Urinary T Cells Identify Renal Antineutrophil Cytoplasmic Antibody-Associated Vasculitis and Predict Prognosis: A Proof of Concept Study

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Introduction: Necrotizing crescentic glomerulonephritis is a major contributor to morbidity and mortality in Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV). Because therapy relies on immunosuppressive agents with potentially severe adverse effects, a reliable noninvasive biomarker of disease activity is needed to guide treatment.

Methods: We used flow cytometry to quantify T cell subsets in blood and urine samples from 95 patients with AAV and 8 controls to evaluate their biomarker characteristics. These were compared to soluble markers, monocyte chemoattractant protein-1 (MCP-1), soluble CD163 (sCD163), soluble CD25 (sCD25), and complement C5a (C5a), measured using multiplex analysis. Available kidney biopsies ($n = 21$) were classified according to Berden.

Results: Patients with active renal AAV (rAAV) showed significantly higher urinary cell counts than those in remission, or those with extrarenal manifestation, or healthy controls. Urinary T cells showed robust discrimination of disease activity with superior performance compared to MCP-1 and sCD163. Patients whose kidney biopsies had been classified as "crescentic" according to Berden classification showed higher urinary T cell counts. Discordant regulatory T cells (T_{reg}) proportions and $CD4^+/CD8^+$ ratio in blood and urine suggested that urinary cells reflect tissue migration rather than mere micro-bleeding. Furthermore, urinary T_{reg} and T helper cells (T_H17) patterns were associated with clinical response and risk of renal relapse.

Conclusion: Urinary T cells reflect the renal inflammatory milieu in AAV and provide further insights into the pathogenesis of this chronic condition. Their promising potential as noninvasive diagnostic and prognostic biomarkers deserves further exploitation.

ANCA cause systemic life-threatening AAV. AAV affects any organ but most prominently the kidneys, causing pauci-immune necrotizing crescentic glomerulonephritis.[1,2] With renal involvement being a major

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contributor to mortality, preventing kidney damage is a main objective of therapy.[3] However, most therapy regimens rely on immunosuppressive agents with considerable side effects potentially contributing to morbidity and mortality. Clinicians are therefore challenged to find the right balance between overtreatment and undertreatment.[4] To aid decision making an adequate assessment of renal involvement, disease activity, and progression is much needed.

Kidney biopsy remains the gold standard for establishing the diagnosis of pauci-immune necrotizing

crescentic glomerulonephritis and offers prognostic value.[5] However, its invasive nature, cost, and sampling error render renal biopsy unsuitable for serial monitoring of disease activity.[6] Assuming the urine space represents a "window into the kidney" because of its proximity to renal pathology, urinary biomarkers could be ideal for noninvasive and cost-effective serial monitoring of disease activity. Urinalysis is widely used by clinicians, yet its implications remain controversial.[7–12] Recently, MCP-1, sCD163, and sCD25 have been shown to be promising urinary biomarkers of renal disease activity in AAV.[13–16] Surprisingly, little is known about urinary leukocyte populations in AAV and whether they reflect disease activity, even though this has been shown in other conditions such as lupus nephritis. In lupus nephritis, urinary leukocytes directly reflect interstitial infiltration, and T cells represent a reliable biomarker.[17–20] Only 3 studies of urinary leukocytes have so far been reported in AAV, and they focused on CD4$^+$ T effector cells and macrophages. These were found to reflect disease activity and histopathological extent of disease; however, their suitability as diagnostic or prognostic biomarkers was never assessed.[21–23]

We hypothesized that urinary leukocyte populations reflect renal vasculitis and can be used as biomarkers in the assessment of disease activity. In particular, we focused on T_{reg} and IL-17 producing T_H17, which have never been studied in urine despite their emerging role in the pathogenesis of AAV.[24–27]

We provide evidence that active renal vasculitis causes distinct leukocyte subpopulations to migrate into the urine space representing a noninvasive prognostic biomarker for rAAV.

METHODS

Patients
Patients with AAV, based on Chapel Hill Consensus Conference criteria,[28] were recruited in the Department of Nephrology and Medical Intensive Care, Charité-Universitätsmedizin Berlin, Germany; Department of Nephrology, Helios Klinikum Berlin-Buch, Berlin, Germany; Department of Nephrology and Endocrinology/Diabetology, Ernst von Bergmann Klinikum, Potsdam, Germany; and Department of Renal Medicine, University College London Royal Free Hospital, London, United Kingdom. Based on disease activity, assessed by Birmingham Vasculitis Activity Score Version 3 (BVAS)[29] and histopathological examination of kidney biopsy, patients were stratified into 3 groups as follows: (i) active vasculitis with renal involvement (rAAV) (histologic proof of pauci-immune necrotizing crescentic glomerulonephritis within 2 weeks and/or

BVAS renal subcategory $\geq$ 10), (ii) active vasculitis without renal involvement nonrenal AAV (nrAAV) (BVAS $\geq$ 1 and renal subcategory $=$ 0), and (iii) remission with previous renal involvement (Rem) (BVAS $=$ 0). Both relapses and first manifestations of AAV were included, whereas patients with anuria, malignancy, menses, or bladder pathology (2 cases of bladder cancer) were excluded. In addition, healthy controls (HC) were recruited. Clinical and routine laboratory data were collected. Abiding by the Declaration of Helsinki and the Declaration of Istanbul, local ethics committees approved the study (EA2_103_17), and all participants gave written, informed consent.

Between November 1, 2017 and December 31, 2019, a total of 112 subjects were recruited. After the completion of the recruitment process, 17 participants had to be excluded from the final analysis because of technical issues (e.g., flow cytometer clogging, $n = 6$; technical failure rate 5 %) and diagnosis of a concomitant kidney condition after initial inclusion ($n = 11$, e.g., proof of concomitant membranous nephropathy, minimal change, etc., on kidney biopsy). The remaining 95 patients (Berlin $n = 67$, London $n = 28$) were split into an inception cohort (between November 1, 2017 and December 31, 2018, $n = 65$) and a validation cohort (between January 1, 2019 and December 31, 2019, $n = 30$). The population demographics are shown in Table 1; an overview of the collected samples are provided in Supplementary Table S1.

Materials
Phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} was procured from Biochrom GmbH (Berlin, Germany), antibodies CD3-PE/Cy5 (catalog number: 300310), CD4-PE/Cy7 (357410), CD8-APC/Cy7 (344714), CD127-BV510 (351332), FoxP3-FITC (320106), Tbet-APC (644814) were procured from BioLegend (San Diego, CA, USA), RORγt-PE (12-6988-82) was procured from Invitrogen by Thermo Fisher Scientific (San Diego, CA, USA), and CD25-APC/Cy7 (557753) was procured from BD Pharmingen (Franklin Lakes, NJ, USA). Live/dead staining was performed using ViobilityTM (Miltenyi Biotec, Bergisch Gladbach, Germany). Luminex Human Magnetic Assay (4-Plex) was used for MCP-1, sCD163, sCD25 and C5a (Luminex Corporation, Austin, Texas, USA).

Sample Processing and Flow Cytometry
Freshly voided urine samples with a volume of at least 20 ml were collected from all patients and within 6 hours, they were centrifuged at 2000 rpm for 8 minutes at 4 °C. Supernatant was stored at −70 °C for later detection of soluble biomarkers. Cell pellets were washed in PBS + 1% bovine serum albumin (BSA),

Table 1. Demographic and clinical information of participants

n	Inception				Validation		
	65				30		
	rAAV	nrAAV	Rem	HC	rAAV	nrAAV	Rem
n	33	4	20	8	19	3	8
Age: yr median (IQR)	57 (48, 64)	44 (38, 51)	63 (55, 71)	47 (40, 54)	67 (56, 73)	50 (44, 58)	64 (52, 68)
Sex: females (%)	13 (39%)	1 (25%)	10 (50%)	5 (63%)	5 (26%)	0	4 (50%)
Diagnosis							
GPA: n, (%)	21 (64%)	4 (100%)	11 (55%)		10 (53%)	2 (67%)	5 (63%)
MPA: n, (%)	12 (36%)	0	9 (45%)		9 (47%)	1 (33%)	3 (37%)
ANCA							
anti-MPO: n (%)	16 (48%)	1 (25%)	9 (45%)		9 (47%)	1 (33%)	3 (38%)
anti-PR3: n (%)	17 (52%)	3 (75%)	11 (55%)		10 (53%)	2 (67%)	5 (62%)
Histologically proven	17 (52%)	1 (25%)	15 (75%)		6 (32%)	1 (33%)	3 (38%)
Number of relapses							
First manifestation	17 (52%)	2 (50%)	9 (45%)		11 (58%)	0	5 (62%)
Median (IQR)	0 (0, 2)	0.5 (0, 1.25)	1 (0, 1)		0 (0, 1)	1 (1, 1.5)	0 (0, 1)
Mo since first diagnosis: median (IQR)	2 (0, 52)	9 (4, 136)	46 (35, 69)		1 (0, 48)	174 (105, 243)	40 (20, 57)
Berden class: count, (%)							
Crescentic	4 (31%)				0 (0%)		
Focal	2 (15%)				4 (44%)		
Mixed	5 (38%)				2 (22%)		
Sclerotic	0 (0%)				1 (11%)		
Too few	2 (15%)				1 (11%)		
Treatment before sample acquisition							
Naive	16 (48%)				9 (47%)		
Steroids	17 (52%)				10 (53%)		
BVAS: median (IQR)	15 (12, 19)	16 (10, 19)	0 (0, 0)		16 (14, 20)	12 (11, 12)	0 (0, 0)
BVAS renal subcategory: median (IQR)	10 (10, 12)	0 (0, 0)	0 (0, 0)		12 (10, 12)	0 (0, 0)	0 (0, 0)
Creatinine: [μmmol/l] median (IQR)	161 (95, 300)	81 (77, 89)	111 (82, 164)		291 (216, 382)	83 (73, 87)	116 (90, 173)
CRP [mg/l]: median (IQR)	20 (6, 46)	17 (6, 30)	3 (2, 6)		12 (6, 27)	15 (9, 28)	4 (2, 7)
Proteinuria [g/l]: median (IQR)	1.21 (0.30, 2.10)	0.11 (0.09, 0.14)	0.18 (0.11, 0.47)		0.75 (0.40, 1.29)	0.13 (0.07, 0.16)	0.21 (0.12, 0.51)
Ery dipstick	3 (3, 3)	0 (0, 0)	0 (0, 1)	0 (0, 0)	3 (3, 3)	0 (0, 0)	0 (0, 1)
Active sediment[a]	11 (33%)	0	0	0	7 (36%)	0	0

AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; CRP, C-reactive protein; IQR, interquartile range; ery dipstick, erythrocytes on urinary dipstick analysis; GPA, granulomatosis with polyangiitis; HC, healthy control; MPA, microscopic polyangiitis; MPO, myeloperoxidase; nrAAV, nonrenal active AAV; PR3, proteinase 3; rAAV, active renal AAV; Rem, remission.
[a]Presence of acanthocytes on phase-contrast microscopy of urinary sediment.

centrifuged as above, resuspended in PBS, and transferred to 96-well plates. Venous blood was collected in lithium heparin tubes, 1 ml of which was lysed for 5 minutes in 10 ml ammonium chloride lysis buffer at 37 °C and centrifuged at 2000 rpm for 5 minutes at 4 °C. Subsequently, the pellet was resuspended in 1 ml PBS and 100 μl of the cell suspension were transferred to 96-well plates. The panel consisted of CD3-PE/Cy5, CD4-PE/Cy7, CD8-APC/Cy7, CD127-BV510, FoxP3-FITC, Tbet-APC, and RORγt-PE; and live/dead stained using Viobility™. In a subset of patients, CD25-APC/Cy7 was substituted for CD8-APC/Cy7.

Live/dead staining was performed using Viobility in 100 μl of PBS for 15 minutes on ice. After washing with 100 μl PBS-BSA 1% and centrifugation at 2000 rpm for 3 minutes at 4 °C, samples were blocked using Fc-block in PBS/BSA for 10 minutes on ice. Subsequently, they were stained for surface markers (CD3, CD4, CD8, and CD127) for 15 minutes on ice. Subsequently, they were washed, centrifuged twice (see above), fixed, and permeabilized (FoxP3 Transcription Factor Staining Buffer Kit, Invitrogen by Thermo Fisher Scientific, San Diego, CA, USA) and stained for intracellular markers (FoxP3, RORγt, and Tbet). CountBright Absolute Counting Beads (Invitrogen by Thermo Fisher Scientific, San Diego, CA, USA) were added, and samples were analyzed on BD FACSCanto II or BD LSR II (both BD Biosciences, Franklin Lakes, NJ, USA). FACS data were analyzed using FlowJo 10 (TreeStar, Ashland, OR, USA), and absolute cell counts were calculated based on bead count and sample size and normalized to 100 ml blood or urine.

Detection of Soluble Biomarkers

Frozen cell-free urine samples were thawed at room temperature, centrifuged, diluted 1:10 with PBS/BSA and analyzed with a Luminex Human Magnetic Assay (4-Plex) for MCP-1, sCD163, sCD25, and C5a (Luminex

Corporation, Austin, Texas, USA). Multiplex assays were analyzed with a Bioplex reader and Bioplex software (Bio-Rad Laboratories, Hercules, CA, USA). When a given molecule was not detectable in a sample, the concentration was determined as half of the lowest detectable value. Protein concentrations were normalized as pg/g urinary creatinine. Creatinine concentration was determined by R&D Creatinine Parameter Assay Kit (R&D Systems Inc., Minneapolis, MN, USA).

Histopathological Analysis

Patients with active renal vasculitis who had received a kidney biopsy at the initial presentation were included for histopathological evaluation. A total of 21 samples were available for analysis by an expert in kidney histopathology and classified according to the Berden classification.[5] Results were compared with urinary T cell counts.

Statistical Analysis

Statistical analysis and creation of graphs were performed in R (R Core Team, Vienna, Austria).[30] Group medians were compared using Kruskal-Wallis and *post hoc* Dunn's test to correct for multiple comparisons from the PMCMRplus package.[31] Bonferroni corrected *P*-values < 0.05 were considered significant. Data were displayed as medians and interquartile range, adding 1 to visualize zeros on logarithmic scales, using the ggplot2 package.[32] To compare results of a single cell population between blood and urine samples, paired Wilcoxon signed-rank test from the ggpubr package was used. Correlation analysis between urine cells and routine clinical parameters was performed using the ggstatsplot package. Pearson's index with *P*-value correction for multiple comparisons using Holm's method was used to identify statistically significant correlations. Biomarker abilities were assessed and compared using receiver operating characteristic curves (ROC) from the pROC and plotROC packages.[33,34] Defining rAAV as cases and nrAAV and remission as controls, Youden's index (i.e., the point on the curve with the maximum sum of sensitivity and specificity) was used to determine ideal cut off values for the diagnosis of active renal vasculitis for each marker. Survival plots were created using the survival and survminer packages and statistical significance was evaluated using logrank test.[35]

RESULTS

Urinary T Cells are Elevated in Active Renal Vasculitis

We recruited a total of 95 patients in 2 separate cohorts. The inception cohort comprised 65 subjects, distributed as follows: 33 active rAAV, 4 active nrAAV, 20 patients

in stable remission with previous rAAV remission. In addition, 8 HCs were recruited. The validation cohort included 30 patients, distributed as follows: 19 rAAV, 3 nrAAV, and 8 remission. Samples were analyzed for the presence of $CD3^+$ T cells by flow cytometry and normalized to 100 ml of blood or urine. Exemplary dot plots illustrating distinct urinary T cell populations found in active rAAV are shown in Figure 1a (see blood sample including fluorescence minus one controls for comparison in Supplementary Figure S1).

We found a significant increase in absolute urinary $CD3^+$, $CD4^+$, and T_{reg} cell counts in rAAV in comparison to most other groups in the inception cohort (Figure 1b, c, and e). The increase of $CD8^+$ and T_H17 cells seen in rAAV only reached significance compared to nrAAV and HC, whereas for T_H1 the only significant difference was between rAAV and HC (Figure 1d, f, and g; Table 2). There were no significant differences when comparing centers, ANCA serotype (i.e., antimyeloperoxidase vs. Antiproteinase 3) or clinical syndrome (i.e., granulomatosis with polyangiits vs. microscopic polyangiitis) (Supplementary Figures S2–S4).

Urinary T Cell Populations Differ From Blood T Cell Populations

In parallel, T cell subpopulations were analyzed in the peripheral blood. Here, we found no significant difference in $CD3^+$, $CD4^+$, T_{reg}, T_H17, or T_H1 T cell subpopulations between the different groups. Only patients in remission in the validation cohort showed a slight increase in $CD8^+$ cells compared to rAAV (Supplementary Figure S5a–f).

To assess whether the finding of higher urinary T cells in rAAV patients was merely a surrogate of transglomerular bleeding, we compared T cell subset proportions in the peripheral blood and urine. Matched comparison revealed a significantly higher frequency of T_{reg} per $CD4^+$ cell in urine than in blood (medians: 13.4% vs. 2.9%, $P < 0.0001$) unique to the rAAV group (Figure 2a). A similar, albeit statistically insignificant increase was found for T_H17 cells (6.1% vs. 2.5%, $P = 0.2$) (Supplementary Figure 6b). Conversely, the $CD4^+/CD8^+$ ratio was significantly lower in urine than in blood in rAAV patients (0.96 vs. 1.91, $P = 0.0008$) (Figure 2b).

Urinary T Cell Count Correlated With Serum C-reactive Protein and Proteinuria

Analysis of urinary cells and routine clinical data in the rAAV group showed a strong correlation between C-reactive protein with urinary $CD4^+$ cell count (Pearson's $r = 0.72$, $P = 0.0015$), and T_{reg}/T_H17 ratio and ANCA titer ($r = 0.60$, $P = 0.032$). Correlations between $CD3^+$, $CD4^+$, and T_{reg} were disregarded because they reflect the gating strategy. All other correlations were statistically
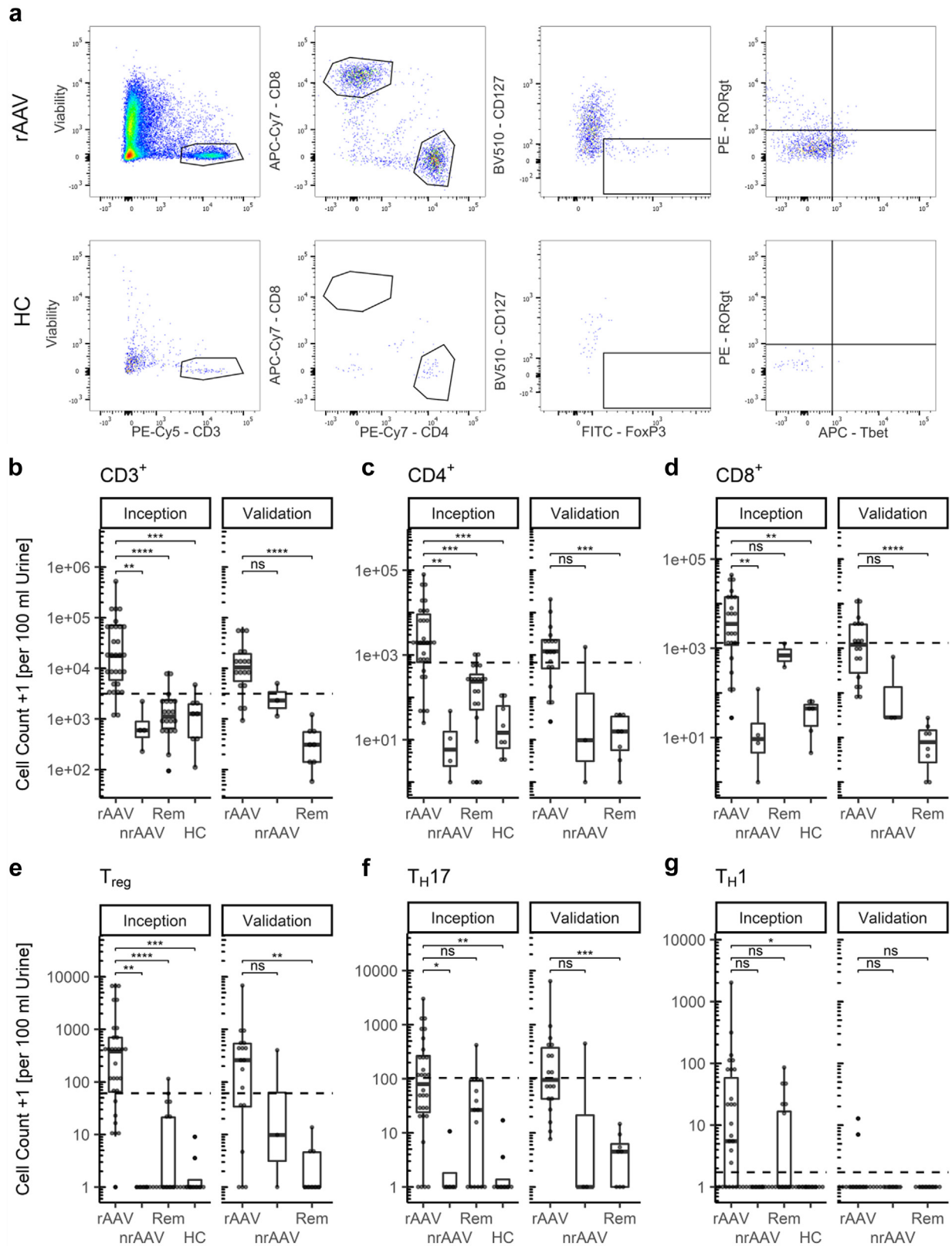
Figure 1. T cells show distinct, highly elevated subpopulations in urine in renal ANCA-associated vasculitis (AAV). (a) Exemplary urinary flow cytometry dot plots of renal active AAV (rAAV) patient and HC, previous gating: even flow, singlets; (b–g) box plots of urinary T cell subpopulations: CD3$^+$, CD4$^+$, CD8$^+$, T$_{reg}$, T$_H$17, and T$_H$1 cells. Cell counts plotted +1 to visualize zeros on logarithmic scale, statistics were determined using Kruskal-Wallis and *post hoc* Dunn's test, P-value summary: ns: $P > 0.05$, *: $P = 0.05 - 0.01$, **: $P = 0.01 - 0.001$, ***: $P < 0.001$. AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibodies; HC, healthy control, dashed line indicates optimal cut off for identification of rAAV; nrAAV, nonrenal active AAV; rAAV, active renal AAV; Rem, remission; T$_{reg}$, regulatory T cell; T$_H$17, T helper 17; T$_H$1, T helper 1.

Table 2. Summary of urinary T cell count

Urinary T cell count [1/100 ml urine] median (IQR)

Inception cohort

	rAAV	nrAAV	P-value	Rem	P-value	HC	P-value
CD3$^+$	17599 (5459, 72194)	597 (383, 1436)	0.0031	1116 (638–2376)	< 0.0001	1263 (406, 2064)	0.0005
CD4$^+$	1917 (667, 9626)	6 (1, 28)	0.0014	236 (45, 400)	0.0007	15 (5, 75)	0.0001
CD8$^+$	3766 (1131, 14658)	8 (3, 66)	0.0061	829 (381, 1277)	1.0000	44 (13, 55)	0.0031
T$_{reg}$	378 (60, 698)	0 (0, 0)	0.0026	0 (0, 21)	<0.0001	0 (0, 1)	0.0001
T$_H$17	82 (22, 281)	0 (0, 5)	0.0320	26 (0, 92)	0.2015	0 (0, 1)	0.0012
T$_H$1	5 (0, 75)	0 (0, 0)	0.1637	0 (0, 17)	0.2510	0 (0, 0)	0.0189
Validation cohort							
CD3$^+$	10519 (5137, 20206)	2324 (1733, 3675)	0.4463	311 (140, 554)	<0.0001	-	-
CD4$^+$	1233 (445, 2303)	9 (4, 777)	0.1369	15 (4, 35)	0.0007	-	-
CD8$^+$	1215 (306, 3440)	28 (27, 334)	0.3797	7 (20, 14)	<0.0001	-	-
T$_{reg}$	259 (33, 535)	9 (4, 205)	0.9543	0 (0, 4)	0.0065	-	-
T$_H$17	97 (41, 403)	0 (0, 224)	0.2861	4 (0, 6)	0.0009	-	-
T$_H$1	0 (0, 0)	0 (0, 0)	1.0000	0 (0, 0)	0.9327	-	-

AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibodies; HC, healthy control; IQR, interquartile range; nrAAV, nonrenal active AAV; rAAV, active renal AAV; Rem, remission.
Statistics: Kruskal-Wallis and post hoc Dunn's test with Bonferroni correction for multiple comparisons.

insignificant after correction for multiple testing using Holm's method (Supplementary Figure S7).

Urinary T Cells Represent a Reliable Biomarker for Active Renal Vasculitis

Using the inception cohort data, we generated ROC curves to examine the ability of urinary T cells to identify active renal vasculitis (Figure 2g). Patients with renal active AAV were compared to those with nonrenal active AAV and those in remission. In short, CD3$^+$ and T$_{reg}$ cells represent the most reliable biomarkers with an area under the curve (AUC) of 0.95 and 0.92, respectively. We defined ideal cut off values (i.e., the point on the curve with maximum sum of sensitivity and specificity) of 3149 CD3$^+$ cells per 100 ml of urine (sensitivity: 90%, specificity: 92%) and 60 T$_{reg}$ (74%, 96%) (Figure 2g, Table 3). These cut-offs were then validated in an independent validation cohort of 30 patients, yielding comparable biomarker characteristics (Table 3). The composition of combined markers i.e., T$_{reg}$/T$_H$17 ratio did not add any benefit (area under the curve: 0.73) (Supplementary Figure S8).

Next, the diagnostic performance of urinary T cells was compared to established markers, such as hematuria, proteinuria, phase-contrast microscopy, and recently reported urinary biomarkers for active rAAV, namely urinary sCD163, sCD25, MCP-1, and complement C5a. Traditional dipstick markers showed robust diagnostic performance (hematuria: ideal cut off: 3+, area under the curve 0.98, sensitivity: 88%, specificity 100%, proteinuria: 1+, 0.81, 96%, 52%), whereas presence of acanthocytes on phase-contrast microscopy performed weakly (0.89, 79%, 100%). sCD163 (0.78), MCP-1 (0.82), and C5a (0.73) showed modest diagnostic

accuracy, whereas sCD25 (0.59) performed poorly (Figure 2h, Table 3).

Urinary T Cells Correspond With Berden Classification

To determine if urinary T cells specifically reflect renal inflammation, available kidney biopsies were classified according to the Berden classification. From all 52 patients included as active rAAV, 21 kidney biopsies were available for evaluation. In the others, no biopsy had been performed, mainly because of previously established clinical diagnosis. Berden classes were as follows (crescentic: 4/21, focal: 6/21, mixed 7/21, and sclerotic 1/21) and 3 of 21 samples contained too few glomeruli to be classified. Urinary CD3$^+$ counts trended higher for the crescentic class (medians: 111,859; focal: 15,843; mixed: 13,765; sclerotic: 20,206; global $P = 0.15$) (Figure 2e). Similar but statistically insignificant differences were also found for urinary CD4$^+$, CD8$^+$, T$_{reg}$, and T$_H$17 counts (Supplementary Figure S9 and Supplementary Table S2). Because of the small sample numbers, we compared crescentic to all other classes pooled. Both urinary CD3$^+$ cell count (crescentic: 111,859 vs. other: 16,142, $P = 0.023$) and CD4$^+$ (crescentic: 10,747 vs. other: 667, $P = 0.032$) were significantly elevated in the crescentic class (Supplementary Figure S10B). Differences were not significant for CD8$^+$, T$_{reg}$ and T$_H$17 (Supplementary Tables S2 and S3).

Urinary T$_{reg}$ and T$_H$17 Cells Predict Clinical Response

Having demonstrated that urinary T cells reflect active renal disease and histopathological classification, we next asked whether the concentration of urinary T cells could predict disease outcome. All patients with active
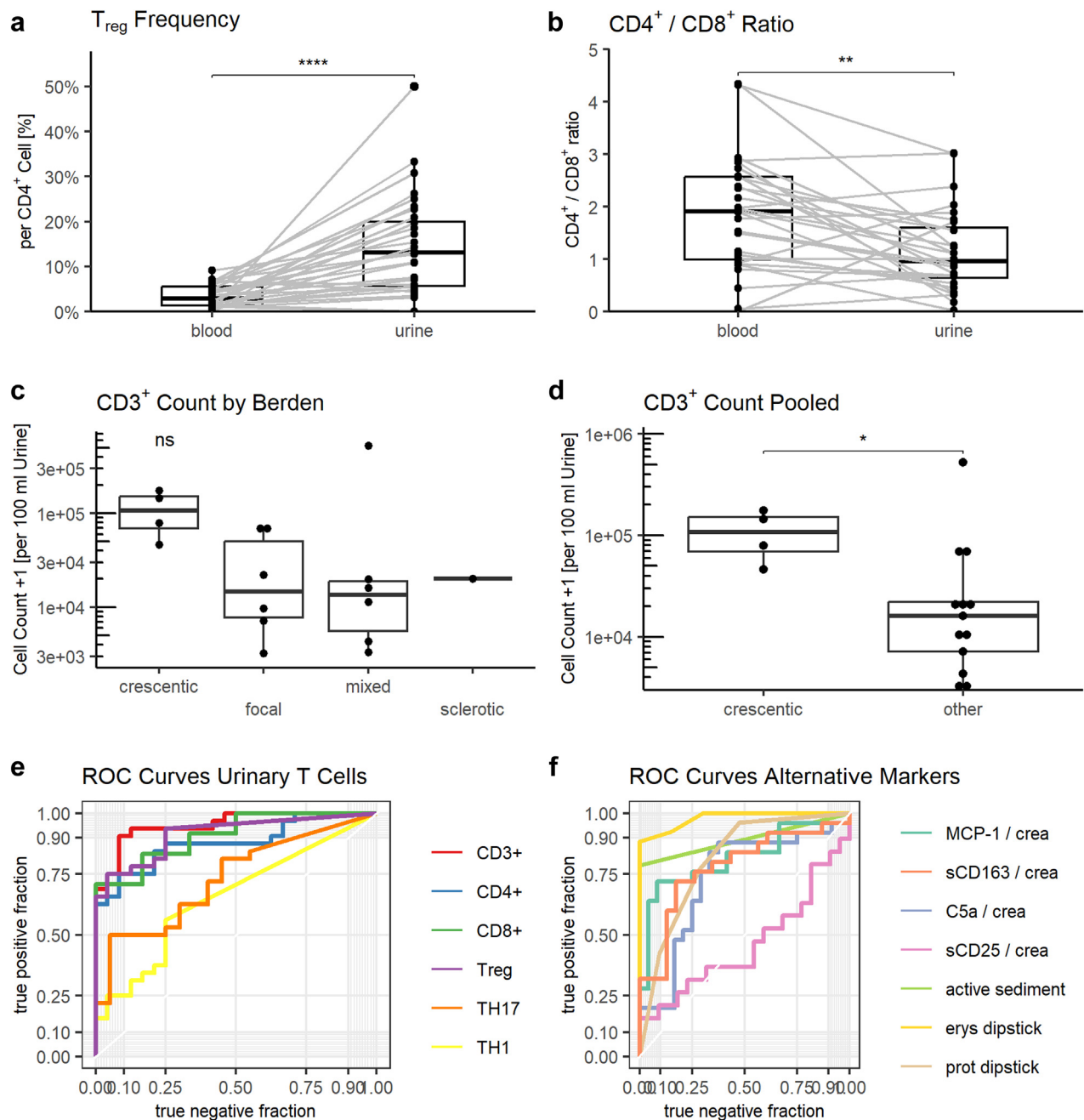
Figure 2. Urinary T cells show an altered phenotype between blood and urine, reflect the Berden classification, and show robust biomarker characteristics. Patients with active renal ANCA-associated vasculitis (AAV) show significantly higher urinary regulatory T cell (T_{reg}) frequency (a) and decreased $CD4^+/CD8^+$ ratio in urine (b). Frequency of chemokine receptor 6 (CCR6) positive $CD4^+$ and T_{reg} is significantly higher in urine than in blood (c and d) in patients with active renal AAV. (e) Urinary CD3+ cells trend higher in patients classified as crescentic according to Berden classification. (f) Urinary CD3+ cells are significantly elevated when comparing crescentic class to pooled other classes. (g–h) receiver operator characteristic (ROC) curves for the diagnosis of active renal AAV of urinary T cells, soluble markers and traditional markers. Statistics: (a–d): Paired Wilcoxon signed-rank test, (e): Kruskal-Wallis, F: Mann-Whitney-Wilcoxon test, P-value summary: ns: not significant, *: $0.05 - 0.01$, **: $P = 0.01 - 0.001$, ***: $P = 0.001 - 0.0001$, ****: $P < 0.0001$. CCR6, chemokine receptor 6; Erys dipstick, erythrocytes on urinary dipstick analysis; HC, healthy control; nrAAV, nonrenal active AAV; Prot dipstick, protein on urinary dipstick analysis; rAAV, active renal AAV; Rem, remission; ROC, receiver operator characteristics; T_{reg}, regulatory T cell.

rAAV were pooled and follow-up data were available for 30 participants. Routine clinical (BVAS, renal BVAS [i.e., sum of BVAS renal subcategory]) and laboratory parameters were determined 2, 4, and 6 months after

the initial assessment; renal remission was defined as a renal BVAS of 0.[29]

Only a single patient (1/30) had not reached complete remission after 6 months (i.e., nonresponder). Out

Table 3. Summary of receiver operator characteristics

Inception cohort

Urinary marker	AUC	cut off	sens	spec	ppv	npv	LR+	LR−
$CD3^+$	0.95	3149	0.94	0.92	0.94	0.92	11.23	0.07
$CD4^+$	0.88	664	0.74	0.92	0.92	0.73	8.90	0.28
$CD8^+$	0.91	1321	0.71	1.00	1.00	0.46	Inf	0.29
T_{reg}	0.92	60	0.74	0.96	0.96	0.74	17.81	0.27
T_H17	0.73	103	0.48	0.95	0.94	0.54	9.68	0.54
T_H1	0.66	1	0.58	0.75	0.75	0.58	2.32	0.56
Erys dipstick	0.98	3+	0.88	1.00	1.00	0.87	Inf	0.12
Prot dipstick	0.81	1+	0.96	0.52	0.71	0.92	2.02	0.07
Active sediment[a]	0.89	y	0.79	1.00	1.00	0.62	Inf	0.21
sCD163/crea	0.78	40.88	0.72	0.83	0.82	0.73	4.14	0.34
MCP1/crea	0.82	0.44	0.72	0.92	0.90	0.76	8.64	0.31
sCD25/crea	0.54	0.52	0.42	0.77	0.62	0.61	1.85	0.75
C5a/crea	0.73	2.93	0.84	0.67	0.72	0.80	2.52	0.24

Validation cohort

			sens	spec	ppv	npv	LR+	LR-
$CD3^+$			0.82	0.89	0.93	0.73	7.41	0.20
$CD4^+$			0.65	0.89	0.92	0.57	5.82	0.40
$CD8^+$			0.47	1.00	1.00	0.50	Inf	0.53
T_{reg}			0.65	0.89	0.92	0.57	5.82	0.40
T_H17			0.47	0.89	0.89	0.47	4.24	0.60
T_H1			0.12	1.00	1.00	0.38	Inf	0.88

AUC, area under the curve; C5a, complement C5a (pg/g creatinine); Erys dipstick, erythrocytes on urinary dipstick analysis; LR−, negative likelihood ratio; LR+, positive likelihood ratio; MCP-1, monocyte chemoattractant protein-1 (pg/g creatinine); npv, negative predictive value; ppv, positive predictive value; Prot dipstick, proteinuria on urinary dipstick analysis; sCD163, soluble CD163 (pg/g creatinine); sCD25, soluble CD25 (pg/g creatinine); Sens, sensitivity; Spec, specificity; TH1, T helper 1; TH17, T helper 17; Treg, T regulatory cell;
[a]Presence of acanthocytes in urinary sediment.
Ideal cut off was determined in the inception cohort using Youden's index (i.e. point on the ROC curve with maximum sum of sensitivity and specificity).

of the responders 18 of 29 had reached a renal BVAS of 0 after 2 months and were considered early responders as opposed to 11 of 29 late responders. Early responders showed higher urinary T cell counts for $CD3^+$, $CD4^+$, T_{reg}, and T_H17 cells, which did not reach statistical significance (Supplementary Table S4). Interestingly, hematuria (>1+) was still present in 15 of 29 responders at 2 months follow-up and persisted even after 6 months in 11 of 29 patients.

Next, we created Kaplan-Meier curves for urinary T cells based on automatically generated cut off values set between these 2 medians and selected those curves with the best stratification based on logrank test. T_H1 cells were disregarded because of the low urinary cell count. $CD3^+$, $CD4^+$, and $CD8^+$ showed no significant difference in cumulative incidence of full response. Conversely, we identified a cut off of 200 T_{reg} cells and 250 T_H17 cells per 100 ml of urine, which significantly predicted early response behavior (P-value: 0.026 and 0.0068, respectively) (Figure 3a and b).

Urinary T Cells Predict AAV Renal Flares

Finally, we tested whether elevated urinary T cells in patients with AAV who are in clinical remission reflects ongoing occult renal inflammation and could therefore predict renal flares in these patients. To this end, we followed patients in remission from the inception cohort, focusing on the first 6 months after initial measurement because of infeasible prediction beyond this time point. Not accounting for therapy adjustment, 3 of 28 patients developed a renal relapse in this period (median time to relapse was 3 months).

Interestingly, all 3 patients with early relapses showed markedly elevated urinary T_H17 counts and a complete absence of urinary T_{reg} cells. Other T cell subpopulations did not show such a marked difference (Figure 3c). Comparison of soluble markers revealed an insignificant increase in urinary MCP-1 and sCD163 concentration in patients with subsequent renal flare (Figure 3c). Of note, all 3 patients who flared within 6 months showed positive erythrocytes on dipstick analysis and 2 patients without flare were also positive (positive predictive value = 60%, negative predictive value = 100%). Only 1 patient showed ANCA above the reference range and did not develop a relapse within the follow-up period, whereas all 3 patients with early relapse were ANCA negative at the time of sampling.

Next, we performed ROC analysis to identify cut off values with maximum positive predictive value i.e., risk of renal relapse without missing any relapses within the first 6 months. T_H17 cells appeared to be the best predictor, with a risk of renal relapse of 30% at a threshold of 10 cells per 100 ml urine. Because patients with equally elevated urinary T_H17 cells as relapsing patients but simultaneously detectable urinary T_{reg} cells showed 0% risk of relapse, we combined these 2 markers. These patients showed a 50% risk of relapse. On this basis, we created a combined predictor of T_H17 greater 10 and T_{reg} count of 0 cells per 100 ml urine (Figure 3d). This combination identified all renal flares within 6 months with a positive predictive value of 50% and negative predictive value of 100% (P-value = 0.0097) (Figure 3d). However, this finding is strongly limited because of the small number of events available for this analysis.

DISCUSSION

We studied patients with AAV from 4 centers using flow cytometry to show that T cells are highly elevated in urine in active renal vasculitis and reflect the Berden classification. Specific migration into the kidney and subsequent release into the urine space, rather than mere transglomerular bleeding, cause this increase. There was no difference in blood T cell subpopulations, and only few correlations with routine clinical parameters were present. Particularly $CD3^+$, $CD4^+$, and
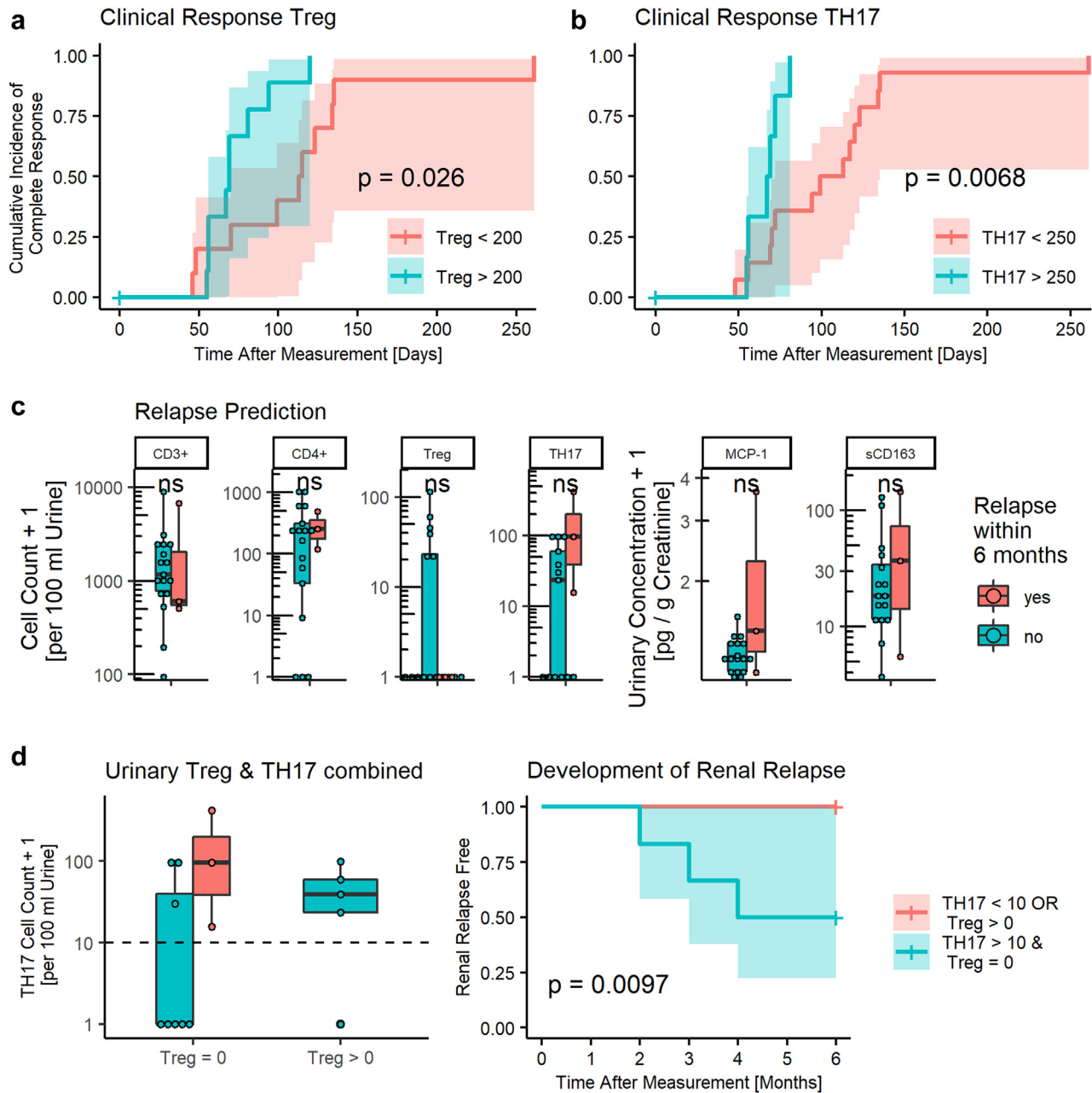
Figure 3. Urinary T cells predict clinical response and early relapses. (a, b) Active renal AAV patients with higher urinary T_{reg} and T_H17 T cells show faster full response (i.e., renal BVAS of 0). (c) Patients in remission who develop a renal flare within 6 months of measurement show no difference in urinary T cell counts or monocyte chemoattractant protein-1, soluble CD163. (d) A combined predictor of elevated T_H17 and absent T_{reg} predicts renal relapse. Dashed line indicates suggest cut off value of 10 TH17 cells per 100 ml urine. Curve of Relapse free survival based on combined predictor $T_H17 > 10/100$ ml urine and $T_{reg} = 0/100$ ml urine. Cell counts and concentrations plotted +1 to visualize zeros on logarithmic scale. Statistics: logrank test, 95% confidence interval indicated by colored area, Mann-Whitney-Wilcoxon test, ns: $P > 0.05$. T_{reg}, regulatory T cell; T_H17, T helper 17; T_H1, T helper 1.

T_{reg} T cells showed robust biomarker characteristics outperforming previously described soluble markers such as sCD163 or MCP-1 and traditional markers, such as hematuria and proteinuria. Finally, we found that T_{reg} and T_H17 cells even hold prognostic value in predicting clinical outcome of patients with active disease and potentially even renal relapse in patients in remission up to 6 months before relapse.

Our findings extend emerging evidence that urinary T cells reflect the renal inflammatory milieu in various inflammatory kidney diseases.[18,36,37] To our knowledge, this is the first report on urinary T cell subpopulations, particularly T_{reg} and T_H17, and their biomarker performance in rAAV. Urinary cell counts reflect the clinical and histopathological disease state. Furthermore, we observed an increased frequency of

urinary T_{reg} cells, discordant $CD4^+/CD8^+$ ratios in urine and blood. Therefore, we hypothesize, that urinary T cells are not merely the product of transglomerular bleeding because of ruptured capillaries but reflect an active selective process. Therefore, it seems viable to use the urine space as a window into the inflammatory milieu of the kidney. However, we cannot distinguish whether these immigrating cells are the cause of renal immunopathogenesis or merely attracted by the ongoing inflammation.

Although $CD3^+$, $CD4^+$, and T_{reg} urinary T cells showed a robust discrimination between active and remission state, there was no significant difference between these groups for $CD8^+$ and T_H17 T cells. At this point, we speculate that the apparent increase of $CD8^+$ and T_H17 urinary T cells in remission compared to HC reflects occult renal inflammation and renal vasculitis activity in patients with AAV, who are considered as clinically inactive. Therefore, the biomarker characteristics may underestimate the true predictive capacity of our T cell biomarkers.

This concept is further supported by our observation that remission patients with increased urinary T_H17 cell counts showed a higher risk of future renal flare. This risk was decreased if patients showed an elevated urinary T_{reg} cell count. These findings are strongly limited by the small number of events and further studies are needed to confirm this. However, this might give further insight into the dual nature of T cell subpopulations, which has been postulated before. Although T_{reg} cells seem protective over the entire course of AAV, T_H17 cells seem to exhibit proinflammatory effects in the early phase of inflammation and protective effects during the subsequent course.[38] Notably, although we only observed 3 renal flares within the first 6 months of follow-up, thus limiting these results, we cannot rule out that clinicians altered remission therapy regimens thereby preventing additional relapses. Despite this caveat, these observations not only reflect experimental results in humans *in vivo* but also underline that we were not measuring an epiphenomenon of inflammation. Finally, increased urinary T_{reg} and T_H17 cell counts were associated with earlier clinical response in active rAAV patients in our study. These hypotheses are supported by various animal models demonstrating the important anti-inflammatory role of T_{reg} cells and damage promoting effects of T_H17 cells in crescentic glomerulonephritis and AAV.[24–26]

Interestingly, we did not find a difference in blood T_{reg} frequency between active and remission AAV. There are contradicting reports on this subject, which are likely caused by varying definitions of the T_{reg} subset.[39]

The traditional biomarker, hematuria on dipstick analysis, showed robust biomarker characteristics, particularly a high area under the ROC curve. In addition, we found that erythrocytes were also able to predict renal relapse with high positive predictive value, as previously described.[9] However, we argue this strong performance might be skewed because of the inclusion of hematuria in the definition of active disease and renal relapse. Furthermore, we cannot rule out that some remission patients with persistent hematuria who were considered as "grumbling disease," were never considered for this study. Although the significance and origin of persistent hematuria in AAV are still highly disputed, it is difficult to differentiate hematuria caused by scarring from active vasculitis.[9,40] Along these lines, acanthocytes have been traditionally used to detect active renal vasculitis in AAV. However, the detection and quantification of acanthocytes is characterized by high interobserver variability and requires rapid processing of samples. This leads to suboptimal test performance in the real world.[41] Conversely, we showed that urinary T cells accurately reflect clinical course with cell counts following clinical response closely. Furthermore, distinct subpopulation patterns in urine enable the differentiation between mere bleeding caused by scarring or active immunologic activity.

Because kidney biopsy remains the gold standard for diagnostic and prognostic evaluation in patients with AAV, we compared urinary T cells to the established and validated Berden classification.[5] We found that patients classified as crescentic showed significantly higher urinary $CD3^+$ and $CD4^+$ cells. This finding is strongly limited by the small number of available biopsies (21 compared to 51 active renal patients). This number was mainly limited by the large number of patients with relapse, in which a re-biopsy is not clinical standard. Although this may not be sufficient to validate urinary T cells as liquid biopsy, we argue it is sufficient additional evidence to support a robust clinical biomarker. Although urinary T cells are the first leukocyte biomarker in AAV, there are several described soluble markers relying on enzyme-linked immunoassays for monocyte proteins such as sCD163 and MCP-1.[13,14] In direct comparison, urinary $CD3^+$ and T_{reg} outperformed these in diagnosing active rAAV. In addition, we identified urinary T_{reg} and T_H17 patterns as predictors of treatment response and renal relapse in our cohort, which has not been shown for soluble markers. This might help identify subsets of disease and also enable tailored treatment such as IL-17 blockade in patients where early T_H17 cells still drive inflammation.

This study is limited by the small number of patients included. Particularly regarding the small number of relapses and the number of available kidney biopsies.

Therefore, it is important to stress that this study does not allow definitive conclusions on the prognostic value and the use as liquid biopsy. However, we see this study as proof of concept that urinary T cells can be used as biomarkers in the assessment of AAV disease activity. Further, larger studies are needed to confirm some of the promising and interesting signals that we found, particularly regarding the prediction of renal relapse.

Besides these caveats, there are technical limitations to the use of urinary T cells in real world practice. It is important to stress that this biomarker is not designed to diagnose AAV specifically rather than assess disease activity of established AAV. We envision a scenario where urinary T cells are quantified as part of the routine follow up in patients with confirmed diagnosis. Until further studies validate the AAV specific nature of this biomarker, confounders such as urinary tract infection, bladder pathology, and bladder catheterization immediately before sample acquisition need to be excluded. Although this study required immediate processing of fresh urine samples, we have developed a protocol which could make this a more realistic tool for routine monitoring of outpatients and use in clinical studies. Using a cup with an extended formaldehyde releaser to which fresh urine is added allows analysis at later time points.[42] One frequent critique against using urinary T cells as biomarker is, that flow cytometry is too complicated, arguing for enzyme-linked immunoassays or similar tests. However, flow cytometry is widely available in most hospitals in high income countries, is already routinely used by other clinical disciplines like hematology, and it offers the advantage of unprecedented, detailed information on the "state" of inflammatory renal diseases.

In summary, urinary T cells directly reflect the renal inflammatory milieu and show reliable biomarker characteristics, outperforming soluble markers. Furthermore, they may even reveal prognostic information, which warrants further study. If confirmed in further validation studies, urinary T cells would represent the first noninvasive biomarker to: (i) assess disease activity, (ii) enable prognosis of clinical response, (iii) predict future flares, and (iv) identify patients who might benefit from tailored treatment strategies such as IL-17 blockade. These characteristics could make urinary T cells a perfect tool for a personalized treatment of ANCA-associated glomerulonephritis.

DISCLOSURE

The authors JS, JK, MB, AR, KE, SE, SP, SB, RK, ADS, and AS declare no conflicts of interest. PE declares that he has submitted a patent using flow cytometry for diagnosing renal transplant rejection and other inflammatory renal disease (EP17190719.9) and a patent for a conservation method for urinary cells (PCT/EP2019/086433).

AUTHOR CONTRIBUTIONS

All authors meet the journal's criteria for authorship.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Blood T cells and fluorescence minus one.
Figure S2. Urinary T cell count by center.
Figure S3. Urinary T cell count by ANCA serotype.
Figure S4. Urinary T cell count by clinical diagnosis.
Figure S5. Blood T cell counts.
Figure S6. T cell subpopulations in blood and urine.
Figure S7. Correlation matrix.
Figure S8. Treg/TH17 ratio.
Figure S9. Urinary T cell counts by Berden classification.
Figure S10. Urinary T cell counts by pooled Berden classification.
Table S1. Summary of collected samples.
Table S2. Summary of Urinary T cell counts according to Berden classification.
Table S3. Summary of Urinary T cell counts crescentic vs. Other Berden classes.
Table S4. Summary of Urinary T cell counts by response behavior.

REFERENCES

1. Hutton HL, Holdsworth SR, Kitching AR. ANCA-associated vasculitis: pathogenesis, models, and preclinical testing. *Semin Nephrol*. 2017;37:418–435. https://doi.org/10.1016/j.semnephrol.2017.05.016

2. Hilhorst M, van Paassen P, Tervaert JW, Limburg Renal Registry. Proteinase 3-ANCA Vasculitis versus myeloperoxidase-ANCA Vasculitis. *J Am Soc Nephrol*. 2015;26:2314–2327. https://doi.org/10.1681/ASN.2014090903

3. Mukhtyar C, Flossmann O, Hellmich B, et al. Outcomes from studies of antineutrophil cytoplasm antibody associated vasculitis: a systematic review by the European League Against Rheumatism systemic vasculitis task force. *Ann*

Rheum Dis. 2008;67:1004–1010. https://doi.org/10.1136/ard.2007.071936

4. Little MA, Nightingale P, Verburgh CA, et al. Early mortality in systemic vasculitis: relative contribution of adverse events and active vasculitis. *Ann Rheum Dis*. 2010;69:1036–1043. https://doi.org/10.1136/ard.2009.109389

5. Berden AE, Ferrario F, Hagen EC, et al. Histopathologic classification of ANCA-associated glomerulonephritis. *J Am Soc Nephrol*. 2010;21:1628–1636. https://doi.org/10.1681/ASN.2010050477

6. Dhaun N, Bellamy CO, Cattran DC, Kluth DC. Utility of renal biopsy in the clinical management of renal disease. *Kidney Int*. 2014;85:1039–1048. https://doi.org/10.1038/ki.2013.512

7. Fujita T, Ohi H, Endo M, et al. Level of red blood cells in the urinary sediment reflects the degree of renal activity in Wegener's granulomatosis. *Clin Nephrol*. 1998;50:284–288.

8. Magrey MN, Villa-Forte A, Koening CL, et al. Persistent hematuria after induction of remission in Wegener granulomatosis: a therapeutic dilemma. *Med (Baltim)*. 2009;88:315–321. https://doi.org/10.1097/MD.0b013e3181c101cc

9. Rhee RL, Davis JC, Ding L, et al. The utility of urinalysis in determining the risk of renal relapse in ANCA-associated vasculitis. *Clin J Am Soc Nephrol*. 2018;13:251–257. https://doi.org/10.2215/CJN.04160417

10. Chen TK, Murakami C, Manno RL, Geetha D. Hematuria duration does not predict kidney function at 1 year in ANCA-associated glomerulonephritis. *Semin Arthritis Rheum*. 2014;44:198–201. https://doi.org/10.1016/j.semarthrit.2014.03.008

11. Hasegawa J, Hoshino J, Sekine A, et al. Clinical and pathological features of ANCA-associated vasculitis in patients with minor urinary abnormalities. *Nephrol (Carlton)*. 2018;23:1007–1012.

12. Geetha D, Seo P, Ellis C, et al. Persistent or new onset microscopic hematuria in patients with small vessel vasculitis in remission: findings on renal biopsy. *J Rheumatol*. 2012;39:1413–1417. https://doi.org/10.3899/jrheum.111608

13. Tam FW, Sanders JS, George A, et al. Urinary monocyte chemoattractant protein-1 (MCP-1) is a marker of active renal vasculitis. *Nephrol Dial Transplant*. 2004;19:2761–2768. https://doi.org/10.1093/ndt/gfh487

14. O'Reilly VP, Wong L, Kennedy C, et al. Urinary soluble CD163 in active renal vasculitis. *J Am Soc Nephrol*. 2016;27:2906–2916. https://doi.org/10.1681/ASN.2015050511

15. Dekkema GJ, Abdulahad WH, Bijma T, et al. Urinary and serum soluble CD25 complements urinary soluble CD163 to detect active renal anti-neutrophil cytoplasmic autoantibody-associated vasculitis: a cohort study. *Nephrol Dial Transplant*. 2018;34:234–242. https://doi.org/10.1093/ndt/gfy018

16. Moran SM, Monach PA, Zgaga L, et al. Urinary soluble CD163 and monocyte chemoattractant protein-1 in the identification of subtle renal flare in anti-neutrophil cytoplasmic antibody-associated vasculitis. *Nephrol Dial Transplant*. 2020;35:283–291. https://doi.org/10.1093/ndt/gfy300

17. Dolff S, Abdulahad WH, van Dijk MC, et al. Urinary T cells in active lupus nephritis show an effector memory phenotype. *Ann Rheum Dis*. 2010;69:2034–2041. https://doi.org/10.1136/ard.2009.124636

18. Enghard P, Humrich JY, Rudolph B, et al. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. *Arthritis Rheum*. 2009;60:199–206. https://doi.org/10.1002/art.24136

19. Enghard P, Rieder C, Kopetschke K, et al. Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response. *Ann Rheum Dis*. 2014;73:277–283. https://doi.org/10.1136/annrheumdis-2012-202784

20. Dolff S, Abdulahad WH, Arends S, et al. Urinary CD8+ T-cell counts discriminate between active and inactive lupus nephritis. *Arthritis Res Ther*. 2013;15:R36. https://doi.org/10.1186/ar4189

21. Sakatsume M, Xie Y, Ueno M, et al. Human glomerulonephritis accompanied by active cellular infiltrates shows effector T cells in urine. *J Am Soc Nephrol*. 2001;12:2636–2644. https://doi.org/10.1681/ASN.V12122636

22. Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA. Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum*. 2009;60:2830–2838. https://doi.org/10.1002/art.24747

23. de Souza AW, Abdulahad WH, Sosicka P, et al. Are urinary levels of high mobility group box 1 markers of active nephritis in anti-neutrophil cytoplasmic antibody-associated vasculitis? *Clin Exp Immunol*. 2014;178:270–278. https://doi.org/10.1111/cei.12422

24. Gan PY, Steinmetz OM, Tan DS, et al. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol*. 2010;21:925–931. https://doi.org/10.1681/ASN.2009070763

25. Ghali JR, Wang YM, Holdsworth SR, Kitching AR. Regulatory T cells in immune-mediated renal disease. *Nephrol (Carlton)*. 2016;21:86–96. https://doi.org/10.1111/nep.12574

26. Schreiber A, Rousselle A, Klocke J, et al. Neutrophil gelatinase-associated lipocalin protects from ANCA-induced GN by inhibiting T(H)17 immunity. *J Am Soc Nephrol*. 2020;31:1569–1584.

27. Krebs CF, Paust HJ, Krohn S, et al. Autoimmune renal disease is exacerbated by S1P-Receptor-1-Dependent intestinal Th17 cell migration to the kidney. *Immunity*. 2016;45:1078–1092. https://doi.org/10.1016/j.immuni.2016.10.020

28. Jennette JC, Falk RJ, Bacon PA, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum*. 2013;65:1–11. https://doi.org/10.1002/art.37715

29. Mukhtyar C, Lee R, Brown D, et al. Modification and validation of the Birmingham Vasculitis Activity Score (version 3). *Ann Rheum Dis*. 2009;68:1827–1832. https://doi.org/10.1136/ard.2008.101279

30. R: a language and environment for statistical computing. R Core Team (2020). The R Foundation for Statistical Computing. http://www.r-project.org/index.html

31. Pohlert T. Calculate pairwise multiple comparisons of mean rank sums extended. PMCMRplus. Published 2020. Accessed October 22, 2022. https://cran.r-project.org/web/packages/PMCMRplus/PMCMRplus.pdf

32. Wickham H. *ggplot2: Elegant Graphics for Data Analysis.* Springer; 2016.

33. Sachs MC. plotROC: A tool for plotting ROC curves. *J Stat Softw.* 2017;79:2. https://doi.org/10.18637/jss.v079.c02

34. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics.* 2011;12:77.

35. Therneau TM. Survival analysis in R Version 2.38. Published 2015. https://cran.microsoft.com/snapshot/2015-06-27/web/packages/survival/survival.pdf

36. Arazi A, Rao DA, Berthier CC, et al. The immune cell landscape in kidneys of patients with lupus nephritis. *Nat Immunol.* 2019;20:902–914. https://doi.org/10.1038/s41590-019-0398-x

37. van Doesum WB, Abdulahad WH, van Dijk MC, et al. Characterization of urinary CD4+ and CD8+ T cells in kidney transplantation patients with polyomavirus BK infection and allograft rejection. *Transpl Infect Dis.* 2014;16:733–743. https://doi.org/10.1111/tid.12273

38. Odobasic D, Gan PY, Summers SA, et al. Interleukin-17A promotes early but attenuates established disease in crescentic glomerulonephritis in mice. *Am J Pathol.* 2011;179:1188–1198. https://doi.org/10.1016/j.ajpath.2011.05.039

39. Miyara M, Gorochov G, Ehrenstein M, et al. Human FoxP3+ regulatory T cells in systemic autoimmune diseases. *Autoimmun Rev.* 2011;10:744–755. https://doi.org/10.1016/j.autrev.2011.05.004

40. Mahoney SL, Nachman PH. Persistent hematuria in ANCA vasculitis: ominous or innocuous? *Clin J Am Soc Nephrol.* 2018;13:201–202. https://doi.org/10.2215/CJN.14101217

41. Crop MJ, de Rijke YB, Verhagen PC, et al. Diagnostic value of urinary dysmorphic erythrocytes in clinical practice. *Nephron Clin Pract.* 2010;115:c203–c212. https://doi.org/10.1159/000313037

42. Freund P, Skopnik CM, Metzke Ds, et al. Addition of formaldehyde releaser imidazolidinyl urea and MOPS buffer to urine samples enables delayed processing for flow cytometric analysis of urinary cells. *medRxiv. Forthcoming.* 2022. https://doi.org/10.1101/2022.04.07.22273579