Flow cytometry protocol and gating strategies

For immune cell isolation, fresh EDTA blood was diluted 1:1 with saline, subjected to a Ficoll-Hystopaque gradient (Ficoll-Paque PLUS™, GE Healthcare, Menlo Park, NJ, USA) and centrifuged at room temperature for 35 minutes at 1500 rpm. The plasma was removed and the two clouds were used: the first consisting of peripheral blood mononuclear cells (PMBC) and the second band enriched by polymorphonuclear (PMN) cells, characteristically neutrophils (approximately 94% purity). The immune cell bands were placed in different falcon tubes to be washed with Ca/Mg-free phosphate-buffered saline (PBS). Contaminant red blood cells were removed from the PMN cloud with hypotonic lysis. Pellets were resuspended in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, USA) supplemented with 1% nutridoma (Roche, Indianapolis, IN, USA) and 1% penicillin/streptomycin.

To evaluate the immunophenotype of monocytes, Natural Killer (NK) cells, Low-Density (LD) neutrophils, and High-Density (HD) neutrophils, $1x10^6$ cells/ml of each immune cloud were incubated with 100μl of blocking serum (2% fetal bovine calf sera and 2% fetal goat) for 20 minutes at 4ºC to inhibit non-specific binding through FCγRs. Then, cells were stained with monoclonal antibodies-containing panels for 20 minutes at 4ºC. The monoclonal antibodies used in this protocol are listed below in Table 1.

Table 1. Description of the antibodies used in flow cytometry experiments.

Antibody	Fluorochrome	Clone	Provider	Catalog number/REF	Dilution factor	Immune Cell
CD14	PerCP-Cy 5.5	M5E2	BD	561116	1:200	Monocytes
CD80	FITC	2D10.4	Invitrogen	11080942		Monocytes
CD163	PE-Cyanine7	EBioGHI/61	Invitrogen	25163942	1:80	Monocytes
CD274	PE	MIH1	BD	557924	1:30	Monocytes
HLA-DR	Alexa Fluor 700	LN3	Invitrogen	56995642	1:80	Monocytes and both LD and HD neutrophils
CD3	PE	HIT3a	BD	555340	1:40	Natural Killer
CD11c	PerCP-Cy 5.5	B-ly6	BD	565227	1:30	Natural Killer and HD neutrophils
CD16	FITC		BD	555406	1:30	Natural Killer
CD56	PE-Cy7	B159	BD	560916	1:100	Natural Killer
CD11b	APC	ICRF44	BD	4330081	1:150	HD neutrophils
CD182	PerCP-eFluor 710	Ebio5EB-C7-F10	Invitrogen	46182942	1:100	HD neutrophils
TREM-1	PE	TREM-26	BioLegend	314906	1:200	HD neutrophils
CD279	FITC	MIH4	BD	557860	1:40	HD neutrophils

After antibody incubation and fixation with formaldehyde, samples were acquired in Attune™ NxT Flow Cytometer software version 2.4 (Life Technologies, CA, USA) considering a minimum of 30,000 events. Data were analyzed using FlowJo software version V10 (Treestar Inc, Ashland, USA). For multiparameter cytometry analysis, singlets were selected for live cells and, then, each immune cell (monocytes, lymphocytes, LD, and HD neutrophils) was gated according to size (Forward Scatter; FSC-A) and granularity (Side Scatter area; SSC-A) parameters. Separately, into the gate of each immune cell targeted, were selected CD14+ cells for monocytes, CD3- cells for NK, CD11b+ for LD neutrophils, and CD11b+ and CD11c+ cells for HD neutrophils from different panels. Figure 1 shows the gating strategy was performed as previously described[1]. Finally, into the gate of CD14+ monocytes, the expression of CD80, CD163, CD274, and HLA-DR were analyzed. Into the gate of CD3- NK cells, the expression of CD11c, CD16, and CD56 was analyzed. Into the gate of CD11b+ LD neutrophils, the expression of CD14, CD274, and HLA-DR was analyzed. Into the gate of CD11b+ HD neutrophils, the expression of CD16, CD182, and TREM-1 was analyzed. Into the gate of CD11c+ HD neutrophils, the expression of CD274, CD279, and HLA-DR was analyzed. Statistic values were used to report the relative frequency of each immune cell population and fluorescence intensity values are reported as MFI, which were determined by FlowJo according to intensity values of events gated from live cells.

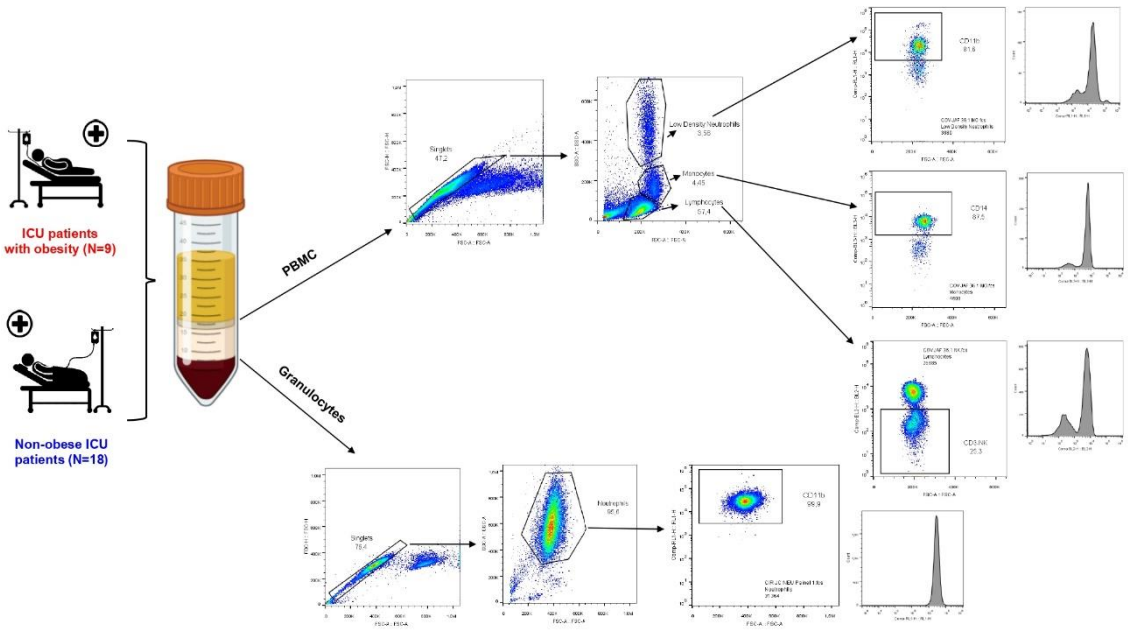

Figure 1. Gating strategy.

References

1 Staats J, Divekar A, McCoy JP, Maecker HT. Guidelines for Gating Flow Cytometry Data for Immunological Assays. 2019, pp 81–104.