Human CD4+ T-cell Isolation, activation, and differentiation

$CD4^+$ T-cells were isolated from human umbilical cord blood as described previously [1-3]. For Th17 cell differentiation, isolated $CD4^+$ cells were activated with a combination of plate-bound anti-CD3 (750 ng/24-well culture plate well; Immunotech/Beckman Coulter REF # IM-1304) and soluble anti-CD28 ((1ug/mL; Immunotech/Beckman coulter REF # IM1376) antibodies in serum-free X-Vivo 20 medium (Lonza), in the absence (Th0) or presence (Th17) of IL-6 (20ng/ml, Roche, Cat# 11138600 001); IL-1β (10ng/ml, R&D Systems Cat # 201 LB); TGF-β1 (10ng/ml, R&D Systems Cat# 240); anti-IL-4 (1 μg/ml) R&D Systems Cat# MAB204) and anti-IFN-γ (1 μg/ml R&D Systems Cat#MAB-285). Differentiation of Th17 cells was confirmed by measuring IL-17 expression by quantitative real-time PCR, at 72 hours of Th17 / Th0 culturing [1].

For iTreg cell culturing, after CD25 depletion, done using LD columns and a CD25 depletion kit (Miltenyi Biotec), $CD4^+CD25^-$ cells were activated with plate-bound anti-CD3 (500 ng/24-well culture plate well) and soluble anti-CD28 (500 ng/mL) at a density of 2×10^6 cells/mL of X-vivo 15 serum-free medium (Lonza). For iTreg differentiation, the medium was supplemented with IL-2 (12 ng/mL), TGF-β (10 ng/mL) (both from R&D Systems), all-trans retinoic acid (ATRA) (10 nM; Sigma-Aldrich), and human serum (10%) and cultured at 37°C in 5% CO_2. Control Th0 cells were stimulated with plate-bound anti-CD3 soluble anti-CD28 antibodies without cytokines. For confirmation of iTreg cell differentiation, we used intracellular staining to measure, at 72 hours of iTreg culturing, expression of FOXP3 which is the major transcription factor driving Treg differentiation. Intracellular staining was performed using buffer sets of Human Regulatory T-cell Staining Kit (eBioscience/Thermo Fisher Scientific), following the manufacturer's protocol. The following antibodies were used: anti-human FOXP3-PE (eBioscience, Cat. No. 12-4776-42) and rat IgG2a isotype control (eBioscience, Cat. No. 72-4321-77A). All samples were acquired by a flow cytometer (LSRII) and analyzed either with FlowJo (FLOWJO, LLC) or with Flowing Software [2].

Th1 and Th2 cell differentiation were done as described previously (*Hawkins RD., et al. Immunity 38, 1271-1284, 2013*). Briefly, purified naive $CD4^+$ T-cells were activated with plate-bound anti-CD3 (500 ng/24-well culture plate well) and 500 ng/ml soluble anti-CD28 and cultured in the absence (Th0) or presence of 2.5 ng/ml IL-12 (R&D Systems) (Th1) or 10 ng/ml IL-4 (R&D Systems) (for Th2). At 48 hours following the activation of the cells, 17 ng/ml IL-2 (R&D Systems) was added to the cultures. Differentiation of Th1 and Th2 cells was confirmed by measuring (using flow cytometry) the expression of T-bet and Gata3 at 72 hours after cell activation. Briefly, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience/Thermo Fisher Scientific), according the manufacturer's protocol. The following antibodies were used: anti-human GATA3-PE (eBioscience, 12-9966), anti-human T-bet-BV711 (BD, 563320) and corresponding isotype controls (BV711 Mouse IgG1, BD, 563044 and PE Rat IgG2b, eBioscience, 12-4031-82). Samples were acquired by BD LSRFortessa™ cell analyzer and data were analyzed using FlowJo software (FLOWJO, LLC).

References

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