In situ Visualization of C3/C5 Convertases to Differentiate Complement Activation

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Deregulated complement activation contributes to or drives the pathogenesis of various kidney diseases. Targeting complement is a therapeutic option.[1,2] Inhibition on the level of C5 is an established therapy in atypical hemolytic uremic syndrome (aHUS), paroxysmal nocturnal hemoglobinuria, and myasthenia gravis, and novel drugs targeting other components of the complement cascade are being developed.[3,4] Currently, the diagnosis of complement activation in kidney diseases is based primarily on immunohistochemical detection of deposited complement activation products in tissue and the detection of consumption of complement components in plasma.[5]

To date, a method to directly identify, localize, quantify, and differentiate complement convertases in tissue has been lacking. We therefore established an *in situ* method to detect and localize assembled C3/C5 convertases of the classical/lectin and alternative pathways (Figure 1; Supplementary Methods; Supplementary Figures S1–S4). We utilized a bright field proximity ligation assay[6,7] because (i) the microanatomic context is preserved, allowing, for example, selection of preserved glomeruli and the exact localization of the signals, and (ii) the signals are stable and easily quantifiable. Close proximity of C2 and C4b was used to identify assembled classical/lectin C3/C5 convertases, and of C3b and Bb, the fragment of factor B, to identify the alternative C3/C5 convertase.

RESULTS

As expected, cases with immune-complex glomerulonephritis in patients with systemic lupus erythematosus revealed higher densities of classical/lectin convertases within the glomerular mesangium and around capillary walls (median, 7685 signals/mm^2), as compared to biopsies from patients with aHUS (median, 393 signals/mm^2) and to normal control biopsies (median, 207 signals/mm^2). The difference between aHUS versus normal controls was not statistically significant. In contrast, aHUS cases showed a predominance of alternative convertases (median, 3032 signals/mm^2), as compared to systemic lupus erythematosus and normal control biopsies (median, 1329 signals/mm^2 and 1418 signals/mm^2, respectively; Figure 2), with most signals being located within the glomerular capillary lumen.

DISCUSSION

Formation of the classical/lectin pathway and alternative pathway C3/C5 convertases is an important step in the activation of the complement cascade.[1] The presented technique allows the detection of direct proximity of proteins of the classical/lectin pathway (C4b and C2b) and the alternative pathway (C3b and Bb fragment of factor B) forming the C3/C5 convertases, indicating convertase formation in the tissue. As expected, in aHUS, there is a predominance of the alternative convertase, and in systemic lupus erythematosus, the classical/lectin convertases dominate. Unexpectedly, in systemic lupus

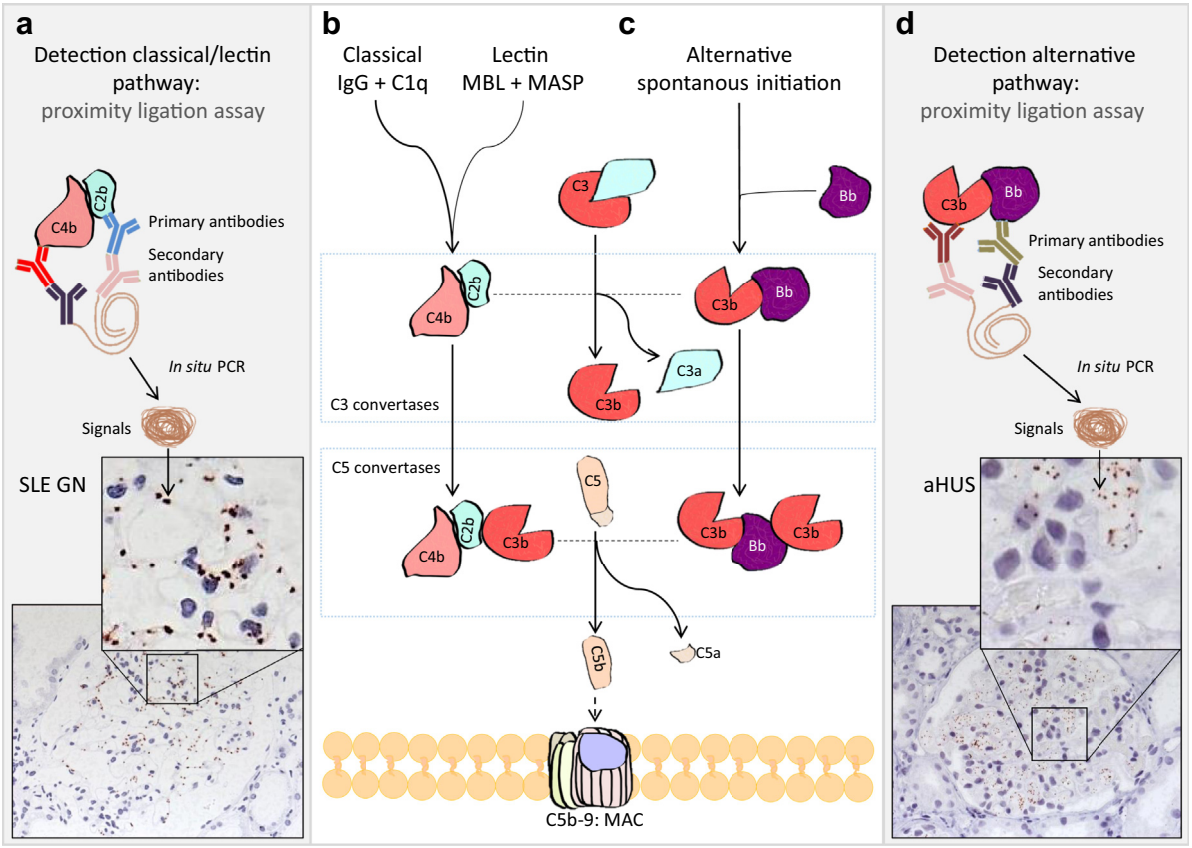

Figure 1. The complement cascade can be initiated by 3 pathways: (b,c) the classical, lectin, and alternative pathways. Classical and lectin pathway activation results in assembly of C4b and C2b, whereas the alternative pathway activation leads to assembly of C3b and fragment of factor B, Bb. Both C4bC2b and C3bBb are C3 convertases, which cleave surrounding C3 from the fluid phase, or after binding of another C3b molecule, act as C5 convertases. (a,d) The assembled complexes were detected by visualization of close proximity of their components by proximity ligation assay. Primary antibody binding was followed by the application of secondary antibodies, which have attached oligonucleotides. The latter, when in close proximity, form rings enabling an *in situ* polymerase chain amplification and detection with labeled probes, resulting in a brown dot–like signal for each (a, bottom) C4b/C2 and (d, bottom) C3b/Bb pair. aHUS, atypical hemolytic uremic syndrome; Bb, fragment of factor B; MAC, membrane attack complex; MASP, mannose-associated serine protease; MBL, mannose-binding lectin; PCR, polymerase chain reaction; SLE GN, systemic lupus erythematosus glomerulonephritis.

erythematosus biopsies, the alternative convertase (likely as amplification loop) level is not higher than in normal controls. The reason for this finding is not clear; unknown negative feedback mechanisms could play a role.

Detection, quantification, and discrimination of the specific complement convertases in tissue allows monitoring of complement-inhibiting therapies, providing an additional tool for future individualized medicine. Such discrimination can be important for the heterogeneous group of C3 glomerulopathies, which have various genetic and autoimmune causes. Mutant complement proteins and autoantibodies modify the activation of the complement system, systemically and locally.[8]

Patients with immune complex–mediated membranoproliferative glomerulonephritis, or C3 glomerulonephritis, show variable response to eculizumab therapy; persistently reduced proteinuria is achieved in just a subgroup.[9] Several other inhibitors of the complement system are either currently in development or undergoing clinical trials.[4]

Complement activation plays an important role in other glomerular diseases also, including membranous nephropathy,[S1,S2] IgA nephropathy,[S3] and ANCA-associated necrotizing glomerulonephritis.[S4] However, complement is involved in not only renal diseases but also other disorders, such as neoplastic diseases. Several studies indicate that the complement system is playing a dichotomous role in the formation of cancer, activating pro-survival pathways while attacking tumor cells.[S5]

Therefore, it is beneficial to detect the formed C3/C5 convertases *in situ* at the primary site of activation and damage in target tissue. The application of the bright field visualization protocol enables the depiction of the convertases in their histopathologic context, allowing direct correlation to the different types of tissue damage.

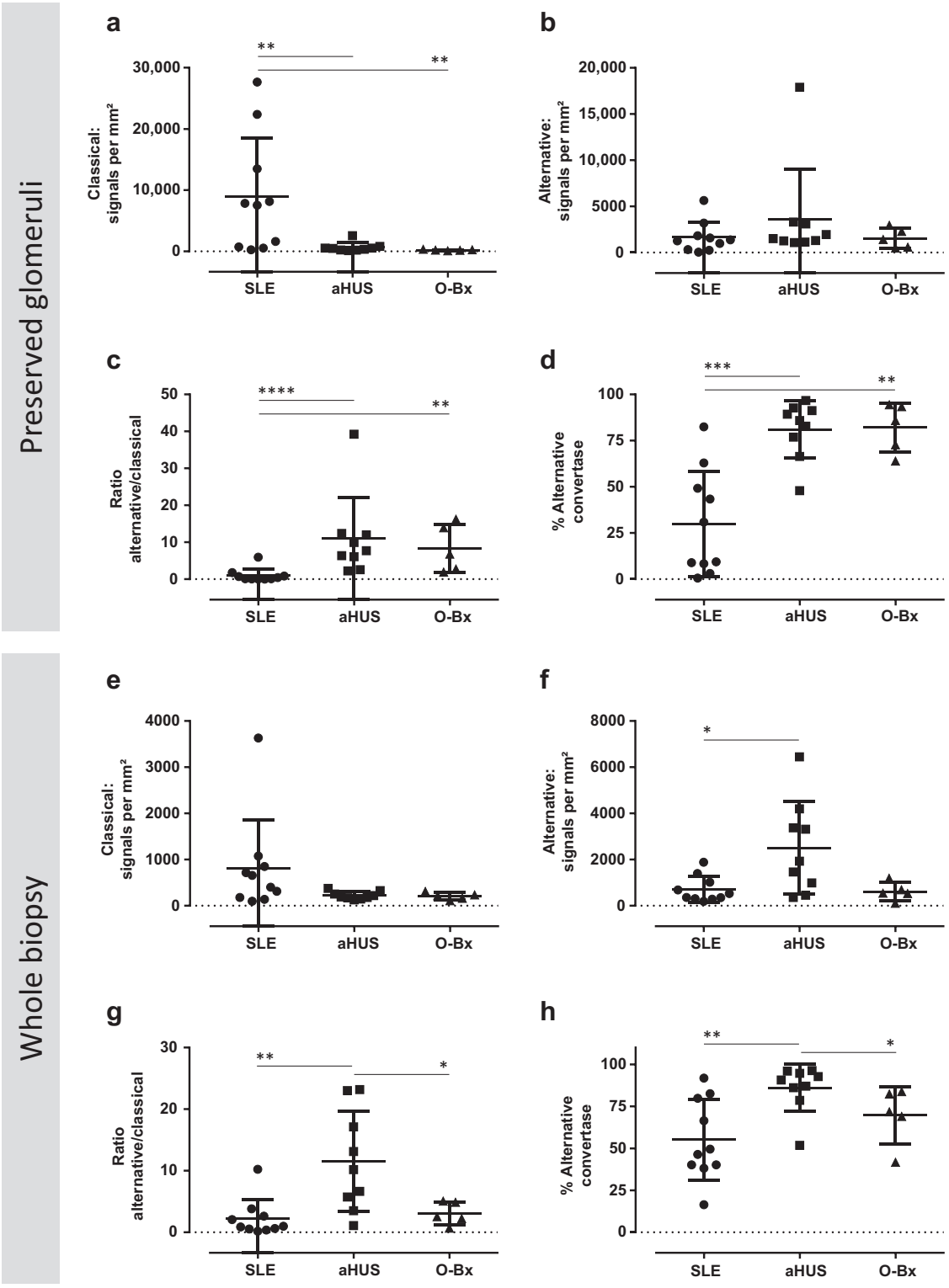

Figure 2. Results of the evaluated signals in the (a–d) preserved, presumably functional glomeruli only and in (e–h) whole biopsies. (a,e) The classical convertases show significantly higher densities in systemic lupus erythematosus (SLE) cases, as compared to atypical hemolytic uremic syndrome (aHUS) cases or zero-hour transplant biopsies (O-Bx) taken as normal controls. (b,c,f,g) In contrast, densities of the alternative convertase as well as the alternative/classical pathway ratios were higher in aHUS biopsies. (d,h) The percentage of alternative pathway signals out of total signals is lower in SLE cases compared to aHUS cases and normal controls. A 2-tailed Mann-Whitney U test was used to evaluate differences between the values (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

We introduce the first methodological workflow for the visualization, differentiation, and quantification of classical/lectin and alternative C3/C5 convertases directly within a tissue specimen. This new approach represents a promising tool to discriminate complement pathways in tissue and show the dynamics of activation,

enhancing diagnosis and potentially allowing future monitoring of efficacy during individualized therapy.[S6]

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

TW, PFZ, FP, and TP designed the research. SW, FP, TP, and FB performed histopathologic and immunohistochemical analysis. JO provided clinical data. FP, TW, CS, PFZ, SB, and WF wrote the manuscript. All the authors approved the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary References.

Figure S1. Immunostaining of the single components of the C3/C5 convertases. (A,B) Complement C2 shows a granular positivity in lupus nephritis (SLE GN) in the mesangium and along the thickened peripheral glomerular basement membranes (GBM). In aHUS biopsies, glomerular C2 positivity does not exceed the background staining. (C,D) Lupus nephritis revealed strong granular positivity for C4b. Also, in aHUS, there is a granular positivity along the thickened GBM. In this staining, real positivity cannot be distinguished from unspecific trapping. (E,F) Factor B staining shows low positivity in SLE and stronger positivity in aHUS, mostly within the capillary lumens. (G,H) Lupus nephritis has a granular positivity for C3b in the mesangium, at the GBM, and in some capillary lumens. In aHUS, there is predominant endocapillary positivity for C3b.

Figure S2. Morphology and proximity ligation assays for C4b/C2 and C3b/factor B in lupus nephritis and atypical hemolytic uremic syndrome. (A–C) Periodic acid–Schiff (PAS) reaction of lupus nephritis (SLE GN), thrombotic microangiopathy due to atypical hemolytic syndrome (aHUS) and zero-hour transplant biopsy as normal controls. (D–F) Proximity ligation assays for C4b/C2 of lupus nephritis, atypical hemolytic uremic syndrome, and zero-hour biopsies. Note brown dot–like PLA signals indicating assembly of the C3/C5 convertase of the classical/lectin pathway, most abundant in SLE (D) being located within the mesangium and along the peripheral glomerular capillary walls. (G–I) Proximity ligation assays for C3b/factor B of lupus nephritis, atypical hemolytic uremic syndrome, and zero-hour biopsy. Brown dot–like PLA signals indicate assembly of the C3/C5 convertase of the alternative pathway with most signals in aHUS (H) being located within the glomerular capillary lumens.

Figure S3. Exemplary depiction of glomeruli included and excluded from analysis. (A) Proximity ligation assay for classical/lectin pathway C3/C5 convertase in an SLE case. Note a preserved glomerulus with numerous signals included in the analysis (○) and a representative scarred glomerulus without signals (*) excluded from the analysis. (B) Proximity ligation assay for alternative pathway C3/C5 convertase in an aHUS case. Note a preserved glomerulus with signals included in the analysis (○) and a representative collapsed glomerulus without signals (Δ) excluded from the analysis.

Figure S4. Overview of the computer-assisted quantification of signals in glomeruli. (A) Definition of a glomerulus of an aHUS case as region of interest (») for subsequent analysis. Note the abundant brown dot–like proximity ligation assay signals for the alternative pathway C3/C5 convertase inside the glomerular capillary lumens. (B) Detection of proximity ligation assay signals inside the defined region of interest. Note the green markup of the signals (>) according to the set parameters. (C,D) Automatized splitting of several confluent or overlapping signals into single signals (→). Note dark-green markup of the overlapping signals (→) and the number of estimated signals inside the respective area, subsequently rounded up, printed below the signals.

REFERENCES

1. Thurman JM. Complement in kidney disease: core curriculum 2015. *Am J Kidney Dis*. 2015;65:156–168.
2. Zipfel PF, Skerka C, Chen Q, et al. The role of complement in C3 glomerulopathy. *Mol Immunol*. 2015;67:21–30.
3. Reddy YNV, Siedlecki AM, Francis JM. Breaking down the complement system : a review and update on novel therapies. *Curr Opin Nephrol Hypertens*. 2017;26:123–128.
4. Ricklin D, Mastellos DC, Reis ES, et al. The renaissance of complement therapeutics. *Nat Rev Nephrol*. 2018;14:26–47.
5. Thurman JM, Nester CM. All Things Complement. *Clin J Am Soc Nephrol*. 2016;11:1856–1866.
6. Söderberg O, Gullberg M, Jarvius M, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods*. 2006;3:995–1000.
7. Hnasko RM. Methods in molecular biology ELISA methods and protocols. In: Hnasko RM, ed. *ELISA Methods and Protocols. Methods In Molecular Biology series*. Vol 1318. 1. New York, NY: Humana Press; 2015:149–159.
8. Smith RJH, Appel GB, Blom AM, et al. C3 glomerulopathy—understanding a rare complement-driven renal disease. *Nat Rev Nephrol*. 2019;15:129–143.
9. Ruggenenti P, Daina E, Gennarini A, et al. C5 convertase blockade in membranoproliferative glomerulonephritis: a single-arm clinical trial. *Am J Kidney Dis*. 2019;74:224–238.