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Technical Note

Effect of peripheral blood mononuclear cell cryopreservation on innate and adaptive immune responses

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ABSTRACT

Cryopreservation of blood-derived immune cells is commonly used in clinical trials to examine immunological responses. However, studies elucidating the effects of cryopreservation on peripheral blood mononuclear cell (PBMC) responses have shown inconsistent results making it difficult to draw meaningful conclusions. Therefore we sought to address this issue by comparing key innate and adaptive immune parameters between freshly-isolated and cryopreserved PBMCs from healthy adults. We examined the effect of cryopreservation on the expression of key markers on innate and adaptive immune cell populations (i.e. CD4+ and CD8+ [T cells], CD14+ [monocytes], CD19+ [B cells], CD56+ [NK cells] or CD19 + CD27+ [memory B cells]), on cytokine secretion (TNF-α, INF-γ, IL-1β, IL-10, IL-6, MCP-1 and RANTES) in cultured PBMC supernatants following stimulation with a range of Toll-like receptor (TLR) agonists, as well as on antigen-specific memory B cell enumeration by ELISpot. We found that cryopreservation had no effect on the expression of immune markers on innate and adaptive immune cells as well on the number of antigen-specific memory B cells. However, the response to TLR ligands such as FLA-ST, CpG and LPS was variable with increased cytokine production by cryopreserved PBMCs observed compared to freshly-isolated PBMCs. Our results suggest that the effect of cryopreservation on the biological response of immune cell populations needs to be carefully considered, particularly in the context of clinical studies that rely on these immune outcomes.

1. Introduction

Clinical trials focusing on immunological responses, particularly in the context of infection and vaccination, rely on the use of PBMCs for downstream functional assays (Spentzou et al., 2010; Bourguignon et al., 2014). Since many of these types of trials are conducted internationally and often across multiple sites, cryopreservation of PBMCs is commonly undertaken to enable efficient transport and analysis at partner laboratories (Golab et al., 2013; Ford et al., 2017; Sambor et al., 2014). Furthermore, cryopreserved PBMCs can be stored for long periods of time, allowing immunological studies able to be conducted years following collection for some large clinical trials.

The use of PBMCs are indispensable to dissect important innate and adaptive immune responses (Truck et al., 2014). A number of immune cells in PBMCs express TLRs. These receptors are a type of pattern recognition receptor (PRR) that recognise conserved pathogen structures and are part of our first line defence against a diverse array of infectious and endogenous agents (Ida et al., 2006; Klonowska-Szymczyk et al., 2014; Wong et al., 2010). The activation of TLRs is an important component of vaccines and vaccine adjuvants as stimulation of PRRs, including TLRs, leads to initiation of innate immune responses causing the release of cytokines such as interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) that recruit innate immune cells (i.e. natural killer (NK) cells and macrophages) (Siednienko & Miggin, 2009) and subsequently activate the adaptive immune response (Coffman et al., 2010). Adaptive immunity consisting primarily of T-cells and B-cells is important in resolving infection and critical to the success of current vaccines. T-cells can be classified into T-helper cells (CD4+) and cytotoxic T-cells (CD8+), which can activate other immune cells (i.e. B-cells) and clear pathogens, respectively. B-cells produce antigen-specific antibodies that neutralise the pathogen or facilitate clearance by phagocytes. A small proportion are memory cells which respond more rapidly and at higher magnitude to previously encountered antigens (Coffman et al., 2010; Iwasaki & Medzhitov, 2015; Sarkander et al., 2016). Therefore, it is important to determine whether cryopreservation has any effects on these responses.

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Immunological assays such as multiplex bead array, flow cytometry and ELISpot are routinely used for examination of functional immunity and rely almost exclusively on the use of PBMCs (Honge et al., 2017). As these assays are usually performed ex vivo, multiple factors can affect the results, including sample processing, cryopreservation, storage and thawing (Nazarpour et al., 2012). There is some evidence that suggests cryopreservation of PBMCs may have unintended consequences on their responsiveness (Li et al., 2009; Luo et al., 2017; Yang et al., 2016; Angel et al., 2016). Therefore, it is important that the data obtained from studies using either freshly-isolated or cryopreserved PBMCs are reliable so that comparison and interpretation of results between studies can be done with confidence.

This study aimed to determine the effect of PBMC cryopreservation on a number of critical innate and adaptive immune parameters using several methodologies that are commonly used in clinical studies.

2. Methods

2.1. Study samples

Blood samples for the flow cytometry and multiplex cytokine data were collected from 12 healthy adults following informed consent, comprising six males and six females, from the Murdoch Children's Research Institute, Melbourne, Australia. A single blood sample (~10 mL) was collected from each individual into a sodium heparin tube. The 'fresh' sample was collected 1–12 months after the 'cryopreserved' sample. Blood samples for the enumeration of memory B cells were collected from 12 month old infants ($N = 6$) who were enrolled in a separate ethically-approved study. Paired samples of freshly-isolated and cryopreserved PBMCs were cultured at the same time for all assays except for the enumeration of memory B cells due to study requirements. Ethical approval for both studies was obtained from the Royal Children's Hospital Human Research Ethics Committee (HREC) - 35253 for the healthy adult study and 31010B for the infant study.

2.2. Peripheral blood mononuclear cell (PBMC) isolation and cryopreservation

The PBMCs were isolated immediately after blood collection by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) at 400 ×*g* for 30 min without brake at room temperature (RT). Isolated PBMCs were then washed twice in RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Utah, USA), 1000 IU penicillin-streptomycin (Sigma-Aldrich, St. Louis, USA) and 200 nM L-glutamine (RPMI-FBS) (Sigma-Aldrich, St. Louis, USA) at 500 ×*g* for 10 min at RT. The cells were resuspended in 1 mL RPMI-FBS and the concentration was determined by trypan blue exclusion. For cryopreservation, PBMCs were diluted in a 1:1 ratio of 15% dimethyl sulfoxide/85% FBS (freezing mix) and RPMI-FBS media and aliquoted at a concentration of 1×10^7 cells/mL. PBMCs were transferred to isopropanol containers (Mr. Frosty™; Thermo-Fischer Scientific, Dreieich, Germany) and stored at -80 °C for 24 h, before being transferred to vapour-phase liquid nitrogen storage until use.

2.3. Flow cytometry

Freshly-isolated and cryopreserved PBMCs were washed with RPMI-FBS by centrifugation at 500 ×*g* for 10 min, and then resuspended in PBS supplemented with 0.1% Sodium azide (Australian Chemical Reagents, Gillman, Australia) and 2% FBS (flow buffer). PBMCs were washed before 100 µL of the Zombie Aqua™ Fixable Viability dye (BioLegend, San Diego, CA, USA) was added and incubated for 10 min in the dark. After this, PBMCs were resuspended in flow buffer and then washed by centrifuging for 10 min at 500 ×*g*. The PBMCs were then incubated with the following fluorescently-conjugated monoclonal antibodies CD4-PE, CD8-FITC, CD14-BV480, CD19-APCH7, CD27-APC, CD56-BV421 (BD Biosciences, San Jose, CA, USA) in the dark for 30 min at RT. After staining, PBMCs were washed and resuspended in flow buffer, then read on the BD LSR Fortessa X-20 flow cytometer and analysed using FlowJo, LLC v10.4.2 software. Compensation of spectral overlap was performed using BD CompBeads, with the individual antibodies used in the experiment, and an unstained tube was used as a control. A minimum of 100,000 events was recorded for each experiment. Representative gating strategies used are shown in Supplementary Fig. 1.

2.4. PBMC stimulation assays

Freshly-isolated and cryopreserved PBMCs were adjusted to a final concentration of 1×10^6 cells/mL and stimulated with 1 ng/mL lipopolysaccharide (LPS), 10 µg/mL CpG, 100 ng/mL FLA-ST, 5 µg/mL imiquimod (IMQ), 100 ng/mL FSL-1 (all from InvivoGen, San Diego, USA) or respiratory syncytial virus (RSV) at a multiplicity of infection = 1 (MOI = 1) (kindly provided by Department of Chemistry and Molecular Biology, University of Queensland, Australia) for 24 h at 37 °C, 5% CO_2. Supernatants were harvested and stored at −80 °C until cytokine assay.

2.5. Multiplex cytokine bead array

Measurement of TNF-α, INF-γ, IL-1β, IL-10, IL-6, MCP-1 and RANTES in PBMC supernatants were analysed using a commercial multiplex bead array kit according to the manufacturer's instructions (Milliplex; Millipore Corporation, WA, Aus). The multiplex bead array plate was read on a Luminex 200 instrument (Luminex; Texas, USA) and results were reported by interpolation from the standard curve in pg/mL from recorded Mean Fluorescence Intensity. Data was analysed using Xponent LX100/LX200 software.

2.6. Enumeration of antigen-specific memory B cells

Enumeration of tetanus toxoid and diphtheria toxoid-specific memory B cells was done according to a previously published method (Licciardi et al., 2016). Briefly, freshly-isolated PBMCs were resuspended in RPMI-FBS at a concentration of 2×10^6 cells/mL and were stimulated with an antigen cocktail (*Staphylococcus aureus* Cowan strain – Pansorbin cells [SAC; 1:5000], 2.5 µg/mL CpG and 83 ng/mL pokeweed mitogen) for 6 days at 37 °C, 5% CO_2. At day 6, cells were harvested and were resuspended in RPMI-FBS at a final concentration of 2×10^6 cells/mL before seeded onto ELISpot plates coated with anti-IgG (10 µg/mL), tetanus toxoid (5 µg/mL) and diphtheria toxoid (10 µg/mL) and incubated overnight (16–20 h) at 37 °C with 5% CO_2. The ELISpot plates were developed using an alkaline phosphatase-conjugated IgG and the reaction developed with alkaline phosphatase substrate solution and counted using an automated ELISpot reader and software (iSpot reader, AID Strassberg, Germany).

2.7. Statistical analysis

For cytokine and flow cytometry data, the geometric mean ± 95% confidence intervals (CI) were presented. Memory B cell data was presented as mean ± 95% CI. Continuous data were compared using a paired *t*-test between freshly-isolated and cryopreserved PBMCs. Memory B-cell data were compared using a Wilcoxon sign-ranked test. The data was graphically represented and statistically analysed using GraphPad prism 7 software (GraphPad Software Inc.; California, USA). All tests performed were two-tailed and a p-value < .01 was considered statistically significant on account of multiple comparisons.

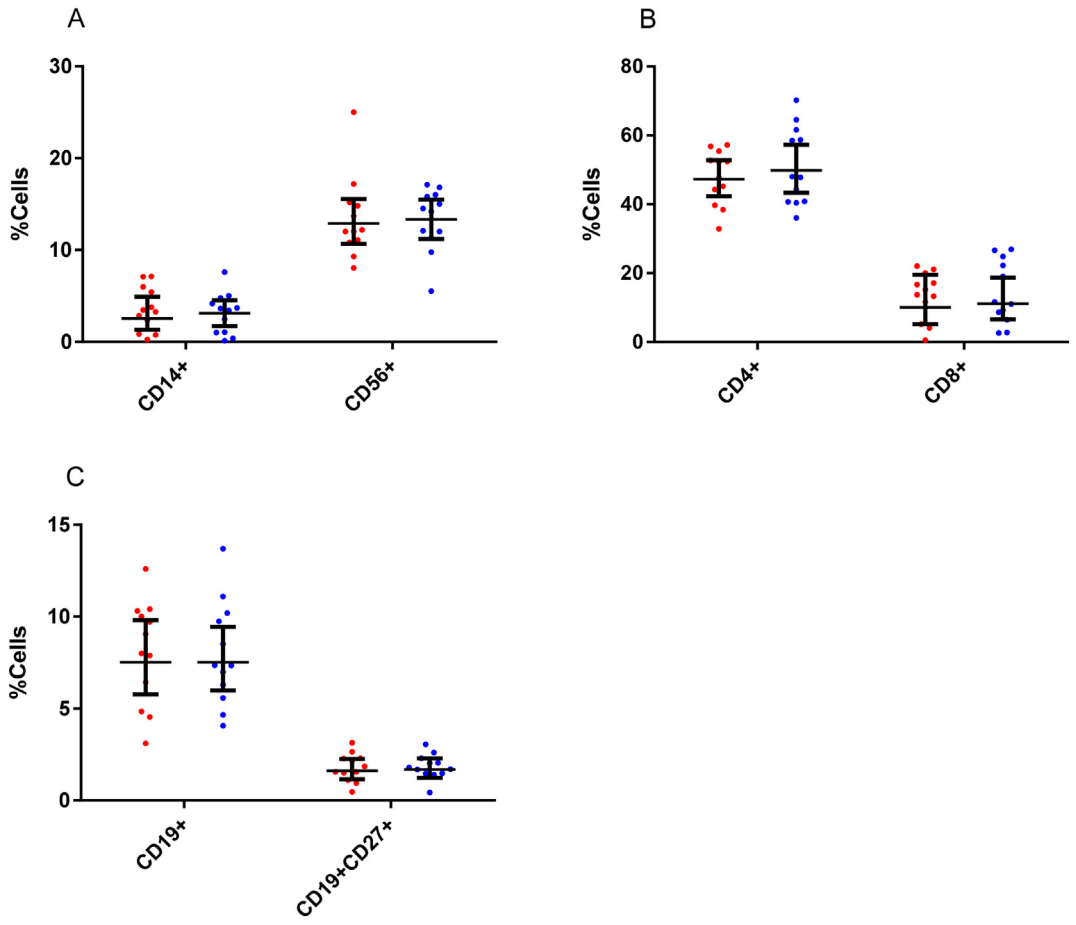

Fig. 1. Cryopreservation of PBMCs does not influence the expression of markers on key cell subsets involved in the innate and adaptive immune response. Paired freshly-isolated and cryopreserved PBMCs were incubated for 24 h prior to phenotyping by flow cytometry. Data points in red represent freshly-isolated PBMCs whilst data in blue represent cryopreserved PBMCs. A) Innate cells, CD14 +, CD56 + B) T cells, CD4 +, CD8 + C) B cells, CD19 +, CD19 + CD27 +. All data were gated on the live lymphocyte population (% Cells) and captured with a minimum of 100,000 events. Data represents geometric mean ± 95% CI; n = 12 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Influence of cryopreservation on the frequency of innate and adaptive immune cell populations

To determine how cryopreservation effects the expression of key immune cell markers, both freshly-isolated and cryopreserved unstimulated PBMCs were phenotyped by flow cytometry. No significant differences were observed in the proportions of each of the immune cell populations examined between freshly-isolated and cryopreserved PBMCs for innate immune cells; monocytes (CD14 +) and NK cells (CD56 +) or adaptive immune cells; T cells (CD4 +, CD8 +), B cells (CD19 +) and memory B cells (CD19 + CD27 +) (Fig. 1A–C). This was also true when comparing median fluorescence intensity data for each of these immune markers between freshly-isolated and cryopreserved PBMCs (data not shown).

3.2. Influence of cryopreservation on cytokine secretion following TLR stimulation

We next examined the effect of cryopreservation on cytokine and chemokine secretion following stimulation with a panel of TLR ligands comprising both bacterial and viral antigens. Cryopreservation in general led to increased cytokine and chemokine responses (Fig. 2). Moreover, the increased cytokine production by cryopreserved PBMCs was associated mainly with stimulation by bacterial ligands (e.g. LPS, CpG, FLA-ST, FSL-1) compared to viral ligands such as RSV or IMQ. In contrast, there were no differences in IFN-γ secretion for any of the TLR ligands used (Fig. 2A). Stimulation with FLA-ST, a TLR5 ligand, led to increased cytokine production for 4 out of the 7 cytokines/chemokines tested, with increased IL-10 ($p = .004$), TNF-α ($p = .004$), MCP-1 ($p = .0007$) and RANTES ($p = .009$) compared to freshly-isolated PBMCs (Fig. 2C, E, F, G). In comparison, CpG stimulation led to 3-fold increases in the level of IL-6 ($p = .002$) and MCP-1 ($p < .0001$) (Fig. 2B, F), whilst LPS stimulation were found to increase IL-1β levels only in cryopreserved PBMCs ($p = .002$; Fig. 2D).

For the viral ligands, cryopreserved PBMCs stimulated with live RSV produced a 2-fold increase in IL-6 ($p = .005$) and 4-fold increase in IL-1β ($p = .006$) levels (Fig. 2B, D) whereas there was no differences in response to IMQ. In the absence of stimulation, cryopreserved PBMCs secreted a higher amount of MCP-1 compared to freshly-isolated PBMCs ($p = .0004$; Fig. 2F).

3.3. Influence of cryopreservation on total and antigen-specific B-cell memory

Given that measurement of antigen-specific memory B cells is a critical aspect of vaccine immunogenicity assessments for clinical trials, we wanted to determine whether cryopreservation had any impact on this response. We found that cryopreservation had no impact on the number of antigen-specific IgG secreting cells (as a marker of circulating memory B cells) for common vaccine antigens diphtheria and

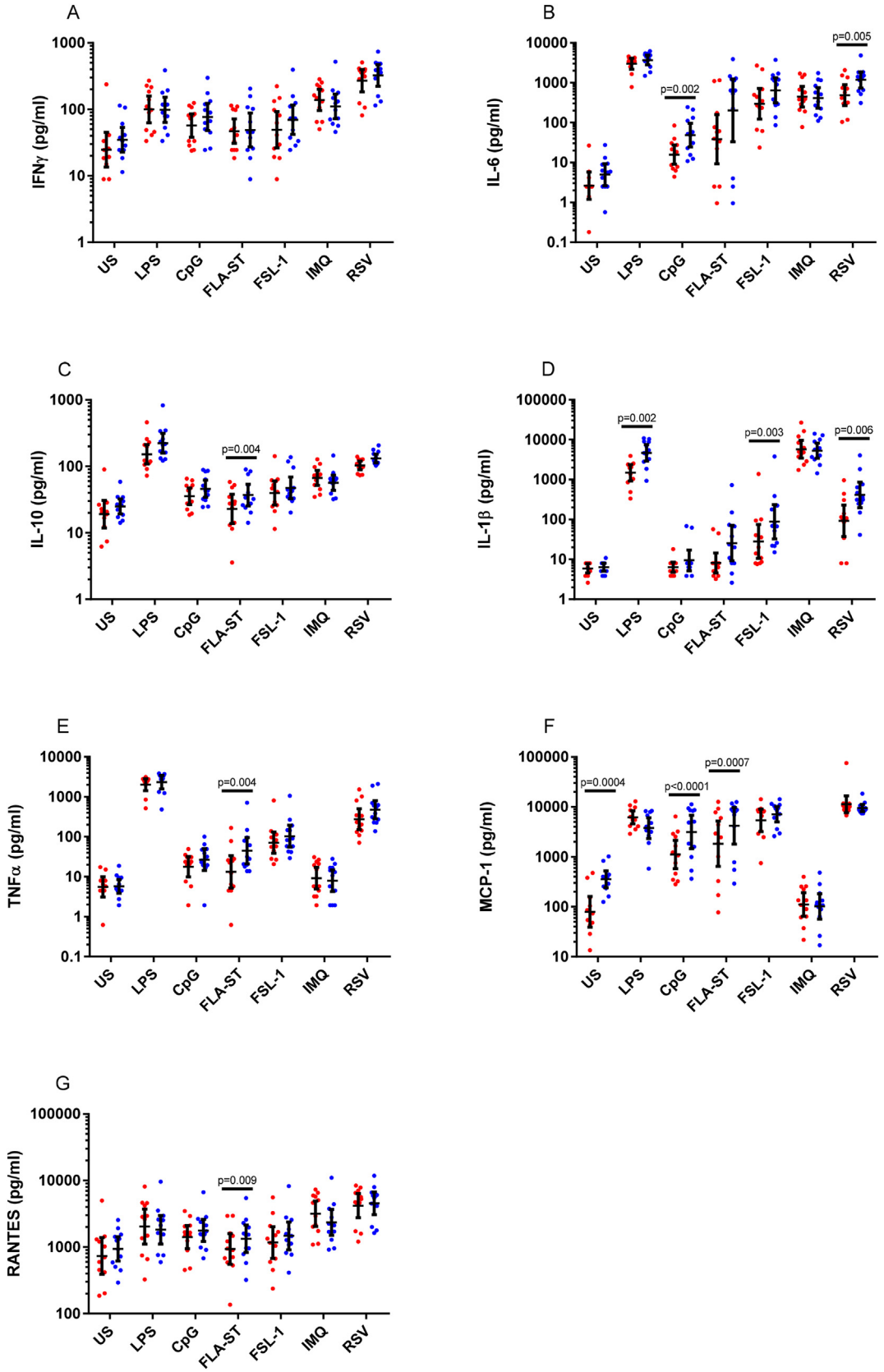

Fig. 2. Cryopreservation of PBMCs increases cytokine responses to TLR ligands. Paired freshly-isolated and cryopreserved PBMCs were stimulated with LPS (1 ng/mL), CpG (10 μg/mL), FLA-ST (100 ng/mL), FSL-1 (100 ng/mL), IMQ (5 μg/mL), or RSV (MOI = 1) for 24 h prior to collection of supernatants. Data points in red represent freshly-isolated PBMCs whilst data in blue represent cryopreserved PBMCs. A) IFN-γ B) IL-6 C) IL-10 D) IL-1β E) TNF-α F) MCP-1 G) RANTES concentration were measured via multiplex assay. Data represents geometric mean concentration ± 95% CI; n = 12 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

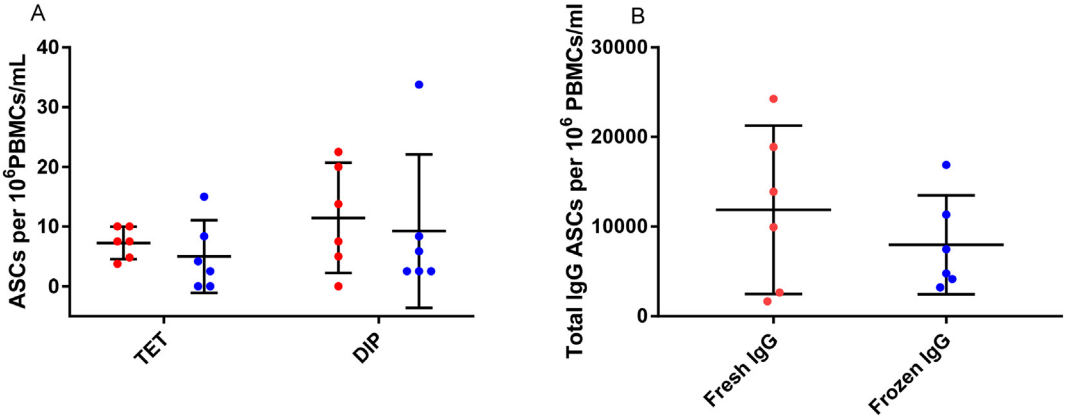

Fig. 3. Cryopreservation of PBMCs does not affect the number of total and antigen-specific IgG antibody-secreting cells (ASCs) detected by Elispot. PBMCs were stimulated with an antigen cocktail and then seeded onto ELISpot plates coated with anti-IgG, tetanus toxoid and diphtheria toxoid. Data points in red represent freshly-isolated PBMCs whilst data in blue represent cryopreserved PBMCs. A) Tetanus- and diphtheria toxoid-specific IgG ASCs and B) Total IgG ASCs per 10^6 cells/mL. Data represents mean ± 95% CI; n = 6 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tetanus toxoids, as well as the total number of IgG secreting B cells (Fig. 3A, B).

4. Discussion

The use of PBMCs is essential in understanding innate and adaptive responses involved in infection and immunity. As clinical trials often require the cryopreservation of PBMCs to enable assay and analysis at partner laboratories, it is important to know the effects of cryopreservation on PBMCs responses, given that there is mixed evidence suggesting cryopreservation affects key cell subsets and their function (Truck et al., 2014; Lemieux et al., 2016).

Reliable detection of T-cell and B-cell responses are an essential aspect of clinical trial research of vaccines and other immunomodulatory therapies. Our data found that cryopreservation did not modulate the expression of key innate and adaptive immune cell markers, finding no differences between freshly-isolated and cryopreserved PBMCs expressing CD4+, CD8+, CD14+, CD19+, CD27+ and CD56+. Similar studies have also observed no effects of cryopreservation in PBMC populations, consistent with our data (Luo et al., 2017; Lemieux et al., 2016). However, a study by Ford et al. (2017) showed that IFN-γ, IL-2 and TNF-α secretion was reduced in frozen CD3 + CD4+ T-cells in individuals immunised with heterologous viral vectors for malaria, suggesting that secretions may be reduced (Ford et al., 2017). We also observed that cryopreservation had no effect on the number of total, tetanus- or diphtheria toxoid-specific IgG ASCs, in infants. These results were in agreement with Truck et al. (2014) who also demonstrated that memory B-cells, from adults, specific to diphtheria and tetanus toxoids did not differ between freshly-isolated and cryopreserved PBMCs (Truck et al., 2014), suggesting that memory-B cell responses remain unaffected by cryopreservation. Many large clinical trials rely on shipment of cryopreserved PBMCs to partner laboratories for downstream immunological analysis of innate and adaptive immune cell enumeration and function. These data therefore lend support to the use of cryopreserved PBMCs in clinical trials, particularly those evaluating vaccine immunogenicity or based on immunophenotyping data (Jeurink et al., 2008; Gilbert, 2012; Koup & Douek, 2011; De Vincenzo et al., 2014).

In contrast to findings on the proportions of innate and adaptive immune cells, we found variable effects of cryopreservation following stimulation of PBMCs with a panel of TLR ligands. Studies have shown that cytokine responses are variable following ligand stimulation of cryopreserved PBMCs or when left unstimulated (Jeurink et al., 2008; Kreher et al., 2003; Mallone et al., 2011). A study by Blimkie et al. (2011), found that stimulation of TLR2, TLR4, TLR7 and TLR8 elicit altered cytokine responses following cryopreservation, with more potent ligands exhibiting greater differences (Blimkie et al., 2011). However, in our study, we observed a larger range of TLRs and found that stimulation of cryopreserved PBMCs led to increased production of cytokines and chemokines in general, irrespective of the stimulus used. This was mostly observed for bacterial ligands, suggesting that bacterial TLRs are more susceptible to cryopreservation than viral TLRs. This is most likely due to the fact that receptors for bacterial ligands are mostly expressed on the cell surface, whilst viral receptors are expressed at the cell surface as well as intracellularly (Grove & Marsh, 2011). This may explain these differences observed as intracellular receptors may be protected from cryopreservation. In contrast, a study by Meijerink et al. (2011) showed that whilst cryopreservation broadly led to increased IL-10 secretion, IL-12, IL-1β and TNF-α from immature dendritic cells and monocytes was decreased following stimulation with multiple bacterial ligands (Meijerink et al., 2011). One explanation for our findings could be that cryopreservation modulated TLR expression. This is supported by a recent study showing that damage to PBMC deoxyribonucleic acid, which occurs through cryopreservation, leads to broad up-regulation of TLR gene expression (Yang et al., 2016; Menendez et al., 2011). Whilst this suggests a mechanism for the increased inflammatory responses observed in our study, the authors did not relate their gene expression data to functional immunological readouts, limiting the interpretation of these results. Changes in immune function at the protein level as a result of cryopreservation is biologically significant and of great interest to researchers working with these samples.

Accurate measurement of cytokine responses is critical. Increased cytokine responses are often important in determining associations with infectious disease outcomes. For example, exacerbated IL-17A during RSV infections has been associated with increased allergic responses in asthmatics (Mukherjee et al., 2011) and increased pro-inflammatory cytokine responses during pneumonia are often associated with increased morbidity and mortality (Bordon et al., 2013). Therefore, our data suggests cytokine responses associated with increased morbidity and mortality should be carefully interpreted as this is inevitable through cryopreservation. This is important as identifying these patterns of immune responses commonly occurs in target populations, usually developing countries, where risk of disease is much higher. As resources to complete full analysis of samples are often limited in these areas, cryopreservation of PBMCs is required to send to partner laboratories, often in different countries (Shi et al., 2017).

One of the advantages of this study was the use of a panel of TLR-specific ligands. Previously, many studies utilised potent TLR agonists

such as LPS or non-TLR ligands such as PMA, which produce excessive cytokine production (Blimkie et al., 2011). These potent agonists may potentially mask any subtle effect of cryopreservation, particularly through non-TLR pathways in the case of PMA. Another strength of this study was the evaluation of innate and adaptive responses that are commonly investigated in many human studies. Determining the effect on both bacterial and viral pathogens has important implications for clinical trials.

A limitation of our study is that TLR expression was not measured in our samples, which would have allowed for a better understanding of the mechanism for the increased cytokine secretion observed in cryopreserved PBMCs. Furthermore, whilst the relatively small sample size for our memory B-cell data limits our ability to draw conclusion on cryopreservation, our results were consistent with previous studies examining this effect (Ford et al., 2017).

5. Conclusion

Cryopreservation of PBMCs is a common method used in clinical studies worldwide to examine immune responses. Using a range of TLR ligands, we found that cryopreservation had variable effects on cytokine secretion, but did not modulate antigen-specific B-cell responses or the expression of innate and adaptive immune cell markers on PBMCs. Further studies aimed at identifying the mechanism for these effects is important and would greatly assist the design and interpretation of future clinical trials.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.11.006.

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