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Granzyme B Is Dispensable for Immunologic Tolerance to Self in a Murine Model of Systemic Lupus Erythematosus

Kareem L. Graham, Donna L. Thibault, Jonathan B. Steinman, Lance Okeke, Peter N. Kao, and Paul J. Utz

Objective. **Proteolytic autoantigen cleavage by the serine protease granzyme B has been implicated in the development of systemic autoimmune disease; however, there has been no conclusive demonstration of a pathogenic role for granzyme B in autoimmunity. In this study, we evaluated the role of granzyme B in a murine model of autoimmunity.**

Methods. **To identify potential novel granzyme B substrates, complementary DNAs encoding nuclear factor 45 (NF45) and NF90 were used to generate ^{35}S-methionine–labeled proteins by coupled in vitro transcription/translation. Radiolabeled proteins were then incubated with purified recombinant granzyme B or caspases, and the cleavage products were analyzed by autoradiography. We also immunized granzyme B–deficient and granzyme B–intact mice with the mineral oil pristane. Production of autoantibodies directed against granzyme B substrates in response to pristane was evaluated by Western blotting, immunoprecipitation, and enzyme-linked immunosorbent assay.**

Results. **The double-stranded RNA–binding protein NF90 was identified as a novel substrate for caspases and granzyme B, both in vitro and in vivo. NF90 is uniquely cleaved by granzyme B in vitro; however, pristane immunization still induced anti-NF90 antibodies in granzyme B–deficient mice. Pristane-treated granzyme B–deficient mice also produced antibodies directed against the U1–70-kd antigen, a previously identified granzyme B substrate. Last, antibodies directed against U1–70 kd arose spontaneously in granzyme B–deficient mice.**

Conclusion. **These results demonstrate that granzyme B is not required for the production of autoantibodies directed against antigens that are granzyme B substrates in vitro. The data also suggest a protective role for this proapoptotic protease in systemic autoimmunity.**

The question of how immunologic tolerance to self antigens is broken is a central issue in autoimmunity. However, the underlying events that are responsible for the initial activation of autoreactive lymphocytes remain poorly understood. One area of extensive focus has been the biochemical properties that are shared among antigens that are targeted in systemic autoimmune disease. There is considerable support for the notion that alterations in protein structure caused by various posttranslational autoantigen modifications may generate "immunocryptic" protein fragments for presentation to autoreactive lymphocytes (1).

Recently, autoantigen cleavage by proapoptotic proteases, particularly caspases and granzyme B, has been implicated in the initiation and propagation of autoimmunity (2–4). Granzyme B, which is found in the cytolytic granules of cytotoxic T lymphocytes and natural killer (NK) cells, is critical for inducing target cell apoptosis during granule exocytosis-induced cytotoxicity. Observations made in both in vitro and in vivo

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Kareem L. Graham, BA, Donna L. Thibault, BS, Jonathan B. Steinman, Lance Okeke, BA, Peter N. Kao, MD, PhD, Paul J. Utz, MD: Stanford University School of Medicine, Stanford, California.

Address correspondence and reprint requests to Paul J. Utz, MD, Stanford University School of Medicine, CCSR Building, Room 2215A, 269 Campus Drive, Stanford, CA 94305. E-mail: pjutz@stanford.edu.

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systems appear to support a deleterious role for granzyme B in autoimmune disease. A screening test performed by Andrade and colleagues (3,4) identified 21 autoantigens (of 29 tested) that were uniquely cleaved by granzyme B both in vitro and in vivo. ("Uniquely cleaved" antigens are defined as those in which the granzyme B cleavage pattern differs from the caspase cleavage pattern.) A unifying feature of these granzyme B substrates is that many have roles in important cellular processes. Examples include poly(ADP-ribose) polymerase (PARP) and the catalytic subunit of DNA-dependent protein kinase, both of which have critical roles in DNA repair (3,4). However, the exact contribution of proteolytic autoantigen cleavage by granzyme B has not been directly tested in an animal model of autoimmunity.

The murine model of pristane-induced systemic lupus erythematosus (SLE) has provided insight into the potential role of environmental triggers in the development of autoantibodies to lupus-associated autoantigens. A single intraperitoneal injection of the hydrocarbon pristane has been shown to induce lupus-specific autoantibodies in mice of virtually any genetic background (5). C57BL/6 (B6) mice exposed to a single intraperitoneal injection of pristane develop antibodies directed against components of the U1 small nuclear RNP (snRNP)/Sm complex, including the U1–70-kd antigen, a granzyme B substrate (6). Serum autoantibodies from a subset of pristane-treated B6 mice also recognize the ribosomal P antigens (5,7), as well as the nuclear factor 45 (NF45) and NF90 proteins (6). The NF45/NF90 protein complex has double-stranded RNA–binding capability, has been implicated in the regulation of transcription (8), and is recognized by a small percentage of human lupus sera (6).

This study had 2 major goals. First, we sought to determine whether the NF45 or NF90 autoantigen could be cleaved by proapoptotic proteases in vitro. Herein we demonstrate that the NF90 autoantigen is a novel substrate for caspase 1, caspase 3, and granzyme B in vitro. NF90 also undergoes proteolysis in vivo (i.e., in cultured cells) during apoptosis induced by a variety of stimuli. Second, we investigated whether autoantibodies specific for granzyme substrates could be induced in granzyme B–deficient ($GB^{-/-}$) mice. In the pristane-induced model of autoimmunity, $GB^{-/-}$ mice developed antibodies directed against the U1 snRNP/Sm complex and ribosomal P antigens at approximately the same rate and frequency as observed in their granzyme B–intact ($GB^{+/+}$) counterparts. Importantly, pristane-treated $GB^{-/-}$ mice produced antibodies directed against the granzyme B substrates U1–70 kd and NF90. Surprisingly, $GB^{-/-}$ mice displayed significantly increased mortality following pristane immunization. Finally, phosphate buffered saline (PBS)–treated $GB^{-/-}$ mice produced U1–70-kd antibodies, indicating that antibodies to granzyme B substrates can arise spontaneously in the absence of functional enzyme. Taken together, these results demonstrate that granzyme B is not required for autoantibody production against antigens that are granzyme B substrates in vitro. Instead, the data suggest that granzyme B activity may promote the maintenance of peripheral tolerance.

MATERIALS AND METHODS

Mice and treatment. C57BL/6 (B6; $GB^{+/+}$) and granzyme B–deficient mice ($GB^{-/-}$; generated on the B6 genetic background) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under standard conditions in a virus-free environment at the Stanford University Research Animal Facility. Female $GB^{+/+}$ and $GB^{-/-}$ mice were given a single 0.5-ml intraperitoneal injection of pristane (Sigma-Aldrich, St. Louis, MO) at 8–10 weeks of age. Age- and sex-matched animals given PBS served as controls. Sera were collected every 4 weeks for the duration of the experiment. All remaining animals were killed 12 months after receiving the injection of pristane or PBS.

Cells and culture media. K562 and Jurkat cells were grown in suspension in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% calf serum (BioWhittaker, Walkersville, MD) and penicillin/streptomycin (Gibco).

Antibodies. NF90-specific rabbit antiserum was generated as previously described (9) and used at a dilution of 1:1,000. Anti-PARP p85 rabbit polyclonal antibodies (Promega, Madison, WI) were used at a dilution of 1:1,000, and anti–β-actin antibodies (Sigma) were used at a dilution of 1:40,000. Monoclonal antibodies 2.73 (anti–U1–70 kd) and 9A9 (anti-U1A/U2B′′) prepared as culture supernatants (kindly provided by W. J. van Venrooij, University of Nijmegen, The Netherlands) were used at a dilution of 1:5.

Plasmids. La complementary DNA (cDNA) was kindly provided by W. J. van Venrooij. NF45 and NF90 cDNA were cloned as previously described (9). Complementary DNAs encoding caspase 1 and caspase 2 were a gift from H. Li and J. Yuan (Harvard Medical School, Boston, MA).

Western blot analysis. Cells were washed and lysed as previously described (10). After boiling lysates in sodium dodecyl sulfate (SDS) loading buffer, proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH), and membranes were blocked with 5% Blotto (Bio-Rad, Hercules, CA) in PBS–Tween (PBS with 0.1% Tween 20) overnight at 4°C. The membranes were then incubated with antibodies at the dilutions indicated above for 1 hour, followed by species-specific antibody conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:5,000, and developed using chemiluminescence performed according to the manufacturer's instructions (Pierce, Rockford, IL).

For immunoblot analysis of anti–U1–70 kd and anti-U1A reactivity in mouse sera, 100 μg of purified human U1–70-kd or U1A antigen (Diarect, Freiburg, Germany) was separated by 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to nitrocellulose, and the membrane was probed with individual mouse sera, at a dilution of 1:250, using a Miniblotter device (Immunetics, Cambridge, MA). After rocking for 2 hours at room temperature, HRP-conjugated anti-mouse secondary antibody (Southern Biotechnology, Birmingham, AL) was added, followed by chemiluminescence detection.

In vitro transcription/translation. Radiolabeled La, NF45, and NF90 were generated by coupled in vitro transcription/translation using a T7 rabbit reticulocyte lysate system (Promega). A reaction mix containing 10 μl rabbit reticulocyte lysate, 0.5 μl nuclease-free water, 20 units RNase inhibitor (Promega), 5 μCi translation-grade ^{35}S-methionine (Perkin-Elmer, Wellesley, MA), and 0.25 μg plasmid DNA was incubated for 90 minutes at 30°C. Reactions were terminated by boiling in 2× SDS sample buffer. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography.

Cleavage assays. All cleavage reactions with in vitro–translated substrates used 1 μl of translation reaction mixture in a total volume of 10 μl. Granzyme B protease (Sigma) was incubated with in vitro–translated protein for 60 minutes at 37°C at a concentration range of 3–6 ng/μl in buffer A (150 m*M* NaCl, 50 m*M* HEPES [pH 7.5]). Recombinant caspase 1, recombinant caspase 2, and a control bacterial lysate were generated as previously described (11,12) and incubated with in vitro–translated protein for 90 minutes at 30°C in buffer B (10 m*M* dithiothreitol [DTT], 1 m*M* EDTA, 100 m*M* Tris [pH 7.5], 1× protease inhibitor cocktail). Recombinant caspases 3 (1 ng/μl), 8 (10 ng/μl), and 9 (250 ng/μl; all from Calbiochem, La Jolla, CA) were incubated with in vitro–translated protein in buffer C (50 m*M* HEPES, 50 m*M* NaCl, 10 m*M* DTT, 10 m*M* EDTA, 5% glycerol, 0.1% CHAPS [pH 7.2]) for 60 minutes at 37°C. Reactions were terminated by boiling in an equal volume of 2× SDS sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and cleavage products were analyzed by autoradiography.

Immunoprecipitation and cell labeling. Immunoprecipitation of ^{35}S-cysteine/methionine–labeled cell extract from K562 cells was performed as previously described (13,14), and immunoprecipitation reactions were boiled in 2× SDS loading buffer. Proteins were then separated by 12% SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography.

Preparation of apoptotic cell extracts. Ultraviolet (UV) irradiation of Jurkat cells was performed as previously described (15). After irradiation, cells were incubated at 37°C for the indicated times prior to harvesting. For other treatments, cells were plated in cell growth medium containing anisomycin (Sigma) at 10 μg/ml or etoposide (Sigma) at 10 μg/ml. 7C11 IgM anti-Fas monoclonal antibody from hybridoma supernatants (kindly provided by M. Robertson, Indiana University, Bloomington, IN) was used at a final dilution of 1:500. The cells were harvested after incubation at 37°C for 5 hours, and the extracts were analyzed by Western blotting.

Serology. For anti–U1–70-kd enzyme-linked immunosorbent assays (ELISAs), 96-well Nunc plates (Nalgene Nunc, Milwaukee, WI) were coated with purified U1–70-kd antigen (Diarect) at a concentration of 1 μg/ml. Wells were incubated with mouse sera diluted 1:250 in a buffer containing 3% calf serum in PBS–Tween, followed by incubation with HRP-conjugated donkey anti-mouse IgG (Southern Biotechnology) at a dilution of 1:5,000. Tetramethylbenzidine substrate (Pierce) was added, and optical density values were determined at 450 nm. Absorbances from blank wells (no serum added) were subtracted.

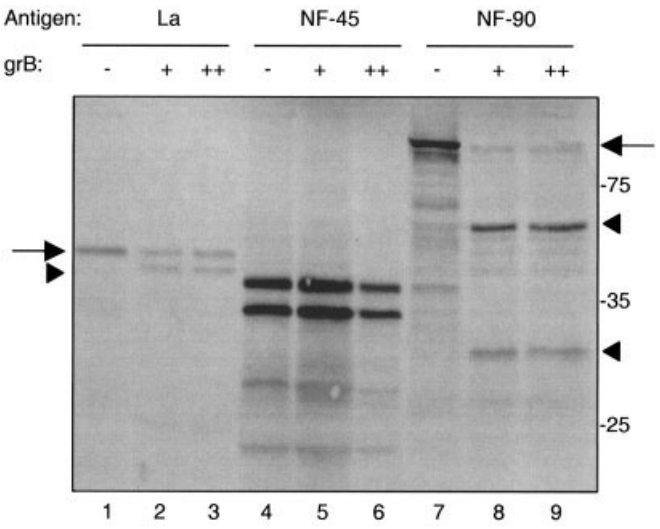

Figure 1. Nuclear factor 90 (NF90), but not NF45, is a substrate for granzyme B. Radiolabeled substrates were generated by coupled in vitro transcription/translation and then incubated in the absence (lanes 1, 4, and 7) or presence of increasing concentrations of purified granzyme B (grB). Reactions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by autoradiography. The relative migration of molecular size markers (in kilodaltons) is shown on the right side of the figure. Arrows indicate full-length La (left) and full-length NF90 (right). Arrowheads indicate La cleavage fragments (left) and NF90 cleavage fragments (right) generated by granzyme B.

RESULTS

NF90 as a novel substrate for granzyme B in vitro. The primary amino acid sequences of NF45 and NF90 contain candidate granzyme B cleavage motifs, implicating these molecules as potential granzyme B substrates. To determine whether the NF45 and NF90 autoantigens are substrates for granzyme B in vitro, plasmids encoding NF45 and NF90 were used to generate ^{35}S-methionine–labeled substrates by coupled in vitro transcription/translation. Radiolabeled substrates were then incubated with purified recombinant granzyme B. Reactions were terminated, and cleavage products were visualized by autoradiography. As shown in Figure 1, the La autoantigen, previously identified as a

granzyme B substrate (4), was cleaved by granzyme B in vitro (lanes 2 and 3). Although NF45 was not cleaved by granzyme B (lanes 4–6), NF90 was a substrate for the protease (lanes 7–9). Cleavage of NF90 by granzyme B appeared to occur at a single site, generating 2 distinct fragments roughly 60 kd and 30 kd in length, respectively (indicated by arrowheads in lanes 8 and 9). The precise granzyme B cleavage site within NF90 is currently unknown. However, results of studies using a truncated form (55 kd) of in vitro–translated NF90 (9) suggested that the granzyme B cleavage site lies within the amino terminus of the protein (data not shown). To determine whether granzyme B could mediate proteolysis of endogenous NF90, we also analyzed protein cleavage in a Jurkat cell lysate treated with granzyme B. Notably, the granzyme B–specific cleavage products generated upon incubation with endogenous NF90 were identical in size to those observed with in vitro–translated protein (data not shown). NF90 was also cleaved in a Fas-treated apoptotic lysate, yielding fragments distinct in size from those generated by granzyme B (data not shown). Taken together, these data establish NF90 as a unique substrate for granzyme B.

Cleavage of NF90 by caspases in vitro and in vivo. Many granzyme B substrates also contain caspase cleavage sites, as both granzyme B and caspases preferentially cleave after aspartate residues (16). We sought to determine whether the NF90 autoantigen is a substrate for caspases in vitro. ^{35}S-methionine–labeled NF90 protein was incubated with purified recombinant caspases, and substrate cleavage was analyzed by autoradiography. As shown in Figure 2, NF90 was efficiently cleaved by caspases 1 and 3 (lanes 2 and 4). We also observed modest, but reproducible, cleavage by caspase 8 (lane 5). Conversely, NF90 was not cleaved by caspases 2 or 9 (lanes 3 and 6). Caspases 1 and 3 each generated cleavage products of approximately the same length (roughly 50 kd and 40 kd, respectively) (open arrowheads; compare lanes 2 and 4). Notably, the cleavage products generated by incubation of in vitro–translated NF90 with granzyme B differed in size from those generated by caspases (lane 7; compare areas indicated by closed arrowheads with areas indicated by open arrowheads). In addition, NF90 was cleaved much more efficiently by granzyme B than by caspases. The recombinant caspases used in these assays were able to cleave in vitro–translated control substrates, verifying that the enzymes were active (data not shown). Taken together, these results demonstrate the existence of a granzyme B cleavage site within the NF90 protein that is distinct from the caspase cleavage site.

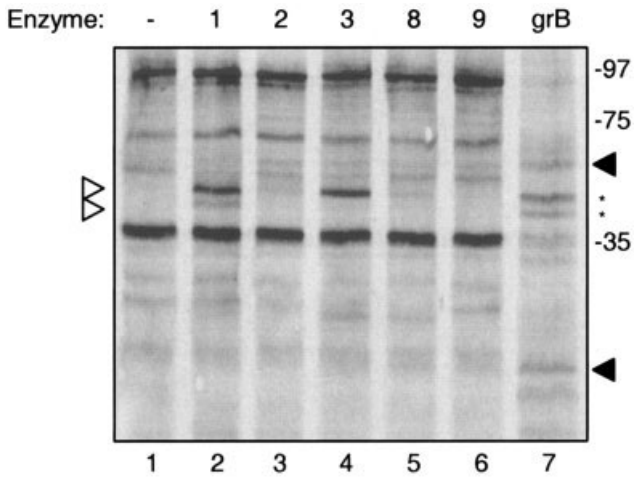

Figure 2. Cleavage of nuclear factor 90 (NF90) by caspases in vitro. Radiolabeled substrates were incubated with recombinant caspases, as described in Materials and Methods. Granzyme B (grB) and caspases 1, 2, 3, 8, and 9 are indicated by number at the top of the figure. The relative migration of molecular size markers (in kilodaltons) is shown on the right side of the figure. Lane 7, showing NF90 incubated in the presence of granzyme B, is used for comparison of granzyme B and caspase cleavage fragments. Solid arrowheads indicate granzyme-specific cleavage products, and open arrowheads indicate caspase-specific cleavage products. ∗ = bands in lane 7 that arise from premature termination of the coupled in vitro transcription/translation reaction.

We next attempted to determine whether cleavage of NF90 is a general consequence of programmed cell death induced by various apoptotic stimuli. Jurkat cells were incubated in the absence or presence of the following: the protein synthesis inhibitor anisomycin, the cell cycle inhibitor etoposide, anti-Fas 7C11 monoclonal antibody, and UV irradiation. Cells were harvested after 5 hours, and the extracts were separated by SDS-PAGE, followed by Western blotting with anti-NF90 antibody. As shown in Figure 3A, NF90 was cleaved in vivo during apoptosis induced by multiple stimuli. Furthermore, the cleavage products observed during programmed cell death (~50 kd and 40 kd, respectively) comigrate precisely with those observed during cleavage of radiolabeled NF90 by caspases 1 and 3 in vitro (compare with Figure 2, lanes 2 and 4), suggesting that proteolysis of NF90 is mediated by one or both of these caspases during apoptosis in vivo.

We next determined the time course of NF90 proteolysis during apoptosis. Jurkat cells were exposed to UV irradiation, and cells were harvested at various times over a 6-hour period. As shown in Figure 3B, NF90 cleavage was detectable between 2 hours and 4

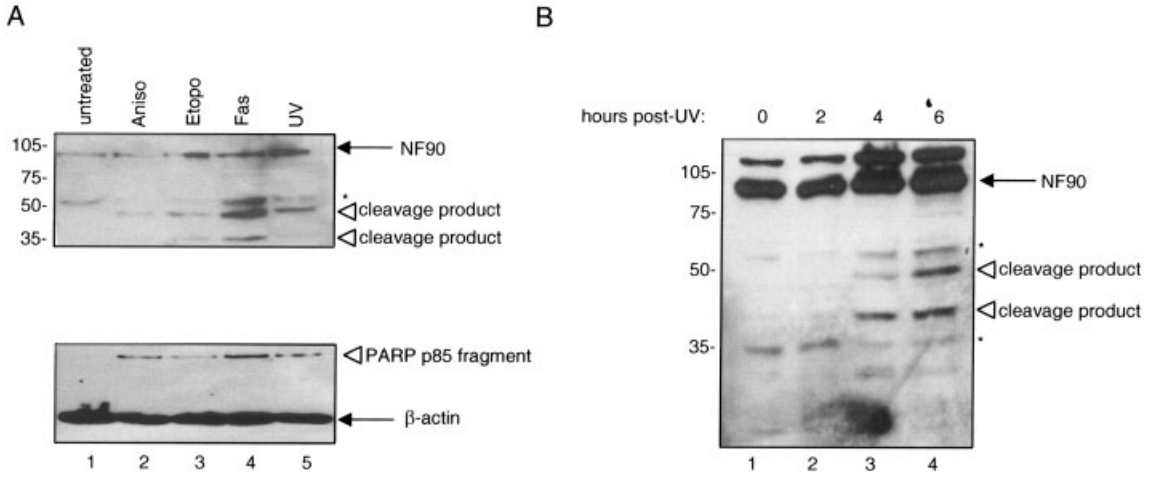

Figure 3. Cleavage of nuclear factor 90 (NF90) by caspases during apoptosis in vivo. **A,** Jurkat cells were subjected to various apoptotic stimuli and harvested for protein analysis after 6 hours. Lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and subjected to Western blot analysis using anti-NF90, anti–poly(ADP-ribose) polymerase (anti-PARP) p85, and anti–β-actin antibodies. The apoptotic stimuli (anisomycin [Aniso], etoposide [Etopo], Fas, and ultraviolet [UV] irradiation) are shown above the top panel. The anti-PARP p85 antibody specifically recognizes the 85-kd PARP cleavage product that is generated during apoptosis. **B,** Jurkat cells were subjected to UV irradiation and harvested by detergent lysis at the indicated times. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-NF90 antibody. $*$ = nonspecific band.

hours after induction of apoptosis (lanes 2 and 3). The kinetics of NF90 proteolysis are slightly delayed in comparison with those observed for PARP and nuclear mitotic-associated protein, 2 autoantigens that are cleaved by caspases early during apoptosis (17). Cleavage of NF90 in response to UV irradiation was prevented in Bcl-2–overexpressing Jurkat cells (data not shown), suggesting a potential role for the mitochondrial pathway in the cleavage of NF90 during programmed cell death in vivo. NF90 cleavage during Fas-mediated apoptosis was also prevented by pretreatment of Jurkat cells with the broad-spectrum caspase inhibitor Z-VAD-FMK (data not shown).

Pristane-induced lupus-associated autoantibodies in granzyme B–intact mice. In order to examine the role of the granzyme B protease in the bypass of tolerance to lupus-associated autoantigens in an animal model of autoimmunity, we administered pristane to female, 8–10–week-old, wild-type B6 ($GB^{+/+}$) mice. Consistent with previous reports (6), pristane induced autoantibodies directed against the ribosomal P antigens, NF45/90, and U1 snRNP/Sm proteins in B6 ($GB^{+/+}$) animals (Figure 4A; sera in lanes 5, 7, and 9 are anti–U1 snRNP/Sm positive and anti–ribosomal P positive; sera in lanes 5, 6, and 7 are anti-NF90 positive).

Production by granzyme B–deficient mice of antibodies directed against autoantigens that are granzyme B substrates in vitro. Pristane was also administered to 8–10–week-old $GB^{-/-}$ mice. As shown in Figure 4B, sera derived from $GB^{-/-}$ pristane-treated mice contained antibodies capable of immunoprecipitating ribosomal P proteins, NF45/90, and U1 snRNP/Sm from radiolabeled K562 extract. (Sera in lanes 6 and 9 are anti–U1 snRNP/Sm positive and anti–ribosomal P positive; sera in lanes 7 and 9 are anti-NF90 positive.) With respect to the frequency of anti–U1 snRNP/Sm, anti–ribosomal P, and anti-NF90 autoantibodies induced by pristane, no significant differences between $GB^{-/-}$ and $GB^{+/+}$ mice were observed (Table 1). Anti-NF90 reactivity in sera derived from pristane-treated $GB^{-/-}$ mice was further validated by Western blot analysis of immunoprecipitated proteins with anti-NF90 antibody (data not shown). Neither $GB^{-/-}$ nor $GB^{+/+}$ mice produced double-stranded DNA antibodies in response to pristane (data not shown). However, we observed increased levels of serum IgG1 in pristane-treated $GB^{-/-}$ mice (data not shown), suggesting that granzyme B may have direct or indirect effects on immunoglobulin isotype switching. Taken together, these data definitively show that tolerance to the NF90 antigen is broken in $GB^{-/-}$ mice.

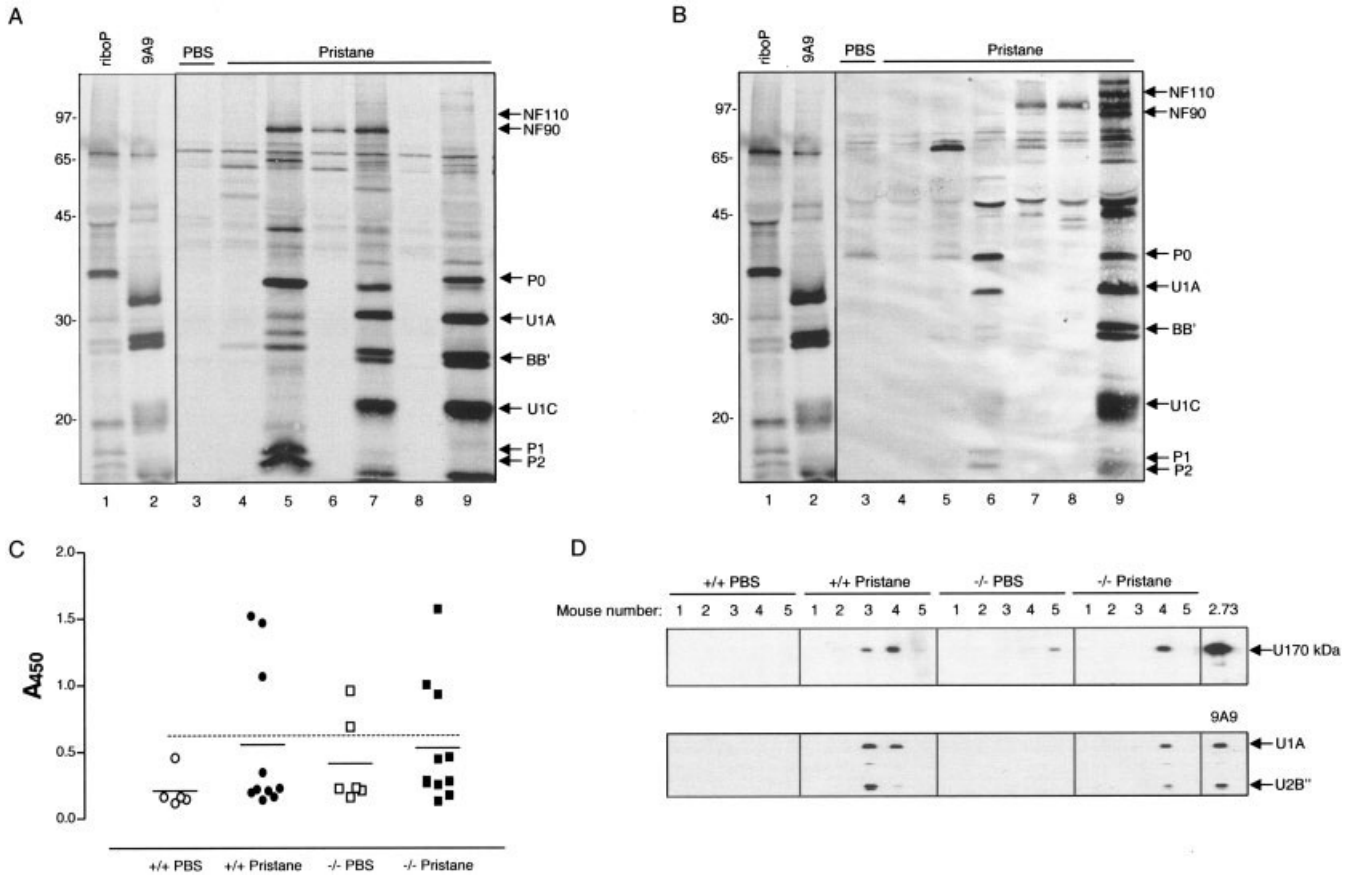

Figure 4. Pristane induces anti–U1 small nuclear RNP (snRNP)/Sm antibodies in granzyme B–deficient mice. **A** and **B,** K562 human erythroleukemia cells were labeled with ^{35}S-cysteine/methionine for 14 hours. Cells were lysed in Nonidet P40 lysis buffer, and the extract was immunoprecipitated with sera derived from granzyme B–intact ($GB^{+/+}$) (**A**) or granzyme B–deficient ($GB^{-/-}$) (**B**) mice 6 months after treatment with phosphate buffered saline (PBS) or pristane. Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by autoradiography. Positions of the nuclear factor 90 (NF90)/NF110 proteins, ribosomal P (riboP) antigens, and U1 snRNP/Sm proteins are shown on the right. **A,** Anti–ribosomal P reference sera and 9A9 anti–U1A/U2B′′ monoclonal antibody immunoprecipitation reactions are shown in lanes 1 and 2, respectively. Sera in lanes 5, 7, and 9 are anti–U1 snRNP/Sm positive and anti–ribosomal P positive; sera in lanes 5, 6, and 7 are anti-NF90 positive. **B,** Sera in lanes 6 and 9 are anti–U1 snRNP/Sm positive and anti–ribosomal P positive; sera in lanes 7 and 9 are anti-NF90 positive. **C,** Antibodies to the U1–70-kd antigen arise spontaneously in $GB^{-/-}$ mice. Sera obtained 12 months after treatment were analyzed for levels of IgG anti–U1–70-kd antibodies by enzyme-linked immunosorbent assay. Data are plotted as absorbance values for individual animals. Bars show the mean optical density values for each group. Broken line represents the mean +3 SD anti–U1-70 level in sera from PBS-treated $GB^{+/+}$ mice. **D,** Individual mouse serum samples were tested for anti–U1–70 kd and anti-U1A reactivity using a slot-blot device.

As shown above in immunoprecipitation experiments, pristane induced antibody responses to the U1 snRNP/Sm complex in both $GB^{-/-}$ and $GB^{+/+}$ mice. We also analyzed antibody responses to the U1–70-kd antigen by ELISA, using sera collected at various time points after PBS or pristane was administered. Compared with the levels in PBS-treated $GB^{+/+}$ animals, anti–U1–70-kd antibody levels were elevated in sera derived from 3 of 11 pristane-treated $GB^{-/-}$ and 3 of 10 $GB^{+/+}$ mice 12 months after treatment. Surprisingly, anti–U1–70 kd reactivity was also elevated in 2 of 6 PBS-treated $GB^{-/-}$ animals at this time point (Table 1 and Figure 4C). These unexpected results were further confirmed by Western blotting. As shown in Figure 4D (top row), serum antibodies derived from select pristane-treated $GB^{+/+}$ and $GB^{-/-}$ mice recognized purified U1–70-kd antigen. As expected, none of the sera derived from PBS-treated $GB^{+/+}$ mice recognized the U1–70-kd protein. However, one of the PBS-treated $GB^{-/-}$ samples (which was also positive by ELISA)

Table 1. Autoantibody production in $GB^{-/-}$ and $GB^{+/+}$ mice*

Strain	Treatment	RNP/Sm†	Ribo-P†	NF90†	U1–70 kd‡
+/+	PBS	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
+/+	Pristane	8/22 (36)	5/22 (23)	8/22 (36)	3/10 (30)
−/−	PBS	0/6 (0)	0/6 (0)	0/6 (0)	2/6 (33)
−/−	Pristane	8/25 (32)	9/25 (36)	8/25 (32)	3/11 (27)

* Values are the number (%). For enzyme-linked immunosorbent assay (ELISA) experiments, positive anti–U1–70 kd reactivity was defined as 3 SD greater than the mean absorbance value in phosphate buffered saline (PBS)–treated granzyme B–intact ($GB^{+/+}$) mice. None of the differences in reactivity between groups were statistically significant, as determined by Student's *t*-test.
† Antibodies to RNP or Sm proteins, ribosomal P (Ribo-P), and nuclear factors 45 and 90 (NF45/NF90) were detected by immunoprecipitation, using sera obtained 6 months after treatment with pristane or PBS.
‡ Antibodies to the U1–70-kd antigen were detected by ELISA, using sera obtained 12 months after treatment.

displayed strong anti–U1–70 kd reactivity. We also used Western blotting to analyze antibody responses to other components of the U1 snRNP/Sm complex. Sera from PBS-treated $GB^{-/-}$ mice did not recognize purified SmBB′ (data not shown) or U1A, which is not a granzyme B substrate in vitro (Figure 4D, bottom panel, and data not shown). Taken together, these data definitively demonstrate that granzyme B is not required for bypass of tolerance to the NF90 and U1–70-kd antigens, both of which are granzyme B substrates in vitro. Furthermore, the presence of U1–70-kd antibodies in PBS-treated $GB^{-/-}$ mice suggests that granzyme B may actually function to promote immunologic tolerance to self antigens in the periphery.

Increased mortality in granzyme B–deficient mice administered pristane. Satoh and colleagues reported that 10–50% of B6 mice die within 1 month of pristane injection, due to pulmonary hemorrhage of unknown origin (7). Death at later time points in B6 mice does not appear to be related to pulmonary hemorrhage or lupus-like complications. In our studies, wild-type B6 ($GB^{+/+}$) and $GB^{-/-}$ mice treated with PBS displayed 100% survival after 1 year (Table 2). Pristane did cause kidney inflammation in a small subset of $GB^{-/-}$ and $GB^{+/+}$ animals. However, there were no significant differences in kidney pathology between pristane-primed $GB^{+/+}$ or $GB^{-/-}$ mice (data not shown).

Table 2. Survival in granzyme B–deficient ($GB^{-/-}$) and $GB^{+/+}$ mice

Strain	Treatment*	n	% survival	
			6 months	12 months
+/+	PBS	5	100	100
+/+	Pristane	35	91.4	85.7
−/−	PBS	6	100	100
−/−	Pristane	50	72†	70‡

* PBS = phosphate buffered saline.
† $P = 0.0339$ versus pristane-treated $GB^{+/+}$ mice.
‡ $P = 0.0939$ versus pristane-treated $GB^{+/+}$ mice.

In our hands, ~10% of B6 mice died within 1–2 months of pristane treatment. Mortality was significantly enhanced in $GB^{-/-}$ animals primed with pristane, as nearly 30% of the mice were dead by 3 months after treatment. However, despite histopathologic analysis of several organs, we were unable to identify the reason(s) for increased mortality in pristane-treated $GB^{-/-}$ mice (data not shown). Thus, it is not entirely clear that the increase in mortality was caused by enhanced autoimmunity. Taken together, these results demonstrate that absence of the granzyme B enzyme exacerbates disease in the pristane-induced model of autoimmunity. The data also suggest that granzyme B may influence immune system responses through mechanisms other than direct proteolysis of target substrates.

DISCUSSION

A hallmark of lupus is the production of IgG autoantibodies directed against primarily intracellular molecules (18). The recent observation that granzyme B can uniquely cleave autoantigens that are targeted across the spectrum of autoimmune disease has focused attention on a possible role for cytotoxic T lymphocyte and NK cell granule components in the initiation and propagation of autoimmunity. In an elegant study, Casciola-Rosen and colleagues identified numerous autoantigens as unique substrates for the granzyme B protease, including the catalytic subunit of DNA-dependent protein kinase, U1–70 kd, and the La antigen (4). In this study, we identify the NF90 antigen as a novel substrate for caspase 1, caspase 3, and granzyme B. We report that proteolytic cleavage by granzyme B is not required for the breakage of tolerance to the NF90 and U1–70-kd autoantigens in a murine model of induced autoimmunity. Strikingly, granzyme B–deficient animals immunized with pristane display higher mortality than their granzyme B–intact counterparts. In addition, autoantibodies to the U1–70-kd antigen arise spontaneously in mice that are genetically deficient in granzyme B.

NK cells and CD8+ cytotoxic T lymphocytes utilize both the Fas/Fas ligand (FasL) and perforin/granzyme pathways to induce target cell death. Ligation of the Fas receptor on a target cell results in cell death, secondary to caspase activation. Whereas Fas-mediated

apoptosis plays a central role in lymphocyte homeostasis (19), the perforin/granzyme pathway appears to be dominant in control of viral infections and tumor surveillance (20). Numerous granzymes are found in the cytolytic granules of NK cells and cytotoxic T lymphocytes, with granzymes A and B being the most abundantly expressed in mice and humans (21). Our data confirm and extend previous reports of a protective role for cytolytic granule components in murine lupus. Investigators have previously examined the role of perforin in the MRL/lpr murine model of spontaneous SLE. MRL/lpr mice contain a transposon insertion in the Fas gene (22), which results in expression of a defective Fas receptor on the cell surface. MRL/lpr mice produce a variety of SLE-specific antibodies and develop severe renal disease, with 50% mortality by 5 months in most colonies (23). Peng and colleagues found that perforin-deficient MRL/lpr mice displayed accelerated mortality compared with their perforin-intact counterparts, suggesting a regulatory role for the perforin/granzyme pathway in systemic autoimmunity (24).

How might granzyme B act to maintain tolerance to self antigens? Granzyme B targets several molecules that have critical roles in cellular repair, and some autoantigens are cleaved in vitro with greater efficiency by granzyme B than caspases (3,4). Thus, we speculate that granzyme B activity is essential for the complete and faithful execution of the apoptotic death pathway in target cells. In this model, absent or reduced granzyme B activity may lead to prolonged survival of cells that have been targeted for destruction. One scenario in which this may be particularly relevant is in the elimination of virus-infected cells. Release of sequestered antigens from host cells is just one of many proposed mechanisms explaining how viruses and other infectious agents may cause autoimmune disease (25).

Caspase dysfunction and impaired ability to clear apoptotic cell debris are 2 prime examples of apoptotic defects that have been implicated in the etiology of lupus. Defects in classical complement pathway proteins are strongly associated with human lupus (26). Inherited death receptor mutations also play a role in human disease. In humans with genetic defects in the Fas receptor, a condition known as autoimmune lymphoproliferative syndrome develops; this disorder is marked by defective lymphocyte apoptosis, lymphadenopathy, splenomegaly, and autoimmunity (27,28). However, our results and those of other investigators highlight the complex role of proapoptotic proteases and the apoptotic death pathway in the regulation of immune responses. The lpr (Fas) and gld (FasL) mutations actually inhibit autoantibody production in the pristane-induced model of lupus (7). Several investigators have suggested an important role for caspases in T cell activation (29,30), and Chun et al noted immunodeficiency in humans with homozygous mutations in caspase 8 (31). The dual nature of caspases, which are participants in both cell death and cell activation, is reminiscent of the cytokine interleukin-2. Interleukin-2 is a critical factor for T cell growth and survival but also promotes Fas-mediated T lymphocyte activation–induced cell death (32). Thus, the exact outcome of caspase or granzyme activation—target cell death, immunity, or tolerance—is likely to depend on a host of factors, including the type of cellular stimulus and the local cytokine milieu.

In summary, our results demonstrate that cleavage by granzyme B is not required for breaking tolerance in the murine model of pristane-induced SLE. It has been reported that cleavage by granzyme B is strongly predictive of autoantigen status. Our data indicate that the relationship is likely to involve a mechanism other than the proteolytic effects of the enzyme. Recently, it was shown that sera from patients with scleroderma can recognize centromere protein C autoantigen fragments that are generated upon incubation with granzyme B (33). However, the role of these granzyme B–generated fragments in the context of cell-mediated cytotoxicity in vivo is unclear. Instead of granzyme fragments representing "immunocryptic" epitopes, we speculate that granzyme fragments may instead reflect the importance associated with inactivation of certain substrates during execution of the apoptotic program. In mice, granzymes A, B, and C are expressed in the fetal liver and the thymus, and granzyme A enzymatic activity is detectable in CD8+ thymocytes (34). Thus, one might predict either that the determinants generated in in vitro systems are not generated in vivo or that lymphocytes are tolerized to these determinants during development.

The apparent contradictions between our findings and those of other groups of investigators may be attributable to fundamental differences in the experimental systems used. We took advantage of a murine disease model to examine the role of granzyme B in autoimmunity, while other investigators primarily addressed this question in the context of human cells (or granule contents derived from human cells) and human autoantigens. Both mouse and human granzyme B cleave after aspartate residues and exhibit similar patterns of cellular expression, but these 2 forms of the enzyme display only ~70% homology at the amino acid level (35). In addition, although many autoimmune diseases are characterized by the production of autoan-

tibodies directed against molecules that are highly conserved (36), it is possible that an autoantibody may specifically target a portion of an autoantigen that is not conserved during evolution (37). It is also important to note that granzymes may have a dual role in the regulation of immunologic tolerance. For instance, granzyme B may serve to promote central tolerance, with environmental stimuli (e.g., viral infection) inducing events in which the enzyme participates in breaking peripheral tolerance. Furthermore, granzymes may play a redundant role in autoantigen cleavage in vivo, and the absence of granzyme B does not necessarily preclude the generation of immunogenic autoantigen cleavage fragments by other granzymes. In support of this viewpoint, granzyme A can also directly cleave a limited number of autoantigens that are targeted in systemic autoimmunity (38,39).

Our data do not rule out a role for autoantigen cleavage by caspases in the development of autoimmunity. Moreover, the observation that granzyme B is not required for bypass of self-tolerance may be unique to the pristane model. However, results of studies on the role of perforin in the MRL/lpr model (24) suggest a general protective role for perforin/granzyme pathway components in this and other murine models of lupus. Although the pristane-induced pathway of autoimmunity in mice likely differs from that triggered in human lupus, the demonstration that granzyme B–expressing CD4+ T regulatory cells can kill target cells in a perforin-dependent manner underscores a potential regulatory role for granzyme B in humans (40). Future studies should be aimed at assessing the immunogenicity of granzyme-generated fragments or analyzing immune responses of mice in which granzyme cleavage sites of granzyme substrates have been mutated. Such studies will shed additional light on the contribution of proteolytic autoantigen cleavage to autoimmune disease.

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