Proportion of T Follicular Helper Cells in Peripheral Blood of Rheumatoid Arthritis Patients: A Systematic Review and Meta-Analysis

Peyman Bemani

Shiraz University of Medical Sciences

Kari K. Eklund

Department of medicine, division of rheumatology, Helsinki university

Mohammad Ali-Hassanzadeh

Department of immunology, Geroft university of medical sciences

Dieter Kabelitz

Institut of immunology, Keil university

Reinhold E. Schmidt

Klinik für immunologie und rheumatologie hannover, Germany

Seppo Meri

Department of immunology and bacteriology, university of Helsinki, Finland

Kurosh Kalantar (✉ kalantark@sums.ac.ir)

Shiraz University of Medical Sciences <https://orcid.org/0000-0002-9160-9449>

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Abstract

Objectives: Follicular helper T (Tfh) cells have critical roles in the development and promotion of humoral immune responses. Accumulating evidence points to alterations in the levels and activity of Tfh which may lead to impaired immune tolerance and development of antibody-mediated autoimmune diseases, such as rheumatoid arthritis (RA). To elucidate the status of Tfh cells in patients with RA, we conducted a systematic review and meta-analysis to assess the proportion and types of Tfh cells in the PB of RA patients.

Method: We systematically searched databases of Embase, Pubmed, Web of sciences (WOS), and Scopus to identify the eligible articles. We performed meta-analysis to assess the proportion of Tfh cells in PB of RA patients compared to healthy controls (HCs). We also assessed the proportion of Tfh cells based on different parameters including Tfh definition markers, disease activity status, disease stage, treatment status, serum markers, and the geographical location of the included articles.

Results: Regardless of definition markers, the proportion of Tfh cells in RA patients was significantly higher than that in HCs (SMD 0.75, [0.56, 0.93], $p < 0.0001$). Based on subgroup analyses, the proportion of Tfh cells with the individual definitions were also significantly higher in PB of RA patients compared to HCs. Tfh cells proportion in untreated-RA and early-RA patients was markedly greater than HCs, when comparisons were made without considering the definition markers, and also when Tfh cells was defined by the specified definition markers. While the proportion of Tfh cells by all definitions was significantly higher in active- and remission-RA compared to HCs, individual analysis of two definitions, $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^+$, didn't show significant differences. In addition, higher proportion of Tfh cells defined by all definitions as well as a specified definition ($CD4^+CXCR5^+PD-1^{high}$) was observed when S^+RA and S^-RA patients were compared to the HCs. Higher Tfh cells proportion in RA patients compared with HCs was observed in the Asian populations (SMD 0.89, [0.68, 1.09], $p < 0.0001$), while there was a trend toward significant in the proportion of Tfh between the two groups in non-Asians (SMD 0.29, [-0.003, 0.59], $p = 0.052$).

Conclusion: Altogether, our results demonstrate that circulating Tfh are highly elevated in RA patients and are also significantly increased in untreated, early- and S^+RA patients, underscoring the potential pathogenic role of this cell population in RA and its potential use as a biomarker and a target for RA therapy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with a prevalence of 0.5–1% of the population, and primarily affecting joint tissues [1]. Typical clinical manifestations include symmetrical inflammation and swelling in particular of the small joints of hands and feet, which can lead to joint destruction, deformity and functional disability [1, 2]. RA imposes a substantial socio-economic burden on patients and societies [2, 3]. Although, the exact etiology and pathogenesis of RA are not completely understood, it is well accepted that the disease is multifactorial caused by multiple genetic, environmental, and microbial factors. These risk factors together trigger uncontrolled immune responses, and development and progression of the disease [2].

Different types of innate and adaptive immune cells including dendritic cells (DCs), monocytes, macrophages, antigen-specific T and B lymphocytes along with cytokines and autoantibodies contribute to the pathogenesis of RA [3]. Among the immune cells, $CD4^+$ T cells have critical roles in the disease induction and progression. Once activated, $CD4^+$ T cell can differentiate into different T helper (Th) subsets including Th1, Th2, Th17, T regulatory or T follicular helper (Tfh) cells, each play an important role in the RA [4].

Tfh cells are a subpopulation of Th cells localized in the B cell follicle of lymph nodes. These cells are essential in humoral immune response by providing help for B cell to develop germinal center, differentiate to memory B cells and long-lived antibody-secreting plasma cell, immunoglobulin class-switching and antibody affinity maturation [5, 6]. Tfh cells can be distinguished from other differentiated $CD4^+$ Th subsets by the expression of B-cell lymphoma 6 (BCL-6) transcription factor [7], the surface expression of CXCR5 chemokine receptor [8–10], CD40 ligand, programmed cell death protein 1 (PD-1), and inducible costimulatory molecule (ICOS) [6, 11], and the production and release of interleukin (IL)-21 [7].

Despite the prominent role of Tfh cells in humoral immunity for protection against infectious pathogens, accumulating evidence has demonstrated that deregulated frequency and activity of Tfh may lead to impaired immune tolerance, generation of high-affinity autoantibodies and hence development of antibody-mediated autoimmune diseases [12–15]. However, anatomical localization of Tfh cells in the secondary lymphoid tissues limits their routine studies in human patients [6, 8]. Recently, studies have introduced a new circulating subpopulation of $CD4^+$ T cells that show both phenotypical and functional characteristics of classical Tfh cells residing in the lymphoid tissues [16, 17]. Based on these studies, circulating Tfh (cTfh) cells, similar to the classical Tfh, could promote differentiation of naïve B cell into plasma cells and immunoglobulin secretion through IL-21 production [16]. In addition studies have shown that cTfh cells increase in proportion to their GC counterparts in secondary lymphoid tissues [17]. Thus, they represent circulating compartment of Tfh cells and evaluation of the status of cTfh would reflect the status of their counterpart in GC of secondary lymphoid tissues [16, 17]. Following the introduction of cTfh, several human studies have demonstrated the increased frequency of Tfh in the peripheral blood (PB) of patients with different autoimmune diseases including systemic lupus erythematosus, autoimmune thyroiditis, myasthenia gravis, Sjogren's syndrome and RA [18].

Several research groups have explored the potential role of cTfh cells in RA pathogenesis by determining their frequency in PB of RA patients compared to the healthy controls (HCs). While some of these studies have shown increase in the frequency of Tfh in PB of patients with RA and its positive correlation with disease scores, some other studies have failed to show any differences in the frequency of these cells in patients with RA compared with

HCs. Hence, in the present work, we systematically searched and reviewed the available studies documenting the proportion of Tfh among CD4⁺T cells in PB of RA patients, and conducted a meta-analysis to elucidate the proportion of Tfh cells in the PB of RA patients compared to the HCs.

2. Materials And Methods

2.1 Search Strategy

This study was carried out in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and was registered at the international prospective register of systematic reviews (PROSPERO) (CRD42020163549). We searched online databases including Embase, PubMed, Scopus and Web of Science (WOS) to identify original articles published until November 2019 reporting the frequency of Tfh among CD4⁺ T cells in PB of patients with RA. The search terms used in this meta-analysis were as follows: ("Arthritis, Rheumatoid"[Mesh] OR "Rheumatoid arthritis" OR RA) AND ("T helper follicular" OR "T follicular helper" OR "Follicular T helper" OR "Follicular helper T" OR "Tfh"). In order to reduce the risk of missing any related study, we also searched manually the reference list of review articles and the included original articles. The searches and selection of articles were conducted by two investigators independently. In the case of any disagreement, it was resolved through discussion with a third investigator.

2.2 Study selection

Inclusion criteria: 1) original human studies; 2) title or abstract containing the terms "rheumatoid arthritis" or "RA" and "T helper follicular" OR "T follicular helper" OR "follicular T helper" OR "follicular helper T" OR "Tfh"; 3) documenting the frequency/proportion of Tfh among CD4⁺ T cells in the PB of RA patients and HCs; 4) available as full-text article on the internet (website or PDF); 5) Available information regarding the number of patients and HCs. No restriction was applied for the subtypes of the RA disease, disease severity score, sex or race of the participant subjects in the study. Also, no time or language restrictions were imposed.

Exclusion criteria: 1) non-original studies, review articles, case reports, letter to editor or conference abstract publications; 2) Animal or in vitro studies; 3) studies without healthy control group; 3) studies without raw data in which data extraction from their graphical results were not feasible; 4) Redundancies among Embase, PubMed, Scopus, and WOS search were removed and each study was counted only once in the meta-analysis

2.3 Data extraction and quality assessment

Two independent researchers carefully reviewed the included studies and extracted data, and disagreements were resolved through discussion with the third investigator. The following data were extracted from the eligible articles: author's name, publication year, country where the authors performed the analysis, number of RA patients and HC controls, Tfh definition, mean and standard deviation (SD) of the frequency of Tfh among CD4⁺ T cells. When Tfh were defined by different patterns of markers in the studies, data were extracted from all of them. If instead of mean and SD, the studies reported mean and standard error (SE), medians and ranges, or median and interquartile ranges we extracted all types of the data. In the case of some of articles presenting only graphical results without raw data in which it was not possible to extract data from their graphs, the raw data (mean and SD) were generously received from the authors after contacting the authors by email. The quality of all included studies was assessed by Newcastle–Ottawa Quality Assessment Scale (NOS) (Case Control Studies) [19].

2.4 Data analysis

All statistical analyses for the Tfh frequency differences between RA and HC subjects were conducted using STATA version 16.0 (Stata Corp., College Station, Texas). The sample size, mean, and SD of the frequency of Tfh cells were used to calculate the standardized mean difference (SMD) between RA and HC groups.

For certain studies reporting only graphical results, the graphs were converted to the numerical data by using GetDat Graph Digitizer software version 2.24 (<http://getdata-graph-digitizer.com/>). When some studies reported SE instead of SD, the SD was calculated from sample size and SE using the following formula: $SD = SE \times \sqrt{n}$. When some studies presented their results as median and range, or median and interquartile range (IQR), the mean and SD were estimated by using a standard method [20]. When there was need to combine data from two subgroups (for example combining mean and SD of "active-RA" and those from "remission-RA" groups), the pooled mean and SD was calculated by using a standard formula according to the Cochrane Handbook for Systematic Reviews of Interventions [21].

The meta-analysis was performed to calculate the SMD of the proportion of Tfh among CD4⁺ T cells in the PB of 1) RA patients versus HCs 2) untreated RA (u-RA) patients versus HCs, 3) early RA (e-RA) patients versus HCs, 4) active-RA (a-RA) patients versus in remission-RA (r-RA) patients, 5) a-RA patients versus HCs, 6) seropositive (S⁺) RA patients versus seronegative (S⁻) RA patients, and 7) S⁺RA patients versus HCs. Furthermore, since the studies used different patterns of markers to define Tfh cells, subgroup analysis was also performed to estimate the results based on each Tfh definition.

In order to evaluate the heterogeneity between the studies, the Q test and I² statistic was used. For the Q test, P-value < 0.1 was considered as statistically significant and I² values of 75%, 50%, and 25% were considered as evidence of high, moderate and low level of heterogeneity, respectively. SMD with 95% confidence interval (CI, Hedges' g) was calculated to assess the proportion of Tfh cells, and random effect model (REM) was used when the heterogeneity was high (I² > 50%), and a fixed effect model (FEM) was chosen when the heterogeneity was low or absent (I² ≤ 50%). In addition, the publication bias was assessed by using funnel plot in which the x- axes and y-axes of the plot were used to show SMD and SE. Egger's test was used for evaluating funnel

plot asymmetry, and in the case of its presence, the “trim and fill” method was applied to assess the effect of publication bias on the calculated results. P-value < 0.05 was considered to be statistically significant except where noted.

3. Results

3.1 Literature Search

A total of 821 potentially eligible studies were retrieved after searching databases along with manual searching. Of these, after primary screening, 405 and 365 studies were respectively excluded due to duplication and not satisfying the inclusion criteria. Fifty one relevant full-text articles were selected and assessed for eligibility, of which 26 articles were excluded because of the following reasons: lack of healthy control group (n = 3), evaluating the Tfh cells in in vitro (n = 4), without raw data and unable to extract data from the graphical result (n = 2), evaluating changes in the proportion of Tfh in autoimmune diseases other than RA (n = 1), reporting only the absolute number of Tfh cells (n = 2), overlapping samples from other studies (n = 2), reporting only Tfh subsets (Tfh-1, -2 and -17) (n = 2), and letter to editor. Additional 10 articles were irrelevant and didn't evaluate proportion of Tfh cells, thus, finally a total of 25 articles were included in the meta-analysis (Fig. 1).

3.2 Characteristics of eligible studies

Of the included articles, 14 were performed in China, 4 in Japan, 2 in Spain, 1 in Sweden, 1 in Italy, 2 in USA, and 1 in Argentina. In total the eligible articles included 1041 RA patients and 626 HCs. According to the NOS risk of bias assessment, all the included studies had a score of 4–8, most of them had high quality (NOS score, 7 and 8), and fewer with moderate quality (NOS score, 4–6). The included studies and their basic characteristics were also listed in Table 1.

3.3 The proportion of Tfh in the PB of RA patients versus HCs

3.3.1 Pooled results

In initial step, we carried out meta-analysis to compare the proportion of Tfh cells between RA and HC groups regardless of the markers used for definition of Tfh cells. Some of these studies reported a significant increase in the proportion of Tfh cells in BP of RA versus HC subjects [22–34], some studies showed no differences [35–39], some studies revealed both increase in RA and no differences between the two groups depending on the definition markers [40–43], and some other reported non-significant decrease in Tfh cells proportion in RA group [44, 45]. Based on the pooled result of meta-analysis, RA patients had a significantly higher proportion of cTfh cells compared with HCs (SMD 0.75, [0.56, 0.93], $p < 0.0001$), although high level of heterogeneity ($I^2 = 80.15$, $p < 0.0001$) was observed between studies (Fig. 2).

Table 1
Basic characteristics of the included studies

Author	Year	Country	RA number	HC number	Study	Tfh definition	% of Tfh cells in PB				NOS
							RA mean	RA SD	HC mean	HC SD	
Liu et al. [22]	2018	China	78	33	a	CD4 ⁺ CXCR5 ⁺	18.905	3.360	15.180	4.190	7
					b	CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	7.070	2.800	3.590	1.010	
Ma et al. [23]	2012	China	33	23	a	CD4 ⁺ CXCR5 ⁺	2.910	3.000	0.900	0.290	4
			29	22	b	CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	0.140	0.180	0.020	0.020	
Han et al. [24]	2018	China	20	12		CD4 ⁺ CXCR5 ⁺	22.720	6.300	13.750	4.880	5
Costantino et al. [35]	2017	Argentina	24	22		CD4 ⁺ CXCR5 ⁺	12.890	7.730	10.480	3.900	8
An et al. [25]	2016	China	40	20	a	CD4 ⁺ CXCR5 ⁺	16.750	3.920	7.490	1.840	7
					b	CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	1.570	1.100	0.240	0.300	
					c	CD4 ⁺ CXCR5 ⁺ ICOS ⁺	8.370	4.280	3.720	1.810	
He et al. [36]	2013	China	38	30	a	CD4 ⁺ CXCR5 ⁺	13.729	5.000	13.478	5.693	6
					b	CD4 ⁺ CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high} CD45RA ⁻	16.363	6.629	14.000	4.100	
Takeshita et al. [26]	2019	Japan	53	30		CD3 ⁺ CD4 ⁺ CXCR5 ⁺	13.649	4.515	11.422	4.449	7
Wang et al. [27]	2013	China	25	15	a	CD3 ⁺ CD4 ⁺ CXCR5 ⁺	15.514	6.329	11.710	4.060	8
			24	15	b	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	10.603	5.382	4.783	2.173	
					c	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	8.600	4.050	4.790	1.570	
					d	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ⁺ ICOS ⁺	4.646	2.554	2.713	1.496	
Arroyo-Villa et al. [37]	2014	Spain	34	34	a	CD3 ⁺ CD4 ⁺ CXCR5 ⁺	16.176	6.660	16.324	7.110	7
					b	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	1.460	0.788	1.177	0.699	
Niu et al. [40]	2018	China	44	20	a	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ Foxp3 ⁻	21.401	7.113	18.200	5.810	7
					b	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	28.810	11.405	13.400	3.577	
					c	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	14.238	8.115	12.300	4.470	
Liu et al. [28]	2012	China	47	47		CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	5.710	3.630	2.320	0.890	7
Cao et al. [29]	2019	China	30	30		CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	2.610	0.970	1.350	0.450	7
Aldridge et al. [38]	2018	Sweden	75	31		CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	13.830	4.105	13.690	5.030	6
Wang et al. [33]	2019	China	24	20		CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	4.090	3.040	0.550	0.510	7
Penatti et al. [44]	2017	Italy	8	8		CD4 ⁺ CXCR5 ⁺ ICOS ⁺	3.910	5.200	5.910	4.720	8
Zhang et al. [30]	2015	China	54	16		CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	1.010	0.630	0.640	0.260	7
Nakayamada et al. [31]	2018	Japan	108	33		CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	1.100	0.500	0.900	0.400	8
Kang et al. [41]	2018	China	29	37	a	CD4 ⁺ CXCR5 ⁺ CD45RA ⁻	14.410	4.993	13.332	5.304	6
					b	CD4 ⁺ CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high} CD45RA ⁻	5.297	3.618	3.170	2.036	

Author	Year	Country	RA number	HC number	Study	Tfh definition	% of Tfh cells in PB				NOS
							RA mean	RA SD	HC mean	HC SD	
Fukuyo et al. [34]	2017	Japan	34	14		CD4 ⁺ CXCR5 ⁺ CD45RA ⁻	8.970	6.600	2.010	0.200	4
Zhou et al. [42]	2019	China	44	37	a	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ CD45RA ⁻	21.102	40.720	22.677	30.590	6
					b	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ⁺ ICOS ⁺ CD45RA ⁻	2.283	2.400	1.200	0.540	
Fortea-Gordo et al. [43]	2019	Spain	55	47	a	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ CD45RA ⁻	17.365	5.970	15.735	5.278	7
			56	47	b	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} CD45RA ⁻	0.498	0.360	0.232	0.139	
			55	47	c	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} ICOS ⁺ CD45RA ⁻	1.008	0.660	0.529	0.405	
Umeda et al. [39]	2018	Japan	9	9		CD4 ⁺ CXCR5 ^{high} ICOS ^{high} PD-1 ^{high} CD45RA ⁻	10.149	9.353	10.547	6.965	5
Chen et al. [32]	2016	China	41	32		CD3 ⁺ CD4 ⁺ CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high}	12.800	5.700	8.700	2.000	7
Singh et al [45]	2016	USA	51	10		CD4 ⁺ CXCR5 ⁺ CD45RO ⁺	23.927	8.882	27.734	13.413	8
RA: rheumatoid arthritis, HC: healthy control, PB: peripheral blood, Tfh: T follicular helper, NOS: Newcastle–Ottawa Quality Assessment Scale for each included article, SD: standard deviation. Study identifier (a, b, c, d) was used to distinguish each study											

3.3.2 Subgroup analysis

In the next step, we performed subgroup meta-analysis based of different definition markers used for Tfh cells. It should be noted that as all Tfh cells express CD3 and due to previous antigen exposure all are CD45A⁻ and CD45RO⁺ memory T cells. Therefore, CD3 and CD45RA/RO were not taken into consideration for subgrouping and meta-analysis. The results of the subgroup analysis based on Tfh definitions are summarized in Table 2.

First we analyzed the studies in which “CD4⁺CXCR5⁺” was used to identify Tfh cells. From a total of 15 studies, 7 studies found higher frequency of Tfh cells in BP of patients with RA compared to HCs [22–27, 34], 7 studies showed no difference [35–37, 40–43], and 1 study reported non-significant decrease in proportion of Tfh cells in RA group [45]. Analysis of the pooled effect size of the 15 studies revealed that RA patients had a significant increased Tfh cells proportion in comparison with the HC group when Tfh cell was defined as “CD4⁺CXCR5⁺” (SMD 0.6, [0.23, 0.96], $p = 0.001$; $I^2 = 86.23\%$, $p < 0.0001$) (Supplementary Fig. 1). Funnel plot (Supplementary Fig. 2) and Egger’s test detected existence of publication bias ($p = 0.015$). However, when the trim and fill test was applied the result did not change, demonstrating that our results was steady. Then we evaluated the proportion of Tfh cells when “CD4⁺CXCR5⁺PD-1^{+/high}” was used for identification. Some of these studies used “CD4⁺CXCR5⁺PD-1^{high}” and some other didn’t consider the intensity of PD-1 expression for definition of Tfh. Of 6 studies, 5 studies revealed the higher proportion of “CD4⁺CXCR5⁺PD-1⁺” Tfh cells in RA patients [25, 27–29, 40] and only 1 study [38] showed no differences. Expectedly, the result of meta-analysis showed higher Tfh cells proportion in RA patients when they defined as “CD4⁺CXCR5⁺PD-1⁺” (SMD 1.18, [0.67, 1.69], $p < 0.0001$, $I^2 = 81.4\%$, $p < 0.0001$) (Supplementary Fig. 3). There was also a significant rise in the proportion of Tfh cells when they were defined as “CD4⁺CXCR5⁺PD-1^{high}” (SMD 1.22, [0.95, 1.5], $p < 0.0001$, $I^2 = 43.80$, $p = 0.168$) (Supplementary Fig. 4). Next we evaluated the Tfh cells proportion when “CD4⁺CXCR5⁺ICOS^{+/high}” was adopted to define Tfh. From 6 studies reporting “CD4⁺CXCR5⁺ICOS⁺” as Tfh cells, 3 found higher frequency of Tfh cells in RA [25, 27, 31], while 2 studies showed no difference [37, 40] and 1 reminder revealed non-significant decrease in RA patients [44]. The meta-analysis result of the Tfh cells proportion when they were defined as “CD4⁺CXCR5⁺ICOS⁺” revealed higher proportion of Tfh cells in PB of RA patients compared to the HCs (SMD 0.54, [0.13, 0.95], $p = 0.0094$, $I^2 = 68.66\%$, $p = 0.0.013$) (Supplementary Fig. 5). In addition there was a significant increase in “CD4⁺CXCR5⁺ICOS^{high}” proportion (SMD 0.75, [0.35, 1.15], $p = 0.0002$, $I^2 = 0\%$, $p = 0.0.58$) when RA patients were compared to the HCs (Supplementary Fig. 6).

We also analyzed the studies in which the “CD4⁺CXCR5⁺” Tfh cells co-expressed PD-1 and ICOS (CD4⁺CXCR5⁺PD-1⁺ICOS⁺ cells). From 4 studies we removed two studies in which high expression of PD-1 and/or ICOS were used for Tfh cell evaluation. According to the pooled SMD result a significant increase in the proportion of Tfh cells co-expressing ICOS and PD-1 was found in RA patients in comparison with the HCs (SMD 0.67, [0.31, 1.04], $p = 0.0003$, $I^2 = 0\%$, $p = 0.518$) (Supplementary Fig. 7).

Finally, we assessed Tfh cells proportion from the studies in which “CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}” was used to identify Tfh. Of these studies 2 reported markedly higher proportion of Tfh cells in patients with RA [32, 41], and 1 reported no difference when RA group was compared with HC group [36]. Our

subgroup meta-analysis revealed significant increase in the proportion of “CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}” cells in PB of RA patients in comparison with HCs (SMD 0.68, [0.4, 0.96], $p < 0.0001$, $I^2 = 4.93\%$, $p = 0.34$) (Supplementary Fig. 8).

Table 2
Subgroup analysis results of the proportion of Tfh cells in RA compared to HC based on definition markers.

Tfh definition	No.	SMD [95% CI]	p	Heterogeneity		Publication bias (Egger's P)	Model
				I ² (%)	P		
CD4 ⁺ CXCR5 ⁺	15	0.6 [0.23, 0.96]	0.001	86.23	$p < 0.0001$	0.015	REM
CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	6	1.18 [0.67, 1.69]	$p < 0.0001$	81.4	$p < 0.0001$	0.088	REM
CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	3	1.22 [0.95, 1.5]	$p < 0.0001$	43.80	0.168	0.197	FEM
CD4 ⁺ CXCR5 ⁺ ICOS ⁺	6	0.54 [0.13, 0.95]	0.0094	68.66	0.013	0.588	REM
CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	2	0.75 [0.35, 1.15]	0.0002	0	0.58	-	FEM
CD4 ⁺ CXCR5 ⁺ PD-1 ⁺ ICOS ⁺	2	0.67 [0.31, 1.04]	0.0003	0	0.518	-	FEM
CD4 ⁺ CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high}	3	0.68 [0.4, 0.96]	$p < 0.0001$	4.93	0.34	0.672	FEM
Tfh: T follicular helper, No: number of studies, SMD: Standardize mean difference, p: p value, REM: random effect model, FEM: fixed effect model, RA: rheumatoid arthritis							

3.4 The proportion of Tfh in PB of untreated-RA patients as compared with HC

Studies in which patients were taking steroids or immune-suppressive drugs were removed from this part of the meta-analysis, since treatments were not uniform across the studies. A total of 12 articles covering 21 studies evaluated the proportion of Tfh cells in patients who did not take any medicine at the time of sample collection. Of these, some of studies included patients who didn't receive any drug for at least 1 month [22], 2 months [30, 35] and 3 months [32, 36, 42] before blood sample collection, and 6 articles covering 12 studies included patients with lack of any treatment history [23, 26, 27, 29, 38, 43]. Therefore, the proportion of Tfh cells was compared between RA patients without immunosuppressive treatments and HCs. Pooled analysis without considering the defined phenotypes for Tfh cells revealed a significant higher Tfh cells proportion in u-RA compared to the HC subjects (SMD 0.71, [0.51, 0.90], $p < 0.0001$, $I^2 = 69.52$, $p < 0.0001$) (Fig. 3). Egger's test also showed no publication bias ($p = 0.08$). Furthermore, we performed subgroup analysis on the studies that defined Tfh cells as “CD4⁺CXCR5⁺”, “CD4⁺CXCR5⁺PD-1⁺”, “CD4⁺CXCR5⁺PD-1^{high}”, “CD4 + CXCR5 + ICOS^{high}”, “CD4⁺CXCR5⁺PD-1⁺ICOS⁺” and “CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}” (Supplementary Fig. 9A-F). As there was a maximum of 1 study per group for other definitions, we couldn't perform subgroup analysis for them. The meta-analysis results revealed a markedly greater proportion of Tfh cells in u-RA compared to HC individuals when Tfh cells were defined by the 4 out of 5 definitions.(for “CD4⁺CXCR5⁺”: (SMD 0.45, [0.18, 0.72], $p = 0.001$, $I^2 = 60.48$, $p = 0.012$; for “CD4⁺CXCR5⁺PD-1^{high}”: SMD 1.17, [0.69, 1.66], $p < 0.0001$, $I^2 = 61.26\%$, $p = 0.108$); for “CD4⁺CXCR5⁺PD-1⁺ICOS⁺” SMD 0.67, [0.31, 1.04], $p = 0.0003$, $I^2 = 0\%$, 0.518, for “CD4⁺CXCR5⁺ICOS^{high}”: SMD 0.75, [0.35, 1.15], $p = 0.0002$, $I^2 = 0\%$, $p = 0.587$ and for “CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}”: SMD 0.66, [0.18, 1.14], $p = 0.0075$, $I^2 = 50.79\%$, $p = 0.154$. While “CD4⁺CXCR5⁺PD-1⁺” cells didn't statistically significant difference, but it has a trend toward significant (SMD 0.96, [-0.02, 1.94], $p = 0.055$, $I^2 = 89.48$, $p < 0.0001$) (Table 3, Supplementary Fig. 9A-F). In addition, based on Egger's test, no evidence of publication bias was found for the studies ($p > 0.1$, Table 3)

Table 3
Subgroup analysis results of Tfh cells proportion in u-RA compared to HC based on definition markers.

Tfh definition	No.	SMD [95% CI]	p	Heterogeneity		Publication bias (Egger's p)	Model
				I ² (%)	P		
CD4 ⁺ CXCR5 ⁺	8	0.45 [0.18, 0.72]	0.001	60.48	0.012	0.524	REM
CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	3	0.96 [-0.02, 1.94]	0.055	89.48	$p < 0.0001$	0.145	REM
CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	2	1.17 [0.69, 1.66]	$p < 0.0001$	61.26	0.108	-	REM
CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	2	0.75 [0.35, 1.15]	0.0002	0	0.587	-	FEM
CD4 ⁺ CXCR5 ⁺ PD-1 ⁺ ICOS ⁺	2	0.67 [0.31, 1.04]	0.0003	0	0.518	-	FEM
CD4 ⁺ CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high}	2	0.66 [0.18, 1.14]	0.0075	50.79	0.154	-	REM
Tfh: T follicular helper, No: number of studies, SMD: Standardize mean difference, p: p value, REM: random effect model, FEM: fixed effect model, RA: rheumatoid arthritis, u-RA: untreated RA							

3.5 The proportion of Tfh cells in PB of early RA compared with HC

Then we questioned whether the proportion of Tfh would differ between patients and HCs when the stage of the disease course was taken into consideration. We included 3 articles [27, 29, 43] covering 8 studies in which patients had disease duration less than 6 months as e-RA. All the eRA patients in these studies were treatment naïve with lake of any history of taking immunosuppressive drug. Pooled result regardless of Tfh definition showed that the proportion of Tfh cells in the untreated e-RA patients was significantly higher than that in healthy subjects (SMD 0.92, [0.62, 1.22], $p < 0.0001$, $I^2 = 60.26\%$, $p = 0.012$) (Fig. 4). The egger's test indicated no evidence of publication bias ($p = 0.178$). Subgroup analysis on "CD4⁺CXCR5⁺" and "CD4⁺CXCR5⁺PD-1⁺" also showed higher proportion of Tfh cells in untreated e-RA compared to HCs by the all three phenotypes (SMD 0.39, [0.05, 0.72], $p = 0.0225$, $I^2 = 0\%$, $p = 0.322$ for "CD4⁺CXCR5⁺"; SMD 1.49, [1.05, 1.93], $p < 0.0001$, $I^2 = 0\%$, $p = 0.419$ for "CD4⁺CXCR5⁺PD-1⁺") (Table 4, Supplementary Fig. 10A, B)

Table 4
Subgroup analysis results of Tfh cells proportion in early RA (e-RA) patients compared to HCs based on definition markers.

Tfh definition	No.	SMD [95% CI]	p	Heterogeneity		Model
				I ² (%)	P	
CD4 ⁺ CXCR5 ⁺	2	0.39 [0.05, 0.72]	0.0225	0	0.322	EEM
CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	2	1.49 [1.05, 1.93]	$p < 0.0001$	0	0.419	FEM
Tfh: T follicular helper, No: number of studies, SMD: Standardize mean difference, p: p value, FEM: fixed effect model, RA: rheumatoid arthritis						

3.6 The proportion of Tfh in the PB of patients with active RA, patients in remission, and HCs

We next asked whether the proportion of Tfh cells of the blood CD4⁺T cells, could be different between patients with active RA (a-RA) and those who were in remission (r-RA). Eight studies compared the proportion of Tfh cells between a-RA and r-RA, from which 4 studies reported higher proportion of Tfh in a-RA, 3 studies reported no differences in the Tfh cells proportion between the two groups, and 1 study reported higher Tfh cells proportion in r-RA (Table 5). The pooled SMD result of Tfh proportion regardless of its definition revealed that there was a significantly higher Tfh cells proportion in the PB of a-RA in comparison with r-RA (SMD 0.27, [0.07, 0.48], $p = 0.007$, $I^2 = 48.83\%$, $p = 0.057$) (Fig. 5A). No risk of publication bias was found based on the egger's test ($p = 0.085$). As evident from Table 5, different set of markers was used by the authors to evaluate the Tfh cells proportion. Thus, we also did sub-group meta-analysis on these studies to compare a-RA with r-RA. Two studies used "CD4⁺CXCR5⁺" [22, 37] and 2 used "CD4⁺CXCR5⁺ICOS⁺" [37, 40]. Four remaining studies evaluated the proportion of "CD4⁺CXCR5⁺PD-1⁺" [40], "CD4⁺CXCR5⁺PD-1^{high}" [22], "CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}" [32], and "CD4⁺CXCR5⁺Foxp3⁻" [40] cells, and because meta-analysis could not be performed on only one study, they were removed from sub-analysis. The sub-group meta-analysis results revealed no difference between a-RA and r-RA when Tfh cells were defined by the two above-mentioned markers (for CD4⁺CXCR5⁺: SMD 0.16, [-0.21, 0.52], $p = 0.39$, $I^2 = 0\%$, $p = 0.4$; and for CD4⁺CXCR5⁺ICOS⁺: SMD 0.03, [-0.41, 0.47], $p = 0.89$, $I^2 = 34.79\%$, $p = 0.21$). (Table 6, supplementary Fig. 11A, B)

We also performed the same analysis to compare a-RA patients with HC group. Total of 8 studies compared the proportion of Tfh cells between a-RA and HC groups. Of these, 6 studies showed significant increase in the Tfh cells proportion in a-RA, and 2 remaining studies showed no differences (Table 5). Pooled meta-analysis result regardless of the Tfh definition revealed significant increase in the PB Tfh cell proportion when a-RA patients were compared to HCs (SMD 0.91, [0.4, 1.42], $p = 0.0004$, $I^2 = 83.4\%$, $p < 0.0001$). Furthermore, egger's test indicated that there was no publication bias ($p = 0.1$). Next, we analyzed sub-group meta-analysis based on different definition markers used for Tfh cells. Of the 8 studies, two used "CD4⁺CXCR5⁺" and 2 used CD4⁺ + CXCR5 + ICOS⁺ to define Tfh cells. Pooled analysis for both definitions demonstrated no differences in the proportion Tfh cells between a-RA and HCs. (For "CD4⁺CXCR5⁺": SMD 0.58, [-0.22, 1.39], $p = 0.15$, $I^2 = 78.37\%$, $p = 0.03$), for "CD4⁺CXCR5⁺ICOS⁺": SMD 0.315, [-0.105, 0.734], $p = 0.141$, $I^2 = 0\%$, $p = 0.72$) (Table 6, supplementary Fig. 12A, B)

Table 5
Characteristics of the studies that compared the proportion of Tfh cells in active- and remission-RA with HCs.

Author	Year	Country	a-RA number	r-RA number	HC number	Tfh definition	a-RA		r-RA		HC	
							mean	SD	mean	SD	mean	SD
Liu et al. α	2018	China	39	39	33	CD4 ⁺ CXCR5 ⁺	19.000	3.540	18.810	3.170	15.18	4.19
Liu et al. β	2018	China	39	39	33	CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	7.110	2.860	7.030	2.750	3.59	1.01
Arroyo-Villa et al. α	2014	Spain	17	17	34	CD3 ⁺ CD4 ⁺ CXCR5 ⁺	17.511	8.189	14.840	4.673	16.32	7.11
Arroyo-Villa et al. β	2014	Spain	17	17	34	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	1.345	0.638	1.574	0.929	1.18	0.70
Niu et al. α	2018	China	20	24	20	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ Foxp3 ⁻	25.000	7.155	18.400	5.388	18.2	5.81
Niu et al. β	2018	China	20	24	20	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ⁺	31.500	9.390	26.500	12.240	13.4	3.577
Niu et al. γ	2018	China	20	24	20	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ⁺	15.500	10.280	13.200	5.870	12.3	4.47
Chen et al.	2016	China	35	6	-	CD3 ⁺ CD4 ⁺ + CXCR5 ⁺ CCR7 ^{low} PD-1 ^{high}	13.339	5.867	7.720	2.466	-	-
Zhang et al.	2015	China	54	-	16	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	1.01	0.63	-	-	0.64	0.26
a-RA: active-RA, r-RA: remission-RA, HC: healthy control, SD: standard deviation												

Table 6
Subgroup analysis results of disease activity status and Tfh cell proportions.

Groups	Tfh definition	No.	SMD [95% CI]	p	Heterogeneity		Model
					I ² (%)	P	
a-RA vs r-RA	CD4 ⁺ CXCR5 ⁺	2	0.16 [-0.21, 0.52]	0.39	0	0.4	FEM
	CD4 ⁺ CXCR5 ⁺ ICOS ⁺	2	0.03, [-0.41, 0.47]	0.89	34.79	0.21	FEM
a-RA vs HC	CD4 ⁺ CXCR5 ⁺	2	0.58 [-0.22, 1.39],	0.15	78.37	0.03	REM
	CD4 ⁺ CXCR5 ⁺ ICOS ⁺	2	0.315 [-0.105, 0.734]	0.141	0	0.72	FEM
Tfh: T follicular helper, No: number of studies, SMD: Standardize mean difference, p: p value, REM: random effect model, FEM: fixed effect model, a-RA: active rheumatoid arthritis, r-RA: remission rheumatoid arthritis, HC: healthy control							

3.7 The proportion of Tfh in PB of seropositive RA patients versus seronegative RA patients and HCs

Seropositive patients were defined as anti-citrullinated protein antibodies (ACPA)⁺ and/or rheumatoid factor (RF)⁺. Whether Tfh cells proportion among PB CD4⁺ T cells is different between S⁺RA and S⁻RA patients was evaluated. Five studies assessed the proportion of Tfh in S⁺RA compared with S⁻RA patients, of which 3 studies showed higher proportion of Tfh in S⁺RA, and 2 other showed no difference (Table 7). Pooled meta-analysis of all 5 studies, regardless of the Tfh definition revealed a significant increase in the proportion of Tfh cells in PB of patients with S⁺RA compared to that of those with S⁻RA (SMD 0.64, [0.36, 0.91], $p < 0.0001$, $I^2 = 3.85\%$, $p = 0.38$) (Fig. 6A). In addition, based on the egger's test, no publication bias was found ($p = 0.82$). It should be noted that 4 out of the 5 studies were evaluated Tfh cells proportion in "early RA" patients. In addition, since only two studies used the same pattern of Tfh definition (CD4⁺CXCR5⁺PD-1^{high}) to compare S⁺RA and S⁻RA patients, we performed a sub-group meta-analysis on these studies. The sub-analysis revealed higher proportion of Tfh cells in PB of S⁺RA compared with S⁻RA patients when Tfh was defined as "CD4⁺CXCR5⁺PD-1^{high}" (SMD 0.67, [0.26, 1.07], $p = 0.001$, $I^2 = 0\%$, $p = 0.93$) (Table 8, supplementary Fig. 13).

We then performed further analyses to compare the proportion of PB Tfh cells between S⁺RA patients and the HCs. Four studies from one article were evaluated PB Tfh proportion in S⁺RA and HCs with different definitions. Pooled SMD result revealed that the proportion of Tfh cells was markedly higher in PB of S⁺RA patients compared to HCs (SMD 0.89, [0.48, 1.3], $p < 0.0001$, $I^2 = 68.92\%$, $p = 0.019$) (Fig. 6.B). Egger's tests indicated no publication bias (p

= 0.178). In addition two studies from this article compared the proportion of “CD4⁺CXCR5⁺PD-1^{high}” Tfh cells between early RA or established RA and HCs. Subgroup analysis of the proportion of “CD4⁺CXCR5⁺PD-1^{high}” cells regardless of the disease stage revealed a significantly higher proportion of Tfh cells in S⁺RA compared with HCs (SMD 1, [0.67, 1.33], $p < 0.0001$, $I^2 = 34.9\%$, $p = 0.215$). (Table 8, supplementary Fig. 14)

Table 7
Characteristics of the studies that compared Tfh cells proportion between S⁺RA and S⁻RA, and S⁺RA and HCs.

Author	Year	Country	S ⁺ RA number	S ⁻ RA number	HC number	Tfh definition	S ⁺ RA		S ⁻ RA		HC	
							mean	SD	mean	SD	mean	SD
Ma et al.	2012	China	19	6	-	CD4 ⁺ CXCR5 ⁺ ICOS ^{high}	0.197	0.209	0.074	0.051	-	-
Fortea-Gordo et al. (α)	2019	Spain	38	17	47	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ CD45RA ⁻	17.748	5.587	16.510	6.868	15.735	5.278
Fortea-Gordo et al. (β)	2019	Spain	38	18	47	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} CD45RA ⁻	0.574	0.393	0.337	0.276	0.232	0.139
Fortea-Gordo et al. (γ)	2019	Spain	38	17	47	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} ICOS ⁺ CD45RA ⁻	1.207	0.674	0.562	0.394	0.529	0.404
Fortea-Gordo et al. (δ)	2019	Spain	47	-	29	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} CD45RA ⁻	0.271	0.177	-	-	0.151	0.095
A. Rao et al. [46]	2017	USA	42	16	-	CD3 ⁺ CD4 ⁺ CXCR5 ⁺ PD-1 ^{high} CD45RA ⁻	0.625	0.500	0.316	0.250	-	-
S ⁺ RA: sero positive rheumatoid arthritis, S ⁻ RA: seronegative rheumatoid arthritis, HC: healthy control, SD: standard deviation												

Table 8
Subgroup analysis results based on serostatus and Tfh cell proportions.

Groups	Tfh definition	No.	SMD [95% CI]	p	Heterogeneity		Model
					I ² (%)	P	
S ⁺ RA vs S ⁻ RA	CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	2	0.67 [0.26, 1.07]	0.001	0	0.93	FEM
S ⁺ RA vs HC	CD4 ⁺ CXCR5 ⁺ PD-1 ^{high}	2	1 [0.67, 1.33]	$p < 0.0001$	34.9	0.215	FEM
Tfh: T follicular helper, No: number of studies, SMD: Standardize mean difference, p: p value, REM: random effect model, FEM: fixed effect model, S ⁺ RA: seropositive rheumatoid arthritis, S ⁻ RA: seronegative rheumatoid arthritis, HC: healthy control							

3.8 Geographic location of studies and Tfh cells proportion in PB of RA patients

Finally, we assessed the proportion of Tfh cells based on the geographical regions where the studies were performed. Regarding the geographic location of the included studies 14 studies were performed in China, 4 in Japan, 2 in Spain, 1 in Sweden, 1 in Italy, 1 in USA, and 1 in Argentina (Table 1) to compare RA with HC. We divided the studies into 2 groups, Asian and non-Asian studies. There were 18 articles covering 30 studies on Asian populations (Japan and China), and 6 articles covering 9 studies on the non-Asians (Table 1). The pooled SMD revealed that there was higher Tfh cells proportion in RA patients compared to HCs in Asian populations (SMD 0.89, [0.68, 1.09], $p < 0.0001$, $I^2 = 77.48$, $p < 0.0001$) (Supplementary Fig. 15), while surprisingly there was no significant difference in the proportion of Tfh between the two groups in non-Asians (SMD 0.29, [-0.003, 0.59], $p = 0.052$, $I^2 = 69.15\%$, $p = 0.0012$) (Supplementary Fig. 16). There was an evidence of publication bias in the Asian analysis based on egger's test ($p = 0.02$) and funnel plot, and implementing the trim-and-fill method did not show any change in the bias-adjusted result. Regarding non-Asians there was also publication bias ($p = 0.039$, Supplementary Fig. 17) and adjustment by trim-and-fill analysis revealed an increase in the pooled SMD.

4. Discussion

The main function of Tfh cell is to help B lymphocytes to proliferate, differentiate and promote antibody production, and regulate humoral immunity [5, 6]. When CD4⁺ naïve-T cells are activated, they increase CXCR5 expression, while decrease CCR7, which allows them to migrate into the B-cell follicles in response to CXCR5 ligand, CXCL13. This enables Tfh to localize in close proximity to and interact with B cell, and promotes GC formation and class switched high affinity antibody production [6, 9]. However, Tfh deregulations can drive abnormal germinal center, B-lymphocyte differentiation and survival, autoantibody generation, and thus could be related to the development of autoimmune diseases [12, 47]. Due to the difficulty in the sampling

from human lymphoid tissue [6, 8], and since PB Tfh cells in terms of phenotypic and B lymphocyte helper functions are similar to the GC Tfh cells [16], analysis of cTfh cells in patients has become an important clinically significant alternative strategy [48]

RA is a systemic autoimmune disease characterized by the production of a large number of autoantibodies, such as ACPAs, RF and others [3], and this autoantibody production may be related to Tfh cell abnormality. In this line, while several studies have evaluated the frequency of Tfh in the PB of patients with RA compared to HCs, conflicting conclusions have been reached in some cases. The purpose of this work was to systematically evaluate the proportion of Tfh in PB of RA patients compared to HCs to clarify the proportion of cTfh in RA patients.

Meta-analysis of all included studies neglecting the definition markers, treatment status, disease activity status and serological status of the RA patients revealed a significantly higher Tfh cells proportion in the PB of patients with RA in comparison with HCs. However, significantly high degree of heterogeneity was found between studies. Thus, sub-group analyses were subsequently performed based on different factors including surface markers used to define Tfh cells, treatment status, disease activity status, and serological status of the patients as well as the geographical location of the included studies from which the participants originated. Our sub-analyses revealed that subgrouping the studies based on these criteria were associated with reduction in the strength of heterogeneity in most of analyses which reached to moderate, low or even no heterogeneity (Tables 2, 3, 4, 6 and 8).

In the sub-group analyses, at first we performed the meta-analysis based on only definition markers used to identify Tfh cells. According to the results, the proportion of Tfh cells with the all definition markers was significantly higher in the PB of RA patients compared to HCs (Table 2). From different definitions, “CD4⁺CXCR5⁺PD-1^{high}” and then “CD4⁺CXCR5⁺PD-1⁺” cells showed the largest SMD, and while “CD4⁺CXCR5⁺” were significantly increased in RA, it showed the lowest SMD, suggesting the importance of considering the expression level of PD-1 in RA patients.

PD-1 can promote GC B cells survival and high-affinity long-lived plasma cell formation through its interaction with programmed death-ligand 1 (PD-L1) and PD-L2 on the surface of GC B cells [49, 50]. Studies have shown that the expression of PD-1 is elevated on Tfh cells in patients with autoimmune diseases including RA [40, 51], and PD-1^{high} Tfh cells have a stronger ability to activate B lymphocytes [51]. Consistently, a positive correlation has been found between the expression of PD-1 on Tfh cells, and disease activity of RA [40]. However, the negative regulatory function of this molecule on T cells, that is essential for maintaining peripheral immune-tolerance, should not be ignored [49, 52]. Blocking the interaction of PD-1 with its ligands leads to increase differentiation and number of Tfh cells [50], suggesting the negative regulatory function of PD-1 signals in the differentiation of Tfh cells. Thus, the expression level of PD-1 could mirror the activation status of the Tfh cells, and the increasing in the expression of PD-1 on Tfh cells in RA patients might represent a spontaneous compensatory regulation mechanisms for controlling its excessive activation. But this regulatory feedback fails to inhibit Tfh cells, which is due to increase in soluble form of PD-1 (sPD-1) in the RA patients [33, 53]. Consistently, there is also a significant positive correlation between serum level of the sPD-1 and the frequency of cTfh cells, titer of auto-antibodies and DAS in RA patients [33, 53]. Indeed, the inhibitory function of the increased membrane-bound PD-1 on Tfh cells is blocked in the presence of its soluble form, while its humoral assistance to antibody producing cells is intact or even hyper activated. The results of our meta-analyses revealed significantly higher proportion of Tfh cells in all the comparisons which have included PD-1^{high} for Tfh cells (RA versus HC, u-RA versus HC, S⁺RA versus S⁻RA and S⁺RA versus HC). Furthermore, based on subgroup analyses, the definitions which included PD-1 (“CD4⁺CXCR5⁺PD-1⁺” and especially “CD4⁺CXCR5⁺PD-1^{high}”) had the largest SMDs in comparison with the other definitions suggesting more associations between the proportion of these phenotypes and RA. Thus, in RA patients, the higher expression of PD-1 on the Tfh cells could be associated with the higher proportion of these cells.

ICOS is another surface molecule that has a pivotal role in the development of Tfh cells and also in the production of IL-21 as signature cytokine of Tfh [54]. In addition, ICOS signaling is essential for maintenance of anatomical localization of Tfh cells in B cell follicles through preserving the expression of homing receptor pattern on Tfh cells. Blocking of ICOS has also been shown to lead to reverse differentiation of Tfh through CCR7 up-regulation and CXCR5 down-regulation, hence relocation of Tfh cells to the T cell zone and subsequently collapsing of the GC response [55, 56]. ICOS also promotes survival and functional maturation of GC B cells [57]. Furthermore, Tfh cells expressing the highest expression level of ICOS have the most capacity of inducing IgG production [58]. Our study demonstrated that Tfh cells expressing ICOS (CD4⁺CXCR5⁺ICOS⁺ and CD4⁺CXCR5⁺ICOS^{high}) (Table 2) were higher in RA versus HCs. In addition, “CD4⁺CXCR5⁺ICOS^{high}” proportion had higher association with RA than “CD4⁺CXCR5⁺ICOS⁺” proportion as was evident from SMDs (Table 2) which, could be justified by the above-mentioned roles defined for ICOS molecule. Considering the importance of ICOS in antibody production, unfortunately there were not at least 2 studies with CD4⁺CXCR5⁺ICOS^{+/high} definitions for comparing Tfh cells proportion based on serostatus subgroup analyses.

We also analyzed the studies that only included untreated patients to exclude the effect of immuno-suppressive drugs. The results further confirmed the elevated proportions of Tfh cells in untreated patients, regardless of their definitions. Furthermore, subgroups analyses demonstrated higher proportions of “CD4⁺CXCR5⁺”, “CD4⁺CXCR5⁺PD-1⁺”, “CD4⁺CXCR5⁺PD-1^{high}”, “CD4⁺CXCR5⁺ICOS^{high}”, “CD4⁺CXCR5⁺PD-1⁺ICOS⁺” and “CD4⁺CXCR5⁺CCR7^{low}PD-1^{high}” cells in u-RA compared with HCs. From these comparisons the highest association was found for “CD4⁺CXCR5⁺PD-1^{high}”, and “CD4⁺CXCR5⁺” had the lowest SMD, again suggesting the importance PD-1 molecules in defining the Tfh cells.

The proportion of Tfh cell was also evaluated based on the stage of the disease course. According to 2015 ACR guideline, RA patients with disease duration less than 6 months are considered “early RA”, and those with disease duration $\geq$ 6 months are considered as “established RA” [59]. Studies have shown that the immunological aberrations during the first few months after the disease onset differ from those during later phases [60], which may explain why response rate to treatment in early RA patients is different from those of established RA (early treatment of the RA patients is beneficial and can improve disease outcome) [61, 62]. Because of the importance of the early diagnosis of RA and also to clarify the status of Tfh cell in the early phase of the disease, we also compared Tfh cells proportion in e-RA patients with that of HCs. All e-RA patients included in the selected articles were treatment

naïve. The pooled results of the all Tfh definitions exhibited that cTfh cells proportion in untreated e-RA patients was significantly greater than that of the HC group. Subgroup analysis of Tfh cells with definitions of “CD4⁺CXCR5⁺”, CD4 + CXCR5 + PD-1⁺ also showed higher Tfh cells proportion in untreated e-RA compared to HCs, suggesting pathogenetic role of Tfh in initial stage of the RA development.

In the next step, Tfh cells proportion in patients with a-RA was compared to r-RA patients and HCs. The pooled results regardless of definition markers revealed that the proportion of Tfh cells in a-RA patients was significantly greater than those of both r-RA patients and HCs. Altogether these results suggest pathogenic association of the Tfh frequency with RA. We then evaluated the Tfh cell proportion based on Tfh definitions. The results revealed that there was no significant difference in the proportion of “CD4⁺CXCR5⁺”, and “CD4⁺CXCR5⁺ICOS⁺” cells when a-RA was compared to the both r-RA and HCs, though higher SMD was observed for comparison of a-RA versus HCs than the SMD derived from comparing a-RA with r-RA. It should be kept in mind that since a maximum of two studies have been used for sub-meta analyses comparing a-RA with r-RA and HCs, the power of the test is low in these comparisons and meta-analysis on higher number of studies in the future could be more valuable.

To evaluate the link between the frequency of Tfh cells and presence of auto-antibodies in RA, we next performed meta-analysis with respect to the serological status of the patients. S⁺RA is defined as positive for RF or ACPAs. Seropositivity is associated with more severe disease, joint destruction, and even higher mortality [3]. The pooled meta-analysis results, regardless of the Tfh definition demonstrated that S⁺RA patients had a significantly higher proportion of Tfh cells in their PB compared to the either S⁻ RA patients or HCs. Also, markers-based subgroup analysis showed that the proportion of Tfh cells was significantly elevated in S⁺RA in comparison with the both S⁻RA patients and HCs when Tfh cells were defined as CD4⁺CXCR5⁺PD-1^{high}. This can further emphasize the previously mentioned importance of PD-1 expression level in the frequency of Tfh cells and autoantibody production in RA patients. Due to insufficient study number, we unfortunately couldn't perform subgroup analysis on the other Tfh definitions especially ICOS-expressing Tfh cells to compare S⁺RA and S⁻RA patients. Altogether, these results demonstrate that the production of auto-antibodies in RA patients is associated with the proportion of CD4⁺CXCR5⁺PD-1^{high} Tfh cells, which provides evidence for the connection between the seropositivity and the proportion of these cTfh cells.

Looking at different definitions used in all comparisons demonstrates that the largest SMDs for proportion of Tfh cells in comparison groups belong to the Tfh cells expressing PD-1 (PD-1⁺ and especially PD-1^{high}), while the lowest association was related to the “CD4⁺CXCR5⁺” cells.

The present work has some limitations. First, as disease duration was inconsistent across the studies we couldn't consider duration of the disease in the analysis. Second, the disease activity score of RA patients was not uniform through the studies. Third, due to heterogeneity of drugs used for treatment of patient, we couldn't evaluate the effect of treatment on the proportion of Tfh cells in RA. Fourth, insufficient number of studies with particular Tfh definitions prevented their evaluation in some of the comparisons.

Conclusions

In conclusion, this is the first systematic review and meta-analysis that has assessed the proportion of PB Tfh cells in RA patients. Our analyses demonstrate that compared to HCs the proportions of Tfh cells in general, and in particular Tfh cells expressing PD-1 (PD-1^{high} and then PD-1⁺) are highly elevated in patients with RA. Therefore, this cell type likely plays a pathogenic role in RA and could potentially be used as biomarker for diagnosis, prognosis, or as a target for treatment.

Declarations

Author Contributions

PB: study design. PB, MAH and KK: data collection. PB: statistical analysis. PB: paper writing. KK, KKE, RES, DK, SM: paper review & editing. All authors approved the submitted version of the manuscript.

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Competing interests

None.

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Ethics approval: This is an observational study. The Shiraz University of Medical Sciences Research Ethics Committee has confirmed that no ethical approval is required.

Consent for publication

This is an observational(Meta-Analysis) study and we were not in contact with patients and there was no need to get a consent form from patients.

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Figures

Figure 1

Flow chart of study selection process

Figure 2

Overall standardized mean differences (SMD) of the proportion of Tfh cells in peripheral blood (PB) of RA versus HC. SMD of Tfh cell proportions in PB of RA patients compared with HCs, regardless of the Tfh cell definitions. Study identifier (a, b, c, d) was used to distinguish each study (table 1).

Figure 3

Standardized mean differences (SMD) of the proportion of Tfh cells in peripheral blood (PB) of untreated-RA (u-RA) patients versus healthy controls (HCs). SMD of Tfh cell proportions in PB of u-RA patients compared with HCs, regardless of the Tfh cell definitions. Study identifier (a, b, c, d) was used to distinguish each study (table 1).

Figure 4

Standardized mean differences (SMD) of the proportion of Tfh cells in peripheral blood (PB) of early RA (e-RA) patients versus healthy controls (HCs). SMD of Tfh cell proportions in PB of e-RA patients compared to HC, regardless of the Tfh cell definitions. Study identifier (a, b, c, d) was used to distinguish each study (table 1).

Figure 5

standardized mean differences (SMD) of the proportion of T follicular helper (Tfh) cells in the peripheral blood (PB) of active-RA (a-RA) compared to remission-RA(r-RA). Tfh cell proportion in PB of a-RA compared with r-RA (A) and a-RA compared with HC (B). Study identifier (α , β , γ) was used to distinguish each study (table 2).

Figure 6

Standardized mean differences (SMD) of the proportion of T follicular helper (Tfh) cells in the peripheral blood (PB) of seropositive RA (S+RA) compared to seronegative RA (S-RA) and healthy control (HC). Tfh cell proportion in PB of S+RA compared to S-RA (A) and S+RA compared to HC (B).