Material and methods

Flow cytometric analysis of mHLA-DR expression

We used two methods to determine mHLA-DR expression. First, we determined Mean Fluorescence Intensities as described earlier [1]. Briefly, EDTA anticoagulated blood was stored at 4°C immediately after withdrawal and analyzed by flow cytometry. After withdrawal, 100 μl blood was incubated with the following fluorochrome-conjugated monoclonal antibodies, for 15 minutes protected from light at 4°C. After erythrocyte lysis (NH₄CL: 180 mL + 20 mL lysis stock dilution [BD Pharm-Lyse, BectonDickinson]), cells were washed three times in PBS and monocytes and lymphocytes were identified in an 8-color immunophenotyping (NAVIOS flow cytometer, Beckman Coulter, Miami). Monocytes and lymphocytes were identified by forward and side scatter and by cell-specific binding. The following monoclonal antibodies were used for monocyte HLA-DR analysis: HLA-DR-PE (Immu-357), CD14-ECD (RMO52), CD45-KO (J33). Lymphocyte subpopulations were identified by gating on the lymphocyte population in the CD45/SS plot followed by a gating on CD3-APC (UCHT1), CD4-PECy5.5 (13B8.2) , CD8-APCAlexa700 (B9.11), CD19-APCAlexa750 (HD37) and CD56-PECy7 (N901) to determine the helper T cells, cytotoxic T cells, B cells and NK cells within the lymphocyte gate (all MoAbs were obtained from Beckman Coulter, Marseille, France). mHLA-DR expression was determined by calculating HLA-DR mean fluorescence intensity (MFI) within CD14-positive cells (Figure A, Supplemental Digital Content 3, http://links.lww.com/INF/C279)

Second, we determined which percentage of CD14-positive monocytes was HLA-DR positive, using HLA-DR positive B-cells as an internal positive control (Figure B, Supplemental Digital Content 3, http://links.lww.com/INF/C279).
Cytokine assays

*Ex-vivo* cytokine production was determined as described earlier [2]. In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640+ (RPMI-1640 Dutch modification supplemented with 10μg/mL gentamicin, 10mM L-glutamine, and 10mM pyruvate) (Gibco, Invitrogen, Breda, The Netherlands). The PBMCs were counted using a particle counter (Beckmann Coulter, Woerden, the Netherlands) and were plated in 96 well round-bottom plates (Corning, NY, USA) at a final concentration of 2,5x10⁶/mL, in a total volume of 200 μL. The PBMCs were stimulated for 24 hours, 48 hours, and 7 days with medium alone, or medium containing *E. coli* lipopolysaccharide (LPS; 10 ng/mL), phytohaemaglutinin (PHA; 10μg/ml), heat-inactivated *Candida albicans* yeast (1x10⁶/ml) or heat-inactivated *Candida albicans* hyphae (derived from 1x10⁶/m yeast). After stimulation, cell culture supernatant was collected and stored at -20°C. When all samples were collected, cytokines were measured using commercially available ELISAs (R&D Systems, MN, USA and Sanquin, Amsterdam, The Netherlands) according to the protocols supplied by the manufacturer. Ex-vivo production of cytokines was assessed at time points at which their production has been shown to peak [3]. Monocyte derived cytokines such as tumour necrosis factor (TNF)-α were measured in culture supernatants of 24 hour cultures, IFN-γ was measured in culture supernatants of 48 hour cultures. T-cell derived cytokine IL-17 was measured in culture supernatants of 7 day cultures.
References

