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Vimentin is a dominant target of *in situ* humoral immunity in human lupus tubulointerstitial nephritis

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Abstract

Objective—In lupus nephritis (LuN), severe tubulointerstitial inflammation (TII) predicts progression to renal failure. Severe TII is associated with tertiary lymphoid neogenesis and *in situ* antigen-driven clonal B cell selection. The autoantigen(s) driving *in situ* B-cell selection in TII are not known. This study aimed to identify the dominant driving autoantigen(s).

Methods—Single CD38⁺ or Ki-67⁺ B cells were laser captured from seven LuN diagnostic biopsies. Eighteen clonally expanded immunoglobulin heavy and light chain variable region pairs were cloned and expressed as monoclonal antibodies. Seven more antibodies were cloned from flow sorted CD38⁺ cells from an eighth biopsy. Antigen characterization was performed using a combination of confocal microscopy, ELISA, screening protoarrays, immunoprecipitation and mass spectrometry. Serum IgG titers to the dominant antigen were determined in 48 LuN and 35 non-nephritic lupus samples using purified antigen-coated arrays. Autoantigen expression was localized by immunohistochemistry and immunofluorescence on normal and LuN kidney.

Results—Eleven of 25 antibodies reacted with cytoplasmic structures, four reacted with nuclei, and none with dsDNA. Vimentin was the only autoantigen identified by both mass spectrometry and by protoarray. Ten of the 11 anti-cytoplasmic TII antibodies directly bound vimentin. Vimentin was highly expressed by tubulointerstitial inflammatory cells, and tested TII antibodies preferentially bound inflamed tubulointerstitium. Finally, high-titers of serum anti-vimentin antibodies were associated with severe TII ($p = 0.0001$).

Conclusion—Vimentin, an antigenic feature of inflammation, is a dominant autoantigen targeted *in situ* in LuN TII. This adaptive autoimmune response likely feeds forward to worsen TII and renal damage.

Introduction

Systemic Lupus Erythematosus (SLE) is the archetypical systemic autoimmune disease in which a break in both B and T cell tolerance enables pathogenic adaptive immunity to

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ubiquitous nuclear self-antigens[1]. In this systemic model, antibodies and lymphocytes disseminate from secondary lymphoid organs (SLOs) to cause damage in end organs including the kidneys, lungs, skin, gastrointestinal track, brain and heart [2]. Renal inflammation is a common [3, 4], severe manifestation of SLE that is often resistant to treatment with cytotoxic therapies [5]. Up to 50% of SLE patients develop nephritis and up to 50% of those affected progress to renal failure within five years [6, 7].

The principal lesion within the kidney associated with systemic autoimmunity is glomerulonephritis (GN). GN is associated with serum anti-dsDNA antibodies that often deposit in glomeruli [8, 9]. In animal models, some anti-dsDNA antibodies can induce GN [10, 11].

In human lupus nephritis (LuN) tubulointerstitial inflammation (TII) is also common. On renal biopsy, severity of TII, rather than severity of GN, predicts progression to renal failure [6, 7, 12]. Furthermore, unlike GN, severe TII is associated with *in situ* adaptive immunity. Tertiary lymphoid organ (TLO)-like structures are common in severe TII, including T:B aggregates, plasmablast foci and GCs [13]. *In situ* antigen-driven selection of B cells occurs in each of these structures. Therefore, human LuN appears to arise from both systemic and *in situ* autoimmune responses, with the latter more closely associated with a poor prognosis [6, 7, 12].

The antigens driving *in situ* adaptive immunity in LuN are not known. Therefore, we characterized a panel of *in situ* selected IgGs from renal biopsies. Vimentin, an antigen induced in TII, was the most commonly targeted autoantigen. Furthermore, high serum titers of anti-vimentin antibodies (AVAs) were restricted to patients with severe TII. These findings suggest that AVAs might be a useful biomarker of an *in situ* adaptive immune mechanism associated with severe TII.

Materials and Methods

Patient samples

Patients meeting revised 1982 ACR criteria for Systemic Lupus Erythematosus at the University of Chicago and Ohio State University were retrospectively selected. All patients provided informed consent and the study was approved by relevant institutional review boards.

Monoclonal antibody generation

Briefly, frozen biopsies [13] were sectioned (7 μ m), adhered to microscope slides, fixed in acetone (-20°C , 10 min), washed with ice cold PBS, and blocked with 10% donkey serum (DS, Jackson ImmunoResearch). Sections were stained with anti-CD38 (DAKO, 2 $\mu\text{g}/\text{ml}$) or anti-Ki-67 (Thermo Scientific, 2 $\mu\text{g}/\text{ml}$) antibodies conjugated with FITC (Life Technologies) in PBS/5% DS. Positively stained single cells were captured using the Arcturus Pixcell II (Molecular Devices) and Capsure HS LCM caps (Molecular Devices) with an infrared laser (810 μm) spot diameter of 7.5 μm , 70 mW pulse power, 5 ms pulse duration and 170 mW voltage [13]. Caps were extracted as described previously [13].

One biopsy was digested at 37°C for 30 min in 5 ml digestion buffer (2 µg/ml collagenase B, 0.2 µg/ml DNaseI, 1% BSA, 25 mM NaHCO₃, 10 mM HEPES in HBSS), passed through a 200 µm nylon cell strainer and resuspended in FACS buffer. Single CD19⁺CD38⁺ cells were sorted into 96 well plates containing lysis buffer. mRNA was reverse transcribed [15] and V_H (IgG) and V_L (κ and λ) regions PCR amplified, and cloned into TOPO vectors (Life Technologies), sequenced and analyzed [13, 16]. Paired V_H and V_L sequences were subcloned into their respective IgG1 heavy, λ, or κ expression vectors and human IgG1 monoclonal antibodies (mAbs) produced [15].

Confocal Microscopy

For Figure 1, ANA staining patterns of TIN mAbs were visualized using an IIF kit (INOVA diagnostics). For subsequent studies, HEp-2 cells were fixed in methanol [17] and incubated with mAbs (50 µg/ml), followed by Alexa Fluor 488-conjugated goat anti-human IgG (2 µg/ml) and Hoechst 33342 (both Life Technologies). Selected slides were sequentially stained with anti-vimentin V9 (2 µg/ml, DAKO) or anti-giantin (1µg/ml, Abcam) followed by Alexa Fluor 568-conjugated donkey anti-mouse antibodies (2 µg/ml, Molecular Probes). Samples were imaged using a Leica SP5 II STED-CW laser scanning confocal microscope.

Protein purification and identification

Cells were lysed on ice in RIPA buffer (5×10⁶ cells/0.5 ml). Clarified lysates were pre-cleared with protein G agarose (Pierce) and then incubated with mAb mixtures (200 µg total pre-bound to protein G agarose) overnight at 4°C. Following washing in ice cold IP wash buffer (25 mM Tris, 150 mM NaCl, pH 7.2), immunoprecipitations were resolved by reducing SDS-PAGE (4–12% Bis-Tris, Life Technologies). Gels were stained with Instant Blue (Expedeon) and gel tranches (T1-4) excised. Tranches were reduced, alkylated and trypsinized, for HPLC-MS/MS [18]. Scaffold (version Scaffold_4.0.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were at greater than 99.0% probability to achieve an FDR less than 1.0% [19]. Protein identifications were accepted if greater than 93.0% probability to achieve an FDR less than 1.0% (Protein Prophet) [20].

Protoarrays

Human Protoarrays (Invitrogen) were blocked with 2% BSA/PBS, rinsed with 0.05% Tween-20/PBS then incubated with 25 µg/ml mAb in 2% BSA/PBS. Bound antibody was detected with Cy5-conjugated goat anti-human IgG (0.5 µg/ml, Jackson) and counter-stained with rabbit anti-GST (Millipore) followed by Cy3-conjugated goat anti-rabbit (0.5µg/ml, Jackson). Antigen signals (MFIs) were quantified using a GenePix 4000B microarray scanner and GenePix Pro 6.0 software (Molecular Devices).

Using raw values for the MFI of each probe spot for Cy5 (F635) and Cy3 (F532), and of the probe background for Cy5 (B635) and Cy3 (B532), initial probe intensities were computed as (F635/B635)/(F532/B532). Raw probe intensities were normalized to internal controls and linearly scaled to median control probe intensity of 1.0. Each array was scanned at two PMT voltages, 500 and 600. At PMT 500, we assessed the ratio and the difference between antibody and control intensities: $R500 = A500/C500$ and $D500 = A500 - C500$ (A=antibody,

C=control), respectively; the same was done for PMT 600. The overall probe reactivity was then computed as the harmonic mean: $4/(1/R500+1/R600+1/D500+1/D600)$.

Vimentin microarrays

Bovine vimentin (SIGMA 100 µg/ml in 0.4M HCl) was diluted 1:1 with printing buffer (ArrayIt) and spotted (20 features/block, 24 blocks/slide) onto SuperEpoxy glass slides using a SpotBot 3 microarrayer (ArrayIt). Slides were washed with 0.05% Tween-20/PBS and then incubated in BlockIt blocking buffer (ArrayIt). Serum (1:50) or mAbs (100 µg/ml) in reaction buffer (ArrayIt) were applied followed by Cy3-conjugated goat anti-human IgG (0.5 µg/ml). Signals were quantified using a GenePix 4000B microarray scanner and GenePix Pro 6.0 software (Molecular Devices). The MFIs for the 20 vimentin features in each probed block were median averaged.

TII mAb staining of kidney tissue

Fresh frozen biopsy sections (3 µm) were adhered to glass slides fixed in acetone (-20°C), washed in PBS and permeabilized in 0.5% NP-40/PBS. Slides were blocked in 10% DS/PBS and probed with TII mAbs directly conjugated to FITC (Pierce). Bound mAb was detected with rabbit anti-FITC antibodies (1 µg/ml, Life Technologies), followed by Alexa Fluor 488-conjugated goat anti-rabbit antibodies (0.5 µg/ml, Life Technologies). Cell nuclei were counter-stained with Hoechst 33342.

Results

Characterization of *in situ* expressed immunoglobulins

In situ antigen-driven B cell selection is evident in LuN including intrarenal GCs, T:B cell aggregates and plasmablast foci [13]. All of these histological features contain Ki-67⁺ B cells and/or plasmablasts. To characterize the antigen(s) driving *in situ* B cell selection in each of these tertiary lymphoid-like structures, we identified a cohort of eight lupus patients (Supplementary Table 1) with TII on diagnostic biopsy. Seven of these biopsies had T:B aggregates and plasma cells while one had a GC.

To identify *in situ* selected B cell populations, five of the biopsies with T:B aggregates and plasma cells (patients 1–5) were sectioned and stained for Ki-67. The biopsy containing a GC (patient 6), and one biopsy with T:B aggregates and plasma cells (patient 7), were stained for CD38. Positive cells were laser captured. From each capture, expressed IgG heavy, lambda or kappa variable (V_H , V_L or V_K) regions were amplified and subcloned into TA vectors [13]. Multiple colonies from each transformation were sequenced. A particular variable chain sequence was considered valid if the same sequence was obtained from at least two separate colonies of a given transformation. A V_H and V_L pair was considered valid if the same immunoglobulin heavy chain rearrangement was obtained from at least two different captured areas from one biopsy. Using this approach, 18 paired V_H and V_L regions were identified (Supplementary Table 2).

For most patient samples, the above approach produced single V_H and V_L pairings. However, in one instance (patient 4) two heavy and two light chains persistently co-

occurred. In this case, all four possible pairings were considered and expressed as respective mAbs (Ki4-1 through Ki4-4). Additionally, three of the cloned heavy chains persistently co-occurred with two light chains leading us to generate two different TII mAbs for each respective heavy chain (patient 2, Ki2-1 and Ki2-2; patient 3, Ki3-1 and Ki3-2; patient 6, GC1 and GC1a).

Finally, one biopsy was digested and single CD38⁺ cells were sorted by FACS [15]. The variable regions were again subcloned into TA vectors and sequenced. Seven distinct V_H and V_L pairings were isolated.

A summary of the nucleotide and predicted amino acid sequences for individual V_H and V_L regions is provided in Supplementary Table 2. The V_H segments were different for each antibody chain, with the exception that both Ki5-1 and PB1 used V_H3–15 but with different D and J_H segments. Overall, the frequency of V_H segments was similar to V_H usage in the peripheral B cells of healthy populations [21], including a bias for V_H3 (12/20) and V_H4 (7/20). The length of the V_H complementarity determining region 3 (H-CDR3) and V_L CDR3 (L-CDR3) varied from 7–23 and 8–12 amino acids respectively. All immunoglobulin heavy and light chains were somatically mutated (Supplementary Table 2) and a distribution of replacement and silent mutations consistent with antigen-driven selection was evident in 13 of 20 V_H chains (Supplementary Table 3) [16].

Antibodies from in situ selected B cells often recognize cytoplasmic antigens

Paired V_H and V_L regions were subcloned and expressed as functional human IgG₁ mAbs. In total, 25 TII mAbs were derived from eight patients; 14 from Ki-67⁺ positive cells, three from CD38⁺ cells within a GC, one from CD38⁺ cells within a T:B aggregate, and seven from a biopsy sorted for CD38⁺ cells (Supplementary Tables 1 and 2).

All 25 TII mAbs were assayed for nuclear reactivity by IIF on HEp-2 cells (Figure 1A–C). Only four reacted preferentially with nuclear antigens; three yielded speckled patterns and one yielded a nucleolar pattern. In contrast, the control human IgG1 mAb, 3H9, which preferentially binds dsDNA, yielded a homogeneous nuclear staining pattern, and reacted to a lesser extent with the cytoplasm.

Eleven antibodies, from seven of the eight patients, bound cytoplasmic antigens. Of these, seven had preferential cytoskeletal reactivity, two yielded a diffuse homogeneous cytoplasmic pattern and two had both cytoplasmic and nuclear binding. One mAb (PB7) co-localized with the golgi apparatus protein giantin (Supplementary Figure S1) [22]. Nine of 25 mAbs demonstrated no detectable reactivity. These data suggest that TII *in situ* adaptive immune responses are more often targeted to cytoplasmic than nuclear self-antigens.

TII antibodies do not frequently bind usual nuclear antigens

To confirm the absence of dsDNA reactivity, *Crithidia luciliae* were stained with each mAb (Supplementary Figure S2). While 3H9 had strong reactivity with the kinetocore, there was no detectable reactivity with the TII mAbs. ELISAs were subsequently performed for ANA, SSA and Sm/RNP reactivity (Supplementary Figure S3). Interestingly, none of the four nuclear reactive mAbs (PB2, Ki1-2, Ki4-2 and Ki5-3) showed strong reactivity with any of

these substrates. Ki4-2 did however have low reactivity with Sm/RNP. These data suggest that the nuclear antigen specificities normally associated with SLE are not commonly selected *in situ* in lupus TII. Furthermore, none of the TII antibodies had appreciable IgG rheumatoid factor activity (Supplementary Figure 3).

Vimentin is an autoantigen commonly targeted in situ

We next sought to identify the antigenic targets of antibodies with cytoplasmic and cytoskeletal reactivities (both collectively grouped as anti-cyto antibodies). We first interrogated Protoarrays (Life Technologies), containing 9400 human proteins, with a selected group of TII mAbs (Figure 2). The measures of respective antigen reactivities were computed as harmonic mean ratios (see Materials and Methods). We validated the Protoarrays with a transglutaminase 2 (TG2) reactive human IgG₁ mAb (4B06), cloned from a patient with Celiac disease [23]. The two TG2 antigens (IVGN:PM_2144 and BC003551) were the most reactive features, giving values of 32.8 and 11.7 respectively.

The TII mAbs used for Protoarray probing provided at least one example of each staining pattern; anti-cyto (Ki5-2, Ki3-2, Ki3-1, Ki5-1), speckled nuclear (Ki1-2), GA (PB7), nucleolar (Ki5-3) and no appreciable HEp-2 reactivity (GC1, GC1a, PB6, Ki2-1, PB1). A subset of antigens was hierarchically clustered using a Pearson correlation distance measure and displayed in a heat-map (Figure 2); the antigens selected were those with reactivity greater than two-fold for the 99% value of the control probes. The most commonly reactive targets included fms-related tyrosine kinase 3 ligand (FLT-3LG), IgG1 and IgM heavy chains, and epidermal growth factor (EGF)-like discoidin I-like domains 3 (EDIL3). Essentially all of the antibodies bound FLT-3LG and therefore this immunospecificity was likely spurious.

Most TII mAbs, comparably to the TG2 specific mAb, displayed restricted poly-reactivity with eight or fewer proteins. In contrast, Ki3-1 displayed greater polyreactivity. Antigens targeted by at least two anti-cyto TII mAbs included EDIL3 and vimentin. In contrast, the nuclei reactive mAb, Ki1-2, only bound weakly to a few Protoarray antigens. A similar pattern of pauci-reactivity was observed with those TII mAbs that did not react with HEp-2 cells.

We next used mass spectrometry to characterize the antigens targeted by the TII anti-cyto mAbs (Figure 3A-B). HEp-2 cell lysates were immunoprecipitated with a mixture of anti-cyto mAbs (Ki3-1, Ki3-2, Ki5-1, Ki5-2) or a mixture of four different anti-TG2 IgG₁ mAbs (4B06, 4A06, 4D03, 4E05) [23]. Lysates were also immunoprecipitated with the anti-nucleolar mAb Ki5-3 (Supplementary Table 4). Samples were resolved by reducing SDS-PAGE, and stained with Coomassie blue (Figure 3A). A few unique bands were detected in the anti-cyto mAb lane including one with a relative molecular weight of approximately 45 kDa (T1). The T1 band, and a region corresponding to the same molecular weight from the Ki5-3 immunoprecipitation (T2), were excised for mass spectrometry. From the T1 band, proteins inferred from peptide spectra were cytokeratin-1, -2, -7 and -9, actin, fructose biphosphate aldolase A, and vimentin (Supplementary Table 5). Among the above proteins identified from T1, the cytokeratins were also observed in T2. Cross-referencing of the T1 proteins identified by mass spectrometry to those on the protoarray heat map (Figure 2)

revealed that only vimentin was common. The two vimentin peptides identified by mass spectrometry (Figure 3B and Supplementary Tables 4 and 5) represented 29 of the 466 amino acids (6%) in the full-length translated protein.

Additional immunoprecipitations were performed with a different mixture of cytoskeleton staining TII mAbs (GC2, Ki4-5, PB4 and PB5) using lysates of HEp-2 or primary renal proximal epithelial cells (RPECs). As full-length vimentin has a relative molecular weight of 54 kDa, gel tranches covering 50–55 kDa were excised (Supplementary Table 5, tranches T3 and T4 respectively) for mass spectrometry. Vimentin peptides were once again identified in both samples. The lower molecular weight forms of vimentin detected in T1 likely represent proteolytic fragments [24, 25]. In total, 27 different vimentin peptides covering 259 amino acids (56% of the full length protein) (Figure 3B) were identified from T1, T3, and T4 (Supplementary Table 5). To confirm that vimentin was targeted by TII anti-cyto mAbs, HEp-2 cells were co-stained with the TII mAb Ki3-2 and the murine anti-vimentin mAb V9 (DAKO, Figure 3C), revealing extensive co-localization.

To determine whether vimentin was a frequent target of antibodies produced *in situ* during TII, all 10 remaining anti-cyto TII mAbs were assayed by confocal microscopy. All 10 had some overlap with V9 staining, with PB4, Ki3-1, GC2, PB3, PB5, and Ki1-1 showing extensive and specific overlap (Figure 4A and Supplementary Figures 4 and 5). Poly-reactivity was observed with the TII mAbs Ki2-2, Ki5-1 and Ki5-2, (Supplementary Figures 4 and 5), while Ki4-5 (Figure 4A) demonstrated preferential staining of cytoskeletal filaments, some of which did not react with V9. As these co-localizations could represent binding to antigens closely neighboring vimentin, direct vimentin immunoreactivity was confirmed using purified bovine vimentin coated Superepoxy glass slides (Figure 4B). Ten of 11 anti-cyto TII mAbs had vimentin immunoreactivity that was greater than that observed for any of the other non-cyto TII mAbs ($p = 3.9 \times 10^{-4}$).

Vimentin is upregulated in inflamed tubulointerstitium

To begin to understand why vimentin was so commonly targeted *in situ*, we examined the distribution of vimentin expression in normal and LuN renal biopsies by immunohistochemistry (Figure 4C). Consistent with previous reports [24, 26], in normal kidney, vimentin is expressed primarily in glomeruli, arterioles, and interstitial fibroblasts, but not in epithelial cells. In contrast to normal kidney, the distribution and intensity of vimentin expression in TII was radically different, with vimentin being abundant throughout the tubulointerstitium. Much of this increased vimentin expression localized to the mononuclear cells infiltrating the tubulointerstitium.

High expression of vimentin by infiltrating mononuclear cells suggested that *in situ* humoral immunity is directed against molecules associated with inflammation. Therefore, we directly examined whether TII mAbs also preferentially bound inflamed tubulointerstitium (Figure 5). Two TII mAbs, Ki3-1 and PB4, both with vimentin immunoreactivity, were directly labeled with FITC and used to stain sections from normal kidney and LuN kidney with severe TII. Samples were then incubated with rabbit anti-FITC antibodies followed by Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Supplementary Figure 6). As expected, no discernable immunoreactivity was detected in the absence of primary antibody (Figure 5). In

contrast, staining with 3H9 yielded a predictable nuclear pattern. In normal kidney, Ki3-1 had some immunoreactivity with glomeruli and minimal immunoreactivity with the tubulointerstitium. PB4 demonstrated little immunoreactivity with normal renal tissue. In contrast, both antibodies bound inflamed tubulointerstitium and Ki3-1 also bound inflamed glomeruli. These data are consistent with a model in which *in situ* immunity targets inflammation.

Serum anti-vimentin antibodies associated with severe TII

As vimentin is frequently targeted by the *in situ* humoral immune response, it was predicted that serum anti-vimentin antibody (AVA) titers would reflect TII severity. Therefore, AVA titers in serum samples from patients with LuN (n=48), or with no history of renal disease (n=35) were assayed as above (Figure 6A–B). High titer AVAs (>180 AU) were almost exclusively found in patients with renal disease, and renal patients had higher average AVA titers ($p=0.01$). The one non-renal patient who had a relatively high AVA titer had very active extra-renal disease with severe discoid lupus, arthritis, vasculitis, decreased serum complements and increased anti-dsDNA antibody titers. Within the renal group, high-titer AVAs strongly correlated with severe (grade 3) TII ($p < 0.0001$) with the five highest AVA titers detected within this group. Only one of 10 patients with severe TII had low titer AVAs. These data suggest that high-titer AVAs might be a useful biomarker of severe TII.

Discussion

Many studies have first identified autoantibody specificities in serum and have subsequently correlated these to disease manifestations [2, 27]. Instead, our study started with the affected end organ, providing immediate pathological context. The relevance of the characterized *in-situ* derived anti-vimentin mAbs to TII was supported by the correlation between high serum AVA titers and severe TII. Our results suggest that tissue-focused studies can identify clinically important biomarkers, and potentially pathogenic mechanisms, not immediately apparent from peripheral blood studies.

In Lupus TII, *in situ* humoral immunity appeared antigenically restricted and primarily focused on cytoplasmic antigens, notably vimentin. Vimentin was expressed by infiltrating inflammatory cells and tested TII mAbs preferentially reacted with the inflamed tubulointerstitium. We postulate that by targeting inflammation the adaptive immune response, and the attendant deposition of antibodies and complement, feed-forward to amplify local inflammation, tissue destruction and renal scarring [28].

Of the other serological specificities associated with SLE, only anti-dsDNA antibody titers provide a similar correlation with disease activity [29, 30]. This is best defined for GN, where high-titer dsDNA antibodies predict renal flare in some patients [2] and anti-dsDNA antibodies can be detected deposited in inflamed glomeruli [9, 31]. While dsDNA antibody titers provide a measure of systemic autoimmunity, we suggest that AVA titers provide complementary measures of organ intrinsic mechanisms of autoimmunity.

Most of the identified antigens targeted by the *in situ* humoral immune response were cytosolic proteins, the immune response to which usually requires T-cell help [32]. Indeed,

T follicular helper (T_{FH}) cells are common in LuN biopsies manifesting severe TII, where they are in apparent cognate pairs with B cells [33]. Therefore, the inflamed tubulointerstitium appears sufficient to propagate cytosolic antigen targeted humoral immunity. In contrast, inflamed glomeruli lack infiltrating B and T_{FH} cells. B cell expressed toll-like receptors might therefore be less important *in situ* than they are in systemic immune responses measured in the periphery (and propagated in secondary lymphoid organs), where these receptors play a role in responses to dsDNA and RNP containing complexes [34, 35].

Some characteristics of vimentin might enhance its antigenicity *in situ*. Vimentin is a highly basic molecule that is secreted by activated macrophages [25]. Therefore, in inflammation, vimentin is abundant and available. Furthermore, vimentin can bind and activate dectin-1 [36], a c-type lectin receptor expressed on cells including macrophages, dendritic cells, and B cells. Vimentin might therefore locally prime antigen-presenting cells. Finally, vimentin can be post-translationally modified which, in RA, provides neo-epitopes and increases antigenicity [37]. It remains to be determined if post-translational modifications enhance *in situ* immunity to vimentin in LuN TII, or whether particular HLA molecules preferentially present vimentin-derived peptides. Mapping dominant vimentin epitopes should provide insights into these questions.

Most AVAs demonstrated polyreactivity. This could reflect a less efficient selection on multiple abundant antigens in the inflamed kidney, in comparison to a predictably stringent selection in GCs, in which antigen is limited and presented in competitive contexts that favor higher affinity and restricted polyreactivity [38]. Alternatively, the heightened polyreactivity/poly-specificity may be due to more than one antigen driving selection, reflecting poly-selection.

Interestingly, anti-cytoplasmic antibodies are a common feature of the normal newly emigrated and mature naïve B cell repertoires [39]. Furthermore, in SLE anti-cytoplasmic antibodies are even more frequent in these pools with at least a third of mature naïve B cells having some cytoplasmic reactivity [40]. The high prevalence of B cells expressing anti-cytoplasmic antibodies may help explain the low-titer AVAs observed in most SLE patients. Presumably, the TII AVAs that we characterized arose from one or more of the above B cell populations expressing anti-cytoplasmic antibodies. Alternatively, it is possible that the AVAs were selected from non-autoreactive or anti-nuclear antigen specific B cell precursor populations.

High-titer AVAs (>225 AU) were invariably associated with high disease activity, usually severe TII. Among the six highest AVA titers, five occurred in severe TII with all four of the very highest titers associated with this manifestation. The only non-nephritic patient with high-titer AVAs (236 AU) had severe extra-renal disease. Intermediate AVA titers (150–220 AU) were preferentially found in patients with nephritis. Therefore, different titers of AVAs might reflect different levels of disease activity and disease manifestations.

Anti-cytoplasmic antibodies and AVAs have been observed in a variety of autoimmune diseases [41–44] and in transplant rejection. Interestingly, in cardiac allograft recipients, IgM AVAs are predictive of allograft vasculopathy [45] and rejection [46]. Furthermore,

AVAs have been observed in renal transplant rejection [47, 48]. In mice, immunization with vimentin accelerates cardiac allograft rejection [49] providing evidence that anti-vimentin immune responses can be pathogenic. These data, with ours, suggest that AVAs represent a common adaptive immune response in chronic organ inflammation which perpetuates disease.

Because of the difficulties inherent in isolating *in situ* selected B cells, our study was restricted to a small number of biopsies and mAbs. Sampling single cells by LCM is imprecise and it is likely that a few V_H and V_L pairings were spurious. Indeed, in some cases a single V_H could not be paired with a single V_L. Furthermore, closed needle biopsies provide a limited sampling of the entire lesion. Despite these limitations, our study revealed consistent themes; antibodies from seven of eight patients were immunoreactive with cytoplasmic antigens and antibodies from six of eight patients had vimentin immunoreactivity. Furthermore, these prevalences correlated with the frequency of high serum AVA titers in patients with severe TII. Therefore, our sample size and approach appeared sufficient to capture common features of the TII *in situ* adaptive immune response.

This study demonstrates that high AVA titers identify lupus patients with severe TII, a population at substantial risk for progression to renal failure [6, 7, 12]. Whether AVAs are directly pathogenic, or if they can be used as a biomarker, requires additional study. Also, it is not known if high AVA titers identify responders to targeted therapies. Regardless, knowing where and when AVAs arise in lupus will provide a strong context for interpreting future clinical and mechanistic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HEp-2 staining patterns using TII mAbs. **A**, Indirect immunofluorescent microscopy of HEp-2 cells following staining with TII mAbs (25 µg/ml). Provided are representative examples. Positive controls included a known ANA⁺ serum sample (ANA pos) and the human 3H9 IgG1 mAb, both of which demonstrate a preferential reactivity for DNA and a characteristic homogeneous nuclear staining pattern. White scale bar = 10 µm. **B**, Pie charts summarizing relative frequencies of HEp-2 staining patterns observed for TII mAb. The smaller chart is a sub-division of the mAbs that preferentially react with nuclear antigens. Colors correspond to representative images in **A**. Color key: Yellow: nuclear speckled (nuc spec); brown: nucleolar; red: cytoskeletal (cytosk); blue: cytoplasmic (cytopl); green: cytoplasmic and nuclear (cytopl & nuc); pink: golgi apparatus (GA); white: no reactivity (N.R.). **C**, Staining patterns of respective TII mAbs.

Figure 2.

Immunoreactivities of TII mAbs with Protoarrays. Antibodies with high reactivity in at least one array (defined as at least two-fold higher than 99% of control probes in the same array) were hierarchically clustered by antigen immunoreactivity. mAb 4B06 has known immunoreactivity with transglutaminase 2 (TG2). The key (top left) indicates the magnitude of immunoreactivity (Materials and Methods).

Figure 3. Identification of vimentin as a putative antigen targeted by TII mAbs. **A**, Immunoprecipitations of HEp-2 cell lysates with either TII mAbs (Ki3-1, Ki3-2, Ki5-1 and Ki5-2), no mAbs, or TG2 reactive mAbs, were resolved by reducing SDS-PAGE and Coomassie stained. Unique TII mAb band (T1) was excised and analyzed by mass spectrometry. Among proteins identified was vimentin. **B**, The vimentin (NP_003371.2) amino acid FASTA sequence is italicized and blue in regions corresponding to peptides identified by mass spectrometry of T1. Tranches cut from analogous immunoprecipitates (Supplementary Tables S4 and S5), generated with other TII mAbs, yielded additional vimentin peptides (bold). **C**, Confocal microscopy of HEp-2 cells demonstrating co-staining of TII mAb Ki3-2 with anti-vimentin mAb, V9. White scale bar=10 μ m.

Figure 4.

Vimentin is frequently targeted by TII mAbs. **A**, HEP-2 cells co-stained with TII mAbs (green), anti-vimentin (V9, red) and Hoechst (blue). Examples of four different patterns of co-localization are shown. Staining with other TII mAbs with cytoplasmic reactivity is provided in Supplementary Figures 4 and 5. **B**, Vimentin protein arrays probed with TII mAbs, detected with anti-hIgG₅₃₂. Data-points are reactivities of individual TII mAbs. Raw MFIs are given. mAbs are grouped by cytoplasmic (cyto) HEP-2 immunoreactivity or not (Mann-Whitney two-tailed test indicated). **C**, Immunohistochemical staining of normal (top left) and TII (other panels) kidney tissue with V9 antibody (DAKO). Red box: area magnified. Red and black scale bars: 10 μ m and 100 μ m respectively.

Figure 5.

Enhanced binding of TII mAbs to inflamed renal tubulointerstitium. Indicated TII mAbs were used to stain normal renal and TII samples. The mAb 3H9 and diluent were used as positive and negative controls respectively. Tissue was counter-stained with Hoechst for nuclei (blue). Representative patterns of tubulointerstitial (TI) and glomerular (glom) binding are given. White scale bar = 10 μ m.

Figure 6.

Association of nephritis with anti-vimentin IgG levels in the serum of lupus patients. Individual serum samples from patients with SLE were used to probe vimentin arrays for IgG reactivity. Data-points represent individual serum samples. **A**, Patients were first grouped as having history of nephritis or not. **B**, Patients with nephritis were further characterized by degree of TII (0 is none, 1 is mild, 2 moderate and 3 severe). Titers are given in arbitrary units (AU), extrapolated from a serially diluted high AVA titrated TII patient serum sample. Shown are p-values of two-tailed Mann-Whitney tests for indicated comparisons. Medians and interquartile ranges are given. For renal patients, the median time difference between biopsy and blood draw was 1.36 years (interquartile range 0.08 – 3.14 years). The mean age at the time of serum draw was 33.1 ± 9.7 years. For the non-renal patients, average age at serum draw was 41.6 ± 10.5 years (vs. renal patients, $p < 0.01$).