$CD3^+CD56^+$natural killer T cells in fibromyalgia syndrome patients: association with the intensity of depression

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ABSTRACT

Objectives. *Fibromyalgia syndrome (FMS) patients have multiple symptoms, including mental symptoms such as depression. Natural killer T (NKT) cells have shown to be correlated with depression. However, up to now there is no information regarding the role of $CD3^+CD56^+$NKT cells in FMS patients, especially in the intensity of mental symptoms. The present study aimed to observe the role of $CD3^+CD56^+$NKT cells level in FMS patients in relation to the intensity of depression.*

Methods. *Ninety-six female patients who matched definition of FMS were divided into subgroups of depressions according to Hospital Anxiety and Depression Scale (HADS) score (HADS-Depression <8; 8–10; and >10). $CD3^+CD56^+$NKT cells from peripheral blood were measured by fluorescence-activated cell sorting methods.*

Results. *ANOVA test in the subgroup of depression of FMS patient showed significant differences. Additionally, the differences were observed in HADS-D <8 and >10. The use of antidepressant significantly altered the level of $CD3^+CD56^+$NKT cells. A blocking variable ANCOVA with antidepressant as covariate showed main effects in the subgroup of depression, however, the interaction of antidepressant and the subgroup of depression did not influence the level of $CD3^+CD56^+$NKT cells.*

Conclusion. *These results suggest that $CD3^+CD56^+$NKT cells could play a role as a mediator in mental symptom such as depression in FMS patients. It seems the role of antidepressant in the mood intensity is not mediated by $CD3^+CD56^+$NKT cells in FMS patients. Additionally, subgrouping FMS patients based on the intensity of mental symptoms may help to optimise the treatments.*

Introduction

Fibromyalgia syndrome (FMS) in the 1990s was defined as a chronic pain syndrome characterised by multiple tender points (1). Its prevalence has been reported around 2% (2). The American College of Rheumatology (ACR) had defined fibromyalgia by the presence of widespread (musculoskeletal) pain of at least 3 months duration and pain upon palpation of at least 11 out of 18 specific tender points (1, 3). However, clinical experience and epidemiological data show that FMS patients frequently report other symptoms such as sleep disturbances, fatigue, irritable bowel syndrome and others (4-7). Therefore, a new definition has been published recently (8). This definition combined the count of tender points and mood and some functional symptoms. It is known that depression and anxiety are symptoms in FMS patients with high prevalence. However, the prevalence has high variety and ranges from 20 to 80% for depression and from 13 to 64% for anxiety (4).

Since FMS patients have multiple symptoms, a multimodal therapy approach has been recommended. It consists of the combination of pharmacological and non-pharmacological interventions (9, 10). Many different types of non-pharmacological therapies have been studied, including balneotherapy (11), combination of aerobic and strengthening exercise (12), aerobic exercise (13), cognitive behavioural therapy (CBT) (14), or combination of aerobic exercise and CBT (15). The most frequently prescribed non-pharmacological treatments are exercise and CBT (16, 17). Pharmacological treatments mostly are analgesics, NSAIDs, and antidepressants; among them, antidepressant treatment is the most prescribed pharmacological approach for FMS patients (10). Tricyclic antidepressants are helpful for FMS patients (18). This suggests

a relationship between FMS and psychological factors.

Abnormalities of immune system have been reported in different types of diseases such as autoimmune diseases, diabetes, cancer, and others. In FMS, the immune system seems to play a role, as well. Some studies reported an alteration of immune mediators such as cytokines and immune cells in FMS (19, 20).

Natural killer (NK) cell is a type of cytotoxic lymphocyte. As it is described by its name, it can induce the death of tumour and virus-infected cells without the presence of specific immunisation (21). NK cells in FMS have been reported to be decreased as compared to healthy controls (22). Additionally, NK cells as an integrated compound of the innate immune system have been suspected to play a role in FMS. Landis (23) showed NK cells in FMS were lower as compared to healthy controls. However, after correction of multiple comparisons, it was no longer significantly different. The authors suggested there is only little evidence that pain, mood, and sleep symptoms are associated with changes in the enumeration of peripheral lymphocytes or function in FMS.

NKT cells have differences with NK cells. Besides by origin, NKT cells express T-cell antigen receptors or Pan T marker cluster of differentiation (CD) 3 or surface immunoglobulin B-cell receptors. NK cells do not express this marker (21). In other words, NKT cells have characteristics of both NK and T cells (24). In humans, these cells are characterised as $CD3^+CD56^+$ (25, 26). Besides their role in immune response to viruses, bacteria, and parasites (27, 28), NKT cells are also involved in various types of diseases such as type 1 diabetes and haematological cancer (29). Furthermore, these T-cell subsets play a role in a general function of suppressing autoimmunity such as rheumatoid arthritis, inflammatory bowel diseases (30), and multiple sclerosis (31). In addition, some studies demonstrated the role of NKT cells as inhibitors of tumour growth. For example, NKT cells are able to lyse human breast cancer, ovarian cancer and lung cancer cells *in vitro* (32). NKT cells have also some relation to psychological functions. For example, they were found to be enhanced in the blood of elderly subjects with high extraversion and social support (33). Additionally, social support was also shown to be related to significantly higher NKT cell percentage in the peripheral blood of patients with ovarian cancer (24). Possibly, NKT cells could share a similar function in FMS. Additionally, to the best of our knowledge, there is no study of NKT cells in FMS patients until now.

In this study, we hypothesised that a percentage of $CD3^+CD56^+$NKT cells in peripheral blood might have a correlation with the intensity of depression in subjects with FMS. In order to observe differences of the level of $CD3^+CD56^+$NKT cells in FMS patients with different levels of depression, they were subgrouped on the basis of the Hospital Anxiety and Depression Scale-D (HADS-D). Additionally, possible influence of the intake of antidepressant drugs on the level of $CD3^+CD56^+$NKT cells in FMS patients, a covariate adjustment with antidepressant as a covariate was performed. Finally, a regression analysis model was applied using antidepressant, age, pain, fatigue, disease duration, anxiety and depression on the level of $CD3^+CD56^+$NKT cells.

Materials and methods

All procedures were carried out with written informed consent of the subjects. The study has been approved by the ethics committee of Hannover Medical School. Additionally, this study has been performed in accordance to the ethical standards laid down in the 1964 Declaration of Helsinki.

Patients

Ninety-six voluntary female FMS patients were recruited from Rheumatologist practices in Hannover, outpatients of the Department of Rehabilitation Medicine of Hannover Medical School and the patient organisation "Rheuma-Liga", Hannover. They were screened based on the definition of ACR criteria for fibromyalgia (1) by physicians of the Department of Rehabilitation Medicine, Hannover Medical School, Hannover, Germany. Additionally, patients had to understand German language. They were not restricted with regard to treatments either pharmacological or non-pharmacological. However, the treatments were recorded and the patients were obliged to stop any food and medicine intake one night before blood collection (only water was allowed).

Patients with recent or past history of psychiatric disorders (*e.g.* major depressive disorder, alcohol dependence, substance abuse, schizophrenic or paranoid disorder, personality disorders, and somatoform disorders), inflammatory, endocrine or clinically significant chronic disease (*e.g.* diabetes, rheumatoid arthritis, inflammatory bowel disease, and organic brain disorders), abnormal function of liver, pregnant and breast feeding women were excluded.

Sample size calculation

Sample size calculation was performed by using PS: Power and Sample Size Calculation version 3.0 (34). To our knowledge, this is the first study that compared NKT cells in FMS patients with and without depression and they were asked not to stop drug treatments. The most comparable study is by Bouhuys *et al.* (33), which was thus used for sample size calculation. This study was planned for elderly depressive patients on antidepressants with 2.3 control(s) per experimental subject. The response within each subject group was normally distributed with standard deviation 58. If the true difference in the patients with depression and without depression means is 55, power calculation demonstrated that 17 FMS patients with depression and 39 FMS patients without depression were able to reach 90% power. The Type I error probability associated with this test of this null hypothesis is 0.05.

Patient assessment

Patients were clinically assessed according to the criteria of ACR 1990 (1). Additionally, the following variables were evaluated: illness duration (years), pain intensity (Visual Analogue Scales [VAS]), and intensity of fatigue (VAS), as well as depression (HADS-D) and anxiety (HADS-A).

Patient grouping

Subgroups of patients were selected according to the HADS-D scores using the classification given by Zigmond and Snaith (35): HADS-D score <8=no depression; HADS-D score 8–10: mild depression; HADS-D score >10: high depression). This resulted in 3 subgroups of depression (no depression [n=45], mild depression [n=23], high depression [n=28]).

For the purpose of this explorative study, sub grouping of patients according to their anxiety score was adopted using the same principle as in the subgroup of depression. This resulted in three subgroups of anxiety (no anxiety [n=22], mild anxiety [n=33], high anxiety [n=41]).

Fluorescence-activated cell sorting (FACS) analyses

Overnight fasting (only water was allowed) peripheral venous blood samples were collected in anticoagulant (K-EDTA) tubes from all patients and controls between 08:00 and 10:00 am.

Erythrocytes were immediately lysed with Schwinzer lysis buffer (8.3 g NH_4Cl, 0.1 g EDTA, 1.0 g $KHCO_3$/l aquadest) for 10 min at room temperature and centrifuged for 10 min at 400 g at 4°C and resuspended in phosphate buffer saline (PBS). Cell suspension was centrifuged at 400 g and 4°C for 10 min. The leukocyte pellet was mixed with PBS-containing 0.1% bovine serum albumin (BSA) and 0.1% Na-azide and centrifuged for 2 min at 400 g and 4°C. The cell count was determined under light microscopy using a Neubauer counting chamber.

Monoclonal antibodies for T cells (CD3-PE-Cy7; BD Biosciences, Heidelberg, Germany) and NK cells (CD56-APC; BD Biosciences) were added and incubated in the dark for 20 min at 4°C. Afterwards, samples were washed twice with 100 µl PBS for 2 min at 400 g and 4°C. After the addition of 100 µl measuring buffer samples were vortexed and measured with a FACSCanto (BD Biosciences).

Statistics analysis

1. Kolmogorov-Smirnov test was used to test normality of the data. Based on this test, except for pain intensity, the others did not have normal distribution. Therefore, ANOVA was used to compare the differences of pain intensity (VAS), while Kruskal-Wallis test was used to test differences of age, disease duration, number of tender points, and fatigue score in the subgroup of depression in FMS patients.
2. Log transformation of %$CD3^+$ $CD56^+$NKT cells into log %$CD3^+$ $CD56^+$NKT cells was performed in order to get normal distribution data. After confirming normal distribution of the resulting data set by Kolmogorov-Smirnov test, ANOVA was used to test significance in the subgroups of depression.
3. Explorative test:
   a. The correlation analysis between $CD3^+CD56^+$NKT cells and HADS-D or HADS-A scores was analysed by means of Spearman's correlation coefficient.
   b. Subgroup analysis: Mann-Whitney U-test was used to compare the level of $CD3^+CD56^+$NKT cells in FMS patients with and without antidepressant drug intake.
   c. Covariate adjustment:
      i. A blocking variable ANCOVA was applied to compare the level of $CD3^+CD56^+$NKT cells in the subgroup of depression with antidepressant as covariate. The same method was used for the subgroup of anxiety.
      ii. A hierarchical stepwise regression model was used to explore the extent to which age, pain, fatigue, tender points, disease duration, anxiety and depression score with antidepressant (at first block) explained the variance of $CD3^+CD56^+$NKT cells in FMS patients.
4. Significance of the results was set as $p<0.05$. Statistic package SPSS 19.0 was used.

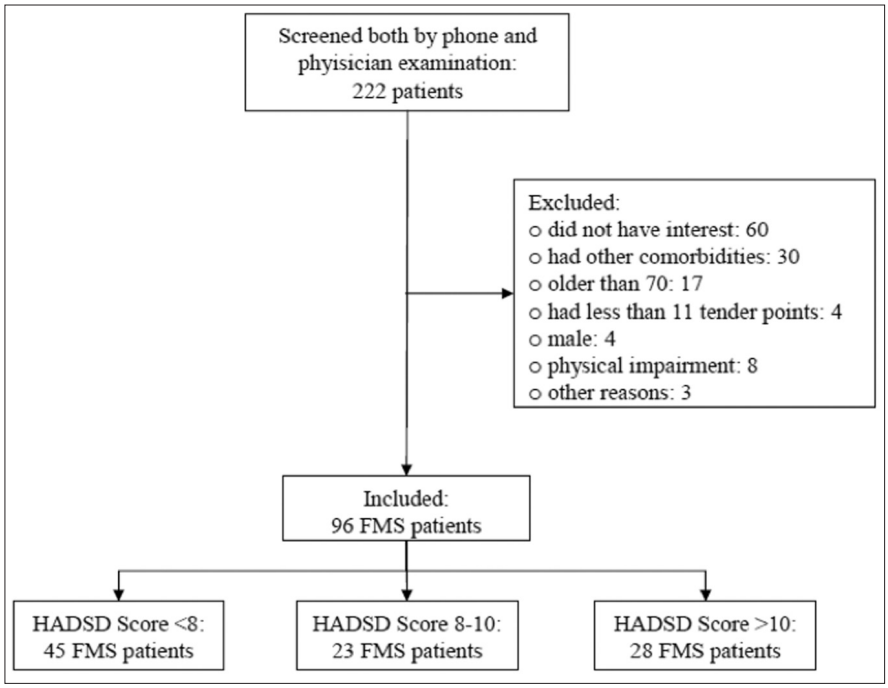

Fig. 1. Flowchart screening of the patients.

Results

Patients were screened both by phone and interview with physicians. Out of 222, 60 did not have interest, 30 had other comorbidities, 17 were older than 70 years old, 4 had less tender points, 4 were male, 8 had physical impairments, and 3 had other reasons. In the end, we had 45, 23, and 28 FMS patients with HADSD score of <8, 8–10, and >10, respectively (Fig. 1).

As summarised in Table I, 47% of patients were without depression, while 24% and 29% of patients had mild and

high depression, respectively. There were no significant differences among the groups with respect to the age, disease duration, tender point count, pain and fatigue scores.

The level of $CD3^+CD56^+$NKT cells showed significant differences in subgroups of depression in FMS patients (Fig. 2) (ANOVA, p=0.02). Furthermore, post-hoc analysis showed significant of difference between depression subgroup with HADS-D score below 8 and more than 10 (Bonferroni test, p=0.018). Additionally, the level of $CD3^+CD56^+$NKT cells is correlated with intensity of depression (R=-0.297; p=0.003) and anxiety (R=-0.277; p=0.006).

Interaction with antidepressants

As demonstrated in Figure 3, the level of $CD3^+CD56^+$NKT cells is significantly higher in FMS patients without antidepressant as compared to the subgroup with antidepressant intake (Mann-Wihtney U-test: p=0.041).

Table II demonstrates the results of ANCOVA test using antidepressant drug intake as a confounding factor. This showed main effects in the depression subgroup. However, the main effect of antidepressant was not significant, nor was interaction of subgroups of depression and antidepressant. The same statistical test was performed in the subgroup of anxiety. It showed no main effect in the anxiety subgroup or antidepressant. Furthermore, no interaction of subgroups of anxiety and antidepressant was found.

Other possible factors that influence the level of $CD3^+CD56^+$NKT cells are age, pain, fatigue, tender points, and disease duration. These factors were analysed by using regression analysis model. The results showed that, in addition to depression, disease duration (p=0.018) is also another factor that can predict the level of $CD3^+CD56^+$NKT cells in FMS patients. In addition, it is shown by its correlation with $CD3^+CD56^+$NKT cells (R=-0.224, p=0.028)

Table I. Clinical characteristics of FMS patients based on the depression score (mean ±SEM).

	n	HADS-D score group			p-value
		<8 (n=45)	8–10 (n=23)	>10 (n=28)	
Age (years)	96	55.7 ± 1.5	58.2 ± 1.8	53.7 ± 1.7	> 0.05
Disease duration (years)	96	7.8 ± 0.8	7.7 ± 1.0	6.9 ± 0.9	> 0.05
Number of tender points	96	14.9 ± 0.3	15.6 ± 0.4	15.0 ± 0.5	> 0.05
Pain intensity (VAS 0-10)	96	5.5 ± 0.3	5.9 ± 0.4	6.1 ± 0.4	> 0.05
Fatigue (VAS 0-10)	96	6.1 ± 0.3	6.0 ± 0.4	6.7 ± 0.4	> 0.05

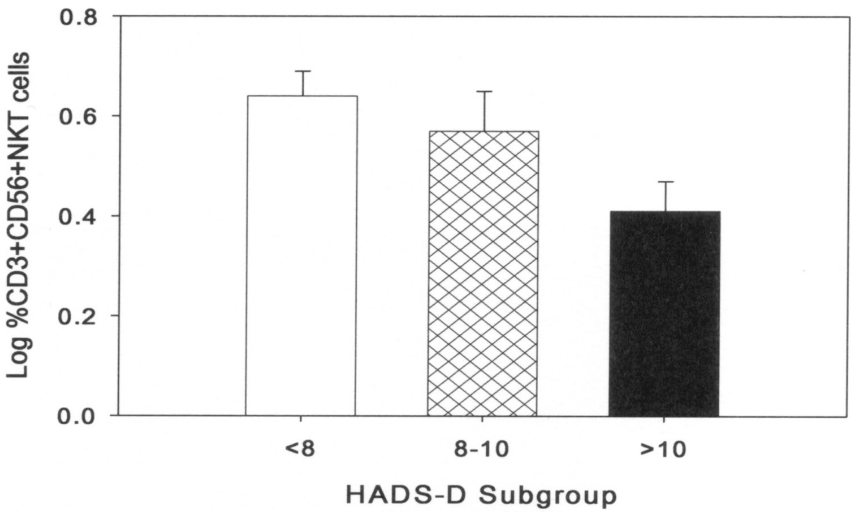

Fig. 2. Mean levels of NKT cells in depression subgroups of FMS patients (ANOVA after log transformation, p=0.02). Data: (Mean±SEM).

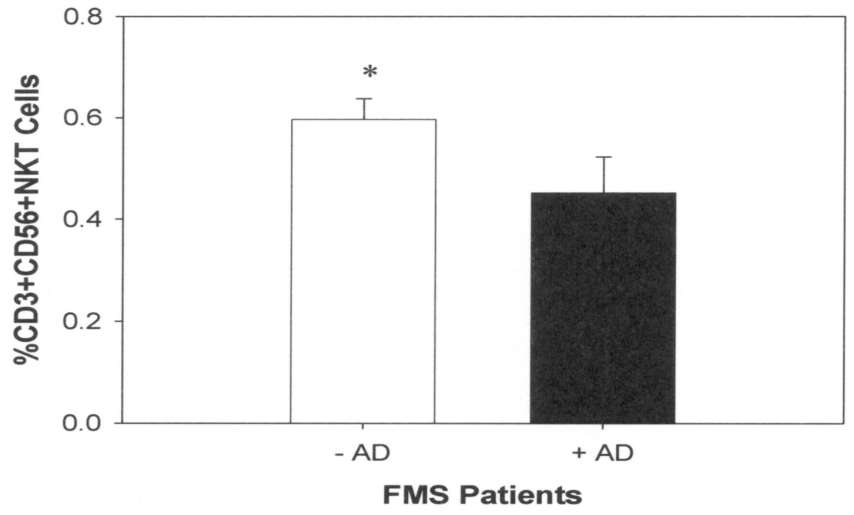

Fig. 3. Level of %$CD3^+$CD56NKT cells in subgroup analysis of FMS with antidepressant (+AD, n=27) and without antidepressant (-AD, n=69). *Mann-Withney U-test (p=0.041). Data: (Mean±SEM).

Discussion

Although a new definition of FMS has been published (8), in this study, the recruitment of FMS patients was based on Wolfe *et al*. (1), because this study was started before 2010. Additionally, for clinical study, this approach demonstrates more homogeneity (8).

In this trial patients with major depressive episodes were excluded. Such episodes are defined in the DSM IV by the presence of five or more symptoms from a 9-item list (36) and not caused by a mixed episode, clinically significant distress, physiological effects of substances or medical condition or accounted by bereavement (so-called reactive depression). These symptoms have been investigated in the patient

Table II. Explorative study with ANCOVA test for subgroup of depression and anxiety in FMS patients.

Subgroup of depression		
Subgroup of depression	F(2, 90) = 3.675	p=0.029
Antidepressant	F(1, 90) = 0.537	p=0.465
Interaction of subgroup of depression and antidepressant	F(2, 91) = 0.988	p=0.376
Subgroup of anxiety		
Subgroup of anxiety	F(2, 90) = 2.226	p=0.114
Antidepressant	F(1, 91) = 1.553	p=0.216
Interaction of subgroup of anxiety and antidepressant	F(2, 91) = 0.533	p=0.589

interview performed by the doctor. Thirty out of 222 FMS patients were excluded because they had other comorbidities, including MDD.

Patients with FMS often show depressive symptoms, even though they do not meet the criteria of a major depressive disorder (MDD) (37). The expression of these symptoms can be measured by the HADS, which is not a tool to diagnose MDD (38), and it can differentiate between patients with low, moderate or severe depressive mood (35).

This recruitment and evaluation strategy on the one hand might be a limitation of the study as patients with MDD have been excluded. On the other hand, it demonstrates that the correlation of the intensity of depressive mood with NKT-cell levels are not caused by the psychiatric disorder of a major depression.

Depression and anxiety are common in many chronic diseases, including chronic pain syndromes. These symptoms are also frequently observed in FMS patients (4). In this study, we observed that our group of patients showed depression and anxiety in 29% and 43% of cases, respectively. This result corresponds to previous studies which reported a prevalence of depression and anxiety of FMS patients at range of 20–80% and 13–64%, respectively (4).

To date, only few studies have reported the impact of altered immune cells in patients with FMS. Most of them studied the differences of T-lymphocytes and NK cells between FMS patients and healthy controls, correlation of symptom intensity of FMS with T lymphocytes (22, 23, 39). To the best of our knowledge, no study was performed previously regarding $CD3^+CD56^+$NKT cells and FMS. Thus, this is the first study demonstrating significant interactions of $CD3^+CD56^+$NKT cells from peripheral blood of FMS patients with FMS-related mental symptoms. In particular, FMS patients who had depression (HADS-D scores >10) had significantly lower level of $CD3^+CD56^+$NKT cells. This result was supported by the significant correlation between the level of $CD3^+CD56^+$NKT cells and intensity of depression as well as anxiety. These suggest that $CD3^+CD56^+$NKT cells could be one important factor in mental symptoms such as depression in FMS patients.

However, since antidepressants can influence the level of $CD3^+CD56^+$NKT cells (33), subgroup analyses were performed and showed a significant higher level of $CD3^+CD56^+$NKT cells in FMS patients without antidepressants as compared to those with antidepressants. This shows conflicting results as compared to a previous study which demonstrated that the level of NKT cells is higher in patients with antidepressants as compared to those without antidepressant intake (33). However, the authors investigated depressive patients in their study (33), while we investigated FMS patients with depressive mood who do not necessarily fulfil the criteria of major depression. Therefore, both studies cannot be directly compared.

The result of subgroup analysis led us to perform another explorative test to verify whether the intake of antidepressants drug is a possible confounding factor. The use of antidepressants as covariate in subgroup of depression and anxiety showed that antidepressants had main effects only in the subgroup of patients with depression, but not in subgroup of patients with anxiety. Additionally, the interaction of antidepressant intake and subgroup of depression as well as anxiety did not influence the level $CD3^+CD56^+$NKT cells. This suggests that the level of $CD3^+CD56^+$NKT cells in FMS patients is independent from antidepressant intake. However, further studies are necessary to observe the correlation among antidepressant intake, mood symptom intensity and $CD3^+CD56^+$NKT cells in FMS patients.

Additionally, it would be of interest not only to measure the level of $CD3^+CD56^+$NKT cells, but also its activity. Further explorative results showed that disease duration also influenced the level of $CD3^+CD56^+$NKT cells.

Several studies have reported a correlation between NKT cells and psychosocial factors. A positive correlation was found between distress and the percentage of NKT cells in healthy adults (40). Furthermore, Bouhuys *et al*. (33) found an association of NKT cells with extraversion and social support in an elderly population. Another study reported that ovarian cancer patients who coped with their illness by reframing it in a positive way and more vigor demonstrated higher percentages of NKT cells in their peripheral blood (24). A study investigating patients with major depressive disorder, NKT cells showed negative correlation with depression but not with anxiety (41). However, in this study, level of $CD3^+CD56^+$NKT cells had correlation with both symptoms. This difference can be due to several reasons. Firstly, the type of cells, although they stated NKT cells, in fact they measured $CD56^+$ as a unique NKT cell marker, while we measured $CD3^+CD56^+$NKT cells. Regarding this, we might refer to the results of Landis (23), who measured similar cells as Park *et al*. (41). However, Landis (23) could observe the difference between FMS and healthy controls regarding depressive symptom. However, after correction of multiple comparisons, the latter author could no longer see the differences. Secondly, Park *et al*. observed patients with major depressive disorders, whereas FMS patients were subjects in this study and in the study by

Landis (23). FMS patients had multiple symptoms, including pain as a major symptom. Therefore, these results cannot be directly compared. Thirdly, the authors used the Hamilton Rating Scale for Depression and Anxiety, while in this study we used HADS.

In order to elucidate the mechanism of the impact of $CD3^+CD56^+$NKT cells for depression in FMS patients, we need to trace back the hypothesis of FMS. One leading hypothesis of FMS is an aberration of the hypothalamic-pituitary-adrenal (HPA) axis (42). Besides locus coeruleus/noradrenalin-sympathetic system, HPA axis is one of principal components of human stress response. The decrease of HPA axis activity is often found in individuals who have fatigue, depressed mood, sleep disturbance, and myalgia (19). Additionally, anxiety disorders have been shown to be correlated with an altered HPA axis (43).

Although FMS is a non-inflammatory disorder, many studies showed that cytokines could play a role in FMS. However, the results are conflicting. Though, the imbalance of Th1/Th2 cytokines could have a role in psychological symptoms. Interestingly, the activity of the HPA axis is also mediated by cytokines homeostasis (44, 45). $CD3^+CD56^+$NKT cells influence the balances of Th1 and Th2 cytokines (46, 47) by taking a part in producing Th1/Th2 cytokines. Therefore, the low level of $CD3^+CD56^+$NKT cells in the subgroup of depression of FMS patients in the present study could partly lead to the imbalance of Th1/Th2 cytokines.

Epinephrine, norepinephrine, and dopamine are sympathetic neurotransmitters belonging to catecholamines. In FMS patients, the level of adrenaline is lower compared to healthy controls (48). However, the authors did not differentiate between the levels of depression in their groups of patients. Current studies report that epinephrine induces an increase of $CD3^+CD56^+$NKT cells (49). In our study, the level of $CD3^+CD56^+$NKT cells in FMS patients with depression is lower than in FMS patients without depression. This may be traced back to a lower level of epinephrine in FMS patients.

Catecholamines can bind to adrenergic receptors. Epinephrine has high binding affinity to the β2-adrenoceptor of peripheral blood mononuclear cells (PBMCs) (50). Adrenoceptors are part of the sympathetic nervous system (SNS). These receptors are important to maintain homeostasis (51). Mobilisation of leukocytes from vascular endothelial cells is promoted by SNS cathecolaminergic activity stimulating PBMCs, which express predominantly β2-adrenoceptors (52). Additionally, activation and differentiation of immune cells increase the expression of β2-adrenoceptors (53). Moreover, a recent study has reported a relationship of adrenoceptor gene polymorphisms to the risk of developing FMS and is also linked to different domains of FMS such as morning stiffness, fatigue, and disability (51). The authors speculated dysautonomia may provide a psychologic explanation for somatisation. Although the authors did not report the correlation of β2-adrenoceptors with depression, however, in our study a lower level of $CD3^+CD56^+$NKT cells in FMS patients with depression seems to correlate with β2-adrenoceptor polymorphism.

Limitations

This study has several limitations. Patients were not allowed to take the medicines for only one night before blood drawing. Besides ethical issues, it is also difficult to predict the washout period of the drug with respect to $CD3^+CD56^+$NKT cells. Therefore, we did not apply the washout period in this study. However, other studies should be performed to confirm the effect of washout period of drug intake.

The effect of antidepressant was the only medicine as covariate, because this is the most prescribed drug for FMS patients. In this study, we only measured the number of $CD3^+CD56^+$NKT cells, however, it would be of interest if activity of $CD3^+CD56^+$NKT cells were also observed. Therefore, more complex confounding factors should be considered for further studies. Although HADS cannot be used to asses clinically depressed patients, we cannot avoid in real clinically situation that there are some FMS patients who have depressive mood but do not meet the criteria of major depression. Patients with past episodes of depression were not included in this study. Therefore, the results only reflected FMS patients with new episodes of depression.

Conclusion

Our results suggest that $CD3^+CD56^+$ NKT cells could be one of the mediators that play a role in mental dysfunction in FMS patients. This can also be interpreted as a part of complex pathomechanisms in FMS. Further studies investigating other symptoms and the mechanisms of immune cell alterations and activities, as well as their interactions in FMS patients, need to be performed in the future, also considering the effect of medicine intake and other confounders. Furthermore, in the future it would be of interest to diagnose subtypes of FMS based on biomolecules parameters. Finally, subgrouping FMS patients based on their symptom intensity may help to optimise the results of treatments.

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