

Investigation of mast cell toll-like receptor 3 in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and Systemic Mastocytosis using the novel application of autoMACS magnetic separation and flow cytometry

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Abstract

Background: Viral infections and hypersensitivities are commonly reported by Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients. Mast Cells (MC) uniquely mediate type 1 hypersensitivities and resolve viral infections via toll-like receptor 3 (TLR3).

Objective: To characterise and compare mast cell progenitors (MCPs) in CFS/ME participants with a known MC disorder, Systemic mastocytosis (SM), and secondly, to investigate the role of MC TLR3 in CFS/ME participants following Polyinosinic:polycytidylic acid (Poly I:C) stimulation.

Methods: A total of 11 International Consensus Criteria defined CFS/ME participants (40.42 ± 10.31), 9 World Health Organisation defined systemic mastocytosis (SM) participants (47.00 ± 10.37) and 12 healthy controls (HC) (36.36 ± 9.88) were included. Following autoMACS magnetic separation, CD117⁺/Lin⁻ MCPs were stimulated with Poly I:C for 24 hr. MCP purity (CD117 and Lin2), maturity (CD34 and FcεRI), interaction receptors and ligands (CD154 and HLA-DR), and SM-specific (CD2 and CD25) markers were measured using flow cytometry.

Results: There was a significant decrease in HLA-DR⁺/CD154⁻ expression between CFS/ME and SM groups pre and post Poly I:C stimulation. There were no significant differences in maturity MCPs, CD154, and CD2/CD25 expression between groups pre and post Poly I:C stimulation.

Conclusion: This pilot investigation provides a novel methodology to characterise MCPs in a rapid, inexpensive and less invasive fashion. We report a significant decrease in HLA-DR⁺/CD154⁻ expression between CFS/ME and SM participants, and an observed increase in HLA-DR⁻/CD154⁺ expression post Poly I:C stimulation in CFS/ME participants. Peripheral MCPs may be present in CFS/ME pathophysiology, however further investigation is required to determine their immunological role.

Key words: Chronic Fatigue Syndrome; Mast Cells; Myalgic Encephalomyelitis; Systemic Mastocytosis; Toll-like Receptor 3

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Introduction

Mast cells (MC) are multifunctional leukocytes of the immune system, proficient in responding to both allergen specific and nonspecific danger stimuli.¹ In the peripheral bloodstream,

MCs circulate as CD34⁺/FcεRI⁻ uncommitted hematopoietic progenitors.^{2,3} Mature MCs do not typically circulate in the peripheral blood. MCs migrate as immature progenitor cells to

vascularised tissue, such as the skin, mucosa, brain and airways, to differentiate and mature into functional connective (or serosal) tissue and mucosal MCs.² Stem cell factor (SCF), also known as CD117 (c-kit), is the primary ligand that mediates MC proliferation, development and survival. MCs are responsive to immune modulators within the MC microenvironment, such as growth factors, cytokines and chemokines that influence their functional, structural and biochemical phenotype.⁴ MC phenotype is also determined by protease content and surface expression levels of Cluster of Differentiation (CD) markers such as c-kit, FcεRI, and CD34. CD34 is a primary immaturity marker of MCs. In contrast, differentiated, mature or activated MCs express FcεRI, HLA-DR and CD154, whereby the former is a primary maturation marker of MCs. HLA-DR is expressed on B and T lymphocytes, monocytes/macrophages and dendritic cells, whereas CD154 is expressed on activated T cells.¹

MC activation and degranulation can occur following IgE cross-linkage to the FcεRI receptor during hypersensitivity responses via pattern recognition receptors following direct pathogen interactions, such as Nod-like receptors, C-type lectins, CD48, and toll-like receptors (TLR). Additional MC activation receptors include: other immunoglobulin receptor (FcγR), cytokines, chemokines, neuropeptides, interaction molecules with surrounding immune cells (MHC-II, CD40L), complement receptors and G-protein-coupled receptors.¹

Traditionally, *in vitro* culture of mast cell progenitors (MCP) have been characterised from the bone marrow and tissue in pathological diseases using predominant methods of microscopic analysis,⁵ histochemical staining⁶ and fluorescence imaging.⁷ However, this method continues to be a challenge in providing adequate numbers for assessing MC maturation and function as isolation of live tissue-resident MCs do not readily proliferate and have a limited survival period following isolation. Alternatively, several scientific groups have developed protocols for *in vitro* differentiation and culture of human MCs from different progenitors to establish a method that could easily provide mature, abundant and functional MCs. Similarly to tissue-resident MCs, MCPs can be strongly influenced by isolation techniques⁸ and require a significantly long development and maturation period *in vitro*. CD34⁺ MCPs have been the predominant precursor cell for MC differentiation *in vitro* from the bone marrow, peripheral blood and cord blood. However, this precursor cell demonstrates phenotype variation dependent on *in vitro* stimulants such as IL-3, IL-6, IL-9, and SCF.⁹

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a debilitating disorder hallmarked by unexplained fatigue that is associated with immune, neurological (including autonomic), musculoskeletal, cardiovascular and gastrointestinal systems.¹⁰ Although the underlying aetiology of CFS/ME is not clearly defined, immunological dysfunction has been consistently implicated in this condition. A significant reduction in natural killer (NK) cell cytotoxicity is a consistent feature in CFS/ME patients compared with healthy controls, as well as atypical pro- and anti-inflammatory cytokines, nitric oxide production, and hypersensitivity responses.¹¹ A high prevalence of viruses has also been commonly reported by CFS/ME patients prior to the onset of CFS/ME symptoms. Although no universal virus or pathogen has been identified in CFS/ME

patients, a number of viruses have been consistently reported by CFS/ME patients such as Herpes human viruses (e.g. Epstein Barr virus), adenoviruses, measles, rubella, influenza, cytomegaloviruses, *Coxiella burnetti*, and Ross River virus. A similar feature exhibited by these viruses is the activation of TLR3.¹²

TLRs are type I membrane receptors that induce antimicrobial immune responses by recognising pathogen-associated molecular patterns (PAMP). Currently, eleven human (TLR1-11) TLRs have been identified and are subclassed on subcellular localisation and selective PAMP recognition. Group one TLRs (1, 2, and 4-6) are present at the plasma membrane, whereas group two (TLR3, and 7-9) localize within intracellular compartments, such as endosomes. All TLRs utilise the universal MyD88-dependent pathway adaptor to initiate TLR signalling, with the exception of TLR3.¹³ Human TLR3 is activated by double stranded ribonucleic acid (dsRNA). The synthetic analogue, Polyinosinic:polycytidylic acid (Poly I:C), has been predominantly used by researchers to mimic the effects of viral dsRNA.^{14,15} Although MC reactivity against bacteria, primarily through TLR2 and TLR4, has been characterised more than viruses; an anti-viral mechanism has recently emerged via TLR3.

MC TLR3 also responds to dsRNA and Poly I:C, which activates the nuclear factor kappa beta pathway following the MyD88 independent TRIF pathway.¹³ Subsequently, various pro-inflammatory cytokines (notably type I and type III interferons) and chemokines are released which can collectively enhance the recruitment of multiple inflammatory cells including eosinophils (eotaxin), NK cells (IL-8), and neutrophils (IL-2 and TNF-α).¹ Interestingly, type I hypersensitivity responses have been reported during viral infections due to a possible synergistic signal and cross-linkage between MC TLR3 and the FcεRI receptor during prolonged viral infections.¹⁶ Given MCs have not been investigated in CFS/ME and are a key innate immune cell involved in both inflammation and hypersensitivities, TLR3 on MCs has been proposed to possibly contribute to the consistent viral reports and inflammation exhibited by CFS/ME patients. To better understand the role of MCs in CFS/ME, it is advantageous to compare this disorder with a known MC disorder, such as Mastocytosis.

Mastocytosis is a heterogeneous group of disorders characterized by a myeloproliferative neoplasm of MCs, with both cutaneous and systemic manifestations.¹⁷ Systemic mastocytosis (SM) is characterised by significant systemic involvement of MCs in one or more extracutaneous organs, a somatic KIT gene mutation (D816V) of the SCF receptor (c-kit), organomegaly, abnormal serum tryptase levels, and atypical CD2 and CD25 expression.¹⁷ SM patients often experience sudden attacks, lasting approximately 15-30 minutes. These attacks are characterised by various clinical manifestations ranging from constitutional signs, mediator-related symptoms, muscular skeletal disease-related, and dermatological symptoms, some of which overlap with CFS/ME including: fatigue, flushing, headache, syncope, abdominal pain, bone pain, arthralgia, and myalgia. Dermatological symptoms such as pruritus, blistering, and urticaria pigmentosa are not commonly exhibited by CFS/ME patients, however are hallmark symptoms of SM.¹⁸

Currently, the bone marrow is recognised as the most useful biopsy site as it is the primary extracutaneous tissue infiltrated

by MCs in mastocytosis patients. Examination of the bone marrow both reveals diagnostic infiltrates and allows evaluation of the hematopoietic marrow, which provides important prognostic information. Immunohistochemical staining of bone marrow biopsies with antitryptase is currently the method of choice to characterise and visualize MCs in paraffin-embedded decalcified specimens.^{17,18} However, these methodologies are poorly reproducible, expensive, extremely invasive and biologically unrepresentative *in vivo*.⁹ As MC leakage from the tissues into the peripheral blood has been found in SM patients,¹⁹ a number of studies have attempted to characterise MCPs from alternative routes, such as the peripheral blood, using flow cytometric methods. Aberrant co-expression of CD2 and/or CD25 by flow cytometry and immunohistochemistry has been found on neoplastic CD117⁺ MCs from bone marrow aspirates, which has further refined the diagnostic options for mastocytosis patients.²⁰ Currently, aberrant expression of CD2 and/or CD25 is acknowledged as one minor inclusion criteria by the WHO case definition of Mastocytosis.^{18,21}

As active MCs are not typically found in the peripheral blood, the primary aim of this project was to identify peripheral MCPs in CFS/ME participants and compare these MCPs with an established MC disorder, such as SM (positive control group), in addition to a HC group. The use of two control groups enabled CFS/ME to be compared with a disorder hallmarked with high MC activity, as well as a normal MC activity group. The rationale for this project was to develop a less invasive and less expensive procedure to characterise and analyse MC activity in CFS/ME and other MC activation disorders. A secondary aim was to analyse the immunological role and contribution of TLR3 on MCs via Poly I:C stimulation to determine the extent of viruses in the pathomechanism of CFS/ME.

Materials and methods

Study Participants

Participants were sourced from the National Centre for Neuroimmunology and Emerging Diseases (NCNED) research database for CFS/ME. Participants aged between 18 and 65 years were recruited from community support networks in the South East Queensland and Northern New South Wales region of Australia. All participants completed a screening questionnaire reporting their sociodemographic details, medical history, and symptoms. All participants provided written consent prior to participation and completed a self-reported questionnaire on their current symptoms and history of illness. CFS/ME participants were aged and sex-matched with SM and HC groups. CFS/ME participants were defined in accordance with the Fukuda and International Consensus Criteria (ICC) symptom requirements.^{10,22} The HC group reported no chronic illness or symptoms of CFS/ME and SM. SM participants were defined by the World Health Organisation (WHO) case definition of SM^{17,18} and diagnosed by a clinician. Participants were excluded if they were pregnant or breastfeeding, or reported a previous history of smoking, alcohol abuse or chronic illness (for example, autoimmune diseases, cardiac diseases and primary psychological disorders).

Participants donated 85 ml of whole blood which was collected in ethylenediaminetetraacetic acid tubes between 8:00 am and 10:30 am. Routine pathology screening was further

performed to exclude participants who demonstrated parameters outside the normal ranges. All participants provided written informed consent and the study was approved by the Griffith University Human Research Ethics Committee (HREC/15/QGC/63).

Peripheral blood mononuclear cells and CD117⁺/Lin⁻ Magnetic Bead Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation over a density gradient medium (Ficoll-Paque Premium; GE Healthcare, Uppsala, Sweden) to separate granulocytes (such as neutrophils, basophils and eosinophils). PBMCs were stained with trypan blue stain (Invitrogen, Carlsbad, CA) to determine total cell count and cell viability and adjusted to a final concentration of 1×10^8 cells in 20 ml. CD117 Microbead and Lineage Cell Depletion kits were used to isolate PBMCs to CD117⁺/Lin⁻ cells by magnetic bead separation on the autoMACS[®] Pro Magnetic Separator as described by the manufacturers' instructions. The CD117 Microbead kit was used to separate CD117⁺ cells, a primary MC marker, and the Lineage Cell Depletion Kit labelled Lin⁻ cells by negative selection with a cocktail of biotin-conjugated antibodies against lineage-specific antigens (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a), followed by magnetic labelling of Anti-Biotin microbeads. Following magnetic bead separation, CD117⁺/Lin⁻ cells were divided into two 5 ml polystyrene round-bottom FACS tubes where one tube was stimulated with 2 ul/1000 ul of Poly I:C. CD117⁺/Lin⁻ cells were incubated for 24 hr at 38°C. Post the 24 hr incubation, stimulated and unstimulated CD117⁺/Lin⁻ cells were pelleted down and resuspended in 200 ul of autoMACS running buffer (bovine serum albumin).

CD117⁺/Lin⁻ cells were labelled with CD117 for 15 minutes, followed by a number of fluorochrome-conjugated antibodies correspondent to MCP: purity (CD117 and Lin2), maturity (FcεRI and CD34), interaction receptors and ligands (CD154 and HLA-DR) and SM-specific markers (CD2 and CD25) for 25 minutes in the dark. A Lin2 monoclonal antibody cocktail (anti-CD3, anti-CD14, anti-CD19, anti-CD20 and anti-CD56) was used as a negative MCP marker to label and exclude additional immune cells (such as, T cells, B cells, NK cells, monocytes, eosinophils and neutrophils). Cells labelled with the myeloid receptor, anti-CD117 were gated as CD117⁺ committed MCPs. CD117⁺/Lin⁻ cells were further labelled with anti-CD34 and anti-FcεRI to distinguish four different MC maturity MCPs, including CD34⁺/FcεRI⁻ (MC-monocyte committed MCP), CD34⁺/FcεRI⁺ (late-committed MCP), and CD34⁺/FcεRI⁺ (mature MCP). Labelled cells were resuspended with stain buffer (BD Bioscience, San Jose, CA) prior to flow cytometric analysis as previously described.²³

Flow cytometry

MCPs were determined using LSR Fortessa[™] X-20 flow cytometry as previously described.²³ All samples were collected at 10,000 events and all antibodies were purchased from BD Bioscience, unless otherwise stated. Four separate panels were designed to investigate MCP: purity, maturity, interaction receptors and ligands, and atypical SM characteristics. MC purity was measured by a Lin2 monoclonal antibody cocktail

and anti-CD117, which were used to gate for CD117⁺/Lin⁻ MCPs. There were no significant differences in MCP purity between groups. The mean MCP purity of all groups was 84.72 ± 7.93. CD117⁺/Lin⁻ MCPs with purity more than 80% were gated for the following maturity MCPs: mature MCP (FcεRI⁺/CD34⁻), late-committed MCP (FcεRI⁺/CD34⁺), MC/monocyte committed MCP (FcεRI⁻/CD34⁺) and non-MC (FcεRI⁻/CD34⁻). Mature MCPs were further characterised by T and B lymphocyte interaction markers, HLA-DR (MHC-II) and CD154 (CD40L). CD2 and CD25 marker expression was used on the total MC population to determine possible atypical SM characteristics.

Statistical analysis

Flow cytometry data were exported directly from BD FACS LSR Fortessa X-20 and the four panels were separated into pre and post Poly I:C stimulation data sets. Data were compared between the three test groups (CFS/ME, SM and HC) with statistical analyses performed based on the distribution of each variable. Data were analysed using IBM SPSS Version 22. Demographics of participants was normally distributed and the one-way ANOVA test was used to test for significance at p < 0.05. Shapiro-Wilk normality tests were conducted to determine the distribution of data, in addition to skewness and kurtosis tests to determine data normality. The Kruskal-Wallis H test was performed to determine the statistical significance of MCP: purity, maturity, interaction receptors and ligands, and atypical SM characteristics. Statistical significance was reported at p < 0.05 and a Bonferroni correction was applied to adjust for multiple test parameters.

Results

Participant Demographics

From a total of 32 participants, 11 participants were defined by the Fukuda and ICC criteria for CFS/ME, 9 participants met the WHO case definition for SM, and 12 participants met the criteria for HCs. There were no significant differences in age and gender between groups (Table 1). Similarly, there were no significant differences between groups for routine pathology tests (Table 2).

Identification of human peripheral maturity mast cell progenitors

There were no significant differences between groups for maturity MCPs pre and post Poly I:C stimulation (Figure 1). However, there was an observed increase in MC/monocyte committed (CD34⁺/FcεRI⁻) and late-committed MCPs (CD34⁺/FcεRI⁺) pre and post Poly I:C stimulation for CFS/ME participants compared with SM and HCs. Conversely, mature (CD34⁻/FcεRI⁺) MCPs were the predominant MCP in SM participants pre and post Poly I:C stimulation. The HC group demonstrated no distinct MCP pre and post Poly I:C stimulation.

Comparison of CD154 and HLA-DR expression between CFS/ME, SM and HC groups

Surface markers CD154 and HLA-DR were compared between groups pre and post Poly I:C stimulation. There was a

Table 1. Demographic results between CFS/ME, SM and HC groups

Parameters	HC	CFS/ME	SM	p value
Age (years)	36.36 ± 9.88	40.42 ± 10.31	47.00 ± 10.37	0.083
Gender				
Male	36.4%	41.7%	77.78%	
Female	63.6%	58.3%	22.22%	

Table 2. Routine pathology blood results between CFS/ME, SM and HC groups

Parameters	HC	CFS/ME	SM	p value
White Cell count (× 10 ⁹ /L)	5.77 × 10 ⁹ ± 4.43 × 10 ⁸	4.70 × 10 ⁹ ± 7.34 × 10 ⁸	6.05 × 10 ⁹ ± 5.99 × 10 ⁸	0.515
Neutrophils (× 10 ⁹ /L)	3.69 × 10 ¹⁰ ± 3.49 × 10 ⁹	2.21 × 10 ¹⁰ ± 6.14 × 10 ⁹	3.44 × 10 ¹⁰ ± 3.79 × 10 ⁹	0.214
Lymphocytes (× 10 ⁹ /L)	1.82 × 10 ¹⁰ ± 1.63 × 10 ⁹	1.77 × 10 ¹⁰ ± 1.19 × 10 ⁹	2.22 × 10 ¹⁰ ± 1.98 × 10 ⁹	0.221
Monocytes (× 10 ⁹ /L)	3.34 × 10 ⁸ ± 2.48 × 10 ⁸	2.98 × 10 ⁸ ± 3.71 × 10 ⁷	3.05 × 10 ⁸ ± 2.00 × 10 ⁷	0.548
Eosinophils (× 10 ⁹ /L)	1.47 × 10 ⁸ ± 2.62 × 10 ⁷	2.11 × 10 ⁸ ± 2.62 × 10 ⁷	1.67 × 10 ⁸ ± 3.93 × 10 ⁷	0.189
Basophils (× 10 ⁹ /L)	4.00 × 10 ⁷ ± 7.07 × 10 ⁶	2.75 × 10 ⁷ ± 4.12 × 10 ⁶	2.27 × 10 ⁷ ± 2.37 × 10 ⁶	0.151
Platelets (× 10 ⁹ /L)	2.51 × 10 ¹¹ ± 1.16 × 10 ¹⁰	2.68 × 10 ¹¹ ± 2.49 × 10 ¹⁰	2.51 × 10 ¹¹ ± 1.10 × 10 ¹⁰	0.635
Haemoglobin (× 10 ⁹ /L)	144.25 ± 4.02	132.25 ± 4.62	143 ± 3.66	0.124
Haematocrit (× 10 ⁹ /L)	0.43 ± 0.01	0.40 ± 0.01	0.43 ± 0.007	0.149
Red Cell count (× 10 ¹² /L)	4.89 × 10 ¹² ± 1.25 × 10 ¹¹	4.44 × 10 ¹² ± 1.46 × 10 ¹¹	4.87 × 10 ¹² ± 1.05 × 10 ¹¹	0.057
MCV (× 10 ⁹ /L)	87.83 ± 1.06	89.88 ± 0.95	87.64 ± 0.93	0.223

significant decrease in HLA-DR⁺/CD154⁻ expression between HC and SM participants, as well as CFS/ME and SM participants pre Poly I:C stimulation. This significant decrease in HLA-DR⁺/CD154⁻ expression remained post Poly I:C stimulation between CFS/ME and SM participants (Figure 2). Although there were no significant differences between groups for mature MCPs (CD34⁻/FcεRI⁺), higher levels of HLA-DR/CD154⁺ were expressed on these mature MCPs (CD34⁻/FcεRI⁺) in CFS/ME participants compared to HCs and SM participants post Poly I:C stimulation.

CD2 and CD25 expression between CFS/ME, SM and HC groups

Across the four SM MCPs, there was no significant difference between groups for CD2 and CD25 expression pre and post Poly I:C stimulation (Figure 3).

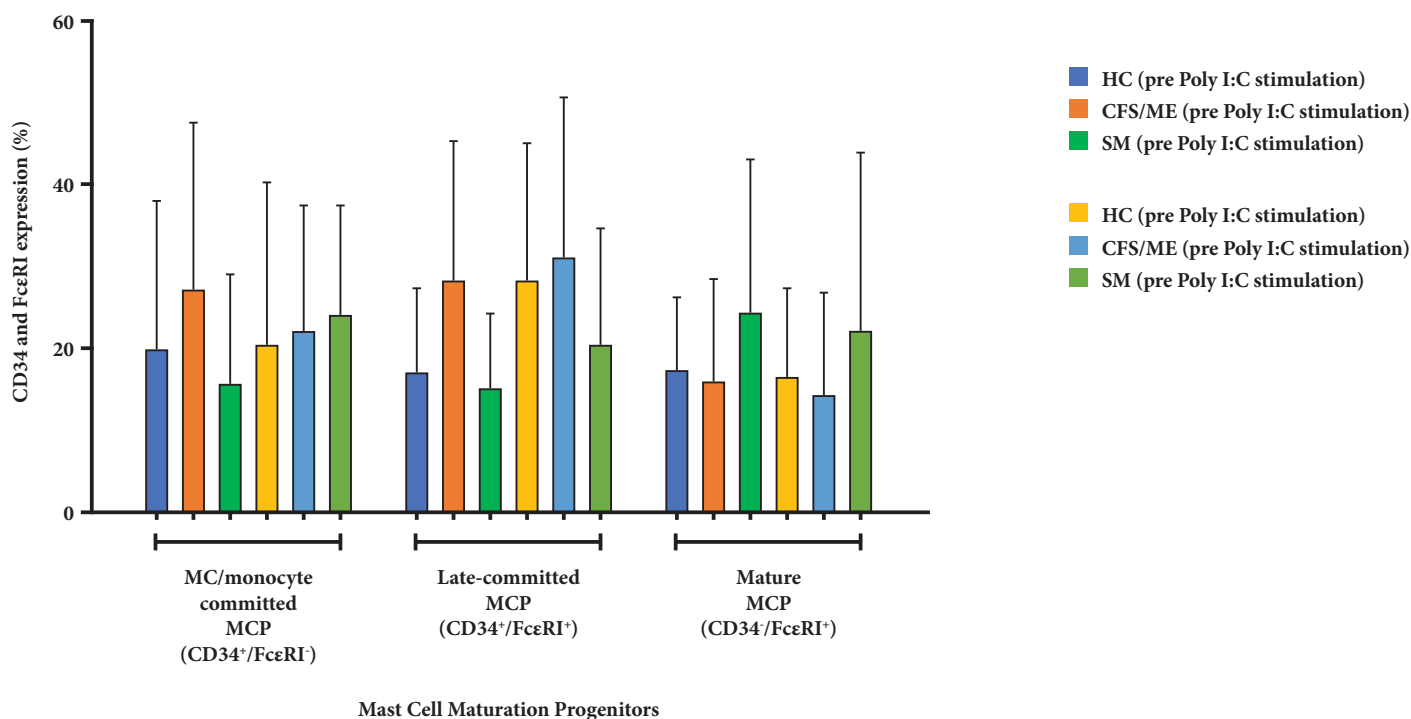


Figure 1. Bar graph plots for CD34 and FcεRI expression are shown as percentage of parent CD117⁺/Lin⁻ cells in CFS/ME, SM and HC groups pre and post 24hr Poly I:C stimulation.

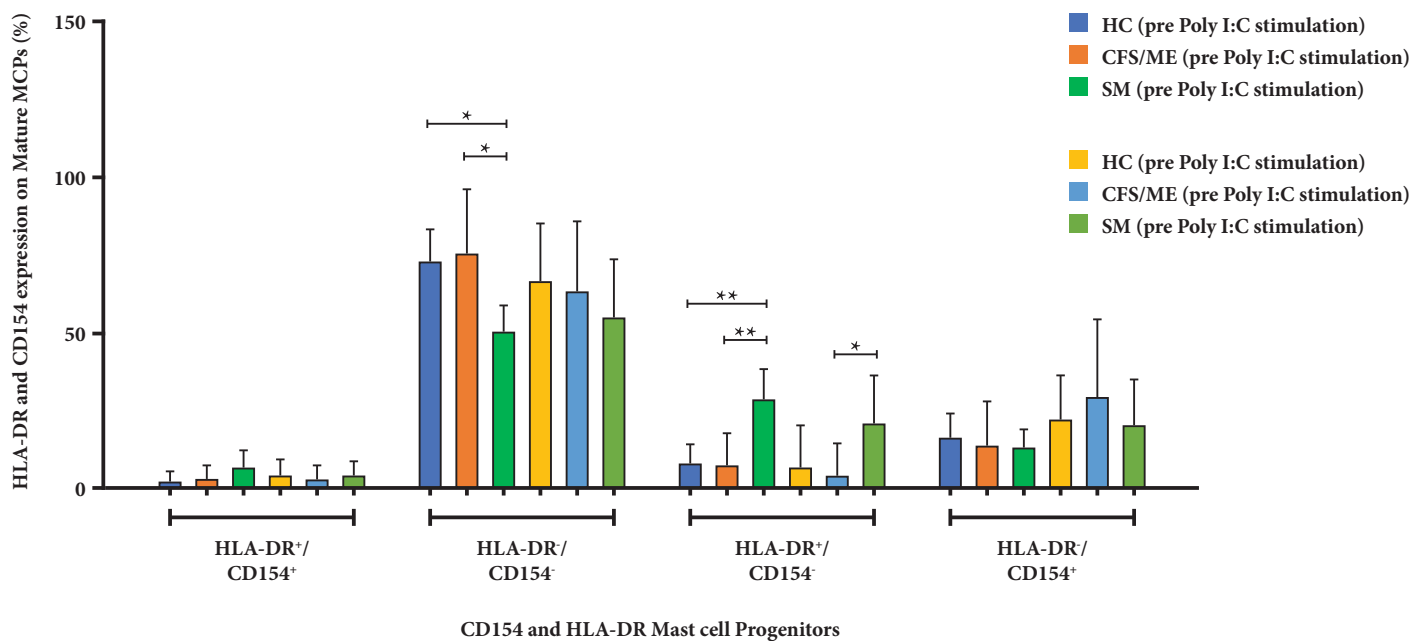


Figure 2. Bar graph plots for CD154 and HLA-DR expression are shown as percentage of parent CD117⁺/Lin⁻ mature MCPs (CD34⁺/FcεRI⁺) in CFS/ME, SM and HC participants pre and post 24hr Poly I:C stimulation.

* refers to significant difference where $p < 0.05$, using Bonferroni post-hoc test.

** refers to significant difference where $p < 0.001$, using Bonferroni post-hoc test.

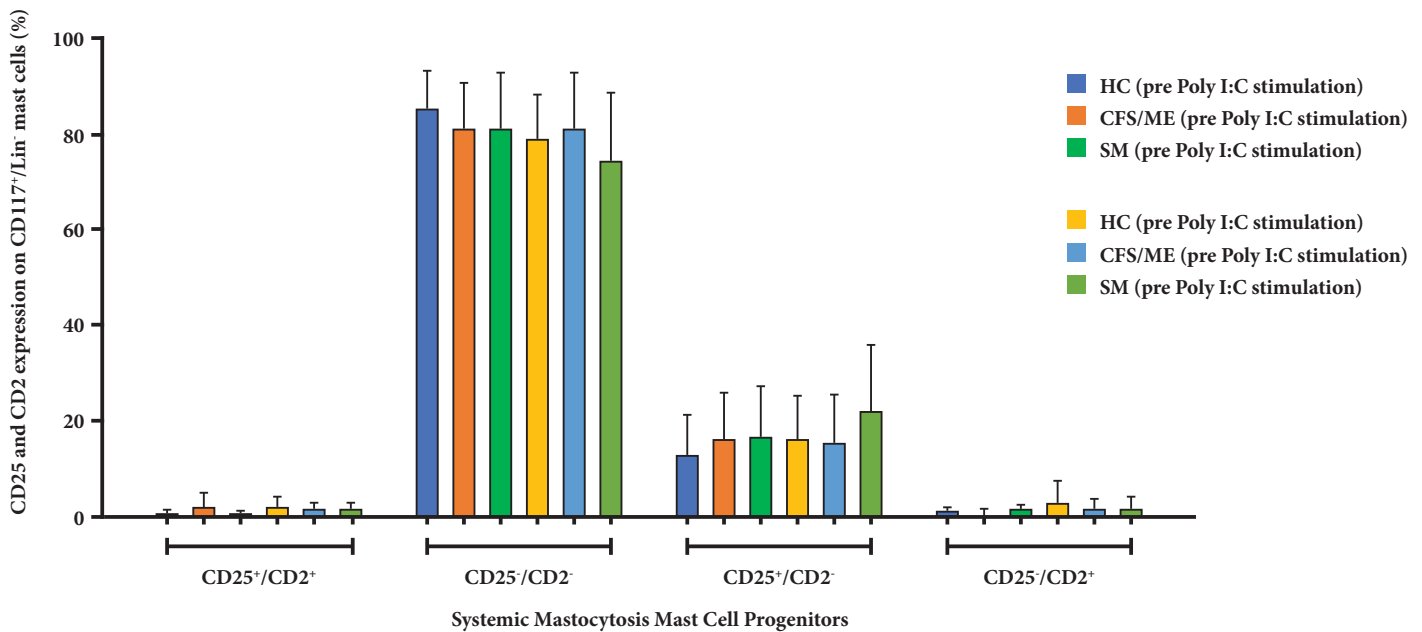


Figure 3. Bar graph plots for CD2 and CD25 expression are shown as percentage of parent CD117⁺/Lin⁻ cells in CFS/ME, SM and HC groups pre and post 24hr Poly I:C stimulation

Discussion

This present study supports our pilot study²³ that identified MCPs in PBMCs from CFS/ME and HC participants. Additionally, this investigation is the first to characterise peripheral MCPs with a known MC disorder, SM.²⁴ Importantly, we report novel findings of MCPs following pre and post Poly I:C stimulation of TLR3 in CFS/ME participants. A significant decrease in HLA-DR⁺/CD154⁻ expression was reported in CFS/ME pre ($p < 0.001$) and post ($p < 0.05$) Poly I:C stimulation compared with SM participants. This pilot investigation identified for the first time peripheral MCPs through flow cytometric methods and possible immunological dysfunction in disease-compromised patients (CFS/ME and SM). This novel method may have significant implications for analysing MCPs compared with traditional methods, such as bone marrow biopsies, that are poorly reproducible, expensive and extremely invasive.²⁰

We confirm our previous findings that identified MC/monocyte committed (CD34⁺/FcεRI⁻) and late committed (CD34⁺/FcεRI⁺) MCPs in CFS/ME participants compared with HCs as demonstrated in **Figure 1**.²³ This observed increase in MCPs pre and post Poly I:C stimulation in CFS/ME participants may suggest increased mobilisation of MCPs following a latent viral infection. MCPs predominantly circulate in the bloodstream and lymphatics and traverse from the bone marrow to peripheral tissues during heightened physiological and inflammatory settings, such as asthma and hypersensitivities.¹¹ MCs recognise viruses by detecting dsRNA or Poly I:C via TLR3. Following PAMP-TLR3 stimulation, a collection of inflammatory cytokines (TNF-α, IL-6, IL-8, IFN-β, IFN-γ, and IL-1α) and chemokines (CCL4, CCL5, CXCL8 and CXCL10) are released following NF-κβ and IFN-regulatory pathway stimulation.¹ An excessive release of these pro- and anti-inflammatory mediators can disrupt the inflammatory homeostasis and induce systemic inflammation following prolonged MC TLR3 stimulation (viral latency). An imbalance in the inflammatory pathways may influence the tissue microenvironment, and consequently affect

the proliferation, differentiation and recruitment of MCPs to these inflammatory sites. This may explain the altered pro-inflammatory cytokine profiles exhibited in CFS/ME patients.^{11,25,26}

MCs are primarily involved in innate immunity. However, MCs also mediate adaptive immune responses with other immune cells, such as B and T lymphocytes, through their extensive collection of cell surface receptors and ligands. Prior to Poly I:C stimulation, HLA-DR⁺/CD154⁻ expression was significantly reduced in HC ($p < 0.001$) and CFS/ME ($p < 0.001$) participants compared with SM participants. This significant decrease remained in CFS/ME participants ($p < 0.05$) compared with SM participants post Poly I:C stimulation as demonstrated in **Figure 2**. Although it has been reported that MCs express HLA-DR following antigen-dependent interactions with effector CD4⁺T cells,²⁷ the current findings do not demonstrate this interaction. A possible rationale is that high HLA-DR expression is a novel occurrence, transpiring between MCs and T cells only after tissue infiltration and localised tissue inflammation,²⁸ such as in SM. As shown in **Figure 1**, the CFS/ME group acquired the lowest percentage of mature MCPs (CD34⁺/FcεRI⁺) compared with SM and HC participants. This observed decrease in mature MCPs (CD34⁺/FcεRI⁺) and significant decrease in HLA-DR⁺/CD154⁻ expression may possibly suggest that MCs in CFS/ME do not acquire a comparable MC abundance to cause tissue infiltration as in SM.

Interestingly, although CFS/ME participants acquired the lowest percentage of mature MCPs (CD34⁺/FcεRI⁺) (refer to **Figure 1**), HLA-DR/CD154⁺ expression on these mature MCPs (CD34⁺/FcεRI⁺) was the highest in CFS/ME than HCs and SM participants post Poly I:C stimulation as demonstrated in **Figure 2**. CD154 is a critical marker for immune and inflammatory response. It is primarily expressed on B lymphocytes and mediates B cell co-stimulation.²⁹ Our data suggest a possible association between MC TLR3 activation and B cell co-stimulation via FcεRI. Binding of CD154 and B cells may cause an

increase in B cell proliferation, subsequently promoting the expression of the MC activating immunoglobulin, IgE.³⁰ Elevations in circulating IgE provides increased stimuli to activate these mature MCPs (CD34⁺/FcεRI⁺). This potentially may explain the type I hypersensitivity responses reported by CFS/ME patients during viral infections.³¹ These MC interactions with B cells may possibly provide a plausible explanation for the elevated B cell populations documented in CFS/ME patients.^{32,33}

SM is characterised by over-proliferative MC populations, causing tissue infiltration and subsequent MC release into the peripheral bloodstream.¹⁷ Aberrant expression of CD2 and/or CD25 expression by bone marrow, peripheral blood or other extracutaneous tissue MCs is currently a minor WHO diagnostic criterion for SM.³⁴ Given no significant differences were observed between groups pre and post Poly I:C stimulation across the four SM MCPs (CD2⁺/CD25⁺, CD2⁻/CD25⁻, CD2⁺/CD25⁻ and CD2⁻/CD25⁺) (refer to **Figure 3**), these findings suggest that MCs in CFS/ME patients may not acquire a comparable abundance as in SM. Given previous studies have characterised MCs from bone marrow tissue aspirates and other extracutaneous organs, the source of these MCPs may rationalise this finding as MCs can phenotypically change with different activation, anatomical sites and cultured settings and only constitute approximately 0.053% of PBMCs.³⁵ Thus, further investigations in cell culturing and immunofluorescence staining of these MCPs may provide additional support to further evaluate the progenitor state of these MCPs.

Conclusion

This pilot investigation identified for the first time, peripheral MCPs in CFS/ME, SM and HC participants following MC TLR3 stimulation. The dual application of autoMACS magnetic separation and flow cytometry with these sample groups demonstrates the potential application to analyse MCPs through an alternative method that is inexpensive, less invasive and hence ethically preferred compared with traditional methods to potentially diagnose other MC activation disorders.

The results of this study present a novel field for immunological MC investigation in CFS/ME. The observed increase in MC/monocyte committed (CD34⁺/FcεRI⁻) and late-committed (CD34⁺/FcεRI⁺) MCPs in CFS/ME pre and post Poly I:C stimulation represents a finding not previously noted in clinical situations other than SM. This increase in MCP mobilization suggests a possible dysregulation of the inflammatory pathways and alteration of the microenvironment following excessive MC TLR3 activation on tissue-resident MCs.

The significant decrease in HLA-DR⁺/CD154⁻ expression suggests that CFS/ME participants may not acquire a comparable MC abundance to cause significant tissue infiltration as in SM. Conversely, the observed increase in HLA-DR/CD154⁺ expression on mature MCPs (CD34⁺/FcεRI⁺) in CFS/ME participants post Poly I:C stimulation suggests possible associations between MCs and B lymphocytes, which may elucidate the hypersensitivities reported by CFS/ME patients during viral infections. Further investigation is required to determine the immunological contribution of MCs in the pathophysiology of CFS/ME.

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Author contributions

The authors in this article were involved in the design, drafting, and development of this manuscript. All authors have reviewed and approved the final version of and declare no conflict of interest in the research presented.

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