The Presence of Survivin on B Cells from Myasthenia Gravis Patients and the Potential of an Antibody to a Modified Survivin Peptide to Alleviate Weakness in an Animal Model

Xiangyang Zhang,* Michael Ciesielski,† Robert A. Fenstermaker,† Henry J. Kaminski,* and Linda L. Kusner‡

Myasthenia gravis (MG) is an autoimmune disease in which Abs target neuromuscular junction proteins, in particular the acetylcholine receptor. We previously identified the antiapoptotic protein survivin in the autoreactive B cells and plasma cells of MG patients. To further define the role of survivin in MG, we have assessed PBMCs from 29 patients with MG and 15 controls. We confirmed the increased expression of survivin in CD20$^+$ lymphocytes from MG patients compared with controls. Furthermore, the CD20$^+$ population of cells from MG patients contained a higher percentage of extracellular survivin compared with controls. The analysis of CD4$^+$ cells showed an increased percentage of intracellular survivin in MG patients compared with controls, whereas the extracellular survivin CD4$^+$ percentage was unaffected. In an experimental mouse model of MG, we assessed the therapeutic potential of an Ab raised to a modified survivin peptide but cross-reactive to survivin. Ab treatment reduced disease severity, lowered acetylcholine receptor–specific Abs, and decreased CD19$^+$ survivin$^+$ splenocytes. The ability to target survivin through Ab recognition of autoreactive cells offers the potential for a highly specific therapeutic agent for MG. ***The Journal of Immunology*****, 2020, 205: 1743–1751.**

The mechanisms that promote the development and maintenance of autoreactive cells are an open question in autoimmunity. A balance of apoptosis and cell proliferation does play an important role in the development and homeostasis of the immune system. Emerging evidence indicates that apoptotic elimination and proliferation of autoreactive cells are defective in autoimmune disorders (1, 2). Inhibitor of apoptosis proteins (IAPs) are a family of proteins originally found to influence apoptosis and restrict the activation of caspases, preventing the cell from undergoing cell death. The inhibitors of apoptosis are also key regulators of cytokinesis, proliferation, differentiation, and signal transduction (3). Survivin, a member of this family, has emerged as a driver of pathology in several autoimmune disorders (4). Studies assessing patients with primary progressive multiple sclerosis (MS) found expression of survivin in resting T cells (5). Furthermore, survivin was over-expressed in mitogen stimulated T lymphocytes from patients with active MS compared with patients with stable disease. Survivin levels correlate with disease activity in MS (6) and its expression can be reduced with IFN-β treatment (7). Several investigations in rheumatoid arthritis have demonstrated survivin to maintain autoreactive T cells and support pathological synovial cells (8). Survivin expression appears to predict the clinical course of rheumatoid arthritis, whereas anti-survivin Abs (a proxy for the anti-survivin immune responses) are associated with less severe disease (9).

Myasthenia gravis (MG) is an autoimmune disorder in which the primary autoantigen is the skeletal muscle acetylcholine receptor (AChR), and the disease serves as the prototypic Ab-mediated autoimmune disorder (10). In our previous study, we found an increased percentage of survivin-expressing B cells among MG patients but a near absence in controls. Furthermore, we found the hyperplastic thymus, which is considered to be the site of disease induction in MG, to contain survivin$^+$ cells. In our animal experiments, we determined that rodents with experimental autoimmune MG (EAMG) also had survivin$^+$ lymphocytes. Using a vaccination approach, disease severity of EAMG was significantly reduced, with an associated reduction of survivin$^+$ CD19 cells (11). Survivin has been regarded as an exclusively intracellular protein. More recently, it has become clear that cell surface survivin expression occurs, although the function of the molecule in this context is not yet defined. Nevertheless, cell surface survivin offers a potential target for Ab-mediated destruction of autoreactive cells (12). In this study, we assess the percentage of lymphocytes with intracellular and extracellular survivin in MG patients compared with controls. Furthermore, we determine the ability of an Ab directed against a modified survivin peptide (12) to moderate the severity of EAMG.

Materials and Methods

Human subjects and ethics statement

Blood specimens (n = 29) were collected from patients of the MG Center and neurology clinics at George Washington University (Table I). For patients with MG, entrance criteria for participation were as follows: 1) previous clinical diagnosis of MG; 2) age >18 y; 3) presence of serum

*Department of Neurology, George Washington University, Washington, DC 20052; †Department of Neurosurgery, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263; and ‡Department of Pharmacology and Physiology, George Washington University, Washington, DC 20037

ORCIDs: 0000-0002-2041-6535 (X.Z.); 0000-0003-0309-8130 (R.A.F.); 0000-0002-5530-2140 (L.L.K.).

Received for publication April 29, 2020. Accepted for publication July 30, 2020.

This work was funded by the Muscular Dystrophy Association (to L.L.K.).

Address correspondence and reprint requests to Dr. Linda L. Kusner, George Washington University, 2300 I Street NW, Washington, DC 20037. E-mail address: lkusner@email.gwu.edu

Abbreviations used in this article: AChR, acetylcholine receptor; α-BTX, α-bungarotoxin; EAMG, experimental autoimmune MG; MG, myasthenia gravis; MS, multiple sclerosis; NMJ, neuromuscular junction; Q, quadrant; TA, tibialis anterior; tAChR, *Torpedo* AChR.

AChR, and 4) willingness to participate and ability to provide informed consent. Exclusion criterion was limited to inability to provide informed consent. The Myasthenia Gravis Foundation of America Clinical Classification (13) was used to historically define the maximal disease severity at the time of blood draw. Table I shows demographic and clinical characteristics of the patients. For those who had a thymectomy, all had thymic hyperplasia, except for the individual who had a thymectomy 25 y prior and for whom records were not available. The World Health Organization classification of thymoma is indicated in the Table. Control subjects ($n = 15$) were recruited from neurology clinics at George Washington University. Control subject inclusion criteria were limited to willingness to participate and ability to provide informed consent. Control subject exclusion criteria were age <18 y, diagnosis of autoimmune disease of any kind, and treatment with any immunotherapy in the previous 12 mo. All participants provided written consent for inclusion in the study. The study was approved by the George Washington University Institutional Review Board.

PBMC isolation

Blood samples were collected in Vacutainer Acid Citrate Dextrose (catalog no. 364606; BD) and then were transferred to a 50-ml Ficoll-loaded LeucoSep tube (catalog no. 07-000-983; Thermo Fisher Scientific) that contains 15.5 ml of Ficoll-Paque PLUS Media (catalog no. 17144003; GE Healthcare). Tubes were centrifuged at room temperature for 30 min at $1000 \times g$ with the break off. PBMCs were isolated and washed three times with HBSS (catalog no. 14-170-161; Life Technologies) and then were resuspended in ice-cold cell culture IMDM (catalog no. 31980030; Life Technologies) that contains 10% FBS (catalog no. 10437028; Life Technologies) and 1% penicillin–streptomycin (catalog no. 15070063; Life Technologies) as 2.5 million per milliliter. Cells were transferred into 96-well plates and placed on ice. All other cells were kept on ice for cell viability staining.

Flow cytometry cell viability staining

The PBMCs–IMDM suspension (25 million cells) was spun down at 4°C $500 \times g$ for 5 min and then was immediately resuspended in 1 ml of ice-cold cell culture IMDM. One microliter from a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (catalog no. L34955; Thermo Fisher Scientific) was added into PBMCs suspension and was mixed gently and then incubated on ice for 30 min protected from light. Cells were washed twice with 10 ml of ice-cold cell culture IMDM and then were resuspended in ice-cold cell culture IMDM as 4×10^6/ml. Cell suspension for one well (100 μl) was plated into 96-well plates as designed wells.

Extracellular staining of PBMCs

Fc receptors on PBMCs were blocked by 5 μl of Human TruStain FcX (catalog no. 422302; BioLegend), placed for 10 min on ice, then stained with Abs CD20–FITC (catalog no. MA110136; Thermo Fisher Scientific), CD4–PE (catalog no. 561844; BD), CD45–PerCP (catalog no. MHCD4531; Thermo Fisher Scientific), survivin–Alexa Fluor 700 (catalog no. NB500-238AF700; Novus Biologicals), or their isotype control Abs Mouse IgG2a–FITC (catalog no. 11-4732-81; Thermo Fisher Scientific), Mouse IgG1-PE (catalog no. 555749; BD), Mouse IgG1-PerCP (catalog no. MG131; Thermo Fisher Scientific), and Mouse IgG2a–Alexa Fluor 700 (catalog no. IC003N; Novus Biologicals) for 30 min on ice. Single-color staining and fluorescent-minus Alexa Fluor 700 were prepared at the same time. Fixation buffer (100 μl) was added into each well for 10 min on ice. Plates were spun down at 4°C, $500 \times g$ for 5 min, washed twice with 220 μl of ice-cold Cell Staining Buffer (catalog no. 420201; Biolegend), then resuspended in 200 μl of ice-cold Cell Staining Buffer. Cell populations were evaluated by flow cytometry within 1 h.

Intracellular staining of PBMCs

For intracellular staining of survivin expression, the extracellular-stained PBMCs were washed twice with 220 μl of ice-cold 1× Intracellular Staining Permeabilization Wash Buffer (catalog no. 421002; BioLegend) and then were resuspended in 100 μl of ice-cold 1× Permeabilization/Wash Buffer. Survivin–Alexa Fluor 700 (catalog no. NB500-238AF700; Novus Biologicals) or Mouse IgG2a–Alexa Fluor 700 (catalog no. IC003N; Novus Biologicals) was added into designated wells for 30 min on ice. One hundred microliters if ice-cold 1× Permeabilization/Wash Buffer was added into each well and then plate was spun down at 4°C, $500 \times g$ for 5 min. Washed twice by 220 μl of ice-cold 1× Permeabilization/Wash Buffer and then were resuspended in 200 μl of ice-cold Cell Staining Buffer. Cell populations were qualified by flow cytometry within 1 h.

Gating strategies for flow cytometry

To analyze the population of monocytes, first, the singlets were gated on dot-plot forward scatter area/forward scatter height, followed by gating the $CD45^+$ monocytes on dot-plot CD45/side scatter. Live monocytes were determined by dot-plot CD45/Live-Dead, then we used the isotype control of either extracellular or intracellular Ab staining of survivin and set the survivin gate in $CD45^+$ live single monocytes on dot-plot survivin/CD45. The average of the duplicate samples was determined for the $survivin^+$ results for the statistical analysis.

To analyze the populations of $CD20^-CD4^+$ lymphocytes and $CD20^+CD4^-$ cells from the region of lymphocytes, we used the existing gates for $CD45^+$ live single lymphocytes on dot-plot CD4/CD20 (quadrant [Q]1: $CD20^+CD4^-$, Q2: $CD20^+CD4^+$, Q3: $CD20^-CD4^+$, and Q4: $CD20^-CD4^-$) to assess the populations $CD20^-CD4^+$ (Q3) and $CD20^+CD4^-$(Q2) for survivin according to the previous gating strategy. The duplicate results for $CD4^+survivin^+$ percentage (Q3) lymphocytes or $CD20^+survivin^+$ percentage (Q2) cells were used in the statistical analysis.

EAMG induction and treatment

All animals were housed in The George Washington University Animal Research Facility in accordance with Institutional Animal Care and Use Committee, American Association for Laboratory Animal Science, and Association for Assessment and Accreditation of Laboratory Animal Care standards regulating housing conditions, cage cleaning procedures, air purity, humidity, temperature, feed quality, and light–dark cycles. A veterinarian was available to monitor the animals during the course of the study. Animal use was approved by The George Washington University Institutional Animal Care and Use Committee (Permit No. A247), and all experimental outcomes are reported using the quality assurance guidelines set by the National Institute of Neurological Disorders and Stroke. Using methods previously described for EAMG induction (14), 27 female C57BL/6J mice were injected with *Torpedo* AChR (tAChR) in CFA (catalog no. F5881; Sigma-Aldrich) on day 0 and were boosted with tAChR in IFA (catalog no. 263910; BD) on both day 28 and day 52. To test the therapeutic potential, we used a hybridoma IgG2a Ab to the modified peptide SVN53-63/M57 (Ab 2C2) (Kd 1/4 0.56 nmol/L) as previously described (12). On day 54, mice were stratified by weight into three groups, and each group was randomly assigned to a treatment (PBS, Ab 2C2 at 100 μg, and Ab 2C2 at 20 μg per mice). Nine C57BL/6J mice, a control group, were injected with PBS–CFA on day 0, day 28, and day 52. All mice received treatment once every 2 d for six total treatments. Evaluations were performed twice weekly and consisted of weight measurement, grip strength (Chatillon Digital Force Gauge, C.S.C. Force Measurement), and determination of a generally accepted disease score (0 = no weakness after exercise; 1 = normal at rest, but weak after exercise; 2 = weakness at rest; 3 = severe weakness with paralysis; and 4 = found dead or euthanized) (15). Blood, spleen, and tibialis anterior (TA) were collected for further investigation at termination on day 66.

Splenic preparation

Spleens were cut to small pieces with RPMI 1640 (catalog no. 22400089; Life Technologies) that contains 5% FBS (catalog no. 10437028; Life Technologies) and were passed through 40-μm cells strainers (catalog no. 352340; Corning). RBC Lysis Buffer (catalog no. J62150AK; Alfa Aesar) was added to cell pellets. After a wash with 10 ml of RPMI 1640 that contains 5% FBS, cells were resuspended in freezing medium (90% FBS, 10% DMSO [catalog no. D2650; Sigma-Aldrich]) as $1–2 \times 10^7$ cells/ml/vial. Samples were stored in at −80°C.

Flow cytometry of splenocytes

Frozen splenocytes were thawed at 37°C in a water bath and resuspended in RPMI 1640 plus 10% FBS plus 1% penicillin–streptomycin (5000 U/ml) (catalog no. 15070063; Life Technologies). After one wash in RPMI 1640 plus 10% FBS plus 1% penicillin–streptomycin (5000 U/ml), cells were resuspended in Dulbecco's PBS (catalog no. 14190144; Life Technologies) with LIVE/DEAD fixable cell viability dye (catalog no. L34955; Thermo Fisher Scientific). Fc sites were blocked on the cells with purified anti-mouse CD16/32 Ab (catalog no. 101302; BioLegend). Cells were treated with the following Abs to cell surface marker panel: CD3ε–FITC (catalog no. 100306; BioLegend), CD19–PE (catalog no. 115508; BioLegend), CD4–allophycocyanin (catalog no. 100412; BioLegend), and their isotype control Abs FITC (catalog no. 400905; BioLegend), PE (catalog no. 400507; BioLegend), allophycocyanin (catalog no. 400611; BioLegend), and Alexa Fluor 700 (catalog no. IC003N; R&D). Single-color staining

and fluorescence minus one were also prepared at the same time. Cells were washed and resuspended in cold Cell Staining Buffer (catalog no. 420201; BioLegend). Cell populations were qualified by flow cytometry within 2 h. The cell intracellular marker panel included the above markers for 30 min on ice. The Fixation Buffer (catalog no. 420801; BioLegend) was added to the well and spun down at 4˚C 500 × *g*. Ab to survivin–Alexa Fluor 700 and isotype control Mouse IgG2a κ Alexa Fluor 700 were added to cells suspension in 100 μl of 1× Intracellular Staining Permeabilization Wash Buffer (catalog no. 421002; BioLegend) for 30 min on ice and protected from the light. After three washes, cells were resuspended in ice-cold Cell Staining Buffer. Cell populations were quantified by flow cytometry within 2 h.

AChR-specific Ab determination by ELISA

ELISAs were performed to determine titers of the following Ig subtypes specific to tAChR: total IgG, IgG1, IgG2a, and IgG2b. To detect Abs to AChR, flat-bottom 96-well Nunc-Immuno MicroWell Plates (catalog no. CLS3590; Sigma-Aldrich) were coated overnight with tAChR. Blocking buffer (1× PBS [catalog no. P5368; Sigma-Aldrich] with 0.5% [v/v)]

Table I. Clinical information

MG Patients

Sample No.	Gender/ Age (y)[a]	MGFA Classification	MGFA Classification[a]	AChR Level, nmol/L[a]	Disease duration (y)[a]	Treatments	Thymectomy	Thymoma	$CD45^+CD20^+CD4^-$ B cells	
									Intracellular SVN^+%	Extracellular SVN^+%
1	F/26	IVa	IIIa	7.0	1	Pred	X		1.52	1.52
2	M/73	IIIb	IIb	9.0	7	Pred			0.99	1.39
3	M/71	IVa	IIa	91.0	0.6	Pred, Pyr			1.26	1.45
4	F/42	IIIa	IIIa	13.1	0.25	Pred, Pyr	X	B2	1.68	1.31
5	F/21	IVa	IIIa	58.0	8	Pred, Pyr	X		1.44	2.91
6	F/41	II	IIa	0.9	3	None			0.37	2.00
7	M/75	IIIa	IIa	26.7	0.25	Pred < 1 mo			3.45	1.72
8	F/77	V	MR	31.6	2	Pred, Pyr	X	B	2.84	3.50
9	M/59	I	I	0.2	0.33	None			0.29	0.56
10	M/87	IVb	IIa	25.0	0.33	Pred		B2	12.80	2.08
11	M/71	IIIb	MR	19.0	11	Myc, IVIG			5.51	4.96
12	M/63	IIIa	R		7	Aza	X	B1	4.14	1.80
13	M/73	IIIa	MR	45.0	2	Myc, Pred			1.11	2.88
14	M/51	IIIb	IIa	0.0	3	Myc, Pred			5.78	1.20
15	F/50	I	I	33.0	10	Pyr			19.00	4.19
16	M/56	IVa	MR	55.0	1	Myc, Pred			0.99	1.87
17	M/65	I	I	2.7	0.2	Pyr			1.17	2.10
18	M/41	IV	II	1.2	25	Pred, Pyr	X		6.59	2.37
19	F/57	IIa	CSR	5.4	2	None	X	AB	15.00	1.61
20	F/49	IIIa	MR	11.9	6	Myc	X	AB	10.70	0.55
21	M/65	I	I	15.0	1.5	Myc, Pred			4.37	1.16
22	M/61	I	I		0.75	Pyr			1.67	0.76
23	F/26	I	I	0.3	17	Pyr			5.49	1.29
24	M/69	I	I		0.9	Pyr			8.87	3.62
25	F/50	IIa	IIa	2.2	23	Pyr			17.50	0.87
26	F/56	I	CSR	2.5	1.2	Pyr			1.20	1.45
27	M/92	I	I	5.2	0.3	None			6.77	1.60
28	F/32	IIIa	IIa	1.0	1.5	Pred, IVIG	X		4.80	1.47
29	M/30	IIa	IIa	18.0	1.5	Pyr			5.52	0.57

Other Neurological Disease Patients

Sample No.	Gender/Age (y)[a]	Diagnosis	$CD45^+CD20^+CD4^-$ B cells	
			Intracellular SVN^+%	Extracellular SVN^+%
1	F/61	No evidence of neurologic disease	0.09	0.95
2	F/48	Radiculopathy	0.86	0.46
3	F/61	Migraine	3.04	1.42
4	F/26	Syncope	2.10	1.76
5	M/28	No evidence of neurologic disease	1.27	0.56
6	M/48	Headaches	0.72	0.92
7	M/45	Migraine	2.51	1.58
8	M/60	Refractive error	1.75	1.55
9	M/43	Ulnar neuropathy	2.33	1.90
10	M/69	Parkinson disease	0.56	1.04
11	M/40	Bell palsy	1.37	0.86
12	M/47	Cervical radiculopathy	2.80	0.47
13	F/47	Chronic cluster headache	2.42	0.99
14	M/54	Bilateral carpal tunnel syndrome	0.57	0.56
15	F/54	Episodic migraine	1.93	0.42

[a]At the time of blood collection.

AChR, acetylcholine receptor; Aza, azathioprine; F, female; IVIG, i.v. Ig; M, male; MGFA, Myasthenia Gravis Foundation of America; Myc, mycophenolate; Pred, prednisone; Pyr, pyridostigmine; SVN, survivin.

Tween 20 [catalog no. P1379; Sigma-Aldrich] and 5% [w/v] BSA [catalog no. A3608; Sigma-Aldrich]) was then added. To determine the tAChR-specific total IgG, IgG1, IgG2a, and IgG2b, serum from each animal was added in duplicate wells. The plate was incubated for 90 min and then 100 μl of 1:1000 anti-mouse IgG–HRP conjugate (catalog no. 40120; Alpha Diagnostic International, San Antonio, TX) was added. Tetramethylbenzidine (catalog no. 50-76-02 and no. 50-65-02; SeraCare Life Sciences, Milford, MA) was then applied for up to 25 min, and the reaction was terminated with 1 M hydrochloric acid (catalog no. 71826-1L; KPL). The OD plate was read at 450 nm using a Varioskan fluorescent and luminescent plate reader (Thermo Fisher Scientific).

Immunohistochemistry TA muscle

Ten micron cryosections of TA were mounted onto Superfrost Plus–treated slides (catalog no. 12-550-15; Thermo Fisher Scientific), air dried for 1 h, and fixed in cold acetone (catalog no. 423240025; Acros Organics) for 10 min. Slides were air dried, washed in PBS, and blocked in 5% BSA/PBS at room temperature. A conjugate of α-bungarotoxin (α-BTX)–Alexa 594 (catalog no. B13423; Invitrogen) and goat anti-mouse IgG (H+L) highly cross-adsorbed secondary and Alexa Fluor 488 (catalog no. A11029; Invitrogen) in 5% BSA/PBS were applied at a 1:500 dilution for 1 h at room temperature. After washing, coverslips were placed on the slides with Fluormount-G (catalog no. 010001; Southern Biotechnology Associates, Birmingham, AL). Sections were viewed on a Carl Zeiss Cell Observer Spinning Disk Confocal Microscope, and images were taken with a color charge-coupled device camera. Neuromuscular junctions (NMJs) were identified by fluorescently labeled bungarotoxin, and pixel intensity measurements were determined with mean values obtained for each animal, as done in previous investigations (14). A minimum of 30 NMJs were analyzed per animal (n = 9 per group). Zen 2 Lite software (Carl Zeiss AG, Oberkochen, Germany) was used to analyze fluorescence intensities.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6.07. The D'Agostino–Pearson omnibus and Shapiro–Wilk normality tests were conducted prior to other tests. The Mann–Whitney U test was used for analysis of MG patient and control samples. The Kruskal–Wallis test and Dunn post hoc test were performed for EAMG disease scores. We used a Student t test on data from FACS analysis of the mouse splenocytes, Ab titers, and fluorescent intensity. For all statistical values, p values <0.05 were considered statistically significant.

Results

Survivin localization in CD20$^+$ PBMCs from MG patients

Our previous study demonstrated that a greater percentage of PBMCs from patients with MG expressed survivin in specific B cell populations (CD20, CD27, CD38, and CD138) compared with healthy controls (11). To validate the result, we examined the presence of survivin in PBMCs from 29 patients with MG and 15 controls (Fig. 1, Table I). We found an increased percentage of intracellular survivin$^+$ in CD20$^+$ cells from MG patients compared with controls (5.27% ± 5.19%, 1.62% ± 0.92%, respectively; $p < 0.05$; Fig. 1A, 1B), confirming the previous investigation. To examine the localization of survivin in CD20$^+$ cells, we also analyzed nonpermeabilized PBMC for cell surface expression of survivin. A greater percentage of CD20$^+$ cells from MG patients localized survivin to the extracellular surface compared with controls (1.89% ± 1.09%, 1.03% ± 0.50%, respectively; $p < 0.005$; Fig. 1C, 1D). In addition, we assessed the expression

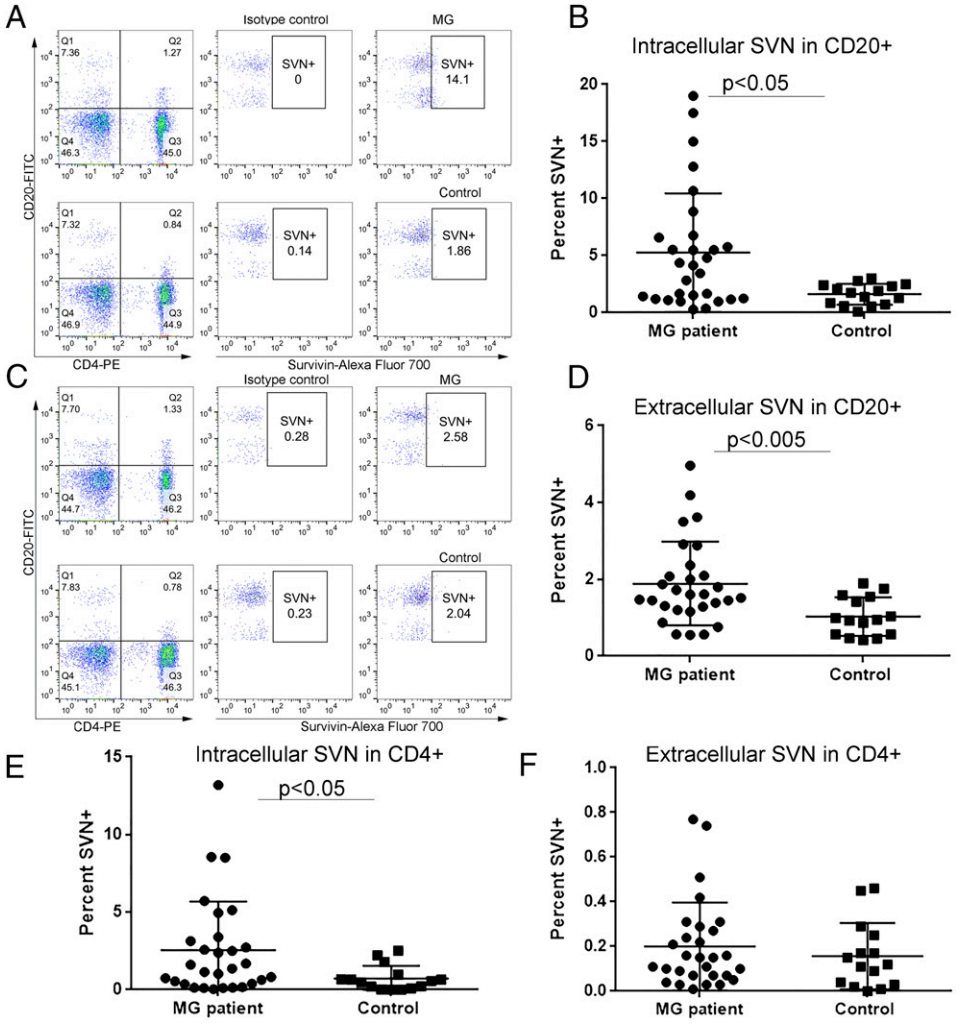

FIGURE 1. Intracellular and extracellular localization of survivin in PBMCs from MG patients. MG patients (n = 29) and controls (n = 15) were assessed for survivin expression by FACS analysis. Samples were viewed on a BD FACSCelesta analyzer, and survivin$^+$ lymphocytes were determined by FlowJo. (**A**) Representative dot plots of intracellular survivin staining from one PBMC sample from MG patient (top) and control (lower) are shown. The area to determine the percentage positive CD20 and survivin cells is shown. (**B**) The graph depicts the percentage of CD20$^+$ cells that were positive for intracellular survivin staining in MG patients and controls. (**C**) Representative dot plot of extracellular staining from one PBMC sample from MG patient (top) and control (lower). (**D**) The graph of extracellular localization of survivin in CD20$^+$ cells from MG patients and controls. (**E**) The percentage CD4$^+$ cells with intracellular localization of survivin from MG patients and controls. (**F**) The graph of extracellular expression on CD4$^+$ cells from MG patients and controls. The data are representative of two independent experiments expressed as mean values. Mann–Whitney U test was used to determine statistical significance. A p value <0.05 was considered significant.

of survivin in $CD4^+$ cells. As with the $CD20^+$ cells, we found an increase in the percentage of $CD4^+$ cells with intracellular expression of survivin from MG patients (2.55% ± 3.13%) compared with controls (0.74% ± 0.81%; $p < 0.05$) (Fig. 1E). The percentage of $CD4^+$ cells that expressed survivin on the extracellular surface was not statistically significant between MG patients and controls (0.20% ± 0.20%, 0.16% ± 0.15%, respectively; Fig. 1F).

2C2 Ab treatment protects mice from EAMG

Animals were induced with tAChR to produce EAMG and monitored for weight and grip strength. On day 54, animals were stratified based on weight and grouped to ensure representative weight in each group. Treatment with cross-reactive survivin Ab 2C2 was initiated 2 d after the second booster of AChR, followed by continuing assessment in a blinded fashion. Over the course of treatment, weight did not vary between groups. At the end of the experiment, no statistical difference was observed between groups based on weight (Fig. 2A). At time of sacrifice, PBS-treated EAMG mice demonstrated significantly higher disease scores (Fig. 2B) compared with both the high-dose (100 μg) and low-dose (20 μg) 2C2 Ab treatment. Grip strength was maintained in the high-dose group, whereas muscle strength was reduced in both the low-dose survivin Ab and PBS-treated groups (Fig. 2C, 2D).

2C2 Ab treatment reduces survivin-expressing B cells in EAMG

The survivin protein negatively regulates apoptosis. Therefore, we postulated that long-lived immune cells in the EAMG mouse model could maintain production of AChR receptor Ab via expression of survivin. In our study, intracellular survivin expression was elevated in $CD3^-CD19^+$ splenic B cells from EAMG animals (Fig. 3).

Anti-survivin treatment effectively suppressed survivin expression in a dose-dependent fashion in splenic B cells (Fig. 3A, 3B). The percentage of splenic $CD3^-CD19^+$ B cells expressing intracellular survivin was 4.85% ± 1.98% in the control CFA only group. The induction of EAMG trended toward an increase in $CD3^-CD19^+survivin^+$ B cells (6.57% ± 2.38%). The addition of 2C2 Ab dramatically reduced $CD3^-CD19^+survivin^+$ B cells in both the high-dose 2C2 group (2.65% ± 1.05%; $p < 0.0005$) and the low-dose 2C2 group (3.72% ± 1.55%, $p < 0.05$). The percentage of $CD19^+$ cells that demonstrated extracellular survivin did not vary significantly regardless of treatment (Fig. 3C). Similarly, the percentage of $CD4^+$ cells with intracellular (Fig. 3D) and extracellular survivin (Fig. 3E) did not vary with the induction of EAMG or with 2C2 treatment. The percentage of $CD3^+CD19^-CD4^-$ T cells, $CD3^+CD19^-CD4^+$ Th cells, and $CD3^-CD19^+$ B cells in the splenic population did not vary among the EAMG-treated groups (data not shown).

Treatment with 2C2 Ab lowers the production of AChR-specific Abs

All EAMG mice induced with tAChR produced detectable Abs to the Ag. The EAMG mice treated with high-dose 2C2 Ab demonstrated statistically lower total IgG, IgG1, and IgG2a ($p = 0.04$ for all; Fig. 4A–C). IgG2b anti-tAChR showed dramatic reduction in the serum of EAMG mice treated with high-dose 2C2 compared with EAMG PBS-treated animals ($p < 0.001$; Fig. 4D). However, EAMG mice treated with low-dose 2C2 Ab demonstrated similar total IgG, IgG1, and IgG2a as the EAMG mice treated with PBS. In contrast, EAMG mice treated with low-dose 2C2 Ab showed statistically lower IgG2b Abs to tAChR compared with the PBS-treated EAMG mice ($p = 0.03$; Fig. 4D).

Analysis of the NMJ demonstrates less Ab binding and greater AChR presence following 2C2 Ab treatment

To determine the binding of autoantibodies to the mouse AChR at the NMJ and the depletion of AChR at the cell surface, TA sections were taken from EAMG mice treated with PBS, high-dose 2C2 Ab, and low-dose 2C2 Ab. Sections were stained with anti-mouse IgG (green) and α-BTX (red) (Fig. 5A). The concentration of mouse IgG Ab bound to the AChR was significantly reduced in the high-dose 2C2 Ab group based on fluorescence (24,205 ± 11,312

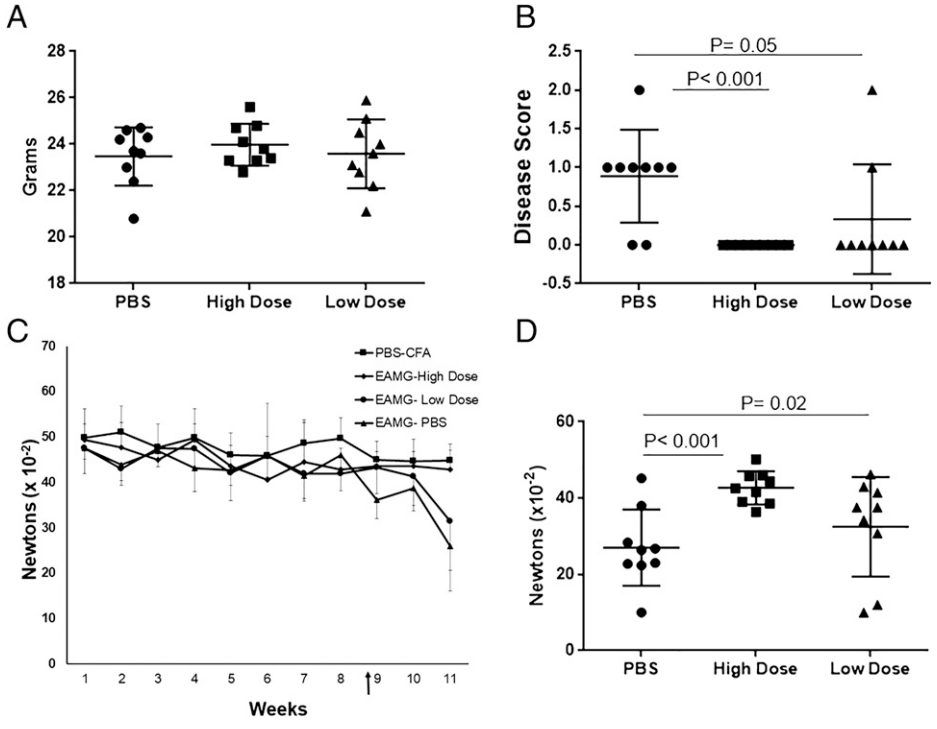

FIGURE 2. Mouse model of EAMG demonstrated efficacy of treatment with Ab to survivin. Mice (n = 27, 9 per group) were injected with AChR in CFA followed by injections with AChR in IFA twice (day 28 and 52). Treatment was initiated on day 54 with i.p. injections of high-dose anti-survivin (100 μg), low-dose anti-survivin (20 μg) or PBS. Treatment was given every other day for a total of six injections. Termination of the experiment occurred on day 67 with final weight (**A**), final disease score (**B**), grip strength over the course of the experiment (**C**), and final grip strength (**D**) noted. Grip strength measurements are mean of five values per animal per group. The data are representative of one independent experiment. (A and D) Student t test was used to confirm statistical relevance. (B) Kruskal–Wallis test and Dunn post hoc test were used to determine statistical significance. Statistical p values < 0.05 were considered significant.

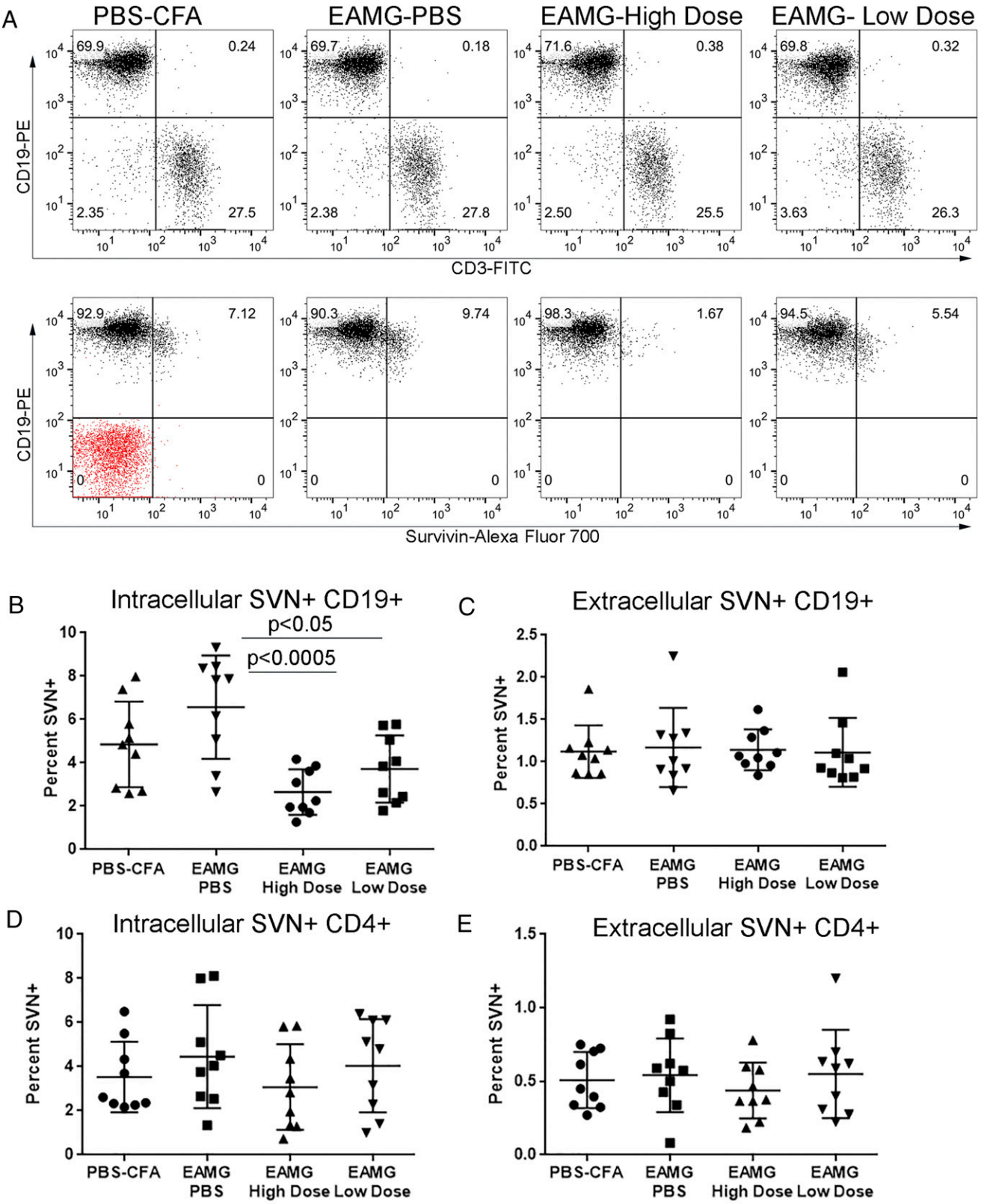

FIGURE 3. Splenocytes from 2C2-treated EAMG animals demonstrate a lower percentage of $CD19^+$ $survivin^+$ cells. Splenocytes were excised from treated animals on day 67. Cells were stained for viability, CD3, CD4, and CD19 markers. Cells were permeabilized and stained for survivin. For extracellular staining of survivin, the permeabilization step was omitted. Samples were viewed on a BD FACSCelesta analyzer, and $survivin^+$ cells were determined by FlowJo. (**A**) Representative dot plot from PBS–CFA (control), and EAMG animals (PBS treated, high-dose anti-survivin, and low-dose anti-survivin) are shown. (**B**) Scatter plot of all samples analyzed for intracellular $survivin^+$ $CD19^+$ cells. (**C**) Percentage of $CD19^+$ cells that demonstrated extracellular survivin. (**D**) Graph of the percentage of $CD4^+$ cells that contained intracellular survivin. (**E**) The percentage of $CD4^+$ cells that showed extracellular localization of survivin. Data were presented as mean ± SD. Results were representative of two independent experiments. $n = 9$ for all groups. Statistical analysis was done by Student t test. Statistical p values < 0.05 were considered significant.

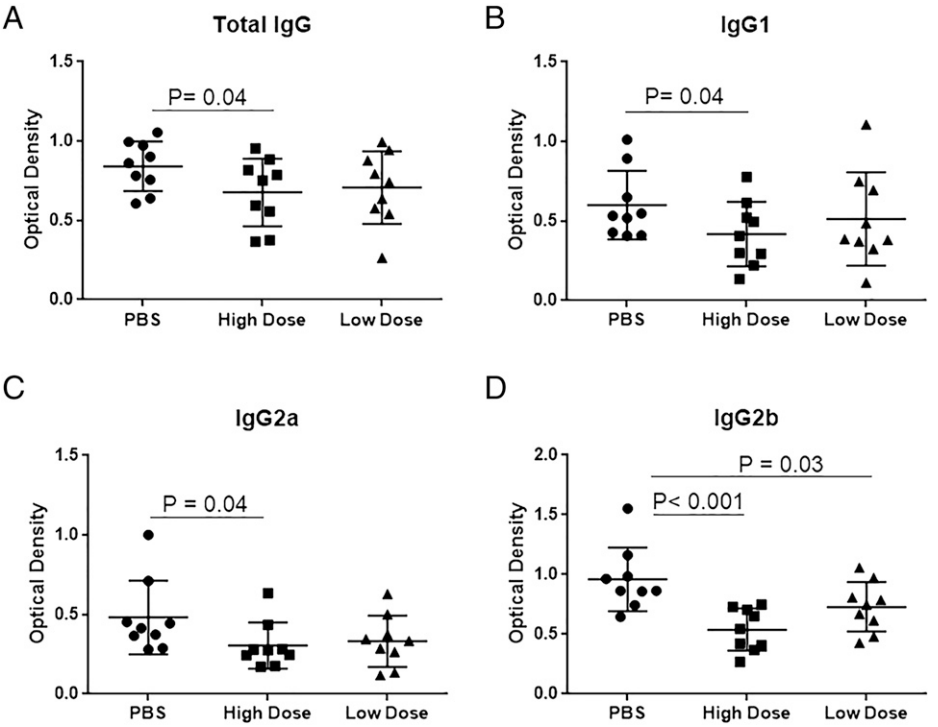

FIGURE 4. Ab levels to AChR were reduced in anti-survivin–treated EAMG animals. Blood was taken from the EAMG animals at time of termination. Serum was collected and assayed by ELISA for tAChR-specific total IgG (**A**), IgG1 (**B**), IgG2a (**C**), and IgG2b (**D**). $n = 9$ for all groups. Student t test was used to determine statistical value. Data were presented as mean ± SD. Results were representative of two independent experiments. Statistical p values < 0.05 were considered significant.

fluorescent intensity) compared with PBS treatment (36,750 ± 8504 fluorescent intensity; $p = 0.009$) (Fig. 5B). AChR expression on the cell surface of the NMJ was significantly increased in both the high- and low-dose 2C2 Ab-treated EAMG mice (38,785 ± 3172, 36,736 ± 6141 fluorescent intensity, respectively) compared with the PBS-treated EAMG mice (32,377 ± 4416 fluorescent intensity; $p = 0.001$, $p = 0.045$) (Fig. 5C).

Discussion

Elevated survivin expression has been found in several autoimmune diseases, although the potential pathogenic mechanism and cell type with survivin expression varies (16). In this study, we analyzed lymphocytes from MG patients and controls for both intracellular and extracellular expression of survivin. As shown in our previous study, intracellular expression for survivin was found in more $CD20^+$ cells from MG patients compared with controls (11). Survivin has been shown to exist in exosomes and on the surface of tumor cells (12, 17, 18). In this study, we saw an increased percentage of $CD20^+$ cells positive for exterior cell membrane expression of survivin from MG patients. We also showed an increase in $CD4^+$ cells that expressed intracellular survivin in MG patients compared with controls, although the extracellular localization of survivin did not vary. Because expression on the extracellular surface suggests a potential therapeutic target, we assessed the ability of an Ab to a modified survivin peptide (2C2) that is immunogenic in both humans and mice to moderate weakness in a mouse model of EAMG. Treatment with 2C2 Ab produced significant improvement in disease score, reduction in $CD19^+$ $survivin^+$ splenocytes, reduced AChR-specific Abs, and retention of AChR at the neuromuscular junction. These results suggest that targeting extracellular survivin directly with a humanized form of 2C2 could be an effective therapeutic approach.

In our previous study, we assessed B cells and plasma cells for survivin expression in an MG patient population (11). We hypothesize that the expression of survivin in autoreactive cells may allow the lymphocytes to escape tolerance. The ability of survivin to transfer to other cells in an autoimmune condition would allow for other autoreactive cells to escape the checkpoint and elimination. In this study, we demonstrated expression of survivin on the outer surface of $CD20^+$ cells, suggesting that survivin may have a role in cell–cell signaling. In the tumor microenvironment, survivin expression in exosomes appears to support cell proliferation and treatment resistance (17, 19, 20). Abs to survivin can reduce the ability of exosomes to internalize on the target cell (20). In our model, the localization of survivin to the external cell surface would allow for recognition by an Ab and could inhibit the role of survivin in supporting persistence of autoreactive cells.

To gain insight into the expression of survivin in the B cells involved in MG, we took advantage of an animal model of MG (15) and a high-affinity Ab to a previously characterized immunogenic-modified survivin peptide (12). This study demonstrated the efficacy of 2C2 in ameliorating weakness and reducing AChR-specific Ab production in an EAMG mouse model. The results mirrored the outcome seen in both rat and mouse EAMG models following vaccination with the modified survivin peptide from which 2C2 was derived (11). Thus, certain Abs induced by vaccination with the peptide conjugate SVN53-67/M57-KLH have a significant ameliorative effect on EAMG. Although we did not observe changes in intracellular survivin in the $CD4^+$ population or the extracellular expression of survivin in $CD19^+$ and $CD4^+$ splenocytes, the ability of the Ab to reduce the $CD19^+survivin^+$ cell population strongly suggests the presence of an available cell surface epitope of the target molecule. Future studies will address the possible communication between autoreactive cells mediated by survivin expression.

Evidence suggests that survivin expression drives pathology in several autoimmune disorders (4). Treatment of these diseases has resulted in the observation of reduced survivin expression along with a reduction in disease severity (7, 9, 21). Our previous study using a vaccine approach to target survivin produced $CD8^+$ cells that recognized the modified survivin peptide, as well as Abs to survivin (11). To obtain a controlled (i.e., reversible) therapeutic effect in MG, Ab delivery offers a more precise method for

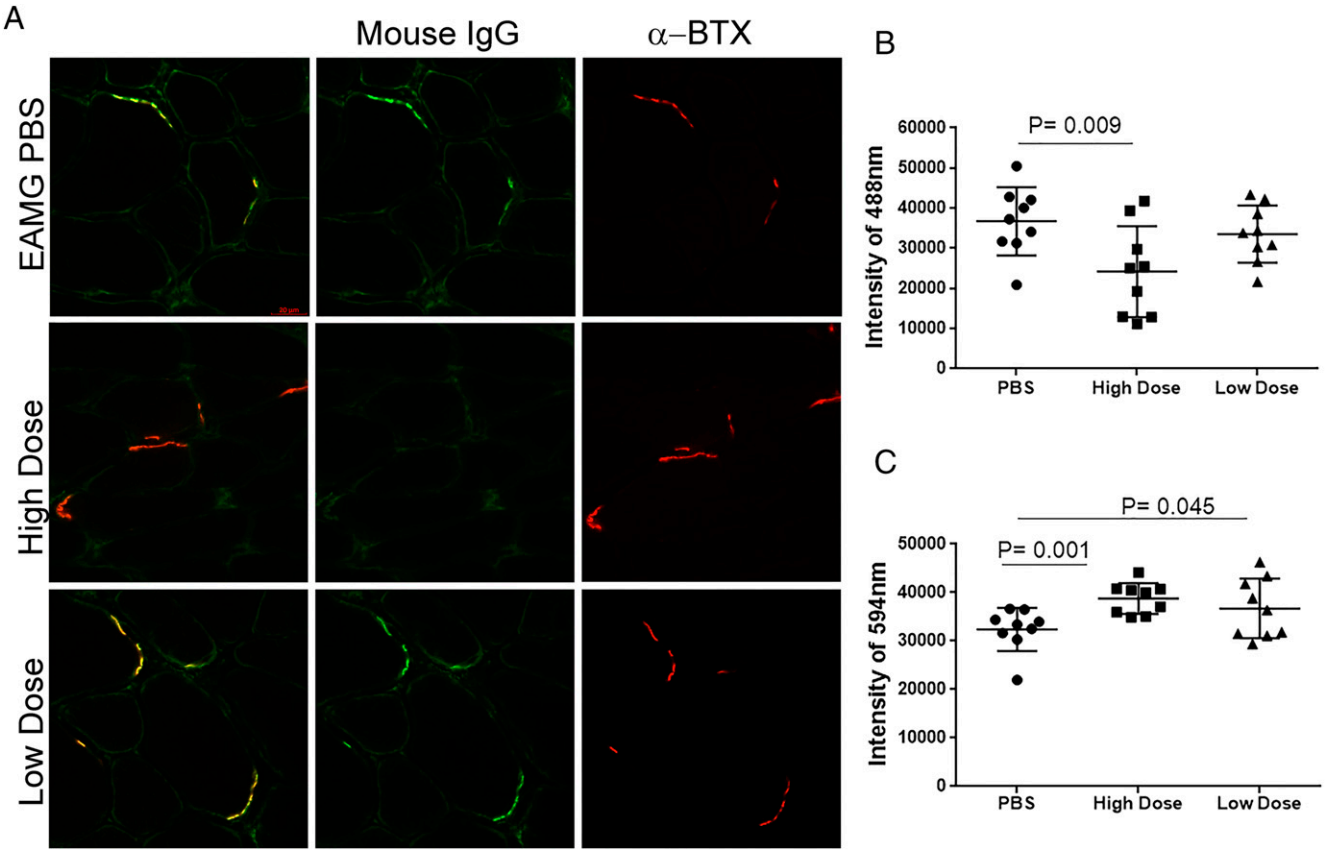

FIGURE 5. Abs that target the NMJ were reduced in high-dose anti-survivin–treated EAMG animals. TA muscle was sectioned and stained for mouse-specific IgG and α-BTX, which labeled AChR. Images were taken on a Carl Zeiss Cell Observer Spinning Disk Confocal Microscope and analyzed for fluorescent intensity by Zen software. (**A**) Representative images of Alexa 488–anti-mouse IgG and Alexa 594–α-BTX for EAMG PBS-treated and EAMG 2C2-treated (high- and low-dose) mice. (**B**) Fluorescent intensity measurements of Alexa 488–anti-mouse IgG. (**C**) Fluorescent intensity measurements of Alexa 594–α-BTX. Data were presented as mean ± SD. Results were representative of two independent experiments of 30 NMJs analyzed per animal. $n = 9$ for all groups. Statistical comparisons were performed by Student t test analysis. Statistical p values < 0.05 were considered significant.

targeting autoreactive cells compared with a vaccine. Other therapeutic agents that target the B cell population are quite broad. By targeting the specific autoreactive cells that produce the disease, survivin Ab therapy may offer a more refined and specific approach. The ability to target survivin through Ab recognition in the autoreactive cells is an attractive therapeutic for MG.

Disclosures

R.A.F. and M.C. are codiscoverers of Ab 2C2 and cofounders of MimiVax, LLC, which is licensed by Roswell Park Comprehensive Cancer Center to develop 2C2 and related biological agents used in the studies described in this work. The other authors have no financial conflicts of interest.

References

1. Lee, J. Y., P. Stathopoulos, S. Gupta, J. M. Bannock, R. J. Barohn, E. Cotzomi, M. M. Dimachkie, L. Jacobson, C. S. Lee, H. Morbach, et al. 2016. Compromised fidelity of B-cell tolerance checkpoints in AChR and MuSK myasthenia gravis. *Ann. Clin. Transl. Neurol.* 3: 443–454.
2. Kinnunen, T., N. Chamberlain, H. Morbach, T. Cantaert, M. Lynch, P. Preston-Hurlburt, K. C. Herold, D. A. Hafler, K. C. O'Connor, and E. Meffre. 2013. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J. Clin. Invest.* 123: 2737–2741.
3. Budhidarmo, R., and C. L. Day. 2015. IAPs: modular regulators of cell signalling. *Semin. Cell Dev. Biol.* 39: 80–90.
4. Gravina, G., C. Wasén, M. J. Garcia-Bonete, M. Turkkila, M. C. Erlandsson, S. Töyrä Silfverswärd, M. Brisslert, R. Pullerits, K. M. Andersson, G. Katona, and M. I. Bokarewa. 2017. Survivin in autoimmune diseases. *Autoimmun. Rev.* 16: 845–855.
5. Hebb, A. L., C. S. Moore, V. Bhan, T. Campbell, J. D. Fisk, H. A. Robertson, M. Thorne, E. Lacasse, M. Holcik, J. Gillard, et al. 2008. Expression of the inhibitor of apoptosis protein family in multiple sclerosis reveals a potential immunomodulatory role during autoimmune mediated demyelination. *Mult. Scler.* 14: 577–594.
6. Sharief, M. K., M. A. Noori, M. R. Douglas, and Y. K. Semra. 2002. Upregulated survivin expression in activated T lymphocytes correlates with disease activity in multiple sclerosis. *Eur. J. Neurol.* 9: 503–510.
7. Sharief, M. K., and Y. K. Semra. 2002. Down-regulation of survivin expression in T lymphocytes after interferon beta-1a treatment in patients with multiple sclerosis. *Arch. Neurol.* 59: 1115–1121.
8. Zafari, P., A. Rafiei, S. A. Esmaeili, M. Moonesi, and M. Taghadosi. 2019. Survivin a pivotal antiapoptotic protein in rheumatoid arthritis. *J. Cell. Physiol.* 234: 21575–21587.
9. Bokarewa, M., S. Lindblad, D. Bokarew, and A. Tarkowski. 2005. Balance between survivin, a key member of the apoptosis inhibitor family, and its specific antibodies determines erosivity in rheumatoid arthritis. *Arthritis Res. Ther.* 7: R349–R358.
10. Conti-Fine, B. M., M. Milani, and H. J. Kaminski. 2006. Myasthenia gravis: past, present, and future. *J. Clin. Invest.* 116: 2843–2854.
11. Kusner, L. L., M. J. Ciesielski, A. Marx, H. J. Kaminski, and R. A. Fenstermaker. 2014. Survivin as a potential mediator to support autoreactive cell survival in myasthenia gravis: a human and animal model study. *PLoS One* 9: e102231.
12. Fenstermaker, R. A., S. A. Figel, J. Qiu, T. A. Barone, S. S. Dharma, E. K. Winograd, P. M. Galbo, L. M. Wiltsie, and M. J. Ciesielski. 2018. Survivin monoclonal antibodies detect survivin cell surface expression and inhibit tumor growth in vivo. *Clin Cancer Res* 24: 2642–2652.
13. Jaretzki, A. , III, R. J. Barohn, R. M. Ernstoff, H. J. Kaminski, J. C. Keesey, A. S. Penn, and D. B. Sanders, Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. 2000. Myasthenia gravis: recommendations for clinical research standards. *Neurology* 55: 16–23.
14. Aguilo-Seara, G., Y. Xie, J. Sheehan, L. L. Kusner, and H. J. Kaminski. 2017. Ablation of IL-17 expression moderates experimental autoimmune myasthenia gravis disease severity. *Cytokine* 96: 279–285.
15. Tuzun, E., S. Berrih-Aknin, T. Brenner, L. L. Kusner, R. Le Panse, H. Yang, S. Tzartos, and P. Christadoss. 2015. Guidelines for standard preclinical experiments in the mouse model of myasthenia gravis induced by acetylcholine receptor immunization. *Exp. Neurol.* 270: 11–17.

16. Ebrahimiyan, H., S. Aslani, N. Rezaei, A. Jamshidi, and M. Mahmoudi. 2018. Survivin and autoimmunity; the ins and outs. *Immunol. Lett.* 193: 14–24.
17. Khan, S., J. M. Jutzy, J. R. Aspe, D. W. McGregor, J. W. Neidigh, and N. R. Wall. 2011. Survivin is released from cancer cells via exosomes. *Apoptosis* 16: 1–12.
18. Khan, S., J. M. Jutzy, M. M. Valenzuela, D. Turay, J. R. Aspe, A. Ashok, S. Mirshahidi, D. Mercola, M. B. Lilly, and N. R. Wall. 2012. Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. *PLoS One* 7: e46737.
19. Khan, S., H. F. Bennit, and N. R. Wall. 2015. The emerging role of exosomes in survivin secretion. *Histol. Histopathol.* 30: 43–50.
20. Gonda, A., J. Kabagwira, G. N. Senthil, H. R. Ferguson Bennit, J. W. Neidigh, S. Khan, and N. R. Wall. 2018. Exosomal survivin facilitates vesicle internalization. *Oncotarget* 9: 34919–34934.
21. Smith, M. D., H. Weedon, V. Papangelis, J. Walker, P. J. Roberts-Thomson, and M. J. Ahern. 2010. Apoptosis in the rheumatoid arthritis synovial membrane: modulation by disease-modifying anti-rheumatic drug treatment. *Rheumatology (Oxford)* 49: 862–875.