B cell stimulation assay

Reagents:

Reagent	Vendor	Cat#	Lot#	Stock konc	Final conc	Final dilution
IL4	R&D	204-IL	NCL0617091	20 ug/ml	0.2 ug/ml	1:100
IL21	R&D	8879-IL	ELF0517041	5 ug/ml	50 ng/ml	1:100
IL10	R&D	1064-IL	EYB0514101	20 ug/ml	0.2 ug/ml	1:100
sCD40L	R&D	6245-CL	DAID1116052	50 ug/ml	500 ng/ml	1:100
ODN2006	Miltenyi	130-100-105	5160209235	100 ug/ul	1 ug/ml	1:100

FACS panel:

Channel	Marker	Flourochrome	Stain index	Density	Laser	Filter	Company	Catalogue number	Dilution	Notes
1	IgD	FITC	moderate	+	488	525/40	BD	555778	1	
2					488	575/30				
3					488	620/30				
4	CD38	PerCPcy5.5	bright	-	488	675/20	BD	561106	0.2	
5	CD19	PECy7	bright	++	488	755/LP	Biolegend	302216	0.5	
6	IgG	APC	brightest	+	638	660/20	Miltenyi	130-093-194	2	
7					638	725/20				
8	Viab IR	APCCy7	dim	++	638	755/LP	Invitroger	L10119	1:1000	
9	CD27	BV421	brightest	+	405	450/50	Biolegend	302824	1	
10	CD45	BV510	moderate	++	405	550/40	BD	563204	3	

Cell culture medium:

- RPMI, 1% PEST, 1% L-glutamine
- 10% of heat inactivated FCS

Procedure:

A. Isolate PBMCs from blood by *Ficoll isolation*[1].
B. Collect PBMCs, wash 2X in PBS
C. Stain 1milj cells for *FACS*[2]
D. Prepare probes and controls in dilution plates diluted to 40x final concentration, add to U-bottomed 96-well cell culture plates 5 ul/well , in triplicates.
E. Dilute cells in cell culture medium to 2 x10^6 cells/ml. Seed cells 100ul/well.
F. Preincubate 30 minutes.
G. Prepare cytokines at double final concentration in cell culture medium. Add 100 ul/well

Conditions

1. Unstimulated
2. IL4, IL21, IL10, sCD40L, ODN2006
3. IL4, IL21, IL10, sCD40L, ODN2006+DMSO
4. IL4, IL21, IL10, sCD40L, ODN2006+Probe 1

5. etc

H. On day 6 Screen 1:
   1. Collect supernatants (centrifuge plate, collect 100 ul from each well) for analysis of IgG & IgM content by ELISA[3] in each of the triplicate wells.
   2. Pool cells from the triplicate wells and stain for FACS[2].

I. On day 6 Screen 2:
   1. Collect supernatants (centrifuge plate, collect 100 ul from each well) for analysis of IgG & IgM content by ELISA[3] in each of the triplicate wells.
   2. Treat the remaining supernatants and cells with CellTiter-Glo® Assay[4] to measure viability. No standard, comparison to control.

***ELISA*[3]**

- Human **IgG** ELISA development kit, Mabtech, #3850-1AD-6 (As instructed by protocol from manufacturer). Day 6 supernatants.
- Human **IgM** ELISA development ELISA, in-house ELISA (As instructed by protocol from manufacturer). Day 6 supernatants.

***Ficoll-separation of PBMC from whole blood*[1]**

Reagents: Ficoll-Paque PLUS GE Healthcare, #17-1440-03

1. Dilute blood with equal volume sterile PBS
2. Add <35ml of the blood:PBS in a 50ml Falcon tube
3. Fill a 10ml pipette with Ficoll, ie ca 13ml
4. Place the full pipette in the Falcon tube and carefully remove the pipette-filler. Let the ficoll slowly fill the tube by gravity. When the ficoll level reaches the level of the blood, slowly lift the pipette until it reaches the top of the ficoll-level underneath the blood. Finally, place your fingertip on top of the pipette before carefully removing it from the Falcon tube.
5. Centrifuge 20min, ca 2000rpm, no break = program 8
6. Collect PBMC
7. Wash PBMC twice in sterile PBS (1500 rpm, 10 min)
8. Count – you should expect at least 1 million PBMC per ml of whole blood, at least 500 million PBMC from one buffy coat. The numbers vary a lot between donors.

***Surface stain mononuclear cells*[2]**

1. Transfer the cells pooled from the 3-plicate wells (day 0 → take 1 milj. cells from PBMCs) to a V-bottomed FACS-staining plate. Spin (1500 rpm, 3 min) and remove supernatant.
2. Wash cells in PBS once to remove serum. (wash=add buffer, spin and discard sup).
3. Add 100 ul viability near infrared (near IR) diluted 1:1000 in PBS, 30 min in fridge.
4. Wash cells once in PBS.

5. Add antibodies for cell surface markers (see table for concentration) in staining buffer buffer, 50ul/well. Suspend the cells!
6. Incubate for 10 minutes at +4C.
7. Wash. Add 150 ul staining buffer, spin and remove supernatant.
8. Resuspend in 200ul BD Fixation buffer and transfer to FACS insert tubes, and store at +4C.
9. Analyze in Gallios FACS machine at the latest the next day.
   (Staining buffer= PBS + 0.05%FCS)

CellTiter-Glo® Assay[4]

1. Take out Celltiter glo reagents from freezer well before use to equilibrate to room temperature.
2. Estimate remaining volume in cell culture plate wells. Calculate total volume. Mix Celltiter glo reagents to corresponding volume.
3. Add the same volume (e.g. volume in well 80 ul, add 80 ul CellTiter reagent) to wells of reagent as cell culture volume remaining.
4. Read Luminescence asap.