Supplementary Materials

An Optimised Method to Differentiate Mouse Follicular Helper T Cells *in Vitro*

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Materials and Methods

In vitro differentiation for mouse Tfh cells

Splenocytes from congenic wild type (WT) mice were left untreated or pre-treated by 1 μg/mL lipopolysaccharide (LPS) (ENZO, ALX-581-014-L002) for 24 hours in the complete RPMI media (10% heat-deactivated FBS (v/v), 100 units/mL penicillin, 100 μg/mL streptomycin, 1mM sodium pyruvate, 1% MEM non-essential amino acids (v/v) and 0.055 mM β-Mercaptoethanol in RPMI 1640 with L-glutamine and 25 mM HEPES). Then 5×10^5 splenocytes were seeded into each well of the 96-well flat-bottom plate. Either untreated or LPS pre-treated splenocytes were then co-cultured with FACS purified naïve OT-II cells at the ratio of 5:1, 50:1 or 500:1 in the presence of 1 μg/mL $OVA_{323-339}$ peptide (Mimotopes, 51023-005), 100 ng/mL IL-6 and 50 ng/mL IL-21 (PeproTech) in the complete RPMI media for 72 hours. No cytokines were added for differentiating Th0 cells. Neutralizing antibodies anti-IL-4, anti-IFN-γ and anti-TGF-β (BioxCell) were used at 10 μg/mL.

T cells and B cells *in vitro* co-culture

In vitro differentiated OT-II cells were FACS purified and treated by 50 μg/mL mitomycin-c (Sigma, M4287-2MG) for 30 minutes before co-culture. 5×10^4 OT-II cells and 5×10^4 LPS pre-treated $B220^+$ cells were seeded into the 96-well U-bottom plate and cultured for 6 days in the presence of 1 μg/mL $OVA_{323-339}$ peptide. On day 6, the phenotype of B cells was analysed by flow cytometry. The culture supernatant was diluted 50 times to analyse the antibody titers by bead-based ELISA (Biolegend, 740493).

Flow cytometric analysis

Surface staining was conducted by incubating the cells with the antibodies under room temperature for 30 minutes. For Bcl6 intranuclear staining, surface staining was performed followed by fix/perm (eBioscience, 00-5523-00) and stained for Bcl6 under room temperature for 45 minutes. For IL-21 staining, cells were stimulated with 50 ng/mL PMA, 1 μg/mL ionomycin and 5 μg/mL brefeldinA for 6 hours, followed by surface staining and were fixed/permed (BD, 554714) and stained for IL-21 under room temperature for 1 hour. CXCR5 was stained by a biotin conjugated antibody, all the other markers were stained by directly conjugated antibodies. Flow cytometry was performed on a FACS analyser (Fortessa X-20, BD) and the data was analyzed by FlowJo (TreeStar).

Quantitative Real-time PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen, 15596026) and RNeasy Mini kit (Qiagen, 74104). cDNA was synthesized (Bioneer, K-2057) and quantitative real-time PCR performed on a 7900HT Fast Real-Time PCR System with SYBR Green PCR Master Mix and primers for Bcl6, CD40L and ICOS. SRP14 was used for normalization. Data in differentiated OT-II T cells was expressed relative Th0 cells in the form of $2^{-\Delta\Delta CT}$.

Antibodies

CXCR5 (L138D7), PD-1 (29F.1A12), CD4 (GK1.5), CD45.1 (A20), CD44 (IM7), CD40L (MR1), ICOS (15F9), Bcl6 (K112-91), IL-21 (FFA21 and mxhal21), B220 (RA3-6B2), CD138 (281-2)

Primers

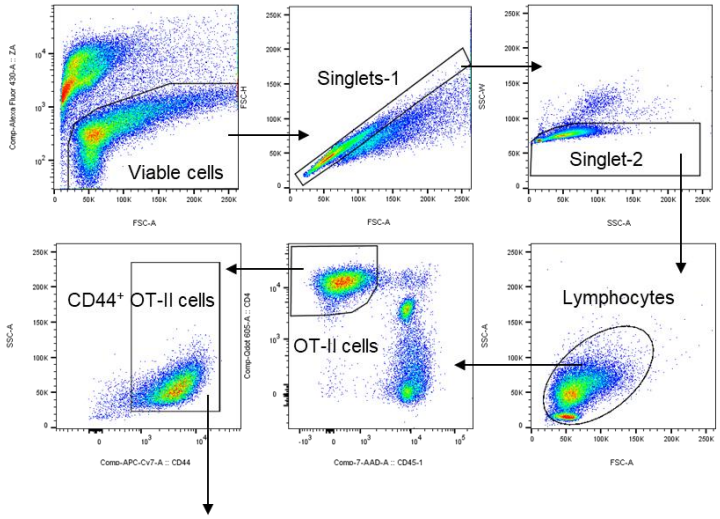
Bcl6: F- CAGAGATGTGCCTCCATACTGC; R- CTCCTCAGAGAAACGGCAGTCA
CD40L: F- GAACTGTGAGCAGATGAGAAGGC; R- TGGCTTCGCTTACAACGTGTGC
ICOS: F- GCAGCTTTCGTTGTGGTACTCC; R- TGTGTTGACTGCCGCCATGAAC

Statistical Analyses

Values between groups were analysed by unpaired student's t-test using Prism software (GraphPad). P values < 0.05 are considered significant.

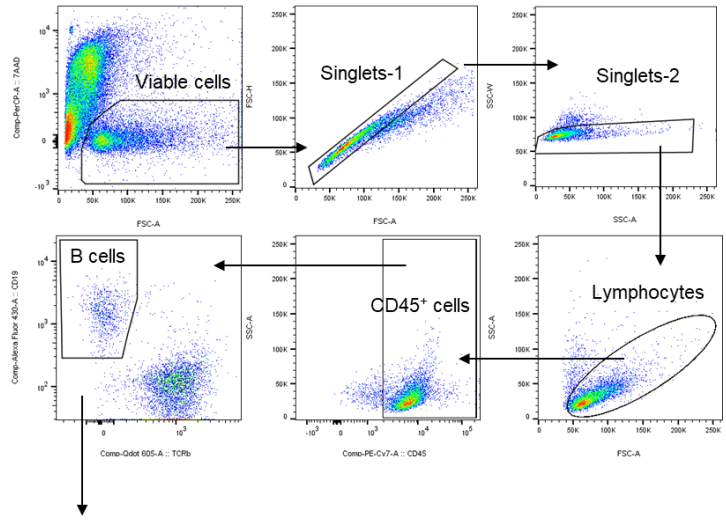
Gating strategies

Gating strategy to identify OT-II cells in *in vitro* differentiation



Proceed to analyse the expression for PD-1, CXCR5, ICOS, CD40L and Bcl6 or IL-21

Gating strategy to identify B cells in T cells and B cells co-culture



Proceed to analyse the expression for B220 and CD138