ARTHRITIS & RHEUMATISM
Vol. 50, No. 10, October 2004, pp 3239–3249
DOI 10.1002/art.20508

Interferon-α–Inducible Proteins Are Novel Autoantigens in Murine Lupus

Wolfgang Hueber, Defu Zeng, Samuel Strober, and Paul J. Utz

Objective. **To investigate the spectrum of B cell autoimmunity in the recently described anti-CD1–autoreactive T cell receptor (TCR)–transgenic murine lupus-like (CD1 lupus-like) model.**

Methods. **Lethally irradiated BALB/c/nu/nu mice were injected intravenously with donor BALB/c bone marrow and spleen cells expressing TCRα and TCRβ transgenes that recognize CD1d. Sera from adoptive host animals that developed lupus (i.e., CD1 lupus mice) were collected at serial time points and analyzed by Western blotting and immunoprecipitation, using protein extracts prepared from NIH3T3 mouse fibroblasts and EL-4 lymphocytes, respectively. Sera obtained from older animals in several models of spontaneous lupus (NZB/NZW, MRL++, and MRL/*lpr* mice), unmanipulated BALB/c/nu/nu mice, and normal BALB/c mice were used as controls.**

Results. **Analyses demonstrated that the prominent targets of autoantibodies in the CD1 lupus-like model are interferon-α (IFNα)–inducible antigens. Biochemical and serologic characterizations identified one antigen as belonging to the interferon-inducible 202 (Ifi202) subfamily of proteins within the Ifi200 family, and a second antigen as a member of the 70-kd heat-shock protein family. Autoantibodies directed against these antigens were rapidly produced at an early stage of disease. Anti-p50 autoantibodies were present in sera from 7 (78%) of 9 CD1 lupus mice that developed severe kidney disease.**

Conclusion. **IFNα-inducible proteins represent a novel class of autoantigens in murine lupus, and the findings suggest additional roles for IFNα in this disease. Since Ifi202 autoantigens are encoded by the murine non–major histocompatibility complex lupus-susceptibility gene locus *Ifi202,* these data provide a link between recent advances in lupus genetics and the formation of autoantibodies.**

Although a wide spectrum of autoantibodies has been identified in systemic lupus erythematosus (SLE), only a few, such as anti-DNA antibodies, have been implicated in the pathogenesis of certain manifestations of this complex disease (1). Other unidentified autoantibodies may play potentially crucial roles with regard to tissue injury, and their identification may provide critical insights into the pathogenesis of connective tissue disease (2).

CD1 is a non–major histocompatibility complex (MHC)–encoded antigen-presenting molecule that is primarily involved in the presentation of glycolipid antigens to subsets of T cells expressing natural killer cell markers (natural killer T cells) (3). Some reports have suggested that CD1 presentation of nonlipid antigens is possible (4,5). The physiologic ligands of CD1 are still unknown. Evidence is accumulating to support the notion that the CD1 molecule is important in the modulation of immune responses in several autoimmune diseases (6). We recently suggested a role for CD1 in the pathogenesis of hereditary murine lupus in NZB/NZW mice (7). In the anti-CD1 T cell receptor (TCR)–transgenic murine lupus-like model (referred to as the CD1 lupus-like model), the transfer of a single-positive

Dr. Hueber's work was supported by a James Klinenberg Fellowship from the Arthritis National Research Foundation. Dr. Zeng's work was supported by an Investigator award from the Arthritis Foundation. Dr. Strober's work was supported by NIH grant AI-400931. Dr. Utz's work was supported by the Northern California Chapter of the Arthritis Foundation, the Dana Foundation, the Stanford Program in Molecular and Genetic Medicine, NIH grants DK-61934, AI-50854, AI-50865, and AR-49328, and NHLBI Proteomics contract N01-HV-28183. Dr. Utz is also recipient of a Donald E. and Delia B. Baxter Foundation Career Development Award, and an Investigator award from the Arthritis Foundation.

Wolfgang Hueber, MD, Defu Zeng, MD, Samuel Strober, MD, Paul J. Utz, MD: Stanford University School of Medicine, Stanford, California.

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

Submitted for publication February 26, 2004; accepted in revised form June 4, 2004.

(SP) (CD4+ or CD8+) subset of transgenic T cells into BALB/c/nu/nu animals activates syngeneic host B cells to produce anti–double-stranded DNA (anti-dsDNA) autoantibodies (8) and induces the rapid onset of glomerulonephritis (GN). The SP transgenic T cells express a Th1-like cytokine pattern. Double-negative (DN) (CD4−,CD8−) transgenic T cells, which express a Th2-like pattern, do not induce lupus GN (8).

To further elucidate the humoral autoimmune response in the CD1 lupus-like model, we investigated the possibility that autoantibodies other than anti-dsDNA antibodies are produced. We used immunoprecipitation (IP) and Western blot (WB) analyses to screen sera of CD1 lupus mice for their ability to recognize novel autoantigens. We identified novel, interferon (IFN)–inducible autoantigens, including a member of the 70-kd heat-shock protein (Hsp70) family as well as 2 other IFN-inducible factors that comigrate, as detected on WB, with the gene products recently identified in a murine lupus-susceptibility gene cluster (9).

Our results suggest an additional role for IFNα in lupus, which is consistent with recent reports that have implicated IFNα as a key cytokine in the pathogenesis of human lupus (10–12) and hereditary murine lupus (13). Of note, the human homolog and close relative of the murine IFN-inducible 200 (Ifi200) protein family, the IFN type I– and type II–inducible Ifi16 protein (14), is a known target of subsets of antinuclear antibodies in human SLE (15). Our results suggest that cytokine-inducible proteins may represent a new class of autoantigen targets in this disease.

MATERIALS AND METHODS

Transgenic mice. Development of SP and DN lines of TCR α- and β-chain gene–transgenic mice has been described in detail previously (16). Briefly, 2 lines of transgenic mice were established using the TCR α-chain (V_α4.4–J_α24) and β-chain (V_β9–D_β1.1–J_β2.1) genes from a cloned CD4−,CD8− α/β+ (DN) T cell line derived from the spleen of a BALB/c mouse (16). The TCR genes were expressed in CD4+,CD8− and CD4−,CD8+ (SP) T cells and double-positive (DP) T cells in the thymus and in SP T cells in the peripheral lymphoid tissues and bone marrow in one transgenic mouse line, and predominantly in DN T cells in the other line (16). In previous in vitro experiments, we demonstrated that this particular clone of transgenic CD4+ and CD8+ cells proliferated in response to syngeneic B cells expressing CD1, but did not proliferate in response to nontransfected control B cells lacking CD1 expression (8). These experiments were performed using a BALB/c B cell line (A20) that had been transfected with complementary DNA encoding CD1 (a gift from M. Kronenberg, La Jolla Institute for Allergy and Immunology, La Jolla, CA). In the experiments reported herein, we used CD1 TCR–transgenic mice that were backcrossed to BALB/c mice for at least 10 generations; 2–3-month-old male mice were used as cell donors.

Induction of autoimmune disease. Lethally irradiated (800 cGy whole-body irradiation), 2–3-month-old male host BALB/c/nu/nu mice received 5×10^6 SP transgenic donor bone marrow cells intravenously, along with 2×10^5 sorted, SP transgenic donor spleen CD4+ or CD8+ T cells within 24 hours after irradiation. Briefly, the bone marrow and spleen cells were stained with saturation concentrations of phycoerythrin (PE)–conjugated anti-CD4 (GK1.5) and PE-conjugated anti-CD8 (anti-Lyt2) monoclonal antibodies (obtained from Caltag, Burlingame, CA), since the TCRβ transgene uses the V_β9 gene segment (8). Counterstaining was performed using fluorescein isothiocyanate–conjugated anti-TCRα/β (H57-597) or anti-V_β9 (MR10-2) monoclonal antibodies, obtained from PharMingen (San Diego, CA). Previous studies showed that the SP transgenic T cells contained within the bone marrow cell inoculum mediate the induction of lupus, and that the addition of sorted splenic T cells increases the rapidity and severity of disease activity (8).

For the serologic studies reported herein, 2 groups of mice were injected in separate experiments. In group A, of the 6 adoptive host mice that were injected with SP transgenic bone marrow and splenic T cells, 4 developed lupus with GN. In group B, of the 8 adoptive host mice that were injected with SP transgenic bone marrow and splenic T cells, 5 developed lupus with GN.

Serum. Serum from the adoptive hosts of group A was obtained serially at days 0, 20, 35, 45, 60, and 75 after the adoptive transfer procedure. Serum from the adoptive hosts of group B was obtained at days 0, 45, and 60. These sera and additional sera from unmanipulated wild-type BALB/c/nu/nu adoptive hosts that received DN transgenic cells, as well as sera from NZB/NZW, MRL++, MRL/*lpr*, and normal BALB/c mice were stored at −80°C until used. Proteinuria was measured as described previously (8). Proteinuria of more than 100 mg/dl, measured in 2 separate determinations, was considered to be the cutoff level for positivity.

Antibodies. The mouse polyclonal antibodies were a gift from Dr. W. J. van Venrooij, University of Nijmegen, Nijmegen, The Netherlands (anti-U1A/U2B′′9A9), from Drs. D. Choubey and P. Lengyel, Yale University, New Haven, CT (rabbit anti-mouse Ifi202 [anti-Ifi202(PL)]), and from Drs. G. Gribaudo and S. Landolfo, University of Turin, Turin, Italy (rabbit anti-mouse Ifi203). Anti-Hsp70/Hsc70 mouse monoclonal antibodies were purchased from StressGen, San Diego, CA. Goat anti-Ifi202 polyclonal antibody (M-15) was purchased from Santa Cruz Biotechnology (anti-Ifi202[SC]; Santa Cruz, CA).

Cell culture. NIH3T3 cells and EL-4 cells were grown in 5% CO_2 at 37°C, using RPMI 1640 (for EL-4 cells) and Dulbecco's modified Eagle's medium (for 3T3 cells) (Gibco BRL, Grand Island, NY). Medium was supplemented with 9% heat-inactivated fetal bovine serum (for EL-4 cells) or bovine calf serum (for 3T3 cells) (BioWhittaker, Walkersville, MD), penicillin, and streptomycin (Gibco BRL). Cells were grown and harvested at mid-log phase (for EL-4) or 80% confluency (for 3T3), unless indicated otherwise.

Metabolic labeling. The 3T3 cells were incubated at 80% confluency, and the EL-4 cells were incubated at a density

of 1×10^6 cells/ml in labeling medium containing the following: 90% RPMI 1640 lacking either phosphate or methionine and cysteine (Gibco BRL), and 10% heat-inactivated fetal calf serum that had been dialyzed to equilibrium against a buffer containing 10 m*M* HEPES and 140 m*M* NaCl (Sigma, St. Louis, MO). Either ^{32}P-orthophosphate or ^{35}S-methionine and cysteine (NEN, Boston, MA) were added at a concentration of 0.15 mCi/ml and 0.25 mCi/ml, respectively. Cells were then incubated at 37°C for 5 hours.

Cell lysis and activation with IFNα. Lysis of cells was performed as described previously (17). Recombinant murine IFNα (Calbiochem, La Jolla, CA) was used for all antigen-induction experiments at a concentration of 750 IU/ml culture medium. NIH3T3 and EL-4 cells were treated with IFNα for 18 hours, beginning at 50% confluency in the case of 3T3 cells or at 8×10^5 cells/ml in the case of EL-4 cells, followed by 5 hours of supplementation of the medium with fresh IFNα at 750 units/ml during metabolic labeling.

IP and WB analyses. Lysates were precleared 3 times for 15 minutes with 15 μl of a 50% solution of protein A–Sepharose (Pharmacia, Uppsala, Sweden) in phosphate buffered saline (PBS) and 2.5 μg rabbit anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). IP was performed in 1% Nonidet P40 (NP40) lysis buffer followed by rotation in a cold room overnight. Precipitates were harvested by centrifugation for 15 seconds at 13,000 revolutions per minute and washed 3 times with NP40 lysis buffer, resuspended in sodium dodecyl sulfate (SDS) loading buffer with 4% 2-mercaptoethanol, boiled for 2 minutes, and electrophoresed on 12% or 7% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels.

Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for WB experiments or to polyvinylidene difluoride (PVDF; Dupont-New England Nuclear, Boston, MA) for phospho–amino acid analysis, and either exposed for autoradiography or subjected to WB analysis. For the IP and WB studies, nitrocellulose membranes were excised and blocked in 3% fat-free dry milk in PBS at room temperature for 1 hour, or at 4°C overnight. Anti-Hsp70/Hsc70 monoclonal antibodies (StressGen) were used at a dilution of 1:5,000 in 3% fat-free dry milk in PBS. Bands were detected using donkey anti-mouse IgG, conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch), and visualized using enhanced bioluminescence (Pierce, Rockford, IL), in accordance with the manufacturer's specifications. For WB detection of autoantibodies against p70 and Hsp70/Hsc70, strips of nitrocellulose membrane were blocked as before and incubated with 1:200 dilutions of the same autoimmune serum or same anti-Hsp70 monoclonal antibodies that had been used to immunoprecipitate p70 or Hsp70, respectively. Bands were visualized as described above.

For Ifi202-preclearing experiments, NIH3T3 cell lysates were first precleared as described above, followed by specific preclearing 2 times, for 1 hour and overnight, using 5 μl of anti-Ifi202(SC) polyclonal antibody and 40 μl protein G–Sepharose. IP from precleared lysates was then performed with CD1 lupus serum or anti-Ifi202(SC), using either the lysate precleared with anti-Ifi202(SC) or a control lysate that was not precleared with specific antibody.

Two-dimensional phospho–amino acid analysis. Immunoprecipitates that had been electrophoresed and transferred to PVDF membranes were rinsed and exposed for autoradiography, and appropriate bands were excised. The radiolabeled bands were then subjected to acid hydrolysis as described previously (18), followed by 2-dimensional electrophoresis performed at 14°C.

RESULTS

Immunoprecipitation of a 70-kd and 50-kd protein from cell lysates using serum derived from CD1 lupus mice. Anti-CD1 TCR–transgenic T cells from donor BALB/c mice were transferred to irradiated BALB/c/nu/nu recipients, and development of lupus was monitored by measuring proteinuria and the presence of anti-dsDNA autoantibodies in the serum (8). To screen transgenic donor and adoptive recipient nu/nu mouse sera for additional autoantibodies, we performed IPs from lysates prepared from radiolabeled mouse cell lines. NIH3T3 fibroblasts and EL-4 lymphocytes were metabolically labeled with ^{35}S-methionine, and lysates were prepared. Sera obtained at day 45 from recipients of SP transgenic T cells (Figure 1, lane 3) and recipients of DN transgenic T cells (Figure 1, lane 4), as well as sera from donors of SP transgenic T cells (Figure 1, lane 2) and from an MRL++ mouse (Figure 1, lane 5) were used to precipitate autoantigens from the lysates. Immunoprecipitated proteins were separated by SDS-PAGE on 12% gels, transferred to nitrocellulose, and exposed for autoradiography.

Two striking patterns of antibody reactivities were observed in the sera from recipient mice with lupus that had been injected with SP transgenic T cells, but not in the sera from recipients of DN transgenic T cells without lupus. When sera from the SP transgenic T cell recipients were analyzed, one of the precipitated antigens migrated at ~70 kd (termed p70), and a second protein migrated as a broad, 50-kd band (termed p50) (Figure 1, lane 3). SDS-PAGE on 7% gels, which allows better resolution in the 50-kd range, revealed that the broad 50-kd band migrated as a distinct doublet of 48/52 kd (see Figure 5B, lane 3). Of the 4 animals of group A that received SP transgenic T cells and showed progression to severe lupus, 2 developed antibodies capable of precipitating p50 and p70 (Figure 1). The other 2 animals with lupus developed antibodies that recognized a novel RNP complex (19).

Serologic findings from both group A mice and group B mice and from control animals are summarized

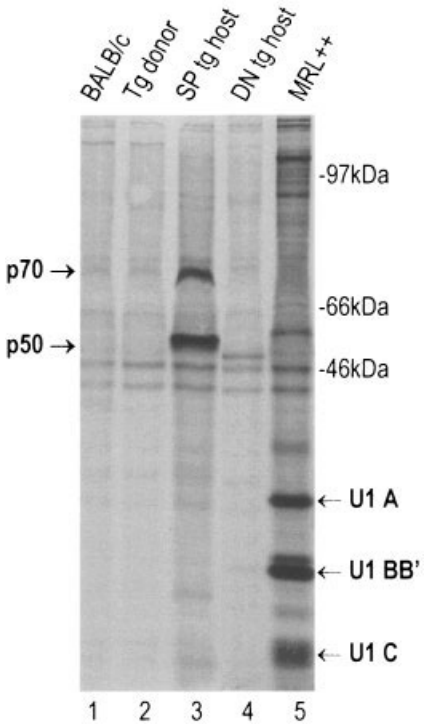

Figure 1. Novel autoantigens recognized by serum derived from a subset of CD1 lupus mice. ^{35}S-labeled EL-4 cells were lysed in Nonidet P40 lysis buffer, and proteins were precipitated using serum from healthy and diseased mice: normal BALB/c, donor of anti-CD1 T cell receptor–transgenic (tg) bone marrow, tg host with severe systemic lupus erythematosus (SLE) that had received single-positive (SP) tg bone marrow, tg host without SLE that had received double-negative (DN) tg bone marrow, and 12-month-old MRL++. The molecular markers are indicated by their relative molecular weight on the right. Arrows on the right indicate proteins of the U1 small nuclear RNP complex that are precipitated by the MRL++ autoimmune serum. Arrows on the left indicate the novel autoantigens (p50 and p70).

in Table 1. Of the 14 mice that were injected with the disease-inducing SP transgenic T cell subset, 9 developed severe GN, and 7 of those 9 animals had anti-p50 antibodies. No anti-p50 antibodies were found in the sera from the 5 animals that were injected with the disease-preventing DN T cells, from the 4 transgenic BALB/c donor animals, and from the 8 healthy control animals (5 normal BALB/c, 3 BALB/c/nu/nu). Anti-p70 autoantibodies were found in the sera from 5 of the 7 anti-p50–positive mice. Anti-p70 alone was also precipitated, albeit as a considerably weaker band, by serum from 2 of the SP transgenic T cell recipients that developed mild proteinuria but not overt lupus, by serum from 1 DN transgenic T cell recipient, and by serum from 1 transgenic healthy donor (Table 1). We conclude that anti-p50 antibodies are strongly associated with GN in the CD1 lupus-like model, and that a majority of CD1 lupus autoimmune sera coprecipitate p50 and p70.

Appearance of antibodies against p50 and p70 as early as 3 weeks after induction of disease. We sought to establish the time course of autoantibody development in the CD1 lupus-like model. CD1 lupus sera (from the recipients of SP transgenic T cells) were obtained at induction and at 5 time points after induction of disease. To minimize interassay variability, all sera were screened simultaneously for the presence of antibodies in a single IP experiment. Results from 2 representative animals with p50 and p70 reactivity are shown in Figure 2. In transgenic host animal A, antibody reactivity could be detected at day 20 after induction of lupus, and this peaked at day 35 and disappeared from day 45 onward. Similarly, in transgenic host animal B, antibodies were detectable as early as day 20, and thereafter remained stable over the observation period. Animal A developed severe ascites between day 30 and day 45; the decline in antibody detection may thus have been caused by sequestration of Igs into the abdominal cavity. Animal B developed severe kidney disease but no significant ascites.

Table 1. Summary of serologic findings in the CD1 lupus model*

	No. of mice	Anti-p50	Anti-p70	Anti-p50 and anti-p70	Anti-dsDNA	Proteinuria
SP Tg recipient mice with severe kidney disease	9	7 (78)	5 (55)	5 (55)	9 (100)	Severe
SP Tg recipient mice with mild disease	5	0	2 (40)	0	1 (20)	Mild
DN Tg healthy recipient mice	5	0	1 (20)	0	0	None
Tg BALB/c healthy donor mice	4	0	1 (25)	0	0	None
Healthy BALB/c mice	5	0	0	0	0	None
Healthy BALB/c/nu/nu mice	3	0	0	0	0	None

* Except where indicated otherwise, values are the no. (%) with positive serologic findings. Anti-dsDNA = anti–double-stranded DNA; SP = single-positive (CD4+ or CD8+); Tg = (anti-CD1 T cell receptor) transgenic; DN = double-negative (CD4−,CD8−).

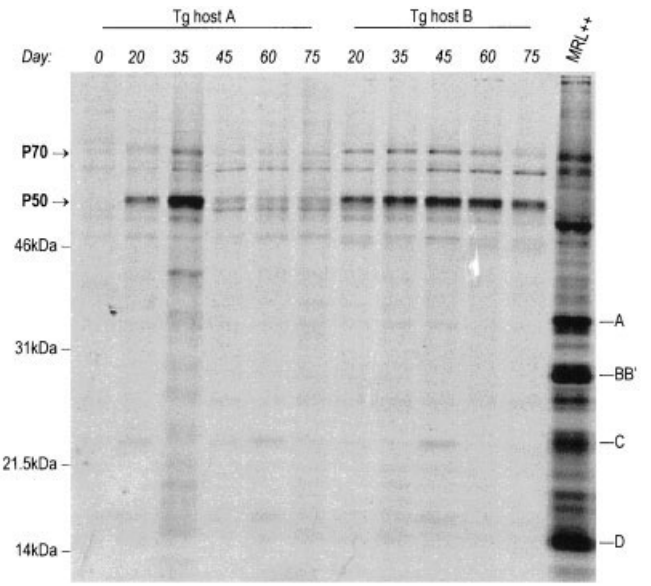

Figure 2. Appearance of anti-p50/p70 antibodies as early as 20 days after induction of disease. Radiolabeled EL-4 cells were lysed and proteins were precipitated using serum obtained at sequential time points after disease induction (in days) from 2 individual anti-CD1 T cell receptor–transgenic (Tg) host mice, A and B. Along with the proteins, the relative migration of molecular mass markers is indicated on the left. Serum from an MRL++ mouse was used to precipitate U1 small nuclear RNP proteins, which are indicated on the right.

Categorizing p70 in the Hsp70 family. A spectrum of proteins belonging to the Hsp70 family migrates around 70 kd, as revealed by SDS-PAGE. Autoantibodies directed against Hsp70 were previously described in older MRL/*lpr* lupus mice (20). To investigate whether p70 might be a member of the Hsp70 family, we performed IP and WB experiments and used a mouse monoclonal antibody that specifically recognizes both constitutive and inducible Hsp70/Hsc70. First, we immunoprecipitated p70 with prototypic CD1 lupus serum and Hsp70 with anti-Hsp70 reference serum as a positive control, respectively. The nitrocellulose membrane containing the 70-kd proteins was then subjected to WB analysis with monoclonal antibody anti-Hsp70/Hsc70. A strong band was seen for both p70 (Figure 3A, lane 1) and Hsp70 (Figure 3A, lane 2), indicating that p70 is a member of the Hsp70 family.

To determine whether the CD1 lupus serum autoantibodies directly recognized Hsp70 on WB, we excised strips of nitrocellulose membrane containing p70 that were immunoprecipitated by 4 additional CD1 lupus sera, as well as Hsp70 that was immunoprecipitated by monoclonal antibody anti-Hsp70. In the subsequent WB step of the experiment, the strips were blocked and then incubated with 1:200 dilutions of the autologous serum (Figure 3B, lanes 1–4 and lane 6) or anti-Hsp70 (Figure 3B, lane 5). Bound serum antibodies were detected using HRP-conjugated donkey anti-mouse antibody, and visualized by chemiluminescence. All sera recognized a 70-kd band that exactly comigrated with Hsp70, suggesting that the autoantibodies directly recognize Hsp70 (Figure 3B, lanes 1–4). We were unable to unequivocally detect autoantibodies directly recognizing the p50 antigen, because of the presence of the comigrating Ig heavy chain (results not shown).

Identification of p50 as a serine phosphoprotein. We attempted to better characterize the 50-kd antigen, first by investigating whether p50 was a phosphoprotein.

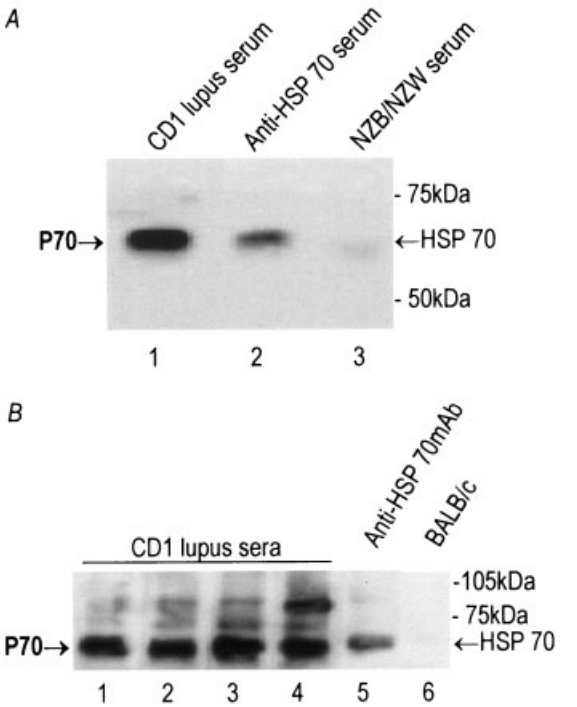

Figure 3. Identifying p70 as a member of the 70-kd heat-shock protein (HSP70) family of proteins. **A,** ^{35}S-labeled proteins were immunoprecipitated using mouse antisera and anti-HSP70 reference immune serum, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 7% gel. Proteins were transferred to nitrocellulose membranes and exposed for autoradiography. The p70 bands were identified, and the membrane was excised accordingly. After overnight blocking, Western blotting with a 1:5,000 dilution of anti-HSP70/HSC70 monoclonal antibody (mAb) was performed. **B,** p70 was immunoprecipitated using 4 different CD1 lupus sera (lanes 1–4) and anti-HSP70 mAb (lane 5). Individual membranes were blocked and then probed with 1:200 dilutions of the same sera and antibody, respectively, that were used for the immunoprecipitation. The relative migration of molecular markers is indicated with their relative molecular weights on the right.

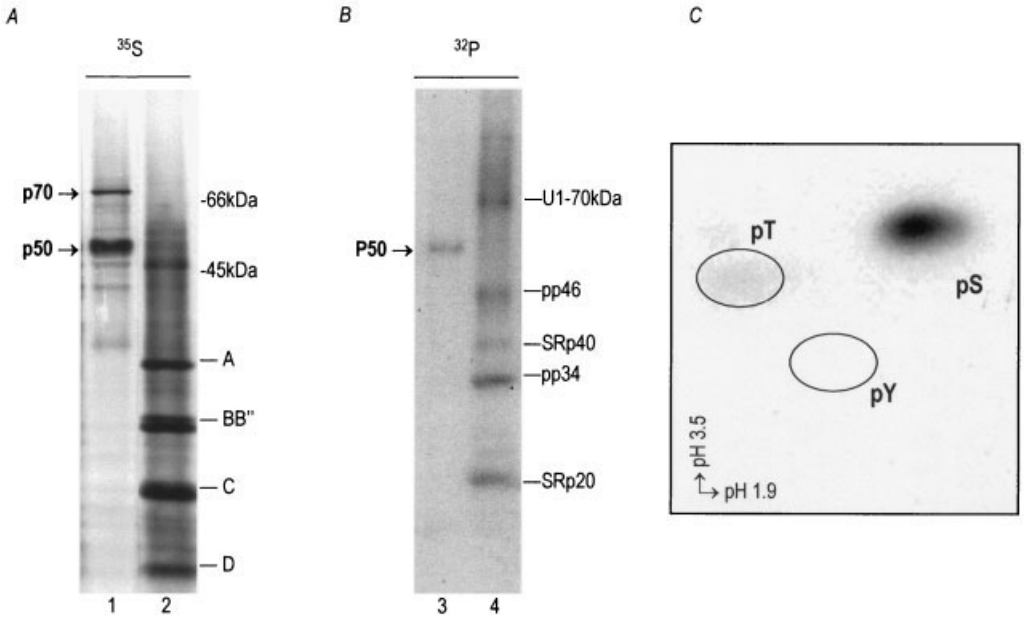

Figure 4. Identifying p50 as a serine phosphoprotein. EL-4 cells were separately labeled with ^{35}S-methionine **(A)** and ^{32}P-orthophosphate **(B)** for 5 hours and subjected to detergent lysis. Proteins were then precipitated from the cell lysate using a prototype sample of CD1 lupus mouse autoimmune serum (lanes 1 and 3), or the mouse monoclonal antibody 9A9 specific for U1-A and U2-B′′ (lanes 2 and 4) as a control. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and exposed for autoradiography. Bands corresponding to the U1 small nuclear RNP proteins A, BB′′, C, and D are indicated on the right in **A.** Phosphoproteins precipitated by the monoclonal antibody 9A9 (SR splicing factors) are indicated on the right in **B.** p50 was excised from the membrane and subjected to acid hydrolysis, in which phospho–amino acids were separated by 2-dimensional electrophoresis in pH 1.9 buffer in the horizontal dimension followed by pH 3.5 buffer in the vertical dimension **(C)**, before autoradiographic analysis. Phospho–amino acid standards are labeled as follows: phosphothreonine (pT), phosphotyrosine (pY), and phosphoserine (pS).

EL-4 cells were metabolically labeled with ^{32}P-orthophosphate for 5 hours, and after preparation of a cell lysate, we immunoprecipitated p50 and p70 with prototypic CD1 lupus sera. Proteins were subjected to SDS-PAGE, transferred to PVDF membrane, and exposed for autoradiography.

We found that p50 was precipitated not only from EL-4 lysates labeled with ^{35}S, but also from lysates labeled with ^{32}P, indicating that p50 is indeed a phosphoprotein (Figures 4A and B, lanes 1 and 3). In contrast, p70 was not precipitated from ^{32}P-labeled protein extract, suggesting that this antigen is not phosphorylated (Figure 4B). The predominant site of phosphorylation was identified on serine residues, using 2-dimensional phospho–amino acid analysis (Figure 4C). Taken together, these results suggest that the p50 antigen is a 50-kd serine phosphoprotein that is coprecipitated along with an Hsp70 family member.

Recognition of IFNα-inducible proteins by CD1 lupus serum. Hsp70 has been identified by other investigators as an autoantigen in murine and human lupus (20,21), mixed connective tissue disease (22), and rheumatoid arthritis (23). Members of the Hsp70 family have been shown to associate with some members of the IFN-inducible Ifi200 proteins, and Hsp70 itself is inducible at an ~2-fold increased level in cells exposed to IFNα (24). In mice, Ifi200 proteins comprise Ifi202a and Ifi202b (~52 kd), Ifi203 (48 kd), Ifi204 (72 kd), and D3 (47 kd) (25,26). Ifi200 proteins recently have been identified as candidate lupus-susceptibility gene products in murine lupus (9). Biochemical characterization of p50 confirmed that molecular weight, phosphorylation, and association with Hsp70 are shared features of the unknown antigen p50 and several members of the Ifi200 family.

To elucidate the role of Ifi200 proteins as poten-

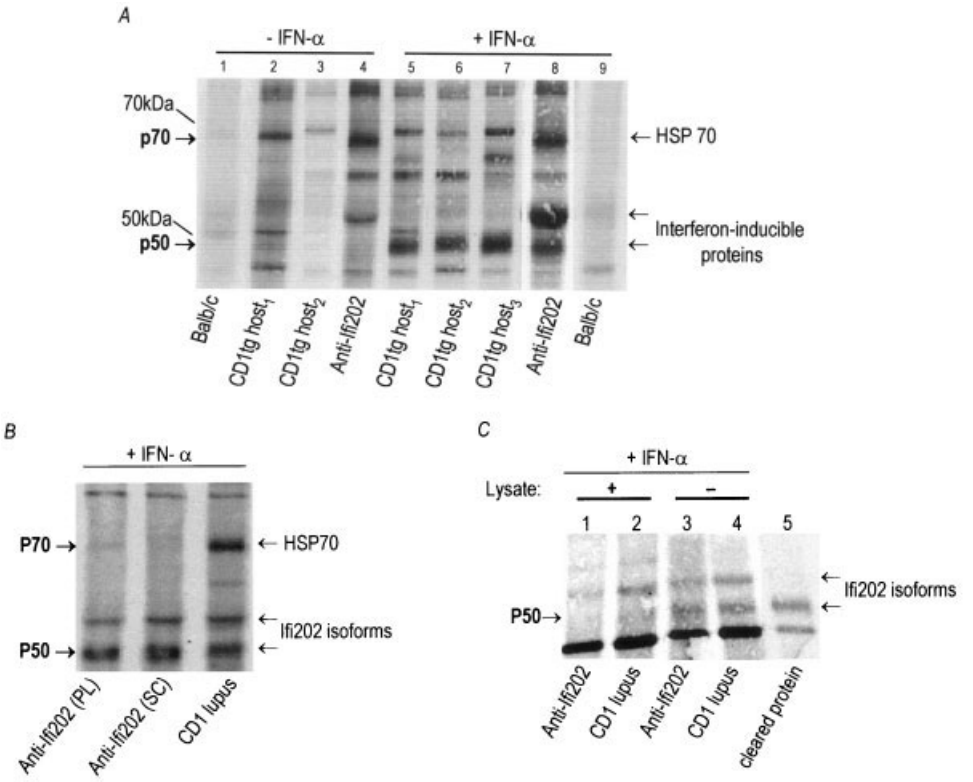

Figure 5. Identifying p50 as an interferon-α (IFN-α)–inducible (Ifi) protein of the Ifi202 subfamily. NIH3T3 cells were separately incubated at 50% confluency without (−) or with (+) IFN-α for 18 hours, followed by metabolic labeling with ^{35}S-methionine for 5 hours. After cell lysis, proteins were immunoprecipitated using 2 μl of mouse sera and anti-Ifi202 polyclonal antibodies. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 7% gel. Arrows on the right indicate proteins precipitated by anti-Ifi202 immune serum, and arrows on the left indicate the antigens. In **A,** lanes 1–4 represent immunoprecipitations from protein extracts prepared from untreated 3T3 cells, and lanes 5–9 from 3T3 cells exposed to IFN-α. For CD1-transgenic (tg) host 3, only the result following IFN-α treatment is shown (lane 7). HSP 70 = 70-kd heat-shock protein. In **B,** 2 different anti-Ifi202 polyclonal antibodies (PL = Peter Lengyel, SC = Santa Cruz Biotechnology) were used to demonstrate precise comigration of IFN-α–inducible proteins recognized by anti-Ifi202 and a prototypic CD1 lupus serum, respectively. In **C,** lysate was either precleared twice with anti-Ifi202 (+) or not precleared (−). Proteins precleared from lysate (+) by anti-Ifi202 were run as a positive control (lane 5 in **C**). The strong unidentified band of ~42 kd serves as a loading control.

tial candidate antigens in the CD1 lupus-like model, we sought to determine whether p50 might be an IFN-inducible antigen. We used recombinant mouse IFNα to induce Ifi200 proteins in NIH3T3 cells. Cells were then metabolically labeled using ^{35}S-methionine, and lysates were prepared. Lysates from untreated 3T3 cells were used for control IPs under equivalent loading conditions. A strong band was detected at ~50 kd in the lanes representing precipitates from CD1 lupus sera, using lysates from IFNα-treated cells (Figure 5A, lanes 5, 6, and 7), whereas no bands were seen in this range when lysates from untreated cells were used (Figure 5A, lanes 2 and 3). Rabbit anti-mouse Ifi202(PL) antiserum weakly precipitated a 52-kd and a 68-kd protein from untreated cell lysate (Figure 5A, lane 4). Those proteins as well as an additional protein of 48–50 kd were efficiently immunoprecipitated from the IFNα-treated cell lysate by the same anti-Ifi202 antiserum (Figure 5A, lane 8). This result demonstrates that serum antibodies derived from CD1 lupus mice immunoprecipitate several different IFN-inducible antigens.

Categorizing p50 in the Ifi200 family. The 48–52-kd and the 68-kd bands precipitated by the anti-Ifi202 sera were previously reported to correspond to Ifi202

and an Hsp70 family member, respectively (24). All CD1 lupus sera precipitated an IFN-inducible autoantigen, and this protein precisely comigrated with the 48-kd protein immunoprecipitated by anti-Ifi202 reference serum (Figure 5A, lanes 5–8). In additional experiments, both p48 and p52 were precipitated by prototypic CD1 lupus sera and precisely comigrated with the 48-kd and 52-kd proteins recognized by 2 different anti-Ifi202 polyclonal antibodies (anti-Ifi202[PL] and anti-Ifi202[SC]) (Figure 5B, and data not shown), suggesting that p50 is an IFN-inducible protein closely related to Ifi202 (see Discussion).

To confirm that anti-Ifi202 is recognized by CD1 lupus autoantibodies, lysates prepared from NIH3T3 lysates were precleared twice with anti-Ifi202 antibodies. When the precleared lysate was used as a source of antigen, the 48-kd protein was no longer precipitated either by CD1 lupus serum (Figure 5C, lane 2) or by anti-Ifi202 antibodies (Figure 5C, lane 1). The protein precleared from the lysate was separated on the same gel (Figure 5C, lane 5), and precisely comigrated with the 48-kd proteins immunoprecipitated by CD1 lupus serum (Figure 5C, lane 4) and anti-Ifi202 (Figure 5C, lane 3). This result indicates that p50 and the 48-kd protein immunoprecipitated by anti-Ifi202 are identical or closely related. The higher 52-kd protein was not removed from the lysate (Figure 5C, lane 5), and correspondingly was detected by both CD1 lupus serum and anti-Ifi202 (Figure 5C, lanes 1 and 2).

DISCUSSION

In the present study, we discovered and characterized 2 autoantigens, p50 and p70, in the anti-CD1 TCR–transgenic lupus-like model. We identified p70 as a member of the Hsp70 family of heat-shock proteins. Biochemical and serologic characterization of p50 revealed that p50 is a member of the murine Ifi202-protein subfamily. Ifi200 family members have not yet been implicated as antigens in murine autoimmune disease, and therefore represent a truly novel class of autoantigens. Preliminary data indicate that IFNα-inducible proteins are targeted by humoral autoimmune responses in older MRL++ and MRL/*lpr* mice, providing further evidence that this class of autoantigens may play a role in murine lupus (results not shown). These findings are consistent with the results in earlier reports which demonstrated autoreactivity against the IFNγ- and IFNα-inducible Ifi16 protein in subsets of lupus patients (15).

Ifi200 proteins are encoded on mouse chromosome 1 within the *Ifi200* gene cluster and are important regulators of cell growth and differentiation (14). Ifi202 is a key inhibitor of various transcription factors and associates with a 68-kd member of the Hsp70 family (24). This study showed that Ifi202 proteins from 3T3 cells were undetectable on WB analysis, unless the proteins were first induced by activation of cells with IFNα, but were weakly detectable by IP from radiolabeled cell lysates. The fact that Ifi200 proteins are expressed at low levels in some cells may explain why our initial experiments using uninduced cells (Figures 1–4) identified an autoantigen by IP.

Herein we show that p50 shares several biochemical features with Ifi202: molecular weight, phosphorylation, IFNα inducibility, and association with Hsp70 (24). Choubey and Lengyel previously demonstrated that anti-Ifi202 polyclonal antibodies coprecipitate 3 proteins of 68 kd, 52 kd, and 48 kd, that the 48-kd and 52-kd bands precisely comigrate with in vitro–translated Ifi202, and that only the 52-kd Ifi202 band is recognized by anti-Ifi202 polyclonal antibodies on WB analysis (24). Since both of the anti-Ifi202 polyclonal antibodies used in our studies are not known to cross-react with Ifi203, it is most likely that the lower molecular weight protein seen in our experiments represents a posttranslational modification of Ifi202, such as a phosphorylated isoform.

Ifi202 exists as 3 variants, Ifi202a, Ifi202b, and Ifi202c (25). Our observation that the 52-kd factor could not be cleared from the lysates by anti-Ifi202 may be explained by the existence of 2 different Ifi202 complexes, whereby only epitopes in one complex are accessible to both CD1 lupus antibodies and anti-Ifi202 antibodies. Taken together, our results suggest that p50 is an Ifi202 isoform or a homolog within the Ifi202 subfamily of proteins.

Ifi202 from the *Ifi200* gene cluster has recently been identified among ~500 genes on mouse chromosome 1 to be a dominant gene locus controlling susceptibility for lupus outside the MHC system (9). It is striking that autoantibodies produced in the CD1 lupus-like model are directed against proteins encoded by the Ifi202 gene locus. The functional significance of this observation, and a potential pathogenic role of autoantibodies directed against IFN-inducible antigens, awaits further experimentation (11). In a hypothetical model, intermittent or chronically sustained increases of IFNα concentrations activate IFN-inducible genes. We are not aware of investigations that implicate increased expression of Ifi202 protein or Hsp70 in the severity of lupus. In a recent study of SLE patients, transcriptional profil-

ing identified up-regulation of IFNα-inducible genes to be associated with more severe disease (27). We hypothesize that repetitive overexpression of certain proteins, i.e., in the course of viral infections or in the context of other known triggers of lupus disease activity, may facilitate the autoimmune response to certain autoantigens. Such a mechanism could complement other mechanisms, for example, the emergence of neoepitopes presented in surface blebs of apoptotic cells (28).

IFN-regulated proteins of the Ifi200 family may play an important role as autoantigens in early disease. We did not observe alleviation of disease with the emergence of anti-p50 and anti-p70 antibodies. On the contrary, the pathogenicity of anti-Ifi202 antibodies was suggested by their strong association with severe kidney disease, the absence of these antibodies in mice with mild disease and in mice without disease (see Table 1), and their appearance early in the course of disease (Figure 2). Even the most prominent antibodies in murine lupus, antichromatin antibodies, are the only ones in which solid evidence exists for a direct pathogenic role (1,2). Antibody transfer experiments are planned to explore whether anti-Ifi202 antibodies are pathogenic. Remarkably, a subset of antinuclear antibodies from SLE patients recognizes the human IFNα- and IFNγ-inducible protein p16, which is most closely related to murine Ifi202 and Ifi204 (14,15). This report provides a link between human SLE and our findings in a murine lupus-like model, underscoring the possible relevance of IFN-inducible autoantigens in this disease.

Our results extend these findings on IFN-inducible autoantigens in a murine lupus-like disease model, and infer that activation of cell lines with IFNs, preceding the performance of cell-based immunoassays for autoantibody screens, may enhance the sensitivity for the detection of autoantibodies against inducible antigens. This hypothesis is currently being tested in our laboratory. Since autoantibodies are clinically important predictors of autoimmune disease, better sensitivity of autoantibody assays is highly desirable in various clinical settings (29).

Several lines of evidence suggest an etiopathogenic role for IFNα in human lupus (for review, see ref. 30). First, elevated serum levels of IFNα are prevalent in SLE and correlate with disease severity and the presence of anti-DNA antibodies (11,31). Moreover, many exogenous factors that activate the IFN system, such as infections, ultraviolet light, or other stressors, are prominent features of current pathogenic models that involve the initiation and exacerbation of lupus. Furthermore, recent studies suggest a pivotal role for IFNα in SLE initiation by virtue of its ability to induce activation and differentiation of dendritic cells (11), and in the perpetuation of disease by providing a positive feedback loop involving endogenous IFNα-inducing DNA/anti-DNA immune complexes (12,30). Finally, autoantibodies, and more rarely autoimmune syndromes, are occasionally induced in patients treated with IFNα for chronic hepatitis B and hepatitis C infection, and in patients with several malignant disorders (for review, see ref. 32). In this context, SLE is the most frequently encountered IFNα-induced autoimmune disease.

In contrast, the role of IFNα in murine lupus is much less rigorously investigated. A recent report demonstrated that disruption of IFNα signaling in NZB mice by deleting type I IFN receptors significantly decreased the spontaneous development of lupus in these mice (13). Other investigators reported that the enzyme RNA helicase A is an autoantigen in NZB/NZW mice (33), and genetic analysis suggested that the gene encoding RNA helicase A is likely to be regulated by IFNs (34). In a recent murine lupus study using the B6/*lpr* background, higher autoantibody levels, enhanced immune complex formation, and more severe kidney disease were reported following sustained induction of type I IFNs (35). In contrast, we did not observe autoantibodies against inducible antigens in a group of NZB/NZW mice (results not shown). However, due to the small sample size in our study, we cannot rule out the possibility of autoreactivity directed against IFNα-inducible proteins in hereditary lupus. In other preliminary experiments, we found that a subset of autoimmune sera from older MRL++ and MRL/*lpr* mice precipitated IFNα-inducible proteins (results not shown).

Future studies will be directed toward the broader characterization of autoreactivity against IFN-inducible proteins, using larger panels of sera from these models of lupus. Moreover, the identification of inducible antigens and the elucidation of a potential link between IFN-driven overexpression of inducible protein antigens and clinical disease are important issues to address. Since IFN biosignatures, as determined by transcriptional profiling, have recently been reported to identify patients with higher disease scores, in terms of the presence of the SLE classification criteria and more severe disease (27), it will be interesting to analyze whether profiles of autoantibodies directed against IFN-inducible proteins are associated with such IFN biosignatures.

Hsp70 is a known autoantigen in murine lupus (20) and human SLE (21). Gsp78, a member of the Hsp70 family, has recently been implicated as a target in

early autoimmune responses in a murine lupus model, and an intimate association with the lupus autoantigens Ro52 and Ro60 has been demonstrated (36). However, anti-Hsp70 antibodies are also detectable in a variety of autoimmune diseases other than lupus, including rheumatoid arthritis (23). Although anti-Hsp70 antibodies have been suggested as markers for mixed connective tissue disease (22), the principal diagnostic value of anti-Hsp70 antibodies in autoimmune diseases remains unclear (for review, see ref. 37).

Is there a role for CD1 in the development of lupus and in breaking tolerance to certain antigens? CD1 has been implicated in both murine (7) and human (38) SLE, and may be a target for therapeutic interventions (7). Moreover, our finding of protein antigens in the anti-CD1 TCR–transgenic lupus-like model suggests that autoantigens derived from protein complexes may be presented by CD1. The therapeutic potential of such putative ligands would be tremendous and certainly warrants further investigation.

In summary, IFNα-inducible antigens represent a new class of inducible autoantigens. We speculate that adjustments of current immunoassays to enable detection of these novel cytokine-inducible antigens may considerably extend our knowledge of the role of autoantigens in lupus and other autoimmune diseases.

ACKNOWLEDGMENTS

We are grateful to Dr. P. Lengyel for providing the anti-p202 antibodies and insightful discussions, and Dr. G. Gribaudo for providing the anti-p203 antibodies. We also thank Yin Ping Liu for excellent technical assistance, and the members of the laboratories of Drs. Utz and Strober for their insights, encouragement, and critical review of the manuscript.

REFERENCES

1. Reeves WH, Satoh M, Wang J, Chou CH, Ajmani A. Systemic lupus erythematosus: antibodies to DNA, DNA-binding proteins, and histones. Rheum Dis Clin North Am 1994;20:1–28.
2. Lefkowith JB, Kiehl M, Rubenstein J, Di Valerio R, Bernstein K, Kahl L, et al. Heterogeneity and clinical significance of glomerular-binding antibodies in systemic lupus erythematosus. J Clin Invest 1996;98:1373–80.
3. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted $\alpha\beta$+T cells. Nature 1994;372:691–4.
4. Castano AR, Tangri S, Miller J, Holcombe HR, Jackson MR, Huse WD, et al. Peptide binding and presentation by mouse CD1. Science 1995;269:223–6.
5. Lee DJ, Abeyratne A, Carson DA, Corr M. Induction of an antigen-specific, CD1-restricted cytotoxic T lymphocyte response in vivo. J Exp Med 1998;187:433–8.
6. Wilson MT, Singh AK, Van Kaer L. Immunotherapy with ligands of natural killer T cells. Trends Mol Med 2002;8:225–31.
7. Zeng D, Lee MK, Tung J, Brendolan A, Strober S. Cutting edge: a role for CD1 in the pathogenesis of lupus in NZB/NZW mice. J Immunol 2000;164:5000–4.
8. Zeng D, Dick M, Cheng L, Amano M, Dejbakhsh-Jones S, Huie P, et al. Subsets of transgenic T cells that recognize CD1 induce or prevent murine lupus: role of cytokines. J Exp Med 1998;187:525–36.
9. Rozzo SJ, Allard JD, Choubey D, Vyse TJ, Izui S, Peltz G, et al. Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. Immunity 2001;15:435–43.
10. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 2003;197:711–23.
11. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-α in systemic lupus erythematosus. Science 2001;294:1540–3.
12. Ronnblom L, Alm GV. A pivotal role for the natural interferon α-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. J Exp Med 2001;194:59–64.
13. Santiago-Raber ML, Baccala R, Haraldsson KM, Choubey D, Stewart TA, Kono DH, et al. Type-I interferon receptor deficiency reduces lupus-like disease in NZB mice. J Exp Med 2003;197:777–88.
14. Johnstone RW, Trapani JA. Transcription and growth regulation of the HIN-200 family of proteins. Mol Cell Biol 1999;19:5833–8.
15. Seelig HP, Ehrfeld H, Renz M. Interferon-γ–inducible protein p16: a new target of antinuclear antibodies in patients with systemic lupus erythematosus. Arthritis Rheum 1994;37:1672–83.
16. Cheng L, Dejbakhsh-Jones S, Liblau R, Zeng D, Strober S. Different patterns of TCR transgenic expression in single-positive and double-negative cells: evidence for separate pathways of T cell maturation. J Immunol 1996;156:3592–601.
17. Utz PJ, Hottelet M, van Venrooij W, Anderson P. Association of phosphorylated SR proteins and the U1-small nuclear ribonuclear protein autoantigen complex accompanies apoptotic cell death. J Exp Med 1998;187:547–60.
18. Coligan J, Kruisbeek A, Margulies D, Shevach E, Strober W. Two dimensional phosphopeptide mapping. Curr Protocols Immunol 1994;3:11.2.1–8.
19. Hueber W, Zeng D, Sharpe O, Robinson WH, Strober S, Utz PJ. Characterization of novel antigens recognized by serum autoantibodies from anti-CD1 TCR-trangenic lupus mice. Eur J Immunol 2004;34:1654–62.
20. Faulds G, Conroy S, Madaio M, Isenberg D, Latchman D. Increased levels of antibodies to heat shock proteins with increasing age in Mrl/Mp-lpr/lpr mice. Br J Rheumatol 1995;34:610–5.
21. Minota S, Cameron B, Welch WJ, Winfield JB. Autoantibodies to the constitutive 73-kD member of the hsp70 family of heat shock proteins in systemic lupus erythematosus. J Exp Med 1988;168:1475–80.
22. Mairesse N, Kahn MF, Appelboom T. Antibodies to the constitutive 73kD heat shock protein: a new marker of mixed connective tissue disease? Am J Med 1993;95:595–600.
23. Hayem G, De Brandt M, Palazzo E, Roux S, Combe B, Eliaou JF, et al. Anti-heat shock protein 70kDa and 90kDa antibodies in serum of patients with rheumatoid arthritis. Ann Rheum Dis 1999;58:291–6.
24. Choubey D, Lengyel P. Interferon action: cytoplasmic and nuclear localization of the interferon-inducible 52-kD protein that is encoded by the Ifi202 gene from the gene 200 cluster. J Interferon Res 1993;13:43–52.
25. Wang H, Chatterjee G, Meyer JJ, Liu CJ, Manjunath NA, Bray-Ward P, et al. Characteristics of three homologous 202 genes (Ifi202a, Ifi202b, and Ifi202c) from the murine interferon-activatable gene 200 cluster. Genomics 1999;15:281–94.
26. Wang H, Liu CJ, Lu Y, Chatterjee G, Ma XY, Eisenman RN, et al. The interferon- and differentiation-inducible p202a protein

inhibits the transcriptional activity of c-myc by blocking its association with max. J Biol Chem 2000;275:27377–85.
27. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 2003;100:2610–5.
28. Casciola-Rosen LA, Andrade F, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 1994;179:1317–30.
29. Leslie D, Lipsky P, Notkins AL. Autoantibodies as predictors of disease. J Clin Invest 2001;108:1417–22.
30. Ronnblom L, Alm GV. An etiopathogenic role for the type I IFN system in SLE. Trends Immunol 2001;22:427–31.
31. Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL, Notkins AL. Immune interferon in the circulation of patients with autoimmune disease. N Engl J Med 1979;301:5–8.
32. Ioannou Y, Isenberg DA. Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy. Arthritis Rheum 2000;43:1431–42.
33. Yoshida H, Satoh M, Behney KM, Lee CG, Richards HB, Shaheen VM, et al. Effect of an exogenous trigger on the pathogenesis of lupus in (NZB $\times$ NZW)F_1 mice. Arthritis Rheum 2002;46:2235–44.
34. Lee CG, Eki T, Okumura K, da Costa Soares V, Hurwitz J. Molecular analysis of the cDNA and genomic DNA encoding mouse RNA helicase. Genomics 1998;47:365–71.
35. Braun D, Geraldes P, Demengeot J. Type I interferon controls the onset and severity of autoimmune manifestations in lpr mice. J Autoimmun 2003;20:15–25.
36. Kinoshita G, Purcell AW, Keech CL, Farris AD, McCluskey J, Gordon TP. Molecular chaperones are targets of autoimmunity in Ro(SS-A) immune mice. Clin Exp Immunol 1999;115:268–74.
37. Tishler M, Shoenfeld Y. Anti-heat shock protein antibodies in rheumatic and autoimmune disease. Semin Arthritis Rheum 1996; 26:558–63.
38. Sieling PA, Porcelli SA, Duong BT, Spada F, Bloom BR, Diamond B, et al. Human double-negative T cells in systemic lupus erythematosus provide help for IgG and are restricted by CD1c. J Immunol 2000;165:5338–44.