Urinary soluble CD163 reflects glomerular inflammation in human lupus nephritis

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ABSTRACT

Background In addition to classically activated macrophages that have effector roles in tissue injury, alternatively activated M2 macrophages are involved in the resolution of inflammation in animal models of kidney disease. To clarify the clinical relevance of macrophage phenotypes in human glomerular diseases, we evaluated renal accumulation of macrophages and plasma and urine levels of CD163, an M2 marker, in lupus nephritis (LN) patients.

Methods Kidney biopsies, plasma and urine samples were obtained from LN patients who underwent renal biopsy between 2008 and 2012. $CD163^+$, $CD68^+$ and $CD204^+$cells were counted in paraffin and frozen sections. LN histological activity was evaluated semiquantitatively with the biopsy activity index. Plasma or urinary soluble CD163 (sCD163) concentrations were also measured and evaluated for their significance as potential LN biomarkers.

Results Immunohistological analysis of glomeruli from LN patients revealed that >60% of $CD68^+$macrophages had merged with $CD163^+$cells. The increased number of glomerular $CD163^+$macrophages was correlated with LN severity, as determined by the biopsy active index (r=0.635). Urinary (u-)sCD163 level was strongly correlated with glomerular $CD163^+$cell counts and histological disease score as well as urinary monocyte chemoattractant protein 1 levels (r=0.638 and 0.592, respectively). Furthermore, u-sCD163 level was higher in patients with active LN patients than in those with other diseases.

Conclusions Glomerular $CD163^+$macrophages are the predominant phenotype in the kidneys of lupus patients. These findings indicate that u-sCD163 level can serve as a biomarker for macrophage-dependent glomerular inflammation in human LN.

KEYWORDS

Biomarker, Histopathology, Surface marker, Lupus nephritis, Macrophage

SHORT SUMMARY

To investigate the significance of $CD163^{+}$macrophages in lupus nephritis, histological analysis to identify $CD163^{+}$macrophages on kidney biopsy samples and measurement of soluble form of CD163 in urine and plasma were performed in lupus patients. Glomerular $CD163^{+}$macrophagesare the predominant phenotype in the kidneys of lupus patients. Moreover, urinary sCD163 level can serve as a biomarker for macrophage-dependent glomerular inflammation in human LN.

INTRODUCTION

Macrophages have been implicated in the pathogenesis of renal diseases[1] and are functionally distinguished as a classically activated, inflammation-promotingM1 type and an alternatively activated M2type that exhibits an anti-inflammatory function and contributes to wound healing[2, 3]. Animal studies have demonstrated that administration of M2 macrophages mitigates renal damage in adriamycin nephropathy, type 1 diabetes, and experimental autoimmune encephalomyelitis[4-7]. In contrast, the functional phenotype of macrophages in human renal diseases is not known[8, 9].

CD163 is a type I transmembrane protein belonging to the cysteine-rich scavenger receptor superfamily type B that acts as a scavenger receptor for the hemoglobin-haptoglobin complex[10, 11]. CD163 is a surface marker expressed by M2 macrophages that accumulate during the healing phase of acute inflammation. It is actively shed from the plasma membrane by metalloproteinases in response to certain inflammatory stimuli and diffuses to inflammatory tissues or enters the circulation in its soluble form (sCD163)[12, 13],which may have anti-inflammatory activity and is a useful marker of macrophage activation in inflammatory diseases such as sepsis, liver disease, malaria, autoimmune disorders, and reactive hemophagocytic syndrome[11, 14, 15]. However, there have been few studies addressing the significance of sCD163 levels in urine[16].

Lupus nephritis (LN) is a feature of systemic lupus erythematosus (SLE) and is linked to poor clinical outcome in patients. Markers of SLE activity, including serum complement (C)3, C4,and anti-double-stranded (ds)DNA antibody, are conventional biomarkers for assessing disease severity[17], but are insufficient for predicting the histological classification of LN. Kidney

biopsy is still regarded as the gold standard for histological assessment of LN. However, given that it is an invasive procedure, alternative indices have been proposed for evaluating nephritis[18, 19].One of these is urinary monocyte chemoattractant protein (MCP)-1,which is a biomarker that reflects disease status and can be used to predict LN flares, although it does not have adequate sensitivity or specificity for clinical applications[20].

Macrophage infiltration into the glomerulus plays a significant role in LN pathogenesis[21],and therefore the identification of macrophage phenotypes and urinary proteins derived from renal macrophages may provide useful information on LN histological activity. In this study, we investigated the renal accumulation of macrophage phenotypes in human LN and the significance of sCD163—particularly in urine—as a biomarker of LN.

MATERIALS AND METHODS

Patients and sample collection

Kidney biopsy, urine and plasma samples were obtained from 74 patients with LN (45.0 ± 16.9 years old; 16 males and 58 females) who underwent renal biopsy at Nagoya University or its affiliated hospitals between 2008 and 2012. LN plasma samples were collected from 68 patients in the cohort. Samples were also obtained in the same manner in 2010 from patients with minimal change nephrotic syndrome (MCNS), membranous nephropathy (MN), diabetic nephropathy (DMN), IgA nephropathy (IgAN) and ANCA-associated vasculitis (AAV) as disease controls and from healthy volunteer as normal controls. Normal kidney samples were obtained from a healthy part of kidneys that were removed due to cancer. Patients who received any renal replacement therapy at the time of sampling were excluded from the cohort. Clinical and laboratory data were collected retrospectively from medical records at the time of renal biopsy. The institutional review board approved this study.

Clinical and laboratory data were obtained at the time of kidney biopsy. The estimated glomerular filtration rate (eGFR) was calculated with the following formula: eGFR (ml/min/1.73 m^2) = 194 × serum Cr (mg/dl)$^{-1.094}$ × age$^{-0.287}$ (× 0.739 if female). Plasma and urine samples were stored at −80°C until use.

Urinary protein and Cr concentrations were measured by Mitsubishi Kagaku BCL Inc. (Tokyo, Japan).

Histological assessment

Histological classification of LN in renal biopsies was performed according to International Society of Nephrology/Renal Pathology Society(ISN/RPS) criteria[22]. Patients exhibiting class III or IV along with class V were categorized as class III and IV, respectively. The biopsy active index (BAI) was evaluated semiquantitatively based on previously reported pathological findings[23].

Immunohistochemical analysis of kidney biopsy samples

Kidney biopsy samples were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm sections for immunohistochemistry. Sections were labeled with mouse anti-human CD68 monoclonal IgG3 (clone PG-M1; Dako, Glostrup, Denmark), mouse anti-human CD163 monoclonal IgG1 (clone 10D6; Abcam, Cambridge, UK), or mouse anti-human CD204 monoclonal IgG1 (clone SRA-C6;TransGenic, Tokyo, Japan).Immunoreactivity was detected with a secondary antibody and 3,3'-diaminobenzidine (DAB) from the Histofine Simple Stain Max PO kit (Nichirei, Tokyo, Japan). The average number of glomerular $CD68^+$, $CD163^+$, and $CD204^+$macrophages per glomerulus was determined by examining at least five glomeruli from each patient. Kidney samples from 64 patients were subjected to histological assessment for $CD163^+$macrophages;62 of the kidney samples were analyzed for $CD68^+$ and $CD204^+$cells,since fewer than five glomeruli were observed in two of the samples. DAB-positive areas in the renal cortex were examined for tubulointerstitial macrophage accumulation by microscopy (BZ 9000; Keyence, Osaka, Japan) in at least 12 consecutive high power fields (400×) using image analysis software (Keyence).

Immunohistochemical analysis of renal tissue was carried to examine whether $CD163^+$cells expressed CD68 or CD204 as M2 macrophages. Mouse anti-human CD68 monoclonal IgG1 (clone KP1; Abcam), mouse anti-human CD163 monoclonal IgG1 (clone EDHu-1; AbduErotic, Raleigh, NC, USA), and mouse anti-human CD204 monoclonal IgG1 were fluorescently labeled using a ZenonAlexa Fluor 488 or 568 mouse IgG1 labeling kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Kidney samples were obtained from 10 class IV LN patients with at least one glomerulus in each section. More than 30 glomeruli were analyzed. Positive cells were counted under a fluorescence microscope(Keyence).

Enzyme-linked immunosorbent assay (ELISA)

Plasma or urinary (u-)sCD163 concentrations were measured using ELISA kits according to the manufacture's instructions (DY1607 Duo Set; R&D Systems, Minneapolis, MN, USA). Plasma and urine samples were diluted 100- and 10-fold, respectively. Urinary MCP-1 concentration was determined with an ELISA kit (R&D Systems) in samples that were diluted 4-fold. All samples were processed in duplicate.

Statistical analysis

Data were analyzed using JMP software. Continuous variables were compared using the Kruskal-Wallis, Steel-Dwass, and Tukey/Kramer tests. Comparisons between cohorts with or without glucocorticoids (GCs) were made with Welch's t test. For comparisons among LN classes,

statistical values were evaluated in class III, IV, and V patients since there were few class II LN patients in the cohort. The coefficient of determination was used to examine the strength of association between two continuous variables. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were used to assess the diagnostic value of each biomarker as well as cut-off value, sensitivity, and specificity, and positive and negative likelihood ratios were calculated with SPSS software (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Serum and renal biopsy samples were collected from 74 patients with LN at the time of their admission to Nagoya University Hospital and its affiliated hospitals for renal biopsies between 2008and 2012. Similarly, samples were collected from patients with MCNS, MN, DMN, IgAN, AAV as disease controls, and from healthy volunteers (except for biopsy samples)as a normal control. Patient profiles and clinical data are shown in Table1. Renal function, as determined by eGFR, was impaired in the DMN and AAV groups and the degree of proteinuria varied among disease groups. Nine of 10 AAV patients were histologically active, as evidenced by an average rate of cellular or fibrocellular crescents > 40%, while 38 LN patients were treated with either steroid or other immunosuppressive agents and three patients received both prior to sample collection. Serological activity was evaluated by serum C3 concentration and anti-dsDNAantibody; clinical activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index; LN histology was determined based on ISN/RPS classification; and histological scores were evaluated according to BAI(Table2).

Glomerular $CD163^+$M2 macrophages are associated with kidney disease

We first determined the presence of $CD163^+$macrophages in glomeruli of patients with a variety of renal diseases by immunohistochemistry using a monoclonal antibody against human CD163. Few $CD163^+$cells were observed in normal and MCNS glomeruli, with slightly higher numbers detected in MN and IgAN. In DMN, the number of glomerular $CD163^+$macrophagesincreasedslightly with expansion of the mesangial matrix in the early phase

of renal injury, but numbers varied considerably in advanced stages, since the development of acellular nodules increased in accordance with DMN severity. In AAV, a massive infiltration of $CD163^+$macrophages was observed in crescent glomeruli but their numbers were negligible in unaffected glomeruli (Fig. 1A). Among patients with LN, glomerular accumulation of $CD163^+$cellswashigh in ISN/RPS LN class IV and was minimal in stage V, which corresponds to MN (Fig. 1B).Disease-specific patterns of glomerular $CD163^+$macrophage accumulation were confirmed by quantitative analysis (Fig. 1C). Additionally, a light microscopy analysis revealed that the number of glomerular $CD163^+$macrophages was correlated with histological LN activity (r=0.635)(Fig. 1D).

In contrast to glomerular macrophage accumulation in LN, tubulointerstitial$CD163^+$macrophages were predominantly observed in patients with impaired renal function, irrespective of SLE disease status or pathological LN classification (Supplemental Figure 1).

CD163is coexpressed with other M2 macrophage markers

The tissue distribution of macrophages and their involvement in inflammatory diseases have been investigated in various types of organ injury[24, 25]. Recent vitro and animal studies have shown that macrophages can be classified into several phenotypes based on the expression of specific markers and their function during the development of inflammation[2, 26-28]. An immunohistochemical analysis of paraffin-embedded LN samples revealed CD68-positive pan-macrophages [2], consistent with the observed number of $CD163^+$ cells (Fig. 2A). The macrophage scavenger receptor CD204, initially identified as an acetylated low-density lipoprotein

receptor [29], is another M2 cell marker in human IgAN [3, 8]. Immunohistochemical detection of CD204 showed a significant correlation with $CD163^+$ cells. We therefore hypothesized that monocytes/macrophages can adopt a particular phenotype according to the microenvironment of the diseased kidney, which can include specific cytokines/chemokines, growth factors, and cell types. We next probed macrophages in frozen sections with antibodies against CD163, CD68,and CD204. The sections were from 1-mm^3 frozen kidney samples containing one to six glomeruli at each renal biopsy from class IV patients, since these samples were expected to have a sufficient number of macrophages with each glomerulus affected to the same extent. In more than 30glomeruli from 10 class IV LN patients labeled with any two of the antibodies CD163, CD68, and CD204, 61.4% and 66.1% of $CD68^+$cells co-expressed CD163 and CD204, respectively(Fig. 2C, D). In the $CD163^+$population, 83.0% of macrophages also expressed CD204 (Fig. 2E). These findings demonstrate the heterogeneity of macrophage populations in inflamed glomeruli of LN.

U-sCD163 is a potential biomarker for LN

Given the molecular dynamics of CD163—including its shedding from the cell surfaceassCD163[12]—and the close correlation between glomerular $CD163^+$macrophage infiltration and histological and clinical LN histological activity, we investigated whether sCD163 can serve as a reliable biomarker for LN. Among the renal diseases shown in Table1, u-sCD163 corrected by urinary creatinine (Cr) concentration was increased in the LN group, particularly in ISN/RPS class IV subpopulation, which was accompanied by enhanced cell proliferation[22] (Fig. 3A). It should be noted that the increase in u-sCD163 observed in LN persisted even after correction by urinary excretion of total protein (TP) to eliminate the influence of massive protein

leakage in nephritic syndrome (Fig. 3B). Urinary MCP-1 corrected by urinary Cr was also increased in active LN patients as previously described[20, 30];however, urinary MCP-1 corrected by urinary TP was high in the AAV but not in the LN group (Fig. 3C,D).These findings strongly suggest that sCD163 in urine is a more specific and therefore useful biomarker for LN activity than urinary MCP-1.Class IV LN patients showed elevated u-sCD163/uCr and MCP-1/uCr compared to class III and V patients. When corrected for urinary excretion of TP, class III and IV LN patients showed increased u-sCD163 relative to class V patients; however, urinary MCP-1 lost its significance as a biomarker for proliferative LN of classes III and IV.

Elevated plasma sCD163 in LN is not associated with ISN/RPS class

We also evaluated plasma sCD163 levels in renal diseases. LN showed significant elevation of sCD163 as compared to IgAN, MCNS, and MN, but did not show a difference relative to AAV or DMN. Moreover, there was weak association between plasma sCD163 concentration and LN histological activity (Fig.4). These findings strongly suggest that plasma sCD163 reflects systemic inflammation or other organ damage of SLE, but not the histological classification of LN.

U-sCD163 is correlated with renal CD163^{+}macrophage infiltration and LN histological activity

To evaluate the robustness of sCD163 as a biomarker for disease activity, we analyzed the correlation between sCD163 concentration and histology in renal specimens from patients with

LN. UrinarysCD163 was highly correlated with glomerular $CD163^+$cellnumber (r=0.580) and to a lesser degree with the CD163-positive area in the tubulointerstitial area (r = 0.370) (Fig. 5A). Moreover, the correlation between urinary but not plasma sCD163and histological activity index was comparable to urinary MCP-1 (r = 0.638 vs. r = 0.592)(Fig. 5B, C). We measured u-sCD163 level as a predictor of histological LN classification. In the ROC curve generated to predict ISN/RPS class III and IV subpopulations—both of which clinically require induction therapy with corticosteroid and immunosuppressants[31]—AUC of u-sCD163 (u-sCD163/u-TP: 0.89 and u-sCD163/u-Cr: 0.825; cut off: 3.93 ng/mg; sensitivity: 83.3%; specificity: 85.7%; and positive and negative likelihood ratios of 5.83 and 0.19, respectively) was greater than that of urinary MCP-1 (Fig. 5D).A similar result was obtained when an ROC curve was generated for class IV LN alone (0.851 and 0.813 in u-sCD163/u-Cr and u-MCP-1/u-Cr, respectively). Taken together, these data suggest that u-sCD163 can be a useful biomarker for predicting histological disease activity in LN.

Systemic GC administration does not affect glomerular CD163 expression and u-sCD163 excretion in class IV LN

GC promotes the conversion of human monocytes to $CD163^+$ macrophages in vitro [32]. We therefore investigated the effect of GC treatment of LN on glomerular accumulation of $CD163^+$ macrophages and u-sCD163 excretion. The number of glomerular $CD163^+$ macrophages and u-sCD163 level between GC-treated and untreated populations were comparable in patients presenting class IV lesions (Table 3). Moreover, there was no association between GC dosage and CD163-related LN parameters (Supplemental Figure 2).

DISCUSSION

Macrophages can be classified into M1 or M2 subtypes. Glomerular infiltrating macrophages represent the inflammatory M1 phenotype at the initiation of glomerular nephrititis (GN), but anM2 macrophage population is involved in immuneregulation, in part during fibrogenesis at the resolution state of glomerular inflammation[3].However, there are few reports of macrophage phenotypes in GN[8, 9]. We investigated the accumulation of macrophages expressing CD163, a representative M2 marker, in human renal biopsy samples of various types of kidney disease as well as the clinical significance of sCD163 in the urine of LN patients.

CD163$^+$macrophages are considered as M2 cells with anti-inflammatory activity. Stimulation with interleukin (IL)-6, IL-10 or macrophage colony-stimulating factor (M-CSF)-induces the differentiation of naïve M0 macrophages into CD163$^+$cells[11]. In healthy as well as diseased individuals, CD163$^+$macrophages are distributed in various organs including the lymph node, spleen, bone marrow, lung, liver, and skin[33]. CD163$^+$macrophages have also been detected in the kidney of patients with IgAN and AAV[8, 9]. We observed that glomerular CD163$^+$cell infiltrates were negligible in MCNS, mild in IgAN, MN, moderate in DMN, and pervasive in the crescentic glomeruli of AAV patients. However, LN exceeded the other renal diseases in terms of the average number of glomerular CD163$^+$macrophages. Interestingly, this varied according to LN class. A marked elevation in the renal levels of IL-6, IL-10, and M-CSF—which are essential for macrophage differentiation into CD163$^+$cells—has previously been reported in human LN[19, 34]. These findings indicate that the glomerular microenvironment modified by inflammation can convert infiltrated macrophages to the CD163$^+$phenotype. In addition to glomeruli, CD163$^+$macrophages were also present in the tubulointerstitial area of LN kidneys. Notably,

tubulointerstitial accumulation of CD163$^+$macrophages was highly correlated with eGFR but not with glomerular CD163$^+$cell number (data not shown). Additionally, CD163$^+$macrophages were frequently distributed in areas with tubulointerstitial fibrosis despite the presence of renal injury (data not shown), indicating that tubulointerstitial accumulation of CD163$^+$macrophages is not a disease-specific feature but instead reflects renal function in various types of chronic kidney disease.

Some in vitro studies have shown that CD163$^+$macrophages exert their anti-inflammatory function by producing IL-10[35]; however, the in vivo roles of CD163$^+$cells are largely unknown. CD163 was observed to co-localize with IL-10 and chemokine (C-C motif) ligand 18—two cytokines with immunosuppressive and fibrotic activity—on macrophages that infiltrated into submandibular glands in IgG4-associated dacryoadenits[36]. It is therefore possible that CD163$^+$macrophages are involved in immunosuppression and fibrosis in inflamed tissue. A functional analysis that includes cytokine profiling of CD163$^+$macrophages will be carried out in future studies to clarify the significance of glomerular CD163$^+$macrophages in LN.

There are few reliable markers for assessing the inflammatory activity of the monocyte/macrophage lineage in GN. The proinflammatory status of macrophages is reflected by the expression of the calcium-binding proteins myeloid-related protein8 and 14[37-39], which are therefore potential M1 macrophage markers. However, both proteins have been detected in monocytes and neutrophils in the glomerular infiltrate of renal diseases, and are frequently expressed in highly proliferative forms of GN including LN and crescentic GN[40, 41]. These findings, along with the abundant glomerular accumulation of CD163$^+$macrophages in proliferative LN observed in the present study suggest that both M1 and M2 macrophage phenotypes coexist

in active glomerular lesions in GN. It is often difficult to estimate the time elapsed after disease onset from histological and clinical information. In LN, histological changes in glomeruli—including immunecomplex deposition—precede clinical symptoms of renal damage[42-44]. Renal biopsy is usually performed when patients show evidence of renal damage; as such, we often observe the subacute phase but not the initiation of glomerular inflammation in LN kidneys, at which time the development and resolution of inflammation may co-exist. This can explain our observation that the $CD163^+$M2 population accounted for over 60% of total glomerular $CD68^+$macrophages in class IV LN patients. Although we did not evaluate M1 macrophages in LN kidneys, M1 and M2 definitions are not always clear, since these cells are plastic and there may be a continuum between M1 and M2 phenotypes during differentiation in a dynamic microenvironment depending on inflammatory status. Contrary to the prevailing view that M2 macrophages are involved in the resolution of tissue inflammation, the histological evidence suggests that glomerular infiltration of $CD163^+$ M2 macrophages preceded crescent formation in AAV patients [9]. Furthermore, a recent study of patients with Th1- or Th2-associated inflammatory diseases demonstrated that $CD163^+$ macrophages co-expressed phosphorylated signal transducer and activator of transcription 1 and recombination signal binding protein for immunoglobulin kappa *j* region—both of which transcriptionally regulate M1 macrophage polarization—inflammatory conditions [45]. Additional studies of transcription factors involved in macrophage polarization would be useful to clarify the role of $CD163^+$ macrophages in glomerular injury, including LN.

Potential LN biomarkers identified in the circulation and urine include autoantibodies, antigens, and cyto-/chemokines[17, 19]; urinary MCP-1 is among the most potent of these biomarkers[46, 47]. In the current study, urinary MCP-1 was highly associated with LN activity but was also elevated in AAV and DMN patients; in these individuals, renal dysfunction was more

severe than in LN patients, as indicated by higher serum Cr levels. Renal function is highly correlated with renal tubular damage; in an inflammatory setting, not only macrophages but also tubular cells secrete MCP-1[48]. We therefore speculate that urinary MCP-1 excretion is not specific to glomerular damage in LN. In contrast, CD163 is expressed exclusively by M2 macrophages and is not secreted by other cell types, including tubular cells. Therefore, u-sCD163 accurately reflect renal conditions involved in macrophage infiltration, as evidenced by a greater AUC value than was determined for urinary MCP-1.

Finally, we did not determine u-sCD163 level in LN patients after immunosupressive therapy. Since almost all cases in our cohort exhibited clinical remission along with elimination of proteinuria, we did not expect sCD163 excretion. Repeated renal biopsies in order to evaluate curative effects is not a common practice unless patients show an unfavorable clinical course; therefore, performing a histological assessment for glomerular $CD163^{+}$cells after treatment is ethically unacceptable in patients with clinical remission. Since our cohort had a small number of class II LN cases, the significance of u-sCD163 in active LN was not fully evaluated and requires validation in a large cohort of LN patients. Nonetheless, u-sCD163 is a biomarker that can be evaluated noninvasively that directly reflects macrophage-mediated glomerular inflammation and can also distinguish between class III/IV and V LN in patients who show relapsed proteinuria during their clinical course.

DISCLOSURES

None.

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Table1. Clinical and demographic data of the study population

	Normal	IgAN	AAV	MCNS	MN	DMN	LN
Age at examination (years)[a]	38.9 ± 8.8	37.2 ± 7.5	70.2 ± 6.5*	48.8 ± 13.1	65.0 ± 11.1*	59.3 ± 9.1*	45.0 ± 16.9
Sex (male : female)	7:7	6:4	5:5	9:8	9:7	7:3	16:58
Immunosupressive treatment(number of patients)	0	0	4	4	1	0	36
Serum creatinine (mg/dl)[a]	0.74 ± 0.16	0.92 ± 0.23	2.87 ± 2.13*	0.98 ± 0.74	0.77 ± 0.30	2.63 ± 1.17*	1.08 ± 1.00
eGFR (ml/min/1.73m^2)[a]	74.4 ± 20.7	60.1 ± 17.1	24.9 ± 20.9*	61.3 ± 22.2	64.8 ± 21.5	18.6 ± 8.7*	72.9 ± 36.2
Proteinuria (g/gCr)[a]	N.D.	1.40 ± 1.76	2.17 ± 1.99	7.94 ± 5.95*	4.26 ± 4.20	9.76 ± 3.74*	3.43 ± 3.44
Urine sample(number of patients)	14	10	10	17	16	10	74
Plasma sample(number of patients)	14	10	10	17	13	10	68
Histology[b](number of patients)	0	9	8	17	15	9	64

IgAN, Immunoglobulin A nephropathy; AAV, Anti-neurotrophil cytoplasmic antibody-associated vasculitis; MCNS, Minimal change nephrotic syndrome; MN, Membranous nephropathy; DMN, Diabetic nephropathy; LN, Lupus nephritis; eGFR, estimated glomerular filtration rate; N.D., not detected

[a]Data are expressed as mean±SD.

[b]Renal specimens with more than five glomeruli were subjected to histological analysis by counting glomerular macrophages.

*The Tukey/Kramer test was used for comparisons to LN at a 5% significant level.

Table2. Clinical and laboratory data and histological findings of LN patients

	Number of patients	Value[a] (normal range)
C3(mg/dl)	72	52.5±25.0 (86–160)
Anti-DNA antibody(IU/ml)	65	102.1±129.4 (< 6.0)
CRP(mg/dl)	68	0.77±1.11 (< 0.3)
Total SLEDAI	67	13.6±5.5
Renal SLEDAI	70	8.2±4.0
ISN/RPS class		
II	4 (3)[b]	
III	8 (8)[b]	
III+V	12 (12)[b]	
IV	27 (21)[b]	
IV+V	13 (10)[b]	
V	10 (10)[b]	
Activity index	74	4.9±3.3

C3, complement C3; CRP, C-reactive protein; SLEDAI, Systemic lupus erythematosus disease activity index; ISN/RPS, International Society of Nephrology/Renal Pathology Society.

[a]Data are expressed as mean ± SD.

[b]Renal specimens with more than five glomeruli were subjected to histological analysis by counting glomerular macrophages.

Table 3. Clinical and laboratory data of class IV LN patients with or without GC treatment

	Non-GC	GC
Number of patients	17	21
Age	48.8±17.3	40.7±16.0
Sex (Male : Female)	03:14	02:19
Dosage of GC	0	36.2±20.7
Serum creatinine (mg/dl)	1.26±0.99	1.40±1.45
eGFR (ml/min/1.73m^2)	53.7±21.1	66.5±39.3
Proteinuria (g/gCr)	5.09±3.85	6.15±4.53
C3 (mg/dl)	35.3±16.0	42.8±16.6
Anti-DNA antibody (IU/ml)	158.0±131.0	134.3±166.5
CRP (mg/dl)	0.41±0.68	0.96±1.27
Total SLEDAI	16.1±4.80	16.6±5.20
Renal SLEDAI	10.0±3.30	10.1±3.50
Activity index	7.47±2.92	7.24±2.62
u-sCD163/u-Cr (ng/mg)	50.4±48.3	43.9±39.2

u-sCD163/u-TP (ng/mg)	12.6±7.40	12.2±9.50
CD163^{+}cells/glomerulus[a]	13.6±5.20	14.5±6.60

Data are expressed as mean ± SD. None of the parameters showed a significant difference.

Two patients receiving GC pulse therapy (> 500 mg/day) at the time of sample collection were excluded.

[a]Kidney samples from 13 patients in the non-GC and 16 in the GC group were evaluated.

FIGURELEGENDS

Figure 1. CD163 expression in patients with various kidney diseases and LN. (A) Microscopic observation of $CD163^+$macrophages (brown) in glomeruli of normal, IgAN, AAV, MCNS, MN, and DMN kidney. Bar:100μm. (B) Light microscopic analysis of LN classified according to ISN/RPS criteria. Bar:100μm. (C) Quantitative analysis of $CD163^+$cell numbers in glomeruli from patients with various kidney diseases (IgAN, n=9; AAV, n=8; MCNS, n=17; MN, n=15; DMN, n=9;LN, n=64;left) and LN (class II, n=3, III, n=20; IV, n=31, V, n=10;right).Each patient is represented by a dot and the mean of each group is shown as a horizontal bar. *P<0.05. (D) Correlation between glomerular $CD163^+$cell numbers from LN patients and BAI; each dot represents a value from a single patient. Coefficients of determination (R^2) and p values are shown.

Figure 2. Association between the expression of CD163 and other M2 markers in LN.(A–C) Glomerular $CD68^+$ (A) and $CD204^+$ (B) macrophages in LN classified according to ISN/RPS criteria (left panels). Bar: 100 μm. Correlations between $CD68^+$or $CD204^+$and$CD163^+$cell numbers in LN glomeruli (right panels)from 62kidney samples. Bar: 100 μm. Coefficients of determination (R^2) and P values are shown. (C–E) Double-immunolabeling of CD68 and CD163 (C), CD68 and CD204 (D), and CD163 and CD204 (E) in LN glomeruli (upper panels). Bar: 100 μm. Quantitative analysis of single cells showing co-localization of markers in more than 30LN glomeruli (lower panels) from a total of 10patients.

Figure 3. UrinarysCD163 and MCP-1 concentrations in various kidney diseases and LN. (A–D) Urinary protein excretion of sCD163(A, B) and MCP-1 (C, D) (u-sCD163 and u-MCP-1, respectively) corrected by urinary Cr or urinary total protein (u-Cr and u-TP) in renal diseases (left) and LN classified according to ISN/RPS criteria (right).Each patient is represented by a dot and the mean of each group is shown as a horizontal bar. *P<0.05. In B and D, data from normal subjects were excluded from the analysis due to the absence of proteinuria.

Figure 4. Plasma sCD163 and MCP-1 concentrations in various kidney diseases and LN. Plasma sCD163 concentrations from kidney diseases (left) and LN classified according to ISN/RPS criteria(right);each patient is represented by a dot and the mean of each group is shown as a horizontal bar. *P<0.05.

Figure 5. Associationbetween sCD163 and histological LN subtype. (A) U-sCD163 levels are more highly correlated with $CD163^{+}$macrophage number in glomeruli (left)as compared to the interstitium (right). (B) sCD163level in urine (left) but not plasma (right) is highly correlated with BAI. (C) Correlation between urinary MCP-1and BAI; each dot represents value from individual patients. Coefficients of determination (R^2) and p values are shown. (D) ROC curves of u-sCD163 and MCP-1 (u-sCD163 and u-MCP-1, respectively) were generated to predict ISN/RPS class III and IV subpopulations. Values were corrected by urinary Cr or TP (u-Cr and u-TP, respectively) in LN patients, along with corresponding AUCs.

Figure 1

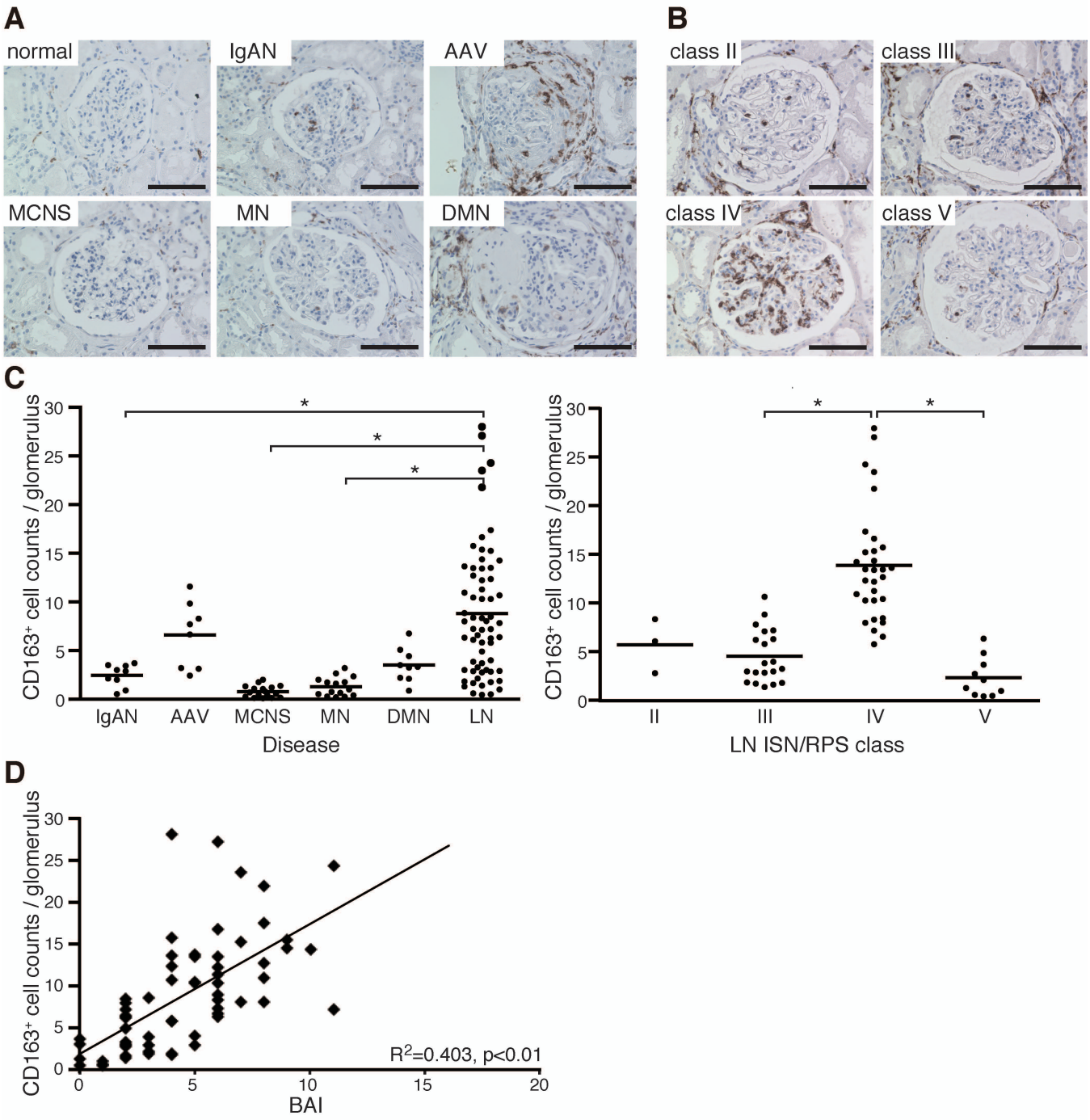

A
normal
IgAN
AAV
MCNS
MN
DMN
B
class II
class III
class IV
class V
C
CD163+ cell counts / glomerulus
IgAN
AAV
MCNS
MN
DMN
LN
Disease
II
III
IV
V
LN ISN/RPS class
D
CD163+ cell counts / glomerulus
BAI
R2=0.403, p<0.01

Figure 2

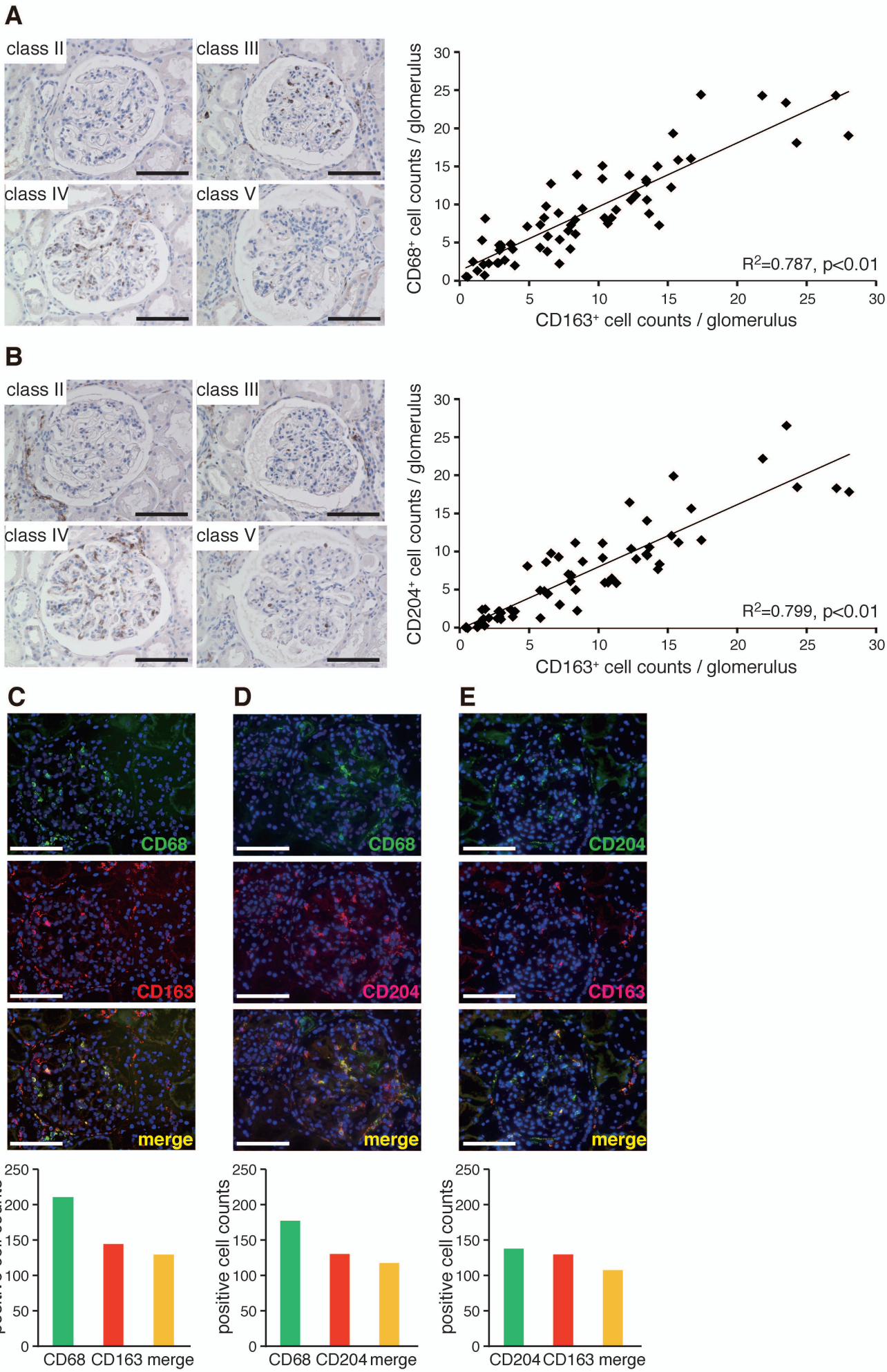

A
class II
class III
class IV
class V
CD68⁺ cell counts / glomerulus
CD163⁺ cell counts / glomerulus
R²=0.787, p<0.01
B
class II
class III
class IV
class V
CD204⁺ cell counts / glomerulus
CD163⁺ cell counts / glomerulus
R²=0.799, p<0.01
C
D
E
CD68
CD163
merge
CD68
CD204
merge
CD204
CD163
merge
positive cell counts
CD68 CD163 merge
positive cell counts
CD68 CD204 merge
positive cell counts
CD204 CD163 merge

Figure 3

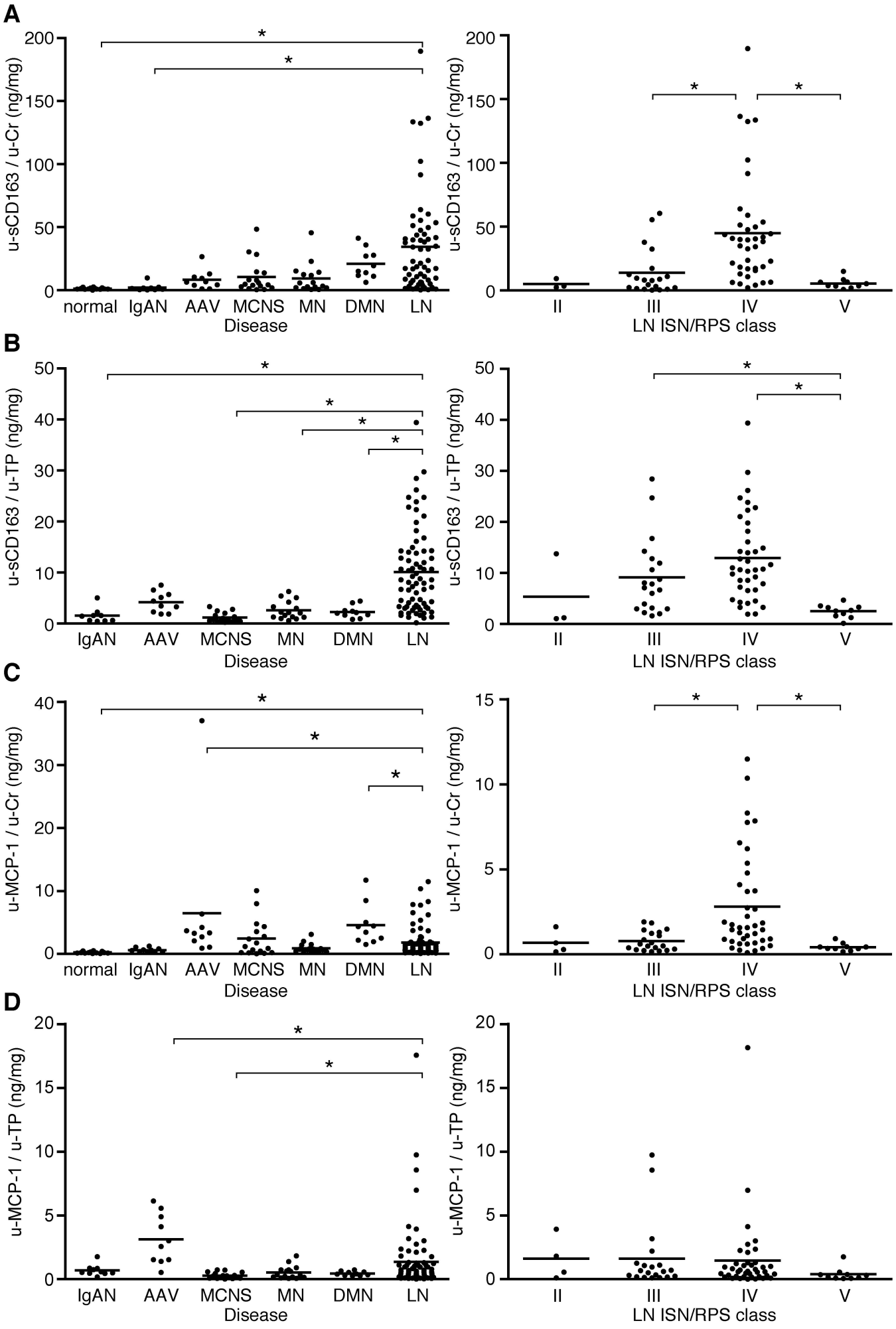

A
u-sCD163 / u-Cr (ng/mg)
200
150
100
50
0
normal
IgAN
AAV
MCNS
MN
DMN
LN
Disease
II
III
IV
V
LN ISN/RPS class
B
u-sCD163 / u-TP (ng/mg)
50
40
30
20
10
0
IgAN
AAV
MCNS
MN
DMN
LN
Disease
II
III
IV
V
LN ISN/RPS class
C
u-MCP-1 / u-Cr (ng/mg)
40
30
20
10
0
15
10
5
normal
IgAN
AAV
MCNS
MN
DMN
LN
Disease
II
III
IV
V
LN ISN/RPS class
D
u-MCP-1 / u-TP (ng/mg)
20
15
10
5
0
IgAN
AAV
MCNS
MN
DMN
LN
Disease
II
III
IV
V
LN ISN/RPS class

Figure 4

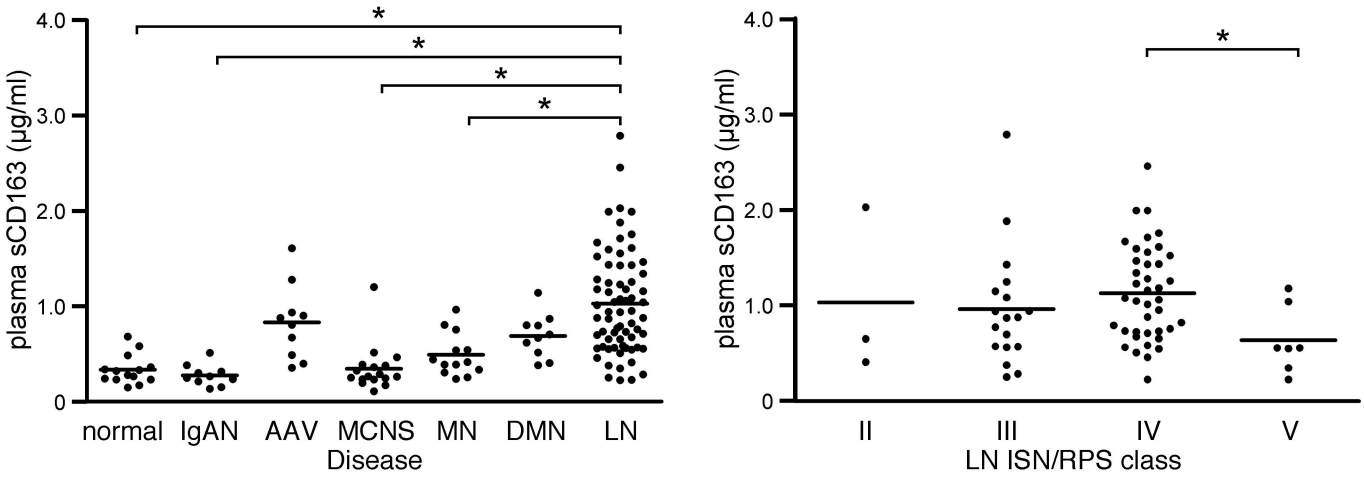

plasma sCD163 (µg/ml)
4.0
3.0
2.0
1.0
0
*
*
*
*
normal
IgAN
AAV
MCNS
MN
DMN
LN
Disease
plasma sCD163 (µg/ml)
4.0
3.0
2.0
1.0
0
*
II
III
IV
V
LN ISN/RPS class

Figure 5

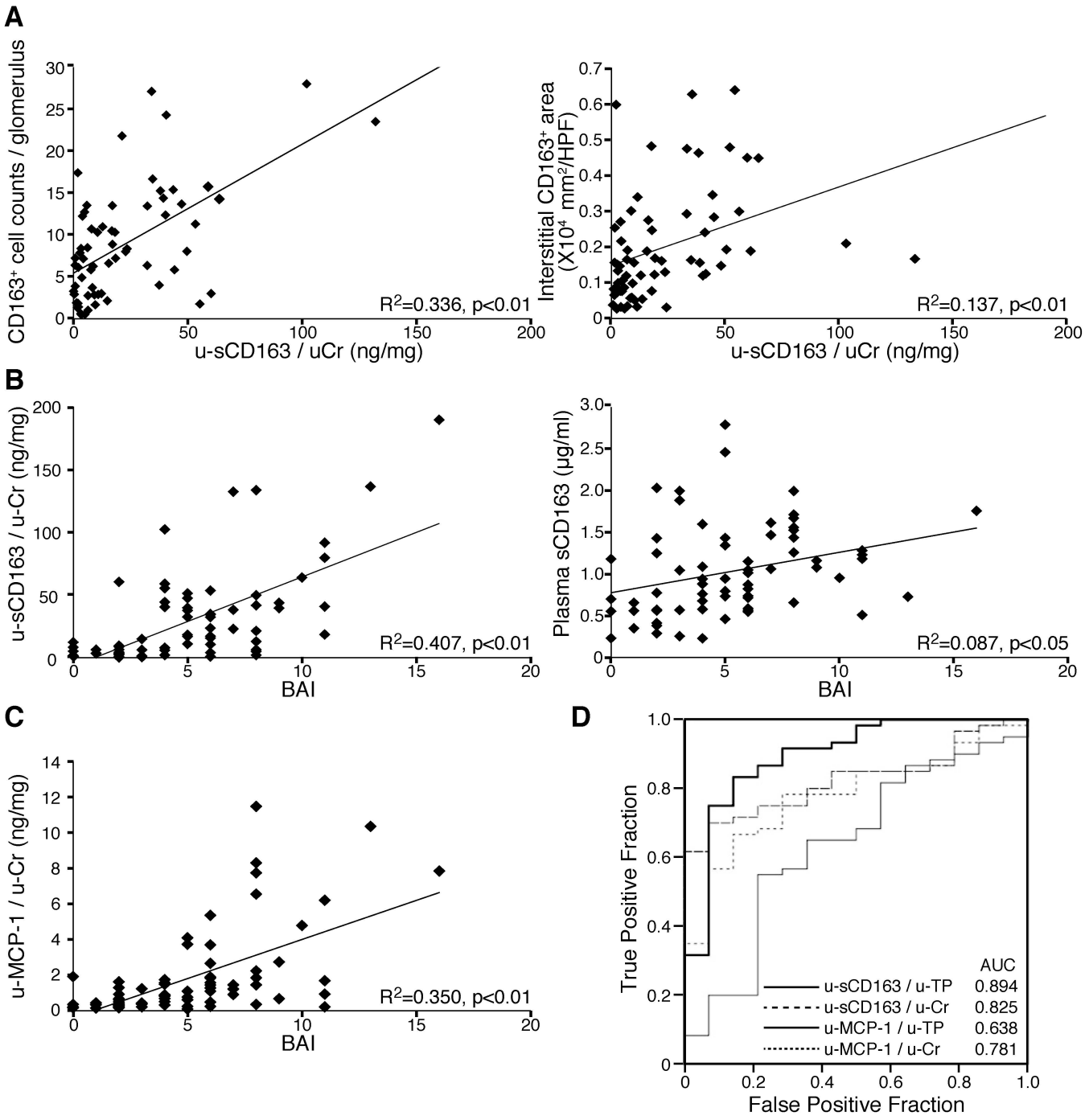
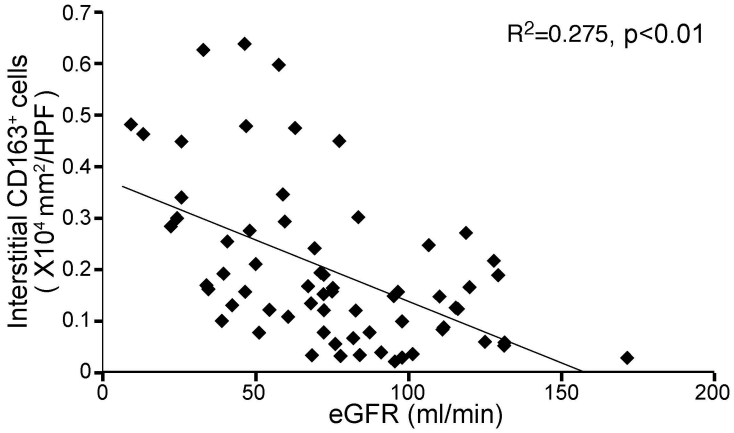

A
CD163+ cell counts / glomerulus
u-sCD163 / uCr (ng/mg)
R2=0.336, p<0.01
Interstitial CD163+ area (X10^4 mm^2/HPF)
u-sCD163 / uCr (ng/mg)
R2=0.137, p<0.01
B
u-sCD163 / u-Cr (ng/mg)
BAI
R2=0.407, p<0.01
Plasma sCD163 (μg/ml)
BAI
R2=0.087, p<0.05
C
u-MCP-1 / u-Cr (ng/mg)
BAI
R2=0.350, p<0.01
D
True Positive Fraction
False Positive Fraction
AUC
u-sCD163 / u-TP 0.894
u-sCD163 / u-Cr 0.825
u-MCP-1 / u-TP 0.638
u-MCP-1 / u-Cr 0.781

Figure S1

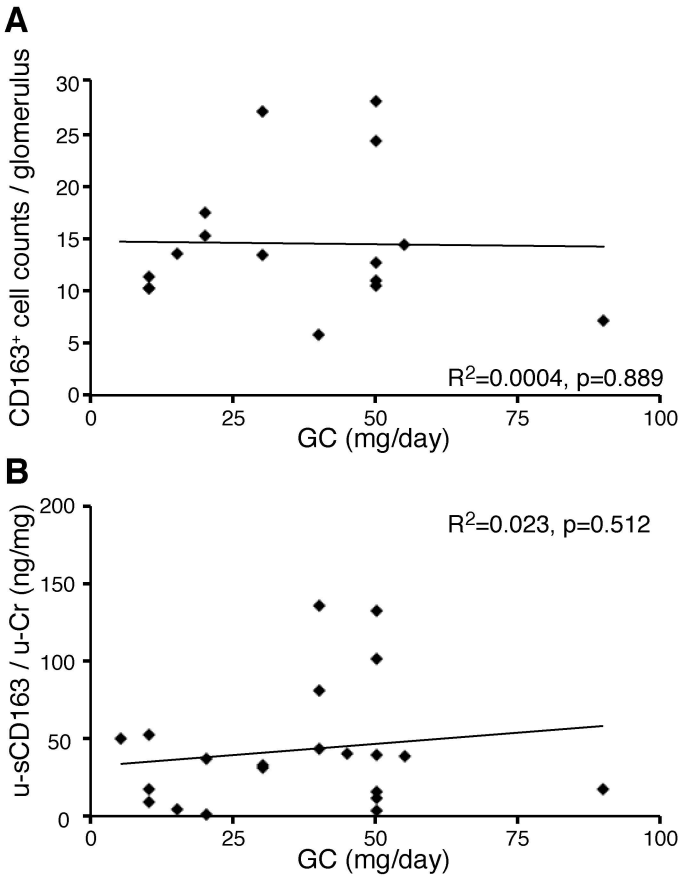


Association of interstitial CD163$^+$macrophage accumulation and eGFR in LN.

Interstitial infiltrates of CD163$^+$macrophage strongly correlated with eGFR of LN patients. Each dot indicates eGFR of individual patients at the time of renal biopsy.

coefficient determination (R^2) and p value are shown.

Figure S2



Association between glucocorticoid (GC) dosage and glmerular CD163 expression or urinary sCD163 in class IV LN.

GC dosages did not correlated with glomerular $CD163^+$macrophage number (A) or u-sCD163/u-Cr level (B). Each dot represents value from individual patients. Coefficient determinations (R^2) and p values are shown.