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Highlights

- Cryopreservation is a common laboratory technique used for long term storage
- Cryopreservation alters the detection of epitopes by flow cytometry
- Care must be taken when interpreting expression of epitopes on thawed samples
Aberrant detection of phenotypic markers in chronic lymphocytic leukemia (CLL) lymphocytes following cryopreservation

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Abstract
The cryopreservation of peripheral blood mononuclear cells (PBMCs) is a routine research laboratory process, enabling long-term storage of primary patient blood samples. Retrospective analysis of these samples has the potential to identify markers which may associate with prognosis and response to treatment. In order to draw valid biological conclusions from this type of analysis, it is essential to ensure that any observed changes are...
directly related to the pathology of the disease rather than the preservation process itself. Therefore, we have investigated 15 cell surface markers which are relevant to chronic lymphocytic leukemia (CLL) on matched fresh and thawed samples to determine the effect of cryopreservation on their detection. We found that the number of CLL cells positive for the markers CD22, CD40, CD49d, CD54, CD69 and CXCR3 was significantly decreased following cryopreservation. In addition, the MFI of 10 of the 15 markers significantly changed following cryopreservation. These findings demonstrate that care must be taken when interpreting this type of analysis on thawed samples.

Introduction
In a diagnostic setting, quantifying the expression of membrane proteins by flow cytometry is generally carried out using fresh blood samples where the conformation of the epitope is likely to be in its native form. However, in a research environment, cells such as peripheral blood mononuclear cells (PBMCs) are often cryopreserved in liquid nitrogen and analysed as needed. Cryogenic preservation requires the addition of cryoprotective reagents, most commonly, dimethyl sulfoxide (DMSO), in order to protect the cells from damage during the preservation process. Despite its widespread use, there are only a limited number of studies analysing the effects of cryostorage of PBMCs (e.g. 2–5).

One of the many blood diseases which relies heavily on flow cytometry assessment is chronic lymphocytic leukaemia (CLL), a common malignancy of B-lymphocytes, with many studies reporting results of membrane analysis carried out on cryopreserved samples (e.g. 6–9). Due to its heterogeneous nature, novel membrane markers are routinely investigated in an attempt to identify those with good concordance in predicting disease course. In addition, new targets for chemoimmunotherapy are frequently based on cell surface molecules, often interrogated using cryopreserved samples from long-term storage.

To date, only one paper has specifically looked at the impact of cryopreservation on the CLL phenotype, albeit for a limited number of surface markers. This study has extended this using a panel of 15 novel markers not currently included in common CLL diagnostic panels, but implicated in playing important roles in cell migration/signalling and have been suggested as new prognostic markers.

Methods
Ethics consent, patient selection and characterisation
Peripheral venous blood samples were obtained in lithium heparin tubes from 30 treatment naïve patients by protocols approved by the Southern Adelaide Clinical Human Research Ethics Committee. Samples were processed within 24 hours.

Sample preparation
We examined whole blood, freshly isolated PBMCs, thawed PBMCs which had undergone cryopreservation for a minimum of 4 weeks and PBMCs which were thawed and cultured at
high density overnight (>1x10^7 cells/mL). Detailed information can be found in Supplementary Methods.

PBMCs were isolated by the addition of Lymphoprep™ (Axis-Shield, Oslo, Norway) followed by density centrifugation. Cells were washed twice with PBS, counted and resuspended in RPMI supplemented with L-glutamine, 1% pen-strep, 25% fetal calf serum (FCS) and 10% DMSO at a concentration of 4x10^7 cells/mL in cryopreservation vials. The cells were slowly frozen overnight at -80°C and transferred to vapour phase of liquid nitrogen.

*Preparation of thawed cells.* Cells were thawed rapidly at 37°C until no ice was present. Pre-warmed RPMI containing 10% FCS was added dropwise to the cells, followed by centrifugation at 500 x g for 5 minutes. The cells were washed with pre-warmed PBS and again centrifuged at 500 x g for 5 minutes. Cell viability was assessed by trypan blue analysis using an automated cell counter (TC20, BioRad, USA). Any samples with poor viability (<80%) were discounted from further analysis.

*Preparation of cultured cells.* The remainder of cells were cultured at high density into 24 well plates containing 1mL of RPMI supplemented with L-glutamine, 1% pen-strep, 10% FCS and left at 37°C in 10% CO₂ for 24-hours, at which point they were centrifuged and checked for viability as detailed above.

**Antibodies and reagents**
All monoclonal fluorochrome-labelled antibodies were titrated to determine the appropriate concentration. Prior to addition to the tubes, antibodies were diluted and 5µL of each dilution was added to a total of 5x10^5 PBMCs or 50µL of whole blood. For further details on the antibodies and staining methods refer to supplementary methods.

**Flow cytometry**
FACSCanto™ II flow cytometer and FACSDiva software (BD Biosciences) were used for acquisition and analysis of data. Spectral compensation was set up by using CompBeads (BD Biosciences). 50,000 total events from each tube were collected. Leukocyte subsets were first defined based on side scatter and CD45+, CLL cells were selected based on their dual expression of CD5+/CD19+. Apoptotic or dead cells, as well as cell debris were excluded from the analysis by using forward scatter to set a threshold.

**Data analysis**
Data was analysed in two ways; each marker was expressed as the percentage of positive (PP) cells and also as the mean fluorescence intensity (MFI). A detailed description of these methods can be found in Supplementary Methods. Differences in the MFI and PP cells between the samples were investigated using the statistical program PRISM (Graphpad Software, California, USA). A paired two-tailed t-test was carried out, and p-values <0.05 were considered significant.

**Results and Discussion**
We noted a slight decrease in CD19 and CD45 expression post-thaw, however this did not reach significance (p=0.135 and p=0.241 respectively, data not shown). Denyes et al<sup>10</sup> noted a decrease in CD5 expression post cryopreservation; our study did not observe a significant
change (p=0.594), possibility attributable to the use of an antibody against a different epitope. We noticed a small decrease in the average viability of the cells pre- and post-thaw from 95% to ~84% respectively which was expected due to the stresses of the cryopreservation process. Resting the cells for 24 hours at 37°C showed no significant change in cell viability. We confirmed that differences in staining intensity were not attributable to the addition of Ficoll®, used to isolate the PBMCs, as confirmed by other studies.

We analysed both MFI and PP cells following cryopreservation (Table 1) as analysing MFI alone is not sufficient due to the large variation which can be found between laboratories. Changes in MFI were noted for CD22, CD40, CD44, CD49d, CD54, CD62L, CD69, CD72, CXCR3 and CXCR4, all of which significantly decreased with the exception of CD44 and CD72 which increased. A statistically significant difference in the average PP cells between fresh and thawed samples was found with 6 markers: CD22, CD40, CD49d, CD54, CD69 and CXCR3. Four markers (CD11c, CD44, CD72 and CXCR5) were positive in all CLL samples analysed, revealing a high level of expression that was not affected by the freeze-thaw process.

Table 1: Cryopreservation induced changes in the PP and MFI of CD19+CD5+ CLL cells. PP values are expressed as the mean number of positive cells per sample for each marker ± SEM (%). MFI values were calculated as described in materials and methods. CD44 was measured with two antibodies targeted against two different epitopes (J.173 immunogen, BJ18 immunogen). Values are expressed as mean ± SEM. Statistically significant differences (p<0.05) are marked in bold. Fresh samples, n=21; thawed samples, n=20.

<table>
<thead>
<tr>
<th>Mean Positive cells (PP) (%)</th>
<th>MFI</th>
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<tbody>
<tr>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>CD11c</td>
<td>83.0 ± 27.9</td>
</tr>
<tr>
<td>CD18</td>
<td>9.9 ± 18.6</td>
</tr>
<tr>
<td>CD22</td>
<td>82.7 ± 23.1</td>
</tr>
<tr>
<td>CD25</td>
<td>6.0 ± 13.9</td>
</tr>
<tr>
<td>CD40</td>
<td>49.5 ± 39.8</td>
</tr>
<tr>
<td>CD44</td>
<td>99.5 ± 0.6</td>
</tr>
<tr>
<td>CD44*</td>
<td>99.0 ± 0.2</td>
</tr>
<tr>
<td>CD49d</td>
<td>41.0 ± 32.0</td>
</tr>
<tr>
<td>CD54</td>
<td>47.6 ± 28.8</td>
</tr>
<tr>
<td>CD62L</td>
<td>18.2 ± 20.2</td>
</tr>
<tr>
<td>CD69</td>
<td>7.7 ± 9.2</td>
</tr>
<tr>
<td>CD72</td>
<td>84.1 ± 13.7</td>
</tr>
<tr>
<td>CD81</td>
<td>17.7 ± 32.1</td>
</tr>
<tr>
<td>CXCR3</td>
<td>59.4 ± 25.1</td>
</tr>
<tr>
<td>CXCR4</td>
<td>21.0 ± 18.3</td>
</tr>
<tr>
<td>CXCR5</td>
<td>93.7 ± 21.3</td>
</tr>
</tbody>
</table>

As each patient sample was analysed both fresh and following cryopreservation, we were able to analyse the change within an individual sample. Importantly, there were marked individual variations that are not necessarily captured by the cohort mean change. In some cases, the
expression of a marker for an individual may increase whilst for others it may stay stable or decrease. Whilst the reasons for variability with the same marker for individual patients is not clear it is possible that the heterogeneous nature of CLL in terms of the different cytogenetic abnormalities and mutational events may render some cells more susceptible to cryoinjury than others.

Overnight resting of cells is known to be important to recover functional ability\(^\text{11}\) and has previously been reported to reverse the low observed frequency of some CD markers in T-cell subsets\(^\text{12, 13}\). Our analysis found that resting the cells for 24 hours post-thawing mitigated the effects of freezing for only 2 markers, CD69 and CXCR4, where the MFI and the number of PP cases increased back to the same levels seen in the fresh samples (Figure 1a and 1b). This was not seen for any of the other markers that showed significant changes following cryopreservation (e.g CD54, Figure 1c).

We observed a dramatic increase in the MFI of CD44 on thawed samples when analysed with the J.173 immunogen (Table 1, Figure 2a). This dramatic increase was present in all patient samples analysed (Figure 2b). When we used a second antibody raised against another epitope, BJ18, we noted a 2-fold decrease in MFI between fresh and thawed samples (Figure 2c) and this decrease was consistent across all patients (Figure 2d). This suggests that the epitope recognised by J.173 may change conformation either in the presence of DMSO or post-thaw, allowing it to be more accessible to the antibody (Figure 2).

This analysis has shown that differences in cell surface markers occur following cryopreservation of CLL PBMCs\(^\text{14-16}\). Conversely, others found that cryopreservation does not alter the antigen expression in leukemia cells from other haematological malignancies\(^\text{17, 18}\) and PBMCs\(^\text{12, 19, 20, 21}\). We can only speculate that the reason for the changes observed here are due to selective loss of cells due to thawing-induced sub-lethal damage, unknown alterations in the fragile surface-expressed molecules, loss of the epitope due to internalisation/cleavage, or a conformational change of the epitope following the thawing process. Other studies have observed similar changes, showing that epitopes are sensitive to the freeze/thaw process\(^\text{22, 23}\), can cleave or shed from the cell surface\(^\text{24}\), can be internalised in the presence of DMSO\(^\text{25}\) and that disruption to the actin cytoskeleton disruption following cryopreservation can interfere with the anchoring of membrane ligands\(^\text{26}\).

**Conclusion:**
We have shown that cryopreservation modifies the immunophenotypic profile of human CLL cells, in most cases resulting in the underestimation of antigen expression. The aberrant expression of several markers following the freeze/thaw process demonstrates that care must be taken when extrapolating results from thawed samples to fresh samples and these differences must be considered when carrying out retrospective analyses.


Figure 1: Resting of cells post-thaw has variable effects on restoring the detection of markers. Only 2 of the 15 markers examined were recovered by the resting process. The MFI of CD69 (A) and CXCR4 (B) both decreased significantly (p<0.05) following thawing but a brief 24-hour resting period in a high density culture returned the MFI levels back to that of fresh sample. A
representative marker where the post-thaw effects were not mitigated by a 24-hour culture period is shown in (C).

**Figure 2:** CD44 was measured in the primary CLL samples using two different antibody clones. First, with J.173 raised against a native purified CD44 from LAZ 221 cells which showed a statistically significant increase from ~7000 MFI to ~25000 MFI (p<0.05). All individual samples showed an increase, however the number of PP cases remained at 100% both pre- and post-thaw (data not shown). A second antibody, BJ18, raised against CD44 from human peripheral blood lymphocytes, showed a decrease in a smaller sample group (n=6) from ~28000 to ~17000 (p<0.05). All individual cases showed a decrease in MFI following cryopreservation. However, whilst the MFIs dropped, all samples again showed 100% positivity (data not shown).

**Supplementary Figure 1.** Flow chart showing a summary of the cell processing methods in this study. After collection of peripheral blood from CLL patients, the expression of all 15 markers was first examined on fresh blood. The PBMCs were then isolated using Lymphoprep™ and the same flow cytometry analysis panel was again carried out. To ensure that the addition of Lymphoprep™ did not cause changes in the detection of the epitope, we compared results from freshly isolated PBMCs with the same sample from whole blood. The PBMC sample then underwent cryopreservation for a minimum of 4-weeks in the presence of DMSO. The samples were then thawed using conventional techniques and again examined using the original method. There is some evidence that overnight resting of the samples at high density can mitigate the damaging effects of cryopreservation and possibly restore the confirmation of surface epitopes. To investigate this, we then repeated the same flow cytometry analysis for a final time.