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Clinical impact of urinary CD11b and CD163 on the renal outcomes of anti-neutrophil cytoplasmic antibody-associated glomerulonephritis

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ABSTRACT

Background. The detection of leukocyte-derived CD11b (α subunit of integrin Mac-1) and CD163 (scavenger receptor) in urine may reflect renal inflammation in antineutrophil cytoplasmic antibody-associated glomerulonephritis (ANCA-GN). The objective of this study was to evaluate the clinical significance of urinary CD11b (U-CD11b) and CD163 (U-CD163) in ANCA-GN.

Methods. U-CD11b and U-CD163 were examined using enzyme-linked immunosorbent assay in ANCA-GN urine samples from our institutional cohort ($n = 88$) and a nationwide cohort ($n = 138$), and their association with renal histology was subsequently analyzed. Logistic regression analyses were performed on a nationwide ANCA cohort to determine the associations of the two urinary molecules with renal remission failure at 6 months or with yearly estimated glomerular filtration rate (eGFR) slope over a 24-month observation period.

Results. U-CD11b and U-CD163 were significantly associated with cellular crescent formation and leukocyte accumulation in glomerular crescents. With regard to interstitial inflammation, both levels of U-CD11b and U-CD163 at diagnosis remarkably increased in ANCA-GN compared with the levels observed in nonglomerular kidney disorders including nephrosclerosis, immunoglobulin G4-related disease and tubulointerstitial nephritis; however, the presence of U-CD11b alone was significantly correlated with tubulointerstitial leukocyte infiltrates. Although neither U-CD11b nor U-CD163 at diagnosis was associated with remission failure at 6 months, multivariate analysis demonstrated that the baseline U-CD11b levels were significantly associated with the increase in eGFR following immunosuppressive therapy.

Conclusions. Although both U-CD11b and U-CD163 reflect renal leukocyte accumulation, U-CD11b at diagnosis provides

KEY LEARNING POINTS

What is already known about this subject?

- Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) presenting clinically as rapidly progressive glomerulonephritis (RPGN) and histologically as crescentic glomerulonephritis is an urgent condition that critically impacts both the renal and the life prognosis of patients.
- Although clinical manifestations including active urine sediment and RPGN with the presence of ANCA in the serum can indicate ANCA-associated glomerulonephritis (ANCA-GN), histopathological evaluation of kidney specimens is essential not only to reach a definitive diagnosis but also for assessing disease activity, renal prognosis and therapeutic indication.
- CD11b (α subunit of integrin Mac-1) on neutrophils and monocytes/macrophages and soluble form of CD163 (a scavenger receptor for the hemoglobin–haptoglobin complex) on alternatively activated macrophages have been shown to be present in urine of patients with lupus nephritis and ANCA-GN.

What this study adds?

- Because renal biopsy is an invasive procedure that is often avoided in elderly patients with AAV who have atrophic kidneys or poor general conditions including respiratory dysfunction and hemorrhagic complications, non-invasive diagnostic methods are clinically desirable for both initial diagnosis and re-evaluation of disease activity.
- This study revealed the association of two urinary biomarkers of CD11b (U-CD11b) and CD163 (U-CD163) with histological manifestations in the Nagoya Kidney Disease Registry and compared the significance of urinary leukocyte-derived molecules on clinical outcomes in nationwide RemIT-JAV-RPGN cohort.

What impact this may have on practice or policy?

- Urinary (U)-CD11b and U-CD163 at diagnosis reflected crescent formation and glomerular leukocyte accumulation in ANCA-GN kidneys.
- Levels of U-CD11b and U-CD163 at diagnosis were remarkably increased in ANCA-GN compared with the levels observed in non-glomerular kidney disorders.
- U-CD11b levels at diagnosis were significantly associated with the increase in eGFR following immunosuppressive therapy, indicating that U-CD11b can be a biomarker to predict the recovery rate in diseased kidneys after the treatment of ANCA-GN.

additional clinical value by predicting the recovery rate after the treatment of ANCA-GN.

Keywords: anti-neutrophil cytoplasmic antibody, biomarkers, inflammation, macrophages, neutrophils

INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) causes damage to multiple organs and is characterized by inflammation and necrosis of blood vessel walls [1]. Particularly, renal involvement presenting clinically as rapidly progressive glomerulonephritis (RPGN) and histologically as crescentic glomerulonephritis (CGN) is an urgent condition that critically impacts both the renal and the life prognosis of patients with AAV [2, 3]. Although clinical manifestations including active urine sediment and RPGN with the presence of ANCA in the serum can indicate ANCA-associated glomerulonephritis (ANCA-GN), histopathological evaluation of kidney specimens is essential not only to reach a definitive diagnosis but also for assessing disease activity, renal prognosis and therapeutic indication [4]. Particularly, the recent European Vasculitis Study (EUVAS) Group classification for glomerular injuries in ANCA-GN kidneys clarified that cellular crescents and glomerular sclerosis impact the recovery of renal function and renal outcomes, respectively [2]. However, renal biopsy is an invasive procedure that is often avoided in elderly patients with AAV who have atrophic kidneys or poor general conditions including pulmonary dysfunction and hemorrhagic complications. Therefore, noninvasive diagnostic methods are clinically desirable for both initial diagnosis and reevaluation of disease activity.

We and others have shown that the soluble form of CD163, a scavenger receptor for the hemoglobin–haptoglobin complex specifically expressed on alternatively activated macrophages [5], in the urine of patients with lupus nephritis (LN) and ANCA-GN reflects histopathological disease activity [6, 7]. More recently, we revealed elevated CD11b, an α subunit of integrin Mac-1 on neutrophils and monocytes/macrophages, in the urine (U-CD11b) of patients with LN and ANCA-GN [8]. Because Mac-1 is released from the leukocyte cell surface following leukocyte activation by inflammatory signals [9], cleaved CD11b can be detected in the urine of patients with ANCA-GN.

In our research institute and its affiliated hospitals, ANCA-GN patients with kidney disease are registered in the Nagoya Kidney Disease Registry (N-KDR) [6, 8]. In addition to the N-KDR, the Japan Research Committee of the Ministry of Health, Labour and Welfare for Intractable Vasculitis and for Intractable Renal Disease collaboratively implemented a nationwide inception cohort study of remission induction therapy in Japanese patients with AAV and RPGN (RemIT-JAV-RPGN) [10], which was prospectively conducted to reveal the epidemiological characteristics of newly diagnosed AAV [3, 11] and subsequently evaluate the histopathological

manifestations [12] and identify AAV serum biomarkers by proteomic analysis [13].

In the present study, which was aimed at developing noninvasive screening tests to identify patients with active ANCA-GN, we validated the association of two urinary biomarkers of CD11b and CD163 with histological manifestations in the N-KDR and compared the significance of urinary leukocyte-derived molecules on clinical outcomes in the nationwide RemIT-JAV-RPGN cohort.

MATERIALS AND METHODS

Patients and sample collection from N-KDR and RemIT-JAV-RPGN cohorts

Urine samples and kidney specimens were obtained from 88 patients with ANCA-GN diagnosed with microscopic polyangiitis (MPA) or granulomatous polyangiitis (GPA) according to the criteria for primary systemic vasculitis as proposed by the European Medicines Agency algorithm [14] and who underwent renal biopsy at Nagoya University or its affiliated hospitals between 1 January 2011 and 31 December 2015 (N-KDR). Clinical and laboratory data were collected retrospectively from medical records at the time of renal biopsy in the N-KDR AAV cohort. To represent disease controls as individuals who presented with tubulointerstitial injuries or leukocyte accumulation but lacked glomerular inflammation, patients histologically diagnosed with nephrosclerosis (NeSc; $n = 20$), immunoglobulin G4 (IgG4)-related disease (IgG4RD; $n = 12$) and tubulointerstitial nephritis (TIN; $n = 15$) in the N-KDR were included in the study.

In the RemIT-JAV-RPGN study population, the clinical, laboratory and histological data of 138 patients with MPA or GPA, whose urine samples were available, were extracted from the database [3, 12]. Patients with eosinophilic granulomatosis with polyangiitis were excluded from the cohort because of the small number of cases available. Additionally, unclassified cases in RemIT-JAV-RPGN were excluded. Because we extracted subpopulations with urine samples, which included 56 cases with paired urine samples at 6 months after diagnosis, from among 321 patients enrolled in RemIT-JAV-RPGN [3], we reevaluated the demographic and clinical information from the RemIT-JAV-RPGN as presented in Table 1.

The N-KDR AAV cohort lacks information on the clinical patient outcomes and urine samples at 6 months, whereas the RemIT-JAV-RPGN lacks paraffin-embedded kidney samples for immunostaining by renal leukocyte identification.

This study was approved by the institutional review boards at Nagoya University and was conducted in accordance with the principles of the Declaration of Helsinki. All patients provided written informed consent regarding participation in N-KDR and RemIT-JAV-RPGN.

Evaluation of clinical disease activity and renal outcomes in ANCA-GN

Renal disease activity in patients with AAV who had urine samples was evaluated by determining renal scores according to the Birmingham Vasculitis Activity Score (BVAS) 2003 [15]. Patients in remission were defined so when their score was 0 after two evaluations using the BVAS criteria in at least 1-month intervals and a daily glucocorticoid (GC) dosage of $\leq$15 mg prednisolone at 6 months. Patients without follow-up data within 6 months were excluded as previously described [3].

Renal remission failure at 6 months was defined as the short-term remission failure, and the estimated glomerular filtration rate (eGFR) slope characteristics were analyzed as the long-term renal outcome. We assumed the change in eGFR over a 24-month follow-up period to be linear and applied the linear mixed-effects models to estimate eGFR slopes for 138 patients. To estimate yearly eGFR changes, the sum of random and fixed effects of the interaction terms between baseline eGFR and the measurement time was multiplied by 12.

Assessment of kidney histopathology and glomerular leukocyte infiltrates

In the N-KDR AAV cohort, periodic acid–Schiff-, hematoxylin and eosin- and periodic acid–methenamine silver-stained kidney samples from 64 patients in the N-KDR obtained from 1 January 2011 to 31 December 2014 were evaluated by light microscopy. Kidney sections in 61 cases (excluding three cases with fewer than five glomeruli) were also stained to detect leukocyte subsets expressing CD11b, CD163, CD68 and esterase as previously described [6, 8]. In glomerular observation, the average number of glomerular leukocyte subsets per glomerulus was determined in 61 cases by examining at least five glomeruli from each patient. To assess leukocyte accumulation in the tubulointerstitial area, the number of leukocyte subsets counted with TissuemorphDP (Visiopharm, Hørsholm, Denmark) on three individual images at $\times$200 magnification was divided by the evaluation area.

Glomerular crescent formation was evaluated as the ratio of the number of cellular or uncategorized crescentic glomeruli divided by the total number of glomeruli. Glomerular crescent classes on renal biopsies were categorized according to EUVAS classification [2] in the N-KDR AAV cohort. Two independent observers examined the renal tissue using light microscopy to diagnose the renal involvement of AAV. In the RemIT-JAV-RPGN, crescent classes in the current study were obtained from the data set of the whole previously described cohort population [12].

Measurement of renal function and urinary proteins

Urine samples were centrifuged at 3000*g* for 10 min at 4°C, and the supernatants were stored at −80°C until use. The concentrations of U-CD11b and U-CD163 in urines diluted 1–20 times their original concentrations were evaluated by enzyme-linked immunosorbent assay kits [MBS702254 for human CD11b (MyBioSource, CA, USA); DY1607 DuoSet for human CD163 (R&D Systems, MN, USA)] and urine creatinine (U-Cr) was measured enzymatically as described in our previous studies [6, 8]. The concentrations of U-CD11b, U-CD163 and U-Alb were normalized to that of U-Cr. The eGFR was calculated using the eGFR equation modified for Japanese patients [16].

Table 1. Patient characteristics of the study population in N-KDR and RemIT-JAV-RPGN cohorts

Cohort	N-KDR				RemIT-JAV-RPGN
Disease	NeSc	IgG4RD	TIN	ANCA-GN	ANCA-GN
Number of patients	20	12	15	88	138
Age at biopsy[a] (years)	44 (37–62)*	73 (66–75)	66 (59–72)	71 (65–76)	71 (62–78)
Male[b]	6 (30.0)	11 (91.7)	9 (60.0)	44 (50.0)	63 (45.7)
Disease onset (number of newly diagnosed/relapsed patients)				87/1	138/0
Treatments at the time of sampling[c] (number of patients treated with none/GC/IS/GC + IS /unknown)				48/36/1/3/0	NA
Number of patients treated with GC/IS/GC + IS/unknown for the remission induction therapy[c]				NA	59/1/77/1
S-Cr, mg/dL[a]	3.45 (1.34–4.80)	1.84 (1.41–2.91)	2.45 (2.03–3.82)	2.46 (1.40–4.23)	1.55 (0.82–3.92)[§]
eGFR, mL/min/1.73 m^2[a]	17.0 (9.20–34.7)	26.6 (17.4–37.5)	20.8 (10.0–26.5)	18.8 (11.0–38.0)	31.3 (11.7–62.3)[‖]
U-P/U-Cr, g/g[a]	1.10 (0.53–2.35)	0.42 (0.26–1.06)	0.54 (0.25–1.04)	1.28 (0.55–2.47)	0.76 (0.27–1.37)[§]
U-CD11b/U-Cr [ng/mg (ng/mmol)][a]	3.28 (371.0) [0.87–6.44 (98.4–728.5)]	4.52 (511.3) [2.29–24.8 (259.0–2805.4)]	10.9 (1233.0) [7.76–45.8 (877.8–5181.0)]	26.6 (3009.0) [12.8–67.4 (1448.0–7624.4)][†]	26.7 (3020.4) [13.0–76.5 (1470.6–8653.8)]
U-CD163/U-Cr [ng/mg (ng/mmol)][a]	0.80 (90.5) [0.34–1.87 (38.5–211.5)]	0.39 (44.1) [0.18–0.95 (20.4–107.5)]	0.36 (40.2) [0.24–0.79 (27.1–89.4)]	4.02 (454.8) [1.91–11.4 (216.1–1289.6)][‡]	3.30 (373.3) [1.36–7.63 (153.8–863.1)]
$CD11b^+$ cell accumulation in tubulo interstitial area, cells/μm^2[a]	28.7 (22.8–46.3)	251.6 (133.7–353.5)	371.3 (92.7–700.4)	86.6 (42.0–153.8)	NA
MPA/GPA				82/6	113/25
ANCA (number of patients of MPO/PR-3/both				82/6/0/0	119/12/4/3
Renal BVAS[a]				12 (10–12)	12 (10–12)
Number of patients with glomerular crescent category by EUVAS classification				88	42
Focal, %[b]				31 (35.2)	22 (52.4)
Crescentic, %[b]				22 (25.0)	6 (14.3)
Mixed, %[b]				26 (29.5)	9 (21.4)
Sclerotic, %[b]				9 (10.2)	5 (11.9)

[a]Data are expressed as the median (IQR).
[b]Data are expressed as the *n* (%).
[c]IS in the N-KDR cohort include cyclophosphamide ($n = 2$), mizoribine ($n = 1$), tacrolimus ($n = 1$) and those in the RemIT-JAV-RPGN include cyclophosphamide ($n = 67$), azathioprine ($n = 7$), mizoribine ($n = 1$), tacrolimus ($n = 1$) and tocilizumab ($n = 2$).
MPO: myeloperoxidase; PR-3: proteinase 3; NA: not applicable; U: urine.
*$P < 0.0001$ versus IgG4RD, TIN and ANCA-GN by the Kruskal–Wallis test.
[†]$P < 0.05$ versus NeSc and versus IgG4RD.
[‡]$P < 0.0001$ versus NeSc, versus IgG4RD and versus TIN by Dunn's multiple comparison test.
[§]$P < 0.01$ versus N-KDR ANCA-GN.
[‖]$P < 0.05$ versus N-KDR ANCA-GN by Mann–Whitney U-test.

Each patient was classified into CKD stages according to the criteria described elsewhere [17]. Urinary concentrations of total protein and Cr from both cohorts were reevaluated at Nagoya University by Mitsubishi Kagaku BCL, Inc. (Tokyo, Japan).

Evaluations of the diagnostic values of U-CD11b and U-CD163 and their associations with renal outcomes

RemIT-JAV-RPGN cases whose remission status data at 6 months ($n = 123$) and on the yearly eGFR slope over the course of the 24-month follow-up period ($n = 138$) were available for the study. Urinary protein (U-P), U-CD11b and U-CD163 values normalized by U-Cr were converted to common logarithm ($\log_{10}$) and then used as continuous variables for the regression analyses. To assess the predictive values of covariates, including baseline levels of biomarkers (U-CD11b, U-CD163, eGFR and U-P) and the basic demographic data (age and sex), logistic and linear regression models were, respectively, employed for renal remission failure at 6 months and the yearly eGFR slope.

Statistical analysis

The patients' clinical characteristics were summarized as medians with interquartile ranges (IQRs) or frequencies with percentages. The Mann–Whitney U-test was used to compare continuous variables between two groups. To compare more than three groups, data showing a significant difference ($P < 0.05$) by the Kruskal–Wallis test were subjected to Dunn's multiple comparison test for comparison between two groups. Wilcoxon matched-pairs signed rank test was used to compare longitudinal continuous variables. Spearman's rank correlation coefficient was used to examine the strength of association between two variables. All statistical analyses were conducted using STATA MP version 16.0 (STATA Corp., College Station, TX, USA).

RESULTS

Characteristics of ANCA-GN patients in N-KDR and RemIT-JAV-RPGN cohorts

Patient profiles, clinical data and kidney histological evaluations in both the N-KDR and the RemIT-JAV-RPGN research populations are summarized in Table 1.

All cases in the N-KDR cohort were newly diagnosed ANCA-GN cases except for one relapsed case maintained with 5 mg/day of GC. The cohort included a rheumatoid arthritis case maintained with tacrolimus prior to renal biopsy. Patients in 9 cases were treated with remission induction therapy using GC alone for 2–4 weeks and 30 cases received GC alone ($n = 27$) or GC plus an immunosuppressant (IS; cyclophosphamide: $n = 2$; and mizoribine: $n = 1$) in the 2 weeks preceding the histological diagnosis of ANCA-GN (Table 1).

All cases enrolled in the RemIT-JAV-RPGN cohort were newly diagnosed ANCA-GN patients, although information regarding the treatment administered prior to renal biopsy was not collected. Remission induction therapies were introduced to all cases in the RemIT-JAV-RPGN cohort, and the number of patients who received each type of remission induction therapy is presented in Table 1.

Renal dysfunction and proteinuria were comparable among all disease control groups, except for the NeSc population which included patients who underwent biopsy at a younger age than those with other diseases. Age at biopsy, sex, disease rate of MPA/GPA, ANCA subtypes and renal BVAS scores were comparable between the AAV populations from the N-KDR and RemIT-JAV-RPGN, but serum creatinine (S-Cr) and U-P were significantly elevated in the N-KDR compared with the RemIT-JAV-RPGN. In histological evaluation of glomerular crescent formation determined as focal, crescentic, mixed and sclerotic according to EUVAS classification [2], the focal class accounted for a much higher proportion than the other three classes in study populations from the RemIT-JAV-RPGN. These data collectively indicate that the N-KDR includes clinically and histologically more advanced cases than the RemIT-JAV-RPGN.

Associations of U-CD11b and U-CD163 with glomerular crescent category by EUVAS classification, proteinuria and eGFR in ANCA-GN

The association of U-CD163 with glomerular crescent formation in ANCA-GN was recently documented in a European AAV population [7]; therefore, we next analyzed elevations in U-CD11b and U-CD163 at histological stages of crescent formation according to the updated EUVAS classification [2]. In our combined study population of the N-KDR and the RemIT-JAV-RPGN cohorts, U-CD11b and U-CD163 levels in the crescentic class were significantly elevated among the four categories (Figure 1A and B). Notably, the U-CD11b level showed a weak correlation with the U-CD163 level in the combined AAV population (Figure 1C) as well as with both U-P and eGFR compared with the U-CD163 level (Figure 1D–G). These findings collectively indicate that U-CD11b and U-CD163 levels individually reflect renal inflammation in ANCA-GN.

Distributions of $CD11b^+$ and $CD163^+$ infiltrates in ANCA-GN glomeruli from N-KDR cohort and their associations with U-CD11b or U-CD163

Because of the dominant expression of CD11b on neutrophils and macrophages and CD163 on macrophages, we assessed glomerular $CD11b^+$ cells, $CD163^+$ cells, $esterase^+$ neutrophils and $CD68^+$ macrophages in AAV kidneys from 61 cases in the N-KDR (Figure 2A). Infiltrations of $CD11b^+$ leukocytes, neutrophils and $CD68^+$ macrophages were dominantly observed in the glomerular tufts (Figure 2B, D and E). In contrast, $CD163^+$ leukocytes were equally distributed both in the glomerular tufts and in the crescents (Figure 2C).

We subsequently analyzed the correlations of U-CD11b and U-CD163 levels with the proportion of cellular crescents and with $CD11b^+$ or $CD163^+$ cell infiltrates in the glomerular tufts or crescents. Notably, U-CD11b or U-CD163 levels were

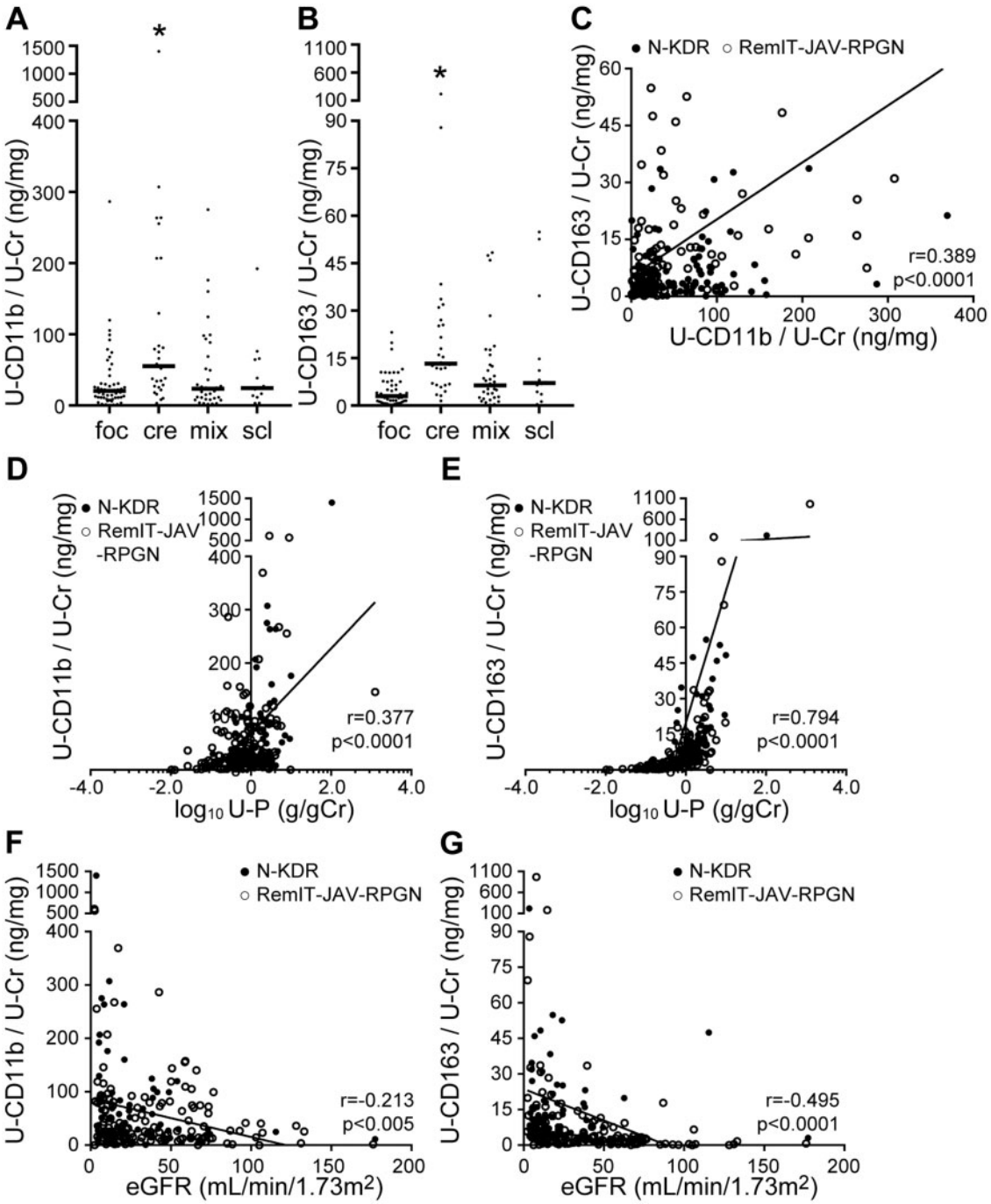

FIGURE 1: Levels of U-CD11b and U-CD163 across the glomerular crescent category and those associations with eGFR and proteinuria in the combined population of N-KDR and RemIT-JAV-RPGN cohorts. (**A** and **B**) Levels of U-CD11b (**A**) and U-CD163 (**B**) across glomerular crescent categories in ANCA-GN patients from the combined cohort of N-KDR ($n = 61$) and RemIT-JAV-RPGN ($n = 42$). Glomerular crescent classes of focal, crescentic, mixed and sclerotic were denoted according to EUVAS classification as foc, cre, mix and scl, respectively. Each patient is represented by a dot, and the median of each group is shown as a horizontal bar. *$P < 0.05$ in comparison among the four categories was determined by the Kruskal–Wallis test. (**C**–**G**) Correlations of U-CD11b levels with U-CD163 levels (**C**), and those of U-CD11b and U-CD163 levels with U-P (**D** and **E**) and eGFR (**F** and **G**) on ANCA-GN biosamples from the combined cohort. Urinary concentrations of both biomarkers and protein were corrected by U-Cr. Values of U-P/U-Cr were converted to common logarithm ($\log_{10}$) for the analysis. Each patient from N-KDR cohort (black circles) and from RemIT-JAV-RPGN cohort (white circles) is represented by a dot. Correlation lines, coefficients of determination (R), and P-values are shown.

significantly associated with cellular crescent formation and were more strongly correlated with cell accumulation in the crescents than in the glomerular tufts or whole area in Bowman's capsules, suggesting that U-CD11b and U-CD163 reflect the active phase in AAV-related glomerular inflammation (Supplementary data, Table S1, Figure 2F–I).

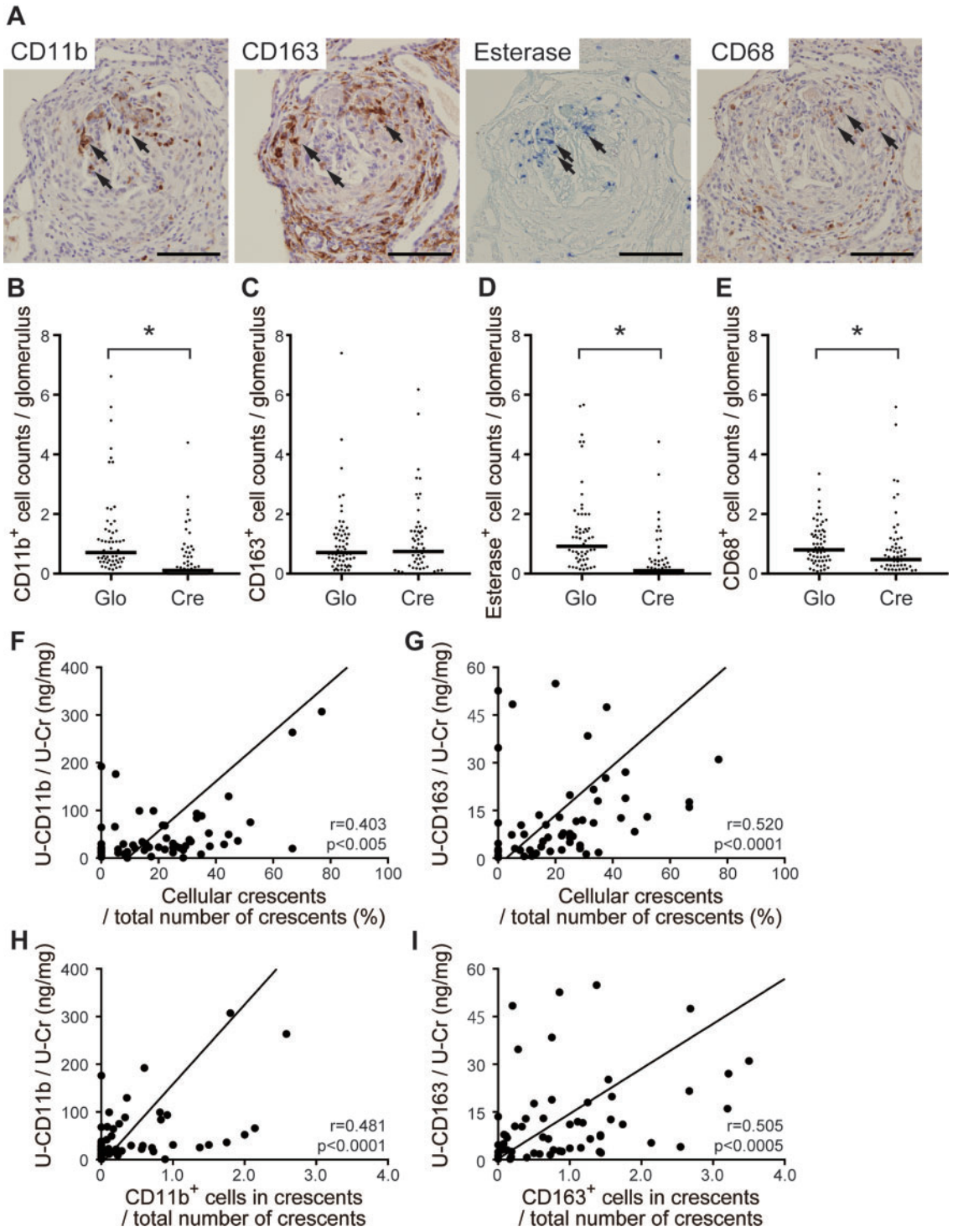

FIGURE 2: Histological analysis of CD11b$^+$ cells, CD163$^+$ cells, neutrophils and macrophages in human ANCA-GN kidneys from N-KDR cohort. (**A**) Light-microscopic observation of CD11b$^+$ cells, CD163$^+$ cells, esterase$^+$ neutrophils and CD68$^+$ macrophages in a crescentic glomerulus of human ANCA-GN kidney from the N-KDR cohort. Arrows represent cells expressing indicated leukocyte markers. Bar: 100 μm. (**B–E**) Histological quantification of CD11b$^+$ cells, CD163$^+$ cells, esterase$^+$ neutrophils and CD68$^+$ macrophages in glomerular tufts (Glo, left) and in a crescent (Cre, right) in an AAV glomerulus from N-KDR cohort ($n = 61$). Each patient is represented by a dot and the median value of each group is shown as a horizontal bar. *$P < 0.05$ by Mann–Whitney U-test. (**F** and **G**) Correlations of U-CD11b (**F**) and U-CD163 (**G**) corrected by U-Cr with the formation rate of glomerular cellular crescents on ANCA-GN biosamples from N-KDR cohort ($n = 61$). (**H** and **I**) Correlations of U-CD11b (**H**) and U-CD163 (**I**) corrected by U-Cr with leukocytes expressing the respective molecules in glomerular crescents on ANCA-GN biosamples from N-KDR cohort ($n = 61$). Each patient is represented by a dot. Although outliers in U-CD11b and U-CD163 levels have been omitted because of scaling, correlation lines, coefficients of determination (R) and P-values analyzed in the whole study population (U-CD11b/U-Cr, median 26.6 ng/mg, range of values 0.72–1405.8 ng/mg; U-CD163/U-Cr, median 7.45 ng/mg, range of values 0.24–220.9 ng/mg) are shown.

U-CD11b, but not U-CD163, is associated with tubulointerstitial leukocyte infiltration in ANCA-GN kidneys of the N-KDR cohort

ANCA-GN often presents as a tubulointerstitial injury including ANCA-related peritubular capillaritis or tubular atrophy and interstitial fibrosis subsequent to glomerular damage. Although previous studies clearly demonstrated increased levels of U-CD11b and U-CD163 in ANCA-GN compared with other glomerular diseases [6–8], associations of U-CD11b or U-CD163 with histological tubulointerstitial manifestations were not evaluated. Therefore, we first compared U-CD11b and U-CD163 among kidney disease controls presenting with tubulointerstitial injuries or inflammation but lacking glomerular inflammation via renal histology, as presented in Table 1. Both U-CD11b and U-CD163 were significantly elevated in patients with ANCA-GN compared with those with NeSc, IgG4RD and TIN (Figure 3A and B). Although the kidney histology of the N-KDR AAV cohort showed tubulointerstitial accumulation of $CD11b^+$ and $CD163^+$ leukocytes (Figure 3C), the U-CD11b levels significantly correlated with tubulointerstitial accumulation of $CD11b^+$ leukocytes (Figure 3D). In contrast, U-CD163 levels did not correlate with $CD163^+$ leukocytes (Supplementary data, Figure S1A), indicating the association of U-CD11b with ANCA-related leukocyte accumulation in the tubulointerstitium and the specificity of U-CD163 for glomerular injuries in ANCA-GN. Tubulointerstitial $CD11b^+$ cell accumulation in NeSc, which accounted for one-third of those with AAV (Table 1), and in IgG4RD and TIN, which accounted for 2.9 and 4.3 times more than those in ANCA-GN, respectively (Table 1), did not correlate with U-CD11b levels (Supplementary data, Figure S3B–D), partly because of the limited number of study populations (NeSc: $n = 20$; IgG4RD: $n = 12$; and TIN: $n = 15$).

U-CD11b and U-CD163 levels in response to remission induction therapy and their associations with the remission status and with CKD stages at 6 months in ANCA-GN patients

Remission induction therapies with corticosteroid alone or in combination with other therapeutic options such as IS, intravenous immunoglobulin and plasma exchange were implemented in all patients in the RemIT-JAV-RPGN cohort. Therefore, S-Cr, U-P, hematuria and urinary casts were significantly improved in the whole population at 6 months after remission induction therapy (Table 2). Although the levels of U-CD163, but not of U-CD11b, in 55 identical patients at diagnosis and at 6 months were significantly reduced after the 6-month follow-up period (Figure 4A and B), 11 patients showed sustained disease activity compared with 45 patients with remission at the 6-month follow-up after remission induction therapy (Table 2).

Next, we evaluated U-CD11b and U-CD163 levels recorded at the time of diagnosis ($n = 123$ including additional 68 patients for whom urine samples were collected only at diagnosis) and 6 months after remission induction therapy ($n = 55$). Patients who did not achieve remission or had Stage 3/4/5 at 6 months showed significant elevation of

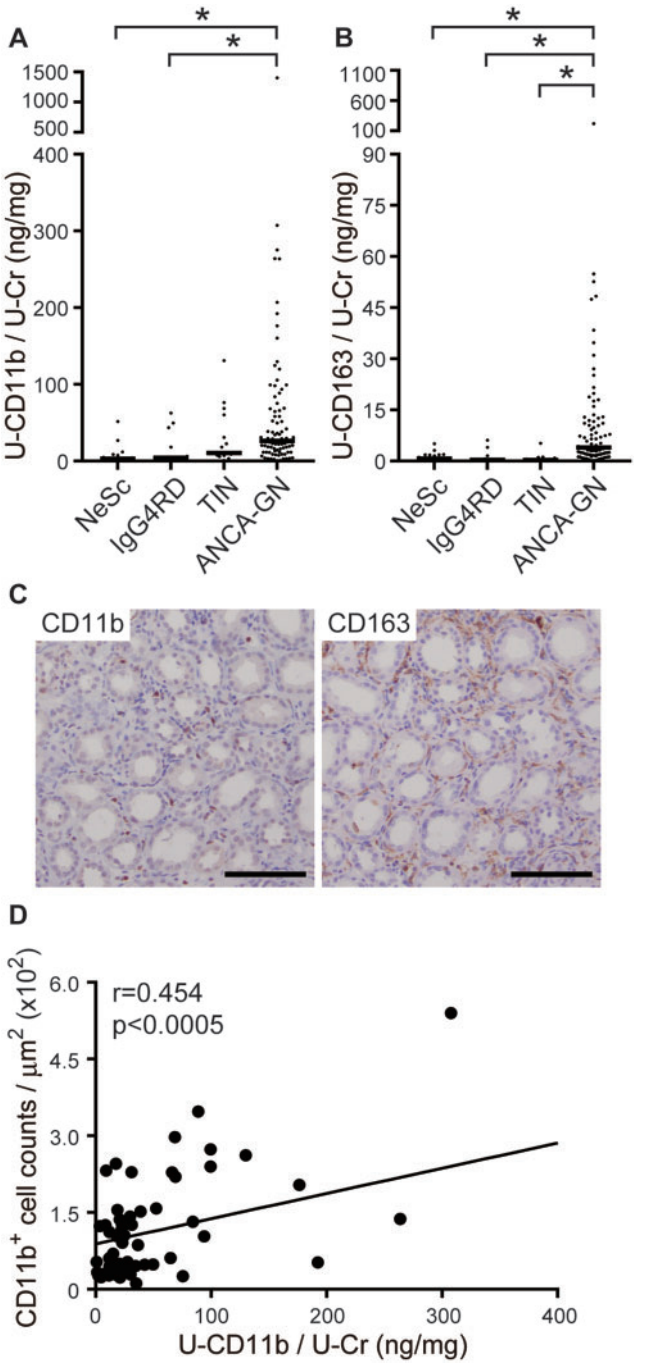

FIGURE 3: Elevation of U-CD11b and U-CD163 and the association of U-CD11b with tubulointerstitial $CD11b^+$ infiltrates in human ANCA-GN kidneys from the N-KDR cohort. (**A** and **B**) Urinary excretion of CD11b (U-CD11b) (**A**) and CD163 (U-CD163) (**B**) corrected by U-Cr in nephrosclerosis (NeSc, $n = 20$), IgG4RD ($n = 12$), TIN ($n = 15$) and ANCA-GN ($n = 88$) from the N-KDR cohort. *$P < .05$ by Dunn's multiple comparison test. (**C**) Representative light-microscopic images of tubulointerstitial $CD11b^+$ (left) and $CD163^+$ (right) infiltrations in ANCA-GN kidneys from the N-KDR cohort. Bar: 100 μm. (**D**) Correlations of tubulointerstitial $CD11b^+$ infiltrations with levels of U-CD11b in ANCA-GN kidneys from N-KDR cohort ($n = 61$). Each patient is represented by a dot. Although outliers in U-CD11b levels have been omitted because of scaling, a correlation line, coefficient of determination (R) and P-value analyzed in the whole study population (U-CD11b/U-Cr; median 26.6 ng/mg, range of values 0.72–1405.8 ng/mg) are shown.

Table 2. Patient characteristics of identical patients at diagnosis and after 6-month follow-up period in RemIT-JAV-RPGN cohort

Characteristic	At diagnosis (0 months)	At 6 months
Age at the biopsy[a] (years)	70 (62–77)	
Male[b]	28 (50.0)	
MPA/GPA	41/15	
Achieved/nonachieved remission (number of patients)	0/56	45/11
Relapse within/later than 6 months (number of patients)	NA	0/7
CKD stage (number of patients of stage 1/2/3/4/5)	6/12/19/4/14[c]	3/17/27/4/4[c]
S-Cr, mg/dL[a]	1.12 (0.72–2.09)[d]	0.96 (0.82–1.29)[d], *
eGFR, mL/min/1.73 m^2[a]	44.6 (18.3–63.5)[d]	49.1 (38.0–64.1)[d]
U-P/U-Cr, g/g[a]	0.53 (0.15–0.99)	0.14 (0.07–0.57)*
Hematuria (score, number of patients)	3+, 29; 2+, 16; 1+, 6; −, 5	3+, 4; 2+, 4; 1+, 10; −, 36[e]
RBC cast (number of patients of +/−/unknown/undescribed)	45/10/1/0	6/41/8/1
granular cast (number of patients of +/−/unknown/undescribed)	28/26/2/0	2/44/9/1
U-CD11b/U-Cr, ng/mg[a]	36.9 (12.9–78.1)	27.1 (11.5–50.0)
U-CD163/U-Cr, ng/mg[a]	2.91 (0.81–6.00)	0.43 (0.23–0.94)*

[a]Data are expressed as the median (IQR).
[b]Data are expressed as the *n* (%).
[c]*n* = 55; one case lacking data was excluded from the study.
[d]*n* = 53; two cases on hemodialysis and one case lacking data at 6 months were excluded from the analysis.
[e]*n* = 54; two cases lacking data at 6 months were excluded from the analysis.
RBC: red blood cell; U: urine; NA: not available.
*P < 0.005 by Wilcoxon matched-pairs signed-rank test.

U-CD163 at diagnosis, but not at 6 months (Figure 4D and F). In contrast, baseline U-CD11b levels did not affect the remission status or CKD stage after 6 months of treatment (Figure 4C and E).

Diagnostic abilities of baseline U-CD11b and U-CD163 levels to predict at 6 months renal outcomes in ANCA-GN patients

To evaluate the clinical significance of U-CD11b and U-CD163 in ANCA-GN and the associations between them, we performed logistic regression analyses with remission failure status data at 6 months in 123 RemIT-JAV-RPGN patients. Univariable and multivariable analyses were performed using U-CD11b (Model 1–1), U-CD163 (Model 1–2) or both (Model 2), which were adjusted for age, sex and baseline eGFR and U-P. These models demonstrated U-P, but neither U-CD11b nor U-CD163, associated with renal remission failure at 6 months (Supplementary data, Table S2).

We next assessed the eGFR slopes of individual patients [median (IQR) value: +5.0 (−6.5 to +17.0) mL/min/1.73 m^2/year] in longitudinal data from RemIT-JAV-RPGN patients to understand whether U-CD11b or U-CD163 levels at diagnosis reflect the improvement or deterioration of renal function after the remission-inducing treatment of ANCA-GN. Both univariable and multivariable linear regression analyses demonstrated that baseline eGFR, U-P and U-CD11b, but not U-CD163, were independently associated with yearly eGFR slope following treatment (Table 3). Most importantly, U-CD11b reflected the increase in eGFR, and in contrast, baseline eGFR and U-P predicted the decrease in eGFR. Thus, these data indicate that U-CD11b may qualify as a predictive biomarker for the functional recovery in response to the therapy in ANCA-GN kidneys.

DISCUSSION

In the current study, we evaluated the diagnostic abilities of U-CD11b and U-CD163 in ANCA-GN. Although our current study and others have demonstrated U-CD163 as a biomarker reflecting crescent glomeruli in ANCA-GN, mild urinary secretion, which is approximately one-third of the value in LN [6], is a clinical disadvantage of using U-CD163 for discriminating ANCA-GN from other glomerular diseases. In our previous study [8], the comparable U-CD11b levels in ANCA-GN and LN, which were greater than in other glomerular diseases, are useful for identifying ANCA-GN at diagnosis without kidney biopsy. Some U-CD11b may originate in CD11b$^+$ cells in the tubulointerstitium, but leukocyte accumulation associated with peritubular capillaritis and necrotizing lesions in MPA or granuloma in GPA is among the histological manifestations in AAV [18]. Therefore, U-CD11b may cover AAV-related renal inflammation in both the glomerulus and tubulointerstitium.

Both U-CD11b and U-CD163 were similarly elevated in active ANCA-GN and reflected the clinical and histological status of AAV-related glomerular inflammation, but our findings in identical patients at 6 months after initial diagnosis indicated differences in the clinical positioning of the two molecules as urinary biomarkers. As demonstrated in LN [6] and ANCA-GN [7], U-CD163 in this study significantly decreased with disease amelioration in response to remission induction therapy. In contrast, reduction of U-CD11b, which was observed in the LN cohort [8], was not striking in the AAV cohort. In previous *in vitro* studies of the molecular dynamics of the two leukocyte surface molecules, CD163 was shed via cleavage by a disintegrin and metalloproteinase 17 in response to certain inflammatory stimuli [19, 20], while shedding of U-CD11b required cell transmigration through the endothelium following cell activation signals [8, 9]. Therefore, U-CD163 may represent glomerular macrophage activation susceptible to immunosuppressive treatment, while U-CD11b may reflect glomerular and partly

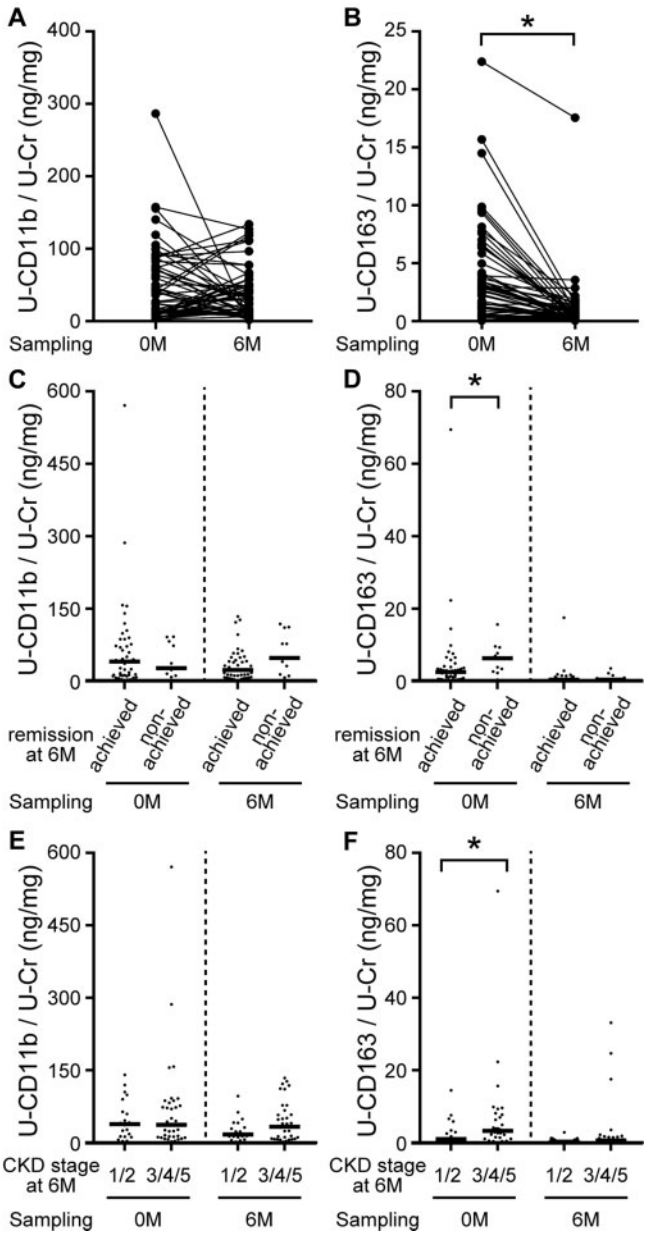

FIGURE 4: U-CD11b and U-CD163 levels before and after remission-induction therapy, and their associations with the remission status and CKD stages at 6 months after treatment in ANCA-GN patients. (**A** and **B**) Evaluations of U-CD11b (**A**) and U-CD163 (**B**) in identical patients at 0 (0M) and 6 months (6M) after treatment in RemIT-JAV-RPGN cohort ($n = 56$). Each patient is represented by a solid line. *$P < 0.05$ by Wilcoxon matched-pairs signed-rank test. (**C** and **D**) U-CD11b (**C**) and U-CD163 (**D**) levels at 0 and 6 months in patients with or without renal remission at 6 months in the RemIT-JAV-RPGN cohort ($n = 56$). *$P < 0.05$ by Mann–Whitney U-test. (**E** and **F**) U-CD11b (**E**) and U-CD163 (**F**) levels at 0 and 6 months in patients according to CKD stages (1/2 and 3/4/5) at 6 months in RemIT-JAV-RPGN cohort ($n = 55$). *$P < 0.05$ by Mann–Whitney U-test. Urinary concentrations of each molecule were corrected using U-Cr in ANCA-GN patients from RemIT-JAV-RPGN cohort.

tubulointerstitial accumulation of $CD11b^+$ cells across glomerular crescent categories in AAV-related CGN.

Although eGFR and U-P at diagnosis were useful for predicting short-term or long-term renal outcomes in ANCA-GN patients, respectively, they cannot be used to indicate the renal inflammatory status in patients undergoing immunosuppressive therapies. Therefore, we expected that leukocyte-derived U-CD11b or U-CD163 at diagnosis would reflect renal remission failure and functional outcome in ANCA-kidneys. However, the multivariable analyses performed in our study could not reveal the predictive values of U-CD163 for the outcomes, which may be due to the close association of U-CD163 with U-P. However, contrary to the predictive values of baseline eGFR and U-P for unfavorable renal outcome, it is noteworthy that multivariable analyses consistently demonstrated the clinical significance of U-CD11b at diagnosis to reflect the future recovery of ANCA-kidneys after the treatment. Unlike the even distribution of $CD163^+$ cells in a glomerulus, CD11b+ cells accumulated more in the glomerular tufts than in the crescent. In addition, the moderate correlation of U-CD11b with the number of cellular crescents was observed. These indicate that the presence of U-CD11b indicates initial inflammation, which may be reversible in response to immunosuppressive therapy rather than U-CD163 in ANCA-GN kidneys. The association of U-CD11b levels with histological renal inflammation at 6 months after treatment was not clarified in the present study. Therefore, future investigations on the associations of U-CD11b with posttreatment renal histology should increase the number of repeated biopsy samples or recurrent cases to reveal the clinical significance of U-CD11b and monitor persistent renal inflammation in ANCA-GN.

Increasing epidemiological evidence has clearly demonstrated that disease and ANCA subtypes in AAV largely differ between Caucasian and Japanese patients. In contrast to European cohorts, in which GPA and proteinase 3-ANCA-positive patients [7, 21] account for most AAV cases, epidemiological studies verified that MPA/renal limited vasculitis and myeloperoxidase-ANCA-positive were the most common form in Japanese AAV populations [3, 11, 22]. Although we observed comparable elevations of U-CD11b and U-CD163 between MPA and GPA (data not shown), the study population was small, particularly the GPA cases. Moreover, associations of U-CD11b and U-CD163 with long-term renal outcomes of patients with ANCA-GN have not been evaluated in the current study because events of renal replacement therapy induction or death were limited during the observation period. Therefore, further validation studies involving large populations with AAV in Western or multiethic cohorts are needed to determine long-term outcomes and the significance of the proposed biomarkers in AAV.

In the present study, we proposed U-CD11b and U-CD163 as urinary biomarkers reflecting renal leukocyte accumulation in ANCA-GN. However, U-CD11b, but not U-CD163, at diagnosis can be used to predict the functional outcomes following the treatment of ANCA-GN kidneys.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

Table 3. Univariable and multivariable linear regression models for assessing the associations of U-CD11b and U-CD163 with yearly eGFR slope in the RemIT-JAV-RPGN cohort

Variables	Univariable		Model 1–1		Model 1–2		Model 2	
	Coefficient (95% CI)	P-value	Coefficient (95% CI)	P-value	Coefficient (95% CI)	P-value	Coefficient (95% CI)	P-value
Age (years)	+0.20 (−0.01 to +0.41)	0.065	−0.22 (−0.35 to −0.09)	0.001*	−0.17 (−0.3 to −0.05)	0.007*	−0.23 (−0.36 to −0.10)	0.001*
Male	+0.71 (−5.35 to +6.78)	0.82	+2.12 (−1.2 to 5.44)	0.21	+1.58 (−1.8 to +4.96)	0.36	+1.93 (−1.4 to +5.25)	0.25
eGFR, mL/min/1.73 m^2	−0.44 (−0.49 to −0.39)	<0.001*	−0.50 (−0.56 to −0.43)	<0.001*	−0.49 (−0.56 to −0.42)	<0.001*	−0.50 (−0.56 to −0.43)	<0.001*
U-P/U-Cr,[a] g/g	+12.5 (+8.3 to +16.8)	<0.001*	−4.3 (−7.9 to −0.8)	0.017*	−5.2 (−10.1 to −0.4)	0.034*	−6.4 (−11.2 to −1.6)	0.010*
U-CD11b/U-Cr,[a] ng/mg	+8.4 (+3.4 to +13.3)	0.001*	+4.7 (+1.4 to +8.0)	0.006*	NA	NA	+4.3 (+1.0 to +7.7)	0.011*
U-CD163/U-Cr,[a] ng/mg	+11.6 (+7.3 to +15.9)	<0.001*	NA	NA	+3.7 (−0.7 to +8.1)	0.10	+2.8 (−1.6 to +7.2)	0.21

[a]Values were $\log_{10}$-converted for the study.
*Statistical significance was determined as $P < 0.05$ by logistic regression analysis.
U: urine; NA: not applicable.

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AUTHORS' CONTRIBUTIONS

Y.Yokoe and N.T. designed the study and drafted the manuscript; Y.Yokoe, A.K., M.K. and N.E. carried out measurements and analyzed the data; Y.Yokoe, T.I. and T.O. performed statistical analyses; Y.S. prepared and stained tissue specimens; T.K. and S.Maruyama diagnosed and assessed renal histology; S.K. managed the ethical application of the study; K.Y., J.U., M.N. and K.S. organized clinical and histological data in the RemIT-JAV-RPGN cohort for the current study; K.Y., J.U., K.S., H.S., Y.A., H.D., M.H., H.M. and S.Matsuo conducted the RemIT-JAV-RPGN study; K.S., K.A., Y.A., T.A., Y.Yuzawa, H.D., Y.T., M.H. and H.H. made a substantial contribution in the collection of clinical/laboratory information and biosamples from the RemIT-JAV-RPGN cohort; all authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

These authors have reported potential conflicts of interest outside the present work: N.T., lecture fees from Chugai Pharmaceutical Company Limited (Co., Ltd.), Eisai Co., Ltd, Kyowa Kirin Co., Ltd, Mochida Pharmaceutical Co., Ltd, Sanofi Kabushiki-Kaisha (K.K.); S.Maruyama, fees from Chugai Pharmaceutical Co., Ltd; T.A., grants and lecture fees from AbbVie Japan Co., Ltd, Astellas Pharma Inc., Bristol-Myers Squibb K.K., Chugai Pharmaceutical Co., Ltd, Daiichi Sankyo Co., Ltd, Eisai Co., Ltd, Eli Lilly Japan K.K., Mitsubishi Tanabe Pharma Co., Otsuka Pharmaceutical Co., Ltd, Pfizer Japan Inc., Takeda Pharmaceutical Co., Ltd, UCB Japan Co., Ltd, grants from Alexion Pharmaceuticals, Inc. and lecture fees from Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Gilead Sciences, Inc., GlaxoSmithKline K.K., Pfizer Japan Inc., UCB Japan Co., Ltd; Y.Yuzawa, grants and lecture fees from Astellas Pharma Incorporated (Inc.), Chugai Pharmaceutical Co., Ltd, Sanofi K.K., lecture fees from GlaxoSmithKline K.K., consultancy fees from Sanwa Kagaku Kenkyusho Co., Ltd; M.H., grants and lecture fees from AbbVie Japan Co., Ltd, Bristol-Myers Squibb K.K., Chugai Pharmaceutical Co., Ltd, Eisai Co., Ltd, Mitsubishi Tanabe Pharma Co., Santen Pharmaceutical Co. Ltd, Takeda Pharmaceutical Co., Ltd, Teijin Pharma, Ltd, lecture fees from Astellas Pharma Inc., Janssen Pharmaceutical K.K. and Pfizer Japan Inc.; H.M., consultancy fees from AbbVie Japan Co., Ltd, Boehringer-Ingelheim Japan, Teijin Pharma Ltd.
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