Distinct immune regulatory receptor profiles linked to altered monocyte subsets in sarcoidosis

Simon D. Fraser [1], Michael G. Crooks [1], Paul M. Kaye [2], Simon P. Hart [1*]

Supplementary material

Immune cell phenotyping

For studying regulatory receptor expression on monocyte subsets, combinations of fluorophore-conjugated antibodies were carefully chosen that would have minimal or no overlap in their emission wavelengths. For all patients and healthy controls, the same combinations of antibodies were used (Supplementary Tables S1 and S2). PBMCs (2×10^5) were suspended in 50µl of FACS blocking solution (0.5% BSA in sterile PBS) for 1 hour at 4°C for blocking. Cells were centrifuged and resuspended in FACS blocking solution containing antibody combinations at optimum concentrations previously determined by titrations, typically 2.5 – 10 µg/ml, and incubated for 1 hour at 4°C in the dark. Cells were washed, resuspended and analysed using a FACS Aria II flow cytometer and FACS Diva software (BD, Oxford, UK). Compensation settings were optimised and kept constant throughout the study. The cytometer was calibrated before each sample analysis run using BD Cytometer Setup & Tracking Beads (ref 642412) to ensure consistency of results over time. Post-acquisition analyses were performed using Flowing Software 2.5.0 (Perttu Terho).

Flow cytometry gating and data analysis

Monocytes were defined by broad FSC and SSC gating followed by CD14+ gating. Lineage markers for T lymphocytes (CD3), B lymphocytes (CD19) and Natural Killer cells (CD3-CD56+) were absent from the monocyte gates. To define monocyte subsets, CD14 (APC) and CD16 (BV421) antibodies were used. Classical monocyte gates were defined using pooled data from controls and further monocyte subset populations were defined as described (1). Combining isotype control plots with density plots to determine the cut-off points allowed us to account for the strong CD16 fluorescence signal obtained from CD16 due to the high

stain index of BV421. All subsequent samples were calibrated using this fixed template to ensure consistent gating. An example of the gating is shown in Figure 1. Regulatory receptor expression was first calibrated using a mean isotype control GMFI in order to account for differences in acquisition between flow cytometer runs. Expression values were calculated as the calibrated GMFI minus the GMFI of the mean isotype control. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, USA).

1. Ziegler-Heitbrock L, Hofer TP. Toward a refined definition of monocyte subsets. Front Immunol. 2013;4:23.

Table S1. Antibodies used for flow cytometry. PBMCs (2×10^5) were suspended in 50µl of FACS blocking solution (0.5% BSA in sterile PBS) for 1 hour at 4°C. Cells were pelleted and resuspended in FACS blocking solution containing antibody combinations at optimum concentrations (previously determined by titrations, typically 2.5–10 µg/ml) and incubated at 4°C in the dark for 1 hour. Cells were washed, re-suspended and analysed using a FACS Aria II flow cytometer and FACS Diva software (BD, Oxford, UK).

Markers	**Fluorescence**	**Reference (Biolegend)**	**Concentration used**
CD3	Brilliant Violet 421	300434	2.5 µg/ml
CD4	APC	300514	5 µg/ml
CD14	PE	325606	10 µg/ml
CD14	APC	325608	10 µg/ml
CD16	Brilliant Violet 421	302038	10 µg/ml
CD19	PerCP/CY5.5	302230	10 µg/ml
CD56	APC	318310	5 µg/ml
CD200R	PE	329306	10 µg/ml
IL-10R	PE	308804	10 µg/ml
SIRP-α	PE	323806	10 µg/ml
CD47	PE	323108	10 µg/ml
Isotype Controls Mouse IgG1	APC	400122	10 µg/ml
	PE	400114	10 µg/ml
	Brilliant Violet 421	400158	10 µg/ml
	PerCP/CY5.5	400150	10 µg/ml
Isotype Control Rat IgG2a	PE	400508	10 µg/ml

Table S2. Antibody combinations used for multi-parameter flow cytometry analysis of peripheral blood mononuclear cells.

Staining Target	BV421	PE	APC	PerCP/CY5.5
Immunophenotyping	CD3	CD14	CD56	CD19
	CD16	CD14	CD4	
CD200R	CD3	CD200R	CD14	CD19
	CD16	CD200R	CD14	

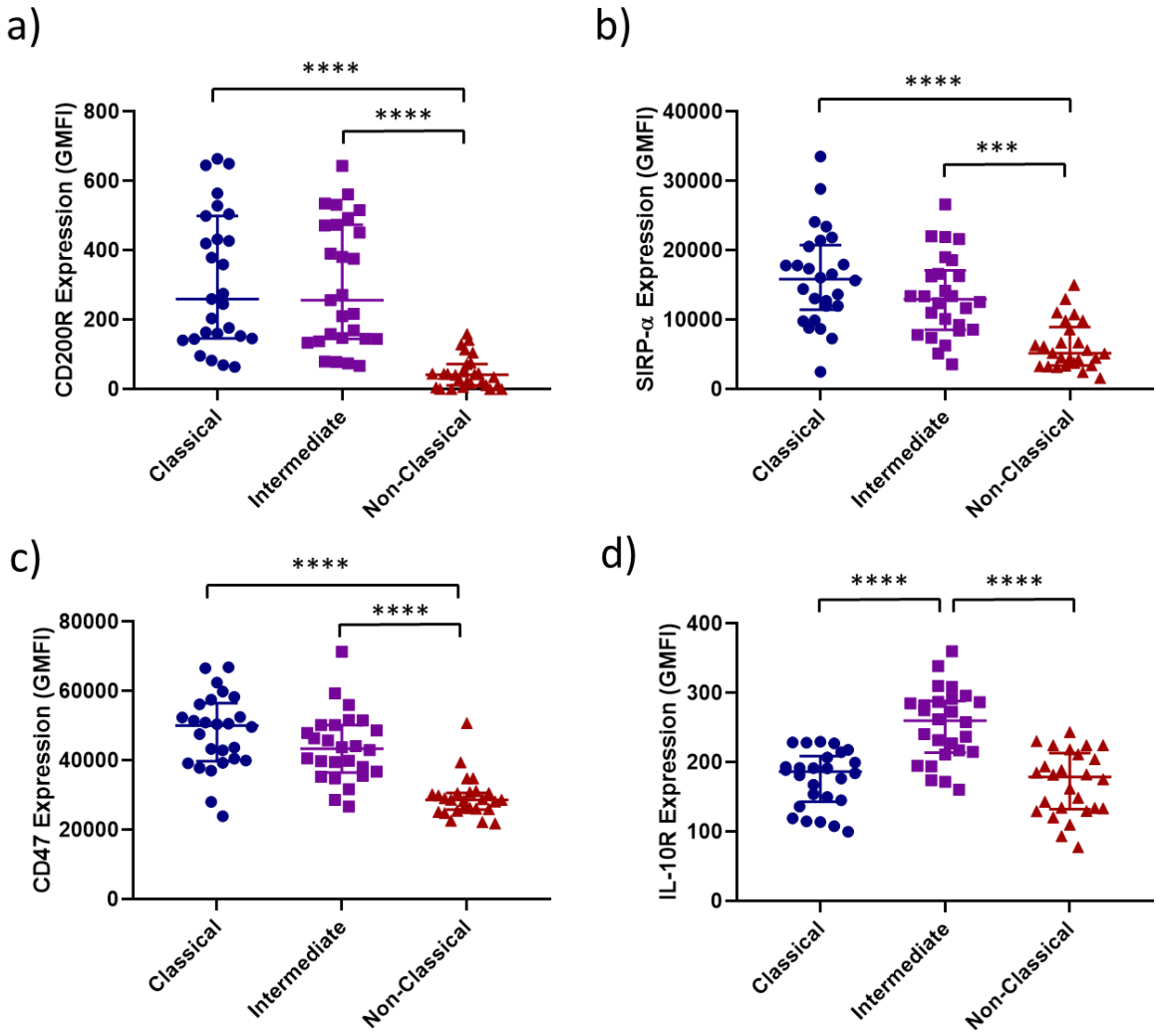

Figure S1. Comparison of regulatory receptor expression on monocyte subsets in patients with sarcoidosis.

Expression of a) CD200R, b) SIRP-α, c) CD47, and d) IL-10 receptor on classical (blue), intermediate (purple), and non-classical (red) monocytes. Receptor expression is presented as the geometric mean fluorescence intensity (GMFI). Individual data points are shown with medians (horizontal lines) with interquartile ranges. **** = $p < 0.0001$, statistics performed using the Kruskal-Wallis test with correction using the Dunn's multiple comparisons test.

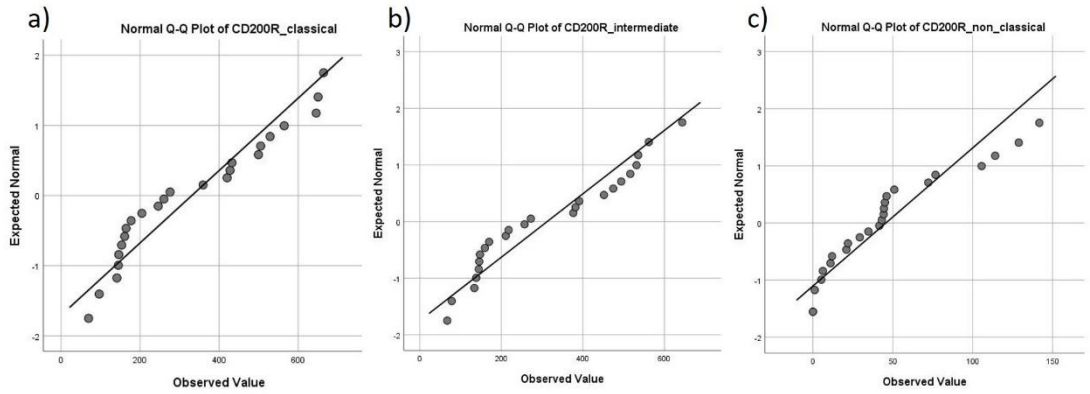

Figure S2. Normal quantile-quantile (Q-Q) plots for CD200R expression on a) classical, b) intermediate, and c) non-classical monocyte subsets from sarcoidosis patients at baseline. The Q-Q plots are consistent of bimodal distributions (with normally distributed data, the points would fall on the straight lines).