Reduced immune system responsiveness in fibromyalgia - A pilot study

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abstract

Fibromyalgia is characterized by widespread musculoskeletal and joint pain, stiffness, fatigue, and sleep and mood disorders. However, the involvement of the immune system in the pathways of fibromyalgia is unclear The aim of this study was to explore the role of the immune system in comparison with healthy controls and in association with clinical symptoms. Thirteen women with fibromyalgia and 14 controls were included. Peripheral blood mononuclear cells were stimulated and analysed by flow cytometry, and interferon gamma (IFN- γ) and interleukins were measured. Among clinical symptoms, the fibromyalgia group showed decreased cold pain threshold. Immunologically, they had a higher percentage of monocytes, a lower percentage of $CD19^{+}$ B - cells and lower secretion of IFN- γ after stimulation. Decreased capacity to secrete IFN- γ was significantly correlated with decreased cold pain threshold in the fibromyalgia group. These results confirm the presence of immune aberrations in fibromyalgia, at least partially responsible for the associated pain.

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1. Introduction

Fibromyalgia (FM) is a syndrome of widespread musculoskeletal pain and hyperalgesia, which affects about 2–4% of the population, with a predominance among women [1 , 2]. For diagnostic purposes, it is important to have simple and accessible biological marker(s), which today do not exist for FM. Hence, laboratory findings are usu- ally normal and exclude evident inflammatory or metabolic condi- tions such as rheumatic disorders, hypothyreosis, immune deficiency, etc. The diagnosis of FM is therefore based solely on clinical symptoms and might be considered as an exclusion diagnosis. FM is also associated with other unspecific symptoms mainly manifested as fa- tigue [3 , 4], sleep disorders [5] , cognitive dysfunction [6–8] , and depression/anxiety [9 , 10].

The role of the immune system in pain remains unclear [11] , al- though some of the FM symptoms have been linked to the secretion of cytokines from immune cells. A study with a large population of FM patients and control-donors showed a pattern of significantly lower concentrations of several cytokines (IFN- γ, IL-5, IL-6, IL-8, IL-10, MIP- 1 β, MCP-1, and MIP-1 α) in FM patients when measured after in vitro stimulation of peripheral blood mononuclear cells (PBMCs) [12 , 13]. Wallace and colleagues suggested a pattern of cytokines as a diag- nostic kit for FM based on overall decreased concentrations of IL- 6, IL-8, MIP-1 α, and MIP-1 β from stimulated PBMCs as compared to healthy controls, patients with rheumatoid arthritis, and patients with systemic lupus erythematosus [13] . However, other studies reported increased levels of pro-inflammatory cytokines after stimulation of PBMCs [14] .

Immunological activity can be studied by investigating the pheno- type and functional responses of immune cells in the peripheral blood. The innate and adaptive cells of the immune system produce a great va- riety of cytokines that can have pro-inflammatory and effector functions (e.g. IL-1, IL-4, IL-6, IL-8, IL-12, IL-13, IL-17, and INF- γ) or regulatory and anti-inflammatory properties (e.g. IL-10) [15] . Cytokines may also be released by other cells. For example, IL-6 belongs to the family of acute phase proteins and is released by hepatocytes [16] , neurons, and glial [17] , endothelial [18] and muscles cells [19] . Concerning IL-6, it is important to note that contractile muscles are the main source of IL-6 in plasma [20–22] . However, many of the studies on the immune system in FM patients have been conducted without considering the relation to clinical symptoms, which complicates the interpretation of immunolog- ical findings.

The aim of this study was to compare the phenotypes and responses of immune cells extracted from the peripheral blood of women with FM and matched controls. When differences were found, the second aim was to further explore the immunological findings in relation to clini- cal symptoms through correlation analysis. The primary hypothesis was that the immune system functions behave differently in FM compared to controls and that aberrations in immune response correlate with clinical symptoms of FM. **2. Methods**

2.1. Participants

Participants were recruited by newspaper advertisements to a multi- center study (clinicaltrials.gov identification number: NCT01226784) where FM participants were randomised to physical exercise or relax- ation therapy [23] . The current study was performed in the Stockholm cohort and relied on baseline data obtained in 2011–2012, i.e. before start of the intervention.

All participants had a physical examination by a specialist in reha- bilitation medicine and completed questionnaires (see below). They had to refrain from analgesics, NSAIDs, or hypnotics for 48 h prior to clini- cal examination and one week before blood sampling. Antidepressants, anti-epileptics, and other important long-term medications, for example, for high blood pressure, asthma, etc., were not suspended.

Thirteen women with FM participated in the study and were of work- ing age (20–65 years) and met the American Colleague of Rheumatology (ACR) −90 classification criteria for FM [2] . During the study period, the ACR-90 criteria were widely accepted in clinical practice, which is still the case in Sweden. Exclusion criteria were high blood pressure (> 160/90 mmHg), osteoarthritis in the hip or knee, other severe somatic or psychiatric disorders, other primary causes of pain than FM, high consumption of alcohol (Audit > 6 for women according to the version used), participation in a rehabilitation program within the past year, regular resistance exercise or relaxation training twice a week or more, and inability to understand or speak Swedish. Fourteen healthy women recruited during the same time period and matched by age served as controls.

2.2. Pain assessment

Pain was rated on a plastic 100 mm Visual Analogue Scale (VAS) with a moveable cursor along a line and anchors only at the extremes. The participant was asked to rate, from “no pain at all ” to “worst imaginable pain ”, their current pain intensity, their global pain intensity, and their average, minimal, and maximal pain intensity during the past week.

2.3. Questionnaires

Both groups answered questionnaires concerning their situation with respect to the impact of FM, health-related quality of life (HRQoL), fa- tigue, and depression/anxiety.

2.3.1. The fibromyalgia impact questionnaire (FIQ)

The FIQ is disease specific and comprises ten subscales with scores ranging from 0 to 100. The total score is the mean of the subscales, and a higher score indicates poorer health status and more expressed FM symptoms [24] .

2.3.2. Short-Form 36 (SF-36)

The SF-36 is a validated and widely used questionnaire to assess HRQoL [25 , 26]. The 36 items in the questionnaire are grouped into the following eight subscales: physical functioning, role limitations caused by physical problems, bodily pain, general health, energy/vitality, social functioning, role limitations caused by emotional problems, and mental health. The subscale scores range from 0 to 100, with a higher score indicating better HRQoL. In addition, the sum of the physical and men- tal scores can also be accumulated creating two additional subscales: physical cumulative and mental cumulative scores.

2.3.3. Multidimensional fatigue inventory (MFI-20)

The Swedish version of the MFI-20 covers the following five dimen- sions of fatigue: general fatigue, physical fatigue, mental fatigue, re- duced motivation, and reduced activity. It contains 20 statements for which the subject must indicate on a five-point scale the extent to which the particular statement applies to him or her. The statements refer to aspects of fatigue experienced during the preceding days, and higher scores indicate a higher degree of fatigue. For each subscale, a total score is calculated by adding up the scores for the four individual items, and thus the scores can range from 4 to 20 for each subscale [27] .

2.3.4. Hospital anxiety and depression scale (HADS)

The HADS is used to determine the levels of anxiety and depression that a person is experiencing [28] . The total sum for both HADS anxiety and HADS depression levels ranges from 0 to 21. A score of 10 or more is indicative of clinically significant anxiety or depression symptoms.

2.4. Nociceptive tests

2.4.1. Quantitative sensory testing (QST)

QST responses to thermal stimulation were examined using a ther- motest (Medoc Pathway ATS, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel). A contact thermode (3 cm × 3 cm) was placed onto the lateral part of the hip on the non-dominant side. QST parame- ters were determined first for cold and warmth detection thresholds and then for cold and heat pain thresholds. For the cold and warmth detec- tion thresholds, the participant was instructed to interrupt stimulation when she perceived the slightest cold or warm sensation. The temper- ature started from the ambient temperature (32 °C) and then increased or decreased by 10 °C/s to a cut-off of either 53 °C (heat stimulation) or 0 °C (cold stimulation). For the cold and heat pain thresholds, the participant was instructed to interrupt stimulation when she first per- ceived a painful sensation, starting again from the neutral temperature of 32 °C and decreasing (cold pain) or increasing (heat pain) in mag- nitude. The measurements were performed three times for every mode and an average temperature was calculated as the threshold temper- ature. The higher the temperature the participant could tolerate, the higher the threshold for heat, while the lower temperature the partici- pant could tolerate, the higher the threshold for cold.

2.4.2. Pressure pain thresholds (PPTs)

PPTs were assessed using a hand-held algometer (Somedic Sales AB, Hörby, Sweden) with a probe area of 1 cm^2 [29] . The pressure was increased at an approximate rate of 50 kPa/s. The participants were familiarized with the procedure beforehand and instructed to press a response button at the first perception of pain. PPTs were assessed bi- laterally at m. supraspinatus, the lateral epicondyle of the humerus, the gluteal area, and the inside of the knee (corresponding to the tender points used in the ACR-90 criteria for FM classification) [2] . The aver- age PPT for these 8 sites for each individual was recorded.

2.5. Procedure for blood sampling and isolation of PBMCs

Blood samples (15–25 ml) were taken by venipuncture, and PBMCs were isolated from the fresh heparinised whole blood by density gradi- ent centrifugation using Ficoll-Paque Plus. In summary, whole blood was first diluted

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with sterile PBS at a ratio of 1:1 and added to 50 ml tubes with Ficoll at a 3:4 ratio of Ficoll to diluted blood. Gradient centrifu- gation was performed at 2500 rpm for 30 min uninterrupted at room temperature. PBMCs were collected and washed twice in 50 ml tubes at 1500 rpm for 10 min at + 4°C. The supernatants were removed, and the cells were re-suspended and diluted with trypan blue and counted using a Bürker chamber. PBMCs were diluted in calf serum at a concentration of approximately 10 million PBMCs/ml and frozen immediately at –80 °C. Within 3 days, the tubes were moved to –140 °C storage.

2.6. In vitro stimulation of PBMCs

PBMCs were thawed and washed twice in RPMI-1640 followed by counting and evaluation of cell viability by trypan blue staining. The cells were diluted to 10^6 cells/ml in culture medium (RPMI-1640 sup- plemented with 20 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), l -glutamine (2 mM) (all from HyClone Laboratories Inc, South Logan, UT, USA), and 10% heat-inactivated FCS (Gibco, Invitro- gen, Auckland, New Zealand). The cells were incubated for 3 h in 48- well plates (for flow cytometry analyses) or for 24 h in 96-well plates (for cytokine measurements) (Costar, Cambridge, UK) either in cell culture medium alone or stimulated with 10 ng/ml of lipopolysaccharide (LPS) (InvivoGen, San Diego, CA, USA) or 25 ng/ml phorbol myristate acetate (PMA) + 1 μg/ml ionomycin (IO) (both from Sigma Aldrich, St Louis, MO, USA) at 37 °C in a 6% CO_2 atmosphere. After incubation, the cells were stained for flow cytometry and the supernatants were collected by centrifugation and stored at –80 °C. Kinetics and titration studies were performed to determine the optimal time points and concentrations for the experiments.

2.7. Flow cytometry

After incubation, the PBMCs were harvested and washed in cold PBS. The cells were pre-incubated with 10% normal human serum in FACS wash buffer (PBS with 0.1% BSA and 2 mM EDTA) for 10 min to block Fc receptors and then stained with titrated amounts of the following mouse anti-human antibodies: CD14-PerCP-Cy5.5 (clone: M5E2), CD16- FITC (clone: 3G8), CD3 PerCP (clone: SK7), CD4 FITC (clone: RPA-T4), CD8 APC (clone: RPA-T8), CD19 PerCP-Cy5.5 (clone: HIB19), and CD69 PE (clone: FN50) (BD Biosciences Pharmingen, San Jose, CA, USA). Cor- responding isotype-matched antibodies were used as negative controls. A minimum of 5000 monocyte or 80,000 lymphocyte events based on forward and side scatter properties were acquired using a BD FACS Cal- ibur flow cytometer (Becton Dickinson). Monocytes were identified by the expression of CD14 and CD16, and B-cells were identified by the expression of CD19. T-cells were first identified as CD3 + cells and then divided into CD4 + or CD8 + cells. CD69 was used as a marker for B- and T-cell activation. Analysis was performed with FLOWJO software 7.6.5 (TreeStar, Ashland, OR, USA), and the gating strategies are presented in Figure S1. The percentage of positive cells and the level of surface expression of each marker, defined as the mean fluorescence intensity (MFI), were used as readouts.

Cell phenotype and function were analysed using absolute values (percentage of cells and MFI intensity) and the stimulation index, which represent a cell's capacity to increase its activation following stimula- tion.

2.8. ELISA

Levels of IL-6, IL-17A (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA), IL-4, IL-10, and IFN- γ (MabTech AB, Nacka Strand, Sweden) were determined in cell culture supernatants by employing the sand- wich ELISA procedure in accordance with the manufacturer's instruc- tions. The optical densities were determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) set at 405 nm (IL-4, IFN- γ) or 450 nm (IL-6, IL-10, IL-17).

Cytokines were analysed using absolute values in pg/ml and the stimulation index (Ratio = stimulated / unstimulated).

2.9. Statistics

Microsoft Excel, SPSS 27, and GraphPad Prism were used for coding, analysing, and presenting the data, respectively. Qualitative parameters (sociodemographic parameters and medications) are presented as num- ber of persons and were analysed by Pearson chi-square between the groups. Shapiro-Wilk test for normality of data preceded the choice of comparison tests between the groups for the quantitative parameters. Data regarding clinical parameters, questionnaires, and immune param- eters are presented as means and 95% confidence intervals (CIs).

Table 1

Data are presented as the mean and 95% confidence interval or as number of persons. Comparisons between the groups were performed using parametric or non-parametric tests according to the Shapiro–Wilk test of normality. Pearson chi-square test was used for the variable "Place of birth ".

	Fibromyalgia (*N* = 13)	*Controls* (*N* = 14)	*P-value*
Age (years)	47.9 (41.1–54.6)	49.6 (43.2–56.0)	0.69
Place of birth:			
Sweden	12	11	0.33
Outside Sweden	1	3	
Education	1	0	0.02
Primary (< 9 years)	5	1	
Secondary (10–12 years)	3	2	
Professional (> 12 years)	4	11	
Higher (> 12 years)			
Marital status:	7	1	0.011
Alone (no partner)	6	10	
Married/living with a partner	0	3	
Having a partner but living apart			
Height (cm)	166.6 (163.0–170.0)	165.0 (161.3–168.8)	0.62
Weight (kg)	82.6 (73.0–92.2)	67.1 (60.6–73.5)	0.01
Body Mass Index (kg/m^2)	29.9 (26.0–34.0)	24.7 (22.1–27.3)	0.03

Independent 2-tailed t-tests or Mann–Whitney U-tests were used for comparisons between groups. Paired 2-tailed t-tests or Wilcoxon rank tests were used for comparisons within the groups for immune param- eters. Spearman's correlation test was used to analyze correlations in the FM group between clinical symptoms and immune parameters that differed significantly from controls. An absolute correlation coefficient (r) of 0 to 0.2 is generally considered a weak correlation, 0.3 to 0.6 a moderate correlation, and 0.7 to 1 a strong correlation [30] . Bonfer- roni correlation was applied for the correlation analysis, $p < 0.05/36 = p < 0.0013$.

An effect size equal to or higher than 0.7 in combination with a p-value below 0.003 was considered acceptable for establishing corre- lation. A p-value below 0.05 was considered statistically significant for the remaining analyses. **3. Results**

3.1. Characteristics of the subjects

The characteristics of the two groups of participants are presented in Table 1 . Regarding the social factors, fewer women with FM had higher education as compared to controls ($p = 0.007$, Mann–Whitney *U test* , $N = 13–14$). Women with FM had higher BMI due to higher weight compared to the controls ($p = 0.03$, Mann–Whitney *U test* , $N = 13–14$). FM participants also had intense pain both at the time of investigation and during the previous week and scored higher for various aspects of fatigue and scored lower for HRQoL compared to controls (Table 2 , $p < 0.001$, a major part of the variables were analysed by Mann–Whitney *U test* , $N = 13–14$). Depressed mood also differed significantly between the two groups (Table 2 , $p < 0.001$, Mann–Whitney *U test* , $N = 13–14$). At the same time, only three FM participants scored 10 or higher on the depression scale of HADS.

The average disease duration of FM was 9.2 years (range 6–13 years, 95% CI, N = 13) and the average number of tender points was 16 out of a maximum of 18 (range 15–18 points, 95% CI, N = 13). Out of 13 FM

Table 2

Self-rated scores in the fibromyalgia group and controls are presented as the mean and 95% confidence interval. Comparisons between the groups were per- formed using parametric or non-parametric tests according to the Shapiro–Wilk test of normality.

	Fibromyalgia PN = 13	*Controls N* = 14	*P-value*
Global VAS	3.8 (2.0–9.2)	0 (0–3.2)	< 0.001
Current pain	5.1 (2 – 10)	0 (0–1.7)	< 0.001
Pain last week	6.4 (3.4–8.7)	0.3 (0–1.7)	< 0.001
Pain minimum	3.9 (2.7–5.1)	0.1 (- 0.–0.3)	< 0.001
Pain maximum	7.4 (6.4–8.4)	1.0 (0.1–1.8)	< 0.001
HADS Depression	6.6 (4.4–8.8)	2.4 (1.3–3.6)	< 0.001
HADS Anxiety	6.6 (4.7–8.5)	4.8 (2.1–7.4)	0.1
General Fatigue	18.2 (117.1–19.4)	9.1 (6.6–11.6)	< 0.001
Physical Fatigue	16.1 (14.5–17.7)	7.9 (5.9–9.9)	< 0.001
Mental Fatigue	16 (13.7–18.3)	8 (6.2–9.8)	< 0.001
Reduced Motivation	9.8 (7.3–12.3)	5.9 (4.4–7.3)	0.06
Reduced Activity	14.4 (11.9–16.9)	7 (5.1–8.9)	< 0.001
FIQ	60.2(53.7–66.6)	12.5 (4–21.1)	< 0.001
SF-36- PCS	30.9 (26.1–35.7)	55.3 (53.4–57.2)	< 0.001
SF-36- MCS	35.2 (10.5)	45.2 (6.6)	< 0.001

Abbreviations: VAS (Visual Analog Scale); HADS (Hospital Anxiety and Depres- sion Scale); MFI-20 (Mental Fatigue Inventory); FIQ (Fibromyalgia Impact Ques- tionnaire); SF-36 (Short Form-36); PCS (Physical Cumulative Score); and MCS (Mental Cumulative Score).

Table 3

Self-reported medication in the fibromyalgia group and controls is presented as numbers per group. In the last line, the total number of medications is pre- sented in parentheses. Comparisons between the groups were performed using Pearson's chi-square test.

	Fibromyalgia N = 13	*Controls N* = 14	*P-value*
Paracetamol	8	0	< 0.001
NSAID	3	0	0.1
Antidepressants	4	1	0.17
Citodon	2	0	0.22
Opioids	3	0	0.1
Sleep medication	4	1	0.14
Antiepileptics	2	0	0.22
Total medication	13 (55)	2 (2)	0.002

Abbreviations: NSAID (Non-Steroidal Anti-inflammatory Drugs).

Table 4

Nociceptive tests in the fibromyalgia group and controls are presented as the mean and 95% confidence interval. Comparisons between the groups were per- formed using parametric or non-parametric tests according to the Shapiro–Wilk test of normality.

	FibromyalgiaN = 10	*ControlsN* = 11	*P-value*
Warmth threshold	36.1 (34.4–37.9)	35.3 (34.0–36.7)	0.34
Warmth pain threshold	41.7 (39.5–43.8)	43.8 (41.5–46.1)	0.29
Cold threshold	28.9 (27.4–30.5)	29.2 (27.8–30.5)	0.9
Cold pain threshold	24.5 (18.9–30.0)	5.9 (0.5–11.3)	< 0.001
Pressure pain threshold	132.6 (80.0–185.2)	351.0 (244.1–457.8)	0.06

participants, four were taking antidepressants, eight were taking parac- etamol, three were taking NSAIDs, five were taking opioids (including codeine), four were taking sleep medication, and two were taking anti- epileptics. Among the 14 controls, one was taking an antidepressant and one was taking a sleep medication (Table 3). The total number of drugs, including non-pain killers, was 55 in the FM group and 2 in the control group (Table 3).

Nociceptive tests performed by QST and algometry showed that only the cold pain thresholds were significantly lower in FM partici- pants compared to controls (Table 4 , $p < 0.001$, Mann–Whitney *U test* , $N = 10–11$). No differences were found between the groups regarding warmth detection and heat pain thresholds and cold detection thresh- olds (Table 4). PPT showed a tendency for lower values in the FM group, but this did not reach statistical significance ($p = 0.06$, Mann–Whitney *U test* , $N = 10–11$).

3.2. Monocyte characteristics and PBMC response after stimulation with LPS

PBMCs from participants with FM and healthy controls were left un- stimulated or were stimulated with LPS for 3 h (for flow cytometry) or 24 h (for ELISA). FM participants had a higher percentage of cells with monocytic properties under unstimulated conditions compared to con- trols, and this tendency was also observed after stimulation ($p = 0.01$ and $p = 0.09$, independent *t* -test, $N = 9$/group). Both groups had a re- duced percentage of monocytic cells after stimulation (Fig. 1 A). Further, the percentage of $CD14^+$ cells among the monocytic cells was higher in the FM group under unstimulated conditions, but this difference was not statistically significant (Fig. 1 B). The percentage of $CD16^+$ cells among the $CD14^+$ cells did not differ between the groups and showed a similar reduction after stimulation (Fig. 1 C). After stimulation, FM participants and controls showed a similar reduction in CD14 MFI on $CD14^+$ $CD16^-$ cells (Fig. 1 D, $p = 0.004$, Wilcoxon rank test, $N = 9$/ group), while only the control group had a significant increase in CD14 MFI on $CD14^+$ $CD16^+$ cells (Fig. 1 E, paired *t* -test, $p = 0.04$, $N = 9$/group.) and only the FM group had a decrease in CD16 MFI on $CD14^+$ $CD16^+$ cells (Fig. 1 F, $p = 0.001$, paired *t* -test, $N = 9$/per group).

PBMC cultures from both groups showed a comparable capacity to secrete IL- 6 and IL-10 after LPS stimulation (Figure S2, A, C); however, the stimulation index for IL-6 tended to be lower in the FM group (Figure S2, B, $p = 0.09$, Wilcoxon rank test, $N = 11–12$/group). The stimulation index for IL-10 was not calculated due to the lack of secreted IL-10 in unstimulated PBMC cultures.

3.3. B-cell and T-cell characteristics and PBMC response after stimulation with PMA + IO

PBMCs from participants with FM and healthy controls were left un- stimulated or were stimulated with PMA + IO for 3 h (for flow cytometry) or 24 h (for ELISA).

Both groups had a similar percentage of lymphocytes based on for- ward and side scatter properties (data not shown). The FM group had lower percentages of $CD19^+$ B - cells under both unstimulated and stim- ulated conditions (Fig. 2 A, $p = 0.08$, $p = 0.03$, independent *t* -test, $N = 13$/group), while CD19 MFI decreased to the same extent in both groups (Fig. 2 B, $p = 0.001$, Wilcoxon rank test, $N = 10–11$/group). $CD19^+$ B - cells from FM participants and controls responded similarly to PMA + IO stimulation with a significant increase in the percentage of $CD69^+$ cells and a significant increase in CD69 MFI among $CD19^+$ cells (Fig. 2 , C and D, $p \leq 0.001$, paired *t* -test. $N = 11–13$/group).

There were no between-group differences in the percentage of $CD3^+$, $CD4^+$, or $CD8^+$ cells within the lymphocyte compartment (Figure S3, A- C). FM and controls responded similarly to stimulation with regard to the percentage of $CD69^+$ cells and CD69 MFI among the $CD4^+$ and $CD8^+$ T - cell populations (Figure S3, d -E, F-G, respectively).

To investigate the potency in secreting cytokines connected to dif- ferent T-helper subsets (T_H) and T-cytotoxic cells (T_C), we analysed the secretion of IFN-γ (T_H1, T_C), IL-4 (T_H2), IL-10 (regulatory subsets), and IL-17A (T_H17). The levels of cytokines did not significantly differ be- tween the groups, even though the levels were consistently lower in the FM group after stimulation (Fig. 3 A (IFN- γ), Figure S2, d -F (IL-4, IL-10, IL17A)). The stimulation index for IFN- γ was significantly lower in the FM group (Fig. 3 B, independent test, $p < 0.001$, N = 9/group). Stimu- lation indices for IL-4, IL-10, and IL-17A were not calculated due to the lack of secreted cytokines in unstimulated PBMC cultures.

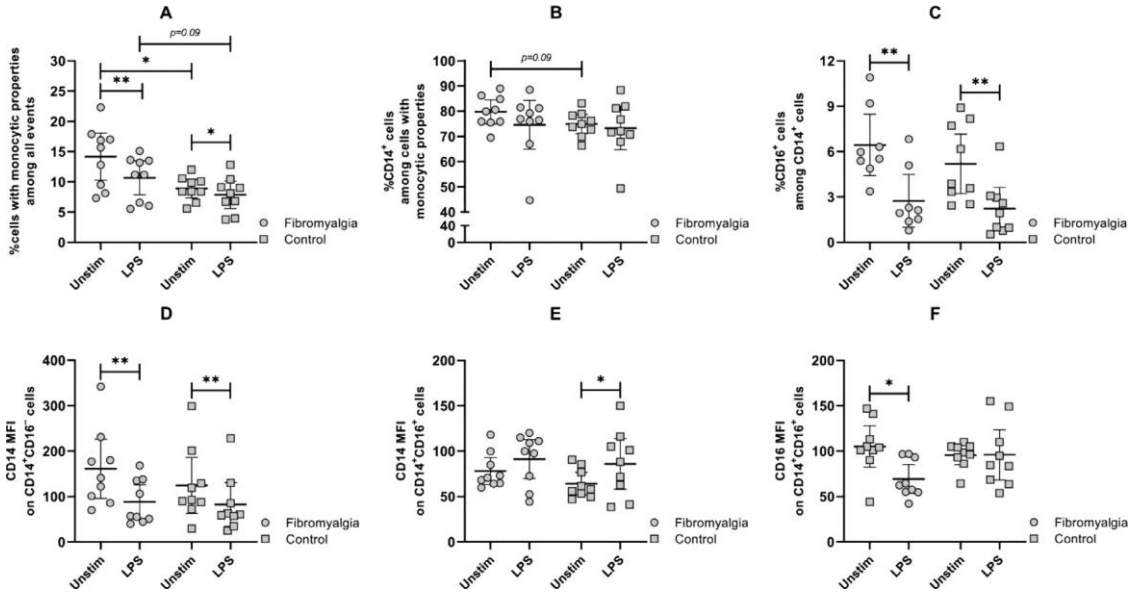

Fig. 1. A) The percentage of cells with monocytic properties among all events. B) The percentage of CD14 $^{+}$ cells among cells with monocytic properties. C) The percentage of CD16 $^{+}$ cells among CD14 $^{+}$ cells. D) The CD14 MFI of CD14 $^{+}$ CD16 $^{-}$ cells. E) The CD14 MFI of CD14 $^{+}$ CD16 $^{+}$ cells. F) The CD16 MFI of CD14 $^{+}$ CD16 $^{+}$ cells. N = 8–9/group. Data are presented as the mean with 95% confidence interval. Comparisons between and within the groups were performed using parametric or non-parametric tests according to the Shapiro-Wilk test of normality. * $p < 0.05$ and ** $p < 0.01$.

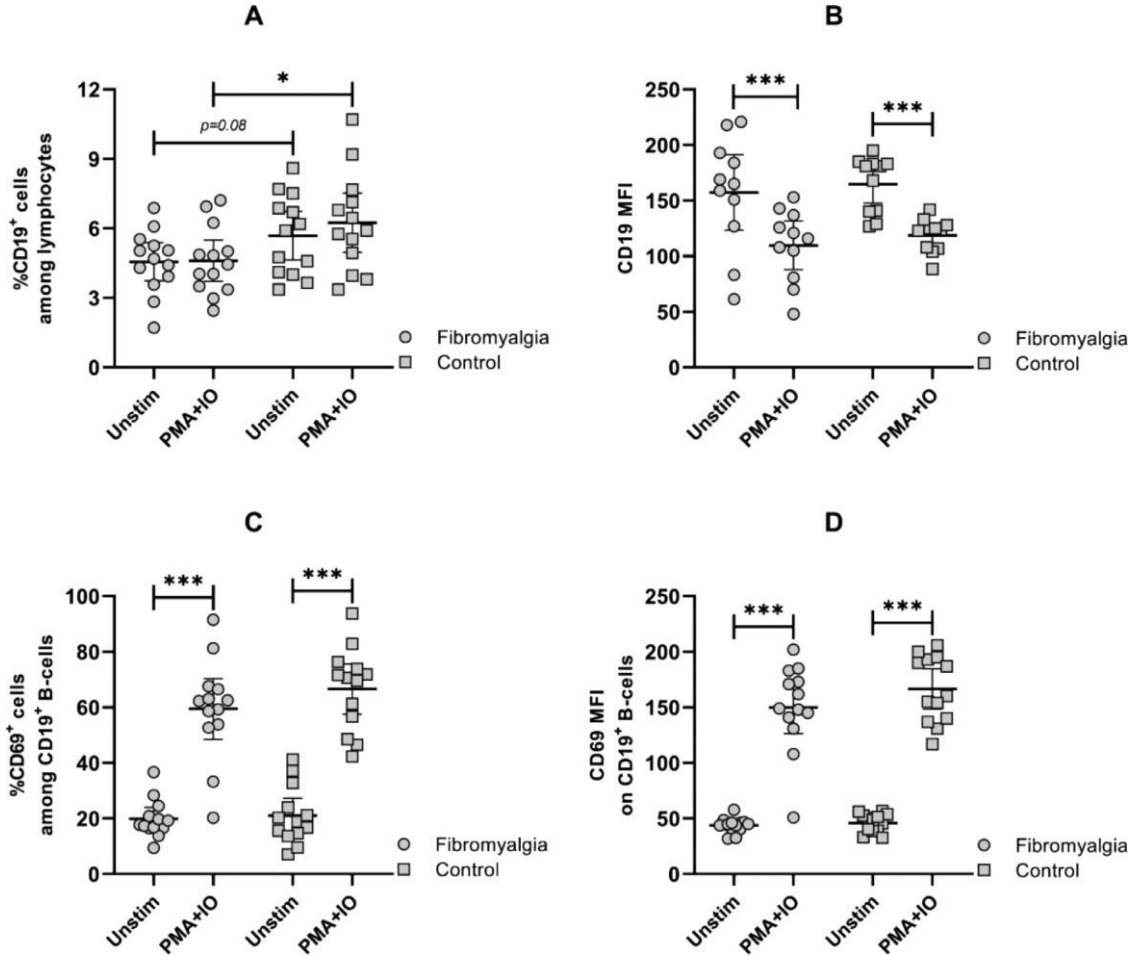

Fig. 2. A) The percentage of CD19 $^{+}$ cells among lymphocytes. B) The CD19 MFI of CD19 $^{+}$ cells. C) The percentage of CD69 $^{+}$ cells among CD19 $^{+}$ cells. D) The CD69 MFI of CD19 $^{+}$ cells. N = 11–13/group. Data are presented as the mean and 95% of confidence interval. Statistics between and within the groups was performed using parametric or non-parametric tests according to the Shapiro–Wilk test of normality. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

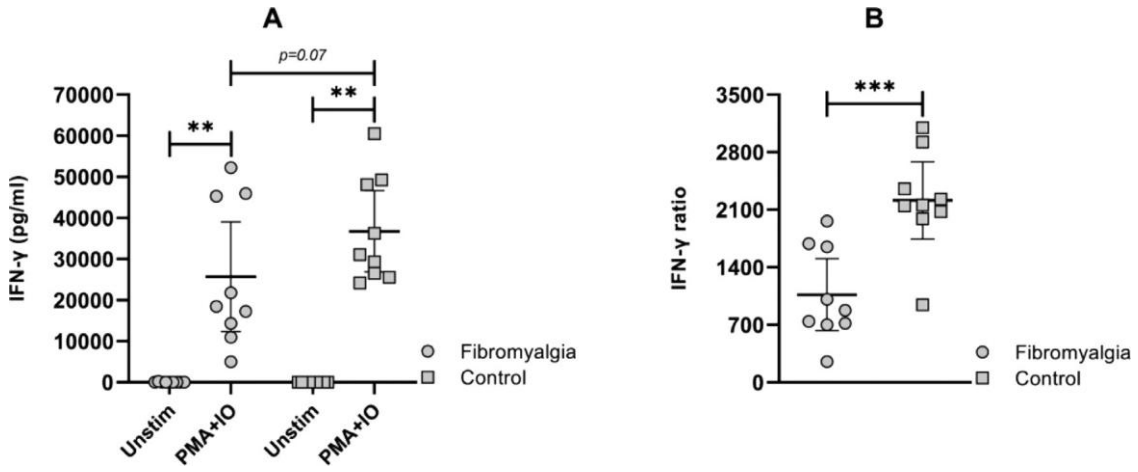

Fig. 3. A) The secreted levels of IFN- γ in cell culture supernatants (pg/ml). B) The IFN- γ stimulation index calculated as the ratio of stimulated to unstimulated. N = 9/group. Data are presented as the mean with 95% of confidence interval. Comparisons between and within the groups was performed using parametric or non-parametric tests according to the Shapiro–Wilk test of normality. * * $p < 0.01$ and * * * $p < 0.001$.

3.4. Correlation analysis between immunological parameters and self-scored symptoms

Correlation analysis was performed between statistically significant clinical symptoms and immune parameters in the FM group and is pre- sented in Table S1. To minimize the number of pain variables in the correlation analysis, only global pain and current pain were included. Likewise, because weight and BMI directly influence each other, only BMI was included. HADS values were not considered to be clinically sig- nificant because only three participants showed increased values above normal, and therefore were not included in the correlation analysis. Thus, the following parameters were analysed: BMI, global pain, cur- rent pain, cold pain threshold, MFI-20 (general fatigue, physical fatigue, reduced motivation, mental fatigue, and reduced activity), FIQ, SF-36 (physical cumulative score and mental cumulative score), immune pa- rameters (percentage of monocytes, percentage of stimulated CD19 + cells, and IFN- γ ratio), and total number of drugs taken. A negative cor- relation, reaching the predefined statistical p-value below 0.0013, was found between the IFN- γ stimulation index and the cold pain threshold ($r_s = -0.95$, $p < 0.001$, $N = 8$), Fig. 4 . None of the remaining correlations reached significance.

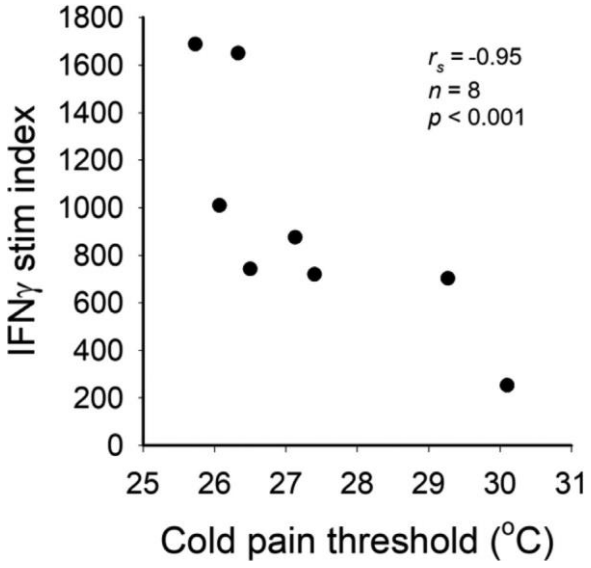

Fig. 4. Correlation plot between cold pain threshold and IFN- γ stimulation in- dex. Spearman's correlation test with correlation coefficient (r_s).

4. Discussion

In the present study, we report that PBMCs from FM partici- pants contained a higher percentage of monocytes, a lower percent- age of B-cells, and a pattern of lower levels of secreted cytokines after stimulation.

Currently, according to the Nomenclature Committee of the In- ternational Union of Immunological Societies, monocytes are divided into CD14 ++ CD16– classical monocytes (representing up to 90% of the blood monocytes), intermediate CD14 ++ CD16 + monocytes, and non-classical CD14 + CD16 ++ monocytes [31] . Classical monocytes (CD14 ++ CD16–) play crucial roles in the mechanisms of innate immune defense against microbial pathogens and in tissue repair [32 , 33]. Inter- mediate CD14 ++ CD16 + monocytes have been shown to predict cardio- vascular events in patients with chronic kidney disease [34] , indicating a possible pro-inflammatory role [35] . CD14 + CD16 ++ monocytes show similarities with tissue macrophages, and therefore they are considered to be more mature than classical and intermediate monocytes [31 , 36]. Non- classical CD14 + CD16 ++ cells display patrolling properties and re- move damaged cells and debris from the vasculature tissue and have been associated with wound healing and with the resolution of inflam- mation in damaged tissues [37] . Both classical and intermediate mono- cytes were found to be significant regressors for pain in FM patients [38] , and that study reported no difference in monocyte subpopulations between FM and control group but did report a negative correlation be- tween pain and classical and intermediate monocytes in the FM patients [38] . Recently, Merriwether and colleagues reported a lower percent- age of intermediate monocytes and a higher percentage of nonclassical monocytes in unstimulated conditions in FM patients compared to con- trols [39] . Although the correlations were weak or moderate, the higher percentage of intermediate monocytes was associated with less pain and fatigue, and the higher percentage of nonclassical monocytes was asso- ciated with more pain and fatigue [39] . Unfortunately, we could not use the gating strategy for monocytes to identify these three subsets. Further studies on monocytic subpopulations are needed to validate the impact of different monocytes in FM pathology.

In the present study, the correlation analysis between immunological parameters and clinical findings was performed only in the FM group, and this showed a strong negative correlation between the IFN- γ ratio and the cold pain threshold. This indicates a possible involvement of IFN- γ in pain pathways in FM. In experimental studies, the ion channel TRPM8 receptors have been demonstrated to mediate painful cold sen- sation [40] . Recently, it has been

shown that responses after exposure to cold might indicate increased sympathetic nervous system activity in FM [41] . However, studies regarding the interplay between IFN- γ and pain mechanisms are scarce and have been performed mostly un- der experimental conditions that might be far removed from the com- plicated in vivo milieu [42] . IFN- γ has been shown to have many pro- tective effects in the host immune system, especially against viruses, bacteria, fungi, and cancer cells as well in autoimmunity, and it is also used in clinical practice with positive effects [43] . Therefore, the role of this cytokine should be further studied in yet unexplained condi- tions such as chronic pain. The reduced ability of PBMCs to secrete cytokines is also confirmed by other studies [12 , 13]. One can spec- ulate that immune cells' responsiveness per se might be a more im- portant issue to study compared to absolute values of immune param- eters. It is known that chronic pain patients, including FM patients, represent a heterogenous group with a broad panorama of symptoma- tology and co-morbidities. Therefore, the absolute values of immune parameters might also be as heterogenous as the clinical symptoms in FM.

The observed weak tendencies for reduced secretion of T-cell re- lated cytokines in FM participants may indicate a generally lower re- sponsiveness of the T-cell compartment. However, there were no sig- nificant differences in the T-cell compartment, including the expression of the activation marker CD69 + after stimulation with PMA + IO. Be- cause we cannot exclude that other cell types such as natural killer (NK) cells are involved in the lower levels of secreted IFN- γ in the FM group, one would need to perform intracellular cytokine staining of different lymphocyte populations to identify the origin of the de- fective IFN- γ production. It is known that the release of IFN- γ from NK and/or T-cells activates monocytes and macrophages resulting in increased microbicidal functions [32] . In a previous study, female FM patients had a tendency for lower NK cell activity, but there was no cor- relation between NK cells and FM symptoms such as sleep quality and mood [44] .

The strength of this study is that it included a randomized population and a broad spectrum of questionnaires used for self-scored symptoms along with standardized nociceptive tests. However, the small number of analysed samples is a clear limitation of the study. Even though medi- cation with analgesics was suspended 1 week before the blood sampling, the long-term effects of anti-inflammatory drugs on the immune system cannot be excluded. At the same time, no significant correlations were found between immune parameters, BMI, and medication. Few of the FM participants consumed antidepressants, which were not suspended prior to the blood sampling. It is important to note that the FM partic- ipants in this study were not totally representative for the FM popula- tion. The participants in the present study were initially recruited for 16 weeks of exercise training, twice per week, with 1 hour of physi- cal exercise at a time. One can speculate that only participants having better physical capacity were able to participate, which partly is con- firmed by the low scores for depression and anxiety in HADS. There- fore, the study population is more representative for less affected FM patients because more severely affected FM patients were not able to participate.

Other limitations of this study are the limited sample size and that only a basic characterization of peripheral T-cells, B-cells, and mono- cytes was performed and we could not explore other immune cells or subsets.

In summary, the results indicate a dysregulation of the immune sys- tem in terms of an increased percentage of monocytes, a decreased per- centage of B-cells, and generally decreased PBMC cytokine secretion af- ter stimulation. This decreased responsiveness might contribute to the further development or persistence of clinical symptoms according to the correlation analysis. Therefore, more research on immune system responsiveness is warranted, including responses in more severely af- fected FM patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clicom.2022.02.003 .

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