A study of TNF-pathway activation in schizophrenia and bipolar disorder in plasma and brain tissue

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Abstract

Objective: A pro-inflammatory imbalance in the tumor necrosis factor (TNF) system may contribute to the pathogenesis of schizophrenia (SCZ) and bipolar disorders (BD) and related comorbidities. We investigated the relative distribution of TNF related molecules in blood and dorsolateral prefrontal cortex (DLPFC) in these disorders.

Method: We measured plasma levels of TNF, soluble TNF receptor 1 (sTNFR1), soluble TNF receptor 2 (sTNFR2) and A Disintegrin And Metalloprotease-17 (ADAM17) using enzyme immunoassays, and calculated the TNF/sTNFRs ratio (TNF/sTNFR1+sTNFR2) in a sample of 816 SCZ and BD spectrum patients and 624 healthy controls (HC). *TNF*, *TNFRSF1A (TNFR1)*, *TNFRSF1B (TNFR2)* and *ADAM17* mRNA levels were determined in whole blood, and post-mortem DLPFC obtained from an independent cohort (n=80 SCZ, n=44 BD, and n=86 HC).

Results: In peripheral blood, we show increased TNF-related measures in patients compared to HC, with an increased TNF/sTNFRs ratio ($p = 6.00x10^{-5}$), but decreased *TNF* mRNA expression ($p=1x10^{-4}$), with no differences between SCZ and BD. Whole blood *ADAM17* mRNA expression was markedly higher in BD *vs*. SCZ patients ($p=1.40x10^{-14}$) and *vs*. HC ($p=1.22x10^{-8}$). In post-mortem DLPFC, we found no significant differences in mRNA expression of TNF pathway genes between any groups.

Conclusions: SCZ and BD patients have increased plasma TNF pathway markers without corresponding increase in blood cell gene expression. *ADAM17* expression in leukocytes is markedly different between the two disorders, while alterations in TNF related gene expression in DLPFC is uncertain. Further studies are necessary to elucidate the aberrant regulation of the TNF pathway in severe mental disorders.

Key words: DLPFC; cytokines; working memory; mRNA; post-mortem.

Introduction

A dysregulation of the immune system due to chronically activated macrophages and T-cells has been proposed to contribute to the pathogenesis of schizophrenia (SCZ)[1] and bipolar disorder (BD)[2]. Several lines of evidence support a pro-inflammatory profile in both diseases, where alterations in the tumor necrosis factor (TNF)-pathway, consisting of soluble and membrane bound TNF (formerly TNF-alpha) and its two receptors, have been reported in peripheral blood[2,3]. However, the regulation and the site of production of TNF related molecules in these disorders are far from clear.

TNF is a pro-inflammatory cytokine expressed by macrophages, other leukocyte subsets and endothelial cells[4-6], as well as by neurons, astrocytes and microglia that have a macrophage phenotype. It signals through two distinct membrane-bound receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) of which TNFR1 is widely distributed in a range of cells and tissues whereas TNFR2 is more selectively expressed by immune cells, endothelial cells, specific neuronal subtypes and glia cells[7]. Under physiological conditions TNF has been known to regulate synaptic transmission, neurotransmission, homeostatic synaptic scaling, neurogenesis and long term potentiation[8-10]. TNF and its receptors are produced as transmembrane proteins with an extracellular domain that can be proteolytically cleaved from the cell surface by metalloproteases like A Disintegrin And Metalloprotease-17 (ADAM17) resulting in soluble TNF, soluble (s)TNFR1 and sTNFR2. Increased circulating TNF, sTNFR1 and sTNFR2 levels have been reported by several studies in SCZ[3] and BD[2,11], but to this end, no large studies have evaluated if this leads to a pro-inflammatory imbalance between TNF and its soluble receptors, i.e., the TNF/sTNFRs ratio that reflects activity in the TNF system and correlates with TNF bioactivity[12].

Previously, modestly sized studies have demonstrated increased *TNF* mRNA expression in monocytes and lymphocytes in SCZ and BD[13-15]. To our knowledge, however, *TNF*, *TNF receptor superfamily [TNFRSF] 1A and TNFRSF1B* (denoted by *TNFR1* and *TNFR2*, respectively, in the

present manuscript), and *ADAM17* mRNA expression in whole blood cells have not been investigated in a well-powered sample of patients with severe mental disorders.

The dorsolateral prefrontal cortex (DLPFC) is associated with a range of complex behaviors frequently referred to as executive functions, including working memory[16], cognitive and behavioral flexibility, and abstract reasoning, all of which have been implicated in SCZ and to a lesser extent in BD[17-19]. We have previously found that general cognitive abilities were negatively associated with plasma TNFR1 levels in adults with SCZ and BD[20]. Moreover, a post-mortem study found elevated levels of *TNFR1* mRNA in the frontal cortex in SCZ and increased transmembrane TNF in BD suggesting alterations in the TNF-pathway in the central nervous system in both illnesses[21]. However, large postmortem studies of TNF-pathway gene expression in the DLPFC are lacking.

The aim of the present study was to further determine the role of TNF-pathway related molecules at the protein and mRNA levels in BD and SCZ patients. First we investigated these markers, including the balance between TNF and its receptors, in peripheral blood in a large cohort (n=1440). We then investigated whether TNF proteins and their proportion is associated with working memory, a task affiliated with the DLPFC. Lastly, mRNA levels of these molecules were examined in the DLPFC in an independent cohort (n=210). We hypothesized that patients with SCZ and BD would present with a distinct systemic and cortical pattern of TNF-pathway markers reflecting the relative contribution of these different compartments to the sustained systemic TNF activation that is proposed to be operating in these patients.

Methods

Plasma and leukocyte cohort

Study Design and Ethics: The TOP Study at the NORMENT Centre, Oslo University Hospital, and collaborating Norwegian hospitals[22] was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate. The biobank was approved by the Norwegian Directorate of Health. All participants provided written informed consent after receiving a description of the study.

Participants: The main inclusion criteria were DSM-IV diagnoses of schizophrenia spectrum disorders or bipolar spectrum disorders, IQ > 70 and age between 18 and 65 years (for details see[22]). Healthy volunteers without any history of severe psychiatric disorders (or in any of their first-degree relatives), or substance/alcohol abuse/dependency from the same catchment area were randomly selected from the National Population Registry (www.ssb.no). (For details see[22]) For the present analyses, patients and controls were not included if they had coexisting autoimmune or inflammatory disease, cancer, ongoing infections, used anti-inflammatory drugs or had C-reactive Protein (CRP) levels above 20 mg/L.

Clinical Assessments: Diagnosis was obtained using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Clinical symptoms were evaluated using the Young Mania Rating Scale (YMRS), Inventory of Depressive Symptoms (IDS), Calgary Depression Scale for Schizophrenia (CDSS), Positive and Negative Syndrome Scale (PANSS), while functioning was measured using the Global Assessment of Functioning split version function (GAF-F) and symptom scale (GAF-S). The clinical assessment team consisted of clinical psychologists and psychiatrists, who were all trained until satisfactory inter-rater reliability was obtained[23,24]. Psychotic symptoms were examined in the SCZ group using the PANSS 5-factorial model[25], while depressive and hypomanic/manic symptoms were investigated in the BD group using YMRS, IDS and CDSS.

Neurocognitive Assessment: Psychologists trained in standardized neuropsychological testing performed neurocognitive assessment. Working memory was assessed with the Digit Span Test—backward (Wechsler Adult Intelligence Scale [WAIS] -III), letter number sequencing (WAIS-III) and the Working Memory—Mental Arithmetic (WM-MA) Test—commissions[26]. For details see [27].

Cytokine Assessment: We used enzyme-linked immunosorbent assay to quantify protein levels due to its high specificity and sensitivity[28], and measured plasma levels of TNF using a high sensitivity enzyme immunoassay (EIA) from Cloud Corp (Housten, TX) while sTNFR1, sTNFR2 and sADAM17 was analyzed using EIAs from R&D systems (Minneapolis, MN). Intra- and inter-assay coefficients of variance for proteins were less than 10%. The ratio between TNF and sTNFRs may provide an estimate of the molar balance in serum between TNF molecules and sTNFRs. In molecular terms, this ratio was defined as TNF (pmol/L)/(sTNFR1 + sTNFR2)(pmol/L) X 100, assuming a molecular mass of (17 x 3) kD and 30 kD for TNF (trimer) and both types of sTNFRs, respectively.

RNA isolation and RT-PCR: Total RNA was isolated from whole blood using the Tempus 12-Port Isolation kit (Applied Biosystems; Ambion, Austin, TX, USA) and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The tubes were stored at (-)80^{o}C. Reverse transcription was performed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with ~0.5 μg total RNA in 96-well PCR plates (Applied Biosystems). Each plate contained 80 samples that were randomly distributed to atone for the differences in reverse transcription or subsequent real-time PCR efficiency.

Quantification of mRNA is described in detail in the Supplementary Text. We used Primer Express software version 3.0 (Applied Biosystems) to design sequence specific mRNA (primer spanning exon-exon junction) oligonucleotide primers for the full-length *TNF, TNFR1, TNFR2* and *ADAM17* mRNA. Melt curves were evaluated for all primers. Data were normalized to ß-actin.

Brain cohort

Post-Mortem Brain sample collection:

Details of tissue acquisition, handling, processing, dissection, clinical characterization, diagnoses, neuropathological examinations, RNA extraction and quality control measures were described previously[29]. Toxicological analysis was performed on every case. For control cases, subjects with evidence of macro- or microscopic neuropathology, drug use, alcohol abuse or psychiatric illness were excluded.

Post-Mortem Brain RNA extraction and sequencing

Details of post-mortem DLPFC RNA extraction and sequencing, and RNA Seq data processing were previously described[30]. Total RNA was extracted from ~100 mg of post-mortem tissue homogenates of DLPFC gray matter approximating BA9/46 in postnatal samples using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The poly(A)-containing RNA molecules were purified from 1 μg DNase-treated total RNA and, following purification, fragmented into small pieces using divalent cations under elevated temperature. Reverse transcriptase and random primers were used to copy the cleaved RNA fragments into first-strand cDNA, and the second-strand cDNA was synthesized using DNA polymerase I and RNase H. We performed the sequencing library construction using the TruSeq RNA Sample Preparation v2 kit by Illumina (See Supplementary Text for details).

Post-Mortem Analyses: RNA sequencing data processing

The Illumina Real Time Analysis (RTA) module performed image analysis, base calling and ran the BCL converter (CASAVA v1.8.2), generating FASTQ files containing the sequencing reads. These reads were aligned to the human genome (UCSC hg19 build) using the spliced-read mapper TopHat (v2.0.4) using the reference transcriptome to initially guide alignment, on the basis of known transcripts of Ensemble Build GRCh37.67 (the "-G" argument in the software). A normalized reads

per kilobase million (RPKM) metric were calculated for each gene by dividing the number of reads mapping to the gene divided by the length of the gene (in kilobases).

Statistical analysis

Plasma and leukocyte cohort analyses: Statistical analyses were performed using the SPSS software package for Windows, version 22.0. Data normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Differences in demographic data between groups were investigated using the chi-square test for categorical variables, the Kruskal-Wallis test for continuous variables, and Tukey and the Mann-Whitney U test for post hoc analyses. We used T-tests for normally distributed variables, and non-parametric tests (Mann Whitney U test) for skewed distributions to investigate differences between groups. Correlations were examined by using Spearman's Rank correlation.

Potential confounders (age, sex, smoking status, body mass index [BMI], time for blood sampling and race) were investigated using non-parametric tests (Spearman's rank correlation and Mann-Whitney U test), and confounders with a p-value lower than 0.2 were controlled for in linear regression models. Results are given as standardized beta or T-test from the regression analyses.

We calculated an aggregate working memory score from the three working memory tasks and controlled for age, gender, diagnosis and investigated interaction effects of diagnosis in general linear models.

We corrected for multiple testing according to Bonferroni, and alpha was set at $p < 0.007$ for the main analyses in the circulation (correcting for 7 tests: TNF, sTNFR2, sADAM17, *TNF* mRNA, *TNFR1* mRNA, *TNFR2* mRNA and *ADAM17* mRNA), and $p < 0.01$ for the aggregate working memory score.

Post-mortem analyses: Differential expression analysis was performed using LIMMA R package[31]. For SCZ versus HC samples, gene RPKM was regressed against a binary diagnosis variable, covarying for 8 principal components and covariates of age, sex, and mitochondrial mapping rate, with the number of components determined for the expression matrix of a set of 562 immune genes (Birnbaum et al, under review) using the sva R package (surrogate variable analysis)[32]. The optimum number of components was determined based on an iterative algorithm to remove the impact of mRNA quality on differences in expression between patients and controls that is not accounted for by controlling only for RIN and demographic variables. For BD versus HC samples, gene RPKM was regressed against a binary diagnosis variable, covarying in this case for 10 principal components and age, sex, and mitochondrial mapping rate. A multiple testing correction, FDR, was employed to correct for all immune genes in the matrix of immune genes from which the principal components were derived.

Results

Demographics and clinical characteristics

The socio-demographic and clinical characteristics of the participants are presented in Tables 1 and 2.

Plasma levels of TNF related molecules

The plasma levels of TNF related molecules in absolute values are summarized in Table 3. The patient group as a whole as well as the SCZ and the BD groups separately had significantly higher levels of TNF, sTNFR1and sTNFR2 as well as the TNF/sTNFRs ratio as an estimate of TNF activity compared to HC, also in adjusted analysis (i.e. confounders). In contrast, patients had lower levels of sADAM17 compared to HC in adjusted analysis. This pattern, however, was restricted to the SCZ group, with no significant difference between the BD and HC group (Table 4).

TNF, TNFR1, TNFR2 and ADAM17 mRNA expression in whole blood

The mRNA expression levels of TNF related molecules in whole blood are summarized in Table 3. The patient group as a whole as well as the SCZ and BD groups independently had lower levels of *TNF* mRNA compared to HC, with no difference between SCZ and BD (Table 4). In contrast, there were no differences in ***TNFR1*** **mRNA** and ***TNFR2*** **mRNA** between patients and controls (Table 4). However, patients had significantly higher levels of ***ADAM17*** **mRNA** compared to HC after controlling for confounding factors. This effect was clearly driven by the BD group (Table 4).

TNF proteins and working memory

Performance on working memory tasks is presented in Supplementary Table 5. We found significant associations between working memory and TNF-ratio and TNF that were significantly stronger in the SCZ group compared to CTR and BD (data not shown), and remained significant after correcting for multiple testing and controlling for age, sex, PANSS score and antipsychotics in

the SCZ group (TNF-ratio: F (5,120)=2.90, adjusted R^2=0.07, β=-0.23, p=0.008) (TNF: F (5,122)=3.35, R^2=0.09, β=-0.26, p=0.003) (Supplementary Table 6).

TNF, TNFR1, TNFR2 and ADAM17 mRNA expression in cortex

As shown in Supplementary Figure 1 and Table 4, there were no differences in mRNA levels of *TNF* (FDR= 0.99), *TNFR1* (FDR=0.84), *TNFR2* (FDR=0.92) or *ADAM17* (FDR=0.95) between SCZ and HC. Similarly, no differences in mRNA levels of *TNF* (FDR=0.76), *TNFR1* (FDR=0.77), *TNFR2* (FDR=0.99) or *ADAM17* FDR=0.39) were detected between BD and HC.

Correlation between TNF-related molecules in plasma (protein) and whole blood (mRNA)

The associations between protein and mRNA of the TNF related molecules in plasma and whole blood, respectively, are presented in Supplementary Table 1. We found moderate but highly significant associations between *TNFR1*, *TNFR2* and *ADAM17* mRNA, and significant weak associations between sTNFR1 and sTNFR2 in both the HC group and the patient group after controlling for confounders. Importantly, however, except for a weak correlation between mRNA and protein levels of TNFR1 in the patient group as a whole and those with SCZ, there was no significant correlation between protein and mRNA levels of the different TNF related molecules (Supplementary Table 1).

Role of medication

Patients using lithium (n=19) had higher levels of *ADAM17* mRNA and sTNFR1 levels compared to non-medicated (n=85) patients in the BD group. Serum levels of lithium were associated with higher TNF levels and increased TNF/sTNFRs ratio (Supplementary Table 2), however, these results do not remain significant after correction for multiple testing. We found no other significant associations between medication groups (antipsychotics, mood stabilizers and antidepressants), medication dosage and cytokines/mRNA.

Clinical characteristics and TNF pathway expression and cytokines

We found weak correlations with small effect sizes between clinical symptoms (i.e., PANSS, GAF and CDSS) and TNF pathway related gene expression and corresponding proteins. These results, however, do not survive correction for multiple testing (Supplementary Table 3-4).

PANSS, GAF and duration of illness in the SCZ group: We found weak negative associations after controlling for confounders between *TNF* mRNA and PANSS excited symptoms, *TNFR1* mRNA and PANSS negative symptoms, TNF and GAF-symptom scale, sTNFR1 and GAF-symptoms scale, and *TNF* mRNA and duration of illness. Thus, it seems that decreased *TNF* and *TNFR1* mRNA expression was associated with increased disease symptom severity, while increased circulating TNF and sTNFR1 was associated with increased symptom severity.

CDSS, IDS, YMRS, GAF and duration of illness in the BD group: We found a weak positive association after controlling for confounders between *TNF* mRNA and duration of illness in the BD group (the opposite of what we found in the SCZ group). Of the clinical symptoms only CDSS showed associations with cytokines and mRNA; increasing sTNFR2 levels and *TNFR2* mRNA expression were associated with decreased depressive symptoms, while increased *TNF* mRNA levels were associated with increased depressive symptoms.

Discussion

We found that patients with SCZ and BD had slightly increased plasma levels of TNF-related molecules with significantly increased TNF/sTNFRs ratio, which has been shown to be a surrogate marker of TNF bioactivity[12], compared to HC suggesting a subtle but potentially biologically relevant pro-inflammatory imbalance in the TNF system in these disorders. However, while TNF levels were elevated in plasma, *TNF* mRNA was decreased in whole blood suggesting other cellular sources of TNF than circulating leukocytes (Figure 1). The increased *ADAM17* mRNA expression detected in BD compared to SCZ in peripheral blood could potentially contribute to increase shedding of membrane-bound TNF in BD, suggesting a differential role for *ADAM17* in BD compared to SCZ. We have previously reported that elevated plasma levels of sTNFR1 were associated with lower scores on several cognitive tests[20,33]. Here, we found that a shift toward a pro-inflammatory imbalance in the TNF pathway was weakly but significantly associated with lower working memory scores potentially suggesting a link between the DLPFC and the TNF pathway, however, we observed no significant alterations in TNF-system mRNA expression in the DLPFC of patients compared to HC.

The increased levels of TNF, sTNFR1 and sTNFR2 in SCZ and BD patients *per se* may not necessarily reflect a pro-inflammatory shift in the TNF-pathway considering that soluble TNF receptors partake in regulating TNF activity by acting as decoy receptors and competing with membrane-bound receptors for TNF[34]. However, our results suggest that while both plasma levels of TNF and its soluble receptors are elevated in SCZ and BD, there is also a pro-inflammatory imbalance as shown by increased TNF/sTNFRs ratio[12]. In spite of this pro-inflammatory imbalance in plasma, and in contrast to previous more small-scaled studies[13,14], *TNF* mRNA was down-regulated in circulating leukocytes from both BD and SCZ patients implying other cellular sources for the elevated plasma levels in these disorders, such as endothelial cells and tissue macrophages (Figure 1). Based on the distinct upregulation of *ADAM17* mRNA in leukocytes in BD and the role of ADAM17 in the release of TNF and its receptor from their membrane to their soluble form, it is,

however, possible that increased shedding of these molecules could contribute to their increased plasma levels in BD patients, potentially representing a distinct pattern of this disorder.

We predicted that these results in peripheral blood would be recapitulated in the DLPFC. Using a conservative method to control for mRNA quality in brain tissue, which is a major confounder of prior studies that control only for RIN and demographics, we find no evidence of the changes in peripheral blood in prefrontal cortical samples from either patients with SCZ or BD. It is important to recognize that our mRNA quality adjustment is rigorous and conservative and though potentially sensitive to type II error, is robust in controlling for Type I error. The lack of changes in the expression of TNF related signaling molecules in the DLPFC *per se* cannot rule out altered TNF-pathway activity in other regions of the brain or the possibility that inflammatory molecules in peripheral blood have CNS effects without changing intrinsic gene expression. Indeed, cytokines in peripheral blood are able to cross the blood brain barrier[35] potentially influencing learning and memory[33], cognition[20] and neural activity and viability[36]. Our findings raise questions, however, about whether TNF signaling in peripheral blood is a marker of a primary pathophysiological process or secondary to other disease mechanisms. Notably, enhanced TNF activity is associated with several co-morbid metabolic conditions such as type 2 diabetes and cardiovascular disease[37,38]. However, it is important to underscore that the cortex analyses were performed on whole cortex samples and not on isolated cells, and an up- or down-regulation for example in microglia may be masked by expression in other cells. Correlations between clinical features, treatment and TNF measures in peripheral blood were largely uninformative showing a rather complex pattern. However, increasing depressive symptoms were associated with a moderate increase in *TNF* mRNA, and lower levels of sTNFR2, *TNFR1* and *TNFR2* mRNA suggesting a pro-inflammatory imbalance in circulating immune cells of these patients, further supporting a potential link between TNF activity and depression as described by others[39]. We found no significant difference in plasma/whole blood cytokine/mRNA levels between patients using antipsychotics in monotherapy and non-medicated patients, and no significant correlation between defined daily

dosage of antipsychotics and cytokine/mRNA levels. This is in contrast to previous smaller studies that have found that antipsychotics may influence the expression of *TNF*[40]. However, plasma levels of TNF and TNF/sTNFRs ratio increased with higher lithium serum concentrations, and patients using lithium had higher sTNFR1 and *ADAM17* mRNA levels compared to non-medicated bipolar patients, suggesting that lithium may promote activation of the TNF system.

There are some limitations to our study. Firstly, there is a difference in the time of blood sampling for our HC group compared to the patient group, but this was controlled for in the analysis of cytokine and mRNA levels. Secondly, current RNA sequencing quantification and mapping methods may lead to potential underestimation of mRNA levels. Further, although previous studies have measured TNF related proteins in postmortem brain[41], we were unable to obtain reliable data on postmortem TNF pathway proteins. Finally, our data on the association of TNF molecules and clinical symptoms should be interpreted with caution as no consistent patterns were observed.

This is by far the largest study that investigates the TNF system in patients with severe mental disorders, including both plasma levels as well as mRNA expression in whole blood and in brain. Our data suggest a complex regulation of TNF related molecules in peripheral blood of BD and SCZ patients with increased TNF/sTNFRs ratio in plasma, reflecting enhanced TNF activity as a major finding. The highly significant difference in *ADAM17* mRNA expression between SCZ and BD may also implicate other ADAM17 substrates and respective systems in the pathology of BD, and requires further elucidation. Our findings also suggest that circulating leukocytes are not a major source of soluble TNF levels in plasma. The lack of findings in the DLPFC leaves unresolved the issue of how peripheral blood alterations relate to the TNF system in the brain. Further studies are needed to investigate other cortical regions of the brain and the impact of our findings on the pathogenesis of severe mental disorders.

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Table 1. Demographic and clinical characteristics of participants.

Parameters	Plasma (cytokine/protein) cohort				Leukocyte (mRNA) cohort			
	SCZ (N = 569)	BD (N = 247)	HC (N = 624)	Post Hoc Analysis	SCZ (N = 224)	BD (N = 143)	HC (N = 184)	Post Hoc Analysis
Male sex, N (%)	340 (59.8)	103 (41.7)	330 (52.9)	SCZ > HC > BD	127 (56.7)	51 (35.7)	108 (58.7)	HC, SCZ > BD
Ethnicity (Caucasian)	453 (79.6)	222 (89.9)	611 (97.9)	HC > BD > SCZ	178 (79.5)	132 (92.3)	184 (100)	HC > BD > SCZ
Tobacco (users)[a]	300 (54.8)	131 (54.6)	55 (19.9)	SCZ, BD > HC	118 (57.3)	66 (56.4)	24 (20.5)	SCZ, BD > HC
Medication:								
Antipsychotics	488 (85.8)	128 (51.8)	-	SCZ > BD	189 (84.4)	76 (53.1)	-	SCZ > BD
Lithium	9 (1.6)	50 (20.3)	-	BD > SCZ	4 (1.8)	20 (14.0)	-	BD > SCZ
Antidepressants	179 (31.5)	95 (38.8)	-	BD > SCZ	36 (16.1)	65 (46.1)	-	BD > SCZ
Mood stabilizers	76 (13.4)	98 (40.2)	-	BD > SCZ	61 (27.2)	54 (38.0)	-	BD > SCZ
Age (years)	27 (15)	32 (18)	32 (13)	BD, HC > SCZ	29 (15)	31 (16)	31.5 (13)	NS
Body Mass Index[b]	25.7 (7.1)	25.4 (5.6)	24.4 (4.4)	BD, SCZ > HC	25.9 (7.4)	25.4 (5)	24.5 (4.29)	SCZ > HC
Duration of Illness (years)	4 (8)	9 (13)	-	BD > SCZ	4.5 (7.8)	8 (10.5)	-	BD > SCZ
Time of blood sampling[c]	09:45 (1:10)	09:27 (0:55)	12:42 (6:50)	HC > SCZ > BD	09:50 (1:00)	09:30 (1:00)	11:10 (6:50)	HC > SCZ > BD
PANSS total score	62 (22)	45 (13)	-	SCZ > BD	63 (22)	44 (13)	-	SCZ > BD
YMRS total score	4.0 (9)	2 (6)	-	SCZ > BD	5 (9)	1 (4)	-	SCZ > BD
IDS total score	17 (20)	15 (18)	-	NS	17 (21)	14 (19)	-	NS
CDSS total score	5 (7)	3 (6)	-	SCZ > BD	5 (8)	2.5 (7)	-	SCZ > BD
GAF-S	40 (13)	55 (16)	-	BD > SCZ	39 (13)	57 (15)	-	BD > SCZ
GAF-F	41 (15)	50 (20)	-	BD > SCZ	40 (13)	51 (17)	-	BD > SCZ

Missing: [a] N = 369, [b] N = 319, [c] N = 422 in the plasma cohort

Abbreviations: HC = Healthy Controls; SCZ = Schizophrenia Spectrum; BD = Bipolar Disorder; NS = Non-Significant; PANSS=Positive and Negative Syndrome Scale; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; CDSS= Calgary Depression Scale for Schizophrenia; GAF-F = Global Assessment of Functioning - Function Scale; GAF-S = Global Assessment of Functioning - Symptom Scale.

Categorical data are given as percent in brackets, while continuous data are given as median with interquartile range. Post hoc analysis is performed using Pearson Chi-square for categorical data, and Kruskal-Wallis and Mann-Whitney tests for continuous data.

Table 2. Post-mortem brain cohort samples descriptives.

Parameters	Brain cohort		
	SCZ (N = 80)	BD (N = 44)	HC (N = 88)
Male sex, N (%)	54 (73.8)	23 (52.3)	69 (78.4)
Ethnicity (Caucasian)	80 (100)	44 (100)	88 (100)
Mean age (SE)	46.5 (1.8)	46.8 (2.1)	44.7 (1.7)
Mean RIN (SE)	8.2 (0.06)	8.3 (0.08)	8.4 (0.05)

Abbreviations: SCZ = schizophrenia; BD = Bipolar Disorder; HC= Healthy Controls, SE = Standard Error, RIN = RNA integrity number

Table 3. Unadjusted data: Protein levels in plasma, relative mRNA expression in peripheral blood, and mRNA expression in the dorsolateral prefrontal cortex.

Parameters	Schizophrenia		Bipolar Disorder		Healthy Controls	
	N	M (IQR)	N	M (IQR)	N	M (IQR)
Plasma cytokine/protein						
TNF (pg/mL)	352	1.20 (1.28)	168	1.16 (1.04)	358	1.09 (1.03)
sTNFR1 (ng/mL)	569	1.76 (0.74)	247	1.67 (0.52)	624	1.60 (0.77)
sTNFR2 (ng/mL)	553	4.76 (1.43)	239	4.60 (1.47)	591	4.51 (1.44)
ADAM17 (pg/mL)	548	189 (282)	240	227 (358)	594	214 (341)
Leukocyte mRNA						
TNF mRNA	224	0.89 (0.44)	142	0.92 (0.41)	184	1.00 (0.58)
TNFR1 mRNA	224	1.02 (0.32)	143	1.08 (0.46)	184	1.00 (0.31)
TNFR2 mRNA	224	0.98 (0.36)	143	1.00 (0.46)	184	1.00 (0.43)
ADAM17 mRNA	224	1.02 (0.29)	142	1.24 (0.46)	184	1.00 (0.38)
DLPFC mRNA						
TNF mRNA	80	0.03 (0.05)	44	0.03 (0.06)	86	0.02 (0.05)
TNFR1 mRNA	80	3.87 (3.10)	44	3.19 (2.43)	86	2.35 (0.77)
TNFR2 mRNA	80	1.74 (1.20)	44	1.37 (0.63)	86	1.44 (0.64)
ADAM17 mRNA	80	3.25 (0.95)	44	2.83 (0.91)	86	3.40 (0.94)

Abbreviations: TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; DLPFC= dorsolateral prefrontal cortex;IQR=interquartile range; M = median, ng = nanogram; pg = pictogram.
Data are given as median with interquartile range due to skewed distributions.

Table 4. Differences in cytokine and mRNA levels between patients and controls after controlling for confounders.

	Plasma cohort (soluble cytokine)			Leukocyte cohort (mRNA)			Brain cohort (mRNA)		
	n	*β*	*t*	*n*	*β*	*t*	*t*	FC	FDR
Patients vs. HC									
TNFR1	1003	.14	**4.47*** **	472	.05	1.11			
TNFR2	1000	.10	**2.94** **	315	.11	1.56			
TNF	459	.17	**3.48** **	564	-.16	**-3.91*** **			
ADAM17	1415	-	**-2.08***	428	.16	**3.15** **			
TNF/sTNFRs ratio	455	.20	**4.07*** **						
SCZ vs. HC									
TNFR1	748	.15	**3.82*** **	309	.01	.15	.79	1.02	.84
TNFR2	828	.09	**2.56***	239	.14	1.71	-.40	.99	.92
TNF	319	.16	**2.38***	323	-.18	**-3.02** **	-.04	1.00	.99
ADAM17	744	-	**-2.93** **	323	.02	.28	-.33	.99	.95
TNF/sTNFRs ratio	317	.16	**2.48***						
BD vs. HC									
TNFR1	479	.17	**3.50** **	264	.05	.81	-.61	.99	.77
TNFR2	516	.15	**3.42** **	234	.04	.57	-.01	1.0	.99
TNF	189	.19	**2.61** **	326	-	**-2.56***	.64	1.01	.76
ADAM17	315	-.03	-.48	222	.40	**5.92*** **	1.62	1.05	.39
TNF/sTNFRs ratio	195	.22	**2.72** **						
BD vs. SCZ									
TNFR1	478	-.07	-1.46	367	.06	1.12			
TNFR2	696	-.06	-1.54	367	-.07	-1.25			
TNF	347	-.07	-1.28	366	.01	.11			
ADAM17	510	.06	1.42	366	.39	**8.03*** **			
TNF/sTNFRs ratio	344	-.02	-.39						

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

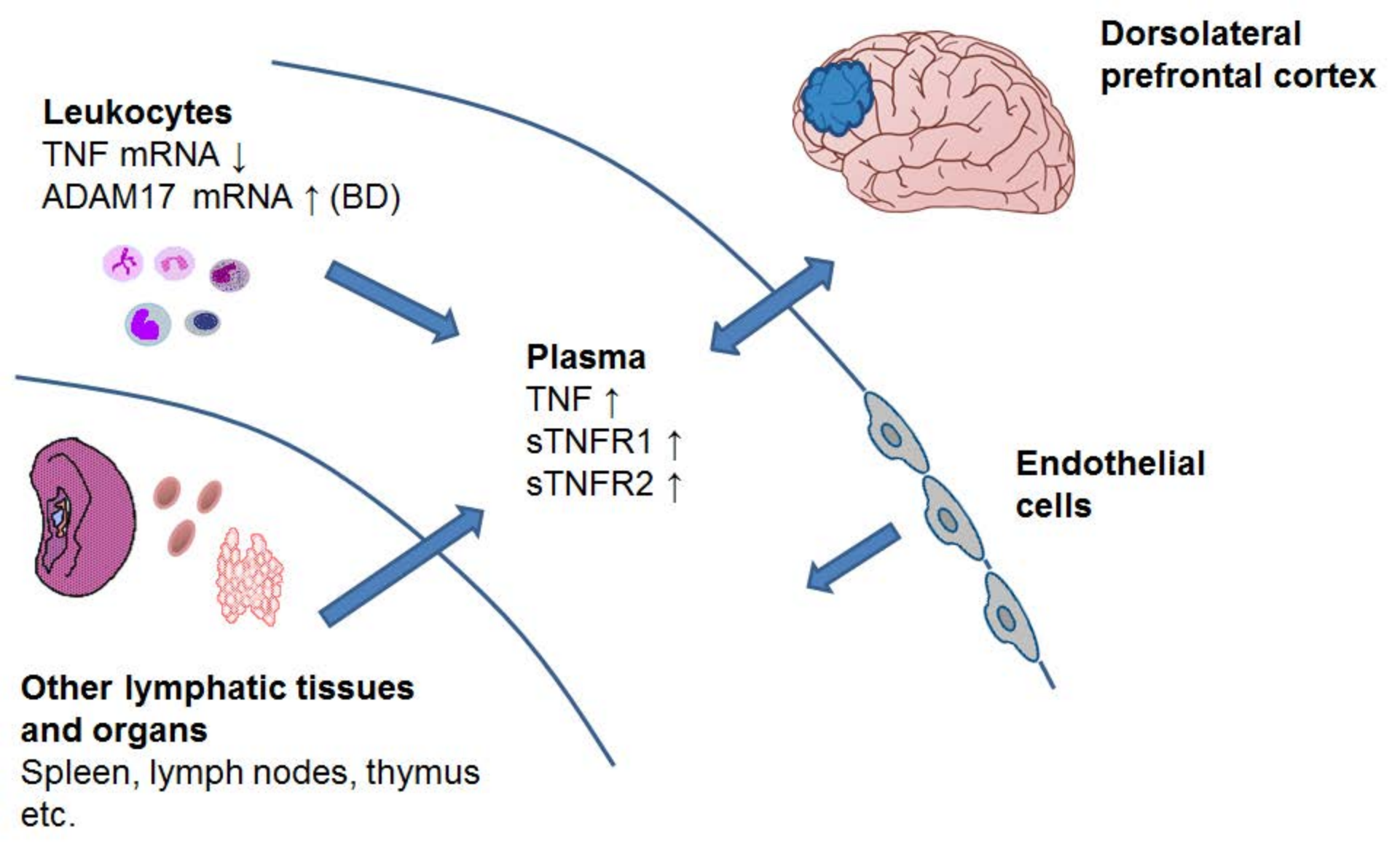
Abbreviations: SCZ=schizophrenia; BD=bipolar disorder; HC=healthy controls; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2)

The brain cohort: *n*(HC) = 86, *n*(SCZ)=79, *n*(BD) = 44.

Results are given as T-values from linear regression analyses after controlling for confounding factors.

Figure Legend

Figure 1. Elevated soluble plasma cytokine levels in SCZ and BD may be a result of increased shedding from their membrane bound form in BD, but blood leukocytes seem an unlikely source as *TNF* mRNA is downregulated in both disorders. There are no strong indications of alterations in TNF related signaling molecules in prefrontal cortex, however, soluble plasma cytokines can pass through the blood brain barrier, and differential expression of these molecules may occur in other regions of the brain. The increase in plasma cytokines may also be a result of heightened immune activity in other lymphatic tissues and organs as well as endothelial cells.


Dorsolateral prefrontal cortex
Leukocytes
TNF mRNA ↓
ADAM17 mRNA ↑ (BD)
Plasma
TNF ↑
sTNFR1 ↑
sTNFR2 ↑
Endothelial cells
Other lymphatic tissues and organs
Spleen, lymph nodes, thymus etc.

Supplementary Text

Quantification of whole blood mRNA

Quantification of mRNA was performed with the q polymerase chain reaction Master Mix for SYBR Green I (Applied Biosystems) in 10 μL duplicate reactions in 384 well plates on an ABI Prism 7900 (Applied Biosystems) using the 2(-Delta Delta C[T]) method with the average of four pools of cDNA. These were included on each plate in the RT-PCR and followed the samples in the real time PCR, as reference. The CV was under 10 % for the average of these calibrators between all the plates analyzed.

Sequencing library construction

The sequencing library construction was performed using the TruSeq RNA Sample Preparation v2 kit by Illumina. Briefly, cDNA fragments undergo an end repair process using T4 DNA polymerase, T4 polynucleotide kinase and Klenow DNA polymerase with the addition of a single adenosine using a Klenow polymerase lacking 3′ to 5′ exonuclease activity, and then ligated to the Illumina paired-end (PE) adapters using T4 DNA ligase. An index/barcode was inserted into Illumina adapters, allowing samples to be multiplexed in one lane of a flow cell. These products were then purified and enriched with PCR to create the final cDNA library for high throughput DNA sequencing using an Illumina HiSeq 2000.

Supplementary Table 1. Associations between protein and mRNA in the blood after controlling for confounders.

	sTNFR1	sTNFR2	sADAM17	TNF mRNA	TNFR1 mRNA	TNFR2 mRNA	ADAM17 mRNA
in HC							
TNF	.10	-.02	.02	-.01	-.03	-.04	-.12
sTNFR1		**.20****	.08	-.21	.03	-.02	-.06
sTNFR2			-.01	.00	-.03	-.12	-.06
sADAM17				-.02	.11	.11	-.01
TNF mRNA					-.13	-.03	-.06
TNFR1 mRNA						**.48*****	**.68*****
TNFR2 mRNA							**.44*****
in SCZ+BD							
TNF	.08	**.15***	**.13***	-.05	-.01	.06	.02
sTNFR1		**.33*****	.01	-.07	**.15***	-.06	-.01
sTNFR2			-.02	**-.18****	.08	-.02	-.05
sADAM17				-.08	.00	-.04	.04
TNF mRNA					-.08	**-.12***	**.16****
TNFR1 mRNA						**.48*****	**.44*****
TNFR2 mRNA							**.39*****
in SCZ							
TNF	-.01	**16***	.12	-.06	-.05	-.05	-.01
sTNFR1		**.41*****	.01	-.04	**.20****	.10	-.02
sTNFR2			.04	-.04	.09	-.02	.05
sADAM17				-.05	.00	-.03	-.03
TNF mRNA					.00	**-.16***	.12
TNFR1 mRNA						**.44*****	**.51*****
TNFR2 mRNA							**.45*****
in BD							
TNF	**.16***	.09	**.19***	-.01	-.04	.11	.02
sTNFR1		**.19***	.07	-.22	.15	.15	.10
sTNFR2			-.06	**-.30****	.15	.02	-.07
sADAM17				-.17	-.04	-.06	-.08
TNF mRNA					**-.27****	-.08	**.26****
TNFR1 mRNA						**.50*****	**.34****
TNFR2 mRNA							**.48*****

*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

Abbreviations: SCZ = Schizophrenia; BD = Bipolar Disroder; HC = Healthy Controls; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2); ADAM17=A Disintegrin And Metalloprotease-17 protein. Results are given as standardized beta from linear regression analyses.

Supplementary Table 2. Associations between daily defined dose of medication and protein/mRNA levels, and group effect of medication vs. non-medicated patients after controlling for confounders and diagnosis.

	Serum cohort					mRNA cohort			
	TNF	sTNFR1	sTNFR2	TNF/sTNFRs ratio	sADAM17	TNF mRNA	TNFR1 mRNA	TNFR2 mRNA	ADAM17 mRNA
DDD associations:									
Antipsychotics	.07	-.07	-.02	-.04	.04	.09	.21	-.04	.11
Lithium	**.42****	-.14	-.01	**.39***	.01	-.40	.15	.15	.42
Mood stabilizers	.02	.08	.11	-.03	.06	-.01	-.08	.04	-.03
Antidepressants	.07	-.01	.14	.03	-.07	-.04	-.02	.08	-.08
Group effects:									
Antipsychotics	-1.11	1.79	.29	-.08	-.15	-1.32	-.82	.02	-1.19
Lithium	.24	**2.34***	.52	.41	-.33	-.37	-.24	-.29	**2.24***
Mood stabilizers	-.10	1.50	.75	-.23	-1.17	-1.24	-.48	-.75	-1.09
Antidepressants	.08	1.69	.45	.28	-1.00	-1.28	-.25	-1.24	-1.52

*$p < 0.05$ **$p < 0.01$

Abbreviations: DDD = daily defined dose; TNF=tumor necrosis factor; TNF=soluble TNF; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2); ADAM17=A Disintegrin And Metalloprotease-17 protein.

Results are given as standardized beta from linear regression analyses for associations and as T-values from linear regression analyses for medication effects.

Only antipsychotics were investigated as monotherapy due to small sample sizes in the other medication groups. We used serum concentration of lithium instead of DDD, and differences in *ADAM17* mRNA levels were examined in the bipolar disorder group.

Supplementary Table 3. Associations between protein/ mRNA and clinical characteristics in **schizophrenia**.

	DOI	Pos.	Neg.	Disorg.	Excited	Depr.	GAF-S	GAF-F
TNF	.05	.03	.04	.10	.05	.09	**-.14***	-.12
sTNFR1	.05	.05	.08	.04	.03	-.03	**-.14****	-.08
sTNFR2	.01	.06	.08	.02	.02	-.02	-.08	-.08
ADAM17	.03	.01	.02	.00	-.06	-.04	-.01	.01
TNF/sTNFRs ratio	.10	.02	-.03	.07	.05	.11	-.11	-.10
TNF mRNA	-.14*	-.07	.02	-.10	**-.17***	.02	.13	.13
TNFR1 mRNA	-.06	-.05	**-.14***	.06	-.07	-.07	.00	-.08
TNFR2 mRNA	-.07	-.08	-.06	.06	.07	-.09	.08	.00
ADAM17 mRNA	-.08	-.09	-.12	-.04	-.12	-.06	.07	.00

*p< 0.1; **p<0.01

Abbreviations: DOI=Duration of illness; PANSS=Positive and Negative Syndrome Scale, 5-factorial model[1]; Pos.=PANSS Positive symptoms; Neg.=PANSS Negative symptoms; Disorg.=PANSS disorganized symptoms; Depr.=PANSS depressive symptoms; GAF-F=Global Assessment of Functioning - Function Scale; GAF-S=Global Assessment of Functioning - Symptom Scale; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2).

Associations are given as standardized beta after controlling for confounders in linear regression analyses. ADAM17 mRNA was not associated with the selected confounders and is therefore investigated using Spearman's Rank correlation.

Supplementary Table 4. Associations between protein/mRNA and clinical characteristics in **bipolar disorder**.

	DOI	CDSS	IDS	YMRS	GAF-S	GAF-F
TNF	.03	-.08	-.04	.07	.01	.07
sTNFR1	-.02	.08	.09	.07	.02	.06
sTNFR2	-.09	**-.22****	-.06	-.08	.09	.05
ADAM17	-.03	-.04	.01	.10	-.10	-.12
TNF/sTNFRs ratio	.05	.04	.01	.12	-.08	-.06
TNF mRNA	.13	**.35****	.14	.01	-.06	.01
TNFR1 mRNA	-.04	-.18	.00	.03	.03	.02
TNFR2 mRNA	-.12	**-.27***	-.07	.04	.06	.01
ADAM17 mRNA	.05	-.17	-.06	.03	.03	.07

*p< 0.1; **p<0.01

Abbreviations: DOI=Duration of illness; YMRS=Young Mania Rating Scale; IDS=Inventory of Depressive Symptoms; CDSS=Calgary Depression Scale for Schizophrenia; GAF-F=Global Assessment of Functioning - Function Scale; GAF-S=Global Assessment of Functioning - Symptom Scale; TNF=tumor necrosis factor; sTNFR1 =soluble TNF receptor 1; sTNFR2=soluble TNF receptor 2; ADAM17=A Disintegrin And Metalloprotease-17 protein; TNF/sTNFRs ratio = TNF/(sTNFR1 + sTNFR2).

Associations are given as standardized beta after controlling for confounders in linear regression analyses.

Supplementary Table 5. Performance on working memory tasks.

Parameters	Schizophrenia		Bipolar Disorder		Healthy Controls		ANOVA/Kruskal-Wallis and Post Hoc analyses
	N	M (SD)	N	M (SD)	N	M (SD)	
Digit Span Test—backward (WAIS-III)	128	4.26 (1.06)	88	4.69 (1.30)	28	4.96 (0.92)	CTR,BD > SCZ
Letter Number Sequencing (WAIS-III)	128	9.54 (2.80)	88	10.07 (2.16)	28	11.54 (2.55)	CTR > BD,SCZ
WM-MA	128	2.68 (1.09)	88	3.00 (0.96)	28	3.46 (0.72)	CTR > BD,SCZ
Working Memory Aggregate Score	128	0.74 (0.77)	88	1.02 (0.71)	28	1.43 (0.63)	CTR > BD > SCZ

Abbreviations: WAIS-III= Wechsler Adult Intelligence Scale-III; WM-MA= Working Memory—Mental Arithmetic. M=mean, SD=standard deviation. Digit span test-backward and letter number sequencing test are given as raw score. WM-MA is given as d-prime. Working memory aggregate score is calculated from z-scores of the 3 tasks. Differences between groups were investigated using ANOVA for working memory aggregate score, and Kruskal-Wallis test for the remaining 3 tests. Post Hoc analyses were performed using Tukey and Mann-Whitney test respectively.

Supplementary table 6. Stepwise linear regression analyses investigating working memory as dependent variable in the schizophrenia group.

	TNF-ratio (*β*)	age (*β*)	sex (*β*)	PANSS total (*β*)	Antipsychotics (*β*)	*adj.* R^2	*F (df)*
model 1	-.25**					.05	$F(1,124) = 8.05$***
model 2	-.26**	-.07	-.10			.05	$F\ (3,122) = 3.40$*
model 3	-.24**	-.10	-.10	-.16		.07	$F\ (4,121) = 3.48$*
model 4	-.23**	-.10	-.10	-.17	-.07	.07	$F\ (5,120) = 2.90$*

	TNF (*β*)	age (*β*)	sex (*β*)	PANSS total (*β*)	Antipsychotics (*β*)	*adj.* R^2	*F (df)*
model 1	-.28**					.07	$F\ (1,126) = 10.61$**
model 2	-.28**	-.06	-.10			.07	$F\ (3,124) = 4.27$**
model 3	-.27**	-.09	-.11	-.15		.09	$F\ (4,123) = 4.02$**
model 4	-.26**	-.09	-.10	-.16	-.07	.09	$F\ (5,122) = 3.35$**

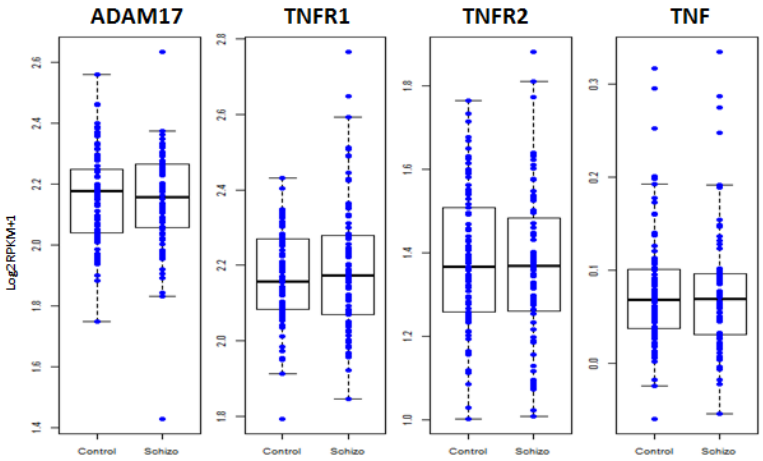
*p<0.05 **p<0.01 ***p<0.001

Abbreviations: TNF=Tumor Necrosis Factor; PANSS=Positive and Negative Syndrome Scale.

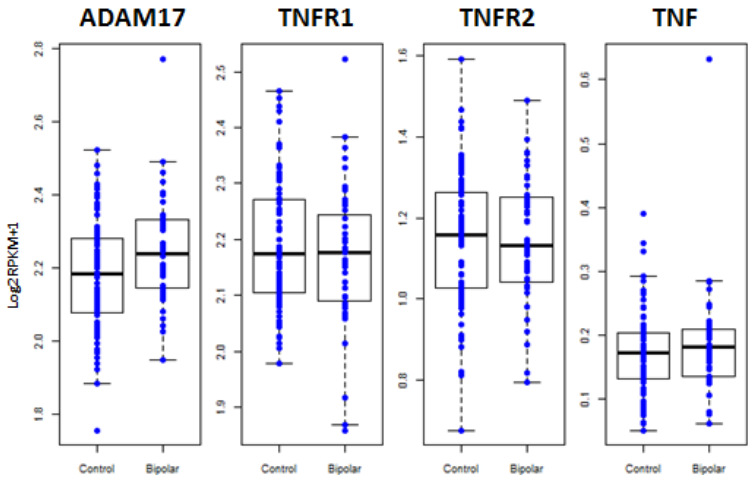
Results are given as standardized β from linear regression analysis.

Supplementary Figure 1. Results from post-mortem prefrontal cortex RNA sequencing.

SCZ vs HC



BD vs HC



No significant difference in *TNFR1*, *TNFR2* and *ADAM17* and *TNF* mRNA expression between patients and controls in the post-mortem sample. SCZ (n=80), BD (n=44) and HC (n=86). Y-axis is Log2RPKM+1 adjusted for principal components, age sex, and mitochondrial mapping rate. FDR is multiple testing correction for expression matrix of n=562 immune genes (elsewhere reported).

Reference to Supplementary Table 3

1. Wallwork RS, Fortgang R, Hashimoto R, Weinberger DR, Dickinson D. Searching for a consensus five-factor model of the Positive and Negative Syndrome Scale for schizophrenia. *Schizophr Res.* 2012;137(1-3):246-250.