Antibodies Produced by CLL Phenotype B Cells in Patients With Myasthenia Gravis Are Not Directed Against Neuromuscular Endplates

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Abstract

Background and Objectives

Myasthenia gravis (MG) can in rare cases be an autoimmune phenomenon associated with hematologic malignancies such as chronic lymphocytic leukemia (CLL). It is unclear whether in patients with MG and CLL, the leukemic B cells are the ones directly driving the autoimmune response against neuromuscular endplates.

Methods

We identified patients with acetylcholine receptor antibody–positive (AChR$^+$) MG and CLL or monoclonal B-cell lymphocytosis (MBL), a precursor to CLL, and described their clinical features, including treatment responses. We generated recombinant monoclonal antibodies (mAbs) corresponding to the B-cell receptors of the CLL phenotype B cells and screened them for autoantigen binding.

Results

A computational immune cell screen revealed a subgroup of 5/38 patients with MG and 0/21 healthy controls who displayed a CLL-like B-cell phenotype. In follow-up hematologic flow cytometry, 2 of these 5 patients were diagnosed with an MBL. An additional patient with AChR$^+$ MG as a complication of manifest CLL presented at our neuromuscular clinic and was successfully treated with the anti-CD20 therapy obinutuzumab plus chlorambucil. We investigated the specificities of expanding CLL-like B-cell clones to assess a direct causal link between the 2 diseases. However, we observed no reactivity of the clones against the AChR, antigens at the neuromuscular junction, or other common autoantigens.

Discussion

Our study suggests that AChR autoantibodies are produced by nonmalignant, polyclonal B cells The new anti-CD20 treatment obinutuzumab might be considered in effectively treating AChR$^+$ MG.

Classification of Evidence

This is a single case study and provides Class IV evidence that obinutuzumab is safe to use in patients with MG.

Glossary

AChR = acetylcholine receptor antibody; BCL = B-cell receptor; CLL = chronic lymphocytic leukemia; mAb = monoclonal antibody; LLN = lower limit of normal; MG = myasthenia gravis; PBMC = peripheral blood mononuclear cell; tSNE = t-distributed stochastic neighbor embedding.

Myasthenia gravis (MG) is an autoimmune disorder caused by antibodies that target structures within the neuromuscular junction, primarily the acetylcholine receptor (AChR). Impaired neuromuscular transmission leads to increased skeletal muscle fatiguability and weakness that can be life threatening. A number of studies have highlighted a rising incidence of MG over the past decade, which is mainly attributed to an increase in elderly patients.[1] Variable incidence rates of 3.1–24.9 cases/1,000,000/year have been reported for MG.[1] In a retrospective study approximately 15% of patients with MG had various extrathymic malignant tumors, with older age at MG onset being the only identified risk factor.[2] In particular, several MG cases accompanied by a lymphoproliferative disease have been documented.[3-11]

Chronic lymphocytic leukemia (CLL), resulting from a clonal expansion of B cells, is one of the most prevalent B-cell malignancies in adults and primarily affects the elderly population. The CLL phenotype of lymphocytes is described by the Matutes criteria as $CD19^+$ $CD5^+$ $CD23^+$ $CD79b^-$ $FMC7^-$ sIg^{dim} cells.[12,13] However, the cellular origin of the CLL clones remains largely enigmatic.[14] Leukemic B cells in patients with CLL have been shown to be often auto- and polyreactive and compromise reactivity against human Ig, DNA, and a range of cytoskeletal autoantigens.[15-17] Accordingly, autoimmune phenomena are well known to complicate CLL and occur in 10%–25% of patients with CLL.[18] These include reports of rare nonhematologic disorders such as single cases of autoimmune MG.[18] The association between CLL and nonhematologic autoimmune phenomena is supported by reports of frequent co-occurrence of CLL progression and autoimmune disease flares.[18] Furthermore, successful CLL treatment and concurrent resolution of MG symptoms with the anti-CD20 agent obinutuzumab and chlorambucil highlight similar cellular drivers during pathogenesis of MG in individual patients.[19]

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Ethics Committee Zurich, Switzerland. Informed written consent was obtained from all participants before inclusion in the study.

MG and CLL Patient Samples

Peripheral blood and serum samples were obtained from 38 patients with AChR MG and 21 age- and sex-matched healthy donors at the Neuromuscular Center, University Hospital Zurich, Switzerland. Patients with AChR MG showed typical clinical and serologic features (Ingelfinger et al., eTable 1, links.lww.com/NXI/A800) and were not treated with immunosuppressive medication including steroids at the time of the blood draw. The CARE Reporting Guidelines were used.[20] Peripheral blood mononuclear cells (PBMCs) were isolated using SepMate 50 tubes (STEMCELL Technologies, Vancouver, Canada) and human Lympholyte Separation Medium (Cedarlane, Burlington, Ontario) and stored in liquid nitrogen until use.

Mass Cytometry Antibody Labeling and Data Acquisition

Mass cytometry data were generated previously as described.[21] In brief, cryopreserved PBMCs were thawed at 37°C in cell culture medium (RPMI-1640, 10% fetal calf serum [Biochrom, Cambridge, United Kingdom], and 1× L-glutamine and 1× penicillin/streptomycin [both Life Technologies]) and divided into 2 aliquots for surface panel antibody labeling and intracellular cytokine panel labeling. The aliquot that underwent intracellular antibody labeling was rested overnight at 37°C and stimulated for 4 hours at 37°C with 50 ng mL^{-1} phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 500 ng mL^{-1} ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of 1× Brefeldin A and 1× Monensin (both BD Biosciences, Franklin Lakes, New Jersey). The sample aliquots for both panels were subsequently barcoded in 2 batches using a restricted 9-choose-3 barcoding scheme using differentially conjugated anti-CD45 monoclonal antibodies (mAbs). The barcoded samples were then labeled with heavy metal–tagged mAbs directed against surface epitopes in cell staining medium (RPMI-1640, 4% fetal calf serum) for 25 minutes at 37°C. Dead cell discrimination labeling was achieved by incubating the sample convolute in 2.5 μM cisplatin (Sigma-Aldrich) for 2 minutes on ice. The barcoded sample aliquot for surface panel antibody labeling was subject to transcription factor detection and fixed and permeabilized in 1x FOXP3 Fixation/Permeabilization Buffer (BioLegend, San Diego, CA) for 40 minutes at 4°C. Transcription factor antibody labeling was performed in permeabilization buffer (0.5% saponin, 2% bovine serum albumin [BSA], 0.01% sodium azide [all Sigma-Aldrich]) for 1 hour at 4°C. The sample aliquot for intracellular cytokine detection was fixed in 1.6% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 1 hour at 4°C and labeled with the antibody mix directed against intracellular epitopes in permeabilization buffer for 1 hour at 4°C. Both aliquots were incubated in 1x iridium intercalator solution (Fluidigm, South San Francisco, CA) at 4°C overnight. Data were acquired at a cytometry by time-of-flight (CyTOF 2.1) mass cytometer (Fluidigm) and preprocessed as described before.[21]

Figure 1 Unbiased Pattern Recognition Approach Discovers Atypical B-Cell Cluster in a Subset of Patients With MG
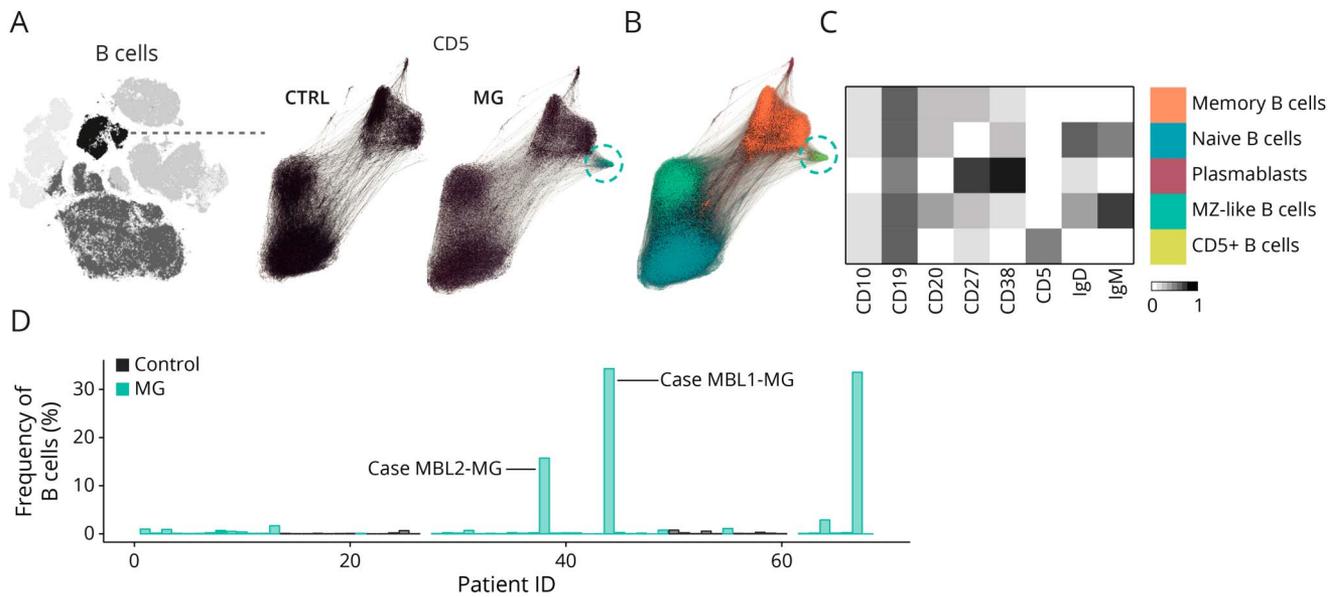
(A) Dimensionality reduction using tSNE was applied to mass cytometry data using all detected markers displaying a random subset of cells from the combined data set (controls and patients with MG, left panel). The highlighted area in black shows the B-cell compartment in the tSNE. A force-directed layout was constructed to display the phenotypic relationship between cells of the B-cell compartment and was displayed separately for healthy controls (CTRL) and patients with MG (right panels). The expression levels of CD5 within the B-cell compartment are displayed in green. The CD5+ B-cell population is highlighted using the green circle. (B and C) Force-directed layout showing the phenotypic relationship of cells within the B-cell compartment (B). Color coding indicates FlowSOM clustering and manual annotation of the B-cell compartment based on the normalized expression profiles as presented in the heatmap in (C). MZ = marginal zone. The green circle highlights the CD5+ B-cell population. (D) Bar graph for the frequency of CD5+ B cells in patients with MG and age-matched controls. The 5 patients with the highest frequency of CD5+ B cells were invited for hematologic follow-up screens. The patients who were diagnosed with an MBL are highlighted. MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis.

Hematologic Flow Cytometry Screen

For flow cytometry analysis, fresh peripheral blood was collected. Samples were prewashed, lysed with VersaLyse Lysing Solution (Beckman Coulter, Krefeld, Germany), washed again, and resuspended in 500 μL phosphate-buffered saline with 0.02% sodium azide and 0.2% BSA. To detect surface protein expression (of CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD34, CD45, CD56, lambda, and kappa light chain), antibody (AB) labeling was performed using the CE-IVD–labeled ClearLLab LS tube according to the manufacturer's protocol. Additional AB (CD22, CD23, CD79a, and FMC7) were combined in a distinct tube and purchased separately from Beckman Coulter. Acquisition was performed on a Navios EX flow cytometer (Beckman Coulter) and analyzed with CXP (Navios) Software. The full list of antibodies is presented in eTable 1 (links.lww.com/NXI/A800).

Isolation and Sequencing of B Cells

Cryopreserved PBMCs were thawed and stained with CD5-PE (Beckman Coulter, A07753), CD19-PeCy7 (BD Biosciences, 341113), and IgG-Alexa Fluor 647 (Jackson ImmunoResearch, 109-606-170). CD5+, CD19+, and IgG+ B cells were sorted into 96-well PCR plates (Eppendorf, 0030129512) filling the wells with 1 cell per well. cDNA was synthesized, and antibody-encoding sequences were amplified by nested PCR and heavy- and light chain variable regions were sequenced using primers specific for the V and J regions of the given antibody.

Sequence Analysis of Antibody cDNA

Variable region of immunoglobulin heavy chain/variable region of immunoglobulin light chain (VH/VL) gene usage and amount of somatic mutations were determined by analyzing the homology of VH/VL sequences of mAbs to known human V, D, and J genes in the IMGT database.[22] Percentage identities of all obtained sequences were investigated using Clustal Omega (Clustal2.1).

Recombinant Production and Purification of mAbs

Heavy chain variability, diversity, and joining and light chain variability and joining regions were cloned into human IgG1, Igκ, and Igλ expression vectors. To amplify the resulting plasmids, TOP10 chemically competent *Escherichia coli* (Thermo Fisher Scientific, Waltham, MA) were transformed and expanded in antibiotics containing LB broth, and amplified plasmids were isolated using the NucleoBond Xtra Midi Plus kit (Macherey-Nagel, Düren, Germany). Amplified plasmids were sequenced to assure identity to original sequence. Expi293F cells (Thermo Fisher Scientific) were transiently transfected with plasmids of corresponding light and heavy chains using polyethylenimine (PEI). Expi293F cells were tested routinely for mycoplasma contamination. mAbs were quantified by ELISA. In short, IgG was quantified using 96-well MaxiSorp plates (Nunc) coated with goat anti-human IgG (SouthernBiotech, 2040-01) using Certified Reference Material 470 (Sigma-Aldrich, ERMs-DA470) as standard. Produced mAbs
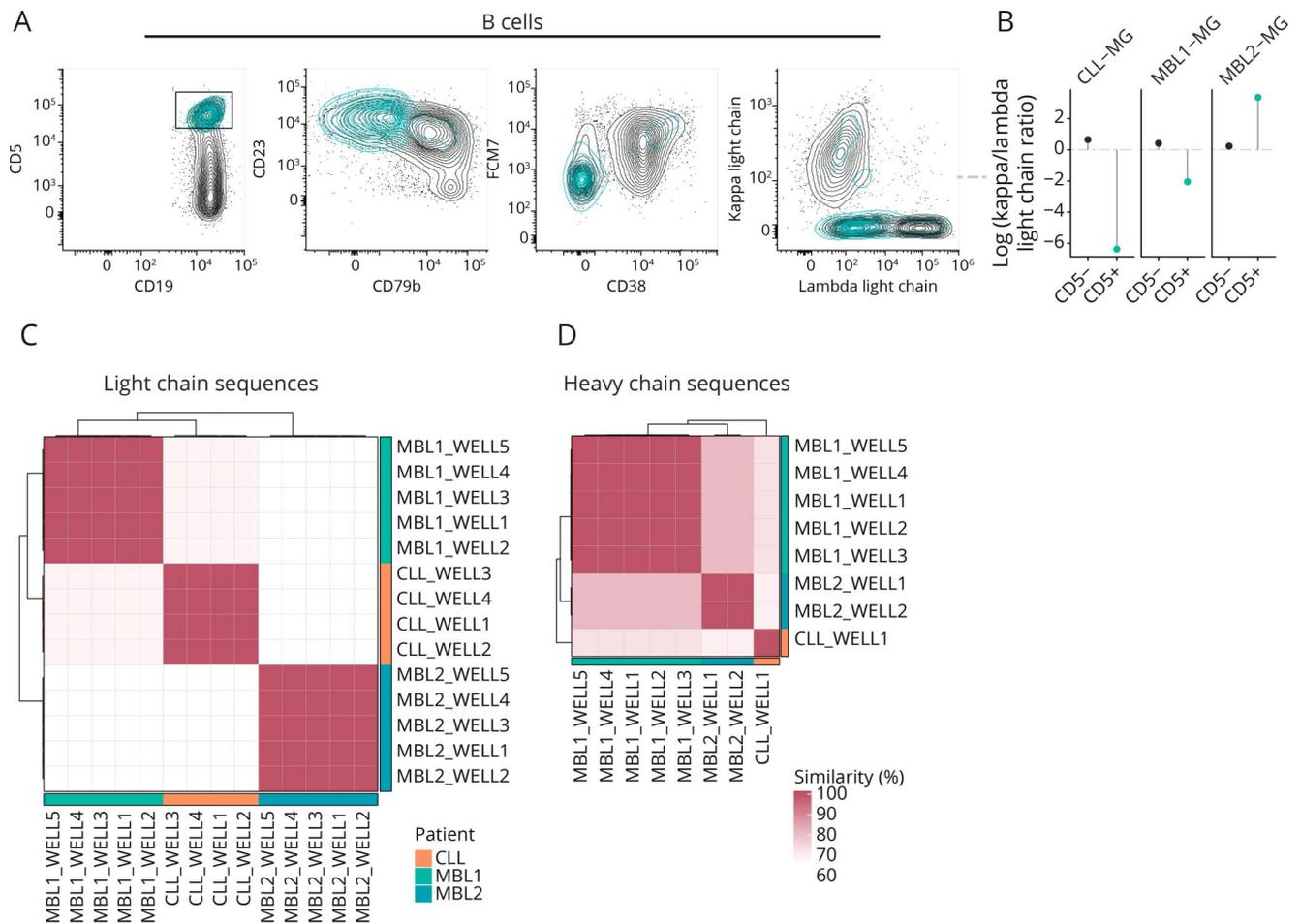
(A) Representative contour plots of the B-cell compartment of case MBL1-MG and case MBL2-MG during hematologic follow-up flow cytometry screens. CD5+ B cells, as gated in the first panel, are highlighted in green to compare the immunophenotype to CD5− B cells of the same patient. The patients met all 5 phenotypic Matutes criteria that are required for a CLL diagnosis. Shown are representative contour plots for 1 patient. (B) Lollipop plot showing the kappa-to-lambda light chain ratio determined by flow cytometry for CD5− B cells (black) and the CD5+ B-cell populations (green) of case CLL-MG, case MBL1-MG, and case MBL2. Data are shown in a log scale, with positive values indicating kappa light chain enrichment and negative values indicating lambda chain enrichment. The dashed line indicates equal kappa-to-lambda ratio as present in polyclonal B cells. (C and D) Heatmap displaying light chain (C) and heavy chain (D) sequence similarities obtained from single cells of case CLL-MG, case MBL1-MG, and case MBL2-MG. Variable sequences were aligned and identity matrices computed using Clustal Omega. The heavy chain sequence of case CLL-MG has been obtained from multiple cells. CLL = chronic lymphocytic leukemia; MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis.

were affinity purified by protein A chromatography (GE Healthcare, Chicago, IL), and concentration of purified recombinant mAbs was quantified by measuring absorption at 280 nm.

Detection of Anti-AChR Reactivity Using a Live Cell-Based Assay

Reactivity against human AChR was assessed in a live cell-based assay as described before.[23] In brief, HEK293T cells were transiently transfected on glass coverslips with human adult AChR subunits and cotransfected with rapsyn to promote clustering. One day after transfection, patient sera or mAbs were added to live cells for 1 hour at room temperature. After washing and fixing in 4% formaldehyde, an Alexa Fluor 568 goat anti-human secondary antibody was added for 1 hour. The coverslips were washed and inverted onto mounting medium on a slide. Coverslips were scored visually by microscopy using a semiquantitative scale.

Testing of Reactivity of mAbs to Antinuclear Antigens (ANAs)

Reactivity of recombinantly produced mAbs to ANAs was performed by indirect immunofluorescence using ORG 870 (ORGENTEC Diagnostika GmbH) and FA 1522-2010 (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) and by immunoblot using DL 1590-1601-3 G (EUROIMMUN Medizinische Labordiagnostika AG). Assays were performed following the manufacturer's instructions.

Immunofluorescence Stainings of Mouse Muscle Sections

Immunofluorescence antibody labeling was performed as described previously.[24] Briefly, sections were incubated overnight at 4°C with the different case 1, 2, or 3 mAbs (at 15 µg/mL each) or patient serum (diluted 1:200) combined with Alexa Fluor 647–conjugated a-bungarotoxin (1:300,

Table Characterization of the Patients With MG With High Frequencies of Cells With a CLL-like Phenotype

	Case CLL-MG	Case MBL1-MG	Case MBL2-MG
Demographics			
Age (when MG was diagnosed)	77 y	42 y	73 y
Sex	Male	Male	Female
Timing between MG and hematologic diagnosis	MG diagnosed 2 mo after CLL	Incidental finding 24 y after MG diagnosis	Incidental finding 1 y after MG diagnosis
MG MGFA classification	I (ocular)	I (ocular)	I (ocular)
MG autoantibody status	Anti-AChR[+]; low anti-titin[+]	anti-AChR[+]	anti-AChR[+]; low anti-titin[+]
Thymus pathology	Chest CT: no thymoma	Chest CT: no thymoma	Chest CT: no thymoma
Hematologic diagnosis	CLL	Monoclonal B-cell lymphocytosis	Monoclonal B-cell lymphocytosis
Autoimmunity, other	Anti-TPO[+], generalized vitiligo, eosinophilic angiocentric fibrosis, and IgG4 associated	Unknown	Unknown
Treatment			
of MG	AChE-Inh.	AChE-Inh.; AZA or MMF (not tolerated, both stopped after max. 2 mo)	AChE-Inh. and Pred.
of hematologic abnormality	Obinutuzumab/chlorambucil (4 cycles)	Follow-up	Follow-up
Response of MG to treatment, outcome	Ocular symptoms improved; CLL remission	Stable	Stable
BCR characteristics			
Isotype	IgG1 (λ)	IgG2 (λ)	IgG3 (κ)
Heavy chain VDJ genes (% identity to GL)	*VH3-74* (88.2)	*VH3-7* (94.1)	*VH3-7* (88.2)
	D3-10	*D2-15*	*D6-25*
	JH6 (64.5)	*JH4* (87.5)	*JH3* (90.0)
Light chain VJ genes (% identity to GL)	*VL3-21* (98.2)	*VL2-23* (96.9)	*VK2-30* (94.6)
	JL1 (89.5)	*JL1* (94.7)	*JK2* (100)
HCDR3 sequence	AGEIVRGHVTSGMDV	ARGHCSGGRCYISAVDY	ARDNWKNNGAFDI

Abbreviations: AChR = acetylcholine receptor antibody; BCR = B-cell receptor; CLL = chronic lymphocytic leukemia; GL = germline; MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis; TPO = thyroid peroxidase; VDJ = variability, diversity, and joining; VJ = variability and joining.
Demographic and clinical characteristics of patients with a CLL-like phenotype identified during mass cytometry screening of patients with MG and a patient with CLL with MG presented at our neuromuscular clinic.

Thermo Fisher Scientific, #B35450) and mouse anti-neurofilament 200 (1:300, Sigma, #N0142). As positive control, the ABCD_AF122 mAb targeting the α-subunit of the AChR was used.[25] Negative control serum was from a donor without MG. Secondary antibodies were Alexa Fluor 488–labeled goat anti-human and Alexa Fluor 555–labeled goat anti-mouse antibodies (1:250 Life, Technologies, #A11013, #A28180), combined with SlowFade Gold antifade reagent (Invitrogen, #S36936, Waltham, MA). Fluorescence images were acquired using a Leica Stellaris 5 upright confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) and analyzed by Imaris imaging software (Bitplane, Zurich, Switzerland).

Autoantibody Screen

The microarray-based autoantibody screen was performed as previously described.[26]

High-Dimensional Analysis

High-dimensional analysis was performed in the statistical programming environment R. Dimensionality reduction using t-distributed stochastic neighbor embedding (tSNE) was accomplished using the Rtsne package (with the parameters perplexity = 50 and theta = 0.1).[27] Clustering was achieved using FlowSOM[28]: 100 clusters were generated using the combined data set, and resulting clusters were metaclustered and manually merged and annotated. Force-directed layouts

Figure 3 CLL-like B-Cell Clones Are Not Directed Against AChR
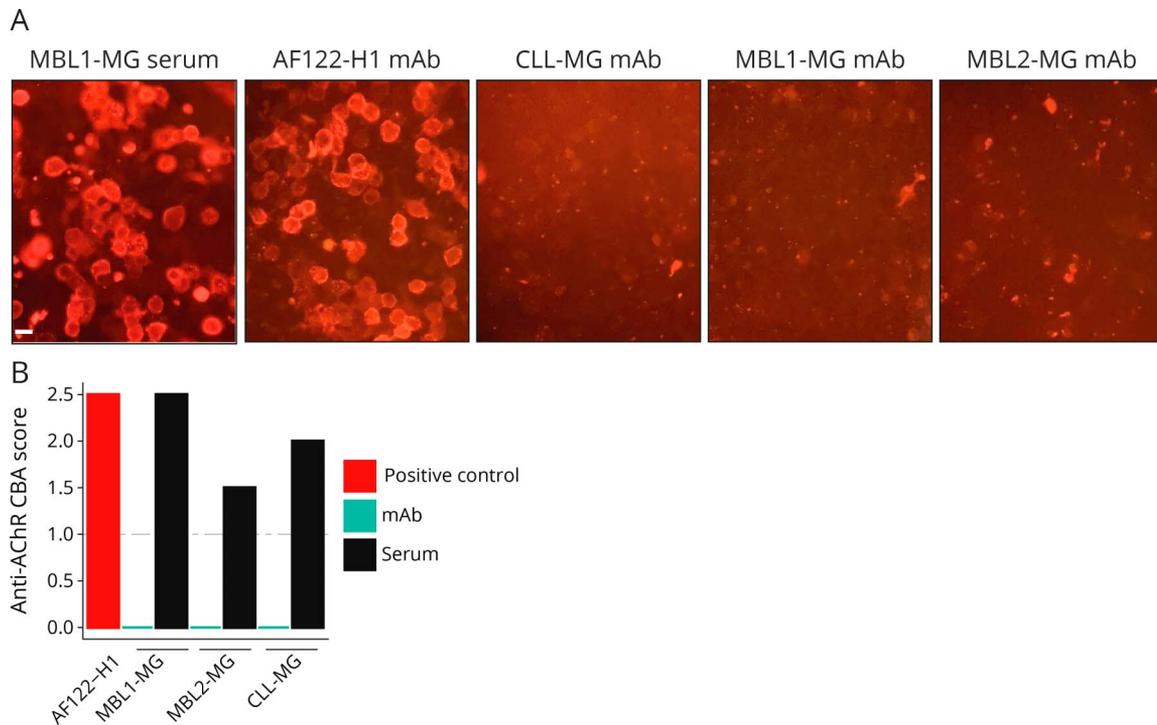
(A) Representative microscopy images of HEK293T cells transiently transfected with human AChR subunits, cotransfected with rapsyn (to facilitate clustering) and labeled with serum or mAbs derived from case CLL-MG, case MBL1-MG, case MBL2-MG, and the mAb AF122 targeting the α-subunit of the AChR (positive control). Bound antibodies were detected using Alexa Fluor 568–conjugated goat anti-human Abs. Scale bar 15 μm. (B) Bar graph showing the binding of mAbs and patient sera from case CLL-MG, case MBL1-MG, and case MBL2-MG determined by rater-blinded semiquantitative scoring of the cell-based assay as shown in (A). 0 = negative; 1–1.5 = low positive; 2–4 = increasing level of positivity based on fluorescence intensity. AChR = acetylcholine receptor antibody; CLL = chronic lymphocytic leukemia; mAb = monoclonal antibody; MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis.

were generated using the ForceAtlas2 algorithm[29] integrated in the VorteX graphical clustering environment creating unweighted edges between the nodes based on the 10 nearest neighbors.[30] Resulting networks were further modified using the open graph visualization platform Gephi 0.9.2. Heatmaps were created using the pheatmap package. All other visualizations were drawn in the ggplot2 package.

Data Availability

Mass cytometry data are publicly available at doi.org/10.17632/nkcb8nc7w8.1 and were previously analyzed.[21]

Results

Mining CyTOF data of PBMC samples from 38 steroid-free patients with seropositive AChR MG,[21] our unbiased pattern recognition approach discovered an atypical B-cell population in some patients (Figure 1A). Dimensionality reduction using tSNE and force-directed layouts revealed the combined expression of memory-like B-cell markers and CD5 (Figure 1A). Application of FlowSOM clustering and concomitant quantification of the CD5$^+$ B-cell cluster revealed high frequencies in 3 patients with MG and detectable frequencies in 2 additional patients with MG (5 of 38 patients with MG and no case in 21 age-matched controls; Figure 1, B–D).

Five patients showing the highest frequency of CLL-like phenotype cells were invited for a confirmatory clinical flow cytometry peripheral blood analysis. In all 5 patients, the immune phenotype could be reproduced and confirmed, although ~1.5 years passed between blood collections for the mass cytometry study and the hematologic examination. In 2 of these 5 patients, a CD5$^+$ B-cell population was detected that met all 5 Matutes criteria as in the diagnosis of CLL (Figure 2A). The lambda and kappa light chain restriction indicated that the CD5$^+$ B cells of each patient were indeed a monoclonal B-cell population with a single B-cell receptor (BCR) reactivity (Figure 2A and B). However, as lymphocytosis was not pronounced (<5 G/L) and there were no other features of a B-cell lymphoproliferative disorder (such as lymphadenopathy, organomegaly, cytopenia, or extramedullary involvement), the patients met the diagnosis of a monoclonal B-cell lymphocytosis (MBL), a premalignant B-cell disorder known to be at risk of progression to CLL (Table, patients MBL-MG1 and MBL-MG2). The absolute clonal B cell counts were 70/μL (MBL-MG1) or 140/μL (MBL-MG2), respectively. Therefore, they were between low-count MBL (<50/μL) and high-count MBL (>2000/μL). For 1 of these 5 patients, a small clonal B-cell population remained questionable, and in 2 cases, no signs of monoclonality in the CD5$^+$ B-cell population could be identified by conventional flow cytometry analysis (not shown). Shortly afterward, an

Figure 4 CLL-like B-Cell Clones Are Not Directed Against Antigens of the Neuromuscular Junction
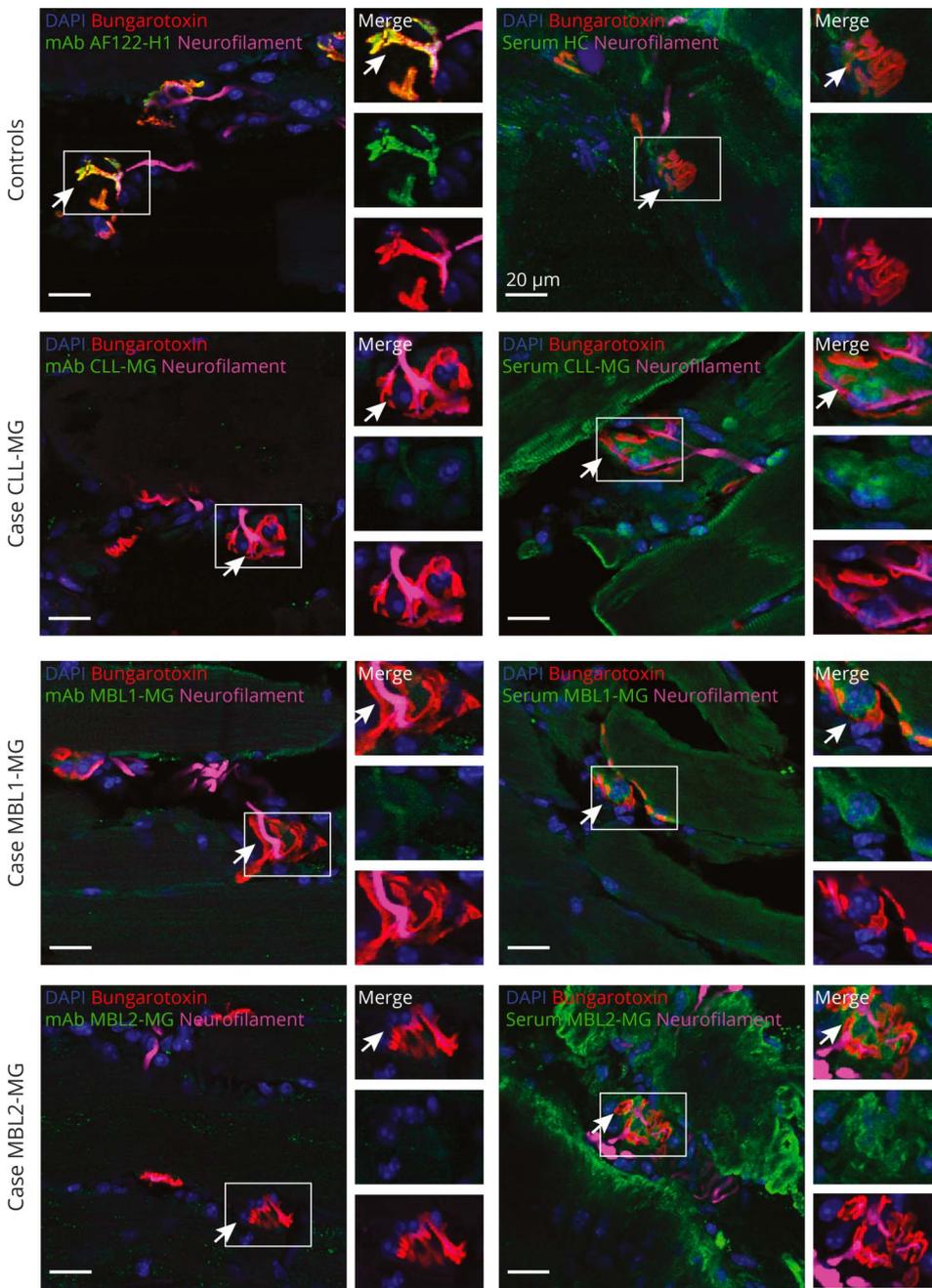

Representative confocal microscopy images of stainings with mAbs (left column) and serum (right column) from case CLL-MG, case MBL1-MG, and case MBL2-MG on murine muscle sections. Bound Abs were detected by Alexa Fluor 488–conjugated goat anti-human Abs. Sections were colabeled with bungarotoxin to identify AChR, anti-neurofilament Abs, and DAPI for cell nuclei. As control, the AF122 mAb targeting the α-subunit of the AChR was used (positive control, left column). Negative control serum (right column) was from a donor without MG. Arrows highlight neuromuscular junctions. Scale bar 20 μm. A zoomed-in view is shown. AChR = acetylcholine receptor antibody; CLL = chronic lymphocytic leukemia; mAb = monoclonal antibody; MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis.

additional patient with MG (Table 1, CLL-MG) presented at our neuromuscular center that had been diagnosed with CLL 2 months after a diagnosis of seropositive AChR MG had been verified. As observed for case MBL-MG1 and MBL-MG2, the CD5+ B-cell population showed a high degree of kappa-lambda light chain restriction, highlighting that the CD5+ B cells of all 3 patients were indeed monoclonal (Figure 2B).

The patient with CLL-MG, a 77-year-old man (at the time of MG diagnosis), had fluctuating ptosis on the left and diplopia. His anti-AChR antibody titers were 10.2 nmol/L (reference lower limit of normal [LLN] <0.5 nmol/L, by radioimmunoassay), anti-titin antibody index of 2 (relative to calibrator sample: reference LLN <1), and anti-MuSK negative at the time of MG diagnosis. Pathologic 3 Hz decrement of the accessory nerve was measured. No thymoma was detected by chest CT. CLL had been diagnosed 2 months before MG with paranasal sinus manifestation. He was treated with 4 cycles of obinutuzumab (2000 mg in cycle 1 and 1000 mg in cycles 2–4) and chlorambucil (0.4–0.5 mg/kg at days 1 and 15 of cycles 1–4 mg/kg). After 4 cycles, the CLL was in remission, and ocular MG symptoms improved with only low-dose pyridostigmine

Figure 5 CLL-like B-Cell Clones Are Not Directed Against Common Autoantigens
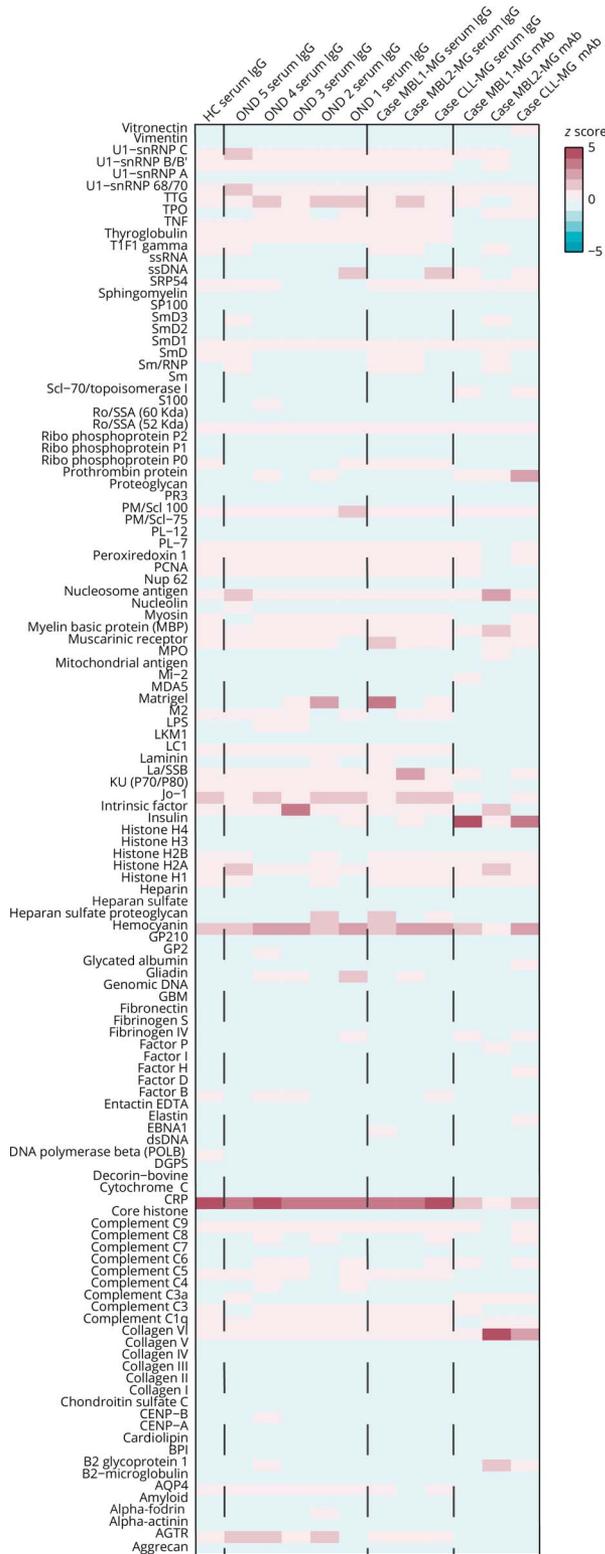
Row-normalized heatmap displaying the binding of the recombinant mAbs derived from the BCR of the CD5$^+$ B cell from case CLL-MG, case MBL1-MG, and case MBL2-MG and the total serum IgGs of the same patients, 5 other neurologic disease controls (OND), and a healthy control to 128 autoantigens. BCR = B-cell receptor; CLL = chronic lymphocytic leukemia; mAb = monoclonal antibody; MBL = monoclonal B-cell lymphocytosis; MG = myasthenia gravis.

required and no signs of clinically significant MG generalization (last follow-up: currently 2 years after the last infusion). The anti-AChR concentration 1 month after the obinutuzumab and chlorambucil treatment cycles was 6.5 nmol/L.

Population-based studies suggest that autoimmune diseases do not predispose patients to CLL.[31] Follow-ups were advised to our 2 patients with MBL by a hematologist to monitor the B-cell clone and size and assessments after 6 months showed no clinical progression of disease. We next investigated how CLL-like phenotype cells or CLL might favor the development of MG as an autoimmune complication. The simultaneous diagnosis of CLL and MG or manifestation within a short period of time in at least some reported patients (Table 1 and eTable 2, links.lww.com/NXI/A800) and concurrent response to CLL-directed therapy (as with our patient with CLL-MG) indicate that they may be related.

To test the hypothesis that the monoclonal expansion of malignant B-cell clones was causally linked to autoimmune MG, we next assessed the specificity of the BCRs expressed by the monoclonal B-cell populations. For this, CD5$^+$ B cells of the patient with MG-CLL and the 2 patients with MG-MBL were sorted into 1 single cell per well and corresponding BCR sequences were retrieved (Table 1 and eTable 3, links.lww.com/NXI/A800). For each patient analyzed, the sequences obtained from single cells were identical, thereby further confirming that the CD5$^+$ B-cell population was indeed monoclonal (Figure 2C and D). Based on the retrieved sequences corresponding to the BCRs of the CD5$^+$ B cells, recombinant mAbs were produced. All 3 antibodies harbored varying degrees of somatic mutations in their variable regions, but no common patterns were discernible. Furthermore, no evidence for stereotypy could be found within the 3 patients analyzed. Opposed to total serum IgGs of these 3 patients, no reactivity was found against recombinant human AChR, the autoantigen at neuromuscular endplates in AChR$^+$ MG, using an ELISA (data not shown) and a clustered AChR cell-based assay (Figure 3A and B). Similarly, no reactivity was detected against nuclear antigens (data not shown). To exclude direct effects of the BCRs of monoclonal B cells on neuromuscular components, we next evaluated binding of the recombinant mAbs to murine neuromuscular endplates of tibialis anterior muscle sections by immunofluorescence (Figure 4). Total serum IgGs of our 3 patients labeled AChRs (determined by counterstaining using α-bungarotoxin) in the neuromuscular endplate, whereas mAbs derived from CLL-like clones did not result in specific stainings (Figure 4). Using a microarray, we next assessed reactivity against 128 autoantigens and found weak reactivity of the recombinant mAbs against collagen VI and insulin (Figure 5).

Discussion

Here, we described the co-occurrence of CLL phenotype B cells in 3 patients with autoimmune MG. Individual rare

cases of the simultaneous presentation of the 2 disease entities have been described before (summarized in eTable 2, links.lww.com/NXI/A800).[4,7,9-11,18] However, large-scale nationwide patient registry studies are required to validate whether the simultaneous manifestation of the 2 diseases occurs significantly more often compared with control groups alone. Instead, in this case series, we demonstrate the absence of a direct causal link between the specificities of the monoclonal CLL phenotype B cells and autoantigens involved in MG, similar to what has been described for CLL-associated autoimmune hemolytic anemia.[32] However, the absence of a shared specificity in this case series does not exclude the possibility that CLL-like B cells in other patients may react against MG autoantigens. The clonality of the B-cell populations in our case series has been demonstrated by both kappa/lambda light chain restriction and single-cell sequencing of BCR variable regions that yielded identical sequences within the CD5$^+$ B-cell population of each patient. The sequences of the mAbs derived from the CD5$^+$ B-cell populations are presented in Table 1 and eTable 3 (links.lww.com/NXI/A800), providing the tools for others to further investigate the specificity of the CLL clones computationally and experimentally even beyond the context of MG.

AChR$^+$ MG is considered a paraneoplastic disorder in approximately 15% of patients who have a thymoma. Furthermore, in patients with AChR$^+$ MG without thymoma, data point to an important role of local B cells in the initiation and sustained production of AChR autoantibodies in the (hyperplastic) thymus.[33] AChR autoantibody–producing B cells have also been identified in the circulation, lymph node, and bone marrow.[34] The patient with MG-CLL and the 2 patients with MG-MBL, described here, were diagnosed with a seropositive AChR$^+$ MG that was not related to thymoma. This is in accordance with the literature, as in most reported cases of CLL in association with MG, antibodies against AChR or other muscle antigens were detected and histologic analysis of thymectomy or autopsy specimens mostly consisted of involuted thymic tissue. This could point to a different pathogenetic mechanism for the development of AChR$^+$ MG in CLL and underlying peripheral rather than central immune dysregulation.[35,36] It may also be that certain genetic and environmental risk factors, for example, genetic risk variants in the *PTPN22* and *TNFRSF11A* loci, predispose these patients to both diseases, (late-onset) AChR$^+$ MG and CLL.[36-38] In addition, the patient with MG-CLL had antibodies against thyroid peroxidase and a history of vitiligo, but no other autoimmune phenomena were noted, in particular, no anemia or thrombocytopenia (Table 1 and eTable 2, links.lww.com/NXI/A800). Although clonal CLL B cells often produce autoantibodies, the immunoglobulins, that they express, generally do not contribute directly to autoimmune disorders such as autoimmune hemolytic anemia or immune thrombocytopenia.[39,40] In contrast, it has been suggested that autoantibodies against erythrocytes or platelets are produced by polyclonal bystander B cells as a consequence of an immune dysregulation associated with CLL. Dysfunctional regulatory T cells,[41] altered control of self-reactive IgG by autologous IgM,[42] or aberrant antigen-presentation[43] are alternative mechanisms that have been suggested in this context. It remains possible that similar processes are involved in the pathogenesis of MG in our patients with MG-CLL and -MBL. Future research, e.g., on antigen presentation to T cells by CLL phenotype B cells isolated from patients with AChR MG, is necessary. Better knowledge of the source of autoantibodies against AChR in individual patients with MG and the immune dysregulation behind it become more important with the increasing availability of more selective therapeutic agents.[44]

Obinutuzumab, a third-generation humanized anti-CD20 therapy approved for CLL, has been suggested to achieve more effective depletion (compared with rituximab) not only of peripheral B cells but also in lymphoid tissue.[45] Here, obinutuzumab and chlorambucil were used as first-line treatment in our patient with CLL-MG, and therefore, we cannot derive a comparative statement regarding anti-CD20 therapies (as it has been done for other CLL-associated/autoimmune diseases, e.g., autoimmune hemolytic anemia, immune thrombocytopenia,[46] rheumatoid arthritis,[47] or anti-MAG neuropathy[48,49]). Nevertheless, our patient with CLL-MG was successfully treated early on, and obinutuzumab and chlorambucil had lasting effects and were safe. Therefore, this therapeutic regimen might be considered in AChR$^+$ MG in selected clinical settings, such as concomitant lymphoid malignancies (or as monotherapy in patients who develop side effects such as rituximab-induced serum sickness[50]).

Disclosure
L.P. is an employee of Vir Biotechnology Inc. and may hold shares in Vir Biotechnology Inc. The other authors report no relevant disclosures. Go to Neurology.org/NN for full disclosures.

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Luca Piccoli, PhD	Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland	Major role in the acquisition of data; analysis or interpretation of data
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Mark Woodhall, PhD	Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom	Major role in the acquisition of data
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Appendix *(continued)*

Name	Location	Contribution
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References

1. Maddison P, Ambrose PA, Sadalage G, Vincent A. A prospective study of the incidence of myasthenia gravis in the east midlands of england. *Neuroepidemiology.* 2019;53(1-2):93-99. doi: 10.1159/000500268
2. Levin N, Abramsky O, Lossos A, et al. Extrathymic malignancies in patients with myasthenia gravis. *J Neurol Sci.* 2005;237(1-2):39-43. doi: 10.1016/j.jns.2005.05.009
3. Zhang Y, Varnadoe C, Tandon A, Forsyth P, Komrokji R, Sokol L. Myasthenia gravis and large granular lymphocytic leukemia: a rare association. *Leuk Res Rep.* 2020;14:100226. doi: 10.1016/j.lrr.2020.100226
4. Cea G, Gallardo VA, Cabrera CME. Myasthenia gravis and its association with lymphoproliferative disorders: a case series. *Rev Med Chil.* 2017;145(12):1626-1630. doi: 10.4067/s0034-98872017001201626.
5. Rezania K, Soliven B, Baron J, Lin H, Penumalli V, Van Besien K. Myasthenia gravis, an autoimmune manifestation of lymphoma and lymphoproliferative disorders: case reports and review of literature. *Leuk Lymphoma.* 2012;53(3):371-380. doi: 10.3109/10428194.2011.615426
6. Davis S, Schumacher MJ. Myasthenia gravis and lymphoma: a clinical and immunological association. *JAMA.* 1979;242(19):2096. doi: 10.1001/jama.1979.03300190036022
7. Cohen SM, Waxman S. Myasthenia gravis, chronic lymphocytic leukemia, and autoimmune hemolytic anemia: 'A spectrum of thymic abnormalities?' *Arch Intern Med.* 1967;120(6):717-720. doi: 10.1001/archinte.1967.00300050073012
8. Bowen JD, Kidd P. Myasthenia gravis associated with T helper cell lymphoma. *Neurology.* 1987;37(8):1405. doi: 10.1212/wnl.37.8.1405
9. Bennett M, Berrebi A, Katz A. Myasthenia gravis and chronic lymphocytic leukaemia. *Eur J Haematol.* 2009;42(1):104. doi: 10.1111/j.1600-0609.1989.tb00257.x
10. Rey J, Attarian S, Disdier P, Feuillet L, Azulay JP, Pouget J. Myasthénie et leucémie lymphoïde chronique: une association fortuite? *La Revue de Médecine Interne.* 2001;22(11):1129-1130. doi: 10.1016/S0248-8663(01)00480-5
11. Vogel JM, Kornfeld P, Forte FA, et al. Myasthenia gravis. Association with chronic lymphocytic leukemia. *N Y State J Med.* 1977;77(14):2252-2256.
12. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia.* 1994;8(10):1640-1645.
13. Moreau EJ, Matutes E, A'Hern RP, et al. Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). *Am J Clin Pathol.* 1997;108(4):378-382. doi: 10.1093/ajcp/108.4.378
14. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood.* 2011;117(6):1781-1791. doi: 10.1182/blood-2010-07-155663
15. Borche L, Lim A, Binet JL, Dighiero G. Evidence that chronic lymphocytic leukemia B lymphocytes are frequently committed to production of natural autoantibodies. *Blood.* 1990;76(3):562-569. doi: 10.1182/blood.v76.3.562.bloodjournal763562
16. Sthoeger ZM, Wakai M, Tse DB, et al. Production of autoantibodies by CD5-expressing B lymphocytes from patients with chronic lymphocytic leukemia. *J Exp Med.* 1989;169(1):255-268. doi: 10.1084/jem.169.1.255
17. Chu CC, Catera R, Hatzi K, et al. Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood.* 2008;112(13):5122-5129. doi: 10.1182/blood-2008-06-162024
18. Jung M, Rice L. Unusual autoimmune nonhematologic complications in chronic lymphocytic leukemia. *Clin Lymphoma Myeloma Leuk.* 2011;11(Suppl 1):S10-S13. doi: 10.1016/j.clml.2011.02.005
19. Russell A, Yaraskavitch M, Fok D, Chhibber S, Street L, Korngut L. Obinutuzumab plus chlorambucil in a patient with severe myasthenia gravis and chronic lymphocytic leukemia. *J Neuromuscul Dis.* 2017;4(3):251-257. doi: 10.3233/JND-170211
20. Gagnier JJ, Kienle G, Altman DG, et al. The CARE guidelines: consensus-based clinical case reporting guideline development. *Case Rep.* 2013;2013(1):bcr2013201554. doi: 10.1136/bcr-2013-201554

21. Ingelfinger F, Krishnarajah S, Kramer M, et al. Single-cell profiling of myasthenia gravis identifies a pathogenic T cell signature. *Acta Neuropathol.* 2021;141(6): 901-915. doi: 10.1007/s00401-021-02299-y

22. Lefranc MP, Giudicelli V, Ginestoux C, et al. IMGT(R), the international ImMunoGeneTics information system(R)®. *Nucleic Acids Res.* 2009;37(Database): D1006-D1012. doi: 10.1093/nar/gkn838

23. Leite MI, Jacob S, Viegas S, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain.* 2008;131(7):1940-1952. doi: 10.1093/brain/awn092

24. Ingelfinger F, Cansever D, Schreiner B. The AF122 antibody recognizes the AChR subunit in murine muscle endplates by immunofluorescence. *Antibody Rep.* 2020; 3(1):e112. doi: 10.24450/journals/abrep.2020.e112

25. Graus YF, De Baets MH, Parren PWHI, et al. Human anti-nicotinic acetylcholine receptor recombinant fab fragments isolated from thymus-derived phage display libraries from myasthenia gravis patients reflect predominant specificities in serum and block the action of pathogenic serum antibodies. *J Immunol.* 1997;158(4):1919-1929. doi: 10.4049/jimmunol.158.4.1919

26. Zhen QL, Xie C, Wu T, et al. Identification of autoantibody clusters that best predict lupus disease activity using glomerular proteome arrays. *J Clin Invest.* 2005;115(12): 3428-3439. doi: 10.1172/JCI23587

27. Van Der Maaten L, Hinton G. Visualizing non-metric similarities in multiple maps. *Machine Learn.* 2012;87(1):33-55. doi: 10.1007/s10994-011-5273-4.

28. Van Gassen S, Callebaut B, Van Helden MJ, et al. FlowSOM: using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A.* 2015;87(7): 636-645. doi: 10.1002/cyto.a.22625

29. Jacomy M, Venturini T, Heymann S, Bastian M. ForceAtlas2, a continuous graph layout algorithm for handy network visualization designed for the Gephi software. *PLoS One.* 2014;9(6):e98679. doi: 10.1371/journal.pone.0098679

30. Samusik N, Good Z, Spitzer MH, Davis KL, Nolan GP. Automated mapping of phenotype space with single-cell data. *Nat Methods.* 2016;13(6):493-496. doi: 10.1038/nmeth.3863

31. Landgren O, Engels EA, Caporaso NE, et al. Patterns of autoimmunity and subsequent chronic lymphocytic leukemia in Nordic countries. *Blood.* 2006;108(1): 292-296. doi: 10.1182/blood-2005-11-4620

32. Centola M, Lin K, Sutton C, et al. Production of anti-erythrocyte antibodies by leukemic and nonleukemic B cells in chronic lymphocytic leukemia patients. *Leuk Lymphoma.* 1996;20(5-6):465-469. doi: 10.3109/10428199609052430

33. Marx A, Pfister F, Schalke B, Saruhan-Direskeneli G, Melms A, Ströbel P. The different roles of the thymus in the pathogenesis of the various myasthenia gravis subtypes. *Autoimmun Rev.* 2013;12(9):875-884. doi: 10.1016/j.autrev.2013.03.007

34. Fichtner ML, Jiang R, Bourke A, Nowak RJ, O'Connor KC. Autoimmune pathology in myasthenia gravis disease subtypes is governed by divergent mechanisms of immunopathology. *Front Immunol.* 2020;11:776. doi: 10.3389/fimmu.2020.00776

35. Garcillán B, Figgett WA, Infantino S, Lim EX, Mackay F. Molecular control of B-cell homeostasis in health and malignancy. *Immunol Cell Biol.* 2018. doi: 10.1111/imcb.12030

36. Chia R, Saez-Atienzar S, Murphy N, et al. Identification of genetic risk loci and prioritization of genes and pathways for myasthenia gravis: a genome-wide association study. *Proc Natl Acad Sci USA.* 2022;119(5):e2108672119. doi: 10.1073/pnas.2108672119

37. Alankus B, Ecker V, Vahl N, et al. Pathological RANK signaling in B cells drives autoimmunity and chronic lymphocytic leukemia. *J Exp Med.* 2021;218(2): e20200517. doi: 10.1084/JEM.20200517

38. Negro R, Gobessi S, Longo PG, et al. Overexpression of the autoimmunity-associated phosphatase PTPN22 promotes survival of antigen-stimulated CLL cells by selectively activating AKT. *Blood.* 2012;119(26):6278-6287. doi: 10.1182/blood-2012-01-403162

39. Kipps TJ, Carson DA. Autoantibodies in chronic lymphocytic leukemia and related systemic autoimmune diseases. *Blood.* 1993;81(10):2475-2487. doi: 10.1182/blood.v81.10.2475.2475

40. Zent CS, Kay NE. Autoimmune complications in chronic lymphocytic leukaemia (CLL). *Best Pract Res Clin Haematol.* 2010;23(1):47-59. doi: 10.1016/j.beha.2010.01.004

41. Giannopoulos K, Schmitt M, Kowal M, et al. Characterization of regulatory T cells in patients with B-cell chronic lymphocytic leukemia. *Oncol Rep.* 1994;20(3):677-682. doi: 10.3892/or_00000059

42. Stahl D, Lacroix-Desmazes S, Heudes D, Mouthon L, Kaveri SV, Kazatchkine MD. Altered control of self-reactive IgG by autologous IgM in patients with warm autoimmune hemolytic anemia. *Blood.* 2000;95(1):328-335. doi: 10.1182/blood.v95.1.328.001k10_328_335

43. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. *Blood.* 2005;105(5): 2007-2015. doi: 10.1182/blood-2003-10-3563

44. Vitale C, Montalbano MC, Salvetti C, et al. Autoimmune complications in chronic lymphocytic leukemia in the era of targeted drugs. *Cancers.* 2020. doi: 10.3390/cancers12020282

45. Mössner E, Brünker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell - mediated B-cell cytotoxicity. *Blood.* 2010;115(22): 4393-4402. doi: 10.1182/blood-2009-06-225979

46. Herishanu Y, Levi S, Kamdjou T, et al. Obinutuzumab in the treatment of autoimmune haemolytic anaemia and immune thrombocytopenia in patients with chronic lymphocytic leukaemia/small lymphocytic lymphoma. *Br J Haematol.* 2021;192(1): e1-e4. doi: 10.1111/bjh.17105

47. Reddy V, Klein C, Isenberg DA, et al. Obinutuzumab induces superior B-cell cytotoxicity to rituximab in rheumatoid arthritis and systemic lupus erythematosus patient samples. *Rheumatology (United Kingdom).* 2017;56(7):1227-1237. doi: 10.1093/rheumatology/kex067

48. Rakocevic G, Martinez-Outschoorn U, Dalakas MC. Obinutuzumab, a potent anti-B-cell agent, for rituximab-unresponsive IgM anti-MAG neuropathy. *Neurol Neuroimmunol Neuroinflamm.* 2018;5(4):e460. doi: 10.1212/NXI.0000000000000460

49. Briani C, Visentin A, Salvalaggio A, Cacciavillani M, Trentin L. Obinutuzumab, a new anti-CD20 antibody, and chlorambucil are active and effective in anti-myelin-associated glycoprotein antibody polyneuropathy. *Eur J Neurol.* 2019;26(2):371-375. doi: 10.1111/ene.13838

50. Blase JR, Frame D, Michniacki TF, Walkovich K. Case report: use of obinutuzumab as an alternative monoclonal anti-CD20 antibody in a patient with refractory immune thrombocytopenia complicated by rituximab-induced serum sickness and anti-rituximab antibodies. *Front Immunol.* 2022;13:863177. doi: 10.3389/fimmu.2022.863177