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ARTICLE

An activated set point of T-cell and monocyte inflammatory networks in recent-onset schizophrenia patients involves both pro- and anti-inflammatory forces

Roosmarijn C. Drexhage[1], Thomas A. Hoogenboezem[1], Dan Cohen[2,3], Marjan A. Versnel[1], Willem A. Nolen[4], Nico J. M. van Beveren[5] and Hemmo A. Drexhage[1]

[1] *Department of Immunology, Erasmus MC, Rotterdam, The Netherlands*
[2] *GZ-NHN, Heerhugowaard, The Netherlands*
[3] *Department of Epidemiology, University Medical Center, Groningen, The Netherlands*
[4] *Department of Psychiatry, University Medical Center, Groningen, The Netherlands*
[5] *Department of Psychiatry, Erasmus MC, Rotterdam, The Netherlands*

Abstract

We recently described a pro-inflammatory gene expression signature in the monocytes of 60% of patients with recent-onset schizophrenia (SCZ). Here we investigated whether the T-cell system is also in a pro-inflammatory state. A detailed fluorescence-activated cell sorting (FACS) analysis, e.g. of $CD3^+CD25^+$ T cells, IFN-γ^+, IL-4^+, IL-$17A^+$ ($CD4^+$) lymphocytes and $CD4^+CD25^{high}FoxP3^+$ regulatory T cells, was performed on peripheral blood of 26 patients with recent-onset SCZ (in 19 of whom the inflammatory gene expression signature of the monocyte had been determined) and in age-/gender-matched healthy controls. Various relevant T-cell cytokines, e.g. sCD25, IFN-γ, IL-17A and IL-4, were measured in serum by a multiplex assay. We detected: (*a*) not only higher percentages of pro-inflammatory-prone monocytes, activated $CD3^+CD25^+$ T cells and pro-inflammatory Th17 cells in patients, but also higher percentages of anti-inflammatory $CD4^+CD25^{high}FoxP3^+$ regulatory T cells and IL-4^+ lymphocytes; (*b*) that this activated T-cell set point was reflected in significantly raised serum levels of sCD25; (*c*) that the up-regulation of IL-4^+-containing lymphocytes was predominantly found in patients characterized by a monocyte pro-inflammatory set point; and (*d*) that regulatory T-cell and Th17-cell numbers were higher in patients irrespective of the pro-inflammatory state of the monocytes. Our data do not support the concept that the T-cell system is in a simple pro-inflammatory state in recent-onset SCZ, but do show that the monocyte and T-cell networks are activated and involve both pro- and anti-inflammatory forces. This suggests control within an activated inflammatory system.

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Introduction

There is accumulating evidence that activation of the inflammatory response system plays an important role in the pathogenesis of schizophrenia (SCZ). In support of this view we recently described an inflammatory gene expression signature, i.e. a coherent aberrant expression of a set of 34 pro-inflammatory genes, in the circulating monocytes of around 60% of patients with recent-onset SCZ (Drexhage *et al*. 2010*b*).

Apart from cells of the monocyte lineage, T cells are important contributors to the inflammatory response, but literature on T-cell numbers and cytokines is scarce and conjectural in SCZ. Although it has been shown (Denicoff *et al*. 1987) that infusion with IL-2, the well-known growth factor for T cells, is capable of inducing psychosis-related symptoms in psychiatrically

Address for correspondence: Mrs R. C. Drexhage, Department of Immunology, Erasmus MC, PO Box 2040, 3000 CA, Rotterdam, The Netherlands.
Tel.: 0031 10 7044091 *Fax*: 0031 10 7044731
Email: r.drexhage@erasmusmc.nl

healthy individuals, there are at present inconclusive results on the levels of IL-2 or its soluble receptor (sIL-2R, synonymous to sCD25) in the circulation of SCZ patients (Drexhage *et al*. 2010*a*). The same holds true for the blood levels of IFN-γ and IL-4, the hallmark cytokines of the $CD4^+$Th1 and Th2 cell subsets, respectively; higher (Kaminska *et al*. 2001; O'Brien *et al*. 2008), lower (Kim *et al*. 2009) or unaltered (Kim *et al*. 2004) levels of these cytokines and T-cell subsets have been described in SCZ patients.

In recent years a new pro-inflammatory T-cell cytokine IL-17, has been discovered predominantly produced by so-called $CD4^+$ 'Th17 cells' (Bettelli *et al*. 2008). Th17 cells protect the host against bacteria and fungi by activating macrophages via the production of IL-17 (and also IL-21 and IL-22). In contrast, they also play a role in the pathogenesis of autoimmune diseases such as psoriasis and rheumatoid arthritis (Bettelli *et al*. 2008). Functions of Th17 cells are thus similar to those of Th1 cells.

The inflammation-inducing effects of Th17, Th1 cells and of monocytes/macrophages are controlled by a special class of T cells, i.e. the regulatory $CD4^+CD25^{high}FoxP3^+$ T cells (Hori *et al*. 2003; Wing & Sakaguchi, 2010). The main function of these inborn, thymus-derived regulatory T cells is to temper the inflammatory response thereby maintaining homeostasis and tolerance to self-antigens. The cytokines involved in this anti-inflammatory action are thought to be IL-10 and TGF-β. Patients lacking these regulatory T cells due to a genetic mutation in the gene coding for FoxP3 suffer from a severe and rapidly lethal autoimmune syndrome (immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome) (Wildin *et al*. 2002).

The aim of this study was to evaluate T-cell-related inflammatory networks in SCZ following the hypothesis that not only the monocyte system, but also the T-cell system would be in a pro-inflammatory state. We therefore determined the percentages of IL-2r ($CD25^+$) T cells and of IFN-γ-, IL-4- and IL-17A containing blood lymphocytes in the circulation of 26 recent-onset patients, using intracellular staining and fluorescence-activated cell sorting (FACS) analysis. In addition we determined with FACS the percentage of the anti-inflammatory regulatory $CD4^+CD25^{high}FoxP3^+$ T cells.

Following these FACS analyses we evaluated the serum levels of the catabolic pro-inflammatory T-cell cytokines IFN-γ, IL-17A, IL-22 (and also of the monocyte/macrophage cytokines CCL2, TNF-α, IL-1β, PTX3) and of the anabolic or anti-inflammatory cytokines sCD25, TGF-β, IL-10, IL-6, IL-4 and IL-5.

In 19 of the tested patients the monocyte inflammatory gene profiles had been determined in the above-mentioned previously reported study (Drexhage *et al*. 2010*b*). This enabled us to not only relate the T-cell pro-inflammatory and anti-inflammatory state to various clinical variables which we measured in the patients [e.g. the global assessment of functioning (GAF) score, medication, smoking], but also to the pro-inflammatory state of their monocytes.

Methods

SCZ patients

Twenty-six acutely psychotic in-patients with SCZ diagnosed according to DSM-IV criteria were recruited at the department of Psychiatry of the Erasmus Medical Centre in Rotterdam. SCZ was diagnosed according to the DSM-IV criteria after a Comprehensive Assessment of Symptoms and History (CASH) interview (Andreasen *et al*. 1992) and by consensus between two senior psychiatrists who were blinded to the results based on all clinical available evidence. In patients with symptoms for <6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterion. All patients were acutely psychotic on admission. The patients level of functioning on admission and at discharge was determined by the GAF score on the fifth axis of DSM-IV.

The SCZ patients were virtually all (except one) recent-onset cases and had a median duration of illness of only 0.3 yr (median, range 0–3 yr). Absence of severe medical illness (including infections and allergies) was established by medical history assessment, physical examination and routine laboratory testing (Hb, Ht, leukocyte count, blood smear, kidney/liver function) on admission. Almost all patients were receiving antipsychotic medication at the time of blood draw; however, with a low lifetime cumulative haloperidol equivalent (median 42 mg Hal equiv.) (Cahn *et al*. 2002) none of the patients were drug naive.

The demographics of the patients are summarized in Table 1.

Healthy controls (*HC*)

Age- and gender-matched HC (Table 1) were recruited from laboratory staff, medical staff and medical students. The inclusion criteria for HC were an absence of any psychiatric and autoimmune disorder in themselves and in first-degree relatives. HC had to be in self-proclaimed good health and free of any obvious medical illness for at least 2 wk prior to blood draw, including acute infections and allergic reactions.

Table 1. Characteristics of patients with schizophrenia and healthy controls

	Schizophrenia	Healthy controls
Group size	26	26
Age (yr)[a]	24 (17–39)	25 (21–39)
Gender		
Male	23 (88%)	23 (88%)
Female	3 (12%)	3 (12%)
Smoking	18 (69%)	9 (35%)
GAF score[a]		
On admission	21–30 (1–10, 41–50)	
At discharge	51–60 (21–30, 71–80)	
Medication		
None	1	
Typical antipsychotics	13	
Atypical antipsychotics	12	

GAF, Global assessment of functioning. GAF range from 0 to 100, higher scores indicate better functioning.
[a] Median with range.

The Medical Ethical Review Committee of the Erasmus MC Rotterdam approved the study. Written informed consent was obtained from all participants after a complete description of the study had been given.

Laboratory methods

Blood collection and preparation

Blood (drawn in the morning hours) was collected in clotting tubes for serum preparation (stored at −80 °C) and in sodium-heparin tubes for immune cell preparation, on average 20 ml blood was collected from each patient. From the heparinized blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation, as described in detail elsewhere (Knijff *et al*. 2006), within 8 h to avoid activation of the monocytes (erythrophagy). PBMCs were frozen in 10% DMSO and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments performed later.

Flow cytometric analyses

FACS analysis was used to measure intracellular cytokine content in PBMC of patients and age- and gender-matched HC. As hallmark intracellular cytokines we used: IFN-γ, IL-4 and IL-17A. To enable the enumeration of regulatory T cells we intracellularly stained for FoxP3. Membrane staining was done for CD3, CD4, CD25 and CD45RO. This enabled us to assign cytokine staining to the enigmatic Th1, Th2 and Th17 cells in either the total population of $CD4^+$ cells or the memory population. It also enabled us to enumerate the enigmatic regulatory $CD4^+CD25^{high}FoxP3^+$ T-cell population.

For the analysis PBMCs were suspended in complete culture medium. Cell suspensions were then stimulated with PMA (Sigma Aldrich, USA), ionomycin (Sigma Aldrich) in the presence of Golgistop (Becton Dickinson, USA) for 4 h in 37 °C under a 5% CO_2 environment for T effector cells, regulatory T cells were not stimulated.

Cells were harvested and stained extracellularly with anti-CD4 (PerCP-Cy5.5; Becton Dickinson) and anti-CD45RO FITC (Dako, Denmark). For determination of regulatory T cells, non-stimulated PBMC samples were stained with anti-CD3 (PerCP; BD Biosciences, USA), anti-CD4 (APC; BD Biosciences) and anti-CD25 (FITC; BD Biosciences) according to standard protocol.

Following extracellular staining, the cells were fixed and permeabilized according to the manufacturer's instructions (eBioscience, USA) and then stained for FoxP3 (PE; Becton Dickinson), IL-4 (PE; Becton Dickinson), IFN-γ (APC; Becton Dickinson), IL-17A (PE; eBioscience).

Isotype antibody controls were used to confirm antibody specificity for CD25. Stained cells were analysed by four-colour flow cytometry (FACSCalibur, BD Biosciences) as described previously and analysed using FlowJo (Tree Star Inc., USA) research software. Supplementary Fig. 1 (available online) gives the dot plots of the stainings and definition of cell populations.

mRNA gene expression in monocytes

The definition and determination of mRNA gene expression fingerprints in monocytes has been previously described in detail (Drexhage *et al*. 2010*b*).

Serum cytokine determinations

Serum cytokines (IFN-γ, IL-17, IL-10, IL-6, IL-4, TNF-α, CCL2, IL-5, IL-1β, PTX3) were measured using the Cytometric Bead Array kits (BenderMedSystems, USA) according to the manufacturer's protocol. Bead flow cytometry allows the simultaneous quantification of various proteins in the same test (http://www.bendermedsystems.com/bm_files/47/BM_Factsheet_3_A4_SCREEN.pdf). Twenty-five μl of serum per test were used. Samples were analysed in a FACSCanto flow cytometer (BD Biosciences) using the

FlowCytomix Pro 2.3 Software (BenderMedSystems). Results are expressed as picograms per millilitre.

For TGF-β (Diaclone, France) and sIL-2r (R & D Systems, USA) commercially available ELISAs were used according to the manufacturer's protocol.

Statistics

Statistical analysis was performed using the SPSS 15.0 package for Windows (SPSS Inc., USA). Data were tested for normal distribution using the Kolmogorov–Smirnov test. Parametric tests were used when the data had a normal distribution. When normal distribution was not reached, data were log-transformed. If the data were still skewed after log-transformation, non-parametric tests were used. In the text, tables and figures the original data have been used. Levels of significance were set at $p=0.05$ (two-tailed). The specific tests used are mentioned in the tables and figure legends.

Results

Percentages of monocytes and lymphocytes

FACS analysis of the samples showed that the percentage of monocytes was higher in SCZ samples compared to those of the gender- and age-matched HC (SCZ: 24.33 ± 7.91; HC: 20.64 ± 4.52; $p=0.049$, Mann–Whitney test). Although the percentage of lymphocytes was smaller in the SCZ samples compared to the HC, values did not reach statistical significance (SCZ: 70.97 ± 7.90; HC: 75.08 ± 5.02; $p=0.077$, Mann–Whitney test). Blood leukocyte counts were within the normal range on admission for all patients (routine clinical determination, mean $6.4\pm1.5\times10.9$/l, reference range for our centre: 3.5–10.0×10.9/l).

IFN-γ-, IL-4- and IL-17A-containing lymphocytes

Using FACS we first analysed the capability of the total lymphocyte fraction to produce IFN-γ, IL-4 and IL-17A. After stimulation with PMA and ionomycin for 4 h (a procedure to re-activate lymphocytes), the percentages of IFN-γ- and IL-17A-containing lymphocytes were not different between SCZ samples and HC samples: IFN-γ (SCZ: 24.47 ± 7.60; HC: 27.63 ± 9.45; $p=0.199$); IL-17A (SCZ: 0.39 ± 0.29; HC: 0.27 ± 0.12; $p=0.137$). However, lymphocytes capable of producing IL-4 were more numerous in SCZ patients compared to lymphocytes of HC (Fig. 1*a*). Differences could not be detected with regard to the mean fluorescence intensity (MFI), which reflects the actual content of the cytokine per lymphocyte.

$CD3^+CD25^+$ cells

Activation of T cells is classically determined by CD25 (IL-2r) expression, illustrating the higher capability of T cells to react to the growth factor IL-2. Percentages of $CD3^+CD25^+$ T cells were higher in SCZ patients compared to HC (Fig. 1*c*). Moreover, serum levels of the shed receptor, sCD25 (=sIL-2r), were also higher in SCZ patients and correlated positively with $CD3^+CD25^+$ T cells ($r=0.394$, $p=0.007$) and IL-4-containing lymphocytes ($r=0.462$, $p=0.001$).

Classical Th1 ($CD4^+$IFN-γ^+), Th2 ($CD4^+$IL-4^+), Th17 ($CD4^+$IL-$17A^+$) and regulatory $CD4^+CD25^{high}FoxP3^+$ T cells

We subsequently studied the percentages of classical Th1 cells ($CD4^+$ T cells capable of producing IFN-γ), classical Th2 cells ($CD4^+$ T cells capable of producing IL-4) or classical Th17 cells ($CD4^+$ T cells capable of producing IL-17A).

The percentage of classical Th1 cells was not different in SCZ patients (SCZ: 7.55 ± 4.19; HC: 7.30 ± 3.50; $p=0.905$), while that of classical Th2 cells showed a trend to be higher in line with the finding that IL-4-producing lymphocytes were higher (Fig. 1*b*).

Statistically, higher percentages of classical Th17 cells were found in the circulation of SCZ patients compared to that of HC (Fig. 1*d*). Virtually all these Th17 cells were of memory type, i.e. they were $CD45RO^+$.

Figure 1*e* also shows that $CD4^+CD25^{high}FoxP3^+$ T-cell (regulatory T cells) percentages were higher in SCZ patients compared to HC (Fig. 1*e*). There was a strong positive correlation between the percentage of regulatory T cells and the serum levels of sIL-2R in the patients ($r=0.447$, $p=0.002$).

Correlations between the different T-cell subsets (Table 2)

The percentages of $CD3^+CD25^+$ cells correlated significantly with the percentages of regulatory T cells, classical Th1 cells and Th17 cells, but not with Th2 cells. Percentages of Th17 cells correlated with those of Th1 cells, but not with Th2 cells. The percentages of regulatory T cells did not correlate with those of Th17 cells. There was also no correlation for regulatory T cells and Th1 or Th2 cells. Although the correlation coefficient was negative for the correlation between Th1 and Th2 cells (as could be expected from the reciprocal character of the subsets), values did not reach statistical significance.

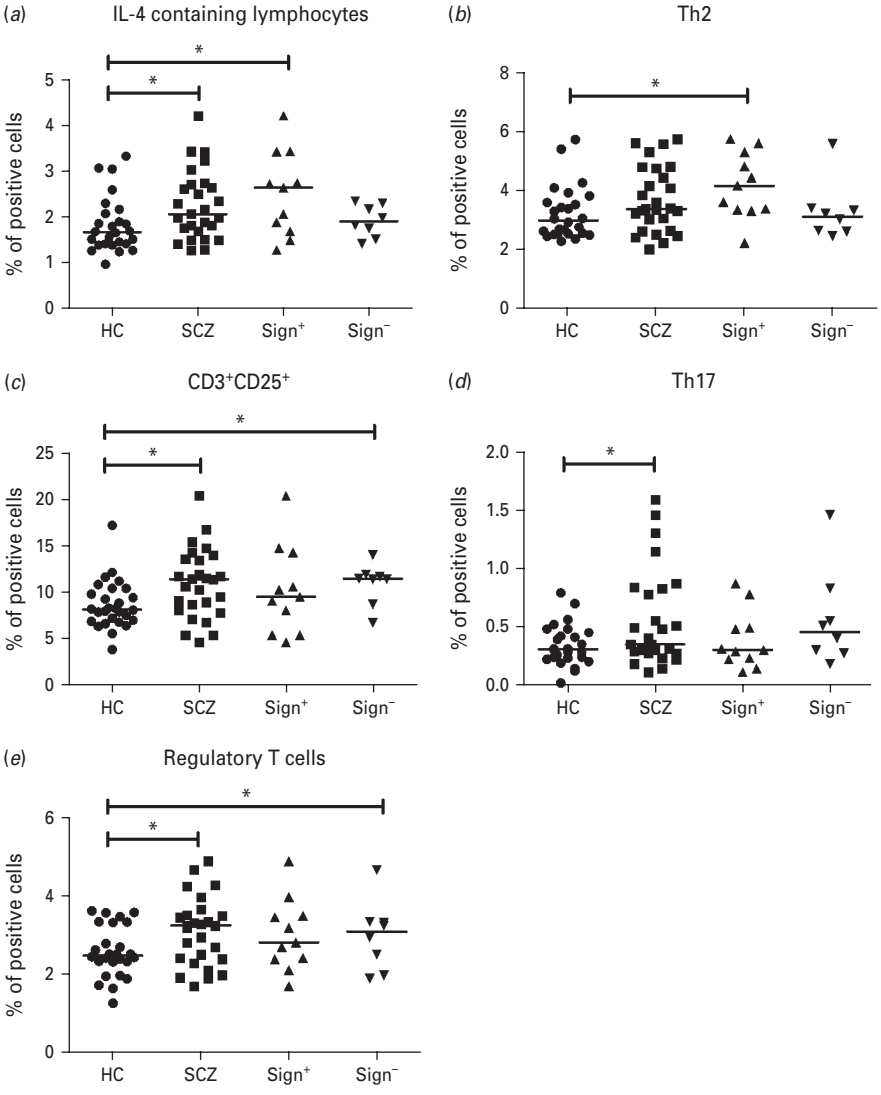

Fig. 1. Fluorescence-activated cell sorting (FACS) analysis data of the different subsets tested. Each symbol represents an individual patient, the bar represents the median. * $p<0.05$; HC, healthy controls; SCZ, schizophrenia; Sign$^+$, SCZ patients with a positive signature; Sign$^-$, SCZ patients with a negative signature. For the comparison of HC *vs.* SCZ p values were tested by linear regression on log-transformed data with age and gender in the model. For the comparison of signature-positive *vs.* signature-negative *vs.* HC p values were tested by linear regression on log-transformed data. Positive monocyte mRNA gene expression inflammatory signature is defined as $\geqslant 9$ genes out of 20 tested genes positive; positivity of a gene is defined as >1 standard deviation (S.D.) higher expressed than the mean expression of the tested gene in HC. Percentages expressed as mean $\pm$ S.D. IL-4-containing lymphocytes (SCZ: 2.27 ± 0.76; HC: 1.82 ± 0.61, $p=0.015$; Sign$^+$: 2.51 ± 0.93; Sign$^-$: 1.91 ± 0.35, $p=0.013$). Th2 (SCZ: 3.65 ± 1.14; HC: 3.23 ± 0.90, $p=0.124$; Sign$^+$: 4.18 ± 1.12; Sign$^-$: 3.29 ± 1.00, $p=0.006$). CD3$^+$CD25$^+$ T cells (SCZ: 11.01 ± 3.79 ; HC: 8.32 ± 2.01, $p=0.002$; Sign$^+$: 10.25 ± 4.75 ; Sign$^-$: 10.95 ± 2.25, $p=0.029$). Th17 (SCZ: 0.56 ± 0.42; HC: 0.34 ± 0.18, $p=0.042$; Sign$^+$: 0.38 ± 0.25; Sign$^-$: 0.56 ± 0.41). CD4$^+$CD25^{high}FoxP3$^+$ T cell (SCZ: 3.01 ± 0.84; HC: 2.57 ± 0.65, $p=0.032$; Sign$^+$: $3.01\pm3.30\pm0.85$, $p=0.043$).

Pro-inflammatory and anti-inflammatory cytokines in serum

Levels of IFN-γ, IL-17, IL-10, IL-6, IL-4 and TNF-α were lower than the detection limit in the serum of patients and controls (Table 3).

Levels of CCL2, IL-5, IL-1β and PTX3 were detectable, but levels were not different in the serum of SCZ patients compared to those of HC. Levels of the Th17-related cytokine IL-22 and of the regulatory T-cell-related cytokine TGF-β were higher in the serum of SCZ patients;

Table 2. Correlations between the different T-cell subsets

Th17	Lymphocytes	Monocytes	CD3CD25$^+$	IL4-containing lymphocytes	Th2	Regulatory T cells		
0.411**	0.202	−0.205	0.460**	0.170	−0.218	0.052	*r*	Th1
0.002	0.155	0.149	0.001	0.223	0.116	0.712	*p*	
	0.081	−0.089	0.441**	0.092	−0.020	0.089	*r*	Th17
	0.571	0.534	0.001	0.512	0.889	0.526	*p*	
		−0.961**	−0.255	−0.097	−0.223	−0.075	*r*	Lymphocytes
		0.000	0.071	0.499	0.116	0.600	*p*	
			0.200	0.063	0.189	0.054	*r*	Monocytes
			0.160	0.662	0.184	0.705	*p*	
				0.548**	0.232	0.516**	*r*	CD3CD25$^+$
				0.000	0.094	0.000	*p*	
					0.653**	0.397**	*r*	IL4-containing
					0.000	0.003	*p*	lymphocytes
						0.123	*r*	Th2
						0.382	*p*	

r = correlation coefficient; *p* values were tested by Pearson's correlation on log-transformed data.

however, levels did not reach statistical significance (Table 3).

We were unable to find correlations between the cytokines listed in this section and the percentages of the various T-cell subsets.

Relation of T-cell inflammatory parameters to clinical variables

We were unable to find any correlation between the T-cell variables reported here and antipsychotic drugs, smoking, positive symptoms, negative symptoms or GAF score on admission.

A positive correlation was found between the level of functioning of SCZ patients at discharge and their regulatory T-cell percentages on admission (Fig. 2). Patients with regulatory T-cell percentages $>3.25\%$ ($n=16$) on admission all had a GAF score of $\geqslant 50$ at discharge, whereas all patients ($n=7$) with regulatory T-cell percentages $<3.25\%$ had a GAF score of $\leqslant 49$ at discharge (GAF score at discharge for three patients was not available). This led to a positive correlation between regulatory T-cell percentage on admission and GAF score at discharge ($r=0.442$, $p=0.035$, with Bonferroni correction for six tests statistical significance was not reached).

Relation of T-cell inflammatory parameters to the pro-inflammatory state of patient monocytes

Samples of 19 of the monocyte pro-inflammatory signature-tested patients could also be used in the present study and in 11/19 of these patients the signature was present compared to 3/23 of the HC [see legend of Supplementary Fig. 2 (online) for definition of a positive signature].

The higher percentages of IL-4-containing lymphocytes and of classical Th2 cells found in our SCZ patients were largely confined to SCZ patients with a positive monocyte pro-inflammatory signature (Fig. 1*a*, *b*, Supplementary Fig. 2).

The higher percentages of Th17 cells, regulatory T cells and CD3$^+$CD25$^+$ cells were found in both monocyte signature-positive and signature-negative SCZ patients. In the signature-negative patients significantly higher percentages of regulatory T cells and CD3$^+$CD25$^+$ cells were found (Fig. 1*c*–*e*).

Discussion

This study shows a strongly activated set point of the T-cell and monocyte inflammatory network in patients with recent-onset SCZ. On the one hand an activation of pro-inflammatory forces was found, i.e. higher percentages of circulating monocytes with a pro-inflammatory gene expression signature, activated (CD3$^+$CD25$^+$) T cells and pro-inflammatory Th17 cells, on the other hand there was also an activation of anti-inflammatory forces, i.e. increases in CD4$^+$ CD25^{high}FoxP3$^+$ T cells and IL-4-containing lymphocytes.

The up-regulation of IL-4-containing lymphocytes and of classical Th2 cells, i.e. the CD4$^+$ population of IL-4-containing lymphocytes, was predominantly found in SCZ patients characterized by a monocyte

Table 3. Cytokine levels in serum

		Schizophrenia	Healthy controls	*p* value
Monocyte activation	CCL2	33.39 (12.46–60.30)	25.74 (14.10–60.90)	0.495
	IL-1β	0.02 (0–0.55)	0.05 (0–2.55)	0.447
	PTX3	66.50 (7.36–381.49)	73.07 (13.90–368.80)	0.493
	IL-6	n.d.	n.d.	
	TNFα	n.d.	n.d.	
T-cell proliferation activity	sIL-2R	1960 $\pm$ 593	1496 $\pm$ 473	0.009
Th1	IFN-γ	n.d.	n.d.	
Th2	IL-5	0.05 (0–0.41)	0.01 (0–2.69)	0.281
	IL-4	n.d.	n.d.	
Th17	IL-22	47.43 (41.17–94.38)	45.34 (41.17–59.98)	0.265
	IL-17a	n.d.	n.d.	
Regulatory T cells	TGF-β	13 872 (7602–68 981)	12 655 (4761–23 198)	0.204
	IL-10	n.d.	n.d.	

n.d., Not detected.

Levels of cytokines are expressed as median with range. All values are in picograms per millilitre. Normal distribution was not obtained after log-transformation. The *p* values were calculated by Mann–Whitney tests.

sIL-2r had a normal distribution and the level is expressed as mean $\pm$ standard deviation in IU/ml. The *p* value was calculated by ANOVA with age and gender in the model.

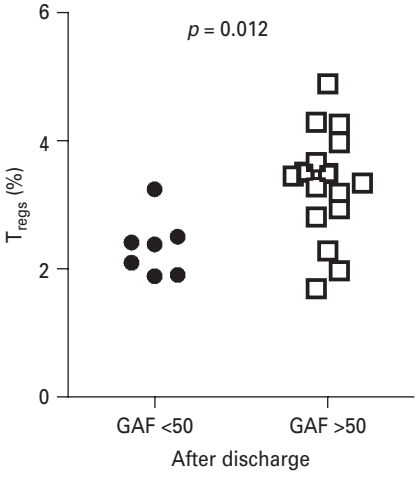

Fig. 2. Relation of regulatory T cell percentage on admission and GAF score at discharge. GAF, Global assessment of functioning; % T_{regs}, percentage of $CD4^+CD25^{high}FoxP3^+$ cells on admission; *p* values were tested by linear regression on log-transformed values ($p = 0.012$, d.f. = 21).

pro-inflammatory gene expression set point. This constellation of the co-occurrence of pro-inflammatory-prone monocytes with higher percentages of IL-4-containing lymphocytes is counterintuitive, since IL-4 dampens the inflammatory state of monocytes and macrophages, turning the cells in so-called M2 cells (Mantovani *et al*. 2004). We interpret our data by hypothesizing that the higher percentage of IL-4-containing lymphocytes in these monocyte gene expression signature-positive patients serves as a control mechanism to counteract monocyte susceptibility for inflammation by keeping an actual higher production of inflammatory monocyte cytokines under control. This interpretation might then explain the normal serum levels of the pro-inflammatory monocyte/macrophage cytokines CCL2, IL-1β and PTX3 we found in our patients. On the other hand, preliminary data of our group (to be published) in a cohort of older (on average 40 yr) patients with chronic naturalistically treated SCZ showed a much lower inflammatory monocyte gene expression than reported here for acute SCZ, while serum levels of inflammatory cytokines were clearly raised in the group of older patients, but – apart from disease – also linked to obesity and serum lipid levels. This indicates a clear and incongruent role for disease duration, treatment, activity and/or diet/obesity in the inflammatory profiles of circulating cells and cytokine levels. This indicates further studies for biomarker development being required to control for these variables, unfortunately in this study we were had no information on metabolic parameters of patients and controls and were therefore unable to adjust for this.

Increases in both the pro-inflammatory subset of Th17 cells and in the anti-inflammatory subset of regulatory T cells in SCZ patients were found irrespective of the monocyte pro-inflammatory state (although a little more obvious in the negative signatures). Increases in the regulatory T cells and Th17

cells also correlated with increased numbers of $CD3^+CD25^+$ T cells and higher levels of serum sCD25. Although we interpret these CD25 data as reflecting a higher tendency for T-cell proliferation, which might then underlie the higher percentages of Th17 and regulatory T cells, there are data in the literature which show that T-cell proliferation is in fact reduced in acute forms of SCZ when T cells were stimulated with anti-CD3 (Craddock *et al*. 2007). That study also found a normal expression of CD25 on $CD4^+$T cells in SCZ patients. Further studies are needed to clarify this issue.

There is strong evidence that Th17 cells are inflammation-promoting cells in chronic inflammatory conditions such as rheumatoid arthritis, asthma, psoriasis and multiple sclerosis and the number of Th17 cells as well as the levels of the Th17-derived cytokines IL-17, IL-21 and IL-22 are higher in affected tissues (Crome *et al*. 2010). Although SCZ patients do have molecular alterations in their brain vasculature (Harris *et al*. 2008), it is unknown whether Th17 cells or the Th17 cytokines pass the blood–brain barrier to exert an effect. In a mouse model of multiple sclerosis Th17 cells do migrate into the brain (Reboldi *et al*. 2009). Studies on post-mortem brains of SCZ patients and animal models of SCZ-like disease could prove instrumental in solving this question of Th17 influence on behaviour.

There is ample evidence that regulatory T cells are capable of dampening the effect of Th17 cells (Crome *et al*. 2010), Th1 cells (Wing & Sakaguchi, 2010) and pro-inflammatory monocytes (Tiemessen *et al*. 2007) in immune reactions. It is therefore tempting to hypothesize that the increase in anti-inflammatory regulatory T cells is meant (like that proposed for the IL-4-containing lymphocytes) to keep under control the pro-inflammatory immune forces in SCZ patients.

Another point of interest with regard to both the rise in Th17 cells and regulatory T cells is that the development of regulatory T cells has been reported to be tightly linked to that of Th17 cells. Both cell lineages share common developmental pathways (Bettini & Vignali, 2009; Crome *et al*. 2010). Also Foxp3-lineage-committed regulatory T cells were directly converted into IL-17-expressing cells by CpG treatment and interestingly IDO acts as a pivotal molecular switch in this process. Only in the absence of IDO does this conversion occur, whereas it was blocked when IDO was active (Baban *et al*. 2009). Although IDO activation and abnormal tryptophan breakdown is thought to play a role in SCZ, we were unable to find in the present study a correlation between percentages of regulatory T cells and those of Th17 cells.

With regard to clinical characteristics we found that a high regulatory T-cell percentage on admission correlated with a better clinical outcome (GAF score) at discharge. Interestingly, in this respect, is that IL-10, one of the key cytokines produced by regulatory T cells, has direct effects on brain function and is able to dampen down sickness behaviour (Richwine *et al*. 2009). However, effects of regulatory T cells could also be mediated by direct suppression of the pro-inflammatory immune forces in the brain, e.g. those exerted by pro-inflammatory microglia.

However, and with regard to this clinical effect, a limitation of our study is that it was not designed to investigate parameters of prediction and p values did not reach significance with Bonferroni correction. Suffice to say that a study on the predictive value of regulatory T cells for outcome in psychosis in a larger group of patients is now imperative.

Our study also has other limitations. We only used peripheral blood samples and we were not able to investigate the cerebrospinal fluid (CSF). Pathological abnormalities of the brain are probably directly reflected in the CSF, and indeed in SCZ there are reports on cell aberrations in CSF. A recent paper investigated T-cell subsets in CSF and peripheral blood of patients with a major psychiatric disorder and found an overlapping low-grade inflammation in CSF as well as in peripheral blood (Maxeiner *et al*. 2008). Unfortunately, we were unable to obtain CSF samples from our patients.

Furthermore, our study has a small sample size and outcomes should be replicated on a larger and independent sample size. The study subjects were the same in which the monocyte signature was determined, and it could be that this sample of SCZ patients has an extreme activation of the immune system. However, we have found a similar strong inflammatory monocyte signature in patients with mania (Drexhage *et al*. 2010*b*; Padmos *et al*. 2008) and in postpartum psychosis (V. Bergink, unpublished observations). In the latter conditions the T-cell system was not activated (V. Bergink, unpublished observations).

Our study is naturalistic and all patients (except one) were on very recently started antipsychotic medication. As antipsychotic medication exerts anti-inflammatory effects (Drzyzga *et al*. 2006; Pollmacher *et al*. 2000; Rybakowski, 2000), this effect on outcome cannot be ruled out (Drexhage *et al*. 2010*b*; Padmos *et al*. 2008). Moreover, there were more male participants than female participants in this study. Although HC were age- and gender-matched, we cannot completely rule out a general effect of gender on the activation state of monocytes and/or T cells. Moreover,

we cannot rule out a general effect of smoking in this study sample because there was a difference in the smoking status of the patients (69%) *vs.* our HC (35%). However, we did not find a correlation for smoking with one of the other immune parameters.

A further limitation is that our FACS data represent percentages, not the number, of cells within the lymphocyte population. We were unable to give absolute cell numbers, since we had not performed a leukocyte count in the same blood sample as used for FACS, although leukocyte counts routinely performed in the patients at the same time did not show abnormalities.

Taken together, our data do not support the concept that the T-cell system is in a simple pro-inflammatory state (our hypothesis), but do show that the monocyte and T-cell networks are activated in recent-onset SCZ and involve both pro- and anti-inflammatory forces.

The question remains as to when and why these activated set points are installed and what the consequences are for the pathology of SCZ. Here the animal models of the disease might become important tools for further study. In rodents chronic immune activation in the peri-natal period by reagents mimicking infections with commensal bacteria and viruses (such as LPS and GpC) do lead to altered set points of the inflammatory system which cause abnormalities in brain development and consequent psychosis-related behaviour (Makinodan *et al*. 2008; O'Mahony *et al*. 2009). Further studies in these animal models into aberrant set points of their macrophage/T-cell system are indicated.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

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References

Andreasen NC, Flaum M, Arndt S (1992). The Comprehensive Assessment of Symptoms and History (CASH). An instrument for assessing diagnosis and psychopathology. *Archives of General Psychiatry* **49**, 615–623.

Baban B, Chandler PR, Sharma MD, Pihkala J, *et al.* (2009). IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. *Journal of Immunology* **183**, 2475–2483.

Bettelli E, Korn T, Oukka M, Kuchroo VK (2008). Induction and effector functions of T(H)17 cells. *Nature* **453**, 1051–1057.

Bettini M, Vignali DA (2009). Regulatory T cells and inhibitory cytokines in autoimmunity. *Current Opinion in Immunology* **21**, 612–618.

Cahn W, Hulshoff Pol HE, Lems EB, van Hare NE, *et al.* (2002). Brain volume changes in first-episode schizophrenia: a 1-year follow-up study. *Archives of General Psychiatry* **59**, 1002–1010.

Craddock RM, Lockstone HE, Rider DA, Wayland MT, *et al.* (2007). Altered T-cell function in schizophrenia: a cellular model to investigate molecular disease mechanisms. *PLoS ONE* **2**, e692.

Crome SQ, Wang AY, Levings MK (2010). Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. *Clinical & Experimental Immunology* **159**, 109–119.

Denicoff KD, Rubinow DR, Papa MZ, Simpson C, *et al.* (1987). The neuropsychiatric effects of treatment with interleukin-2 and lymphokine-activated killer cells. *Annals of Internal Medicine* **107**, 293–300.

Drexhage RC, Knijff EM, Padmos RC, Heul-Nieuwenhuijzen L, *et al.* (2010*a*). The mononuclear phagocyte system and its cytokine inflammatory networks in schizophrenia and bipolar disorder. *Expert Reviews of Neurotherapeutics* **10**, 59–76.

Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, van Beveren N, *et al.* (2010*b*). Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *International Journal of Neuropsychopharmacology* **13**, 1369–1381.

Drzyzga L, Obuchowicz E, Marcinowska A, Herman ZS (2006). Cytokines in schizophrenia and the effects of

antipsychotic drugs. *Brain, Behaviour, and Immunity* **20**, 532–545.

Harris LW, Wayland M, Lan M, Ryan M, *et al.* (2008). The cerebral microvasculature in schizophrenia: a laser capture microdissection study. *PLoS ONE* **3**, e3964.

Hori S, Nomura T, Sakaguchi S (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.

Kaminska T, Wysocka A, Marmurowska-Michalowska H, Dubas-Slemp H, *et al.* (2001). Investigation of serum cytokine levels and cytokine production in whole blood cultures of paranoid schizophrenic patients. *Archivum Immunologiae et Therapiae Experimentalis* (*Warsz*) **49**, 439–445.

Kim YK, Myint AM, Lee BH, Han CS, *et al.* (2004). Th1, Th2 and Th3 cytokine alteration in schizophrenia. *Progress in Neuropsychopharmacology and Biological Psychiatry* **28**, 1129–1134.

Kim YK, Myint AM, Verkerk R, Scharpe S, *et al.* (2009). Cytokine changes and tryptophan metabolites in medication-naive and medication-free schizophrenic patients. *Neuropsychobiology* **59**, 123–129.

Knijff EM, Breunis MN, van Geest MC, Kupka RW, *et al.* (2006). A relative resistance of T cells to dexamethasone in bipolar disorder. *Bipolar Disorders* **8**, 740–750.

Makinodan M, Tatsumi K, Manabe T, Yamauchi T, *et al.* (2008). Maternal immune activation in mice delays myelination and axonal development in the hippocampus of the offspring. *Journal of Neuroscience Research* **86**, 2190–2200.

Mantovani A, Sica A, Sozzani S, Allavena P, *et al.* (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology* **25**, 677–686.

Maxeiner H, Rojewski M, Schmitt A, Tumani H, *et al.* (2008). Flow cytometric analysis of T cell subsets in paired samples of cerebrospinal fluid and peripheral blood from patients with neurologocal and psychiatric disorders. *Brain, Behavior, and Immunity* **23**, 134–142.

O'Brien SM, Scully P, Dinan TG (2008). Increased tumor necrosis factor-alpha concentrations with interleukin-4 concentrations in exacerbations of schizophrenia. *Psychiatry Research* **160**, 256–262.

O'Mahony SM, Marchesi JR, Scully P, Codling C, *et al.* (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological Psychiatry* **65**, 263–267.

Padmos RC, Hillegers MH, Knijff EM, Vonk R, *et al.* (2008). A discriminating messenger RNA signature for bipolar disorder formed by an aberrant expression of inflammatory genes in monocytes. *Archives of General Psychiatry* **65**, 395–407.

Pollmacher T, Haack M, Schuld A, Kraus T, *et al.* (2000). Effects of antipsychotic drugs on cytokine networks. *Journal of Psychiatric Research* **34**, 369–382.

Reboldi A, Coisne C, Baumjohann D, Benvenuto F, *et al.* (2009). C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nature Immunology* **10**, 514–523.

Richwine AF, Sparkman NL, Dilger RN, Buchanan JB, *et al.* (2009). Cognitive deficits in interleukin-10-deficient mice after peripheral injection of lipopolysaccharide. *Brain, Behaviour, and Immunity* **23**, 794–802.

Rybakowski JK (2000). Antiviral and immunomodulatory effect of lithium. *Pharmacopsychiatry* **33**, 159–164.

Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, *et al.* (2007). CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proceedings of the National Academy of Sciences USA* **104**, 19446–19451.

Wildin RS, Smyk-Pearson S, Filipovich AH (2002). Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *Journal of Medical Genetics* **39**, 537–545.

Wing K, Sakaguchi S (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nature Immunology* **11**, 7–13.