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Original article

Distinct features between HLA-DR+ and HLA-DR− PD-1hi CXCR5– T peripheral helper cells in seropositive rheumatoid arthritis

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Abstract

Objectives. PD-1hi CXCR5– T peripheral helper (Tph) cells are newly identified pathogenic CD4 helper T cells in RA. We evaluated the usefulness of Tph cell subsets as biomarkers of RA.

Methods. RA patients who visited our rheumatology department between May 2015 and September 2017 and met the 2010 ACR/EULAR classification criteria were included. We compared the correlation of DAS28-ESR between Tph cell subsets and 40 immune cell subsets. We also explored which subsets reflected the chronological changes in the disease activity after treatment.

Results. Thirty-four seropositive RA patients, 11 seronegative RA patients and 34 healthy controls were included. Tph cell subsets that correlated with the DAS28-ESR were HLA-DR+ Tph cells ($r_s = 0.50$, $P = 0.002$), HLA-DR– Tph cells ($r_s = 0.39$, $P = 0.03$) and Tph1 cells ($r_s = 0.41$, $P = 0.02$). Among the other 40 immune cell subsets, HLA-DR+ Th1-17 cells ($r_s = 0.38$, $P = 0.03$), activated B cells ($r_s = -0.35$, $P = 0.04$), plasma cells ($r_s = 0.43$, $P = 0.01$) and CD14++ CD16+ monocytes ($r_s = 0.36$, $P = 0.04$) correlated, but not strongly as HLA-DR+ Tph cells. However, MTX treatment reduced the proportion of HLA-DR+ Tph cells independently of the disease activity. In contrast, HLA-DR– Tph cells accurately reflected the change in the DAS28-ESR during MTX treatment.

Conclusion. HLA-DR+ Tph cells were decreased with MTX treatment, independent of the disease activity, while HLA-DR– Tph cells reflected the disease activity accurately during the treatment.

Key words: rheumatoid arthritis, T peripheral helper cells, HLA-DR, biomarker

Rheumatology key messages

- HLA-DR+ T peripheral helper cells more strongly reflected baseline disease activity than 40 other immune cells.
- MTX treatment decreased HLA-DR+ T peripheral helper cells, independent of the disease activity.
- HLA-DR– T peripheral helper cells reflected the disease activity during treatment more accurately than HLA-DR+ T peripheral helper cells.

Introduction

RA is an immune-mediated rheumatic disease characterized by persistent inflammation of the synovium, resulting in joint destruction [1]. Recent investigations have indicated that T cells, B cells, monocytes and fibroblasts are the pathogenic drivers for RA [2, 3]. Identifying unique cell subsets is essential not only for understanding the pathogenesis but also for differentiating RA from similar diseases and assessing the efficacy of treatment in clinical settings.

Among immune cell subsets, T cells perform a variety of pro- and anti-inflammatory functions, such as releasing cytokines, helping B cell subsets, directly killing cells

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CLINICAL SCIENCE

via cytotoxic molecules and regulating autoreactive cells. Several T cell subsets have been reported to be involved in the pathogenesis of RA [3–5], including the recently identified pathogenic CD4 helper T cell subset, T peripheral helper (Tph) cells [6]. Tph cells are defined as PD-1hi CXCR5– helper T cells and were discovered in synovium and fluid samples of RA patients. These cells were found to preferentially infiltrate synovial tissue through the expression of CCR2, CCR5 and CX3CR1. Tph cells in the peripheral blood (known as circulating Tph cells) also expand and reflect the disease activity of RA [6, 7]. However, a recent study classified Tph cells into even more detailed subsets [8], but it is unclear which of these Tph cell subsets actually reflects disease activity. Further, several other immune cells have also been reported to reflect disease activity, raising the question of whether or not circulating Tph cells are more useful for evaluating disease activity than other immune cell subsets.

In this study, we compared the correlation of the baseline disease activity between Tph cell subsets and 40 immune cell subsets. We also revealed the association between the proportions of Tph cell subsets and the disease activity after treatment. Here, we report the usefulness of Tph cell subsets as biomarkers of seropositive RA.

Methods

Patients

RA patients who visited our rheumatology department between May 2015 and September 2017 and met the 2010 ACR/EULAR classification criteria [9] were included. All patients were newly diagnosed RA who had not received treatment prior to diagnosis. We confirmed that the healthy controls (HCs) did not have any autoimmune disease, severe allergic disorder, malignancy or infection. As susceptible ages are different between seropositive and seronegative RA, age- and sex-matching was not performed. Treatment was started immediately after the patients were diagnosed as RA. Biologics were initiated according to the treating physician's judgement if MTX alone was insufficient to treat or if it was not possible to use MTX because of a contraindication.

The study protocol was approved by the ethics committee of Keio University School of Medicine (#20140335) and carried out in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients and HCs.

Data collection

Clinical information, including patient age and sex, disease duration, treatment, and the CRP, ESR, MMP-3, RF and ACPA levels were obtained from patients' medical records. Seropositive RA was defined as the elevation of either RF (>15 IU/ml) or ACPA (≥4.5 U/ml). Disease activity was mainly assessed using the DAS28-ESR. MTX inadequate response (MTX-IR) was defined as not reaching the DAS28-ESR <3.2 despite receiving MTX monotherapy for ≥6 months or the initiation of biological or targeted synthetic DMARDs due to the inefficacy of MTX monotherapy.

Flow cytometry (FACS) analysis

Peripheral blood samples were collected from the patients at weeks 0 (time of diagnosis), 12, 24 and 52 of treatment. Immuno-phenotyping with LSRFortessa TM X-20 (BD Biosciences, San Jose, CA, USA) was carried out in accordance with the manufacturers' instructions concerning the antibodies used (BD Biosciences and BioLegend, San Diego, CA, USA; supplementary Table S1, available at *Rheumatology* online). Flow-count Fluorospheres (Beckman Coulter, CA, USA) were used to acquire standardized number of immune cells. Data analyses were performed using FlowJo v.10.1 (Tree Star, OR, USA). The phenotypes of immune cell subsets were defined based on the Human Immunology Project protocol [10]. Details of the gating strategy are shown in supplementary Figs S1–S4, available at *Rheumatology* online. Regarding the gating for markers that did not have a distinct positive and negative population, except for PD-1 and HLA-DR on T cells, we used the isotype control as a negative control to determine cut-off lines. A cut-off line of PD-1 high is defined by the tangent line adjacent to top of the 5% contour line of CXCR5+ cells in biaxial flow cytometry plot using CXCR5 and PD-1 (supplementary Fig. S5, available at *Rheumatology* online). As for the cut-off line of HLA-DR on T cells, we used CD3– CD4– cells as a control to determine a cut-off line of HLA-DR (supplementary Fig. S6, available at *Rheumatology* online). As for the gating for Tph1 cells, Tph2 cells, Tph17 cells and Tph1–17 cells, we determined the cut-off line according to Th1 cells, Th2 cells, Th17 cells and Th1–17 cells classification, which was gated by CXCR3, CCR4 and CCR6 positive or negative. The definition of the proportion of each subset is shown in supplementary Table S2, available at *Rheumatology* online.

Statistical analyses

Continuous data are presented as the mean and S.E. Differences between unpaired samples were compared using the Mann–Whitney *U* test with Bonferroni correction. Categorical variables were analysed using Fisher's exact test. Wilcoxon's signed-rank test was used between paired samples. A correlation analysis was performed using Spearman's rank correlation coefficient. Lines of best fit were drawn when statistically significant. *P*-values <0.05 (two-sided) were considered statistically significant. All statistical analyses were performed with GraphPad Prism 8 (GraphPad, La Jolla, CA, USA).

Table 1 Clinical characteristics of patients with RA

Clinical characteristic	RA ($n = 45$)	Seropositive RA ($n = 34$)	Seronegative RA ($n = 11$)	HCs ($n = 34$)	*P*-value
Age, mean (S.E.), years	57.0 (2.6)	53.4 (2.7)	68 (5.0)	41.0 (2.4)	0.02
Female, *n* (%)	34 (75.6)	26 (76)	8 (72.7)	23 (67.6)	1.00
Disease duration, mean (S.E.), weeks	17.4 (5.9)	21.3 (7.7)	5.3 (2.3)		0.35
DAS28-ESR, mean (S.E.)	4.7 (0.2)	4.3 (0.2)	5.7 (0.2)		0.002
DAS28-CRP, mean (S.E.)	3.8 (0.2)	3.5 (0.2)	4.8 (0.3)		0.001
CDAI, mean (S.E.)	17.2 (1.5)	15.3 (1.6)	23.0 (2.5)		0.01
ESR, mean (S.E.), mm/h	50.2 (5.6)	44.8 (5.8)	66.7 (13.1)		0.10
CRP, mean (S.E.), mg/dl	1.8 (0.5)	1.4 (0.5)	3.3 (1.1)		0.04
MMP-3, mean (S.E.), ng/ml	156.4 (37)	98.5 (18.5)	335.3 (123.7)		0.04
RF-positive, *n* (%)	34 (75.6)	34 (100.0)	0 (0)		<0.001
APCA-positive, *n* (%)	31 (68.9)	31 (91.1)	0 (0)		<0.001
MTX, *n* (%)	37 (84.4)	30 (88.2)	8 (72.7)		0.33
MTX dose, mean (S.E.), mg/week	10.0 (0.4)	10.3 (0.4)	9.0 (1.1)		0.35
Other csDMARDs, *n* (%)		BUC 1 (2.9), PSL 4 (11.7), SASP 4 (11.7), TAC 1 (2.9)	PSL 2 (18.2)		
b/ts DMARDs, *n* (%)		ADA 3 (8.8), IFX 3 (8.8), TCZ 1 (2.9), ABT 1 (2.9), TOF 1 (2.9)	IFX 2 (18.2), TCZ 3 (27.3)		

Continuous variables were compared between seropositive and seronegative RA using Mann–Whitney *U* test. Categorical variables were compared between seropositive and seronegative RA using Fisher's exact test. Treatment was started immediately after diagnosed as RA. b/ts DMARDs were initiated by the physician's judgement such that MTX alone was insufficient to treat or that it was not able to use MTX. HCs: healthy controls; CDAI: Clinical Disease Activity Index; csDMARDs: conventional synthetic DMARDs; b/ts DMARDs: biological or targeted synthetic DMARDs; BUC: bucillamine; PSL: prednisolone; SASP: salazosulfapyridine; TAC: tacrolimus; ADA: adalimumab; IFX: infliximab; TCZ: tocilizumab; TOF: tofacitinib.

Results

Baseline characteristics of patients and HCs

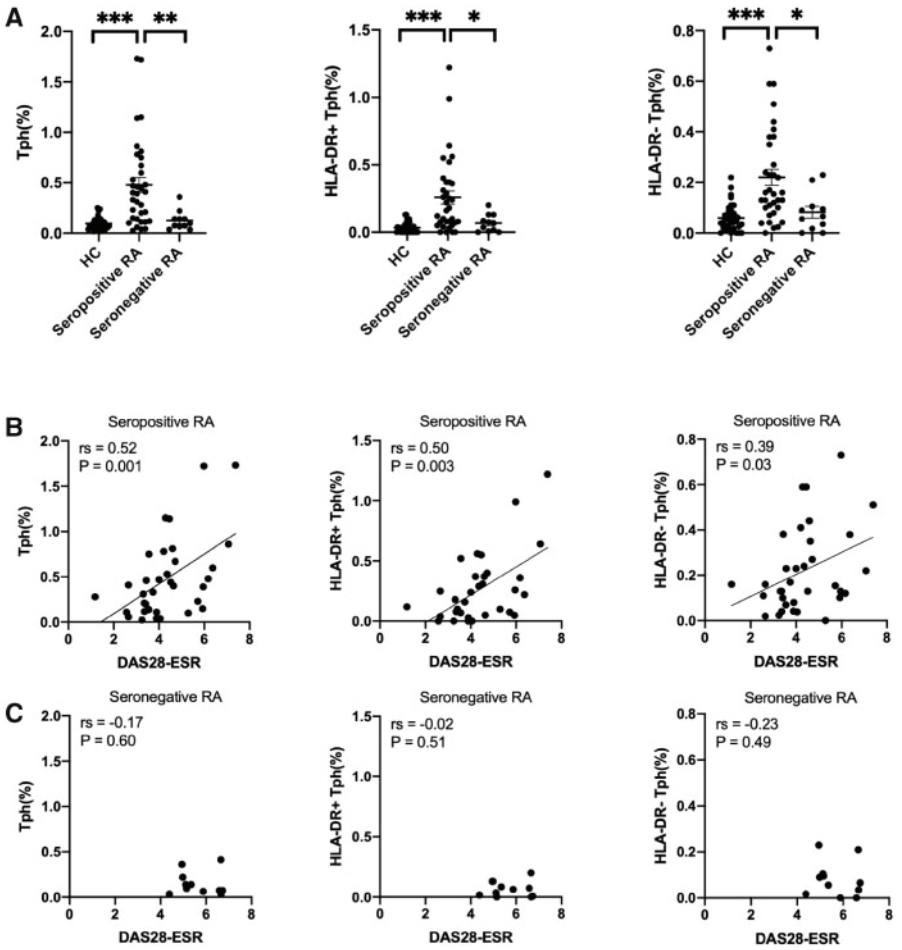
Forty-five RA patients (seropositive $n = 34$, seronegative $n = 11$) and 34 HCs were included in this study (Table 1). Seronegative RA patients were significant older and had higher disease activities (DAS28-ESR, DAS28-CRP, Clinical Disease Activity Index) and levels of CRP and MMP-3 than seropositive RA patients.

Association between the proportions of Tph cell subsets and baseline disease activity of RA

A previous study revealed that the proportions of Tph and HLA-DR+ Tph cells were increased and reflected the disease activity in seropositive RA [6]. Thus, we first assessed the proportions Tph and HLA-DR+ Tph cells as well as HLA-DR– Tph cells in seropositive and seronegative RA patients. As we expected, the proportions of Tph cells and HLA-DR+ Tph cells were only increased in seropositive RA patients (Fig. 1A). Since seronegative RA patients were older and had lower proportions of Tph cell subsets than seropositive RA patients, we assessed the correlation between age and the proportions of Tph cells, HLA-DR+ Tph cells and HLA-DR– Tph cells in seropositive and seronegative RA, but none of them was significantly correlated (supplementary Fig. S7, available at *Rheumatology* online). In addition, Tph cells and HLA-DR+ Tph cells were strongly correlated with DAS28-ESR in seropositive RA but not seronegative RA (Tph cells: $r_s = 0.52$, $P = 0.001$; HLA-DR+ Tph cells: $r_s = 0.50$, $P = 0.002$) (Fig. 1B). Consistent with these findings, the proportion of Tph cells was found to be significantly correlated with DAS28-CRP, Clinical Disease Activity Index, ESR and RF levels in seropositive RA patients but not in seronegative RA patients (Fig. 1C). Interestingly, the proportion of HLA-DR– Tph cells was also increased and correlated with DAS28-ESR in seropositive RA ($r_s = 0.39$, $P = 0.03$) (Fig. 1A and B). Absolute cell counts of Tph, HLA-DR+ Tph and HLA-DR– Tph cells were also increased in seropositive RA, and absolute cell counts of these subsets were strongly correlated with the proportions (supplementary Fig. S8, available at *Rheumatology* online).

Next, to investigate whether Tph cell subsets were useful biomarkers compared with the other immune cell subsets, we assessed the proportions of other 40 immune cell subsets and disease activity in seropositive RA. HLA-DR+ Th1-17 cells ($r_s = 0.38$, $P = 0.03$), activated B cells ($r_s = -0.35$, $P = 0.04$), plasma cells ($r_s = 0.43$, $P = 0.01$) and CD14++ CD16+ monocytes ($r_s = 0.36$, $P = 0.04$) showed a significant correlation with the DAS28-ESR in seropositive RA (Fig. 2), but not as strongly as Tph cells and HLA-DR+ Tph cells did.

Fig. 1 Comparison and correlation analysis of Tph, HLA-DR+ Tph and HLA-DR− Tph cells



Tph in seropositve RA	DAS28-CRP	CDAI	CRP	ESR	MMP-3	RF	ACPA	IgG
Correlation (rs)	0.53	0.40	0.29	0.35	0.21	0.36	-0.07	0.07
p value	0.001	0.02	0.09	0.04	0.24	0.03	0.70	0.69

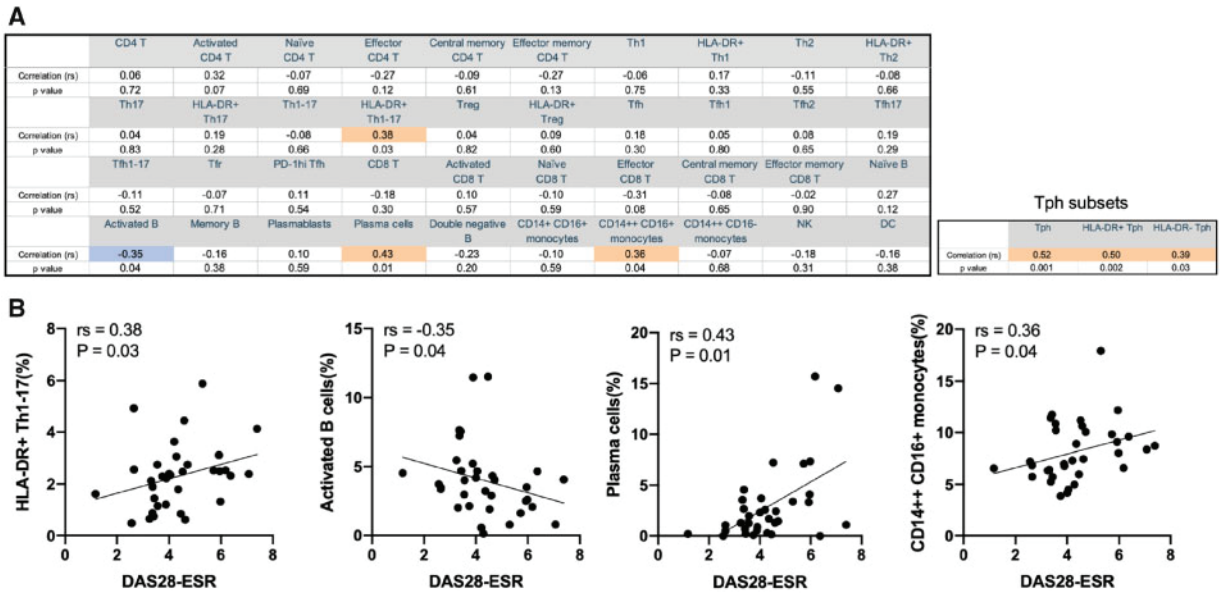
Tph in seronegative RA	DAS28-CRP	CDAI	CRP	ESR	MMP-3	RF	ACPA	IgG
Correlation (rs)	-0.05	-0.05	0.22	0.14	-0.14	-0.09	-0.40	-0.15
p value	0.89	0.89	0.51	0.68	0.69	0.80	0.36	0.66

(**A**) Comparison of the proportions of Tph, HLA-DR+ Tph and HLA-DR− Tph cells among HCs ($n = 34$), seropositive RA ($n = 34$) and seronegative RA ($n = 11$). (**B**) Correlation analysis of Tph, HLA-DR+ Tph and HLA-DR− Tph cells with DAS28-ESR in seropositive RA ($n = 34$) and seronegative RA ($n = 11$). (**C**) Correlation analysis between the proportion of Tph cells and clinical characteristics in seropositive and seronegative RA patients. Numbers of patients were as follows: HCs, $n = 34$; seropositive RA, $n = 34$; seronegative RA, $n = 11$. Orange indicates a significant correlation with the proportions of Tph cells. Data are presented as the mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Mann–Whitney *U* test with Bonferroni correction was performed in (A). Spearman correlation statistics shown in (B) and (C). Lines of best fit were drawn when significant. HC: healthy control; Tph: T peripheral helper.

Tph1, Tph2, Tph17 and Tph1–17 cells as biomarkers for seropositive RA

As a recent study classified Tph cells into Tph1, Tph2, Tph17 and Tph1–17 subsets using CXCR3, CCR4 and CCR6 [8] (Fig. 3A), we also assessed the association between these Tph cell subsets and disease activity in seropositive RA. All four of these Tph cell subsets expanded in seropositive RA patients compared with HCs, and the proportions of Tph1 and Tph1–17 cells were also significantly increased compared with seronegative RA patients (Fig. 3B). In this classification, Tph1 cells showed a significant correlation with DAS28-ESR in seropositive RA ($r_s = 0.41$, $P = 0.02$), but not

Fig. 2 Correlation analysis of other 40 immune subsets with the disease activity in seropositive RA



(**A**) Correlation analysis of other 40 immune subsets with DAS28-ESR in seropositive RA ($n = 34$). Orange and blue colour indicate positive and negative correlations with significance ($P < 0.05$). (**B**) Correlation between HLA-DR+ Th1–17 cells, activated B cells, plasma cells and CD14++ CD16+ monocytes, and DAS28-ESR. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Spearman correlation statistics shown in (A) and (B). Lines of best fit were drawn when statistically significant. Tph: T peripheral helper.

superior to Tph and HLA-DR+ Tph cells. The ratios of Tph1, Tph2, Tph17 and Tph1–17 cells to total Tph cells did not markedly differ among HCs and seropositive and seronegative RA patients (supplementary Fig. S9, available at *Rheumatology* online), meaning that Tph1 cells expanded in seropositive RA patients as a result of an increase in the proportion of total Tph cells.

Association between changes of Tph populations and response to the treatment

We next investigated the chronological changes in the DAS28-ESR as well as the populations of Tph, HLA-DR+ Tph and HLA-DR– Tph cells in seropositive RA patients treated with MTX monotherapy. In the present cohort study, the mean of DAS28-ESR had gradually decreased by weeks 12 and 24, but slightly increased at week 52 after treatment. Interestingly, the proportion of HLA-DR– Tph cells increased between weeks 24 and 52, while no such trend was noted for HLA-DR+ Tph cells (Fig. 4A). Reflecting the fact that DAS28-ESR, Tph cells, HLA-DR+ Tph cells and HLA-DR– Tph cells decreased by week 24, change in DAS28-ESR and change in the proportions of all three Tph cell subsets from baseline to week 24 were correlated (supplementary Fig. S10, available at *Rheumatology* online). However, in week 52, whereas a significant correlation was observed between the change in DAS28-ESR and change in the proportion of Tph and HLA-DR– Tph cells from baseline, no such association was noted with the change in the proportion of HLA-DR+ Tph cells (Fig. 4B). Regarding the seropositive RA patients treated with biological or targeted synthetic DMARDs, the Tph proportions tended to decrease after treatment, but not to a significant degree because of the small sample size (Fig. 4C). In seronegative RA, while DAS28-ESR was gradually decreased with MTX treatment in weeks 12, 24 and 52, the proportion of Tph, HLA-DR+ Tph and HLA-DR– Tph cells did not show such a trend (supplementary Fig. S11, available at *Rheumatology* online).

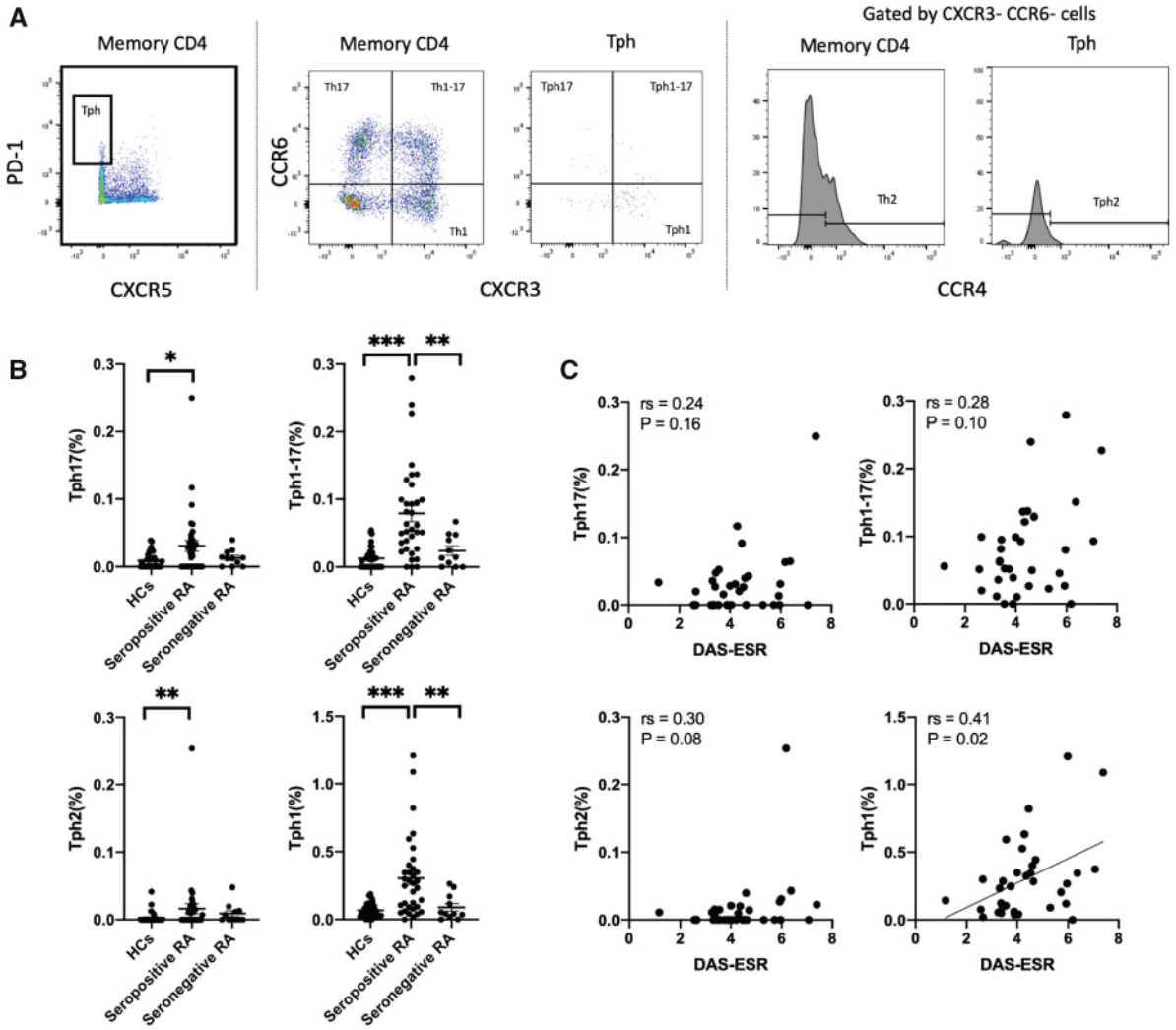
Changes in the proportion of HLA-DR+ Tph cells in cases with an inadequate MTX response

We speculated that the proportion of HLA-DR+ Tph cells would be reduced by MTX treatment, independently of disease activity. We therefore collected and analysed the data of eight patients with MTX-IR (Fig. 5A). In these patients, while the proportions of total Tph and HLA-DR– Tph cells did not change markedly after MTX treatment, the proportion of HLA-DR+ Tph cells decreased significantly (Fig. 5B). These data indicated that the proportion of HLA-DR+ Tph cells did not reflect the disease activity during treatment, as MTX treatment suppressed the proportion, independently of clinical response.

Discussion

In this study, we demonstrated that HLA-DR+ Tph cells were strongly correlated with the baseline disease

FIG. 3 Correlation analysis of Tph1, Tph2, Tph17 and Tph1–17 cells with the disease activity in seropositive RA

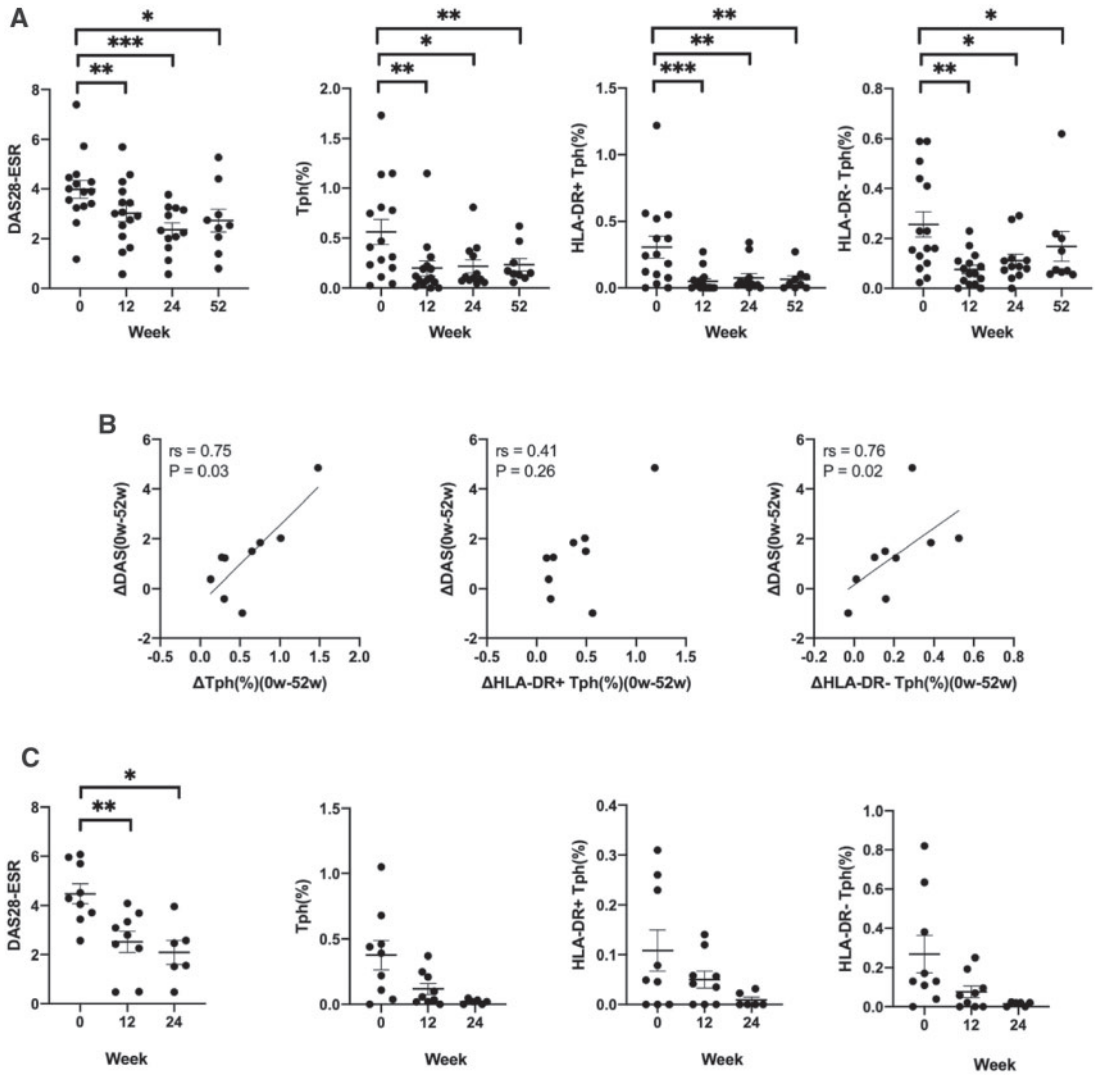


(**A**) Gating strategy of Tph cells using CXCR3, CCR4 and CCR6. Left: representative dot plots show the expression of CXCR5 and PD-1 on memory CD4 T cells gated by CD3+ CD4+ CD8− CD45RA−. A cut-off line of PD-1 high is defined by the tangent line adjacent to top of the 5% contour line of CXCR5+ cells in biaxial flow cytometry plot using CXCR5 and PD-1. Middle: the cut-off lines of CXCR3 and CCR6 were determined by those for gating of Th1, Th17 and Th1–17 cells, which was classified by CXCR3 and CCR6 positive or negative in CD3+ CD4+ CD8− CD45RA– memory CD4 T cells. Right: the cut-off line of CCR4 was determined by that for gating of Th2 cells, which was classified by CCR4 positive or negative in CD3+ CD4+ CD8− CD45RA– memory CD4 CXCR3– CCR6– T cells. (**B**) Comparison of the proportions of Tph1, Tph2, Tph17 and Tph1–17 subsets between HCs ($n = 34$) and seropositive RA ($n = 34$) and seronegative RA ($n = 11$). (**C**) Correlation analysis of Tph1, Tph2, Tph17 and Tph1–17 with DAS28-ESR in seropositive RA. Data are presented as the mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Mann–Whitney *U* test with Bonferroni correction was performed in (B). Spearman correlation statistics shown in (C). Lines of best fit were drawn when statistically significant. HC: healthy control; Tph: T peripheral helper.

activity in seropositive RA. Tph1, Tph2, Tph17 and Tph1–17 classification did not have additional benefit for evaluating the baseline disease activity. In addition, longitudinal analyses revealed that the change in the proportion of HLA-DR– Tph cells reflected the change in the DAS28-ESR, while no such association was noted for HLA-DR+ Tph cells because of their non-specific reduction by MTX treatment.

We compared the correlation of the baseline disease activity between Tph cell subsets and 40 other immune cell subsets in seropositive RA. Tph cells and, among the subsets, HLA-DR+ Tph cells were more strongly correlated with baseline disease activity compared with 40 other immune cell subsets. However, HLA-DR+ Tph cells were decreased independently of the disease activity during the treatment, meaning that HLA-DR+ Tph

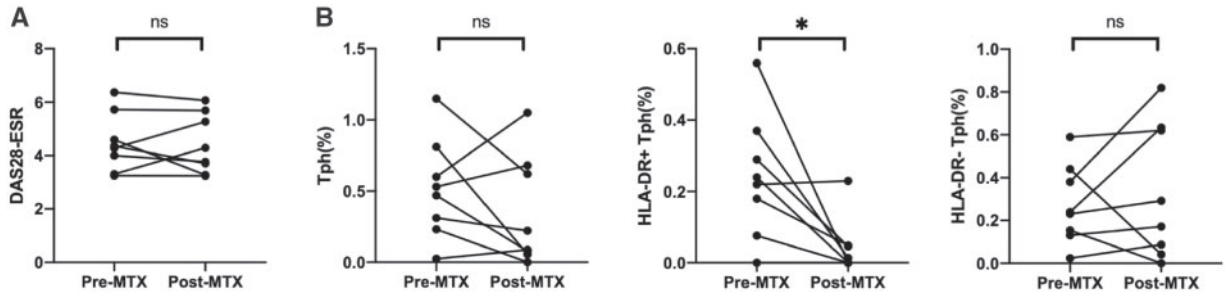
FIG. 4 Chronological changes of Tph, HLA-DR+ Tph and HLA-DR− Tph cells in seropositive RA after treatment



(**A**) Chronological changes in the DAS28-ESR and the proportions of Tph, HLA-DR+ Tph and HLA-DR− Tph cells after MTX treatment in seropositive RA patients receiving MTX monotherapy. Numbers of patients at each time point were as follows: week 0, $n = 15$; week 12, $n = 15$; week 24, $n = 12$; week 52, $n = 9$. (**B**) Correlation between the changes in the DAS28-ESR and those in the proportions of Tph, HLA-DR+ Tph and HLA-DR− Tph cells after MTX treatment. Δ (0–52 weeks) means the difference of the data between baseline and week 52. Numbers of patients were as follows: $n = 9$. (**C**) Chronological changes in the proportions of Tph, HLA-DR+ Tph and HLA-DR− Tph cells after b/ts DMARD treatment. Numbers of patients at each time point were as follows: week 0, $n = 9$; week 12, $n = 9$; week 24, $n = 6$. Numbers of patients treated with each b/ts DMARD were as follows: ADA, $n = 3$; IFX, $n = 3$; TCZ, $n = 1$; ABT, $n = 1$; TOF, $n = 1$. Data are presented as the mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Wilcoxon's signed-rank test was used in (A) and (C). Spearman correlation statistics shown in (B). Lines of best fit were drawn when statistically significant. ABT: abatacept; Tph: T peripheral helper; b/ts DMARDs: biological or targeted synthetic DMARDs; ADA: adalimumab; IFX: infliximab; TCZ: tocilizumab; TOF: tofacitinib.

cells may not the optimal biomarker for evaluating treatment response. Although the change in the proportion of HLA-DR+ Tph cells showed the correlation with the change in DAS28-ESR by week 24, we speculate it is because most of the cases treated with MTX improved by week 24. As for Tph1, Tph2, Tph17 and Tph1–17 classification, Tph1 cells only showed correlation with DAS28-ESR, because Tph1 cells were the major population of Tph cells. However, the ratios of Tph1 cells to total Tph cells were not different between HCs and seropositive and seronegative RA, meaning that the increased population of Tph1 cells in seropositive RA is a result of the increase in the proportion of total Tph cells. Recent studies have shown that Tph cells strongly

Fig. 5 Proportions of Tph, HLA-DR+ Tph, and HLA-DR– Tph cells in MTX-IR before and after treatment



(**A**) The DAS28-ESR in MTX-IR patients before and after MTX treatment. (**B**) Proportions of Tph, HLA-DR+ Tph and HLA-DR− Tph cells in MTX-IR patients before and after MTX treatment ($n = 8$). Duration of MTX treatment is 19.6 ± 5.4 weeks. Data are presented as the mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant. Wilcoxon's signed-rank test was used in (A) and (B). Tph: T peripheral helper; MTX-IR: MTX inadequate response.

express CXCR3 [8, 11, 12], suggesting that these cells may intrinsically have characteristics of Th1 cells.

Our study highlights the importance of HLA-DR– Tph cells as biomarkers of RA; HLA-DR– Tph cells accurately reflected the disease activity at 12, 24 and 52 weeks after initiating the treatment. Biomarkers reflecting disease activity are helpful to assess the disease state and to determine the treatment strategy. Considering Tph cell subsets are responsible for the pathogenic antibody production, the proportions of HLA-DR– Tph cells may be changed prior to the change in the disease activity, meaning that this population could be the predictors of the disease state of seropositive RA. To validate our hypothesis, a more detailed study is needed.

While both HLA-DR and PD-1 are known to be expressed on activated T cells [13, 14], their functions differ: HLA-DR on T cells transduces activation signals via tyrosine phosphorylation and mobilization of intracellular calcium [15, 16], whereas PD-1 is an inhibitory receptor that binds to PD-L1 and PD-L2 expressed on dendritic cells, stromal cells and cancer cells [17, 18]. However, a recent study showed that PD-1 also performs an essential role in the retention of the germinal centre response by promoting the accumulation of T cells in germinal centres and affinity-based selection [19]. In addition, our data revealed that MTX treatment decreased HLA-DR+ Tph cells, but not HLA-DR– Tph cells and total Tph cells in seropositive RA patients with MTX-IR, meaning that reduction of HLA-DR expression on Tph cells is insufficient to treat RA. Also, the findings indicate that cells expressing PD-1 rather than HLA-DR are crucial for the pathogenicity of seropositive RA. We speculate that HLA-DR– Tph cells have the pathogenic function for enhancing B cells response at inflammatory sites, as with HLA-DR+ Tph cells. Tph cells have a high capacity to secrete IL-21 and CXCL13 [5, 20], which enhance the B cell response and recruit B cells [21, 22]. A previous study indicated that expression of B cell helper function transcriptomes, such as BATF and MAF, which regulate IL-21 [23, 24], is comparable between HLA-DR+ and HLA-DR– Tph cells [6]. In addition, the productivities of IL-21 and CXCL13 are not markedly different between HLA-DR+ and HLA-DR– Tph cells [6]. Taken together, these findings support the importance of HLA-DR– Tph cells in the pathogenesis of RA as well as the notion that targeting HLA-DR+ Tph cells alone is not sufficient to treat RA.

As Tph cells were discovered quite recently, a more detailed characterization of these cells is needed. In the present study, we classified Tph cells using CXCR3, CCR6, CCR4 and HLA-DR, and evaluated the involvement of the Tph cell subsets in RA. One question arising from our study is whether or not classification using inducible co-stimulator-positive (ICOS) is useful, as some Tph populations express this molecule [6]. A previous study regarding tumour-infiltrating lymphocytes showed that the CXCR5– ICOSint CD4+ tumour-infiltrating lymphocyte population expressed CXCL13 much more strongly than the CXCR5– ICOShi CD4+ tumour-infiltrating lymphocyte population [25]. Given our present findings underscoring the importance of HLA-DR– Tph cells, we intend to examine the differences between ICOS+ and ICOS– Tph cells at the next stage.

Our results showed the usefulness of Tph cells as biomarkers in seropositive RA. However, some limitations should be noted. First, the number of patients, especially those with MTX-IR and those treated with biologics, was relatively small. A larger cohort study is needed to validate our results. Second, although the previous paper validated the cut-off line of PD-1 by synovial fluid and tissue samples as well [6], we could not include these samples in this study. However, we confirmed the findings described previously such that Tph cells highly increased in seropositive RA but not seronegative RA and HCs [6], and thus, we believe that our PD-1 high cut-off line is convincing.

Conclusion

In summary, we have found that HLA-DR+ Tph cells are decreased by MTX treatment, independently of treatment response. During the treatment, HLA-DR– Tph cells reflect

the disease activity more accurately than HLA-DR+ Tph cells. We believe that HLA-DR– Tph cells is important for the pathogenic antibody production in seropositive RA. Our results suggest the potential utility of Tph cell subsets as biomarkers and new therapeutic targets for RA.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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