Clinical Study

The Phenotype of Circulating Follicular-Helper T Cells in Patients with Rheumatoid Arthritis Defines CD200 as a Potential Therapeutic Target

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Rheumatoid arthritis (RA) is a systemic autoimmune disease primarily affecting synovial joints in which the development of autoantibodies represents a failure of normal tolerance mechanisms, suggesting a role for follicular helper T cells (T_{FH}) in the genesis of autoimmunity. To determine whether quantitative or qualitative abnormalities in the circulating T_{FH} cell population exist, we analysed by flow cytometry the number and profile of these cells in 35 patients with RA and 15 matched controls. Results were correlated with patient characteristics, including the presence of autoantibodies, disease activity, and treatment with biologic agents. Circulating T_{FH} cells from patients with RA show significantly increased expression of the immunoglobulin superfamily receptor CD200, with highest levels seen in seropositive patients ($P = 0.0045$) and patients treated with anti-TNFα agents ($P = 0.0008$). This occurs in the absence of any change in T_{FH} numbers or overt bias towards Th1, Th2, or Th17 phenotypes. CD200 levels did not correlate with DAS28 scores ($P = 0.887$). Although the number of circulating T_{FH} cells is not altered in the blood of patients with RA, the T_{FH} cells have a distinct phenotype. These differences associate T_{FH} cells with the pathogenesis of RA and support the relevance of the CD200/CD200R signalling pathway as a potential therapeutic target.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterised by inflammation of synovial joints [1]. The aetiology of RA is both complex and poorly understood, and while the formation of autoantibodies such as rheumatoid factors (RFs) or anticitrullinated protein antibodies (ACPAs) is common [2, 3], their role in disease pathogenesis remains unclear. Autoantibodies in RA are usually of the IgG subclass and demonstrate high affinity for their targets, characteristics consistent with production by B-cells that have undergone T-cell-dependent germinal centre (GC) maturation [4]. A role for CD4$^+$ T cells in disease development is further supported by their presence in the synovium of affected patients, often with evidence of ectopic germinal centre formation RA [5] and the association of RA with HLA-DR4 [6, 7].

Within germinal centres, the fate of developing B cells is determined by their ability to present antigen to a specialised subset of CD4$^+$ T cells termed follicular helper T cells (T_{FH}), located in the B-cell follicle by virtue of expression of the chemokine receptor CXCR5 [8]. Through a combination of cytokine secretion and highly regulated cell-cell interactions, T_{FH} cells guide the maturation of B cells, facilitating class-switching and somatic hypermutation [9]. T_{FH} cells also provide critical censoring functions, withdrawing help

TABLE 1: Characteristics of the study population*.

Characteristic	Seropositive ($n = 20$)	Seronegative ($n = 15$)	Controls ($n = 15$)	P values
Age	59.4 (33.2–90.4)	60.2 (32.7–80.9)	56.3 (22.4–84.8)	0.87
% Male	50	46.7	53.3	0.94
Lymphocytes†	1.94 (0.84–3.26)	1.95 (0.67–3.18)	2.1 (1.0–3.24)	0.95
% CD4$^+$	50.2 (26.8–66.9)	56.2 (37.6–68.3)	44.43 (28.5–65.4)	0.02
% CD8$^+$	22.3 (8.21–59.1)	17.3 (4.53–51.4)	25.97 (11.2–39.6)	0.03
CRP (mg/L)	33.7 (2–132)	30.29 (2–101)	N/A	0.86
ESR (mm/hr)	24.8 (2–105)	22.2 (1–84)	N/A	0.75
Creatinine (μmol/L)	80.4 (49.3–105.2)	82.8 (50.4–139.7)	N/A	0.95
DAS-28 (CRP)	4.20 (0.97–6.66)	4.74 (2.10–8.63)	N/A	0.84
Duration of RA	52.6 (1–232)	46.5 (1–135)	N/A	0.87
Steroid therapy	6/20	6/15	N/A	0.72
DMARD therapy	16/20	9/15	N/A	0.14
Anti-TNF therapy‡	9/20	6/15	N/A	0.77

* values shown are the mean and range (brackets).
† $\times 10^6$/mL.
‡ 5 patients received etanercept (3 in the seropositive and 2 in the seronegative group); the remaining patients received adalimumab.

from B cells with autoreactive potential thereby preventing autoimmunity [10, 11]. A central role for T_{FH} cells in the development of autoimmune diseases has been confirmed in animal models, where dysregulated T_{FH} function can promote autoantibody formation [12, 13], and in humans, with increased T_{FH} cell numbers identified in some patients with SLE and RA and an altered T_{FH} phenotype demonstrable in patients with juvenile dermatomyositis [14–17].

One of the difficulties of systematically studying T_{FH} cells in human autoimmune conditions is that historically T_{FH} cells were defined not only by the receptors they express but also by their anatomical location: secondary lymphoid organs, making routine analysis of these cells impractical [8, 18]. However, recently circulating populations of T-helper cells that express CXCR5 and have similar functionality to tissue-resident T_{FH} cells (provision of B-cell help, expression of the transcription factor Bcl6 and the cytokine IL-21) have been defined [16, 17]. Analysis of these cells therefore provides an opportunity to interrogate the T_{FH} compartment through sampling of peripheral blood.

To determine whether T_{FH} cells might be relevant to the pathogenesis of RA, we examined whether quantitative or qualitative abnormalities exist in the circulating T_{FH} population in patients with RA and whether these differences might be more pronounced in seropositive patients (the presence of class-switched autoantibodies being indicative of T_{FH} cell-induced maturation). In contrast to previous work, we did not find increased numbers of circulating T_{FH} cells in patients with RA; however the phenotypic profile of these cells was abnormal, with increased expression of the inhibitory receptor CD200. Improved understanding of the spatial and temporal regulation of stimulatory and inhibitory receptors present on T_{FH} cells may provide new insights into the development of autoimmunity in RA.

2. Materials and Methods

2.1. Patient Recruitment and Clinical Samples. Patients attending rheumatology and orthopaedic clinics were recruited to donate whole blood following written informed consent. Healthy controls were recruited through advertisement and donated blood following written informed consent. Research was conducted in accordance with the Declaration of Helsinki. Ethical approval for the study was granted by the Berkshire Research Ethics Committee (REC reference 08/H0607/50). A total of 50 subjects were recruited (35 patients with RA and 15 controls). All patients fulfilled the American Rheumatological Association's criteria for the diagnosis of RA [19], and disease activity scores (DAS28-CRP) were recorded for each patient at the time of recruitment. Patients with RA were further subdivided for analysis based on the presence of circulating autoantibodies and treatment with anti-TNFα agents. 20 patients were autoantibody positive; 19 had rheumatoid factor, 8 had ACPA, and 7 patients were positive for both. Characteristics of the study population are shown in Table 1.

2.2. Reagents. Directly conjugated anti-human antibodies against CD3, CD4, CXCR5, CD45RO, CD69, CD95, CD134

(OX-40), ICOS, CD200, CD150, CXCR3, CCR6, and HLA-DR were purchased from BD Biosciences (San Jose, California).

2.3. Phenotypic Analysis of T_{FH} Cells from Whole Blood.

3 mL of whole blood was washed twice with 10 mL PBS (Fisher Scientific) before centrifugation at 400 g for 5 minutes. Following aspiration of the PBS/plasma, 100 μL aliquots were transferred into polystyrene FACS tubes (BD Biosciences) for staining. Directly conjugated surface antibodies were added and cells incubated at 4°C for 30 minutes in the dark. Red blood cells were lysed with 2 mL of BD Lyse/Fix (BD Biosciences) for 10 minutes at 37°C. Samples were then washed in 3 mL of 2% BSA (Fisher Scientific)/PBS and resuspended in 150 μL of 2% Paraformaldehyde/PBS before acquisition on a BD FACSCanto flow cytometer.

2.4. Serum Exchange Experiments.

Serum was aspirated from clotted blood following centrifugation at 1500 g before being snap frozen on dry ice and stored at −80°C. For serum exchange experiments, serum from two seropositive patients with high T_{FH} cell CD200 levels and two seropositive patients with negligible T_{FH} CD200 levels was thawed and added to fresh PBMCs from healthy controls (without significant CD200 expression on their T_{FH} cells). Cells were cultured (1×10^6/well) in RPMI media containing 5% patient serum, 5% human serum (Sigma-Aldrich, Dorset, UK), or 5% human serum supplemented with PHA (5 μg/mL).

2.5. Data Analysis.

Flow cytometry was performed on a BD FACSCanto machine using BD FACSDiva software. Flow cytometry data were analysed using FlowJo 8.8.3 (Tree Star, Inc., Ashland, OR). Statistical analyses were performed with Prism 5 software (GraphPad Software, Inc. La Jolla, CA). Normality was assessed with the D'Agostino and Pearson test. Nonparametric data were analysed using the Mann-Whitney or Kruskal-Wallis tests. Significance is shown as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

3. Results/Figures

3.1. Follicular Helper T-Cell Numbers Are Not Increased in Patients with RA.

Data from animal studies and from patients with SLE or juvenile dermatomyositis have suggested important roles for T_{FH} cells in the development of autoimmunity. As ectopic germinal centres are common in the synovium of patients with RA and a previous study in treatment-naïve patients with RA demonstrated increased numbers of circulating T_{FH} cells, we sought to confirm whether increased numbers of circulating T_{FH} cells are present in patients with established disease, potentially contributing to the breakdown in tolerance. To answer this question, we analysed T_{FH} numbers in whole blood from 35 patients with active RA (average age 59, range 33–90) and 15 controls (average age 56, range 33–85) ($P = 0.50$). Detailed characteristics of the study population are shown in Table 1. As the most appropriate way to define T_{FH} cells in peripheral blood continues to be debated, we calculated

T_{FH} cell numbers using four different phenotypic definitions: (i) CD4+/CXCR5+, (ii) CD4+/CD45RO+/CXCR5+, (iii) CD4+/CXCR5+/PD-1hi, and (iv) CD4+/CXCR5+/ICOShi (Figure 1), as absolute cell counts (per mL of whole blood) and as a % of total CD4+ cells (data not shown). There was no difference in T_{FH} cell numbers between controls or patients with RA (including the subgroup of seropositive patients) regardless of the definition used ($P \geq 0.4$ in all cases).

3.2. T_{FH} Subsets Are Not Polarized toward Th1, Th2, or Th17 Phenotypes in RA.

Circulating T_{FH} cells can be further subdivided into three subsets based on the expression patterns of the inflammatory chemokine receptors CXCR3 and CCR6 [17]. These subsets display functionality associated with other T-helper cell classes (Th1, Th2, and Th17), including expression of their characteristic cytokines and transcription factors [17]. To determine whether T_{FH} subsets were biased in patients with RA, we analysed the expression of CCR6 and CXCR3 on CD4+/CXCR5+ T cells, using the definitions of Morita et al. [17]. Th1 T_{FH} cells were defined as being CXCR3+/CCR6−, Th2 T_{FH} cells as CXCR3−/CCR6−, and Th17 T_{FH} cells as CXCR3−/CCR6+. No biases toward a particular T_{FH} cell subset were detected in patients with RA or in the subgroup of RA patients with autoantibodies ($P > 0.65$ for all subsets) (Figure 2).

3.3. Qualitative Differences in T_{FH} Cells Exist in Patients with RA.

The balance between the positive and negative signals received by a cell is critical in determining its fate. T_{FH} cells express both stimulatory and inhibitory receptors on their surface that can regulate B-cell maturation and responses to antigen [10, 20]. Therefore we asked next if qualitative differences in receptor expression might contribute to the development of autoreactive B cells in RA. To explore this possibility we reviewed the expression of receptors displayed by T_{FH} cells, including CD200, CD150, CD134 (OX-40), CD69, CD95, and HLA-DR. These assays revealed significantly increased levels of the inhibitory receptor CD200 were present in patients with RA ($P = 0.0079$) (Figure 3(a)). As the presence of autoantibodies defines a distinct subset of patients [21], we also assessed the differences in receptor expression between seropositive and seronegative patients, which demonstrated increased levels of both CD200 and CD150 on T_{FH} cells from seropositive patients with RA ($P = 0.0045$ and $P = 0.0088$ resp.) (Figure 3(b)). Although CD200 and CD150 upregulation could be detected on other CD4+ T cell populations, only CD200 expression was more pronounced on CXCR5+ cells, suggesting selective enrichment in the T_{FH} compartment (Figure 3(c)). Although there was a trend towards increased expression of the activating receptor OX40 (CD134) (see Supplementary Figure 1(a) available online at doi:1155/2012/948218), there was no enrichment in the T_{FH} compartment or in seropositive patients, and the difference was not statistically significant ($P > 0.05$). There were no differences in the expression of CD69, CD95, or HLA-DR (Supplementary Figures 1(a)–1(d)).
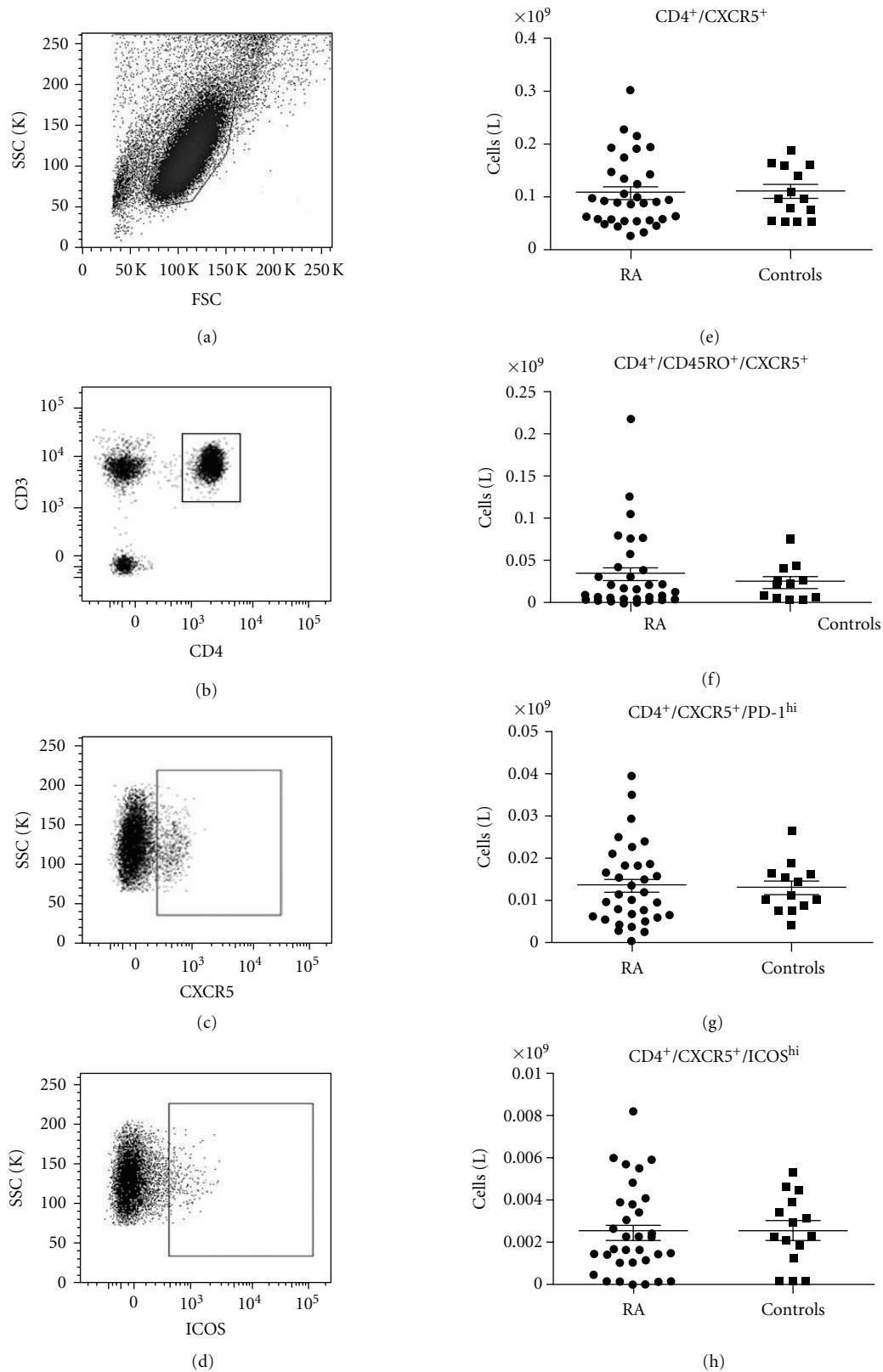
FIGURE 1: Enumeration of circulating T_{FH} cells in whole blood. T_{FH} cells were counted in whole blood using four different phenotypic definitions. (a)–(d) Representative gating strategy. (e) CD4^{+}/CXCR5^{+}; (f) CD4^{+}/CD45RO^{+}/CXCR5^{+}; (g) CD4^{+}/CXCR5^{+}/PD-1^{hi}; (h) CD4^{+}/CXCR5^{+}/ICOS^{hi}. No differences were detected between patients with RA and controls ($P \geq 0.4$ in all cases). Spots represent individual patients. Mean ± SEM are shown.
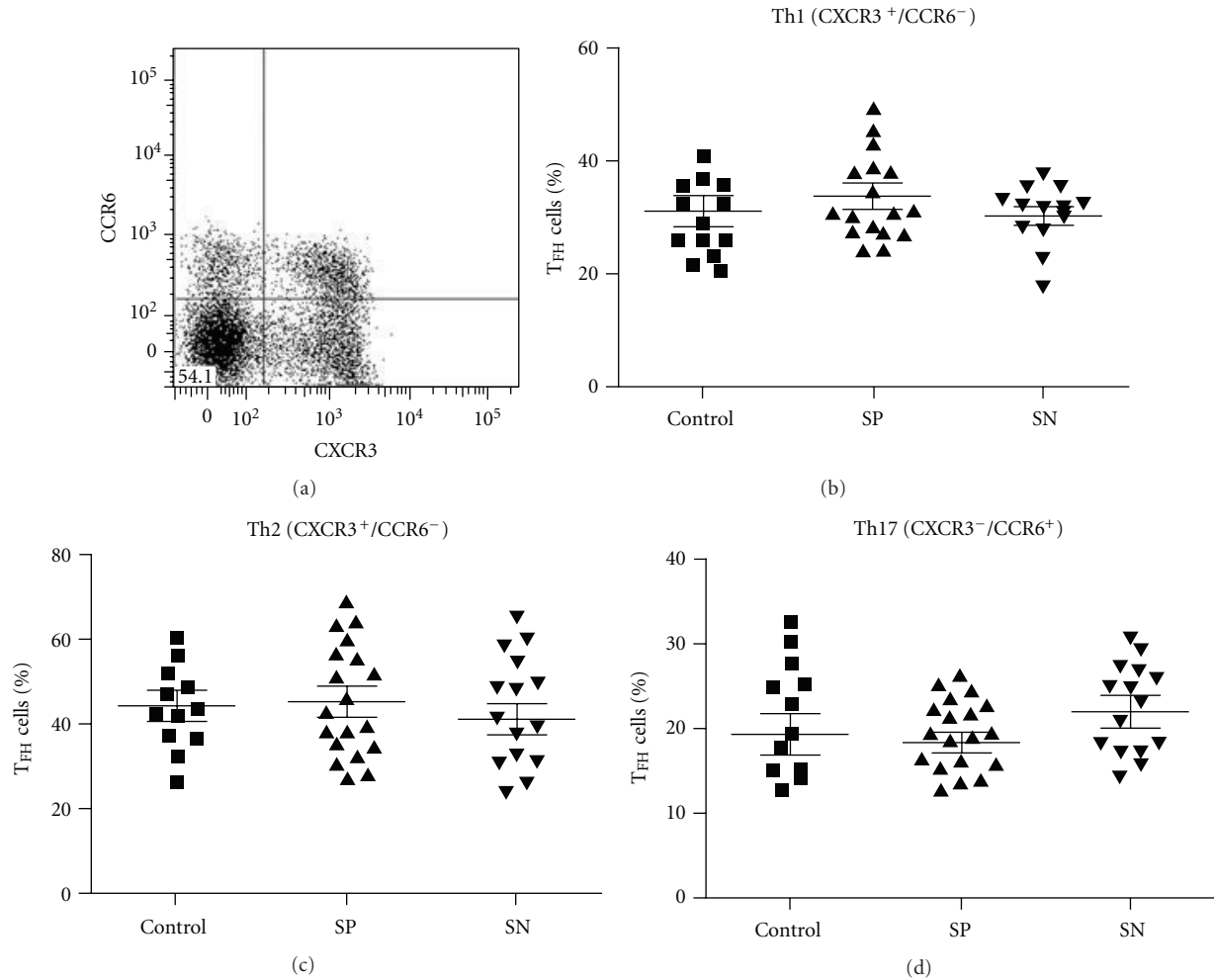
(a)

(b)

(c)

(d)

FIGURE 2: T_{FH} cell subsets in RA. The % of T_{FH} cells with Th1, Th2, or Th17 phenotypes was assessed through expression of the chemokine receptors CXCR3 and CCR6 (see text for details). SP: seropositive, SN: seronegative. Spots represent individual patients. Mean ± SEM are shown.

3.4. CD200 Expression Correlates with Treatment. CD200 expression is induced by inflammation, with increased levels detected on T cells following stimulation with inflammatory mediators, including TNFα [22]. CD200 has been proposed to be part of a negative feedback loop (via CD200R), in which the induction of CD200 suppresses further cytokine release by macrophages [23]. Given the dramatic benefits that anti-TNFα therapies have on disease progression in RA we wondered if CD200 expression on T_{FH} cells might itself reflect treatment with these agents or disease activity. Levels of CD200 on T_{FH} cells were significantly higher in patients receiving treatment with anti-TNFα agents ($P = 0.0008$) (Figure 4(a)), regardless of whether they were seropositive ($P = 0.053$-data not shown). CD200 expression was not related to disease activity as calculated by the DAS-28 (CRP) score ($P = 0.887$, $r^2 = 0.0009$) (Figure 4(b)) or to the ACPA titre ($P = 0.896$, $r^2 = 0.003$ (data not shown)).

3.5. CD200 Expression on T_{FH} Cells Is Not Induced by Circulating Factors. To determine whether a soluble factor could be

responsible for the increased expression of CD200, PBMCs from healthy controls were incubated with serum from two seropositive patients with RA known to have high levels of CD200 on their T_{FH} cells or two seropositive patients with minimal T_{FH} cell CD200 expression. CD200 levels were analysed at baseline and then every 24 hours until 72 hours. CD200 expression increased slightly over time under all conditions, but there was no difference in the percentage of T_{FH} cells expressing CD200 at any time point following addition of serum from high or low expressors (Supplementary Figure 2, $P > 0.05$). However, significantly increased expression of CD200 on T_{FH} cells was detected on freshly purified peripheral blood mononuclear cells PBMC (F) when compared with whole blood from the same donors ($P = 0.004$), suggesting that the manipulation of T_{FH} cells itself may alter expression of the receptor (Supplementary Figure 3).

4. Discussion

This study is the first to describe an alteration in the phenotype of circulating follicular helper T cells in patients
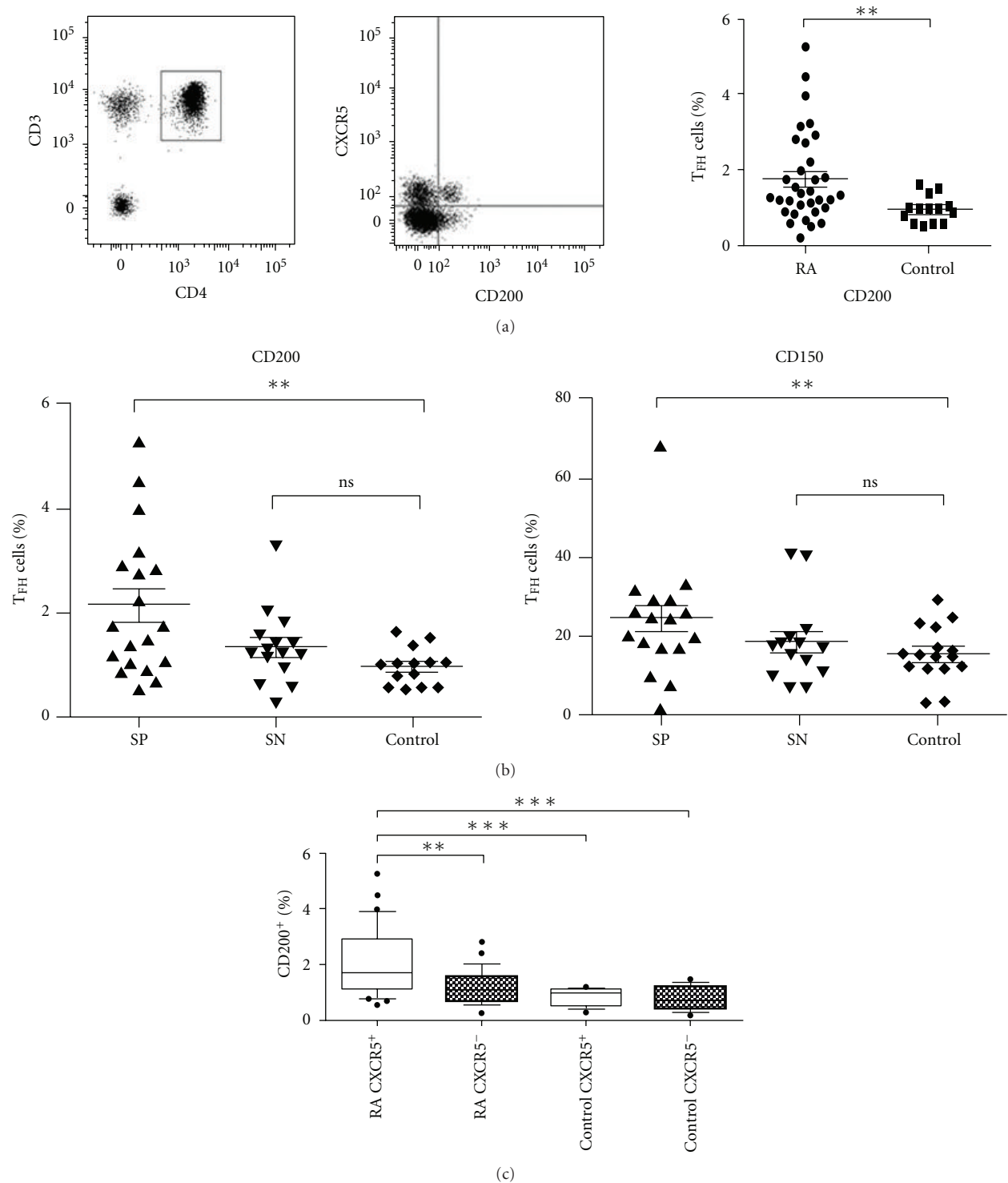
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FIGURE 3: Analysis of receptors expressed by T_{FH} cells. (a) Representative gating strategy showing the increased expression of CD200 on T_{FH} cells from patients with RA. (b) Significantly increased expression of CD200 and CD150 was detected on circulating T_{FH} cells from seropositive RA patients (SP: seropositive, SN: seronegative) ($P = 0.0045$ and $P = 0.0088$, resp.). Each spot represents an individual patient, and the mean ± SEM are shown. (c) CD200 expression is enriched on CXCR5 positive CD4+ T cells in patients with RA.
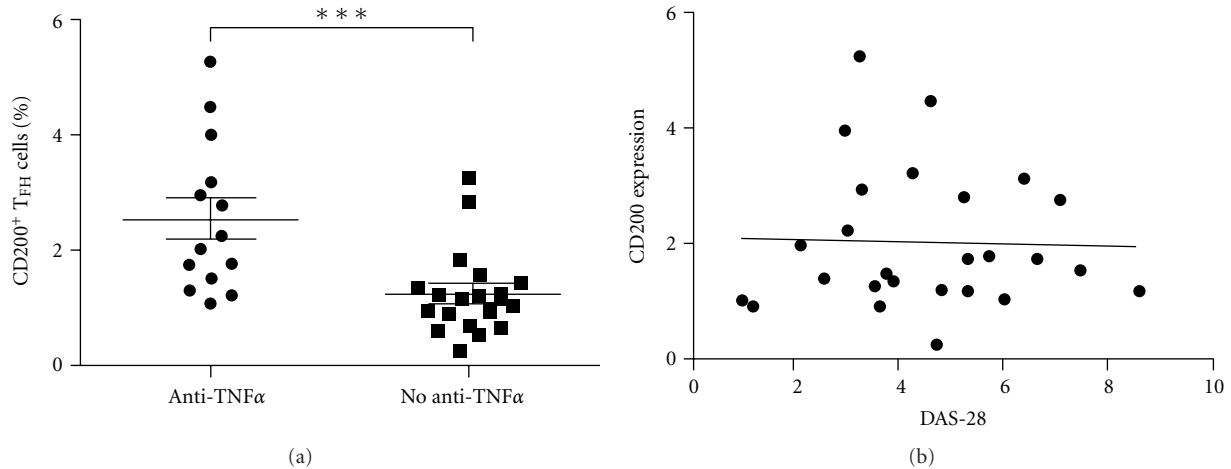
(a)

(b)

FIGURE 4: Relationship between CD200 expression on T_{FH} cells, treatment, and disease activity. (a) The % of T_{FH} cells expressing CD200 in patients being treated with anti-TNFα therapy was significantly increased compared with patients not on anti-TNFα therapy ($P = 0.0008$). The mean DAS of patients receiving anti-TNFα therapy was 4.679, compared with 4.418 in patients who were not ($P = 0.875$). Each spot represents an individual patient, and the mean $\pm$ SEM are shown. (b) Correlation between T_{FH} cell CD200 expression and DAS-28 score ($r^2 = 0.0009, P = 0.887$).

with rheumatoid arthritis. T_{FH} cells are a distinct subset of CXCR5-expressing CD4$^+$ T cells that can localise to germinal centres and are thought to maintain tolerance by censoring B cells with specificity for self-antigens by failing to provide the necessary cytokine and costimulatory receptor support for their maturation [13, 24]. Dysregulation of this process has been reported in animal models and in humans with autoimmune diseases, particularly in conditions associated with autoantibodies [11, 13, 14, 16, 24, 25]. Aberrant T_{FH} cell function is also implicated in the development of autoimmune phenomena in patients with angioimmunoblastic T-cell lymphoma, a malignant proliferation of lymphocytes that possess a T_{FH} phenotype [26].

Given the common findings of autoantibodies and ectopic germinal centres in patients with RA [5, 27], we hypothesized that quantitative or qualitative abnormalities of T_{FH} cells may be central to the autoimmune process. As an accepted phenotype for defining the circulating counterparts of GC T_{FH} cells remains controversial, we measured T_{FH} cell numbers in patients with RA and controls using a series of different phenotypic definitions. In each case T_{FH} cell numbers were not increased in patients with RA. This is in contrast to the findings of Simpson et al. in patients with systemic lupus erythematosus (SLE), where a distinct subset of patients (~30%) was identified as having increased T_{FH} cell numbers, a stable phenotype that appeared unrelated to disease activity [16], and Ma et al. who found increased T_{FH} cell numbers in treatment-naïve patients with RA [15]. Although SLE and RA are both systemic autoimmune diseases in which autoantibody production is prominent, they preferentially affect different organ systems, and different genetic loci have been implicated in their pathogenesis [28, 29]. As the average duration of RA in our patient population was 51 months, whereas the patients in the study of Ma et al. were newly diagnosed, one explanation for the difference

between our data and that of Ma et al. is that excess T_{FH} cells from the circulation may be recruited into sites of ectopic GC formation that develop over time, resulting in normal circulating numbers. The effects of different therapeutic regimens on T_{FH} development and trafficking may also be relevant, and future prospective studies will help answer these questions.

Aberrant expression of costimulatory molecules can sustain the development of autoreactive cells causing autoimmunity, with blockade of these receptors a proven therapeutic strategy [30–34]. As T_{FH} cells are known to express multiple receptors that mediate their interactions with B cells [13, 24], we also investigated whether qualitative differences in T_{FH} cell stimulatory or inhibitory receptor expression were present in patients with RA. Using a panel of antibodies we identified significantly elevated levels of the inhibitory receptors CD200 and CD150 in patients with autoantibodies (Figure 3).

CD200 (previously known as OX-2) is a member of the immunoglobulin gene superfamily of receptors that displays a restricted tissue distribution, including activated T and B cells [35]. CD200 is induced by inflammatory cytokines, including TNFα [22], and binds to CD200R, a nonclassical immunoglobulin family receptor found predominantly on macrophages and dendritic cells, but also identified in the lymph nodes and synovium of animals with collagen-induced arthritis [36]. Binding of CD200 to CD200R causes phosphorylation of the cytoplasmic domain of CD200R and signalling through a series of adaptor proteins and the MAPK pathway [37]. The consequence of this interaction is to dampen the inflammatory response, with ligation of CD200R a therapeutic target in collagen-induced arthritis [23]. The importance of the CD200-CD200R axis in autoimmunity has been confirmed in mice genetically engineered to lack expression of CD200 or where the CD200-CD200R

interaction is blocked, and in patients with multiple sclerosis or androgenetic alopecia, where reduced CD200 expression is associated with disease [38–41]. As well as limiting the expansion of activated macrophages [42], CD200 can inhibit NK cell function, which may be important in the pathogenesis of NK-mediated bone destruction in patients with RA [43], while the expression of CD200R on B-cells suggests the potential for CD200$^+$ T cells to directly regulate B-cell function [44].

Current models propose that the CD200-CD200R axis provides a link between adaptive and innate immune responses by regulating tissue-specific tolerance set points, high CD200 expression serving to increase the threshold for activation [45]. The increased expression of CD200 on T_{FH} cells in patients with RA may therefore represent a physiological response to inflammation designed to contain autoreactivity [46]. To assess whether a circulating factor or factors may be present in the serum of patients with high T_{FH} CD200 levels, that could be a potential biomarker, we performed a series of serum exchange experiments (Supplementary Figure 2). Serum from patients with high T_{FH} CD200 expression did not induce CD200 on control T_{FH} cells, suggesting that serum factors alone are insufficient for the induction of CD200.

As CD200 can be upregulated by TNFα [22], we assessed whether treatment with anti-TNFα agents was related to CD200 expression. Contrary to expectations, T_{FH} CD200 expression was significantly higher in patients with RA who were receiving anti-TNFα therapy (Figure 4(a)). One potential explanation for this finding is that patients whose disease is associated with higher TNF levels may be more likely to respond to anti-TNFα therapy and were therefore receiving these treatments at the time of recruitment. The expression of CD200 on T_{FH} cells might therefore identify patients suited to these agents, a hypothesis that could be tested prospectively. Although CD200 is upregulated during inflammation, most studies have focussed on acute inflammation [45]. Therefore, the failure of T_{FH} CD200 expression to correlate with disease activity (Figure 4(b)) may reflect the chronicity of the inflammatory process in RA or the presence of more complex interactions between treatment and systemic versus local synovial TNFα concentrations.

As the trafficking of T_{FH} subsets is still poorly understood, one possibility is that T_{FH} cells with proinflammatory phenotypes are trapped within GCs, biasing the profile seen in the peripheral circulation. Although skewing of the distribution of T_{FH} subsets has been implicated in the pathogenesis of autoimmune disease [17], we were unable to detect a bias toward a particular T_{FH} subset in patients with RA (Figure 2). As significant alterations in T cell profiles can occur following density-gradient purification (Supplementary Figure 3) [47], these effects may influence T_{FH} cell characterization, and further studies comparing T_{FH} subsets in whole blood versus purified PBMC will be important.

In conclusion, results presented here provide evidence that T_{FH} cells have a role in the pathogenesis of RA and suggest that qualitative differences in the expression of inhibitory receptors may be important to the immune response

and efficacy of treatment with anti-TNF agents. The upregulation of CD200 on T_{FH} cells in RA patients with autoantibodies and those receiving treatment with anti-TNFα therapies supports a causal link between inflammation and induction of this receptor. Future studies examining CD200 and its ligand will contribute further to our understanding of the pathogenesis of RA and may help to identify new therapeutic targets in this disease.

Conflict of Interests

All authors declare they have no conflict of interests with regard to this work.

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