Standard operating procedure (SOP) for reception and handling of RITPBC samples (12/11/2012 to 02/11/2016)

Blood samples for each of visits 1, 16, 17, 18 & 19 were physically collected from the Clinical research facility (CRF) (RVI Level 6) as soon as contacted (Jennifer Bainbridge and team).

A single SST II Advance tube (5ml whole blood) with Gold Hemogard Closure (BD 367954) and two K2EDTA tubes (10ml whole blood) with Lavender Hemogard Closure (BD 367525) were handed over and transported (in a covered polystyrene box) 300 metres to the laboratory (M3063 3rd Floor William Leech Building, The Medical School) where the samples were immediately processed.

At this point the subject identifier (eg 01JC), date of collection, NHS number, MRN Number and subject's date of birth were recorded on an Excel spreadsheet database.

Serum preparation:

The Gold top tube containing 5ml of whole blood was centrifuged @ 1300xg for 10min at RT°C and the serum lying above the gel plug was drawn off to a 7ml bijou tube. This serum was mixed and aliquoted into 1.5ml eppendorf tubes (4 x 500ul). The tubes were then immediately stored at -80°C.

Peripheral Blood Mononuclear Cell (PBMC) purification:

The remaining whole blood from the K2EDTA tubes was pooled (~16mls) into a 50ml Flacon tube and diluted (1:1) with Phosphate Buffered Saline (PBS).

15ml Lymphoprep (AXIS-SHIELD, Oslo, Norway) was dispensed on top of the frit of a 50ml Leucosep tube (227290 Greiner Bio-one) and centrifuged for 5min @400xg at RT°C.

The diluted blood was then poured on top of the frit and the tube centrifuged for 25min @ 800xg with no braking at RT°C.

The "buffy (PBMC containing) layer" was then carefully aspirated (avoiding taking a significant amount of lymphoprep) using a sterile Pasteur pipette to a fresh 50ml Falcon tube containing PBS. This tube was topped up to 50ml with PBS and centrifuged for 5mins @400g at RT°C.

The supernatant was carefully decanted and the pellet resuspended in PBS and topped up to 50ml and re-centrifuged as above.

Following this wash spin the supernatant was decanted and the pellet resuspended in 3ml of freezing medium (10% DMSO + 90% FCS). The PBMCs were aliquoted into Cryovials (3 x 1ml) and placed in a "Mr Frosty" freezing vessel and placed at -80°C overnight. The following day the tubes were taken out and stored long term in liquid nitrogen.

Flow cytometry:

Flow Cytometry Buffer was made (PBS + 2% (v/v) FBS (10mls in 500mls) + 1mM EDTA (1ml of 0.5M). This buffer acts as antibody diluent, wash buffer and Flow Cytometry running buffer.

Whole blood (100ul) (collected in K2EDTA tubes) was dispensed into clear polystyrene FACS tubes.

Add 5ul Human TruStain FcX™ (Fc Receptor Blocking Solution) (Biolegend 422302). Gently mix and incubate 10-15min RT°C.

Add specific antibodies (see panel below) and incubate 30min RT°C away from direct light. Add 2mls 1xFACSLyse Solution (Dilute from 10x - Becton Dickinson Cat No. 349202)

Gently mix and incubate 10min RT°C.

Centrifuge 400xg for 5min and discard supernatant.

Resuspend cells with 2ml Flow Buffer and centrifuge 400xg for 5min. Repeat for 3x washes

Resuspend cells in 150ul Flow Buffer and run immediately or store 4°C for no longer than 12hours.

Samples were analysed in the Flow Cytometry Core Facilty (William Leech Building (2nd floor Room M2.099) using the FACSCanto II (machine 3) analyser throughout the period of study).

Data was securely stored and analysed usingFlowJo10.

Antibody Pa

Unstained	No antibodies	
Tube 1	CD19-FITC/CD69-PE/CD45-PerCP	10ul
Tube 2	CD20-PE	10ul
	CD27-APC	10ul
	CD38-PerCP-Cy5.5	40ul of 1:50 in Flow Buffer (<2wks)
	IgD-FITC	20ul
	CD19-APC-Cy7	5ul
Tube 3	CD19-PerCP-Cy5.5	3.5ul
	CD80-PE	10ul
	CD86-APC	10ul
	CD268-FITC	40ul of 1:50 in Flow Buffer (<2wks)
Isotype Controls:		
Tube 5	IgG2b-PE	10ul
	IgG1-PerCP-Cy5.5	40ul of 1:50 in Flow Buffer (<2wks)
Tube 6	IgG1-FITC	10ul
	IgG1-PE	10ul
	IgG1-APC	10ul
	IgG1-PerCP	10ul