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Cell Survival Failure in Effector T Cells From Patients With Systemic Lupus Erythematosus Following Insufficient Up-Regulation of Cold-Shock Y-Box Binding Protein 1

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Objective. The importance of cold-shock Y-box binding protein 1 (YB-1) for cell homeostasis is well-documented based on prior observations of its association with certain cancer entities. This study was undertaken to explore the role of YB-1 in T cell homeostasis and survival and the potential contribution of YB-1 to the pathogenesis of systemic lupus erythematosus (SLE).

Methods. In the peripheral blood from 25 SLE patients and 25 healthy donors, the expression of YB-1 and frequency of T cell apoptosis was analyzed by quantitative polymerase chain reaction (qPCR) and fluorescence-activated cell sorting of CD4+ T cells ex vivo and also analyzed in T cells in vitro after 6 days of stimulation with anti-CD3–coupled or anti-CD3/anti-CD28–coupled microspheres. YB-1 was overexpressed using lentiviral transduction with wild-type green fluorescent protein (wtGFP) YB-1, and knockdown of YB-1 was achieved using specific short hairpin RNA (shRNA) (3-fold reduction; $P < 0.0001$).

Results. YB-1 expression was significantly lower in apoptosis-prone T cells and in activated T cells from SLE patients compared to YB-1 expression in nonapoptotic T cells and activated T cells from healthy donors ($P = 0.001$). Knockdown of YB-1 in T cells consequently led to expression of proapoptotic molecules and caspase 3 activation (1.6-fold), and subsequently, to apoptosis. Furthermore, YB-1 promoted survival pathways involving enhanced protein expression of the kinase Akt (2-fold) and Bcl-2 (3-fold), even when Fas/CD95 was triggered. YB-1–mediated T cell survival was reversed by Akt and phosphatidylinositol 3-kinase (PI3K) inactivation. In SLE patients, rescue of YB-1 expression strongly promoted survival of T cells and even prevented cell death in T cells that were extremely apoptosis-prone.

Conclusion. Our data show that failure of YB-1 up-regulation in T cells from SLE patients led to enhanced apoptosis. These findings imply that YB-1 plays a crucial role in the disturbed homeostasis of activated T cells leading to hematopoietic alterations in SLE. These insights may help facilitate the development of new treatment strategies for SLE.

INTRODUCTION

Active control of survival and apoptotic cell death pathways is crucial for T cell homeostasis, and thus, balanced immune responses. Mechanisms that support T cell survival ensure efficient responsiveness to acute immune challenges and are required for the generation of T cell memory. Apoptosis, in return, serves to eliminate dysfunctional or infected T cells and is required to terminate immune response (1). Disturbed T cell homeostasis has been implicated in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE). The underlying pathophysiologic mechanisms, however, remain

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incompletely understood. SLE, similar to rheumatoid arthritis and other chronic inflammatory diseases, has been linked to a dysregulation of apoptosis (2–4). Interestingly, both reduced and increased rates of T cell apoptosis have been reported in SLE. For instance, anergy-resistant T cells from SLE patients were found to escape apoptosis by up-regulating cyclooxygenase 2. Moreover, T cells from SLE patients were reported to express elevated levels of the survival factor Bcl-2 (5). In contrast, experiments on peripheral blood mononuclear cells (PBMCs) from SLE patients with infections and fever revealed enhanced apoptosis in activated T cells (6), a fact that may also apply to other autoimmune diseases (7). Consistent with this finding, at least some SLE patients have a reduced amount of peripheral CD4+ T cells and exhibit abundant circulating apoptotic material that promotes autoantibody production (8,9).

Two distinct apoptosis pathways are engaged to maintain homeostatic control of activated T cell populations (10). The extrinsic pathway is triggered by binding of FasL to its cell death–inducing surface receptor Fas/CD95/APO-1 or by tumor necrosis factor (TNF) to related TNF receptors (TNFRs). As both ligands and receptors are progressively up-regulated during T cell activation, they eventually counteract excessive T cell expansion through activation-induced cell death (AICD) (11). The intrinsic pathway reflects the existence of T cell–intrinsic prosurvival/antiapoptotic and proapoptotic factors. The balance may be shifted toward the proapoptotic factors under conditions of severe cell stress or at the end of T cell clonal expansion (ACAD) (12). DNA damage induces enhanced expression of the Bcl-2 interacting, proapoptotic p53 up-regulated modulator of apoptosis (PUMA), which in turn unleashes the proapoptotic protein Bax. This leads to the release of mitochondrial cytochrome C into the cytosol, where it activates caspase 9, which initiates the apoptotic execution cascade at the point which the extrinsic and intrinsic pathways converge (13). Of note, apoptosis may also be abrogated at the level of, for example, death receptors (14), caspases (15), or mitochondria (16). The kinase Akt phosphorylates and thereby inactivates proapoptotic molecules (17), whereas Bcl-2 and Bcl-xl act as prominent antiapoptotic factors at the mitochondrial level (13,18). In fact, Bcl-2 or Bcl-xl gain-of-function mutations are often encountered in T cell leukemia (19).

We recently showed that nuclear enrichment of cold-shock Y-box binding protein 1 (YB-1) tightly correlates with the proliferation of both activated T cells and T cell acute lymphoblastic leukemia (20). YB-1 expression is required for cell cycle progression in primary and Jurkat T cells (20). The pleiotropic functions of YB-1 include transcriptional and translational activities (16,21). Moreover, YB-1 has been implicated in the regulation of cell homeostasis and apoptosis in endothelial and epithelial cells (22–25). In this study, we identified YB-1 as a crucial determinant for the disturbed homeostasis of activated T cells in SLE.

PATIENTS AND METHODS

Patients and healthy donors. PBMCs were obtained from LRS cones (Terumo), which were provided by the Institute of Transfusion Medicine and Immunohematology at Otto von Guericke University Magdeburg, or from fresh peripheral blood samples from healthy donors at the Department of Rheumatology and Clinical Immunology, University of Freiburg or Otto von Guericke University Magdeburg. Peripheral blood from SLE patients was collected at the Clinic for Dermatology and Venereology at the University Hospital Magdeburg and the Department of Rheumatology and Clinical Immunology at the University of Freiburg. Approval for these studies was obtained from the Ethics Committees of the Medical Faculties at the Otto von Guericke University Magdeburg (MD 141/11, 71/14) and at Albert Ludwig University of Freiburg (507/16). All patients provided informed written consent in accordance with the Declaration of Helsinki.

The study was carried out in 45 SLE patients (with SLE Disease Activity Index [SLEDAI] [26] scores ranging from 0 to 10), of whom 37 were women and 8 were men, with ages of female SLE patients ranging from 20 to 79 years and ages of male SLE patients ranging from 21 to 68 years (mean age of 48 years for all SLE patients) and 24 age-matched healthy donors, of whom 18 were women and 6 were men, with ages of female controls ranging from 23 to 53 years and ages of male controls ranging from 32 to 56 years (mean age of 43 years for all controls). All SLE patients met ≥4 American College of Rheumatology (ACR) criteria for SLE (27). The patients received either monotherapy or combination therapy as follows: 9 patients received mycophenolic acid (daily median ± SD doses of 1,080 ± 288 mg daily with monotherapy or 1,250 ± 599.02 mg with combination therapy), 5 patients received azathioprine (daily median ± SD dose of 100 ± 15.81 mg), 6 patients received methotrexate (weekly median ± SD dose of 11.25 ± 3.03 mg), 23 patients received hydroxychloroquine (daily median ± SD dose of 300 ± 97.6 mg), 7 patients received intravenous belimumab (10 mg/kg of body weight once a month), and 16 patients received prednisone (daily median ± SD dose of 4.5 ± 2.31 mg). Five patients had received antibiotic treatment for *Pneumocystis jiroveci* prophylaxis within the last 3 weeks before sampling. No correlation was observed between these treatments and YB-1 expression in activated T cells from SLE patients (data not shown).

Cell isolation and culture. PBMCs were isolated using a density gradient separation with Pancoll human solution (PAN-Biotech). PBMCs were washed for 1 day prior to stimulation. CD4+ T cells were purified using CD4 MicroBeads (Miltenyi Biotec) according to the manufacturer's instruction. CD4+ T cells were cultured in complete RPMI supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin (Thermo Fisher Scientific), and 10% fetal calf serum (Biochrom) in an atmosphere of 5%

CO_2. CD4+ T cells were activated with either anti-CD3 alone or anti-CD3 and anti-CD28 antibodies immobilized on microspheres (Molecular Probes Inc.) at a 1:1 ratio. As previously described (28), 10^8 microspheres were coated with 1 μg/ml of anti-CD3 antibody plus 2 μg/ml of anti-CD28 or isotype control antibody.

Plasmids and cloning. For the knockdown of YB-1, the plasmids pLKO and pLKO–YB-1–short hairpin RNA (shRNA) (Sigma-Aldrich) were genetically modified by replacing the puromycin-resistance gene with the cytoplasmic domain–depleted nerve growth factor receptor gene (ΔNGFR) as a selection marker, as previously described (20). The plasmid pCCLsin.PPT.hPGK.ΔNGFR was kindly provided by R. Bacchetta and L. Passerini (San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy). To overexpress YB-1, the plasmid FuGW was modified with the open-reading frame of green fluorescent protein (GFP)–tagged YB-1. The DNA constructs pCG-HΔ24 and pCG-FΔ30 for the measles virus transduction system were kindly provided by F. L. Cosset and F. Fusil (International Center for Infectiology Research, University of Lyon, Lyon, France). To overexpress Akt1, the plasmids pLenti-Akt1-GFP (RC220257L2) and GFP control (PS100071) were obtained from Origene (Herford, Germany). All constructs were verified by sequencing.

Viral transduction of CD4+ T cells. To generate pseudo measles viral particles, HEK 293T cells were transfected with the expression vector (pLKO or pLKO_YB-1shRNA; both containing ΔNGFR as selection marker), the packaging plasmid psPAX2, and envelope plasmids pCG-HΔ24 and pCG-FΔ30 using calcium phosphate precipitation. Forty-eight and 72 hours after transfection, supernatants containing pseudo viral particles were collected and passed through 45-μm filters (Sarstedtz), with a 42% polyethylene glycol (PEG) solution added at a ratio of 1:5. After incubation for 16 hours at 4°C, concentrated particles were concentrated by centrifugation and the pellet resuspended in RPMI. For transduction of CD4+ T cells, culture plates were coated with 80 μg/ml of RetroNectin (Clontech Laboratories) for 16 hours at 4°C and blocked with 2% bovine serum albumin. After being washed in phosphate buffered saline, the concentrated viral particles were spin-occulated to the wells at 2,000*g* for 2 hours, and CD4+ T cells were added. After 16 hours' incubation, T cells were stimulated as described above.

Antibodies and inhibitors. All antibodies, with clone names, used for flow cytometry and Western blotting can be found in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract. For flow cytometry, Akt inhibitor VIII (Calbiochem), Q-VD-OPH (ApexBIO), and Rapamycin (Sigma-Aldrich) were used.

Protein and RNA quantification. For Western blotting and quantitative reverse transcription–polymerase chain reaction (qRT-PCR), NGFR-positive cells were sorted with a FACSAria III flow cytometer (BD Biosciences). Extraction of total RNA, reverse transcription of isolated messenger RNA (mRNA), and quantification of mRNA by qRT-PCR were performed as previously described (29). Oligonucleotides were obtained from TIB MolBiol (see Supplementary Table 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract). Data analyses were carried out using CFX96 Manager Software (Bio-Rad). Fold change in expression of each gene was normalized to the expression of GAPDH using the $2^{-\Delta\Delta Ct}$ method (30).

Lysates of NGFR-positive T cells were prepared (29), and electrophoresis (12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE] gels) and blotting onto nitrocellulose membranes were performed, as described previously (20). Blots were probed with antibodies and visualized and quantified using an Odyssey scanner and software (Li-Cor).

Flow cytometric analysis. Infected CD4+ T cells were identified by ΔNGFR staining and noninfected CD4+ T cells were used as infection control. For intracellular staining of YB-1, Ki-67, Noxa, Bcl-2, Bcl-xl, and active caspase 3, cells were fixed using the FoxP3 Intracellular/Nuclear Staining Kit (eBiosciences). Cytometric measurements were performed on a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star). For the measurement of apoptotic cells, cells were stained with propidium iodide (PI) and annexin V in binding buffer (10 m*M* Hepes, pH 7.4, 140 m*M* NaCl, 2.5 m*M* $CaCl_2$) or in blocking buffer (10 m*M* Hepes, pH 7.4, 140 m*M* NaCl, 2 m*M* EGTA) as a control. For the detection of dead cells in combination with intracellular staining, the Zombie Violet Fixable Viability Kit (BioLegend) was used according to the manufacturer's instructions.

Statistical analysis. For statistical analyses, a Student's 2-tailed *t*-test was performed. For multiparametric experiments, the analysis of variance test was applied using GraphPad Prism version 6. Pearson's correlation test was used to analyze relationships between YB-1 levels and homeostasis of activated T cells in SLE patients and healthy donors.

RESULTS

Sensitivity of CD4+ T helper cells to apoptosis in SLE patients. The cold-shock protein YB-1 is expressed in T cells, especially after activation (20), and has been implicated in cell survival (23,31). Therefore, we hypothesized that YB-1 might contribute to the altered homeostasis and survival of SLE T cells. As leukopenia is characteristic of SLE patients (32), we first evaluated the contribution of CD4 and CD8 populations to leukopenia. T helper (CD4+) cell numbers especially showed significant reduction (Figure 1A). In order to assess the susceptibility of CD4+ T cells to apoptosis, CD4+ T cells enriched from PBMCs of SLE patients and healthy donors were incubated for 24 hours prior

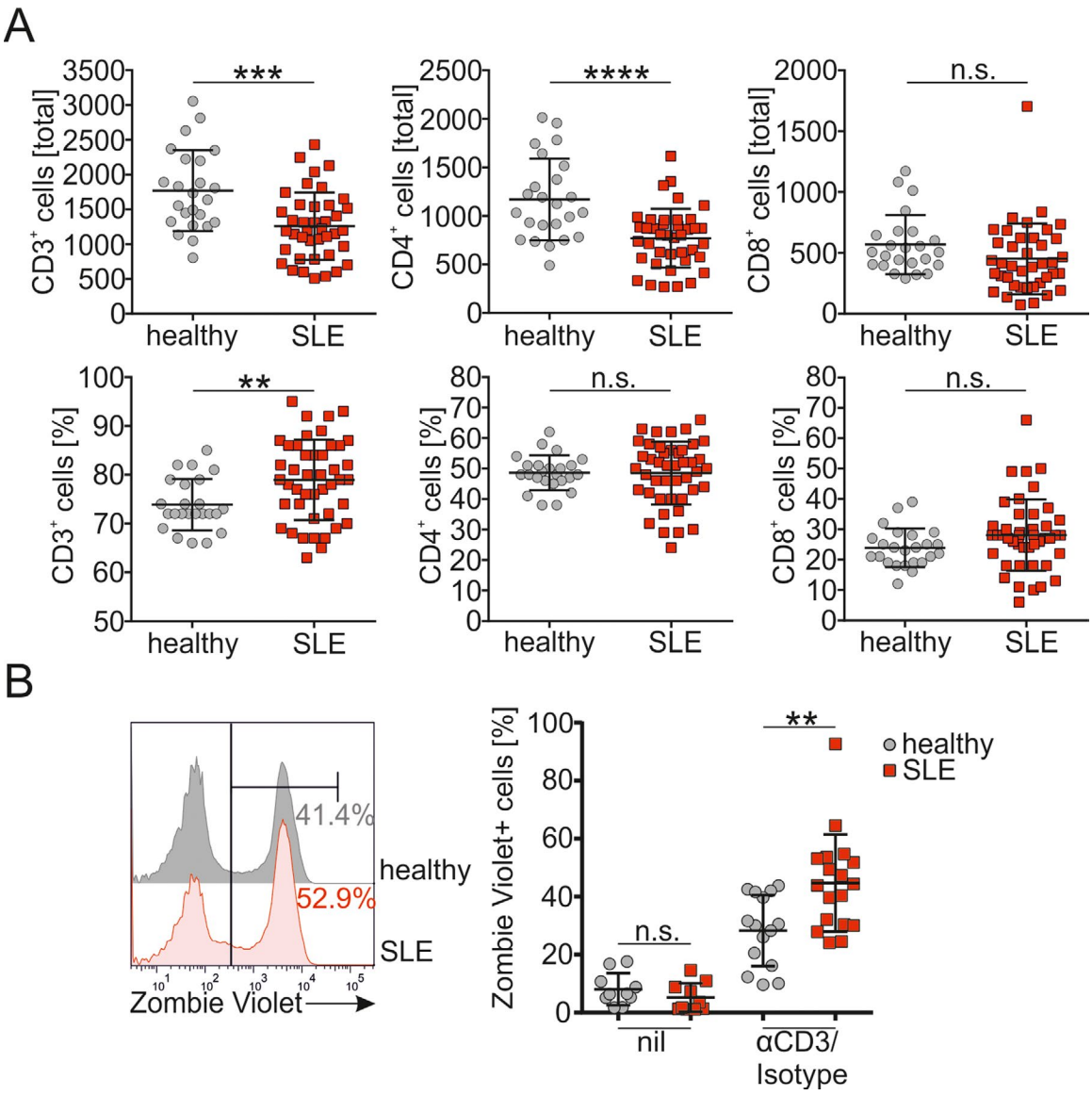

Figure 1. Reduced viability of CD4+ T cells obtained from patients with systemic lupus erythematosus (SLE). **A**, Cellular immune status of EDTA blood obtained from SLE patients (n = 40) or healthy donors (n = 22) was quantitatively analyzed for abundance of lymphocytes (CD3, CD4, and CD8). **B**, Primary CD4+ T cells from SLE patients (n = 18) or healthy donors (n = 15) were stimulated ex vivo with anti-CD3–coated microspheres or unstimulated or resting [nil], and after 6 days of incubation, the cells were assessed by flow cytometry using Zombie Violet (ZV) staining. Representative histograms show anti-CD3–activated CD4+ T cells from each group, as well as the cumulative percentages of ZV+ CD4+ T cells in each group. Symbols represent individual samples; bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$. NS = not significant. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract.

to polyclonal stimulation with anti–CD3-coated microspheres (Figure 1B). Nonviable T cells where identified by flow cytometry using Zombie Violet (ZV) staining. After 2 days of incubation, the frequencies of ZV+CD4+ T cells obtained from SLE patients and healthy donors were comparable. However, after 6 days of incubation, the percentage of apoptotic/dead cells within the CD4+ T cell populations was increased in samples derived from SLE patients (mean ± SD 44.7 ± 3.168%) compared to those obtained from healthy donors (mean ± SD 28.31 ± 3.946%) ($P = 0.0036$).

Correlation of YB-1^{high} expression with viability of activated primary CD4+ T cells. Next, we investigated whether YB-1 counteracts apoptosis in activated CD4+ T cells. Therefore, we first analyzed YB-1 expression in healthy donors (Figure 2A). T cells stimulated with anti-CD3–coated microspheres had YB-1–expressing T cell frequencies of 60–80% on day 4, with frequency of YB-1–expressing T cells dropping to half on day 6 (Figure 2B). This decline was precluded when interleukin-2 (IL-2) was added. In turn, IL-2 dependency was overcome when microspheres coated with both anti-CD3 and anti-CD28 were used

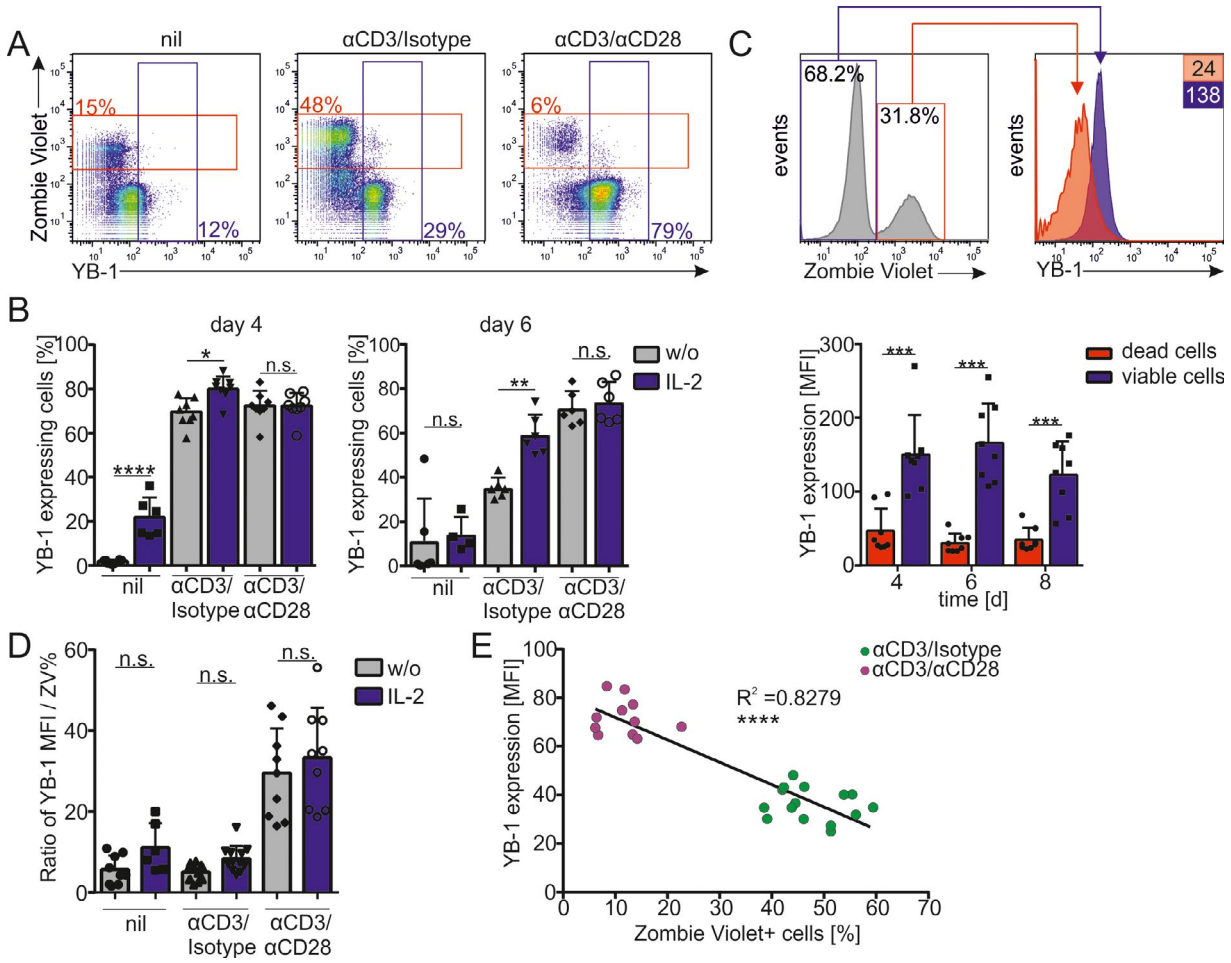

Figure 2. Correlation of increased Y-box binding protein 1 expression (YB-1^{high}) with viability of activated primary CD4+ T cells. **A**, Representative dot plots show YB-1 expression on day 4, as analyzed by flow cytometry, in viable primary CD4+ T cells from a healthy donor activated with either anti-CD3/isotype–coated or anti-CD3/anti-CD28–coated microspheres (or unstimulated or resting [nil]) (as described in Figure 1B). **B**, Histograms show YB-1 expression by activated CD4+ T cells on days 4 and 6 (as described in **A**) in the absence (w/o) or presence of 20 ng/ml of interleukin-2 (IL-2). **C**, Representative histograms show YB-1 expression (mean fluorescence intensity [MFI]) on day 8 in viable CD4+ T cells (Zombie Violet–negative [ZV–]; blue gate) and dead CD4+ T cells (ZV+; red gate) activated with anti-CD3–coated microspheres. Cumulative data for YB-1 expression levels on days 4, 6, and 8 are also shown (n = 8). **D**, Ratio of YB-1 expression to percentage of ZV+ CD4+ T cells is shown for each group of cultures with or without IL-2. **E**, Correlation of YB-1 expression with extent of cell death (percentage ZV+) among activated CD4+ T cells on day 6 is shown. Pearson's correlation coefficient was calculated using 15 individual samples acquired in 6 independent experiments. In **B** and **D**, symbols represent individual samples; bars show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Student's *t*-test. NS = not significant. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract.

for stimulation, most likely due to sufficient IL-2 production by T cells (Supplementary Figure 1A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract). Of note, under each condition, YB-1^{high} cells were almost exclusively found within the ZV– population (Figures 2C and D). In fact, based on the Pearson's correlation coefficients, we observed a strong positive correlation between YB-1 expression levels and T cell viability (Figure 2E).

Effects of YB-1 inhibition by shRNA and apoptosis in CD4+ T cells, and promotion of T cell survival by ectopic overexpression of wild-type YB-1 (YB-1^{wt}). Next, we examined whether reduced levels of YB-1 in dying CD4+ T cells are just a consequence of apoptosis, or whether a decrease in YB-1 expression contributes to apoptosis. To test the possibility of whether reduced YB-1 expression contributes to apoptosis, we used an shRNA knockdown approach. Specifically, constructs expressing YB-1–specific or control shRNA were virally transduced into primary T cells. Truncated NGFR expressed from the same constructs served as a surface marker for the detection and enrichment of transduced cells by flow cytometry and MACS technology. Upon stimulation, T cells transduced with YB-1–specific shRNA displayed a dramatic reduction of YB-1 mRNA and protein expression when compared to controls (Figure 3A and

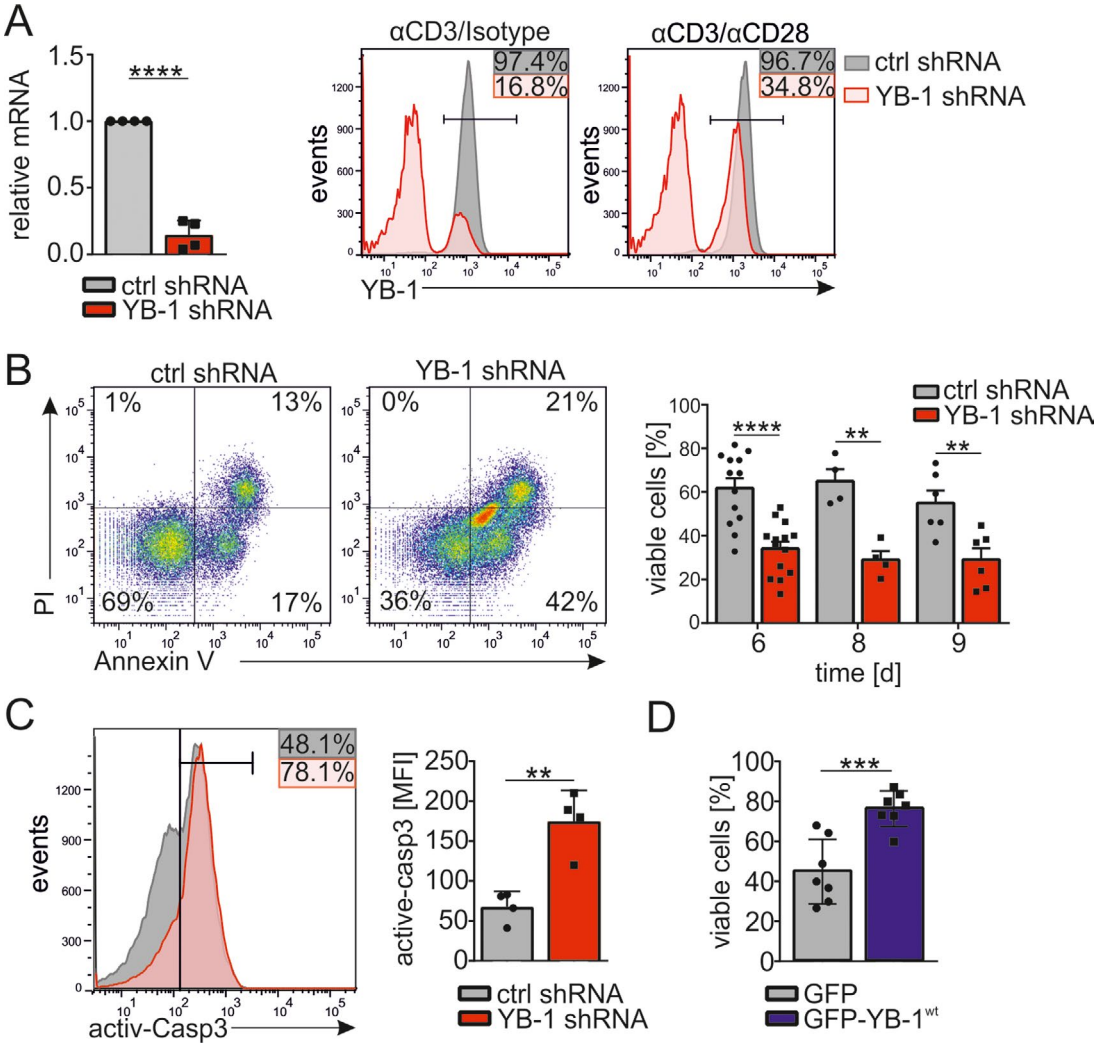

Figure 3. Correlation of Y-box binding protein 1 (YB-1) loss and apoptosis in activated CD4+ T cells, and promotion of CD4+ T cell viability by YB-1 overexpression. **A**, YB-1 expression was knocked down in activated CD4+ T cells by measles virus–mediated transduction of YB-1–specific short hairpin RNA (shRNA) (red) or control shRNA (gray). On day 4, anti-CD3/anti-CD28–activated CD4+ T cells were analyzed for YB-1 mRNA expression by real-time polymerase chain reaction (left) and YB-1 accumulation by flow cytometry (right). **B**, Primary CD4+ T cells were transduced with YB-1–specific shRNA or control shRNA, followed by ex vivo stimulation with anti-CD3/anti-CD28–coated microspheres and then stained with propidium iodide (PI) as well as annexin V. The cells were assessed by flow cytometry on days 6, 8, and 9. Representative dot plots from day 6 are shown (left), along with cumulative percentages of viable cells (n ≥ 4) (right). **C**, Active caspase 3 (activ-Casp3) levels were assessed in CD4+ T cells transduced with YB-1–specific shRNA or control shRNA after stimulation ex vivo with anti-CD3/anti-CD28–coated microspheres. Representative histograms from flow cytometry analysis on day 6 are shown (left), along with cumulative expression (n ≥ 4) (right). **D**, CD4+ T cells were activated with anti-CD3/anti-CD28–coated microspheres and transduced with an overexpressing retroviral wild-type green fluorescent protein–YB-1 (GFP–YB-1^{wt}) vector or control vector (GFP). The amount of viable CD4+ T cells on day 6 was analyzed by flow cytometry. In **A**, **B**, and **D**, symbols represent individual samples; bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Student's *t*-test. MFI = mean fluorescence intensity. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract.

Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract). In order to monitor the frequencies of preapoptotic and apoptotic/necrotic cells, CD4+ T cells were stained with annexin V and PI on days 6, 8, and 9. Compared to controls, YB-1 depletion caused an increase in the frequencies of preapoptotic cells (annexin V+ and PI–) and apoptotic cells (annexin V+ and PI+) by almost 100% (Figure 3B). A hallmark of apoptosis is the activation of caspases and, as depicted in Figure 3C, active caspase 3 was unambiguously increased upon YB-1 knockdown, validating the mediated cell death as an apoptotic process.

To determine whether elevated YB-1 expression is sufficient to enhance T cell survival, we overexpressed GFP-tagged YB-1 (GFP–YB-1^{wt}) from a lentiviral construct. Using a GFP-expressing construct as a control, we found that on day 6 of stimulation, the frequencies of viable CD4+ T cells expressing GFP–YB-1^{wt} exceeded

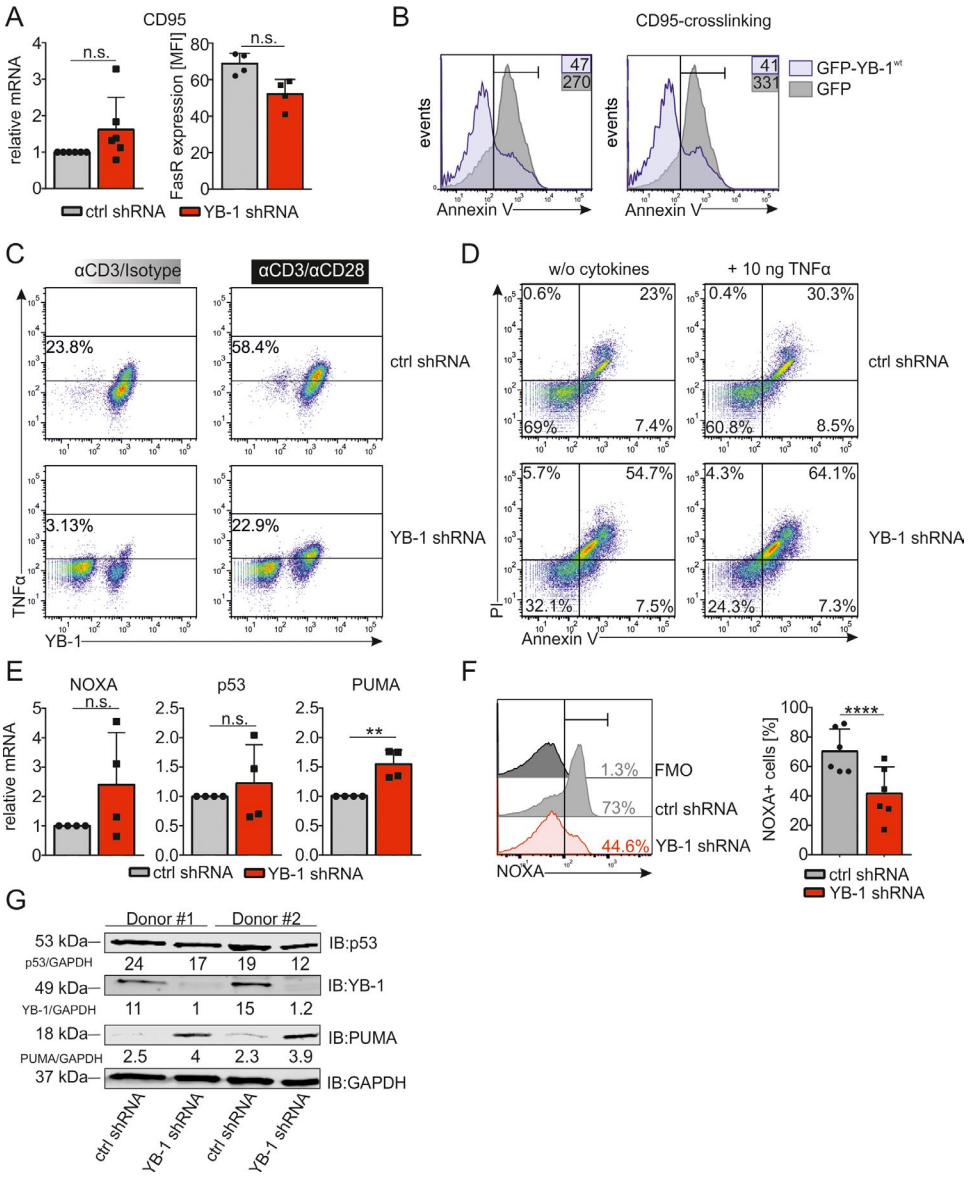

Figure 4. Correlation of Y-box binding protein 1 (YB-1) expression and control of proapoptotic molecules in primed CD4+ T cells. **A**, Primary CD4+ T cells were transduced with YB-1–specific short hairpin RNA (shRNA), followed by ex vivo stimulation with anti-CD3/anti-CD28–coated microspheres and then stained with necrotic propidium iodide (PI) as well as annexin V. The cells were assessed by quantitative polymerase chain reaction or flow cytometry on days 4 and 6, respectively. Expression of FasR (CD95) mRNA on day 4 (left) and surface protein on day 6 (right) in CD4+ T cells is shown. **B**, Representative histograms show primary CD4+ T cells that were activated with anti-CD3/anti-CD28–coated microspheres, followed by transduction with an overexpressing retroviral wild-type green fluorescent protein–YB-1 (GFP–YB-1^{wt}) vector or control vector (GFP). Control cells (left) and CD95-crosslinked CD4+ T cells (right) were stained with annexin V on day 6 and then analyzed by flow cytometry. Numbers indicate percentages of annexin V+ cells. **C**, Representative dot plots show YB-1 and tumor necrosis factor (TNF) expression analyzed by flow cytometry on day 6. Primary CD4+ T cells were transduced by YB-1–specific shRNA or control shRNA (ctrl shRNA), followed by ex vivo stimulation with anti-CD3/anti-CD28–coated microspheres. The cells were analyzed by flow cytometry. **D**, Representative dot plots show untreated control CD4+ T cells (left) or TNF-treated CD4+ T cells (right) that were activated and transduced with shRNA as described in **C** and then stained with annexin V and PI for flow cytometry analysis of cell viability/apoptosis on day 6. **E**, The relative expression of NOXA, p53, and p53 up-regulated modulator of apoptosis (PUMA) mRNA was assessed by quantitative polymerase chain reaction on day 4 in primary CD4+ T cells (n = 4) that were active and transduced as described earlier. **F**, Representative histogram (left) shows intracellular NOXA staining or Fluorescence Minus One (FMO) control staining of CD4+ T cells that were active and transduced as described earlier, and analyzed by flow cytometry on day 4. Bar graph (right) shows cumulative data from analysis of NOXA staining (n = 6). **G**, Representative Western immunoblots (IBs) show p53, YB-1, PUMA, and GAPDH levels in primary CD4+ T cells (n = 2) on day 4 after activation and transduction as described earlier. Values under blots represent the relative protein amounts normalized as indicated. In **A**, **E**, and **F**, symbols represent individual samples; bars show the mean ± SD. ** = $P < 0.01$; **** = $P < 0.0001$, by Student's *t*-test. NS = not significant; MFI = mean fluorescence intensity; w/o = without. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract.

those of just GFP-expressing controls by nearly 100% (Figure 3D). Taken together, the results demonstrate that up-regulation of YB-1 tightly correlates with viability of CD4+ T cells and that knockdown of YB-1 strongly promotes apoptosis of activated T helper cells, whereas YB-1 overexpression increases CD4+ T cell viability. Therefore, we propose that YB-1 acts as a crucial determinant for the death and survival of T cells.

Enhancement of mRNA and protein expression of the proapoptotic molecule PUMA by YB-1 inactivation. We next searched for factors that mediate apoptosis downstream of YB-1 knockdown. The extrinsic pathway for induction of apoptosis in T cells either involves crosslinking of the Fas receptor (FasR) or triggers binding of TNF to its death domain–bearing receptors. In Jurkat and HeLa cells, YB-1 was found to suppress the *fas* promoter (33). We therefore monitored FasR mRNA and cell surface receptor expression in activated T cells transduced with YB-1–specific shRNA or control shRNA. Neither qRT-PCR analysis on day 4 of stimulation nor assessment of surface FasR (CD95) on days 4 and 6 of stimulation revealed significant changes in FasR in YB-1–depleted T cells compared to controls (Figure 4A).

We also addressed the possibility that differential FasR-crosslinking by FasL might account for YB-1–related changes in frequencies of apoptotic cells. To this end, we forced crosslinking of FasR by anti-FasR combined with protein A on activated T cells ectopically expressing GFP or GFP–YB-1^{wt}, respectively. Compared to untreated controls, apoptosis remained unaffected by FasR crosslinking (Figure 4B). We therefore conclude that differences in FasL expression do not account for YB-1–dependent protection against apoptosis.

To evaluate a potential role of YB-1 in TNF-mediated apoptosis in activated T helper cells, we determined TNF protein expression in YB-1–depleted cells and control cells upon stimulation with anti-CD3 alone or with anti-CD3 plus anti-CD28. Flow cytometric analysis revealed that in both cases, the level of intracellular TNF was substantially lower in YB-1–knockdown T helper cells in comparison to control samples (Figure 4C).

It was speculated that the reduced levels of intracellular TNF might reflect prior enhanced secretion of this cytokine, and therefore we tested the effect of adding TNF to the cultures. However, the effect of adding TNF on the existing increased frequency of apoptotic CD4+ T cells was similar between YB-1 shRNA–expressing T helper cells and control shRNA–expressing T helper cells (Figure 4D). Thus, FasL and TNF are unlikely to be the decisive factors in the induction of apoptosis in T helper cells with reduced amounts of YB-1 expression.

Consequently, we concluded that YB-1 is involved in the control of intrinsic mitochondrial apoptosis pathways and analyzed the expression of proapoptotic marker molecules, including NOXA, PUMA, and p53, in anti-CD3/anti-CD28–stimulated CD4+ T cells in the absence or presence of YB-1 knockdown. On day 4 of stimulation, normalized mRNA levels of NOXA and p53 had not significantly increased when YB-1 was depleted. In contrast, PUMA mRNA showed a robust increase of ~50% compared to controls (Figure 4E). Flow cytometric analysis demonstrated a clear reduction of the frequencies of NOXA+ T helper cells upon YB-1 knockdown (Figure 4F).

To quantify protein expression of p53 and PUMA, we performed Western blot analysis on extracts from NGFR+ T helper cells (Figure 4G). While p53 protein expression was slightly reduced in the absence of YB-1, PUMA protein expression was strongly increased in YB-1–depleted cells compared to controls (Figure 4G).

Correlation between YB-1 depletion and reduced expression of antiapoptotic molecules Bcl-x_L and Bcl-2 and Akt in activated CD4+ T cells. Given that YB-1 overexpression protects against apoptosis, we hypothesized that YB-1 might be involved in the control of antiapoptotic and/or prosurvival molecules. Accordingly, we performed qRT-PCR analyses on YB-1–depleted and control cells to determine transcript levels of the BAX antagonists Bcl-x_L and Bcl-2 and transcript levels of Akt as a key regulator in the main survival pathway (Figure 5A). At day 4 of anti-CD3/anti-CD28 stimulation, we found a reduction in Bcl-x_L transcript levels by ~50% in YB-1 knockdown cells.

As YB-1 may control translation (21), protein expression of the same molecules was also analyzed. Indeed, monitoring protein expression by flow cytometry revealed that the frequencies of Bcl-x_L–, Bcl-2–, and Akt-expressing cells were significantly reduced when YB-1 was knocked down (Figures 5B and C). Whereas the percentage of Bcl-x_L– and Akt-positive cells dropped to ~50% compared to controls, YB-1–depleted, Bcl-2 protein–expressing cells were reduced by ~85% compared to controls (Figure 5B).

To further analyze the possible involvement of YB-1 in the Akt-dependent survival pathway, Akt itself or its activating kinase PI3K was inhibited during stimulation using AKT inhibitor VIII or Ly294002, respectively (Figures 5D and E). To evaluate the extent of inhibitor activity, both inhibitors were tested on activated T cells; at 24 hours and 5 days after the initiation of treatment with either inhibitor during stimulation, we observed that Akt phosphorylation was down-regulated in comparison to that in DMSO-treated activated T cells (Figure 5D). For YB-1–depleted T cells, both treatments had little, if any, effect beyond the already high level of apoptosis in untreated controls (Figure 5E). In contrast, a profound increase in apoptosis was observed in control shRNA–transduced YB-1–expressing cells. With inhibition of PI3K, virtually the same levels of apoptotic cells as in YB-1–depleted cell populations were achieved (Figure 5E).

Correlation between low YB-1 expression in activated CD4+ T cells from SLE patients and enhanced apoptosis. Various studies have shown that SLE T helper cells exhibit enhanced susceptibility to apoptosis (7,34). Based on our

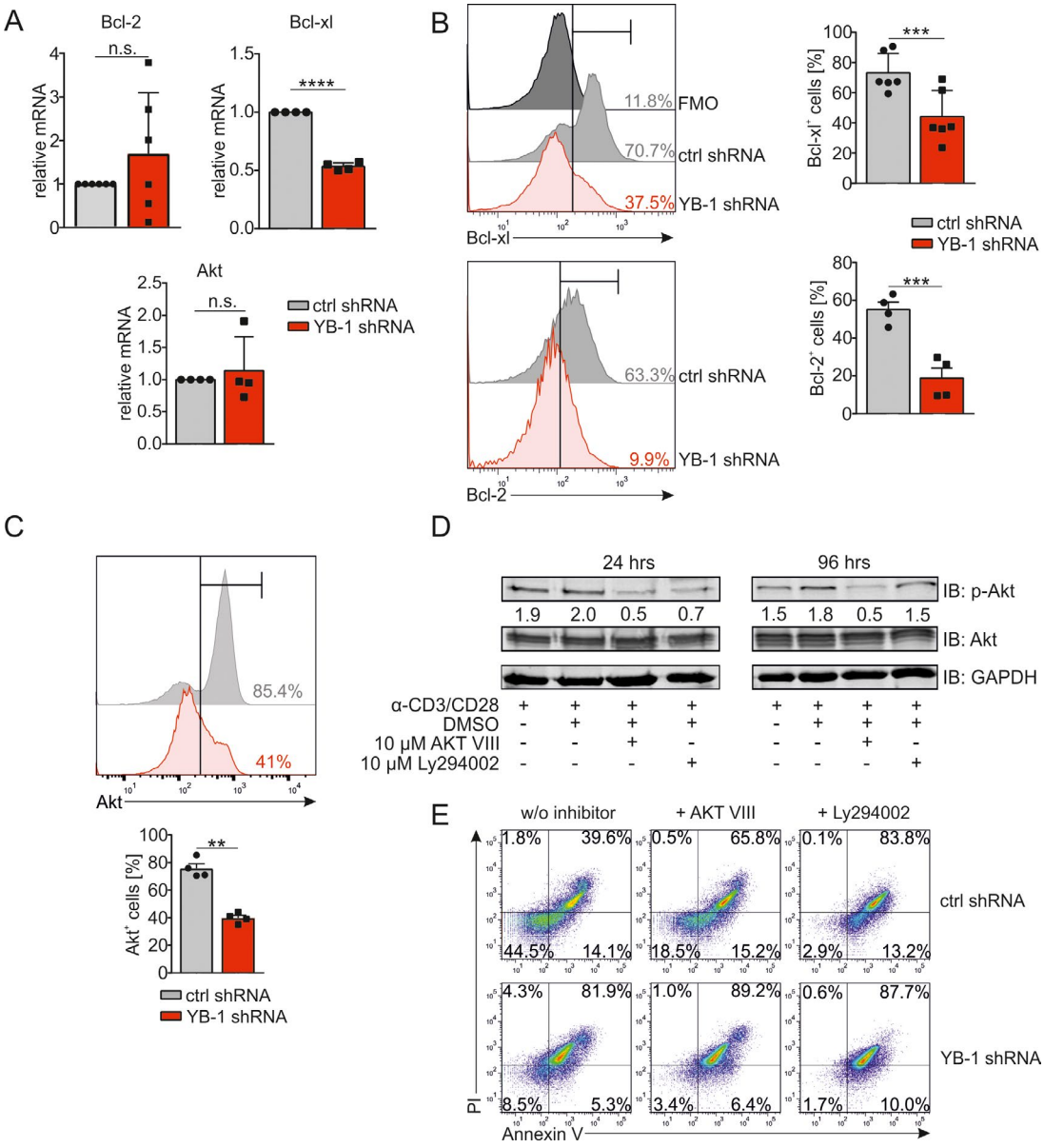

Figure 5. Regulation of antiapoptotic molecules by Y-box binding protein 1 (YB-1) in primed CD4+ T cells. **A**, Primary CD4+ T cells 4 were stimulated ex vivo with anti-CD3/anti-CD28–coated microspheres and then transduced with YB-1-specific short hairpin RNA (shRNA) (red) or control shRNA (ctrl shRNA) (gray). Bar graphs show relative Bcl-2, Bcl-xl, and Akt mRNA levels on day 4. The cells were analyzed by quantitative polymerase chain reaction (n = 4–6). **B**, Representative histograms show intracellular Bcl-xl or Bcl-2 staining or Fluorescence Minus One (FMO) control on day 4 in CD4+ T cells that were transduced and activated as described in **A** and analyzed by flow cytometry. Cumulative data are also shown (n = 4). **C**, Representative histogram shows Akt expression on day 4 in CD4+ T cells, treated as described in **A** and analyzed by flow cytometry (n = 4). **D**, CD4+ T cells were stimulated with anti-CD3/anti-CD28–coated microspheres after preincubation with inhibitors AKT VIII and Ly294002 for 30 minutes and then analyzed by immunoblotting (IB) for Akt and p-Akt expression. Values under the blots represent the relative protein amounts normalized to the levels of GAPDH. **E**, Representative dot plots show control CD4+ T cells without (w/o) inhibitor or CD4+ T cells treated with 10 μ*M* of AKT VIII or Ly294002 after the cells had been activated and transduced as described in **A**. The cells were stained with annexin V and propidium iodide (PI) and assessed by flow cytometry on day 5. In **A–C**, symbols represent individual samples; bars show the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Student's *t*-test. NS = not significant. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41382/abstract.

results, we hypothesized that YB-1 up-regulation in SLE T cells is not induced upon stimulation. Therefore, we isolated CD4+ T cells from SLE patients and quantified YB-1 expression. In analyses of YB-1 expression by CD4+ T cells, no significant differences were found between healthy donors and SLE patients when the CD4+ T cells were analyzed ex vivo (data not shown) or in a resting state after 4 days of culture (Figure 6A). Next, CD4+ T cells were stimulated with either anti-CD3/isotype or anti-CD3/anti-CD28–

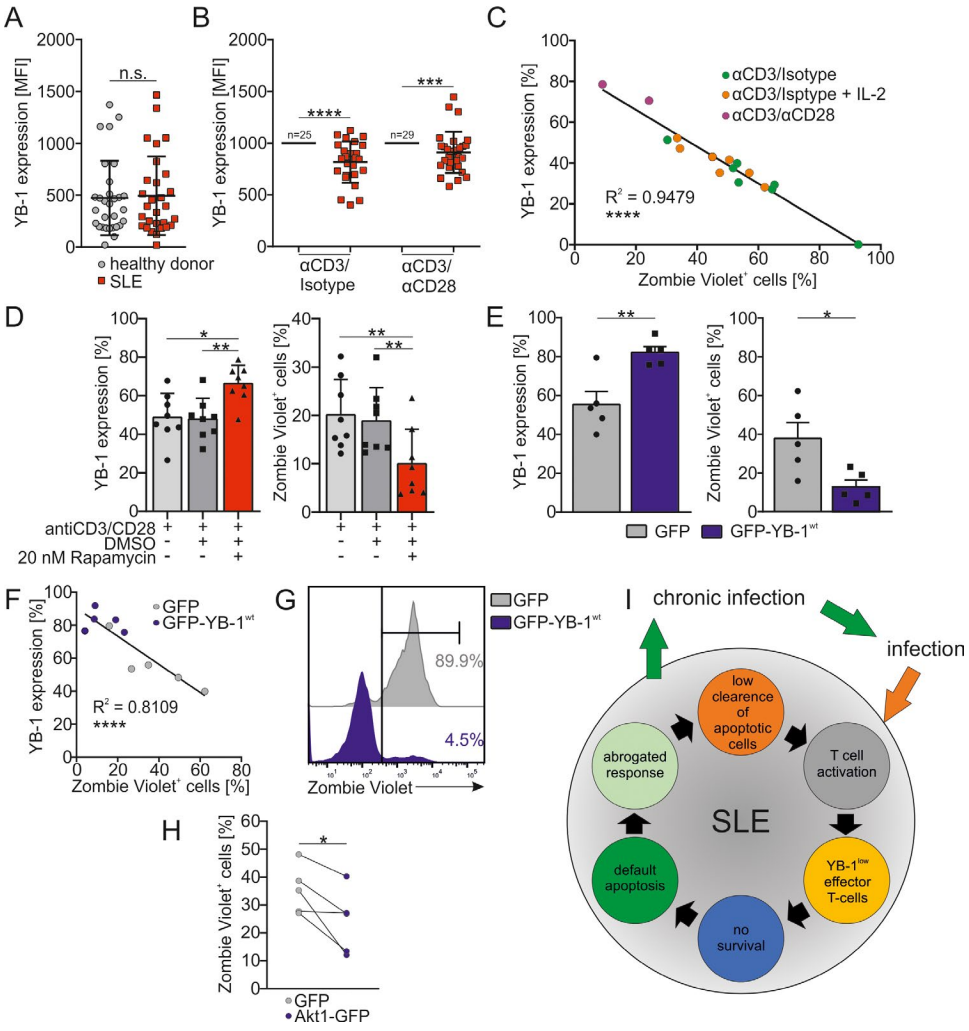

Figure 6. Correlation between reduced Y-box binding protein 1 (YB-1) levels in activated CD4+ T cells from systemic lupus erythematosus (SLE) patients and loss of viability that can be restored upon re-expression of YB-1 or Akt-1. **A**, YB-1 expression levels in resting CD4+ T cells are shown. **B**, Normalized expression levels of YB-1 were assessed by flow cytometry on day 4 in CD4+ T cells that were activated with either anti-CD3/isotype–coated microspheres (n = 25) or anti-CD3/ anti-CD28–coated microspheres (n = 29). **C**, Pearson's correlation tests were used to assess the correlation between YB-1 expression and percentage of dead cells (by Zombie Violet staining) among CD4+ T cells from SLE patients in cultures with either anti-CD3/isotype–coated microspheres in the absence or presence of interleukin-2 (IL-2) or anti-CD3/anti-CD28–coated microspheres. **D**, YB-1 expression and percentage of dead cells (by Zombie Violet staining) were assessed by flow cytometry on day 6 in CD4+ T cells from SLE patients that were activated with anti-CD3/anti-CD28–coated microspheres and left untreated or treated with DMSO and/or 20 n*M* of rapamycin. **E**, YB-1 expression and percentage of dead cells (by Zombie Violet staining) were assessed by flow cytometry on day 6 in viable CD4+ T cells from SLE patients that were activated with anti-CD3/anti-CD28–coated microspheres and transduced with a wild-type green fluorescent protein–YB-1 (GFP–YB-1^{wt}) vector or GFP control vector. **F**, Pearson's correlation tests were used to assess the correlation between YB-1 expression and the percentage of dead cells (by Zombie Violet staining) among CD4+ T cells from SLE patients after activation with anti-CD3/anti-CD28–coated microspheres and transduction with GFP–YB-1^{wt} vector or GFP control vector. **G**, CD4+ T cells from SLE patients were stimulated with anti-CD3–coated microspheres and with IL-2 for 2 days. After stimulation, T cells were transduced as described in **E**, and YB-1 and cell death were analyzed by flow cytometry. **H**, The percentage of dead cells (by Zombie Violet staining) was determined by flow cytometry on day 6 among CD4+ T cells from SLE patients that were activated as described in **E** and transduced with an overexpressing Akt-1–GFP vector or GFP control vector. **I**, Diagram depicts the impact of reduced YB-1 levels in effector T cells on the development and progression of SLE. Upon infection, effector T cells from SLE patients hardly up-regulate YB-1 to a level that would ensure survival. Consequently, apoptosis is initiated as a result of missing survival signals. Defective clearance of apoptotic cell bodies and debris may activate autoreactive T cells. These T cells again barely up-regulate YB-1, feeding into a vicious circle of apoptosis, activation, autoimmunity, and chronic inflammation that drives SLE pathology. In **A**, **B**, **D**, and **E**, symbols represent individual samples; bars show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Student's *t*-test in **A** and **D** and by analysis of variance in **B**. MFI = mean fluorescence intensity; NS = not significant.

coated microspheres for up to 6 days and then analyzed for YB-1 expression and frequency of apoptotic cells. On day 4 of stimulation, CD4+ T cells from SLE patients showed a significant decrease of intracellular YB-1 expression compared to control cells from healthy donors (Figure 6B). As shown above for healthy donors (Figure 2E), a highly significant correlation between YB-1 expression and cell survival was observed using Pearson's test (R^2 = 0.9479; P = 0.0001), even when assessed across different modes of stimulation in SLE (Figure 6C). Since rapamycin treatment is used in SLE patients, T cells were incubated during anti-CD3/anti-CD28 stimulation to rescue apoptotic T cells (Figure 6D). Indeed, rapamycin induced a significant increase in YB-1 expression in SLE T cells and a 50% reduction in cell death.

To further evaluate the relationship between reduced YB-1 levels and the susceptibility of SLE T helper cells to stimulation-induced apoptosis, we performed a rescue experiment, in which we overexpressed GFP-tagged YB-1 in SLE T cells using lentiviral transduction (Figure 6E). In fact, viability of GFP-tagged YB-1–expressing SLE T helper cells increased by 20–30% compared to GFP-tagged controls. Thus, survival of SLE T helper cells was rescued by YB-1 overexpression (Figure 6F) (R^2 = 0.8109; $P < 0.0001$). Even in an SLE patient (defined as an individual with a SLEDAI score of >4) with 90% of T cells that were YB-1^{low} ZV+ 4 days after onset of stimulation (GFP control cells), ectopic overexpression of YB-1 (GFP-tagged YB-1^{wt}) enhanced survival of T cells (Figure 6G).

To elucidate the proposed signaling pathway through Akt by which YB-1 stimulates cell survival, we overexpressed Akt-1 GFP and GFP control in SLE T cells (Figure 6H). Indeed, in SLE patients, Akt-1 overexpression rescued the apoptosis-prone T cells, and this was achieved, at least partly, by enhancing T cell survival (P = 0.0184 versus controls; n = 5).

DISCUSSION

In this study, we identified YB-1 as a central factor in the regulation of survival induction in activated T cells, and we established its malfunction as a characteristic of T cells from SLE patients. Our data show that the frequency of viable activated T cells is high as long as cells retain YB-1 expression. Mechanistically, YB-1 acts on the proapoptotic PUMA–Bcl-2 pathway and enhances the PI3K–Akt axis to mediate survival. Forced up-regulation of YB-1 in activated T cells of SLE patients indeed rescued the T cells from apoptosis.

We show for the first time that YB-1 down-regulation in activated T cells ultimately leads to apoptosis. The expression of the proapoptotic molecules NOXA and PUMA in activated T cells requires YB-1 down-regulation. NOXA up-regulation is usually closely connected to nutritional deprivation, but since nutrition levels in our experiments were not limited, YB-1 likely operates at a connecting point of downstream apoptosis pathways (35). PUMA is also strongly up-regulated upon YB-1 knockdown. It has been shown that YB-1 interacts with p53, thereby hampering each other's function. Thus, in the absence of YB-1, free p53 might up-regulate the expression of the downstream target PUMA (36–38). Therefore, the absence of YB-1 induces apoptosis as a default pathway.

The present study shows that overexpression of YB-1 markedly enhances survival of activated T cells. In fact, consistent with earlier studies in malignant melanoma cells (39), we demonstrated that YB-1 is necessary in order to express the antiapoptotic molecules Bcl-2 and Bcl-xl (40). Our data also demonstrate that YB-1 induces Akt expression, a major player in the survival pathway of CD4+ T cells (41). In cells with YB-1 knockdown, Akt expression is reduced. Moreover, Akt inhibition virtually abolished the survival-promoting effect of YB-1 (i.e., led to rates of apoptosis in YB-1^{high} T cells similar to those observed in T cells with YB-1 shRNA or in T cells of SLE patients deficient in YB-1) (Figures 5E and 6E) (42). Although Akt-1 overexpression rescued SLE T cells at least partially from apoptosis (Figure 6H), inhibition of PI3K entailed an even stronger induction of apoptosis in the control shRNA cells, suggesting that beyond YB-1 Akt signaling, other pathways might be involved in regulating survival in CD4+ T cells (Figure 5D). Since rapamycin treatment yields a marked increase in expression of YB-1 in SLE T cells, the survival pathway could also include aspects of autophagy, which would less likely involve necrosis, as PI+ annexin V– T cells (Figure 3B) are not often observed upon YB-1 reduction in activated T cells (43,44). As rsk is the major kinase in T cells for YB-1 phosphorylation (20,45), we thus propose an extended Akt survival pathway encompassing a p90 rsk–YB-1–Akt–Bcl-2 axis.

Our results provide an explanation for the malfunction of T cell responses in SLE patients due to insufficient up-regulation of YB-1 (Figure 6I). Compared to T cells from healthy donors, activated T cells from SLE patients were hardly able to enhance or maintain the expression of YB-1 upon activation. Consequently, YB-1^{low} T cells will undergo apoptosis, and responses against pathogens may thus be terminated prematurely. Thereby, failure to up-regulate YB-1 leads to abrogated immune responses and enhanced appearance of apoptotic waste. Taken together with previous findings showing that apoptotic cells and RNA/DNA debris are often insufficiently cleared in SLE patients (46,47), this may result in a vicious cycle of prolonged inflammation during which T cells get activated but often fail, and self-reactive T cells might get activated from the debris. On a few waves of T cell activation caused by infections, followed by failure of YB-1–mediated survival, accumulation of debris might contribute to starting and maintaining the autoimmune process. This model is supported by the fact that flairs of SLE often start with infections (48) and that SLE patients experience infections 5 to 10 times more frequently compared to individuals without SLE (8).

Our data show that upon suboptimal stimulation of T cells, IL-2 increases survival and frequencies of YB-1^{high} T cells from SLE patients but does not impact YB-1 expression of optimally activated T cells from healthy donors (Figure 2B and Supplementary Figure

1). Currently, low-dose IL-2 is under investigation as a potential treatment in SLE, and YB-1 stability might explain why IL-2–treated patients seem to experience fewer infections than conventionally treated patients (49). It is tempting to speculate that expansion of Treg cells following low-dose IL-2 therapy may be attributable to stabilized Treg cell survival via recovery of YB-1 up-regulation (50). Indeed, other likely successful treatment strategies in SLE, such as the one shown herein for rapamycin application, may unknowingly aim for YB-1 stabilization in T cells (Figure 6D) (44).

Our data show that YB-1 expression correlates strongly with survival of CD4+ T cells. The observed effects of modulation of YB-1 expression appear to confirm its antiapoptotic function. Therefore, it can be postulated that YB-1 behaves like a sensor for survival, which is applied to apoptosis-prone T cells from SLE patients. So far, autoantibody production, organ involvement, or disease severity does not correlate with the frequency of YB-1^{low} T cells upon activation (data not shown). Whether a certain threshold for YB-1 expression may be used as a new biomarker for the prediction of certain disease manifestations or as a biomarker for the progression of SLE (or even other autoimmune diseases) has to be examined in a larger patient cohort and in a prospective longitudinal study, as previously shown for IL-10 (51). Also, several other biomarkers have been suggested for SLE, including cytokines of the IL-1 family, which are increased in SLE (52). YB-1 expression level inversely correlated with the diagnosis of SLE in general, and thus, might even contribute to more systemic effects in disease. Since increased rates of infections did not correlate with the SLEDAI scores (8), the infection rates may well be correlated with the abrogation of YB-1 expression in SLE patients. Indeed, T cells from 1 SLE patient with extremely low YB-1 expression upon stimulation could be rescued by enhancing YB-1 expression (Figure 6E). Therefore, YB-1 may be a new target molecule in the treatment of SLE.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Brunner-Weinzierl had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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