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Technical Note

CLEC4C gene expression can be used to quantify circulating plasmacytoid dendritic cells

Liisa Murray[a], Yang Xi[a], John W. Upham[a,b,*]

[a] The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia
[b] Department of Respiratory Medicine, Princess Alexandra Hospital, Woolloongabba, QLD, Australia

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ABSTRACT

Plasmacytoid dendritic cells (pDC) are an important type I interferon producer that play an important role in the first line of host defence during viral infection. Abnormalities in pDC numbers and function have been associated with several health conditions. Quantifying pDC is important for understanding pDC related immune responses in viral infections and other diseases, however the current methods for quantifying pDC using flow cytometry have limited utility in large cohort studies involving multiple centres with limited access to flow cytometry. We reasoned that examining gene expression of the pDC marker C-type lectin domain family 4 member C (CLEC4C, also known as CD303 and BDCA2) in combination with pDC exclusive leukocyte immunoglobulin like receptor A4 (LILRA4, also known as CD85g and ILT7) might provide a more practical method that could be applied to multi-centre studies. Our results show a moderate correlation between pDC numbers measured by surface staining and *CLEC4C* gene expression in whole blood (rho = 0.39, $P = .037$, as well as a high correlation between *CLEC4C* gene expression in whole blood and peripheral blood mononuclear cells (rho = 0.79, $P < .001$). *LILRA4* gene expression did not provide additional useful information. Our results indicate that measuring *CLEC4C* gene expression can provide an alternative method for quantifying pDC numbers in human samples.

1. Introduction

Plasmacytoid dendritic cells (pDC) are recognised as the predominant type I interferon producing cell type in the primary antiviral response. Not only do pDC constitutively express viral nucleic acid-sensing pattern recognition receptors such as toll-like receptors, pDC contribute well over 90% of the type I interferon produced during a viral infection (Xi et al., 2015), which makes them highly relevant to host defence and pathogenesis in a variety of specific diseases. Alterations in numbers and function of pDC have been associated with delayed viral clearance (Dhamanage et al., 2016), autoimmunity, allergic disease and cancer (Swiecki and Colonna, 2015).

While there are a number of methods to study pDC function in experimental animals, studying pDC in human diseases presents several challenges, given that they are a rare leukocyte population in peripheral blood, accounting for < 0.4% of peripheral blood mononuclear cells (PBMC). Both pDC purification and pDC depletion (Xi et al., 2017) with immuno-magnetic beads or fluorescence activated cell sorting (FACS) (Kassianos et al., 2010) have been used to study pDC function. Numbers of circulating pDC can be measured accurately with flow cytometry, but this usually requires fresh blood samples which is not practical in large multi-centre cohort studies where there may not be ready access to flow cytometry. Recently, small volume blood samples have been used to analyse the transcriptome, proteome and metabolome (Urrutia et al., 2016) in various human diseases. With the advent of methods to collect and stabilise blood samples for subsequent gene expression analysis, there is now a convenient and accessible tool which can be used in large scale research studies, especially where there is limited capacity for sample processing in the field. We therefore reasoned that measuring the expression of pDC specific genes might provide a more convenient method for researchers to estimate pDC numbers in blood samples, especially in situations where flow cytometry is not readily available.

CLEC4C and LILRA4 are cell surface proteins that are expressed

Abbreviations: pDC, plasmacytoid dendritic cell; CLEC4C, C-type lectin domain family 4 member C.; LILRA4, leukocyte immunoglobulin like receptor A4.; PBMC, Peripheral blood mononuclear cell.; RT-PCR, Reverse transcription polymerase chain reaction.; FACS, Fluorescence-activated cell sorting.; TLR, Toll-like receptor.; cDNA, complementary DNA.; B2M, Beta-2-Microglobulin.; UBC, Polyubiquitin-C precursor

* Corresponding author at: The University of Queensland Diamantina Institute, Translational Research Institute, Princess Alexandra Hospital, 37 Kent Street, Woollongabba, QLD, 4102, Australia.
E-mail address: j.upham@uq.edu.au (J.W. Upham).

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exclusively on pDC (Cao and Bover, 2010; Xi et al., 2017). CLEC4C is a C-type lectin domain family 4 member C (also known as CD303 and BDCA-2) known to regulate antigen presentation and type I interferon production (Dzionek et al., 2001). Leukocyte immunoglobulin like receptor A4 (LILRA4; also known as ILT7 and CD85g) regulates TLR-mediated type I interferon production in pDC (Cao and Bover, 2010) providing an important negative feedback loop for type I interferon production. We hypothesised that the relative gene expression of *CLEC4C* and *LILRA4* measured by real time PCR would correlate with pDC cell numbers ($CLEC4C^+LILRA4^+CD14^-$ cells) measured by flow cytometry. Our findings show a moderate correlation score of 0.39 ($P = .037$) between *CLEC4C* gene expression and pDC cell numbers measured with flow cytometry, thus providing an alternative method for estimating the pDC numbers in circulating blood.

2. Methods

2.1. Participants

We recruited 29 participants, of which 19 were female, with a mean age of 38.6 ± 13 years. All participants were healthy without respiratory infections within 4 weeks prior to their study visit. Metro South Health Human Research Ethics Committee approved the study, and all participants provided written consent.

2.2. Sample preparation

10 ml of heparinised blood and a PAXgene blood tube were collected from each participant. An aliquot of 1 ml of heparinised blood was used to evaluate the percentage of the pDC in the whole blood using flow cytometry (Section 2.3). PBMC were isolated from the remaining blood by density gradient centrifugation using Lymphoprep (Stemcell technologies, Vancouver, Canada) as described previously in (Roponen et al., 2010). 1×10^6 cells were used to evaluate the percentage of pDC in PBMC (Section 2.4) and 4×10^6 PBMC were stored in RNAprotect (Qiagen, Hilden, Germany) for RNA extraction and subsequently RT-PCR (Section 2.5).

2.3. Whole blood cell preparation

300ul of whole blood per sample was washed once with 1 ml of FACs buffer (1% heat-inactivated foetal bovine serum in phosphate-buffered saline; foetal calf serum; Bovogen Biologicals, Keilor East, VIC, Australia). The red blood cells were then lysed with 2 ml of 1× BD FACS lysing solution (BD FACS™ lysing solution, BD Cat#349202, San Jose CA, USA) for 30mins at dark and room temperature. After centrifuging, the supernatant was removed and cell pellet was washed with 1 ml of FACs buffer followed by surface staining (Section 2.4).

2.4. Surface staining and flow cytometry analysis

The prepared cells (Section 2.3) and isolated PBMC (Section 2.2) were washed with FACs buffer and then stained with CLEC4C-PE (CD303, Miltenyi Biotec Cat# 130–090-511, RRID: AB_244168), CD14-PerCP (BioLegend Cat# 301824, RRID: AB_893251) and LILRA4-APC (CD85g, Thermo Fisher Scientific Cat# 17–5179-41, RRID: AB_10597138) in FACs buffer for 30 min at 4 °C in dark. The cells were then washed twice with the FACs buffer and fixed in 2% paraformaldehyde. A total of ~500,000 gated events were acquired using LSRFortessa X-20 (BD Biosciences, San Jose, CA). Data was analysed using the FlowJo Tree Star software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

2.5. RNA extraction and quantitative RT- PCR analysis

RNA was extracted from the PAXgene tube using Preanalytix blood RNA kit (Qiagen, Maryland, USA) or from the PBMC stored in RNAprotect using RNeasy mini kit (Qiagen, Maryland, USA) plus DNase II digestion protocol. RNA was reverse-transcribed to cDNA with sensiFAST cDNA synthesis kit (Bioline, London, UK). Relative quantitative RT- PCR was performed from the cDNA with PowerUp SYBR Green (Applied Biosystems, CA, USA) mastermix with Lightcycler 480 (Roche) machine. Custom primers were purchased from Geneworks (SA, Australia) with sequences shown in Appendix A. Delta Ct of *CLEC4C* and *LILRA4* gene expression was obtained against the mean Ct of the two reference genes *B2M* (Beta-2-Microglobulin) and *UBC* (Polyubiquitin-C precursor) (primer sequences in Table A1).

2.6. Statistical analysis

Non-parametric (for FACS measurements) or parametric rank correlation test were performed between paired samples in R version 3.4.2 (R Core Team, 2018) with cor.test() function. To compare paired samples from the same individuals, the Wilcoxon signed rank test was performed using GraphPad Prism 6 for Windows (GraphPad Software, San Diego, CA, USA). A two-sided p-value < .05 was considered significant.

3. Results and discussion

To validate gene expression quantification as a measurement tool we compared relative quantification by RT-PCR to the current 'gold standard', namely surface staining with FACS. In theory, the pDC frequency in each of the measurements should correlate but sample processing and technical variability were expected to produce variations.

LILRA4 was previously identified as being stably expressed in unstimulated pDC compared to other immune cell types (Cho et al., 2008). CLEC4C was recognised as a pDC specific cell marker in 2001 (Dzionek et al., 2001), and we previously showed that it is markedly reduced in pDC depleted cell populations (Xi et al., 2017), therefore we used those two markers to detect pDC population (Fig. 1A). We compared whole blood to PBMC, and as expected the pDC frequency was higher in PBMC than in whole blood (Fig. 1B).

LILRA4 and CLEC4C expression were more strongly correlated in PBMC than in whole blood (Fig. 2). In fact, the correlation coefficient for LILRA4 versus CLEC4C was similar with both the RT-PCR method (cor = 0.84) and FACS method (rho = 0.89) in PBMC. However, in whole blood, the correlation co-efficient for LILRA4 versus CLEC4C was higher for FACS (rho = 0.82) than for RT-PCR (cor = 0.7). It seems likely that the lower pDC frequency in whole blood, and the presence of additional cell types, increases the variability between samples and reduces the correlation coefficient.

Overall, correlation scores were high (Fig. 2), so we reasoned that the combined use of the two markers might further increase the precision of the method. To compare the two methods for pDC measurement, we correlated the mean frequency of $CLEC4C^+LILRA4^+CD14^-$ cells against the mean *LILRA4* and *CLEC4C* gene expression for each sample in PBMC and whole blood. Correlation analysis (Fig. 3A) showed that both samples correlated highly for FACS measurements (rho = 0.76, $p = 1.6e\text{-}06$), and similarly for RT-PCR samples (cor = 0.7, $p = 2.6e\text{-}05$). We concluded that both methods are accurate and that is likely to be relevant for studies with large sample sizes.

However, we saw no significant correlation between mean RT-PCR and FACS measurements in PBMC (cor = −0.019, $P = .31$) or whole blood samples (rho = 0.35, $P = .067$) to compare the relative pDC count of individuals. Since the whole blood sample correlation reached near significance, we then examined how individual gene expressions perform in whole blood. *CLEC4C* gene expression alone in whole blood samples correlated moderately with the mean frequency of $CLEC4C^+LILRA4^+CD14^-$ cells measured by FACS (Fig. 3B upper panel, rho = 0.39, $P = .037$). *LILRA4* gene expression was not significantly correlated with pDC measured by FACS (rho = 0.29,

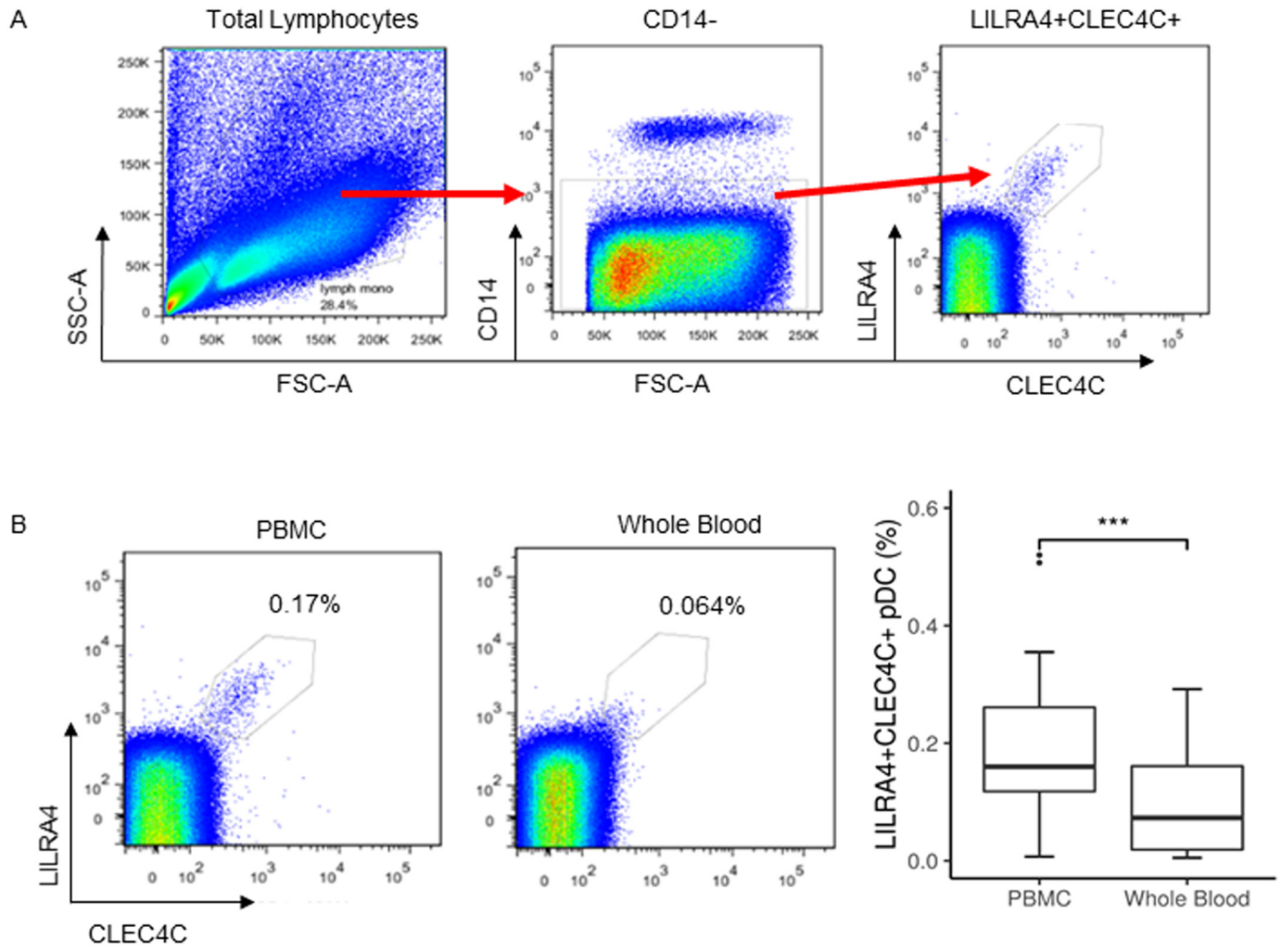

Fig. 1. Frequency of LILRA4 and CLEC4C expressing pDC in PBMC and whole blood. A) Top panels shows the gating strategy. Firstly, we gated on lymphocytes and monocytes based on forward and side scatter properties. $CD14^+$ cells were then excluded, and the percentage of $CLEC4C^+$ $LILRA4^+$ cells was analysed. B) The percentage of $CLEC4C^+LILRA4^+$ cells was enumerated in PBMC and whole blood. Note that LILRA4 + CLEC4C+ pDC were more frequent in PBMC than in whole blood (Mann–Whitney *U* test, *p*-value 1.2e-07, $n = 29$).

$P = .13$). *CLEC4C* gene expression alone correlated strongly between PBMC and in whole blood samples (cor = 0.72, $P = 2.8e\text{-}07$, Fig. 3B lower panel)) – at similar level to that seen with FACS (rho = 0.76, $p = 1.6e\text{-}06$, Fig. 3A upper panel. These results indicate that using both pDC markers for gene expression analysis is not beneficial. *CLEC4C* appeared to be a more robust marker than *LILRA4*, as its use alone showed the best correlation between the two methods (FACS vs RT-PCR) and showed stronger correlation between the two samples types (whole blood vs PBMC).

Because of research interest in pDC biology during viral infection, it is important that selected pDC markers are unaffected by the state of the pDC activation. In our results, CLEC4C performed consistently across the two samples types and methods but LILRA4 did not, especially when using RT-PCR on whole blood samples. The available evidence suggests that *LILRA4* gene expression may be more influenced by pDC activation status than *CLEC4C* gene expression (Cho et al., 2008; Xi et al., 2017) thereby making *LILRA4* gene expression unstable as a marker for estimating pDC numbers. Therefore technical factors and/or biological variability (e.g. subtle changes in cell activation induced by sample processing) could explain why *CLEC4C* gene expression appears to provide a more reliable estimate of pDC numbers than *LILRA4* gene expression. Moreover, we recognise that a low participant number could have limited the study. For use with larger studies, the method requires optimisation and measurement of a known cDNA standard measurement across batches as an internal control.

In conclusion, while flow cytometry remains the 'gold standard' for quantifying blood pDC, analysis of *CLEC4C* gene expression does provide a valid method for assessing relative pDC numbers across samples. Assessing *LILRA4* gene expression does not provide additional useful information. Here we provide evidence that FACS measurements of pDC and RT-PCR measurements of *CLEC4C* gene expression correlate to a reasonable degree, so we recommend the use of CLEC4C and LILRA4 surface staining or *CLEC4C* gene expression analysis for estimating pDC numbers in human blood samples. Our findings are relevant to researchers with an interest in measuring pDC numbers in large patient cohorts, especially in field studies where flow cytometry is not readily available.

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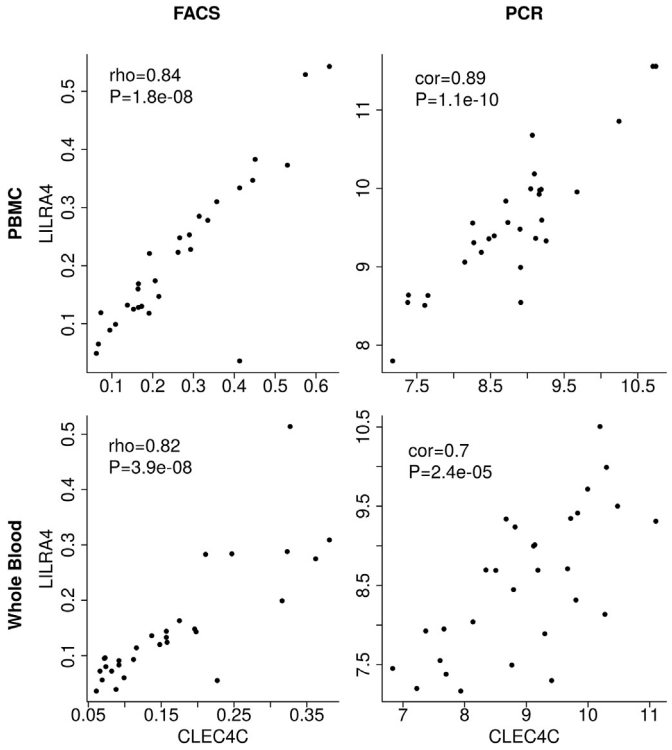

Fig. 2. Correlations between CLEC4C and LILRA4 as measured with FACS or with RT-PCR in PBMC or whole blood. Correlation coefficient is cor for Pearson correlation test performed for normally distributed PCR data and Rho for Spearman correlation test of non-parametric FACS data.

Declarations of interest

Authors have no conflicts of interest to disclose.

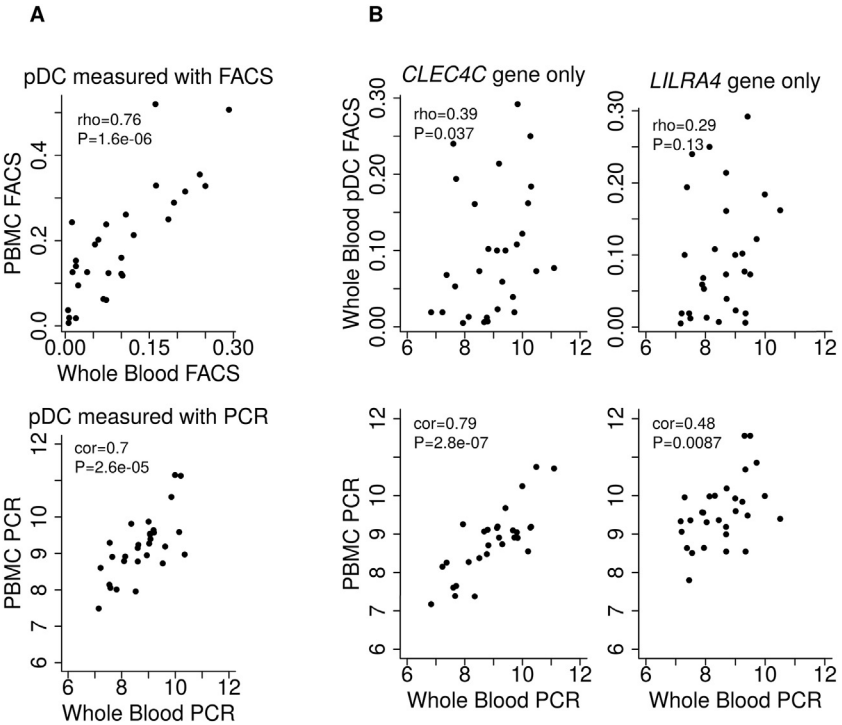

Fig. 3. Correlations between PBMC or whole blood with combined or single use of gene expression. A) Correlation between measures of pDC numbers in whole blood and PBMC with FACS above and PCR below. B: Correlation of pDC measurements using *CLEC4C* gene expression alone (left) or *LILRA4* gene expression alone (right) between whole blood PCR and FACS above or PBMC PCR below. (rho = Spearman correlation coefficient, cor = Pearson correlation coefficient, P = *P*-value, sample size $n = 29$).

Appendix A. Appendix

Table A1
Relative quantity of CLEC4C and LILRA4 gene expression was determined using delta Ct of CLEC4C and LILRA4 gene expression against the mean Ct of two reference genes B2M and UBC.

Gene	Direction	Primer sequence 5′–3′
LILRA4	Forward	*CTCGTGGTCTCAGGAGCAACT*
	Reverse	*CAGTCTTGGAATCTGACTTCTTTTGT*
CLEC4C	Forward	*CCTCTGTCTGACCCTGCAT*
	Reverse	*GCCAAGCCCTTAGATCCTT*
UBC	Forward	*GCAGTTCTTGTTTGTGGATCGCT*
	Reverse	*TGACATTCTCGATGGTGTCACTGG*
B2M	Forward	*AGGCTATCCAGCGTACTCCAAAGA*
	Reverse	*CGGATGGATGAAACCCAGACACAT*

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