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Accelerated Macrophage Apoptosis Induces Autoantibody Formation and Organ Damage in Systemic Lupus Erythematosus

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Increased monocyte/macrophage (Mφ) apoptosis occurs in patients with systemic lupus erythematosus (SLE) and is mediated, at least in part, by an autoreactive CD4+ T cell subset. Furthermore, autoreactive murine CD4+ T cells that kill syngeneic Mφ in vitro induce a lupus-like disease in vivo. However, it is unclear whether increased Mφ apoptosis in SLE per se is sufficient to accelerate/promote autoimmunity. We have investigated whether increased Mφ apoptosis in vivo, induced by the administration of clodronate liposomes, can exacerbate the autoimmune phenotype in NZB × SWR (SNF1) lupus-prone mice, and induce autoantibody production in haplotype-matched BALB/c × DBA1 (DBF) non-lupus-prone mice. Lupus-prone mice SNF1 mice that were treated with clodronate liposomes, but not mice treated with vehicle, developed significant increases in autoantibodies to dsDNA, nucleosomes, and the idiotypically related family of nephritic Abs IdL-SNF1, when compared with untreated SNF1 mice. Furthermore, clodronate treatment hastened the onset of proteinuria and worsened SNF1 lupus nephritis. When compared with vehicle-treated controls, clodronate-treated non-lupus-prone DBF1 mice developed significantly higher levels of anti-nucleosome and IdL-SNF1 Abs but did not develop lupus nephritis. We propose that Mφ apoptosis contributes to the pathogenesis of autoantibody formation and organ damage through both an increase in the apoptotic load and impairment in the clearance of apoptotic material. This study suggests that mechanisms that induce scavenger cell apoptosis, such as death induced by autoreactive cytotoxic T cells observed in SLE, could play a pathogenic role and contribute to the severity of the disease. The Journal of Immunology, 2006, 176: 2095–2104.

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Human systemic lupus erythematosus (SLE) is an autoimmune disease of unclear etiology that can affect multiple organs. Dysregulation of apoptosis has been implicated in the pathogenesis of SLE by increasing the source of autoantigens (reviewed in Ref. 1). Indeed, nucleosomes generated during apoptosis are crucial in the emergence of antinuclear Abs, and anti-nucleosome Abs appear to be involved in the nephritogenic process in murine lupus (2). Increases in apoptosis and altered expression of cell surface receptors and ligands involved in programmed cell death have been reported in a variety of SLE cells, including monocytes/macrophages (Mφ) (3, 4), both in vitro and in vivo (3, 5). This phenomenon correlates with disease activity (3). One of the mechanisms responsible for accelerating apoptosis of monocytes/Mφ in lupus patients is an autoreactive CD4+ T cell subset that kills autologous monocytes in the absence of added Ag (3, 5). In addition, lupus sera can induce monocyte/Mφ death by yet-uncharacterized mechanisms (6). Among scavenger-type cells in the body, Mφ have been implicated in the key functions of uptake and clearance of apoptotic and necrotic cells, with phagocytosis mediated by a variety of cell surface molecules (7).

We propose that accelerated monocyte/Mφ apoptosis in SLE increases the apoptotic load by reducing the clearance of apoptotic cells in addition to contributing to additional apoptotic material (1, 3). Previous studies suggest that high levels of apoptotic and necrotic cells, such as those seen in SLE, can also induce secondary in vivo apoptosis, with the Mφ the most sensitive to die because of their scavenger activity (8). Cell death can therefore induce additional Mφ apoptosis, which in turn leads to the release of nuclear contents, both from the engulfed cell and its own, in a cleaved form into the blood (8). However, whether the enhanced monocyte/Mφ apoptosis observed in SLE plays a pathogenic role or represents an epiphenomenon has not been clarified.

The function of Mφ in lymphoid tissues has been studied in vivo by using the liposome-mediated Mφ depletion model (9–11). Injected liposomes containing the biphosphonate clodronate (dichloromethylene bisphosphonate) kill phagocytic cells by apoptosis, by the release of this compound following uptake into phagosomes. Liposomes are readily engulfed by Mφ and, once delivered into...
phagocytic cells, lysosomal phospholipases disrupt the phospholipid bilayers of the liposomes resulting in the intracellular release of clodronate. The phosphonate accumulates intracellularly and, after exceeding a threshold concentration, the cell is irreversibly damaged and dies by apoptosis (12). In vivo, clodronate liposomes are taken selectively by MΦ, predominantly from liver and spleen (10, 13, 14). In the mouse spleen, MΦ close to the bloodstream can be depleted completely with little harm to other cell populations (9, 14).

Using this system, we have examined whether accelerated MΦ apoptosis per se is sufficient to trigger autoimmunity in non-lupus-prone mice and augment autoimmunity and organ damage in lupus-prone mice. We now report that accelerated MΦ apoptosis aggravates lupus nephritis and increases autoantibody formation in lupus-prone mice, and is associated with the development of autoantibodies in non-lupus-prone mice.

Materials and Methods

Mice and cell lines

Six- to 8-wk-old female NZB, SWR, DBA1, and BALB/c mice were obtained from The Jackson Laboratory. The F1 progeny of NZB × SWR (SNF1) and BALB/c × DBA1 (DBF1) mice were bred and aged at the State University of New York Upstate Medical University at Syracuse, Department of Laboratory Animal Resources, and shipped to the Unit of Laboratory Animal Medicine, University of Michigan, where they were housed in a specific pathogen-free facility. SNF1 mice develop a lupus like disease with autoantibody formation and severe nephritis (15). DBF1 were used as non-autoimmune haplotype-matched controls, which do not develop lupus-like features (15). Protocol was reviewed and approved by the University of Michigan’s Committee on Use and Care of Animals.

The monocytic/MΦ cells lines RAW and P388D1 were grown in DMEM with 4 mM l-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and supplemented with 10% FBS.

Preparation of liposomes

Egg phosphatidylcholine and distearoylphosphatidylethanolamine (DS (PEG2000) PE) were purchased from Avanti Polar Lipids. Cholesterol was obtained from Sigma-Aldrich. Clodronate is a gift from Roche Diagnostics. All of the liposomes were composed of egg phosphatidylcholine, DS (PEG2000) PE, and cholesterol, at a molar ratio of 1.90:1:1 and prepared using a previously established method, with some modifications (16, 17). Briefly, a chloroform solution of the above lipids, containing 10 μmol of total phospholipids, was dried in a round-bottom tube under vacuum using a rotary evaporator for 2 h, to form a thin lipid film. The lipid film was hydrated with 1 ml of isotonic HEPES-buffered saline (HBS, 10 mM HEPES, 140 mM NaCl (pH 7.4)) and vortexed thoroughly to form the liposome suspension (HBS liposomes, called vehicle liposomes). A 100 mM isotonic clodronate solution in HBS was used instead as the hydrating solution, to form the clodronate liposome suspension (clodronate liposomes). The resulting liposome suspensions were freeze-thawed four times and extruded four times, respectively, through 0.4- and 0.2-μm pore size Poretics polycarbonate filters (Osmonics Lab Products). Any un-encapsulated clodronate was removed from the liposome suspension by size exclusion chromatography using a 1 × 20-cm Sepharose CL-4B (Column) (Pharmacia) containing isotonic HBS as the elution buffer, and again monitored under argon and used in the experiments within 3 days after preparation. The liposomal phospholipid concentration was measured by a standard phosphate assay (18), and the lipid concentration of clodronate liposomes was determined using a FACSscan flow cytometer (BD Biosciences). Cells were considered apoptotic when they underwent apoptosis or necrosis was determined using a FACScan flow cytometer (BD Biosciences). Cells were considered apoptotic when they stained positive for annexin V and negative for PI, and late apoptotic or early necrotic when they stained positive for both annexin V and PI.

Injection of liposomes and characterization of MΦ apoptosis in vivo

To determine the kinetics of clodronate liposome-mediated MΦ apoptosis, 8-wk-old lupus-prone and non-lupus-prone mice were given clodronate or HBS liposome injections and then euthanized at different time points. Splenic apoptotic MΦ were identified in frozen sections by staining with rat anti-mouse CD11b (BD Pharmingen) and by TUNEL (Roche Molecular Biochemicals) following published methodology (20). The prevalence of apoptotic cells was assessed as the mean number of apoptotic cells/high-power field, and five to six high-power fields were analyzed in each mouse.

Once the kinetics of MΦ apoptosis were established, 8-wk-old SNF1 mice and 14-wk-old DBF1 mice were bimonthly tail vein injections of 0.2 ml of either clodronate liposomes or HBS (vehicle) liposomes for a total of 10–15 injections/mouse. Sera and urine samples were collected periodically. SNF1 mice were euthanized between 30 and 32 wk of age, and DBF1 mice were euthanized at 40 wk of age. At euthanasia, organs were weighed and processed for histological examination. One kidney from each mouse was fixed in 4% paraformaldehyde and processed for conventional histology, while the other kidney and the spleen were embedded in OCT and frozen by immersion in liquid nitrogen.

Immunofluorescence microscopy of tissue sections

Frozen sections of spleen (7 μm) or kidney (4 μm) were cut using a cryostat, dried to a microscopic slide, and stored at −20°C until stained. For analysis of MΦ apoptosis in the spleen and kidney, sections were dried at 37°C for 1 h, fixed in 4% paraformaldehyde in PBS, and rinsed in 50 mM Tris (pH 8.0). Splenic and kidney MΦ were identified based upon expression of the marker CD68, and cell death was evaluated by TUNEL assay. Briefly, sections were fixed in 4% paraformaldehyde in PBS for 5 min at room temperature and washed in distilled water, and apoptotic cells were labeled with tetramethylrhodamine-labeled dUTP and TdT (TMR Tunel Assay kit; Roche Biotech). Sections were rinsed once in 50 mM Tris, blocked for nonspecific binding of biotin and avidin with blocking kit from Zymed, and incubated with 0.01 mg/ml biotinylated-anti-C3-FITC (Cappel) for 1 h at 4°C. Glomerular staining was graded similarly by staining a kidney section with a 1/200 dilution of mouse IgG F(ab’)2 (Sigma-Aldrich). Sections were washed twice with 50 mM Tris, nuclei were counterstained with 10 μg/ml Hoechst 33342 (Molecular Probes) for 10 min, anti-C3-FITC was quenched, and sections were rinsed in distilled water and mounted in medium containing 3% glycerin. Sections were examined similarly by staining a kidney section with a 1/200 dilution of anti-C3-FITC (Cappel) for 1 h at 4°C. Glomerular staining was graded according to intensity on a 0 to 4+ scale (0, no staining; 4+, maximum intensity staining), and the pattern of staining (predominantly mesangial vs capillary) was recorded. Background was defined as the strongest level of staining observed in DBF1 mice, and only staining above background was considered positive. All images were acquired using a fluorescence microscope equipped with fluorescein, rhodamine, and DAPI filters (Olympus) and a digital camera. Image acquisition software settings were identical for all fluorescein or rhodamine images and processing was done using Adobe Photoshop, version 7.0.

Assessment of lupus nephritis

Proteinuria was measured at different time points during the liposome injections using Uristix 6 (Boehringer Mannheim). Proteinuria level was scored as follows: 0.5+ 15–30 mg/dl; 1+ 30–100 mg/dl; 2+ 100–300 mg/dl; 3+ >2000 mg/dl. Kidney damage was evaluated in 4-μm paraffin-embedded sections using H&E and Masson trichrome staining. All samples were evaluated by a renal pathologist (P. D. Killen) in a blinded

2096 MΦ APOPTOSIS WORSENS AUTOIMMUNITY

buffer, and stained with 5 μl of Annexin V-FITC, and 10 μl of propidium iodide (PI) (BD Pharmingen), as described previously (19). The percentage of cells that underwent apoptosis or necrosis was determined using a FACScan flow cytometer (BD Biosciences). Cells were considered apoptotic when they stained positive for annexin V and negative for PI, and late apoptotic or early necrotic when they stained positive for both annexin V and PI.

In vitro characterization of MΦ apoptosis

To verify that the clodronate liposome preparation used for the in vivo studies induced MΦ apoptosis, we initially tested its potential to induce apoptosis in vitro. RAW and P388D1 cells were treated with varying dilutions (1/20 to 1/500) of clodronate liposomes or HBS liposomes for 24–48 h, or left untreated. Cells were harvested, resuspended in Annexin V binding
manner. Mice were scored as having glomerulonephritis only when >50% of their glomeruli showed a qualitative (segmental and/or global mesangial, or proliferative) lesion on multiple sections.

**Assessment of organ damage**

Lungs, heart, and brain were fixed in 4% paraformaldehyde, and 7-μm paraffin-embedded sections were stained with H&E. For spleen, frozen sections were used for H&E staining.

**ELISA determination of total IgG, Abs to dsDNA, to ssDNA, to nucleosomes, and IdLNF1, Abs**

Only autobodytides with titers significantly elevated on more than one occasion throughout the study were considered for analysis (21). Total serum IgG concentrations were measured using commercially available kits from Alpha Diagnostic International following the manufacturer’s instructions. In brief, samples were diluted to 1/20,000. Twenty microtiter of samples or standards (ranging from 0 to 1000 ng/ml) were added to precoated wells in combination with 80 μl of sample buffer. Plates were incubated for 1 h and washed five times, 100 μl of HRP-labeled anti-mouse IgG conjugate was added, and plates were incubated for 30 min. Plates were washed five times, and TMB substrate solution was added to each well. Plates were further incubated for 15 min, and 100 μl of stop solution was added into each well and mixed. Absorbance was measured at 450 nm using a microplate reader (Biotek). All samples were within the range of standards and in the linear portion of the curve.

Anti-ssDNA and anti-dsDNA Ab titers were determined by coating Immunol 4 plates with purified calf thymus ssDNA (Sigma-Aldrich) or dsDNA (Connaught Laboratories, Montreal) (21) at 10 μg/ml. HRP-conjugated goat anti-mouse polyclonal (IgG, IgM, IgA) Ab (Sigma-Aldrich) was used as the secondary Ab, and plates were developed with Sigma Fast tablets. Controls included identical determinations performed in the absence of 25 μg/ml purified dsDNA or ssDNA as competitive inhibitor. Results are presented as the mean of triplicate determinations on sera from individual mice. Positive control included pooled ascites from 6- to 8-mo-old female SNF1 mice, which was a gift from Dr. G. Glick (University of Michigan, Ann Arbor, MI).

For detection of serum Id-3F-Ig, purified IgG eluted from the kidneys of 6- to 8-mo-old female SNF1 mice was emulsified in CFA and used to immunize rabbits as described previously (22). Anti-allotypic and anti-rheumatoid factor reactivities were removed from the rabbit sera by adsorption with myeloma Ig of all isotypic classes, and IgG was then purified using protein A affinity chromatography. The specificity of the rabbit anti-Id-3F-Ig reagent was confirmed by ELISA using plates coated with either SNF1 kidney eluate Ig or control myeloma Ig, as described (22). Nonspecific binding was not detectable in the purified rabbit anti-Id-3F-Ig reagent, indicating that the adsorptions with myeloma Ig were sufficient to render the anti-Id-3F-Ig reagent Id specific.

A direct binding ELISA was used to detect Id-3F-Ig Abs as previously described (22). Briefly, Immunol I plates (Dynatech) were coated overnight at 4°C with 1 μg/ml rabbit anti-Id-3F-Ig. IgG in 0.05 M borate buffer (pH 9.5), washed twice with PBS-1% Tween (PBS-T) and twice with PBS, and blocked for 1 h at room temperature with PBS-1% BSA. The plates were again washed twice with PBS-T; twice with PBS, and 50 μl of diluted serum was added well per well in duplicate. A standard curve was constructed using pooled aged (>24-wk-old) SNF1 female serum with 2-fold dilutions ranging from 1/50 to 1/6400 in PBS-T. The plates were incubated overnight, washed twice with PBS-T and twice with PBS, and incubated with 50 μl of goat anti-mouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim) for 2 h at room temperature. After two washes with PBS-T and two washes with PBS, 50 μl of p-nitrophenyl phosphate solution (Sigma-Aldrich) was added for 15 min, and substrate development corresponding to bound anti-mouse-IgG was determined by measuring absorbance at 405 nm using an ELISA microplate reader (Bio-Rad). A standard curve for Id-3F-Ig was constructed using the absorbance values from the diluted SNF1 sera, with the 1/50 dilution of SNF1 corresponding to 50 U of Id-3F-Ig. The Id-3F-Ig concentration in the test sera was determined from this standard curve, and the data were expressed as Id-3F-Ig units.

To verify that the reactivity was due to idiotropic recognition of Id-3F-Ig and not rheumatoid factor activity present in the sera of mice prone to autoimmune disorders, ELISA plates coated with 1 μg/ml normal rabbit IgG instead of anti-Id-3F-Ig, IgG, were incubated with pooled sera from 8-wk-old BALB/c mice, SNF1, or DBF1 test sera. SNF1 and DBF1 sera yielded OD values of ~0.2, values that were significantly lower than those attained using anti-Id-3F-Ig-coated plates (range: 0.38-2.0), and similar to those of BALB/c sera. In contrast, MRL/lpr mouse sera generated OD values >1.2, consistent with the high titers of rheumatoid factors found in this strain of autoimmune-prone mice. These results excluded the possibility that the activity of the Id-3F-Ig assay could be secondary to rheumatoid factor activity in SNF1, or DBF1.

Detection of anti-nucleosome Abs was also performed by ELISA as described previously (23, 24). In brief, mononucleosomes were obtained by micrococcal nuclease digestion of isolated mouse liver nuclei and subsequent purification with a 5-20% sucrose gradient as described (25). The sucrose fraction containing only mononucleosomes was confirmed by SDS-gel electrophoresis; the integrity of mononucleosomes was evaluated by the content of core histones via sulfuric acid extraction. Poly-L-lysine-treated plates were then coated with 1 μg/ml mononucleosomes. Mice sera were tested at a 1:1000 dilution and adsorbed Abs were detected using biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and alkaline phosphate-conjugated streptavidin. Serum from a diseased MRL/lpr mouse with a high titer of autoantibodies was also tested at 10 different dilutions from 1/250 to 1/1,280,000, as a positive control.

**Cytokine determination**

Serum TGF-β1 was measured by ELISA using a Quantikine mouse/rat porcine TGF-β1 immunoassay (R&D Systems) following the manufacturer’s instructions. TNF-α was determined using an ELISA kit from Pepro-Tech. In brief, ELISA plates were coated overnight at room temperature with 1 μg/ml affinity-purified goat anti-mouse TNF-α in PBS. The wells were washed, blocked with 1% BSA in PBS, and incubated for 2 h with samples and murine TNF-α standards. Plates were washed and incubated for 2 h with 0.25 μg of biotinylated goat Ag-affinity-purified anti-murine TNF-α. Samples were washed, incubated with avidin peroxidase (1:2000; Sigma-Aldrich) for 30 min, washed, and developed with ABTS substrate solution (Sigma-Aldrich) at room temperature. The plate was read at 405 nm with wavelength correction set at 650 nm.

**Statistical analysis**

Statistical significance was assessed using Student’s t test or Mann-Whitney U test unless otherwise noted, and data are given as mean ± SEM unless otherwise noted. Values of p were considered statistically significant at <0.05.

**Results**

**Clodronate liposomes induce MΦ apoptosis in vitro and in vivo**

Previous studies have demonstrated that clodronate-induced apoptosis is specific for MΦ (9) and is not detected in other cells, including T and B lymphocytes and neutrophils (16, 17, 26, 27). To verify that clodronate preparations induce significant MΦ apoptosis, MΦ cell lines P388D1 and RAW were incubated with different concentrations of vehicle or clodronate liposomes for 24–48 h. Clodronate liposomes, but not vehicle liposomes, caused significant P388D1 apoptosis, as measured by annexin V/PI staining (Fig. 1A). Similar results were obtained using RAW cells (vehicle liposomes, 15 ± 3.5%; clodronate liposomes, 76 ± 5.6%; untreated cells, 11 ± 4%; results representing percentage of apoptotic cells ± SEM of three independent experiments; p < 0.05 when comparing clodronate-treated cells with vehicle-treated or untreated cells; p = NS between vehicle-treated and untreated cells).

In vivo, confirming previous reports (28), the most marked and significant increase in MΦ death was detected in the spleen within 24 h postinjection (Fig. 1B). Significant apoptosis was restricted to the red pulp and marginal zone areas (Fig. 1, D and F). In addition, clodronate-treated mice displayed decreased numbers of CD11b+ MΦ in the red pulp and marginal zone areas (Fig. 1H), again consistent with previous studies demonstrating that red pulp and marginal zone MΦ decrease with a single treatment with liposome-encapsulated clodronate within 24 h postadministration (29). No differences in degree of MΦ apoptosis induced by clodronate treatment were detected between lupus-prone and non-lupus-prone mice (data not shown). Similarly, we saw no differences in the degree of MΦ apoptosis between mice treated with HBS liposomes and untreated mice.
Autoimmune and non-autoimmune mice develop higher levels of autoantibodies after clodronate treatment

**Autoimmune-prone mice.** SNF1 mice that received clodronate liposomes developed levels of anti-dsDNA Abs that were significantly higher than untreated (not shown) and vehicle-treated SNF1 mice (Fig. 2A). The increases were first observed at 8 wk postinjection when mice were ~16 wk old and were sustained throughout the study, even 4 wk after the last injection and just before euthanasia. Titers of anti-nucleosome Abs were also higher in the clodronate-treated lupus-prone mice starting at 12 wk after the first injection when mice were ~20 wk old and were also sustained throughout the study. (Fig. 2B). The pathogenic Abs deposited in the renal lesions of SNF1 mice bear idiotypic determinants collectively referred to as IdLNF1 (30). These Abs are primarily IgG and cationic in charge, characteristic of nephritogenic Abs. Increased levels of IdLNF1 Abs were transiently observed in clodronate-treated SNF1 mice, appeared rapidly following the initiation of clodronate injections (5 wk after first injection) when mice were ~13 wk old, and then decreased to levels below those of untreated or vehicle-treated mice by 13 wk after the first injection when mice were ~21 wk old (Fig. 2C). This pattern of increased IdLNF1 Ab expression is consistent with previous reports in which the ratio of CD4+ IdLNF1-reactive to CD8+ IdLNF1-reactive T cells increases allowing for enhanced IdLNF1 IgG production before deposition in the kidney and onset of nephritis. As deposition occurs, the serum levels of IdLNF1 decrease (31). No significant elevations were seen in total IgG or ssDNA Abs when clodronate-treated SNF1 mice were compared with HBS-treated or untreated SNF1 mice (data not shown).

**Non-autoimmune-prone mice.** DBF1 mice that received clodronate treatment, but not liposome vehicle controls, developed higher levels of anti-nucleosome and IdLNF1 Abs (Fig. 3, A and B, respectively). Increases in anti-nucleosome Ab titers were first detected at 10 wk postinjection when mice were ~24 wk old and elevations were sustained until euthanasia. Indeed, higher levels were detected even 4 wk after the final clodronate injection. Significant increases in IdLNF1 Abs were first detected at 10 wk postinjection when mice were ~25 wk old and lasted for 4 additional weeks. Afterward, levels decreased to similar or lower levels to the ones seen in the vehicle-treated mice. There were no differences in levels of total IgG, anti-ssDNA and anti-dsDNA Abs between the HBS-treated and clodronate-treated DBF1 mice (data not shown). Thus, administration of clodronate liposomes to the non-lupus-prone DBF1 is sufficient to induce the generation of specific autoantibodies. As expected, the levels of all autoantibodies were significantly higher in the group of SNF1 mice relative to DBF1 mice even after clodronate treatment (p < 0.05; Figs. 2 and 3 and data not shown). Total IgG levels ranged from 6 to 9 mg/ml in the DBF1 mice and from 9.5 to 12 mg/ml in the SNF1 mice, and no significant differences were observed between treated and untreated mice.

![Figure 1. Clodronate liposomes induce Mφ apoptosis in vitro and in vivo. A, P388D1 cells were treated with a 1/20 dilution of clodronate liposomes or vehicle liposomes for 24 h, or left untreated. Cells were harvested, incubated with Annexin V-FTTC and PI, and the percentage of apoptotic or early necrotic cells was determined by flow cytometry. Results represent the mean ± SEM of two independent experiments performed in P388D1 cells. The y-axis represents the percentage of cells that were either Annexin V + PI (early apoptotic) or Annexin V + PI (late apoptotic, early necrotic). Background staining on untreated cells was 17 ± 4%, and no statistical differences were detected between untreated cells and vehicle-treated cells. B–H, Non-lupus-prone mice were administered either 200 μl of clodronate or the vehicle liposomes and euthanized 24 h later. Mφ apoptosis was evaluated in the spleen by staining with anti-mouse-CD11b Ab and performing a TUNEL assay. Similar results were obtained in lupus-prone mice. B, Results represent the mean number of apoptotic Mφ nuclei at 24 h postinjection per high power field in four mice/group ± SEM. *, p < 0.05. A total of five to six high-power fields were evaluated in each mouse. C–H, Results display representative spleen microphotographs of vehicle-treated (C, E, and G) or clodronate-treated (D, F, and H) non-lupus-prone mice (n = 4/group) at 24 h post-liposome injection at ×100 or ×200 total magnification. No differences were seen between lupus-prone and non-lupus-prone mice in the capacity of clodronate liposomes to induce Mφ apoptosis. W, Representative white pulp area. Arrows point to representative red pulp area.](http://www.jimmunol.org/content/early/2017/08/31/jimmunol.1700115/F1.large.jpg)
Accelerated Mφ apoptosis increases severity of lupus nephritis

SNF1 mice develop a lethal accelerated glomerulonephritis that peaks at ~24–30 wk of age, which is due to the deposition of immune complexes, including anti-dsDNA Abs and IdLNF1 Abs (15, 32). Administration of clodronate liposomes to SNF1 mice significantly hastened the onset of nephrotic range proteinuria by several weeks relative to untreated or vehicle-treated mice (Fig. 4). This acceleration in severe proteinuria development was accompanied by a readily apparent increase in the severity of lupus nephritis by histology (Fig. 5). Indeed, when compared with untreated or vehicle-treated mice, clodronate-treated mice displayed marked increase in mesangial cellularity, mesangial matrix, cellular crescents, and capillary loop deposits, as well as an increase in focal perivascular interstitial infiltrates (Fig. 5). There was a marked increase in thickness of the capillary loops with subendothelial fuchsinophilic deposits in addition to the expansion of the mesangium. There were no significant differences in the severity of nephritis between the vehicle-treated and untreated SNF1 mice. All SNF1 mice displayed significant and equivalent deposition of immune complexes, irrespective of treatment (Fig. 6, C and D). Likewise, clodronate-treated, vehicle-treated, or untreated SNF1 mice had similar levels of CD68⁺ Mφ infiltrates within their glomeruli (Fig. 6, G and H). Mφ apoptosis was not observed in the glomeruli of SNF1 mice, indicated by the absence of TUNEL-positive cells, suggesting that the increase in organ damage was not due to increased cell death within the glomeruli. There were no differences in the histology of spleen, heart, brain, and lungs between untreated, vehicle-treated, and clodronate-treated mice (data not shown).

DBF1 mice that received clodronate did not develop any histological or clinical feature of nephritis (Fig. 5). The glomeruli of DBF1 mice displayed neither immune complex deposition nor CD68⁺ Mφ infiltrates regardless of treatment (Fig. 6, A and B, and E and F, respectively). Similarly, no histological changes were seen in the spleen, lungs, heart, or brain of DBF1 mice.

As noted above, clodronate induced splenic Mφ apoptosis and decreased the number of tissue-resident Mφ within 24 h both in lupus-prone and non-lupus-prone mice (Fig. 1). As previously described in the SNF1 model, the spleens from these mice showed significant disruption of the typical H&E staining pattern distinguishing red and white pulp (data not shown) (32). Mφ in the DBF1 and SNF1 spleens were localized predominantly within the red pulp and marginal zones as determined by staining with anti-CD68 Ab (Fig. 7), and the pattern of disruption of the splenic architecture in SNF1 mice was also appreciated using this method. Previous studies have shown that, after an initial significant splenic Mφ depletion at 24–48 h following clodronate administration, levels of these cells gradually rise and might actually exceed initial levels within 7 days postclodronate administration (29, 33, 34). Because at least 2 wk elapsed from the final administration of

Accelerated Mφ apoptosis increases autoantibody titers in lupus-prone mice.

Results represent the mean ± SEM of representative experiments. *, p < 0.05 between treatment groups. Six mice were studied per group. A. Anti-dsDNA OD readings at 405 nm absorbance in vehicle (HBS) or clodronate-treated mice at different time points. Arrows represent liposome injection time points. B. Anti-nucleosome Abs. OD readings at 405 nm absorbance in vehicle or clodronate-treated mice at different time points. Arrows represent liposome injection time points.

FIGURE 3. Accelerated Mφ apoptosis induces autoantibodies in non-lupus-prone mice. Results represent the mean ± SEM of representative experiments. *, p < 0.05 between treatment groups. Six mice were studied per group. A. Anti-nucleosome Abs. OD readings at 405-nm absorbance in vehicle- or clodronate-treated mice at different time points. Arrows represent liposome injection time points. B. IdLNF1 Abs. OD readings at 405 nm absorbance in vehicle- or clodronate-treated mice at different time points. Arrows represent liposome injection time points. C. IdLNF1 Abs. Results compare levels after vehicle or clodronate treatment at 5 and 13 wk post-initial injection (corresponding to 13 and 21 wk of age, respectively) and are expressed as units ± SEM of representative experiments. Experiments were performed in triplicate.
clodronate to euthanasia, confirming previous reports (33, 34), there was no decrease of splenic Mφ or increase in Mφ apoptosis when spleens were analyzed at that specific time point (Fig. 7 and data not shown). This finding suggests that, while the increase in the level of autoantibodies was maintained in the clodronate-treated mice, repopulation of the splenic Mφ/H9278 population occurred once clodronate injections were discontinued before euthanasia.

**FIGURE 4.** Accelerated Mφ apoptosis hastens the onset of proteinuria in lupus-prone mice. Results are presented as the mean proteinuria ± SEM measured in milligrams per deciliter on untreated, vehicle-treated, and clodronate-treated SNF1 mice (n = 6 for HBS and for clodronate-treated mice and n = 4 for untreated mice). The x-axis represents the age of the mice. *, p < 0.05 when comparing clodronate-treated mice with other groups of mice. Arrows represent liposome injection time points.

**FIGURE 5.** Accelerated Mφ apoptosis worsens lupus nephritis. Representative trichrome-stained sections of murine kidney. A and B are DBF1 mice treated with vehicle or clodronate, respectively. The glomeruli have normal size and cellularity. No differences in the cellularity or matrix of the glomeruli were discerned. C–F are representative sections of SNF1 mice treated with vehicle (C) or clodronate (D–F). Compared with the DBF1 mice, the glomeruli are enlarged and hypercellular. In C, there is expansion of the mesangium with prominent fuchsinophilic deposits and mild mesangial hypercellularity. In contrast to the vehicle-treated SNF1 mice, clodronate-treated SNF1 mice demonstrated marked thickening of the capillary loops with subendothelial fuchsinophilic deposits (D, arrowheads; E; and F) in addition to expansion of the mesangium. These were associated with a marked increase in mesangial cellularity, and in occasional instances, there were cellular crescents (F, arrowhead). A, B, C, E, and F are magnified at ×1000 total magnification. D is magnified at ×500.

**FIGURE 6.** Glomerular immune complex deposition and Mφ infiltration in lupus-prone and non-lupus-prone mice. A–D, Images are representative of four to six mice per group and represent IgG deposition at ×400 total magnification in kidneys from DBF1 and SNF1 mice treated with vehicle or clodronate or vehicle, counterstained with Hoechst 33345. No significant differences were seen between vehicle- and clodronate-treated mice. No significant glomerular immune complex deposition was observed in the DBF1 mice. Similar findings were seen for IgM and C3. E–H, Images are representative of four to six mice per group and represent staining of CD68+ cells in kidney glomeruli at ×1000 total magnification in DBF1 and SNF1 mice untreated or treated with clodronate. No differences between untreated, vehicle-treated, and clodronate-treated mice were observed. DBF1 mice had higher tubular background staining than SNF1 mice.

**FIGURE 7.** Splenic Mφ accumulation and distribution at euthanasia. Images are representative of four to six mice per group and represent staining of CD68+ cells in the spleen at ×1000 total magnification in DBF1 and SNF1 mice untreated or treated with clodronate. No significant differences were observed between untreated and vehicle-treated mice. W, Representative area of white pulp. Yellow arrows, Representative red pulp area.
We evaluated the levels of two serum Mφ cytokines that may be relevant to kidney damage in SLE, TGF-β and TNF-α. There were no significant differences in levels of TGF-β between clodronate-treated and vehicle-treated DBF1 and SNF1 mice. TNF-α levels were decreased in both SNF1 and DBF1 mice treated with clodronate relative to vehicle-treated mice (mean ± SEM, 405 ± 42 vs 490 ± 55 pg/ml for SNF1 mice and 208 ± 32 vs 360 ± 28 pg/ml for DBF1 mice, respectively), but statistical significance was attained only in the DBF1 mice (p = 0.04).

Discussion

Extensive evidence indicates that dysregulated apoptosis contributes to autoimmunity in SLE (23, 35–39). Indeed, in vitro and ex vivo data suggest that mononuclear cells of patients with lupus, as well as lupus-prone mice, exhibit increased susceptibility toward apoptosis (3, 5, 21, 37, 40–43). Additional evidence indicates that the clearance of apoptotic material by scavenger Mφ is defective in SLE (24, 41, 44). Although a current model proposes that cells that die as part of the normal cell turnover are rapidly phagocytosed by scavenger Mφ without inflammation or immune activation (7, 45, 46), recent data suggest that, under certain circumstances, apoptotic cells may also promote proinflammatory immune responses by eliciting the release of chemokines and pro-apoptotic molecules from Mφ (47), or caspase-1-mediated release of inflammatory cytokines from dying cells (48–50). Therefore, apoptotic cells may be immunogenic in vivo, and this immunogenicity appears regulated by different factors, including the apoptotic load, the APCs involved in engulfment, processing and presentation of Ags contained in the apoptotic cells, and the cytokines released in the microenvironment (50). Apoptotic cell death also generates oxidatively modified moieties, which can induce autoimmune and local inflammatory responses by recruiting monocytes via monocyte-endothelial cell interactions (51). Apoptotic material represents a source of autoantigens, because intracellular and nuclear Ags are modified during apoptosis and translocated into the cell membrane (39). Indeed, nucleosomes are generated primarily by apoptosis and are considered one of the main autoantigens to which Abs are produced in human and murine lupus (2, 52, 53). Nucleosomes are also present in kidney eluates of lupus-prone mice with proteinuria (2). Recognition of nucleosomes, with Ag spreading to other chromatin components, may be the initiating event leading to the development of other autoantibodies. Although nucleosomes appear to be ignored by immunocompetent cells under normal physiological conditions, the development of CD4+ T cells primed to nucleosomal Ags in lupus-prone mice precedes other abnormalities that develop (23, 54), and apoptotic material presented to autoreactive lymphocytes by APCs can promote autoimmunity. This may be especially relevant in settings of excessive cellular apoptosis whereby the scavenger system becomes overwhelmed.

Patients with SLE have evidence of accelerated monocyte/Mφ apoptosis, a phenomenon that correlates with disease activity (3). Increased monocyte/Mφ apoptosis has been considered secondary to a number of different factors. Indeed, SLE serum can induce Mφ apoptosis and impair Mφ clearance by uncharacterized mechanisms (4). Lupus patients also have an autoreactive CD4+ T cell subset that kills autologous monocytes in the absence of added Ag (3, 5). In this case, autoactivity is defined by the capacity to kill autologous APCs in the absence of Ag (5). This type of autoreactivity has been described in the DNA hypomethylation model (55) and in the model of LFA-1-overexpressing lupus T cells (5). In addition, T cells observed in chronic graft-vs-host disease also cause a lupus-like disease by responding to host MHC molecules in vivo (56). Indeed, this phenomenon is MHC restricted, and it is not found in healthy controls or using allogeneic lupus monocytes (3, 5). We have reported that these autoreactive lupus T cells have increased expression of the apoptotic ligands TRAIL, TWEAK, and Fas ligand and that blockade of these apoptotic pathways significantly decreases monocyte cytotoxicity, with the residual cytotoxicity attributable to the perforin pathway (3, 57). The increased expression of these proapoptotic molecules appears to play a significant role in the induction of APC cytotoxicity in SLE, and this phenomenon might be important in inducing autoimmunity (3, 57). Indeed, it appears that cells killed by cytotoxic T cell-assisted apoptosis are preferential sources of autoantigens (58) and that autoreactive T cells play an important role in inducing this increase in the apoptotic load (3). When Th2 and Th1 cell lines are induced to overexpress the β2-integrin LFA-1, they become autoreactive and are cytolytic to Mφ without Ag (59). These cells also induce anti-DNA Abs in vivo, suggesting that this killing of Mφ could contribute to the autoantibody response by providing a source of autoantigen (21, 55, 60, 61). Furthermore, when autoreactive T cell clones are transferred to syngeneic female recipients, these cells (but not non-autoreactive T cells) preferentially localize to the red pulp Mφ without Ag (62). This therapy is known to induce autoantibodies and in some cases overt SLE. Although it has been proposed that the main mechanism for this complication is promotion of Th2 responses when TNF-α is blocked, an additional possibility is that increased Mφ apoptosis contributes to autoantibody formation (62). Supporting this hypothesis, accumulation of plasma nucleosomes upon treatment with anti-TNF-α Abs has been reported (63).

Despite all these studies, it has been unclear whether accelerated monocyte/Mφ apoptosis is important in the pathogenesis of lupus or represents an epiphenomenon. We now report that accelerated Mφ apoptosis in lupus-prone mice results in increased and sustained elevations of anti-nucleosome and anti-dsDNA Abs, hastens proteinuria onset, and increases severity of lupus nephritis. In addition, we report elevation of the autoantibody IgG2A Fcγ. This is relevant, because all of these autoantibodies have been proposed to be pathogenic in kidney damage in murine SLE (15, 23, 30, 64–70), and their significant increases in the clodronate-treated mice further supports the notion that they were involved in worsening renal disease. The mechanisms by which accelerated Mφ apoptosis induces worsening nephritis in lupus-prone mice are not entirely clear. The most likely explanation is that increased levels of pathogenic Abs leads to increased deposition of immune complexes in the kidney. This is substantiated by the fact that nephritogenic IgG2A Fcγ Abs were initially increased in the clodronate-treated group and then dropped below the levels seen in the nontreated mice at
around the time when clinical nephritis developed, suggesting depo-
sition in the kidneys, as described in previous work (31). It is
likely that we did not detect differences in the amount of renal
immune complexes because these organs were examined late in
the course of the disease, when end-stage had occurred in all the
lupus-prone mice and maximum immune complex deposition had
been achieved. It is also possible that local effects mediated by
cytokines could be involved in the increased severity of the disease
and future studies will address this possibility. However, no sig-
nificant changes in the serum levels of specific cytokines involved
in lupus nephritis were detected in the SNF₁ mice.

In non-lupus-prone mice, accelerated MΦ apoptosis led to ele-
vations of a variety of autoantibodies, but the kidneys of these
mice displayed no evidence of autoimmunity, suggesting that the
genetic background and other immunological factors might be cru-
cial for the development of autoimmune manifestations, subse-
quent to accelerated MΦ death. In addition, the levels of autoan-
tibodies seen in the non-lupus-prone mice, albeit increased in the
clodronate-treated group, might not be sufficient to promote organ
damage mediated by immune complexes. Our data on non-auto-
immune prone mice are consistent with what other groups have
reported, where exposure to increased apoptotic cells is not a suf-
ficient explanation for induction of high-titer, high-affinity, patho-
genic autoantibodies without the right genetic background. Indeed,
given our results, it is likely that additional immunoregulatory de-
fects would be required for the full induction of systemic autoim-
une disorders (35).

Accelerated MΦ apoptosis might be particularly important in
autoimmunity for a number of reasons. First, an increase in apo-
ptotic load may overwhelm the normal mechanisms of apoptotic
cell clearance. Second, accelerated MΦ death also impairs clear-
ance of apoptotic material. These two factors may act in concert to
greatly enhance autoimmunity in SLE. For example, when levels of
apoptosis exceed the clearance capacity, as is the case in SLE, the
apoptotic cells are allowed to become necrotic and release
intracellular components to the circulation. Previous studies have
suggested that high levels of apoptotic and necrotic cells can fur-
ther promote a secondary round of apoptosis, particularly in the
MΦ due to its scavenger activity, which results in the release of
nuclear contents both from the engulfed cell and the affected MΦ
(8). Therefore, MΦ apoptosis is crucial in the generation of circu-
lating nucleosomes. One potential consequence of enhanced MΦ
apoptosis is that the immunostimulatory dendritic cell may then be
permitted to take up and present autoantigenic material and initiate
proinflammatory pathways. Favoring this hypothesis, histological
analysis of lymph nodes in a subgroup of SLE patients showed that
autoaggressive T cells are not properly cleared by tingible body MΦ of the
germinal centers. Consequently, nuclear autoantigens bind to fol-
licular dendritic cells and may thus provide survival signals for
autoaggressive B cells (71). In addition to capturing and presenting
exogenous Ags, immature as well as mature dendritic cells are
able to phagocytosing endogenous apoptotic cells and present-
ing self-Ags to T cells (72–75), particularly when dendritic cells
are exposed to high doses of apoptotic cells (76). This phenome-
on could be important because dendritic cells, but not MΦ, effi-
ciently cross-present Ag derived from apoptotic cells to autologous
CTLs and activate autoreactive T cells in the lymph node. Fur-
thermore, an increase in proinflammatory cytokines during sec-
ondary necrosis may favor dendritic cell maturation and migration
to secondary lymphatic tissues, where activation of a specific im-
une response would take place. Importantly, a number of previ-
ous studies have shown that clodronate liposome administration
does not affect dendritic cell function or in vivo viability (13, 14,
33). Because mature dendritic cells that have migrated to the
spleen are significantly less efficient at uptaking Ag, it is ex-
pected that they will be much less susceptible to damage by the
clodronate. Upon Ag engulfment, MΦ normally provide sup-
pressor cytokine signals to nearby cells, such as TGF-β and
IL-10. Under specific circumstances, such as a cytokine-driven
proinflammatory environment or hypothetically when MΦ are
undergoing accelerated cell death, mature dendritic cells could
then gain the necessary tools to drive an autoimmune response.
Furthermore, apoptotic monocyte cell lines in the presence of
autoantibodies can cause production of a clearly immunostimu-
latory cytokine, IFN-α, which among other functions can direct
monocyte differentiation into dendritic cells rather than MΦ
(77, 78).

Liposomal clodronate appears to have a very selective effect
because neutrophils and lymphocytes have not been found to be
directly affected by the drug (79). In our study, we did not find
significant changes of pro- and anti-inflammatory cytokines in
blood in the SNF₁ mice. This might be explained by the fact that,
unlike other methods that induce MΦ death, clodronate liposomes
do not lead to the secretion of proinflammatory cytokines by the
dying MΦ (80). This might explain the lack of significant in-
creases, and even a slight decrease, in TNF-α seen in the mice that
we studied. TGF-β is a cytokine secreted by MΦ that are engulfing
apoptotic material (81), is up-regulated in lupus nephritis, and
plays a role in end-stage renal disease in SLE (82). We did not see
an increase in the levels of this cytokine in serum, probably re-
flecting a decrease in the numbers of MΦ that were capable of
engulfing apoptotic material.

Additional studies have shown that clodronate administration
causes significant decreases of marginal zone MΦ as well as red
pulp MΦ within 24 h, and thereafter, these areas gradually repopu-
late to even higher levels than before the injection (28, 34, 83–
86). Furthermore, tissue MΦ in the lungs and kidneys are not de-
pleted (33). Similarly, in our study, the absolute number of MΦ
was not reduced when mice were examined >2 wk after the last
clodronate administration, but significant MΦ apoptosis was de-
tected when spleens were harvested 24 h after clodronate admin-
istration. Therefore, an insult that contributes to prolonged MΦ
apoptosis (such as autoreactive T cells or a cytokine) would be
necessary to induce a chronic increase in apoptotic load and a
decrease in clearance mechanisms. Because the bone marrow pro-
duces >10 billion monocytes/day (87, 88), it is very likely that the
total MΦ number in vivo does not diminish significantly but that
the increased apoptotic load impairs the ability of new MΦ to
process apoptotic material in an efficient manner. Interestingly,
previous work has also shown that MΦ incubated with clodronate
liposomes release DNA into the medium after undergoing apopto-
sis, increasing the apoptotic load (8).

We propose that MΦ apoptosis contributes to the pathogenesis of
autoantibody formation and organ damage. We hypothesize that
this is secondary to both an increase in the apoptotic load and
impairment in the clearance of apoptotic material. This study sug-
gests that autoreactive cytotoxic T cells and other mechanisms that
contribute to scavenger cell apoptosis in SLE, could play a patho-
genic role and contribute to the severity of the disease.

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