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Stabilization of leukocytes from cerebrospinal fluid for central immunophenotypic evaluation in multicenter clinical trials

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ABSTRACT

Analysis of cerebrospinal fluid (CSF) represents a valuable window into the pathogenesis of neuroinflammatory diseases, such as multiple sclerosis (MS). However, analysis of the cellular fraction of CSF is often neglected because CSF cells die rapidly ex vivo. Immunophenotyping of CSF cells in multicenter clinical trials requires sample preservation and shipping to a centralized lab. Yet, there is no consensus on the best method to preserve intact CSF cells and no detailed evaluation of subset-specific cell loss.

We used flow cytometry to compare major leukocyte populations in fresh CSF (processed within 2 h) to cells fixed for 48 h with TransFix-EDTA® or cryopreserved and thawed after 96 h. We observed a statistically significant loss of total mononuclear cells, total T cells, CD3+ CD8- T cells, and CD3+ CD8+ T cells after cryopreservation compared to fresh or fixed ($p < 0.001$), with no significant difference between fresh and fixed. Thus, our results demonstrate that TransFix-EDTA® was superior to cryopreservation for preserving intact CSF T cells. Surprisingly, neither cryopreservation nor fixation had a significant effect on recovery of low frequency cell subsets in CSF, including B cells, NK cells, NKT-like cells, CD14+ monocytes, or CD123+ DCs, versus fresh CSF.

To determine the effect of prolonged fixation on cell recovery, we analyzed major CSF cell subsets by flow cytometry after 24, 48, or 72 h of fixation with TransFix-EDTA®. We observed a consistent and progressive loss in the absolute counts of all subsets over time, although this effect was not statistically significant.

We conclude that for immunophenotyping of major CSF cell subsets by flow cytometry, fixation with TransFix-EDTA®, shipment to a central lab, and analysis within 48 h is a feasible method to ensure stability of both absolute cell number and relative frequency. This method is a valuable alternative to fresh CSF analysis and can be implemented in multicenter clinical trials.

1. Introduction

Cerebrospinal fluid (CSF) surrounds the parenchyma of the central nervous system (CNS) and sampling the CSF by lumbar puncture (LP) represents a feasible method to characterize mediators of inflammatory diseases of the CNS. (Craig et al., 2011; Stangel et al., 2013; Ousman and Kubes, 2012; Stolp et al., 2013; Rahimi and Woehrer, 2018). Immunophenotyping of CSF cells presents a valuable opportunity to characterize the immune profile of multiple sclerosis (MS) and other neuroinflammatory diseases, with the potential to elucidate mechanisms of

Abbreviations: BEAT-MS, Best Available Therapy Versus Autologous Hematopoietic Stem Cell Transplant for MS, ClinicalTrials.gov # NCT04047628; CNS, central nervous system; CSF, cerebrospinal fluid; FBS, fetal bovine serum; FDR, false discovery rate; IIH, Idiopathic Intracranial Hypertension; LP, lumbar puncture; MAIT, mucosal-associated invariant T cells; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SOP, standard operating procedure.

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pathophysiology, improve diagnostics, and find predictors of disease progression or response to therapy, and as such should become common practice in clinical research. As a variety of new immunomodulatory therapies become available for MS, a precision medicine approach that enables evidence-based, individualized treatment decisions is needed, but progress hinges on biomarker discovery and validation (Rotstein and Montalban, 2019). A growing body of literature has demonstrated abnormalities in CSF cellular composition during CNS disease using flow cytometry (Alvermann et al., 2014; Han et al., 2014; Rodriguez-Martin et al., 2015; Schafflick et al., 2020), but technical hurdles have limited standardized application to large multicenter studies, resulting in lost opportunities to gain valuable insight (Stuve et al., 2006; Kowarik et al., 2011; Alvermann et al., 2014; Harrer et al., 2015; Komori et al., 2017).

Characterization of the cellular fraction of CSF remains challenging due to the invasiveness of LP, limited sampling volume, low cell density, and most notably the fragility of CSF cells. CSF cells die rapidly ex vivo and must be processed immediately to accurately capture the frequency and phenotype of major leukocyte populations (Subira et al., 2002; Kivisakk et al., 2003; Kraan et al., 2008; Craig et al., 2011; de Graaf et al., 2011a). The importance of timely processing was shown by de Graaf et al. (de Graaf et al., 2011b), where approximately 50% of leukocytes were lost from fresh CSF in as little as one hour, both by flow cytometric and microscopic cell counts. While cryopreservation is commonly used to preserve peripheral blood mononuclear cells, similar methods are not widely used for CSF cells. In rare instances where cryopreserved CSF cells are used there is no consensus on the best method and no detailed evaluation of the effects of cryopreservation on cell yield as compared to fresh CSF (reviewed in Oh et al., 2021). For this reason, most studies utilizing flow cytometry of CSF cells are limited to a single site, and only occur at sites with a clinic in close proximity to a research lab with the ability to analyze samples within a few hours of collection. Furthermore, for multicenter clinical trials to produce reliable data with minimal batch effect, best practice suggests all samples should be stained and analyzed by one centralized flow cytometry facility, which requires a reliable standardized cell preservation method with minimal loss of cell count and staining intensity of immunophenotyping markers.

Several studies have evaluated stabilization of CSF cells with TransFix-EDTA® (Cytomark, Buckingham, UK) for detection of leptomeningeal localizations of hematological malignancies by flow cytometry (Sedek et al., 2020), but this method has yet to be evaluated or adopted within the neuroinflammatory disease field. Moreover, literature evaluating methods of cell preservation in CSF is sparse and contradictory. According to de Jongste et al., the absolute number of leukocytes in CSF was 2.3 times higher after 18 h in TransFix-EDTA® versus fresh CSF, and lymphocytes were selectively lost from fresh CSF faster than monocytes or granulocytes (De Jongste et al., 2014). These findings contradict those of Dux et al., which suggest that lymphocytes are the most stable subset in CSF (Dux et al., 1994). Before TransFix-EDTA® stabilization can be implemented in large multicenter studies of neuroinflammatory disease, additional work is needed to determine the potential for skewing of relative cell frequencies due to selective loss of specific lymphocyte subsets over a longer timeline. Similarly, while cryopreservation has some advantages over fixation, no published data is available comparing the effects of cryopreservation and fixation on recovery of specific CSF cell subsets by flow cytometry.

Our goal was to establish a standardized method of preserving CSF cells for immunophenotyping by flow cytometry at a centralized facility that could be incorporated across multiple sites and multiple clinical trials studying neuroinflammatory diseases such as MS. We compared absolute counts and relative frequencies of CSF cells stabilized with TransFix-EDTA® or cryopreserved to the "true" cellular makeup of fresh CSF leukocytes. We further compared results from 24, 48, and 72 h after CSF cell fixation to determine the relative cell loss over time. Based on our observations in MS, we propose the following guidelines, which have been successfully implemented in a multicenter study of MS (Best Available Therapy Versus Autologous Hematopoietic Stem Cell Transplant for MS (BEAT-MS), ClinicalTrials.gov # NCT04047628) (i) immunophenotyping CSF cells after the cryopreservation method tested here is not recommended due to substantial cell loss as compared to fresh CSF; (ii) we recommend analysis of CSF cells in TransFix-EDTA® within 48 h due to increased cell loss between 48 h and 72 h. Importantly, 48 h should allow sufficient time for shipment to a centralized flow cytometry facility, staining, and acquisition.

2. Materials and methods

2.1. Patients and CSF samples

CSF was collected from patients undergoing diagnostic LP for MS or therapeutic LP for Idiopathic Intracranial Hypertension (IIH) at the neurology clinic of the Perelman Center for Advanced Medicine (Philadelphia, Pennsylvania, USA) and from patients undergoing diagnostic LP for MS at the Ramon y Cajal University Hospital (Madrid, Spain). CSF cell yields were higher, on average, for samples from the Spanish site than for the US site, which is consistent with the expectation that MS patients have higher CSF cellularity than IIH patients (Supplementary Table A1). All patients provided written informed consent. CSF samples ranged from 5 to 10 mL in total volume. A range of 300–30,000 CSF cells per condition was used to assess three different processing conditions: fresh ($n = 12$), TransFix-EDTA® (n = 12), and frozen ($n = 11$). A range of 5000–30,000 CSF cells per condition was used to assess three different TransFix-EDTA® conditions: 24 h ($n = 6$), 48 h (n = 6), and 72 h (n = 6).

2.2. Antibodies and staining

For cell surface staining, the following eight-colour flow cytometry panel was used (all antibodies from BD Biosciences): PerCP-Cy5.5 anti-CD3 (clone SK7), FITC anti-CD14 (clone MøP9), APC-H7 anti-CD8 (clone SK1), APC anti-CD56 (clone NCAM16.2), V500 anti-CD45 (clone HI30), BV421 anti-HLA-DR (clone G46–6), PE-Cy7 anti-CD19 (clone SJ25C1), and PE anti-CD123 (clone 7G3). Antibody clones and fluorophores were selected based on published compatibility with TransFix-EDTA®, available on the manufacturer's website (www.Cytomark.co.uk/transfix).

All CSF cells were stained for 20 min on ice, washed once with 500 μl phosphate buffered saline (PBS), and centrifuged. Cell pellets were resuspended in 300 μl of FACS buffer (1× PBS + 1% heat-inactivated fetal bovine serum (FBS, Thermo-Fischer)) and transferred to a FACS tube to be acquired with an LSR Fortessa (Pennsylvania) or a BD FACS Canto II (Spain) and analyzed using FlowJo and FACSDiva V8.0 software (Fig. 1). To ensure consistency in staining, acquisition, and analysis across both sites, both labs strictly followed harmonized standard operating procedures (SOPs), as described in previous publications from the authors (Mexhitaj et al., 2019; Li et al., 2021; Zuroff et al., 2022). Both labs used the same source of antibodies at the same dilutions, both labs ran the same calibration and compensation beads each time, both labs set gates based on FMO using the same gating strategy, and all samples at each site were stained, acquired, and analyzed by the same operator.

2.3. Fresh CSF processing

Fresh CSF processing methods were adapted from published consensus protocols (Teunissen et al., 2009). Standard operating procedures for CSF processing were harmonized between the two sites and strictly enforced to ensure reproducibility. CSF was collected into sterile Falcon High Clarity Propylene conical tubes precooled at 4 °C, immediately placed into a SCIENCEWARE® 0 °C Cryo-Safe™ Junior Cooler (VWR) and transported to the lab for processing within 15 min of collection. CSF was centrifuged at 400 ×g for 10 min at 4 °C with no brake. The supernatant was removed, and the cell pellet was

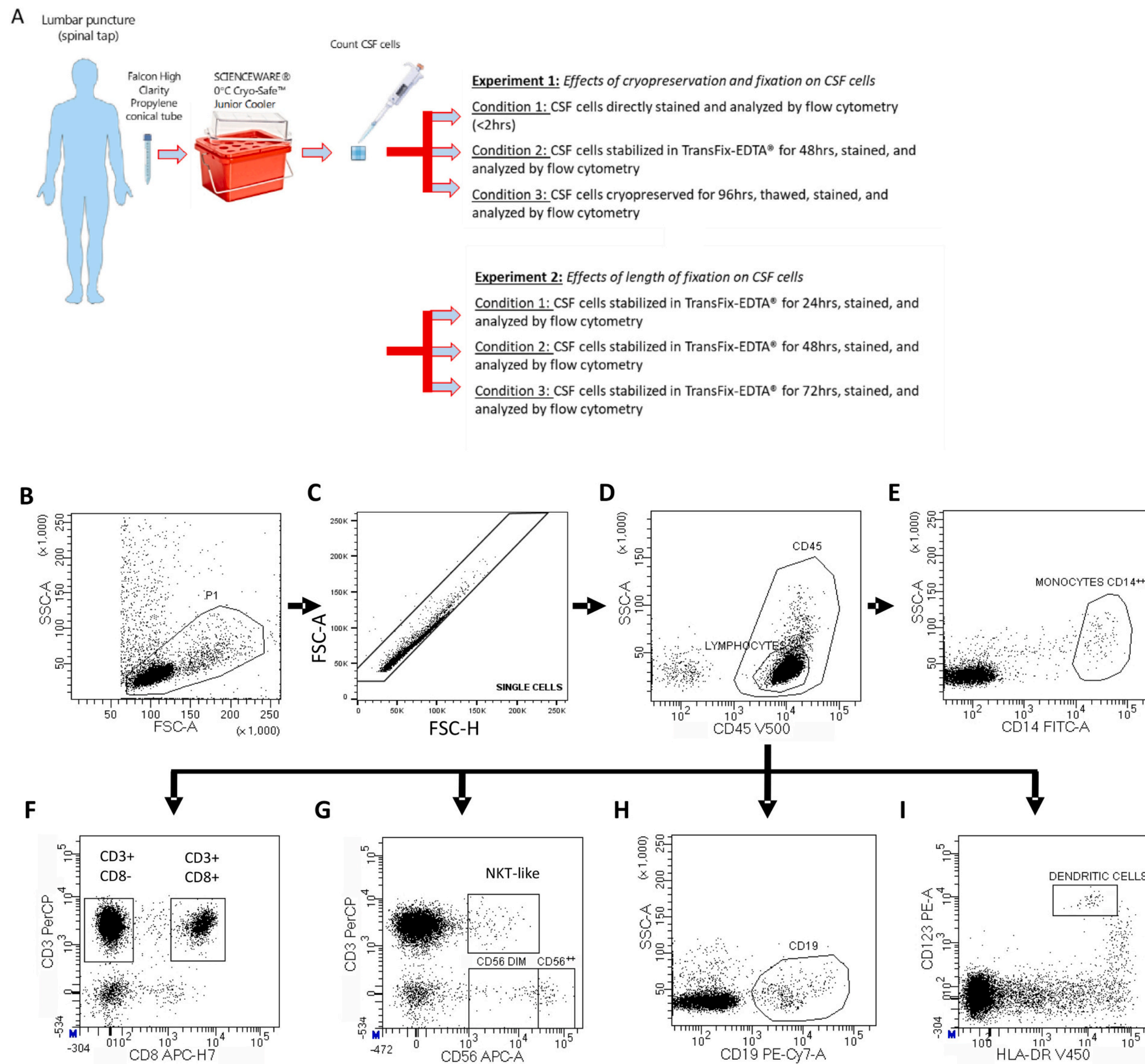

Fig. 1. Schematic representation of the workflow and gating strategy. A. CSF samples were processed as described in Materials & Methods, within 2 h of lumbar puncture. CSF cells were counted, and each sample was divided into three fractions for comparison of preservation methods (Experiment 1) or comparison of length of fixation (Experiment 2). B. Total mononuclear cell gate. C. Singlet gate (parent gate = total mononuclear cells). D. Total CD45+ mononuclear cells and total lymphocyte gates (parent gate = singlets) E. CD14+ monocyte gate (parent gate = total CD45+ mononuclear cells). F. CD3+ CD8- T cell gate and CD3+ CD8+ T cell gate (parent gate = total lymphocytes) G. NKT-like cells (CD3+ CD56+), CD3- CD56dim NK cells, CD3- CD56bright NK cells (parent gate = total lymphocytes). H. CD19+ B cells (parent gate = total lymphocytes). I. CD123+ HLA-DR+ dendritic cells (parent gate = total CD45+ mononuclear cells).

resuspended in 500 μl cold X-Vivo (Lonza Bioscience) and placed on ice. Cells were counted using a hemocytometer with Trypan Blue exclusion. In case of a bloody tap, determined visually by the presence of a red rim on the pellet, cells were excluded from further analysis. Presence or absence of red blood cell contamination was further confirmed by high power hemacytometer counts. Samples included in analysis average < 1 red blood cell per μl, and all were far below the recommended limit of 500 red blood cells per μl, per the consensus protocol (Teunissen et al., 2009). Thus, CSF samples used in this study were unlikely to have been meaningfully impacted by blood contamination. After counting, cells were centrifuged at 400 ×*g* for 10 min at 4 °C. Samples were split into three equal parts for the following treatments (Fig. 1): *Experiment 1* (a) fresh (within 1–2 h of LP), (b) TransFix-EDTA® (48 h) and (c) cryopreserved (96 h) or *Experiment 2* TransFix-EDTA® for (a) 24 h, (b) 48 h, and (c) 72 h.

2.4. CSF cell fixation

After counting, CSF cells were immediately transferred to a TransFix-EDTA® CSF Sample Storage Tube (Cytomark) containing 2.5 mL of CSF supernatant, to replicate a standard operating procedure where whole CSF is fixed immediately after LP. The TransFix tube was mixed gently by inversion ≥10 times and stored at 2–8 °C for up to 72 h. Prior to staining, the fixed CSF sample was allowed to return to room temperature, washed with 2 mL sterile filtered PBS, and centrifuged at 400 ×*g* for 5 min at room temperature. Supernatant was removed and the cell pellet was re-suspended, counted using a hemocytometer, and stained as above.

2.5. CSF cell cryopreservation and thawing

After counting, cells were resuspended in 500 μl of cold heat-inactivated FBS (Hyclone GE Healthcare), transferred to a cryovial,

and placed on ice. An equal volume of 2× freezing medium (heat-inactivated FBS with 20% dimethyl sulfoxide (Merck)) was added dropwise. The cryovial was transferred to a freezing container with 100% isopropyl alcohol and stored at −80 °C for a maximum of 24 h before being transferred to liquid nitrogen.

To thaw the cells, the cryovial was extracted from liquid nitrogen and placed on ice for 1 min, then placed in a 37 °C water bath until thawed, followed by dropwise addition of 1 mL cold complete medium (RPMI 1640 without glutamine (Gibco), with 1% L-Glutamine (200 mM, Gibco) and 10% FBS). Cells were transferred to a 10 mL Falcon High Clarity Propylene conical tube on ice and 9 mL of cold complete medium was added. After centrifugation at 400 ×*g* at 4 °C for 10 min, the supernatant was discarded, and the cell pellet was counted using a hemacytometer and stained as above.

2.6. Statistical analysis

For the assessment of each cell population, values were log transformed and analyzed using a mixed model for repeated measures. The estimated means were compared between fresh, fixed, and frozen. Linear trends over time were also tested using a mixed model. All *p* values were corrected for multiple testing in each set of analysis by false discovery rate (FDR). Statistical significance was set at 5% ($p < 0.05$). All analyses were conducted using R 4.0.2 software.

2.7. Data availability

All CSF processing protocols and data are publicly available through the Immune Tolerance Network and Trial Share websites (www.immunetolerance.org, www.itntrialshare.org).

3. Results

3.1. Effects of fixation and cryopreservation on major CSF cell populations

We first compared fresh CSF cells (<2 h) ($n = 12$) to TransFix-EDTA® stabilization (48 h) (n = 12) and cryopreservation (96 h) ($n = 11$) by

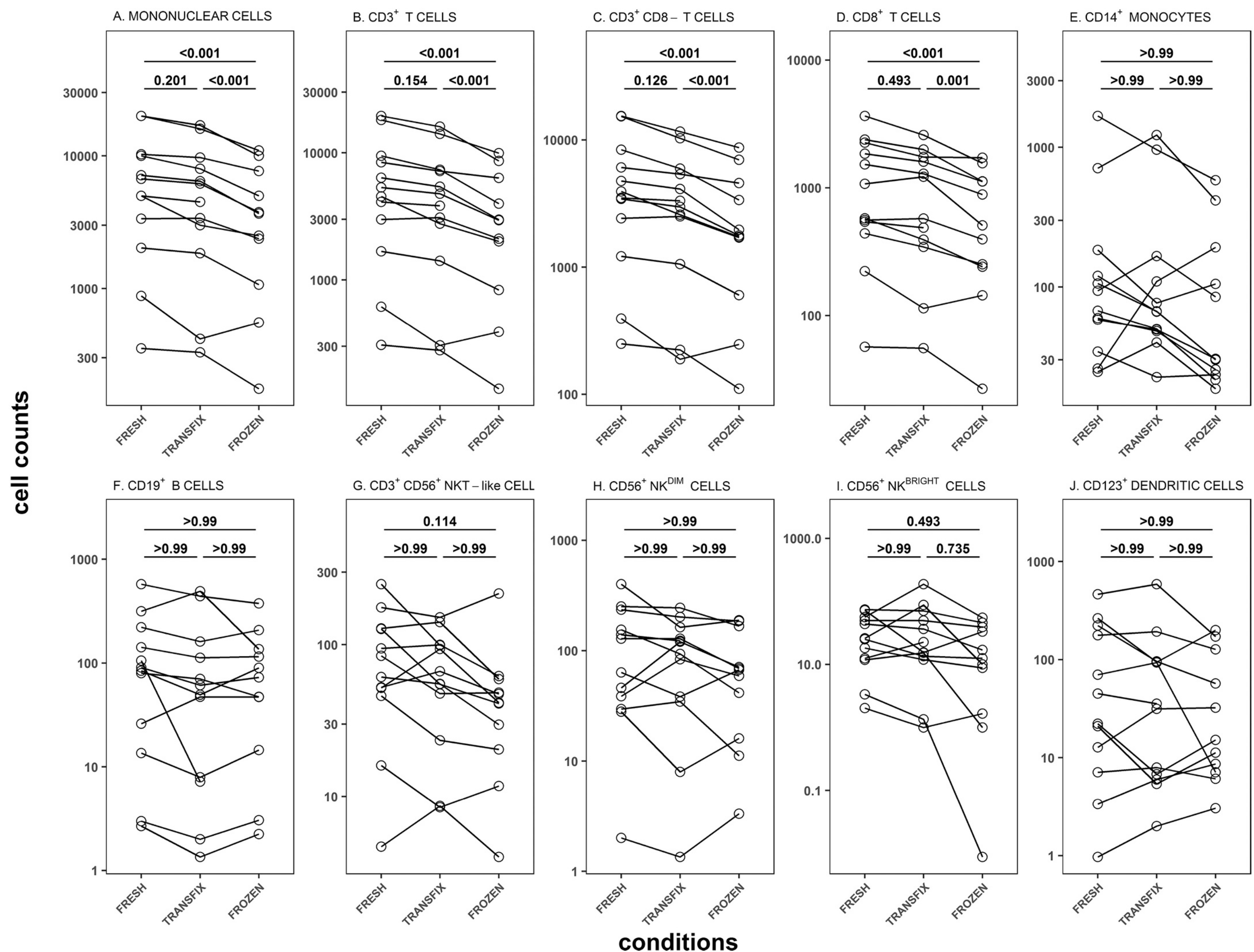

Fig. 2. Effects of fixation and cryopreservation on absolute count of major CSF cell populations. CSF cells ($n = 12$) were split into three equal fractions and counted and stained fresh (⟨2h), after 48 h in TransFix-EDTA®, or after cryopreservation for 96 h. Absolute cell counts of total live mononuclear cells (A) defined by hemacytometer and trypan blue exclusion. Absolute cell counts of total CD3+ T cells (B), CD3+ CD8- T cells (C), CD3+ CD8+ T cells (D), CD14+ monocytes (E), CD19+ B cells (F), CD3+ CD56+ NKT-like cells (G), CD3- CD56dim NK cells (H), CD3- CD56bright NK cells (I), and HLA-DR+ CD123+ DCs (J), defined using gates from Fig. 1 and back calculated using frequency of total mononuclear cells and total live cell count by hemacytometer and trypan blue exclusion as in (A). Statistical analysis represents FDR using a mixed model for repeated measures, with correction for multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flow cytometry to determine the effects of preservation on broad cell subsets. Samples were collected from two unique patient cohorts at two different centers using the same SOP for sample processing, staining, and gating. There was no significant difference in the count of total mononuclear cells fixed with TransFix-EDTA® versus processed fresh ($p = 0.20$) (Fig. 2A). This suggests that fixation and processing within 48 h can be used to preserve intact CSF cells for analysis by flow cytometry. Notably, this method was successful for a wide range of cell yields, including as low as 300 cells (range 300–30,000 cells). Cryopreservation, on the other hand, resulted in a significant loss of total mononuclear cell counts versus fresh CSF and versus fixed ($p < 0.001$) (Fig. 2A), suggesting that the cryopreservation method tested is not suitable for immunophenotyping of CSF cells by flow cytometry.

Previous literature suggests certain subsets of mononuclear cells may survive processing and preservation better than others, resulting in artificial skewing of relative cell frequencies. Thus, we extended our analysis to examine preservation of specific cell subsets, including total CD3+ T cells, CD3+ CD8- T cells (which is expected to consist primarily of CD4 T cells but likely also contains a small percentage of double negative T cells, γδ T cells, and other innate-like T cells (Han et al., 2014)), CD3+ CD8+ T cells, CD19+ B cells, CD3+ CD56+ NKT-like cells (which may include NKT cells, as well as a small percentage of mucosal-associated invariant T (MAIT) cells, γδ T cells, and others), CD3-CD56^{bright} NK cells, CD3-CD56^{dim} NK cells, CD14+ monocytes, and HLA-DR + CD123+ dendritic cells. CD123+ plasmacytoid dendritic cells were chosen as a focus due to published data from Han et al. (2014) demonstrating the plasmacytoid DCs are over-represented in CSF of MS patients versus blood, while conventional myeloid DCs are not.

Compared to fresh CSF, there was no significant change in the absolute counts of total mononuclear cells, total CD3+ T cells, CD3+ CD8- T cells, or CD3+ CD8+ T cells after fixation ($p = 0.15$, $p = 0.13$, $p = 0.49$, respectively) (Fig. 2). Similarly, there was no significant change in relative frequency of total T cells or their subsets after fixation (Supplementary Fig. A1). Conversely, cryopreservation resulted in a significant loss of cell counts for total mononuclear cells, total CD3+ T cells, CD3+ CD8- T cells, and CD3+ CD8+ T cells, as compared to either fresh or fixed ($p < 0.001$) (Fig. 2). There was no significant change in the relative frequency of T cell subsets after cryopreservation, suggesting

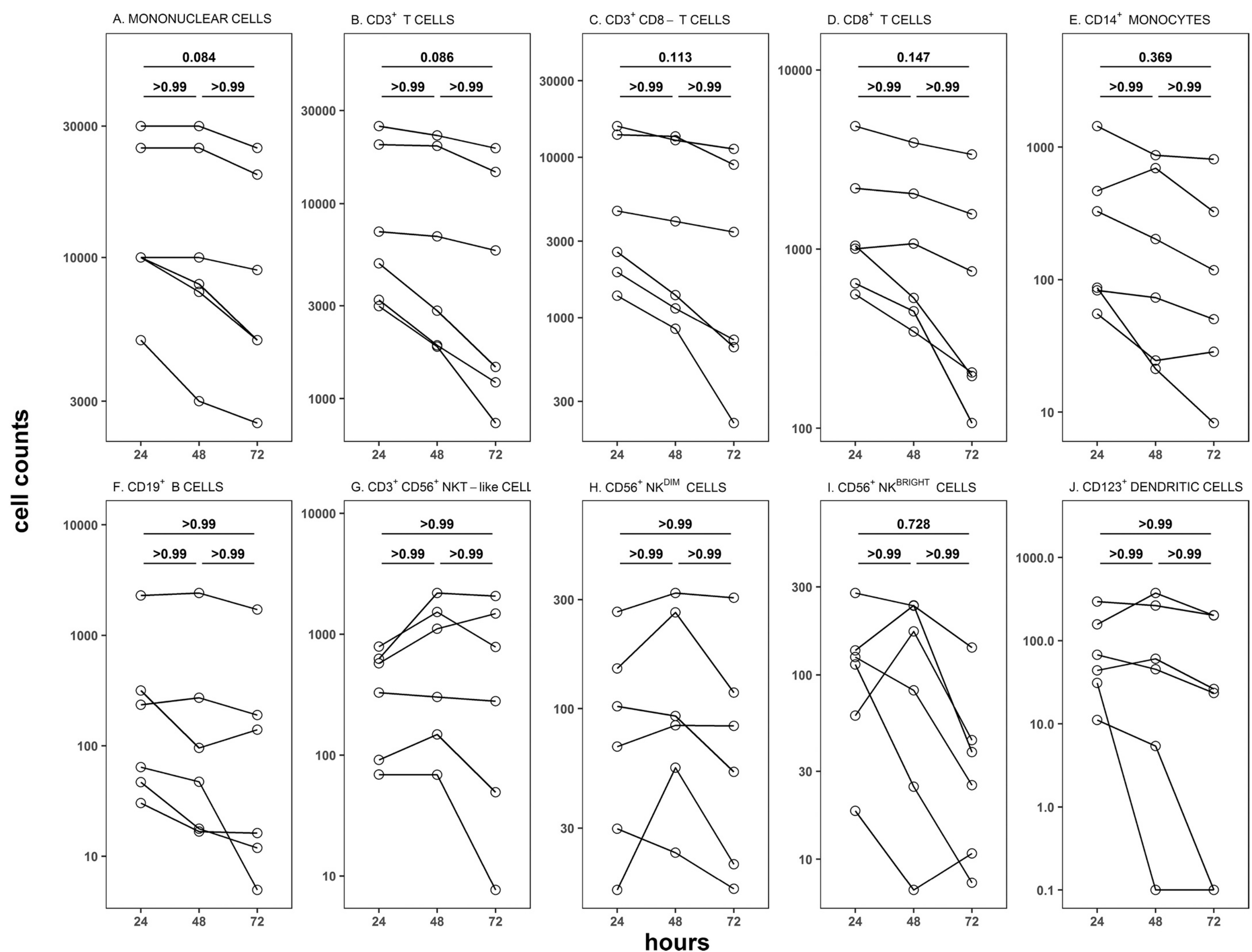

Fig. 3. Effects of fixation time on absolute count of major CSF cell populations. CSF cells ($n = 6$) were collected, counted, split into three equal fractions, and treated with TransFix-EDTA® for 24, 48, or 72 h, followed by counting, staining, and flow cytometry. Absolute cell counts of total live mononuclear cells (A) defined by hemacytometer and trypan blue exclusion. Absolute cell counts of total CD3+ T cells (B), CD3+ CD8- T cells (C), CD3+ CD8+ T cells (D), CD14+ monocytes (E), CD19+ B cells (F), CD3+ CD56+ NKT-like cells (G), CD3- CD56dim NK cells (H), CD3- CD56bright NK cells (I), and HLA-DR+ CD123+ DCs (J), defined using gates from Fig. 1 and back calculated using frequency of total mononuclear cells and total live cell count by hemacytometer and trypan blue exclusion as in (A). Statistical analysis represents FDR using a mixed model for repeated measures, with correction for multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that CD8- and CD8+ T cells are equally susceptible to cell loss (Supplementary Fig. A1).

We further examined the effects of fixation and cryopreservation on absolute cell counts and relative frequencies of CD19+ B cells, CD3 + CD56+ NKT-like cells, CD3-CD56^{bright} NK cells, CD3-CD56^{dim} NK cells, CD14+ monocytes, and CD123+ dendritic cells. Interestingly, there were no significant differences in total cell count (Fig. 2) or relative frequency (Supplementary Fig. A1) for any of these populations after fixation or cryopreservation compared to fresh CSF.

3.2. Effects of fixation time on major CSF cell populations

CSF cell fixation is a practical method to allow clinical sites in multicenter studies to ship samples to a central lab for standardized flow cytometric evaluation, but there is no consensus on the time constraints for this process and no published data on differential effects of fixation on major cell subsets over time. CSF cells were split into three equal fractions and treated with TransFix-EDTA® for 24, 48, or 72 h, followed by analysis of absolute and relative cell recovery by flow cytometry. We observed a progressive loss in the absolute cell number recovered with longer fixation for every subset (Fig. 3). Compared to 24 h, the average recovery of total mononuclear cells, total CD3+ cells, CD3+ CD8- T cells, and CD3+ CD8+ T cells at 48 h was 84.3%, 74.6%, 71.7%, and 75.0%, respectively. At 72 h, the average recovery of total mononuclear cells, total CD3+ cells, CD3+ CD8- T cells, and CD3+ CD8+ T cells dropped to 65.0%, 48.2%, 41.8%, and 40.2% of the yield at 24 h, respectively. The consistent loss in cell yield at 72 h, however, was not statistically significant ($p = 0.08$ for total mononuclear cells, $p = 0.09$ for total CD3+ cells), potentially due to small sample size and our conservative approach to statistical analysis that corrected for multiple comparisons. Similarly, when comparing recovery of minor cell populations after 48 or 72 h of fixation, average recovery at 72 h was consistently less than average recovery at 48 h but did not reach statistical significance (Fig. 3). Compared to 24 h, average recovery at 48 h for CD19+ B cells, CD3+ CD56+ NKT-like cells, CD3-CD56^{bright} NK cells, CD3-CD56^{dim} NK cells, CD14+ monocytes, and CD123+ DCs was 62.0%, 164.0%, 78.2%, 136.5%, 61.2% and 38.2%, respectively. At 72 h, the average recovery for these same populations dropped to 37.5%, 86.8%, 30.5%, 86.1%, and 13.2%, respectively, as compared to 24 h. In addition, there was a trend towards decreased relative frequency of total CD3+ T cells with longer fixation (Supplementary Fig. A2), suggesting that T cells are more sensitive to extended fixation than other CSF cell types.

In all the populations examined, there is a consistent trend of decreasing cell recovery with increasing hours of fixation, with the most dramatic loss in total mononuclear cells and total CD3+ cells, the most common cell in CSF. These data suggest that despite fixation, CSF cells continue to be highly labile.

4. Discussion

Lymphocytes infiltrating the CNS play an important role in the pathogenesis of neuroinflammatory diseases such as MS, and previous reports have demonstrated significant differences in the frequency and phenotype of lymphocytes in CSF versus peripheral blood (Han et al., 2014; Schafflick et al., 2020). Thus, detailed immunophenotyping of CSF cells is a critical step towards a better understanding of disease pathogenesis, characterizing effects of immunotherapy, discovery of prognostic biomarkers, and development of personalized medicine approaches. There are many barriers to analysis of CSF cells that make them more challenging to analyze than peripheral blood mononuclear cells (PBMC). Fresh CSF cells are extremely labile and die rapidly after LP, preventing clinics from shipping fresh CSF overnight to a central lab for analysis. As a result, most studies of fresh CSF cells rely on samples from a single clinical site located in close proximity to a research lab, and studies of CSF cells from multicenter clinical trials have been hindered by the lack of a consensus on methods for preserving intact CSF cells with minimal cell loss. Although both fixation and cryopreservation of PBMCs are common practice, similar procedures are not well established for CSF cells. Thus, developing an SOP for processing and preserving CSF cells that is easily implemented in the clinic and allows for immunophenotyping at a centralized lab with minimal cell loss will fill an important gap. The goal of this study was to compare methods of CSF cell preservation to assess the feasibility of using a small volume of CSF, as available in clinical settings, for accurate evaluation of CSF cell immunophenotypes by flow cytometry.

We compared absolute counts and relative frequencies of major leukocyte populations in fresh CSF (processed within 2 h) to cells fixed for 48 h with TransFix-EDTA® or cryopreserved and thawed after 96 h. Cryopreservation, but not fixation, resulted in statistically significant loss of total mononuclear cells, total CD3+ T cells, CD3+ CD8- T cells, and CD3+ CD8+ T cells (Fig. 2). Thus, our results demonstrate that fixation was superior to the cryopreservation method tested for preserving intact CSF cells with minimal cell loss or skewing from the “true” values of fresh CSF.

Although there are advantages to cryopreservation versus fixation, such as allowing for batched analysis at later time points and a wider array of downstream assays, the average loss of ~50% of total intact mononuclear cells or total T cells upon thawing represents a significant disadvantage, particularly for any study focused on T cells. However, cryopreservation may provide a complementary or parallel approach for studies that rely on other methodologies, such as RNA sequencing or intracellular cytokine analysis, and a combination of CSF cell processing methods may be the best approach if feasible. For example, in the currently active BEAT-MS study, fresh CSF samples are split, with a small fraction treated with TransFix-EDTA® for flow cytometry of major leukocyte populations at a central core facility, and the larger cell fraction cryopreserved for batched RNA sequencing. Importantly, fixation and staining of CSF cells was successful with small numbers of cells, suggesting that this approach is feasible even with small volumes of CSF or CSF samples with low cellularity. In the BEAT-MS example, flow cytometry of fixed cells is regularly successful with only 2.5 mL of CSF, although this may be aided by the fact that MS patients with active disease are likely to have increased CSF cellularity. Of note, Oh et al. used an adapted freezing medium for long-term cryopreservation of CSF cells and successfully conducted single cell RNA sequencing of the recovered cells, consisting largely of T cells (Oh et al., 2021). However, in the absence of comparison to fresh CSF cells, it’s unclear if the recovered population was representative or whether specific populations were lost. The authors note that they recovered fewer myeloid cells and NK cells than expected, which they attribute to differences in cell subset sensitivity to cryopreservation but have no data to corroborate this conclusion in their samples. Thus, flow cytometry of a fraction of fixed CSF cells could aid in interpretation of data from RNA sequencing analysis of cryopreserved cells from the same sample.

Before implementing an SOP for CSF cell fixation across multiple sites, followed by flow cytometry at a central lab, it was important to determine the effects of fixation time on sample stability. Previous reports suggest that fixed CSF cells may be stable for up to 72 h, but detailed data was lacking. We found a consistent decline in absolute numbers of total mononuclear cells and T cells with increasing length of fixation. Similarly, there was a decline in yields of other cell types, including B cells, dendritic cells, NK cells, and NKT-like cells, with reduced recovery of all cell types at 72 versus 48 h. As a result of these findings, we propose CSF samples be analyzed within 48 h of collection, which is a feasible time frame for sample fixation at the site of LP, shipment to a central lab, staining, and analysis by flow cytometry in a multicenter clinical trial.

Interestingly, T cells were more sensitive to both freezing and extended fixation than other cell subsets examined, including B cells, CD123+ DCs, monocytes, NK, and NKT-like cells. Our findings further clarify the conflicting results of de Jongste et al. (2014), which suggested

that CSF lymphocytes are lost faster than monocytes, and Dux et al. (1994), which suggested that lymphocytes are the more stable CSF subset. Differences between the findings of these earlier studies may be attributed to differences in the CSF processing and preservation methods and/or differences in the immunophenotyping protocol. Unlike T cells, there was no significant loss in the recovery of other broad cell types, including B cells, after cryopreservation. This suggests that cryopreservation may be a viable option for preserving CSF cells for downstream studies of B cells and other cell subsets without significant cell loss, although further studies are needed to determine if cryopreservation has a significant effect on function or gene expression. (Lanz et al., 2019; Schafflick et al., 2020; Ramesh et al., 2020; Restorick et al., 2017; Wuest et al., 2014).

This study focused on a limited core panel of surface markers to discriminate major leukocyte populations, thus one limitation of this study is that cell type-specific loss of other subpopulations not included in this panel cannot be ruled out. In future studies, this panel can be expanded to determine if additional subpopulations that have been identified in CSF, such as T helper subsets, double negative T cells, γδ T cells, MAIT cells, B cell subsets, distinct myeloid cell types, innate lymphoid cells, and rare populations of microglia-like cells, are effectively preserved using this method (Schafflick et al., 2020; Esaulova et al., 2020). Since these abnormal cell populations are shown to be present in the CSF and have been reported to contribute to the pathogenesis of neuroimmunological disorders and/or modulation of disease process, monitoring the frequency and activation status of additional relevant cell subsets will be important in future clinical trials.

In conclusion, we demonstrate a method for preserving intact CSF cells for centralized immunophenotyping by flow cytometry with minimal loss of broadly-defined cell types. The results of this study will aid in the implementation of standardized CSF flow cytometric procedures across multicenter clinical trials and can feasibly be combined with other methods of CSF cell analysis, such as RNA sequencing, when there is sufficient CSF volume.

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Declaration of Competing Interest

LAC, NL, TZ, JIF-V: None.

IM: Currently employed by Sanofi.

LMV: Served on scientific advisory boards, participated in meetings sponsored by, received speaking honoraria, travel funding, or research grants from Roche, Sanofi, Merck, Biogen, Bristol Myers, and Novartis.

PAM: Received travel support and speaker honoraria from unrestricted educational activities organized by Novartis, Bayer HealthCare, Bayer Pharma, Biogen Idec, Merck-Serono and Sanofi Aventis. Consulted for Magenta Therapeutics and Jasper Therapeutics.

KMH: Currently employed by and holds equity in Rubius Therapeutics.

AB-O: Participated as a speaker in meetings sponsored by and received consulting fees from Accure, Atara Biotherapeutics, Biogen, BMS/Celgene/Receptos, GlaxoSmithKline, Gossamer, Janssen/Actelion, Medimmune, Merck/EMD Serono, Novartis, Roche/Genentech, Sanofi-Genzyme; and has received grant support to the University of Pennsylvania from Biogen Idec, Roche/Genentech, Merck/EMD Serono and Novartis.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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