ARTICLE - Coagulation & its Disorders

Complement protein C3a enhances adaptive immune responses towards FVIII products

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Supplementary methods

Isolation and differentiation of cells

Buffy coats or freshly drawn venous citrate-blood of healthy adult volunteers were used for isolation of peripheral blood mononuclear cells (PBMC) by Ficoll density gradient centrifugation. The age of donors ranged from 18 to 65 years; male and female donors were included. Monocytes were purified from freshly isolated PBMC, autologous T cells either from thawed PBMC (buffy coats) or from thawed $CD14^-$ PBMC (fresh blood) as previously described (Miller *et al.* 2018) using CD14 MicroBeads, Pan T cell Isolation kits, or CD4 MicroBeads (all from Miltenyi Biotec, Bergisch Gladbach, Germany), respectively.

Differentiation of monocyte-derived DC was performed as previously described. Briefly, purified monocytes were cultured for 5 days in 24-well plates (1×10^6 cells in 1 mL) using either serum-free X-Vivo 15 white supplemented with 1% penicillin/streptomycin or red medium (both Lonza, Verviers, Belgium) in the presence of GM-CSF and IL-4 (1,000 IU/mL each; CellGenix, Freiburg, Germany).

Stimulation of DC

In vitro generated DC were harvested and detached with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 1 mM EDTA, resuspended (5×10^5/mL) in serum-free X-Vivo 15 medium, and serially diluted in 96-well round-bottom plates resulting in 5×10^4, 2.5×10^4, 1.25×10^4, and 0.6×10^4 DC in 100 µL per 96-well. DC were treated for 24 hours at 37°C with 1 IU/mL pdFVIII or rFVIII, 0.1 µg/mL LPS (*Salmonella abortus equi*, Sigma-Aldrich, St. Louis, USA), or 2.5 µL/mL foreign, autologous, or FVIII-deficient plasma.

Plasma

Plasma used in this study was prepared from ten buffy coats (DRK Blutspendedienst, Frankfurt am Main, Germany) by collecting and pooling the plasma following Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation. For autologous plasma, individual plasma of each donor was collected. FVIII-deficient plasma was purchased from Siemens Healthcare Diagnostics GmbH (Marburg, Germany). Heat-treatment of plasma was performed for 1 hour at 56°C prior to use. If not stated otherwise, within all experiments, DC were treated with 2.5 µL/mL of the indicated plasma. Plasma was stored at -80°C. To avoid repeated freeze-thaw cycles, plasma and FVIII aliquots were used only once.

HLA typing

Genomic DNA was extracted from citrate-blood using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. In order to identify HLA-DR1*11 positive donors, the DR low resolution typing kit from Olerup (Vienna, Austria) was used following the manufacturer's protocol with Taq DNA polymerase from Qiagen. Data were analyzed with Helmberg-Score software version 5.00.80.02 T (Olerup).

Statistical analysis and calculations

Statistical analyses were performed using GraphPad Prism software version 9.2.0 (San Diego, USA). Paired comparisons were performed applying the Wilcoxon's matched-pairs signed-rank test. Significance was defined as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Within experiments done with FVIII-tetramers, the numbers of events measured by flow cytometry varied throughout the different stimulations. To enable comparisons between donors, the percentages of FVIII-specific proliferated T cells were calculated in events using the following formula:

FVIII-specific proliferated T cells [events] = (FVIII-specific proliferated T cells [%] x proliferated T cells [events]) / 100 [%].