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Title	CyBorD-DARA is potent initial induction for MM and enhances ADCP: Initial results of the 16-BCNI-001/CTRIAL-IE 16-02 study
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Supplementary Methods for Translational Studies

Blood and Bone Marrow Samples Collection, processing and total cell counts

Freshly drawn peripheral blood (PB) samples and bone marrow (BM) samples were collected in 10ml ethylene diamine tetraacetic acid (EDTA) Vacutainer® tubes (BD Medical Supplies, Crawley, UK). Blood and bone marrow sampling was performed for each participant at study enrolment, and 24 hours after treatment with cyclophosphamide. For enumeration of the major subtypes of PB leukocytes (PBL), 100µl of anti-coagulated blood collected in EDTA vacutainers were incubated with anti-human CD45-APC (BD Biosciences, San Jose, CA) and mouse anti-human CD14-PerCP-Cy5.5 (Miltenyi Biotec, Cologne, Germany) as previously described[25]. Red blood cells (RBC) were lysed using RBC lysis buffer (BD Biosciences) according to manufacturer's instructions. The remaining cell were washed and suspended in PBS (Ca^{2+} and Mg^{2+} free) (Gibco-Life Technologies, Carlsbad, CA). Total cell number was determined using the BD Accuri C6 CFlow Sampler Software (BD Biosciences), by timed acquisition of events per unit volume. The absolute numbers of circulating neutrophils, monocytes, and lymphocytes were calculated and expressed as cells per ml of whole blood from the proportion of total cells recorded within gates based on forward and side scatter profiles, in addition to cell surface staining profiles.

Peripheral Blood and Bone Marrow Mononuclear Cell Isolation and Multi-Colour Flow Cytometry Analysis

Isolation of mononuclear cells (MC) from PB and BM aspirates were performed by layering 3mL EDTA-anti-coagulated sample over 3mL endotoxin-free Ficoll Plaque density-gradient medium (GE Healthcare, Little Chalfont, United Kingdom) in 15-mL Falcon tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged at 420 relative centrifugal force (RCF) for 22 minutes at 4˚C without brake. The visible "buffy coat" layer of mononuclear cells was removed using a plastic Pasteur pipette (Sarstedt). The PBMC were transferred to a new 15ml falcon tube (Sarsedt) and washed twice with 10ml Fluorescence-activated cell sorting (FACS buffer) [2% foetal calf serum (Lonza, Basel, Switzerland), PBS and 0.05% NaN3 (Sigma-Aldrich, St. Louis, MO, USA)]. The mononuclear cells were then pelleted by centrifugation at 300 RCF for 10 minutes at 4°C with full acceleration and brake. The supernatant was discarded and the pellet was suspended in 1ml FACS Buffer. Cells were counted using

a haemocytometer and $5x10^5$ cells per sample were aliquoted and stained for phenotypic characterisation by flow cytometry.

The mononuclear cell aliquots were incubated with optimised concentrations of fluorochrome-conjugated antibodies (mouse anti-human CD45- V500, CD16- FITC, CD14-PerCP, CX3CR1-Pe-Cy7, HLA-DR-APC, CD33-Vio770, CD56-V450, CD32-PE, CD64-APC, PD-1-PE, SIRP-α – APC, CCR2-APC, CD47-FITC, CD3, PE, CD4 – PerCP-APC, CD8- APC) for 20 min at 4°C to allow identification of plasma cells, lymphocytes, neutrophils, monocytes/macrophages, as well as lymphocytes and monocyte/macrophage subsets. All antibodies were purchased from BD Biosciences unless otherwise stated. Each aliquot of mononuclear cells was washed with 1ml FACS buffer, centrifuged at 300 RCF for 5mins at 4°C and finally resuspended in 200µl FACS buffer. Flow cytometric analysis was performed using a BD FACS Canto II flow cytometer (BD Biosciences) which was calibrated according to manufacturer's recommendations. Fluorescence compensation was set using single-stained controls, and matching median compensation algorithms were applied. Fluorescence minus one controls were used to set analysis gates controls. The mean fluorescent intensity (MFI) was calculated by expressing the fluorescent intensity of each sample relative to the cell size. Data were analyzed using Diva v8.0.1 acquisition software (BD Biosciences) or FlowJo® 7.6.5 software (TreeStar Inc., Olten, Switzerland).

Serum Preparation

At baseline and 24 after treatment, peripheral blood samples from participants from the CyBorD-Dara clinical trial collected into Serum Separator Tubes were allowed to coagulate for 2 hours at room temperature (RT) in a horizontal position. The tubes were then centrifuged at 800 RCF for 15 minutes at RT with full acceleration and brake setting 2. The serum was isolated and stored at -80° C for analysis.

Cytokine quantification by multiplex immunoassay

For quantification of inflammatory cytokines in peripheral blood serum samples, a human high sensitivity Panel A Magnetic Luminex kit (cat number: glbMQXfw) and a human human high performance Panel A Magnetic Luminex kit (cat number: rTdpz7A6) was used respectively (R&D Systems, MN, US). This product consists of magnetic microsphere technology enabling detection and quantification of multiple cytokines present in a single sample and was carried out in accordance with the

manufacturer's recommended protocol Prostaglandin E2 was quantified using a competitive enzyme immunoassay (Biotechne, Catalog # KGE004B). Assay was performed according to the manufactures instructions. Serum samples were used undiluted in all of the assays.

Statistical analysis

Statistical analysis was carried out using GraphPad® Prism Version 6 (GraphPad Software, CA, USA) and Microsoft® Excel 2010 (Microsoft Corporation, Washington, USA). To detect differences across groups, Wilcoxon matched pairs signed rank test and paired two-tailed t-tests was used. Statistical significance was considered at $p < 0.05$.

Supplementary Figure Legends

Supplementary Figure 1. Gating strategies for the identification of lymphocyte

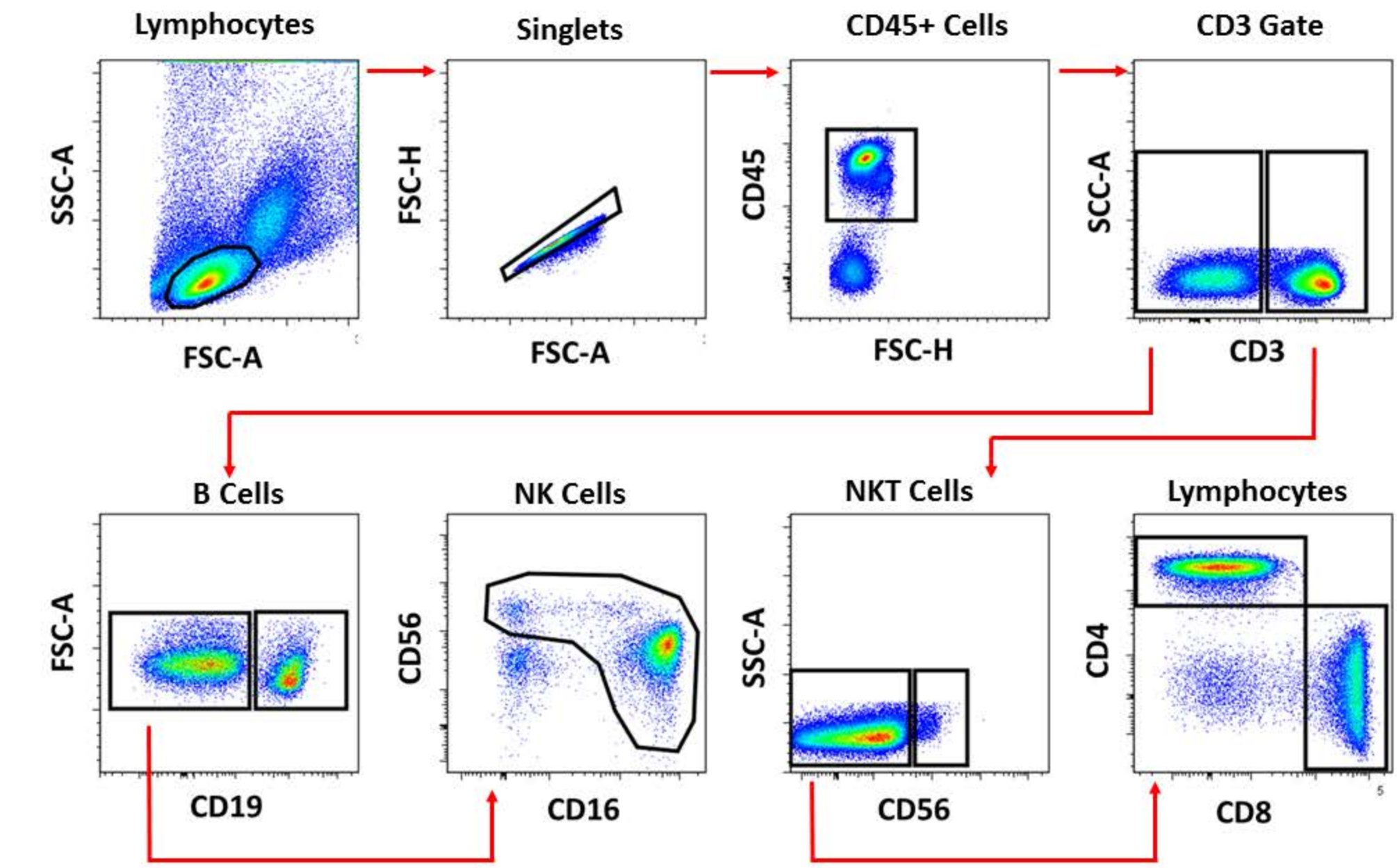
Representative dot plots are shown to illustrate an optimized gating strategy for the identification of lymphocytes in peripheral blood and bone marrow samples. **A**. B cells, NK cells, NKT cells and lymphocytes were identified by staining with CD19, CD56, CD16, CD4 and CD8 antibodies.

Supplementary Figure 2. Multi-color flow cytometry for the identification and expression of SIRP-α on peripheral blood and bone marrow monocyte subsets.

A. Representative dot plots are shown to illustrate the gating strategy for the identification of total monocytes. Monocytes were identified by CD33+CX3CR+CD56-CD14+ expression. **B**. Representative histogram of SIRP-α expression on CD33+CX3CR+CD56-CD14+ peripheral blood monocytes/macrophages from a Multiple Myeloma patient pre- and post- treatment. Black histogram is the fluorescence minus one control (FMO), The grey filled histogram identifies the pre-treatment peripheral blood sample and the dotted line represents the post treatment peripheral blood sample (Left). Dot plots indicating the mean fluorescence intensity of SIRP-α expression pre and post treatment on CD33+CX3CR+CD56-CD14+ peripheral blood monocytes/macrophages (n=6) (Fig 3B, Right). **C**. Representative histogram of SIRP-α expression on CD33+CX3CR+CD56-CD14+ bone marrow

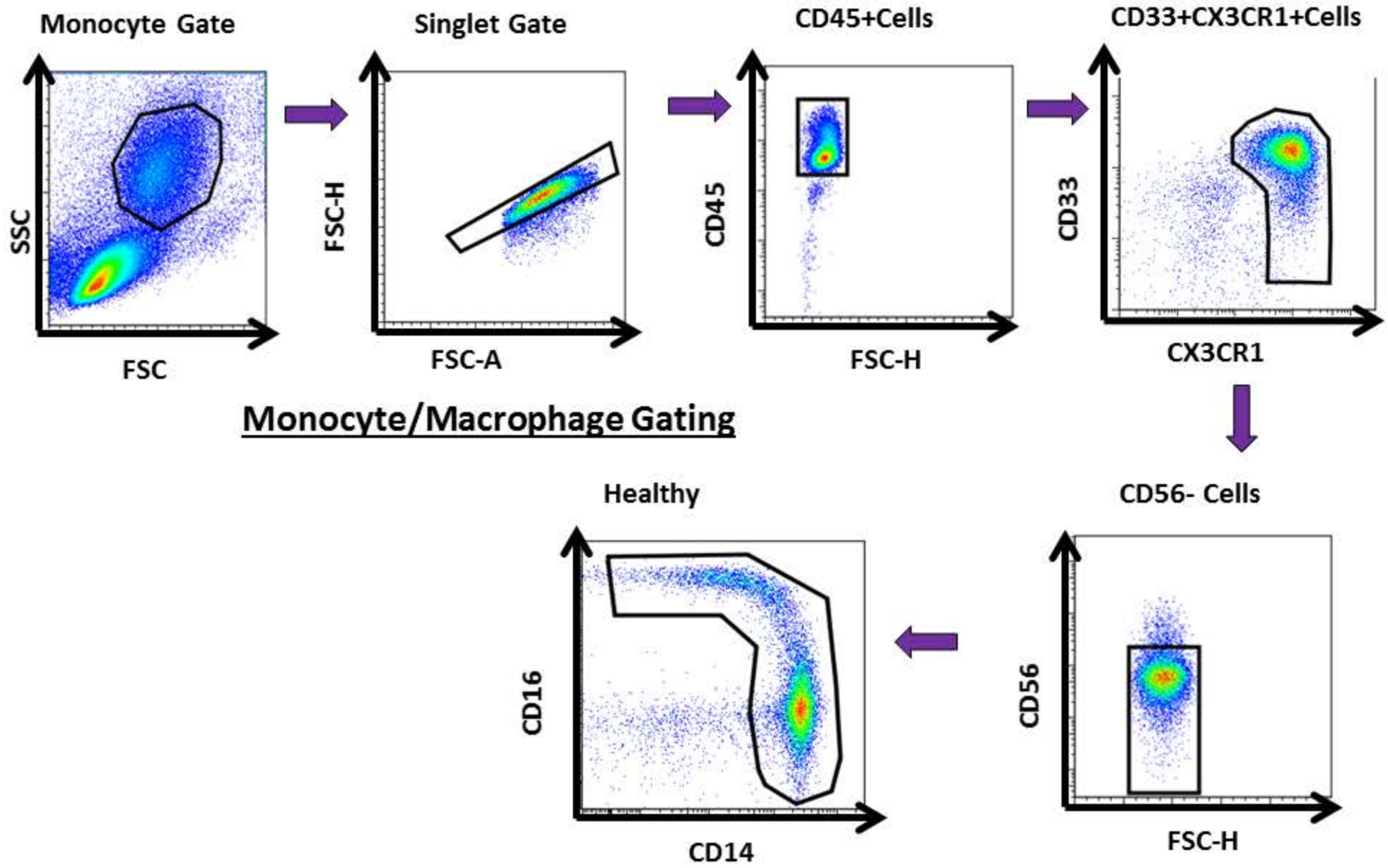
monocytes/macrophages from a Multiple Myeloma patient pre- and post- treatment. Black histogram is the fluorescence minus one control (FMO), The grey filled histogram identifies the pretreatment bone marrow sample and the dotted line represents the post treatment bone marrow sample (Left). Dot plots indicating the mean fluorescence intensity of SIRP-α expression pre and post treatment on CD33+CX3CR+CD56-CD14+ monocytes/macrophages (n=6) (Right).Lines between dots indicate paired samples. Wilcoxon matched pairs signed rank test and paired t tests were used to detect statistically significant differences between pre and post treatment samples; ns (not significant)

Lymphocyte Gating Strategy



Supplementary Figure 1

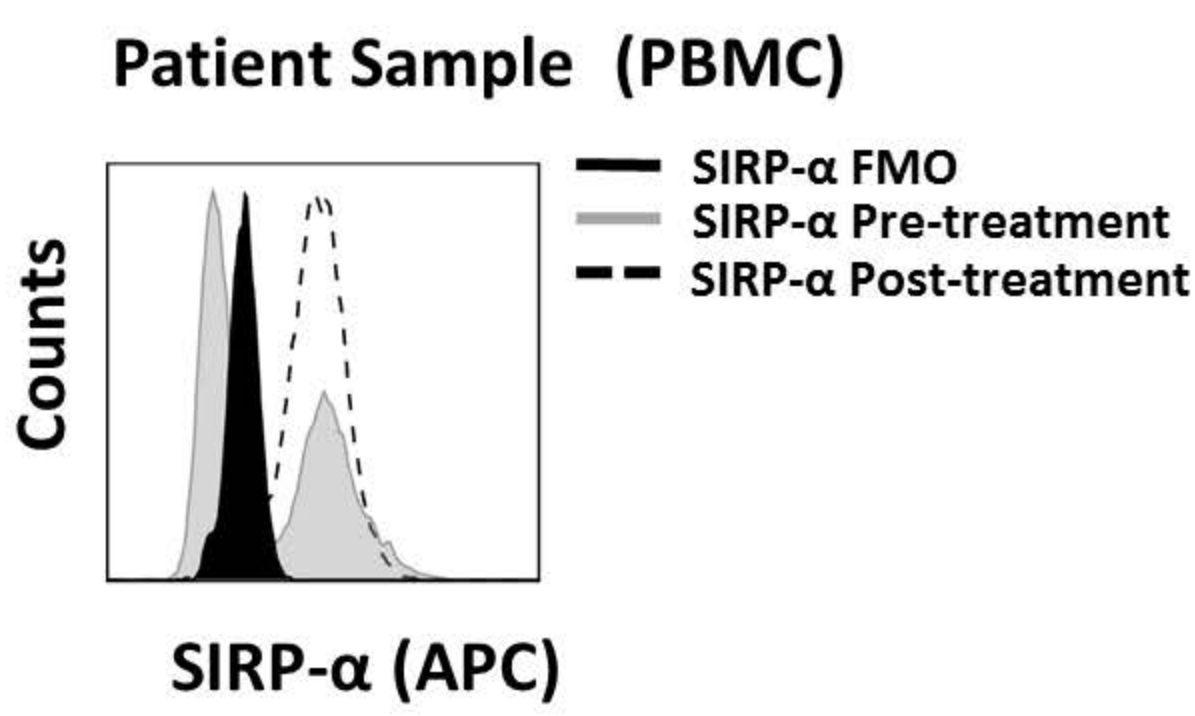
A



Monocyte/Macrophage Gating

Gated on: CD33^{+} CX3CR^{+}CD56^{-} CD14^{+} Macrophages

B



Supplementary Figure 2