Table 1 Baseline characteristics of patients in three institutions of rheumatology in Japan

	Mean	SD	Min	25%	Median	75%	Max
Female (%)	87.6	–	–	–	–	–	–
Age	53.1	12.7	19	46	55	62	80
Duration	9.4	8.8	0	3	6.6	13	54
Stage	3.0	1.0	1	2	3	4	4
Class	2.2	0.5	1	2	2	2	4
RF positive (%)	87.6	–	–	–	–	–	–
RF (titer)	213	331	1	39	96	241	2980
MTX dose	7.8	2.0	0	6	8	8	20
PSL dose	3.8	3.7	0	0	4	5	22.5
CRP	3.3	2.8	0	1.18	2.7	4.7	13.7
TJC28	10.5	7.3	0	5	9	15	28
SJC28	10.6	6.1	0	6	10	14	28
GH	63.1	21.9	0	49.3	66	80	100
DAS28-CRP	5.5	1.1	1.9	4.8	5.6	6.3	8.0

MTX methotrexate, *PSL* prednisolone, *RF* rheumatoid factor, *CRP* C-reactive protein, *GH* general health, *TJC* tender joint count, *SJC* swollen joint count, *DAS* disease activity score

patients. All multivariate analyses were conducted using the variables gender, age, duration of disease, positive/negative rheumatoid factor (RF), concomitant MTX dose, concomitant prednisolone (PSL) dose, and DAS28-CRP at baseline. All reported *P*-values are two-sided and are not adjusted for multiple testing. The significance level was set at a *P* value of <0.05.

Results

Baseline demographic and clinical characteristics

The baseline demographic and clinical characteristics of 410 patients receiving infliximab therapy are summarized in Table 1. Patients had active disease at baseline as evidenced by high mean counts of tender and swollen joints, CRP levels, and the DAS28. Age, sex, and disease duration were similar among these three institutes, while the %user and dose of MTX or PSL were divergent and the disease activities as assessed by TJC, SJC, GH, and DAS28 was also different.

Continuation of infliximab therapy

Infliximab was discontinued in 100 cases (24.4%) among 410 patients during a 54-week period and the survival rate for infliximab use was comparable among three institutes by week 54 after the treatment according to Kaplan–Meier analysis (Fig. 1a). Cumulative hazards of the discontinuation during the 54-week infliximab therapy were different among the causes of discontinuation; discontinuation due to adverse events, inefficiency, remission and other causes (such as change of hospitals/clinics and economic reasons), were 0.093, 0.081, 0.007 and 0.063, respectively (Fig. 1b). Although the cause of the discontinuation was similar among three institutes, adverse events were higher in Center 1 than in the other two institutes, and remission and other causes including economic problems of the patient were greater in Center 3 than in the other two (data not shown). In 100 patients who terminated infliximab treatment, stratified Cox regression was performed to analyze factors associated with the discontinuation of the infusion. Male, older age and RF-negativity were significantly associated with the discontinuation of infliximab due to adverse reactions, whereas there was no significant factor responsible for the discontinuation due to maintained remission or a lack of efficacy (Table 2).

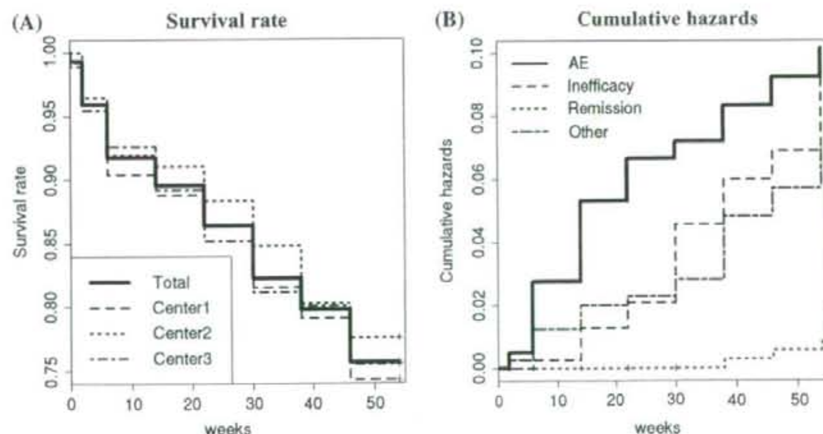

Fig. 1 Continuation of the infliximab therapy in RA patients for 54 weeks. **a** Survival rate of RA patients treated with infliximab ($n = 410$, total and three institutes) during the 54-week therapy. **b** Cumulative hazards of the discontinuation of infliximab therapy by week 54 of the treatment

Table 2 Results from a Cox regression analysis performed to examine the factors related to the discontinuation of infliximab therapy

Variable	AE ($n = 38$)					Inefficacy ($n = 33$)					Remission ($n = 3$)				
	Coef.	HR	CL	CU	*P*	Coef.	HR	CL	CU	*P*	Coef.	HR	CL	CU	*P*
Age	0.037	1.038	1.006	1.070	0.020	−0.014	0.987	0.957	1.017	0.38	−0.021	0.980	0.888	1.081	0.68
Gender	−0.940	0.391	0.177	0.863	0.020	0.160	1.173	0.352	3.910	0.79	–	–	–	–	–
RA duration	0.018	1.018	0.983	1.054	0.320	0.005	1.005	0.964	1.046	0.83	−0.114	0.893	0.622	1.280	0.54
RF (±)	−0.949	0.387	0.171	0.878	0.023	1.545	4.686	0.635	34.573	0.13	−1.150	0.317	0.023	4.291	0.39
MTX dose	0.096	1.101	0.920	1.318	0.290	−0.142	0.867	0.709	1.060	0.16	0.339	1.403	0.799	2.465	0.24
PSL dose	−0.066	0.936	0.848	1.033	0.190	0.003	1.003	0.908	1.109	0.95	−0.636	0.529	0.187	1.500	0.23
DAS (0 week)	0.205	1.227	0.890	1.692	0.210	0.113	1.120	0.815	1.537	0.49	−0.474	0.623	0.164	2.367	0.49

Coef. coefficient, *HR* hazard ratio, *CL* 95% confidence lower limit of HR, *CU* 95% confidence upper limit of HR

Efficacy of infliximab therapy

The average DAS28-CRP before starting infliximab was 5.5 ± 1.1, and this decreased to 3.4 ± 1.2, 3.2 ± 1.4, 3.1 ± 1.3 at weeks 2, 22 and 54, respectively, after the infliximab therapy (Fig. 2). Disease characteristics at baseline and after 2, 22 and 54 weeks of the inflilximab therapy were as follows: tender joints count (10.5, 4.2, 3.2 and 2.9), swollen joints count (10.6, 4.2, 2.7 and 2.3), GH (63, 34, 33 and 33 mm), and CRP (3.3, 1.0, 1.5 and 1.1 mg/dl) (Fig. 3). Before starting infliximab, the proportions of patients showing low, moderate, and high disease activity were 1.0, 9.0 and 90.0%, respectively. At week 22, patients in remission (defined as DAS28-CRP <2.3) and those showing low (<2.7), moderate (2.7–4.1), and high disease activity (>4.1) had changed to 27.8, 12.0, 32.4 and 27.8%, respectively, and at week 54 patients in remission and those showing low, moderate, and high disease activity were 27.6, 11.7, 34.4 and 26.3%, respectively (Fig. 4). Thus, approximately 27–28% of the patients satisfied the remission criteria at week 22 and still remained at week 54 after the infliximab therapy. Also, when the responses were evaluated by the EULAR response criteria, the proportions for good, moderate, and no response to infliximab as measured by DAS28-CRP were 37.3, 43.7 and 19.0%, respectively, at week 22 and 37.0, 41.7 and 21.2%, respectively, at week 54 (Fig. 5).

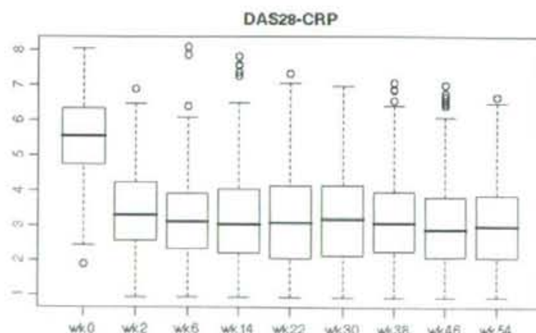

Fig. 2 Longitudinal analysis of DAS28 values during the 54-week study of patients using infliximab. *Line* in the *box* represents the median and the *upper* and *lower* ends of the *box* show the 25th and 75th percentiles of the population

Demographic factors related to the clinical efficacy of infliximab therapy

In order to clarify demographic factors related to the clinical efficacy of infliximab therapy, we performed a multivariate analysis adjust for institutional differences. Younger age, RF-negativity and lower levels of DAS28-CRP were significantly associated with the clinical remission induced by infliximab therapy at week 54, whereas gender, duration of the disease, dose of MTX and dose of PSL did not show a significant association (Table 3). On the other hand, most of the demographic factors, except for older age, did not affect secondary insufficiency from week 22 to 54 after the infliximab therapy according to the logistic regression analysis (Table 4).

Discussion

The RECONFIRM-2 study was designed to fully evaluate the effect of infliximab used in combination with MTX on the clinical results during a 54-week study period in DMARD-resistant RA patients of a RA management group in Japan. The safety profile of infliximab therapy for a total of 5,000 cases was investigated in Japan using an all-case registered post-marketing surveillance system, and the entire profile of adverse events related to infliximab therapy was clearly identified [14]. The assessment of efficacy, however, was based only on the physician's general evaluation and not on quantitative measures such as EULAR criteria. In this report, therefore, efficacy data

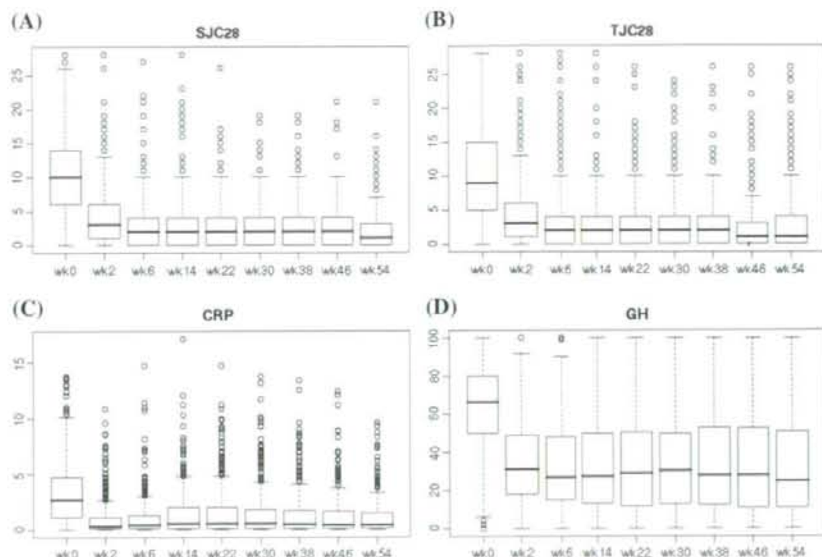

Fig. 3 Longitudinal analysis of **a** SJC28, **b** TJC28, **c** CRP, and **d** GH values during the 54-week study of patients using infliximab. *Line* in the *box* represents the median, and the *upper* and *lower* ends of the *box* show the 25th and 75th percentiles of the population

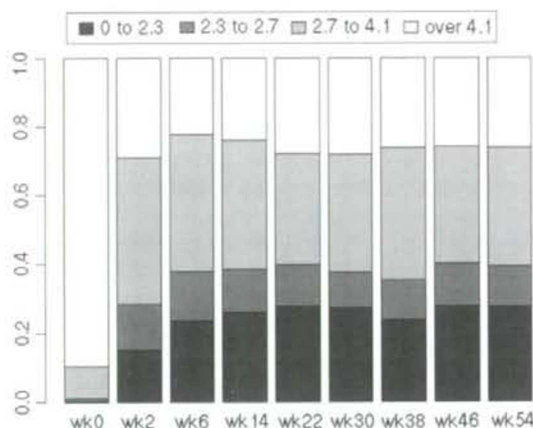

Fig. 4 Changes in DAS28 values during the 54-week study of patients using infliximab. The ratios of patients who demonstrated high disease activity (defined as DAS28-CRP >4.1), moderate activity (2.7–4.1), low activity (<2.7) and remission (<2.3) at each observation point during the 54-week study are shown

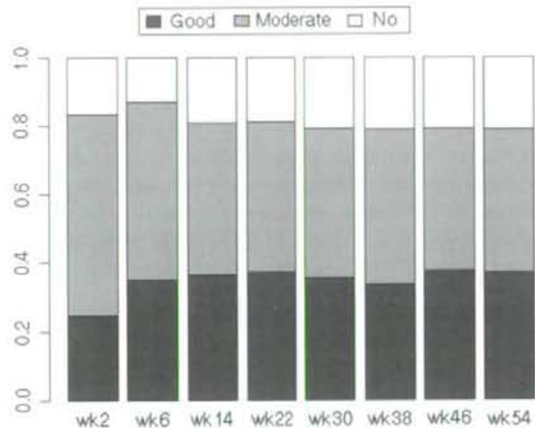

Fig. 5 The response to infliximab therapy during the 54-week study. The ratios of patients whose responses were evaluated by the European League Against Arthritis (EULAR) response criteria are shown

based on DAS28-CRP and EULAR improvement criteria were intensively assessed. The average DAS28-CRP before starting infliximab was 5.5, it decreased to 3.2 at week 22 and it remained steady at 3.1 by week 54 after the infliximab therapy. At week 22 after the therapy, about 28% and 40% of patients satisfied the remission and low disease activity criteria, respectively, and these effects remained (28 and 39%, respectively) by week 54. Also, about 37% of the patients exhibited good response according to EULAR criteria continuously from week 22 to 54. These results reconfirmed that the clinical efficacy of infliximab at week 22 was maintained until week 54 in most of the patients treated with infliximab. Thus, appropriate treatment with infliximab and MTX in this study group could minimize secondary insufficiency of the therapy for at least one year.

On the other hand, the discontinuation of infliximab was observed in approximately 24% of the patients during the 54-week period. This rate of discontinuation of the therapy was comparable to that obtained in other countries: 26.7%

Table 3 Results from the logistic regression analysis used to examine the factors related to clinical remission at weeks 22 and 54 induced by infliximab therapy

Variables	Week 22 ($n = 113$)						Week 54 ($n = 112$)					
	Coef.	OR	SE	CL	CU	*P*	Coef.	OR	SE	CL	CU	*P*
Intercept	1.530	4.617	1.034	0.608	35.067	0.139	1.770	5.868	1.010	0.811	42.445	0.080
Center 2 vs. Center 1	0.174	1.190	0.359	0.588	2.405	0.629	0.564	1.758	0.350	0.886	3.488	0.107
Center 3 vs. Center 1	1.072	2.922	0.324	1.549	5.510	0.001	0.989	2.689	0.325	1.423	5.082	0.002
Age	−0.014	0.986	0.010	0.967	1.006	0.168	−0.024	0.976	0.010	0.957	0.995	0.014
Gender	0.563	1.755	0.392	0.813	3.787	0.152	0.154	1.166	0.370	0.565	2.409	0.677
RA duration	−0.007	0.993	0.015	0.964	1.023	0.640	0.010	1.010	0.014	0.982	1.039	0.491
RF (±)	−0.413	0.662	0.339	0.340	1.287	0.224	−0.742	0.476	0.329	0.250	0.908	0.024
MTX dose	0.042	1.043	0.068	0.913	1.193	0.535	0.005	1.005	0.066	0.884	1.143	0.936
PSL dose	−0.063	0.939	0.035	0.876	1.005	0.070	−0.058	0.944	0.034	0.882	1.009	0.092
DAS28-CRP	−0.464	0.629	0.116	0.501	0.789	0.000	−0.276	0.759	0.112	0.609	0.945	0.014

Coef. coefficient HR, *OR* odds ratio, *SE* standard error, *CL* 95% confidence lower limit of OR, *CU* 95% confidence upper limit of OR

Table 4 Results from the logistic regression analysis used to examine the factors related to the secondary inefficiency from week 22 to 54 during infliximab therapy

Variables	Week 54–22 >0.6 ($n = 85$)						Week 54–22 >1.2 ($n = 47$)					
	Coef.	OR	SE	CL	CU	*P*	Coef.	OR	SE	CL	CU	*P*
(Intercept)	−1.461	0.232	1.079	0.028	1.923	0.176	−4.331	0.013	1.439	0.001	0.221	0.003
Center 2 vs. Center 1	−0.008	0.992	0.350	0.499	1.970	0.981	0.074	1.077	0.440	0.455	2.549	0.866
Center 3 vs. Center 1	0.071	1.073	0.333	0.559	2.060	0.831	0.054	1.056	0.426	0.458	2.433	0.899
Age	0.019	1.019	0.011	0.998	1.042	0.079	0.032	1.032	0.014	1.004	1.062	0.028
Gender	−0.283	0.753	0.358	0.373	1.520	0.429	0.250	1.284	0.511	0.471	3.500	0.625
RA duration	−0.029	0.972	0.017	0.941	1.004	0.083	−0.020	0.980	0.020	0.942	1.019	0.313
RF (±)	−0.233	0.792	0.366	0.387	1.622	0.524	0.164	1.178	0.512	0.432	3.212	0.749
MTX dose	−0.001	0.999	0.069	0.873	1.144	0.990	0.039	1.040	0.086	0.879	1.230	0.649
PSL dose	−0.015	0.985	0.036	0.918	1.057	0.682	0.019	1.019	0.045	0.934	1.112	0.676
DAS28-CRP (0 week)	−0.033	0.967	0.118	0.767	1.219	0.779	−0.011	0.989	0.148	0.739	1.323	0.941

Coef. coefficient HR, *OR* odds ratio, *SE* standard error, *CL* 95% confidence lower limit of OR, *CU* 95% confidence upper limit of OR

in the Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy (ATTRACT) international study carried out in the USA and the EU, 34.6% in a German domestic study and 26% in a French group study during the first one-year period [7, 15, 16]. Cumulative hazards differed among causes of discontinuation; discontinuations due to adverse events, inefficiency, remission and other causes (including changes of hospital/clinic and economic problems) were 0.093, 0.081, 0.007 and 0.063, respectively. Male gender, older age and RF-negativity were significantly correlated with discontinuation of infliximab due to adverse reactions, including infusion reactions ($N = 7$), toxicoderma (4), bacterial pneumonia (3), *pneumocystis jirovecii* pneumonia (3), but more than two-thirds of the withdrawals due to adverse events were observed within the first 14 weeks after the therapy.

In this study, to clarify how predisposing factors from the demographic characteristics of RA patients were related to the clinical efficacy of infliximab therapy, a multivariate analysis using a logistic regression was performed. In this study, multiple variables including sex, age, duration of disease, stage, class, positive/negative RF, concomitant MTX dose, concomitant PSL dose, and initial levels of CRP, TJC, SJC and GH were assessed. It is worth noting that, among multiple variables, younger age, RF-negativity and lower levels of DAS28-CRP at the baseline were significantly correlated with the clinical remission induced by infliximab therapy at week 54. These results imply that the timely use of MTX and infliximab can be strongly recommended for younger RA patients that show RF-negativity in order to efficiently achieve clinical remission. On the other hand, male gender, older age and

RF-negativity were significantly correlated with discontinuation of infliximab due to adverse reactions, whereas there was no significant factor responsible for discontinuation as a result of inefficacy, and only older age affected secondary insufficiency from week 22 to 54 after the infliximab therapy. Although it is intriguing that male gender predisposes for discontinuation as a result of adverse events, these results provide the first information that can be used to facilitate the more efficacious use of infliximab and MTX in the daily practice of rheumatologists.

Taken together, this REOCNFIRM-2 study reconfirms the clinical efficacy of treatment with infliximab and MTX in Japanese RA patients using the DAS28-CRP and EULAR response criteria. Among 410 patients with active RA, approximately 28% and 39% of the patients satisfied the remission and low disease activity criteria, and good response according to the EULAR criteria was achieved in 37% of the patients treated with infliximab plus MTX during the 54-week study period. The clinical efficacy of infliximab was maintained from week 22 to 54 after the treatment, and secondary insufficiency after week 22 was marginal after the appropriate treatment in this study group. Several demographic factors, including male gender, RF-negativity and lower scores of DAS28-CRP, were significant predisposing factors for remission. The promising effectiveness of infliximab at improving measures of disease activity in RA patients has led to this therapy becoming one of the key advances in the management of RA. Thus, this study is important because it provides obvious and invaluable evidence concerning the efficacy of the combinational use of infliximab and MTX, and can guide the real clinical use of infliximab in the future.

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Autoimmune disease after autologous hematopoietic stem cell transplantation

Toshiyuki Bohgaki *,[1], Tatsuya Atsumi, Takao Koike

Department of Medicine II, Hokkaido University Graduate School of Medicine N-15 W-7, Kita-ku, Sapporo 060-8638, Japan

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Abstract

Hematopoietic stem cell transplantation (HSCT) is an effective treatment for refractory autoimmune diseases. The safety and long-term outcome have been also acceptable. Infectious diseases under immune suppressive state after autologous HSCT are common transplantation related complications whereas autoimmune diseases are uncommon. Organ specific autoimmune diseases, such as immune mediated thrombocytopenia and thyroid dysfunction, are the most common after autologous HSCT. Systemic autoimmune diseases can also develop after autologous HSCT in patients with hematological disorders with genetic predisposition to autoimmune diseases. Although the mechanism of autoimmunity after HSCT is not well-known, long-term follow-up is essential in patients with autoimmune diseases treated with autologous HSCT.
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Keywords: Hematopoietic stem cell transplantation; Immune reconstitution; Autoimmune disease

Contents

1. Introduction 198
2. HSCT for autoimmune diseases. 200
3. Autoimmune disease after autologous hematopoietic stem cell transplantation 200
4. Discussion. 201
Take-home messages 202
References 202

1. Introduction

Autologous hematopoietic stem cell transplantation (HSCT) has been an effective treatment for refractory autoimmune diseases. Many patients with multiple sclerosis, systemic sclerosis or systemic lupus erythematosus have benefited from this treatment [1–3]. Autologus HSCT has been well tolerated in patients

* Corresponding author. Tel.: +81 11 706 5915; fax: +81 11 706 7710.

E-mail address: tbohgaki@uhnres.utoronto.ca (T. Bohgaki).

[1] Present address: Ontario Cancer Institute, University Health Network, 610 University Avenue, Suite 10-622, Toronto, Ontario, Canada M5G 2M9.

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Table 1
Organ specific autoimmune diseases after autologous hematopoietic stem cell transplantation

Patient (Age Sex)	Primary disease	Mobilization, Conditioning	Graft	Autoimmune disease after autologous HSCT				Remarks	Reference
				Diagnosis	Onset	Therapy	Outcome		
61 Male	DLBL	ETP+G, MCVC	PBSC	AITP	49 day	Nothing	CR	PAIgG(+)	[15]
45 Male	Germ cell tumor	CY+G, CEC	PBSC	AITP	70 day	PSL, IVIG, splenectomy	CR		[16]
45 Male	FL	CY+G, CY+ETP+TBI	PBSC	AITP	8 month	PSL	CR	Viral infection	[5]
42 Female	FL	CY+G, CY+ETP+TBI	PBSC	AITP	6 month	PSL, IVIG, splenectomy	CR		[5]
51 Female	Anap T	CY+G, BEAM	PBSC	AITP	14 month	PSL, IVIG, splenectomy	CR	Viral infection	[5]
50 Male	T in B	CY+G, CY+ETP+TBI	PBSC	AITP	21 month	PSL	CR		[5]
23 Female	Burkitt	CY+G, BEAM	PBSC	AITP	1 month	PSL, IVIG	CR		[5]
56 Male	Mantle	CY+G, CY+ETP+TBI	PBSC	AITP	5 month	PSL, IVIG, splenectomy	CR	Influenza virus vaccine	[5]
19 Male	AML	?, Bus+CY	PBSC	AITP	76 day	PSL	CR	Autoantibody (−), infection (−)	[17]
58 Female	Breast	ETP+CY+G, CTCb	PBSC	AITP	41 day	PSL, IVIG	CR	Sinus fullness, VZV	[18]
22 Female	AML	?, Bus+CY	PBSC	AITP	50 day	PSL	CR		[19]
8 Male	Ewing's	(−), Melphalan	BM	AITP	1 month	?	?	PAIgG (+)	[20]
36 Female	MDS	(−), AraC+CY+TBI	BM	AITP AIHA	35 month	PSL, IVIG, CyA	CR	IIb/IIIa (+), LAC (+)	[21]
36 Female	SLE, APS	CY+G, CY+ATG	CD34+	Hemop	30 month	aVII, PSL, Rx	CR	VIII inhibitor (+)	[6]
28 Female	SLE	CY+G, CY+ATG	CD34+	Hemop	9 month	aVII, PSL, Rx, IVCY, MMF	CR	VIII inhibitor (+)	[6]
46 Female	MS	CY+G, CY+ alemtuzumab	PBSC	AITP	8 month	PSL, IVIG, Rx, CY	CR		[6]
45 Female	SSc	CY+G, CY+ alemtuzumab	PBSC	AIHA	5 month	PSL, IVIG, Rx, MMF, IVCY	PR		[6]
45 Female	SLE	CY+G, CY+ alemtuzumab	PBSC	ANeutro AIHA	2 month	G, PSL, IVIG, ATG, CY, Rx, MMF, tacrolium, splenectomy	CR	anti-Neutro (+)	[6]
51 Female	MS	CY+G, CY+ alemtuzumab	PBSC	AITP	14 month	PSL, IVIG, Rx, MMF			[6]
34 Female	B-NHL	ETP+G, ETP+ Carbo+CY	PBSC	Evans'	49 day	PSL, pepleo, VCR	PR	dCoombs' (+), PAIgG (+), CMV	[22]
3 Female	Neuro	?, Bus+melphalan	PBSC	Evans'	20 day	PSL, IVIG	CR	dCoombs' (+), anti-Plt (+)	[23]
28 Female	HD	(−), CY+ETP+ carmustine	BM	Evans'	42 month	PSL, IVIG, splenectomy, CY, CVR, CyA, AZP, PE	PR	dCoombs' (+), iCoombs' (+)	[24]
35 Female	MM	CY+G, TBI+melphalan	PBSC	Hyper Thyroid	34 day	Nothing	CR	PAIgG (+), ANA (+)	[25]

(continued on next page)

Table 1 (*continued*)

Patient (Age Sex)	Primary disease	Mobilization, Conditioning	Graft	Autoimmune disease after autologous HSCT				Remarks	Reference
				Diagnosis	Onset	Therapy	Outcome		
46 Male	MultP	CY+G, Melphalan	BM	Hyper Thyroid	28 month	Carbimazole	CR	Mobilization failure	[26]
54 Female	Peri-T	AraC+G, CY+ETP+ ranimustine	PBSC	UC	110 day	PSL, CyA, mesalazine	CR	anti-trop (+)	[27]

Abbreviations: diffuse large B-cell lymphoma (DLBL), follicular lymphoma (FL), anaplastic T cell lymphoma (Anap T), T cell rich B-cell lymphoma (T in B), Burkitt lymphoma (Burkitt), Mantle cell lymphoma (Mantle), acute myelogeneous leukemia (AML), breast cancer (Breast), Ewing's sarcoma (Ewing's), myelodysplastic syndrome (MDS), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), multiple sclerosis (MS), systemic sclerosis (SSc), B-cell non-Hodgkin lymphoma (B-NHL), neuroblastoma (Neuro), Hodgkin disease (HD), multiple myeloma (MM), multiple plasmacytome (MultiP), peripheral T cell lymphoma (Peri-T), etoposide (ETP), granulocyte-colony-stimulating factor (G), ranimustine+carboplatin+ETP+cyclophosphamide (CY) (MCVC), carboplatin+ETP+CY (CEC), total body irradiation (TBI), carmustine+ETP+cytarabine+melphalan (BEAM), busulphan (Bus), CY+thiotepa+carboplatin (Carbo) (CTCb), cytarabine (AraC), anti-thymocyte globulin (ATG), peripheral blood stem cell (PBSC), bone marrow (BM), autoimmune thrombocytopenia (AITP), autoimmune hemolytic anemia (AIHA), Evans' syndrome (Evans'), autoimmune neutropenia (ANeutro)hyperthyroidism (Hyper Thyroid), ulcerative colitis (UC), prednisolone (PSL), intravenous immunoglobulin (IVIG), cyclosporine A (CyA), activated factor VII (aVII), rituximab (Rx), mycophenolate mofetil (MMF), intravenous CY (IVCY), pepleomycin (pepleo), vincristine (VCR), azathioprine (AZP), plasma exchange (PE), partial response (PR), complete response (CR), platelet associated immunoglobulin G (PAIgG), herpes zoster virus infection (VZV), anti-IIb/IIIa autoantibody (IIb/IIIa), lupus anticoagulant (LAC), anti-neutrophil autoantibody (anti-Neutro), direct Coombs' test (dCoombs'), cytomegalovirus antigenemia (CMV), antiplatelet autoantibody (anti-Plt), indirect Coombs' test (iCoombs'), antinuclear autoantibody (ANA), anti-tropomyosin autoantibody (anti-trop).

with autoimmune diseases and common transplantation related complications are infectious diseases during immune suppressive state at the early stage after autologous HSCT [4]. Although the role of HSCT in autoimmune diseases is not well-known, immune modulation with severe immune suppressive treatment can contribute disease outcome. Autoimmune disease after autologous HSCT is relatively-uncommon transplantation related complication and 2% of patients treated autologous HSCT develop autoimmune thrombocytopenia which is the most common organ specific autoimmune diseases after autologous HSCT [5]. Secondary autoimmune diseases after autologous HSCT for autoimmune diseases have been reported [6], but systemic autoimmune diseases have been rarely reported [7,8]. We reported a patient with systemic sclerosis who developed systemic lupus erythematosus after CD34$^+$-selected autologous hematopoietic stem cell transplantation [9]. We described clinical findings about autoimmune diseases after autologus HSCT from our experience and the review of literature.

2. HSCT for autoimmune diseases

HSCT is categorized into several procedures depending on the graft condition and conditioning regimen. Among them autologous HSCT has been usually performed in autoimmune diseases based on the safety and clinical efficacy. Clinical benefit has been obtained in two third patients with autoimmune diseases. About 5% of transplantation related mortality has been also reported in phase I–II studies [1–3]. Clinical effect including stabilization of autoimmune diseases has persisted for up to 7 years after autologous HSCT [1]. Although it is still unclear how autologous HSCT improves autoimmune diseases it is believed that lymphocytes, progenitor cells and hematopoietic stem cells in the graft modify the disease condition and/or the immune system. In peripheral immunity, regulatory T cells can play a role in disease improvement in patients with adult onset juvenile idiopathic arthritis and cytokine balance may influence the disease development [10,11]. In addition, thymic function can contribute to immune modulation leading to clinical benefit in patients with systemic lupus erythematosus or multiple sclerosis [12,13]. In allogenic HSCT, immune reconstitution against autoimmunity may also play a role in improvement of autoimmune diseases [14].

3. Autoimmune disease after autologous hematopoietic stem cell transplantation

Several autoimmune diseases have been reported. Among them, autoimmune thrombocytopenia is the most common organ specific autoimmune diseases after autologous HSCT. Autoimmune thrombocytopenia with the platelet counts drop following an apparent normal initial recovery after autologous HSCT (secondary

Table 2
Systemic autoimmune diseases after autologous hematopoietic stem cell transplantation

Patient (Age Sex)	Primary disease	Mobilization, Conditioning	Graft	Autoimmune disease after autologous HSCT				Remarks	Reference
				Diagnosis	Onset	Therapy	Outcome		
51 Male	DLBL	ETP+G, MCVC	PBSC	RA	40 day	NSAIDs, PSL	PR	RF (+), HLA-DRB1 0405 (+)	[7]
40 Male	FL	Dexa-BEAM, BEAM	PBSC	AS	3 month	NSAIDs, SASP	PR	HLA-B27 (+)	[8]
39 Male	DLBL	Dexa-BEAM, BEAM	PBSC	AS	4 month	NSAIDs	PR	HLA-B27 (+)	[8]
29 Female	AML	(−), CY+TBI	BM	AS	1 year	NSAIDs	PR	HLA-B27 (+), *Yersinia enterocolitica* (+)	[8]
19 Female	SSc	CY+G, CY	CD34+	SLE	4 year	PSL, CyA	CR		[9]

Abbreviations: diffuse large B-cell lymphoma (DLBL), follicular lymphoma (FL), acute myelogeneous leukemia (AML), systemic sclerosis (SSc), etoposide (ETP), granulocyte-colony-stimulating factor (G), ranimustine+carboplatin+ETP+cyclophosphamide (CY) (MCVC), dexamethasone (Dexa), carmustine+ETP+cytarabine+melphalan (BEAM), total body irradiation (TBI), peripheral blood stem cell (PBSC), bone marrow (BM), selected CD34+ cells (CD34+), rheumatoid arthritis (RA), spondylarthropathy (AS), systemic lupus erythematosus (SLE), non steroidal anti-inflammatory drugs (NSAIDs), prednisolone (PSL), salazosulfapyridine (SASP), cyclosporine A (CyA), partial response (PR), complete response (CR), rheumatoid factor (RF).

autoimmune thrombocytopenia) seems to be rare [15]. More than 15 cases of secondary autoimmune thrombocytopenia have been reported (Table 1) [5,6,16–22]. Nine of 15 cases occurred within 6 months after autologous HSCT. Some cases might relate to infectious diseases after autologous HSCT. Most patients with autoimmune thrombocytopenia were treated with corticosteroid with feasible clinical response. Acquired hemophilia, autoimmune hemolytic anemia, Evans' syndrome, hyperthyroidism and ulcerative colitis have been also reported (Table 1) [6,23–28]. Intensive immunosuppressive treatments were sometimes needed for treatment with these conditions.

Rheumatoid arthritis and spondylarthropathy have been reported as systemic autoimmune diseases after autologous HSCT (Table 2) [7,8]. Although their primary diseases were hematological malignancy without autoimmune diseases before autologous HSCT, positive for rheumatoid factor, HLA-DRB1 0405 or HLA-B27 before treatment suggested their genetic predisposition to autoimmune diseases. They had suffered from systemic autoimmune diseases within one year after autologous HSCT during incomplete immune reconstitution. One of them, infectious disease might contribute to the onset of systemic autoimmune disease. These findings suggest that the transient immune suppression following autologous HSCT and infectious disease post-transplant period may play a role in the development of systemic autoimmune diseases in susceptible patients.

4. Discussion

Autoimmune diseases such as sicca syndrome or scleroderma occasionally develop after allogenic HSCT while autoimmunity after autologous HSCT is uncommon [29]. Especially, systemic autoimmune diseases have been rarely reported [7,8]. Although the exact mechanism of autoimmunity after autologous HSCT is not known, several possibilities have been proposed, which include the impairment of peripheral T cell reconstitution as typified by markedly delayed regeneration of the $CD4^+$ subset, clonal expansion of peripheral T cell, stem cell damage during clinical procedure, homeostatic proliferation in patients with autoimmune background or an impaired development of regulatory T cells and generation of interleukin-17-producing helper T cells under the instability of immune system after autologous HSCT [9,16,19,30]. The genetic predisposition to autoimmune disease can also play a role in the development of autoimmune disease after autologous HSCT [7,8,31]. Autoimmune diseases can be treated only high-dose chemotherapy without stem cell rescue and the importance of conditioning regimen has been also reported in patients with secondary autoimmune diseases after autologous HSCT [6,32]. The necessity of stem cell rescue and its impact on immune reconstitution is obscure. The efficacy and safety of autologous HSCT for autoimmune diseases has been accepted, while long-term prognosis is not concluded. Systemic autoimmune diseases developed within one year after autologous

HSCT in patients with hematological disorders unlike our patient. Previous chemotherapies in patients with hematological disorders might result in profound immune suppression leading to the early appearance of systemic autoimmune diseases. Although autoimmune diseases are uncommon condition in patients treated with autologous HSCT, secondary autoimmune diseases can develop after autologous HSCT in patients with autoimmune diseases.

Take-home messages

- Hematopoietic stem cell transplantation is effective for refractory autoimmune diseases and well tolerated in patients with autoimmune diseases.
- Secondary autoimmune diseases can develop after autologous hematopoietic stem cell transplantation.
- Immune instability after autologous hematopoietic stem cell transplantation may lead to development of autoimmunity in patients with immune prone background.
- Long-term follow-up is essential for patients with autoimmune diseases treated with autologous hematopoietic stem cell transplantation.

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BAFF and MyD88 signals promote a lupus-like disease independent of T cells

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of autoantibodies. However, the underlying cause of disease appears to relate to defects in T cell tolerance or T cell help to B cells. Transgenic (Tg) mice over-expressing the cytokine B cell-activating factor (BAFF) develop an autoimmune disorder similar to SLE and show impaired B cell tolerance and altered T cell differentiation. In this study, Groom JR, et al. (J Exp Med 2007; 204: 1959-71) generated BAFF Tg mice that were completely deficient in T cells, and surprisingly, these mice developed an SLE-like disease indistinguishable from that of BAFF Tg mice. Autoimmunity in BAFF Tg mice did, however, require B cell-intrinsic signals through the Toll-like receptor (TLR)-associated signaling adaptor MyD88, which controlled the production of pro-inflammatory autoantibody isotypes. TLR7/9 activation strongly up-regulated expression of transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), which is a receptor for BAFF involved in B cell responses to T cell-independent antigens. Moreover, BAFF enhanced TLR7/9 expression on B cells and TLR-mediated production of autoantibodies. Therefore, autoimmunity in BAFF Tg mice results from altered B cell tolerance, but requires TLR signaling and is independent of T cell help.

The opposite-direction modulation of CD4+CD25+ Tregs and T helper 1 cells in acute coronary syndromes

Different subsets of T lymphocytes have different functions in atherosclerosis advancement. T helper 1 cells and T regulatory 1 cells have been demonstrated to play opposite roles in rupture of atherosclerotic lesions. However, the role of novel subsets of T regulatory cells, known as CD4+CD25+foxp3+ T cells remains largely unknown in coronary artery disease (CAD). In this study, **Shu-fang H. et al (Clin Immunol 2007; 124: 90-7)** investigated the peripheral CD4+CD25+Foxp3+ T cells of patients with CAD and controls. The patients submitted were divided into three groups: stable angina pectoris (SA) group, unstable angina pectoris (UA) group and acute myocardial infarction (AMI) group. The authors analyzed the frequencies of peripheral CD4+CD25+Foxp3+ T cells and T helper 1/T helper2 cells, expression of Foxp3 in CD4+CD25+ T subsets and cytokines pattern in patients and controls. It was found that the reduction of CD4+CD25+Foxp3+ T lymphocytes was consistent with the expansion of Th1 cells in patients with unstable CAD. The reversed development between CD4+CD25+ Tregs and Th1 cells might contribute to plaque destabilization.

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Crucial Role of the Interleukin-6/Interleukin-17 Cytokine Axis in the Induction of Arthritis by Glucose-6-Phosphate Isomerase

Keiichi Iwanami,[1] Isao Matsumoto,[2] Yoko Tanaka-Watanabe,[1] Asuka Inoue,[1] Masahiko Mihara,[3] Yoshiyuki Ohsugi,[4] Mizuko Mamura,[1] Daisuke Goto,[1] Satoshi Ito,[1] Akito Tsutsumi,[1] Tadamitsu Kishimoto,[5] and Takayuki Sumida[1]

Objective. **To clarify the glucose-6-phosphate isomerase (GPI)–specific CD4+ T cell lineage involved in GPI-induced arthritis and to investigate their pathologic and regulatory roles in the induction of the disease.**

Methods. **DBA/1 mice were immunized with GPI to induce arthritis. CD4+ T cells and antigen-presenting cells were cocultured with GPI, and cytokines in the supernatant were analyzed by enzyme-linked immunosorbent assay. Anti–interferon-γ (anti-IFNγ) monoclonal antibody (mAb), anti–interleukin-17 (anti–IL-17) mAb, or the murine IL-6 receptor (IL-6R) mAb MR16-1 was injected at different time points, and arthritis development was monitored visually. After MR16-1 was injected, percentages of Th1, Th2, Th17, and Treg cells were analyzed by flow cytometry, and CD4+ T cell proliferation was analyzed using carboxyfluorescein diacetate succinimidyl ester.**

Results. **GPI-specific CD4+ T cells were found to be differentiated to Th1 and Th17 cells, but not Th2 cells. Administration of anti–IL-17 mAb on day 7 significantly ameliorated arthritis ($P < 0.01$), whereas administration of anti-IFNγ mAb exacerbated arthritis. Neither anti–IL-17 mAb nor anti-IFNγ mAb administration on day 14 ameliorated arthritis. Administration of MR16-1 on day 0 or day 3 protected against arthritis induction, and MR16-1 administration on day 8 significantly ameliorated existing arthritis ($P < 0.05$). After administration of MR16-1, there was marked suppression of Th17 differentiation, without an increase in Th1, Th2, or Treg cells, and CD4+ T cell proliferation was also suppressed.**

Conclusion. **IL-6 and Th17 play an essential role in GPI-induced arthritis. Since it has previously been shown that treatment with a humanized anti–IL-6R mAb has excellent effects in patients with rheumatoid arthritis (RA), we propose that the IL-6/IL-17 axis might also be involved in the generation of RA, especially in the early effector phase.**

Rheumatoid arthritis (RA) is characterized by symmetric polyarthritis and joint destruction. Although the etiology of RA is considered to be an autoimmune reactivity to antigens that are specifically expressed in joints, this remains a controversial hypothesis. It has been reported that autoimmune reactivity to a ubiquitous cytoplasmic enzyme, glucose-6-phosphate isomerase (GPI), provokes joint-specific inflammation in K/BxN mice (1,2). This finding highlights the potential role of systemic autoreactivity to certain ubiquitous autoantigens in the pathogenesis of RA.

More recently, it was reported that arthritis can also be induced in DBA/1 mice by immunization with GPI (3). GPI-induced arthritis is different from collagen-induced arthritis (CIA) with regard to the priority of T cells and B cells. In CIA, treatment with anti-CD4 monoclonal antibodies (mAb) is ineffective after the mice have produced antibodies to type II collagen (4,5), and CD4-deficient mice can develop CIA at the same incidence and severity as untreated mice (6). Adoptive transfer of IgG antibodies purified from mice

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[1]Keiichi Iwanami, MD, Yoko Tanaka-Watanabe, MSc, Asuka Inoue, BSc, Mizuko Mamura, MD, PhD, Daisuke Goto, MD, PhD, Satoshi Ito, MD, PhD, Akito Tsutsumi, MD, PhD, Takayuki Sumida, MD, PhD: University of Tsukuba, Tsukuba, Japan; [2]Isao Matsumoto, MD, PhD: University of Tsukuba, Tsukuba, and PRESTO, Japan Science and Technology Agency, Saitama, Japan; [3]Masahiko Mihara, PhD: Chugai Pharmaceutical Company, Ltd., Shizuoka, Japan; [4]Yoshiyuki Ohsugi, PhD: Chugai Pharmaceutical Company, Ltd., Tokyo, Japan; [5]Tadamitsu Kishimoto, MD, PhD: Osaka University, Osaka, Japan.

Address correspondence and reprint requests to Isao Matsumoto, MD, PhD, Division of Clinical Immunology, Major of Advanced Biochemical Applications, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan. E-mail: ismatsu@md.tsukuba.ac.jp.

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with CIA can induce arthritis even in strains that are not susceptible to CIA induction by conventional immunization. In GPI-induced arthritis, administration of anti-CD4 mAb after arthritis onset rapidly ameliorates the arthritis, despite the absence of changes in the anti-GPI antibody titers. Fcγ receptor–deficient mice are resistant to GPI-induced arthritis, and adoptive transfer of purified IgG antibodies alone is not able to induce arthritis in these mice (3). These findings indicate that although autoantibodies are necessary for GPI-induced arthritis, CD4+ T cells are indispensable even after antibody production.

The present study was designed to further characterize the importance of CD4+ T cells in GPI-induced arthritis. Specifically, we investigated the CD4+ T cell lineage involved in GPI-induced arthritis and the regulatory mechanisms of pathogenic T cells. The results demonstrated that GPI-specific CD4+ T cells shifted to Th1 and Th17 cells and that Th17 played a crucial role in the development of GPI-induced arthritis. We also found that blockade of interleukin-6 receptor (IL-6R) significantly suppressed the arthritis and inhibited Th17 differentiation. The main message of this study is that the IL-6/IL-17 axis may be essential for the development of T cell–dependent autoimmune arthritis.

MATERIALS AND METHODS

Mice. Male DBA/1 mice were purchased from Charles River Laboratories (Yokohama, Japan). All mice were maintained under specific pathogen–free conditions, and all experiments were conducted in accordance with the institutional ethics guidelines.

GPI-induced arthritis. Recombinant human GPI was prepared as described previously (7). Briefly, human GPI complementary DNA was inserted into plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione S-transferase–tagged proteins. The *Escherichia coli*–harboring pGEX-hGPI plasmid was allowed to proliferate overnight at 37°C before the addition of 0.1 m*M* IPTG to the medium, which was followed by a further culture overnight at 30°C. The bacteria were lysed with a sonicator, and the supernatant was purified with a glutathione–Sepharose column (Pharmacia). The purity was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Mice were immunized intradermally with 300 μg of recombinant human GPI in Freund's complete adjuvant (Difco, Detroit, MI). Recombinant human GPI and Freund's complete adjuvant were emulsified at a 1:1 ratio (volume/volume). For induction of arthritis, 150 μl of the emulsion was injected intradermally into the base of the tail. For intracellular staining and cell proliferation assay, 50 μl was injected into each footpad of the hind paw. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3, where 0 = no evidence of inflammation, 1 = subtle inflammation or localized edema, 2 = easily identified swelling that was localized to either the dorsal or ventral surface of the paw, and 3 = swelling of all aspects of the paw.

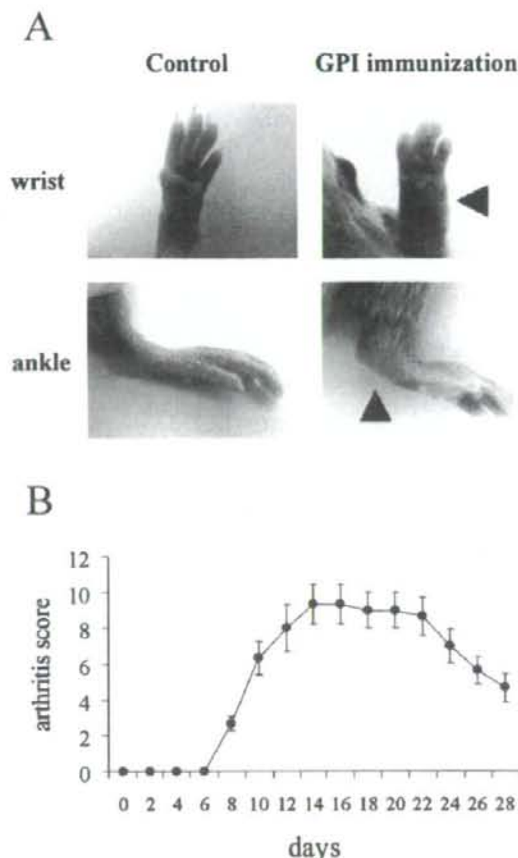

Figure 1. Induction of severe polyarthritis by immunization with recombinant human glucose-6-phosphate isomerase (GPI). DBA/1 mice were immunized with 300 μg of recombinant human GPI, and the development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details). Arthritis was clinically apparent beginning on days 7–8, peaked in severity on day 14, and then gradually subsided. **A,** Severe swelling of the wrist and ankle joints **(arrowheads)** in mice immunized with GPI as compared with control mice. **B,** Mean ± SEM arthritis scores on days 0–28 in 10 mice from a representative experiment.

Analysis of cytokine profiles. Mice were killed on day 7 or day 14. Spleens were harvested and hemolyzed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$, and 0.004% disodium EDTA in phosphate buffered saline (PBS). Single-cell suspensions were prepared in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin,

and 50 μM 2-mercaptoethanol. CD4+ T cells were isolated by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity (>97%) was confirmed by flow cytometry. Splenic feeder cells treated with 50 μg/ml of mitomycin C were used as antigen-presenting cells (APCs). Purified CD4+ T cells and APCs were cocultured with 5 μg/ml of GPI at a ratio of 5:1 for 24 hours at 37°C in an atmosphere containing 5% CO_2. The supernatants were assayed for interferon-γ (IFNγ), IL-4, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Treatment of arthritis with antibodies. To neutralize IL-17 and IFNγ, mice were injected intraperitoneally with 100 μg of neutralizing antibody or isotype control on day 7 or day 14. Anti–IL-17 mAb MAB421 (IgG2a) and anti-IFNγ mAb MAB485 (IgG2a) were purchased from R&D Systems. IgG2a isotype control was purchased from eBioscience (San Diego, CA). For IL-6 neutralization, mice were injected intraperitoneally with 2 mg or 4 mg of MR16-1 (an IgG1-specific mAb against murine IL-6R) or control IgG (purified from the serum of nonimmunized rats) on day 0, 3, 8, or 14. MR16-1 was a gift from Chugai Pharmaceutical (Tokyo, Japan), and control IgG was purchased from Jackson ImmunoResearch (West Grove, PA).

Anti-GPI antibody analysis. Sera were obtained on day 28 or day 35 and diluted 1:500 in blocking solution (25% Block-Ace [Dainippon Sumitomo Pharma, Osaka, Japan] in PBS) for analysis of antibody. Then, 96-well plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 5 μg/ml of recombinant human GPI for 12 hours at 4°C. After washing twice with washing buffer (0.05% Tween 20 in PBS), the blocking solution was applied for 2 hours at room temperature to block nonspecific binding. After 2 washes, 150 μl of diluted sera was added, and the plates were incubated for 2 hours at room temperature. After 3 washes, alkaline phosphatase (AP)–conjugated anti-mouse IgG was added at a final dilution of 1:5,000 for 1 hour at room temperature. After 3 washes, color was developed with substrate solution, consisting of 1 tablet of AP tablet (Sigma-Aldrich) per 5 ml of AP reaction solution (9.6% diethanolamine and 0.25 mM $MgCl_2$, pH 9.8). Plates were incubated for 20 minutes at room temperature, and the optical density was read at 405 nm using a microplate reader.

Intracellular cytokine staining and flow cytometric analysis. Mice were killed on day 7. Popliteal lymph nodes were harvested, and single-cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 100 μg/ml of recombinant human GPI in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) for 24 hours. GolgiStop (BD PharMingen, San Diego, CA) was added during the last 2 hours of each culture. Cells were stained extracellularly, fixed, and permeabilized with Cytofix/Cytoperm solution (BD PharMingen), then the cells were stained intracellularly. A mouse Treg cell staining kit with forkhead box P3 (FoxP3) (eBioscience) was used to stain Treg cells according to the protocol supplied by the manufacturer. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Cell proliferation assay. Mice were killed on day 10. Popliteal lymph nodes were harvested, and single-cell suspensions were prepared as described above. Cells (2×10^7/ml) in PBS were stained with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Molecular Probes, Eugene, OR) for 8 minutes. Stained cells were cultured with 25 μg/ml of recombinant human GPI at 1×10^6/ml in 96-well round-bottomed plates (Nunc) for 60 hours and then analyzed by flow cytometry.

Statistical analysis. Data are expressed as the mean ± SEM or mean ± SD. Differences between groups were examined for statistical significance using the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Induction of severe symmetric polyarthritis by immunization with GPI. For the induction of arthritis, we immunized DBA/1 mice with 300 μg of recombinant human GPI emulsified with Freund's complete adjuvant. Of the 177 mice immunized with recombinant human GPI, 167 (94.4%) developed severe swelling of the wrist and ankle joints (Figure 1A). The arthritis appeared on

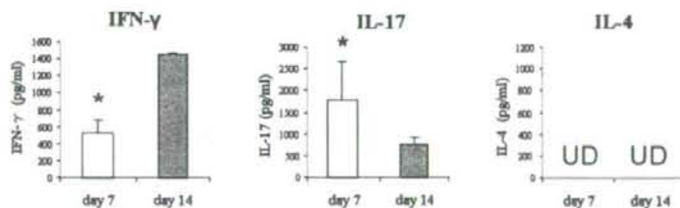

Figure 2. Differentiation of glucose-6-phosphate isomerase (GPI)–specific CD4+ T cells into Th1 and Th17 cells. CD4+ T cells and mitomycin C–treated antigen-presenting cells were stimulated for 24 hours with GPI on either day 7 (induction phase) or day 14 (effector phase) and then assessed for the production of interferon-γ (IFNγ), interleukin-17 (IL-17), and IL-4 by enzyme-linked immunosorbent assay. Values are the mean and SD of 3 independent experiments (n = 3 mice per experiment). * = $P < 0.05$ versus cells stimulated on day 14, by Mann-Whitney U test. UD = undetectable (<2 pg/ml).

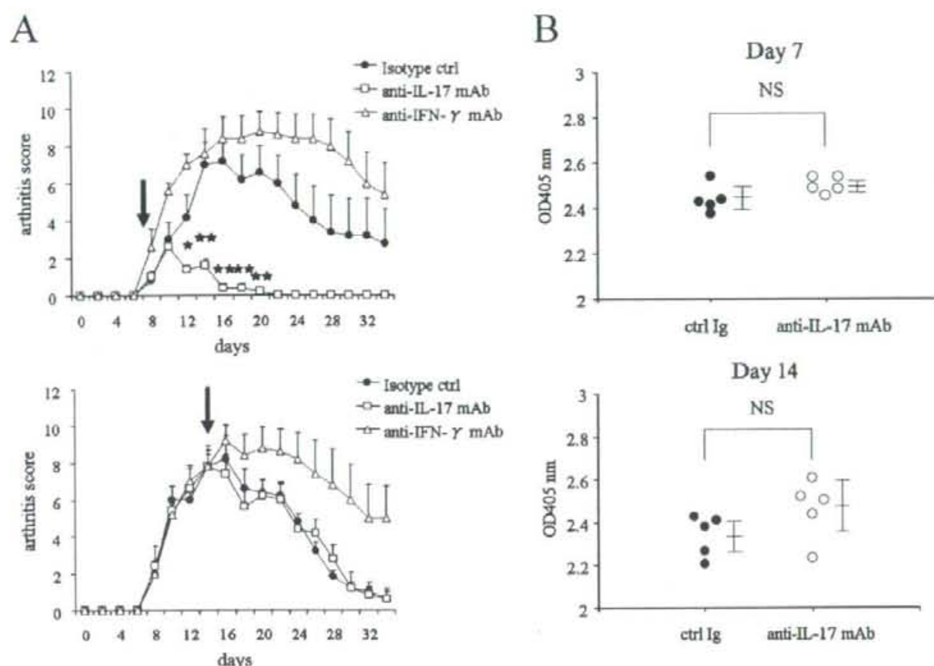

Figure 3. Suppression of the development of glucose-6-phosphate isomerase (GPI)–induced arthritis by treatment with anti–interleukin-17 (anti–IL-17) monoclonal antibody (mAb). **A,** Arthritis scores following intraperitoneal injection of 100 μg of anti–IL-17 mAb or anti–interferon-γ (anti-IFNγ) mAb on day 7 or day 14 after GPI immunization (arrow). Values are the mean and SEM of 5 mice per group. Results are representative of 2 independent experiments. * = $P < 0.05$; ** = $P < 0.01$ versus isotype control at the same time point, by Mann-Whitney U test. **B,** Titers of anti-GPI antibody in sera obtained on day 35 following intraperitoneal injection of 100 μg of anti–IL-17 mAb on day 7 or day 14 after GPI immunization, as determined by enzyme-linked immunosorbent assay. Each symbol represents a single mouse. Bars show the mean ± SD optical density (OD) at 405 nm. NS = not significant (by Mann-Whitney U test).

days 7–8, showed peak severity on day 14, then gradually subsided (Figure 1B).

Differentiation of GPI-specific CD4+ effector T cells to Th1 and Th17 cells, but not Th2 cells. CD4+ T cells are indispensable for both the induction phase and the effector phase of GPI-induced arthritis (3); however, the lineage to which GPI-specific CD4+ effector T cells are differentiated remains to be elucidated. To determine the lineage, we stimulated CD4+ T cells with recombinant human GPI on day 7 (induction phase) or day 14 (effector phase) in vitro and then assessed cytokine production by ELISA. GPI-specific CD4+ T cells produced IFNγ and IL-17, but not IL-4, on days 7 and 14 (Figure 2). Interestingly, IFNγ production was lower on day 7 than on day 14 ($P < 0.05$), whereas IL-17 production was higher on day 7 than on day 14 ($P < 0.05$). These data demonstrated that GPI-specific CD4+ effector T cells are differentiated to Th1 and Th17 and are regulated differently during the development of arthritis.

Crucial role of Th17 cells in the induction phase. If GPI-specific CD4+ T cells produce both IFNγ and IL-17, then which of these two cytokines affects the development of arthritis? To answer this question, we injected 100 μg of anti-IFNγ mAb or anti–IL-17 mAb intraperitoneally on day 7 or day 14 after immunization with recombinant human GPI. Injection of anti–IL-17 mAb on day 7 resulted in significant improvement in the arthritis scores as compared with injection of isotype control ($P < 0.01$), but injection of anti–IL-17 mAb on day 14 did not affect the course of the disease (Figure 3A). In contrast, injection of anti-IFNγ mAb on day 7 and day 14 did not ameliorate arthritis, but rather, tended to exacerbate it (Figure 3A).

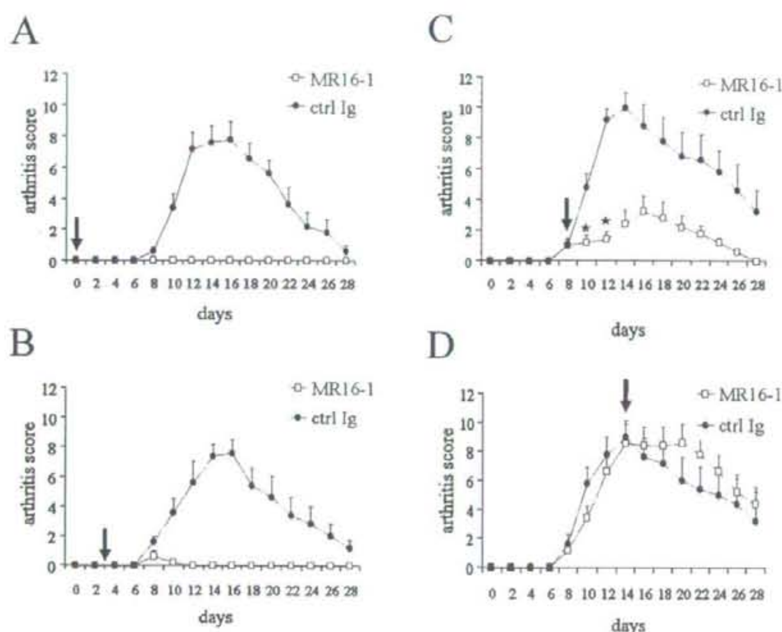

Figure 4. Inhibition of the development of arthritis by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or control Ig on day 0 (**A**), day 3 (**B**), or day 8 (**C**) or with 4 mg of MR16-1 or control Ig on day 14 (**D**) after GPI immunization. The development of arthritis was monitored visually and scored on a scale of 0–3 (see Materials and Methods for details). Arrow indicates the date of mAb injection. Values are the mean and SEM of 5 mice per group. Results are representative of 2 independent experiments. * = $P < 0.05$ versus controls, by Mann-Whitney U test.

Next, we explored whether anti–IL-17 mAb affects the production of anti-GPI antibodies. Treatment of mice with anti–IL-17 mAb on day 7 or on day 14 did not appreciably affect the titers of anti-GPI antibody (Figure 3B). These results indicate that Th17 cells are involved in the development of GPI-induced arthritis independently of anti-GPI antibody titers.

Inhibition of arthritis by anti–IL-6R mAb. It has been reported that IL-6 plays an important role in the differentiation of Th17 cells from naive T cells (8,9). We speculated that blockade of IL-6 might inhibit the development of arthritis, and we examined the effects of anti–IL-6R mAb MR16-1 on the development of arthritis. We injected 2 mg of MR16-1 intraperitoneally on day 0, 3, or 8 after immunization with recombinant human GPI, or we injected 4 mg on day 14 after immunization. As we anticipated, injection of MR16-1 on day 0 completely blocked the development of arthritis (Figure 4A), and injection on day 3 showed an almost complete inhibition (Figure 4B). Even after the development of arthritis, injection of MR16-1 on day 8 significantly suppressed the progression of arthritis (Figure 4C); however, injection of 4 mg of MR16-1 on day 14, at the peak of arthritis, did not ameliorate arthritis (Figure 4D). These results suggest that blockade of IL-6R has protective effects and some therapeutic effects on GPI-induced arthritis.

Inhibition of the development of Th17 cells, without an increase in Th1, Th2, or Treg cell populations, by anti–IL-6R mAb. To examine whether MR16-1 affects Th1, Th2, and Treg cells, we cultured cells from draining lymph nodes obtained on day 7 in the presence of recombinant human GPI for 24 hours. Since the majority of cells that produce IL-17 are of the $CD4^{high}$ population, we analyzed IFNγ and IL-4 production gating on the $CD4^{high}$ population. We found that the majority of cells that produced cytokines such as IL-17 expressed $CD4^{high}$ cells (data not shown).

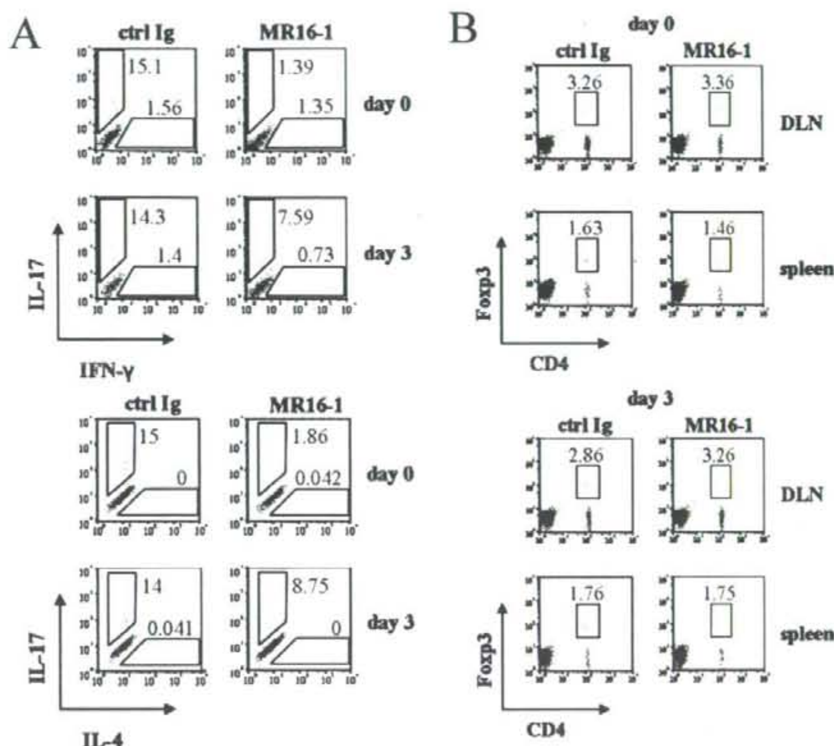

Figure 5. Inhibition of the differentiation of draining lymph node cells into Th17 cells by treatment with anti–interleukin-6 receptor (anti–IL-6R) monoclonal antibody (mAb). Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti–IL-6R mAb MR16-1 or with rat IgG (control) on day 0 or day 3 after GPI immunization. **A,** Cells from draining lymph nodes obtained on day 7 were cultured in the presence of 100 μg of recombinant human GPI. GolgiStop was added during the last 2 hours of each culture, and flow cytometric analysis of IL-17 and either interferon-γ (IFNγ) or IL-4 was performed, gating on $CD4^{high}$ cells. Results are representative of 3 independent experiments (n = 2 mice per experiment). **B,** Cells from draining lymph nodes (DLN) and spleen obtained on day 7 were stained with forkhead box P3 (FoxP3), and flow cytometric analysis of FoxP3 and CD4 was performed. Results are representative of 3 independent experiments (n = 2 mice per experiment). Values shown in the dot plots are the percentages of positive cells in the compartment.

We performed intracellular cytokine staining for IL-17, IFNγ, and IL-4 without nonspecific stimulants, such as phorbol myristate acetate or ionomycin, to assess physiologic cytokine production. Injection of MR16-1 on day 0 resulted in a significant decrease in IL-17 production by $CD4^{high}$ T cells (1.39%) as compared with injection of control Ig (15.1%) ($P < 0.05$), and there was a similar tendency with injection on day 3 (7.59% versus 14.3%; $P < 0.05$) (Figure 5A). IFNγ production was not significantly increased by MR16-1 injection on day 0 (1.35% versus 1.56%) or on day 3 (0.73% versus 1.4%) (Figure 5A). There was no difference in IL-4 production (Figure 5A).

We also used intracellular staining methods to examine FoxP3 expression after treatment with MR16-1. FoxP3-positive CD4+ T cells were essentially unaffected by MR16-1 treatment on day 0 or day 3 (Figure 5B). These data indicate that MR16-1 prevents the differentiation of naive T cells to Th17 cells, but does not affect other cell lineages.

Inhibition of the production of antigen-specific antibodies and antigen-specific proliferation of CD4+ T cells by anti–IL-6R mAb. We next explored whether MR16-1 affects the production of anti-GPI antibodies. Treatment of mice with MR16-1 resulted in significant

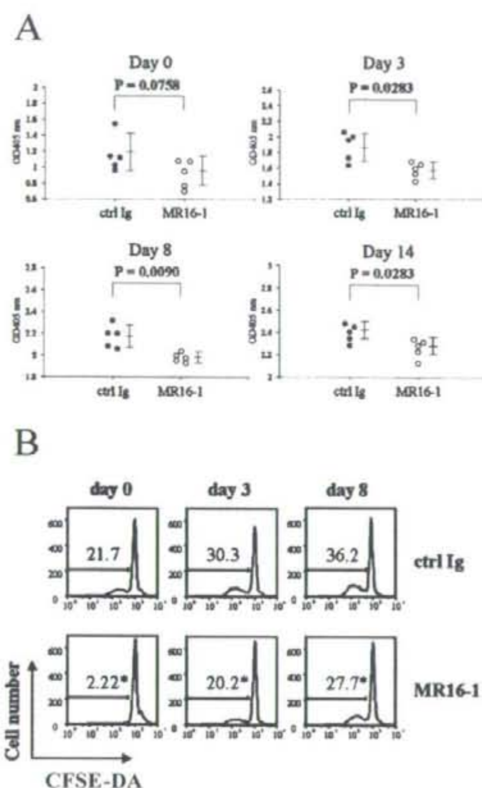

Figure 6. Inhibition of the production of anti-glucose-6-phosphate isomerase (anti-GPI) antibodies and the proliferation of CD4+ T cells by treatment with anti-interleukin-6 receptor (anti-IL-6R) monoclonal antibody (mAb). **A,** Mice were immunized with glucose-6-phosphate isomerase (GPI) and injected intraperitoneally with 2 mg of the anti-IL-6R mAb MR16-1 or rat IgG (control) on day 0, 3, or 8, or with 4 mg of mAb MR16-1 or control Ig on day 14 after GPI immunization. Sera were obtained on day 28, and the titers of anti-GPI antibodies were analyzed by enzyme-linked immunosorbent assay. Each symbol represents a single mouse. Bars show the mean ± SD optical density (OD) at 405 nm. *P* values were determined by Mann-Whitney U test. **B,** Mice were injected intraperitoneally with 2 mg of mAb MR16-1 or rat IgG (control) on day 0, 3, or 8 after immunization. Cells from draining lymph nodes (DLN) obtained on day 10 were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE-DA), cultured with 25 μg of recombinant human GPI for 60 hours, and cell proliferation was analyzed by flow cytometry. Values are the percentage of proliferating cells. Results are representative of 3 independent experiments (n = 2 mice per experiment). * = $P < 0.05$ versus controls, by Mann-Whitney U test.

reductions of anti-GPI antibody titers on days 3, 8, and 14 ($P < 0.0283$, $P < 0.0090$, $P < 0.0283$, respectively) as compared with mice injected with control Ig (Figure 6A). These results emphasize the inhibitory effects of MR16-1 on the production of anti-GPI antibodies irrespective of the phase of arthritis when treatment is administered.

In addition to antibody production, IL-6 is involved in T cell proliferation (10). Therefore, we explored whether MR16-1 affects antigen-specific proliferation of CD4+ T cells. Mice were injected intraperitoneally with 2 mg of MR16-1 on day 0, 3, or 8 after immunization of recombinant human GPI. Popliteal lymph nodes were harvested on day 10, cells stained with CFSE-DA were cultured with recombinant human GPI for 60 hours, and cell proliferation was analyzed by flow cytometry. As expected, CD4+ T cells treated with MR16-1 in vivo proliferated significantly less than those treated with control IgG (21.7% versus 2.22% on day 0, 30.3% versus 20.2% on day 3, 36.2% versus 27.7% on day 8) ($P < 0.05$) (Figure 6B). These data suggest that MR16-1 inhibits antigen-specific proliferation of CD4+ T cells, leading to a reduced population of antigen-specific CD4+ T cells in draining lymph nodes.

DISCUSSION

GPI, a ubiquitous glycolytic enzyme, is a new candidate autoantigen in the initiation of autoimmune arthritis (2). The arthritogenicity of GPI was first described in T cell receptor–transgenic K/BxN mice (2). In K/BxN mice, CD4+ T cells (especially KRN T cells) were required for the development of arthritis, although they appeared to be dispensable after the mice produced arthritogenic autoantibodies to GPI (11). While the K/BxN mouse is a striking model of spontaneous arthritis, the effectiveness of biologic agents used to treat the arthritis is limited. Tumor necrosis factor α (TNFα) blockade had no effect on the development and progression of arthritis in K/BxN mice (12), and serum transfer from arthritic K/BxN mice into IL-6–deficient mice did not affect the course of arthritis as compared with that in wild-type mice (13).

GPI-induced arthritis is produced by immunization of genetically unaltered DBA/1 mice with GPI. In GPI-induced arthritis, administration of either anti-TNFα mAb or CTLA-4Ig after the onset of arthritis shows a significant amelioration of the arthritis (Matsumoto I, et al: unpublished observations). This model is different from the CIA model in a T cell–dependent manner. In GPI-induced arthritis, administration of anti-CD4 mAb around the time of immunization was shown to completely prevent arthritis, and more noteworthy, administration of anti-CD4 mAb on day 11 and

on day 14 was shown to induce rapid remission of the arthritis (3). These findings highlight the importance of CD4+ T cells in the induction phase and the effector phase of GPI-induced arthritis. In contrast, in CIA, CD4+ T cells are indispensable only until the B cells produce autoantibodies, since anti-CD4 mAb treatment is ineffective when administered after anti-GPI antibodies have appeared (4,5). Judging from these findings, GPI-induced arthritis is considered a useful murine model for analyzing the role of CD4+ T cells in the effector phase of the arthritis.

Several studies have examined the roles of Th17 cells, a distinct lineage of CD4+ effector T cells, in various arthritis models (14–17). CIA was shown to be partially suppressed in IL-17–deficient mice (16), whereas it was exacerbated in IFNγ-deficient mice or IFNγ receptor–deficient mice (18–20). Despite the similarity of Th1 and Th17, the efficacy of anti–IL-17 mAb treatment in GPI-induced arthritis was more marked than in CIA. In the CIA model, administration of anti–IL-17 antibodies during the induction phase of arthritis was shown to only partially inhibit the development of arthritis (21). This difference between GPI-induced arthritis and CIA may reflect a more substantial contribution from cells of the Th17 lineage. In our experiments, the production of IL-17 on day 7 was higher than that on day 14, and for IFNγ, the inverse was true, with lower production of IFNγ on day 7 than on day 14. It has been reported that IFNγ suppresses the production of IL-17 by inhibiting IL-23R (22,23); therefore, a cytokine milieu in which little IFNγ is present during the induction phase of arthritis might boost the production of a large amount of IL-17, and conversely, a milieu in which large amounts of IFNγ are present during the effector phase of arthritis might inhibit the production of IL-17. This might also account for the fact that spontaneous remission began on day 14 in mice with GPI-induced arthritis.

Recent in vitro studies indicated that IL-6 is an essential inducer of the differentiation of Th17 cells (8,9). In our experiments, blockade of IL-6R on days 0 and 3 markedly suppressed the production of IL-17 and the proliferation of GPI-specific CD4+ T cells in vivo. In contrast, GPI-induced arthritis was suppressed by MR16-1 administration on days 0 and 3, and when MR16-1 was administered on day 8, the arthritis was ameliorated, which likely occurred through inhibition of T cell proliferation and autoantibody production, rather than blockade of Th17 differentiation. MR16-1 also suppressed autoantibody production most significantly when administered on day 8. This effect was probably mediated through a direct action on B cells (24,25) because the production of anti-GPI antibodies was highest around day 8 (Matsumoto I, et al: unpublished observations).

In the present experiments, the dose of MR16-1 we administered was 20–40 times higher than the dose of the anti–IL-17 mAb. MR16-1 is a mAb against murine IL-6R, and for there to be sufficient inhibition of the biologic activity of IL-6 in vivo, soluble IL-6 receptors, which are consistently present in the blood, would have to be blocked. Therefore, a relatively high dose would be needed compared with the titer of antibodies to the cytokine itself. This idea is supported by our unpublished data (Matsumoto I, et al: unpublished observations) showing that MR16-1 inhibited the biologic activity of IL-6 in vitro when administered at the same concentration as other antibodies to the cytokine itself.

Are these scenarios applicable to RA in humans? The therapeutic effects of a humanized anti–IL-6Rα antibody (tocilizumab) on RA have recently been reported (26,27). Patients with severe forms of RA retained high titers of anti-GPI antibodies (7,28,29), although a few control subjects also had these antibodies. In anti-GPI antibody–positive individuals, GPI-reactive CD4+ T cells, especially Th1-type cells, were specifically detected in peripheral blood mononuclear cells from RA patients who shared either the HLA–DR*0405 or *0901 haplotype (30). What about mice with GPI-induced arthritis? High titers of anti-GPI antibodies have been found to be produced by arthritis-resistant C57BL/6 mice as well, although their T cells exhibited weak GPI responses (ref. 3 and Matsumoto I, et al: unpublished observations) as compared with the responses of T cells from arthritis-susceptible DBA/1 mice.

These findings indicate that anti-GPI antibodies are not sufficient for the induction of arthritis; it is probable that the support of antigen-specific T cell activation is indispensable. In this regard, GPI-induced arthritis seems to be a useful model for analyzing the pathology of RA in humans. In addition, it has been shown that TNF antagonists clearly inhibit the progression of GPI-induced arthritis (3), even after clinical onset of disease (Matsumoto I, et al: unpublished observations). In our present study, administration of anti–IL-17 mAb or MR16-1 on day 14 (late effector phase) was not able to ameliorate GPI-induced arthritis. However, both the IL-6/IL-17 axis and TNFα might play a crucial role in established RA, since both tocilizumab and TNF antagonists have shown marked therapeutic efficacy in humans with established RA (26,27,31–34), although administration of MR16-1 or anti-TNF mAb has shown no effect or only a weak effect on fully established CIA in mouse models (35,36). Further ana-

lysis is necessary to determine whether GPI-reactive Th17 cells exist in the peripheral blood or joints of patients with RA who have anti-GPI antibodies.

In conclusion, the findings of our study highlight the importance of the IL-6/IL-17 axis in GPI-induced arthritis, a murine model of RA. Blockade of IL-6R might be a useful therapeutic strategy in Th17-mediated arthritis. Since a humanized anti–IL-6R mAb has been shown to have an excellent therapeutic effect on RA, further studies are needed to confirm that the IL-6/IL-17 axis is also crucial in RA.

AUTHOR CONTRIBUTIONS

Dr. Matsumoto had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Iwanami, Matsumoto, Sumida.
Acquisition of data. Iwanami, Matsumoto, Tanaka-Watanabe, Inoue, Mihara, Ohsugi, Mamura, Goto, Ito, Tsutsumi, Kishimoto, Sumida.
Analysis and interpretation of data. Iwanami, Matsumoto, Sumida.
Manuscript preparation. Iwanami, Matsumoto, Sumida.
Statistical analysis. Iwanami, Matsumoto.

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