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EDITED BY
Jörg Hamann,
University of Amsterdam, Netherlands

REVIEWED BY
Lidia Garcia-Bonilla,
Cornell University, United States
Alanna Gabrielle Spiteri,
The University of Sydney, Australia

*CORRESPONDENCE
Matthew J. Farrer
m.farrer@ufl.edu

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Experimental procedures for flow cytometry of wild-type mouse brain: a systematic review

Robert C. Sharp, Dylan T. Guenther and Matthew J. Farrer*

Department of Neurology, McKnight Brain Institute, University of Florida, Gainesville, FL, United States

Objective: The aim of this study was to systematically review the neuroimmunology literature to determine the average immune cell counts reported by flow cytometry in wild-type (WT) homogenized mouse brains.

Background: Mouse models of gene dysfunction are widely used to study age-associated neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease. The importance of the neuroimmune system in these multifactorial disorders has become increasingly evident, and methods to quantify resident and infiltrating immune cells in the brain, including flow cytometry, are necessary. However, there appears to be no consensus on the best approach to perform flow cytometry or quantify/report immune cell counts. The development of more standardized methods would accelerate neuroimmune discovery and validation by meta-analysis.

Methods: There has not yet been a systematic review of 'neuroimmunology' by 'flow cytometry' via examination of the PROSPERO registry. A protocol for a systematic review was subsequently based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) using the Studies, Data, Methods, and Outcomes (SDMO) criteria. Literature searches were conducted in the Google Scholar and PubMed databases. From that search, 900 candidate studies were identified, and 437 studies were assessed for eligibility based on formal exclusion criteria.

Results: Out of the 437 studies reviewed, 58 were eligible for inclusion and comparative analysis. Each study assessed immune cell subsets within homogenized mouse brains and used flow cytometry. Nonetheless, there was considerable variability in the methods, data analysis, reporting, and results. Descriptive statistics have been presented on the study designs and results, including medians with interquartile ranges (IQRs) and overall means with standard deviations (SD) for specific immune cell counts and their relative proportions, within and between studies. A total of 58 studies reported the most abundant immune cells within the brains were TMEM119^{+} microglia, bulk CD4^{+} T cells, and bulk CD8^{+} T cells.

Conclusion: Experiments to conduct and report flow cytometry data, derived from WT homogenized mouse brains, would benefit from a more

standardized approach. While within-study comparisons are valid, the variability in methods of counting of immune cell populations is too broad for meta-analysis. The inclusion of a minimal protocol with more detailed methods, controls, and standards could enable this nascent field to compare results across studies.

KEYWORDS

neuroimmune, flow cytometry, mouse studies, immunity, systematic review, inflammation, neurological disorders, methodology

1 Introduction

The mouse has been used to model neurological disorders for many decades, whether employing lesion models (i.e., inducing a stroke in a mouse specimen) or transgenic or more physiologic gene knock-out or mutant knock-in approaches (1–3). Some illustrations of mouse modeling for neurologic and neurodegenerative disorders include work in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), psychological/intellectual disabilities (i.e., depression or Down's syndrome), traumatic brain injuries (TBI), and prion diseases (1–3). Most of these disorders are multifactorial, with genetic and environmental components, for which the immune system may provide some integration. Consequently, there has been growing interest in neuroimmunology that is not just focused on resident immune cells within the brain but also on infiltrating peripheral immune cells (4–7). Recent studies of the gut microbiota have highlighted nervous and immune communication between the gut and central nervous system (CNS) (8–10). With this resurgence, researchers have employed traditional (i.e., immunofluorescence/histology slide staining and Western blotting) and contemporary methods (i.e., single-cell genomics and single-cell sorting/staining via flow cytometry) to better characterize specific immune cell subsets within the body and brain.

Specifically, brain resident immune cells, including microglia and astrocytes, have been comprehensively examined in many mouse models of neurologic disorders (4–10). However, the characterization of other immune cell subsets in peripheral blood, the CNS, and within the brain (both residential and infiltrated) has yet to be fully described (4–10). These immune cell subsets include, but are not limited to, natural killer cells (NK cells), T cells, and B cells (4–10). However, validation and comparison through meta-analysis of immunophenotypes of these mouse models might be enabled if researchers utilize more standardized methods and reporting.

Technologic developments for single-cell isolation and analysis, including single-cell RNAseq, mass cytometry (CYTOF), fluorescence-activated cell sorting (FACS), and multi-color flow cytometry-derived immunophenotyping, have illuminated a wide variety of scientific fields (11, 12). Of these techniques, FACS and flow cytometry immunophenotyping are most frequently used. Reasons include the ease of use in setting up a flow cytometer or sorter for a variety of applications, the sensitivity of the assay, the specificity of the antibodies used, the potential of those antibodies to be used for both flow cytometry and Western blotting for the same target, and the ability to produce qualitative and quantitative data (12, 13).

Nevertheless, flow cytometry has its pitfalls as analytic interpretation of the data depends on the user's preference for gating and target choices (13). Experimentally, flow cytometry is also dependent on the fluorophores and cytometers used, and variation between these instruments may result in false positives and negatives. Nevertheless, such issues can be circumvented by providing multiple controls, such as unstained, isotype antibody-stained, and fluorescence-minus-one (FMO) controls, more rigorously described methods, and standardization of flow cytometry protocols.

FACs and flow cytometry immunophenotyping have been insightful and widely used in basic immunology, and other scientific fields, including neurology, have begun to adopt these methods (14–16). However, now that their utility has been demonstrated, the use of flow cytometry for detecting cell types within the brain and CNS warrants more standardized protocols and reporting. Although mouse immune profiles within the brain have been identified by high-dimensional single-cell mapping using techniques such as mass cytometry (CYTOF) (17), at the time of writing and despite the numerous publications, the expected cell counts and proportions of each immune subset within the brain of a wild-type (WT) mouse have not yet been clearly defined by standard FACS and flow cytometry. Consequently, we have performed a systematic review focused on neuroimmunology and the use of flow cytometry to detect immune cells derived from WT homogenized mouse brains. In our results, we summarize the number of immune cells overall and estimate the immune subsets that can be detected via flow cytometry immunophenotyping. Our findings demonstrate a critical need for more standardized methods and reporting and lead to best-practice recommendations for future publications.

2 Methods

2.1 Study design

The study design was informed by prior literature (18–20) and based on the Preferred Reporting Items for Systematic Reviews and

Meta-Analyses (PRISMA) criteria (21) and the Cochrane Handbook of reporting methodology reviews, employing the SDMO (Types of Studies, Types of Data, Types of Methods, and Types of Outcome Measures) criteria (22, 23). Bias assessment for each individual study selected for systematic review inclusion was also conducted using the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) (24) methodology, and subsequent reporting used the *robvis* R package and Shiny web app (25).

2.2 Search strategy

For this systematic review, the Google Scholar and PubMed databases were used to identify all studies published between January 2013 and July 2023. The search protocol and study design were also assessed within the National Institute of Health (NIH) PROSPERO registry database, which confirmed that a review of this topic has not been previously conducted. For database searches, the following combination of keywords was used to identify eligible studies: "flow cytometry" AND "immune subset name examined in study" AND "mouse brain." For example, a keyword search containing "flow cytometry CD4 T cells mouse brain" was used. "Immune subset name examined in study" is defined as one of the following immune cell subsets: "CD4 T cells," "CD8 T cells," "double negative DN T cells," "regulatory T cells TREG," "follicular helper T cells TFH," "T helper 1 T cells TH1," "T helper 2 T cells TH2," "T helper 17 T cells TH17," "naïve T cells," "naïve CD4 T cells," "naïve CD8 T cells," "naïve-like T cells," "central memory T cells TCM," "central memory CD4 T cells TCM," "central memory CD8 T cells TCM," "effector memory T cells TEM," "effector memory CD4 T cells TEM," " effector memory CD8 T cells TEM," "effector memory T cells re-expressing CD45RA TEMRA," "effector memory CD4 T cells re-expressing CD45RA TEMRA," "effector memory CD8 T cells re-expressing CD45RA TEMRA," "TEMRA-like T cells," "natural killer cells NK cells," "dendritic cells DC," "B cells," "monocytes," "macrophages," "M1 macrophages," "M2 macrophages," "TMEM119 microglia," or "neutrophils.".

2.3 Selection and exclusion criteria

Studies were selected from the search results employing the following inclusion criteria: 1) any study performed between 2013 and 2023; 2) any study that contained flow cytometry data identifying immune cell subsets and counts in mouse brains; 3) any study that reported total cell numbers or total live cell percentages for one or more immune cell subsets; and 4) any study that had WT mice or a non-treated control (when reporting on transgenic mouse models). Studies were excluded from search results based on the following criteria: 1) studies performed in 2012 and prior; 2) any study not focused on flow cytometry of homogenized mouse brains; 3) rat studies; 4) human studies; and 5) studies not reporting controls or the background mouse strain.

2.4 Data extraction

For each study, information on the mouse strain used, age, and sex was recorded. In addition, methodological information on mouse perfusion, brain extraction, and homogenization was recorded. Flow cytometry methods were recorded when the following was reported: 1) cytometer make and model; 2) software for data collection/analysis; 3) full gating strategy; 4) total cells collected per sample; 5) total live cell counts; 6) total immune cell subset percentages calculated directly from live cell counts; and 7) methods for determining cell counts and/or mean fluorescence intensity (MFI) readings.

Studies were subsequently examined for immune cell subset counts from WT/control mouse brains directly from the main text, Supplementary Materials, and/or extrapolated from the figures and graphs presented. In this systematic review, "raw" total immune cell subset counts were reported for each immune cell subset as a median with an interquartile range (IQR; defined as 75th percentile upper quartile [Q3] – 25th percentile lower quartile [Q1]) and as the combined mean with standard deviation (SD) of multiple studies (n). The "raw" total overall cell count collected per sample by flow cytometry was also reported from these studies.

2.5 Data analysis

After the median with IQR and combined means with SD from the "raw" total immune cell subset counts and from the "raw" total overall cell count collected per sample were recorded from each study, we standardized an approach to estimate how many immune cells of each subset would be counted if the total homogenized mouse brain cells collected by flow cytometry equaled a total of 1 x 10^5 collected cells. The value of cells required per sample for an accurate flow cytometry reading is stated to be between 1 x 10^4 total cells (minimum) and 1 x 10^6 total cells (maximum); hence, we used the median (26).

The standardized total cell counts for each immune cell subset reported in this systematic review were calculated using the following equation:

$$\frac{a}{b} = \frac{X}{1 \times 10^5 \, Total\ Cells\ Collected}$$

Where "a" is the [Raw Total Immune Cell Subset Count], "b" is the [Raw Total Cell Count Collected] per sample, and "X" is the [Standardized Total Immune Cell Subset Count]. Rearranging to solve for X gives the following equation:

$$\frac{(a)(1 \times 10^5 \, Total\ Cells\ Counted)}{b} = X$$

Once all total immune cell subset counts were standardized, we determined the theoretical standardized percentages of each immune cell subset within the entire WT mouse brain and reported those results as means with SD.

3 Results

3.1 Literature search and study selection

A PRISMA-based flow diagram illustrates our screening methodology for study identification (Figure 1) (21). The two databases (Google Scholar and PubMed) were searched using keywords, as defined in the Methods, and 900 articles were highlighted for systematic review. Of those 900 reports, 223 were removed as they supplied only an abstract or were duplicated between databases, and 677 studies remained. Of these, an additional 240 studies were removed as they were published in 2012 or prior. This cut-off is arbitrary, but it was used to identify more contemporary flow cytometry immunophenotyping publications and resulted in 437 eligible studies. Further inclusion and exclusion criteria removed 133 studies in which flow cytometry of homogenized mouse brains was not a main focus, 129 studies with insufficient information and/or results from controls or in which the background mice strain was not specified, 94 human studies, and 23 rat studies. Consequently, 58 studies that passed our inclusion and exclusion criteria were incorporated into this systematic review (27–84).

3.2 Reporting of mouse information and perfusion/tissue processing is inconsistent between studies

Of the 58 studies selected, we reviewed the basic characteristics of the mice strains used (Figure 2A; Supplementary Table 1). The inbred congenic C57BL/6 mouse line was used the most (26/58 studies [44.8%]) as a control and as the background for genetically modified mice (27, 28, 30, 31, 36, 41–47, 49, 51, 54, 58, 66–69, 72, 74–76, 79, 82). However, other C57BL/6 mice sub-strains were used throughout the 58 studies, which include C57BL/6J (19/58 studies [32.8%]) (29, 32, 33, 39, 52, 56, 60, 62–65, 70, 71, 73, 78, 80, 81, 83, 84); C57/BL6 (2/58 studies [3.44%]) (34, 40); C57BL/6 (H-2b) (2/58 studies [3.44%]) (35, 53); and C57BL/6J (B6) (2/58 studies [3.44%]) (38, 61). The majority of studies that reported mouse sex (Figure 2B; Supplementary Table 1) used only male mice (33/58 studies [56.9%]) (29, 30, 33, 34, 38, 40, 41, 44, 45, 47, 49, 50, 54, 58–63, 65, 67–71, 74, 77–80, 82–84), although 10/58 studies [17.2%] reported mixed results of male and female animals together (27, 31, 32, 36, 37, 39, 43, 51, 72, 75). The age of the mice (Figure 2C; Supplementary Table 1) within the studies varied, but the majority reported findings at 8-12 weeks (2-3 months) (9/58 studies [15.5%])

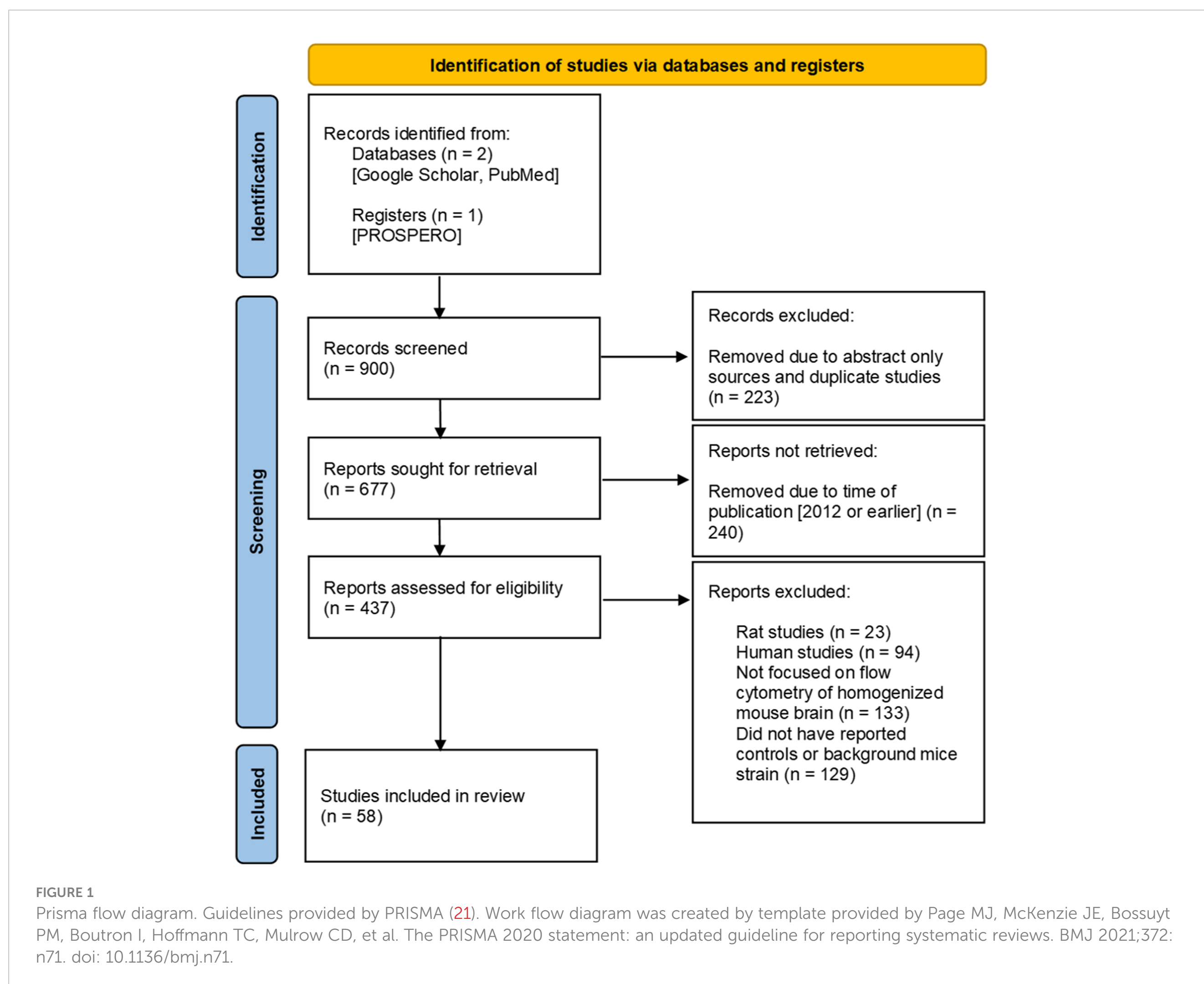

FIGURE 1
Prisma flow diagram. Guidelines provided by PRISMA (21). Work flow diagram was created by template provided by Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372: n71. doi: 10.1136/bmj.n71.

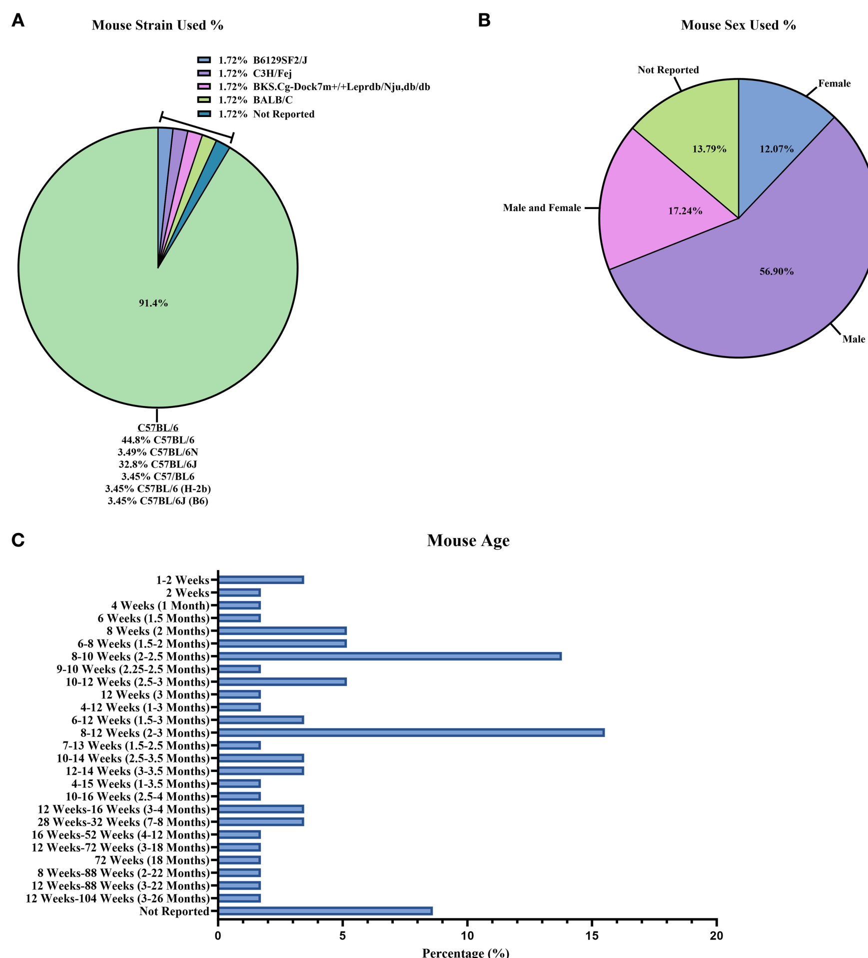

FIGURE 2
Mouse information reported between studies. Reported baseline mouse information described as percentages out of the 58 studies: **(A)** mouse strains; **(B)** mouse sex; and **(C)** mouse age.

(29, 33, 56, 57, 65, 72, 75, 80, 83). However, studies characterized animals over a wide range of ages, from 1 to 2 weeks (32, 39, 81) and between 3 and 26 months (37).

Perfusion (Supplementary Figure 1; Supplementary Table 1) and brain tissue processing methods (Figures 3A–C; Supplementary Table 1) also varied across the 58 studies. A majority of studies (36/58 studies [62.1%], Supplementary Figure 1; Supplementary Table 1) used cold PBS for perfusion (27, 29, 31–33, 35, 39, 43–48, 50–58, 61, 63–67, 70, 73–76, 79, 81, 84). Of the 58 studies reviewed, 6/58 [10.3%] (Figure 3A; Supplementary Table 1) used a commercial kit, such as the Neural Tissue Dissociation Kit P (Miltenyi Biotec), to isolate immune cells from mouse brain (34, 40, 41, 61, 67, 71). Nevertheless, in neurology, it remains unclear how to best process mouse brain to dissociate the tissue and leave cell types intact (85–87). Researchers may use mechanical homogenization, enzymatic homogenization, or both homogenization techniques (85–87). For the studies reviewed, 18/58 [31.0%] (Supplementary Table 1) used some type of mechanical homogenization (glass–Teflon homogenizer, an 18-gauge needle, etc.) before filtering through a cell strainer prior to enzyme treatment and the use of a myelin removal/immune isolation gradient (i.e., Percoll gradient) (31, 33, 37, 44, 48, 50, 53, 55–57, 59, 60, 63, 64, 66, 69, 81, 84). The enzymatic solutions used in the reports also vary widely (Figure 3B; Supplementary Table 1). Most studies used collagenase (I, II, IV, D, I-S, or Liberase) alone or combined with additional enzymes (30/58 studies [51.7%]) (27–31, 33, 46–48, 53, 54, 60, 62–64, 66–68, 70, 73–80, 82–84). The enzyme most used in combination with collagenase (or another tissue digestion enzyme) was DNase I (27/58 studies [46.6%]) (27–31, 47, 48, 51, 53, 54, 60, 62, 63, 66, 68, 70, 73–80, 82–84). After the homogenization of mouse brain, cell strainers are generally used to remove dead cells and myelin (Figure 3A; Supplementary Table 1), and a 70 µm filter was used in the majority (23/58 studies [39.7%]) of studies (15, 30, 32, 33, 38, 39, 43, 45, 46, 48, 52, 53, 56, 58, 59, 62–64, 67, 72, 74, 82, 84). To further remove myelin from mouse brain homogenate along with isolating immune cells, researchers employ a myelin removal kit or Percoll gradient solutions. Specific cell types can be isolated while the myelin layer rises to the top of the sample tube with a centrifuge, depending on the percentage of Percoll. Again, in the studies reviewed, the percentages of Percoll (Figure 3C; Supplementary Table 1) varied, with most using a 30%/70% Percoll gradient solution (17/58 studies [29.3%]) (15, 27,

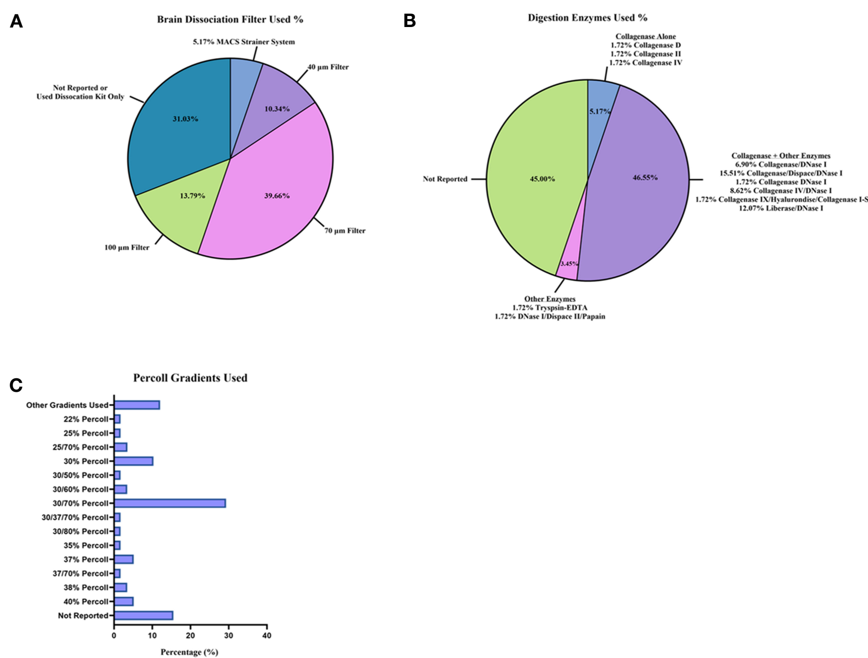

FIGURE 3
Brain tissue dissociation and cell isolation methods reported between studies. Tissue processing techniques were reported as percentages from the 58 studies: **(A)** brain dissociation filters utilized; **(B)** digestion enzymes used for brain homogenization; and **(C)** Percoll gradients used to remove myelin layer and isolate immune cells.

29, 33, 43, 45, 53, 57, 62, 63, 71, 72, 74, 77, 79, 81, 84). Overall, the age of mice and methodology for isolating immune cell counts from the brain for flow cytometry varied greatly between the studies.

3.3 Flow cytometry methodology used and cell counts are inconsistently reported between studies

After considering mice strain and brain homogenization methods, we examined the flow cytometry instruments used and data reporting (Figures 4A–C; Supplementary Figures 2A, B, 3A, B; Supplementary Table 2). The make and model of the flow cytometer/FACS sorter (Supplementary Figure 2A; Supplementary Table 2) and analysis software (Supplementary Figure 2B; Supplementary Table 2) used in each study also varied greatly. The flow cytometer most reported was the BD LSRII Flow Cytometer (12/58 studies [20.7%]) (30, 32, 33, 35, 36, 39, 43, 51, 60, 61, 63, 65), whereas the FACS sorter was the BD FACS Aria III (8/58 studies [13.8%]) (31, 38, 47, 49, 54, 55, 64, 83). The analysis software most generally used was a version of FlowJo (Tree Star) for flow cytometry immunophenotyping (38/58 studies [65.5%]) (27, 29–31, 33–36, 38, 41, 43–45, 47, 49–54, 56, 58, 62, 63, 65, 68–74, 76, 77, 79, 81, 83, 84) and BD FACSDiva specifically for FACS analysis (11/58 studies [19.0%]) (32, 35, 37, 39, 42, 45, 60–62, 76, 77).

After reviewing the methodology in all 58 studies, we assessed the quality of flow cytometry reporting (Figures 4A–C; Supplementary Figures 3A, B; Supplementary Table 2). For each study, we scored the following parameters: 1) whether a full gating strategy was reported; 2) the total cells collected per sample for flow cytometry; 3) the total live cell counts during flow cytometry sample collection; 4) if the total immune cell subset percentage was

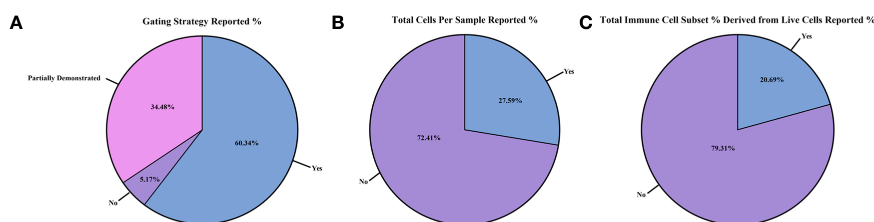

FIGURE 4
Reporting of flow cytometry results and cell counts between the studies. Differences in flow cytometry results and cell counts reported as within study percentages (n=58 studies): **(A)** gating strategy reported; **(B)** total cells per sample collected from flow cytometer/sorter reported; and **(C)** total immune cell subset counts derived from the live cell gate reported.

calculated directly from the live cell count reported or if it was derived from another gate (i.e., if the immune cell subset percentage reported was derived from the $CD45^+$ gate or from the live/dead gate); and 5) whether the methods used to determine cell counts and/or MFI readings were documented. Of the 58 studies reviewed, 35 [60.3%] included a full gating strategy (Figure 4A; Supplementary Table 2) (27, 30, 32–34, 36, 37, 41, 43–45, 47, 50, 51, 54, 55, 58, 60–64, 67, 71–79, 81, 82, 84), while 20 [34.5%] reported a partial gating strategy (28, 29, 35, 38, 40, 42, 46, 48, 49, 52, 53, 56, 57, 59, 66, 68–70, 80, 83)., and 3 [5.17%] did not include this information (31, 39, 65). When documented, the flow antibody clone and gating strategy were reported (44/58 studies; 75.9%) (Supplementary Table 2) (27, 29–33, 35–40, 42, 43, 45–55, 57, 58, 60–62, 66–69, 74–80, 82–84). Most studies used similar clones to identify specific immune cell subsets (Supplementary Table 2).

On reporting the total cells collected per sample during flow cytometry collection (Figure 4B; Supplementary Table 2), only 16/58 [27.6%] of studies provided this data (30, 36, 37, 41, 44, 47, 52, 57, 58, 61, 63, 67, 71, 73, 74, 82). Total live cell counts collected during flow cytometry (Supplementary Figure 3A; Supplementary Table 2) were only reported in 9/58 [15.5%] of the studies reviewed (30, 37, 57, 58, 61, 67, 71, 73, 74). Out of the 58 selected studies, only 12/58 [20.7%] expressed their results in terms of total immune cell subset percentages derived from live cells only (Figure 4C; Supplementary Table 2) and not from another gate (such as deriving from the $CD45^+$ gate) (35, 37, 43, 47, 52, 58, 67, 68, 71, 72, 74, 76). Lastly, 35/58 [60.3%] of studies (Supplementary Figure 3B; Supplementary Table 2) reported methods on how cell counts and/or MFI readings were recorded (27, 28, 30, 32, 33, 35, 37, 39, 41–44, 49–52, 54, 55, 57–59, 61–64, 67, 70–77, 82). Overall, the cytometer/FACS sorter used and the reporting of total cells collected, total live cells, and immune cell subset percentages were not standardized in the 58 studies examined.

3.4 Reported immune cell counts from WT/control homogenized mouse brain are highly variable between studies

Across all the studies, we then examined the total immune cell counts reported in WT/control mouse models, as detected by flow cytometry immunophenotyping and/or by FACS (Supplementary Table 3). As described in the Methods and Supplementary Table 3, studies were selected based on their reporting of a wide variety of immune cell subsets. These included T cell subsets/memory T cells (naïve-like, central memory [T_{CM}], effector memory [T_{EM}], and effector memory re-expressing CD45RA [T_{EMRA}]), natural killer cells (NK cells), dendritic cells (DCs), B cells, monocytes, macrophages (M1 [predominately $CD86^+$] and M2 [predominately $CD163^+$] phenotypes), $TMEM119^+$ microglia, and neutrophils. Of note, the M1/M2 nomenclature for macrophages is currently being updated in the immunology field (88).

Many immune subsets were examined, but relatively few were reported in a sufficient number of studies to be able to calculate representative medians with IQR and means with SD (Figure 5; Supplementary Table 3). Immune cell subsets with at least two or more references to derive a median and mean cell count for WT/control mice included bulk $CD4^+$ T cells (27–33), bulk $CD8^+$ T cells (27, 28, 30, 34–37), double negative (DN) T cells (33, 38), regulatory T cells (T_{REG}) (29, 39–42), T helper 1 cells (T_{H1}) (27, 40), T helper 17 cells (T_{H17}) (44, 45), NK cells (33, 41, 46, 47, 49–51), DCs (33, 52–55), B cells (33, 56–60), monocytes (61–67), bulk macrophages (64, 65, 68–72), $TMEM119^+$ microglia (73–76), and neutrophils (46, 77–84). Immune cell subset counts that were derived from only one study, such as follicular T cells (T_{FH}) (43) and T_{EM} bulk $CD4^+$ and $CD8^+$ T cells (30), were still included in this review as a representation of the possible median/average cell count for these subsets.

We first calculated the overall medians for each examined immune subset by collecting all of the "raw" total immune subset cell counts from each of the 58 studies (Figure 5). By doing this, we discovered that out of these studies, there were some that were outliers (outside of the IQR) that appear to have heavily altered the overall total immune cell counts determined by flow cytometry (27, 33, 36, 41, 47, 53, 58, 61, 69, 72, 76, 79). Out of all the immune cell subsets from the 58 studies, the highest median was $TMEM119^+$ microglia (72,300 [IQR=194,038]; n = 4 studies) (73–76). The lowest median that was calculated was T_{FH} T cells (8 [IQR=0]; n = 1 study) (43). Memory T cell subsets found within both $CD4^+$ and $CD8^+$ T cells were not reported as an immune cell subset in any study. Similarly, T helper 2 cells (T_{H2}) were not reported in any studies.

The 58 studies had highly variable ranges for each immune cell subset found within mouse brain, for which most of the SD calculated was greater than the means (Supplementary Table 3). As with the calculated medians, the highest mean cell count reported from the 58 studies was $TMEM119^+$ microglia (90,323 ± 104,555; n = 4 studies) (73–76). The lowest mean cell counts reported were for T_{FH} T cells (8 ± 0; n = 1 study) (43). Subsequently, we also calculated the means of total overall cell counts collected per sample, as reported in the 58 studies reviewed (Supplementary Table 3). Although not as variable as the immune cell subset counts, the total overall cell counts collected by flow cytometry ranged from 1 x 10^4 to ~3 x 10^6 cells (Supplementary Table 3). In some studies, the actual number of cells collected by flow cytometry was not specified, but the total cell counts could be extrapolated from data given in the main text, figures, and/or Supplementary Materials. Overall, we conclude the immune cell subset counts collected by flow cytometry immunophenotyping are highly variable between studies, potentially due to the processing method, technical skills, and experience of the researcher. Moreover, there are currently insufficient data on specific T cell subsets/memory subsets and specific macrophage phenotype counts within the WT mouse brain.

3.5 Standardizing immune cell counts and percentages within WT/control mouse brain

We devised a method to standardize the counts of immune cells and subset percentages within WT/control mouse brains in each

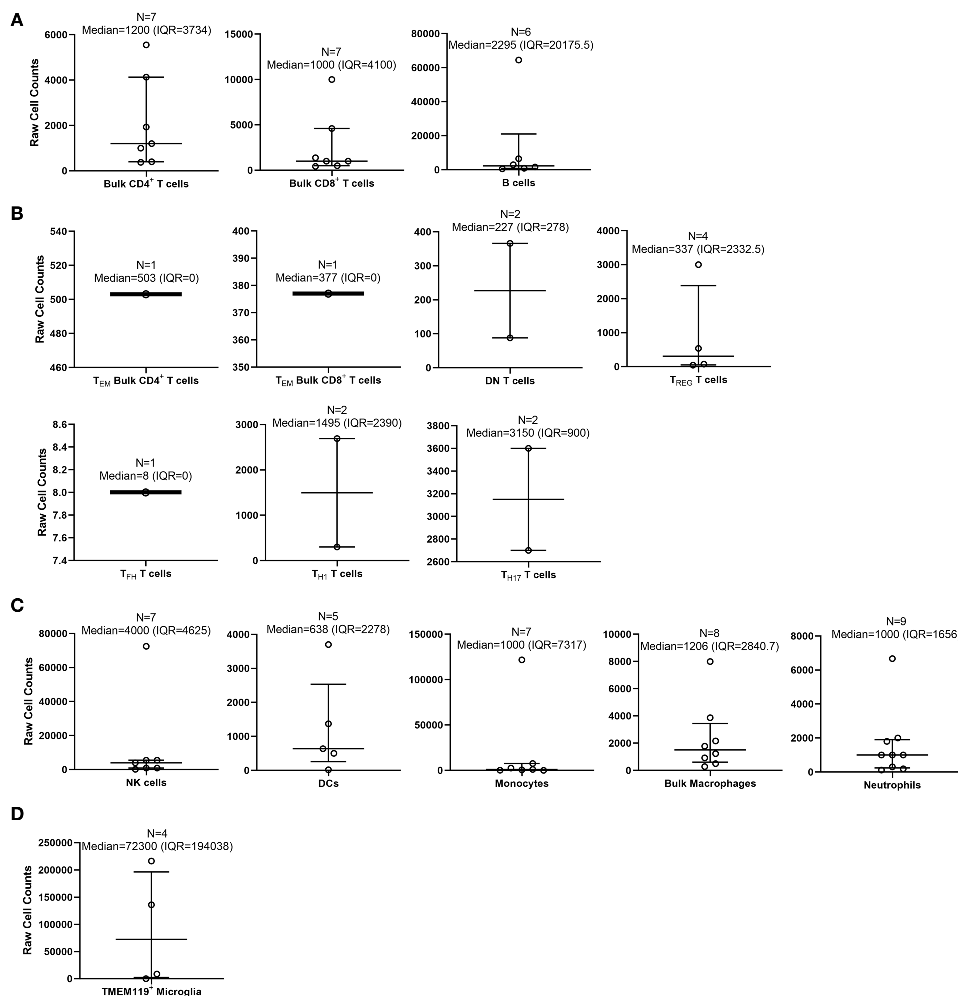

FIGURE 5
Calculated medians with interquartile ranges (IQRs) of immune cells quantified by flow cytometry within wild-type/control mouse brains. Medians with IQRs (defined as: 75th percentile upper quartile [Q3] – 25th percentile lower quartile [Q1]) of immune cell subset counts found with wild-type (WT)/control homogenized mouse brains were calculated from data extrapolated from the 58 studies selected for inclusion in this systematic review. Immune cell subsets were organized as follows: **(A)** bulk adaptive immune cells; **(B)** specialized T cells; **(C)** innate immune cells; and **(D)** microglia. Total n-values (number of studies for each identified subset) are reported along with the median and IQR for each immune subset above each bar graph.

study (Figure 6; Supplementary Table 3). In flow cytometry, cell type percentages should be reported as values from the total number of cells collected (on average 1 x 10^5 cells) rather than from a sub-partition within the gating strategy (26, 89). Thus, based on the assumption that the total cells collected per sample was 1 x 10^5 cells, we were able to estimate a standardized total immune cell subset count for the data provided in each study.

From these standardized total immune cell subset counts, we were able to determine the proportion of immune cell subsets within the brain by simply dividing the standardized count by 1 x 10^5 total cells collected to obtain percentages (Figure 6; Supplementary Table 3). The immune subset with the highest total cell percentages that were found within WT/control mouse brains was TMEM119$^+$ microglia (28.5% ± 33.0). As for non-neural/glial specific immune cells, bulk CD4$^+$ T cells (6.41% ± 6.15) and bulk CD8$^+$ T cells (4.00% ± 5.34) were most often counted within mouse brains compared to other adaptive/innate immune cells. Overall, we were able to calculate the average percentage of immune cells found within WT/control mouse brains from the 58 selected studies. Hence, we are able to report a more reliable estimate of the immune cell composition within the mouse brain despite the wide SD.

3.6 Evaluating risk of bias of all included studies

As per the PRISMA and Cochrane criteria for systematic reviews, it is important to evaluate the risk of bias for all the cited studies (20, 21, 24, 90). Here, we utilize the SYRCLE's risk of bias tools for animal studies (24) to create a summary graph and "stop-light" figure (Figure 7) to highlight the overall bias of each study assessed within the following domains: D1: Sequence Generation (randomization methods used to choose animals for comparable

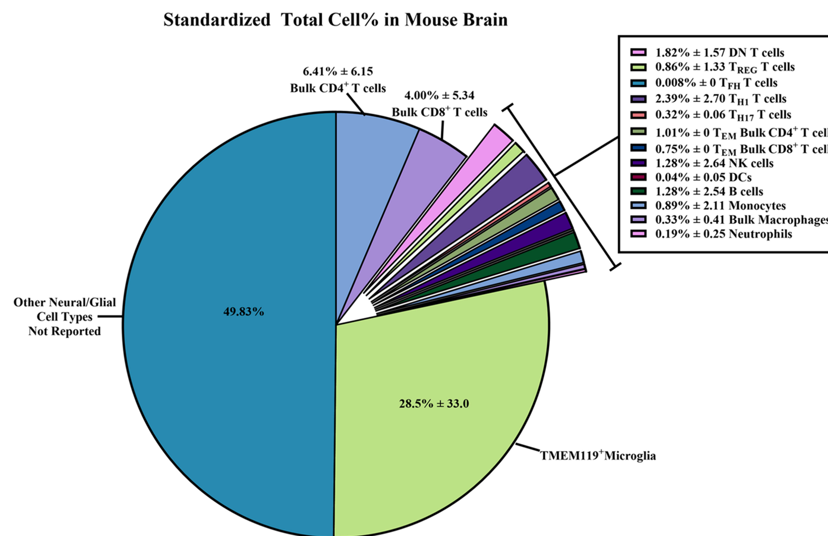

FIGURE 6
Standardized total cell percentages of immune cells quantified by flow cytometry within wild-type/control mouse brains. Estimated percentages of immune cell subset counts found within wild-type (WT)/control homogenized mouse brains were calculated from data extrapolated from the 58 studies selected for inclusion in this systematic review. The equations used to determine the standardized cell counts can be found in the Methods section. Briefly, "raw" total immune cell subset count and "raw" total cell count collected via flow cytometry were standardized assuming 1 x 10^5 total cells were collected. Results were reported as combined means of percentages with standard deviations (± SD) from the standardized totals from each study, assuming 1 x 10^5 total cells were collected.

groups); D2: Baseline Characteristics (full description of animal characteristics from all comparable groups); D3: Allocation Concealment (methods used to conceal how animals are distributed to researchers, i.e., using a coding method for each animal); D4: Random Housing (housing all animal groups randomly within the animal room); D5: Blinding (blinding methods used on researchers, such as blinding the knowledge of intervention or transgenic model used and blinding the outcome assessors); D6: Random Outcome Assessment (methods on how the animals were selected at random for outcome assessment); D7: Incomplete Outcome Data (description of the completeness of the data outcome, i.e., stating if data were excluded or if animals were removed from the study at any point); D8: Selective Outcome Reporting (the completeness of the study protocols); D9: Other Sources of Bias (examples include confounders, contamination problems, analysis errors, and design-specific risk of bias, etc.).

The overall bias of all the selected studies was deemed predominantly "unclear" (~over 50%) due to lack of reporting on specific data/methodology required to pass the "high" or "low" bias questionnaire in each study (Figure 7). The most biased domains (~over 50% high bias scoring) from the selected studies were Baseline Characteristics (i.e., it was largely unclear how sex, age, weight, or other baseline characteristics or confounders were adjusted for in each analysis) and Incomplete Outcome Data (i.e., it was generally unclear whether all animals were included in each analysis and, if not, whether there was any report on why they were missing outcome data or how that missing data influenced the study). The lowest biased domain (~over 75% low bias scoring) was Selective Outcome Reporting (i.e., whether the results reported reflected the methods described in the selected studies). For further clarification of the methods used in order to clear up bias reporting, we contacted all 58 corresponding authors to request more information (all names and affiliations of the authors who responded are included in the Acknowledgements section). Overall, the reported bias from all the included studies was largely considered to be "unclear" due to the lack of reporting and transparency in the methods and results described. As such, this could be a possible reason for the high variability in the immune cell counts that were reported across multiple studies. Reliable reporting and including confounding factors within experimental procedures/data analysis is necessary for the meta-analysis of immunophenotypes found within the mouse brain.

4 Discussion and recommendations

The prominent role of the innate and acquired immune system in brain health and neurologic and age-associated neurodegenerative disorders has become increasingly apparent. In part, this has been driven by the immunologic role of several variant gene discoveries, including triggering receptor expressed on myeloid cells 2 (*TREM2*) in Alzheimer's disease, granulin (*GRN*) in frontotemporal dementia, and leucine-rich repeat kinase 2 (*LRRK2*) in Parkinson's disease (91–94). Despite this burgeoning interest in neuroimmunology and the many published studies, results from flow cytometry immunophenotyping from homogenized mouse brain are highly variable. Although this does not invalidate 'within study' comparisons of specific immune subsets, such variability is a challenge for reproducibility, meta-analysis across studies, and interpretation (27–84). Reliable data on residential and infiltrating immune cells within WT/control mouse brains would be of benefit (1–10). One step toward that goal is

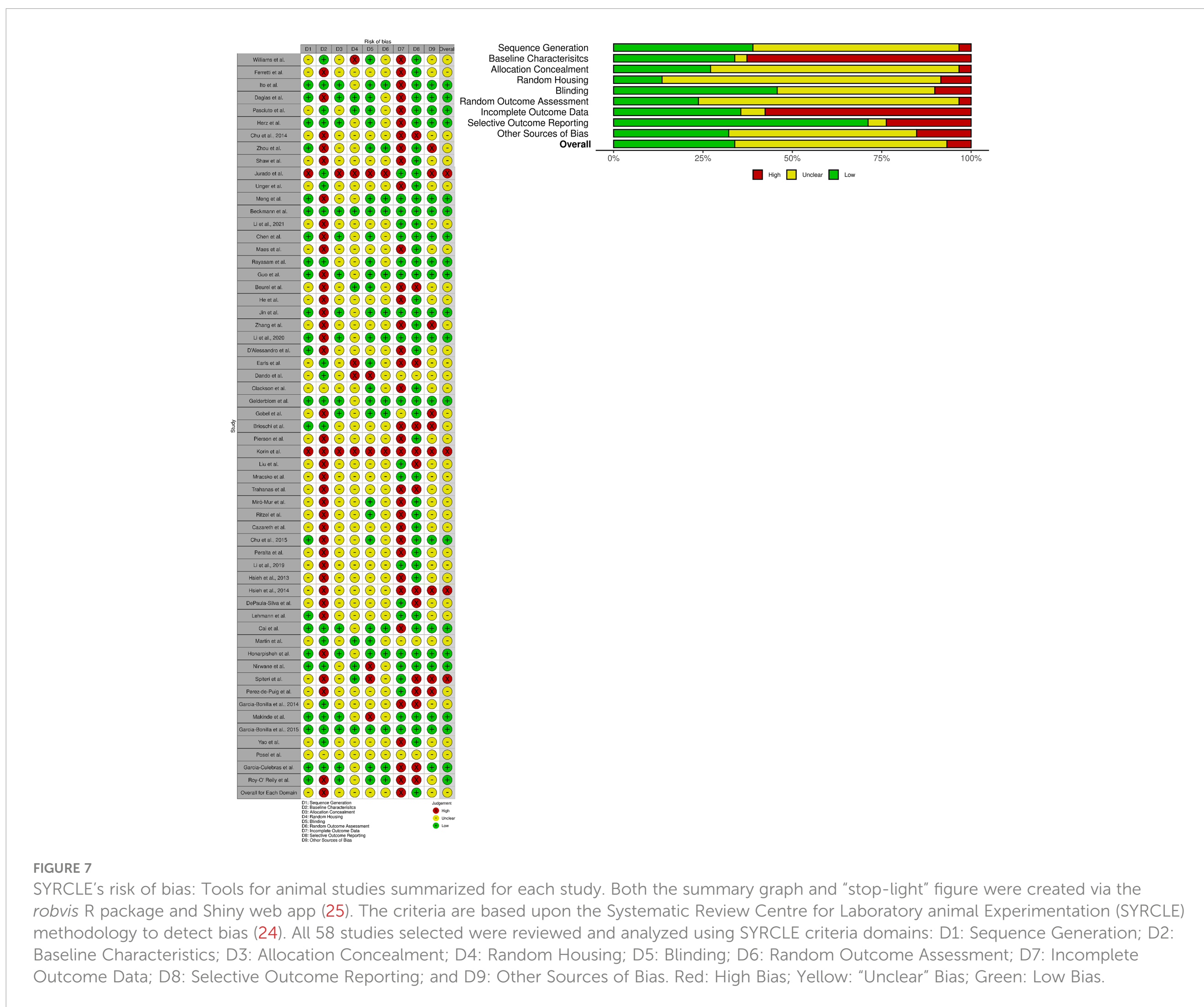

FIGURE 7
SYRCLE's risk of bias: Tools for animal studies summarized for each study. Both the summary graph and "stop-light" figure were created via the *robvis* R package and Shiny web app (25). The criteria are based upon the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) methodology to detect bias (24). All 58 studies selected were reviewed and analyzed using SYRCLE criteria domains: D1: Sequence Generation; D2: Baseline Characteristics; D3: Allocation Concealment; D4: Random Housing; D5: Blinding; D6: Random Outcome Assessment; D7: Incomplete Outcome Data; D8: Selective Outcome Reporting; and D9: Other Sources of Bias. Red: High Bias; Yellow: "Unclear" Bias; Green: Low Bias.

standardized reporting of flow cytometry methods and results and this being required to become a prerequisite for peer-reviewed publications. In this systematic review, we demonstrate most studies that apply flow cytometry methods to neurology and neuroimmunology, specifically to homogenized mouse brain, share little to no consensus on methods, analysis, or results. Here, we summarize our findings and produce a series of recommendations for future studies (Table 1).

We retrieved 58 neurological/neuroimmunology studies that utilized flow cytometry to identify or sort multiple immune cell subsets from WT/control mouse brains, which were generally compared within the study results to an experimental mouse model (27–84). We compared mouse strains, perfusion, and tissue processing methods and noted that the age of mice and methods for tissue homogenization are variable (96–98, 102). Vivarium conditions, such as group housing within ventilated racks in a pathogen-free barrier facility versus more conventional non-barrier non-ventilated caging, were seldom documented. Corresponding authors from 10/58 studies (17.2%) indicated that the majority of studies utilized a barrier facility with HEPA-filtered air, where each cage was individually ventilated and had sterilized bedding and chow (27, 39, 45, 51, 52, 58, 62, 73, 75, 76). Housing conditions, age, and sex influence the immune cell subsets that can be identified by flow cytometry (95–98) and should be carefully considered, documented in experimental protocols, and adjusted for as a covariate in subsequent analyses. When deciding on immune cell isolation methods for mouse brains, both mechanical homogenization or/and enzymatic tissue digestion are appropriate. Nevertheless, each approach has pros and cons on immune cell retrieval and phenotypic expression and, depending on the specific research question, must be carefully considered (102–104).

Once the mouse lines and homogenization/isolation methods were analyzed, we compared flow cytometry techniques and data reporting across the 58 studies. Cytometers/FACS sorters come in a variety of makes and models but essentially perform the same function and should be calibrated using universal standards (89, 99, 105). Many useful guidelines exist for reporting and include the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) criteria (26, 89, 99, 106). These recommend researchers present flow cytometry data and methods by reporting: 1) the sample/specimen used for experiments; 2) how the samples were treated (storage, processing, and staining, etc.); 3)

TABLE 1 Minimum recommendations and sources for standards for future mouse brain flow cytometry reports (4, 5, 13, 15, 26, 89, 95–101).

Criteria	Recommendations and Notes
Mouse Strain	• Report background mouse strain being used as different mouse strains have different immune backgrounds and responses. • Ensure a WT or a non-treated mouse is included and housed in the same vivarium as transgenic/treated mice in order to enable cross-study comparison. • Report specific details of the vivarium and the caging conditions used. • DO NOT switch mouse strains in the middle of an experiment as immune profiles will vary drastically. • Ensure genetic, phenotypic, and supplier information is provided for each mouse strain used.
Mouse Age	• Report the ages of the mice used for the study as immune profiles are altered by age. • For a more naïve, younger immune cell population, it is best to utilize mice aged ~3 months. • For a more mature, older immune cell population, it is best to utilize mice aged ~18 months. • Age can affect neurological phenotype and behavior; thus, it needs to be included as a confounder.
Mouse Sex	• Report the sex of the mice used, as sex contributes towards different immune responses. • Ensure that the same sex of mice is used throughout all experiments within the study unless the study is focused on sex differences. • Use power analysis based upon sex-specific variances to estimate male to female ratios.
Perfusion Techniques	• Perfusion is recommended before brain homogenization to avoid circulating blood/immune cell contamination. • Use cold Hank's balanced salt solution (HBSS) with 10% serum (heat-inactive fetal bovine serum [FBS], mouse serum, etc.) via cardiac perfusion to ensure residential immune cells and neurological cells remain intact and viable for flow cytometry. • Use of 4% paraformaldehyde (PFA) in any perfusion mixture will affect flow cytometry staining and should only be included AFTER staining for surface antigens.
Brain Dissociation and Cell Isolation Techniques	• Mechanical homogenization (Dounce tissue grinder, bead beating, sonication, etc.), enzymatic homogenization (collagenase, DNase I, trypsin-EDTA, etc.), or the combination of both can alter the phenotypes of immune cells; thus, it is important to describe the technique used. • Use one or more cell strainers (preferably using a 100 µm filter first, then a 70 µm filter) in order to remove as much excess dead cells, fats, and myelin, etc., as possible as these components will alter flow cytometry collection and readings. • Use an isotonic Percoll gradient in order to isolate immune cells and other cells of interest. At minimum, a 37% isotonic Percoll gradient is required to remove all myelin (top layer) and isolate all brain cells (bottom layer/cell pellet). • A 30%/37%/70% isotonic Percoll solution is also recommended if microglia isolation is the only objective.
Flow Cytometer/ Sorter Equipment and Analysis Software Reporting	• Report the exact specifications of the flow cytometer/sorter used in the study. • DO NOT switch between cytometers/sorters throughout experimentation as different models can produce different results. Ideally, calibration and optimization of the machine should be performed daily to ensure accurate results. • Report flow cytometry antibody information, such as clone ID, fluorophore-conjugated, targets, and source, etc. • Analysis software and version number should be reported.

(Continued)

TABLE 1 Continued

Criteria	Recommendations and Notes
Flow Cytometry Result Reporting	• Include a representation of the entire gating strategy (from forward scatter/side scatter to single cells, to live dead, to beginning of gating strategy, and beyond) for each type of experiment conducted. These figures should come from a WT/ non-treated control mouse. • State that the results provided are standardized and comparable to multiple flow cytometry controls, such as unstained, FMO, and single-color controls. At a minimum, either unstained and/or FMO control gating should be included in the report. • Provide cell counts and cell percentages for each gate reported. • Histograms should include unstained and FMO results along with the subjects' data. Indicate MFI readings for each histogram included. • Report exact or estimated total cells per sample collected by flow cytometry either as a unified cell count (i.e., all samples had 1 x 10^5 cells collected during flow cytometry) or for each individual sample. Report the total number of $CD45^+$ cells (all infiltrating/residential immune cells) and $CD45^{-/lo}$ cells (all microglia and other glial cells) when counting immune cells within mouse brain. • Use a live/dead dye to separate living cells for counts. Include the total amount of live cells counted on average and/or from each sample. • Report cell subsets as either total cell counts or by percentages derived from either live cells or from total cells collected. • Methods used to describe how cell counts were reported (by use of flow cytometry cell counting beads, counts provided by the machine, counts calculated, etc.) or how MFI's were calculated are recommended. • Deposit raw data, such as FCS files, in a database such as the FlowRepository (http://flowrepository.org/) after full analysis is completed.

what reagents were used and which antibody clones; 4) what controls were used (unstained controls, FMOs, and single-color controls, etc.) with a demonstration of the full gating strategy for each panel; 5) what instrument was used and details about it; 6) how many total cells and/or live cells were collected in each sample (either exact cell counts or overall estimated counts); 7) what analysis software was used and how compensation was calculated. Although these guidelines do recommend reporting the total cells and/or live cells collected in each sample, this can be misleading for brain homogenate studies that use different tissue dissociation and cell isolation techniques. For example, a 30%/70% Percoll gradient solution will preferentially isolate immune cells, whereas a 30% solution will isolate immune cells and other residential brain cells (neurons and astrocytes, etc.). Hence, it would be beneficial to report all $CD45^+$ (infiltrating/residential immune cells within the brain) and $CD45^{-/lo}$ (microglia and other glial/brain cells). Better documentation would enable replication and more reliable and accurate results and enable subsequent meta-analysis on the immunophenotyping of neurogenerative mouse models.

In the 58 selected studies, we next examined the median and average count and percentages of immune cell subsets reported from WT/control mouse brains. The immune cell subsets selected (Figures 5, 6; Supplementary Table 3) consisted of brain/CNS-only residential immune cells (microglia) and immune cells considered

to be both residential and peripheral (i.e., T cells, B cells, macrophages, and NK cells) (1–10). As expected, of all the immune subsets examined, microglia are the most populous immune cells within WT/control mouse brains (15, 74–76). For microglia markers, we searched for publications that showed expression of TMEM119, which is expressed in more stable, non-reactive microglia (107–110). Traditional methods of detecting microglia by flow cytometry use CD11b$^+$ CD45$^{lo/-}$ phenotyping. However, we favored microglia-specific markers such as TMEM119 as these differentiate microglia from other phagocytic cell types.

For other immune cell subsets, T cells make up the second most abundant immune cell within WT/control mouse brains, with more bulk CD4$^+$ T cells (27–37). In the brain, it is likely that both CD4$^+$/CD8$^+$ T cells are comprised of resident memory T cells (T_{RM}) and might be classified as an even more specialized subset (i.e., T_{CM}, T_{EM}, and T_{EMRA}) (111–113). For the innate immune cell populations (excluding microglia), NK cells were observed more frequently in WT/control mouse brains than other innate immune cells (33, 46–51). Many studies report NK cells as most abundant within the brain's parenchyma and more often than other innate immune populations (excluding microglia) or adaptive immune cells (T cell and B cells) (114–116). NK cells were the most reported innate immune cell subset within WT/control mouse brains in the 58 studies reviewed, but there were still more bulk CD4$^+$/CD8$^+$ T cell counts reported. We cautiously included neutrophils in our systematic review (46, 77–84). These polynucleated cells are challenging to detect with flow cytometry as neutrophil extracellular traps (NETs) cause them to be extremely "sticky", to bind onto each other, and to bind non-specifically to flow antibodies (causing false positives) (117–120). The variability in nomenclature/targets to identify neutrophils, their short life span, and sensitivity toward purification methods are additional limitations (117–120).

The presence of less abundant immune cell subsets found within the brain or infiltrating the brain, including specific CD4$^+$ T cell subsets, T cell memory subsets, and DCs, was also assessed. Unpredictably, it appears that T_{H1} T cells are more abundant in WT/control mouse brains than T_{H2} T cells (not reported in the 58 selected studies) (27, 40). Conventionally, the T_{H1}/T_{H2} ratio is used to determine whether an individual has a bacterial/viral infection (higher ratios are indicative of greater infection), although higher ratios of these subsets are also found in aged subjects, once again highlighting the importance of defining age in studies of immunity (121). None of the 58 studies reported specific T cell memory subsets besides bulk T_{EM} CD4$^+$/CD8$^+$ T cells (30) within WT/control mice. Given the importance of specific T cell memory subsets to overall immunity, future flow cytometry analysis of the brain may benefit from their inclusion. Surprisingly, a few reports listed migratory/residential DCs within WT/control mouse brains, albeit at extremely low levels (33, 52–55) as microglia are thought to mediate brain immune surveillance (122–124).

Our systematic review has some limitations. Our analysis and database search were not automated to update figures from more recent research (125, 126), and potentially, this may be considered a selection bias. The keyword searches we conducted for each immune cell subset have been reported in our methods, but different variations of these names or use of other abbreviations when searching could alter what literature is identified in each database; thus, this could also be considered some level of bias. As several of the selected studies failed to report sufficient details within their main text, figures, or Supplementary Materials, whenever possible, data were extrapolated. We attempted to avoid any author bias or incorrect statements as two independent reviewers assessed all the manuscripts. The original authors were also contacted when further clarifications were required.

Bias of each individual study was reported as per the guidelines created by multiple organizations that review and conduct systematic reviews (18–24). With a majority of the studies examined, very few were able to clearly state if there was any bias or not within the experimental design. As such, we deemed most of the studies as "unclear" bias due to the lack actual reporting on specific data/methodology that could pass as "high" or "low" bias. This can be very problematic as, for example, the Baseline Characteristics category of bias was unclear or high in a majority of the 58 studies. Not including mouse baseline characteristics such as sex, age, weight, and housing conditions, etc., as confounders of experimentation is extremely problematic and can lead to high bias due to the dramatic effect of these factors affecting the immune system of each individual mouse model used. As such, factors such as these can heavily affect the results of flow cytometry testing.

We recommend that supplementary data should include all raw cell counts and document which data are used in the main figures and text to provide more transparency and enable reproducibility in flow cytometry experiments. Overall, there was tremendous variability in the immune cell subset counts in the 58 reviewed studies, such that the SD often exceeded the mean estimates. Hence, medians with IQRs have been provided throughout this review. This could be due to a wide variety of reasons, such as the technical skills/experience of the researcher, reporting bias, and unconsidered confounding factors. There are also methods reporting variability that can contribute toward immune cell count variance across studies, such as the following: mouse strain/age/sex, perfusion, and brain tissue processing techniques conducted, flow cytometer used, fluorescent antibodies used, and gating strategy used. Although heterogeneity in instrumentation and procedures can be unavoidable at some points and is dependent upon the facility where the research is conducted, it would be helpful to standardize some aspects of how immunophenotyping and cell counts are reported. While within-study comparisons are still valid, researchers should report more robust information about mouse parameters, brain tissue processing, and flow cytometry procedures in order to be replicated in the field. It would be insightful to compare flow cytometry results across studies, not only within them. As such, in our analysis, we have included a series of recommendations to aid the interpretation of results, reproducibility, and meta-analysis (Table 1). Adherence to reporting guidelines will ultimately improve our understanding of the dynamic role of immunity in mouse brain.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

RS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. DG: Data curation, Formal Analysis, Methodology, Writing – review & editing. MF: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1281705/full#supplementary-material

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