%CD204$^+$)	51.5±25	11.4±6	15.7±3	17.2±13	6.5±3	<0.0001
CD204$^+$CD163$^+$ (%leucocytes)	0.50±0.6	0.08±0.05	0.012±0.04	0.11±0.14	0.04±0.03	<0.0001
CD204$^+$CD163$^+$CD206$^+$ (%CD204$^+$)	21.8±11	3.47±3	4.5±2	5.9±6	1.4±1.2	<0.0001
CD204$^+$CD163$^+$CD206$^+$ (%leucocytes)	0.22±0.29	0.023±0.021	0.035±0.02	0.03±0.05	0.01±0.01	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$ (%CD204$^+$)	21±11	2.5±3	3±1	4.9±6	0.6±0.6	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$ (%leucocytes)	0.21±0.27	0.014±0.014	0.022±0.01	0.03±0.05	0.004±0.004	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD14$^+$ (%CD204$^+$)	2.92±1.6	0.76±1.1	1.21±0.84	0.89±1	0.31±0.38	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD14$^+$ (%leucocytes)	0.028±0.036	0.005±0.007	0.009±0.007	0.005±0.005	0.002±0.003	0.001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD14$^-$ (%CD204$^+$)	18.14±10	1.75±2	1.86±1.5	4.09±6	0.34±0.41	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD14$^-$ (%leucocytes)	0.18±0.24	0.009±0.01	0.012±0.008	0.025±0.05	0.002±0.002	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD80$^+$CD86$^+$ (%CD204$^+$)	15.1±10	1.1±1.7	1.03±1.59	2.96±4.6	0.21±0.29	<0.0001
CD204$^+$CD163$^+$CD206$^+$TLR4$^+$CD80$^+$CD86$^+$ (%leucocytes)	0.16±0.22	0.005±0.008	0.008±0.004	0.017±0.038	0.001±0.001	<0.0001
CD14$^+$CD206$^+$CD163$^+$CD204$^+$TLR4$^+$ (%leucocytes)	0.35±0.36	0.005±0.004	0.007±0.006	0.10±0.11	0.003±0.002	0.002

B

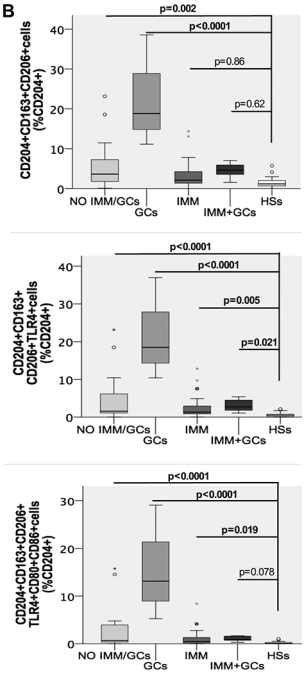

Figure 2 Graphic representation of the subgroup analysis according to treatment regimens. For sample size reasons, patients were stratified according to different treatment regimens. Treatments that could theoretically influence the variables of interest, that is, immunosuppressants, glucocorticoids or endothelin-1 receptor antagonists (ERAs), were considered in the analysis. Patients were defined 'on immunosuppressant' if they were steadily under treatment with: ciclosporin 3–5 mg/kg per day, methotrexate 7.5 mg per week or 2.5 mg every 12 hours for three sequential doses per week, mycophenolate 1500–2000 mg per day; 'on glucocorticoids' if they were steadily under treatment with prednisone 2.5–5 mg per day; 'on ERAs' if they were steadily under treatment with bosentan 250 mg per day or macitentan 10 mg per day. Each enrolled patient was under steady treatment regimen from at least 6 months. (A) Means and SD of the percentage of each cell subset are reported for patients treated with glucocorticoids (GCs), immunosuppressants (IMM), immunosuppressants and glucocorticoids (IMM + GCs) or not treated with any of the two drugs (NO IMM/GCs), and healthy subjects (HSs). (B) Graphic representation of the subgroup analysis of M2 and M1/M2 cell percentages in patients with SSc stratified according to treatment regimens and in HSs. Percentages are calculated over total CD204$^+$cells (%CD204$^+$) or over the total leucocyte population (%leucocytes). PTs, patients with SSc.

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Correction notice This article has been corrected since it published Online First. The equal contribution statement has been added.

Handling editor Josef S Smolen

Acknowledgements The authors thank Dr Sara De Gregorio for designing and making figure 1 and supplementary file 1 of the manuscript and Fabio Gallo for supporting the statistical analysis.

Contributors SS, ACT and MC designed the study, analysed the data, carried out the statistical analysis and wrote the manuscript; PC performed the flow cytometry analysis; VT and BR enrolled the patients with systemic sclerosis and healthy subjects and collected the demographic and clinical parameters of the patients with SSc; RB and PM managed and prepared the samples for the flow cytometry analysis; AS, SP and CP supervised the enrolment of the patients with SSc and the healthy subjects and the clinical data collection; VS revised the critical and important intellectual contents of the manuscript.

Competing interests None declared.

Patient consent Not required.

Ethics approval Local Ethics committee (protocol number 273-REG-2015).

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-213648).

SS and ACT contributed equally.

To cite Soldano S, Trombetta AC, Contini P, *et al*. *Ann Rheum Dis* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/annrheumdis-2018-213648

Received 24 April 2018
Revised 28 June 2018
Accepted 2 July 2018

Ann Rheum Dis 2018;**0**:1–3. doi:10.1136/annrheumdis-2018-213648

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