Validation of immune cell analysis in whole blood by flow cytometry for clinical biomarker investigation

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Introduction

Liquid biopsies are important samples for providing biomarkers in clinical studies in a non-invasive manner. They are particularly relevant for immuno-oncology trials, where regulation of circulating immune cells may reflect immune changes in tumours in response to immune targeting therapies. For example, RXC004, a potent and selective inhibitor of the Wnt pathway regulator porcupine is hypothesised to have immunomodulatory anti-cancer functions [1-5]. Therefore as part of an RXC004 safety and tolerability study in cancer patients with solid tumours (NCT03447470), we aim develop methods to analyse immune response liquid biomarkers. This includes analysis of whole blood by flow cytometry to quantify a range of immune cell subsets and functional markers.

Methods

Flow cytometry analysis is carried out in house using 7 multi-colour panels, analysed on the ACEA Biosciences Novocyte 3000 flow cytometer (Fig 1). Antibodies included in these panels have been validated using healthy donor peripheral blood mononuclear cells (PBMCs), healthy donor whole blood and Biolegend VeriCells™.

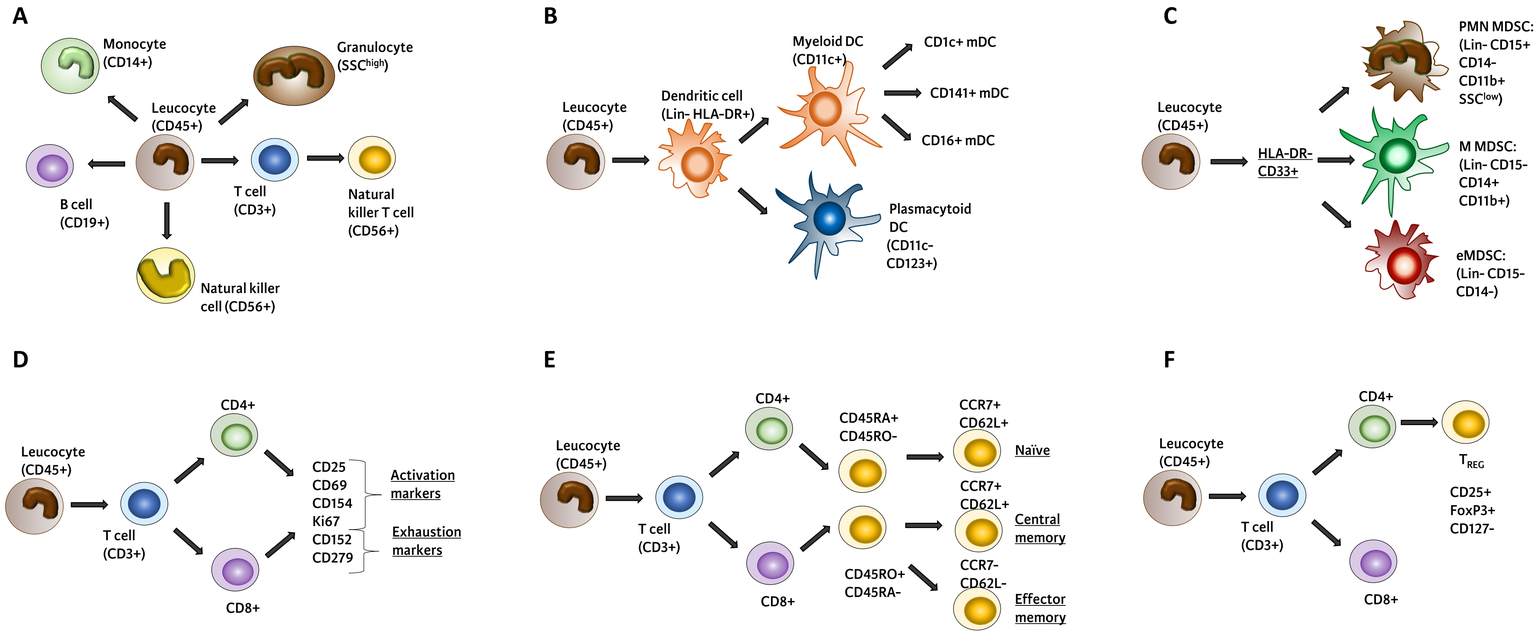


Figure 1. Summary of immunophenotyping panels

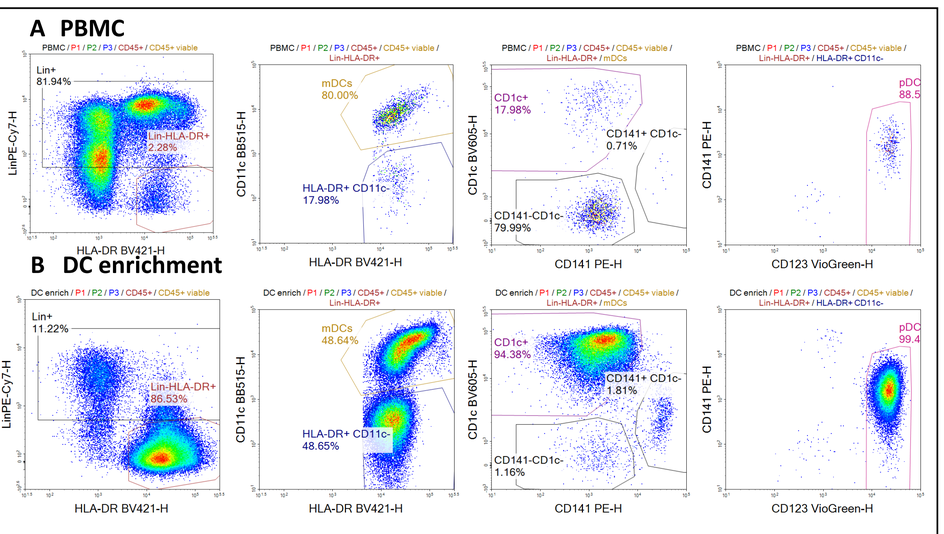
A General immune cell subsets: Exclusive expression of CD14, CD3, CD19 and CD56 identify monocytes, T and B lymphocytes and natural killer cells respectively. CD56 and CD3 co-expression identifies natural killer T cells. Granulocytes are identified based on FSC/SSC.
B Dendritic cells (DCs): High expression of HLA-DR and no lineage marker (CD14/CD3/CD19/CD56) expression identifies DCs. CD11c expression within this population identifies myeloid DCs, while lack of CD11c with CD123 expression identifies plasmacytoid DCs. Further myeloid DC subsets can be separated based on exclusive expression of CD1c, CD141 and CD16.
C Myeloid-derived suppressor cells (MDSCs): Expression of CD33 and lack of HLA-DR and lineage marker (CD3/CD19/CD56) expression can be used together with additional markers to identify polymorphonuclear (PMN)-MDSCs (CD15+ CD11b+), monocytic (M)-MDSCs (CD14+ CD11b+) and early (e)MDSCs (CD14-CD15-).

D-F T cell subset analysis: Expression of CD3 together with exclusive expression of CD4 or CD8 identifies the main T cell subsets
D Functional markers: CD69 and CD25 expression can indicate activation status, Ki67 denotes proliferating cells, and CD154 (CD40L) expression indicates activated antigen-specific T cells. CD279 (PD-1) and CD152 (CTLA-4) can indicate exhausted T cells. The activation markers make up one panel and exhaustion markers another panel.
E T cell memory: CD45RA and CD45RO can be used to identify naïve and memory T cell subsets respectively. CCR7 and CD62L expression can separate further memory subsets, central (double positive) and effector (double negative).
F Regulatory T cells (T_{REGS}): Expression of CD25, FoxP3 and lack of CD127 expression identifies T_{REGS}

Results

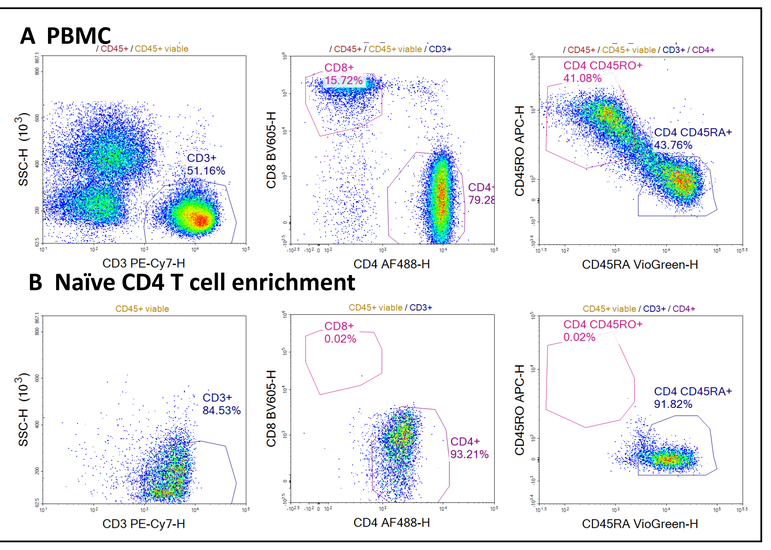
Dendritic cell panel validation

Figure 2. Healthy donor purified PBMCs enriched for DC populations, identified by DC flow cytometry panel (as shown in Fig 1B).
A DCs are a rare subset in PBMCs, and more so in whole blood, making accurate gating of sub-populations a challenge
B A magnetic bead based enrichment kit for specific DC subsets (CD1c mDC, CD141 mDC and pDC) was used to validate the gating strategy for DCs.



T cell memory panel validation

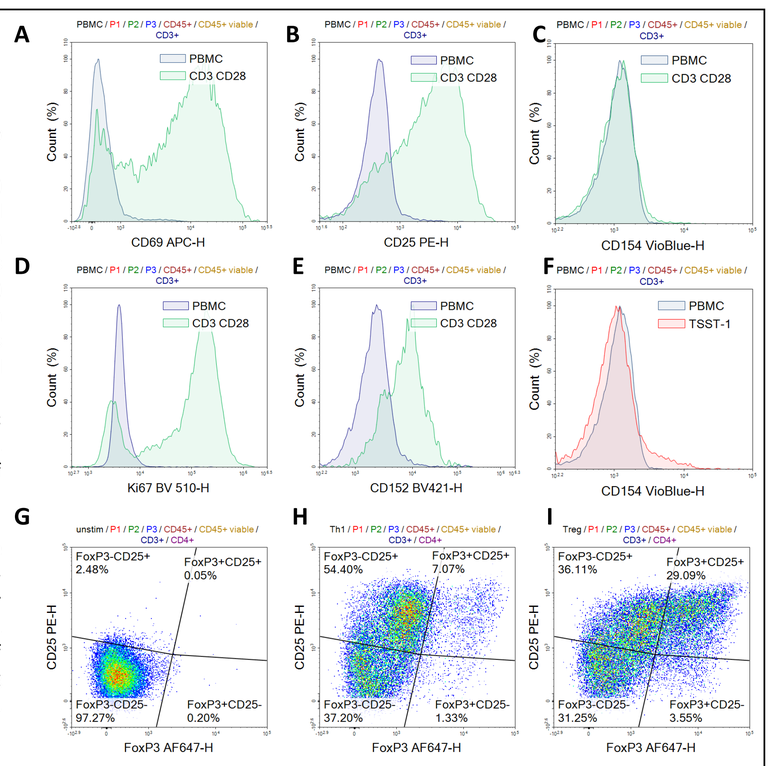
Figure 3. Healthy donor purified PBMCs enriched for naïve CD4 T cells, identified by T cell memory flow cytometry panel (as shown in Fig 1E).
A PBMCs stained with the panel identify a mixed population of CD4 and CD8 T cells, expressing CD45RA (naïve marker) and CD45RO (memory marker)
B A magnetic bead based enrichment kit for naïve CD4 T cells was used to validate the panel, which identifies only CD4 T cells expressing CD45RA following purification.



Functional T cell marker validation

Figure 4. Healthy donor purified PBMCs cultured *in vitro* to induce expression of functional markers
A-E PBMCs were cultured without stimulation (blue) or with CD3/CD28 tetramer stimulation (green) and stained with the T cell activation or exhaustion panels (as shown in Fig 1D). Stimulation for 6 hours induces upregulation of activation markers CD69 (A) and CD25 (B) but not the antigen-specific activation marker CD154 (C). Stimulation for 96 hours induces expression of proliferation marker Ki67 (D) and exhaustion marker CD152 (E).
F PBMCs were cultured with superantigen toxic shock syndrome toxin-1 (TSST-1, red) for 6 hours. Unlike CD3/CD28, TSST-1 induces upregulation of CD154 in a small population.

G-I Naïve CD4 T cells (as shown in Fig 2B) were cultured without stimulation (G), with CD3/CD28/IL-2 and IL-12 (H) or CD3/CD28/IL-2 and TGF-β (I) for 96 hours then stained with the T_{REG} panel (Fig 1F). CD3/CD28/IL-2 stimulation induces upregulation of activation marker CD25, while inclusion of polarising cytokine TGF-β stimulates *in vitro*-derived T_{REGS}, indicated by CD25 and FoxP3 co-expression.



Healthy donor whole blood

Whole blood samples were collected from 3 healthy donors every 3-4 weeks to generate a longitudinal dataset.

Samples were stained with all 7 flow cytometry panels (Fig 1) to assess detectable immune cell populations in whole blood and variability over time in healthy donors.

All populations described in Fig 1 are detectable, with the exception of MDSCs, CD141 mDCs and T_{REGS}. The expression levels of functional T cell markers are also low.

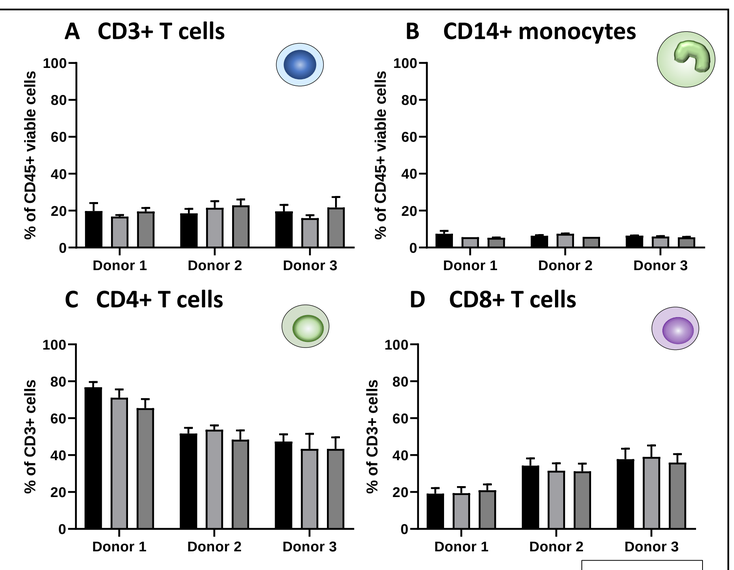
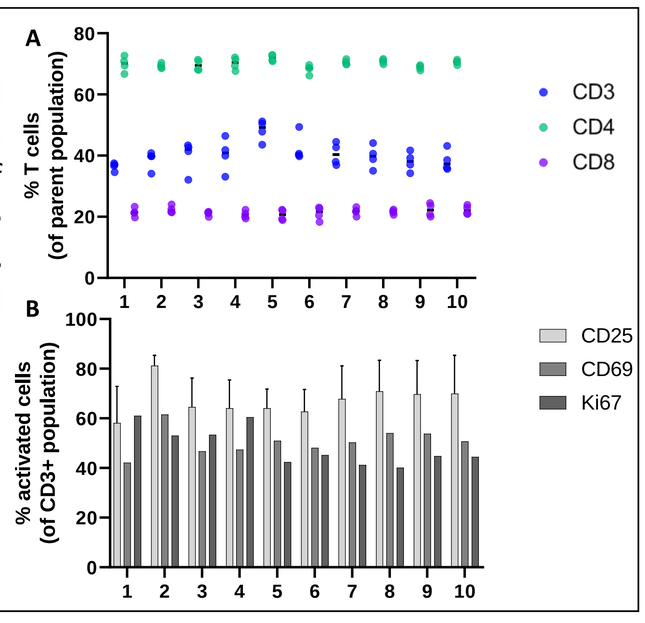


Figure 5. Healthy donor whole blood immune cell subset analysis by flow cytometry
Healthy donor whole blood was stained with the 7 flow cytometry panels described in figure 1. The summarised data show stability of key immune cell subsets over time quantified across multiple panels (mean+SD).
A CD3+ T cells (5 panels) and **B** CD14+ monocytes (2 panels), as a percentage of CD45+ viable cells (2 panels). **C** CD4+ T cells, and **D** CD8+ T cells (4 panels) as a percentage of CD3+ T cells.

VeriCells™

VeriCells™ are lyophilised unstimulated or activated PBMCs, batch produced in lots with specified proportions of immune cell subsets. These cells are included in each staining protocol as quality control for staining reagents and flow cytometer performance.

Figure 6. Biolegend VeriCells™ immune cell subset analysis by flow cytometry
Stability of key immune cell subsets over 10 staining dates, quantified across multiple panels (mean+SD).
A CD3+, CD4+ and CD8+ T cells, as a percentage of parent population (4 panels) in PBMC VeriCells™
B Activation marker expression on CD3+ T cells; CD25 (2 panels), CD69 and Ki67 in activated PBMCs VeriCells™.



Conclusion

We have established a protocol for the analysis of immune cell subsets by flow cytometry, suitable to assess potential therapy-induced changes in circulating immune cells as an exploratory end-point in patients with solid tumours enrolled in the RXC004 clinical study.

References

1. Li X *et al*; *Front Immunol*, 2019, 10:2293; **2.** Phillips C *et al; AACR; Cancer Res*, 2019, 79(13 Suppl), Abstract nr 506; **3.** Luke *et al; Clin Cancer Res*, 2019, 10:1158; **4.** Spranger S, Gajewski T; *Nat. Rev. Cancer*, 2018, 18: 139-147; **5.** Wang *et al; Trends Pharmacol Sci.*, 2018, 39 (7):648-658