**Marked enhancement of the immunogenicity of Ig-Fc fusion proteins by inclusion of cholera toxin non-toxic B subunit within the single polypeptide**

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*moDC differentiation and activation*

CD14+ monocytes were enriched from PBMCs using MojoSortTM Human Pan Monocyte isolation kit (BioLegend) according to the manufacturer’s recommendations. Monocyte isolation yielded >90 % purity and >95 % viability of CD14+ monocytes (data not shown). Right after isolation, enriched monocyte fractions were plated in 24-well tissue culture plates (Corning Inc.) at a density of 1x106 cells per well. Complete RPMI 1640 media supplemented with 70 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech) and 50 ng/mL interleukin-4 (IL-4) (BioLegend) was used for 7 days to induce differentiation of monocytes to monocyte-derived dendritic cells (moDCs). Fresh differentiation media was added to wells every 2 days. At day 7, cells were detached from wells using an enzyme-free dissociation solution (Merck) and replated at a density of 5x104 cells/well in 96-well U bottom tissue culture plates (Corning Inc.) in complete RPMI-1640. Cells were subsequently treated with either lipopolysaccharide (LPS) (100 ng/mL) (Sigma), human IgG (Sigma, 10 µg/mL), unfractionated, HMw or LMw D-PCF (10 µg/mL). At day 9, cells were harvested to measure activation-induced cell-surface markers (MHC-I/HLA-ABC, MHC-II/HLA-DR, CD80, CD86 and PD-L1) by flow cytometry. Briefly, wells were washed with DPBS (Sigma) and then incubated for 45 minutes at 4°C with 1:500 eBioscienceTM fixable viability dye eFluor780TM (Invitrogen), 1:250 Human TruStain FcXTM (BioLegend) 1:200 APC anti-human HLA-DR (BioLegend), 1:200 PE anti-human HLA-A,B,C (BioLegend), 1:200 PE/Cyanine 7 anti-human CD86 (BioLegend), 1:200 Brilliant Violet 510TM anti-human CD80 (BioLegend), and 1:200 Brilliant Violet 42TM anti-human PD-L1 (BioLegend) in DPBS. After incubation, cells were washed and resuspended in DPBS. Cell acquisition was done using CytoFLEX S (Beckman Coulter) flow cytometer. FlowJoTM v10.8.1 (TreeStar) was used for analysis of FACS data.

*THP-1 and moDC internalisation assays*

THP-1 or moDCs were plated in 96-well U bottom plates at a density of 5x104 cells/well. Cells were than treated with either D-PCF (10µg/mL) or D-PIGS (10µg/mL) and incubated for 12 h in a humidified incubator set to 37°C and 5% CO2. Afterwards, cells were washed with PBS and stained for 20 min with 1:500 eBioscienceTM fixable viability dye eFluor780TM (Invitrogen, 65-0865-14). Cells were subsequently fixed and permeabilised using the IC fixation buffer (Invitrogen, 00-8222-49) for 15 minutes, followed by intracellular staining with 1:100 FITC anti-human IgG Fc (Biolegend®, Clone M1310G05, 410720) for 45 min. Stained cells were acquired using CytoFLEX S flow cytometer (Beckman-Coulter) and analysed using FlowJoTM v10.8.1 (BD Life Sciences, Ashland, OR, USA). Data were expressed as mean fluorescence intensity (MFI). For imaging experiments, cells were seeded at 1x105 cells/well in 96 black well plates (Corning Inc., 3603). Cells were left to attach for 2 h, then treated with either monomeric IgG (10µg/mL), D-PCF (10µg/mL) or D-PIGS (10µg/mL) for 12 h. Wells were washed with PBS, followed by fixation and permeabilization using an IC fixation buffer (Invitrogen, 00-8222-49). Afterwards, cells were stained with 1:100 FITC anti-human IgG Fc (Biolegend®, Clone M1310G05, 410720) and 1:2000 Phalloidin-iFluor 647 (abcam, ab176579) for 45 minutes. Cells were then stained with 1:1000 DAPI (Thermo Scientific, 62248) for 5 minutes. Confocal image acquisition was done using Nikon A1R Microscope and Nikon NIS-Elements C software.

*Human alveolar epithelial cells binding and uptake of D-PCF*

Primary human alveolar epithelial cells (hAECs) obtained from Generon were maintained in Endothelial cell growth medium-2 (Promocell) at 37°C in a 5% CO2 humidified atmosphere. hAECs were seeded at a density of 50,000 cells per well in a 96-well tissue culture plate. Cells were then treated with either human IgG or D-PCF overnight. The following day, cells were harvested for confocal imaging and flow cytometry analysis.

For confocal imaging, hAECs were washed twice with PBS and fixed with Fix/Perm buffer (ThermoFisher Scientific) according to the manufacturer’s instructions. Cells were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes and stained with phalloidin, DAPI, and anti-human IgG-FITC (Biolegend). After washing and resuspension in PBS, cells were imaged using a confocal microscope (Nikon A1R).

For flow cytometry analysis, human IgG or D-PCF-treated cells were washed twice with PBS and stained with eFluor870 fixable viability dye (ThermoFisher Scientific) for 20 minutes at 4°C. The cells were then fixed with Fix/Perm buffer for 20 minutes at 4°C, permeabilized with 0.1% Triton X-100 for 10 minutes and stained with anti-human IgG-FITC for 1 hour at 4°C. Flow cytometry acquisition was performed using the Cytoflex (Beckman Coulter) and analysed using FlowJo V10.