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佐川, 文彬

https://hdl.handle.net/2324/7329430

出版情報：Kyushu University, 2024, 博士（医学）, 課程博士
バージョン：
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ORIGINAL RESEARCH

Determination of the factors associated with antigen-specific CD4+ T-cell responses to BNT162b2 in patients with rheumatoid arthritis

Fumiaki Sagawa,[1] Hisakata Yamada ,[2] Masahiro Ayano,[1] Yasutaka Kimoto,[3] Hiroki Mitoma,[1] Nobuyuki Ono,[1] Yojiro Arinobu,[1] Masakazu Kondo,[4] Yasuharu Nakashima,[5] Koichi Akashi,[1] Takahiko Horiuchi,[6] Hiroaki Niiro[7]

To cite: Sagawa F, Yamada H, Ayano M, *et al*. Determination of the factors associated with antigen-specific CD4+ T-cell responses to BNT162b2 in patients with rheumatoid arthritis. *RMD Open* 2024;**10**:e003693. doi:10.1136/rmdopen-2023-003693

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/rmdopen-2023-003693).

Received 6 September 2023
Accepted 19 December 2023

Check for updates

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For numbered affiliations see end of article.

Correspondence to
Dr Hisakata Yamada; yamada.hisakata.579@m.kyushu-u.ac.jp

ABSTRACT

Objectives Understanding interpatient variation in CD4+T-cell responses is the bases for understanding the pathogenesis and management of rheumatoid arthritis (RA). We examined immune responses to SARS-CoV-2 vaccine in a cohort of patients with RA and determined factors associated with the responses.

Methods Four hundred and thirty-one patients with RA having received two doses of BNT162b2, a messenger RNA-based vaccine for SARS-CoV-2, were included. Vaccine antigen-specific IgG was detected by ELISA, and antigen-specific CD4+T cells were detected by CD154 expression in response to antigenic stimulation. Expression of cytokines was concomitantly detected by intracellular staining. Associations among background variables, antigen-specific antibody production and the CD4+T-cell responses were analysed. Unsupervised hierarchical clustering was performed based on the profiles of antigen-specific cytokine production by CD4+T cells to stratify patients with RA.

Results Multivariate analysis indicated that ageing negatively affects CD4+T-cell response as well as antibody production. No association was detected between the presence or the levels of rheumatoid factor/anti-cyclic citrullinated peptide antibody and anti-vaccine immune responses. Methotrexate and prednisolone reduced B cell but not T-cell responses. Conventional immunophenotyping by the expression of chemokine receptors was not associated with the actual CD4+T-cell response, except for T helper cells (Th1). Functional immunophenotyping based on the profiles of antigen-specific cytokine production of CD4+T cells stratified patients with RA into three clusters, among which Th1-dominant type less frequently underwent joint surgery.

Conclusions Clinical and immunological variables that are associated with antigen-specific CD4 T-cell responses in patients with RA were determined by analysing immune responses against SARS-CoV-2 vaccine.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ A variety of CD4+T-cell responses are implicated in the pathogenesis of rheumatoid arthritis (RA).
⇒ Clinical variables predicting antibody, but not CD4 T cell, response to BNT162b vaccine have been reported.

WHAT THIS STUDY ADDS

⇒ Clinical and immunological variables that are associated with CD4+T-cell responses to BNT162b2 have been identified in patients with RA.
⇒ Functional immunophenotyping based on the antigen-specific CD4+T-cell responses stratified patients with RA.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The results of this study might be the basis for understanding immune pathogenesis and the management of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly targeting synovial joint with heterogeneity in clinical manifestations and prognosis. While some patients are refractory to the treatment with multiple biologics, such as the recently defined difficult-to-treat RA,[1] others show no erosive changes despite milder treatment. Hence, precision treatment strategy should be introduced into RA. Rheumatoid factor (RF) or anti-citrullinated protein antibody (ACPA) seropositivity has been established as one of the poor prognostic factors.[2 3] Given that RA is a CD4+T cell-mediated autoimmune disease, individual variation in CD4+T-cell responses might also affect the clinical presentation. In fact, ACPA-positive RA associates with genes that regulate T-cell responses, such as protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[4] However, the extent and the

patterns of individual variations in antigen-specific T-cell responses among patients with RA is not well understood. In addition, there has been insufficient information about the influence of medications, including biologics, on CD4+T-cell responses in patients with RA, which is required for safer management of patients with RA and understanding the immune-modulatory effects of anti-rheumatic reagents.

The diversity in T-cell responses includes both quantity and quality. The former is the extent of clonal expansion, while the latter is functional differentiation reflected by the expression profiles of effector molecules. For the case of CD4+T cells, which are thought to play pivotal roles in RA, they are largely divided into three subsets: T helper cells (Th1), Th2 and Th17, characterised by the production of interferon (IFN)-γ, interleukin (IL)-4 and IL-17, respectively. It has been shown that the T-cell subsets can be discriminated by the expression pattern of chemokine receptors: Th1 (CXCR3+CCR6−), Th2 (CXCR3−CCR6−), Th17 (CXCR3−CCR6+) and an additional subset, Th17.1 (CXCR3+CCR6+), which is originated from Th17 but produce IFN-γ like Th1. Although this discrimination system has widely been used for immunophenotyping,[5] it is unclear to what extent it predicts the actual CD4+T-cell responses elicited by an antigen. Examining cytokine production of T cells against antigens derived from previously or persistently infected pathogens (ie, cytomegalovirus) might be an alternative. However, different history of infection makes it difficult to interpret the individual variations, unlike animal experiments in which immunisation can be performed in equal conditions.

COVID-19 caused by SARS-CoV-2 was the emerging infectious disease first reported in Wuhan in 2019 and then spread worldwide. Thereafter, vaccination for SARS-CoV-2 was recommended for all individuals including patients with RA. In Japan, vaccination started about early 2021, for most cases with BNT162b2, a messenger RNA (mRNA)-based vaccine.[6] Because all humans were basically naive to SARS-CoV-2, this was a valuable opportunity to examine the immune response of humans to an antigen immunised under equal conditions. T and B-cell responses of healthy subjects to the viral peptides derived from the vaccinated mRNA have been detected.[7–9] There have also been studies on patients with RA, most of which showed reduced antibody production by methotrexate (MTX), anti-CD20, CTLA-4 Ig and high-dose glucocorticoid, but not tumor necrosis factor (TNF-α) or IL-6 inhibitors.[10–12] However, so far only a few studies have examined CD4+T-cell response to BNT162b2 in RA, and detailed profiles of cytokine production have not been analysed.[13–15]

In this study, we examined antibody and CD4+T-cell responses to BNT162b2 in a cohort of patients with Japanese RA who had received two doses of vaccination. We performed multivariate analysis to determine clinical and immunological variables, including medications, that are associated with the antigen-specific immune responses. We also performed unsupervised hierarchical clustering based on the profiles of antigen-specific cytokine production by CD4 T cells, which we called functional immunophenotyping, to stratify patients with RA.

METHODS

Patients

Four hundred and thirty-one consecutive patients with RA who had received two doses of BNT162b2 injection were recruited from July to September 2021. All patients met the 1987 American College of Rheumatology (ACR) or the 2010 ACR/EULAR classification criteria for RA at the time of diagnosis. They signed informed consent before participation in the study. This study complies with the Declaration of Helsinki.

Sample collection

Blood samples were taken from the patients between 1 and 3 months after the second vaccination, because the levels of serum antibody and T-cell responses were reported to be stable from 1 month after immunisation.[7] Serum was separated from the whole blood samples by centrifugation, and peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (PROGEN, Heidelberg, Germany). Serum and PBMC were stored at −80°C until use for the experiments.

Cell culture for flow cytometric detection of antigen-specific CD4+ T cells

PMBC were thawed and rested in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2 overnight. The next day, the cells were stimulated with PepTivator SARS-CoV-2 Prot_S (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of costimulatory antibodies against CD28 and CD49d (BD Biosciences, California, USA) for 1 hour.[16] After adding brefeldin A (eBioscience, Massachusetts, USA), the cells were incubated for another 4 hours. CD4+T cells that responded to the antigen were detected by the expression of CD154.[17] The analysis was performed for all patient samples.

Flow cytometric analysis

The combinations of fluorochrome-labelled monoclonal antibodies used for the analysis are listed in online supplemental table 1. The antibodies were added to single cell suspension of PBMC in phosphate-buffered saline and 2% FBS. Intracellular staining of cytokines was performed by using BD Cytofix/Cytoperm solution and BD Perm/Wash solution following the manufacturer's instruction (BD Biosciences). Data were acquired on an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Massachusetts, USA) and analysed using FlowJo software (BD Biosciences).

ELISA for anti-SARS-CoV-2 receptor-binding domain IgG

Ninety-six-well plates were coated with SARS-CoV-2 receptor-binding domain (RBD) of S1 Subunit Protein

(RayBiotech, Georgia, USA) overnight at 4°C. After blocking the plates with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour, serum samples were added to the wells for 1 hour. Plate wells were washed and added with anti-IgG F(ab')2 Human Goat-Poly Horseradish Peroxidase (Rockland Immunochemicals, Pennsylvania, USA) for 30 min. Bound antibody was detected by using Tetramethylbenzidine Substrate Reagent Set (BD Biosciences). The plates were read at 450 nm and 620 nm with an ELISA reader.

Statistical analysis

Continuous variables are expressed as average and SD, and categorical variables are expressed as counts and percentages. Spearman's rank correlation coefficient was used to analyse the correlations between continuous variables, and Mann-Whitney U test between continuous and categorical variables. Variables showing association in univariate analysis with p values<0.1 were selected for multivariate analysis (multiple regression analysis), in which p values<0.05 were considered statistically significant. To stratify the patients based on the pattern of antigen-specific cytokine production by CD4+T cells, hierarchical clustering was performed by using Ward's method, because there had been no prediction about the number of clusters, all parameters are continuous variables and this method minimises the variance within each cluster. The resulting tree dendrogram visually separated the patients into three clusters which showed a distinct pattern of cytokine production.[18 19] One-way analysis of variance or χ^2 test was performed to detect overall difference in each variable among the three clusters. To detect the difference between two of the three clusters, multiple χ^2 tests with Bonferroni correction (p<0.017) or Tukey's Honestly Significant Difference analysis were applied. All statistical analyses were performed on JMP Pro V.16.0.0 (SAS Institute, Cary, North Carolina, USA).

Table 1 Characteristics of the patients

Total number, n	431
Age, years	64.1±11.5
Sex (female), n (%)	399 (92.6)
Disease duration, years	16.7±10.4
Stage (I/II/III/IV), n (%)	87/152/51/141 (20.2/35.3/11.8/32.7)
28-tender joint count	0.38±1.03
28-swollen joint count	0.54±1.09
History of surgery, n (%)	71 (16.5)
ESR, mm/h	16.6±12.9
CRP, mg/dL	0.22±0.48
RF positive, n (%)	331 (76.8)
RF, IU/mL	125.9±255.8
anti-CCP antibody positive, n (%)	355 (82.4)
anti-CCP antibody, U/mL	243.5±357.2
MTX use, n (%)	329 (76.3)
MTX dose, mg	6.03±4.32
MTX hold, n (%)	48 (14.6)
PSL use, n (%)	184 (42.7)
PSL dose, mg	1.38±1.90
Biologics/JAK inhibitors, n (%)	133 (30.9)
TNF-α inhibitors	86 (20.0)
IL-6 inhibitors	29 (6.7)
CTLA-4-Ig	5 (1.2)
JAK inhibitors	13 (3.0)

Values are mean±SD or number (%) of patients.
CCP, cyclic citrullinated peptide; CRP, C reactive protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ESR, erythrocyte sedimentation rate; IL, interleukin; JAK, Janus kinase; MTX, methotrexate; PSL, prednisolone; RF, rheumatoid factor; TNF, tumor necrosis factor.

RESULTS

Patient background and study design

Four-hundred and thirty-one patients with RA who had injected two doses of BNT162b2 were included in this study. The patients' background is shown in table 1. About 30% of patients were receiving biologics, and most patients are in remission or low disease activity, as shown by small number of swollen and tender joints and by low levels of C reactive protein and erythrocyte sedimentation rate. MTX and prednisolone (PSL) was prescribed to about 80% and 40% of patients, respectively. About 15% of patients with MTX hold it for 1 week after vaccination. Blood sample was taken between one to 3 months after the last immunisation, when the levels of serum antibody and T-cell responses past the peak and kept the steady state according to the data of previous studies.[7]

We analysed the association among three parameter groups, namely background variables, vaccine antigen-specific antibody level and vaccine antigen-specific CD4+T-cell responses (figure 1). Background variables include clinical valuables, such as age and sex, medications and immunological parameters which include not only positivity and the levels of RF and anti-cyclic citrullinated peptide (CCP) antibody but also the frequency of helper CD4+T-cell subsets defined by the expression pattern of chemokine receptors (Th-type) (online supplemental figure 1), which has widely been used for immunophenotyping.[5] We confirmed the proportion of Th-type did not differ before and after vaccination (data not shown). Based on the data of antigen-specific cytokine production by CD4+T cells, which we called functional immunophenotyping, unsupervised hierarchical clustering of the patients was performed.

Background variables that are associated with antigen-specific antibody production

We first analysed the association between the background valuables and vaccine antigen-specific antibody

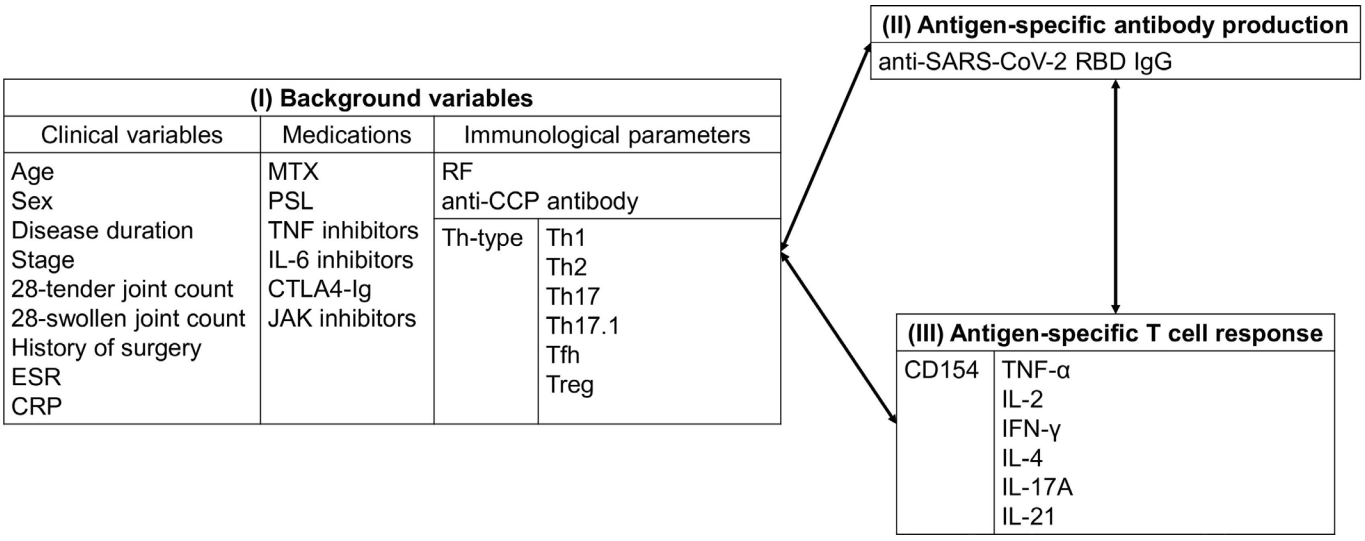

Figure 1 Overview of the association analysis. Three groups of parameters tested for association analysis are depicted in the figure. CCP, cyclic citrullinated peptide; CRP, C reactive protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ESR, erythrocyte sedimentation rate; IFN, interferon; IL, interleukin; JAK, Janus kinase; MTX, methotrexate; PSL, prednisolone; RBD, receptor-binding domain; RF, rheumatoid factor; TNF, tumor necrosis factor.

production (table 2). Multivariate analysis identified ageing negatively affects antigen-specific antibody production. In terms of medication, the use and dose of MTX and PSL significantly inhibited antigen-specific antibody production. One-week hold of MTX increased antibody levels. However, neither biologics or JAK inhibitors significantly affected antibody production. Among immunological parameters, the frequency of CXCR3+CCR6– Th1-type cells positively correlated antibody levels. Other parameters including positivity or the levels of RF and anti-CCP antibody, as well as the frequency of circulating follicular helper T cells, showed no association.

Background variables that are associated with antigen-specific CD4+ T-cell responses

We next examined the association between patient background variables and vaccine antigen-specific CD4+T-cell responses (table 3 and online supplemental tables 2–8). Antigen-specific CD4+T cells were detected by the expression of CD154 after brief stimulation with a mixture of SARS-CoV-2 peptides.[17] Intracellular staining enables antigen-specific cytokine production at the same time, and the results were shown as the percentage of cytokine producing cells among CD154+cells (online supplemental figure 2). Expression of CD154 or cytokines was hardly detected in samples without stimulation or samples taken before vaccination (data not shown).

For the overall frequency of antigen-specific CD4+T cells (total CD154+), which reflects the extent of clonal expansion, only the frequency of CXCR3–CCR6– Th2-type cells was the background variable showing positive correlation. No clinical valuables or medication, including MTX and PSL, associated with CD4+T-cell clonal expansion, in contrast to the case of antibody production. For the antigen-specific cytokine production of CD4+T cells, both IL-2 and IFN-γ production was negatively correlated with age but positively correlated with the frequency of CXCR3+CCR6– Th1-type cells. Antigen-specific IL-4 production was negatively correlated with the use and dose of PSL. However, TNF-α and IL-17 production showed no association with any clinical or immunological variables.

Association between antigen-specific antibody production and CD4+ T-cell responses

The association between antigen-specific antibody production and antigen-specific CD4+T-cell response was also analysed. Only antigen-specific IFN-γ production showed significant correlation with the antibody production by univariate analysis (ρ=0.1389, p=0.0039), but not in multivariate analysis. No correlation was detected between IL-4 or IL-21 production and antibody production.

Hierarchical clustering of patients with RA based on functional immunophenotyping of CD4+ T cells

We lastly performed unsupervised hierarchical clustering analysis of the patients with RA based on the pattern of antigen-specific production of the six cytokines (TNF-α, IL-2, IFN-γ, IL-4, IL-17A and IL-21) by CD4+T cells, functional immunophenotyping. As shown in figure 2A, the patients were classified into three clusters as follows: (1) 'Th1-dominant' cluster with high production of TNF-α, IL-2 and IFN-γ but low production of the others, (2) 'All high' cluster with high production of all cytokines and (3) 'All low' cluster with poor production of all cytokines (figure 2B, online supplemental table 9). We thereby analysed the association of the background variables with the three clusters (table 4). There was no difference in the age, sex or disease activity among the three clusters. There was also no difference in the use or dose of MTX and PSL as well as the use of biologics or JAK inhibitors.

Table 2 Background variables associated with antigen-specific antibody levels

	Univariate analysis		Multivariate analysis		
(n=431)	ρ score (mean antibody titre)	P values	β	T values	P values
Clinical variables					
Age	−0.1161	0.0159	−0.14	−3.07	**0.0023**
Sex		0.0002	−0.09	−1.94	0.0529
Male (n=32)	(509.2)				
Female (n=399)	(1195.0)				
Disease duration	−0.0392	0.4172			
Stage (I/II/III/IV)	(1352.2/1032.3/ 1182.8/1122.2)	0.4952			
28-swollen joint count	0.0209	0.6647			
28-tender joint count	−0.0358	0.4590			
History of surgery		0.0640	0.01	0.09	0.9279
Yes (n=71)	(955.5)				
No (n=360)	(1181.3)				
ESR	0.0691	0.1523			
CRP	−0.0303	0.5306			
Medication					
MTX dose	−0.1207	0.0121	−0.18	−3.73	**0.0002**
MTX user		0.0154	0.18	3.85	**0.0001**
User (n=329)	(999.1)				
Non-user (n=102)	(1611.7)				
PSL dose	−0.2307	<0.0001	−0.15	−3.25	**0.0013**
PSL use		<0.0001	0.14	2.95	**0.0033**
User (n=184)	(863.3)				
Non-user (n=247)	(1353.2)				
TNF-α inhibitors use		0.6538			
User (n=86)	(1350.0)				
Non-user (n=345)	(1092.7)				
IL-6 inhibitors use		0.2023			
User (n=29)	(1408.3)				
Non-user (n=402)	(1125.0)				
CTLA-4-Ig use		0.0756	0.06	1.36	0.1733
User (n=5)	(384.9)				
Non-user (n=426)	(1153.0)				
JAK inhibitors use		0.0829	0.04	0.86	0.3891
User (n=13)	(779.0)				
Non-user (n=418)	(1155.4)				
MTX-withdrawal		0.0240	−0.16	−3.03	**0.0026**
MTX-hold (n=48)	(1364.8)				
MTX-maintain (n=281)	(936.6)				
Immunological variables					
RF level	0.0609	0.2767			
RF positivity		0.4944			
Positive (n=331)	(1157.9)				
Negative (n=100)	(1098.2)				

Continued

Table 2 Continued

(n=431)	Univariate analysis ρ score (mean antibody titre)	Univariate analysis P values	Multivariate analysis β	Multivariate analysis T values	Multivariate analysis P values
Anti-CCP antibody level	–0.0473	0.3585			
Anti-CCP antibody positivity		0.3801			
Positive (n=355)	(1129.1)				
Negative (n=76)	(1214.0)				
Th1-type (%)	0.1807	0.0002	0.13	2.45	**0.0147**
Th2-type (%)	–0.0787	0.1027			
Th17-type (%)	–0.1328	0.0057	–0.03	–0.59	0.5569
Tfh-type (%)	0.0374	0.4390			
Treg-type (%)	–0.0469	0.3316			

p <0.05, statistilally significant
CCP, cyclic citrullinated peptide; CRP, C reactive protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ESR, erythrocyte sedimentation rate; IL, interleukin; JAK, Janus kinase; MTX, methotrexate; PSL, prednisolone; RF, rheumatoid factor; Tfh, follicular helper T ; Th, T helper cells; TNF, tumor necrosis factor.

Interestingly, the frequency of patients with the history of joint surgery significantly differed among the clusters. Multiple testing revealed the patient cluster with Th1 dominant response (Th1-dominant) have undergone joint surgery significantly less frequently than the cluster with overall low cytokine production (All low), which likely reflects less severe joint destruction. Thus, our clustering analysis based on functional immunophenotyping of CD4+T cells stratifies patient with RA with different clinical phenotypes.

Table 3 Background variables associated with antigen-specific CD4+T-cell responses (summary results of multivariate analysis*)

CD4+T-cell response	Variables	Multivariate analysis β	Multivariate analysis T values	Multivariate analysis P values
%CD154+				
	Th2-type	0.12	2.33	0.0204
%CD154+TNF-α+				
	None			
%CD154+IL-2+				
	Age	–0.22	–4.38	<0.0001
	Th1-type	0.15	2.57	0.0105
%CD154+IFN-γ+				
	Age	–0.21	–4.30	<0.0001
	Th1-type	0.16	2.70	0.0072
%CD154+IL-4+				
	PSL dose	–0.13	–2.38	0.0177
	PSL use	0.14	2.61	0.0095
%CD154+IL-17A+				
	None			
%CD154+IL-21+				
	Disease duration	0.11	2.27	0.0236

*Covariates used for the analysis were the same as table 2, and only those showing significant association by multivariate analysis are listed in this table (see online supplemental tables 2–8).
IFN, interferon; IL, interleukin; PSL, prednisolone; TNF, tumor necrosis factor.

DISCUSSION

Because individual variation in immune response might affect clinical manifestation of immune-mediated diseases, such as RA, it would be the basis for precision medicine. However, unlike animal experiments, comparing human immune responses in equal condition is practically a difficult task. We here took advantage of analysing antigen-specific immune-response after vaccination against an emerging viral infection, SARS-CoV-2, and identify clinical variables that affect the immune responses. Functional immunophenotyping thus performed was used to stratify patients with RA.

Ageing is associated with an increased risk of infection and negatively affects immune responses. It is well known that ageing reduced antibody production.[20] Age-related changes in CD4+T-cell dysfunction, including decreased IL-2 production, has also been known.[21] Furthermore, Collier *et al* demonstrated age-related decline of IL-2 and IFN-γ production in response to BNT162b2 in healthy subjects.[22] Our multivariate analysis also demonstrated that ageing is an independent prediction factor for poor antibody production and reduced IL-2 and IFN-γ production by CD4+T cells, while it did not significantly affect production of other cytokines. Thus, age-associated changes of immune response are similarly observed in patients with RA.

ACPA is specifically produced in RA with a large variation in the levels among patients, although the underlying mechanism remains unclear. ACPA is a class-switched IgG with somatic hypermutation in the variable region,[23] indicating an involvement of CD4+T cells in ACPA production. Genetic association of major histocompatibility

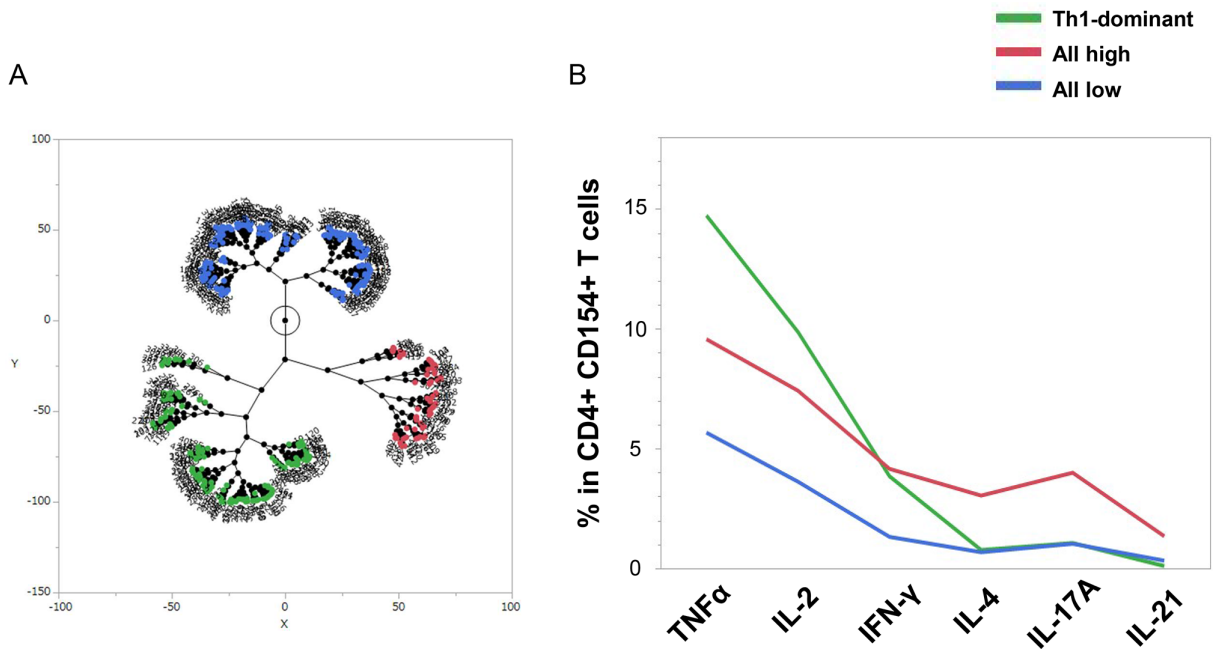

Figure 2 Stratification of patients with rheumatoid arthritis by unsupervised hierarchical clustering based on functional immunophenotyping of CD4+T cells. (A) Unsupervised hierarchical clustering analysis identified three patient clusters. The corresponding tree diagrams were represented in the form of constellation plots. n=431. (B) Profiles of cytokine production by CD4+T cells of each cluster are indicated by line graphs. IFN, interferon; IL, interleukin; Th, T helper cells.

complex class II with ACPA-positive RA further implies a key role for CD4+T cells in ACPA production.[24] Interestingly, several genes that can affect T-cell responses, such as PTPN22 and CTLA-4, have association with ACPA-positive RA.[4] It is thus possible that ACPA positive RA are potentially hyper-reactive in immune responses at T and/or B-cell levels. However, our data indicated that the presence and the level of anti-CCP antibody was not associated with antigen-specific antibody production or any of CD4+T cell-responses. Therefore, T or B cell-intrinsic factors may not be essential for the regulation of ACPA production.

Since several chemokine receptors are differentially expressed on different subsets of CD4+T cells, they have been used as the surrogate marker of helper T-cell subsets. This is mechanistically relevant because the expression of chemokine receptors is under the control of master transcription factors of helper T-cell subsets, such as T-bet, RORγt and GATA3.[25] Thereafter, the expression pattern of chemokine receptors has been used for immunophenotyping of CD4+T cells.[5] In fact, we detect a correlation in the frequency between antigen-specific IFN-γ producing cells and CXCR3+CCR6– Th1-type cells. However, for other T-cell subsets, we do not detect correlation in the frequency between Th types defined by the expression of chemokine receptors and the 'true' antigen-specific Th subsets. There are two important issues concerning this point. First, it is unclear if total number (frequency) of Th subsets, which likely reflect past immune responses, can predict the future immune response to an antigen. Second, the expression pattern of chemokine receptors may not be relevant to detect Th subsets. Although a certain subset of T cells expresses discrete combinations of chemokine receptors, it does not ensure it is applicable verse versa. Thus it is possible that not all T cells expressing a certain pattern of chemokine receptors are functionally homogeneous. In fact, at a single cell level, substantial discrepancy was observed in the expression of chemokine receptors and marker cytokines.[26] Further progress in single cell analysis might find better markers for the typical Th cell subset. Furthermore, recent studies showed a continuum of functionally different Th, and only a minority belong to the classical Th subsets in vivo.[27]

A variety of reagents that potentially affect immune cell function are used for the treatment of RA, but, in most cases, detailed influence on antigen-specific immune response has not been clarified. MTX, the key drug in RA treatment, is often categorised as immunosuppressive drugs, because it potentially inhibits DNA synthesis. However, it has been unclear to what extent MTX suppresses proliferation of lymphocytes in vivo. We here demonstrated that MTX suppresses antigen-specific antibody production without affecting T-cell functions including clonal expansion. The reduced antibody production to BNT162b2 was also observed in several studies,[10 12] although it is unknown at which process during antibody production MTX suppresses antibody production. Andreica *et al* also reported that CD4+T-cell response was not reduced in patients with RA treated with MTX.[11] As has been suggested, an increase of extracellular adenosine, which has an anti-inflammatory effect, might be the main pharmacological action of MTX in RA.[28]

Table 4 Clinical characteristics of patients with rheumatoid arthritis in the three clusters by functional immunophenotyping

	Th1-dominant	All high	All low	P values
Number	151	65	215	
Age, years	62.8±11.7	63.5±11.7	65.3±11.1	
Sex (female), n (%)	135 (89.4)	62 (95.4)	202 (94.0)	
Disease duration, years	15.2±9.6	16.8±10.6	17.8±10.8	
Stage (I/II/III/IV), n (%)	34/62/13/42 (22.5/41.1/ 8.6/27.8)	15/23/8/19 (23.1/35.4/ 12.3/29.2)	38/67/30/80 (17.7/31.2/ 14.0/37.2)	
28-swollen joint count, n	0.38±1.14	0.42±1.20	0.36±0.90	
28-tender joint count, n	0.49±1.03	0.52±1.08	0.57±1.15	
History of surgery, n (%)	16 (10.6)*	11 (16.9)	44 (20.5)*	**p=0.0431**
ESR, mm/h	15.5±11.7	17.5±14.2	17.2±13.2	
CRP, mg/dL	0.14±0.17	0.34±0.65	0.24±0.56	
RF positive, n (%)	120 (79.5)	53 (81.5)	158 (73.5)	
RF level, IU/mL	112.8±168.3	105.9±142.5	141.0±323.6	
anti-CCP antibody positive, n (%)	124 (82.1)	54 (83.1)	177 (82.3)	
anti-CCP antibody level, U/mL	264.0±340.7	248.1±463.2	228.1±331.1	
MTX use, n (%)	110 (72.9)	45 (69.23)	174 (80.9)	
MTX dose, mg	5.76±4.46	5.35±4.36	6.43±4.18	
PSL use, n (%)	70 (46.4)	24 (36.9)	90 (41.9)	
PSL dose, mg	1.54±2.01	1.00±1.56	1.37±1.90	
Biologics/JAK inhibitors, n (%)	46 (30.5)	16 (24.6)	71 (33.0)	
TNF-α inhibitors	32 (21.2)	9 (13.8)	45 (20.9)	
IL-6i inhibitors	13 (8.6)	3 (4.6)	13 (6.0)	
CTLA-4-Ig	0 (0.0)	0 (0.0)	5 (2.3)	
JAK inhibitors	1 (0.7)	4 (6.2)	8 (3.7)	
Antigen-specific IgG, U/mL	1072.4±1369.1	1398.1±1600.1	1117.6±1240.3	

p <0.05, statistilally significant
*Significant difference by multiple χ^2 testing with Bonferroni correction (p<0.017).
CCP, cyclic citrullinated peptide; CRP, C reactive protein; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ESR, erythrocyte sedimentation rate; IL, interleukin; JAK, Janus kinase; MTX, methotrexate; PSL, prednisolone ; RF, rheumatoid factor; Th, T helper cells; TNF, tumor necrosis factor.

PSL is well known for its anti-inflammatory effects on innate immune cells but also inhibits antibody production and downregulates cytokine production from T cells.[29] Our analysis also showed the use and the dose of PSL were negatively correlated with antigen-specific antibody production and IL-4 production from CD4+T cells. These fit well with the clinical effect of PSL in the treatment of allergic diseases.[30] We did not observe a clear effect of PSL on the production of other cytokines, although it might exert a suppressive effect at higher doses.

In contrast to the case of MTX and PSL, we did not observe any effect of TNF-α inhibitors on antigen-specific antibody production or CD4+T-cell responses. In support of our results, other studies examining anti-SARS-CoV-2 vaccine immune responses in patients with RA did not show a difference in immune response between patients with RA with and without TNF-α inhibitors.[10 11] In fact, the guidelines from ACR did not recommend avoiding the use of TNF-α inhibitors during vaccination.[31] It has also been reported that TNF-inhibitors did not increase the risk of SARS-CoV-2 infection.[32] IL-6 is an important cytokine for Th17 differentiation, and IL-17+CD4+ T cells have been reported to decrease in patients treated with tocilizumab.[33] We observed the decreased IL-17 production in patients with anti-IL-6 inhibitors in univariate analysis (data not shown), but not in multivariate analysis. Increasing the sample size may empower the statistical significance. Similarly, the numbers of patients using CTLA-4-Ig and JAK inhibitors in our cohort are also too small for statistical analysis.

High dimensional analysis on multiple parameters can lead to the discovery of previously unappreciated subgroups among the subjects. We found in this study that patients with RA can be stratified into three clusters based on the profiles of antigen-specific cytokine

production by CD4+T cells, which we called functional immunophenotyping. Importantly, the patient clusters did not simply reflect disease activity or different usage of anti-rheumatic drugs, as there was no difference in these factors among the clusters. It might rather be an intrinsic difference in the immune system that forms the clusters, and it would be of interest to test if there is any association between genetic polymorphisms and our clusters. Notably, one of the patient clusters showing Th1-dominant response is associated with less experience of joint surgery, which is considered as less progression of joint damage. Given the lack of difference in disease activity, this suggests that the quality of inflammation caused different prognosis, although different histories of disease activity could not be excluded from our cross-sectional analysis. In support of our finding, it has been known that IFN-γ inhibits osteoclastogenesis.[34] Importantly, multivariate analysis could not identify the association between the history of joint surgery and IFN-γ production by CD4+T cells. This highlights the usefulness of unsupervised high dimensional analysis in finding potentially distinct but unappreciated populations.

There are several limitations in our study. First, as the timing of blood sample collection was not at the peak of immune response, we might underestimate some responses. However, this study was performed in daily practice, it is difficult to fix the day after vaccination for all patients. Instead, we collected samples 1 month after vaccination, when antibody levels and T-cell responses stabilise.[7] Lack of blood samples from healthy subjects is another limitation, which disables generalisation of our findings. As mentioned above, the small number of patients with CTLA-4-Ig and JAK inhibitors in our cohort results in insufficient information about the effect of these reagents. However, at least for the case of CTLA-4-Ig, there have already been many studies about its immune-modulatory functions.[35] Therefore, we could not expect much information if we have more patients with CTLA-4-Ig. Last, the immune response to SARS-CoV-2 may include the cross-reactivity to pre-exposed conventional COVID-19.[36 37] Although this potentially affects our results, none of pre-vaccination patients showed clear antigen-specific responses in our preliminary experiments (data not shown).

In summary, our study demonstrated the variation in antigen-specific immune responses in a large number of human subjects with information about factors associated with the responses. It also suggests the usefulness of functional immunophenotyping based on the actual antigen-specific CD4+T-cell responses for stratifying the subjects in human immune-mediated disorders and understanding the immunopathogenesis.

Author affiliations
[1]Department of Medicine and Biosystemic Science, Kyushu University, Fukuoka, Japan
[2]Department of Clinical Immunology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan
[3]Department of Internal Medicine, Kyushu University Beppu Hospital, Beppu, Japan
[4]Kondo Clinic for Rheumatology and Orthopaedics, Fukuoka, Japan
[5]Department of Orthopedic Surgery, Kyushu University, Fukuoka, Japan
[6]Fukuoka City Hospital, Fukuoka, Japan
[7]Department of Medical Education, Kyushu University, Fukuoka, Japan

Contributors Study design: FS, HY. Sample collection: FS, MK, HY. Experiments and data analysis: FS, HY. Data interpretation: FS, HY, MA, YK, HM, NO, YA, HN. Writing the manuscript: FS, HY. Critical proofreading of the manuscript: all authors. HY is responsible for the overall content as guarantor.

Funding This work was supported in part by The Clinical Research Foundation, Fukuoka, Japan.

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by the Regional Committee of Ethics for Human Research at the Faculty of Medicine of the Kyushu University (2021-315). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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ORCID iD
Hisakata Yamada http://orcid.org/0000-0002-6329-7222

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