Table 6 Suvival rates at 6, 12, and 24 months

	6 months		12 months		24 months	
	Number of follow-ups	%	Number of follow-ups	%	Number of follow-ups	%
Primary						
Anti-GBM antibody-associated crescentic GN						
Group A	39	83.5	29	80.5	28	80.5
Group B	18	88.9	15	88.9	15	82.5
Group C	22	81.1	14	81.1	14	81.1
RLV[a]						
Group A	342	81.1	254	76.2	230	72.4
Group B	147	84.5	113	81.5	103	75.6
Group C	240	89.7	165	87.4	134	82.1
Systemic disease-associated						
Goodpasture's syndrome						
Group A	14	71.4	9	63.5	6	54.4
Group B	5	60.0	3	60.0	3	60.0
Group C	8	62.5	5	37.5	1	37.5
Systemic lupus erythematosus						
Group A	50	85.9	42	85.9	42	83.8
Group B	5	60.0	3	60.0	3	60.0
Group C	10	90.0	9	90.0	8	78.8
Wegener's granulomatosis						
Group A	23	78.3	18	78.3	18	78.3
Group B	9	66.7	6	66.7	5	53.3
Group C	13	67.7	8	67.7	6	67.7
MPA[a]						
Group A	157	68.4	102	65.7	90	61.2
Group B	57	77.9	39	77.9	37	73.6
Group C	126	82.6	81	79.3	57	73.3
Total[a,b]						
Group A	883	79.2	643	75.5	586	72.0
Group B	321	80.1	228	78.3	211	72.8
Group C	556	86.1	365	82.8	281	77.7

GN glomerulonephritis

[a] $p < 0.05$ between Groups A and C

[b] $p < 0.05$ between Groups B and C

prednisolone (0.6–0.8 mg/kg/day) for the initial treatment of MPO-ANCA-positive MPA or RLV with or without intravenous pulse methyl prednisolone treatment [7]. A significant reduction of the mean serum creatinine level at presentation among RPGN patients and a significant reduction of oral prednisolone dose were observed in patients with RLV and MPA in Groups B and C. Finally, the survival rates of RLV and MPA patients have recently shown a significant improvement. Furthermore, even with mild immunosuppressive treatment in recent years, the renal survival of these patients has also significantly improved. The main reasons for such an improvement in renal survival are early referral to nephrologists and early treatment initiation in those patients. After announcing the results of our survey, we believe that recognition of RPGN in general practice has improved.

Based on the prognostic analysis of these patients, we created an RPGN patient clinical grading system (Table 8) based on scoring the age, renal function, CRP level, and involvement of lung disease at the start of immunosuppressive treatment using data on Group A patients. This clinical grade category also predicts patient prognoses for Groups B and C quite well. The Birmingham Vasculitis Activity Score (BVAS) is useful tool for evaluating disease activity and predicting the survival of patients with systemic vasculitis [25]. However, when BVAS is used for RPGN patients, the BVAS renal score is always maximized, since RPGN involves proteinuria, hematuria and renal dysfunction by definition. Consequently, it is difficult to predict a prognosis for renal vasculitis patients or RPGN patients with BVAS.

Table 7 Renal survival rates at 6, 12, and 24 months

	6 months		12 months		24 months	
	Number of follow-ups	%	Number of follow-ups	%	Number of follow-ups	%
Primary						
Anti-GBM antibody-associated crescentic GN						
Group A	31	44.7	12	44.7	11	44.7
Group B	17	58.8	8	58.8	8	51.5
Group C	18	48.5	8	48.8	8	41.6
RLV[b]						
Group A	320	72.4	189	70.9	175	66.7
Group B	137	87.9	99	85.2	87	79.8
Group C	233	83.1	136	81.0	134	75.2
Systemic disease-associated						
Goodpasture's syndrome						
Group A	8	60.0	3	40.0	2	40.0
Group B	2	0.0	0	0.0	0	0.0
Group C	5	20.0	1	0.0	0	0.0
Systemic lupus erythematosus						
Group A	47	89.1	39	86.8	38	84.4
Group B	3	66.7	2	66.7	1	66.7
Group C	11	80.8	8	80.8	8	80.8
Wegener's granulomatosis						
Group A	20	85.0	15	85.0	15	85.0
Group B	7	85.7	5	85.7	4	85.7
Group C	12	83.3	7	83.3	5	83.3
MPA[a,b]						
Group A	144	74.0	81	72.1	72	69.9
Group B	53	88.0	32	85.1	29	85.1
Group C	118	89.0	71	89.0	50	87.0
Total[a,b]						
Group A	812	73.3	483	71.9	442	68.7
Group B	288	81.3	183	78.6	163	75.4
Group C	521	81.8	296	80.5	226	76.7

GN glomerulonephritis

[a] $p < 0.05$ between Groups A and B

[b] $p < 0.05$ between Groups A and C

Although the benefits of an early start to treatment and a reduced dose of oral prednisolone were recognized, the most frequent cause of death was infectious complications in Group C. The main reason for this high frequency of infectious complications in Group C was the shorter duration of observations in Group C. During the late follow-up period, the dose of immunosuppressant was further reduced, and the possibility of opportunistic infection was reduced. Several previous studies have suggested that prophylactic treatment with trimethoprim/sulfamethoxazole significantly reduces the occurrence of opportunistic infections [26]. Recent improvements in prognosis during initial treatment may relate to prophylactic treatment with trimethoprim/sulfamethoxazole in our subjects. Further studies are needed to clarify this point.

For the treatment of active renal vasculitis, to avoid relapses, and to improve long-term renal outcomes, treatment with cyclophosphamide is one choice; however, prolonged immunosuppression with a safer immunosuppressive agent, such as azathioprine [27], mycophenolate mofetil [28], or mizoribine [29], should also be considered.

Furthermore, the prognoses for anti-GBM antibody-associated RPGN, Goodpasture's syndrome and Wegener's granulomatosis are still poor in Japan. An early diagnosis system and effective treatment methods should be established as soon as possible for Japanese patients with these diseases. However, the average age at presentation of the patients with those diseases increased by ten years during our study. The higher age at

Table 8 Clinical grading for predicting RPGN patient prognosis

Clinical score	Serum creatinine (mg/dl)	Age (years old)	Lung involvement	Serum CRP (mg/dl)
0	<3	≤59	Negative	<2.6
1	3–6	60–69		2.6–10.0
2	≥6	≥70	Positive	>10
3	Dialysis			

Clinical grade	Total score
I	0–2
II	3–5
III	6–7
IV	8–9

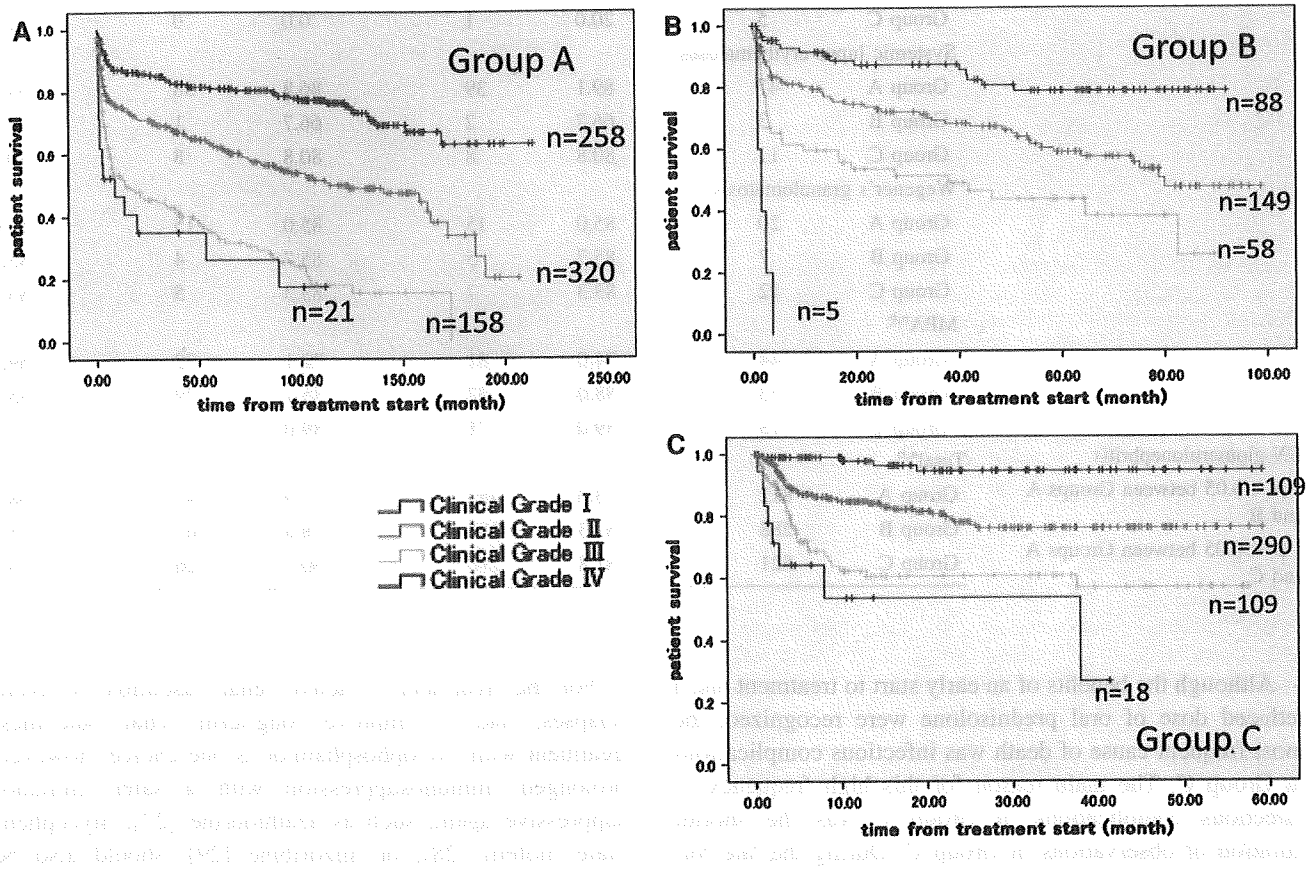

Fig. 2 Clinical grading system for predicting patient prognosis. The RPGN patient clinical grading system for predicting RPGN patient prognosis was produced based on Group A patients (**a**). However, this clinical grading system also fitted recent RPGN cases very well (**b**, **c**)

presentation may relate to the poor prognosis of those patients.

In summary, the prevalence of RPGN patients and primary renal disease were surveyed and compared among Group A (retrospective data), Group B (prospective data during the preparation of the clinical guidelines), and Group C (prospective data after the Japanese RPGN clinical guidelines were published). Following the recommendations of the clinical guidelines, both early referrals to nephrologists and mild immunosuppressive treatments were observed. These changes resulted in a significant improvement in the outcomes of Japanese patients with RPGN.

Table 9 Institutions that provided data for this survey

Department of Nephrology, Tsukuba Central Hospital	Department of Nephrology, National Tochigi Hospital	Internal Medicine I, Chiba University Hospital	Internal Medicine I, Hamamatsu Medical University Hospital	Department of Pediatrics, Shinshu University Hospital	Internal Medicine II, School of Medicine, Toho University
Department of Nephrology, Tsuruoka Kyoritsu Hospital	Department of Pediatric, Shimoshizu National Hospital	Department of Pediatrics, Chiba University Hospital	Department of Pediatrics, Hamamatsu Medical University Hospital	Department of Pediatrics, Niigata Prefectural Yoshida Hospital	Department of Pediatrics, School of Medicine, Toho University
Department of Nephrology and Rheumatology, Toyota Memorial Hospital	Kagawa Children's Hospital	Department of Nephrology, Kawasaki Medical University	Department of Pediatrics, Hamamatsu Red Cross Hospital	Department of Internal Medicine, Niigata Prefectural Central Hospital	Department of Nephrology, Toho University Omori Hospital
Nephrology and Dialysis Unit, MisatoKenwa Hospital and Clinic	Department of Pediatric, National Mie Hospital	Department of Pediatrics, Kawasaki Medical University	Department of Nephrology, Hamamatsu Rosai Hospital	Department of Pediatrics, Niigata City Hospital	Blood Purification Center, Tohoku University Hospital
Internal Medicine I, Aichi Medical University Hospital	Department of Pediatric, National Niigata Hospital	Department of Pediatrics, Kawasaki Kyodo Hospital	Internal Medicine II, Toyama Medical University Hospital	Blood Purification Center, Niigata University Medical and Dental Hospital	Department of Internal Medicine, Fujita Health University
Department of Pediatrics, Aichi Medical University Hospital	Department of Pediatric, National Nishisapporo Hospital	Department of Pediatrics, Kawasaki City Hospital	Department of Pediatrics, Toyama Medical University Hospital	Department of Nephrology, Niigata University Hospital	Department of Pediatrics, Department of Pediatrics, Fujita Health University
Pediatric Department, Ehime University Medical School Hospital	Department of Pediatric, National Nishitaga Hospital	Department of Pediatrics, Kurashiki Central Hospital	Department of Internal Medicine, Toyama Prefectural Central Hospital	Department of Invernal Medicine, Niigata Minami Hospital	Department of Invernal Medicine, Tokushima Prefectural Central University
Internal Medicine I, Asahikawa Medical College Hospital	Department of Pediatric, National Chiba-Higashi Hospital	Department of Internal Medicine, Sagamihara Kyodo Hospital	Internal Medicine, Toyama Red Cross Hospital	Department of Pediatric Nephrology, Nippon Steel Yawata Memorial Hospital	Department of Pediatrics, Tokushima University Hospital
Department of Pediatric, Asahikawa Medical College Hospital	Department of Pediatric, National Chubu Hospital	Department of Nephrology, Inoue Hospital	Department of Internal Medicine, Toyama Prefectural Central Hospital	Department of Invernal Medicine, Shinnittetsu Hachiman Memorial Hospital	Department of Internal Medicine, Tochigi Saiseikai Utsunomiya Hospital
Department of Nephrology, Anjo Kose Hospital	Department of Nephrology, Kurobe City Hospital	Internal Medicine I, Osaka Medical University Hospital	Department of Pediatrics, Fukui Medical University Hospital	Kobe University School of Medicine Faculty of Health Sciences	Internal Medicine II, Nara Medical University Hospital

Table 9 continued

Department of Urology, Kyorin University School of Medicine	Department of Internal Medicine, Saga University	Department of Pediatrics, Osaka Medical University Hospital	Department of Internal Medicine, Fukui Red Cross Hospital	Department of Nephrology, Kanagawa Prefectural Children's Medical Center	Department of Pediatrics, Nara Medical University Hospital
Internal Medicine I, Kyorin University School of Medicine	Department of Pediatric, Saga University	Internal Medicine I, Osaka City University Hospital	Department of Nephrology, Fukui Red Cross Hospital	Department of Nephrology, Kandatsu Hospital	Department of Nephrology, Minami Ichijo Hospital
Department of Pediatric, Kyorin University School of Medicine	Department of Internal Medicine, Kosei-kan, Saga Prefectural Hospital	Internal Medicine II, Osaka City University Hospital	Internal Medicine IV, Fukuoka University Hospital	Department of Nephrology, Mito Saiseikai General Hospital	Department of Nephrology, Nikko Memorial Hospital
Department of Clinical Genetics, Faculty of Health Sciences, School of Medicine, Kyorin University	Department of Internal Medicine, Saku Sogo Hospital	Department of Pediatrics, Osaka City University Hospital	Internal Medicine I, Fukuoka University Hospital	Department of Internal Medicine, Mito Central Hospital	Department of Nephrology, Hidaka Hospital
Department of Pediatric, Isezaki Municipal Hospital	Department of Internal Medicine, Sano Kosei Sogo Hospital	Osaka Red Cross Hospital	Department of Nephrology, Fukuoka University Hospital	Department of Nephrology, Mizushima Kyodo Hospital	Department of Pediatrics, Red Cross Medical Center
Department of Pediatric, Ibaraki Children's Hospital	Department of Internal Medicine, Saiseikai Yokohama-shi Nambu Hospital	Internal Medicine I, Osaka University Hospital	Department of Pediatrics, Fukuoka University Tsukushi Hospital	Internal Medicine I, St. Marianna School of Medicine Hospital	Internal Medicine I, Nippon Medical School Hospital
Department of Nephrology, Ibraki Prefectural Central Hospital	Department of Nephrology, Saiseikai Shimonoseki Sogo Hospital	Department of Pediatrics, Osaka University Hospital	Internal Medicine IV, Fukushima Prefectural Medical University Hospital	Yokohama Seibu Hospital, St. Marianna School of Medicine	Internal Medicine II, Nippon Medical School Hospital
Department of Nephrology, Ibaraki Seinan Medical Center Hospital	Department of Pediatrics, Saiseikai Kurihashi Hospital	NTT West Osaka Hospital	Department of Pediatrics, Fukushima Prefectural Medical University Hospital	Department of Pediatrics, St. Marianna School of Medicine Hospital	Department of Pediatrics, Nippon Medical School Hospital
Department of Nephrology, Utsunomiya Socialinsurance Hospital	Department of Nephrology, Saiseikai Nakatsu Hospital	Department of Nephrology, Osaka Prefectural Hospital	Internal Medicine V, The Hospital of Hyogo College of Medicine	Department of Pediatrics, Seirei Hamamatsu Hospital	Internal Medicine II, Nippon Medical School Hospital
Department of Internal Medicine, Urasoe Sogo Hospital	Department of Nephrology, Saitama Medical University Hospital	Department of Nephrology, Kumamoto Chuo Hospital	Department of Pediatrics, The Hospital of Hyogo College of Medicine	Department of Pediatrics, St. Lukes International Hospital	Department of Pediatrics, Nippon Medical School Chiba Hokusoh Hospital
Department of Internal Medicine, Yokosuka Kyosai Hospital	Department of Pediatric, Saitama Medical University	Department of Internal Medicine, Oita Medical University Hospital	Department of Pediatric Nephrology, The Hospital of Hyogo College of Medicine	Department of Pediatrics, Seirei Sakura Citizen Hospital	Department of Nephrology, Nihon Red Cross Medical Center

Table 9 continued

Department of Internal Medicine, Yokosuka City Hospital	Department of Internal Medicine IV, Saitama Medical Center, Saitama Medical University	Department of Urology, Oita Medical University Hospital	Hyogo Prefectural Children's Hospital	Department of Invernal Medicine, Seirei Sakura Citizen Hospital	Internal Medicine II, Nihon University Hospital
Internal Medicine II, Yokohama City University Hospital	Department of Nephrology, Saitama Children's Medical Center	Department of Pediatrics, Oita Medical University Hospital	Department of Internal Medicine, Hyogo Prefectural Amagasaki Hospital	Department of Urinology, Seirei Sakura Citizen Hospital	Department of Pediatrics, Nihon University Surugadai Hospital
Department of Pediatric, Yokohama City University Medical Center	Department of Internal Medicine II, Sapporo Medical University Hospital	Department of Pediatrics, Yamato City Hospital	Department of Internal Medicine, Toyohashi City Hospital	Department of Invernal Medicine, National Cardiovascular Center	Department of Nephrology, Mito General Hospital
Department of Urology, Yokohama Minami Kyosai Hospital	Department of Pediatric, Sapporo Medical University Hospital	Takeshita Hospital	Internal Medicine II, National Defence Medical College	National Health Center for Children's Health and Development	Department of Nephrology, Hitachi General Hospital
Internal Medicine III, Okayama University Hospital	Department of Internal Medicine, Mitsui Memorial Hospital	Department of Nephrology, Tsukuba Gakuen Hospital	Department of Nephrology, Hokkaido Kinrosha Iryo Kyokai Chuo Hospital	Department of Internal Medicine, Sendai Red Cross Hospital	Department of Pediatrics, Hakodate Goryokaku Hospital
Department of Pediatrics, Okayama University Hospital	Department of Pediatrics, Mitsui Memorial Hospital	Department of Metabolism, Nakadori Sogo Hospital	Department of Pediatrics, Hokkaido University Hospital	Department of Nephrology, Senboku Kumiai Hospital	Department of Nephrology, Hashiro General Hospital
Department of Pediatrics, Okinawa Prefectural Chubu Hospital	Department of Internal Medicine I, Mie University Hospital	Department of Nephrology, Nakagami Hospital	Internal Medicine II, Hokkaido University Hospital	Department of Internal Medicine, Nishi Clinic	Internal Medicine II, Kochi Medical School
Department of Nephrology, Okinawa Prefectural Chubu Hospital	Department of Pediatric, Mie University Hospital	Department of Internal Medicine, Chubu Rosai Hospital	Department of Internal Medicine, Kitamatsu Central Hospital	Department of Nephrology, Nishikobe Medical Center	Department of Pediatrics, Kochi Medical School
Department of Internal Medicine, Okinawa Prefectural Chubu Hospital	Department of Internal Medicine, Saiseikan, Yamagata City Hospital	Department of Internal Medicine, Nakano Sogo Hospital	Department of Nephrology, Hokushin Sogo Hospital	Department of Nephrology, Shizuoka Children's Hospital	Department of Nephrology, Asahi Chuo Hospital
Internal Medicine II, Kansai Medical University	Department of Urology, Yamagata University Hospital	Internal Medicine II, Ngasaki University Hospital	Department of Nephrology, Kitazato University Hospital	Department of Nephrology, Shizuoka Saiseikai General Hospital	Department of Nephrology, Kasumigaura Medical Center
Department of Pediatrics, Kansai Medical University	Department of Pediatric, Yamaguchi University Hospital	Internal Medicine II, Nagasaki University Hospital	Department of Pediatrics, Kitazato University Hospital	Department of Nephrology, Shizuoka City Hospital	Department of Bacteriology, National Institute of Infectious Diseases

Table 9 continued

Department of Nephrology, Kansai Rosai Hospital	Department of Nephrology, Yamamoto Kumiai Sogo Hospital	Department of Pediatrics, Nagasaki University Hospital	Department of Nephrology, Hokuriku Central Hospital	Department of Nephrology, Sendai Shakai Hoken Hospital	Department of Internal Medicine I, Kanazawa Hospital
Kidney Dialysis Center, Kanto Hospital	Department of Pediatrics, Yamanashi Medical University Hospital	Department of Internal Medicine, Nagano Red Cross Hospital	Department of Nephrology, Horinouchi Hospital	Department of Nephrology, Chiba Children's Hospital	Department of Nephrology, Kure Medical Cemter,
Department of Pediatrics, Iwate Medical University	Cardiovascular Internal Medicine at Yamanashi Prefecural Central Hospital	Department of Internal Medicine II, Tottori University Hospital	Department of Nephrology, Honjo Daiichi Hospital	Department of Pediatrics, Medical Center East, Tokyo Women's Medical University	Department of Nephrology, Takasaki Hospital
Department of Pediatrics, Iwate Prefectural Central Hospital	Department of Internal Medicine, Yamanashi Red Cross Hospital	Department of Pediatrics, Tottori University Hospital	Department of Nephrology, Iizuka Hospital	Department of Internal Medicine, Tokyo Sembai Hospital	Department of Pediatrics, International Medical Center of Japan
Department of Pediatrics, Gifu University Hospital	Department of Nephrology, University of Occupational and Environmental Health, Japan Hospital	Department of Nephrology, Teikyo University Hospital	Department of Pediatrics, Mino City Hospital	Department of Nephrology, School of Medicine and Faculty of Medicine, The University of Tokyo	Department of Nephrology, Department of Pediatrics
Internal Medicine III, Kurume University Hospital	Department of Pediatric, University of Occupational and Environmental Health, Japan Hospital	Department of Pediatrics, Teikyo University Hospital	Department of Preventive Medicine, Nagoya University Hospital	Internal Medicine II, School of Medicine and Faculty of Medicine, The University of Tokyo	Internal Medicine II, Hirosaki University Hospital
Department of Pediatrics, Kurume University Hospital	Department of Internal Medicine I, University of Occupational and Environmental Health, Japan Hospital	Department of Internal Medicine III, Teikyo University Hospital	Internal Medicine III, Nagoya University Hospital	Department of Pediatrics, School of Medicine and Faculty of Medicine, The University of Tokyo	Department of Pediatrics, Hirosaki University Hospital
Internal Medicine I, Miyazaki Medical University Hospital	Department of Internal Medicine II, University of Occupational and Environmental Health, Japan Hospital	Department of Pediatrics, Tenri Yorozu Sodanjo Hospital	Department of Internal Medicine, Nagoya University Daiko Medical Center	Department of Urology, Tokyo University Branch Hospital	Department of Pediatrics, Faculty of Medicine, Kagawa University
Department of Pediatrics, Miyazaki Medical University Hospital	Department of Nephrology, Sapporo City Hospital	Department of Nephrology, Tenri Yorozu Sodanjo Hospital	Department of Pediatrics, Nagoya Daiichi Red Cross Hospital	Department of Nephrology, Tokyo Teishin Hospital	Department of Invernal Medicine, Faculty of Medicine, Kagawa University
Department of Pediatrics, Kyoto City Hospital	Department of Internal Medicine IV, Akita City Dogo Hospital	Department of Nephrology, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital	Department of Nephrology, Nagoya Daiichi Red Cross Hospital	Department of Nephrology, Tokyo Saiseikai Chuo Hospital	Department of Nephrology, Kagawa Prefectural Chuo Hospital

Table 9 continued

Cardiovascular Science and Medicine, School of Medicine, Kyoto University	Department of Pediatric, Itoigawa Sogo Hospital	Department of Nephrology Tokyo Metropolitan Kiyose Children's Hospital	Department of Nephrology, Nagoya Daini Red Cross Hospital	Department of Nephrology, Tokyo Fuchu Hospital	Department of Internal Medicine, Takaoka City Hospital
Department of Pediatrics, Onomichi City Hospital	Department of Internal Medicine, Teraoka Memorial Hospital	Department of Pediatrics, Metropolitan Bokutoh Hospital	Department of Pediatrics, Nagoya Daini Red Cross Hospital	Department of Internal Medicine, Tokyo Rosai Hospital	Department of Urology, Takamatsu City Hospital
Department of Pediatrics, School of Medicine, Kyoto University	Department of Internal Medicine III, Shiga University of Medical Science	Department of Internal Medicine, Metropolitan Bokutoh Hospital	Department of Pediatrics, Tachikawa Sogo Hospital	Department of Nephrology, Tokyo Medical University Hachioji Medical Center	Department of Pediatrics, Showa University Hospital
Kyoto University Health Service	Department of Pediatric, Shiga University of Medical Science	Department of Internal Medicine IV, Shimane Medical University	Internal Medicine III, Ryukyu University Hospital	Department of Nephrology, Tokyo Police Hospital	Department of Internal Medicine, Showa University Fuigaoka Hospital
Internal Medicine II, University Hospital, Kyoto Prefectural University of Medicine	Department of Nephrology, Jichi Medical University	Department of Pediatrics, Shimane Medical University Hospital	Department of Pediatrics, Ryukyu University Hospital	Internal Medicine II, The Jikei University	Department of Nephrology, Showa University Hospital
Department of Pediatrics, University Hospital, Kyoto Prefectural University of Medicine	Department of Pediatric, Jichi Medical University	Department of Nephrology, Shimada Memorial Hospital	Internal Medicine III, Wakayama Prefectural Medical University Hospital	Department of Pediatrics, The Jikei University	Department of Nephrology, Kamitsuga General Hospital
Internal Medicine III, Kinki University Hospital	Department of Nephrology, Saitama Medical Center, Jichi Medical University	Department of Nephrology, Tokai University Hospital	Department of Pediatrics, Wakayama Prefectural Medical University Hospital	Department of General Medicine, The Jikei University Kashiwa Hospital	Shinrakuen Hospital
Department of Pediatrics, Kinki University Hospital	Department of Pediatric, Kagoshima City Hospital	Department of Pediatrics, Tokai University Hospital	Department of Nephrology, Komatsu City Hospital	Internal Medicine IV, Tokyo Women's Medical University Hospital	Department of Pediatrics, Juntendo University Hospital
Department of Nephrology, Kanazawa Medical University	Department of Internal Medicine II, Kagoshima University Hospital	Department of Internal Medicine VII, Tokai University Oiso Hospital	Department of Pediatrics I, Dokyo Medical Unviersity Hospital	Department of Urology Kidney Center, Tokyo Women's Medical Unviersity	Department of Internal Medicine III, Gumma University Hospital
Department of Pediatrics, Kanazawa Medical University	Department of Pediatrics, Shakaihoken Chukyo Hospital	Department of Internal Medicine II, Tokyo Medical and Dental University	Department of Nephrology, Tsukuba University Hospital	Department of Internal Medicine, School of Medicine, Keio University	Department of Nephrology, Nihon Red Cross Medical Center

Table 9 continued

Internal Medicine I, Kanazawa University Hospital	Toride Kyodo Hospital	Department of Nephrology, Tokyo Medical University	Internal Medicine III, Hiroshima Red Cross Hospital & Atomic-Bomb Survivors Hospital	Department of Pediatrics, School of Medicine, Keio University	Department of Nephrology, Juntendo University Hospital
Department of Pediatrics, Kanazawa University Hospital	Department of Nephrology, Akita Kumiai Sogo Hospital	Department of Pediatrics, Tokyo Medical University	Internal Medicine II, Hiroshima University Hospital	Department of Pediatrics, Gunma University Hospital	Department of Pediatrics, Kumamoto Central Hospital
Department of Blood Purification Therapy, Medical School of Kanazawa University	Department of Internal Medicine III, Akita University Hospital	Department of Nephrology, Tokyo Medical University Kasumigaura Hospital	Department of Pediatrics, Hiroshima University Hospital	Department of Nephrology, Toranomon Hospital	Department of Pediatrics, Onomichi City Hospital
Internal Medicine II, Kyushu University Hospital	Department of Pediatrics, Akita University Hospital	Department of Nephrology, Kensei Sogo Hospital	Department of Nephrology, Showa University Fujigaoka Hospital	Department of Pediatrics, Toranomon Hospital	
Department of Pediatrics, Kyushu University Hospital	Department of Nephrology, Akita Rosai Hospital	Department of Nephrology, Hara Urological Clinic	Department of Nephrology, Matsuyama Red Cross Hospital	Department of Pathology I, Shinshu University School of Medicine	
Internal Medicine III, Kumamoto University Hospital	Department of Pediatrics, Sumitomo Hospital	Department of Nephrology, Koga Hospital	Department of Pediatrics, Matsuyama Red Cross Hospital	Department of Nephrology, Shizuoka City Hospital	
Department of Pediatrics, Kumamoto University Hospital	Department of Pediatrics, Shigei Medical Research Center Hospital	Department of Nephrology, Showa Hospital	Internal Medicine II, Shinshu University Hospital	Department of Nephrology, Sendai Shakai Hoken Hospital	
		Department of Pediatric Nephrology, Osaka Medical Center and Research Institute for Maternal and Child Health			

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ORIGINAL ARTICLE

Analysis of T-cell receptor usage in myeloperoxidase–antineutrophil cytoplasmic antibody-associated renal vasculitis

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Abstract
Background Bacterial superantigens produced by *Staphylococcus aureus* may be associated with the onset of proteinase-3 antineutrophil cytoplasmic antibody (PR3-ANCA)-associated vasculitis, including Wegener's granulomatosis. We investigated T-cell subsets to assess the superantigens present in patients with myeloperoxidase–antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis.
Methods Peripheral-blood mononuclear cells (PBMC) obtained from 40 normal controls and ten patients with MPO-ANCA-associated vasculitis were stained with fluorescence-labeled monoclonal antibodies against T-cell markers, including 17 variable regions of T-cell receptor β-chains (TCR-Vβ) and were then analyzed using flow cytometry.
Results Among PBMCs, the percentage of $CD3^+$ cells from patients with MPO-ANCA-associated vasculitis was significantly lower than that from normal controls, but there were no differences between the two groups in the percentage of $CD19^+$ cells or $CD16^+$ cells. Although there were no differences regarding the overall percentage of $CD4^+$ cells between the two groups, the percentage of $CD4^+CD45RO^+$ cells in patients with MPO-ANCA-associated vasculitis was significantly higher than that in normal controls, and percentages of $CD4^+CD45RO^+HLA\text{-}DR^+$ and $CD4^+CD45RO^+CD62L^{low}$ cells in patients with MPO-ANCA-associated vasculitis were also significantly increased. There was no significant difference between the two groups in terms of the usage of the 17 different TCR-Vβ regions.
Conclusion There was no difference in bacterial superantigens between controls and MPO-ANCA-associated vasculitis patients because of the absence of specific usage of TCR-Vβ regions. Given the elevated levels of memory T cells, conventional antigens rather than superantigens may be associated with the pathogenesis of MPO-ANCA-associated vasculitis.

Keywords MPO-ANCA-associated vasculitis · Memory T cells · T-cell receptors

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Introduction

Microscopic polyangiitis (MPA), Wegener's granulomatosis (WG), and Churg–Strauss syndrome (CSS) form a group

of systemic immune-mediated diseases with a strong and highly specific association with antineutrophil cytoplasmic autoantibodies (ANCAs). ANCAs are circulating autoantibodies directed against different target antigens located in azurophilic granules of polymorphonuclear leucocytes and the peroxidase-positive lysosomes of monocytes. In MPA, the target antigen is mostly myeloperoxidase (MPO) and the type of immunofluorescence is perinuclear (p-ANCA). In WG, ANCAs are usually directed against proteinase 3 (PR3) and have a cytoplasmic type of immunofluorescence (c-ANCA). In the pathogenesis of ANCA-associated vasculitis, the interaction of ANCAs and neutrophils results in premature neutrophil activation, subsequent endothelial cell damage, and further leucocyte recruitment [1]. T cells play a crucial role in regulating immune responses, and alteration of T-cell responses may be associated with the pathogenesis of autoimmune diseases. In ANCA-associated vasculitis, T cells infiltrate vasculitic and granulomatous lesions [2, 3], activation markers on T cells are up-regulated [4, 5], and serum markers of T-cell activation, such as soluble forms of interleukin (IL)-2R, CD4, and CD8, are elevated [6, 7]. Furthermore, T cells proliferate in the presence of MPO and PR3 in vitro [8]. Therefore, failure to adequately control T-cell activity may result in ANCA-associated vasculitis.

In ANCA-associated vasculitis, respiratory tract or other infections frequently precede or accompany the initial symptoms. Moreover, treatment of proteinase-3 antineutrophil cytoplasmic antibody (PR3-ANCA)-associated vasculitis limited to the airways with the antibiotic cotrimoxazole often leads to the achievement of stable remission [9]. In WG patients, chronic nasal carriage of *Staphylococcus aureus* is approximately three times higher than that in healthy individuals [10]. Moreover, the risk for relapse of WG depends on the presence and type of superantigen, with toxic shock syndrome toxin 1 (TSST-1) associated with a higher risk [relative risk (RR) 13.3, 95% confidence interval (95% CI) 4.2–42.6) of relapse [11]. Thus, the association with PR3-ANCA-associated vasculitis and staphylococcal superantigens in local lesions, such as lymphoid organs, was evident. On the other hand, in MPO-ANCA-associated vasculitis, several substances, such as silica dust exposure [12], air pollution [13], and antithyroid drugs [14], were reported as triggers. Although there have been some case reports of MPO-ANCA-associated vasculitis following staphylococcal infection [15, 16], staphylococcal infections are not usually verified. Bacteriological examinations of blood, sputum, or urine are performed before the initial treatment for MPO-ANCA-associated vasculitis because of the patients' inflammation, but staphylococcal species are usually not detected. Reportedly, staphylococcal species are also not detected on bacteriological examinations of WG lesions but are detected in WG nasal carriages. Nasal carriages are pathogenic lesions of WG, but there is no obvious pathogenic lesion of MPA or MPO-ANCA-associated vasculitis. Even though it would be difficult to detect the bacteria themselves in MPO-ANCA-associated vasculitis, bacterial infections cannot be ruled out as a pathogenic source of MPO-ANCA-associated vasculitis. One method of evaluating the association with bacterial infection—in particular, bacterial superantigens—is to identify peripheral or local T-cell receptor (TCR) usages. T-cell expansions were present at a significantly higher rate in WG patients than in healthy individuals [17]. On the other hand, T-cell expansions were generally of small extent and did not appear simultaneously in CD4 and CD8 subsets [17]. Thus, the roles of staphylococcal superantigens in ANCA-associated vasculitis are controversial. Several researches have explored expansions of peripheral blood T-cell subsets expressing TCRs in connection with PR3-ANCA-associated vasculitis [17–19], but MPO-ANCA-associated vasculitis was not satisfactorily investigated. In this study, we investigated T-cell subsets, including a repertoire of TCRs, to assess superantigens in peripheral blood mononuclear cells (PBMCs) of patients with MPO-ANCA-associated renal vasculitis.

Materials and methods

Patients

We evaluated ten patients (six women and four men) with MPO-ANCA-associated renal vasculitis. Their mean age was 67.0 ± 3.9 (range 62–73) years. The diagnosis of MPO-ANCA-associated renal vasculitis was based on the characteristic clinical and histological features of microscopic polyangiitis as defined by the Chapel Hill Consensus Conference [20], clinical evidence of rapidly progressive glomerulonephritis, and a positive test for MPO-ANCA. Patients with other types of systemic small-vessel vasculitis, such as Henoch–Schönlein purpura, essential cryoglobulinemic vasculitis, drug-induced vasculitis, systemic lupus erythematosus, rheumatoid arthritis, or malignancy-associated vasculitis, and those with antiglomerular basement membrane disease, were excluded. Patients with PR3-ANCA-associated renal vasculitis, WG and CSS were also excluded. ANCA was examined by MPO-specific enzyme-linked immunosorbent assay (ELISA), and normal ranges were defined as values <10 ELISA units (EU). Only patients with biopsy-proven pauci-immune necrotizing and crescentic glomerulonephritis were accepted for participation.

All patients had proteinuria (mean 24-h excretion of urinary protein was 1.1 ± 1.1 g/day; range 0.2–3.9 g/day)

and microscopic hematuria. Mean serum creatinine level was 4.5 ± 3.7 mg/dl (range 0.7–11.6 mg/dl). All patients had anemia, as well (the mean hemoglobin concentration was 8.6 ± 1.5 g/dl; range 6.2–11.7 g/dl), and the mean white blood cell count was elevated to 11130 ± 5254/mm^3 (range 4000–18900/mm^3). Hypoalbuminemia was observed in all patients (2.7 ± 0.4 g/dl; range 2.0–3.3 g/dl). In most patients, serum C-reactive protein (CRP) levels were elevated (6.9 ± 6.3 mg/dl; range 0.1–19.8 mg/dl), but they were within the normal range in two patients. MPO-ANCA was detected in all patients, and its titers ranged from 27 to 923 EU. Renal biopsies were examined in all patients, and pauci-immune necrotizing and crescentic glomerulonephritis were observed in all patients. The mean percentage of crescent formation in obtained glomeruli was 69.0 ± 29.1% (range 15.4–100%). The main crescent forms were cellular in six patients and fibrocellular in four patients. Interstitial pneumonitis was revealed by chest X-ray examinations in four patients, but alveolar hemorrhage was observed in none. Lymphocyte subsets were analyzed in all ten patients and 40 normal volunteers. The healthy subjects had normal renal function without microscopic hematuria or proteinuria. These control subjects had not had any diseases and had not taken any drugs. No microscopic hematuria or proteinuria was detected in their samples in the one month prior to this study using dip-and-read sticks (Multistix®: Sankyo Co. Ltd., Tokyo, Japan). The study protocol was accepted by the ethics committee of our institution, and informed consent was obtained from all patients or their immediate family members, as well as from normal volunteers. This study also conformed to the provisions of the Declaration of Helsinki as revised in Edinburgh in 2000.

Flow cytometry

Heparinized venous blood was collected, and within a few hours, PBMCs were separated by density gradient centrifugation using a Ficoll-Paque density gradient (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice with ice-cold phosphate-buffered saline and pelleted at 200×g for 10 min. After harvesting and washing, PBMCs were stained with biotin, phycoerythrin (PE), or fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, anti-45RO, and anti-CD62L (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). To analyze the variable regions of the human TCR, PBMCs were also stained using the following T-cell receptor β-chain (TCR-Vβ)-specific monoclonal antibodies: Vβ5.1 (Vβ5c), Vβ5.2 + 5.3 (Vβ5a), Vβ 5.3 (Vβ5b), Vβ6.7 (Vβ6a), Vβ8 subfamily (Vβ8a), and Vβ12.1 (Vβ12a) from T Cell Diagnostics, Inc. (Cambridge, MA, USA) and Vβ2, Vβ3 (Vβ3a), Vβ11, Vβ13 (Vβ13a), Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ20, Vβ21.3, and Vβ22 from Pharmingen (San Diego, CA, USA). PBMCs stained with a biotinated antibody were incubated with a conjugate of streptoavidin-RED613 (Gibco BRL Grand Island, NY, USA). PBMCs were also stained with isotype monoclonal antibodies, such as biotinylated anti-mouse immunoglobulin (Ig)G antibody, PE-conjugated anti-mouse IgG antibody, and FITC-conjugated anti-mouse IgG antibody (Pharmingen) as negative controls.

Stained cells were analyzed via flow cytometry equipped with a 15-mW air-cooled 488-nm argon-ion laser (FACScan®: Becton-Dickinson Immunocytometry Systems) using CELL Quest software (Becton-Dickinson Immunocytometry Systems). Voltage and spectral compensation were initially set using single fluorescein (FITC alone, PE alone, or biotin-streptavidin-RED 613 alone)-conjugated antibody-stained cells. The overlapping fluorescent emissions of FITC, PE, and RED 613 were corrected by electronic compensation. Dead cells and monocytes were excluded by forward and side-scatter gating. Typically, 20,000 events were acquired depending on the antigen being studied in duplicate. As a negative control, parallel staining was performed with the fluorescein (FITC, PE, and biotin-streptavidin-RED 613)-conjugated isotype monoclonal antibodies, and 0.1% or fewer positive cells of conjugated-isotype antibodies were allowed beyond the statistical markers of the controls.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistically significant differences in mean values between patients and controls were evaluated by the Mann–Whitney U test. Correlation analysis was performed by Pearson's correlation method, and linear regression analysis was carried out to determine whether percentages of CD4$^+$ T-cell subpopulation were related to clinical parameters. Statistical significance was defined as a P value <0.05. Statistical analyses were performed on a computer using the StatView® program, version 5.0J (SAS Institute, Cary, NC, USA) for Windows.

Results

T-cell subset analysis

In terms of PBMCs, the percentage of CD3$^+$ cells among T cells from patients with MPO-ANCA-associated vasculitis was significantly lower than that from normal controls (58.8 ± 17.4 vs. 68.4 ± 7.1%, P = 0.036). There were no differences between the two groups in terms of percentage

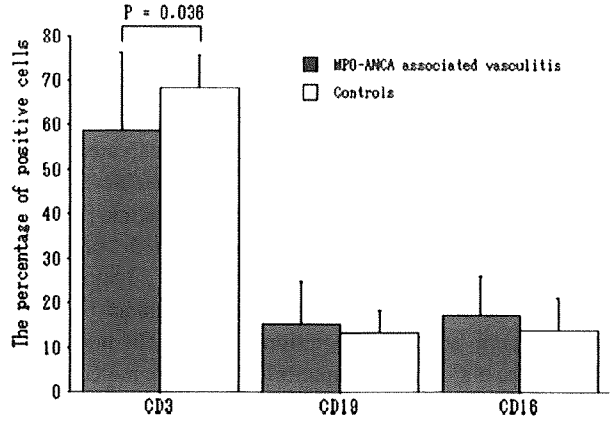

Fig. 1 Percentage of $CD3^+$, $CD16^+$, and $CD19^+$ cells among peripheral blood lymphocytes. *Shaded squares* show the mean percentages of those cells in patients with myeloperoxidase–antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis, *open squares* show those in controls, and *bars* indicate the standard deviation of positive cells

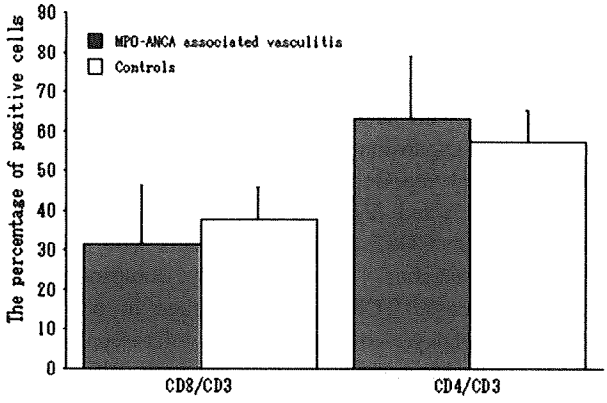

Fig. 2 Percentage of $CD4^+$ and $CD8^+$ cells among peripheral T cells ($CD3^+$ cells). *Shaded squares* show the mean percentages of cells in patients with myeloperoxidase-antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis, *open squares* show those in controls, and *bars* indicate the standard deviation of positive cells

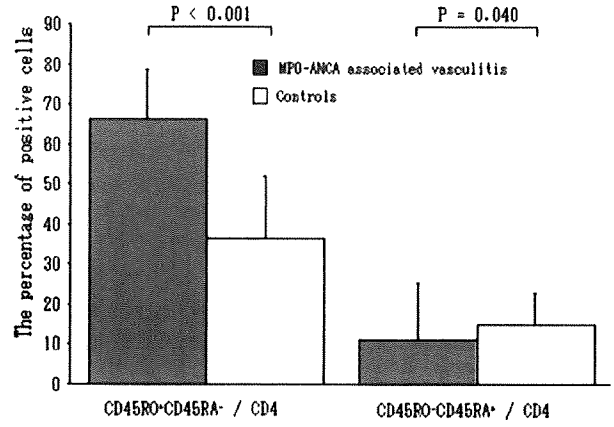

Fig. 3 Percentage of $CD45RO^+$ and $CD45RA^+$ cells among peripheral helper T cells ($CD4^+$ cells). *Shaded squares* show the mean percentages of cells in patients with myeloperoxidase–antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis, *open squares* show those in controls, and *bars* indicate the standard deviation of positive cells

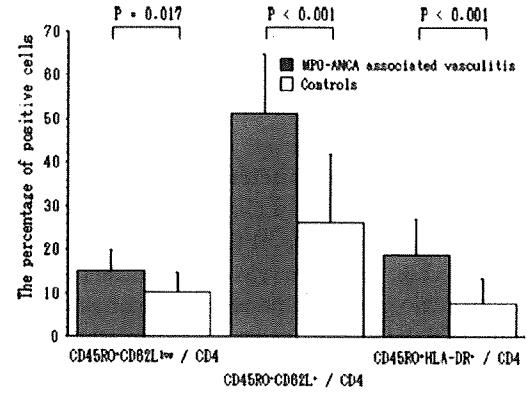

Fig. 4 Percentage of $CD62L^+$, $CD62L^{low}$ and $HLA\text{-}DR^+$ cells among peripheral helper T cells ($CD4^+$ cells). *Shaded squares* show the mean percentages of cells in patients with myeloperoxidase–antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis, *open squares* show those in controls, and *bars* indicate the standard deviation of positive cells

of $CD19^+$ cells (B cells) or $CD16^+$ cells (monocytes) (Fig. 1). Among $CD3^+$ cells, there were no differences as regards the overall percentage of $CD4^+$ cells (helper T cells) and $CD8^+$ cells (suppressor/cytotoxic T cells) between the two groups (Fig. 2). However, among $CD4^+$ cells, the percentage of $CD4^+CD45RO^+$ cells (memory T cells) from patients with MPO-ANCA-associated vasculitis was significantly higher than that from normal controls (66.3 ± 12.6 vs. $36.5 \pm 15.7\%$, $P < 0.001$), and the percentage of $CD4^+CD45RA^+$ cells (naïve T cells) from patients with MPO-ANCA-associated vasculitis was significantly lower than that from normal controls (11.1 ± 14.2 vs. $15.0 \pm 7.9\%$, $P = 0.040$) (Fig. 3).

Memory T-cell subpopulation analysis

The subpopulations of $CD4^+CD45RO^+$ cells were analyzed (Fig. 4). The percentage of $CD4^+CD45RO^+CD62L^+$ cells (central memory T cells) among $CD4^+$ cells was higher in MPO-ANCA-associated vasculitis patients than in normal controls (51.1 ± 13.6 vs. $26.3 \pm 15.3\%$, $P = 0.017$). Moreover, the percentage of $CD4^+CD45RO^+CD62L^{low}$ cells (effector memory T cells) in patients with MPO-ANCA-associated vasculitis was significantly higher than that in normal controls (15.2 ± 4.6 vs. $10.2 \pm 4.4\%$, $P < 0.001$), and the percentage of $CD4^+CD45RO^+HLA\text{-}DR^+$ cells (activated memory T cells) in patients with

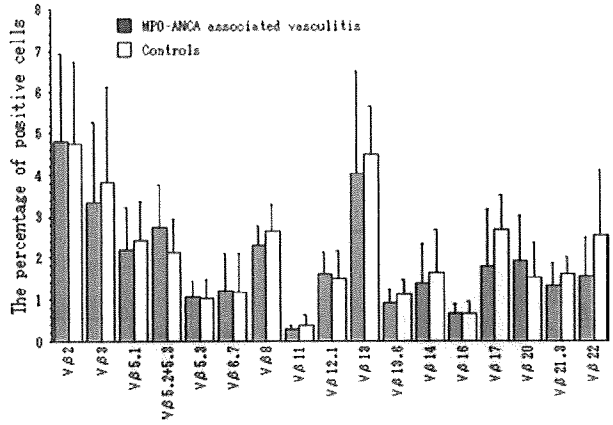

Fig. 5 Percentage of 17 T-cell receptor β-chain (TCR-Vβ^+) cells among $CD3^+$ T cells. *Shaded squares* show the mean percentages of cells in patients with myeloperoxidase–antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis, *open squares* show those in controls, and *bars* indicate the standard deviation of positive cells

MPO-ANCA-associated vasculitis was also significantly higher than that in normal controls (18.9 ± 8.1 vs. $7.8 \pm 5.6\%$, $P < 0.001$).

T-cell receptor analysis

There was no significant difference between patients with MPO-ANCA-associated vasculitis and normal controls in the percentage usage of any of the 17 TCR-Vβ regions in $CD3^+$ cells (Fig. 5). There was no significant difference between the two groups in the percentage of any of the 17 types of TCR-Vβ^+CD4^+ cells. Moreover, there was no significant difference between the two groups in the percentage of any of the 17 types of TCR-Vβ^+CD8^+ cells.

Association percentages of $CD4^+$ T-cell subpopulation and clinical parameters

There were no significant correlations between the percentage of $CD4^+CD45RO^+$ cells and age ($r = 0.223$, $P = 0.595$), WBC ($r = 0.447$, $P = 0.267$), hemoglobin concentration ($r = 0.158$, $P = 0.709$), platelet count ($r = 0.524$, $P = 0.183$), serum albumin level ($r = 0.489$, $P = 0.219$), creatinine level ($r = 0.201$, $P = 0.633$), serum CRP level ($r = 0.713$, $P = 0.052$), ANCA titer ($r = 0.494$, $P = 0.213$), or percentage of crescent formation ($r = 0.279$, $P = 0.503$). There were also no significant correlations between those clinical parameters and the percentage of $CD4^+CD45RA^+$ cells, the percentage of $CD4^+CD45RO^+CD62L^+$ cells, the percentage of $CD4^+CD45RO^+CD62L^{low}$ cells, or the percentage of $CD4^+CD45RO^+HLA\text{-}DR^+$ cells.

Discussion

We previously analyzed peripheral T-cell subsets in patients with glomerulonephritis with methicillin-resistant *S. aureus* (MRSA) infections and found increases in activated $CD4^+$ T cells, activated $CD8^+$ T cells, and some specific TCR-Vβ usages in patients with staphylococcal infection [21, 22]. Staphylococcal enterotoxins have been called "superantigens" because of their efficient activation of T lymphocytes and because of the similarity of the mechanism of T-cell stimulation to antigen recognition [23, 24]. The interaction sites on the major histocompatibility complex molecule and on the TCR are different from the peptide-binding site; i.e., on the TCR, the interaction sites are in the variable part of the β-chain. Staphylococcal enterotoxins have been shown to play a role in the pathogenesis of numerous diseases, including multiple sclerosis [25], toxic shock syndrome [26], rheumatoid arthritis [27], acquired immunodeficiency syndrome [28], Sjögren's syndrome [29], and Kawasaki disease [30]. In these diseases, an increase in certain TCR-Vβ usages is thought to be a marker of superantigen-related disease. Therefore, we speculated that staphylococcal enterotoxins may be associated with the pathogenesis of glomerulonephritis with MRSA infections.

In patients with necrotizing vasculitis, several groups have reported expansion of various T-cell subsets [18, 19]. Giscombe et al. reported lower expressions of TCR-Vβ2, -Vβ5.1, -Vβ6.7, and -Vβ17 in $CD4^+$ cells and TCR-Vβ2, and -Vβ13.1 in $CD8^+$ cells from patients with systemic necrotizing vasculitis [18]. Conversely, Simpson et al. reported increased TCR-Vβ2 gene usage in peripheral blood T lymphocytes of patients with microscopic polyarteritis [19]. Meanwhile, in patients with WG, T-cell expansions were generally of small extent and were not associated with the presence of either *S. aureus* or its superantigens [17], and there was no difference in TCR-Vβ usage in peripheral blood and bronchoalveolar lavage in most WG patients [31]. Thus, expansion of peripheral blood T-cell subsets expressing TCRs with ANCA-associated vasculitis is controversial. One reason for the different results for expansion of peripheral blood T-cell subsets expressing TCRs may be that the investigated subjects included patients with different diseases, such as MPA, WG, and CSS, or PR3- and MPO-ANCA. Another reason may be that expansion of peripheral blood T cells were investigated in different T-cell subpopulations, such as $CD3^+$, $CD4^+$, and $CD8^+$ cells. Recently, TCR-Vβ20$^+$ $CD8^+$ T-cell expansion in CSS was recently reported [32]. In that study, TCR-Vβ20$^+$ T-cell expansion was observed in $CD8^+$ cells but not in $CD3^+$ cells. However, in our study, expansion of peripheral blood T-cell subsets expressing TCRs with MPO-ANCA-associated vasculitis were not observed in $CD3^+$, $CD4^+$, or $CD8^+$ cells.

Therefore, MPO-ANCA-associated vasculitis other than CSS may be different from MRSA-associated glomerulonephritis, which is associated with circulating staphylococcal superantigens.

Although no expansion of peripheral blood T-cell subsets expressing TCRs was observed, the percentages of $CD4^+CD45RO^+$ T cells (memory T cells) in patients with MPO-ANCA-associated vasculitis were significantly elevated, and those of $CD4^+CD45RA^+$ T cells (naïve T cells) were significantly decreased in our study. Subpopulations of memory T cells were originally divided by the expression of chemokine receptor CCR7, $CD4^+CD45RO^+$ $CCR7^+$ cells, being central memory T cells [33]. Usually, central memory T cells express the homing receptor CD62L (L-selectin), and $CD4^+CD45RO^+CD62L^{low}$ cells could be regarded as effector memory T cells [33]. In our study, percentages of activated memory T ($CD4^+$ $CD45RO^+HLA\text{-}DR^+$) cells and effector memory T ($CD4^+$ $CD45RO^+CD62L^{low}$) cells were also increased. The naïve/memory T-cell balance in ANCA-associated vasculitis has been reported; there was a significant decrease in naïve $CD4^+CD45RO^-$ T cells, and the expression of HLA-DR in memory T cells was significantly increased [34]. Therefore, conventional antigens recognized by memory T cells may be associated with MPO-ANCA-associated vasculitis. Several groups have demonstrated clearly that T cells from ANCA-associated vasculitis patients are able to proliferate in response to PR3 and MPO [35–37]. MPO-deficient mice immunized with MPO developed immune responses to MPO but failed to recruit effector cells to glomeruli or develop significant crescent formation, but effector $CD4^+$ cell depletion in this model attenuated crescentic glomerulonephritis and effector-cell influx without altering ANCA titers. Moreover, B cell deficient mice, with no ANCA, still developed severe crescentic glomerulonephritis with the accumulation of effector cells [38]. MPO-specific effector T cells are important in pauci-immune crescentic glomerulonephritis, and increase of memory T cells may be reflected in the activation of MPO-specific effector T cells. However, similar in vitro T-cell proliferative responses to PR3 and MPO are also detected in healthy individuals, and differences in peripheral blood T-cell proliferation and frequencies as measured in response to PR3 and MPO have not been consistently detected in patients with ANCA-associated vasculitis compared with normal controls [39].

In this study, there was no relation between the percentages of memory T cells or memory T cell subpopulations and clinical parameters, such as ANCA titers or serum CRP levels. Therefore, memory T cells or memory T cell subpopulations alone may not necessarily regulate the degrees of inflammation or ANCA production. The actual trigger for T-cell activation in ANCA-associated vasculitis patients remains to be elucidated. Further accumulation of data from more patients and further investigations to probe regulatory molecules for ANCA production are needed.

In conclusion, we demonstrated elevated levels of memory T cells and no expansion of peripheral blood T-cell subsets expressing particular TCRs in patients with MPO-ANCA-associated vasculitis. Therefore, we speculate that conventional antigens rather than superantigens may be associated with the pathogenesis of MPO-ANCA-associated vasculitis.

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Laser microdissection-based analysis of cytokine balance in the kidneys of patients with lupus nephritis

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Summary

To determine the cytokine balance in patients with lupus nephritis (LN), we analysed kidney-infiltrating T cells. Renal biopsy samples from 15 systemic lupus erythematosus (SLE) patients were used. In accordance with the classification of International Society of Nephrology/Renal Pathology Society, they were categorized into Class III, Class III+V (Class III-predominant group, $n = 4$), Class IV, Class IV+V (Class IV-predominant group, $n = 7$) and Class V ($n = 4$) groups. The single-cell samples of both the glomelular and interstitial infiltrating cells were captured by laser-microdissection. The glomerular and interstitial infiltrating T cells produced interleukin (IL)-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. Interferon-gamma was detected only in the glomeruli of the Class III-predominant and Class V group samples. The expression level of IL-17 was correlated closely with clinical parameters such as haematuria, blood urea nitrogen level, SLE Disease Activity Index scores in both glomeruli and interstitium, urine protein level in glomeruli and serum creatinine and creatinine clearance levels in interstitium. This suggests that the glomerular infiltrating T cells might act as T helper type 1 (Th1), Th2 and Th17 cells while the interstitial infiltrating T cells, act as Th2 and Th17 cells in the Class III-predominant and Class V groups. In contrast, both the glomerular and interstitial infiltrating T cells might act as Th2 and Th17 cells in the Class IV-predominant group. The cytokine balances may be dependent upon the classification of renal pathology, and IL-17 might play a critical role in SLE development.

Keywords: laser-microdissection, lupus nephritis, SLEDAI, Th17

Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease characterized by various clinical manifestations. T cell-derived cytokine production plays a determinant role in SLE development. Previous studies have reported that an imbalance in cytokine production between T helper type 1 (Th1) and Th2 T cells (predominance of Th2 cytokine) in the peripheral blood of SLE patients is associated with the pathogenesis of the disease [1–3]. In contrast, Akahoshi *et al.* [4] demonstrated that a substantial predominance of Th1-type response took place in the peripheral blood samples of lupus nephritis (LN) patients categorized in WHO Class IV. Not only T cells in the peripheral blood, but also the balance in cytokine production between Th1 and Th2 cells in the kidney has drawn a great deal of attention. Masutani *et al.* [5] analysed the expression levels of interferon (IFN)-γ and interleukin (IL)-4 on intrarenal T cells as well as those in the peripheral blood samples from SLE patients with diffuse proliferative LN by immunohistochemistry, demonstrating the predominance of Th1 type response. They suggested that the Th1 : Th2 ratio in the peripheral blood might directly reflect the local histopathological findings. However, Murata *et al.* [6] indicated that the kidney-infiltrating T cells could produce Th2 type cytokines such as IL-4 and IL-10 through reverse transcription–polymerase chain reaction (RT–PCR), and made an assumption that this discrepancy might arise from a difference in sensitivity between the methods used in detection of cytokines. The expression level of IL-13, one of the Th2 type cytokines, was reported to be higher in the serum from the rheumatoid arthritis (RA), SLE, Sjögren's syndrome and

systemic sclerosis patient groups than that in the normal healthy control group [7]. Morimoto *et al.* [8] also showed elevated expression level of IL-13 in SLE patients. Recently, it has been reported that naive murine $CD4^+$ T helper cells can be induced to differentiate into Th1, Th2, Th17 and regulatory phenotypes [9]. IL-17 is a proinflammatory cytokine, as possibly known from the pathological conditions of various inflammatory diseases in both humans and mice [9]. We have reported previously that both IL-13 and IL-17 were produced in the murine LN (MRL/lpr mice) cells; however, we did not analyse them at a single-cell level [10]. The laser microdissection (LMD) technique has been adopted recently to obtain tissue samples exclusively from specific regions of interest. This new technique has been used successfully in various fields, including oncology [11], endocrinology [12], gastroenterology [13], rheumatology [14–16] and nephrology [10,17–19]. With this technique, attempts to analyse single-cell gene expression were made [13,16,20]. In our study, we analysed the single-cell expression levels of cytokines, including IL-13 and IL-17, by infiltrating T cells in the kidneys of LN patients.

Patients and methods

Renal biopsy samples were obtained from 15 SLE patients, two minor glomerular abnormalities (MGA) patients (female, 16 years old; male, 14 years old) and one minimal change nephrotic syndrome (MCNS) patient (male, 14 years old), and used in our experiments. In accordance with the classification criteria defined by International Society of Nephrology/Renal Pathology Society (ISN/RPS) [21,22], renal pathologies were diagnosed as: Class III, three cases; Class III+V, one case; Class IV, two cases; Class IV+V, five cases; and Class V, four cases. To ensure consistency with the World Health Organization (WHO) classification criteria, a further membranous lesion (Class V) may be added to Class III or Class IV in ISN/RPS. They were categorized as Class III-predominant group (Class III-predominant group included patients with both Class III and Class III+V, $n = 4$) and Class IV-predominant group (including patients with both Class IV and Class IV+V, $n = 7$). The patients, who had underwent renal biopsy before 2004, had already been classified in accordance with the WHO classification criteria [23] at the time of biopsy, but in this study were re-evaluated by nephrologists in accordance with the ISN/RPS classification criteria. The SLE Disease Activity Index (SLEDAI) scores [24], histological activity index (AI) and chronicity index (CI) scores [25] at renal biopsy are shown as Table 1. This study was approved by the ethical committee of Tsukuba University Hospital (no. 392). Prior written consent was given by the patients.

Immunohistological examinations

Five-μm-thick sections were obtained from the renal biopsy specimens of the SLE patients. Immunohistochemical

Table 1. Clinical characteristics of patient and positivity of dissected T cells.

No.	Age	Sex	Classification	Pre-s	UP (g/day)	Haematuria (RBC/HPF)	Urinary cast	Pyuria	BUN (mg/dl)	Cr (mg/dl)	Ccr (ml/min)	ADNA (U/ml)	CH50 (U/ml)	SLEDAI	TCR-cβ/ β-actin (%)	AI	CI
1	45	F	III (A)	No	5·0	10–19	+	–	10·1	0·54	92·1	> 300	4·8	17	18/28 (64·3%)	5	2
2	52	F	III (C)	Yes	0·46	1–4	–	–	20·7	1·01	38·9	< 2	40·2	0	15/34 (44·1%)	1	8
3	52	F	III (A) + V	No	0·3	1–4	–	–	10·0	0·57	84·0	64·7	18·2	9	18/26 (69·2%)	0	3
4	29	F	IV-G (A/C)	No	8·0	56	+	+	31·1	1·10	48·1	100·0	12·0	21	28/36 (77·8%)	16	3
5	25	F	IV-G (A/C)	Yes	5·0	5–9	+	–	15·0	0·60	93·0	> 300	2·1	19	18/25 (72·0%)	11	3
6	58	F	IV-S(A/C)+V	No	6·1	1–4	+	–	10·0	0·56	159·0	45	24·5	16	53/67 (79·1%)	12	4
7	59	F	IV-G(A/C)+V	No	2·3	1–4	–	+	26·1	0·95	51·5	64·7	18·2	9	19/28 (67·9%)	5	2
8	55	F	IV-G (A) + V	No	0·8	1–4	–	–	13·1	0·49	100·0	94·6	7·4	13	14/27 (51·9%)	10	0
9	18	F	IV-S (A) + V	Yes	0·46	1–4	–	–	12·3	0·48	108·5	58·1	9·5	18	22/31 (70·1%)	6	3
10	28	F	V	No	7·7	0–1	–	–	13·8	0·70	96·7	16·7	41·6	5	18/26 (69·2%)	1	2
11	39	F	V	Yes	5·3	0–1	–	–	12·4	0·51	138·1	5·4	50·7	4	22/32 (68·8%)	0	2
12	26	F	V	Yes	2·1	5–9	–	–	13·5	0·60	95·4	23·1	42·9	19	46/62 (74·2%)	0	0
13	22	M	IV-G(A/C)+V	Yes	4·15	30–50	+	–	16·0	0·90	110·8	< 5·0	20·5	10	7/20 (35·0%)	8	6
14	38	F	III (A/C)	No	0·85	1–4	+	–	18·7	0·62	141·7	> 300	23·2	13	18/31 (58·1%)	6	4
15	60	F	V	No	0·49	20–99	–	–	16·6	0·73	99·6	20·3	54·0	22	27/40 (67·5%)	0	3

S: segmental; G: global; A: active; C: chronic; Pre-s: pretreatment with steroid; UP: urine protein; RBC/HPF: red blood cell/high power field; BUN: blood urea nitrogen; Cr: serum creatinine; Ccr: creatinine clearance; Anti-ds: anti-double-stranded DNA; CH50: 50% haemolytic unit of complement serum; SLEDAI: systemic lupus erythematosus Disease Activity Index; AI: activity index score; CI: chronicity index score.

staining was performed by the avidin–biotin complex technique. Primary antibodies used included murine anti-human IFN-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-IL-4, 10 (Research & Diagnostics Systems, Minneapolis, MN, USA); and polyclonal rabbit anti-human IL-17 and IL-13 (Santa Cruz Biotechnology). Staining was performed on the sections using normal murine IgG or rabbit immunoglobulin (Ig)G, a primary antibody, as a negative control. We also performed staining on sections of the renal biopsy samples of MGA and MCNS patients using anti-human IL-17 as the control.

Tissue sampling by laser microdissection

Frozen sections (10 μm thick) from the renal biopsy specimens of the SLE patients were stained with 0·05% toluidine blue solution (pH 7·0) (Wako Pure Chemical Industries, Osaka, Japan) and the individual single cells infiltrating into glomeruli and interstitiums were selected and dissected with laser-microdissection system (AS-LMD; Leica Microsystems Japan, Tokyo, Japan) (Fig. 2A).

RNA extraction and nested RT–PCR

Total RNA was extracted from the LMD samples by the Isogen method (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA using the ThermoScript RT–PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA) and amplified with primers specific to β-actin, T cell receptor β chain (TCR-Cβ), IL-2, IL-4, IL-10, IL-13, IL-17 and IFN-γ for nested RT–PCR (Table 2).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Statistically significant differences between groups were determined using the Mann–Whitney *U*-test. A simple linear regression analysis was used to evaluate the correlation between the two parameters. The statistical significance was defined as $P < 0.05$.

Results

Detection of T cells in glomeruli and interstitium

Stained IL-4, IL-10 and IL-13 were observed in the glomerular and interstitial areas of the specimens from the SLE patients of the Class III-predominant, IV-predominant and Class V groups, especially in the latter area of the Class IV-predominant group (Fig. 1A) (the immunohistochemical data for the Class III-predominant and Class V groups are not shown). Many IL-4 cells were observed predominantly, mainly in the glomerular and interstitial cells, especially in intraglomerular infiltrating cells, in the Class IV-predominant group, while there were only a few IL-4-positive cells in the tubular epithelial cells (TEC) (Fig. 1Aa, b). IL-10- and IL-13-positive cells were observed prominently in the glomerular and interstitial infiltrating cells (Fig. 1Ac–f). Some stained IL-10-positive cells were observed in TEC (Fig. 1Ac, d). IL-17-positive cells were observed mainly in the glomerular and interstitial infiltrating cells and TECs, especially in intraglomerular cells of the Class IV-predominant group (Fig. 1Ag, h). Almost no IL-17-positive cells were observed in the glomeruli of the Class III-predominant (Fig. 1Ba) and Class V group (not shown) samples. However, IFN-γ cells were not observed in all the specimens (Fig. 1Bb) (the immunohistochemical data for the Class III-predominant groups are shown). Normal rabbit IgG was used as a negative control (Fig. 1Bc). IL-17-positive cells were not observed in all the specimens from the MGA and MCNS patients (Fig. 1C). This demonstrates that IL-17 may be produced preferentially in SLE patients.

Analysis of gene expression by laser microdissection and nested RT–PCR

Of 622 glomerular and interstitial infiltrating cells, 513 (82·5%) were β-actin-positive, among which 343 (66·7%) were TCR-Cβ-positive; these 343 cells were deemed to be T cells and used for cytokine analysis (Table 1). The number of positive samples for each cytokine/ TCR-Cβ$^+$ cells was expressed as a percentage.

The glomerular and interstitial infiltrating T cells produced IL-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. The positivity of cytokines is shown in Table 3 and Fig. 2B. The percentages of positive IL-4, IL-10 and IL-13 samples were more than 70%, 67% and 41%, respectively, in all the groups. The expression levels of IL-2 were low in each of the predominant groups. IFN-γ was detected only in the glomeruli of the Class III-predominant and Class V groups (32·3 ± 12·9% and 24·0 ± 10·0%, $P < 0.05$) (Table 3 and Fig. 2B). In the glomerular lesions, the percentage of positive IL-17 samples was 64·7 ± 10·1% and 70·7 ± 6·0% in the Class IV-predominant and V groups, while it was significantly greater than in the Class III-predominant group (44·7 ± 5·9%, $P < 0.05$) (Fig. 2Bb). In the interstitial lesions, the positivity of IL-17 (48·0 ± 4·2%) was also significantly lower in the Class III-predominant groups than that in the Class IV-predominant group (69·1 ± 8·9%, $P < 0.05$) (Fig. 2Bc).

Correlation between the expression levels of cytokines and clinical parameters in SLE patients

We analysed the correlation between the expression levels of Th1 (IL-2), Th2 (IL-4, IL-10, and IL-13) and Th17 (IL-17)