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Lack of Chromatin and Nuclear Fragmentation In Vivo Impairs the Production of Lupus Anti-Nuclear Antibodies

Lorenza Frisoni,* Lenese McPhie,* Sun-Ah Kang,† Marc Monestier,† Michael Madaio,† Minoru Satoh,‡ and Roberto Caricchio‡*

Nuclear autoantigens in systemic lupus erythematosus are thought to derive primarily from apoptotic cells, yet there is no direct evidence that interfering with apoptosis impairs the generation of lupus autoantibodies. Here we use a mouse model that lacks the endonuclease caspase-activated DNase (CAD), resulting in an absence of chromatin and nuclear fragmentation during apoptotic cell death. We show that in this mouse, production and release into circulation of chromatin is impaired after exposure to several apoptotic triggers, but that the absence of CAD does not interfere with upstream steps of apoptosis or immune system function. Finally we show that in CAD-mutant mice, impaired lupus autoimmunity is skewed toward known cytoplasmic components, and autoimmune toward membrane autoantigens is preserved, while autoimmunity toward chromatin and other lupus nuclear targets is severely impaired or absent. We also show, as control, that the induction of experimental autoimmune encephalomyelitis is not affected by the absence of CAD. Thus, our work in vivo strongly suggests that apoptotic molecular steps during cell death generate nuclear autoantigens to sustain the specific autoimmune response in systemic lupus erythematosus. The Journal of Immunology, 2007, 179: 7989–7996.

Dying cells have been proposed as a critical reservoir of autoantigens in systemic lupus erythematosus (SLE) (1). Indeed, impaired clearance or excessive production of apoptotic cells or their debris can lead to SLE-like disease in certain mouse models (2–7). Moreover, lupus patients exhibit increased circulating apoptotic debris that correlates with disease activity (8).

Both necrotic and apoptotic cells release chromatin (9, 10). In particular, an important mechanism by which apoptotic cells are thought to produce nuclear autoantigens in SLE is through chromatin fragmentation (11), which allows subsequent fragmentation of the nucleus (12). Fragmented nuclei and their contents concentrate into characteristic blebs and bodies, which are extrusions of the cytoplasmic membrane and are of suitable size to be taken up and cross-presented by immature dendritic cells (1, 13). Finally, chromatin fragmentation allows apoptotic cells to release nuclear material such as fragmented chromatin itself and RNA/protein complexes into the circulation, two major lupus autoantigens, which provide the autoantigens that sustain an ongoing autoimmune response (14–16).

Many studies have supported, directly or indirectly, the relevance of apoptotic molecular steps, such as chromatin fragmentation, in lupus autoimmunity; however no direct in vivo evidence has yet been presented to support such a hypothesis. The apparent difficulty of creating a fragmented-chromatin free immune system has seriously limited studies in such direction. The recent development of a new mutant mouse deficient for caspase-activated DNase (CAD), also known as DNA fragmentation factor 40 (17), has offered the opportunity to study the source of nuclear autoantigens (18) in vivo. Indeed, this mouse displays an impaired ability to fragment chromatin at the internucleosomal level, to fragment the nucleus, and to induce apoptotic membrane blebbing and body forming (for a complete review see Refs. 19, 20). Nevertheless, important upstream apoptotic steps, such as exposure of membrane phospholipids, are preserved (21).

Therefore, we chose this mouse model to study, directly, the in vivo role of apoptotic molecular steps in promoting or sustaining SLE autoimmunity. We used, for our studies, an inducible model of lupus triggered by a single injection of pristane (2,6,10-tetramethylpentadecane) oil (22, 23). Pristane-induced lupus is characterized by many typical lupus features, including production of anti-chromatin Abs and other anti-nuclear Abs (ANA), polyclonal B cell activation, and mild to severe glomerulonephritis, depending on the mouse strain (22, 23). Moreover, apoptotic cell death is an important mechanism in the pristane-induced lupus model (24).

Indeed, we found that in this lupus model, in the absence of apoptotic fragmented chromatin, production of anti-chromatin, anti-small nuclear ribonucleoproteins (snRNPs), and other nucleus-directed autoantibodies was severely impaired or completely absent, while production of Abs directed at membrane lupus autoantigens, such as cardiolipin (25), was preserved. Finally, in this lupus model, the absence of chromatin fragmentation did not protect the mice from developing kidney immune-complexes deposition and mild lupus nephritis.
Thus, our work in vivo strongly suggests that apoptotic molecular steps during cell death generate nuclear autoantigens to sustain the specific autoimmune response in SLE.

Materials and Methods

Mice

CAD−/− mice were generated on the 129 background as previously described (18) and were a generous gift of Dr. Nagata (Osaka University, Japan). They were then backcrossed at least eight generations with C57BL/6 mice to eliminate the 129 background. Mice were bred and maintained in accordance with the guidelines of the University Laboratory Animal Resource Office of the University of Pennsylvania, an American Association for the Accreditation of Laboratory Animal Care accredited facility.

Induction of lupus-like autoimmunity

CAD−/+ and CAD−/− mice were injected i.p. with 0.5 ml of pristane to induce a lupus-like syndrome (22). Serum samples were collected before the injection, at 2 wk, and then monthly until the mice were sacrificed.

Induction of cell death

In vivo, systemic apoptosis was induced by exposure to 600 or 1500 Rads using a Cs-137 emission source of 220°C, washed three times with PBS and NET/Nonidet P-40, and incubated with 100 U/ml anti-mouse IgG, Fcγ-fragment (Jackson ImmunoResearch Laboratories, dilution 1:1000) in blocking buffer for 1.5 h at 22°C. After washing, the plates were developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich). OD405 was converted to units based on a standard curve produced by serial dilutions of pooled sera from MRL/Mp-lpr/lpr (MRL/lpr) mice: 1/500 dilution = 625 U; 1/12500 = 125 U; 1/62500 = 5 U; 1/312500 = 1 U; and 1/1600000 = 0.2 U. Usually, the standard is clearly positive at a 1/312500 dilution. For the ELISA to measure total Ig levels, a similar protocol was used: plates were coated with three μg/ml goat anti-mouse/kA L chain Abs in ratio 9:1, sera were diluted 1:200,000, and as secondary Ab a 1:1000 dilution of alkaline phosphatase-labeled goat anti-mouse Abs specific for IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were used. Standard curves were generated by dilution of commercially available Ig isotype standards from 100 ng/ml to 0.5 ng/ml.

Anti-cardiolipin ELISA

The cardiolipin (CL) serum levels were determined according to a previously described protocol (25). In brief, polystyrene microtiter plates (BD Biosciences) were coated with 20 μg/ml CL in ethanol. After blocking, sera were diluted 1/100 in PBS containing 1% BSA and 0.05% Tween 20 and incubated for 2 h at RT. Serial dilutions of the purified anti-CL mAb FB1 (mouse IgG2b) obtained from a 5-mo-old female (New Zealand White × BSXB) F1 mouse were used as a standard curve (25). After washing, binding was detected with goat anti-mouse IgG-alkaline phosphatase (Southern Biotechnology Associates) followed by color development with the appropriate substrate.

Immunofluorescent ANA

Staining was performed on prefixed HEP-2 cells on glass slides following manufacturer instruction (Antibodies Incorporated). In brief, mouse sera were used at 1/40 and 1/160 dilution in PBS and incubated for 30 min at RT in a humidified chamber. After washing, ANA were detected with a goat anti-mouse IgG (Fcγ specific) FITC-conjugated Ab, incubated for 30 min in the dark. Slides were washed again, dried, and mounted with Vectashield (Vector Laboratories). Images were taken with a Nikon TE300 scanning confocal microscope, equipped with Nomarski differential interference contrast optics, coupled to the Bio-Rad Radiance 2000 laser. A >60 magnification was used.

Immunoprecipitation

Autoantibodies to cellular proteins in murine sera were analyzed by immunoprecipitation of [35S]methionine/cysteine-radio labeled K562 cell extract using 5 μl of murine serum and SDS-PAGE (22, 23). In brief, cells were labeled with [35S]methionine/cysteine (93 μCi/ml), lysed in 0.5 M NaCl NET/NP40 buffer containing 0.5 mM PMSF and 0.3 treysin inhibitor units/ml aprotinin. After centrifugation, the cleared extract was immunoprecipitated with protein A-Sepharose beads coated with 5 μl of mouse sera. Immunoprecipitates were washed three times with 0.5 M NaCl NET/NP40, washed once with NET buffer, and finally analyzed with SDS-PAGE (12.5% SDS) and autoradiographed. Abs specificity was assessed using reference sera.

Flow cytometry

Single cell suspension was washed in cold PBS and FcYR was blocked with 2-4G2 Ab for 10 min on ice. The following mAbs purchased from BD Pharmingen were used for staining: Rat IgG2a PerCP or alkalophocyanin-conjugated anti-CD11c. Cells were incubated with directly labeled Abs for 30 min in the dark for 15 min at room temperature (RT). Samples were read within 1 h. In some experiments, flow cytometric scatter (forward light scatter vs side light scatter) was used as a measure of apoptotic cell death and debris generation (27, 28).

DNA isolation

Plasma samples were obtained by collecting blood in a microcentrifuge tube containing 50 IU of heparin followed by spinning at 2000 rpm for 5 min. The pellet was saved and the plasma was filtered through a 0.2 μm filter to collect debris residual cells. DNA was isolated from cells pellet supernatant or plasma using the Qiagen DNA isolation kit (Qiagen) and was analyzed by electrophoresis on a 2% agarose gel containing 0.5 μl/ml ethidium bromide.

ELISA

Anti-OVA ELISA

Mice were primed IP with 100 μg per mouse of OVA in PBS emulsified with CFA. To assess the titer of specific anti-OVA Abs produced, microtiter plates were coated O/N at 4°C with 100 μl of 10 μg/ml OVA (Sigma-Aldrich) solution in 0.05 M carbonate buffer. All washes were performed in PBS/0.05% Tween 20. To avoid nonspecific binding, plates were incubated in 3% BSA in PBS/0.05% Tween 20 solution for 1 h at RT. Sera were diluted 1:2000 in PBS/Tween and 50 μl were incubated for 90 min at RT. Dilutions of a monoclonal anti-OVA Ab (Sigma-Aldrich) were used to generate a standard curve. After washing, a secondary F(ab′)2 goat anti-mouse IgG (Fcγ), alkaline phosphate conjugated (Jackson ImmunoResearch Laboratories) was used at 1:10000 dilution in PBS/Tween20.
CAD demethylation was detected in either cell or supernatant extracts from mice treated with either anti-Fas Ab. DNA was extracted from both cell pellet and supernatant, and run on a 2% agarose gel. H&E staining was performed and a previously described method was used to assess the disease severity. A grade higher than 2+ is considered disease (31).

**Statistical analysis**
Mann-Whitney or Wilcoxon matched pair test and \( \chi^2 \) test were used to determine the statistical significance of differences in values and frequency between groups, respectively. Analyses were conducted with GraphPad Prism 4.0x software for Mac (GraphPad).

**Results**
Apoptotic chromatin and nuclear fragmentation, and formation of apoptotic bodies are absent in CAD/−/− mice

Release of nuclear contents during apoptotic cell death has been suggested as a fundamental step to provide autoantigens in SLE (2). To demonstrate the validity of the CAD−/− mouse as a model in which nuclear contents are not released, we induced apoptosis in vitro in splenocytes from CAD+/+ and CAD−/− mice. Intracellular chromatin fragmentation and its extracellular release were evaluated over time by the presence of DNA laddering, a direct measure of fragmented chromatin (11) (Fig. 1A). Results in Fig. 1A show that, in contrast to CAD+/+ splenocytes, no DNA laddering (magnified inset). C. Splenocytes isolated from CAD+/+ and CAD−/− mice were treated to induce cell death and incubated overnight (serum-free medium for starvation; 20 mJ/cm² for UV B irradiation). In CAD+/+ mice, a large population of cells with low forward and side scatter (forward light scatter and side light scatter) indicates that the cells underwent fragmentation into smaller blebs and bodies (\( p < 0.05 \) for both treatments). This population is less represented in CAD−/− mice, showing that cellular breakup does not occur in vitro. D. Mice received 600 rads of \( \gamma \) radiation to induce systemic apoptosis in vivo. Thymocytes and splenocytes from CAD+/+ and CAD−/− mice were evaluated for DNA fragmentation and nuclear appearance before and after the \( \gamma \)-irradiation. CAD+/+ and CAD−/− mice were treated with either \( \gamma \)-irradiation or LPS/IFN-\( \gamma \)-Galactosamine. E. CAD+/+ and CAD−/− mice were treated with either \( \gamma \)-irradiation or LPS/IFN-\( \gamma \)-Galactosamine. F. Plasma DNA was collected from 10 CAD+/+ and 10 CAD−/− mice 8 h after \( \gamma \)-irradiation. Plasma was pooled, filtered to eliminate residual cells, and DNA was extracted. DNA fragments were released in the circulation of CAD+/+ mice but were not detectable in CAD−/− mice.
was detectable in the CAD−/− splenocytes at any time-point, indicating that intracellular chromatin fragmentation is impaired in this mouse. Moreover, apoptotic chromatin was detected only in the supernatant from CAD+/+ splenocytes, while in the CAD−/− splenocytes there was no detectable DNA laddering, even 24 h after the induction of cell death.

Apoptotic bodies concentrate nuclear content, expose autoantigens, and are of the appropriate size for uptake by professional APCs (13). For these reasons, apoptotic bodies are an important source of autoantigens in lupus. We therefore evaluated whether CAD−/− mice showed an equal capacity of binding for annexin V (Fig. 1B, left panel), an important marker of membrane flipping during apoptotic cell death (34). These results, along with previous published data (35), suggest that the impairment of DNA fragmentation does not affect the triggering of phagocytosis by professional scavengers, one of the basic features of apoptosis (35, 36). Apoptotic splenocytes in both mice also showed similar caspase-3 activation (Fig. 1D, right panel), demonstrating comparable activation of this fundamental apoptotic molecular step (37).

We also evaluated chromatin fragmentation in vivo using gamma-irradiation and D-galactosamine/LPS to induce apoptosis. DNA laddering was used to assess intracellular fragmentation of apoptotic chromatin in the tissues and the circulation. Several organs were harvested before and after the apoptotic stimuli. As shown in Fig. 1E, in CAD+/+ mice, massive chromatin fragmentation was induced in the thymus, spleen, and bone marrow by γ-irradiation, and by D-galactosamine/LPS in the liver (septic shock). In contrast, no in vivo fragmentation was detectable in the CAD−/− mouse. To confirm that the lack of chromatin fragmentation also leads to impaired release of intranuclear components, DNA laddering was evaluated in plasma from CAD+/+ and CAD−/− mice 8 h after γ-irradiation treatment. Again, circulating fragmented chromatin was detected only in CAD+/+ mice, demonstrating that in CAD−/− mice, extra cellular release of nuclear content is absent or extremely reduced (Fig. 1F).

### Table I. Lymphocyte subpopulation in CAD−/− mice

<table>
<thead>
<tr>
<th>Lymphocyte Subpopulation</th>
<th>CAD+/+</th>
<th>CAD−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>46.4 ± 1.5</td>
<td>44.0 ± 5.9</td>
</tr>
<tr>
<td>B220</td>
<td>57.4 ± 5.0</td>
<td>53.7 ± 1.0</td>
</tr>
<tr>
<td>CD3</td>
<td>26.0 ± 5.3</td>
<td>33.9 ± 3.2</td>
</tr>
<tr>
<td>CD4</td>
<td>17.3 ± 0.8</td>
<td>18.1 ± 3.7</td>
</tr>
<tr>
<td>CD8</td>
<td>11.3 ± 2.5</td>
<td>13.6 ± 1.2</td>
</tr>
<tr>
<td>CD11b</td>
<td>9.4 ± 3.5</td>
<td>7.12 ± 0.3</td>
</tr>
<tr>
<td>CD19/CD21+</td>
<td>10.4 ± 1.0</td>
<td>12.3 ± 3.5</td>
</tr>
<tr>
<td>CD19/CD23+</td>
<td>66.6 ± 8.2</td>
<td>67.7 ± 3.6</td>
</tr>
<tr>
<td>CD11b/CD11c-</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CD19/MHCII+</td>
<td>60.6 ± 19.1</td>
<td>55.2 ± 10.3</td>
</tr>
<tr>
<td>CD19/CD80+</td>
<td>6.3 ± 0.9</td>
<td>8.1 ± 2</td>
</tr>
<tr>
<td>CD19/CD86+</td>
<td>29.9 ± 1.4</td>
<td>25.7 ± 4.5</td>
</tr>
<tr>
<td>CD4/CD69+</td>
<td>72 ± 17.7</td>
<td>64.7 ± 32.3</td>
</tr>
<tr>
<td>CD8/CD69+</td>
<td>85 ± 2.2</td>
<td>80.8 ± 8.7</td>
</tr>
</tbody>
</table>

*Indicates percentage of positive cells ± SD.

Kawane et al. have previously reported that CAD null mice, as opposed to DNase II null mice, have normal thymic development (18). Nevertheless, the immune system in the absence of chromatin fragmentation has been only partially investigated (18). We therefore first determined whether CAD−/− mice differed from their CAD+/+ littermates in the general composition of immune competent cells, and then if the absence of CAD interfered with a T cell-dependent humoral immune response. No statistically significant differences in percentage were seen among numbers of splenic T cells, B cells, and macrophages from CAD+/+ and CAD−/− mice (Table I). Likewise, no differences emerged when more specific populations were targeted, i.e., follicular (CD23/CD19 positive) and marginal zone (CD21/CD19 positive) B cells (Table I). B, T, and dendritic cell activation parameters before or after stimulation in vivo were also equally represented (Fig. 2A, Table I).

Next, we tested the capacity of CAD−/− mice to mount an immune response against a foreign Ag. We immunized CAD+/+ and CAD−/− mice with OVA in CFA and evaluated the production of anti-OVA Abs by specific ELISA at 2, 3, and 4 wk after immunization. Both CAD+/+ and CAD−/− mice were able to build a vigorous immune response as indicated by the high IgG anti-OVA Ab levels as early as 2 wk after immunization (Fig. 2B). We also tested the ability of CAD−/− mice to develop EAE, a model of autoimmune disease that does not depend on apoptotic cells as a source of Ag (38). We found no statistical differences in the EAE clinical score between the CAD+/+ and the CAD−/− mice (Fig. 3). Although abnormalities are still possible, based on our results, we concluded that the absence of chromatin fragmentation does not grossly interfere with the immune functions of a normal immune system and that the absence of CAD does not prevent development of EAE.

**FIGURE 2.** CAD−/− mice have a functional immune system. CAD−/− and CAD+/+ mice were injected with LPS to induce systemic apoptosis. We investigated splenic B cell in vivo activation 24 h later by staining for activation markers. A, The statistical analysis shows no difference in up-regulation of the activation marker CD69 between the CAD−/− and CAD+/+ CD19 positive subsets. B, Five CAD−/− and 5 CAD−/− mice were immunized with 100 μg of OVA in PBS emulsified with an equal volume of CFA. Mice were bled at the times indicated and sera were tested by ELISA for anti-OVA Ab levels. CAD−/− mice responded to the immunogenic challenge as well as CAD+/+ mice, as shown by the comparable levels of Abs produced (p = n.s.).
Induction of SLE-like autoimmunity disease promotes comparable increases of Ig levels in both CAD+/+ and CAD−/− mice

The mineral oil pristane induces an autoimmune syndrome similar to SLE when injected in healthy mice, accompanied by a B cell polyclonal response to the initial pristane challenge. As shown in Fig. 4, in this experiment, the levels of all Ig subclasses increased after pristane injection in both CAD+/+ and CAD−/− mice. Although some degree of variability existed from mouse to mouse in each group, the only statistical difference detectable between the two groups before or after treatment (p > 0.05) was in IgA levels, which was higher in the CAD−/− mice (p = 0.014). Similar results were obtained in the other two experiments. These results demonstrate that CAD−/− mice reacted as expected, with a B cell polyclonal response to the initial pristane challenge.

CAD−/− mice challenged with pristane fail to develop lupus autoantibodies toward nuclear components but maintain autoimmunity toward membrane phospholipids

To determine in vivo the relevance of apoptotic chromatin fragmentation and the subsequent nuclear fragmentation, apoptotic body formation, and release of nuclear contents into the circulation, we investigated the occurrence and specificity of pristane-induced lupus autoantibodies in CAD+/+ and CAD−/− mice.

We first tested anti-chromatin Abs by ELISA in CAD+/+ and CAD−/− mice 4 mo after pristane injection. Although the levels of autoantibodies were overall low, although, CAD+/+ mice showed a statistically significant increase in anti-chromatin Abs (p = 0.0078), while CAD−/− mice did not (p = 0.9375) (Fig. 5A). As expected, both CAD+/+ and CAD−/− mice were negative for anti-dsDNA Abs (data not shown) (23). To determine whether the absence of chromatin and nuclear fragmentation impaired the generation of other lupus nuclear-targeted autoantibodies, we used IP (23). Radiolabeled K562 cell extract was incubated with sera 6 mo after pristane injection. Sera were obtained in three separate experiments from a total of 27 CAD+/+ and 24 CAD−/− mice. The results showed that 12 of 27 CAD+/+ mice but only 3 of 24 CAD−/− mice became positive for known lupus autoantibodies (Fig. 5B, Table II, p < 0.05). Autoantibodies toward nuclear components such as the snRNPs, the pathognomonic target of lupus autoantibodies, and NF110/90/45 complexes were identified only in CAD+/+ mice (p < 0.05). In contrast, autoantibodies against cytoplasmic components such as Su and signal recognition particle were generated in both CAD−/− and CAD+/+ mice (Fig. 5B and Table II, p = n.s.).

To confirm and extend these findings, we performed ANA staining on HEp-2 cells with sera from pristane-injected mice. Nine of twenty-seven CAD+/+ mice were positive for ANA, while only two of the CAD−/− mice were positive (Fig. 5C and Table II), consistently showing a reduction of autoantibodies (p = 0.01). These results confirm the impairment of humoral autoimmunity in CAD−/− mice.

Interestingly, similar to the IP results, ANA staining in the CAD−/− mice showed absence of Abs against nuclear components (p < 0.05) but also a similar prevalence for cytoplasmic targets (Table II and Fig. 5C, p = n.s.).

We have shown that the absence of CAD does not interfere with membrane phospholipid exposure during cell death (Fig. 1D). Therefore we tested whether anti-cardiolipin (aCL) Abs, lupus Abs that target membrane phospholipids exposed during cell death (25), were still generated in the absence of CAD. Indeed, 4 mo after pristane injection, both CAD+/+ and CAD−/− mice demonstrated significant increases of aCL levels (Fig. 5D, p = 0.003, respectively). Although aCL increased in CAD−/− mice, their levels were lower than in CAD+/+ mice. Perhaps the absence of nuclear fragmentation and blebbing partially interferes with CL Ag recognition but is not sufficient to impair the development of autoantibodies.

Overall, these results demonstrate that cell death molecular steps, such as fragmentation of chromatin and phospholipids exposure, interfere with the generation of specific autoantibodies. Moreover, the absence of autoantibodies to known major nuclear autoantigens in the CAD−/− mice was accompanied by autoimmunity toward cytoplasmic targets, as shown by the generation of autoantibodies to Su and SRP (Fig. 5, B and C, and Table II).

Both pristane treated CAD+/+ and CAD−/− mice develop mild kidney disease

We next evaluated renal disease in the pristane model of SLE (23). Seven months after injection, mice were sacrificed and kidneys were evaluated for the severity of nephritis and the presence of immune deposits. Glomerulonephritis was scored according to established guidelines previously published (41).

As shown in Fig. 6A, CAD−/− and CAD+/+ mice scored similarly (p = n.s.). The intensity of immune deposits was also very similar (p = n.s., data not shown). Particularly noteworthy,
since we found increased levels of IgA in the CAD−/− mice, we also stained glomeruli for IgA. However, there were comparable IgA deposits in CAD+/+ and CAD−/− mice (data not shown). Examples of kidney sections from the pristane induced lupus and spontaneous lupus murine model MRL/lpr are shown in Fig. 6B.

Discussion

We report that mice lacking the capacity of fragmenting chromatin and nuclei during apoptosis failed to generate ANA, including anti-chromatin and anti-snRNPs, following induction of an SLE-like disease.

Our data clearly indicate that CAD−/− mice are unable to fragment DNA and nuclei during apoptosis both in vitro and in vivo. We also found that chromatin is not released systemically in vivo and in the supernatant of CAD−/− cultured cells in vitro. Our results thus concur with the literature that emphasizes the role of CAD over that of other nucleases in causing chromatin fragmentation in vivo following a variety of apoptotic stimuli (42). Other critical steps of the apoptosis process, such as exposure of phospholipids and caspase-3 activation, are however intact in the absence of CAD. Since we tested both immune and nonimmune tissues, we conclude that CAD−/− mice are a suitable model to investigate autoimmunity in the absence of the generation of apoptosis-induced lupus autoantigens.

We induced lupus by injecting a single i.p. dose of pristane oil (22). In this experimental model, lupus develops within months and is characterized by production of ANAs and mild glomerulonephritis (43). Recently, it has been proposed that apoptotic cell

Table II. Nuclear and cytoplasmic Ags after pristane-induced lupus

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pattern</th>
<th>CAD+/+</th>
<th>CAD−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>Nuclear</td>
<td>12/27 (44.4)&quot;</td>
<td>3/24 (12.5)&quot;</td>
</tr>
<tr>
<td></td>
<td>NF/snRNP</td>
<td>8/27 (29.6)&quot;</td>
<td>0/24 (0)&quot;</td>
</tr>
<tr>
<td>ANA (1:160)</td>
<td>Cytoplasmic</td>
<td>7/27 (25.9)&quot;</td>
<td>3/24 (12.5)&quot;</td>
</tr>
<tr>
<td></td>
<td>Ef</td>
<td>9/27 (33.3)&quot;</td>
<td>2/24 (8.3)&quot;</td>
</tr>
<tr>
<td></td>
<td>Nucl</td>
<td>7/27 (25.9)&quot;</td>
<td>0/24 (0)&quot;</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic</td>
<td>2/27 (7.4)&quot;</td>
<td>2/24 (8.3)&quot;</td>
</tr>
</tbody>
</table>

" Percentages of positive mice.
" Statistical significance (p < 0.05).
' Not significant.
death supplies autoantigens because pristane induces cell death of lymphoid cells in the peritoneal cavity along with proinflammatory cytokines and secondary necrosis (24).

Following pristane injection, Ig levels increased in both strains, except for IgA that showed significantly higher levels in the absence of CAD. Nevertheless, the IgA increase did not result in increased IgA-specific immune complex deposition in the glomeruli (data not shown).

In the present study, the drastic reduction of ANAs in the absence of CAD cannot be ascribed to an impaired production of Abs. Indeed, we found that CAD−/− mice have a functional immune system and produce a normal Ag-dependent humoral immune response. Moreover, they are susceptible to EAE and respond to pristane by increasing serum Ig levels similarly to the response observed in +/+ mice. In contrast, we showed that CAD−/− mice also lack the capability of generating blebs (20) and bodies upon induction of apoptotic cell death. These subcellular structures can concentrate lupus autoantigens and are the most suitable material to be phagocytosed by dendritic cells (1, 13).

The absence of CAD led to the production of autoantibodies with cytoplasmic specificity as demonstrated by the immunoprecipitation studies and the ANA patterns. The lack of auto-Ab and their skewing toward a cytoplasmic repertoire is in agreement with the results obtained by Christensen et al. in lupus-prone mice that have been rendered TLR7−/− or TLR9-deficient (44, 45). In these mice, the generation of anti-snRNPs and anti-chromatin autoantibodies was dependent on the TLR triggered by the specific autoantigen (45). It is therefore possible that, in our model, a reduced triggering of TLR7 and TLR9, due to the lack of circulating Ag, contributed to the impairment of autoimmunity.

Finally, our study and those on lupus models dependent on impaired clearance of apoptotic cells raise a critical question: is there a particular cell type or tissue more prone than others to provide lupus autoantigens? Indirect evidence suggests that B lymphocytes (46) and macrophages (47) are the “culprits,” and experiments are underway in our laboratory to determine whether immune cells or other cell types are the primary suppliers of autoantigens.

In conclusion, our results strongly suggest that during apoptotic cell death and in the absence of CAD-dependent chromatin and nuclear fragmentation, specific lupus nuclear autoantibodies do not develop, indicating that apoptotic cells and their by-products are indeed a source of lupus autoantigens in vivo. Importantly, the lack of exposure to major nuclear lupus autoantigens induces the generation of different auto-Ab specificities, especially to cytoplasmic targets. Our results also show that the absence of chromatin fragmentation specifically interferes with nuclear lupus autoimmunity but does not interfere with cytoplasmic membrane lupus autoantibodies (aCL) and with other forms of autoimmunity, such as EAE. Experiments are underway to determine whether apoptotic Ags specifically generated by the cell death machinery also drive autoimmune in spontaneous murine lupus models.

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Disclosures
The authors have no financial conflict of interest.

References

FIGURE 6. CAD+/+ and CAD−/− showed similar kidney damage. Glomerulonephritis was assessed as previously described in the materials and methods. A, H&E stained sections from three groups of CAD+/+ and CAD−/− mice were scored showing comparable kidney damage. Concomitantly, the kidney involvement in the pristane model of lupus was limited for all groups and the severity of the disease was mild. B, Frozen sections of kidneys from pristane-treated CAD+/+ and CAD−/− mice were stained for immune complex deposition using an FITC-conjugated goat anti-mouse IgG (heavy and light) Ab. As positive control, kidney sections from an old MRL/lpr mouse that spontaneously developed autoimmunity and kidney disease were used. As negative control, an age-matched untreated C57Bl/6 mouse was used. H&E staining on formalin fixed kidney sections showed as well that the absence of CAD and chromatin fragmentation did not interfere with the development of renal disease.