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Citation for published version (APA):

Aendekerk, J. P., Jiemy, W. F., Raveling-Eelsing, E., Bijnens, N., Abdul-Hamid, M. A., Strating, I. M., Dekkema, G. J., Sanders, J.-S. F., Stegeman, C. A., Damoiseaux, J. G. M. C., Little, M. A., Heeringa, P., & van Paassen, P. (2022). CD163 and CD206 expression define distinct macrophage subsets involved in active ANCA-associated glomerulonephritis. *Journal of Autoimmunity*, *133*, Article 102914. https://doi.org/10.1016/j.jaut.2022.102914

Document status and date:
Published: 01/12/2022

DOI:
10.1016/j.jaut.2022.102914

Document Version:
Publisher's PDF, also known as Version of record

Document license:
Taverne

Please check the document version of this publication:

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Download date: 19 Apr. 2024

Journal of Autoimmunity 133 (2022) 102914

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

CD163 and CD206 expression define distinct macrophage subsets involved in active ANCA-associated glomerulonephritis

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A R T I C L E I N F O

Keywords:
Vasculitis
Macrophages
ANCA glomerulonephritis
Kidney pathology
Acute kidney injury

A B S T R A C T

Introduction: Macrophages are key players in the immunopathology of anti-neutrophil cytoplasmic antibody (ANCA) mediated-vasculitis (AAV) with glomerulonephritis (ANCA GN). Different macrophage phenotypes are expected to play distinct roles in ANCA GN. Macrophages expressing CD163 and CD206 are found in lesions associated with ANCA GN. Hence, we aimed to investigate the clinicopathological significance of CD206 and CD163 in ANCA GN in a multicenter retrospective cohort study.

Material and methods: Patients with ANCA-associated vasculitis, with clinical data, serum and urine samples were included from three cohorts. Serum soluble CD206 (ssCD206) and urinary soluble CD163 (usCD163) levels were measured. Human kidney tissue samples ($n = 53$) were stained for CD206 and CD163 using immunohistochemistry and immunofluorescence, and findings were correlated with clinical and pathological data.

Results: In total, 210 patients were included (i.e., ANCA GN, $n = 134$; AAV without GN, $n = 24$; AAV in remission $n = 52$). Increased levels of both ssCD206 and usCD163 were seen in ANCA GN. High levels of ssCD206 declined after reaching remission, however, ssCD206 did not improve the accuracy of usCD163 to detect ANCA GN. Soluble markers correlated with histopathological findings. CD163^{+}CD206^{-} macrophages were found in the glomerulus and may play pivotal roles in glomerulonephritis, whereas CD206^{+}CD163^{-} and CD206^{+}CD163^{+} macrophages were located tubulointerstitially and likely play a more prominent role in ANCA-associated tubulointerstitial inflammation. In ANCA GN patients increasing levels of ssCD206 increased the risk for end-stage renal disease and mortality.

Conclusions: Our results confirm and extend the notion that CD206^{+} and CD163^{+} macrophages are prominent components of the cellular infiltrate in ANCA GN. We found distinct macrophage phenotypes that may play distinct roles in the immunopathology of ANCA GN and elaborate on a potential mechanism underlying the findings of this study. usCD163 remains an excellent marker to detect active ANCA GN, whereas ssCD206 seems a more prominent marker for risk prediction.

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https://doi.org/10.1016/j.jaut.2022.102914
Received 26 June 2022; Received in revised form 6 September 2022; Accepted 12 September 2022
Available online 29 September 2022

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1. Introduction

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is the predominant form of small vessel vasculitis characterized by systemic inflammation, mainly affecting the kidney tissue and upper airways [1]. Up to 70% of all AAV patients develop glomerulonephritis (ANCA GN) warranting immediate treatment to prevent chronic kidney damage [2,3]. However, there are still patients who progress to end-stage kidney disease (ESKD) [2,3] highlighting that establishing true disease quiescence remains a challenge. Therefore, there is a pressing need to decipher the mechanisms underlying inflammation and resolution in ANCA GN.

The histological hallmark of ANCA GN, extracapillary crescentic GN, is characterized by proliferating parietal epithelial cells and the early infiltration of neutrophils and macrophages [4,5]. Macrophages are crucial players in the immunopathology of ANCA GN [4,5]. Macrophages are incredibly plastic cells that can change their phenotypes depending on cues from the surrounding tissues [6,7]. Hence, their functional response varies from amplifying the inflammatory response and elimination of invading pathogens to promoting tissue repair. Due to their high degree of plasticity, different macrophage phenotypes are expected to exist and play different roles in the immunopathology of ANCA GN.

Macrophages expressing the markers CD163 and CD206 (also known as mannose receptor/MR) have been documented in lesions associated with ANCA GN [8,9]. Infiltration of $CD163^+$ macrophages in the tubulointerstitium and glomeruli in AAV patients with active crescentic GN has been reported [10–12]. Interestingly, the extent of $CD163^+$ macrophage infiltration in the glomerulus is associated with increased proteinuria [12], cementing the role of these macrophages in ANCA GN. Furthermore, upon activation, macrophages shed CD163 and urinary soluble CD163 (usCD163) has been identified as a potent marker for active ANCA GN [10,11,13], correlating tightly with glomerular lesions [11]. CD206, like CD163, is shed by macrophages upon activation and, although their mechanisms of shedding are thought to be different [14], this suggests that levels of sCD206 may also reflect ANCA GN.

In a mouse model of crescentic GN, CD206-deficiency protected mice against severe crescent formation [15]. In another mouse model of ischemia/reperfusion injury $CD206^+$ macrophages were implicated in kidney fibrosis progression [16]. Furthermore, a recent *in vitro* study has shown that recombinant soluble CD206 (sCD206) promotes secretion of proinflammatory cytokines by human monocyte-derived macrophages [17]. Collectively, these studies have shown that distinct phenotypes of macrophages may play different roles in the immunopathology of ANCA GN. The knowledge regarding their spatial distribution and their involvement in active ANCA GN may aid in identifying novel biomarkers, treatment targets and patient stratification.

In this study, we aim to investigate the clinicopathological significance of $CD163^+$ and $CD206^+$ macrophages in active ANCA GN. We first measured sCD206 levels in three independent cohorts of AAV patients and analyzed its potential to detect active ANCA GN. Next, we performed a detailed immunohistochemical analysis of CD206 and CD163 expression to assess the presence and spatial distribution of $CD206^+$ and $CD163^+$ macrophages in kidney biopsies of AAV patients, and correlated these findings with clinical and pathological parameters from patients with active ANCA GN.

2. Materials and methods

2.1. Patients

Patients with ANCA-associated vasculitis, fulfilling the Chapel Hill Consensus Conference classification criteria [1], were included from three independent cohorts (C1-3). Patients and clinical data were recruited from the Limburg Renal Registry [18] from Maastricht University Medical Center (MUMC), Maastricht, the Netherlands (C1), University Medical Center Groningen (UMCG), Groningen, The Netherlands (C2), and the Rare Kidney Disease (RKD) Biobank, Trinity Healthy Kidney Center, Dublin, Ireland (C3). Nine healthy control (C1) kidney tissue samples with concurrent urine and serum samples were included. Disease activity was determined using the Birmingham Vasculitis Activity Score version 3 (BVAS) [19]. Remission was defined as BVAS of zero. Creatinine level post-treatment was defined as a creatinine during stable remission, or last known creatinine before lost-to-follow-up, ESKD or death. Active kidney vasculitis was assessed using kidney biopsy findings or, when kidney tissue was unavailable, according to clinical practice as new or increasing erythrocyturia, and/or proteinuria, and/or a rise in serum creatinine. The study was approved by the local ethics committees and informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

2.2. Patient sample collection and preparation

Ten mL of blood was collected and allowed to clot at room temperature for 30–60 min. Samples were then centrifuged for 10 min at 1500 g and serum was collected and stored at either −80 °C (C1, C3) or −20 °C (C2). Urine samples were collected and stored at −80 °C (C1, C3) or −20 °C (C2, diluted in phosphate-buffered saline [PBS] 1:1). All urine samples were centrifuged at 1200–2000g for 10–15 min and supernatants were used for the analyses.

2.3. Detection of soluble CD206 and urinary soluble CD163

Soluble CD206 (sCD206) levels (1:10 dilution) and urinary soluble CD163 (usCD163) levels (1:4 dilution) were assessed by enzyme-linked immunosorbent assay (ELISA) (human soluble Mannose Receptor HK381/CD206; HycultBiotech, Uden, The Netherlands and DY1607; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. sCD206 levels are expressed as nanogram per milliliter. usCD163 levels were corrected for urinary creatinine levels to correct for urinary dilution and expressed as nanograms per millimole. We expand on usCD163 data that have previously been published independently [10,11,20] and are clustered here to determine the utility of sCD206 and usCD163 to detect active ANCA GN.

2.4. Microarray gene expression analysis

We utilized the publicly available glomerular and tubulointerstitial transcriptome microarray datasets from the Gene Expression Omnibus (GEO) database (European Renal cDNA bank, GEO accession GSE104948 – glomerular and GSE104954 - tubulointerstitial) [21,22]. The GEO2R online analysis tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to detect the difference in the expression of macrophage markers (CD68, CD206, and CD163) between AAV samples ($n = 22$ for glomerular dataset, $n = 21$ for tubulointerstitial dataset) and HC ($n = 18$).

2.5. Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded kidney tissue sections were processed according to routine clinical practice [18]. Kidney tissue sections were stained for CD163 and CD206. For immunohistochemistry (IHC), three μm sections were deparaffinized and rehydrated, followed by antigen retrieval with Tris-EDTA buffer (pH 9) at 98 °C for 60 min and blocking with 3% H_2O_2. Rabbit anti-CD163 monoclonal antibody (mAb) (ab182422, Abcam, Cambridge, United Kingdom) was diluted 1:300 in PBS and incubated overnight at 4 °C. Mouse *anti*-MMR/CD206 mAb (MAB25341, R&D systems, Minneapolis, MN, USA) was diluted 1:50 in PBS and incubated for 30 min. EnVision anti-rabbit HRP (Agilent, Santa Clara, CA, USA) was used to visualize CD163 and CD206. For co-staining of CD163 and CD206 using immunofluorescence, three μm sections were

deparaffinized and rehydrated, followed by antigen retrieval with Tris-EDTA buffer (pH 9) at 98 °C for 60 min and blocking with 10% goat serum/PBS. Sections were incubated overnight at 4 °C with rabbit anti-CD163 mAb (ab182422, Abcam, Cambridge, United Kingdom) and mouse *anti*-MMR/CD206 mAb (MAB25341, R&D systems, Minneapolis, MN, USA) diluted 1:100 and 1:20, respectively, in 10% goat serum/PBS. Subsequently, sections were incubated with secondary antibodies Goat anti-rabbit IgG-AF488 (ThermoFisher, Waltham, MA, USA) and Goat anti-mouse IgG-AF544 (ThermoFisher, Waltham, MA, USA) diluted both 1:200. Slides were sealed with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Inc, Burlingame, CA, USA). Images were captured using a Zeiss Axioplan 2 Fluorescent Microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

IHC sections with ≥10 glomeruli were used to score the presence of $CD206^+$ and $CD163^+$ cells. CD206 and CD163 were quantitively scored in three compartments, i.e., (1) glomeruli, (2) sclerosed glomeruli, and (3) cortical tubulointerstitial areas, using digital image analysis software QuPath [23]. $CD206^+$ or $CD163^+$ cells were quantified as a mean percentage or mean number of cells per mm^2 per biopsy for all three compartments.

2.6. Kidney tissue section

Formalin-fixed paraffin-embedded and snap-frozen kidney tissue sections were processed according to routine clinical practice and scored as previously described [11]. In short, kidney tissue sections were scored by two separate investigators; discrepancies were resolved by a third investigator. In all biopsies glomeruli were scored for %glomeruli presenting with (fibro)cellular crescents, fibrinoid necrosis, breaks in the glomerular basement membrane (GBM break), fibrotic crescents and sclerotic glomeruli, and tubulointerstitial areas were scored for interstitial fibrosis and/or tubular atrophy (IFTA) and arteriosclerosis. All biopsies were classified according to the histological classification of ANCA GN [24] and ANCA renal risk score (ARRS) [25].

2.7. Statistical analysis

Statistical analyses were performed with Prism Graphpad 8 for Windows (GraphPad Software, La Jolla, CA, USA) and RStudio v2022.02.0 (R Foundation for Statistical Computing, Vienna, Austria). Categorical variables, expressed as numbers (percentages), were compared using chi-squared or Fisher exact test, as appropriate. Continuous variables, expressed as mean ± standard deviation (SD) or median (interquartile range [IQR]), were analyzed using the independent samples *t*-test or two-sided Mann-Whitney *U* test, as appropriate, or in case of ≥3 groups the Kruskal-Wallis test with post-hoc one-sided Mann-Whitney *U* test with Bonferroni correction for multiple testing was used. Wilcoxon matched-pairs testing was used for paired analysis. Receiver operating characteristics (ROC) analysis was used to assess the diagnostic accuracy of sCD206 and usCD163. The optimal cut-off points were identified by the maximum of the sum of sensitivity and specificity subtracted by 1 (sensitivity + specificity – 1), according to the Youden Index. Correlations were tested using Pearson's correlation coefficient using RStudio. A $P < 0.05$ was considered significant. Unadjusted HR (95%CI) were calculated using univariable Cox regression analysis, and significant variables were adjusted for age and serum creatinine using Cox multiple regression analysis. For microarray gene expression analyses, the adjusted *P*-value were calculated using Benjamini & Hochberg (false discovery rate) test and logFC and a $P < 0.01$ was considered significant.

Table 1
Patient characteristics.

Characteristics	ANCA cohorts			Healthy controls
	Cohort 1 Maastricht	Cohort 2 Groningen	Cohort 3 Dublin	Cohort 1 Maastricht
Number of patients	85	28	97	9
Male/Female, n/n	57/28	12/16	57/40	4/9
Age, years, median (range)	65 (23–85)	53 (33–82)	60 (19–87)	36 (18–65)
ANCA specificity, n (%)				
MPO	44 (52)	7 (25)	52 (54)	
PR3	35 (41)	21 (75)	42 (43)	
Double positive	2 (2)	0 (0)	0 (0)	
ELISA negative	4 (5)	0 (0)	3 (3)	
Disease state, n (%)				
Active ANCA GN	70 (82)	17 (61)	47 (48)	
Extrarenal AAV	9 (11)	3 (11)	12 (12)	
Remission	6 (7)	8 (29)	38 (39)	
Diagnosis/relapse n/n				
Active ANCA GN	46/24	8/9	41/6	
Extrarenal AAV	4/5	2/1	4/8	
BVAS, median (IQR)				
Active ANCA GN	14 (12–18)	15 (14–20)	15 (13–19)	
Extrarenal AAV	9 (6–13)	18 (15–19)	10 (7–14)	
Serum creatinine, µmol/L, median (IQR)				
Active ANCA GN	206 (150–310)	253 (134–345)	272 (142–356)	
Extrarenal AAV	102 (74–124)	90 (77–128)	85 (64–100)	
Remission	190 (147–262)	85 (74–133)	117 (94–220)	
IST at inclusion, n (%)	27 (32)	12 (43)	61 (63)	

AAV, ANCA associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; BVAS, Birmingham Vasculitis Activity Score v3; EGPA, eosinophilic granulomatosis with polyangiitis; ELISA, enzyme-linked immunosorbent assay; GN, glomerulonephritis; GPA, granulomatosis with polyangiitis; IQR, interquartile range; IST, immunosuppressive therapy; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PR3, proteinase 3; SD, standard deviation.

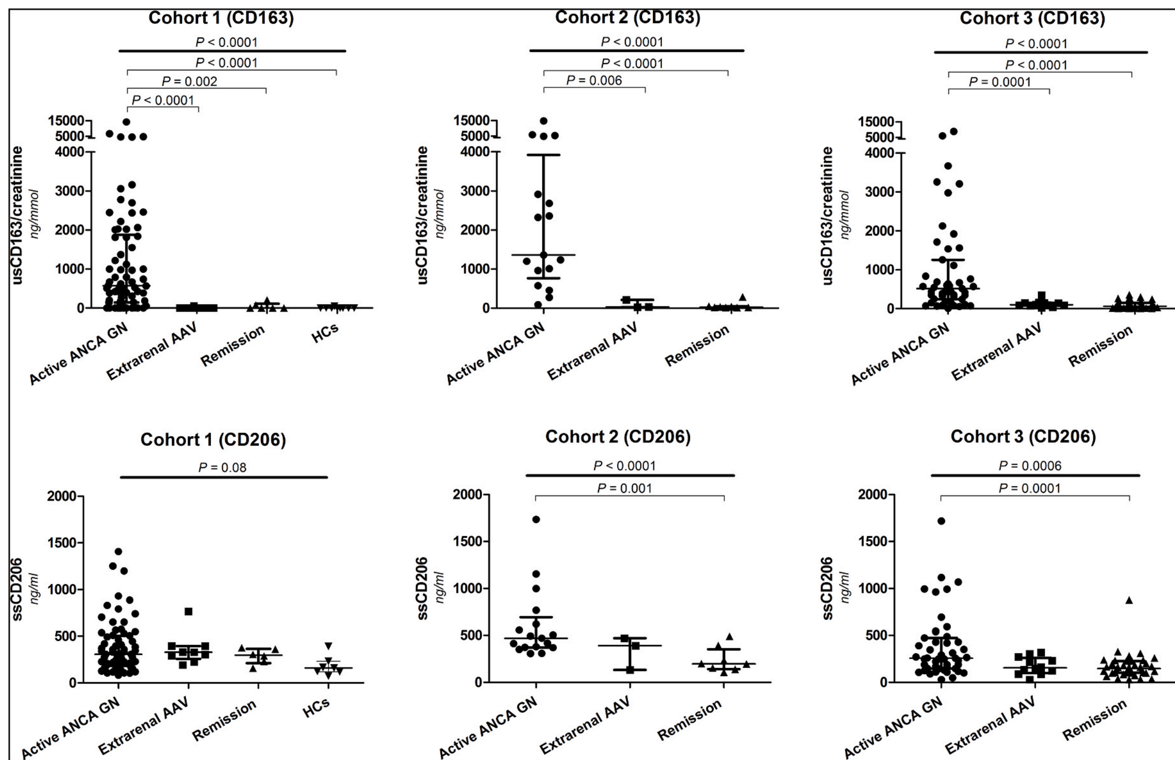

Fig. 1. Urinary sCD163 and serum sCD206 levels in the three independent AAV cohorts. Active ANCA GN compared to extrarenal AAV patients, patients in remission (C1-3), and healthy controls (C1). usCD163 levels were significantly higher in active ANCA GN compared to extrarenal AAV patients and patients in remission (C1-3), which was previously published in part independently [10, 11, 20]. ssCD206 levels were significantly higher in active ANCA GN compared to patients in remission (C2 and C3). Lines show median and interquartile range. Differences between the groups were tested using the Kruskal-Wallis test and post hoc one-tailed Mann-Whitney U test with Bonferroni correction, where $P \leq 0.017$ was considered significant. AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; GN, glomerulonephritis; ssCD206, serum soluble CD206; usCD163, urinary soluble CD163.

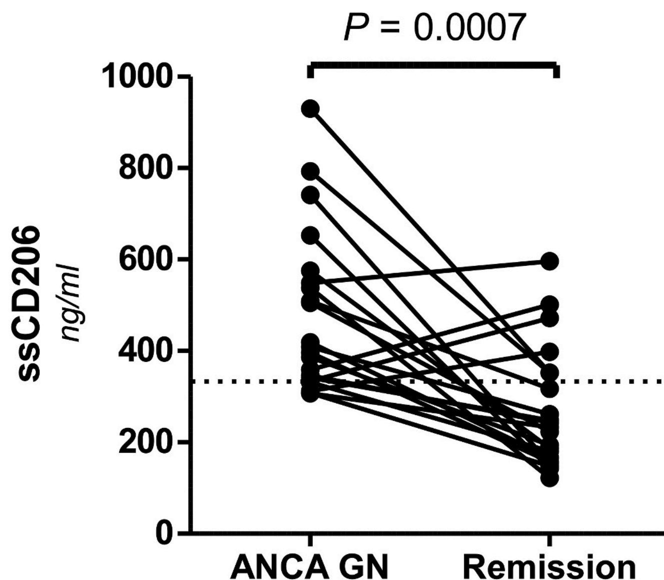

Fig. 2. ssCD206 decreased at the time of remission. In 21 active ANCA GN patients (C1) samples at the time of remission were available. At the time of remission ssCD206 decreased significantly. Dotted line: cut-off level at 332.5 ng/ml. ANCA GN, anti-neutrophil cytoplasmic antibody mediated glomerulonephritis; ssCD206, serum soluble CD206.

3. Results

3.1. Patient cohorts

Three cohorts were included in the study. Cohort 1 (C1) consisted of 85 AAV patients, i.e., patients with active ANCA GN, $n = 70$ (82%), active extrarenal AAV, $n = 9$ (11%), or in remission $n = 6$ (7%). Cohort 2 (C2) consisted of 28 patients (active ANCA GN, $n = 17$ [61%], extrarenal AAV, $n = 3$ [11%], and remission $n = 8$ [29%]). Cohort 3 (C3) consisted of 97 patients (active ANCA GN, $n = 47$ [48%], extrarenal AAV, $n = 12$ [12%], and remission $n = 38$ [39%]). In the total cohort ($N = 210$), male to female ratio was 1.5 and most patients had active ANCA GN ($n = 134$, [64%]). Nine healthy controls (C1) with kidney tissue samples and concurrent urine ($n = 9$ [100%]) and serum ($n = 7$ [78%]) were included. Detailed patient characteristics are listed in Table 1.

3.2. usCD163 and serum sCD206 are elevated in active ANCA GN

We first assessed usCD163 and serum sCD206 (ssCD206) levels in the three independent cohorts of AAV patients. Of note, analysis of urinary sCD206 levels ($n = 8$; biopsy-proven ANCA GN) revealed that sCD206 could hardly be detected and therefore we did not pursue this further (data not shown). Patients with active ANCA GN had significantly higher levels of usCD163 compared to extrarenal AAV patients and patients in remission in all cohorts (Fig. 1A–C). ssCD206 levels were elevated in active ANCA GN patients compared to remission patients (C2&3, Fig. 1D–F). However, no significant difference in ssCD206 levels was detected in cohort 1. Interestingly, ssCD206 levels declined significantly after reaching remission (C1; $n = 21$, median [IQR], 409 [338–562] vs. 233 [162–352], $p = 0.0007$, Fig. 2).

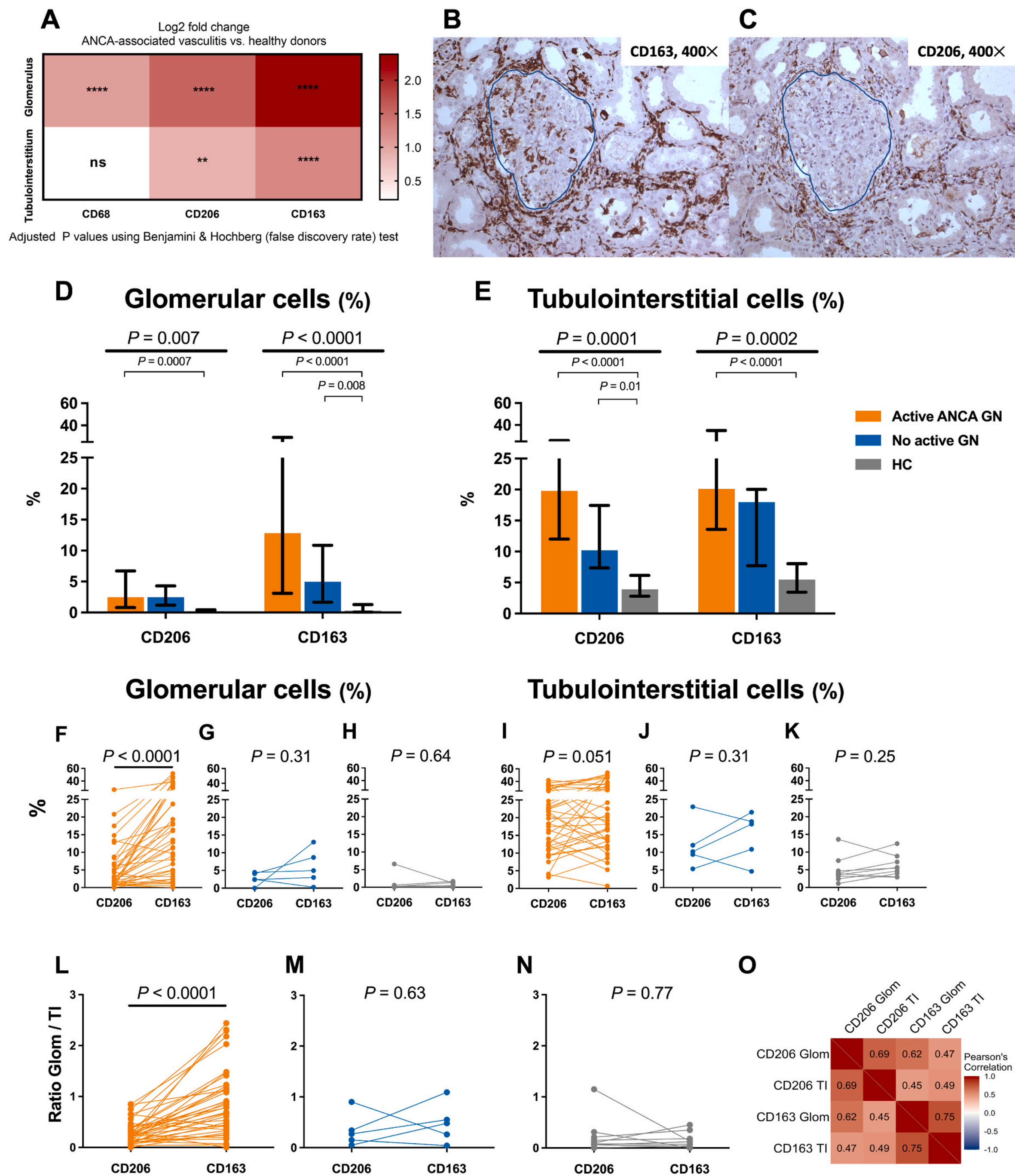

Fig. 3. Expression of CD206 and CD163 in kidney biopsies. (**A**) mRNA expression of macrophage markers CD68, CD206 and CD163 was higher in kidney tissue of AAV patients compared to healthy controls. $^{**}P < 0.01$, $^{****}P < 0.0001$. (**B–C**) Distribution pattern of (**B**) CD163^{+} and (**C**) CD206^{+} macrophages using immunohistochemical staining in consecutive kidney tissue sections from a representative active ANCA GN patient. CD163^{+} macrophages were detected in the affected glomeruli (blue outline in **B**) and tubulointerstitial compartment, whereas CD206^{+} macrophages were mainly detected in the tubulointerstitial compartment in contrast to glomeruli (blue outline in **C**). (**D-K**) Mean percentage of CD206^{+} and CD163^{+} cells in (**D**) glomeruli and (**E**) tubulointerstitial areas in kidney biopsies of patients with active ANCA GN ($n = 39$), no active GN ($n = 5$) and HCs ($n = 9$). Individual paired mean percentage of CD206^{+} and CD163^{+} cells from **D-E** in (**F–H**) glomerular and (**I–K**) tubulointerstitial areas. (**L-N**) The ratio between glomeruli vs. tubulointerstitial areas for number of CD206^{+} and CD163^{+} cells per mm^2. Sclerosed glomeruli were not included and were assessed separately (Suppl. Fig. 1). (**O**) Correlation matrix of glomerular and tubulointerstitial expression of CD206 and CD163. D–E: Bars represent median with interquartile range, compared using the Kruskal-Wallis test and post hoc one-tailed Mann-Whitney *U* test with Bonferroni correction. $P \leq 0.017$ was considered significant. F–N: Paired CD206 and CD163 percentages compared using Wilcoxon signed rank test. O: Pearson's correlations shown when statistically significant. ANCA GN, anti-neutrophil cytoplasmic antibody mediated glomerulonephritis; AAV, ANCA-associated vasculitis; Glom, glomerular; HCs, healthy controls; Ratio Glom/TI, ratio of CD206^{+} or CD163^{+} cells in glomerular vs. tubulointerstitial areas expressed in positive cells/mm^2; TI, tubulointerstitial.

Table 2
Characteristics of analyzed kidney tissue samples.

Characteristics	ANCA GN	No active GN	Healthy controls	*P*-value
Number of patients	39	5[a]	9	
Male/Female, n/n	23/16	4/1	4/5	0.43
Serum creatinine, μmol/L, median (IQR)	65 (23–85)	53 (33–82)	60 (19–87)	0.001
Erythrocyturia, n (%)	36 (95)[b]	5 (100)	3 (33)	<0.0001
Proteinuria, g/L, median (IQR)	0.38 (0.1–1.19)	0.07 (0.04–1.00)	0.04 (0.02–0.12)	0.007
ANCA GN class[c], n (%)				
Focal class	12 (31)	–	–	
Crescentic class	8 (21)	–	–	
Mixed class	11 (28)	–	–	
Sclerotic class	8 (21)	–	–	
ANCA Renal Risk Score[d], n (%)				
Low	22 (56)	–	–	
Intermediate	13 (33)	–	–	
High	4 (10)	–	–	
usCD163, ng/mmol, median (IQR)	508 (117–2027)	0 (0–104)	0 (0–2)	0.0002
ssCD206, ng/L, median (IQR)	278 (177–565)	304 (259–364)	159 (119–232)	0.07
Kidney expression of:				
CD163				
%Glomerular	12.8 (4.0–29.2)	5.0 (1.7–10.9)	0.3 (0.1–1.3)	0.0001
%Tubulointerstitial	20.1 (13.4–34.9)	18.0 (7.7–20.0)	5.5 (3.5–8.0)	0.0002
CD206				
%Glomerular	2.3 (0.8–6.7)	2.5 (1.2–4.3)	0.2 (0.2–0.5)	0.007
%Tubulointerstitial	19.8 (12.0–25.9)	10.2 (7.3–17.5)	3.9 (2.8–6.2)	0.0001

AAV, ANCA associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibody; GN, glomerulonephritis; IQR, interquartile range; ssCD206, serum soluble CD206; usCD163, urinary soluble CD163.
[a] Negative tissue samples from patients with extrarenal AAV ($n = 3$) and in remission ($n = 2$).
[b] Erythrocyturia not available, $n = 1$.
[c] Classification system for active ANCA GN according to Berden et al. [24].
[d] Classification system according to Brix et al. [25].

3.3. Utility of usCD163 and ssCD206 in detecting active ANCA GN

In all cohorts usCD163 was highly specific at $\geq$ 0.96, however, sensitivity rates ranged from 0.69 to 0.88. Overall, ssCD206 alone performed poorly. Combining usCD163 and ssCD206 increased sensitivity, however at the cost of specificity. Thus, combining both markers did not significantly improve diagnostic accuracy when compared to usCD163 alone (Suppl. Table 1).

3.4. CD163 and CD206 are expressed in kidney tissue of active ANCA GN patients

Since we found different associations of the two soluble markers sCD163 and sCD206 with disease activity in ANCA GN patients, we assessed the spatial distribution of CD163$^+$ and/or CD206$^+$ macrophages in kidney tissue using gene expression data analysis, IHC staining, and IF double staining.

Gene expression data analysis of publicly available glomerular and tubulointerstitial transcriptome datasets [22] showed significant elevation of CD206 and CD163 expression in both the tubulointerstitium (n = 21) and glomeruli (n = 21) of AAV patients compared to HC (n = 18). In contrast, expression of the pan macrophage marker CD68 was elevated in the glomerulus, but not in the tubulointerstitium of the kidney tissue from AAV patients compared to HC (Fig. 3A).

Fifty-three kidney tissue sections (C1; active ANCA GN, n = 39, no active GN, n = 5 [i.e., extrarenal AAV, $n = 3$; remission, $n = 2$], HC, $n =$ 9) were stained using IHC (Table 2, Fig. 3B–C). This revealed both CD206$^+$ and CD163$^+$ cell expression in glomeruli and tubulointerstitium to be higher in ANCA GN patients compared to HC, however not compared to no active GN (Fig. 3D–E). There were strong correlations between expression of CD206 and CD163 in both glomeruli and tubulointerstitium (Fig. 3O). Glomerular expression of CD206 in active ANCA GN was lower than CD163 (Fig. 3F), whereas tubulointerstitial expression of CD206 and CD163 was equal (Fig. 3I). Accordingly, the ratio of glomerular vs. tubulointerstitial expression was lower for CD206 compared to CD163 (0.24 [0.11–0.34] vs. 0.78 [0.35–1.21], resp. Fig. 3L). Sclerotic glomeruli showed higher mean percentages of CD206$^+$ and CD163$^+$ cells compared to non-sclerotic glomeruli (Suppl. Fig. 1). Differences in glomerular and tubulointerstitial expression were not found in patients with no active GN and HC patients (Fig. 3G–H, J–K, M–N).

Immunofluorescent double staining revealed CD163$^+$ and CD206$^+$ single positive macrophages, as well as CD163$^+$CD206$^+$ double positive macrophages in the kidney tissue of ANCA GN patients (Fig. 4). CD163$^+$ single positive macrophages were most abundant in affected glomeruli whereas CD163$^+$CD206$^+$ double positive, as well as CD206$^+$ single positive macrophages were predominantly detected in the tubulointerstitium. Our IF data comply with our IHC findings and strengthens our notion that CD206 is expressed mainly in tubulointerstitial areas, whereas CD163 expression locates in both glomerular and tubulointerstitial areas.

3.5. CD163 and CD206 expression in kidney tissue correlate with clinical and pathological parameters

Kidney pathology and clinical parameters (Table 2) from the active ANCA GN patients were correlated with IHC data. Both ssCD206 and usCD163 correlated strongly with glomerular and tubulointerstitial CD163 expression, and less pronounced also with CD206 glomerular and tubulointerstitial expression (Fig. 5). Glomerular CD163, but not CD206,

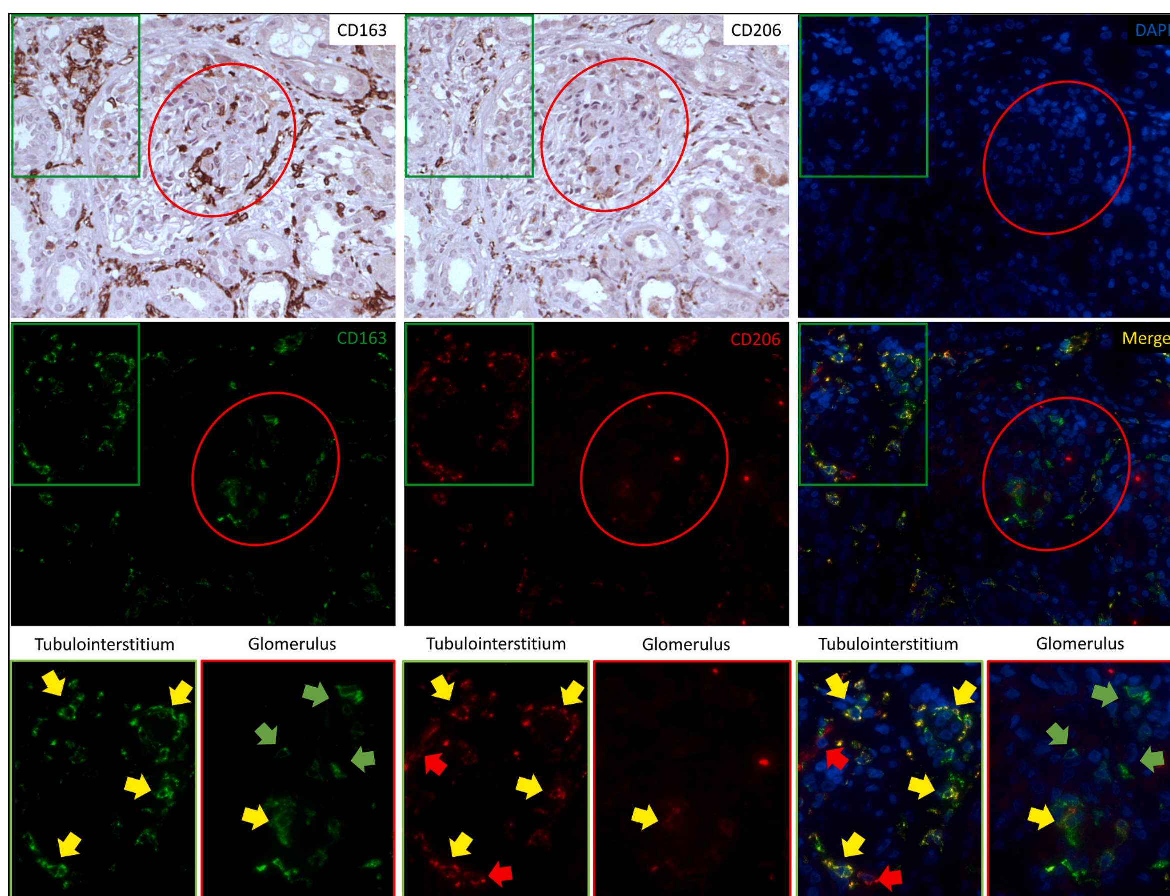

Fig. 4. Presence of CD163$^+$, CD206$^+$ and CD163$^+$CD206$^+$ macrophages in the kidney tissue of ANCA GN patients. Representative immunohistochemical and -fluorescence staining of CD163 (green) and CD206 (red) in a kidney tissue section of an active ANCA GN patient. Images in green and red boxes represent magnified areas of the tubulointerstitial compartment and glomerulus respectively. CD163$^+$CD206$^+$ macrophages (yellow arrows) are found abundantly in the tubulointerstitial compartment but are less prevalent in the glomerulus. CD163$^+$CD206$^-$ macrophages (green arrows) are found abundantly in the glomerulus while a small number of CD206$^+$CD163$^-$ (red arrows) macrophages are detected in the tubulointerstitial compartments.

expression correlated with abnormal glomeruli, whereas both tubulointerstitial CD163 and CD206 expression did correlate with abnormal glomeruli. Concordantly, cellular crescents correlated strongly with glomerular and tubulointerstitial CD163 expression, but less so with CD206. Additionally, fibrinoid necrosis correlated only with CD163 expression. eGFR at presentation showed a negative correlation with tubulointerstitial CD206 and CD163. Other histopathological or clinical parameters showed weak, if any, correlation with IHC findings (Fig. 5, Suppl. Fig. 2). These data suggest that the different macrophage phenotypes, as found by IHC and IF, contribute to different stages of the glomerular and tubulointerstitial inflammatory processes in ANCA GN.

3.6. CD206 and CD163 relate to patient outcome

In patients with active ANCA GN from cohort C1 outcome data were available. Creatinine levels at the time of remission were higher with increasing rates of percentages of abnormal and/or sclerotic glomeruli, IFTA, as well as percentage of glomerular CD163$^+$ cells. Additionally, usCD163 showed an inverse correlation with Δserum creatinine, suggesting a relation between increased glomerular CD163 and adverse glomerular outcome (Fig. 6).

3.6.1. End-stage kidney disease

Eight (11%) patients developed ESKD after a median (IQR) of 30 (19–45) months. After adjusting for age and serum creatinine levels at active disease, serum creatinine at active disease (μmol/L, HR [95%CI], 1.007 [1.004–1.010], $P < 0.001$), usCD163 (ng/mmol, HR [95%CI], 1.000 [1.000–1.000], $P = 0.044$), ssCD206 (ng/mL, HR [95%CI], 1.003 [1.001–1.006], $P = 0.011$), Δcreatinine at remission (HR [95%CI], 1.013 [1.002–1.025], $P = 0.021$), ANCA GN score (HR [95%CI], 27.375 [2.667–281.014], $P = 0.005$), %normal glomeruli (HR [95%CI], 0.911 [0.840–0.987], $P = 0.023$) and %sclerotic glomeruli (HR [95%CI], 1.040 [1.002–1.080], $P = 0.041$) were predictors for ESKD (Suppl. Table 2).

3.6.2. Relapse

Fifteen (21%) patients developed relapsing AAV after a median (IQR) of 29 (15–39) months. No predictors for relapsing disease were found (Suppl. Table 2).

3.6.3. Death

Thirteen (19%) patients died after a median (IQR) of 32 (21–53) months. Patients with active AAV who died during follow-up presented with higher levels of ssCD206 compared to patients who did not (ng/mL, median [IQR], 396 [346–642] vs. 277 [178–417], resp, $P = 0.004$, Suppl. Fig. 3). After adjusting for age and serum creatinine levels at active disease, age (HR [95%CI], 1.136 [1.049–1.231], $P = 0.002$) and ssCD206 (ng/mL, HR [95%CI], 1.003 [1.001–1.005], $P = 0.009$) were predictors for death (Suppl. Table 2).

4. Discussion

Macrophages, recruited and activated by the initial damaging inflammatory effects of neutrophils, are the most prominent inflammatory

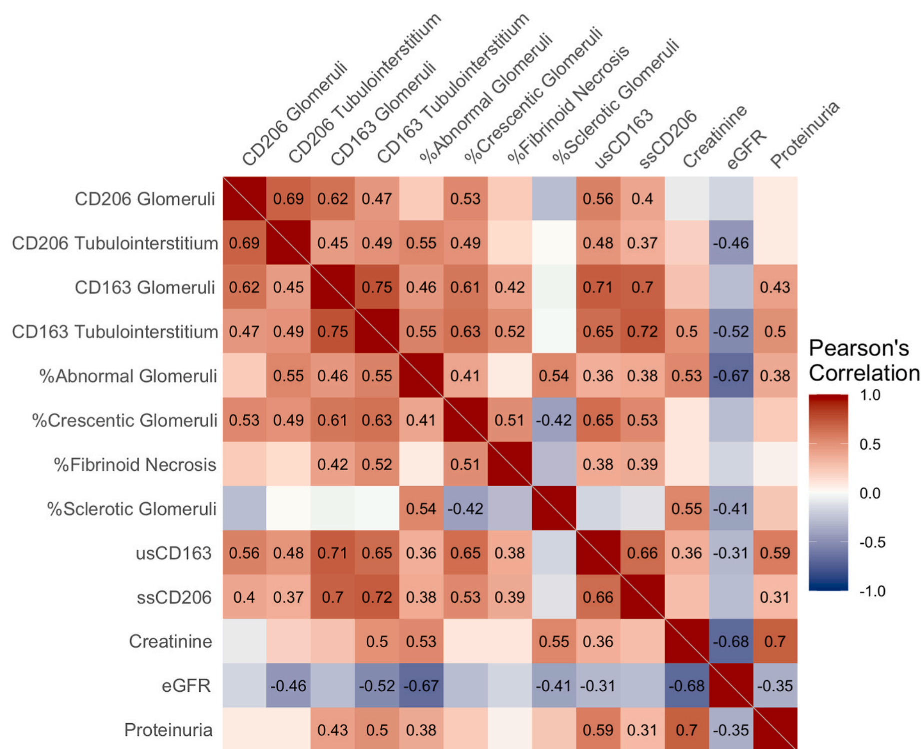

Fig. 5. Correlation matrix of immunohistochemistry parameters correlated with clinical and biopsy parameters from ANCA GN patients. Only significant Pearson's correlations are displayed in the matrix. %glomeruli/crescents, calculated percentage of all glomeruli in the tissue specimen; ANCA, anti-neutrophil cytoplasmic antibody; eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; ssCD206, serum soluble CD206; usCD163, urinary soluble CD163.

cell types in the pathology of AAV and are expected to play pivotal roles in the immunopathology of ANCA GN. Here we evaluated the clinicopathological significance of CD206-and CD163-expressing macrophages in ANCA GN. We found that both usCD163 and ssCD206 were elevated in patients with active ANCA GN, however, as a biomarker for active ANCA GN usCD163 proved superior. Histopathological analysis revealed different subsets of CD206$^+$ and/or CD163$^+$ macrophages. CD206$^+$ macrophages were found mainly tubulointerstitially, whereas CD163$^+$ macrophages were found in both glomeruli and tubulointerstitially. Likewise, these macrophages showed varying correlation profiles with clinicopathological parameters and outcome measures. Our results confirm and extend the notion that different macrophage subsets are present in ANCA GN. It also suggests that these macrophages play versatile roles in different kidney compartments which could aid in assessing treatment responses, as well as risk stratification for adverse outcomes in ANCA GN patients.

SsCD206 levels are elevated in patients with active ANCA GN. However, ssCD206 proved to be a less reliable indicator for active ANCA GN. Combining ssCD206 with usCD163 did not significantly improve diagnostic accuracy for ANCA GN compared to usCD163 alone. CD206$^+$ macrophages likely also contribute to extrarenal manifestations, implying that ssCD206 does not solely reflect GN but extrarenal inflammation as well. Indeed, elevated ssCD206 levels have been reported in patients with autoimmune and inflammatory conditions, e.g., rheumatoid arthritis and inflammatory liver disease [26–30]. Ideally, a biomarker panel consisting of kidney function (e.g., serum creatinine, proteinuria), kidney injury (e.g., KIM-1, NGAL), and macrophage (e.g., usCD163, ssCD206, MCP-1) markers is required to effectively discern ANCA GN, extrarenal AAV and quiescent disease, potentially obviating the need for repeated kidney biopsy.

During follow-up, ssCD206 levels in ANCA GN patients declined after reaching remission following immunosuppressive treatment, similar to usCD163 [11]. Additionally, our data show that patients with increased levels of ssCD206 during active vasculitis were at increased risk for ESKD and death during follow-up. Similar findings regarding survival in pneumococcal bacteraemia and alcoholic cirrhosis patients show increased ssCD206 levels in non-survivors [30,31]. Hence, we suggest ssCD206 can potentially be helpful in AAV to assess treatment response and identify patients in whom vigilance regarding adverse events is needed.

Similar to usCD163, we attempted to measure sCD206 in urine from ANCA GN patients but found this to be near undetectable. Histopathological findings indeed show low numbers of glomerular CD206$^+$ macrophages. Although sCD206 is relatively stable in the blood [31], we detected only ~80–85% of recombinant CD206 spiked into healthy donor urine (Suppl. Fig. 4), suggesting that sCD206 is unstable in urine upon long-term storage. Thus, urinary sCD206 will likely not be a viable marker for glomerulonephritis.

In line with previous findings, we found distinct macrophage phenotypes including CD206$^+$CD163$^-$, CD163$^+$CD206$^-$ and CD163$^+$CD206$^+$ macrophages, throughout the kidney tissue from active ANCA GN patients [10]. CD163$^+$CD206$^-$ macrophages were abundantly found in the affected glomeruli. In the early stages of ANCA-associated necrotizing glomerulonephritis, CD163$^+$ macrophages are the most prominent infiltrating cell type at sites of fibrinoid necrosis [4,9]. Furthermore, we previously demonstrated that (1) CD163$^+$ macrophages are abundant in the glomeruli of active ANCA GN patients, (2) usCD163 levels correlate strongly with the presence of glomerular crescents and fibrinoid necrosis and (3) that usCD163 can signal relapsing ANCA GN [11]. Tubulointerstitial CD163$^+$ macrophages have been shown to be important in predicting ESKD [32]. On the other hand, CD163$^+$CD206$^+$ and CD206$^+$CD163$^-$ macrophages were mainly found in the tubulointerstitial compartment, but were less prevalent in affected glomeruli, corroborating previous observations in crescentic GN [8]. Massive infiltration of CD206$^+$ macrophages has previously been found in acute interstitial nephritis and tubular necrosis [9]. Additionally, in an experimental mouse model, exogenous CD206$^+$ macrophages ameliorated antibody-mediated GN by attenuating proinflammatory

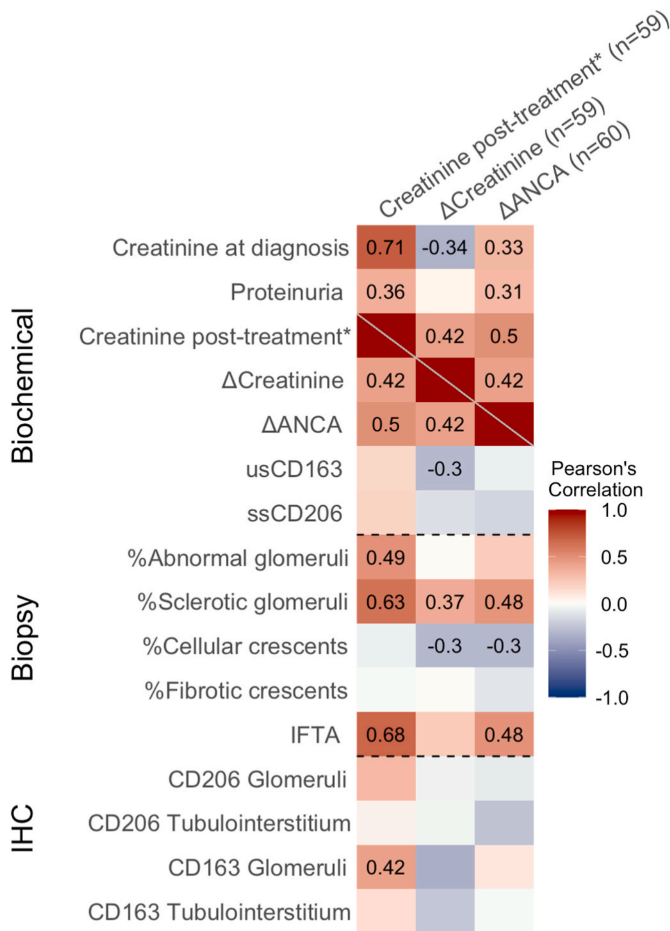

Fig. 6. Correlation matrix of outcome measures correlated with biochemical, biopsy, and IHC parameters from ANCA GN patients. Only significant Pearson's correlations are displayed in the matrix. *Creatinine level during stable remission, or last known creatinine before lost-to-follow-up, ESKD, or death. Δ, difference in parameter comparing levels post-treatment vs. active disease; % glomeruli/crescents, calculated percentage of all glomeruli in the tissue specimen; ANCA, anti-neutrophil cytoplasmic antibody; GN, glomerulonephritis; IFTA, interstitial fibrosis and tubular atrophy; IHC, immunohistochemistry parameters from ANCA GN patients ($n = 39$) as described in Table 2; n, number of patients with available outcome for the parameter; ssCD206, serum soluble CD206; usCD163, urinary soluble CD163.

mediators, inflammatory cell infiltration and crescent formation [33]. Furthermore, Xie et al. showed that glomerular CD206$^+$ cells in IgA nephropathy patients predict a favorable response to immunosuppressive treatment [34]. Therefore, the absence of CD206$^+$ macrophages in glomeruli may indicate a hampered anti-inflammatory mechanism and/or hampered resolution of GN. Collectively, our data and other studies suggest the presence of diverse macrophage phenotypes with distinct roles in the immunopathology of active ANCA GN. The glomerular presence of CD163$^+$ macrophages, and possibly the absence of CD206$^+$ macrophages, may play complementary roles in glomerular damage, while both CD163$^+$ and CD206$^+$ macrophages seem to play a prominent role in acute tubulointerstitial inflammation.

The heterogeneous macrophage populations in different kidney compartments may be a consequence of differences in local cytokine production, e.g., granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). Elevated expression levels of CD163 and CD206 have been reported in macrophages skewed by M-CSF and GM-CSF, respectively [35–37]. In various forms of GN, the expression of both M-CSF and GM-CSF has been documented [38,39]. Elevated M-CSF expression has been shown in both glomerular and tubulointerstitial compartments [38–40]. Moreover, in the MRL-Fas^{lpr} model of lupus nephritis, M-CSF was shown to enhance macrophage recruitment, proliferation and activation, exacerbating kidney damage [41,42]. In human glomerulonephritis, expression of tubular and glomerular M-CSF has been reported to correlate with macrophage proliferation and kidney dysfunction [39]. Indeed, by blocking the M-CSF receptor (M-CSFR) in rodent GN models, glomerular macrophage infiltration and kidney injury was ameliorated [40,43] accompanied by a significant reduction of glomerular CD163 expression, but not CD206 [40]. On the other hand, in experimental mouse GN models, increased expression of GM-CSF has been reported in the tubulointerstitial compartment [44,45]. In a mouse model of renal ischemia/reperfusion injury, the expression of GM-CSF by tubular epithelial cells was found to activate STAT5-mediated macrophage activation leading to elevated expression of CD206 [46]. Ablation of GM-CSF expression attenuated tubulointerstitial inflammation and glomerular injury in renal ischemia/reperfusion injury and *anti*-GBM kidney disease models [44,45,47]. GM-CSF polarized macrophages display increased production of proinflammatory cytokines, chemokines and metalloproteinases that may contribute to prolonged kidney inflammation, chronic remodeling, and fibrosis [35–37,48–54]. Collectively, these mechanistic studies suggest that these distinct macrophages play different roles in the immunopathogenesis of ANCA GN (Fig. 7). The recruitment and polarization of CD163$^+$ macrophages, triggered by M-CSF signaling, is an important driver of the acute inflammatory process in the glomerulus that leads to crescent formation. On the other hand, the high expression of GM-CSF in the tubular cell polarized

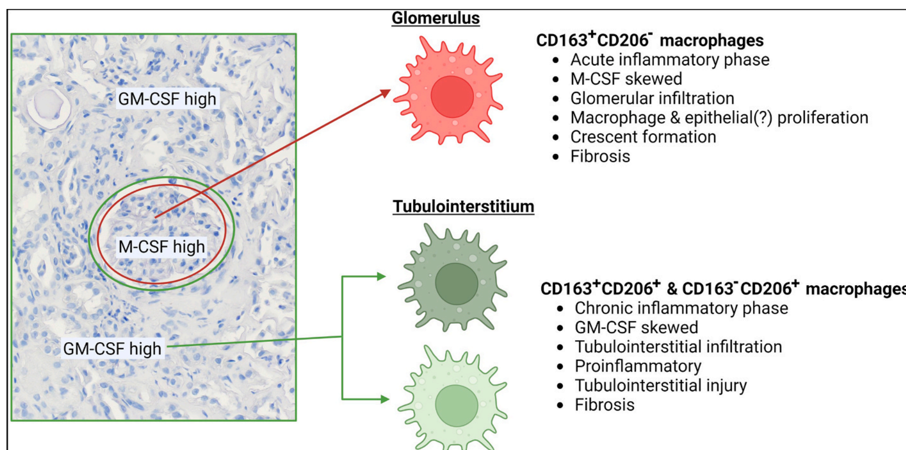

Fig. 7. Macrophage plasticity in ANCA GN. The difference in M-CSF and GM-CSF expression gradients in the different compartments of the kidney may promote different macrophage polarization. The high glomerular expression of M-CSF promotes CD163$^+$ macrophage differentiation that promotes glomerular crescent formation. The high GM-CSF expression by tubular cells promotes differentiation of CD206$^+$ macrophages, leading to tubulointerstitial inflammation, tissue damage and fibrosis; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor (Figure made with: BioRender.com).

CD206^{+} macrophages which contributes to tubulointerstitial inflammation, chronic remodeling, and fibrosis. This notion is perfectly in line with our findings, where we found that usCD163 and ssCD206 correlated with acute inflammatory ANCA GN activity versus ANCA GN outcome, i.e., ESRD and death, respectively.

The strengths of our study include the combined investigation of macrophage markers in serum and urine samples and kidney tissue. Furthermore, three AAV cohorts were studied, albeit in different centers with different equipment. This could explain the differences in the performance for detecting ANCA GN in the individual cohorts with a cut-off value based on the total cohort. Additional variation likely originates from inherent differences in the cohorts, e.g., use of immunosuppressants at the time of sampling and criteria for active ANCA GN (biopsy/traditional markers [C1] vs. traditional markers [C2&3]). Validation of our findings in prospective cohorts is thus warranted. Another limitation of our study is that no mechanistic studies could be performed to elucidate the pathological mechanism by which these macrophages contribute to the pathobiology of ANCA GN. Although we elaborately discuss these potential mechanisms by leveraging data available in the literature, future research should take advantage of novel high-plex spatial multi-omics platforms to further understand their distinct roles in the immunopathology of ANCA GN.

5. Conclusions

Our results confirm that CD206^{+} and CD163^{+} macrophages are prominent components of the cellular infiltrate in ANCA GN. We found distinct macrophage phenotypes which may play a distinct role in the immunopathology in ANCA GN. usCD163 remains an excellent marker to detect active ANCA GN, whereas ssCD206 may be important for risk prediction.

Author statement

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Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. M.A.L. received funding from the Meath Foundation (grant number 208591) and Health Research Board (HRA-POR-2015-1205).

Data availability

Data will be made available on request.

Acknowledgements

We gratefully thank R. Theunissen and H. van Rie (Laboratory of Clinical Immunology, Maastricht University Medical Center, Maastricht, the Netherlands) for their technical assistance. Furthermore, we thank S. Timmermans, M. Busch, R. Ysermans, J. Potjewijd and L. Frenken (Clinical Immunology and Nephrology, Maastricht University Medical Center, Maastricht, The Netherlands) and all participating nephrologists of the Limburg Renal Registry for providing data for the present study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2022.102914.

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