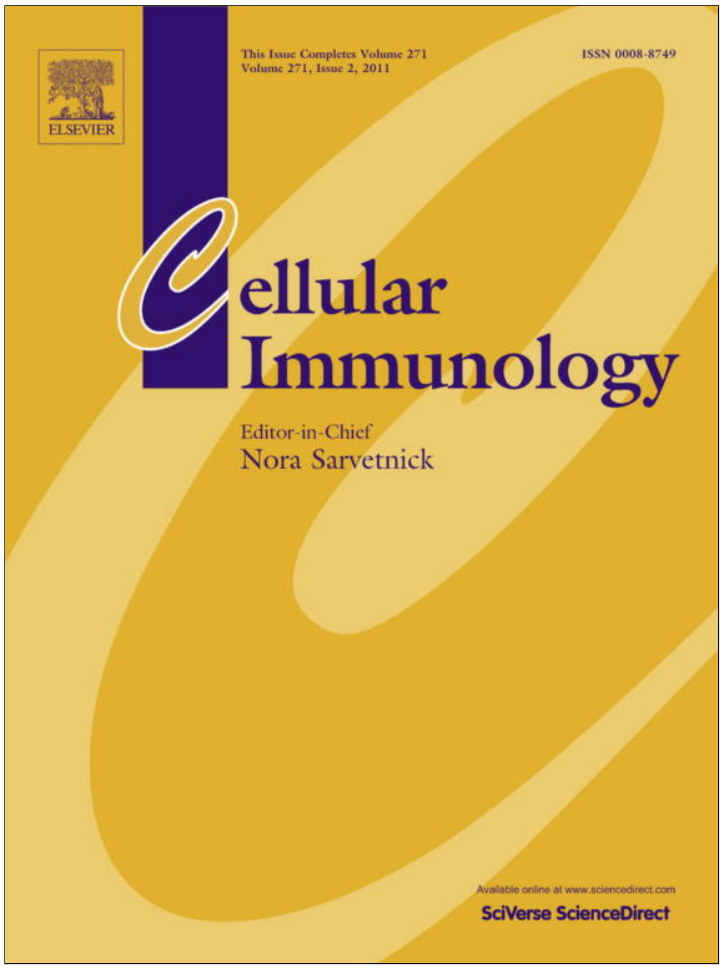

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Characterization of peripheral blood acetylcholine receptor-binding B cells in experimental myasthenia gravis

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ABSTRACT

In myasthenia gravis (MG), the neuromuscular transmission is impaired by antibodies (Abs) specific for muscle acetylcholine receptor (AChR). Anti-AChR Abs can be detected in the serum of MG patients, although their levels do not correlate with disease severity. In this study, we developed a flow cytometric assay for the detection of peripheral blood AChR-specific B cells to characterize B cell phenotypes associated with experimental autoimmune myasthenia gravis (EAMG). Alexa-conjugated AChR was used as a probe for AChR-specific B cells (B220+Ig+). Mice with EAMG had significantly elevated frequencies of AChR-specific IgG2+ and IgM+ B cells. While the frequencies of IgG2+ B cells and plasma anti-AChR IgG2 levels significantly correlated with the clinical grades of EAMG, the frequencies of IgM+ B cells and plasma anti-AChR IgM levels did not. These results indicate that the frequency of AChR-specific and IgG1+ (mouse IgG2 equivalent) peripheral blood B cells and anti-AChR IgG1 levels could be potential biomarkers for MG disease severity.

1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune neuromuscular disorder, and in 85–90% of patients, the disease is mediated by antibodies that bind acetylcholine receptors (AChR) located at the postsynaptic membranes of neuromuscular junctions (NMJ) [1,2]. Rodents immunized with *Torpedo californica* AChR develop experimental autoimmune myasthenia gravis (EAMG), a disease which closely resembles MG [3,4]. AChR is a transmembrane glycoprotein consisting of five subunits, two α, one β, one δ, with either an ε or γ subunit, organized to form a gated-ion channel which depolarizes muscle membranes when the neurotransmitter acetylcholine is bound [5]. AChR-specific B cells are involved in the pathogenesis of MG and EAMG by producing anti-AChR antibodies, which activate the complement cascade [6]. This antibody-mediated immune response at the NMJ reduces the number of functional AChR and destroys the morphology of the NMJ, thereby impairing neuromuscular transmission, causing muscle weakness and increasing fatigability [2,7]. Therefore, in most cases MG is diagnosed by radioimmunoassay, ELISA or ELISPOT-based detection of sera anti-AChR antibodies [8,9]. However, although serum anti-AChR levels are decreased in response to immunosuppression in individual patients, the anti-AChR antibody levels are not correlated with MG severity in patient groups and therefore are not reliable predictors/markers of disease severity in MG [10–13].

Since B cells express both surface and/or secreted forms of immunoglobulins, which are capable of recognizing AChR, measuring antibody secretion alone is an indirect and limited means of determining B cell activation. We developed a new potential biomarker flow cytometry assay for MG which identifies AChR-binding B cells. We used the EAMG mouse model in a strategy to employ Alexa fluor-conjugated AChR as a probe for identifying potentially pathogenic peripheral blood B cells from mice with EAMG. Labeled antigens have been previously used by several investigators to determine frequencies of antigen-specific B cells by flow cytometry [14–17]. Similar techniques have also been used to study protective antigen-specific B cell responses towards tetanus toxin, rotavirus, influenza and autoreactive specific B cell responses in diseases such as SLE and pemphigus vulgaris [18–20]. But in these studies the significance of antigen-specific B cell frequencies was never evaluated directly for protection from infection or for induction of pathogenic autoimmune B cell responses. In our study, we demonstrate, for the first time, the frequency of a subset of AChR-binding B cells which express IgG2b in the peripheral blood of EAMG mice, and that the population size also correlates with clinical grades of disease severity.

2. Materials and methods

2.1. Mice and induction of EAMG

C57BL/6 (B6) mice were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA). AChR extracted from *Torpedo califor-*

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nica was purified on a neurotoxin affinity column, as previously described [21]. EAMG was induced by emulsifying 100 μl CFA with 100 μl *Torpedo californica* AChR (20 μg) in PBS. A separate group of mice were immunized with 100 μl of LPS (5 mg) emulsified in CFA for comparison. Both groups were anesthetized, and then immunized (200 μl/animal) three times, 30 days apart with s.c. injections in shoulders and foot pads. Naïve mice were neither immunized with an antigen nor treated with a mock reagent. All animals were housed in a barrier facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee guidelines.

2.2. Clinical evaluation of EAMG

Evaluation of disease severity and muscle weakness was performed immediately prior to blood draw and at 2 weeks after each immunization and measured as follows: Grade 0**,** normal mobility, posture and grip strength; Grade 1**,** hunchback posture, restricted mobility and decreased muscle grip strength after paw grip exercises; Grade 2, without exercise, observed hunchback posture, restricted mobility, and decreased muscle grip strength; Grade 3, dehydrated and moribund with Grade 2 weakness, death, or euthanasia due to paralysis [22].

2.3. Conjugation of Alexa fluor 647 to AChR

AChR was purified from *Torpedo californica* electric organs (Aquatic Research Consultants, CA) according to published methods [21]. AChR was concentrated by centrifugation with Centricon YM (10,000 molecular weight) centrifugal filters (Millipore, MA). AChR was then dialyzed in PBS by using Spectra/Por dialysis tubing (12–14,000 molecular weight). The final concentration of AChR was 1.5 μg/μl. AChR was labeled with Alexa fluor 647 Protein Labeling Kit (Invitrogen) according to the manufacturer's instructions.

2.4. Blood collection and flow cytometry

Approximately 200 μl of blood was collected from the tail vein into K_2EDTA microtubes at 2 weeks following each immunization. Whole blood was then treated with BD Pharm Lyse Buffer to lyse erythrocytes. Cells were washed three times in PBS containing 2% FBS and 0.1% sodium azide. Fcγ receptors were blocked with anti-CD16/32 Ab (1/100) (Ab 93, eBioscience, San Diego, CA). One hundred thousand of whole blood cells were stained with Alexa fluor 647-AChR (0.1 μg for 100,000 cells) or Alexa fluor 647-Ovalbumin (0.1 μg for 100,000 cells) (Ova) in combination with PE-Cy7-anti B220 (1:200) (RA3–6B2, a mouse pan B cell marker, BD Biosciences, Franklin Lakes, NJ), FITC-anti-IgG2b (1:100) (R12-3, BD Biosciences) and PE-anti-IgM (1:200) (eB121-15F9, eBioscience). Cells were first stained for B220 expression and surface immunoglobulin AChR binding, then fixed and permeabilized by using a Cytoperm/Cytofix kit (BD Biosciences) to determine IgM or IgG. Cells were then analyzed using a BD FACS Canto, cells were acquired until 20,000 B220+ B cells were collected. Data was processed using FlowJo v 7.2 (Tree Star) to determine cell populations and prepare graphics.

2.5. ELISA for anti-mouse muscle AChR antibodies and isotypes

In keeping with previously published ELISA protocols, blood was centrifuged at 500*g* for 15 min, and plasma was removed for analysis of secreted anti-AChR antibodies [22]. IgM and IgG2b (IgG2) antibody isotypes to mouse muscle AChR were evaluated by ELISA, using a previously described method [22]. Mouse AChR was purified as previously described with a concentration of 1.25 μg/μl [21]. Briefly, muscle tissue from B6 mice was homogenized and then ultracentrifuged. The pellets were homogenized in detergent-containing buffer and centrifuged to yield supernatants containing crude AChR. This crude preparation was then applied to a neurotoxin 3 affinity column to prepare AChR for use as an immunogen. Affinity-purified mouse AChR (0.5 μg/ml) was coated onto a 96-well microtiter plates in 0.1 M carbonate bicarbonate buffer overnight at 4 °C. Diluted plasma samples of 100 μl (1:500) were added and incubated at 37 °C for 90 min. Horseradish peroxidase-conjugated anti-mouse IgM and IgG2 (Caltag Laboratories, Burlingame, CA) (1:1000) were added and then incubated at 37 °C for 90 min. Subsequently, the peroxidase indicator substrate 2,2′-azino-bis-(3-ethylbenzothiazoline 6-sulfonate) substrate (ABTS) solution in 0.1 M citric buffer (pH 4.35) was added in the presence of H_2O_2, and mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of 405 nm. Normal mouse plasma (collected from mice before immunization) was used for the background determination.

2.6. Statistics

Cell phenotypes were analyzed using an ANOVA with Tukey's post hoc test. Correlations between clinical grades, AChR-specific B cell frequencies and plasma secreted anti-AChR antibodies were determined by a Spearman correlation test with a two-tail *p* value.

3. Results

3.1. Detection by flow cytometry of AChR-binding B cells among peripheral blood cells of mice with EAMG

Given the pathological significance of complement activation in MG and EAMG [23], we conducted a comparative flow cytometry study of AChR-binding B cells which express IgM or IgG2b (IgG2) (complement binding mouse immunoglobulin isotypes) in mice. To activate AChR-specific B lymphocytes, we immunized 5 B6 mice three times with AChR in CFA. The protocol was optimized by using whole blood drawn from mice with EAMG 2 weeks post third immunization with AChR emulsified in CFA; these results were then compared to those in naïve ($n = 5$) or LPS-immunized ($n = 5$) controls. Alexa fluor 647-AChR was used as a probe for potentially autoreactive AChR-specific B cells, while staining with Alexa fluor 647-OVA was used as a negative control. Shown in Fig. 1A–C is the typical staining patterns observed from blood stained with Alexa fluor-AChR, anti-B220, anti-IgM, and anti-IgG2. Alexa fluor-AChR preferentially bound to B220-expressing cells, which indicated to us that B cells are the main subset of peripheral lymphocytes capable of binding AChR (Fig. 1B). Furthermore, B220+ AChR-binding lymphocytes are most prominent in mice with EAMG. There is no significant increase in B220+ OVA-binding lymphocytes in mice with EAMG. These data suggest that the expansion of B220+ lymphocytes in mice with EAMG is specific to AChR-binding cells. To characterize these cells further, lymphocytes from the upper quadrants (B220+) were gated on and evaluated for expression of IgM or IgG2 and AChR-binding (Fig. 1C). Although all mice had B220+IgM+ AChR-binding cells, these cells appeared at the highest frequencies in mice with EAMG (Fig. 1C). Conversely, only mice with EAMG had elevated frequencies of B220+IgG2+ AChR-binding cells (Fig. 1C). Background staining of blood lymphocytes with Alexa-OVA provided results showing the AChR-binding B cells are responsible for the increase in B cell frequencies. As shown in Table 1, the frequencies of AChR-specific B cell populations but not the whole B220+ B cell populations were significantly higher in AChR-immunized mice as compared to controls. To confirm the specificity of this assay, inhibition of Alexa fluor 647-AChR binding to B cells was shown by incubating the cells with a 10-fold

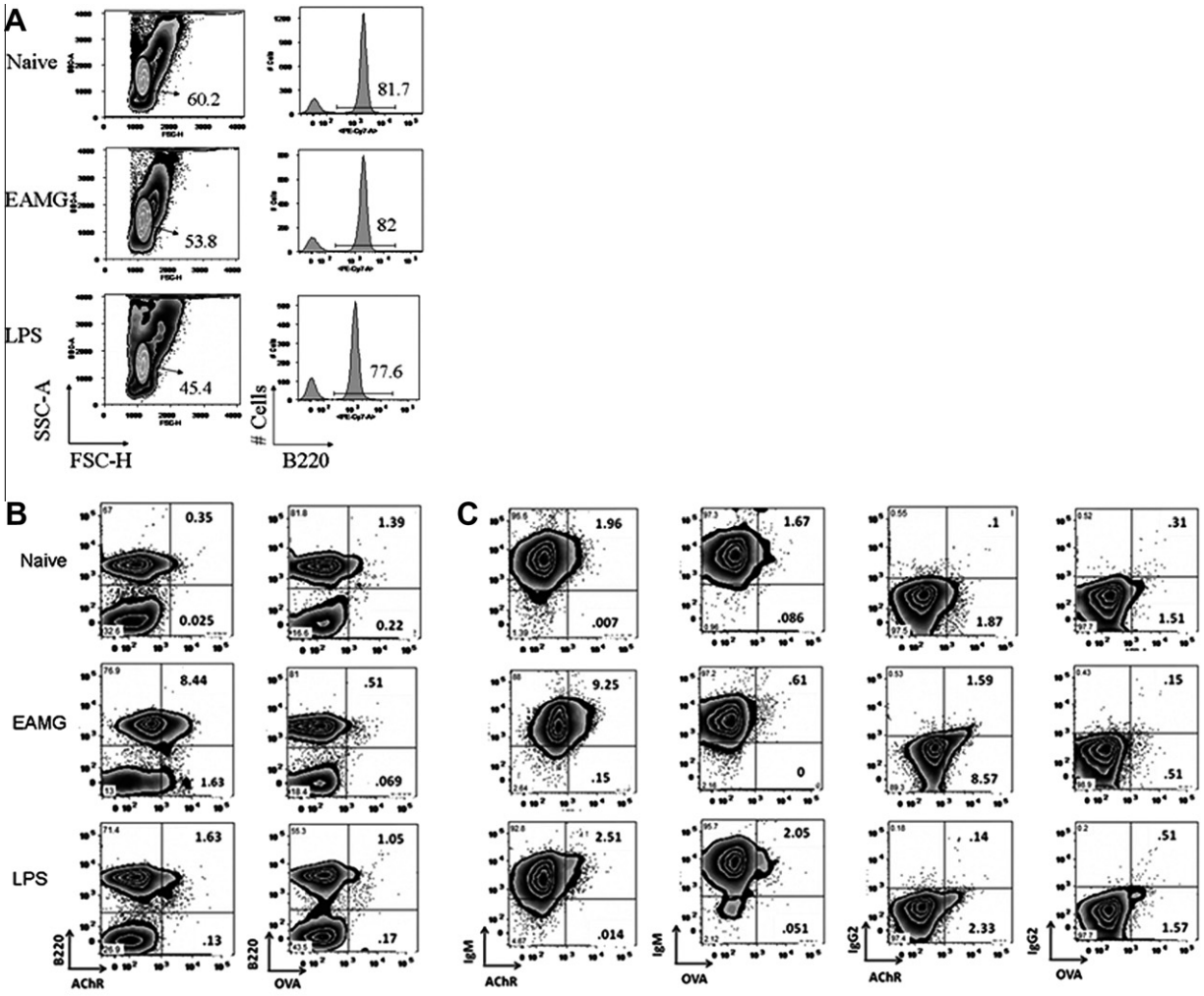

Fig. 1. The characterization of AChR-binding peripheral blood lymphocytes by flow cytometry. Representative flow cytometry analysis of peripheral blood lymphocytes from naïve ($n = 5$), LPS-immunized ($n = 5$) or AChR+ CFA-immunized (EAMG) ($n = 5$) mice, 75 days postprimary immunization. Cells were first gated on lymphocytes and then analyzed for B220 expression (A) and either AChR binding or OVA binding (B). Then lymphocytes were gated on B220+ cells to characterize IgM or IgG2 (C) expression and either AChR binding or OVA binding. The numbers shown in bi-exponential plots indicate the relative percentage of cells in each quadrant. The experiment was repeated five times with similar results.

excess of unlabeled AChR prior to fluorescent labeling (Fig. 2). Ten B6 mice were immunized with AChR in CFA for three times and peripheral blood cells were obtained 2 weeks after last immunization. The blood cells of mice were then incubated in the presence ($n = 5$) or absence ($n = 5$) of unlabeled AChR. Alexa fluor-AChR staining of total B220+ cells, and IgM+ and IgG2+ B cells was significantly reduced by inhibition with unlabeled AChR.

To determine the significance of the differences observed for AChR-binding B cell frequencies between mice with EAMG and controls, we evaluated AChR-binding B cell frequencies at different time points following immunization with AChR in CFA (16 mice) (Fig. 3, Table 2). Five LPS-immunized and 10 non-immunized naïve mice were used as controls. Using the analysis scheme described in Fig. 1, we found no significant differences in AChR-binding B cell frequencies two weeks following the primary AChR immunization. After the second AChR immunization, the frequencies of AChR-binding peripheral blood B cells began to rise. Both B220+IgG2+ and B220+IgM+ AChR-binding B cell frequencies were significantly elevated compared to healthy and LPS immunized mice. After the third immunization, all subsets (B220+, B220+IgM+, and B220+IgG2+) of AChR-binding B cells analyzed were significantly elevated compared to findings with subsets in healthy naïve or LPS immunized mice (Fig. 3, Table 2).

Table 1
Frequencies[a] of AChR-binding peripheral blood B cells.

	% of Lymphocyte B220+	% of B cells[b]		
		AChR+	IgM+AChR+	IgG2+AChR+
Naïve $n = 5$	82.56 ± 1.536	1.51 ± 0.195	1.52 ± 0.191	0.05 ± 0.017
LPS $n = 5$	81.42 ± 1.214	1.61 ± 0.184	1.94 ± 0.195	0.08 ± 0.019
EAMG $n = 5$	81.85 ± 0.195	13.45 ± 1.405	13.24 ± 1.363	1.69 ± 0.308
p-Value[c]	NS[d]	<0.0001	<0.0001	0.0008

[a] Frequencies are shown as the mean percent of lymphocytes ± standard error of the mean.
[b] Cells were gated using the pan B cell marker B220.
[c] p-Values were obtained using ANOVA.
[d] NS, not significant.

3.2. The frequency of peripheral blood AChR-specific B cells correlates with the severity of EAMG

Although the presence of serum antibodies to AChR indicates a possible diagnosis of MG, anti-AChR antibody concentrations are not reliable markers for disease severity [10–13]. Clinical grades of EAMG severity were used to determine whether the frequencies of peripheral blood AChR-specific B cells correlate with disease severity. The clinical grade of EAMG is a combination of several observed

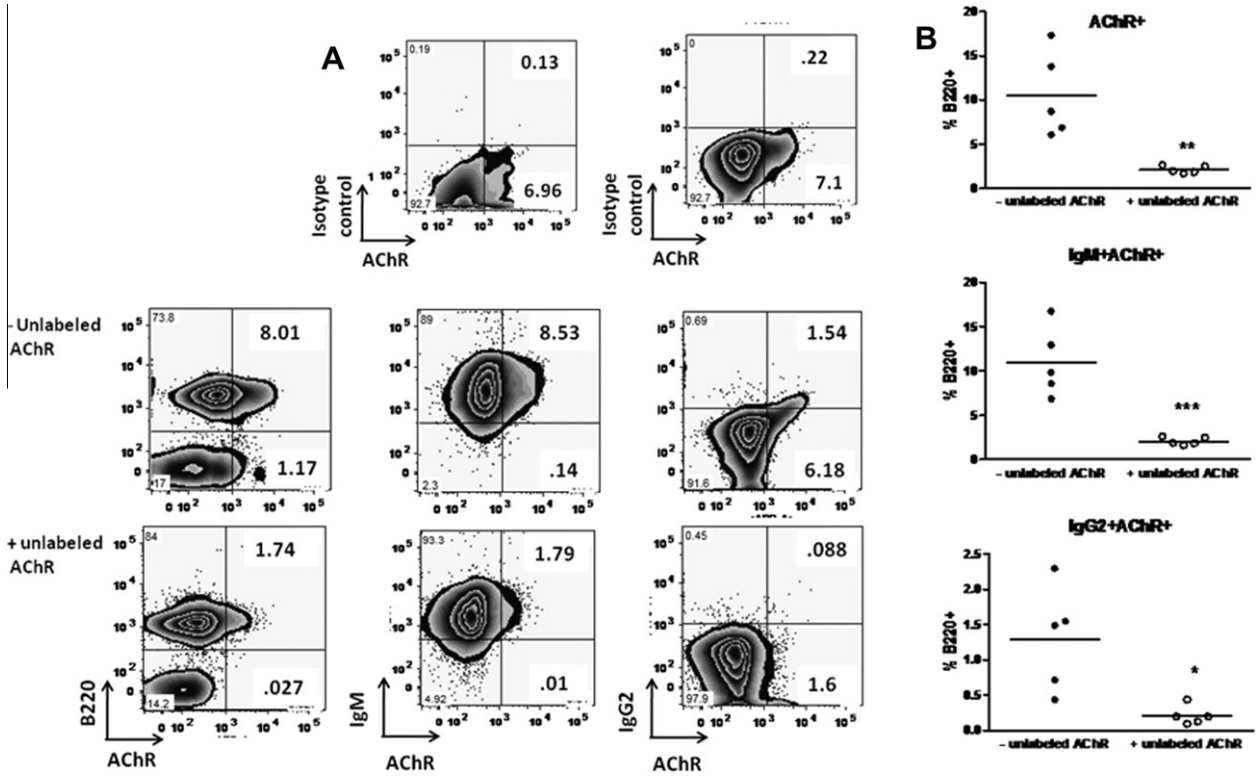

Fig. 2. Inhibition of Alexa fluor-AChR-binding to peripheral blood B cells with unlabeled AChR. Representative flow cytometry staining of peripheral blood lymphocytes with (+) or without (−) blocking by incubating cells with (+) unlabeled AChR prior to staining with Alexa fluor-AChR; anti-B220 and anti-IgM, anti-IgG2, or isotype controls (A). The numbers shown in bi-exponential plots indicate the relative percentage of cells in each quadrant. The mean percentage of B-cell, AChR-binding subsets with (+) or without (−) blocking with unlabeled AChR (B). Each circle represents the frequency of AChR-binding B cells after three immunizations with CFA+ AChR from individual mice having EAMG ($n = 5$).The bar indicates the mean frequency of AChR-binding B cells. The data shown are from one experiment that was repeated three times.$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, *t*-test.

parameters of EAMG, such as posture, mobility, and muscle strength. Mice with no EAMG symptoms are assigned a clinical score of 0. A score of 1 is associated with no signs of EAMG prior to exercise or mild disease, a 2 indicates overall moderate symptoms of limb weakness, a score of 3 is associated with significant signs of muscle weakness without exercise and severe disease. Following the immunization of 25 B6 mice three times (days 0, 30, and 60) with AChR in CFA, 11 mice developed mild grade 1 disease, whereas 10 mice developed moderate-severe grade 2 or 3 disease and 4 mice had no symptoms (Grade 0). The frequencies of peripheral blood AChR-specific IgM+ and IgG2+ B cells and plasma levels of anti-AChR IgM and IgG2 were measured in blood cells and plasma samples obtained 2 weeks after third immunization (day 75) and were compared with the clinical grades of disease and each other (Fig. 4A–F). Earlier time points were not evaluated due to the lack of animals with severe disease. While IgM+ AChR-specific B cells in blood were not significantly correlated with the clinical grades of EAMG (Fig. 4B), IgG2+ AChR-specific B cells in blood had a strong correlation with clinical grades ($R = 0.7421$, $p = 0.0001$) (Fig. 4E). These results indicate that increased frequencies of peripheral blood AChR-specific and IgG2+ B cells correspond to loss of limb muscle strength and to higher clinical grades of disease. Therefore, AChR-specific IgG2 expressing B cells might be a good biomarker of disease severity.

3.3. Plasma secreted immunoglobulins specific for mouse AChR corresponds with Torpedo AChR-specific B cell frequencies

It is well established that the immunization of B6 mice with *Torpedo californica* AChR emulsified in CFA leads to the production of anti-AChR immunoglobulins with high-affinity to mouse AChR [3,12,21–23]. Therefore, we evaluated the association between mouse AChR-specific immunoglobulins from plasma and disease severity. Plasma anti-mouse AChR IgM levels did not correlate with disease severity (Fig. 4A), whereas plasma anti-mouse AChR IgG2 levels were significantly correlated with clinical grades ($R = 0.6265$, $p = 0.0001$) (Fig. 4D). We also evaluated the association between *Torpedo* AChR-specific B-cell frequencies and plasma anti-mouse AChR concentrations. Both anti-AChR IgM and IgG2 levels were significantly correlated with IgM+ ($R = 0.6055$, $p = 0.0015$) and IgG2+ B cell ($R = 0.7848$, $p = 0.0001$) frequencies (Fig. 4C and F), respectively. These results indicate that plasma anti-AChR antibody levels are correlated with AChR specific B cell frequencies and anti-AChR IgG2 levels may also be used as a biomarker of MG severity.

4. Discussion

Our data demonstrate that Alexa fluor-AChR conjugates could be used to identify and characterize subsets of B cells with specificity for AChR. The vast majority of cells which bound AChR express a B cell phenotype, B220+IgM+ or B220+IgG2+. B220 is a member of the protein tyrosine phosphate family and a major cell surface glycoprotein, represents a restricted form of the CD45 family which primarily recognizes the cells of B-lineage from pro-B cell through mature B lymphocytes and is commonly used as a pan B-cell marker in mice [24]. It also reacts with certain activated T cells [25], however, in our experiments, less than 1% of Alexa fluor-AChR-binding cells were B220− but CD3+ (data not shown). Also, in Figs. 1 and 2, small populations of B220-AChR+ blood cells were observed in EAMG mice. These cells might be memory B cells, which bind whole protein antigens through their B cell receptors. The sig-

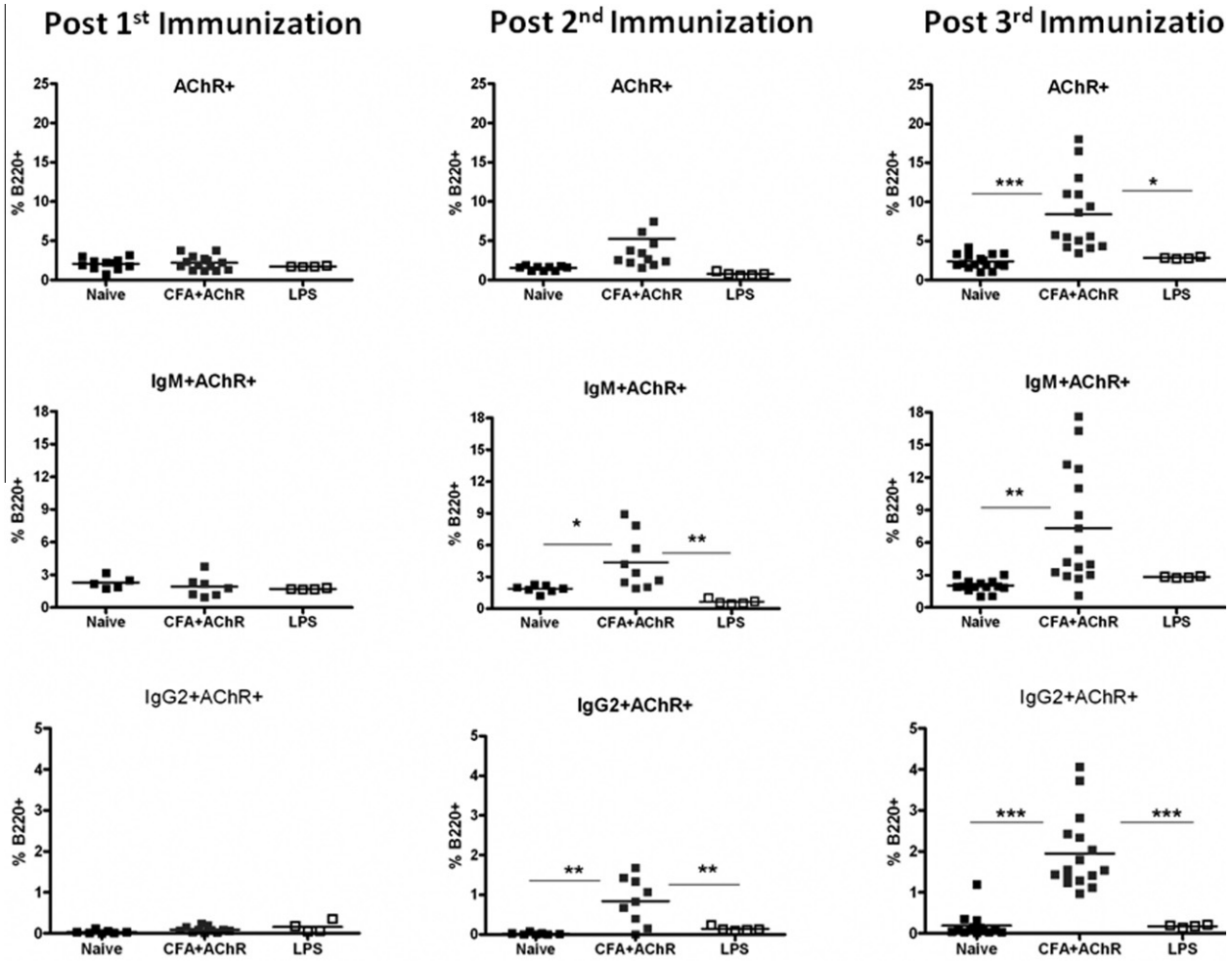

Fig. 3. The kinetics on the frequencies of AChR-binding B cells. Shown is the mean percentage of subsets of peripheral blood B cells which are AChR-binding from naïve ($n = 10$), LPS-immunized ($n = 5$) or CFA+ AChR-immunized ($n = 16$) (EAMG) mice. Cells were analyzed as shown in Fig. 1. Each square represents the frequency of the total AChR-binding B cells (top row), AChR-binding IgM+ B cells (middle row) or AChR-binding IgG2+ B cells (bottom row) from individual mice with EAMG after each immunization. Significant differences between populations were determined by ANOVA with Tukey's post hoc test and represented by a $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Each data point represents an individual mouse. Results shown are representative of multiple flow cytometry experiments.

Table 2
Frequencies of peripheral blood AChR-binding (AChR+) B cells and B cell receptor expression (IgM/IgG2).

		B220+	*p*-Value	ACHR+	*p*-Value	AChR+IgM+	*p*-Value	AChR+IgG2+	p-value
Day 15	Naïve ($n = 10$)	66.72 ± 12.97	0.9176	1.91 ± 0.69	0.1899	2.31 ± 0.56	0.3928	0.03 ± 0.04	0.0921
	LPS ($n = 5$)	79.23 ± 5.34	0.1418	2.64 ± 0.35	0.4911	1.75 ± 0.06	0.8749	0.15 ± 0.13	0.2495
	CFA-AChR ($n = 16$)	67.33 ± 14.67		2.36 ± 0.86		1.81 ± 0.15		0.09 ± 0.07	
Day 45	Naïve ($n = 10$)	64.05 ± 18.27	0.6599	1.72 ± 0.45	0.0913	1.86 ± 0.36	**0.0251**	0.02 ± 0.02	**0.0018**
	LPS ($n = 5$)	66.71 ± 7.49	0.3445	0.97 ± 0.38	0.0615	0.77 ± 0.21	**0.0093**	0.14 ± 0.26	**0.0014**
	CFA-AChR ($n = 16$)	61.24 ± 11.41		5.62 ± 1.81		4.35 ± 2.58		0.94 ± 0.63	
Day 75	Naïve ($n = 10$)	61.92 ± 19.71	0.3349	2.06 ± 0.78	**<0.0001**	2.32 ± 0.44	**0.0092**	0.21 ± 0.29	**<0.0001**
	LPS ($n = 5$)	49.22 ± 4.77	0.8103	2.84 ± 0.10	**0.0117**	2.67 ± 0.12	0.1014	0.17 ± 0.31	**<0.0001**
	CFA-AChR ($n = 16$)	52.29 ± 27.55		9.59 ± 4.49		7.31 ± 5.27		1.94 ± 0.91	

Mean (%) ± standard deviation for respective groups are shown. *p*-Values were determined by ANOVA with Tukey's post hoc test (CFA-AChR group versus other groups). $p < 0.05$ was considered statistically significant and is indicated in bold. Day 15; post first immunization, Day 45; post second immunization, Day 75; post third immunization.

nificance of these populations was not investigated further due to small population frequencies. Instead, we focused our attention on B220+ AChR-binding cells.

Our data suggest that B220+IgM+ AChR-binding B cells are part of the B cell repertoire in B6 mice, since these cells were detected in naïve, LPS stimulated and EAMG mice. However, frequencies of B220+IgM+ AChR-binding cells were significantly elevated in peripheral blood of mice with EAMG. Nevertheless, we found that the frequency of B220+IgG2+ AChR-binding cells and plasma levels of anti-AChR IgG2 are better indicators of disease than B220+IgM+ cells and plasma anti-AChR IgM levels. The correlations between frequencies of B220+IgG2+ AChR-binding cells, anti-AChR IgG2b antibodies and EAMG severity corroborate the previously postulated significance of the complement-fixing IgG2b isotype antibodies in EAMG pathogenesis [26]. *Torpedo* and mouse AChR have around 70% amino acid sequence similarity and therefore it is

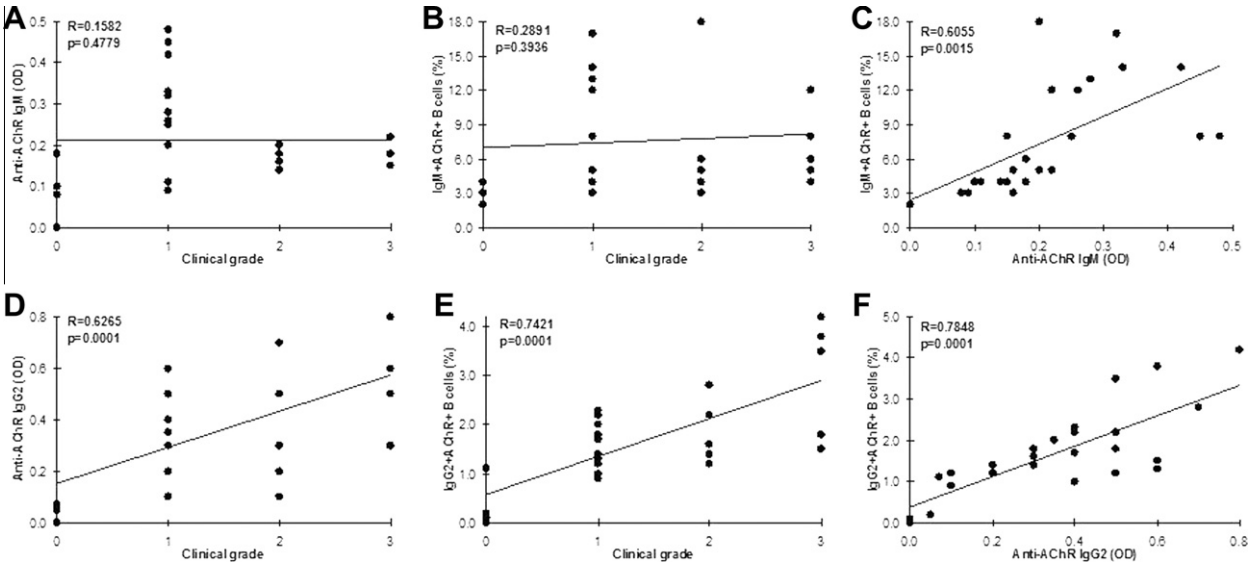

Fig. 4. Correlations between AChR-binding peripheral blood B cells, plasma anti-AChR IgGs and clinical EAMG grades (A–F) of 25 AChR-CFA immunized mice. Anti-AChR IgM or IgG2 OD values (A, C, D, and F) and the frequencies of AChR-binding IgM+ or IgG2+ B cells (B, C, E, and F) were obtained after the third immunization (day 75) at the same day that the clinical grades were evaluated (A, B, D, and E). *R* is the Spearman correlation coefficient between clinical and peripheral blood parameters and *p* is the *p* value for Spearman correlation test. Each data point represents an individual mouse. The experiment was repeated four times with similar results.

not surprising that anti-mouse AChR antibody levels correlated with *Torpedo* AChR binding B cell frequencies. Overall, these data are in agreement with previous reports of B cell immunoglobulin isotypes in MG patients indicating that at onset of disease there appears to be a conversion of IgM to IgG AChR specific cells [10].

Currently, diagnosis of MG involves a combination of clinical history, nerve stimulation tests, and blood test for serum Abs against AChR [1]. It is well established that anti-AChR titer alone is not a reliable predictor of disease severity in human MG [10–13]. This might be due to the fact that only a certain and random fraction of the whole anti-AChR antibody population in a given individual activates the complement cascade and/or reacts with epitopes that alter AChR functions and thus are pathogenic. Therefore, it is impossible to predict the alterations in the levels of pathogenic AChR antibodies by simply measuring total anti-AChR levels. In keeping with this assumption, in our experiment, levels of a complement activating isotype IgG2b were found out to be correlated with EAMG severity. These results suggest that measurement of AChR-antibodies of the complement fixing human IgG1 and IgG3 isotypes might predict disease severity in MG patients better than total IgG levels. By contrast, there was no correlation between IgM levels and disease severity. One likely interpretation of this discrepancy could be that the appearance of clinical symptoms is not linked to primary responses (IgM) but rather linked to memory responses (IgG2), which are obtained after at least two immunizations in parallel with the development of clinical muscle weakness. Other potential reasons for the IgG2 isotype's being a better indicator of EAMG severity could be that this isotype is more substantially involved in several immunological functions. The murine IgG2 is not only a strong complement activator but it also avidly binds to protein antigens such as AChR. Moreover, phagocytosing cells express Fc receptors that strongly interact with murine IgG2, which makes this isotype an important contributor of antibody-dependent cellular cytotoxicity (ADCC) [27,28].

Another intriguing question is why AChR-reactive IgG2+ B cells have a more robust correlation (higher *R* value) with EAMG severity than anti-AChR IgG2 levels. Data from several studies support the theory that B cells may contribute to the pathogenesis of EAMG by means other than that of antibody secretion. Studies in μMT mice indicated that B cells are essential for inducing EAMG and are also important for activating T cells [29,30]. The production of anti-AChR antibodies is capable of destroying the NMJ and activating the complement component system, which causes inflammation and immune complex deposition at the NMJ [1]. Activated B cells can produce cytokines, such as IL-4, IL-10, TNF-α, IFN-γ, and IL-6, which can activate dendritic cells (DCs) and other mononucleated cells, skew immune responses, and cause inflammation [31]. B cells are also capable of antigen presentation and, therefore, have the ability to activate autoreactive CD4+ T cells [32,33]. All of these B cell attributes might be making the AChR binding B cell frequency measurement a stronger predictor of EAMG severity than anti-AChR antibody levels.

Here, we have developed a simple assay for detecting peripheral blood AChR-specific B cells by flow cytometry. This assay uses no radioactivity and can screen blood samples in slightly more than one hour for increased frequency of AChR-specific B cells. This assay is more accessible to clinical labs and offers the ability to analyze autoreactive B cell function (by characterizing different cytokines, receptors ect. and function (cell sorting using FACS and then in vitro experiments). Since mouse AChR-specific immunoglobulins correlate with disease severity and corresponds with *Torpedo* AChR-specific B cell frequencies, this data confirms that *Torpedo* AChR can be used to characterize autoreactive B cells in EAMG, and perhaps in MG patients as well. The characterization of pathogenic AChR-specific B cells will also be a valuable tool for understanding autoantibody-mediated disease pathogenesis. The use of flow cytometry will enable us discern more information about the types of cells producing anti-AChR antibodies, such as Ig class, frequency, activation, cell signaling, and cytokines. It has recently been demonstrated that a fraction of seronegative MG patients display low-affinity AChR antibodies, which can currently be only detected by a time-consuming cell-based assay [34]. Whether our assay can be used as an alternative quick method to detect B cells producing low-affinity AChR antibodies in seronegative patients remains to be elucidated.

5. Conclusion

Alexa-conjugated AChR was used as a probe for AChR-specific B cells (B220+Ig+). Mice with EAMG have significantly elevated fre-

quencies of AChR-specific IgG2+ and IgM+ B cells. The frequencies of AChR-specific IgG2+ B cells and plasma IgG2 levels significantly correlated with the clinical grade of disease. These results indicate that significantly elevated frequencies of AChR-specific peripheral blood B cells and anti-AChR IgG1 (mouse IgG2 equivalent) levels could be potential biomarkers for MG disease severity.

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