Analysis method for the analysis of the leukocyte subset distribution in peripheral blood

Appendix A

The participant's blood sample 1 and 2, which are collected before and after intravenous treatment with albumin or immunoglobulin, respectively, are analysed in the exact same manner.
First, a total leucocyte count (TLC) will be measured using the Sysmex XN-1000 blood analyser. The Sysmex provides absolute count for white blood cells (WBC), lymphocytes, monocytes, and neutrophil granulocytes.

The EDTA blood sample collected from the participant will be prepared for flow cytometric analysis using two different protocols. These flow cytometer analyses will be performed throughout the study and the analyses are initiated within 1 hour from blood samples were collected. The gating will be checked in the end of the study on all analyses.

The first flow cytometric protocol will provide the concentration of T helper cells (Th), cytotoxic T cells (Tc), B cells, and natural killer (NK) cells, and the fraction of each subgroup of the total lymphocyte population using two different antibody panels. These two panels contain 10 µL of fluorochrome-conjugated-antibodies against CD3, CD4, CD8, and CD45 (BD Bioscience) (tube 1) or CD3, CD16, CD56, and CD45 (BD Bioscience) and CD19 (BD Bioscience) (tube 2) which will be added to FACS tube 1 and 2, respectively, followed by 50 µL EDTA blood from the participant. Then the tubes will incubate in the dark in room temperature 15 minutes before a lysing solution (BD Biosciences) will be added to the tubes removing the red blood cells (RBC). The samples are run through the Novocyte 3000 flow cytometer (Acea Biosciences, Inc., San Diego, USA).
The collected data includes all WBC. Thus, to analyse only the lymphocytes, the $CD45^{+}SSC^{low}$ cells will be gated. In the tube 1, the $CD3^{+}$ T cells will further be divided into $CD3^{+}CD8^{+}$ Tc-cells and $CD3^{+}CD4^{+}$ Th-cells. In the tube 2, $CD3^{-}CD56^{+}CD16^{+}$ NK cells and $CD3^{-}CD19^{+}$ B cells are gated.

The second protocol will provide the percentage of Th1-, Th2-, Th17-cells, T regulatory (Treg) cells, and $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells of the total peripheral blood mononuclear cell (PBMC) population using two different antibody panels.
The first panel includes fluorochrome-conjugated-antibodies against CD3, CD4, CD16, CD25, CD56, and CD127 (all from BD Bioscience). These markers can differentiate $CD56^{dim}CD16^{+}$ NK cells from $CD56^{bright}CD16^{-}$ NK cells. This antibody panel have CD16 and CD56 conjugated to different fluorochromes to be able to distinguish the NK subgroups, while the panel used in tube 2 in the first flow cytometric protocol contained anti-CD16 and anti-CD56 antibodies conjugated to the same fluorochrome to provide the total NK cell count. Anti-CD25 and anti-CD127 antibodies will be used to gate Treg cells ($CD25^{+}CD127^{low}$).
The second panel will differentiate Th1, Th2 and Th17 cells. These groups will be distinguished using the CCR4 (Biolegend), CCR6(BD Bioscience), CCR10(BD Bioscience) and CXCR3(BD Bioscience) chemokine receptors in combination with the anti-CD3 and anti-CD4 antibodies. This analysis will provide the percentage of each Th-cell subgroup.
All antibody concentrations will be optimized for this second protocol before the antibodies will be included in this project. The optimization is lot number specific, which means different dilution factors are

used throughout the project. Of the optimized antibody concentration, we will use 5µL of all antibodies except CD25, of which we will use 20 µL.

300 µL EDTA blood will be added to each FACS tube labelled full panel 1, full panel 2 or necessary controls for each panel. The blood will be lysed and washed to remove RBCs. Then each antibody panel will be added to different tubes and the samples will incubate in the dark in room temperature 15 minutes followed by two washes. The samples will be resuspended in 300µL wash buffer containing phosphate buffered saline (PBS) (VWR Chemicals) + 1% FCS (Gibco) before running the samples on the flow cytometer within 6 hours after the blood sample was taken.

The remaining of the blood sample will be frozen and saved for future analysis. This research biobank for future research will consist of three 6 ml collection tubes containing serum, EDTA plasma, and citrate plasma, respectively. After immediate centrifugation, the remaining serum and plasma will be stored in tubes at -80^{o}C.

Analysis methods for analysing stimulus-specific induced immune responses in peripheral blood ex vivo

Appendix B

TruCulture® is a whole blood assay with directly ex vivo human immune stimulation which permits evaluation of individual and stimulus-specific induced immune responses through measurements of released cytokines as a proxy for immune function in vivo against a range of stimuli. TruCulture tubes (Myriad RBM, Austin, TX, USA) are pre-loaded with cell culture media and immune stimulants. In this trial, the tubes are pre-loaded with one of the following immune stimuli: CD3/CD28, R848, lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (polyIC), and a no-stimulus null control.[1]

Peripheral blood is drawn by vein puncture and collected into a Lithium-Heparin tube. The analysis is initiated in the laboratory within 1 hour after sampling. 1 ml is transferred to each TruCulture® tubes and incubated in a dry heat block for 22 (+/- 10 min) hours at 37 °C. Following the 22 hour long immune stimulation, supernatants are collected by inserting a valve separator to separate cells from the culture supernatant as described previously described.[2]

Cytokines will be quantified in TruCulture® tube supernatants using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel II (MilliporeSigma, Burlington, Massachusetts) on the Luminex 200 platform following manufacturer protocols.

Ten cytokines will be measured: IFN-α2, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-12 (p40), IL-13, IL-17, and Monocyte Chemoattractant Protein-1 (MCP-1). The limit of detection (LoD) will be 6,56 pg/ml for IFN-α2, 0,86 pg/ml for IFN-γ, 5.39 pg/ml for TNF-α, 0,28 pg/ml for IL-2, 0.20 pg/ml for IL-4, 0.14 pg/ml for IL-6, 3.24 pg/ml for IL-12 (p40), 2.58 pg/ml for IL-13, and 3.05 pg/ml for MCP-1.

Reference list

1. Duffy D, Rouilly V, Libri V et al. Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. *Immunity.* 2014;40:436–450.

2. Urrutia A, Duffy D, Rouilly V et al. Standardized whole-blood transcriptional profiling enables the deconvolution of complex induced immune responses. *Cell Rep.* 2016;16:2777–2791.