Schizophrenia Bulletin vol. 50 no. 2 pp. 403–417, 2024
https://doi.org/10.1093/schbul/sbad171
Advance Access publication December 15, 2023

Toll-Like Receptor mRNA Levels in Schizophrenia: Association With Complement Factors and Cingulate Gyrus Cortical Thinning

Thomas W. Weickert[1–3], Ellen Ji[4,5], Cherrie Galletly[6–8], Danny Boerrigter[1], Yosuke Morishima[9], Jason Bruggemann[1,2,10,11], Ryan Balzan[12], Maryanne O'Donnell[1,13], Dennis Liu[6–8], Rhoshel Lenroot[1,2,14], Cynthia Shannon Weickert[1–3,16], and Jochen Kindler[*,1,15,16]

[1]Neuroscience Research Australia, Schizophrenia Research Institute, Randwick, NSW 2031, Australia; [2]School of Psychiatry, University of New South Wales, Randwick, NSW 2031 Australia; [3]Department of Neuroscience and Physiology, Upstate Medical University, Syracuse, NY 13210, USA; [4]Psychiatric University Hospital Zurich, Zurich, Switzerland; [5]Neuroscience Research Australia, Sydney, NSW, Australia; [6]Discipline of Psychiatry, School of Medicine, University of Adelaide, Adelaide, SA, Australia; [7]Ramsay Health Care (SA) Mental Health, Adelaide, Australia; [8]Northern Adelaide Local Health Network, Adelaide, SA, Australia; [9]Translational Research Center, University Hospital of Psychiatry, University of Bern, Bern, Switzerland; [10]Edith Collins Centre (Translational Research in Alcohol Drugs and Toxicology), Sydney Local Health District, Sydney, Australia; [11]Speciality of Addiction Medicine, Central Clinical School, Faculty of Medicine and Health, University of Sydney, Sydney, Australia; [12]School of Psychology, Flinders University, Adelaide, SA, Australia; [13]Kiloh Centre, Prince of Wales Hospital, Randwick, New South Wales, Australia; [14]Department of Psychiatry, University of New Mexico, Albuquerque, NM 87131-0001, USA; [15]University Hospital of Child and Adolescent Psychiatry and Psychotherapy, University of Bern, 3000 Bern, Switzerland

[16]Equally contributing senior authors.

*To whom correspondence should be addressed; University Hospital of Child and Adolescent Psychiatry and Psychotherapy, Bolligenstrasse 111, Bern, Switzerland; e-mail: jochen.kindler@upd.unibe.ch, Tel.: +41319309111, Fax: +41319309888 jochen.kindler@upd.unibe.ch, Tel.: +41319309111, Fax: +41319309888

***Background and Hypotheses*: Previous studies revealed innate immune system activation in people with schizophrenia (SZ), potentially mediated by endogenous pathogen recognition receptors, notably Toll-like receptors (TLR). TLRs are activated by pathogenic molecules like bacterial lipopolysaccharides (TLR1 and TLR4), viral RNA (TLR3), or both (TLR8). Furthermore, the complement system, another key component of innate immunity, has previously been linked to SZ. *Study Design*: Peripheral mRNA levels of TLR1, TLR3, TLR4, and TLR8 were compared between SZ and healthy controls (HC). We investigated their relationship with immune activation through complement expression and cortical thickness of the cingulate gyrus, a region susceptible to immunological hits. TLR mRNA levels and peripheral complement receptor mRNA were extracted from 86 SZ and 77 HC white blood cells; structural MRI scans were conducted on a subset. *Study Results*: We found significantly higher TLR4 and TLR8 mRNA levels and lower TLR3 mRNA levels in SZ compared to HC. TLRs and complemental factors were significantly associated in SZ and HC, with the strongest deviations of TLR mRNA levels in the SZ subgroup having elevated complement expression. Cortical thickness of the cingulate gyrus was inversely associated with TLR8 mRNA levels in SZ, and with TLR4 and TLR8 levels in HC. *Conclusions*: The study underscores the role of innate immune activation in schizophrenia, indicating a coordinated immune response of TLRs and the complement system. Our results suggest there could be more bacterial influence (based on TLR 4 levels) as opposed to viral influence (based on TLR3 levels) in schizophrenia. Specific TLRs were associated with brain cortical thickness reductions of limbic brain structures.**

Key words: magnetic resonance imaging/innate immunity/toll like receptors/schizophrenia/complement factors/cortical thickness

Introduction

Recent studies have highlighted the role of inflammation in the pathophysiology of schizophrenia.[1–4] While it is debated whether neuroinflammation is causative or consequential or neither, increased cytokines typically signal pathology and are known to be damaging to cells and tissue. The immune system consists of innate and adaptive components that are separated functionally, cellularly, and molecularly.[5] Activations of both the innate and adaptive immune system have been reported to be involved in the pathogenesis of schizophrenia.[4,6,7]

The toll-like receptors (TLRs) are an essential part of the innate immune system being involved in first line recognition of common pathogen associated molecular patterns (PAMPs). They induce a cascade of intracellular signaling and activation of transcription factors such as NF-κB that lead to increased production of proinflammatory cytokines, such as interleukin (IL) 1β, IL6, tumor necrosis factor (TNF) α, interferon gamma (IFN-γ), oxidative stress markers, and the release of C-reactive protein (CRP).[8] Hence, TLRs are considered as primary activators of the innate immune response that may be responsible for activating cytokines in people with schizophrenia. TLRs are primarily expressed by macrophages, monocytes, and dendritic cells, but are also located on endothelial cells, neural and glia cells and putatively contribute to neuronal development and plasticity.[9,10] Importantly, specific TLRs such as TLR4 and TLR1 (together with TLR2) are expressed on the cell surface and are primarily activated by bacterial lipopolysaccharides (LPS), whereas TLRs such as TLR3 and TLR8 are located in the cytoplasm on endosomes. While TLR3 is specifically activated by viral double stranded RNA,[11] TLR8 is activated by both bacterial LPS and viral and bacterial RNA.[12] Activation of TLRs increases mRNA expression of the respective TLR in differential patterns depending on the trigger (ie, viral RNA or bacterial LPS) of the immune activation.[13–17] Thus, TLR gene expression can be used as an index of innate immune cell activation in schizophrenia and it also provides information regarding the general pathogen that initiated the response.

To date, most available studies investigated the levels of TLR3 and TLR4 mRNA and/or protein in peripheral blood and brain of people with schizophrenia. TLR4 is the primary receptor for LPS from Gram-negative bacteria. Further, TLR4 is a trans-membrane protein with an extracellular domain harboring leucine-rich repeats (LRRs) and a cytoplasmic tail known as Toll/IL1 receptor domain.[8] TLR4 is up-regulated during exposure to stress and is involved in various neuropsychiatric disorders.[18,19] Within the brain, TLR4 protein is increased in the prefrontal cortex of people with schizophrenia.[19] Additionally, flow cytometry indicates increased numbers of TLR4 positive monocytes are found in the circulation of people with schizophrenia.[20,21] Further, stimulation of whole blood with a selective TLR4 agonist induces significantly higher IL1β in people with schizophrenia compared to healthy controls (HC).[22] This raises the possibility that increased TLR4 synthesis and activation may be upstream of cytokine changes.

TLR3 recognizes viral double strand RNA. Stimulation of TLR3 using poly(I:C), an immunostimulant which simulates viral infections, induces antiviral and inflammatory responses mediated by transcription factors, such as interferon regulatory transcription factor 3 and the nuclear factor-κB, and leads to massive secretion of proinflammatory cytokines in rodents.[23] In murines, TLR3 activation of hippocampal and cortical neuronal cultures via poly(I:C) downregulates the disrupted-in-schizophrenia-1 (Disc1) gene causing neuronal impairments.[24] Further research has found that increased TLR3 levels in rodents were partially reversed by the antipsychotic paliperidone.[25] Using exome sequencing, de novo missense mutations of the TLR3 gene were reported in a large sample of schizophrenia parent-offspring trios.[26] Although one study showed increased expression of TLR3 receptors in monocytes in people with schizophrenia, in vitro stimulation of monocytes using poly(I:C) yielded a lower increase of expression of TLR3 in people with schizophrenia compared to HC.[21] While no difference was detected in TLR3 gene expression levels compared to HC, in people with schizophrenia who were medication free,[27] downregulation of TLR3 mRNA was reported in antipsychotic treated, chronically ill people with schizophrenia.[28] Hence, while studies on schizophrenia models in rodents suggested increased TLR3 levels, results in people with schizophrenia are inconsistent.

Studies on the other TLRs (TLR1 and TLR 8) in schizophrenia are also inconclusive.[22,29] The structurally and functionally related TLR1 and TLR2 both recognize lipoproteins of diverse microbial ligands, such as Gram-positive bacteria, whereas, TLR8 binds bacterial and viral single stranded (ss)RNA and detects Gram-positive bacteria.[12] Regarding neurodevelopment, TLR8 is critical for dendritic arborization and later, for dendritic pruning and shortening.[30] In general, however, TLR8 has been described as a negative regulator of neuronal growth and may cause neuronal apopotosis.[31]

Like TLRs, the complement system is another key component of the innate immune system and has been increasingly implicated in schizophrenia over the past decade.[6,32] TLRs and the complement system play distinct but interconnected roles in detecting and responding to pathogens. Together, they enhance and amplify immune responses,[33,34] and complement activation can influence expression of TLRs on immune cells.[35] Further, hyperactivation or dysregulation of either could lead to excessive unwarranted inflammation, which has been repeatedly reported in some individuals with schizophrenia.[1,36,37] However, understanding the relationship between TLRs and the complement system in schizophrenia is in its infancy. We therefore aimed to study the complement-TLR relationship in individuals with schizophrenia in order to better understand how they may co-contribute to inflammatory dysfunction.

Gray matter reductions in the cingulate cortex have consistently been reported in people with schizophrenia, people with first episode psychosis and in individuals at risk of psychosis who later convert to psychosis[38] and have been implicated in cognitive deficits in people with schizophrenia.[39,40] However, the pathphysiological mechanisms associated with these volume reductions remain elusive. We and others have shown an association

between gray matter volume and peripheral inflammation in schizophrenia.[2,36,41–44] Postmortem studies in schizophrenia indicate increased measures of inflammation, predominantely in the prefrontal cortex and the anterior cingulate cortex.[1,45–47] Our previous work in rodents suggests that the cingulate cortex may be specifically affected by a maternal immune activation that triggers TLR activation.[48] To our knowledge no study has determined the relationship between peripheral TLR levels and anterior cingulate cortex thickness in people with schizophrenia. It is also unclear whether innate immune system activation in people with schizophrenia is specifically related to either the viral or the bacterial arm of the system, or equally affects both. Further, no study has linked TLR mRNA expression with inflammation status (ie, "high" vs "normal" levels of complement) in people with schizophrenia. Importantly, an unconstrained immune response through activation of TLRs may contribute to cortical gray matter loss.

In light of these previous findings, we measured TLR1, TLR3, TLR4, and TLR8 mRNA levels in the plasma of people with schizophrenia compared to HC. We hypothesized that people with schizophrenia would have significantly higher peripheral TLR mRNA levels. We expected that peripheral TLR levels would be highest in those people with schizophrenia with increased complemental factors. Further, since the cingulate cortex is a neuroanatomical structure particularly affected by inflammatory activation in schizophrenia, we hypothesized that peripheral TLR mRNA expression would be associated with gray matter thickness of the cingulate cortex in people with schizophrenia. Specifically, we predicted that elevated peripheral TLR expression levels would be associated with reduced gray matter thickness.

Materials and Methods

Participants

Eighty-six chronically ill patients meeting the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria for schizophrenia or schizoaffective disorder on the basis of the Structured Clinical Interview for DSM-IV Axis 1 disorders (SCID) and 77 adult HC were recruited from 2 sites (Adelaide and Sydney). Demographics on the sample are provided in table 1.

All participants were screened for exclusion criteria, which included a concurrent DSM-IV Axis I diagnosis other than schizophrenia or schizoaffective disorder for patients or any personal history or first-degree relative with a DSM-IV Axis I disorder for HC. Exclusion criteria for all participants included a history of uncontrolled diabetes or cardiovascular disease including hypertension, central nervous system infection, recent alcohol/substance abuse (within the past 5 years), head injury with loss of consciousness, epileptic seizures, structural brain abnormalities, developmental disorders, and/or mental retardation. All people with schizophrenia were receiving antipsychotic medication (95% receiving second-generation antipsychotics) for at least 1 year prior to participation. The mean daily dose of antipsychotic medication for each person with schizophrenia was converted to approximate daily mean chlorpromazine milligram equivalents (CPZ) dose using standard guidelines.[49] Symptom severity in people with schizophrenia was assessed using the Positive and Negative Syndrome Scale (PANSS)[50] by a psychologist or psychometrician trained in administration and scoring. Additionally, the body mass index (BMI) and smoking status were assessed, as both might influence immunological parameters. As part of a battery of baseline psychometric assessments and previously described in,[51] all participants were administered cognitive subtests that measured working memory (Letter-Number Sequencing and Arithmetic from the Wechsler Adult Intelligence Scale-Third Edition [WAIS-III])[52] and tests of language abilities (letter fluency from the Controlled Oral Word Association Test[53] and WAIS-III Similarities). The 2 working memory test scores were summed and converted to *z*-scores, and the 2 language test scores were summed and converted to *z*-scores to provide separate overall scores for working memory and language, respectively.

All procedures were approved by the UNSW (07-121, 09-187) and South-Eastern Sydney and Illawarra Area Health Service (07-259) Human Research Ethic Committees, Sydney and the Queen Elizabeth Hospital (2010188) Ethics and Human Research Committee, Adelaide. Written informed consent was obtained from each participant before entry into the study.

RNA Isolation

Samples of fasting peripheral blood were collected from all participants between 9 am and 11 am on the day of the magnetic resonance imaging (MRI) scan. Blood samples from all participants were collected in 9 ml ACD-B yellow-topped tubes (BD Biosciences, North Ryde, NSW, Australia) before being processed. RNA isolation was done according to the TRIZOL (Life Technologies, Melbourne, Victoria, Australia) method. One microgram of total RNA per sample was used in 20 μl reverse-transcriptase reactions using the SuperScript IV First-Strand Synthesis System to create cDNA (Invitrogen, Carlsbad, CA, USA).

TLR mRNA Levels, NF-kB Pathway, Interleukins, and Complemental Receptors

TLR1, 3, 4 and TLR8 mRNA expression levels were assayed on the BioMark HD platform (Fluidigm Corperation, South San Francisco, CA, USA) at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia) utilizing predesigned TaqMan probes (Thermo Fisher Scientific,

Table 1. Sample Description

	People with Schizophrenia			Healthy Controls					
	Mean	*SD*	*n*	Mean	*SD*	*n*	Test Value	*df*	*P*-value
Age at sampling (years)	35.83	8.31	86	31.73	8.54	77	−3.10	161	.002**
Sex (m/f)	53/33			38/39			1.99	1	.158
BMI (kg/m^2)	31.0	6.33	74	23.22	3.25	4	—	—	—
Smoking (y/n)	36/32	—	68	1/7	—	8	—	—	—
NF-κB1 mRNA	0.95	0.18	81	0.97	0.23	74	1.1	152	.349
NF-κB2 mRNA	0.98	0.15	81	1.00	0.26	74	0.42	152	.660
C5ar mRNA	1.11	0.27	82	0.98	0.26	75	−4.8	154	.01*
CR1 mRNA	0.95	0.16	85	0.87	0.11	77	−5.6	159	.005**
ITGAM/C3Ra mRNA	0.97	0.12	86	0.92	0.09	77	−5.2	160	.006**
C3 Protein (mg/dl)	4.09	2.93	68	2.59	1.12	56	−6.2	121	.003**
C3b Protein (mg/dl)	4.27	3.78	68	3.32	2.47	57	−2.8	122	.063
C4 Protein (mg/dl)	3.34	1.29	68	2.90	1.40	57	−3.1	122	.051
Cortisol (nmol/l)	304.4	121.97	86	303.67	117.79	74	−0.036	158	.971
NLR	2.47	1.09	82	1.87	0.72	24	−2.52	104	.013*
IL1 β mRNA	1.077	0.440	86	0.878	0.323	77	−5.3	160	.006*
IL6 mRNA	1.137	0.669	86	0.976	0.485	77	−3.1	160	.079
IFN-γ mRNA	1.407	1.481	86	0.916	0.531	77	−3.8	160	.025**
CRP (mg/l)	4.75	5.31	75	2.06	3.45	65	−6.5	137	.002**
Working memory domain	16.11	5.18	86	22.01	5.30	77	29.45	160	<.001**
Language domain	46.36	11.93	86	53.10	12.34	77	6.74	160	.002**
Rost. Ant. CC (mm)	2.96	0.19	51	3.11	0.14	57	4.76	106	<.001**
Caud. Ant. CC (mm)	2.70	0.18	51	2.81	0.20	57	3.16	106	.002**
Post. CC (mm)	2.61	0.15	51	2.71	0.14	57	3.29	106	.001**
Isthmus CC (mm)	2.55	0.20	51	2.65	0.17	57	2.81	106	.006**
Age of onset (years)	22.94	5.75	86	—	—	—	—	—	—
Illness duration (years)	12.88	7.70	86	—	—	—	—	—	—
CPZ (mg/d)	554.74	463.41	86	—	—	—	—	—	—
PANSS pos	15.20	4.44	86	—	—	—	—	—	—
PANSS neg	14.48	6.15	86	—	—	—	—	—	—
PANSS gen	30.79	8.76	86	—	—	—	—	—	—
PANSS total	60.47	16.39	86	—	—	—	—	—	—

Note: Means ± standard deviations. Age in years, M/F male/female in absolute numbers.
BMI, body mass index. Smokers yes/no in absolute numbers; NF-κB1/2, kappa-light-chain-enhancer' of activated B-cells (mRNA, log transformed); C5ar, complement receptor 5 a (mRNA log transformed); CR1, complement receptor type 1 (mRNA, log transformed); ITGAM/C3a, integrin alpha M/complement factor 3 receptor alpha (mRNA log transformed); C3/C3b/C4 Prot., complement component 3/3b/4 protein; cortisol in nmol/l; NLR, neutrophil/lymphocyte ratio; IL1 β, interleukin1 beta; IL6, interleukin 6; IFN-γ, interferon gamma; CRP, c-reactive protein; Rost. Ant. CC, rostral anterior cingulate cortex thickness; Caud. Ant. CC, caudal anterior cingulate cortex thickness; Post. CC, posterior cingulate cortex thickness; Isthmus CC, isthmus cingulate cortex thickness. Thickness in mm. Age of onset = Age of illness onset and illness duration in years; CPZ, mean daily chlorpromazine equivalent dose; PANSS, Positive And Negative Syndrome Scale; *df*, degrees of freedom; Test value referring to independent *t*-test or chi-square tests.
*Significant at $P < .05$.
**Significant at $P < .01$.

Waltham, MA, USA): TLR1(Hs00413978_m1), TLR3 (Hs01551078_m1), TLR4 (Hs01060206_m1) and TLR8 (Hs00152972_m1). NF-κB1 (Hs00765730_m1), and NF-κB2 (Hs00174517_m1), the proinflammatory cytokines Interleukin-1β (Hs01555410_m1), Interleukin 6 (Hs00174131_m1), and IFN-γ (Hs00989291_m1), as well as complement receptors C5aR1 (Hs00383718_m1), CR1 (Hs00355835_m1), and ITGAM/CR3a (Hs00355885_m1) were measured as previously described.[7,11] Controls included no reverse transcriptase and no template to rule out genomic DNA contamination and reagent contamination, respectively. Gene expression levels were normalized to the geometric mean of 4 housekeeper genes; Beta-2 microglobulin (Hs00984230_m1), Glyceraldehyde 3-phosphate dehydrogenase (Hs99999905_m1), TATA-binding protein (Hs00427620_m1), and Ubiquitin C (Hs00824723_m1) assayed within the same experiment that did not differ between any of the groups compared (diagnosis: $t_{160} = -0.93$, $P = .35$; cytokine subgroup: $F_{3,136} = 0.31$, $P = .82$). Further information is provided in the Supplementary material.

Additional Peripheral Blood Measures

Cortisol, neutrophil/lymphocyte ratio (NLR), and CRP were measured in clotted and heparinized blood that was delivered on ice immediately following collection. CRP was measured in plasma using a high-sensitivity

Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IBL-international, Hamburg, Germany). Cortisol was assayed using a chemiluminescent immunometric assay (Siemens Immulite 2000). Additionally, 3 complement protein components C3, (i)C3b and C4 from the Human Complement Panel 2 (HCMP2MAG-19 K, Merck Millipore, Billerica, MA, USA) were analysed as previously described.[11] Bacterial genomic DNA encoding 16S rRNA was measured from plasma using TaqMan qPCR in all subjects and normalized to the geomean. Values >2 standard deviations were excluded. Normality of the data was checked using the Kolmogorov–Smirnov test ($P < .001$) and then log transformed. A linear regression model was run with normalized 16S rRNA as the dependent variable, group (control/ schizophrenia) as the predictor variable and age as a covariate, to test for a group difference between people with schizophrenia and HC. Finally, an ANCOVA with 16S rRNA levels as dependent variables and inflammatory group (HC normal/high, schizophrenia normal/high) as grouping factors with age as a covariate was run.

Further information on analyses are provided in the Supplementary material.

Inflammatory Subgrouping

We previously found complement mRNA expression for 3 complement mRNAs to be increased in people with schizophrenia compared to HC.[11] Using K-means clustering, participants were classified into one of 2 subgroups based on the 3 mRNA expression levels (C5AR1, CR1, and ITGAM/CR3a). Because of too many missing values K-means clustering was not performed based on complement proteins that were also found to be significantly increased in schizophrenia (C3, C3b, and C4). See Supplementary methods for further information on complement subgrouping.

Gray Matter Volume Data Collection and Processing

Magnetic resonance imaging was performed on a subset of participants consisting of 54 people with schizophrenia and 59 HC using a 3-T Philips Achieva MRI scanner at Neuroscience Research Australia, Randwick, Australia. We obtained T1-weighted, high-resolution anatomical scans using an 8 channel head coil, with a repetition time (TR) 5.4 ms, echo time (TE) 2.4 ms, field of view (FOV) 256 mm, matrix 256 × 256, sagittal plane, slice thickness 1 mm, no gap, 180 slices. All scans were screened for anatomical abnormalities, which excluded 1 HC and 1 person with schizophrenia, excessive motion, successful normalization, and artifacts which excluded 1 additional HC and 2 people with schizophrenia. The final sample comprised 51 people with schizophrenia and 57 HC. The standard recon-all pipeline in FreeSurfer v 5.1.0 (http://surfer.nmr.mgh.harvard.edu) was carried out and processed scans were visually evaluated to check for accurate segmentation and manually edited if necessary. Measurements of cortical thickness based on the Desikan-Killiany atlas were extracted. The present study focused on the cingulate cortex separated into 4 sections: rostral anterior, caudal anterior, posterior, and isthmus cingulate cortex. Statistics were calculated on right and left hemisphere mean gray matter cortical thickness, combined.

Statistics

All analyses were performed using SPSS (version 24, IBM, Armonk, NY, USA).

Demographic and immunological factors were compared between people with schizophrenia and HC in 2 sample *t*-tests. To evaluate the relation between TLR mRNA levels and demographic (BMI, age) or immunological factors [IL levels, neutrophil/lymphocyte ratio (NLR), cortisol, CRP, complental factors, NF-κB] and cognition [language and working memory domain], Pearson correlations were calculated in the total sample and for people with schizophrenia and HC, separately. Differences of TLR mRNA levels according to sex and smoking status were calculated using *t*-tests.

To detect differences of TLR mRNA levels between people with schizophrenia and HC, ANCOVAs were performed with TLR1, 3, 4 and 8 mRNA levels as dependent variables and diagnostic group (people with schizophrenia vs HC) as grouping factors with age as a covariate. A further ANCOVA including sex as an additional covariate was conducted.

To evaluate the influence of inflammatory subgroups on TLR mRNA levels, ANCOVAs were calculated with TLR mRNA levels as dependent variables and inflammatory group (HC normal/high, schizophrenia normal/high) as grouping factors with age as a covariate. Post hoc LSD comparisons were used to follow up significant main effects or interaction effects. All *P* values underwent FDR correction.

Regarding the relationship of peripheral TLRs to cingulate cortical thickness, for TLRs with a significant diagnostic group differences, stepwise linear regressions were performed with cortical thickness within each region of interest (rostral anterior cingulate, dorsal anterior cingulate, posterior cingulate, and isthmus cingulate cortex) as dependent variables and TLRs (mRNA levels log transformed) as independent variables, for people with schizophrenia and HC, separately.

Significant results ($P < .05$, two-tailed) are reported both, uncorrected and false discovery rate (FDR) corrected for multiple testing.

Results

Cohort Description

As previously reported, people with schizophrenia had a significantly higher NLR, elevated CRP levels and higher

IL1β and IFN-γ mRNA levels.[12] While NF-κB mRNA did not differ across diagnostic groups, significantly higher complement mRNAs (C5ar, CR1, ITGAM/C3Ra) and significantly higher C3 protein levels were found in patients as compared to controls as reported previously.[7,11] Here, we found significantly reduced thickness of all sections of the cingulate cortex (rostral anterior, caudal anterior, posterior, and isthmus cingulate) and a lower performance in working memory and language domains in people with schizophrenia relative to HC. The group of people with schizophrenia was also significantly older compared to HC, see table 1.

Associations Between TLRs and Demographic Factors

No significant correlations were detected between TLR mRNA levels and age, BMI, interleukin levels, CRP, age of onset, duration of illness, or antipsychotic dose (chlorpromazine equivalents). Significantly higher TLR3 mRNA levels were detected in smokers as compared to non-smokers in people with schizophrenia. Further information is provided in Supplementary table S1.

Associations of TLR Levels and the Complement System and Other Inflammatory Measures

Overall, we found consistent correlations between all TLR mRNAs and complement factor mRNAs in people with schizophrenia and in HC. In HC, TLR 1 mRNA was significantly positively correlated with CR1 mRNA and C3b protein levels, while TLR 4 and TLR8 mRNAs were significantly positively correlated with all complement transcripts (C5AR, CR1, and ITGAM/C3Ra) mRNAs. In people with schizophrenia, TLR1, TLR4, and TLR8 mRNAs were significantly positively correlated with all complement transcripts (C5AR, CR1, and ITGAM/C3Ra). Specially, we discovered a strong relationship between TLR4 and CR1 mRNAs, where the level of TLR4 mRNA predicted ~50% of the variability in CR1 mRNA (table 2). In contrast, TLR3 mRNA was significantly inversely correlated with all complement transcripts (C5AR, CR1, and ITGAM/C3Ra) mRNAs.

Significant associations between TLR mRNAs and cytokine mRNA were also found. In HC, TLR1, TLR4, and TLR8 mRNAs all significantly positively correlated with IL1β mRNA levels (table 2). TLR4 mRNA correlated with IL-6 mRNA and TLR3 mRNA strongly and negatively correlated with IFN-γ mRNA levels. Similar to controls, TLR1, TLR4, and TLR8 mRNAs all significantly positively correlated with IL1β mRNA levels in people with schizophrenia. But in contrast to controls, TLR4 mRNA did not correlate with IL6 mRNA levels in people with schizophrenia. In schizophrenia, IFN-γ mRNA levels correlated with not just one, but 3 TLR mRNAs, TLR1, TLR3, and TLR4 mRNAs, but not TLR8 mRNA.

Interestingly, most of the TLR mRNAs correlated positively with our immune cell marker of inflammation (*N*/*L* ratio) in both HC and people with schizophrenia (table 2). The one exception was TLR3 mRNA which showed a strong negative correlation to *N*/*L* ratio in people with schizophrenia and no significant correlation in controls. We did not detect any correlations between TLR mRNAs and cortisol levels.

Further information on associations between TLRs and other inflammatory markers are shown in table 2 and in the Supplementary material.

Diagnostic Group Differences

We found significantly lower TLR3 mRNA levels in people with schizophrenia as compared to HC [$F_{(1,160)} = 13.9$, $P < .001$ (uncorrected), $P < .001$ (FDR corrected), figure 1A]. Further, we found significantly higher TLR4 mRNA levels in people with schizophrenia as compared to HC [$F_{(1,\ 160)} = 8.10$, $P = .005$ (uncorrected), $P < .01$ (FDR corrected), (figure 1B)] and significantly higher TLR8 mRNA levels in schizophrenia as compared to HC [$F_{(1,160)} = 20.4$, $P < .001$ (uncorrected), $P < .001$ (FDR corrected), figure 1C]. We did not detect a significant diagnostic difference in TLR1 mRA levels between people with schizophrenia and HC [$F_{(1,\ 160)} = 0.611$, $P = .436$ (figure 1D)]. Including sex as a covariate did not influence results (TLR3 $F_{(1,159)} = 15.6$, $P < .001$, TLR4 $F_{(1,159)} = 8.7$, $P = .004$, TLR8 $F_{(1,159)} = 22.2$, $P < .001$, TLR1 $F_{(1,159)} = 0.8$, $P = .38$).

Inflammatory Subgroups

A chi-square test of independence was performed to examine the relation between diagnosis (control/schizophrenia) and complement mRNA subgroup. The relation between these variables was significant, X2 (1, $n = 157$) = 12.0, $P < .001$, where there was a significantly higher proportion of individuals with schizophrenia in the high complement mRNA subgroup compared to controls (controls high: $n = 19$, controls normal: $n = 56$, schizophrenia high: $n = 43$, schizophrenia normal: $n = 39$).

TLR1 mRNA by Diagnostic/Inflammatory Subgroup

The mean level of TLR1 mRNA was highest in the high inflammation schizophrenia subgroup. When separated into inflammatory subgroups, a non-significant difference for TLR1 mRNA levels was detected among the 4 groups [$F_{(3,\ 156)} = 2.37$, $P = .055$ (uncorrected), $P > .05$ FDR (corrected)]. We found significantly higher TLR1 in people with schizophrenia in the high complement group as compared to HC in the normal complement group ($P = .027$) and compared to people with schizophrenia in the normal complement group ($P = .010$), (figure 2A).

Table 2. Correlations Between TLR mRNAs and Other Immunological Markers

	nfkb2	nfkb1	CRP	C5AR	CR1	ITGAM	CR3	C3b	C4	IL1B	IL6	IFNy	NL ratio	Cortisol
HC														
TLR1														
Pearson *R*	.17	−.09	.05	.18	.389**	.02	.04	.279*	.22	.258*	−.07	−.02	.485*	−.19
P-value	.14	.44	.70	.13	.00	.86	.75	.04	.10	.02	.54	.87	.02	.11
n	74	74	65	75	77	77	56	57	57	77	77	77	24	74
TLR3														
Pearson *R*	−.06	−.18	−.18	−.15	−.05	−.17	−.02	−.13	−.05	−.13	.13	−.594**	−.21	−.15
P-value	.62	.13	.15	.19	.69	.13	.90	.33	.69	.24	.27	.00	.32	.20
n	74	74	65	75	77	77	56	57	57	77	77	77	24	74
TLR4														
Pearson *R*	.10	−.01	.23	.323**	.564**	.300**	.02	.14	.23	.390**	−.231*	−.13	.576**	−.19
P-value	.42	.95	.06	.00	.00	.01	.86	.29	.08	.00	.04	.27	.00	.10
n	74	74	65	75	77	77	56	57	57	77	77	77	24	74
TLR8														
Pearson *R*	.14	−.01	.07	.415**	.383**	.379**	.01	−.04	.11	.413**	−.05	.19	.435*	−.15
P-value	.23	.97	.61	.00	.00	.00	.96	.77	.42	.00	.68	.09	.03	.22
n	74	74	65	75	77	77	56	57	57	77	77	77	24	74
SZ														
TLR1														
Pearson *R*	.07	−.18	.12	.281*	.470**	.248*	.10	.15	.17	.278**	.14	.298**	.234*	.07
P-value	.51	.10	.32	.01	.00	.02	.41	.22	.16	.01	.20	.01	.03	.52
n	81	81	75	82	85	86	68	68	68	86	86	86	82	86
TLR3														
Pearson *R*	.11	−.09	−.01	−.357**	−.317**	−.268*	−.23	−.15	−.09	−.17	.18	−.500**	−.572**	.00
P-value	.33	.43	.94	.00	.00	.01	.06	.21	.47	.11	.10	.00	.00	1.00
n	81	81	75	82	85	86	68	68	68	86	86	86	82	86
TLR4														
Pearson *R*	.04	−.19	.10	.481**	.710**	.484**	.19	.22	.13	.386**	.00	.314**	.509**	.21
P-value	.72	.08	.41	.00	.00	.00	.13	.07	.30	.00	.98	.00	.00	.05
n	81	81	75	82	85	86	68	68	68	86	86	86	82	86
TLR8														
Pearson *R*	−.13	−.04	.16	.230*	.228*	.320**	.05	.15	.16	.235*	.08	.12	.309**	.05
P-value	.24	.75	.18	.04	.04	.00	.69	.22	.21	.03	.46	.28	.00	.63
n	81	81	75	82	85	86	68	68	68	86	86	86	82	86

Note: Pearson *R* correlations between TLR mRNA and immunological factors in healthy controls (HC) and people with schizophrenia (SZ).
NF-κB1/2, kappa-light-chain-enhancer' of activated B-cells (mRNA, log transformed); CRP, c-reactive protein; C5ar, complement receptor 5 a (mRNA log transformed); CR1, complement receptor type 1 (mRNA, log transformed); ITGAM/C3a, integrin alpha M/complement factor 3 receptor alpha (mRNA log transformed); C3/C3b/C4 Prot., complement component 3/3b/4 protein; IL1 β, interleukin 1 beta; IL6, interleukin 6; IFN-γ, interferon gamma; NLR, neutrophil/lymphocyte ratio, cortisol in nmol/l.
*$P < .05$ level,
**$P < .01$ (2-tailed).

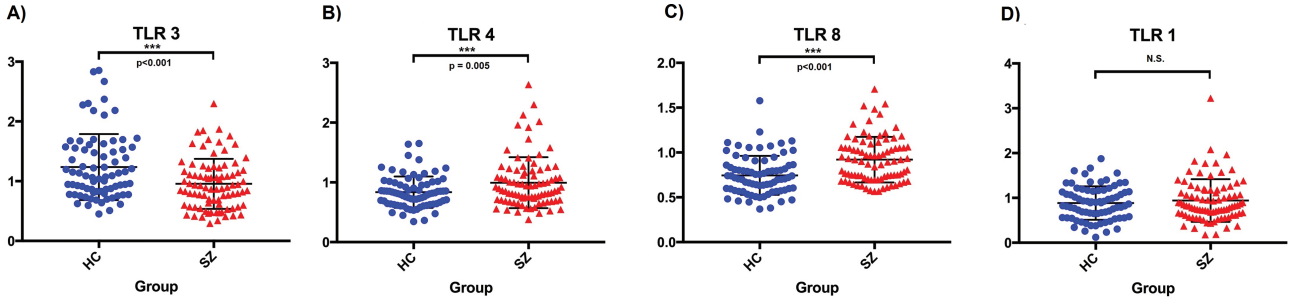

Fig. 1. TLR mRNA levels in healthy controls (HC) and patients with schizophrenia (SZ). (A) Significantly lower TLR3 mRNA levels were detected in SZ as compared to HC. (B) Significantly higher TLR4 mRNA levels were detected in SZ as compared to HC. (C) Significantly higher TLR8 mRNA levels were detected in SZ as compared to HC. (D) No significant differences in TLR1 mRNA levels were detected between SZ and HC. n.s., not significant; TLR, toll-like receptor level, ***significant at $P \leq .005$, two-sided.

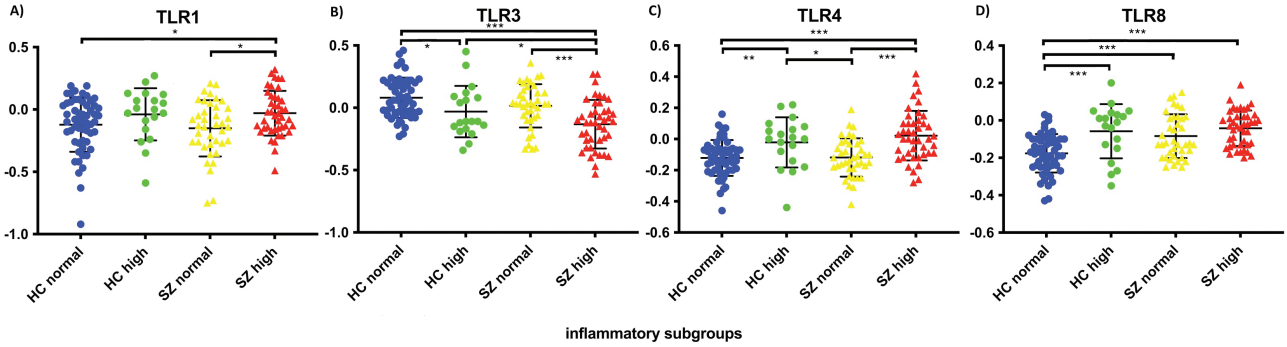

Fig. 2. Inflammatory subgroups and TLR mRNA levels in people with schizophrenia (SZ) and healthy controls (HC). Inflammatory subgroups were based on complement C5AR1, CR1, ITGAM/CR3a mRNA expression. The 2 subgroups which we entitled high and normal based on the expression pattern, was characterized such that the high complement mRNA subgroup had a signficantly higher average expression across all 3 mRNAs compared to the normal complement mRNA subgroup. TLR mRNA levels were log transformed. Significant pairwise differences of TLR1 (A), TLR3 (B), TLR4 (C) and TLR8 (D) mRNA levels are marked with * indicating $P < .05$, ** $P < .01$, *** $P < .001$.

TLR3 mRNA by Diagnostic/Inflammatory Subgroup

The mean level of TLR3 mRNA was lowest in the high inflammation schizophrenia subgroup. A significant difference among the 4 groups was detected for TLR3 mRNA: [$F_{(3,\ 156)} = 8.65$, $P < .001$ (uncorrected), $P < .05$ (FDR corrected)]. We found lower TLR3 transcript levels in the people with schizophrenia in the high complement group compared to all of the 3 other groups: compared to HC in the normal complement group ($P < .001$); compared to HC in the high complement group ($P = .047$) and also people with schizophrenia in the normal complement group ($P < .001$). Additionally, the levels of TLR3 mRNA in HC in the high complement group were lower than HC in the normal complement group ($P = .022$), even though this high complement group of HC still had higher TLR mRNA levels as compared to high complement schizophrenia group.

TLR4 mRNA by Diagnostic/Inflammatory Subgroup

The mean level of TLR4 mRNA was highest in the high inflammation schizophrenia subgroup. A significant difference for TLR4 mRNA was detected among the 4 groups: [$F_{(3,\ 156)} = 9.00$, $P < .001$ (uncorrected), $P < .05$ (FDR corrected)]. We found higher TLR4 mRNA in people with schizophrenia in the high complement group as compared both normal complement groups [HC, ($P < .001$) and people with schizophrenia ($P < .001$, figure 2C)]. Additionally, the HC in the high complement group had higher TLR4 mRNA as compared to both normal complement groups [HC, ($P = .007$, figure 2C) and people with schizophrenia ($P = .018$)].

TLR8 mRNA by Diagnostic/Inflammatory Subgroup

The mean level of TLR8 mRNA was highest in the high inflammation schizophrenia subgroup. A significant difference for TLR8 mRNA was detected among the 4 groups: [$F_{(3,\ 156)} = 11.67$, $P < .001$ uncorrected, $P < .01$ FDR (corrected)]. We found significantly higher TLR8 mRNA in people with schizophrenia in both the normal complement group ($P < .001$) and the high complement group ($P < .001$) relative to HC in the normal complement group, (figure 2D). TLR8 mRNA was also significantly higher in HC in the high complement group relative to HC in the normal complement group ($P < .001$).

Further information on demography, clinical and immunological features, and cortical thickness separated for inflammatory subgroups is provided in Supplementary table S2.

Bacterial 16S rRNA

There was no significant difference between levels of genomic bacterial 16S rRNA between people with schizophrenia vs HC ($P = .31$) and no differences in genomic bacterial 16S rRNA amongst subgroups when investigating high vs low complement subgroups ($F_{3,131} = 0.85$, $P = .47$). While no significant correlations between 16S rRNA and IL1b, CRP, NL ratio, or NF-κB were detected, a negative correlation with IFN-γ mRNA and 16S rRNA was found in HC only ($R = -.37$, $P = .001$, $n = 74$). Importantly, 16S rRNA showed a significant positive correlation with CR3a/ITGAM mRNA in the total sample ($R = .223$, $P = .005$, $n = 154$), and in people with schizophrenia ($R = .25$, $P = .03$, $n = 74$) and HC ($R = .26$, $P = .02$, $n = 80$), separately. Further information is provided in Supplementary table S3.

Gray Matter Thickness and TLR mRNA Levels in People with Schizophrenia and HC

In people with schizophrenia, stepwise linear regression yielded a significant negative association between caudal anterior cingulate thickness and TLR8 mRNA levels

($F_{1,50}$ = 7.2, P = .01, beta = −.36, t = −2.7, figures 3 and 4A), between posterior cingulate thickness and TLR8 mRNA levels ($F_{1,50}$ = 5.4, P = .024, beta = −.32, t = −2.3, figures 3 and 4B) and between isthmus cingulate thickness and TLR8 mRNA levels ($F_{1,50}$ = 5.3, P = .025, beta = −.31, t = −2.3, figures 3 and 4C). In HC, a separate stepwise linear regression yielded a significant negative association between caudal anterior cingulate thickness and TLR8 mRNA levels ($F_{1,56}$ = 4.97, beta = −.29, P = .03, figures 3 and 4D) and between the rostral anterior cingulate thickness and TLR8 and TLR4 mRNA levels ($F_{2,56}$ = 8.83 P < .001; TLR8 beta = −.37, t = −3.1, P = .003, figure 4E; TLR 4 beta = −.31, t = −2.6, P = .01, figures 3 and 4F).

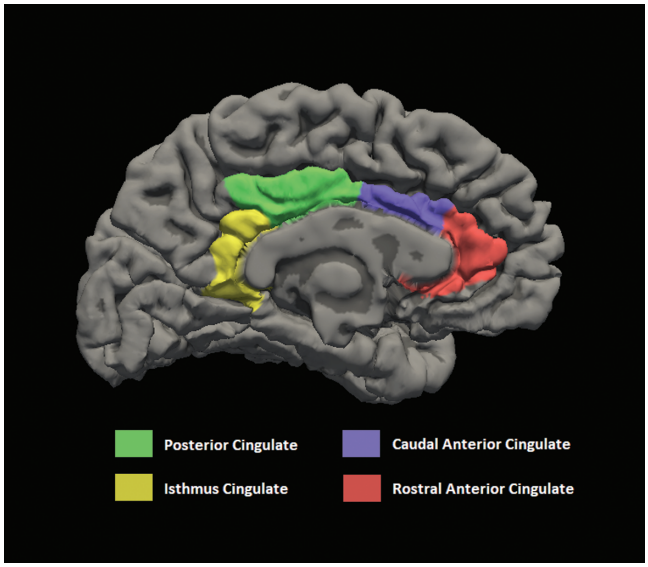

Fig. 3. Cingulate cortex (gyrus cinguli) separated into 4 regions of interest: Rostral Anterior Cingulate, Caudal Anterior Cingulate, Posterior Cingulate, and Isthmus Cingulate cortex, according to the Desikan-Killiany atlas.

The negative association between TLR8 mRNA and caudal anterior cingulate thickness in people with schizophrenia and between TLR8 and TLR4 mRNA and rostral anterior cingulate cortex thickness in HC survived FDR correction for multiple comparisons (P < .05). When separated for inflammatory subgroups, significant correlations between rostral anterior cingulate cortex thickness and TLR8 mRNA in high complement HC subgroup (P = .004, R = −.684, n = 16) were found. In high complement schizophrenia subgroup, we detected significant correlations between TLR8 mRNA and isthmus cingulate cortex (P = .03, R = −.46, n = 22), posterior cingulate (P = .03, R = −.45, n = 22), and a trend with caudal anterior cortex thickness (P = .058, R = −.41, n = 22).

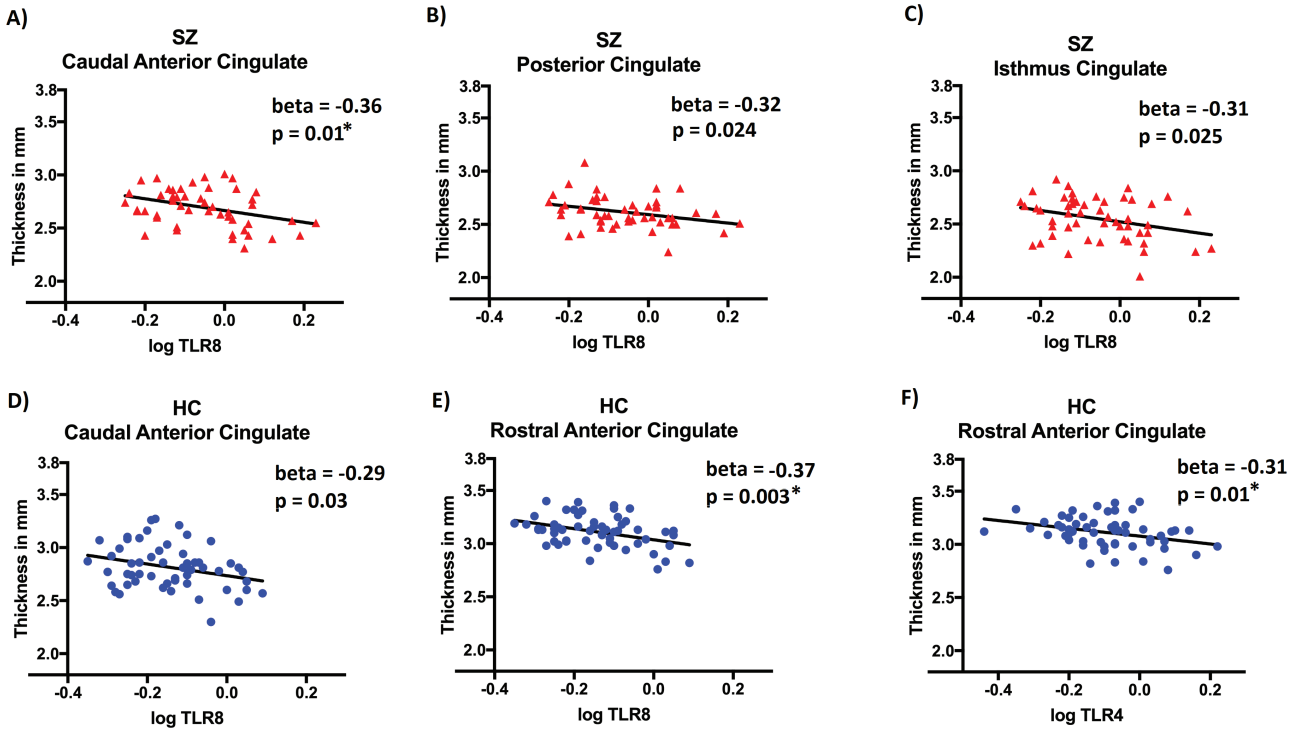

Fig. 4. Associations of gray matter cingulate cortex thickness and TLR4 and TLR8 mRNA levels in people with schizophrenia (SZ) and healthy controls (HC): In SZ a significant negative association was detected between TLR8 levels and the caudal anterior cingulate cortex (A, P = .01), between TLR8 levels and the posterior cingulate cortex (B, P < .05) and between TLR8 levels and the isthmus cingulate (C, P < .05). In HC, a significant negative association was detected between TLR8 levels and the caudal anterior cingulate cortex thickness (D, P < .05), between TLR8 levels and the rostral anterior cingulate (E, P < .005) and between TLR4 levels and the rostral anterior cingulate cortex (F, P = .01). Cortical thickness in mm. TLR (toll-like receptor) mRNA levels, log transformed. *Significant at P < .05, FDR corrected.

No other significant associations between cortical thickness and TLR mRNA levels were detected in HC or people with schizophrenia (all *P*'s > .05).

Discussion

In the white blood cells of people with schizophrenia, we find seemingly opposing mRNA changes in the innate immune receptors for microogranisms. In particular, 2 peripheral toll-like receptor transcripts encoding proteins integral to bacterial defense are increased in people with schizophrenia (TLR4, TLR8), whereas one transcript encoding a key toll-like receptor involved in sensing viral RNA (TLR3) is decreased in people with schizophrenia.

Our finding of significantly increased TLR4 mRNA in the peripheral blood cells of people with schizophrenia compared to HC is consistent with previous work showing increased TLR4 positive immune cells in people with schizophrenia.[20,21] Further, these results are in line with evidence showing increased TLR4 mRNA expression in post mortem brain samples[18] and previously reported increased TLR4 protein in monocytes in living people with schizophrenia.[20,21] Our results are compatible with the notion of TLR4 induced by bacterial antigens.[8,54] Another endogenous receptor for bacteria, TLR1 mRNA was not found to differ at the diagnostic group level. However, TLR1 was elevated in the high complement expression schizophrenia subgroup. In support of these findings, there is accumulating evidence that the gut microbiome may contribute to the pathogenesis of schizophrenia.[55–57] While TLR4 is specific to Gram-negative bacteria, such as gut enterobacteria, TLR1 recognizes Gram-positive bacteria,[8] suggesting that the host defense against Gram-negative bacteria may be more active in schizophrenia as compared to the defense against Gram-postitive bacteria. In fact, the gastrointestinal tract harbors large numbers of diverse Gram-negative enterobacteria, comprising a large part of the gut microbiome of an individual, and Gram-negative enterobacteria can impact brain structure and function via the gut–brain axis.[56] As primary receptors for enterobacterial LPS, TLR4 has been shown to interact with the gut microbiome[58] and thus, can modify the gut–brain axis. Importantly, it has been demonstrated that TLR4 regulates gut barrier permeability and function.[59] Thus, increased TLR4 activity[17] might lead to, or be caused by, a "leaky gut."[60] Although there is mixed evidence for "leaky gut" syndrome in people with schizophrenia, some work suggests bacterial translocation and thus, a passage of bacteria from the gastrointestinal tract to extraintestinal locations, such as lymphatic nodes, the blood and, eventually, to the central nervous system.[54] If these gut bacteria reach the brain they may be responsible for the upregulation of TLR4 proteins found in the brains of people with schizophrenia.[18] However, in addition to a leaky gut also other putative sources of LPS such as the oral microbiome may stimulate TLR4 expression and could therefore be responsible for our findings.[61]

Further, in our relatively large sample, we find decreased peripheral TLR3 mRNA levels in people with schizophrenia. Previous studies indicate negative feedback loops between TLR3 and TLR4 within the brain.[62] Thus, we speculate that TLR3 mRNA downregulation is compensatory, caused by an overproduction of bacterial TLR activity. Interestingly, there is evidence that higher TLR3 in astrocytes has protective effects (eg, in cases of ischemic brain damage).[63] Thus, lower TLR3 levels may indicate less protection against viral infection. Our finding of lower peripheral TLR3 mRNA levels in people with schizophrenia is in contrast to studies of rodent models of schizophrenia that use poly(I:C) to mimic maternal immune activation that show an increase of TLR3.[25,64] In general, evidence of TLR3 alterations within the brains of people with schizophrenia is still lacking and should be investigated in future studies.

We found higher TLR8 mRNA levels in people with schizophrenia. In addition to recognizing single strand viral RNA, TLR8 is also important in the recognition of bacteria such as *Staphylococcus aures*, *Pseudomonas aeruginosa*, and *Escherichia coli* which can be found in the human colon and thus are a part of the gut microbiome.[12] Overall, the downregulation of viral TLRs concurrently with increased bacterial TLRs indicates that the innate immune system is perhaps less poised to respond to viral infection, yet more responsive to bacterial infections in schizophrenia.

While bacterial DNA encoding the 16s rRNA was found in all samples of human blood, both in HC and people with schizophrenia, no significant differences in levels were detected between groups. This might indicate that there is no difference in total bacterial levels in plasma between patients and controls, but it does not provide information about the levels of pathogenic vs commensal bacteria. Importantly, none of the included subjects had a known bacterial infection at the time of the study. Moreover, and contrary to our expectations, 16s rRNA did not correlate with CRP levels or proinflammatory cytokines in patients or in controls, suggesting either that this mRNA may not represent true bacteria, or that these putative bacteria are sufficiently low in number or not the type to trigger a generalized immune response. Thus, 16s rRNA as a measure of global bacterial concentration in blood may be insufficiently specific to differentiate between people with schizophrenia and controls. However, it is possible that specific bacteria could trigger the innate immune system and this could be reflected by changes in complement mRNAs. In support of the latter, we found significantly higher levels of complement receptor 3 mRNA (ITGAM/CR3a)[11] in patients, a receptor known to be central for host defense against bacteria. Further, we show for the first time that bacterial 16s rRNA and ITGAM/CR3a mRNA levels were significantly positively correlated in both patients and controls. Interestingly, ITGAM/CR3a mRNA levels were linked to all 4 TLR

mRNA levels in schizophrenia, suggesting that bacteria levels, complement receptor, and TLRs expression are related in this disease state. While there is no difference in plasma total bacterial load on a diagnostic level, differences in the immunological response to bacterial antigens within the innate immune system pathway might contribute to the pathophysiology of schizophrenia. Thus, while it is still speculative, we believe our data support that there may be a bacterial influence in the pathogenesis of schizophrenia. However, we cannot rule out that viral pathogens could also change TLRs mRNA levels in schizophrenia. The TLR8 mRNA increase we find could be linked to viruses and we do find a significant down regulation of TLR3 mRNA that negatively correlates with IFN-γ levels, both of which are involved in responses to viruses. Future studies will have to continue to attempt to separate bacterial compared to viral activation of immunological pathways to support or refute if either of these or other microorganisms can play a role in schizophrenia.

Previous studies have indicated that only a subset of people with schizophrenia show mRNA increases in proinflammatory cytokines in white blood cells.[37] Defining a high inflammatory biotype of schizophrenia may help to identify immunological mechanisms that contribute to the pathophysiology of some forms of schizophrenia and to identify individuals with schizophrenia who could benefit from anti-inflammatory therapies.[2,65] Focusing on this group of people with schizophrenia could help us to zero in on changes restricted to an active pathological state. Here, for the first time a high inflammatory biotype based on complement factors has been defined.

In addition to correlations between TLRs with proinflammatory interleukins that have previously been described,[51] we show for the first time significant associations between TLRs and complemental factors in people with schizophrenia and/or HC. While these associations suggest a coordinated immune response of these separated key components of the innate immune system they could also indicate shared activation triggers. In schizophrenia, correlating levels of TLR and complement factors might point toward a shared role in disease pathophysiology, for example, a joint pathological influence contributing to inflammatory processes within the brain. Further studies will have to concurrently investigate both components of the innate immune system and whether there are meaningful differences between people with schizophrenia and HC.

Our findings demonstrate that TLR mRNA levels may be partially explained by increased complement mRNA expression, and that for some TLRs this relationship may differ between schizophrenia and controls. Specifically, our finding of increased TLRs mRNAs in high inflammatory groups (TLR1, TLR4, TLR8 in schizophrenia, and TLR4 and TLR8 in controls) may reflect the capacity of complement and TLRs to coordinate immune and inflammatory responses through synergistic interactions. On the other hand, interestingly and unlike the other TLRs, TLR3 was significantly lower in high inflammatory groups (both SZ and control) as compared to the normal inflammatory groups, possibly reflecting antagonistic regulatory interactions. Interestingly, TLR3 mRNA was even more suppressed in high inflammation schizophrenia than expected. This finding was the only TLR mRNA to discriminate between controls and schizophrenia groups under conditions of heightened inflammation. The significance of this decrease is unknown, but it may suggest a weakened response to viral pathogens. Interestingly, when monocytes from people with schizophrenia are stimulated with a viral mimic, they show less TLR3 and lower IL-1b as compared to controls.[8] Complement-TLR relationship differences between schizophrenia and HC within inflammatory subgroups may be related to a number of factors including the co-regulation of TLRs and complement receptors, the receptors cellular location (cell surface vs endosomal compartments within cells), expression level within immune cell types and ligand specificity. Since our understanding of the immunology of schizophrenia is continually evolving, and our findings suggesting that the overall relationship between TLRs and the complement system is strong yet, complex; more experiments aiming to tease out the specific interactions between microbes and immune cells and immune cells and the brain in schizophrenia, particular including TLRs and complement components are warranted.

We found that TLR4 and 8 mRNA levels were inversely related to cingulate cortex thickness in people with schizophrenia and HC. The strongest effects that survived correction for multiple comparisons were detected for TLR8 and the anterior cingulate cortex (anterior and caudal rostral), and being most consistent in HC and people with schizophrenia. The maternal immune activation hypothesis of schizophrenia suggests that an early immunological hit negatively affects brain development.[66] Consequently, brain structural abnormalities related to immunological factors would already be evident before the emergence of clinical psychotic symptoms. Reduced cingulate cortex thickness is amongst the most robust findings in people at clinical high risk for psychosis, and predicts conversion to psychosis.[67] Indeed previous studies have shown that the anterior cingulate is particularly vulnerable to immunological hits.[46,48] However, several studies have demonstrated that the prefrontal cortex is another cortical area affected by immunological activation in schizophrenia[2,36,46,68] and our study did not investigate involvement of the prefrontal cortex.

Importantly, the mechanisms by which inflammation can cause cortical volume loss remain undetermined. It may be that increased TLR mRNAs in blood directly correlates with TLR mRNA levels in the cerebral cortex and since increased brain TRL4 has been implicated in glutamatergic excitotoxicity due to NMDA receptor activation.[69] In support of this, TLR4 mRNA is increased in the

prefrontal cortex of people with schizophrenia compared to HC.[18] Additionally, peripheral TLR activation during pregnancy inhibits neurogenesis and disrupts normal neurodevelopment in the offspring.[70] While these last experimental findings are from rodent studies, our investigation is the first to show an association between TLRs and cortical thickness in humans. As mentioned previously, TLR8 has been shown to inhibit neuronal growth and induce apoptosis in rodents,[31] which could explain the negative relation between TLR8 mRNA levels and cingulate gray matter thickness. Interestingly, negative relationships between TLR levels and gray matter thickness were also detected in HC, which suggests similar relationships between immune activation and cingulate brain structure in people with schizophrenia and HC. Thus, the effects of increased TLR4 and TLR8 mRNA on cingulate cortical thickness seem to be independent of psychiatric diagnosis but rather related to immune activation itself. However, people with schizophrenia more often show immune activation and higher levels of proinflammatory markers.[37] Hence, while TLR4 and TLR8 activation negatively impacts cortical thickness in both people with schizophrenia and HC, the detrimental effect appears to be more common in schizophrenia.

While our study measured TLRs in the peripheral blood, it is unknown how this may relate to TLR signaling in the brain. TLRs are expressed in various tissues throughout the body and the expression patterns of TLRs can differ between tissues (eg, peripheral immune cells vs microglia). In brain, higher TLR4 protein levels, along with no change in TLR4 mRNA has been found in the prefrontal cortex of people with schizophrenia.[5,6] Thus, although we found significant differences in TLR mRNAs in peripheral blood in people with schizophrenia, we cannot conclude that elevated TLR is a direct reflection of neuroinflammation as it could suggest dysregulation of a systemic immune response.

Indeed, Muller et al[21] found increased TLR4 protein on the surface of monocytes derived from the blood of people with schizophrenia, however, these same monocytes appeared less responsive to a bacterial mimic suggesting an overall blunting of immune response related to elevated chronic inflammation. In contrast to our findings of reduced TLR3 mRNA, Muller et al[21] found increased TLR3 protein on monocytes, suggesting a discrepancy between transcript levels and protein abundance or that the reduction in TLR3 we find schizophrenia may involve other types of immune cells.

Previous studies have shown an influence of antipsychotics on TLR levels.[20] However, in our study there were no significant correlations between TLR mRNAs and doses of antipsychotic medication. This is consistent with one previous study investigating people with schizophrenia before and after starting treatment with antipsychotics and finding no significant effect of antipsychotics on TLR gene expression levels.[27] The innate and adaptive immune systems interact and are influenced by hormones and lifestyles such as nutrition or smoking. We did not have BMI and smoking data on most of the HC group so were not able to undertake between-group comparisons to explore the potential impact of these factors in our analysis, which is a significant limitation of this study. While previous studies have indicated an association between immune activation and obesity[71,72] in the present study, TLR mRNA levels were not associated with BMI in people with schizophrenia (Supplementary table S1), suggesting that body fat did not contribute to our findings. Further, smoking may affect inflammatory status. Here, in people with schizophrenia, smoking status did not appear to influence on TLR 1, 4 and 8 mRNA levels, whereas smokers had increased TLR3 mRNA levels. The latter finding is consistent with another study showing increased TLR3 expression in smokers.[73]

Importantly, our study is preliminary and replication in larger samples is critical. Furthermore, we emphasize that it is not yet clear which inflammatory transcripts should be used when attempting to classify people with schizophrenia into a normative/low compared to an elevated/high inflammatory status. Here we have used complement mRNAs to stratify patients. Future, longitudinal studies will be needed to demonstrate whether TLR mRNA deviations remain stable or fluctuate over time in order to differentiate whether the gray matter volume reductions are due to single immunological hits or a tonically activated immunological system. Longitudinal studies will have to explore whether immunoactivation induces gray matter volume reduction via developmental changes or by causing gray matter loss due to degenerative/neurotoxic processes at maturation or in adulthood.

In summary, our data suggests that the bacterial arm of the innate immune system could be activated in people with schizophrenia. TLR4 and TLR8, which are important for the recognition of Gram-positive and Gram-negative bacteria, were elevated in people with schizophrenia. These findings were most pronounced in schizophrenia patients with high expression of complemental factors. Gram-negative bacteria are harbored by the gastrointestinal tract and thus are part of the gut microbiome. Finally, our study links specific TLRs and brain cortical thickness reductions of limbic brain structures for the first time, suggesting that the cingulate cortex might be particularly susceptible for immunological hits in schizophrenia.

Supplementary Material

Supplementary material is available at https://academic.oup.com/schizophreniabulletin/.

Acknowledgments

We would like to thank Loretta Moore, Nicholas Vella, Merribel Kyaw, Selena Hu, Richard Morris, Daniel

Pellen, Jackie Curtis, Beverly Hisee, Stan Catts and Alice Rothwell for assistance with recruitment and administration and scoring of neuropsychological, symptom and/or medical assessments. We thank Hayley North for making the CRP measurements.

Funding

This work was supported by the University of New South Wales School of Psychiatry; National Health and Medical Research Council Project Grant no. 568807; Neuroscience Research Australia; the Schizophrenia Research Institute (utilizing infrastructure funding from the New South Wales Ministry of Health and the Macquarie Group Foundation); and the Australian Schizophrenia Research Bank, which is supported by the National Health and Medical Research Council of Australia; the Pratt Foundation; Ramsay Health Care; and the Viertal Charitable Foundation. CSW is a recipient of a National Health and Medical Research Council (Australia) Senior Research Fellowship (#1021970). JK was supported by the Swiss National fund (SSMBS, Advanced Postdoc Mobility, No. P3SMP3 148381). GJG is a recipient of an Australian Research Council Future Fellowship and CKL was a recipient of the Society of Mental Health Research Early Career Fellowship and the Ramaciotti Perpetual Foundation. The authors report no competing financial interests or potential conflicts of interest relevant to this project.

Conflict of interest

The authors declare no conflict of interest.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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