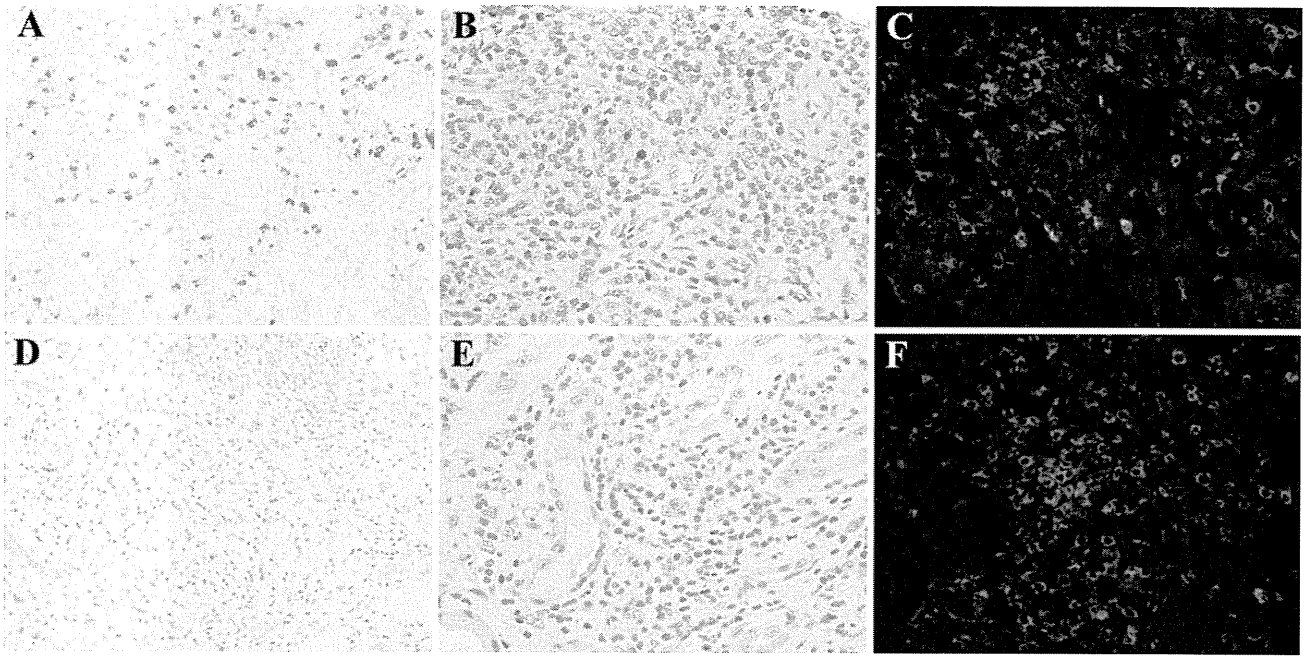

Fig. 5 Changes in immunoglobulin (Ig)G4-positive plasma cell, Foxp3+ cell, and CD4+CD25+ cell infiltration before (**a**–**c**) and 1 month after (**d**–**f**) corticosteroid therapy in patient 6. Compared with the pretreatment specimens (**a**), the number of IgG4-positive plasma cells in the lesions was markedly diminished in the posttreatment specimens (**d**). In the same way, compared with the pretreatment specimens (**b**), the number of Foxp3+ cells obviously decreased in the posttreatment specimens (**e**). Some CD4+CD25+ cells in the lesions were observed before treatment (**c**), whereas almost none were detected after therapy (**f**) [**a**, **d** IgG4 ×200, **b**, **e** Foxp3 ×400, **c**, **f** CD4 (*red*) and CD25 (*green*) ×400]

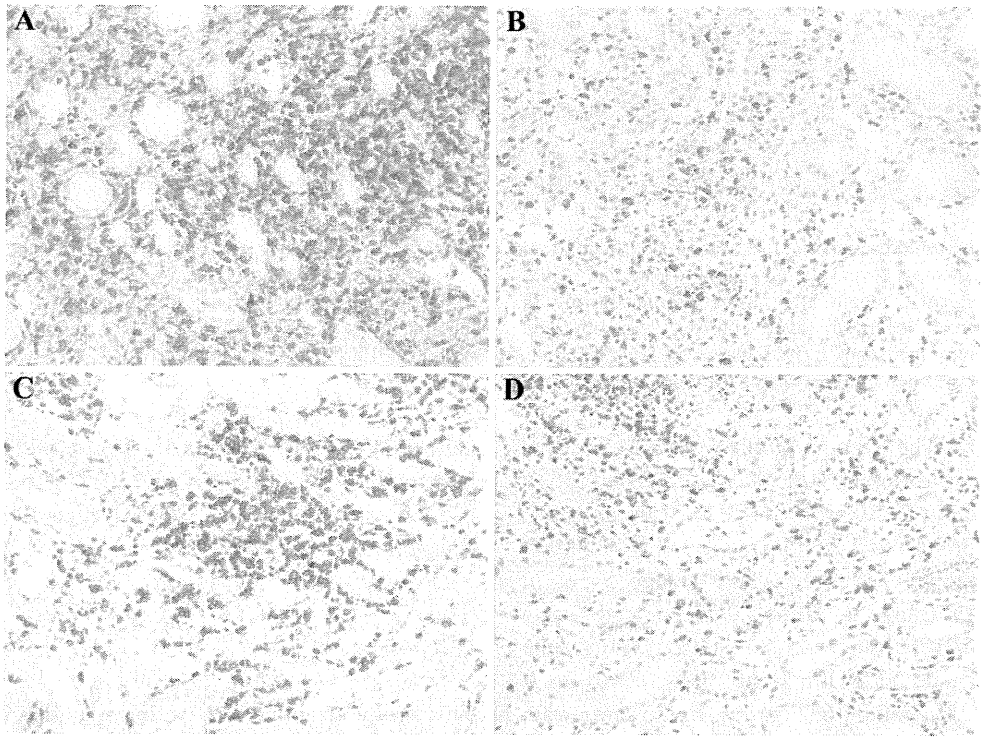

Fig. 6 Changes in CD4+ and CD8+ T cell infiltration before (**a**, **b**) and 4 months after (**c**, **d**) corticosteroid therapy in patient 5. The extent of CD4+ T cell (**a**, **c**) and CD8+ T cell (**b**, **d**) infiltration in the lesions remained largely constant, even after therapy (**a**, **c** CD4 ×100, **b**, **d** CD8 ×100)

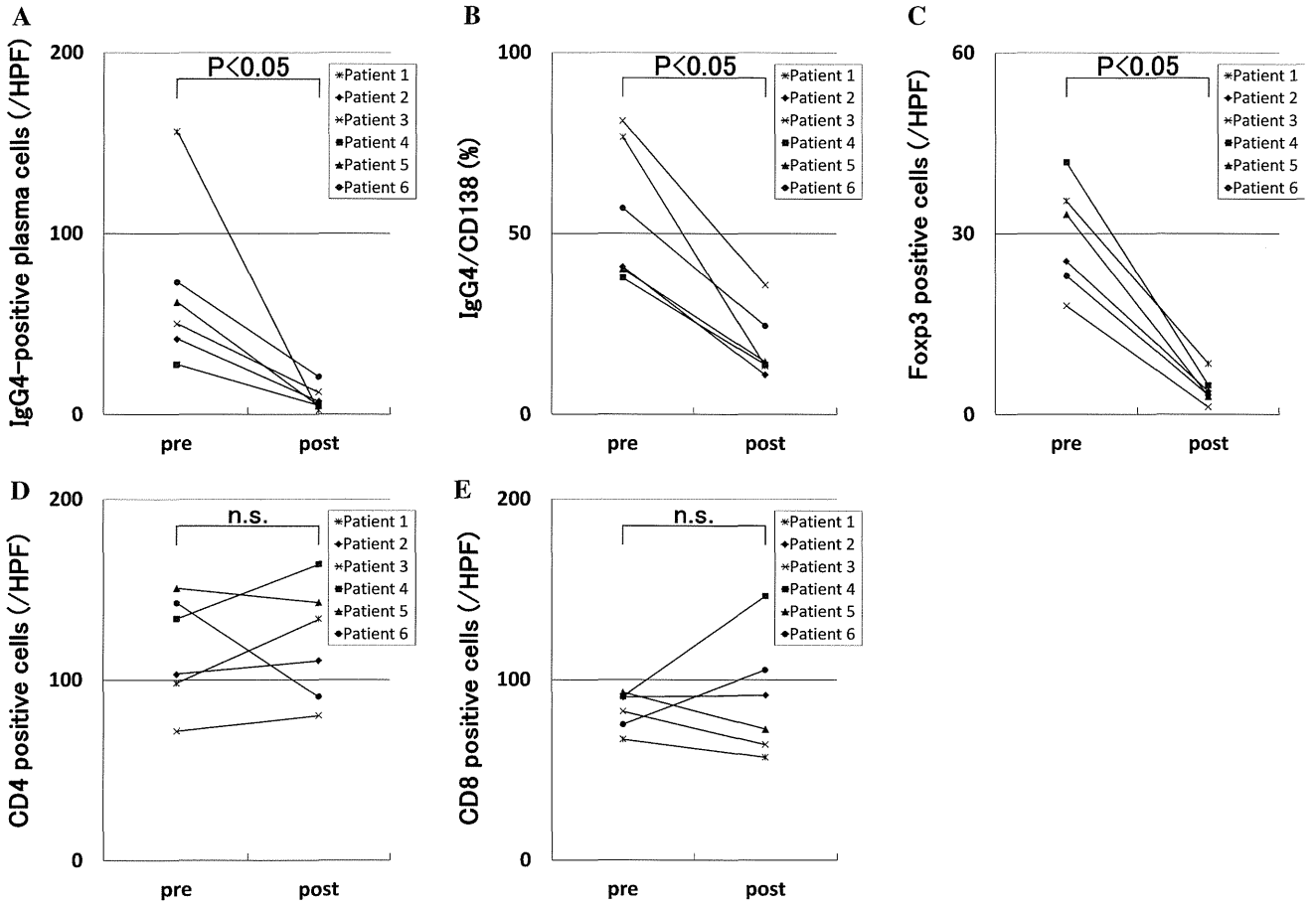

Fig. 7 Changes in numbers of infiltrating cells before and after corticosteroid therapy. In six IgG4-related TIN cases, the average of IgG4-positive plasma cell numbers and the ratio of IgG4-positive cells to CD138-positive cells in the lesions significantly decreased after compared with before corticosteroid therapy (**a**, **b**, respectively). Similarly, average of Foxp3+ cell numbers significantly decreased (**c**). However, average of CD4+ and CD8+ T cell numbers did not change (**d**, **e**, respectively)

microglobulinuria. Renal insufficiency was also frequent, and almost all patients with IgG4-related TIN had some radiographic abnormalities. Therapy with 20–30 mg/day of prednisolone rapidly improved renal function in three patients with severe renal dysfunction, whereas some renal insufficiency persisted in two. Radiographic abnormalities persisted in half of the patients. Histologically, infiltration of CD4+CD25+ T cells and Foxp3+ cells in addition to IgG4-positive plasma cells was characteristic in IgG4-related TIN. Notably, IgG4-positive plasma cells, Foxp3+ cells, and CD4+CD25+ cells had already decreased 1 month after corticosteroid therapy in one case (patient 6), whereas small to moderate numbers of CD4+ T cells and CD8+ T cells infiltrated where inflammation persisted in all patients. In the later stage after treatment, patchy or regional fibrosis remained to some extent.

In this study, radiographic findings of renal lesions were almost the same as those noted in past reports [21, 30]. Multiple low-density lesions on enhanced CT, diffuse bilateral renal swelling, and thickening of the renal pelvic wall were the major features. Past reports mentioned that corticosteroid therapy was effective in improving the appearance of renal lesions on imaging study, although cortical scar or renal capsular dimpling persisted in a small number of cases [30–33]. In our study, although some renal parenchymal lesions showed recovery of contract enhancement, others progressed to scar-like atrophy in three of six cases. In contrast to past studies [30–33], both imaging study and histological analysis were performed before and after corticosteroid therapy in all patients in this study. Our data might suggest that these atrophic lesions in imaging study correspond to the histological fibrotic lesions. In addition, recovery of contrast enhancement might relate to the partially normalized renal interstitium observed in the posttreatment specimens of patients 5 and 6, who showed diffuse TIN and renal tubular atrophy before therapy. In this way, IgG4-related TIN could leave macroscopic atrophy and microscopic fibrosis, which might explain why renal function did not totally recover after corticosteroid therapy in our patients with severe renal insufficiency. The possibility of these sequelae must be considered when determining the corticosteroid dose and optimal timing of the initiation of treatment.

The main histological findings before corticosteroid therapy were consistent with previously published histological features of IgG4-related TIN [21, 22, 34]. In addition, extension of inflammation beyond the renal capsule reported by Yamaguchi et al. [29] was also observed and seemed to correspond to the extension of lesions beyond the pancreatic capsule in autoimmune pancreatitis (AIP). Zen et al. [35] reported infiltration of CD4+CD25+ T cells and Foxp3+ cells in the lesions of IgG4-related pancreatitis and cholangitis and that those lesions had significantly increased levels of Th2 and regulatory T-cell cytokines. Nakashima et al. [36] also reported increased Th2 and regulatory cytokines in the lesions of IgG4-related TIN. In this study, we similarly confirmed the presence of CD4+CD25+ T and Foxp3+ cells in the renal interstitium where lymphocytes and IgG4-positive plasma cells infiltrated. Accordingly, the presence of CD4+CD25+ T or Foxp3+ cell infiltration might be another distinctive finding of IgG4-related TIN. As Houghton and Troxell [37] reported that an abundant infiltration of IgG4-positive plasma cell is not so specific for IgG4-related TIN, it will be necessary to survey the presence or absence of these regulatory cells in TIN diseases other than IgG4-related TIN.

We focused on histological and immunohistochemical changes of IgG4-related TIN during the clinical course of corticosteroid therapy. The area with infiltrating cells in the renal interstitium decreased with the passage of time after therapy was initiated. Conversely, more obvious regional fibrosis was observed in the re-biopsy specimens, although there was the possibility of sampling bias due to randomly performed biopsies. Reflecting these histological findings, the radiological lesions to some degree showed recovery following therapy, whereas in some parts, there was progression to scar formation. These findings suggest the need to search for ways to prevent fibrosis in addition to control of inflammation. Whether early initiation of corticosteroid therapy can prevent fibrosis remains to be verified, as there was no untreated control group in this study and so this point could not be concluded definitively. However, comparing posttreatment findings of patients 3 and 5, both of whom underwent re-evaluation 4 months after the start of therapy, macroscopic atrophy and microscopic fibrosis were more marked in patient 5 with severe renal dysfunction before therapy than in patient 3 with normal renal function then. This difference implies that early initiation of corticosteroid therapy prevents fibrosis to some extent. The possibility should also be considered that corticosteroid therapy alone is insufficient to prevent residual fibrosis despite early initiation. Other treatment options should be examined, as rituximab therapy, for example, has been reported to be effective in achieving clinical improvement in IgG4-RD [38, 39]. The characteristic cells, including CD4+CD25+ T cells, Foxp3+ cells, and IgG4-positive plasma cells, seemed to disappear quickly after corticosteroid initiation and did not reappear as long as corticosteroid was administered, whereas other CD4+ T and CD8+ T cells persisted for a long time in lesions where cell infiltration was still observed. This finding suggests that we cannot precisely evaluate IgG4-positive plasma cell infiltration in diagnosing IgG4-related TIN if corticosteroid therapy has already been initiated. The pathogenic significance of this finding remains unclear. More accumulation of clinical and histological data of IgG4-related TIN, including recurrences during corticosteroid tapering or after cessation of corticosteroid therapy and basic research based on those data, are essential to elucidate the roles of these cells in the pathogenesis of IgG4-related TIN.

Biomarkers that could be used as a goal for treating IgG4-related TIN have not yet been established. It was reported that in AIP, serum IgG4, IgG, and circulating immune complex decreased after corticosteroid therapy and increased at relapse, whereas serum C3 and C4 levels showed reciprocal changes [40]. Tabata et al. [41] suggested that the measurement of serial serum IgG4 levels was useful to determine disease activity of IgG4-RD. Nevertheless, whether normalization of serum IgG4 levels could be a treatment goal is unclear, as it is not always observed despite apparent clinical remission [41]. Cutoff values of these markers as a goal should be further examined. Nishi et al. [42] reported that elevated urine NAG and/or α1-microglobulin concentrations were useful markers for detecting renal abnormalities in their AIP patients with or without clinically detected renal involvement. However, neither urine β2-MG nor NAG entirely responded to corticosteroid therapy and fluctuated despite the continuation of treatment in our study. Thus, reliable markers showing a goal for treatment remain to be identified. Further studies are required to seek such markers, including novel candidates.

The small number of cases is a limitation of our study that relates to the rarity of this disease, and the inconsistent follow-up times of radiological and histological data in each patient is another limitation. However, with increasing worldwide awareness of IgG4-related TIN as a distinct disease entity [21, 22], we can expect larger studies that include many more cases and anticipate that, with more patients enrolled in each stage of corticosteroid therapy, especially in the very early and later stages, the dynamics of various infiltrating cells during the clinical course will be better clarified.

In conclusion, our investigations suggest that, clinically, persistent renal insufficiency associated with macroscopic atrophy and microscopic fibrosis is not so rare in IgG4-related TIN. Pathologically, the behavior of regulatory T cells during the clinical course of corticosteroid therapy is

quite similar to that of IgG4-positive plasma cells, and the distinctive behavior pattern of those cells may provide a clue to the mechanisms underlying this disease. Further studies are required to elucidate the total picture of this disease, including its clinical and pathogenic aspects.

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Conflict of interest None.

References

1. Hamano H, Kawa S, Horiuchi A, Unno H, Furuya N, Akamatsu T, et al. High serum IgG4 concentrations in patients with sclerosing pancreatitis. N Engl J Med. 2001;344:732–8.
2. Hamano H, Kawa S, Ochi Y, Unno H, Shiba N, Wajiki M, et al. Hydronephrosis associated with retroperitoneal fibrosis and sclerosing pancreatitis. Lancet. 2002;359:1403–4.
3. Yamamoto M, Harada S, Ohara M, Suzuki C, Naishiro Y, Yamamoto H, et al. Clinical and pathological differences between Mikulicz's disease and Sjögren's syndrome. Rheumatology (Oxford). 2005;44:227–34.
4. Takahira M, Kawano M, Zen Y, Minato H, Yamada K, Sugiyama K. IgG4-related chronic sclerosing dacryoadenitis. Arch Ophthalmol. 2007;125:1575–8.
5. Cheuk W, Yuen HK, Chan JK. Chronic sclerosing dacryoadenitis: part of the spectrum of IgG4-related sclerosing disease? Am J Surg Pathol. 2007;31:643–5.
6. Uchiyama-Tanaka Y, Mori Y, Kimura T, Sonomura K, Umemura S, Kishimoto N, et al. Acute tubulointerstitial nephritis associated with autoimmune-related pancreatitis. Am J Kidney Dis. 2004; 43:e18–25.
7. Takeda S, Haratake J, Kasai T, Takaeda C, Takazakura E. IgG4-associated idiopathic tubulointerstitial nephritis complicating autoimmune pancreatitis. Nephrol Dial Transplant. 2004;19:474–6.
8. Nakamura H, Wada H, Origuchi T, Kawakami A, Taura N, Aramaki T, et al. A case of IgG4-related autoimmune disease with multiple organ involvement. Scand J Rheumatol. 2006;35:69–71.
9. Watson SJ, Jenkins DA, Bellamy CO. Nephropathy in IgG4-related systemic disease. Am J Surg Pathol. 2006;30:1472–7.
10. Saeki T, Saito A, Yamazaki H, Emura I, Imai N, Ueno M, et al. Tubulointerstitial nephritis associated with IgG4-related systemic disease. Clin Exp Nephrol. 2007;11:168–73.
11. Saeki T, Nishi S, Ito T, Yamazaki H, Miyamura S, Emura I, et al. Renal lesions in IgG4-related systemic disease. Intern Med. 2007;46:1365–71.
12. Katano K, Hayatsu Y, Matsuda T, Miyazaki R, Yamada K, Kawano M, et al. Endocapillary proliferative glomerulonephritis with crescent formation and concurrent tubulointerstitial nephritis complicating retroperitoneal fibrosis with a high serum level of IgG4. Clin Nephrol. 2007;68:308–14.
13. Murashima M, Tomaszewski J, Glickman JD. Chronic tubulointerstitial nephritis presenting as multiple renal nodules and pancreatic insufficiency. Am J Kidney Dis. 2007;49:e7–10.
14. Yoneda K, Murata K, Katayama K, Ishikawa E, Fuke H, Yamamoto N, et al. Tubulointerstitial nephritis associated with IgG4-related autoimmune disease. Am J Kidney Dis. 2007;50:455–62.
15. Cornell LD, Chicano SL, Deshpande V, Collins AB, Selig MK, Lauwers GY, et al. Pseudotumors due to IgG4 immune-complex tubulointerstitial nephritis associated with autoimmune pancreatocentric disease. Am J Surg Pathol. 2007;31:1586–97.
16. Aoki A, Sato K, Itabashi M, Takei T, Yoshida T, Arai J, et al. A case of Mikulicz's disease complicated with severe interstitial nephritis associated with IgG4. Clin Exp Nephrol. 2009;13: 367–72.
17. Morimoto J, Hasegawa Y, Fukushima H, Uesugi N, Hisano S, Saito T, et al. Membranoproliferative glomerulonephritis-like glomerular disease and concurrent tubulointerstitial nephritis complicating IgG4-related autoimmune pancreatitis. Intern Med. 2009;48:157–62.
18. Saeki T, Imai N, Ito T, Yamazaki H, Nishi S. Membranous nephropathy associated with IgG4-related systemic disease and without autoimmune pancreatitis. Clin Nephrol. 2009;71:173–8.
19. Dhobale S, Bedetti C, Killian P, Ilyas M, Liput J, Jasnosz K, et al. IgG4 related sclerosing disease with multiple organ involvements and response to corticosteroid treatment. J Clin Rheumatol. 2009;15:354–7.
20. Kuroda N, Nakamura S, Miyazaki K, Inoue K, Ohara M, Mizuno K, et al. Chronic sclerosing pyelitis with an increased number of IgG4-positive plasma cells. Med Mol Morphol. 2009;42:236–8.
21. Saeki T, Nishi S, Imai N, Ito T, Yamazaki H, Kawano M, et al. Clinicopathological characteristics of patients with IgG4-related tubulointerstitial nephritis. Kidney Int. 2010;78:1016–23.
22. Raissian Y, Nasr SH, Larsen CP, Colvin RB, Smyrk TC, Takahashi N, et al. Diagnosis of IgG4-related tubulointerstitial nephritis. J Am Soc Nephrol. 2011;22:1343–52.
23. Tsubata Y, Akiyama F, Oya T, Ajiro J, Saeki T, Nishi S, et al. IgG4-related chronic tubulointerstitial nephritis without autoimmune pancreatitis and the time course of renal function. Intern Med. 2010;49:1593–8.
24. Saida Y, Homma N, Hama H, Ueno M, Imai N, Nishi S, et al. A case of IgG4-related tubulointerstitial nephritis showing the progression of renal dysfunction after a cure for autoimmune pancreatitis. Nippon Jinzo Gakkai Shi. 2010;52:73–9 (in Japanese, abstract in English).
25. Masaki Y, Dong L, Kurose N, Kitagawa K, Morikawa Y, Yamamoto M, et al. Proposal for a new clinical entity, IgG4-positive multiorgan lymphoproliferative syndrome: analysis of 64 cases of IgG4-related disorders. Ann Rheum Dis. 2009;68:1310–5.
26. Kawano M, Saeki T, Nakashima H, Nishi S, Yamaguchi Y, Hisano S, et al. Proposal for diagnostic criteria for IgG4-related kidney disease. Clin Exp Nephrol. 2011;15:615–26.
27. Kim F, Yamada K, Inoue D, Nakajima K, Mizushima I, Kakuchi Y, et al. IgG4-related tubulointerstitial nephritis and hepatic inflammatory pseudotumor without hypocomplementemia. Intern Med. 2011;50:1239–44.
28. Ito K, Yamada K, Mizushima I, Aizu M, Fujii H, Mizutomi K, et al. Henoch–Schönlein purpura nephritis in a patient with IgG4-related disease: a possible association. Clin Nephrol. 2012 (in press).
29. Yamaguchi Y, Kanetsuna Y, Honda K, Yamanaka N, Kawano M, Nagata M. Characteristic tubulointerstitial nephritis in IgG4-related disease. Hum Pathol. 2011 [Epub ahead of print].
30. Takahashi N, Kawashima A, Fletcher JG, Chari ST. Renal involvement in patients with autoimmune pancreatitis: CT and MR imaging findings. Radiology. 2007;242:791–801.
31. Khalili K, Doyle DJ, Chawla TP, Hanbidge AE. Renal cortical lesions in patients with autoimmune pancreatitis: a clue to differentiation from pancreatic malignancy. Eur J Radiol. 2008;67:329–35.
32. Sohn JH, Byun JH, Yoon SE, Choi EK, Park SH, Kim MH, et al. Abdominal extrapancreatic lesions associated with autoimmune pancreatitis: radiological findings and changes after therapy. Eur J Radiol. 2008;67:497–507.
33. Triantopoulou C, Malachias G, Maniatis P, Anastopoulos J, Siafas I, Papailiou J. Renal lesions associated with autoimmune pancreatitis: CT findings. Acta Radiol. 2010;51:702–7.

34. Nishi S, Imai N, Yoshida K, Ito Y, Saeki T. Clinicopathological findings of immunoglobulin G4-related kidney disease. Clin Exp Nephrol. 2011 [Epub ahead of print].
35. Zen Y, Fujii T, Harada K, Kawano M, Yamada K, Takahira M, et al. Th2 and regulatory immune reactions are increased in immunoglobin (sic) G4-related sclerosing pancreatitis and cholangitis. Hepatology. 2007;45:1538–46.
36. Nakashima H, Miyake K, Moriyama M, Tanaka A, Watanabe M, Abe Y, et al. An amplification of IL-10 and TGF-β in patients with IgG4-related tubulointerstitial nephritis. Clin Nephrol. 2010;73:385–91.
37. Houghton DC, Troxell ML. An abundance of IgG4+ plasma cells is not specific for IgG4-related tubulointerstitial nephritis. Mod Pathol. 2011;24:1480–7.
38. Khosroshahi A, Bloch DB, Deshpande V, Stone JH. Rituximab therapy leads to rapid decline of serum IgG4 levels and prompt clinical improvement in IgG4-related systemic disease. Arthritis Rheum. 2010;62:1755–62.
39. Zaidan M, Cervera-Pierot P, de Seigneux S, Dahan K, Fabiani B, Callard P, et al. Evidence of follicular T-cell implication in a case of IgG4-related systemic disease with interstitial nephritis. Nephrol Dial Transplant. 2011;26:2047–50.
40. Muraki T, Hamano H, Ochi Y, Komatsu K, Komiyama Y, Arakura N, et al. Autoimmune pancreatitis and complement activation system. Pancreas. 2006;32:16–21.
41. Tabata T, Kamisawa T, Takuma K, Egawa N, Setoguchi K, Tsuruta K, et al. Serial changes of elevated serum IgG4 levels in IgG4-related systemic disease. Intern Med. 2011;50:69–75.
42. Nishi H, Shibagaki Y, Hirano K, Akahane K, Kido R, Nangaku N, et al. Laboratory and imaging features of kidney involvement in autoimmune pancreatitis: incidence, correlation, and steroid therapy response. Clin Nephrol. 2010;73:253–9.

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Original Articles

Light-microscopic characteristics of IgG4-related tubulointerstitial nephritis: distinction from non-IgG4-related tubulointerstitial nephritis

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Abstract

Background. IgG4-related disease is a multi-organ disorder characterized by a high level of serum IgG4 and dense infiltration of IgG4-positive cells into affected organs. In routine studies, however, IgG subclasses are not estimated. In the present study, we attempted to clarify the light-microscopic characteristics of IgG4-related tubulointerstitial nephritis (TIN) to facilitate distinction from non-IgG4-related TIN in specimens obtained by renal biopsy using routine staining.

Methods. In specimens from 34 cases of TIN (13 IgG4-related and 21 non-IgG4-related), 9 nephrologists independently reviewed the following histological features of interstitial lesions: (i) cell infiltration extending into the renal capsule, (ii) cell infiltration into the renal medulla, (iii) regional lesion distribution, (iv) lymphoid follicles, (v) granulomatous lesions, (vi) necrotizing angiitis, (vii) eosinophil infiltration, (viii) neutrophil infiltration, (ix) tubulitis, (x) peritubular capillaritis, (xi) storiform fibrosis and (xii) the stage of interstitial fibrosis. The modified nominal group technique was applied to obtain a consensus in the pathological interpretation.

Results. Consensus was successfully attained among the diagnosticians for all but one pathological feature (regional lesion distribution). Storiform fibrosis was demonstrated in 12 of 13 (92.3%) cases of IgG4-related TIN but in none of the cases of other types of TIN. Cell infiltration extending into the renal capsule was also observed only in IgG4-related TIN. Conversely, neutrophil infiltration, severe tubulitis, severe peritubular capillaritis, granulomatous lesions and necrotizing angiitis were evident only in non-IgG4-related TIN.

Conclusions. This study revealed some useful and characteristic features for distinguishing IgG4-related from non-IgG4-related TIN on the basis of light-microscopic observation.

Keywords: IgG4; light microscopy; storiform fibrosis; tubulointerstitial nephritis

Introduction

IgG4-related disease (IgG4-RD) is a new clinical entity that has been attracting worldwide attention, being characterized by a high level of serum IgG4 and dense infiltration of IgG4-positive cells into affected organs [1–3]. The prototype of this condition was sclerosing pancreatitis [4] (also known as Type 1 autoimmune pancreatitis [5]), but it is known to affect various organs including the salivary glands, hepatobiliary tract, lymph nodes, lungs, retroperitoneum and kidneys [1, 2, 6, 7]. Recently, we reported that the major renal parenchymal lesion associated with IgG4-RD is tubulointerstitial nephritis (TIN) [8]. Because steroid therapy is usually quite effective, diagnosis of IgG4-related TIN is important. However, IgG4-related TIN is difficult to recognize in the absence of autoimmune pancreatitis or Mikulicz's disease, which are representative conditions of the disease [1–3] because serum IgG subclasses are not examined routinely and immunostaining for IgG subclasses is not a routine part of renal pathologic studies. Furthermore, recent studies have revealed that high serum IgG4 levels and/or IgG4-positive plasma cells can also be present in some inflammatory conditions that are not associated with IgG4-RD,

including anti-neutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitis [9–11]. On the other hand, several studies have revealed that certain common pathological features of IgG4-RD are evaluable in routine examinations, such as storiform fibrosis, eosinophilic infiltration, lymphoid follicles and obliterative phlebitis, and that these are also useful for diagnosis [5–8, 12–16]. In fact, we have observed that some pathological features including storiform fibrosis in IgG4-related TIN were similar to those observed in Type 1 autoimmune pancreatitis [8]. However, it is still unclear whether the pathological features of IgG4-related TIN are actually characteristic in comparison with those of non-IgG4-related TIN and whether a consensus can be obtained among diagnosticians in the interpretation of pathological features such as storiform fibrosis, because tubulointerstitial lesions have not been fully examined from this perspective in ordinary TIN. These background factors prompted us to examine the light-microscopic characteristics of IgG4-related TIN, in which pathological consensus is attainable among diagnosticians, to allow its distinction from non-IgG4-related TIN using routine staining.

Materials and methods

Patients

This study included 13 patients with IgG4-related TIN (IgG4 group) and 21 patients with the other types of TIN (non-IgG4 group). The cases in both groups were selected from the renal biopsy pathology files at the Division of Clinical Nephrology, Niigata University (including cases seen at Nagaoka Red Cross Hospital) and the Division of Rheumatology, Kanazawa University Hospital, between 1998 and 2010 (IgG4 group) and between 2004 and 2010 (non-IgG4 group), respectively. Cases in which the specimens included over 100 glomeruli (obtained by surgery, open biopsy or autopsy) were excluded. The diagnosis of IgG4-related TIN was based on both (i) a high serum IgG4 level (>135 mg/dL) [17] and (ii) infiltration of numerous IgG4-positive plasma cells into the renal interstitium (IgG4-positive plasma cells/IgG-positive plasma cells >40% and/or IgG4-positive plasma cells >10/high power field), along with clinical features [18, 19]. The patients in the IgG4 group were all Japanese (11 males and 2 females) with an average age of 69.2 years (range 55–83 years). Twelve of the 13 patients had some IgG4-related extra-renal lesions (autoimmune pancreatitis, sialadenitis, dacryoadenitis, lymph node swelling, lung lesion, prostatitis or pseudo-tumor of the liver). The serum creatinine levels were within the range 0.9–6.17 mg/dL (average 2.55 ± 1.89). The levels of serum IgG4 before steroid therapy were 486–1860 (mean 1091, normal range <105). Hypocomplementemia was evident in 61.5% of the patients. None of them met the criteria for systemic lupus erythematosus, ANCA-associated vasculitis or sarcoidosis. Nine of the 13 patients had been included in our earlier study [8] and four were newly enrolled and had not been previously described.

The patients in the non-IgG4 group had a main renal pathological diagnosis of TIN, excluding IgG4-related TIN, renal allograft rejection and TIN associated with progressive chronic primary glomerular disease. The etiology of TIN included drug use (n= 6), vesicoureteral reflux (n= 1), malignant hypertension (n = 1), sarcoidosis (n = 2), Sjögren's syndrome (n = 3), ANCA-related vasculitis (n = 3) and idiopathic (n = 5). They were all Japanese (11 males and 10 females) with an average age of 55.7 years (range 11–80 years). Sialadenitis was present in three patients with Sjögren's syndrome and a patient with sarcoidosis showed lung lesions. The other 17 patients had no extra-renal lesions. Serum creatinine levels were 0.96–14.4 mg/dL (average 3.52 ± 3.07). Serum IgG4 levels were examined in three patients (two idiopathic and one Sjögren's syndrome) and were within the normal range in all of them. No patient showed hypocomplementemia. In all cases of idiopathic TIN, immunostaining for IgG4 revealed no or only few IgG4-positive plasma cells in the renal interstitium (data not shown).

Histological evaluation

Renal tissues were obtained by needle biopsy from all patients, except one patient with vesicoureteral reflux. For routine light-microscopic studies, renal biopsy specimens were fixed in formalin or alcohol-Bouin, embedded in paraffin and stained with hematoxylin and eosin, periodic acid-Schiff, periodic acid-methenamine silver (PAM) and Masson's trichrome or Azan. All tissue slides were anonymized and reviewed independently by nine nephrologists (all experts in renal pathology and routinely involved in the diagnosis of renal biopsy samples), who were blinded to the clinical and serological data and the IgG4/non-IgG4 status of the patients.

The estimated items pertaining to histological features were decided on the basis of previous pathological studies of IgG4-RD [5–8, 12–16]: (i) cell (inflammatory cell) infiltration extending into the renal capsule (absent, present or not evaluable due to lack of a renal capsule in the specimens), (ii) cell (inflammatory cell) infiltration into the renal medulla (absent, present or not evaluable due to lack of a renal medulla in the specimens), (iii) regional lesion distribution, well defined and excluding small patchy lesions (absent or present), (iv) lymphoid follicles with germinal centers (absent or present), (v) granulomatous lesions (absent or present), (vi) necrotizing angiitis (absent or present), (vii) eosinophil infiltration (0, no; 1+, occasional and 2+, numerous), (viii) neutrophil infiltration (0, no; 1+, occasional and 2+, numerous), (ix) tubulitis (0, no inflammatory cells in tubules; 1, mild; 1–4 cells per tubule cross section; 2, moderate; 5–10 cells per tubule cross section; 3, severe; >10 cells per tubule cross section, judged from the highest number of all types of cells infiltrating each tubule in the whole specimen), (x) peritubular capillaritis (0, no; 1, mild; 2, moderate; 3, severe; assigned a 'ptc' score of 0–3 in the Banff 07 classification [20]), (xi) storiform fibrosis (absent or present), (xii) stage of interstitial fibrosis (0, no fibrosis; 1, mild; scattered fibrosis; 2, moderate; degree of fibrosis less predominant than that of cell infiltration; 3, severe; degree of fibrosis more predominant than that of cell infiltration; 4; only fibrosis). In each specimen, all stages and the main stage were described respectively.

'Storiform fibrosis' is a characteristic swirling pattern of fibrosclerosing inflammation consisting of inflammatory cells and irregular fibrosis evident in Type 1 autoimmune pancreatitis [12]. In our earlier study, we demonstrated a similar pattern of fibrosis in IgG4-related TIN and showed that the irregular fibrosis surrounded nests of inflammatory cells in PAM-stained preparations [8]. In the present study, we defined 'storiform fibrosis in IgG4-related TIN' as a pattern of fibrosclerosing inflammation consisting of both (i) dense collagen fibers, into which inflammatory cells had infiltrated, exhibiting a swirling or arabesque pattern in the renal interstitium and (ii) irregular fibers surrounding nests of inflammatory cells in PAM-stained preparations. Representative photographs of storiform fibrosis in IgG4-related TIN are shown in Figure 1. Reference photographs of the stages of interstitial fibrosis (Stage 4 was not evident in any of the cases examined) are shown in Figure 2. Storiform fibrosis was evaluable in Stages 2 and 3. In our earlier study of IgG4-related TIN [8], obliterative phlebitis was not evident although phlebitis was shown in some patients (data not shown). Therefore, obliterative phlebitis was not investigated in this study.

The modified nominal group technique developed by the RAND Corporation [21] was applied to obtain consensus in the histopathological interpretation of renal biopsy specimens among the nine nephrologists. Briefly, each nephrologist recorded his/her assessments according to the rating system for TIN described later. These ratings were collected centrally, and the most frequent ratings were chosen as tentative interpretations. Then, the nephrologists anonymously rated the tentative interpretations on a 9-point scale, in which 1 = extremely inappropriate, 5 = uncertain and 9 = extremely appropriate. After anonymous feedback of the distribution of the ratings made by their colleagues and anonymous discussion via electronic mail, a second round of ratings was undertaken confidentially for all specimens regardless of the initial degree of agreement. To determine the final degree of agreement and disagreement, a statistical definition using a binomial distribution was applied. Consensus was considered to exist when no more than two individuals rated a particular indication outside a 3-point range (i.e. 1–3, 4–6 and 7–9). Items for which the nine nephrologists were unable to reach an agreement were discussed further in a group meeting. Attempts were made to modify the wordings of the rating system, and subsequently the third anonymous rating round was undertaken for these particular items. If a consensus was still not attainable after the third round of ratings, the item was described as 'consensus failure'.

Statistical analysis

Statistical analyses were done using the Fisher's exact probability test or the Mann–Whitney's *U*-test. A probability of $P < 0.05$ was considered to indicate statistical significance.

Results

Renal pathology

The histological features of the IgG4 and non-IgG4 groups are summarized in Table 1. Consensus was successfully attained among the nine nephrologists for all but one pathological feature (a regional lesion distribution) in two cases. The features that were evident only in the IgG4 group were storiform fibrosis and 'cell infiltration extending into the renal capsule'. Storiform fibrosis was evident in 12/13 patients (92.3%) in the IgG4 group but in none of the non-IgG4 group ($P < 0.0001$) (Figure 3). Cell infiltration extending into the renal capsule, although this feature was not evaluable in many patients in the both groups, was evident in two patients in the IgG4 group (Figure 4). Conversely, neutrophil infiltration, granulomatous lesions and necrotizing angiitis were evident only in the non-IgG4 group [neutrophil infiltration = 12/21 ($P = 0.0010$), granulomatous lesions = 5/21 ($P = 0.1317$) and necrotizing angiitis = 5/21 patients ($P = 0.1317$)]. The grades of tubulitis were significantly lower in the IgG4 group (in the IgG4 group, stage of tubulitis was 0 in 7.7%, 1 in 76.9%, 2 in 15.4% and 3 in 0%, whereas in the non-IgG4 group, it was 0 in 0%, 1 in 33.3%, 2 in 47.6% and 3 in 19.0%, $P = 0.0026$). Severe tubulitis was evident only in the non-IgG4 group. Although the grades of peritubular capillaritis were not significantly different between the two groups, severe peritubular capillaritis was also evident only in the non-IgG4 group. A regional lesion distribution was observed more frequently in the IgG4 group (5/12 in the IgG4 and 1/20 in the non-IgG4, $P = 0.0185$); however, consensus in the pathological interpretation was not attainable in two patients. Cell infiltration into the renal medulla was observed in both groups (5/6 in the IgG4 and 12/15 in the non-IgG4, P = not significant). Eosinophil infiltration was evident in 30.8% of the patients in the IgG4 group and 9.5% in the non-IgG4 group, but the difference was not significant. Lymphoid follicles were evident in only one patient in the non-IgG4 group.

The results of interstitial fibrosis staging are summarized in Table 2. The stages of fibrosis were mixed in most cases in both groups. The IgG4 group had significantly higher stages of fibrosis than the non-IgG4 group (in the IgG4 group, main stage of fibrosis was mild in 15.4%, moderate in 61.5% and severe in 23.1%, whereas in the non-IgG4 group, it was mild in 57.1%, moderate in 42.9% and severe in 0%, $P = 0.0054$).

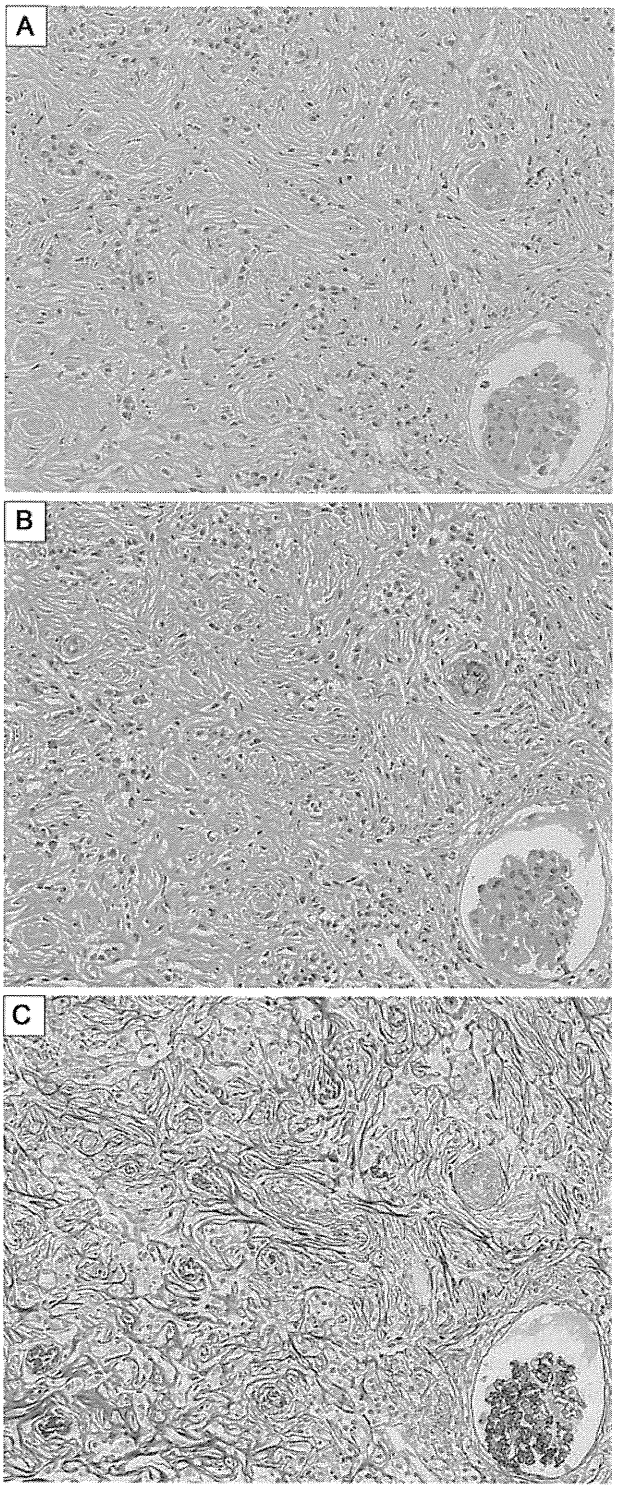

Fig. 1. Storiform fibrosis in IgG4-related TIN. A swirling or arabesque pattern consisting inflammatory cells and collagen fibers, corresponding to storiform fibrosis in Type 1 autoimmune pancreatitis, is evident (**A** and **B**). In PAM stain, irregular fibers surround nests of inflammatory cells (**C**). (**A**) Hematoxylin and eosin, (**B**) Elastica–Masson trichrome, (**C**) PAM. All images ×200 in almost the same field .

Discussion

Although the etiology of IgG4-RD has not been elucidated, some common pathological characteristics of this disease have been demonstrated. Dense lymphoplasmacytic infiltration with fibrosis and infiltration of numerous IgG4-positive plasma cells are the most characteristic

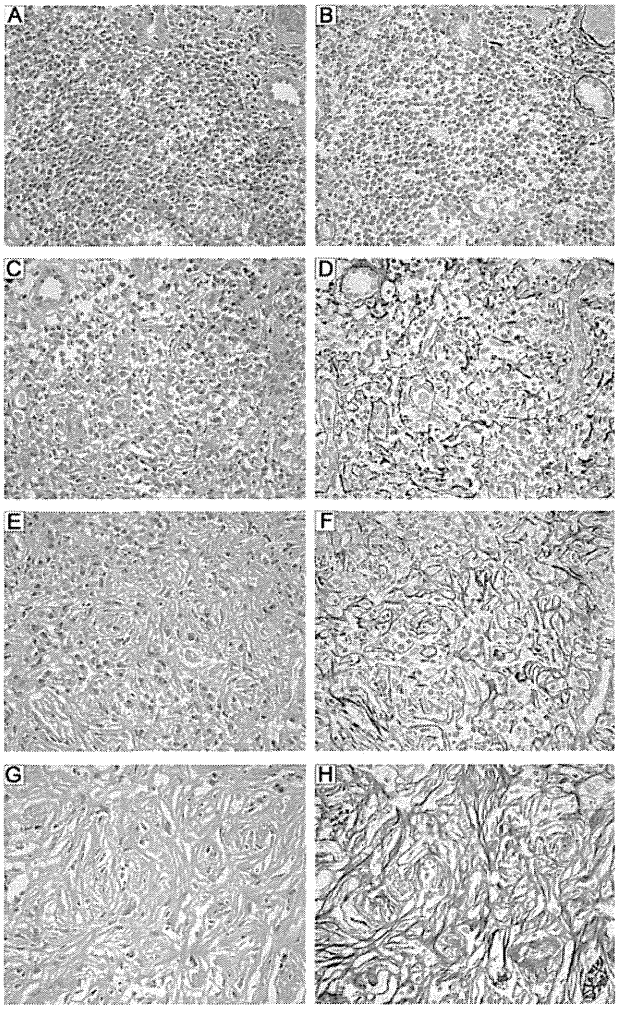

Fig. 2. The stages of interstitial fibrosis. Stage 0, no fibrosis (**A** and **B**); 1, mild; scattered fibrosis (**C** and **D**); 2, moderate; degree of fibrosis less predominant than that of cell infiltration (**E** and **F**); 3, severe; degree of fibrosis more predominant than that of cell infiltration (**G** and **H**). (**A**, **C**, **E** and **G**): Elastica–Masson trichrome, (**B**, **D**, **F** and **H**) PAM (×400). Each stage is from almost the same field in IgG4-related TIN.

features [1–8, 12–16, 18, 19] and storiform fibrosis, eosinophil infiltration, lymphoid follicles, inflammation around the margins of affected tissues, a regional lesion distribution and obliterative phlebitis have also been considered to be common features [5–8, 12–16, 22]. On the other hand, neutrophil infiltration and inflammation of the duct epithelium in affected organs are rare [7, 12, 13, 16]. In IgG4-related TIN, lymphoplasmacytic infiltration with numerous IgG4-positive plasma cells and fibrosis are also very important features [8]. Recently, Raissian *et al.* [18] revealed that the presence of plasma cell-rich TIN with numerous IgG4-positive plasma cells has diagnostic utility, with a sensitivity of 100% and specificity of 92%, for IgG4-related TIN, excluding pauci-immune necrotizing and crescentic glomerulonephritis. However, lymphoplasmacytic infiltration is a non-specific finding in TIN, and immunostaining is not performed routinely. Furthermore, recent studies have revealed that numerous IgG4-positive plasma cells can also be present in conditions not associated with IgG4-RD [9–11]. Houghton *et al.* [9] described that the presence of numerous IgG4-positive plasma cells is essential to, but not sufficient for, the diagnosis of IgG4-related TIN. In addition, there is organ specificity in the pathological features of IgG4-RD [7]. For example, storiform fibrosis is not evident in the lymph nodes, minor salivary glands [7] or lung lesions [23]. It is important to examine the pathological findings closely in each organ and elucidate points of similarities and differences. In this study, we have revealed some useful and characteristic features for distinguishing IgG4-related from non-IgG4-related TIN on the basis of light-microscopic observation using routine staining, with consensus among diagnosticians. In particular, storiform fibrosis was revealed to be quite characteristic and useful for diagnosis of IgG4-related TIN. Because renal biopsy is usually applied to non-atrophic kidneys, dense interstitial fibrosis is noted relatively rarely in specimens obtained by biopsy. Interestingly, however, most of the patients with IgG4-related TIN showed high grades of fibrosis, even those with mild renal dysfunction, and the characteristic pattern was easy to recognize.

Cell infiltration extending into the renal capsule was evident in two patients in the IgG4 group. Because this feature is ordinarily not evident in other types of TIN, it might also be diagnostic of IgG4-related TIN. However, this feature was not evaluable in many of the patients in both groups, because specimens obtained by renal biopsy and subjected to light microscopy often lack the renal capsule. Therefore, further study to confirm this will be necessary. Neutrophil infiltration and severe tubulitis were not evident in IgG4-related TIN. Neutrophil infiltration has been described as rare in head and neck, hepatic and pancreatobiliary and retroperitoneal lesions in IgG4-RD [7]. Also, rarity of inflammation of the duct epithelium has been demonstrated in Type 1 autoimmune pancreatitis and IgG4-related sclerosing cholangitis [6, 12], suggesting these features are common in IgG4-RD. A regional lesion distribution was evident more frequently in the IgG4 group, suggesting that this may also be useful for the diagnosis of IgG4-related TIN. However, pathological interpretation of this feature was difficult in some patients, and a consensus could not be reached in two patients. Lymphoid follicles were evident in only one of all patients. Because specimens obtained by needle renal biopsy are quite small, lymphoid follicles with germinal centers might be difficult to discern. Eosinophil infiltration was often evident in IgG4-related TIN and so, IgG4-related TIN should be considered in the differential diagnosis of TIN with eosinophils. But there was no significant inter-group difference in this respect. Because eosinophil infiltration has also been reported as a common feature of non-IgG4-related TIN [24], it should be considered as not being diagnostic but rather a supportive feature of IgG4-related TIN. Granulomatous lesions and necrotizing angiitis, which are sometimes observed in sarcoidosis or ANCA-related vasculitis, were not evident in IgG4-related TIN, suggesting that they are useful features for distinguishing between these diseases, although

Table 1. Histological features of IgG4-related and non-IgG4-related TIN[a]

	IgG4 ($n=13$)	Non-IgG4 ($n=21$)	P
Cell infiltration extending into the renal capsule	2/7 (NE 6)	0/7 (NE 14)	0.4615
Cell infiltrations into the renal medulla	5/6 (NE 7)	12/15 (NE 6)	>0.9999
Regional lesion distribution	5/12 (CF 1)	1/20 (CF 1)	0.0185
Lymphoid follicles	0/13 (0%)	1/21 (4.8%)	>0.9999
Granulomatous lesions	0/13 (0%)	5/21 (23.8%)	0.1317
Necrotizing angiitis	0/13 (0%)	5/21 (23.8%)	0.1317
Storiform fibrosis	12/13 (92.3%)	0/21 (0%)	<0.0001
Eosinophil infiltration			
Grade 0	9/13 (69.2%)	19/21 (90.5%)	0.1084
Grade 1	3/13 (23.1%)	2/21 (9.5%)	
Grade 2	1/13 (7.7%)	0/21 (0%)	
Neutrophil infiltration			
Grade 0	13/13 (100%)	9/21 (42.9%)	0.0010
Grade 1	0/13 (0%)	9/21 (42.9%)	
Grade 2	0/13 (0%)	3/21 (14.3%)	
Tubulitis			
Grade 0	1/13 (7.7%)	0/21 (0%)	0.0026
Grade 1	10/13 (76.9%)	7/21 (33.3%)	
Grade 2	2/13 (15.4%)	10/21 (47.6%)	
Grade 3	0/13 (0%)	4/21 (19.0%)	
Peritubular capillaritis			
Grade 0	5/13 (38.5%)	5/21 (23.8%)	0.0649
Grade 1	5/13 (38.5%)	4/21 (19.0%)	
Grade 2	3/13 (23.1%)	8/21 (38.1%)	
Grade 3	0/13 (0%)	4/21 (19.0%)	

[a]NE, not evaluable; CF, consensus failure; grade of eosinophil and neutrophil infiltration: Grade 0, no; 1+, occasional and 2+, numerous; grade of tubulitis and peritubular capillaritis: Grade 0, no; 1, mild; 2, moderate and 3, severe.

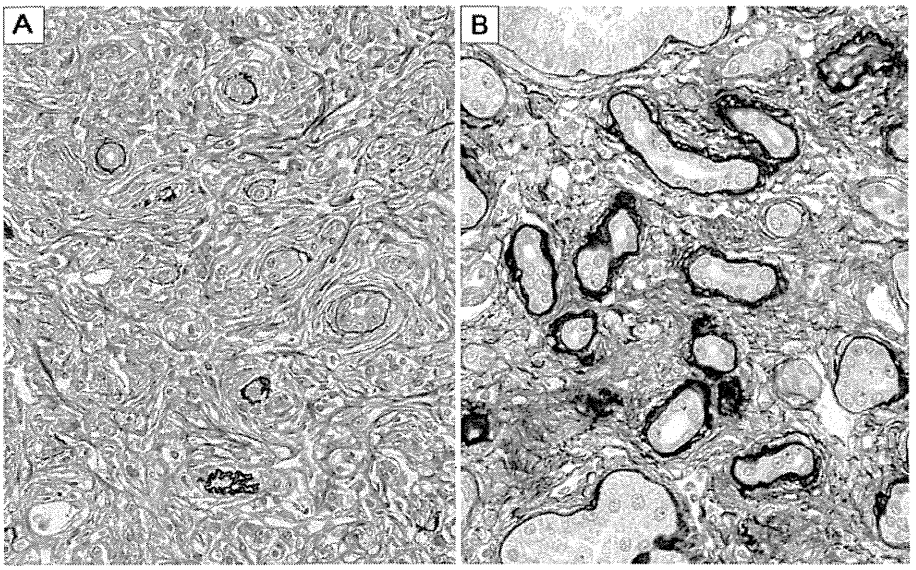

Fig. 3. Interstitial fibrosis of IgG4-related TIN and non-IgG4-related TIN. Characteristic storiform fibrosis is evident in IgG4-related TIN (**A**) but not in non-IgG4-related TIN (**B**) (PAM–Masson trichrome, ×400).

granulomas have rarely been observed in extra-renal organs associated with IgG4-RD [7].

Except for the items examined in this light-microscopic study using routine staining, immune complex deposits in the renal tubule basement membranes by immunofluorescence, immunohistochemistry and/or electron microscopy have been shown to be a characteristic feature of IgG4-related TIN [18, 25]. On the basis of these pathological features, nephrologists might be able to recognize IgG4-related TIN in specimens obtained by renal biopsy using routine methods, even when no data for IgG subclass are available.

In this study, each item was examined closely by nine diagnosticians in a blinded manner, and a modified nominal group technique was used to elucidate whether consensus can be obtained among diagnosticians in the interpretation of pathological features. For this purpose, the study employed a relatively small cohort size, and

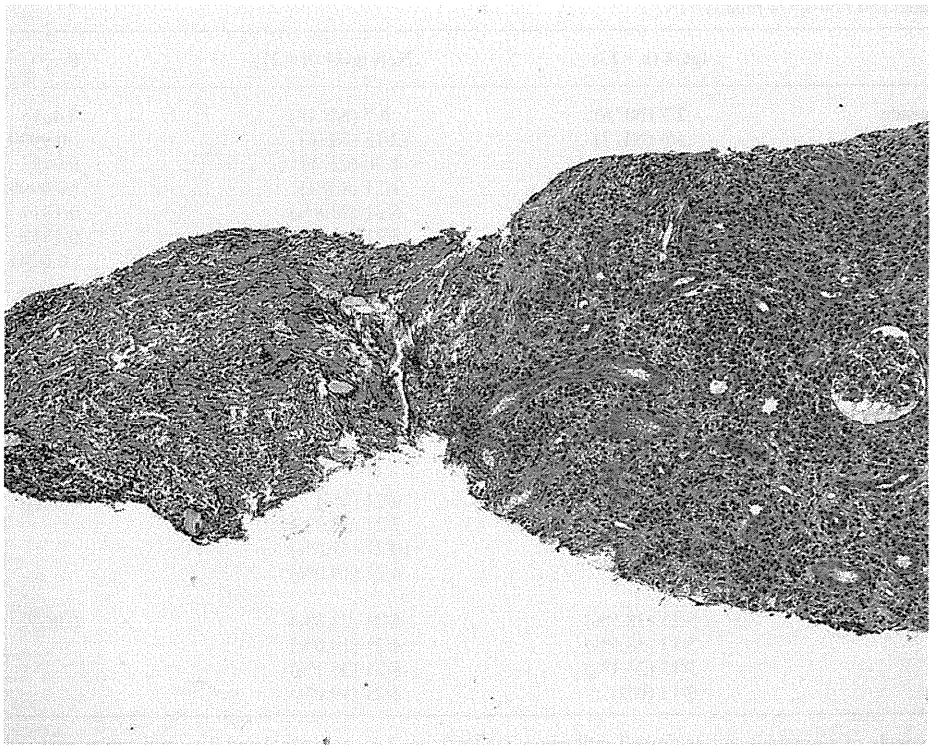

Fig. 4. Cell infiltration extending into the renal capsule. Inflammatory cells are evident within the renal capsule (Masson's trichrome, ×100).

Table 2. Stages of interstitial fibrosis of IgG4 and non-IgG4-related TIN[a]

	IgG4 ($n=13$)	Non-IgG4 ($n=21$)	P
Mean s-Cr (mg/dL) (range)	2.55 (0.9–6.17)	3.52 (0.96–14.4)	0.2719
Main stage			
1	2/13 (15.4%)	12/21 (57.1%)	0.0054
2	8/13 (61.5%)	9/21 (42.9%)	
3	3/13 (23.1%)	0/21 (0%)	
All stages			
0+1	1/13	1/21	
1	0/13	2/21	
0+1+2	0/13	4/21	
1+2	2/13[b]	5/21	
0+1+2+3	2/13[b]	3/21	
1+2+3	8/13[b]	6/21	

[a]s-Cr, serum creatinine; Stage 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis and 3, severe fibrosis.
[b]Storiform fibrosis was evident.

addition of TIN cases proven to have non-IgG4 status as controls was unfortunately impossible. However, including more non-related TIN cases would make the P-values more significant for certain features (granulomatous lesion or necrotizing angiitis). Further studies employing a larger cohort size and including all types of TIN as a control will be necessary to clarify the positive and negative predictive values of each item for diagnosis of IgG4 TIN.

In conclusion, the present study identifies some useful and characteristic features for distinguishing IgG4-related TIN from non-IgG4-related TIN in specimens examined by light microscopy. However, the significance of these pathological findings and the etiology of IgG4-RD remain poorly understood. Further studies will be necessary to elucidate the underlying mechanisms.

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Conflict of interest statement. None declared.

References

1. Kamisawa T, Okamoto A. IgG4-related sclerosing disease *World J Gastroenterol* 2008; 14: 3948–3955.
2. Masaki Y, Dong L, Kurose N *et al.* Proposal for a new clinical entity, IgG4-positive multi-organ lymphoproliferative syndrome: analysis of 64 cases of IgG4-related disorders *Ann Rheum Dis* 2009; 68: 1310–1315.
3. Yamamoto M, Takahashi H, Ohara M *et al.* A new conceptualization for Mikulicz's disease as an IgG4-related plasmacytic disease *Mod Rheumatol* 2006; 16: 335–340.
4. Hamano H, Kawa S, Horiuchi A *et al.* High serum IgG4 concentrations in patients with sclerosing pancreatitis *N Engl J Med* 2001; 344: 732–738.
5. Chari ST, Kloeppel G, Zhang L *et al.* Histopathologic and clinical subtypes of autoimmune pancreatitis. The Honolulu consensus document *Pancreas* 2010; 39: 549–554.
6. Cheuk W, Chan JK. IgG4-related sclerosing disease: a critical appraisal of an evolving clinicopathologic entity *Adv Anat Pathol* 2010; 17: 303–332.
7. Zen Y, Nakanuma Y. IgG4-related disease. A cross-sectional study of 114 cases *Am J Surg Pathol* 2010; 34: 1812–1819.

8. Saeki T, Nishi S, Imai N *et al.* Clinicopathological characteristics of patients with IgG4-related tubulointerstitial nephritis *Kidney Int* 2010; 78: 1016–1023.
9. Houghton DC, Troxell ML. An abundance of IgG4 + plasma cells is not specific for IgG4-related tubulointerstitial nephritis *Mod Pathol* 2011; 24: 1480–1487.
10. Yamamoto M, Takahashi H, Suzuki C *et al.* Analysis of serum IgG subclasses in Churg-Strauss syndrome—the meaning of elevated serum levels of IgG4 *Intern Med* 2010; 49: 1365–1370.
11. Strehl JD, Hartmann A, Agaimy A. Numerous IgG4-positive plasma cells are ubiquitous in diverse localized non-specific chronic inflammatory conditions and need to be distinguished from IgG4-related systemic disorders *J Clin Pathol* 2011; 64: 237–243.
12. Notohara K, Wani Y, Fujisawa M. Pathologic findings of autoimmune pancreatitis and IgG4-related disease *Curr Immunol Rev* 2011; 7: 212–220.
13. Zen Y, Inoue D, Kitao A *et al.* IgG4-related lung and pleural disease: a clinicopathologic study of 21 cases *Am J Surg Pathol* 2009; 33: 1886–1893.
14. Zen Y, Onodera M, Inoue D *et al.* Retroperitoneal fibrosis: a clinicopathologic study with respect to immunoglobulin G4 *Am J Surg Pathol* 2009; 33: 1833–1839.
15. Nakanuma Y, Zen Y. Pathology and immunopathology of immunoglobulin G4-related sclerosing cholangitis: the latest addition to the sclerosing cholangitis family *Hepatol Res* 2007; 37: S478–S486.
16. Deshpande V, Gupta R, Sainani N *et al.* Subclassification of autoimmune pancreatitis: a histologic classification with clinical significance *Am J Surg Pathol* 2011; 35: 26–35.
17. Okazaki K, Kawa S, Kamisawa T *et al.* Clinical diagnostic criteria of autoimmune pancreatitis: revised proposal *J Gastroenterol* 2006; 41: 626–631.
18. Raissian Y, Nasr SH, Larsen CP *et al.* Diagnosis of IgG4-related tubulointerstitial nephritis *J Am Soc Nephrol* 2011; 22: 1343–1352.
19. Kawano M, Saeki T, Nakashima H *et al.* Proposal for diagnostic criteria for IgG4-related kidney disease *Clin Exp Nephrol* 2011; 15: 615–626.
20. Solez K, Colvin RB, Racusen LC *et al.* Banff 07 classification of renal allograft pathology: updates and future directions *Am J Transplant* 2008; 8: 753–760.
21. Murphy MK, Black NA, Lamping DL *et al.* Consensus development methods, and their use in clinical guideline development *Health Technol Asess* 1998; 2: i–iv, 1–88
22. Saeki T, Nishi S, Ito T *et al.* Renal lesions in IgG4-related systemic disease *Intern Med* 2007; 46: 1365–1372.
23. Shrestha B, Sekiguchi H, Colby TV *et al.* Distinctive pulmonary histopathology with increased IgG4-positive plasma cells in patients with autoimmune pancreatitis *Am J Surg Pathol* 2009; 33: 1450–1462.
24. Nadasdy T, Sedmak D. Acute and chronic tubulointerstitial nephritis. In: Jennette JC (eds). *Heptinstall's Pathology of the Kidney.* 6th edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2007 pp. 1083–1137.
25. Cornell LD, Chicano SL, Deshpande V *et al.* Pseudotumors due to IgG4 immune-complex tubulointerstitial nephritis associated with autoimmune pancreatocentric disease *Am J Surg Pathol* 2007; 31: 1586–1597.

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Timing of initiation of renal replacement therapy for acute kidney injury: a survey of nephrologists and intensivists in Canada

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Abstract

Background. Little is known about factors that influence the timing of initiation of renal replacement therapy (RRT) for acute kidney injury (AKI). We sought to better describe these factors for Canadian physicians that prescribe RRT for AKI.

Methods. A web-based survey was conducted of physicians involved in the decision to initiate RRT for critically ill patients in Canada. Participants were asked about the factors that prompt them to initiate RRT for AKI both directly and using scenario-based questions.

Results. Surveys completed by 180 physicians at 32 different sites were included for analysis. Serum potassium level and severity of pulmonary edema were the most commonly utilized factors for deciding when RRT should be started. For all clinical and laboratory factors inquired about, there

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MyD88-dependent interleukin-10 production from regulatory CD11b$^+$Gr-1^{high} cells suppresses development of acute cerulein pancreatitis in mice

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ABSTRACT

We explored the role of the MyD88 signaling pathway. This pathway mediates the recognition of pathogen-associated molecular patterns and damage-associated molecular patterns via Toll-like receptors (TLRs) and/or IL-1/IL-18 via each cytokine receptor in a murine model of acute pancreatitis induced by cerulein administration. Our analysis revealed that: various TLRs and MyD88 molecules were constitutively expressed in the pancreas of cerulein-treated and untreated wild-type (WT) mice. MyD88$^{-/-}$ mice administered cerulein developed severe pancreatitis as compared with MyD88$^{+/+}$ WT mice. The number of IL-10-expressing CD11b$^+$Gr-1^{high} cells in cerulein-administered MyD88$^{-/-}$ mice was significantly decreased. This was in accordance with a reciprocal increase in the infiltration of CD4$^+$ T cells as compared with that in control MyD88$^{+/+}$ mice. WT mice pretreated with antibiotics and administered cerulein developed milder pancreatitis as compared with control cerulein-administered mice without antibiotic treatment. The MyD88 signaling pathway contributes to the induction of regulatory IL-10-producing macrophages/myeloid-derived suppressor cells, possibly in response to non-bacterial components in the damaged pancreas. These results provide a new concept for therapeutic strategies against acute pancreatitis.

1. Introduction

Severe acute pancreatitis is an intractable disease, which has an approximate 20% mortality rate because of multiple organ failure [1,2]. The pathogenesis of acute pancreatitis begins in an autodigestive manner [3], but the subsequent inflammatory responses may be critically involved in the pathogenesis of acute pancreatitis [4,5]. As such, innate immune cells such as macrophages possibly play a crucial role in acute pancreatitis [6,7]. We have recently demonstrated sequential steps of macrophage migration and activation in the pathogenesis of cerulein-induced acute pancreatitis in mice. CCL2/CCR2-dependent migration of pathological CD11b$^+$Gr-1^{low} macrophages in the damaged pancreas, and SOCS3-dependent activation of these macrophages aids with production of TNF-α [8]. The activated macrophages in the inflamed pancreas may crosstalk with the eventual effect or cells to establish acute pancreatitis [9]. However, the precise immunological mechanisms involved in suppressing acute pancreatitis remain largely unclear. To clarify this issue, we investigated the role of innate immune responses via MyD88 molecules using a murine acute pancreatitis model induced by intraperitoneal cerulein administration [10,11]. These MyD88 molecules are adaptor molecules for the main pathway of Toll-like receptors (TLRs) [12–14] and receptors for IL-1 and IL-18 [15].

2. Materials and methods

2.1. Mice

MyD88$^{-/-}$ mice were kindly provided by S. Akira (Osaka University) [16]. C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). MyD88$^{-/-}$ mice were backcrossed with C57BL/6 wild-type (WT) mice, and the F1 mice were intercrossed to generate the littermate MyD88$^{+/+}$ and MyD88$^{-/-}$ mice in our animal facility. All animals were maintained under specific pathogen-free conditions in the Animal Care Facility of Keio University School of Medicine. All experiments were approved by the regional animal study committees and were performed according to institutional guidelines and Home Office regulations.

Abbreviations: APC, antigen presenting cell; MDSC, myeloid-derived suppressor cell; MyD88, myeloid gene 88; TLR, Toll-like receptor; WT, wild-type.

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2.2. Cerulein-induced pancreatitis model

Cerulein (Sigma–Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS; Wako, Osaka, Japan) and was injected intraperitoneally (8 doses of 50 μg/kg, 1 h apart). Mice were euthanized by cervical dislocation 1 h after the last cerulein injection. Before euthanasia, whole blood was collected from the ocular artery under light ether anesthesia. The pancreas was excised and weighed immediately. For histological analysis, the pancreas was fixed with 10% formaldehyde (Wako). Serum amylase and lipase levels were determined with commercially available assays (SRL, Tokyo, Japan). Harvested small tissues from the pancreas tail were immediately placed in a sufficient volume of RNA later RNA Stabilization Reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations and used for RNA extraction.

2.3. Antibiotic treatment

C57BL/6J mice were orally administered a cocktail of antibiotics (1 g/l ampicillin, 500 mg/l vancomycin, 1 g/l neomycin sulfate, and 1 g/l metronidazole) for 2 weeks prior to the first administration of cerulein [17]. Control mice received only water without antibiotics.

2.4. Preparation of pancreatic mononuclear cells

Preparation of pancreatic mononuclear cells was performed as described previously [8]. Briefly, harvested pancreas was cut into small fragments and digested with collagenase A (3 mg/ml in Hank's balanced salt solution) while stirring for 15 min. The tissue was then passed through a pipette and filtered through a 40-μm pore strainer (BD Falcon, Franklin Lakes, NJ). Acinar cells were excluded by centrifugation (50 × *g*, 30s) and cells further purified using a 40–75% Percoll gradient method.

2.5. Flowcytometry

After blocking with anti-FcR (CD16/32; BD Bioscience, Franklin Lakes, NJ) for 15 min, cells were incubated with a specific fluorescence-labeled monoclonal antibody (mAb) at 4 °C for 20 min [18]. The following mAbs were used: CD3e-APCCy7; CD4-PECy7; CD11b-PECy7; Ly6G/Ly6C-APCCy7 (Gr-1, clone RB6-8C5); CD80-PE; CD86-PE; corresponding isotype IgGs (BD Bioscience); and CD69-FITC; (eBioscience, San Diego, CA). Dead cells were eliminated by 7-aminoactinomycin D (BD Bioscience) and stained cells were analyzed by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). Data were reanalyzed using FlowJo software (v7.2.5; Tree Star Inc., Ashland, OR). The mean fluorescence intensity (MFI) was calculated as follows:

MFI = (CD80 or CD86 geometric mean) − (isotype geometric mean)

2.6. Intracellular cytokine staining

Pancreas mononuclear cells were cultured with or without 1 μg/ml lipopolysaccharide in 10% fetal calf serum-containing RPMI1640 medium for 5 h. After staining with CD11b-PECy7, Ly6G/Ly6C-APCCy7, and CD3e-PerCPCy5.5 antibodies, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocols. For cytokine staining, TNFα-PE and IL-10-APC antibodies (BD Bioscience) were used.

2.7. Cell counts

Cells were counted using a light microscope. To avoid counting dead cells, cells were incubated with 0.4% (w/v) trypanblue (Invitrogen, Carlsbad, CA). The absolute number of mononuclear cells in the pancreas was estimated as follows:

Total cell count = (isolated cell count) × (total wet weight)/(wet weight used for cell isolation). The cell number for each leukocyte subset was calculated by observing 7-AAD$^-$ staining, characteristic forward scatter, and side scatter.

2.8. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from pancreas tissue preserved in RNA later using an RNeasy Mini Kit (QIAGEN) [19]. Complementary DNA was synthesized from 1 μg of total RNA by reverse transcription. To determine the expression of TLRs in the pancreas, PCR was performed using an AmpliTaq Gold Fast PCR MasterMix (Applied Biosystems, Carlsbad, CA) as per the manufacturer's protocol. Primer sequences were described as follows:

TLR1, 5′-AGAGACTTCCGAAACAGCTATGT-3′,
5′-GACAGAGCCTGTAAGCATATTCG-3′;
TLR2, 5′-AGATTTCAGCTAGGCGCTGTA-3′,
5′-TACCCAGCTCGCTCACTACGT-3′;
TLR3, 5′-AACAGAAGACGTGCTTGGAC-3′,
5′-CGCAACGCAAGGATTTTATT-3′;
TLR4, 5′-GTTCTTGTATCTAGACTCGAAGTTGGG-3′,
5′-GCTGTCCAATAGGGAAGCTTTCTAGAG-3′;
TLR5, 5′-TGACTTAAGGAATTCGCTGCAT-3′,
5′-AGAAGATAAAGCCGTGCGAAA-3′;
TLR6, 5′-TTGTCCTATGCCTCGGAACT-3′,
5′-CCAGGAAAGTCAGCTTCGTC-3′;
TLR7, 5′-AAGGCTATGCTACTTATACGTGC-3′,
5′-TGAGTTTGTCCAGAAGCCGTAAT-3′;
TLR8, 5′-CCGTGTTGAGGGAACACTAA-3′,
5′-CATTTGGGTGCTGTTGTTTG-3′;
TLR9, 5′-GGCGTTCTGAGATAAACACGACC-3′,
5′-TGTCCCTAGTCAGGGCTGTACTCAG-3′;
MyD88, 5′-CCGGAACAATCTGGCACTCC-3′,
5′-TCATCTTCCCCTCTGCCCTA-3′.

After PCR amplification, products were detected by 2.0% agarose gel electrophoresis and stained with ethidium bromide.

2.9. Quantitative PCR

IL-6 (Mm00446190_m1), TNF-α (Mm00443258), and proIL-1beta (Mm01336189_m1), and proIL-18 (Mm00434225) mRNA expression was assessed by reverse transcription followed by real-time PCR using the TaqMan Gene Expression Assays kit according to the manufacturer's recommendations (Applied Biosystems). Briefly, 1 μg of total pancreatic RNA was reverse transcribed into cDNA using a TaqMan Universal Master Mix. Quantitative PCRs were performed using the StepOnePlus™ System (Applied Biosystems) and TaqMan technology. Data were analyzed using the relative standard curve method, and expression levels were normalized to the expression of hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1, Mm00446973_m1)**.

2.10. Histological score

Photomicrographs were acquired with the aid of a light microscope (Nikon, Tokyo, Japan) and ACT software (Nikon). Histological scoring was performed using a point-counting morphometry method as described previously [8]. Briefly, a 10 × 10-μm grid was superimposed on three randomly chosen fields of view, and the interstitial space under each grid point was counted.

2.11. Statistical analysis

The Mann–Whitney *U* test was used for statistical analysis of histological scores, and an unpaired Student's *t* test was used for other analyses. All analyses were performed with GraphPad Prism 5 for Windows software (GraphPad Software Inc., San Diego, CA), and all data are presented as mean ± SEM.

3. Results

3.1. Development of severe cerulein-induced pancreatitis in MyD88$^{-/-}$ mice

To investigate the role of the innate immune system in a murine model of cerulein-induced acute pancreatitis [8,10,11], we examined mRNA expression of TLR1–9 and their adaptor molecule, MyD88, in the pancreas. RT-PCR analysis revealed that pancreas tissue samples expressed all members of TLR1–9, and MyD88, irrespective of whether cerulein or PBS was administered to WT mice (Fig. 1A).

We then assessed the severity of pancreatitis in cerulein-administered littermate MyD88$^{+/+}$ and MyD88$^{-/-}$ mice (Fig. 1B). PBS-administered MyD88$^{+/+}$ and MyD88$^{-/-}$ mice did not exhibit any pathological changes. The concentrations of serum amylase and lipase in those mice were at baseline levels (Fig. 1E). Microscopic analysis revealed that the pathology of MyD88$^{-/-}$ mice given cerulein injections was more severe when compared with MyD88$^{+/+}$ mice given cerulein (Fig. 1C). This was statistically confirmed by objectively assessing the interstitial space (Fig. 1D). Consistent with this, the concentrations of serum amylase and lipase in cerulein-injected MyD88$^{-/-}$ mice were significantly increased as compared with those in cerulein-injected MyD88$^{+/+}$ mice (Fig. 1E). The level of IL-6 mRNA expression in the pancreas of cerulein-injected MyD88$^{-/-}$ mice was significantly increased as compared with that in cerulein-injected MyD88$^{+/+}$ mice, but the levels of TNF-α and proIL-1β mRNA expression were inversely decreased in MyD88$^{-/-}$ pancreas (Fig. 1F). The level of pro-IL-18 mRNA expression was comparable between MyD88$^{+/+}$ mice and MyD88$^{-/-}$ mice (Fig. 1F).

3.2. Reduced numbers of pancreatic CD11b^{+}Gr-1^{high} cells in cerulein-injected MyD88$^{-/-}$ mice

We focused on pancreatic antigen-presenting cells (APCs), as we have previously shown that pancreatic APCs are involved in pathogenesis in this model [8]. We categorized CD11b^{+} cells in the pancreas as CD11b^{+}Gr-1^{-} (resident macrophages), CD11b^{+}Gr-1^{low} (migratory macrophages), and CD11b^{+}Gr-1^{high} (macrophages/granulocytes/myeloid-derived suppressor cells) subpopulations. We designated these subsets as S1, S2, and S3, respectively (Fig. 2A) [8]. Statistical analysis revealed that the absolute number of CD11b^{+}Gr-1$^{negative\text{-}low}$ S1 and S2 macrophages was comparable between MyD88$^{+/+}$ and MyD88$^{-/-}$ mice given cerulein injections, while that of the CD11b^{+}Gr-1^{high} S3 subpopulation was significantly decreased in cerulein-administered MyD88$^{-/-}$ mice as compared with cerulein-administered MyD88$^{+/+}$ mice (Fig. 2B). These results suggest that a decreased S3 subpopulation in the pancreas of cerulein-administered MyD88$^{-/-}$ mice would contribute to functions as suppressor cells, resulting in the exacerbation of pancreatitis in those mice.

3.3. IL-10-producing CD11b^{+}Gr-1^{high} cells were significantly decreased in the pancreas of cerulein-injected MyD88$^{-/-}$ mice

To explore the suppressive activity S3 cells that were decreased in cerulein-administered MyD88$^{-/-}$ mice, we assessed IL-10 production in those cells, as we previously reported the possibility that

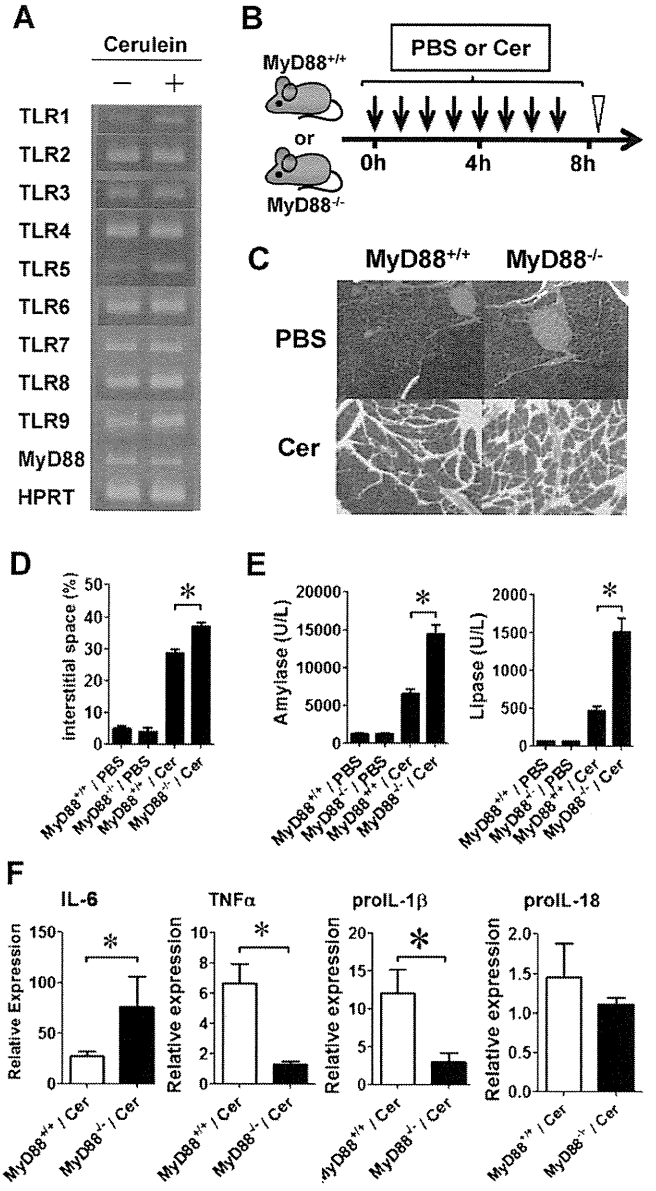

Fig. 1. MyD88$^{-/-}$ mice developed more severe cerulein-induced pancreatitis. (A) The mRNA expression of various TLRs and MyD88 in mouse pancreas treated with or without cerulein. (B) Study design for acute cerulein pancreatitis. MyD88$^{-/-}$ and MyD88$^{+/+}$ mice were given eight intraperitoneal doses of cerulein (50 μg/kg) or PBS, spaced 1 h apart. All mice were euthanized 1 h after the last injection. (C) Representative hematoxylin and eosin staining. (D) Histological score (each group, $n = 9$). (E) Serum amylase and lipase ($n = 9$) (F) IL-6, TNFα, proIL-1β, proIL-18 mRNA expression in the pancreas given cerulein injections ($n = 7$). HPRT, hypoxanthine phosphoribosyltransferase; Cer, cerulein.

the S3 subpopulation includes IL-10-producing myeloid-derived suppressor cells (MDSCs) [8]. IL-10 production appeared to be more prominent in the S3 subpopulation than in S1 or S2 (Fig. 3C and D). IL-10 production by APCs from cerulein-administered MyD88$^{-/-}$ mice was significantly reduced as compared with that in cerulein-administered MyD88$^{+/+}$ mice (Fig. 3C and D). This suggests that the S3 subpopulation, including MDSCs, may have greater contribution to the suppression of acute pancreatitis development.

3.4. MyD88 deficiency results in abnormal increase and activation of CD4^{+} T cells

The CD4^{+} T cells in the pancreas, which have been reported to be terminal effector cells for this model [9], are possibly regulated

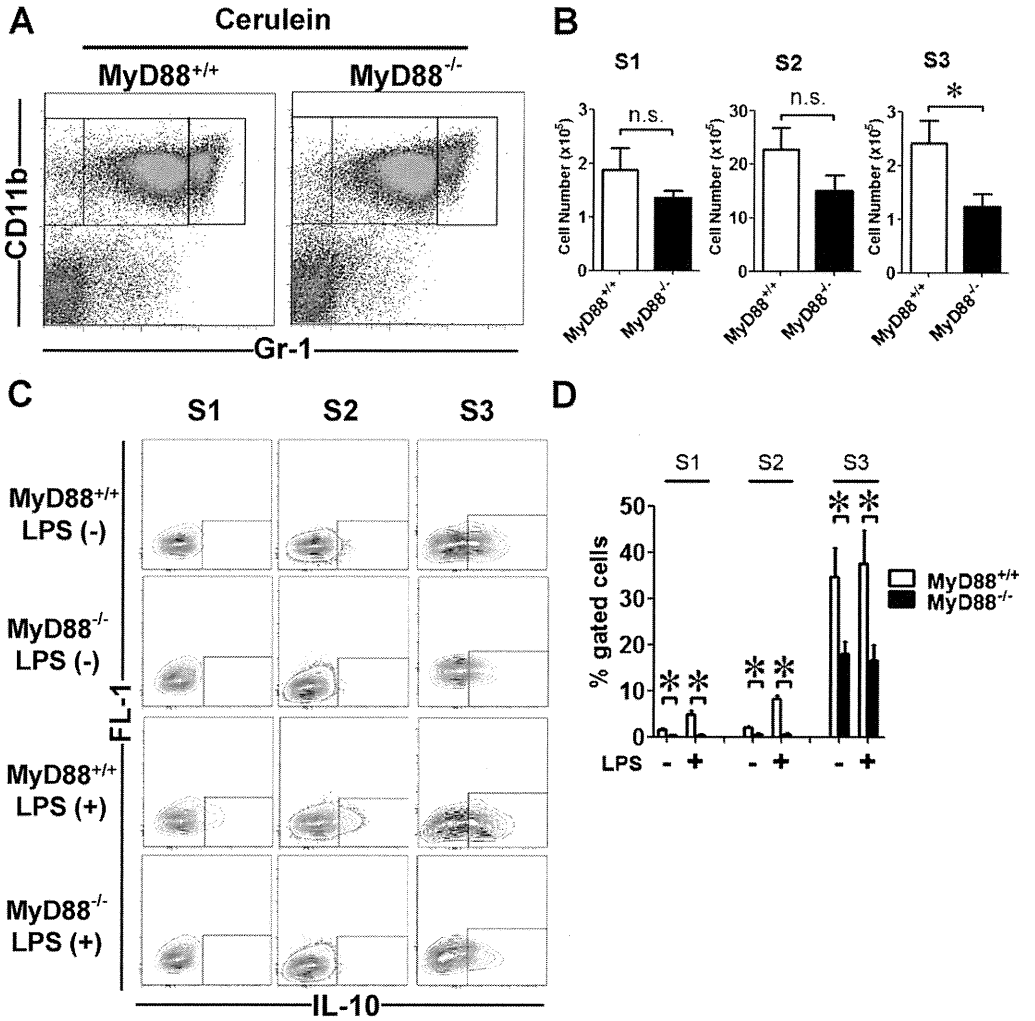

Fig. 2. Reduction in the number of pancreatic IL-10-producing CD11b$^+$Gr-1^{high} cells in cerulein-injected MyD88$^{-/-}$ mice. (A) Flow cytometry of pancreas mononuclear cells stained with CD11b and Gr-1 (n=4). (B) Absolute cell number of the indicated subsets (n=4). (C) Intracellular IL-10 expression of each subpopulation cultivated with or without 1 μg/ml LPS for 5 h. (D) Ratio of the indicated subsets (n=4). LPS, lipopolysaccharide.

by IL-10-producing CD11b$^+$Gr-1^{high} cells. The majority of inflammatory APCs are contained in the S2 subpopulation [8] were not positively involved in the exacerbation of acute cerulein pancreatitis. The macrophages in cerulein-administered MyD88$^{-/-}$ mice were not activated in terms of CD80 and CD86 expression (Fig. 3A) and produced lower levels of TNF-α in cerulein-injected MyD88$^{-/-}$ mice as compared with control cerulein-injected MyD88$^{+/+}$ mice (Fig. 3B and C). We finally explored pancreatic CD4$^+$ T cells in MyD88$^{-/-}$ mice and MyD88$^{+/+}$ mice given cerulein injections. The absolute cell number of CD4$^+$ T cells was significantly increased in MyD88$^{-/-}$ mice given cerulein injections, compared with control MyD88$^{+/+}$ mice given cerulein injections (Fig. 3D). The ratio of CD69$^+$ cells among CD4$^+$ T cells was significantly higher in cerulein-administered MyD88$^{-/-}$ mice than in MyD88$^{+/+}$ mice (Fig. 3E). This suggests a contribution of activated CD4$^+$ T cells because of a lack of IL-10 secretion by the CD11b$^+$Gr-1^{high} S3 subpopulation, including MDSCs.

3.5. Antibiotic treatment ameliorates cerulein-induced pancreatitis

We examined whether the presence of commensal bacteria, major activators of the MyD88-TLRs signaling pathway, affect the development of cerulein-induced acute pancreatitis in mice. The WT mice in the group given antibiotics were administered drinking water including ampicillin, vancomycin, neomycin sulfate, and metronidazole, prior to the administration of cerulein (Fig. 4A). These antibiotics were chosen as they almost completely deplete commensal bacterial as previously verified by bacteriologic analysis of colonic feces [17,20]. Unlike the results using MyD88$^{-/-}$ mice, antibiotic-pretreated WT mice developed less severe pancreatitis after cerulein administration as assessed by pancreas histology (Fig. 4B and C) and serum levels of amylase and lipase (Fig. 4D). Even in the presence of antibiotics (therefore excluding the stimulating role of the microflora), there is IL-10 production by myeloid cells.

4. Discussion

In the present study, we demonstrated that: various TLRs are expressed in the pancreas of mice irrespective cerulein administration; MyD88$^{-/-}$ mice develop more severe cerulein-induced pancreatitis with increased pancreas pathology and greater elevation of serum amylase and lipase; the IL-10-producing CD11b$^+$Gr-1^{high} subpopulation is reduced in the pancreas of cerulein-administered MyD88$^{-/-}$ mice; and stimuli not related to bacteria may participate in the induction of IL-10-produing CD11b$^+$Gr-1^{high}cells in the pancreas of cerulein-administered mice.

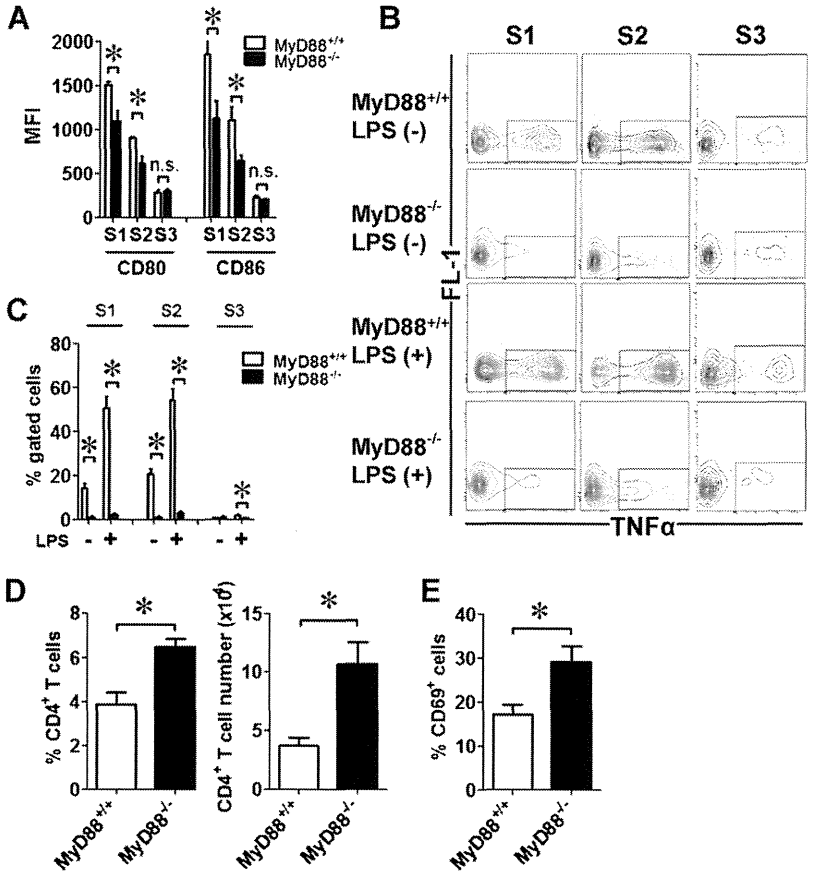

Fig. 3. MyD88 deficiency results in an abnormal increase and activation of CD4⁺ T cells in the pancreas of mice with acute cerulein pancreatitis. (A) Mean fluorescence intensity of CD80 and CD86 expression in pancreatic S1, S2, and S3 cells ($n=4$). (B) Intracellular TNF-α expression for each subpopulation. (C) Ratio of the indicated subpopulation ($n=4$). (D) The ratio (left) and the absolute number (right) of pancreas CD4⁺ T cells. (E) The ratio of CD69⁺ cells to the gated subset. All data were representative of two independent experiments ($n=4$ per group).

Collectively, these results suggest that MyD88-dependent IL-10 production by CD11b$^+$Gr-1^{high} cells controls immune homeostasis in the pancreas to avoid dysregulated inflammation in the pancreas.

The pathogenesis of acute pancreatitis begins in an autodigestive manner [3]. Trypsinogen activation is involved in the primary pathogenesis and leads to subsequent autodigestion. Based on these findings, a number of randomized controlled studies have assessed various protease inhibitors for the treatment of human acute pancreatitis [21,22], but no significant reduction in mortality was achieved. Hence, subsequent inflammation may be critically involved in the pathogenesis of acute pancreatitis. It has been reported that macrophages [6,8], granulocytes [7], and CD4$^+$ T cells [9] are involved in the pathogenesis of acute pancreatitis, and chemokines produced by injured acinar cells attract these immune cells to the pancreas [23]. We recently demonstrated that two consecutive immunological events are required for the development of cerulein-induced acute pancreatitis. These are the migration of CCL2/CCR2-dependent TNF-α-producing pathological CD11b$^+$Gr-1^{low} macrophages from the bone marrow to the pancreas, and SOCS3-dependent macrophage activation after their migration to become TNF-α-producing pathological macrophages [8]. Although these previous findings reveal a positive involvement of immune cells in the development of acute pancreatitis, no reports have suggested a regulatory immune mechanism to maintain pancreatic immune homeostasis. In this study, we showed that CD11b$^+$Gr-1^{high} cells preferentially produce IL-10 in a MyD88-dependent manner, suggesting these cells may control the immune tolerance of pancreas. It still remains unknown which migration mechanisms are involved in the migration of those cells. We previously showed that both S2 and S3 subpopulations emerge in the pancreas of cerulein-injected mice, but only the migration of pathological TNF-α-producing CD11b$^+$Gr-1^{low} cells is dependent upon the CCR2–CCL2 axis. This results in the amelioration of acute cerulein pancreatitis in CCL2$^{-/-}$ mice [8]. Therefore, as yet undetermined migration mechanisms, with the exception of the CCL2/CCR2 axis, may be involved in the migration of regulatory CD11b$^+$Gr-1^{high} cells in this model.

The current finding that MyD88$^{-/-}$ mice developed more severe pancreatitis was surprising, as it has been previously reported that almost all TLR-deficient mice, such as TLR3$^{-/-}$ [24], TLR4$^{-/-}$[25], and TLR9$^{-/-}$[26] mice, developed less severe acute cerulein pancreatitis. In accordance with the results from TLR-deficient mice in this model, we have shown that antibiotic treatment ameliorated this model. Although it remains unknown whether antibiotic prophylaxis prevents secondary infection and reduces the mortality of patients with acute pancreatitis in humans [27,28], our results provide theoretical evidence that the reduction in bacteria, including commensal bacteria, by a cocktail of antibiotics may be beneficial for the treatment of acute pancreatitis. In this study, however, it remains largely unknown how bacteria or bacteria-derived products access the pancreas, as it is believed to be sterile. It is interesting that these kinds of mechanisms, through bacterial translocation and/or the circulation of their products, may not participate in the induction of regulatory IL-10-producing CD11b$^+$Gr-1^{high} cells. Further studies will be needed to clarify the role of viable bacteria in the pathogenesis of acute pancreatitis.

Apart from the involvement of bacteria in this model, it is particularly notable that IL-18$^{-/-}$ mice develop more severe acute

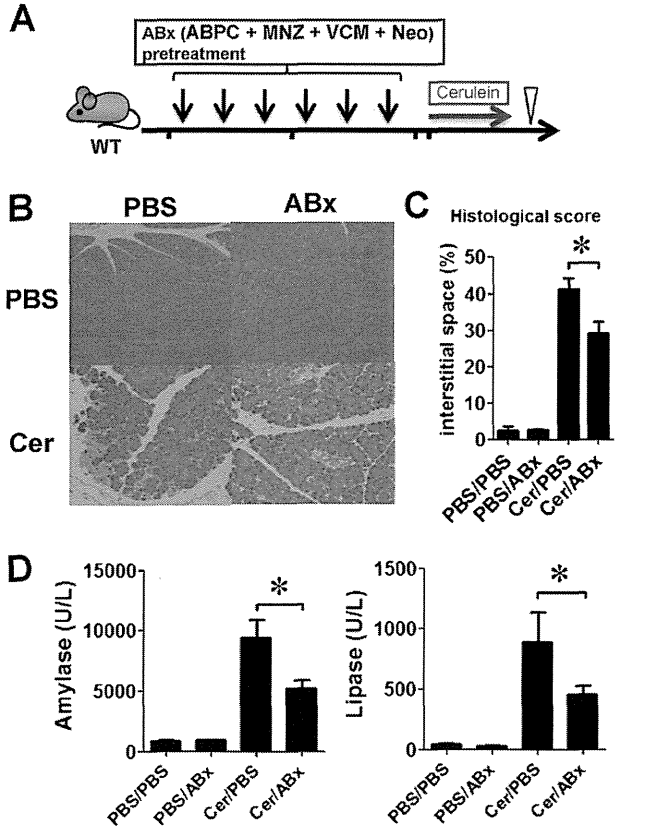

Fig. 4. Antibiotic treatment ameliorates cerulein-induced pancreatitis. (A) Study design. WT mice in the group given antibiotics were administered ampicillin, vancomycin, neomycin sulfate, and metronidazole for 2 weeks prior to the first administration of cerulein, then given eight intraperitoneal doses of cerulein (50 μg/kg) or PBS, spaced 1 h apart. All mice were euthanized 1 h after the last injection. (B) Representative hematoxylin and eosin staining. (C) Histological score ($n = 6$). (D) Serum amylase and lipase levels ($n = 9$). ABx, antibiotics; ABPC, aminobenzylpenicillin; MNZ, metronidazole; VCM, vancomycin; and Neo, neomycin.

cerulein pancreatitis as compared with WT mice [29]. This is in accordance with results using MyD88$^{-/-}$ mice, although it has been reported that IL-1R$^{-/-}$ mice were resistant to cerulein-induced acute pancreatitis [30]. Consistently, it has been reported that the expression of IL-1β and IL-18 was markedly upregulated in the pancreas after cerulein injection [29,30]. Therefore, our MyD88-dependent regulatory mechanism in acute pancreatitis may be mediated by IL-18-directed protective mechanisms, through a MyD88 pathway in this model of acute pancreatitis. Further studies will be needed to address this issue.

The MyD88 signaling pathway contributes to the induction of protective IL-10-producing macrophages, including MDSCs, possibly in response to pancreas damage via non-bacterial components. This provides a new concept for the pathogenesis of acute pancreatitis in terms of immunological aspects and its therapeutic strategies.

Conflicts of interest

No conflicts of interest exist.

Acknowledgments

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References

[1] Baron TH, Morgan DE. Acute necrotizing pancreatitis. N Engl J Med 1999;340:1412–7.
[2] Sand J, Nordback I. Acute pancreatitis: risk of recurrence and late consequences of the disease. Nat Rev Gastroenterol Hepatol 2009;6:470–7.
[3] Saluja AK, Lerch MM, Phillips PA, Dudeja V. Why does pancreatic overstimulation cause pancreatitis? Annu Rev Physiol 2007;69:249–69.
[4] de Beaux AC, Palmer KR, Carter DC. Factors influencing morbidity and mortality in acute pancreatitis; an analysis of 279 cases. Gut 1995;37:121–6.
[5] Al Mofleh IA. Severe acute pancreatitis: pathogenetic aspects and prognostic factors. World J Gastroenterol 2008;14:675–84.
[6] Sakai Y, Masamune A, Satoh A, Nishihira J, Yamagiwa T, Shimosegawa T. Macrophage migration inhibitory factor is a critical mediator of severe acute pancreatitis. Gastroenterology 2003;124:725–36.
[7] Sandoval D, Gukovskaya A, Reavey P, Gukovsky S, Sisk A, Braquet P, et al. The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. Gastroenterology 1996;111:1081–91.
[8] Saeki K, Kanai T, Nakano M, Nakamura Y, Miyata N, Sujino T, et al. CCL2-induced migration and SOCS3-mediated activation of macrophages are involved in cerulein-induced pancreatitis in mice. Gastroenterology 2012;142:1010–20.
[9] Demols A, Le Moine O, Desalle F, Quertinmont E, Van Laethem JL, Devière J, et al. CD4$^+$ T cells play an important role in acute experimental pancreatitis in mice. Gastroenterology 2000;118:582–90.
[10] Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. Gastroenterology 2007;132:1127–51.
[11] Chan YC, Leung PS. Acute pancreatitis: animal models and recent advances in basic research. Pancreas 2007;34:1–14.
[12] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;24:783–801.
[13] Seki E, Brenner DA. Toll-like receptors and adaptive molecules in liver disease: update. Hepatology 2008;48:322–35.
[14] Brodsky IE, Medzhitov R. Targeting of immune signalling networks by bacterial pathogens. Nat Cell Biol 2009;11:521–6.
[15] Boraschi D, Tagliabue A. The interleukin-1 receptor family. Vitam Horm 2006;74:229–54.
[16] Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11:115–22.
[17] Nemoto Y, Kanai T, Kameyama K, Shinohara T, Sakamoto N, Totsuka T, et al. Long-lived colitogenic CD4$^+$ memory T cells residing outside the intestine participate in the perpetuation of chronic colitis. J Immunol 2009;183:5059–68.
[18] Mikami Y, Kanai T, Sujino T, Ono Y, Hayashi A, Okazawa A, et al. Competition between colitogenic Th1 and Th17 cells contributes to the amelioration of colitis. Eur J Immunol 2010;40:2409–22.
[19] OjiroK, Ebinuma H, Nakamoto N, Wakabayashi K, Mikami Y, Ono Y, et al. MyD88-dependent pathway accelerates the liver damage of concanavalin A-induced hepatitis. Biochem Biophys Res Commun 2010;399:744–9.
[20] Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229–41.
[21] Yang CY, Chang-Chien CS, Liaw YF. Controlled trial of protease inhibitor gabexelatemesilate (FOY) in the treatment of acute pancreatitis. Pancreas 1987;2:698–700.
[22] Büchler M, Malfertheiner P, Uhl W, Schölmerich J, Stöckmann F, Adler G, et al. Gabexatemesilate in human acute pancreatitis German Pancreatitis Study Group. Gastroenterology 1993;104:1165–70.
[23] Bhatia M, Ramnath RD, Chevali L, Guglielmotti A. Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 2005;288:1259–65.
[24] Richer MJ, Lavallée DJ, Shanina I, Horwitz MS. Toll-like receptor 3 signaling on macrophages is required for survival following coxsackievirus B4 infection. PLoS ONE 2009;4:e4127.
[25] Sharif R, Dawra R, Wasiluk K, Phillips P, Dudeja V, Kurt-Jones E, et al. Impact of toll-like receptor 4 on the severity of acute pancreatitis and pancreatitis-associated lung injury in mice. Gut 2009;58:813–9.
[26] Hoque R, Sohail M, Malik A, Sarwar S, Luo Y, Shah A, et al. TLR9 and the NLRP3 inflammasome link acinar cell death with inflammation in acute pancreatitis. Gastroenterology 2011;141:358–69.
[27] Isenmann R, Rünzi M, Kron M, Kahl S, Kraus D, Jung N, et al. Prophylactic antibiotic treatment in patients with predicted severe acute pancreatitis: a placebo-controlled, double-blind trial. Gastroenterology 2004;126:997–1004.
[28] Røkke O, Harbitz TB, Liljedal J, Pettersen T, Fetvedt T, Heen LØ, et al. Early treatment of severe pancreatitis with imipenem: a prospective randomized clinical trial. Scand J Gastroenterol 2007;42:771–6.
[29] Ueno N, Kashiwamura S, Ueda H, Okamura H, Tsuji NM, Hosohara K, et al. Role of interleukin 18 in nitric oxide production and pancreatic damage during acute pancreatitis. Shock 2005;24:564–70.
[30] Fu K, Sarras Jr MP, De Lisle RC, Andrews GK. Expression of oxidative stress-responsive genes and cytokine genes during cerulein-induced acute pancreatitis. Am J Physiol 1997;273:696–705.

CCL2-Induced Migration and SOCS3-Mediated Activation of Macrophages Are Involved in Cerulein-Induced Pancreatitis in Mice

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BACKGROUND & AIMS: Acute pancreatitis is a common inflammatory disease mediated by damage to acinar cells and subsequent pancreatic inflammation with recruitment of leukocytes. We investigated the pathologic roles of innate immune cells, especially macrophages, in cerulein- and L-arginine–induced acute pancreatitis in mice. **METHODS:** Acute pancreatitis was induced by sequential peritoneal administration of cerulein to mice. We determined serum concentrations of amylase and lipase, pancreatic pathology, and features of infiltrating mononuclear cells. We performed parabiosis surgery to assess the hemodynamics of pancreatic macrophages. **RESULTS:** Almost all types of immune cells, except for $CD11b^{high}CD11c^{-}$ cells, were detected in the pancreas of healthy mice. However, activated $CD11b^{high}CD11c^{-}$ cells, including $Gr\text{-}1^{low}$ macrophages and $Gr\text{-}1^{high}$ cells (granulocytes and myeloid-derived suppressor cells), were detected in damaged pancreas after cerulein administration. $CCL2^{-/-}$ mice given cerulein injections developed significantly less severe pancreatitis, with less infiltration of $CD11b^{high}CD11c^{-}Gr\text{-}1^{low}$ macrophages, but comparable infiltration of myeloid-derived suppressor cells, compared with cerulein-injected wild-type mice. Parabiosis and bone marrow analyses of these mice revealed that the $CD11b^{high}CD11c^{-}Gr\text{-}1^{low}$ macrophages had moved out of the bone marrow. Furthermore, mice with macrophage-specific deletion of suppressor of cytokine signaling 3 given injections of cerulein developed less severe pancreatitis and $Gr\text{-}1^{low}$ macrophage produced less tumor necrosis factor-α than wild-type mice given cerulein, although the absolute number of $CD11b^{high}CD11c^{-}Gr\text{-}1^{low}$ macrophages was comparable between strains. Induction of acute pancreatitis by L-arginine required induction of macrophage migration by CCL2, via the receptor CCR2. **CONCLUSIONS: Cerulein induction of pancreatitis in mice involves migration of $CD11b^{high}CD11c^{-}Gr\text{-}1^{low}$ macrophage from the bone marrow (mediated by CCL2 via CCR2) and suppressor of cytokine signaling 3–dependent activation of macrophage. These findings might lead to new therapeutic strategies for acute pancreatitis.**

Keywords: Immune Response; Signaling; Mouse Model; Chemokine.

Severe acute pancreatitis is an intractable disease. It has a mortality rate of approximately 20% because of severe complications, such as systemic inflammatory syndrome, acute respiratory distress syndrome, and renal failure, and requires intensive care treatments.[1,2] The pathogenesis of acute pancreatitis begins in an autodigestive manner.[3] Trypsinogen activation by cathepsin B[3] and autophagy via Atg5 activation[4,5] are involved in the primary pathogenesis and lead to subsequent autodigestion. Based on these findings, a number of randomized controlled studies have assessed various protease inhibitors for the treatment of human acute pancreatitis.[6–8] Unfortunately, no significant reduction in mortality was achieved. Activation of proteases is not solely responsible for the initiation of acute pancreatitis associated with the high mortality, and subsequent inflammation might also be critically involved.[9,10]

In this regard, it has been reported that macrophages,[11] granulocytes,[12] and $CD4^{+}$ T cells[13] are involved in the pathogenesis of acute pancreatitis, and it has been suggested that chemokines produced by injured acinar cells attract these immune cells to the pancreas.[14] With this background, we used a cerulein-induced pancreatitis model[15,16] in the present study to comprehensively clarify the types of macrophages/mononuclear phagocytes involved in the pathogenesis of acute pancreatitis and the mechanisms of their recruitment and activation.

Materials and Methods

Detailed methods are described in the Supplementary Materials and Methods.

Induction of Acute Pancreatitis

Cerulein (Sigma-Aldrich, St Louis, MO) was dissolved in phosphate-buffered saline (PBS) and administered intraperito-

Abbreviations used in this paper: APC, antigen-presenting cell; BM, bone marrow; cDC, classical dendritic cells; cKO, conditional knockout; IL, interleukin; L-Arg, L-arginine; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; NF-κB, nuclear factor–κB; NK, natural killer; NKT, natural killer T cell; PBS, phosphate-buffered saline; RAG-2, recombinase-activated gene-2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activation of transcription; TCR, T-cell receptor; TNF-α, tumor necrosis factor–α; WT, wild-type.

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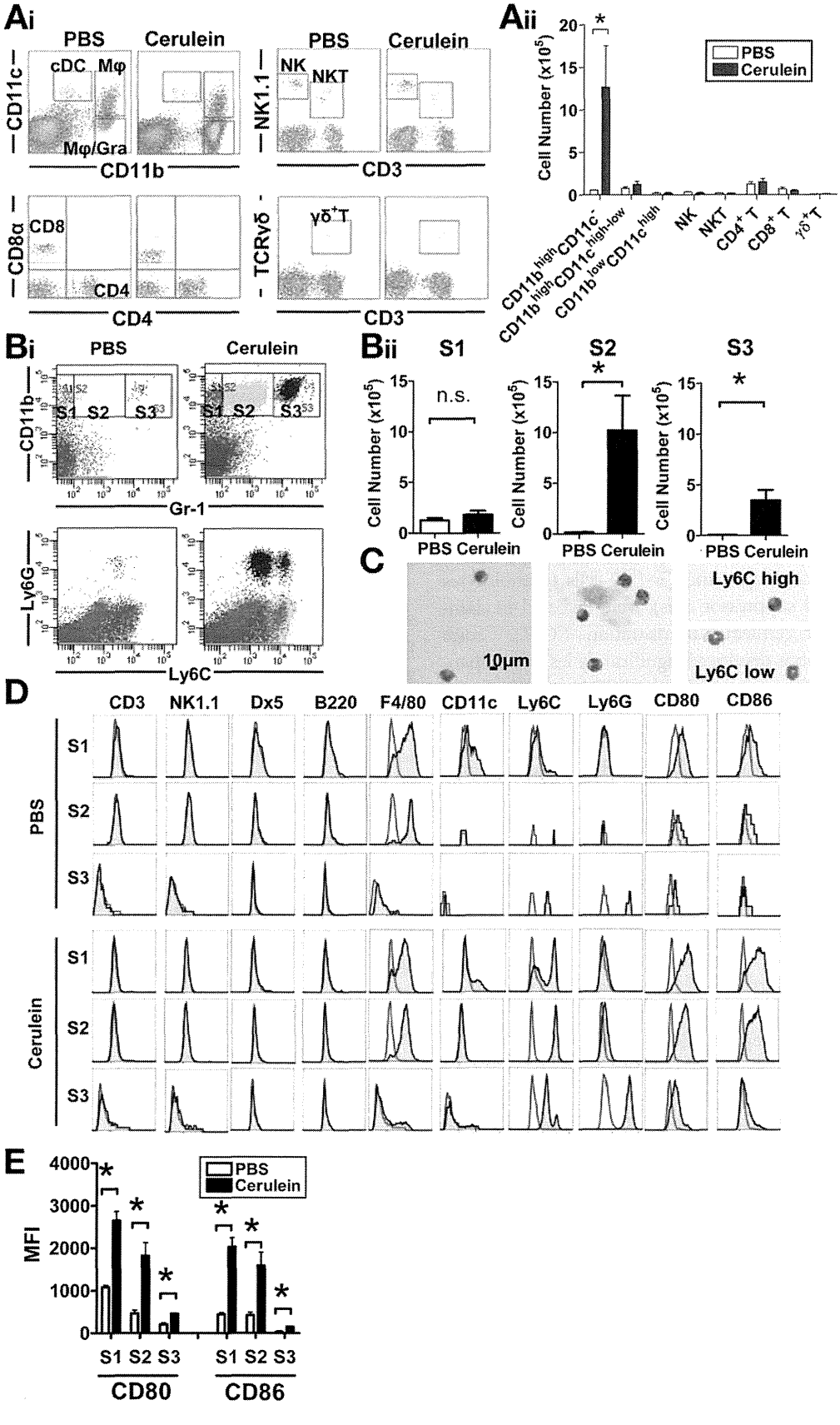
Ai
PBS
Cerulein
cDC
Mφ
Mφ/Gra
CD11c
CD11b
NK
NKT
NK1.1
CD3
CD8α
CD8
CD4
γδ+T
TCRγδ
Aii
Cell Number (x10^5)
PBS
Cerulein
CD11b^high CD11c^-
CD11b^high CD11c^high-low
CD11b^low CD11c^high
NK
NKT
CD4+ T
CD8+ T
γδ+T
Bi
S1
S2
S3
Gr-1
Ly6G
Ly6C
Bii
n.s.
C
10μm
Ly6C high
Ly6C low
D
CD3
NK1.1
Dx5
B220
F4/80
CD11c
Ly6C
Ly6G
CD80
CD86
E
MFI