Immunophenotyping IMPC_IMM_001

Purpose

This test differentiates immune cell sub-populations via flow cytometry.

Description: increased CD4-positive T cell number (MP:0008074), decreased CD4-positive T cell number (MP:0008075), etc..

Experimental Design

Minimum number: 3M + 3F

Age at test: 16 weeks

Sex: Both (sexually dimorphic)

Equipment

- Scissors
- Precision balance
- Calibrated single and multichannel pipettes
- Plate shaker
- Refrigerated centrifuge
- Flow Cytometer (capable of distinguishing a minimum of 8 colours per well)
- Tissue dissociator: Equipment for manual dissociation
- Cell counter equipment: Countess Automated Cell Counter (Invitrogen)

Supplies

- 96-well U-bottomed plates (Falcon# 353077)
- Dispensing troughs
- Extra long 10 µl pipette tips for antibody solutions
- 5 ml tubes
- 12 x 75 mm tubes
- Cell strainers e.g. Nylon mesh
- Cell counter recipients (slides for cell counter)
- CS (calf serum)
- HBSS with Mg2+, with Ca2+
- PBS without Mg2+, without Ca2+
- EDTA (final concentration 2mM)
- Collagenase D (Roche) (10x Stock solution: 2000 Mandl Units/ml collagenase D in <u>enzyme buffer</u>, aliquoted and stored at –20°C)
- DNAse I (Sigma) (10x Stock solution: 0.1 mg/ml DNAse I in <u>enzyme buffer</u>, aliquoted and stored at –20°C)
- 10x RBC lysis buffer (eBioscience)
- HEPES (pH7.2)

Procedure

This protocol requires several steps in the collection, preparation and analysis of the samples. Each one is detailed separately below.

Reagent preparation

- **Enzyme buffer**

- o   HBSS with Ca2+ and Mg2+
- o   2% CS (v/v)
- o   10mM HEPES
- **1x Enzyme cocktail (working solution):** 3 ml per each spleen, containing final concentration of:
    - o   Enzyme buffer
    - o   DNAse I (0.01 mg/ml)
    - o   Collagenase D (200 Mandl Units/ml)
- **FACS buffer**
    - o   1x PBS *without* Ca2+ and Mg2+
    - o   EDTA 2mM
    - o   2% CS (v/v)
    - o   10mM HEPES
    - o   0.025% NaN_3
- **RBC Lysis buffer:** Prepare a1x solution in ddH_2O from lysis buffer.
- **Stopping buffer**
    - o   1x PBS without Ca2+ and Mg2+
    - o   0.1M EDTA
- **Antibody cocktails for Panels 1 & 2**
    - o   Protect antibodies and prepared cocktails from direct light.
    - o   Mastermix concentration, storage temperature and stability to be determined after panels 1 and 2 have been finalised and tested.
    - o   Each sample will require 50 µl of diluted 1X antibody cocktail.
    - o   Antibody cocktails should be gently but thoroughly mixed or quickly vortexed to ensure homogeneity of the solutions.
- **Read buffer / dead cell exclusion dye**
    - o   SytoxBlue at 1:10000 concentration in FACS buffer
    - o   Require 200 µl per well (i.e. 400 µl for each spleen).

Other preparations on the day

- Bring RBC lysis buffer to room temperature.
- Prepare wet ice box, label tubes, etc.

Spleen collection

- Collect the spleen from euthanized mice.
- Remove all fat from the spleen and weigh the organ on a petri dish (do not hydrate the organ before weighing it as this would lead to substantial errors in measurement).
- Place the spleen in a 5 ml tube with 3 mL of 1x enzyme cocktail on ice.

Spleen dissociation / digests

Performing manual digests:

- Mince into fine pieces using small scissors, place on ice until all samples are minced.
- Incubated in a 37ºC water bath for 30 min.
- Add 300 µl of stopping buffer and mix by inversion to block enzymatic digestion and dissociate T cell-dendritic cell interactions.
- Cells were filtered through a nylon mesh to 12 x 75 mm tube.

- Spin at 500 x g in a swing bucket rotor for 5 min at 10⁰C.
- Decant the supernatant, rack the tubes to resuspend the pellet.
- Add 2 ml FACS buffer, mix well by vortexing, take 10 µl for the cell counting step.

Cell counting

- Dilutions for counting: 2 serial 1:10 dilutions (10 µl cells + 90 µl FACS buffer, then 10 µl of the 1:10 dilution + 90 µl buffer).
- Perform a cell count on an aliquot of the re-suspended cells (adjust concentration according to the cell counter method used).
- Note down the cell count, correct for dilution and calculate the concentration in cells per µl.
- Cell count:
    - Pipette the volume containing approximately 4 million cells per well to a 96 well plate in horizontal fashion starting from A1 onwards for panel 1 staining.
- Do the same for panel 2 staining in separate wells leaving a few empty rows between the panels to avoid cross contamination.
- Top up to final volume of 150 µl using FACS buffer, centrifuge, discard supernatant and keep plate on wet ice.

Red blood cell lysis, Blocking & staining

- Remove plate from ice and add 50 µl of 1X RBC lysis buffer (at room temperature) to each cell pellet from the previous step.
- Vortex the edges of the plates and ensure complete lysis.
- Incubate for 1 minute at room temperature and then return to ice and add 100 µl of FACS buffer (to stop lysis) to each well.

Note plate centrifuge steps are: 3 min, 510 x g at 4°C

- Centrifuge, discard supernatant and resuspend in 100 µl FACS buffer.
- Again centrifuge and discard supernatant and resuspend in 100 µl of anti-CD16/32 antibody (diluted 1/50) and incubate on ice for 10 min.
- Centrifuge the plate, discard supernatant and resuspend in 50 µl 1X AB mix in appropriate wells for individual panels followed by incubation on ice and in the dark for 60 min.
- If using Sytox Blue as live/dead discriminator:
    - Top up to 100 µl with FACS buffer after incubation. Centrifuge, discard supernatant and resuspend in 200 µl FACS buffer.
    - When ready to read plate, centrifuge again and discard supernatant. Resuspend the pellet in 200 µl of read buffer (Sytox Blue diluted 1:10000 in FACS buffer).

General Recommendations for Setting up Cytometer

Set up the analyser to aim acquire 300,000 viable events (live cells) for each of Panels 1 and 2. 500,000 are recommended for panel 2 in order to increase robustness of myeloid population of low frequencies (macrophages, DCs).

Gating Panel 1

Parameters	Gating steps						
Panel A live leukocyte count							
T cells (panel A)	number of live leukocytes	CD5+	CD161-				
NKT cells (panel A)	number of live leukocytes	CD5+	CD161+				
NK cells (panel A)	number of live	CD5-	CD161+				

	leukocytes						
Others	number of live leukocytes	CD5-	CD161-				
CD4 T cells	number of live leukocytes	CD5+	CD161-	CD4+	CD8-		
CD8 T cells	number of live leukocytes	CD5+	CD161-	CD4-	CD8+		
DN T cells	number of live leukocytes	CD5+	CD161-	CD4-	CD8-		
DP T cells	number of live leukocytes	CD5+	CD161-	CD4+	CD8+		
CD4 NKT cells	number of live leukocytes	CD5+	CD161+	CD4+	CD8-		
CD8 NKT cells	number of live leukocytes	CD5+	CD161+	CD4-	CD8+		
DN NKT cells	number of live leukocytes	CD5+	CD161+	CD4-	CD8-		
CD4 CD25+ T cells		number of CD5+	CD161-	CD4+	CD8-	CD25+	
CD4 CD25- T cells		number of CD5+	CD161-	CD4+	CD8-	CD25-	
CD8 CD25+ T cells		number of CD5+	CD161-	CD4-	CD8+	CD25+	
CD8 CD25- T cells		number of CD5+	CD161-	CD4-	CD8+	CD25-	
DN CD25+ T cells		number of CD5+	CD161-	CD4-	CD8-	CD25+	
DN CD25- T cells		number of CD5+	CD161-	CD4-	CD8-	CD25-	
CD4 CD25+ NKT cells		number of CD5+	CD161+	CD4+	CD8-	CD25+	
CD4 CD25- NKT cells		number of CD5+	CD161+	CD4+	CD8-	CD25-	
CD8 CD25+ NKT cells		number of CD5+	CD161+	CD4-	CD8+	CD25+	
CD8 CD25- NKT cells		number of CD5+	CD161+	CD4-	CD8+	CD25-	
DN CD25+ NKT cells		number of CD5+	CD161+	CD4-	CD8-	CD25+	
DN CD25- NKT cells		number of CD5+	CD161+	CD4-	CD8-	CD25-	
CD4 CD44+CD62L- T cells		number of CD5+	CD161-	CD4+	CD8-	CD44+	CD62L-
CD4 CD44+CD62L+ T cells		number of CD5+	CD161-	CD4+	CD8-	CD44+	CD62L+
CD4 CD44-CD62L+ T cells		number of CD5+	CD161-	CD4+	CD8-	CD44-	CD62L+
CD4 CD44-CD62L- T cells		number of CD5+	CD161-	CD4+	CD8-	CD44-	CD62L-
CD8 CD44+CD62L- T cells		number of CD5+	CD161-	CD4-	CD8+	CD44+	CD62L-
CD8 CD44+CD62L+ T cells		number of CD5+	CD161-	CD4-	CD8+	CD44+	CD62L+
CD8 CD44-CD62L+ T cells		number of CD5+	CD161-	CD4-	CD8+	CD44-	CD62L+
CD8 CD44-CD62L- T cells		number of CD5+	CD161-	CD4-	CD8+	CD44-	CD62L-

DN CD44+CD62L- T cells		number of CD5+	CD161-	CD4-	CD8-	CD44+	CD62L-
DN CD44+CD62L+ T cells		number of CD5+	CD161-	CD4-	CD8-	CD44+	CD62L+
DN CD44-CD62L+ T cells		number of CD5+	CD161-	CD4-	CD8-	CD44-	CD62L+
DN CD44-CD62L- T cells		number of CD5+	CD161-	CD4-	CD8-	CD44-	CD62L-
CD4 CD44+CD62L- NKT cells		number of CD5+	CD161+	CD4+	CD8-	CD44+	CD62L-
CD4 CD44+CD62L+ NKT cells		number of CD5+	CD161+	CD4+	CD8-	CD44+	CD62L+
CD4 CD44-CD62L+ NKT cells		number of CD5+	CD161+	CD4+	CD8-	CD44-	CD62L+
CD8 CD44+CD62L- NKT cells		number of CD5+	CD161+	CD4-	CD8+	CD44+	CD62L-
CD8 CD44+CD62L+ NKT cells		number of CD5+	CD161+	CD4-	CD8+	CD44+	CD62L+
CD8 CD44-CD62L+ NKT cells		number of CD5+	CD161+	CD4-	CD8+	CD44-	CD62L+
DN CD44+CD62L- NKT cells		number of CD5+	CD161+	CD4-	CD8-	CD44+	CD62L-
DN CD44+CD62L+ NKT cells		number of CD5+	CD161+	CD4-	CD8-	CD44+	CD62L+
DN CD44-CD62L+ NKT cells		number of CD5+	CD161+	CD4-	CD8-	CD44-	CD62L+

Gating Panel B

Parameters	Gating steps					
Panel B live leukocyte count						
Neutrophils	Live	CD11b+	Ly6G+			
Monocytes	Not Granulocytes	CD11b+	Ly6C High			
Eosinophils	Not Monocytes	CD11b+	SSC-H High			
NK Cells (panel B)	Not Eosinophils	CD161+	CD19-	CD5-		
NK Subsets (Q1)	Not Eosinophils	CD161+	CD19-	CD5-	CD11b-	Ly6C+
NK Subsets (Q2)	Not Eosinophils	CD161+	CD19-	CD5-	CD11b+	Ly6C+
NK Subsets (Q3)	Not Eosinophils	CD161+	CD19-	CD5-	CD11b-	Ly6C-
NK Subsets (Q4)	Not Eosinophils	CD161+	CD19-	CD5-	CD11b+	Ly6C-
NKT Cells (panel B)	Not Eosinophils	CD161+	CD19-	CD5+		
NKT Subsets (Q1)	Not Eosinophils	CD161+	CD19-	CD5+	CD11b-	Ly6C+
NKT Subsets (Q3)	Not Eosinophils	CD161+	CD19-	CD5+	CD11b-	Ly6C-
T Cells (panel B)	Not Eosinophils	CD161-	CD5+			
T Subset	Not Eosinophils	CD161-	CD5+	Ly6C+		
B Cells	Not Eosinophils	MHCII+	CD19+			
B1B Cells	Not Eosinophils	MHCII+	CD19+	CD5+		
B2B Cells	Not Eosinophils	MHCII+	CD19+	CD5-		
Follicular B Cells (CD21/35+)	Not Eosinophils	MHCII+	CD19+	CD5-	CD21/35+	
pre-B Cells (CD21/35 low)	Not Eosinophils	MHCII+	CD19+	CD5-	CD21/35 low	
MZB (CD21/35 high)	Not Eosinophils	MHCII+	CD19+	CD5-	CD21/35 High	
cDCs	Not Eosinophils	MHCII+	CD19-	CD11c+		

cDCs CD11b Type	Not Eosinophils	MHCII+	CD19-	CD11c+	CD11b+	
pDCs	Not Eosinophils	Not T Cells	Ly6C+	CD317+		
RP Macrophage (F4/80+)	Not Eosinophils	MHCII+	F4/80+			

Parameters

	Version	Type	Increment	Option	Derived	Unit	Data Type
Spleen weight IMPC_IMM_001_001	1.0	simpleParameter				g	FLOAT
Total number of acquired events in Panel A IMPC_IMM_026_001	1.3	simpleParameter					INT
Percentage of live gated events in Panel A IMPC_IMM_002_001	1.5	simpleParameter					FLOAT
T cells (panel A) IMPC_IMM_003_001	1.5	simpleParameter					INT
NKT cells (panel A) IMPC_IMM_004_001	1.5	simpleParameter					INT
NK cells (panel A) IMPC_IMM_005_001	1.5	simpleParameter					INT
Others IMPC_IMM_006_001	1.4	simpleParameter					INT
CD4 T cells IMPC_IMM_007_001	1.4	simpleParameter					INT
CD8 T cells IMPC_IMM_008_001	1.4	simpleParameter					INT
DN T cells IMPC_IMM_009_001	1.4	simpleParameter					INT
CD4 NKT cells IMPC_IMM_011_001	1.4	simpleParameter					INT
CD8 NKT cells IMPC_IMM_012_001	1.4	simpleParameter					INT
DN NKT cells IMPC_IMM_013_001	1.4	simpleParameter					INT
CD4 CD25+ T cells IMPC_IMM_014_001	1.4	simpleParameter					INT
CD4 CD25- T cells IMPC_IMM_015_001	1.4	simpleParameter					INT

	Version	Type	Increment	Option	Derived	Unit	Data Type
CD8 CD25+ T cells IMPC_IMM_016_001	1.4	simpleParameter					INT
CD8 CD25- T cells IMPC_IMM_017_001	1.4	simpleParameter					INT
DN CD25+ T cells IMPC_IMM_018_001	1.4	simpleParameter					INT
DN CD25- T cells IMPC_IMM_019_001	1.4	simpleParameter					INT
CD4 CD25+ NKT cells IMPC_IMM_020_001	1.4	simpleParameter					INT
CD4 CD25- NKT cells IMPC_IMM_021_001	1.4	simpleParameter					INT
CD8 CD25+ NKT cells IMPC_IMM_022_001	1.4	simpleParameter					INT
CD8 CD25- NKT cells IMPC_IMM_023_001	1.4	simpleParameter					INT
DN CD25+ NKT cells IMPC_IMM_024_001	1.4	simpleParameter					INT
DN CD25- NKT cells IMPC_IMM_025_001	1.2	simpleParameter					INT
CD4 CD44+CD62L- T cells IMPC_IMM_028_001	1.2	simpleParameter					INT
CD4 CD44+CD62L+ T cells IMPC_IMM_029_001	1.2	simpleParameter					INT
CD4 CD44-CD62L+ T cells IMPC_IMM_030_001	1.2	simpleParameter					INT
CD8 CD44+CD62L- T cells IMPC_IMM_032_001	1.2	simpleParameter					INT
CD8 CD44+CD62L+ T cells IMPC_IMM_033_001	1.2	simpleParameter					INT
CD8 CD44-CD62L+ T cells IMPC_IMM_034_001	1.2	simpleParameter					INT
DN CD44+CD62L- T cells IMPC_IMM_036_001	1.2	simpleParameter					INT
DN CD44+CD62L+ T cells IMPC_IMM_037_001	1.2	simpleParameter					INT

	Version	Type	Increment	Option	Derived	Unit	Data Type
DN CD44-CD62L+ T cells IMPC_IMM_038_001	1.2	simpleParameter					INT
CD4 CD44+CD62L- NKT cells IMPC_IMM_040_001	1.2	simpleParameter					INT
CD4 CD44+CD62L+ NKT cells IMPC_IMM_041_001	1.2	simpleParameter					INT
CD4 CD44-CD62L+ NKT cells IMPC_IMM_042_001	1.2	simpleParameter					INT
CD8 CD44+CD62L- NKT cells IMPC_IMM_043_001	1.2	simpleParameter					INT
CD8 CD44+CD62L+ NKT cells IMPC_IMM_044_001	1.2	simpleParameter					INT
CD8 CD44-CD62L+ NKT cells IMPC_IMM_045_001	1.2	simpleParameter					INT
DN CD44+CD62L- NKT cells IMPC_IMM_046_001	1.2	simpleParameter					INT
DN CD44+CD62L+ NKT cells IMPC_IMM_047_001	1.2	simpleParameter					INT
DN CD44-CD62L+ NKT cells IMPC_IMM_048_001	1.2	simpleParameter					INT
Total number of acquired events in Panel B IMPC_IMM_027_001	1.1	simpleParameter					INT
Percentage of live gated events in Panel B IMPC_IMM_049_001	1.3	simpleParameter					FLOAT
Neutrophils IMPC_IMM_050_001	1.2	simpleParameter					INT
Monocytes IMPC_IMM_051_001	1.2	simpleParameter					INT
Eosinophils IMPC_IMM_052_001	1.2	simpleParameter					INT
NK Cells (panel B) IMPC_IMM_053_001	1.3	simpleParameter					INT
NK Subsets (Q1) IMPC_IMM_054_001	1.3	simpleParameter					INT

	Version	Type	Increment	Option	Derived	Unit	Data Type
NK Subsets (Q2) IMPC_IMM_055_001	1.2	simpleParameter					INT
NK Subsets (Q3) IMPC_IMM_056_001	1.2	simpleParameter					INT
NK Subsets (Q4) IMPC_IMM_057_001	1.2	simpleParameter					INT
NKT Cells (panel B) IMPC_IMM_058_001	1.2	simpleParameter					INT
NKT Subsets (Q1) IMPC_IMM_059_001	1.2	simpleParameter					INT
NKT Subsets (Q3) IMPC_IMM_060_001	1.2	simpleParameter					INT
T Cells (panel B) IMPC_IMM_061_001	1.2	simpleParameter					INT
T Subset IMPC_IMM_062_001	1.2	simpleParameter					INT
B Cells IMPC_IMM_063_001	1.2	simpleParameter					INT
B1B Cells IMPC_IMM_064_001	1.2	simpleParameter					INT
B2B Cells IMPC_IMM_065_001	1.2	simpleParameter					INT
Follicular B Cells (CD21/35+) IMPC_IMM_067_001	1.1	simpleParameter					INT
pre-B Cells (CD21/35 low) IMPC_IMM_069_001	1.1	simpleParameter					INT
MZB (CD21/35 high) IMPC_IMM_071_001	1.1	simpleParameter					INT
cDCs IMPC_IMM_072_001	1.2	simpleParameter					INT
cDCs CD11b Type IMPC_IMM_073_001	1.2	simpleParameter					INT
pDCs IMPC_IMM_074_001	1.2	simpleParameter					INT
RP Macrophage (CD19- CD11c-) IMPC_IMM_076_001	1.1	simpleParameter					INT

Metadata

	Version	Type	Increment	Option	Derived	Unit	Data Type
Equipment name	1.0	procedureMetadata		Fortessa_1			TEXT

	Version	Type	Increment	Option	Derived	Unit	Data Type
IMPC_IMM_077_001							
Equipment manufacturer IMPC_IMM_078_001	1.0	procedureMetadata		BD Biosciences			TEXT
Equipment model IMPC_IMM_079_001	1.0	procedureMetadata		BD LSRFortessa Cell Analyzer			TEXT
CS&T Bead lot IMPC_IMM_080_001	1.0	procedureMetadata					TEXT
Anesthesia IMPC_IMM_081_001	1.0	procedureMetadata		Injection narcosis with Sodium Pentobarbital (Somnopentyl)			TEXT
Cell digestion IMPC_IMM_082_001	1.0	procedureMetadata		manual			TEXT
Cell digestion agent IMPC_IMM_083_001	1.0	procedureMetadata		Collagenase D			TEXT
Cell digestion agent manufacturer IMPC_IMM_084_001	1.1	procedureMetadata		Roche			TEXT
Cell digestion agent catalog number IMPC_IMM_085_001	1.2	procedureMetadata		#11088858001			TEXT
Cell counting IMPC_IMM_086_001	1.1	procedureMetadata		pre-lysis			TEXT
Cell counting equipment manufacturer IMPC_IMM_087_001	1.0	procedureMetadata		Life Technologies			TEXT
Cell counting equipment model IMPC_IMM_088_001	1.0	procedureMetadata		Countess Automated Cell Counter			TEXT
Cell counting equipment name IMPC_IMM_089_001	1.0	procedureMetadata					TEXT
Cell lysis buffer manufacturer IMPC_IMM_090_001	1.2	procedureMetadata		eBioscience			TEXT
Cell lysis buffer catalog number	1.2	procedureMetadata		00-4300-54			TEXT

	Version	Type	Increment	Option	Derived	Unit	Data Type
IMPC_IMM_091_001							
Date and time of sacrifice IMPC_IMM_092_001	1.0	procedureMetadata					DATETIME
Date and time of sample preparation IMPC_IMM_093_001	1.0	procedureMetadata					DATETIME
Sample storage temperature until analysis (in Celsius) IMPC_IMM_094_001	1.0	procedureMetadata				C	FLOAT
Balanced salt solution type IMPC_IMM_096_001	1.0	procedureMetadata		PBS			TEXT
Balanced salt solution manufacturer IMPC_IMM_097_001	1.0	procedureMetadata		Wako			TEXT
Balanced salt solution catalog number IMPC_IMM_098_001	1.0	procedureMetadata		041-20211			TEXT
DNAse I manufacturer IMPC_IMM_101_001	1.1	procedureMetadata		Sigma			TEXT
DNAse I catalog number IMPC_IMM_102_001	1.2	procedureMetadata		DN25			TEXT
Dead cell exclusion dye IMPC_IMM_103_001	1.0	procedureMetadata		Sytox Blue			TEXT
Dead cell exclusion dye manufacturer IMPC_IMM_104_001	1.0	procedureMetadata		Life Technologies			TEXT
Dead cell exclusion dye catalog number IMPC_IMM_105_001	1.0	procedureMetadata		S11348			TEXT
Cell digestion temperature IMPC_IMM_106_001	1.0	procedureMetadata		37			TEXT