SCIENCE CHINA
Life Sciences

Special Topic: Organ/Tissue-specific immunity
• RESEARCH PAPER •

December 2016 Vol.59 No.12: 1282–1289
doi: 10.1007/s11427-016-5030-9

T cell infiltration is associated with kidney injury in patients with anti-glomerular basement membrane disease

Shui-Yi Hu[1,3], Xiao-Yu Jia[1], Jian-Nan Li[1], Xin Zheng[1], Jie Ao[1], Gang Liu[1], Zhao Cui[1*] & Ming-Hui Zhao[1,2]

[1]*Renal Division, Department of Medicine, Peking University First Hospital; Institute of Nephrology, Peking University; Key Laboratory of Renal Disease, Ministry of Health of China; Key Laboratory of CKD Prevention and Treatment, Ministry of Education of China; Beijing 100034, China;*
[2]*Peking-Tsinghua Center for Life Sciences, Beijing 100871, China;*
[3]*Department of Nephrology, Tianjin Medical University General Hospital, Tianjin 300052, China*

Received November 12, 2015; accepted January 6, 2016; published online April 13, 2016

Cell-mediated autoimmunity, particularly that involving autoreactive T cells, participates in mediating anti-glomerular basement membrane (GBM) disease. However, direct kidney injury mediated by renal infiltrated T cells has not been clearly elucidated in humans. The T cell profile (CD3, CD4, CD8, IL-17, and foxp3) and macrophage (CD68) were examined by immunohistochemistry on renal biopsy tissues from 13 patients with anti-GBM disease. The correlation between cell infiltration and clinical data was also analyzed. We found that the distribution of T cell infiltration was predominant in the peri-glomerular and interstitial areas. $CD3^+$ T cell infiltratrion around the glomeruli with cellular crescent formations was significantly higher than that around the glomeruli with mild mesangial proliferation. $CD8^+$ T cells significantly accumulated around the glomeruli with cellular crescents without IgG deposits compared to those with IgG deposits. The prevalence of infiltrating $CD8^+$ T cells was correlated with the percentage of ruptured Bowman's capsules. In conclusion, cellular immunity may play a crucial role in the inflammatory kidney injury in anti-GBM patients. The periglomerular infiltration of T cells, especially $CD8^+$ T cells, may participate in the pathogenic mechanism of glomerular damage.

anti-GBM disease, T cell, IL-17, CD8, kidney injury

Citation: Hu, S.Y., Jia, X.Y., Li, J.N., Zheng, X., Ao, J., Liu, G., Cui, Z., and Zhao, M.H. (2016). T cell infiltration is associated with kidney injury in patients with anti-glomerular basement membrane disease. Sci China Life Sci 59, 1282−1289. doi: 10.1007/s11427-016-5030-9

INTRODUCTION

Anti-glomerular basement membrane (GBM) disease is an autoimmune disorder characterized by rapidly progressive glomerulonephritis and is often accompanied by lung hemorrhage (Kalluri, et al., 1995). Although the pathogenic roles of anti-GBM autoantibodies against the non-collagenous domain 1 of α3 chain of type IV collagen (α3(IV)NC1) have been fully demonstrated (Sado et al., 1989; Wilson and Dixon, 1973), there is strong evidence indicating that the full expression of this disease is dependent on cell-mediated autoimmunity, particular autoreactive T cells.

In rodent models, α3(IV)NC1-specific $CD4^+$ T cells alone are sufficient to induce glomerular injury in the absence of autoantibodies against GBM, revealing a direct pathogenic role (Wu et al., 2002). The disease cannot be induced in $CD4^+$ and $CD8^+$ T cell knockout mice (Tipping et al., 1998) and is inhibited by CD28-B7 and CD154-CD40 co-stimulatory blockades (Reynolds et al., 2000, 2004) as well as anti-CD8 monoclonal antibodies (Reynolds et al., 2002). Additionally, a Th17 cell subset,

*Corresponding author (email: cuizhao@bjmu.edu.cn)

which is maintained by IL-23, plays a dominant role in the development of anti-GBM disease. IL-23-deficient mice immunized with α3(IV)NC1 may develop anti-GBM disease. While the autoantibody titers are lower in these mice, cellular reactivity is reduced and renal injury is less severe than that in non-IL-23-deficient mice (Ooi et al., 2009). In human anti-GBM disease, T cell involvement can be implied from strong human leukocyte antigen (HLA) associations (Phelps and Rees, 1999). $CD4^+$ and $CD8^+$ T cell infiltration has been observed in the affected glomeruli (Bolton et al., 1987). However, the contributions of T cell infiltration to kidney injury in humans remain unclear.

In the current study, we detected different types of T cells and macrophages in the renal biopsy tissues from anti-GBM patients. We analyzed the associations between the infiltrating cells in different areas and the corresponding clinical and pathological data to elucidate the role of infiltrated T cells in the development of kidney injury in patients with anti-GBM disease.

RESULTS

Clinical features and pathological data of patients

Thirteen patients with biopsy-proven anti-GBM disease were enrolled in this study. Patients with positive anti-neutrophil cytoplasmic antibody (ANCA) or secondary anti-GBM disease were excluded. The demographic data and clinical features are shown in Table 1. At the time of diagnosis, the mean concentration of serum creatinine was (907.7±339.0) μmol L^{-1}. All patients underwent a kidney biopsy, and were diagnosed as crescentic glomerulonephritis for large crescent formation in over 50% of glomeruli by light microscopy and confirmed by electron microscopy. The mean percentage of crescents in glomeruli was (93.8±9.0)%. As measured by direct immunofluorescence, 61.5% of the patients exhibited linear IgG deposition along the GBM, with or without C3 deposits. All patients possessed circulating anti-GBM IgG antibodies at an average level of (76.2±42.2) U mL^{-1}, which was confirmed by enzyme-linked immunosorbent assay (ELISA) using recombinant human α3(IV)NC1. During the 1-year follow-up, 79.6% of the patients were alive, but all patients had progressed to end stage renal disease (ESRD).

T cell and macrophages infiltration in the kidneys

Positive staining for $CD3^+$, $CD4^+$, $CD8^+$, IL-17-producing, $foxp3^+$, and $CD68^+$ cells were detected in the kidney tissues of all patients (Figure 1). $CD3^+$ cells infiltration was more common than macrophages infiltration (Table 1). Inside the glomeruli with crescents, macrophages were detected at a higher frequency than T cells. Outside the glomeruli in the periglomerular and interstitial areas, T cells infiltrated more predominantly than macrophages. T cell infiltration, including the $CD3^+$, $CD8^+$, $CD4^+$, and IL-17-producing cells, exhibited differential distributions in the kidney, with predominance in the periglomerular area, followed by the interstitial area and then the intraglomerular area (Table 2). $Foxp3^+$ cells were detected only in the interstitial area and not in the intra- or periglomerular areas. No significant differences were observed in the distributions of macrophages in the different areas of the kidney (Table 2).

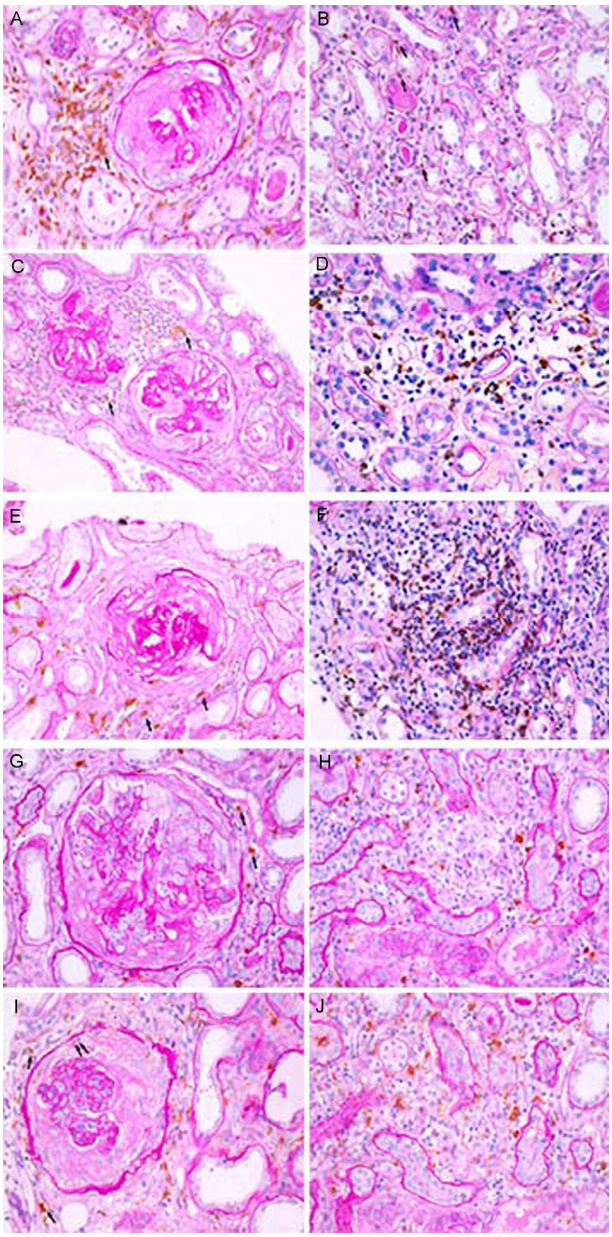

Figure 1 T cell and macrophage infiltration in the kidney tissues of patients with anti-GBM disease. Immunohistochemistry + PAS, magnification 400×, ↑ positive cells in peri-glomerular area, ↑ ↑ positive cells in intraglomerular area. A, $CD3^+$ cells infiltrated in the peri-glomerular area. B, $CD3^+$ cells in the interstitial area. C, $CD4^+$ cells infiltrated in the peri-glomerular area. D, $CD4^+$ cells in the interstitial area. E, $CD8^+$ cells infiltrated in the peri-glomerular area. F, $CD8^+$ cells in the interstitial area. G, IL-17^+ cells infiltrated in the peri-glomerular area. H, IL-17^+ cells in the interstitial area. I, $CD68^+$ cells infiltrated in the intra-glomerular area. J, $CD68^+$ cells in the interstitial area.

Table 1 Demographics, clinical parameters and outcomes of patients with anti-GBM disease

Parameter	*N*=13
Age (year)	30.5±11.8
Gender (male/female)	9/4
Prodromal infection, *n* (%)	5 (38.5)
Hydrocarbon exposure, *n* (%)	1 (7.7)
Smoking, *n* (%)	7 (53.8)
Hemoptysis, *n* (%)	7 (53.8)
Hemoglobin (g L^{-1})	75.2±19.9
Oliguria/anuria, *n* (%)	5 (38.5)
Urinary protein (g 24 h^{-1})	5.3±4.1
Nephrotic syndrome, *n* (%)	6/8 (75.0)
Gross hematuria, *n* (%)	5 (38.5)
Serum creatinine on diagnosis (μmol L^{-1})	907.7±339.0
Level of anti-GBM antibodies (U mL^{-1})	76.2±42.2
Percentage of crescents in glomeruli	93.8±9.0
Cellular crescents	66.2±31.8
Fibro-cellular crescents	28.6±24.0
Fibrotic crescents	5.2±15.2
Cells infiltration in kidneys	
CD3 positive cells (cells mm^{-2})	1776.4±1209.0
CD4 positive cells (cells mm^{-2})	268.8±234.0
CD8 positive cells (cells mm^{-2})	610.8±484.2
IL-17 positive cells (cells mm^{-2})	180.9±39.1
Foxp3 positive cells (cells mm^{-2})	7.0±7.1
CD68 positive cells (cells mm^{-2})	993.9±628.2
Renal survival at one year, *n* (%)	0/13 (0)
Patient survival at one year, *n* (%)	10/13 (76.9)

The association between cell infiltration and crescent formation

Crescent formation is a classical glomerular injury in anti-GBM disease. Following kidney injury, crescents present as cellular, fibrocellular, or fibrous crescents.

The infiltration of $CD3^+$ T cells was more severe around the glomeruli with cellular crescents than those with mild mesangial proliferation ((11.3±8.4) vs. (3.5±2.1) cells gcs^{-1}, P=0.040). The infiltration of $CD4^+$ T cells around the glomeruli with cellular crescents was positively correlated with the serum creatinine level on diagnosis (r=0.786, P=0.021) (Figure 2).

The number of IL-17-producing cells that infiltrated around the glomeruli with fibrocellular crescents was significantly higher than that with mild mesangial proliferation ((1.28±0.58) vs. 0 cells gcs^{-1}, P=0.032). The number of infiltrating IL-17-producing cells in the glomeruli with cellular crescents was positively correlated with the percentage of cellular crescents in the glomeruli (r=0.968, P=0.007).

The correlation between cell infiltration and IgG deposition in kidneys

Linear IgG deposition along the GBM was observed in the renal biopsies of 61.5% of the patients; however, all patients presented circulating antibodies against human α3(IV)NC1. No significant differences in the clinical data and kidney injuries were observed between the patients with and without IgG deposition (Table 3). However, cell infiltration in the kidneys was different. When compared with the patients with classical IgG deposition on the GBM, the patients lacking IgG deposition showed significantly more T cell infiltration in the interstitial area of the kidney ((786.0±316.2) vs. (305.0±196.3) cells mm^{-2}, P=0.017) (Figure 3A) and more $CD8^+$ T cells in the glomerular area (i.e., the intra- and periglomerular areas) with cellular crescents ((10.7±6.4) vs. (1.1±1.0) cells gcs^{-1}, P=0.027) (Figure 3B). No correlation was found between circulating anti-GBM antibodies and infiltrating T cells.

The association between cell infiltration and the rupture of Bowman's capsule

The rupture of Bowman's capsule represents severe damage of the glomerulus. Much more T cells were observed in the interstitial area around the glomeruli with ruptureed Bow-

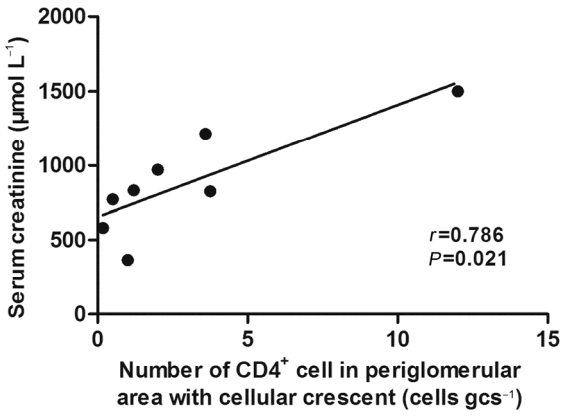

Figure 2 The correlation between the number of $CD4^+$ cells in the periglomerular area with cellular crescent and the level of serum creatinine at diagnosis.

Table 2 Cell infiltration in the intra-glomerular, peri-glomerular, and interstitial areas of the kidneys of patients with anti-GBM disease

Marker	Glomeruli per section	Intra-glomerular area (cells mm^{-2})	Peri-glomerular area (cells mm^{-2})	Interstitial area (cells mm^{-2})	*P* value
$CD3^+$	6.6±3.3	43.6±42.8	1317.6±1033.7	497.4±341.2	0.001
$CD4^+$	7.4±4.5	1.7±4.8	176.3±162.4	119.4±121.2	0.021
$CD8^+$	5.7±2.6	13.9±14.6	456.4±391.7	161.4±118.4	0.002
$IL\text{-}17^+$	6.2±2.9	10.5±13.0	114.5±54.4	67.1±54.5	0.004
$Foxp3^+$	7.6±3.5	0	0	7.0±7.1	0.028
$CD68^+$	6.3±1.5	119.7±21.1	661.6±598.6	186.7±56.8	0.143

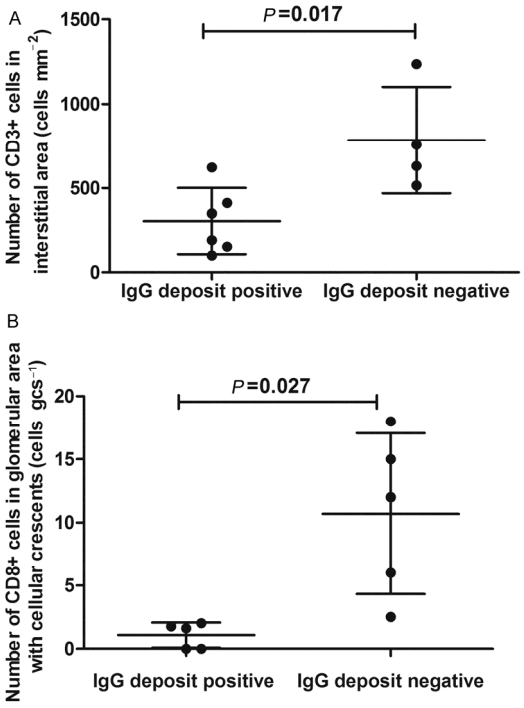

Figure 3 The differences in cell infiltration numbers between patients with IgG deposition in kidneys and those without IgG deposition. A, More $CD3^+$ cells infiltrated in the interstitial area of the kidneys with IgG linear deposition than those lacking the deposition ((786.0±316.2) vs. (305.0±196.3) cells mm^{-2}, P=0.017). B, More $CD8^+$ T cells in the glomerular area of cellular crescents were observed in the kidneys with IgG linear deposition than those without IgG deposits ((10.7±6.4) vs. (1.1±1.0) cells gcs^{-1}, P=0.027).

man's capsule. The number of T cells in the interstitial area was positively correlated with the percentage of glomeruli with ruptured Bowman's capsules (r=0.709, P=0.022) (Figure 4A). When compared with the glomeruli with intact Bowman's capsules, the glomeruli presenting with ruptured Bowman's capsules had significantly higher numbers of $CD8^+$ cytotoxic T cells infiltrating the glomerular area (i.e., the intra- and peri-glomerular areas) ((9.0±6.8) vs. (2.7±2.0) cells gcs^{-1}, P=0.040) (Table 4). Furthermore, the number of infiltrating $CD8^+$ T cells in the glomerular area was positively correlated with the percentage of glomeruli with ruptured Bowman's capsule (r=0.708, P=0.022) (Figure 4B). The number of IL-17-producing cells was also significantly higher in the glomerular area of the glomeruli with ruptured Bowman's capsules than those with intact capsules ((2.1±1.3) vs. (0.5±0.3), P=0.016) (Table 4).

DISCUSSION

In the current study, we identified remarkable T cell infiltration in the kidneys of patients with anti-GBM glomerulonephritis. $CD8^+$ and IL-17^+ cells were the most predominant subtypes and were closely associated with damage to the glomerular structures. This study first identified the infiltration of IL-17-producing cells in the kidneys in human anti-GBM disease. More severe T cell infiltration was observed in the kidneys without IgG deposits, highlighting the pathogenic role for these autoreactive T cells.

T cells were predominantly distributed in the periglom-

Table 3 Demographics, clinical parameters and outcomes of anti-GBM patients, with comparison between patients having IgG linear deposits on GBM and those without deposits

Parameter	IgG deposit positive N=8	IgG deposit negative N=5	P value
Age (year)	28.8±12.4	33.4±11.9	0.522
Gender (male/female)	6/2	3/2	1.000
Prodromal infection, n (%)	1 (12.5)	4 (80)	0.032
Hydrocarbon exposure, n (%)	0	1 (20)	0.385
Smoking, n (%)	4 (50)	3 (60)	1.000
Hemoptysis, n (%)	5 (62.5)	2 (40)	0.592
Hemoglobin (g L^{-1})	77.9±23.8	71.0±12.5	0.567
Oliguria/anuria, n (%)	2 (25)	3 (60)	0.293
Urinary protein (g 24 h^{-1})	6.0±4.5	4.3±3.7	0.532
Nephrotic syndrome, n (%)	4/6 (66.7)	2/2 (100)	1.000
Gross hematuria, n (%)	2 (25)	3 (60)	0.293
Serum creatinine on diagnosis (μmol L^{-1})	1038.3±337.7	698.8±237.8	0.077
Level of anti-GBM antibodies (U mL^{-1})	67.2±44.5	90.6±38.3	0.351
Percentage of crescents in glomeruli	96.6±5.1	89.4±12.4	0.273
Cellular crescents	59.5±26.5	76.8±39.6	0.362
Fibro-cellular crescents	33.8±16.5	20.4±33.5	0.352
Fibrotic crescents	6.9±19.1	2.8±6.3	0.667
Renal survival at one year, n (%)	0/8 (0)	0/5 (0)	–
Patient survival at one year, n (%)	6/8 (62.5)	4/5 (80)	1.000

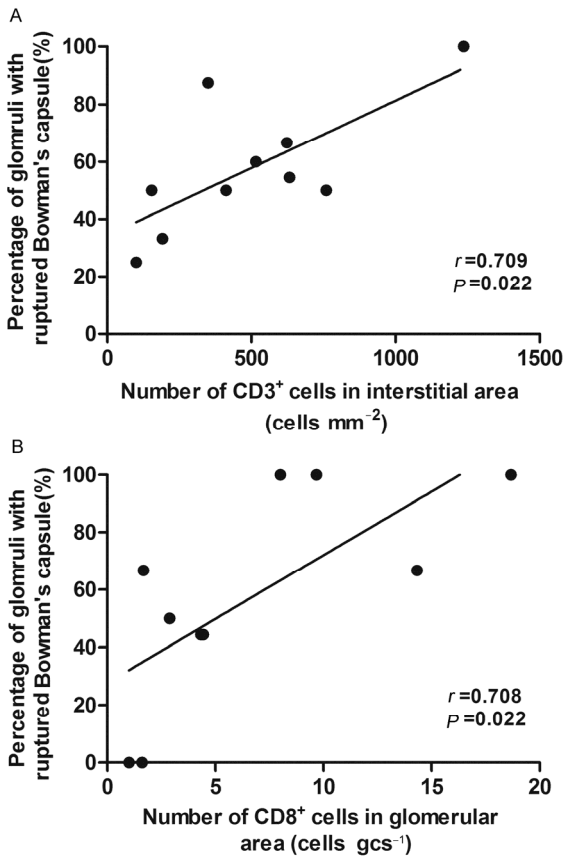

Figure 4 The relationship between cell infiltration and the percentage of ruptured Bowman's capsules. A, The number of T cells in the interstitial area was positively correlated with the percentage of glomeruli with a ruptured Bowman's capsule (r=0.709, P=0.022). B, The number of $CD8^+$ T cells in the glomerular area was positively correlated with the percentage of glomeruli with ruptured Bowman's capsule (r=0.708, P=0.022).

Table 4 Kidney infiltration of T cells and macrophages with and without ruptured Bowman's capsule in patients with anti-GBM disease[a)]

Marker	iBC (cells gcs^{-1})	rBC (cells gcs^{-1})	*P*
$CD3^+$			
Intraglomerular area	0.4±0.5	1.0±1.1	0.127
Periglomerular area	15.3±23.0	16.7±11.3	0.121
Glomerular area	15.7±23.0	17.7±12.1	0.131
$CD8^+$			
Intraglomerular area	0.1±0.1	0.4±0.3	0.031
Periglomerular area	2.6±2.0	8.6±6.8	0.063
Glomerular area	2.7±2.0	9.0±6.8	0.040
$CD4^+$			
Intraglomerular area	0.0±0.1	0.5±1.4	1.000
Periglomerular area	1.6±1.8	3.3±2.9	0.210
Glomerular area	1.6±1.9	3.8±3.9	0.200
IL-17^+			
Intraglomerular area	0.1±0.2	0.4±0.8	0.295
Periglomerular area	0.4±0.4	1.6±0.9	0.036
Glomerular area	0.5±0.3	2.1±1.3	0.016
$CD68^+$			
Intraglomerular area	1.5±0.9	2.6±0.6	0.268
Periglomerular area	3.0±3.5	10.1±9.7	0.248
Glomerular area	4.7±2.5	12.7±10.2	0.379

a) iBC, intact Bowman capsule. rBC, ruptured Bowman capsule. gcs, glomerular cross-section.

erular and interstitial areas, especially around severely injured glomeruli with crescent formation or a ruptured Bowman's capsule. Rutgers et al. also observed that vasculitis patients with periglomerular granulomatous inflammation presented with higher proteinuria and higher activity index scores (Rutgers et al., 2005). These findings indicate that T cell stimulation participates in damaging the kidney structure in human anti-GBM disease.

The first mechanism for the pathogenic role of T cells in kidney injury may be mediated by $CD4^+$ helper T cells. We found that the infiltration of $CD4^+$ cells was much higher in the areas around the glomeruli with crescents and were positively correlated with serum creatinine levels. Previous studies have implied that glomerular-derived antigenic peptides that enter the urine can be captured and degraded by tubular cells and subsequently presented to interstitial dendritic cells, leading to the stimulation of infiltrating T cells (Wilde et al., 2009; Krüger et al., 2004; Macconi et al., 2009). However, the dendritic cells in the interstitia do not natively recognize autoantigens on the GBM. Other resident glomerular cells may have more significant roles in the activation of T cells around the glomeruli. Recent studies have indicated that podocytes from parietal epithelial cells were the predominant cellular components of crescents (Appel et al., 2009); these podocytes function as local professional antigen-presenting cells in the kidney (Goldwich et al., 2013) and may destroy its structure (Li et al., 2015). Further studies are required to determine the mechanisms of interaction between T cells and podocytes to elucidate the activation of $CD4^+$ cells in the kidney *in situ*.

The second mechanism of kidney structure damage may be directly mediated by $CD8^+$ effector T cells. This is supported by the close association between $CD8^+$ T cell infiltration around the glomeruli and the rupture of the Bowman's capsule, as observed in the current study. Such rupturing of the capsule results in the release of glomerular antigens to the intersititia, which initiates a positive feedback cycle involving antigen capture and presentation by renal dendritic cells to the Th cells, the subsequent production of chemokines and cytokines, the recruitment of additional $CD8^+$ T cells and macrophages, and aggravated renal damage. The rupture of the Bowman's capsule may also facilitate the entry of these activated periglomerular T cells and fibroblasts into the Bowman's space, enabling crescent formation and a progressive fibrous organization of cellular crescents (Boucher et al., 1987).

This study was the first to investigate IL-17^+ cells in human anti-GBM disease patients. We provided evidence of IL-17^+ cell infiltration in the kidneys. The IL-17^+ cells were observed more frequently than $CD4^+$ T cells in the glomerular area, indicating that other IL-17-expression cells such, as γδ T cells, neutrophils, and mast cells, may participate in local inflammation (Velden et al., 2012). IL17-producing Th17 cells accounted for much of the T cell-induced inflammation (Miossec et al., 2009; Kitching and Holdsworth,

2011). The presence of Th17 cells has been demonstrated in inflamed kidneys, and the IL23/Th17 axis is considered central to the mediation of kidney injury in anti-GBM models (Ooi et al., 2009). Paust et al. provided mechanistic evidence for a cytokine–chemokine-driven feedback loop that orchestrated the differential Th1 and Th17 cell infiltrations into inflamed kidneys (Paust et al., 2005). A recent study found that renal γδ Tcell was the major source of IL-17 in experimental cresentic glomerulonephritis and promoted kidney injury through IL-23 mediated neutrophil recruitment (Turner et al., 2012). All of the above findings highlight the crucial role of IL-17-producing cells in the pathogenesis of kidney injury in human anti-GBM disease.

In the current study, nearly forty percent of the patients with positive serologic anti-GBM autoantibodies lacked IgG deposition along the GBM in the kidney. Several mechanisms have been suggested for the absence of antibody detection (Cui et al., 2005; Sheerin et al., 2001); however, the mechanism of crescent formation without antibody deposition was unclear. We observed that in the kidneys without IgG deposition, more T cell infiltration was detected than in the kidneys with antibody deposition. In rodent models of anti-GBM nephritis, nearly half of the rats immunized with rCol4α3NC1 developed severe glomerulonephritis and lacked IgG deposition in the kidneys (Wu et al., 2001). rCol4α3NC1-specific T cells have been identified as having a direct pathogenic role in initiating glomerular injury in the absence of autoantibodies (Wu et al., 2002). The therapeutic intervention by the depletion of $CD8^+$ cells using monoclonal antibodies could block crescent formation and glomerulonephritis development in the models without significantly affecting the circulating levels of antibodies (Reynolds et al., 2002; Kawasaki et al., 1992). These findings imply that autoreactive T cells might lead to crescent formation without the need of autoantibodies.

A few unique features of the T cell response to GBM include the appearance of long-living Tregs and the inversion of T cell effector/regulatory cell ratios in later stages of the disease in animal models (Wolf et al., 2005; Ooi et al., 2011; Tan et al., 2013). These features may account for the uncommon recurrences of anti-GBM disease compared with other autoimmune glomerulonephritis (Wolf et al., 2005; Ooi et al., 2011; Tan et al., 2013). In this study, few Tregs were observed in the kidney. This may be due to severe the kidney damage and active disease profile in our patients.

In conclusion, cellular immunity may play a crucial role in the inflammatory kidney injury of anti-GBM patients. The periglomerular and interstitial infiltrations of T cells, especially $CD8^+$ T cells, likely participate in the pathogenic mechanisms of glomerular damage in anti-GBM disease.

MATERIALS AND METHODS

Patients

Renal biopsy samples from 13 patients with biopsy-proven anti-GBM disease were collected at Peking University First Hospital. All patients were positive for serum anti-GBM autoantibodies as determined by commercial ELISA kits (Euroimmun, Germany) using purified bovine α(IV)NC1 as the solid phase antigen, with confirmation of antibody specificity for recombinant human α3(IV)NC1. ANCAs were negatively-screened by both indirect immunofluorescence assay (Euroimmun) and antigen-specific ELISA against myeloperoxidase (MPO) and proteinase 3 (PR3). Patients with other coexisting renal diseases were excluded. Clinical and pathological parameters were collected from medical records at the time of presentation and during follow-up. Biopsy samples were obtained before immunosuppressive treatment. The experimental protocols complied with the Declaration of Helsinki and approved by the ethics committee of Peking University First Hospital. Written informed consent was obtained from each participant.

Routine renal histopathology

Renal biopsy was performed at the time of diagnosis. For direct immunofluorescence measurements, frozen sections were examined by a fluorescent microscopy (Nikon, Japan) after staining with fluorescein isothiocyanate (FITC)-conjugated antibodies specific for human IgG, IgM, IgA, C3c, C1q, fibrinogen, and albumin (Dako, Denmark). For light microscopy observations, paraffinized sections were stained with hematoxylin and eosin, periodic acid-Schiff, periodic acid-silver methenamine, and Masson's trichrome staining. For electron microscopy observations, the biopsy materials were fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in graded acetone, and embedded in Epon 812 resin (Zhongshan Golden Bridge Biotechnology, China). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by a JEM-1230 transmission electron microscope (JEOL, Japan).

Renal immunohistochemistry

To assess the cellular infiltration in renal tissues, immunohistochemical staining was performed for CD3, CD4, CD8, IL-17, foxp3, and CD68 using the primary antibodies listed in Table 5. Four-micron formaldehyde-fixed paraffin-embedded renal tissue sections were deparaffinized in xylene-ethanol, rehydrated in graded alcohol, and immersed in freshly prepared 3% hydrogen peroxide in methanol solution for 10 min at room temperature to quench endogenous peroxidase activity. After washing in phosphate buffered saline (PBS), the sections were immersed in citric acid buffer (0.01 mol L^{-1}, pH 6.0). The sections were then treated at 800 Watts by microwave for 2 min and subsequently at 200 Watts for 8 min for antigen retrieval. To block non-specific staining, the sections were incubated with 3% bovine serum albumin in PBS at 37°C for 30 min. The primary antibodies were added to each section and incubated overnight at 4°C. EnVision horseradish peroxidase (HRP) (Dako A/S, Denmark) was used as a detection system. This

Table 5 Primary antibodies used for immunohistochemistry.

Markers	Cells	Specificity	Dilution	Source
CD3	T lymphocytes	rabbit anti-CD3 monoclonal antibody	1∶300	Lab Vision, Newcastle, UK
CD4	Helper T cells	rabbit anti-CD4 monoclonal antibody	1∶100	Zhongshan Golden Bridge Biotechnology, Beijing, China
CD8	Cytotoxic T cells	rabbit anti-CD8 monoclonal antibody	1∶100	Lab Vision, Newcastle, UK
Foxp3	Regulatory T cells	mouse anti-Foxp3 monoclonal antibody	1∶100	Abcam, Cambridge, UK
IL-17	IL-17-producing cells (especially Th17 cells)	goat anti-IL-17 polyclonal antibody	1∶100	R&D, Wiesbaden, Germany
CD68	Macrophages	mouse anti-CD68 monoclonal antibody	1∶500	Abcam, Cambridge, UK

method uses an avidin-free, two-step indirect method with anti-rabbit, anti-mouse, and anti-goat immunoglobulin conjugated with HRP as secondary antibodies. The sections were incubated with the secondary antibodies for 30 min at 37°C before being developed in fresh hydrogen peroxide plus 3-3-diaminobenzidine tetrahydrochloride solution for 2 min. Finally, the sections were counterstained with periodic acid-Schiff and hematoxylin, and dehydrated using graded alcohols and xylene.

Appropriate dilutions of the primary and secondary antibodies were determined on tissue sections of human lymph nodes and tonsils. The lymph node sections were used as positive controls. Renal tissues obtained from the normal part of a nephrectomized kidney due to renal carcinoma were used as negative controls. They were considered normal, as indicated by light microscopy, immunofluorescence and electron microscopy. The primary antibodies were replaced with normal rabbit or mouse IgG as blank controls.

Tissue analysis and evaluation of positive stained cells in histopathology

Two pathologists independently evaluated all kidney sections and were blind to the clinical data. Differences between the evaluations for the same sections were resolved by reviewing the sections and reaching consensus conclusions.

The number of glomeruli with lesions was expressed as the percentage of injured glomeruli in all the glomeruli in renal biopsy tissues. Each glomerulus was separately scored for the presence of fibrinoid necroses, crescents (i.e., cellular, fibrocellular, and fibrous) and glomerular sclerosis. The periglomerular and interstitial areas were defined as previously described (Couzi et al., 2007). Briefly, the area between the Bowman's capsule and adjacent tubules was defined as the periglomerular area. The peritubular area was defined as the interstitial area excluding the periglomerular area.

The numbers of stained cells in the glomerular and periglomerular areas were counted in each glomerulus and expressed as the number of cells per glomerular cross-section (gcs). Interstitial infiltrating cells were counted in 10 fields at 400× magnification, referring to an area of 0.069 mm^2 $field^{-1}$. The results were expressed as the number of positive cells per square millimeter.

Statistical analysis

Differences in quantitative data were assessed using Student's *t* test (for normally distributed data), or Mann-Whitney *U* test or Wilcoxon 1-sample test (for non-normally distributed data). Differences in semi-quantitative data were evaluated using Kruskal Wallis H one-way analysis and Mann-Whitney *U* test. Differences in qualitative data were compared using Chi square tests. Pearson's correlation test was used to measure the correlation between two normally distributed variables. Spearman's correlation test was used to measure the correlation between two non-normally distributed variables or one normally and one non-normally distributed variables. All statistical analyses were two-tailed, and $P<0.05$ was considered significant. SPSS 13.0 (SPSS, USA) was employed.

Compliance and ethics *The authors declare no conflicts of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.*

Acknowledgements *The authors greatly appreciate the technical support provided by Miao Wang from Renal Division, Department of Medicine, Peking University First Hospital. This work was supported by the National Basic Research Program of China (2012CB517702), the Natural Science Foundation of China (81321064,81330020, 81370801, 81400703).*

Appel, D., Kershaw, D.B., Smeets, B., Yuan, G., Fuss, A., Frye, B., Elger, M., Kriz, W., Floege, J., and Moeller, M.J. (2009). Recruitment of podocytes from glomerular parietal epithelial cells. J Am Soc Nephrol 20, 333–343.

Bolton, W.K., Innes, D.J., Sturgill, B.C., and Kaiser, D.L. (1987). T-cells and macrophages in rapidly progressive glomerulonephritis: clinicopathologic correlations. Kidney Int 32, 869–876.

Boucher, A., Droz, D., Adafer, E., and Noël, L.H. (1987). Relationship between the integrity of Bowman's capsule and the composition of cellular crescents in human crescentic glomerulonephritis. Lab Invest 56, 526–533.

Couzi, L., Merville, P., Deminière, C., Moreau, J.F., Combe, C., Pellegrin, J.L., Viallard, J.F., and Blanco, P. (2007). Predominance of $CD8^+$ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis. Arthritis Rheum 56, 2362–2370.

Cui, Z., Zhao, M.H., Xin, G., and Wang, H.Y. (2005). Characteristics and prognosis of Chinese patients with anti-glomerular basement membrane disease. Nephron Clin Pract 99, c49–c55.

Goldwich, A., Burkard, M., Olke, M., Daniel, C., Amann, K., Hugo, C., Kurts, C., Steinkasserer, A., and Gessner, A. (2013). Podocytes are

nonhematopoietic professional antigen-presenting cells. J Am Soc Nephrol 24, 906–916.
Kalluri, R., Wilson, C.B., Weber, M., Gunwar, S., Chonko, A.M., Neilson, E.G., and Hudson, B.G. (1995). Identification of the alpha 3 chain of type IV collagen as the common autoantigen in antibasement membrane disease and Goodpasture syndrome. J Am Soc Nephrol 6, 1178–1185.
Kawasaki, K., Yaoita, E., Yamamoto, T., and Kihara, I. (1992). Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. Kidney Int 41, 1517–1526.
Kitching, A.R., and Holdsworth, S.R. (2011). The emergence of TH17 cells as effectors of renal injury. J Am Soc Nephrol 22, 235–238.
Krüger, T., Benke, D., Eitner, F., Lang, A., Wirtz, M., Hamilton-Williams, E.E., Engel, D., Giese, B., Müller-Newen, G., Floege, J., and Kurts, C. (2004). Identification and functional characterization of dendritic cells in the healthy murine kidney and in experimental glomerulonephritis. J Am Soc Nephrol 15, 613–621.
Li, X.Z., and He, J. C. (2015). An update: the role of Nephrin inside and outside the kidney. Sci China Life Sci 58, 649–657.
Macconi, D., Chiabrando, C., Schiarea, S., Aiello, S., Cassis, L., Gagliardini, E., Noris, M., Buelli, S., Zoja, C., Corna, D., Mele, C., Fanelli, R., Remuzzi, G., and Benigni, A. (2009). Proteasomal processing of albumin by renal dendritic cells generates antigenic peptides. J Am Soc Nephrol 20, 123–130.
Miossec, P., Korn, T., and Kuchroo, V.K. (2009) Interleukin-17 and type 17 helper T cells. N Engl J Med 361, 888–898.
Ooi, J.D., Phoon, R.K., Holdsworth, S.R., and Kitching, A.R. (2009). IL-23, not IL-12, directs autoimmunity to the Goodpasture antigen. J Am Soc Nephrol 20, 980–989.
Ooi, J.D., Snelgrove, S.L., Engel, D.R., Hochheiser, K., Ludwig-Portugall, I., Nozaki, Y., O'Sullivan, K.M., Hickey, M.J., Holdsworth, S.R., Kurts, C., and Kitching, A.R. (2011). Endogenous foxp3$^+$ T-regulatory cells suppress anti-glomerular basement membrane nephritis. Kidney Int 79, 977–986.
Paust, H.J., Turner, J.E., Riedel, J.H., Disteldorf, E., Peters, A., Schmidt, T., Krebs, C., Velden, J., Mittrücker, H.W., Steinmetz, O.M., Stahl, R.A., and Panzer, U. (2012). Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis. Kidney Int 82, 72–83.
Phelps, R.G., and Rees, A.J. (1999). The HLA complex in Goodpasture's disease: a model for analyzing susceptibility to autoimmunity. Kidney Int 56, 1638–1653.
Reynolds, J., Khan, S.B., Allen, A.R., Benjamin, C.D., and Pusey, C.D. (2004). Blockade of the CD154-CD40 costimulatory pathway prevents the development of experimental autoimmune glomerulonephritis. Kidney Int 66, 1444–1452.
Reynolds, J., Norgan, V.A., Bhambra, U., Smith, J., Cook, H.T., and Pusey, C.D. (2002). Anti-CD8 monoclonal antibody therapy is effective in the prevention and treatment of experimental autoimmune glomerulonephritis. J Am Soc Nephrol 13, 359–369.
Reynolds, J., Tam, F.W., Chandraker, A., Smith J., Karkar, A.M., Cross, J., Peach, R., Sayegh, M.H., and Pusey, C.D. (2000). CD28-B7 blockade prevents the development of experimental autoimmune glomerulonephritis. J Clin Invest 105, 643–651.
Rutgers, A., Slot, M., van, Paassen, P., van, Breda, Vriesman, P., Heeringa, P., and Tervaert, J.W. (2005). Coexistence of anti-glomerular basement membrane antibodies and myeloperoxidase-ANCAs in crescentic glomerulonephritis. Am J Kidney Dis 46, 253–262.
Sado, Y., Naito, I., and Okigaki, T. (1989). Transfer of anti-glomerular basement membrane antibody-induced glomerulonephritis in inbred rats with isologous antibodies from the urine of nephritic rats. J Pathol 158, 325–332.
Sheerin, N.S., Springall, T., Abe, K., and Sacks, S.H. (2001). Protection and injury: the differing roles of complement in the development of glomerular injury. Eur J Immunol 31, 1255–1260.
Tan, D.S., Gan, P.Y., O'Sullivan, K.M., Hammett, M.V., Summers, S.A., Ooi, J.D., Lundgren, B.A., Boyd, R.L., Scott, H.S., Kitching, A.R., Chidgey, A.P., and Holdsworth, S.R. (2013). Thymic deletion and regulatory T cells prevent antimyeloperoxidase GN. J Am Soc Nephrol 24, 573–585.
Tipping, P.G., Huang, X.R., Qi, M., Van, G.Y., and Tang, W.W. (1998). Crescentic glomerulonephritis in CD4- and CD8-deficient mice. Requirement for CD4 but not CD8 cells. Am J Pathol 152, 1541–1548.
Turner, J.E., Krebs, C., Tittel, A.P., Paust, H.J., Meyer-Schwesinger, C., Bennstein, S.B., Steinmetz, O.M., Prinz, I., Magnus, T., Korn, T., Stahl, R.A., Kurts, C., and Panzer, U. (2012). IL-17A production by renal γδ T cells promotes kidney injury in crescentic GN. J Am Soc Nephrol 23, 1486–1495.
Velden, J., Paust, H.J., Hoxha, E., Turner, J.E., Steinmetz, O.M., Wolf, G., Jabs, W.J., Özcan, F., Beige, J., Heering, P.J., Schröder, S., Kneißler, U., Disteldorf, E., Mittrücker, H.W., Stahl, R.A., Helmchen, U., and Panzer, U. (2012). Renal IL-17 expression in human ANCA-associated glomerulonephritis. Am J Physiol Renal Physiol 302, 1663–1673.
Wilde, B., van, Paassen, P., Damoiseaux, J., Heerings-Rewinkel, P., van, Rie, H., Witzke, O., and Tervaert, J.W. (2009). Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis. Nephrol Dial Transplant 24, 2151–2156.
Wilson, C.B., and Dixon, F.J. (1973). Anti-glomerular basement membrane antibody-induced glomerulonephritis. Kidney Int 3, 74–89.
Wolf, D., Hochegger, K., Wolf, A.M., Rumpold, H.F., Gastl, G., Tilg, H., Mayer, G., Gunsilius, E., and Rosenkranz, A.R. (2005). CD4$^+$CD25$^+$ regulatory T cells inhibit experimental anti-glomerular basement membrane glomerulonephritis in mice. J Am Soc Nephrol 16, 1360–1370.
Wu, J., Hicks, J., Borillo, J., Glass, W.F. II., and Lou, Y.H. (2002). CD4$^+$ T cells specific to a glomerular basement membrane antigen mediate glomerulonephritis. J Clin Invest 109, 517–524.
Wu, J., Hicks, J., Ou, C., Singleton, D., Borillo, J., and Lou, Y.H. (2001). Glomerulonephritis induced by recombinant collagen IV alpha 3 chain noncollagen domain 1 is not associated with glomerular basement membrane antibody: a potential T cell-mediated mechanism. J Immunol 167, 2388–2395.