Regular article

The impact of antipsychotic medication on IL-6/STAT3 signalling axis in peripheral blood mononuclear cells of drug-naïve schizophrenia patients

Manjula Subbanna, M.Sc.^{a,b}, Venkataram Shivakumar, Ph.D^{b,c}, Deepthi Venugopal, Ph.D^{a,b}, Janardhanan C. Narayanaswamy, MD^{b,c}, Michael Berk, MD, PhD^{d,e}, Shivarama Varambally, MD^{b,c}, Ganesan Venkatasubramanian, MD, PhD^{b,c}, Monojit Debnath, PhD^{a*}

a. Department of Human Genetics, National Institute of Mental Health and Neurosciences, Bangalore, Karnataka, India.

b. Translational Psychiatry Laboratory, Neurobiology Research Centre, National Institute of Mental Health and Neurosciences, Bangalore, Karnataka, India

c. Department of Psychiatry, National Institute of Mental Health and Neurosciences, Bangalore, Karnataka, India.

d. School of Medicine, IMPACT Strategic Research Centre, Barwon Health, Geelong, Victoria, Australia.

e. Orygen, The Centre of Excellence in Youth Mental Health, the Department of Psychiatry and the Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Australia.

Running title: Th17 pathway in schizophrenia

***Corresponding author:**

Dr. Monojit Debnath

Department of Human Genetics

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National Institute of Mental Health and Neurosciences

Hosur Road, Bangalore-560029, India.

Tel.:+91-80-26995121; FAX:+91-80-26564830

Email: monozeet@gmail.com

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Abstract:

Aim: Immunopathogenesis remains a widely appreciated etiopathological model of schizophrenia. Persistent efforts have aimed to identify schizophrenia biomarkers indexing immune system abnormalities and also immuno-dampening effects of antipsychotic medications. Although data arising from published reports are encouraging, such studies are limited to few immune parameters and also not focused on a specific pathway.

Th17 cells mediated immuno-inflammatory responses have emerged as a potential mechanism in various neuropsychiatric conditions, including schizophrenia. Th17 pathway is distinctly regulated through a coordinated action of multiple cytokines and transcription factors. In this study, we wanted to explore whether antipsychotic medication has any effect on the cytokines and transcription factors of Th17 pathway.

Methods: A total of 27 drug naïve schizophrenia patients were recruited and followed up for three months after initiation of antipsychotic medication. Lymphocyte gene expression levels of two transcription factors (STAT3 and RORC) and one of their upstream regulators, IL6 were quantified before and after treatment. Plasma levels of cytokines, such as IL-1 β , IL-6, IL-17A, IL-23, and IL-33 were also analyzed before and after treatment.

Results: Treatment with antipsychotic medication for three months resulted in significant downregulation of STAT3 gene expression as well as reduction in plasma levels of IL-1 β , IL-6 and IL-17A. Significant reduction in total SAPS and SANS score was also observed in schizophrenia patients after 3 months of antipsychotic treatment.

Conclusion: Our findings suggest possible immuno-modulatory effects of antipsychotic medication on the critical regulators such as IL-6 and STAT3 of Th17 pathway in schizophrenia patients. IL-6/STAT3 signalling axis involved in the transcriptional regulation of Th17 cells might appear as important target of antipsychotic treatment in schizophrenia patients. Alternatively, IL-6/STAT3 signalling axis irrespective of the effect of antipsychotic drugs might be crucially involved in ameliorating psychotic symptoms.

Keywords: Schizophrenia; inflammation; Th17 pathway; STAT3; antipsychotic treatment.

INTRODUCTION

Schizophrenia is a complex neurodevelopmental disorder with inadequately explained etiopathology. The notion that schizophrenia is a multi-system disorder is gaining momentum in recent times. In addition to abnormalities in the central nervous system (CNS), immune system dysfunction has persistently been implicated in schizophrenia pathophysiology based on the data derived from genomic (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), transcriptomic and proteomic studies (1, 2). The strength of this connection was further enhanced by studies showing close cross-talk between immune system and other pathologically relevant systems or pathways like CNS, cardiovascular, metabolic and hypothalamic-pituitary-adrenal (HPA) axis in schizophrenia.

Altered expressions of immune parameters in the cerebrospinal fluid (CSF), and post-mortem brain as well as findings correlating immune abnormalities with brain morphometric changes in patients with schizophrenia has arguably made the immune hypothesis as one of the predominant elements of the etiology of schizophrenia (3-5). It is noteworthy that some of the immune parameters have also been linked to core psychopathology like cognitive deficits in schizophrenia (5, 6). Given such functional relevance, immune component(s) and pathway(s) are increasingly envisaged as a target for schizophrenia treatment (7). Agents including celecoxib, aspirin, minocycline, davunetide, estrogen, selective estrogen receptor modulators, pregnenolone, N-acetylcysteine, and erythropoietin have been explored (8). However, few studies exist on the impact of antipsychotic drugs specifically on the critical regulators of the immune cells and mediators. Amongst the immune system's cells, T lymphocytes are a crucial player in mediating disorders of CNS including schizophrenia (9). Much of the support for this notion was garnered from studies in human and/or experimental animals demonstrating the ability of T lymphocytes to a) transmigrate to the brain, b) release inflammatory cytokines in the brain, c) access the brain compartments through lymphatics, d) modulate neurogenesis (10), and e) contribute to cognitive deficits as well as altered behavior (11). Multiple studies over the past few years have demonstrated links between altered T cell numbers, T cell network, T cell receptor repertoires and schizophrenia pathobiology (12, 13).

Th17 cells possess pronounced encephalitogenic potential and are increasingly being implicated in diseases originating in the CNS. Earlier, we had proposed hypothetical mechanisms through which Th17 pathway might mediate immunopathogenesis of schizophrenia (14, 15). Higher proportions of Th17 cells were reported in drug naïve, first episode schizophrenia patients (16), suggesting activation of Th17 cells in schizophrenia. Besides this, plasma levels of Th17 pathway related cytokines such as TGF- β 1, IL-17 and IL-23 were associated with severity and aggressive behavior in patients with schizophrenia (17).

Our recent findings on the altered gene expression level of a key transcription factor, RORC, involved in the development and differentiation of Th17 cells as well as plasma levels of key signature cytokines of Th17 pathway, such as IL-6 and IL-22 add further support towards the involvement of Th17 pathway in schizophrenia (18). These data suggest that the aberrant Th17 pathway in schizophrenia could be transcriptionally regulated. In a previous study, 4 weeks of risperidone treatment significantly reduced the proportions of Th17 cells in schizophrenia patients (16). However, it is essential to understand the effect of antipsychotic medication on the transcription factors as well as cytokines regulating Th17 pathway. To address this knowledge gap, this study was aimed to explore the effect of antipsychotic medication on the lymphocyte gene expression profile of three critical regulators of the Th17 pathway such as IL6, STAT3 and RORC as well as plasma levels of a panel of related cytokines in drug-naïve schizophrenia patients.

PATIENTS AND METHODS

Study population

Drug-naïve patients with schizophrenia (n=27) attending the clinical services of the Department of Psychiatry at the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India and fulfilling Diagnostic and Statistical Manual of Mental Disorders –IV (DSM-IV) criteria were included in this study. Mini International Neuropsychiatric Interview Plus (19) was used to establish a diagnosis of schizophrenia. The Scale for Assessment of Positive Symptoms (SAPS) (Andreasen, 1984) and the Scale for Assessment of Negative Symptoms (SANS) (Andreasen, 1983), were used to evaluate symptoms of schizophrenia.

Demographic and clinical characteristics (history of presenting illness, any other medical illness, family & personal history as well as antipsychotic-naïve/ antipsychotic-free status) of the patients were ascertained

by the information provided by the first-degree relatives (**Table-1**). Exclusion criteria included patients who had a recent history of high-grade fever/infection within the past six weeks or any co-morbid medical disease that could potentially influence the immune system as well as co-morbid substance abuse or dependence. The study was conducted after obtaining approval from the institutional ethics committee and written informed consent from the patients.

Blood sample collection, RNA extraction and cDNA conversion

Approximately 10 ml of peripheral blood was collected from drug-naïve schizophrenia patients on the same day of the clinical assessments into Ethylenediaminetetraacetic acid (EDTA)-coated vacutainers and before starting antipsychotic treatment. Patients were not prescribed benzodiazepines or other holding treatment. Patients were then started on treatment as usual by the attending clinician. The patients were treated with adequate doses of various antipsychotics (Risperidone=19, Olanzapine=4, and Aripiprazole=4) which were converted into chlorpromazine equivalents for statistical analysis. After 3-months of antipsychotic medication, patients were assessed again with the SANS and SAPS and 10 ml of peripheral blood sample was collected during this follow-up visit. The plasma was separated from whole blood by centrifugation at 3500 RPM for 10 mins from 5ml of blood while peripheral blood mononuclear cells (PBMCs) were isolated from remaining 5 ml of blood using density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Sweden). The total RNA was extracted from PBMCs by commercially available spin column method (Qiagen, Inc, Limburg, Netherlands). RNA was then reverse transcribed to single-stranded complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

Quantification of gene expression

Gene expressions of IL6, STAT3 and RORC were quantified in all 27 schizophrenia patients before and after 3 months of antipsychotic treatment. Adherence was explored in clinical interview confirmed by the accompanying first-degree relatives of the patients. Quantitative PCR (qPCR) was performed with a commercially available TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California) containing-AmpliTaq Gold® DNA polymerase. Pre-formulated and -designed TaqMan probes and primers for IL6 (Assay ID: Hs00985639), RORC (Assay ID: Hs01076112) and STAT3 (Assay ID: Hs01047580) with primers spanning exon boundaries to avoid non-specific amplification were used. The PCR amplification of IL-6, STAT3 and RORC genes in each sample was normalized to endogenous control, 18S rRNA (Assay ID: Hs99999901).

qPCR reaction was carried out in Quantstudio 6 Real-Time PCR System (Applied Biosystems, Foster City, California) using a 96-well format. Reactions were performed in a final volume of 10 μ L containing TaqMan Universal PCR Master Mix, probes (final concentration of 250 nM) and primers for IL-6, RORC, and STAT3 (final concentration of 900 nM) and 18S rRNA (final concentration of 150 nM) as well as 1 μ L of cDNA. The assay for every sample was run in triplicates. Standard PCR conditions of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C were followed. The $2^{-\Delta\Delta C_t}$ method of relative quantification was used for data interpretation. The final result was presented as the fold change of target gene expression relative to a reference sample, normalized to the endogenous control.

Measurement of plasma levels of cytokines

Plasma levels of 5 cytokines (IL-1 β , IL-6, IL-17A, IL-23, and IL-33) were estimated in 27 drug naïve schizophrenia patients, before and after 3-months of antipsychotic medication. These cytokines were analyzed by using Bio-Plex Pro Human Th17 Cytokine 5-plex panel in a Bio-Plex 200 analyzer (Bio-Rad, Hercules, CA, USA). The sensitivity of the panel was high (pg/ml) and the limit of detection (LOD) for various cytokines were as follows: IL-1 β (0.02 pg/ml), IL-6 (0.67 pg/ml), IL-17A (0.49 pg/ml), IL-23 (1.55 pg/ml), and IL-33 (0.58 pg/ml). All the samples were assayed in duplicate. The standard curve of each cytokine was generated by using the standards provided in the kit by the manufacturer. Bioplex Manager software was used to analyse the data according to manufacturer's instructions.

Statistical analysis

Statistical analyses were executed by the Statistical Package for the Social Sciences software (version-24) [SPSS Inc.,]. A two-tailed P value less than 0.05 was considered to be statistically significant. Paired sample t test and Wilcoxon signed rank test were used to analyze pre- and post-treatment differences of gene expression depending on the distribution of the data. The plasma cytokines were not normally distributed and consisted of non-detects. Cytokine concentrations falling below the lower limit of detection were considered as non-detectable and were left-censored. Hence, Wilcoxon signed rank test was performed without loss of information. Pearson's and Spearman's correlations were performed based on the distribution pattern of the data.

RESULTS

The clinical and demographic data of schizophrenia patients are summarized in **Table-1**.

Gene expression

We did not observe any significant difference in the levels of gene expression of IL6 and RORC in schizophrenia patients before and after antipsychotic treatment (**Table-2**). However, using a paired sample t-test, a statistically significant difference was observed in the levels of gene expression of STAT3 at baseline and after antipsychotic treatment ($p < 0.001$) (**Figure-1**). There was a significant reduction in total SAPS and SANS score in schizophrenia patients after antipsychotic treatment (**Table-3**). The levels of expression of IL6, STAT3 and RORC genes were then correlated with psychopathology, however, the correlation was not found to be statistically significant.

Plasma levels of Th17 pathway cytokines

Amongst these cytokines (IL-1 β , IL-6, IL-17A, IL-23 and IL-33), the plasma levels of IL-23 were below the detectable range both in drug naïve and treated schizophrenia patients, while the levels of remaining four cytokines were modulated by antipsychotic treatment (**Table-4**). Interestingly, the plasma levels of IL-1 β and IL-17A that were in detectable range in drug naïve patients became undetectable after antipsychotic treatment. Levels of IL-6 also dropped significantly after three months of antipsychotic treatment (**Figure-2 & Table-4**). The plasma levels of cytokines were then correlated with psychopathology, but none of them turned out to be statistically significant.

DISCUSSION

The pathobiological relevance of Th17 pathway is becoming increasingly apparent in various neuropsychiatric diseases including major depression, obsessive compulsive disorder, autism spectrum disorder and in particular, schizophrenia (16, 20-22). Divergent lines of evidence suggest important functional implications of Th17 cells in schizophrenia pathophysiology, psychopathology and treatment response (16, 17). Maternal immune activation (MIA) has consistently been linked to enhanced risk of schizophrenia in offspring by both experimental animal and epidemiological studies (23, 24). Interestingly, maternal immune stimulation during pregnancy was shown to shape the immunological phenotype of offspring, especially through preferential development of Th17 cells (25, 26). This implies that Th17 cells can preserve the attributes of immunological memory across the neurodevelopmental window as well as in adult life. This process could be primed through transcriptional regulation of genes such as STAT3 and RORC that are involved in the development and differentiation of Th17 cells. Altered activation and/or regulation of these transcription factors by upstream cytokines could lead to the generation of pathogenic Th17 cells. The significance of these transcription factors has not been examined adequately with respect to treatment response in patients with schizophrenia.

In the current study, we examined the effect of antipsychotic drug on the gene expression levels of IL6, STAT3 and RORC in drug naïve schizophrenia patients. In addition, the effects of antipsychotics on plasma levels of five Th17 pathway related cytokines were also assessed. The most important finding was significant downregulation of STAT3 gene expression after 3 months of antipsychotic medication. Besides this, antipsychotic medication also led to significant reduction in the plasma levels of IL-1 β , IL-6 and IL-17A. It is a well-documented fact that there exist potential functional interactions between cytokines and STAT3/RORC and *vice versa*. For example, IL-6 in conjunction with TGF- β can induce RORC expression and drive the lineage commitment of Th17 cells (27). Further to this, IL-6 along with IL-21 and IL-23 was

found to preferentially activate STAT3 expression and enhance the secretion of IL-17 by Th17 cells (28). Notably, generation of pathogenic Th17 cells was shown to be mediated by IL-6 and IL-23 dependent activation of STAT3 signalling (29). These findings suggest important functional interactions between IL-6 and transcription factors (STAT3 and RORC). Contextually, a growing body of evidence suggests crucial role of IL-6/STAT3 signalling in autoimmune diseases (30). We observed a non-significant decrease in the gene expression levels of IL6 (**Table-2**) and a significant reduction in the plasma levels of IL-6 in schizophrenia patients after 3 months of antipsychotic treatment. When we examined the correlation between IL6 and STAT3 gene expression, there was a non-significant positive correlation (data not shown). Importantly, antipsychotic medication had modulatory effects on three important signatures of Th17 pathway, i.e. IL-6, STAT3 and IL-17A in schizophrenia patients. The effects of antipsychotic medication on this pathway may form part of the biological effects of the agent in addition to the known effects of the medication through pathways like dopamine receptor system. Based on the above findings, it is suggested that IL-6/STAT3 signalling might play important role in the immunopathogenesis of schizophrenia and these components may serve as potential predictor of treatment response or theragnostic marker (31) of antipsychotic treatment.

Several studies over the past few years have demonstrated immuno-modulatory attributes of antipsychotic drugs. A recent meta-analysis has suggested that antipsychotic treatment could normalize immune balance dysfunction through its anti-inflammatory effect and a cytokine, IL-6 was envisaged as a marker of illness equilibration (32). This is possibly achieved by downregulating the levels of pro-inflammatory cytokines, upregulating the levels of anti-inflammatory cytokines and through inhibition of microglial activation (33, 34). Inflammatory responses are proposed to be relevant and linked to distinct symptom classes of schizophrenia, particularly in the development and treatment of the negative and cognitive symptoms.

These notions are empirically supported by data published in recent studies. For example, a positive correlation was reported between plasma levels of C-reactive protein (CRP), a non-specific marker of systemic inflammation and negative symptoms of schizophrenia (35). In addition, elevated plasma levels of IL-6 and TNF- α were linked to deficit syndrome and negative symptoms in chronic schizophrenia patients (36). Further support was garnered from another study highlighting the relationships between baseline levels of pro-inflammatory cytokines like IL-6 and TNF- α and negative symptom trajectories assessed longitudinally over a period of 12 months in individuals at clinical high risk for psychosis (37). Similarly, chronic inflammation due to elevated levels of CRP was linked with cognitive impairment in patients with schizophrenia (38). Based on these findings, the authors of the above studies suggested that inflammatory markers might influence/predict the development of negative and cognitive symptoms in some individuals. The effects of antipsychotic drugs, especially when used in combination with anti-inflammatory agents like minocycline, aspirin etc. in suppressing the inflammatory response systems and improving the symptoms, mainly negative and cognitive symptoms of schizophrenia are well documented(39, 40). Therefore, immune normalization capacity of antipsychotic drugs might be considered as one of the key processes in determining their clinical efficacy in the symptomatologic treatment of schizophrenia. In this study, we observed a significant reduction in the total SANS and SAPS score. The alteration of IL-6/STAT3 signalling pathway in schizophrenia patients might also be associated with improvement of symptoms. There is a possibility that IL-6/STAT3 signalling axis might directly be involved in improving schizophrenia symptoms, irrespective of the effects of antipsychotic drugs. This could happen as a bystander effects of antipsychotic drugs, or through some intermediate elements involved in translating the effects of antipsychotic drugs. However, it is premature to make such overarching conclusion.

Comprehensive functional studies, either on cell lines or on experimental animals are required to explore these possibilities.

This study was conducted only on drug naïve schizophrenia patients and this is the main strength of this study. The limitations included the relatively small sample size and the length of the follow up. Besides this, information about the childhood trauma was not available for most participants.

CONCLUSIONS

Th17 pathway has taken centre stage of immunology research given its increasing implications in pathogenic pathways of autoimmune/inflammatory diseases as well as its growing relevance as a therapeutic target (41, 42). There are multiple novel therapeutic targets along the Th17 pathway. Studies using inhibitors of the various Th17 pathway components like STAT3, IL-12, IL-23, IL-17A, IL-17RA etc. have demonstrated beneficial effects in several autoimmune diseases like psoriasis, Crohn's disease, rheumatoid arthritis, ankylosing spondylitis etc. (43, 44) Besides this, IL-6/STAT3 signaling is also emerging as a promising therapeutic target in autoimmune diseases and cancer (45, 46).

In the present study, antipsychotic medication reduced levels of various key elements such as STAT3, IL-1 β , IL-6 and IL-17A. Based on these findings, it appears that antipsychotic medication could suppress IL-6/STAT3 signalling in schizophrenia patients. Further *in vitro* studies are required to precisely delineate the effects of antipsychotic drugs on various other components of Th17 pathway.

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Author contribution:

MD contributed to study concept and design. **GV, SV, and JCN evaluated, recruited and treated the patients. MS, VS, DV, JCN, SV, GV and MD** contributed to data acquisition and analysis. MD has written the first draft. GV, SV and MB have provided expert opinion as well as revisions. MD had full access to all study data and final responsibility for submission for publication.

Figure legends

Figure-1: Differences in STAT3 gene expression in schizophrenia patients before and after treatment.

Figure -2: Differences in plasma IL-6 levels in antipsychotic naïve and antipsychotic treated schizophrenia patients.

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PCN_12938_Figure-2.jpg

Table 1:Demographic and clinical profile of the study participants

Characteristic (n=27)	Mean $\pm$ SD
Age (in years)	30.63 $\pm$ 7.53
Sex (M:F)	27(16:11)
Age at onset (in years)	27.97 $\pm$ 7.92
Duration of untreated illness (in months)	46.00 $\pm$ 66.79
Total SAPS score	27.85 $\pm$ 12.91
Total SANS score	33.63 $\pm$ 26.01

Table 2: Differences in the gene expression of IL6, STAT3 and RORC genes in schizophrenia patients at baseline and during follow up.

Gene	Mean $\pm$ SD		Stats	P
	Baseline (n=27)	Follow up (n=27)		
IL6	0.66 $\pm$ 0.91	0.53 $\pm$ 0.48	-0.92 [^]	0.36
STAT3	1.18 $\pm$ 0.32	0.82 $\pm$ 0.22	4.79 [#]	< 0.001
RORC	0.93 $\pm$ 0.27	0.96 $\pm$ 0.39	-0.41 [#]	0.69

(t) Paired sample t test

[^](Z)Wilcoxon signed ranks test

Table-3: Differences in total SANS and total SAPS of schizophrenia patients at baseline and during follow up.

Symptoms	Mean $\pm$ SD		Stats [#]	P
	Baseline (n=27)	Follow up(n=27)		
SANS	27.85 $\pm$ 12.91	7.74 $\pm$ 10.79	9.84 [#]	< 0.001
SAPS	33.63 $\pm$ 26.01	20.04 $\pm$ 17.77	2.89 [#]	0.008

(t) Paired sample t test

Table-4: Differences in plasma cytokine levels before and after antipsychotic treatment in schizophrenia patients.

Cytokines	Mean± SD (n=27)		Stats*	P
	Before antipsychotic treatment	After 3 months of Antipsychotic treatment		
IL-1 β	0.24 $\pm$ 0.20	0.0	-3.83	< 0.001
IL-6	6.29 $\pm$ 4.51	3.14 $\pm$ 3.17	- 2.21	0.027
IL-17A	3.04 $\pm$ 2.37	0.0	- 4.45	< 0.001
IL-33	26.21 $\pm$ 35.71	15.56 $\pm$ 26.52	- 1.37	1.71

*Wilcoxon Signed -Rank Test

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Author/s:

Subbanna, M;Shivakumar, V;Venugopal, D;Narayanaswamy, JC;Berk, M;Varambally, S;Venkatasubramanian, G;Debnath, M

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