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Original Article

Clinical-pathologic significance of CD163 positive macrophage in IgA nephropathy patients with crescents

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Abstract: Background: CD163, a marker of M2 macrophages, express anti-inflammatory properties. This study aims to investigate the difference of CD163 positive macrophages expression between IgA nephropathy patients with and without crescents. Methods: Renal tissues from IgAN patients (n = 24), including IgAN with crescents (n = 10), IgAN without crescents (n = 14), minimal change disease (MCD, as disease control, n = 8) and normal control kidneys (negative control, n = 3), were included in this study. Expressions of CD163 and CD68 in renal tissues were detected by immunohistochemistry or immunofluorescence. Results: Compared with IgAN without crescent, IgAN patients with crescents have lower serum albumin and poor renal function. CD163 was mainly expressed in acute tubulointerstitial lesions. CD163 positive cells accumulate in areas around tubules with RBC casts. CD163 positive cells can also be seen in tubular lumen. CD163 positive cells can be seen in glomerular lesions, including endocapillary hypercellularity, cellular crescent and fibrous-cellular crescent. There were more CD163 positive cells in tubulointerstitial and glomerular lesions in IgAN patients with crescents. CD163 positive cells number in tubulointerstitial tissue was positive correlated with percentage of crescents, proteinuria, and negative correlated with serum albumin, eGFR. CD163 positive cells number in glomeruli was positive correlated with percentage of crescents, and was negative correlated with eGFR. Percentage of crescents was negative correlated with serum albumin, eGFR, and positive correlated with proteinuria. Dual staining showed that CD163 positive cells also expressed CD68. Conclusions: CD163 positive macrophages were involved in active crescent disease, acute tubular injury and glomerular lesions of IgAN with crescents.

Keywords: CD163, macrophage, IgA nephropathy, acute tubular injury

Introduction

Macrophage infiltration is a universal feature of glomerular injury in both human and experimental disease [1-4]. Macrophages comprise a heterogeneous population of cells, with diverse functions and phenotypic plasticity. M1 macrophages are characterized by a pro-inflammatory phenotype, while M2 macrophages display regulatory functions in tissue repair and remodeling [5-7]. One characteristic of M2 macrophages is the increase in CD163 expression [5, 6]. Eduardo Gutiérrez et al reported that oxidative stress, CD163 positive macrophages infiltration are determinants of long-term renal outcome in macro hematuria-induced acute kidney injury of IgA nephropathy (IgAN) [8]. Lei Zhao et al reported that CD163+ macrophages accumulated in glomerular capillaries and Bowman's space in normal-appearing glomeruli and the interstitium of anti-neutrophil cytoplasmic antibody-associated vasculitis, and were more numerous in glomeruli with fibrinoid necrosis and those with cellular crescents [9]. However, there is still unknown about the clinicopathologic significance of CD163 positive macrophage in IgAN patients and the difference of CD163 positive macrophage expression in IgAN patients with crescents from those without crescents. This study aims to investigate the difference of CD163 positive macrophages expression between IgA nephropathy patients with and without crescents.

Subjects and methods

Subjects

Formalin-fixed, paraffin-embedded renal tissues from core needle biopsies in IgAN patients

Table 1. Clinical data of IgAN patients before renal biopsy

	IgAN patients with crescents	IgAN patients without crescents	*P*
Gender (male/female)	3/7	6/8	0.388
Age (y)	35 ± 20	42 ± 10	0.195
Serum albumin (g/l)	34 ± 7	41 ± 7	0.016*
Urinary protein (g/d)	1.8 ± 1.1	1.0 ± 0.7	0.099
eGFR (ml/min	58 ± 27	88 ± 22	0.003*
CD163/tubulointerstitium	26 ± 8	9 ± 4	0.0001**
CD163/glomeruli	4 ± 1	2 ± 1	0.0001**

Footnote: *$P < 0.05$, **$P < 0.01$; IgAN: IgA nephropathy; eGFR: estimated glomerular filtration rate.

(n = 24), including IgAN with crescents (n = 10), IgAN without crescents (n = 14), minimal change disease (MCD, as disease control, n = 8) and normal control kidneys (negative control, n = 3), were included in this study.

For IgAN patients with crescents, cellular crescents or fibrous-cellular crescents ≥ 30%, for patients of IgAN without crescents, there is no cellular crescent, fibrous-cellular crescent or fibrous crescent in their renal tissues. Percentage of global glomerular sclerosis in all IgAN patients was within the range of corresponding age, that is, no more than [(age/2)-10] %.

According to oxford classification of IgA nephropathy, their pathologic characters were scored respectively, including mesangial proliferation, endocapillary hypercellularity, percentage of global glomerular sclerosis, percentage of focal glomerular sclerosis, percentage of crescents and score of interstitial fibrosis [10].

All the cases exclude acute inflammatory disease such as fever, urinary infection and so on. All biopsies were obtained before any medical intervention. Informed consent was obtained from each patient. The research was in compliance with the Declaration of Helsinki. The study protocol has been approved by the institute's committee on human research.

Methods

Immunohistochemistry detection of CD163 and CD68: Thin sections (2 μm) of the renal biopsies were deparaffinized and dehydrated by a series of xylene and alcohol washes. Following quenching of endogenous peroxidase activity with 3% (vol/vol) H_2O_2 in methanol × 10 minutes, for antigen retrieval, slides were immersed in buffer solution, then placed in pressure cooker (100°C, 2 min). The tissues were blocked with 1% (wt/vol) bovine serum albumin (BSA) and incubated with monoclonal antibodies directed against CD163 (1:200, ab156769, Abcam, USA), monoclonal antibodies directed against CD68 (1:50, ZM-0464, ZSGB-BIO, Beijing, China) respectively, at 4°C for overnight in a humidified chamber. Anti-mouse antibody and anti-rabbit antibody (Ready to Use, GK500705, GENETECH) applied for 30 minutes. The chromogen reaction was developed with DAB and the slides were counterstained with hematoxylin and Periodic Acid Schiff (PAS) staining. Negative controls for all antibodies included incubation of sections with preimmune serum and deletion of the primary antibody, a process that completely prevented immunostaining.

Immunofluorescence detection of co-location of CD163 and CD68: Dual staining using paraffin sections was used to examine the co-localization of CD163 and CD68. Heating of the section at 100, for 2 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) was performed for antigen retrieval. Sections were first stained with mouse anti-CD68 monoclonal antibody as described above, followed by incubation with rabbit anti-CD163 polyclonal antibody (1:200, ab-100909, Abcam, USA). Sections were sequentially incubated with Rhodamine (TRITC) -conjugated goat-anti-rabbit IgG (1:50, Jackson Immuno Research Inc, USA) and FITC -conjugated goat-anti-mouse-IgG (1:50, Jackson Immuno Research Inc, USA). Cover slips were applied to the sections, using Glycergel to which 2.5% 1, 4-diazabicyclo octane (DABCO; Sigma) was added as fading retardant.

Immunohistochemical assessment of biopsies: The numbers of CD163 and CD68 positive cells in glomerular and interstitium are counted at a magnification of × 400. Glomerular infiltrate is expressed as number of positive cells/glomerulus. Interstitial infiltrate is expressed as number of positive cells/high power field (HPF). Ten fields are selected; results are expressed as mean ± standard deviation (SD).

Table 2. Pathologic data of IgAN patients

	Mean rank		
	With crescent	Without crescent	*P*
Mesangial proliferation	12.55	12.46	1.000
Endocapillary hypercellularity	13.1	12.07	1.000
% Glomerular Sclerosis	11.93	12.11	0.963
% Focal Sclerosis	12.65	12.39	0.943
% Glomeruli with crescents	19	7.86	< 0.0001**
Interstitial fibrosis	12.7	12.36	0.930

Footnote: ***P* < 0.01; IgAN: IgA nephropathy.

Statistical analysis

Comparisons between groups were tested by Kruskal-Wallis test. A comparison between two groups was assessed by Mann-Whitney U for nonparametric data. Correlation analysis was assessed by spearman for nonparametric data. A *P* value < 0.05 was considered to be significant.

Results

*Clinical data from these patients before renal biopsy (**Table 1**)*

Compared with IgAN patients without crescents, those with crescents have lower serum albumin and poor renal function. However, there is no difference in their gender, age and proteinuria measurement.

Pathologic data from IgAN patients are shown in **Table 2**. There is no difference in their most pathologic characters, including mesangial proliferation, endocapillary hypercellularity, percentage of global glomerular sclerosis, percentage of focal glomerular sclerosis, score of interstitial fibrosis, glomerular deposits of IgA and complement3.

After renal biopsy, IgAN patients with crescents had received methylprednisolone pulse therapy or oral prednisone (1 mg/kg.d) combined with cyclophosphamide or Tripterygium wilfordii therapy. For IgAN patients without crescents, if proteinuria ≥ 1 g/24 h, medium dose of prednisone (0.5 mg/kg.d) were suggested. The average follow-up period was 2 years. Except for 4 IgAN patients (IgAN patients without crescents n = 2, IgAN patients with crescents n = 2) who did not receive amelioration, the remaining 20 IgAN patients were all received remission of different degree, including partial remission (n = 10), complement remission (n = 10). In IgAN patients who had received immunosuppressive or steroid regimen, severe side effect occurred in 3 cases, including empyema, herpes zoster and severe pneumonia.

*Expression of CD163 and CD68 in renal tissue of negative controls and MCD (**Figure 1A**)*

In normal kidney, CD68 was occasionally expressed in tubulointerstitial tissue, staining of CD163 was almost negative. In negative controls (rabbit serum or mouse serum substitute for primary antibody), staining of CD163 and CD68 were all negative. In MCD, CD163 and CD68 were occasionally expressed in tubulointerstitial tissue.

*Expression of CD163 in tubulointerstitial lesions and glomeruli of IgAN patients (**Table 1; Figures 1B-F, 2** and **3**)*

CD163 was mainly expressed in acute tubulointerstitial lesions. CD163 positive cells accumulate in areas around tubules with RBC casts. CD163 positive cells can also be seen in tubular lumen. CD163 positive cells can be seen in glomerular lesions, including endocapillary hypercellularity, cellular crescents and fibrous-cellular crescents.

Compared with IgAN without crescents, IgAN patients with crescents have more CD163 positive cells in tubulointerstitial lesions ($P < 0.0001$) and glomeruli ($P < 0.0001$).

Dual staining showed that CD163 positive cells also expressed CD68.

*Correlation of tubulointerstitial and glomerular CD163 positive cells number with clinical and pathologic index (**Table 3**)*

CD163 positive cells number in tubulointerstitial tissue was positive correlated with percentage of crescents ($r = 0.821$, $P < 0.0001$), proteinuria measurement ($r = 0.520$, $P = 0.009$), and was negative correlated with serum albumin ($r = -0.574$, $P = 0.003$), GFR ($r = -0.718$, $P < 0.0001$). There was no correlation between tubulointerstitial CD163 positive cells number with other pathologic characters, including mesangial proliferation, endocapillary hypercellularity, percentage of global glomerular

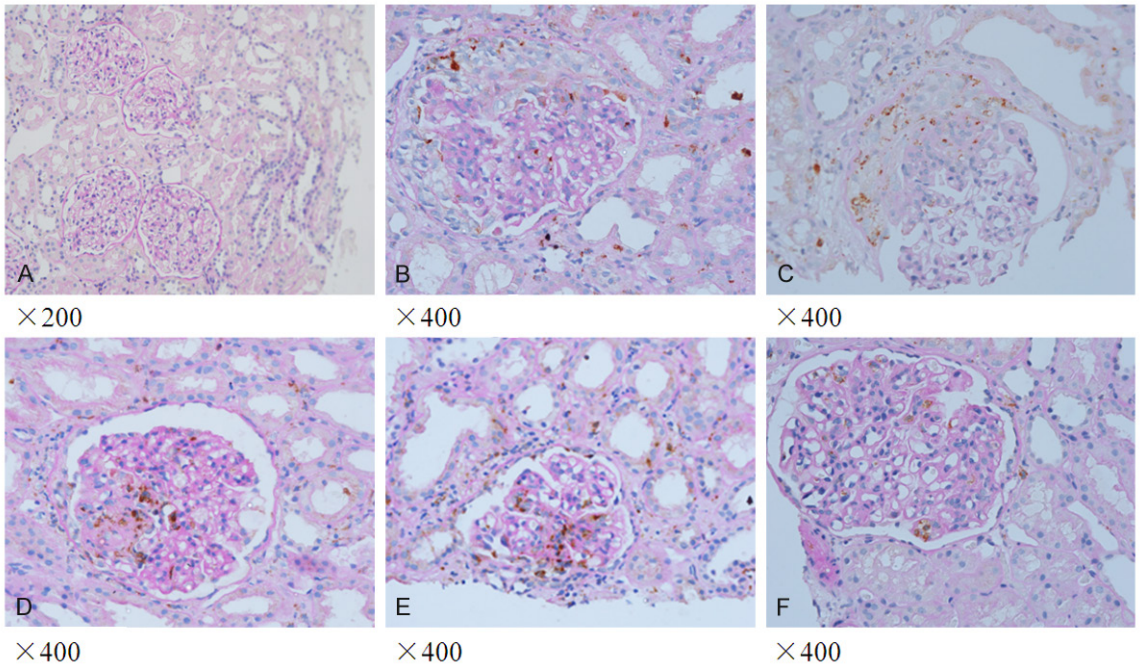

Figure 1. A. Location of CD163 in normal renal tissue and IgA nephropathy with crescents. A. Location of CD163 in normal renal tissue. B-F. Localization of CD163 in IgAN with crescents; B, C. CD163 was expressed in cellular crescent and fibrous-cellular crescent. D-F. L CD163 was expressed in lesions of focal sclerosis, mesangial proliferation, endocapillary hypercellularity. Immunohistochemistry (brown).

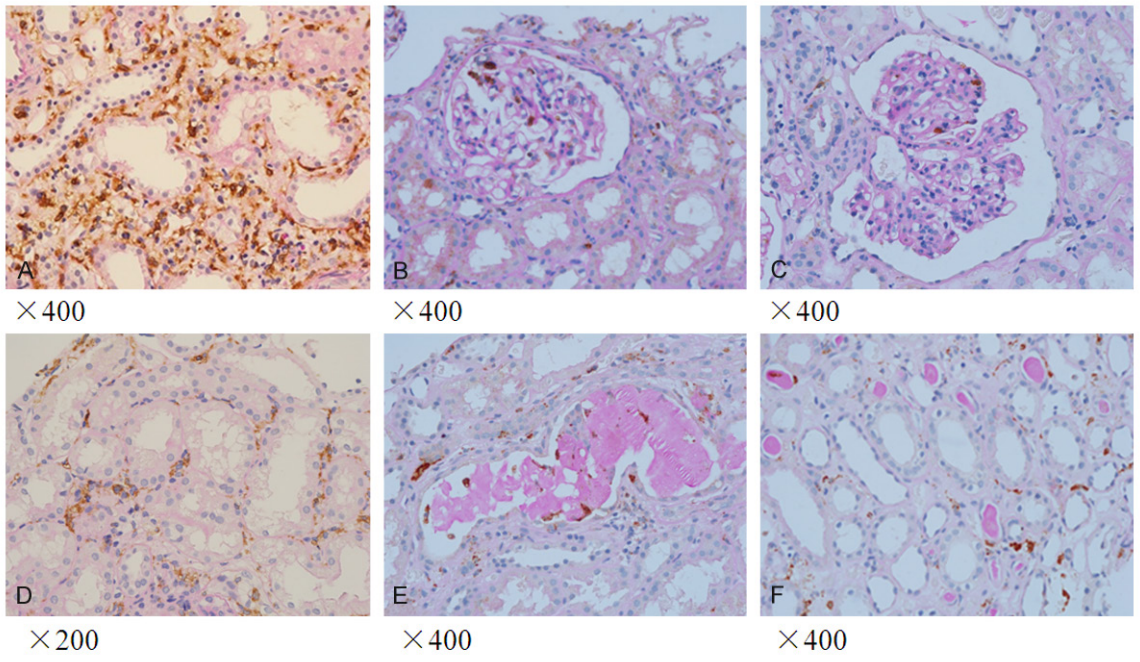

Figure 2. Localization of CD163 in IgA nephropathy with or without crescents. A, B. Localization of CD163 in IgAN with crescents. CD163 was expressed in acute tubulointerstitial lesions and normal-appearing glomeruli. C, D. Localization of CD163 in IgAN without crescents. E, F. CD163 was expressed in tubular with protein casts. Immunohistochemistry (brown).

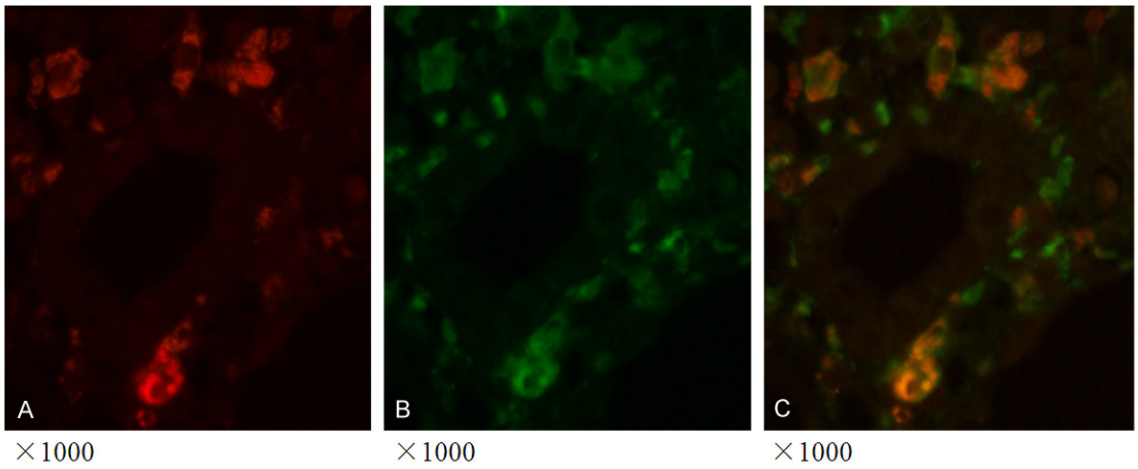

Figure 3. Colocation of CD163 and CD68. A-C. Localization of CD163 (red), CD68 (green), and co-location (yellow) of CD163 and CD68 in tubulointerstitial lesions of IgAN. Immunofluorescence.

sclerosis percentage of focal glomerular sclerosis and score of interstitial fibrosis. CD163 positive cells number in glomeruli was positive correlated with percentage of crescents (r = 0.730, $P < 0.0001$), and was negative correlated with GFR (r = -0.523, $P = 0.009$). There was no correlation between tubulointerstitial or glomerular CD163 positive cells number with other pathologic characters, including mesangial proliferation, endocapillary hypercellularity, percentage of global glomerular sclerosis percentage of focal glomerular sclerosis, score of interstitial fibrosis, deposits of IgA and complement 3.

Percentage of crescents was negative correlated with age (r = -0.408, $p = 0.048$), serum albumin (r = -0.486, $P = 0.016$), GFR (r = -0.596, $P = 0.002$), and positive correlated with proteinuria measurement (r = 0.568, $P = 0.004$).

Discussion

Through complement-dependent and independent pathway, macrophage infiltrate glomeruli of IgAN and involved in pathologic lesions [11]. Dual staining showed that CD163 positive cells were subpopulation of macrophage.

Macrophages express different surface molecules that involved in different function in different stage of renal disease [4-7]. CD163, a member of cysteine-rich scavenger receptor family, was identified as a maker of M2 macrophage. CD163 positive macrophages play a central role in hemoglobin clearance and limit oxidative heme toxicity, which are significant prognostic factors for an incomplete recovery (IR) of renal function in IgA nephropathy patients with macro hematuria-associated AKI, warfarin coagulopathy and paroxysmal nocturnal hemoglobinuria [8, 12, 13].

In our study, CD163 positive macrophages also accumulate in acute tubulointerstitial lesions without erythrocyte casts in IgAN patients. It implies a role of CD163 other than oxidative stress.

In our study, CD163 positive cells infiltrate in glomeruli and tubulointerstitial lesions, mainly at sites of proliferative glomerular lesions, active crescents and acute tubular injury, which is of note in IgAN with crescents; and CD163 positive cells numbers were negative correlated with eGFR; at the end of follow-up, most patients received remission of proteinuria and amelioration of renal function. It implies that CD163 positive macrophage might be involved in acute tubulointerstitial injury and glomerular lesions of IgAN, especially those with crescents, and active glomerular and acute tubular injury could be reversible only if it is treated timely [17].

Studies reported that CD163 help to amelioration of renal injury in animal models of crescent nephritis (anti-glomerular basement membrane glomerulonephritis) and adriamycin nephropathy [2, 3, 14]. And tubular cells can instruct macrophage activation by secreting GM-CSF,

Table 3. Correlation of CD163 counts (tubulointerstitial area/HPF) with clinical and pathologic data

Spearman's rho r *P*	CD163/tubulointerstitium	Percentage of crescents	CD163/glomeruli
Serum albumin (g/l)	-0.574	-0.486	-0.196
	*0.003***	*0.016**	*0.360*
eGFR (ml/min)	-0.718	-0.596	-0.523
	*< 0.0001***	*0.002***	*0.009**
Proteinuria (g/24 h)	0.520	0.568	0.178
	*0.009***	*0.004***	*0.406*
Mesangial proliferation	0.092	0.101	0.092
	0.669	*0.637*	*0.669*
Endocapillary hypercellularity	0.125	0.122	-0.129
	0.559	*0.572*	*0.547*
% Global glomerular sclerosis	-0.023	-0.001	0.034
	0.919	*0.996*	*0.877*
% Focal glomerular sclerosis	-0.043	-0.058	0.090
	0.840	*0.787*	*0.676*
% Glomeruli with crescents	0.821	-	0.730
	*< 0.0001***		*< 0.0001***
Interstitial fibrosis	0.061	-0.143	0.198
	0.777	*0.056*	*0.353*

Footnote: *$P < 0.05$, **$P < 0.01$; HPF: high power field; eGFR: estimated glomerular filtration rate.

leading to a unique reparative M2 macrophage phenotype that supports tubular proliferation after injury [15]. Studies in vitro also showed that steroids contribute to upregulate the expression of CD163 in macrophage [16]. It imply that steroid regimen in IgAN with crescents might contribute to upregulate the expression of CD163 positive macrophage, which is involved in the restoration of tissue integrity and amelioration of renal function.

A study reported that M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN patients and accumulated in glomerular capillaries and Bowman's space in normal-appearing glomeruli [9]. In our study, there is more CD163 positive cells in normal-appearing glomeruli of IgAN patients with crescents, whose pathologic significance need further research.

CD163 positive cells were also expressed in tubules with proteinuria casts, which suggest that proteinuria might induce chemotaxis of CD163 positive cells. Previous study reported that CD163 positive cells or macrophages were correlated with chronic tubulointerstitial fibrosis [18, 19]; however, in our study, the expression of CD163 was not correlated with chronic tubulointerstitial fibrosis. The reason might be that there were more acute tubulointerstitial lesions in our study.

The reason why the prognosis of IgAN patients in our study was good might be as follows: firstly, the global glomerular sclerosis was within range of corresponding age associated global glomerular sclerosis. There was less chronic lesions in renal tissue of the patients. Secondly, there were mostly cellular crescents, which is effective to steroid and immunosuppressive regimen. Thirdly, Tubulointerstitial injury was acute and reversible. Therefore, for IgAN patients with proteinuria and/or renal failure, renal biopsy help to find active kidney injury earlier, which has good response to immunosuppressive therapy.

Future studies need to investigate the efficacy of steroid and on the expression of CD163 and clinical-pathologic index in IgAN animal models.

In conclusion, there were more numerous CD163 positive macrophages in glomeruli and acute tubulointerstitial lesions of IgAN with crescents. CD163 positive macrophages were involved in active crescent disease, acute tubular injury and glomerular lesions of IgAN with crescents. Therefore, for IgAN patients with proteinuria and/or acute kidney injury, renal biopsy help to find active kidney injury earlier, which has good response to immunosuppressive therapy.

Disclosure of conflict of interest

None.

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