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# Anti-DNA autoantibodies initiate experimental lupus nephritis by binding directly to the glomerular basement membrane in mice

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The strongest serological correlate for lupus nephritis is antibody to double-stranded DNA, although the mechanism by which anti-DNA antibodies initiate lupus nephritis is unresolved. Most recent reports indicate that anti-DNA must bind chromatin in the glomerular basement membrane or mesangial matrix to form glomerular deposits. Here we determined whether direct binding of anti-DNA antibody to glomerular basement membrane is critical to initiate glomerular binding of anti-DNA in experimental lupus nephritis. Mice were co-injected with IgG monoclonal antibodies or hybridomas with similar specificity for DNA and chromatin but different IgG subclass and different relative affinity for basement membrane. Only anti-DNA antibodies that bound basement membrane bound to glomeruli, activated complement, and induced proteinuria whether injected alone or co-injected with a non-basement-membrane-binding anti-DNA antibody. Basement membrane-binding anti-DNA antibodies co-localized with heparan sulfate proteoglycan in glomerular basement membrane and mesangial matrix but not with chromatin. Thus, direct binding of anti-DNA antibody to antigens in the glomerular basement membrane or mesangial matrix may be critical to initiate glomerular inflammation. This may accelerate and exacerbate glomerular immune complex formation in human and murine lupus nephritis.

*Kidney International* (2012) **82**, 184–192; doi:10.1038/ki.2011.484;  
published online 1 February 2012

**KEYWORDS:** complement; immune complexes; lupus nephritis; proteinuria; systemic lupus erythematosus

The contribution of anti-DNA antibody to glomerulonephritis in mouse<sup>1</sup> and human<sup>2</sup> systemic lupus erythematosus is well established. Although anti-double-stranded DNA (dsDNA) antibody is the best serological correlate for lupus nephritis,<sup>3,4</sup> the frequent lack of correlation between serum anti-dsDNA and glomerulonephritis is a long recognized conundrum in the clinical evaluation of individual systemic lupus erythematosus patients.<sup>3,5,6</sup> The lack of correlation between anti-dsDNA and lupus nephritis within individual patients may be a consequence of how anti-dsDNA antibodies bind in the glomerulus and initiate glomerulonephritis,<sup>6</sup> a process not yet fully resolved.<sup>7</sup> Mechanisms proposed to explain glomerular deposition of anti-DNA antibody include glomerular binding of soluble immune complexes of nucleosomes and immunoglobulin G (IgG) anti-DNA,<sup>2,8–10</sup> *in situ* formation of immune complexes when anti-DNA antibody binds to chromatin that has bound to glomerular basement membrane (GBM) or mesangial matrix (MM),<sup>11–17</sup> and direct binding of anti-DNA antibody that cross-reacts with GBM or cell surface antigens.<sup>18–25</sup> Recent morphological studies<sup>12–14,16</sup> have identified chromatin and IgG within the glomerular subendothelial and subepithelial electron-dense deposits (EDS) in nephritic kidneys from lupus patients<sup>26</sup> and lupus-prone mice.<sup>27</sup> The recent results were interpreted to indicate that anti-DNA antibody could form glomerular deposits only when bound to chromatin or nucleosomes.<sup>28–30</sup>

The present experiments were designed to test the hypothesis that initial glomerular binding of anti-DNA antibody in lupus nephritis is a function of direct, cross-reactive binding to glomerular antigens, particularly in GBM or MM, and independent of DNA, nucleosomes, or chromatin. The experiments took advantage of a panel of anti-DNA monoclonal antibodies (mAbs) with similar relative affinities for DNA and chromatin, but different relative affinities for basement membrane (BM) antigens in GBM and MM. Only anti-DNA mAbs that also bound BM antigens bound glomeruli *in vivo* and induced proteinuria. Glomerular binding of the anti-DNA mAbs was independent of DNA, nucleosomes, or chromatin. The results may explain why some anti-DNA mAbs are very effective at inducing lupus nephritis, but others are not. Similarly, the results may

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Received 28 September 2011; revised 11 November 2011; accepted 15 November 2011; published online 1 February 2012

**Table 1 | Specificity of monoclonal antibodies**

Group <sup>a</sup>	Number mAbs	Competitive ELISA (ng/ml competitor) <sup>b</sup>		Direct ELISA (ng/ml IgG) <sup>c</sup>		
		ssDNA	dsDNA	DNA	Chromatin	BM
A	14	111 ± 84 <sup>d</sup>	NI	75 ± 61	826 ± 904	NB
B	21	560 ± 351	5770 ± 2580	6730 ± 6110	1810 ± 2590	NB
C	18	658 ± 302	4200 ± 2580	7040 ± 5560	81 ± 94	5510 ± 1490
D	16	957 ± 469	1570 ± 690	971 ± 1990	52 ± 60	94 ± 780

Abbreviations: BM, basement membrane; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; mAbs, monoclonal antibodies; NB, no binding with ≥10,000 ng/ml mAb; NI, no inhibition with 10,000 ng/ml competitor; ssDNA, single-stranded DNA.

<sup>a</sup>Sixty-nine mAbs were stratified according to BM binding into: (A) NB to BM (14 mAb), (B) NB to BM but binding to dsDNA (21 mAb), (C) BM binding with ≥1000 ng/ml IgG (18 mAbs), and (D) BM binding with ≤1000 ng/ml IgG (16 mAbs).

<sup>b</sup>ng/ml competitor is the amount of dsDNA or ssDNA competitor required to produce 50% inhibition of mAb binding to solid phase DNA in a competitive ELISA.<sup>24</sup>

<sup>c</sup>ng/ml mAb that yields 50% maximum binding in a direct ELISA.

<sup>d</sup>The values are means ± 95% confidence intervals. Analysis of variance among groups for the category of binding to: ssDNA,  $P=0.025$ ; dsDNA,  $P=0.033$ ; DNA,  $P=\text{nonsignificant}$  (n.s.); chromatin,  $P=\text{n.s.}$ ; BM,  $P=3.6 \times 10^{-8}$ . Linear regression with BM binding as dependent variable ( $R^2=0.465$ ,  $P=4.3 \times 10^{-8}$ ): chromatin,  $B=0.381$  and  $\beta=0.290$ ,  $P=0.00298$ ; ssDNA,  $B=-0.496$  and  $\beta=-0.301$ ,  $P=0.0022$ ; and dsDNA,  $B=0.606$  and  $\beta=0.423$ ,  $P=0.00010$  ( $B=\text{slope}$  and  $\beta=\text{correlation coefficient}$ , PASW Statistics18).

**Table 2 | Monoclonal antibody binding to DNA, chromatin, and basement membrane antigens**

mAb <sup>a</sup>	Isotype <sup>b</sup>	pl <sup>c</sup>	Direct binding ELISA				Competitive ELISA		In vivo activity	
			DNA (ng/ml IgG)	Chromatin (ng/ml IgG)	Nucleosome (ng/ml IgG)	BM (g/ml IgG)	ssDNA (ng/ml ssDNA)	dsDNA (ng/ml dsDNA)	Glomerular binding <sup>d</sup>	Induces proteinuria <sup>e</sup>
163p.64	2a	8.4	60	8.0	19,700	30	470	1030	Yes	Yes
163p.77	2b	8.5	20	30	11,500	20	1420	700	Yes	Yes
163p.124	2a	8.4	30	4.0	1880	90	1690	470	Yes	Yes
DNA6	2a	7.6	10	10	ND	200	2900	1000	Yes	Yes
DNA5	2a	8.7	1000	10	ND	3380	1500	10,800	Yes	Yes
163p.132	2b	8.5	50	10	50	8600	660	NI	No <sup>f</sup>	No <sup>f</sup>
DNA3	2a	6.5	11,000	10	ND	NB	54	1600	No	No
452s.46	2b	7.6	10	200	2300	NB	730	490	No	No
452s.160	2a	7.3	70	90	ND	NB	80	4400	No	ND
3H9	2b	8.3	4720	50	ND	NB	ND	ND	No <sup>g</sup>	No <sup>g</sup>

Abbreviations: BM, basement membrane; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; mAb, monoclonal antibody; NB, no binding; ND, not done; NI, no inhibition; pl, isoelectric point; ssDNA, single-stranded DNA.

<sup>a</sup>163p.64, 77, 124 and DNA6: Group D, Table 1; DNA 5 and 163p.132: Group C, Table 1; 452s.46, 160 and DNA3: Group B, Table 1.

<sup>b</sup>IgG subclass of hybridoma mAb.

<sup>c</sup>pl of the respective mAb (calculated using the Swiss Institute of Bioinformatics ExPASy pl calculation tool, [http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)).

<sup>d</sup>Glomerular binding of mAb was determined by immunofluorescence on kidney cryosections 24 h after mice were injected with 1 mg purified mAb(s).

<sup>e</sup>Summary of results presented in Table 3.

<sup>f</sup>MAb 163p.132, IgG<sub>2b</sub> produced minimal glomerular fluorescence and no proteinuria 5 days after the injection of hybridoma cells, but readily detected immunofluorescence and proteinuria 8 days after injection.

<sup>g</sup>Gilkeson et al.<sup>31</sup> only 2/5 mice had glomerular-bound IgG, and the glomerular disease score was not different from the negative control.

help explain why systemic lupus erythematosus patients with similar serum anti-dsDNA antibody may have different susceptibility for lupus nephritis.

## RESULTS

### In vitro binding of anti-DNA mAb to BM

Culture supernatants from 69 autoimmune anti-DNA mAbs from eight different (NZB × NZW)F<sub>1</sub> mice (BWF1) were randomly selected for analysis (Table 1). Total IgG and relative affinity for binding to single-stranded DNA (ssDNA), dsDNA, chromatin, and BM were quantified for each supernatant. The mAbs were stratified by relative affinity for BM into four different specificity groups (Table 1). There is a significant difference among the four specificity groups for competitive binding to ssDNA and dsDNA, and direct binding to BM, but not for direct binding to chromatin.

There is a strong and highly significant correlation between binding to BM and binding to dsDNA, and a moderate, highly significant inverse correlation between binding to BM and binding to ssDNA. Anti-DNA mAbs that bound best to dsDNA are generally the mAbs that also bound best to BM. The correlation between BM and chromatin binding, although significant, was low compared with that for BM and dsDNA. The results indicate that mAbs with high relative affinity for dsDNA are more likely to bind BM than mAbs with high relative affinity for ssDNA. The results also indicate that anti-DNA mAb binding to BM is unrelated to relative affinity for chromatin.

The correlations between mAb binding to DNA and chromatin vs. their potential to bind BM were further confirmed with purified mAbs (Table 2). BM binding by purified mAbs was independent of relative binding affinity

for dsDNA, chromatin, or nucleosomes, as 163p.132, 452s.160, DNA3, and 3H9 mAbs bound nucleosomes and/or chromatin with high relative affinity but bound poorly or not at all to BM. MAb 452s.46 bound dsDNA with high relative affinity but did not bind BM. DNA6 mAb bound chromatin in a manner similar to 163p.132 and DNA3, but, unlike 163p.132 and DNA3, DNA6 also bound to BM. MAbs 163p.64, 163p.77, and 163p.124 had 20- to 650-fold higher relative affinity for BM than for nucleosomes. Binding to BM was also independent of mAb isoelectric point. These results further indicate that anti-DNA mAb binding to BM is correlated with dsDNA binding and to lesser extent with chromatin binding, but is independent of both for binding to BM.

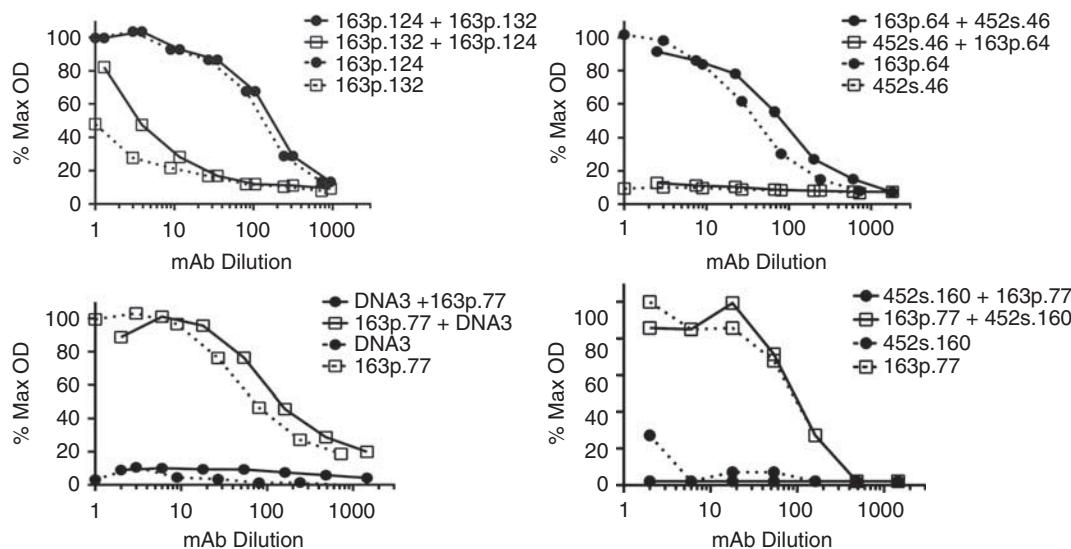
As previous investigators had concluded that anti-dsDNA mAb binding to BM was consequential to nucleosome contamination of hybridoma supernatants and purified mAbs,<sup>10</sup> we performed co-incubation assays to ensure that differential binding of anti-dsDNA mAbs to BM was not simply a consequence of contaminating chromatin in some, but not all, hybridoma supernatants. When hybridoma supernatants of mAb pairs 163p.132 and 163p.124, 452s.46 and 163p.64, 163p.77 and DNA3, and 163p.77 and 452s.160 were assayed for binding to BM, only the mAb that bound to BM in the individual assays, 163p.124, 163p.64, and 163p.77, bound to BM when co-incubated with a non-BM-binding mAb (Figure 1 and Table 2). MAb 163p.132 does bind BM, but with 100- to 500-fold less relative affinity than mAbs 163p.64, 77, and 124. The results in Figure 1 corroborate the conclusion that anti-DNA mAb binding to BM is independent of dsDNA or chromatin.

MAb 163p.64 was tested by direct enzyme-linked immunosorbent assay (ELISA) for binding to individual components of BM, including laminin, perlecan, entactin, and agrin. The mAb bound perlecan, entactin, and agrin (59, 250, and 220 ng IgG/ml, respectively, for 50% maximum binding) but not laminin. The recombinant agrin did not include the amino-terminal extracellular matrix interaction domains (R&D Systems). Binding to collagen IV was not tested. The results indicate that a BM-binding mAb may also bind to some but not all of the individual components of GBM.

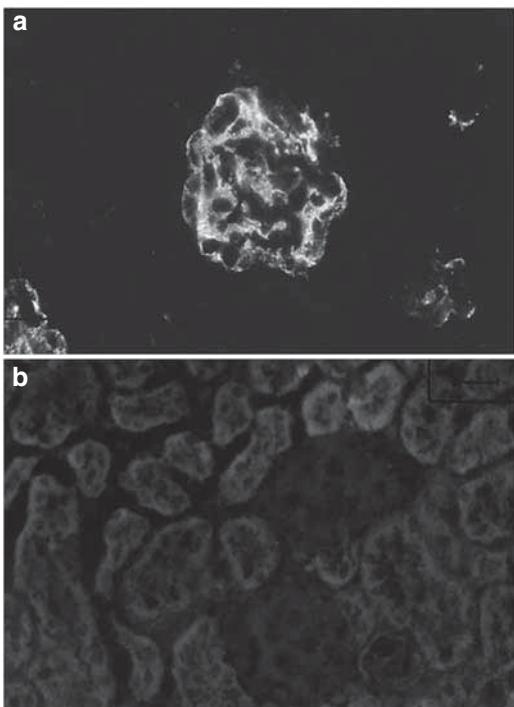
#### ***In vivo* glomerular binding of anti-DNA mAbs**

Six purified mAbs were further tested for glomerular binding when injected into non-autoimmune-prone BALB/c mice alone or co-injected with a mAb with different BM-binding potential and different IgG subclass. The co-injected pairs were 163p.77, IgG<sub>2b</sub> with 452s.160, IgG<sub>2a</sub>; 163p.64, IgG<sub>2a</sub> with 452s.46, IgG<sub>2b</sub>; and 163p.124, IgG<sub>2a</sub> with 163p.132, IgG<sub>2b</sub> (Table 2). The co-injection experiments were included to exclude the possibility that co-purified chromatin or nucleosomes influenced glomerular binding.<sup>10</sup> Only mAbs that bound BM by ELISA, 163p.77, 16p.64, and 163p.124, bound glomeruli *in vivo* when injected either alone or co-injected with a mAb of different IgG subclass (Table 2 and Figure 2). Glomerular binding was unrelated to relative affinity of the mAbs for DNA, chromatin, or mononucleosomes, or to IgG subclass.

Confocal microscopy indicated that 163p.64 mAb chronically injected over a 3-month period was co-localized with heparan sulfate proteoglycan (HSPG) in GBM and MM, but



**Figure 1 | Supernatant monoclonal antibodies (mAbs) that do not bind basement membrane (BM) when assayed alone do not bind BM when combined with supernatant mAbs that do bind BM.** Supernatant mAbs from the indicated hybridoma pairs were assayed by direct enzyme-linked immunosorbent assay (ELISA) for BM binding. Titration curves represent serial dilution of supernatants assayed independently for IgG<sub>2a</sub> or IgG<sub>2b</sub> binding to BM: solid circles, IgG<sub>2a</sub>; open squares, IgG<sub>2b</sub>; solid lines, IgG<sub>2a</sub> and IgG<sub>2b</sub> mAbs co-incubated; and broken lines, IgG<sub>2a</sub> or IgG<sub>2b</sub> mAb incubated alone. Supernatant concentrations of mAbs: 163p.124, 12.1 µg/ml; 163p.132, 34.7 µg/ml; DNA3, 29.1 µg/ml; 163p.77, 23.5 µg/ml; 163p.64, 10.0 µg/ml; 452s.46, 6.4 µg/ml; and 452s.160, 18.7 µg/ml. Maximum OD<sub>405</sub> (optical density at 405 nm) = 2.600. IgG, immunoglobulin G; Max, maximum.



**Figure 2 | Only purified basement membrane (BM)-binding anti-dsDNA monoclonal antibody (mAb) binds in glomeruli when injected intravenously into mice.** Detection of glomerular (a) IgG<sub>2b</sub>, 163p.77 but not (b) IgG<sub>2a</sub>, 452s.160 in kidney serial cryosections 24 h after co-injecting 1 mg of each purified monoclonal antibody (mAb) into a BALB/c mouse. Serial cryosections had granular IgG<sub>2b</sub> but no IgG<sub>2a</sub> within mesangial matrix (MM). Mice injected with 163p.64, IgG<sub>2a</sub> and 452s.46, IgG<sub>2b</sub> (see Figure 3a and c), and 163p.124, IgG<sub>2a</sub> and 163p.132, IgG<sub>2b</sub> had IgG<sub>2a</sub> but no IgG<sub>2b</sub> staining. Results were similar in replicate mice. IgG, immunoglobulin G.

minimally with chromatin (Figure 3a). Glomerular IgG was also co-localized with HSPG and minimally with chromatin in autoimmune BWF1 kidneys (Figure 3b). As expected, there was no glomerular binding of anti-DNA mAb 452s.46 after similar 3-month chronic injection (Figure 3c). Complement C3 was co-localized with mAb 163p.64 in glomeruli from chronically injected mice (Figure 3e). These results indicate that BM-binding anti-DNA antibodies also bind directly to MM and GBM antigens independently of DNA, chromatin, or nucleosomes and initiate complement activation. The small regions of chromatin and IgG co-localization and perlecan, chromatin, and IgG co-localization in the kidneys from BALB/c mice chronically injected with 163p.64 mAb (Figure 3a) were more numerous in kidneys from autoimmune BWF1 (Figure 3b). Those regions of co-localization may be the glomerular EDS identified by electron microscopy in the kidneys from autoimmune BWF1<sup>13</sup> and anti-DNA mAb-injected mice.<sup>11</sup>

#### Only BM-binding anti-DNA mAbs induce proteinuria in non-autoimmune-prone mice

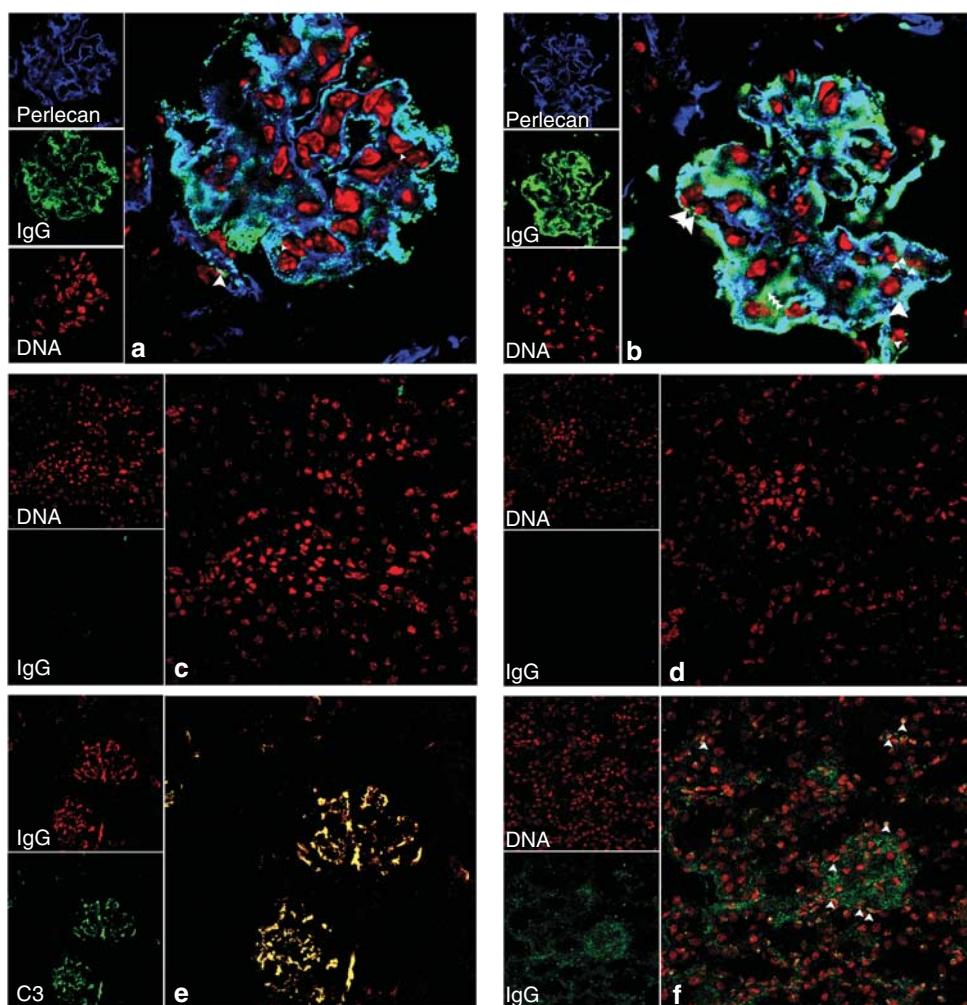
Ascites tumors were induced in non-autoimmune BALB/c mice by injecting hybridoma cells either individually or as

co-injected pairs, one producing IgG<sub>2a</sub> and the other producing IgG<sub>2b</sub> (Table 3). Only mice injected with hybridomas-producing mAbs that bound BM, 163p.64, 77, or 124 or DNA 5 or 6, had glomerular-bound IgG of the expected IgG subclass and moderate to severe proteinuria 5 days after hybridoma injection. Mice injected with 163p.64 or 163p.124, IgG<sub>2a</sub> hybridoma cells with either 452s.46 or 163p.132, IgG<sub>2b</sub> hybridoma cells had only glomerular-bound IgG<sub>2a</sub>. Glomerular IgG binding was not IgG<sub>2</sub> subclass dependent, nor was glomerular binding simply a correlate of circulating mAb titers. The average serum anti-DNA titer after 5 days was 25,568 (range 12,000–36,000) for glomerular-bound mAbs and 31,272 (range 14,000–41,000) for mAbs that did not bind in glomeruli. Only BM-binding mAbs initiated glomerular disease detected as proteinuria.

Gilkeson *et al.*<sup>31</sup> observed that mice injected with 163p.77 and 163p.132 hybridoma cells developed glomerular IgG deposits and proteinuria after the injected mice developed pronounced ascites. The results with 163p.77 are similar to those in Figure 2 and Table 3. We extended the time before killing of mice injected with 163p.132 from 5 days to 8 days, and observed results similar to those of Gilkeson *et al.* After 8 days, mice injected with 163p.132 cells had glomerular IgG deposits (Figure 3f) and moderate proteinuria (Table 3). The difference between 163p.132-injected mice at 5 and 8 days are likely a consequence of much higher mAb serum titer after 8 days. MAb 163p.132 does bind to BM, but with 300-fold less relative affinity than mAb 163p.64 (Table 2). Alternatively, 163p.132 mAb deposition after 8 days may have been due to circulating immune complexes. There was co-localization of 162p.132 mAb with DNA (yellow pixels in Figure 3f), although most of the glomerular 163p.132 IgG was not co-localized with DNA.

#### DISCUSSION

The present results demonstrate that some but not all anti-DNA mAbs bind directly to BM antigens and that direct binding of anti-dsDNA antibody to GBM or MM is critical for the initiation of experimental lupus nephritis. Glomerular binding of IgG and complement, and the initiation of glomerular disease, identified as proteinuria, were independent of mAb binding to DNA or chromatin whether the mAbs were injected or produced *in situ*. MAb binding to GBM and MM was correlated with relative affinity for dsDNA, but independent of binding to dsDNA or chromatin. Only anti-dsDNA mAbs that bound BM antigens bound to GBM and MM *in vivo*. These results and conclusion are consistent with previous reports that anti-dsDNA antibodies may initiate glomerulonephritis after binding directly to glomerular antigens.<sup>18–24</sup> The results and conclusion contrast with results<sup>10–14,16,17,32</sup> interpreted to indicate that anti-DNA antibodies can only bind to GBM or MM as immune complexes of anti-DNA antibody and nucleosomes or by binding to chromatin already bound to GBM or MM.<sup>28–30</sup> The results from co-injection of mice with a hybridoma producing a BM-binding anti-DNA mAb with a hybridoma



**Figure 3 | Confocal micrographs of kidney cryosections from an autoimmune BWF1 mouse or BALB/c mice injected with purified anti-DNA monoclonal antibody (mAb).** (a, e) One hundred micrograms of 163p.64 mAb administered twice weekly for 3 months; (b) uninjected BWF1; (c) 100 µg 452s.46 mAb administered twice weekly for 3 months; (d) uninjected BALB/c; and (f) BALB/c with 163p.132 hybridoma-induced ascites 8 days after hybridoma injection. Images a and b show chromatin as red, perlecan in glomerular basement membrane (GBM) and mesangial matrix (MM) as dark blue, and immunoglobulin G (IgG) as green. Co-localization of IgG with heparan sulfate proteoglycan (HSPG) is clearly identified as turquoise; co-localization of IgG with chromatin, yellow; and co-localization of IgG and chromatin with HSPG, white. The large white arrowheads in a and b indicate areas of IgG, chromatin, and perlecan co-localization. Small arrowheads indicate IgG and chromatin co-localization. Image e shows IgG as red and C3 as green with co-localization of 163p.64 mAb and C3 as yellow. Confocal images a and b: 512 pixels<sup>2</sup>, 180 nm/pixel (92 µm<sup>2</sup>), optical sections collected at 0.6-µm intervals; (c-e) 512 pixels<sup>2</sup>, 450 nm/pixel (230 µm<sup>2</sup>), optical sections collected at (c-e) 0.8 µm intervals and (f) 0.5 µm. All images are from optical sections near the center of respective z-stacks. Replicate mice yielded similar results.

producing a non-BM-binding anti-DNA mAb are difficult to reconcile with the previous interpretation. MAbs produced by the co-injected hybridomas had similar relative affinity for DNA, nucleosomes, or chromatin, but only the MAbs that bound BM also bound glomeruli *in vivo*. The results cannot be explained by potential absence of circulating nucleosomes or chromatin in non-autoimmune-prone BALB/c mice. Circulating or glomerular-bound chromatin or nucleosomes, including that released from necrotic or apoptotic hybridomas, would have been equally accessible to the two MAbs.

The present results may explain why autoimmune female BWF1 transgenic for V<sub>H</sub> of the 3H9 anti-DNA mAb<sup>33,34</sup> do not develop nephritis.<sup>35</sup> 3H9 mAb binds DNA and

chromatin<sup>36</sup> but does not bind BM. Autoimmune, 3H9 V<sub>H</sub> transgenic BWF1 had similar serum IgG<sub>2a/b</sub> anti-DNA titers as non-transgenic BWF1 of similar age but did not develop proteinuria even after 1 year of age. Similar outcome was reported for D42 V<sub>H</sub><sup>37</sup> and 3-32 V<sub>H</sub><sup>38</sup> transgenic BWF1. Non-transgenic, female BWF1 invariably produce anti-DNA autoantibody and develop glomerulonephritis with proteinuria by 10 months of age.<sup>1</sup> BALB/c mice injected with the 3H9 hybridoma had relatively low glomerular immuno-fluorescence and disease scores compared with the mice injected with 163p.77 or 163p.132 hybridomas.<sup>31</sup> The majority of anti-DNA hybridomas from V<sub>H</sub>3H9 transgenic BWF1 had V<sub>H</sub>3H9 H chains.<sup>39</sup> It is likely that those MAbs could not bind BM and could not initiate disease.

**Table 3 | Hybridomas producing BM-binding mAb induce proteinuria**

Hybridoma(s) injected <sup>a</sup>	mAb isotype	Glomerular isotype <sup>b</sup>	Days <sup>c</sup>	Anti-DNA serum titer (2a/2b) <sup>d</sup>	Proteinuria (mg/dl) <sup>e</sup>
163p.64	2a	2a	4	1601/<90	100
		2a	5	11 842/<90	300
163p.77	2b	2b	5	<90/36 000	100
		2a	5	24 000/<90	100
163p.124	2a	2a	5	36 000/<90	100
DNA5	2a	2a	5	36 000/<90	100
DNA6	2a	2a	5	36 000/<90	100
452s.46	2b	None	5	<90/28 024	<30
DNA3	2a	None	5	36 000/<90	<30
163p.132	2b	~2b <sup>f</sup>	5	<90/32 938	<30
		2b	8	<90/>200 000	100
163p.64+163p.132	2a+2b	2a	4	2578/4546	100
		2a	5	24 704/25 202	300
163p.77+DNA3	2b+2a	2b	5	36 000/36 000	30
163p.124+163p.132	2a+2b	2a	5	24 000/24 000	100
163p.64+452s.46	2a+2b	2a	5	12 001/41 470	100

Abbreviations: BM, basement membrane; mAb, monoclonal antibody.

<sup>a</sup>Ten mice per group were injected with the indicated hybridomas on day 0 and monitored daily for proteinuria. Two mice per group were terminated daily. Results are presented from one mouse in each group. Similar results were obtained with the other mouse in each group on the respective day.

<sup>b</sup>The subclass of IgG detected within serial kidney cryosections was determined by immunofluorescence from kidneys excised on the indicated days after hybridoma injection.

<sup>c</sup>The number of days after injection of hybridoma cells.

<sup>d</sup>Serum IgG<sub>2a</sub> and IgG<sub>2b</sub> anti-DNA titers were determined on the indicated days after hybridoma injection.

<sup>e</sup>Proteinuria measured on the indicated days after hybridoma injection.

<sup>f</sup>Weak immunofluorescence only slightly above background. Two mice were separately injected with 163p.132 in a later experiment. Sera and kidneys were collected and proteinuria was measured 8 days after hybridoma injection. See Figure 3f.

Essentially three experimental systems have described nucleosome-dependent glomerular binding of anti-DNA antibodies. Schmiedeke *et al.*<sup>32</sup> and Termaat *et al.*<sup>17</sup> allowed soluble DNA to bind to histones after the histones were perfused into kidneys or added to isolated glomeruli or GBM. Anti-DNA mAb bound to the immobilized DNA but not to GBM, histone-coated GBM, or DNA added to GBM. Although interesting, the experiments do not accurately reflect the physical and chemical properties of intact nucleosomes, nor how nucleosomes or chromatin may interact with GBM or MM. Kramers *et al.*<sup>10</sup> reported that purified anti-DNA mAbs perfused into kidneys may only bind in glomeruli as immune complexes with histones or nucleosomes, presenting as example mAb 32. Nucleosomes in the immune complexes were presumed to promote binding to GBM through histone-dependent charge interaction. Nucleosomes in physiological saline have a net negative charge with more exposed acidic than basic regions.<sup>40,41</sup> The basic termini of H2B and H3 that protrude from the octamer cores through the DNA superhelix bind with the acidic patches on the octamer surface of consecutive nucleosomes and with linker DNA to organize the nucleosomes into chromatin.<sup>40,42</sup> Nucleosomal organization into chromatin precludes surface availability of positive charge contributed by histones.<sup>41</sup> The net charge of the GBM lamina rara interna and externa initially accessible to chromatin or nucleosomes is anionic<sup>43,44</sup> and unlikely to promote binding. Although nucleosomes bound isolated collagen IV, laminin,<sup>15</sup> and agrin<sup>16</sup> on laboratory sensor chips, radiolabeled nucleosomes<sup>45,46</sup> were rapidly cleared from blood into the liver with

insignificant localization to kidneys unless nucleosome injections were preceded by injection of soluble histones.<sup>45</sup> DNA-anti-DNA immune complexes were rapidly cleared from the circulation in a similar manner.<sup>47,48</sup> Perfusion into the renal artery<sup>10</sup> would bypass initial circulation to the liver. An alternative explanation for why mAb 32-nucleosome immune complexes bound GBM, but mAb alone did not, might be that the mAb 32 in nucleosome immune complexes had increased relative avidity for GBM. The mAb 32-nucleosome immune complexes were created at 15:1 molar ratio of mAb to mononucleosome.<sup>10</sup> Multiple unbound antibody combining sites in mAb 32-nucleosome immune complexes prepared in antibody excess may have created higher avidity of the complexes for GBM than mAb 32 alone. The DNA, nucleosome, and BM-binding characteristics of mAb 32 were similar to those for mAb 163p.132 in the present study. MAb 163p.132 bound glomeruli only after reaching a serum concentration of ~10 mg/ml. MAbs 163p.64 and 163p.77, which bind with high relative affinity to BM, both bound glomeruli at serum concentrations of ≤720 µg/ml. MAb 163p.132 binds BM, but with low relative affinity. Alternatively, the additional 3 days of 163p.132 hybridoma growth from 5 to 8 days may have produced sufficient chromatin or nucleosomes from dying cells to produce immune complexes, likely in mAb excess. There was more glomerular co-localization of DNA with 163p.132 mAb than with the BM-binding 163p.64 mAb.

GBM-associated EDS in kidneys from nephritic BWF1,<sup>13</sup> nephritic lupus patients,<sup>12</sup> and BALB/c mice chronically injected with an anti-DNA mAb<sup>11</sup> contained both chromatin



**Figure 4 | Hypothetical mechanism for the initiation of lupus nephritis by basement membrane (BM)-binding anti-double-stranded DNA (dsDNA) antibody.** The stage I to II transition is likely to be reversible.<sup>62</sup> The stage II to III transition associated with the progressive accumulation of antibody and chromatin into immune complexes will eventually reach a threshold for which the immune complex deposition is no longer reversible. This stage would yield chronic inflammation and lupus nephritis. Electron-dense substance (EDS)<sup>11,12,27</sup> is predicted to be formed by the stage II to III transition. —, GBM or MM; chromatin; Y, BM-binding anti-dsDNA; Y, non BM-binding anti-dsDNA; \*, activated complement.

and IgG. The EDS chromatin was presumed to have originated from mesangial cells undergoing apoptosis.<sup>13</sup> The released chromatin was presumed to bind GBM and present target antigens to chromatin-binding antibody. Caspase 3-positive mesangial cells were detected in kidneys from nephritic but not pre-nephritic BWF1,<sup>13</sup> and chromatin was never detected in EDS that did not contain IgG as well.<sup>11,13</sup> Direct binding of nucleosomes or chromatin to GBM was not tested. If chromatin binding to GBM determines when and where anti-DNA antibody binds GBM to initiate EDS, mAbs with similar relative affinity for chromatin should have similar potential to initiate nephritis, which our results show not to be true. Nucleosomes do not bind GBM as discussed above. The recent morphological studies have elegantly refined our understanding of glomerular EDS in nephritic kidneys<sup>12–14,16,49</sup> but fail to clarify how EDS are initiated in lupus nephritis.

Earlier experiments determined that small lattice immune complexes prepared with a cationized antibody were retained within glomerular subendothelial EDS but persisted only when they were able to form larger lattice immune complexes.<sup>50</sup> Glomerular mAb deposition in the present study was independent of mAb isoelectric point or immune complexes. Immune complex deposition in GBM can also be initiated by GBM-binding antibody that also binds circulating free antigen to produce immune complexes and EDS.<sup>51</sup> Our results are most consistent with this latter mechanism to explain how anti-DNA antibody can initiate glomerular EDS (illustrated in Figure 4). The initial event toward glomerular IgG binding and initiation of EDS is direct binding of anti-DNA antibody to GBM or MM (Figure 4 I). Complement activation and the ensuing inflammation could provide a source for locally released oligonucleosomes. If the locally released oligonucleosomes are bound by GBM or MM-bound antibody (Figure 4 II),<sup>51</sup> both BM-binding and non-BM-binding anti-DNA antibodies could bind the progressively accumulating complex and induce more complement activation, inflammation, and oligonucleosome release (Figure 4 III). Reduced glomerular DNase I would contribute to and

accelerate stage III (Figure 4).<sup>52</sup> The progressive accumulation of immune complexes would eventually produce chronic inflammation and lupus nephritis. BM-binding and non-BM-binding anti-DNA mAbs were not co-localized in mice 5 days after co-injection with respective hybridomas. There may have been insufficient circulating oligonucleosomes or chromatin from the ascites tumors to generate the glomerular complexes depicted in Figure 4 II. Similarly, 3H9 transgenic BWF1 may fail to develop glomerulonephritis not only because the transgenic anti-DNA antibody cannot bind glomerular antigens, but also because locally released oligonucleosomes are unavailable to create large lattice immune complexes.

Anti-DNA antibodies that bind directly to glomerular endothelial, mesangial, or other cell surface antigens can function in a manner similar to anti-DNA antibodies that bind GBM or MM.<sup>19–21,23,53–55</sup> Cell-bound anti-DNA antibodies can initiate inflammation by directly altering cell function, inducing apoptosis or necrosis, or interrupting cell-cell or cell-matrix interactions.<sup>6,56</sup> Oligonucleosomes released from apoptotic or necrotic cells as a consequence of the induced inflammation can form large lattice immune complexes locally,<sup>50</sup> which persist as subendothelial EDS.<sup>13</sup>

Our results do not exclude the potential for GBM binding of circulating nucleosome-antibody immune complexes<sup>10</sup> or antibody binding to GBM chromatin, but they do indicate that neither is necessary for BM-binding anti-DNA antibody to bind GBM or MM.<sup>21,23,52</sup>

We did not directly test whether injected anti-DNA mAbs would bind differently in nephritic or pre-nephritic BWF1 kidneys compared with BALB/c mouse kidneys. Confocal images of IgG co-localization with GBM and MM in the kidneys from 9-month-old BWF1 were similar to those from BALB/c mice chronically injected with BM-binding anti-DNA mAb.

The present results provide additional insight to explain why lupus patients with similar serum antibody to dsDNA or nucleosomes can have different antibody-dependent disease outcomes.<sup>6</sup>

## MATERIALS AND METHODS

### Mice

BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained within the UTHSC Laboratory Animal Care Unit. All experimental protocols were approved by IACUC.

### Antibodies and antigens

The generation, DNA specificity, and V-region sequences for the mAbs used in these studies have been described.<sup>34,57–59</sup> All hybridomas were derived from autoimmune (NZB × NZW)F<sub>1</sub> mice (BWF1), except for 3H9,<sup>34</sup> which was provided by Drs M Weigert and M Radic (Chicago, IL and Memphis, TN). Matrigel (BM; BD Biosciences, Bedford, MA) is a soluble basement membrane matrix of laminin, collagen IV, HSPG, and entactin (nidogen 1). Only high-molecular-weight bands corresponding to laminin, collagen IV, entactin, and HSPG were detected after high-sensitivity staining of an SDS-PAGE of 12.5 µg of Matrigel.

DNA, ssDNA, and dsDNA were prepared as described.<sup>58</sup> Chromatin and mononucleosomes were isolated from mouse liver or from cultured P3x63-Ag8.653 cells as described.<sup>60</sup> Perlecan (HSPG2) and heparan sulfate were purchased from Sigma-Aldrich (St Louis, MO), and recombinant human nidogen (entactin) and C-terminal recombinant rat agrin from R&D Systems (Minneapolis, MN). Agrin is a heparan sulfate proteoglycan in GBM.<sup>61</sup> Biotinylated goat anti-mouse IgG, IgG<sub>2a</sub>, and IgG<sub>2b</sub>, fluorescein isothiocyanate (FITC)-goat anti-mouse IgG, and FITC-streptavidin were purchased from Southern Biotechnology (Birmingham, AL); alkaline phosphatase-streptavidin from Jackson Immunoresearch Laboratories (West Grove, PA); biotinylated rat anti-perlecan mAb (clone A7L6) from Lab Vision (Thermo Fisher Scientific, Fremont, CA); Alexa 546-streptavidin and TO-PRO3 DNA dye from Molecular Probes (Invitrogen, Carlsbad, CA); and anti-C3-FITC from BD Bioscience.

### MAb isolation

MAbs were isolated from hybridoma supernatants by affinity chromatography on protein G-Sepharose 4B (Invitrogen) essentially as described.<sup>10</sup> MAbs were eluted with glycine-HCl, pH 2.8, and immediately neutralized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of eluted MAbs stained with a high-sensitivity Coomassie (Biorad, Hercules, CA) yielded bands corresponding only to immunoglobulin H and L polypeptides. DNA was not detected in purified MAbs by ethidium bromide staining after agarose electrophoresis, but was detected in the high-salt eluate.

### ELISA for DNA, chromatin, nucleosome, and BM binding

Direct and competitive ELISAs for DNA binding were performed as described.<sup>59</sup> ELISAs for mAb binding to chromatin, nucleosomes, BM, and the BM constituents HSPG, heparan sulfate, and entactin were performed identically to the direct DNA ELISA. Plates (Immulon I, Thermo-Fisher) were coated with DNA, chromatin, or mononucleosomes at 1 µg/well DNA; 1/250 dilution of Matrigel, ~5 µg/well, estimated as 2.8 µg/well laminin, 1.5 µg/well collagen IV, 0.4 µg/well entactin, and 0.25 µg/well HSPG (BD Bioscience assay); or 0.2 µg/well of purified BM proteins. Bound IgG from serially diluted supernatant, purified mAb, or serum antibody were detected as described.<sup>59</sup> A biotinylated rat anti-laminin mAb (clone A5; Neomarkers, Thermo Fisher Scientific, Kalamazoo, MI) was used as a positive control for the anti-BM ELISA. Statistical analyses were performed with PASW Statistics 18 (SPSS Statistics, IBM, Armonk, NY).

### In vivo glomerular binding of anti-DNA mAb and measurement of proteinuria

BALB/c mice, 8–12 weeks old, were injected once intravenously with 1 mg of a single, purified mAb or 1 mg each of two purified MAbs, one IgG<sub>2a</sub>, the other IgG<sub>2b</sub>. After 24 h, injected mice were killed and their kidneys were removed and snap-frozen in optimum cutting temperature embedding medium (Tissue-Tek, Miles Laboratories, Elkhart, IN). Serial 1-µm cryosections were fluorescently stained with biotinylated goat anti-mouse IgG<sub>2a</sub> or IgG<sub>2b</sub> and FITC-streptavidin. In separate experiments, mice were chronically injected with 100 µg per intraperitoneal injection of a single mAb twice weekly for 3 months or injected intraperitoneal with hybridoma cells 5–7 days after intraperitoneal injection with 0.5 ml pristane (Sigma). The hybridoma injection consisted of 10<sup>7</sup> cells from one hybridoma or 10<sup>7</sup> cells each from two hybridomas, one producing IgG<sub>2a</sub> and the other producing IgG<sub>2b</sub>. Kidneys were removed and embedded for cryosection after 3 months chronic injection of purified mAb or

1–5 days after hybridoma injection. Serial cryosections from the same kidney were stained for detection of mouse IgG<sub>2a</sub> or IgG<sub>2b</sub>. For confocal microscopy, 4–12 µm cryosections were stained with TO-PRO3 for DNA, goat anti-mouse IgG-FITC, and rat anti-perlecan and streptavidin Alexa 546, or anti-C3-FITC and biotinylated goat anti-mouse IgG and streptavidin-Alexa 546. Confocal images were collected with a Zeiss LSM510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY). Proteinuria was measured with Ames Uristix (Miles) according to the manufacturer's instructions.

### DISCLOSURE

All the authors declared no competing interests.

### ACKNOWLEDGMENTS

The research was supported by NIH NIAID Grant AI26833, NIH NCRR Grant RR301812, and a grant from the UTHSC Center of Excellence for Diseases of Connective Tissue. The authors acknowledge Ryle Holder for technical assistance in mAb purification, Tim Higgins for help in figure preparations, Dr Michael Madaio for help in interpreting glomerular immunofluorescence, and the Microbiology, Immunology, and Biochemistry Confocal Microscope Facility for assistance with confocal microscopy. We also acknowledge Drs David Isenberg, Marc Monestier, Marko Radic, and Ole Petter Rekvig for critical comment on the manuscript.

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