Identification of auto-antigens in Myasthenia Gravis by using B cell immortalization

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List of abbreviations

BCIP – 5-bromo-4-chloro-3-indolyl phosphate
BSA – Bovine serum albumin
CDR – Complementarity determining region
CPM – Counts per minute
CS – Culture supernatants
Da – Dalton
DAB – Di-aminobenzidine
EBV – Epstein-barr virus
ELISA – Enzyme-linked immunosorbent assay
EOMG – Early-onset myasthenia gravis
FACS – Fluorescence-activated cell sorting
FSC – Forward scatter
HRP – Horseradish peroxidase
Ig – Immunoglobulin
IHC – Immunohistochemical
IL-2 – Interleukin-2
IVIg – Intravenous immunoglobulin
MG – Myasthenia gravis
MuSK – Muscle-specific kinase
MWPA – Mega western protein array
AChR – Acetylcholine receptor
NBT – Nitro blue tetrazolium
NC – Negative control
NFDM – Non-fat dry milk
NMJ – Neuromuscular junction
PBMC – Peripheral blood mononuclear cell
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
RIA – Radioimmunoassay
SNMG – Seronegative myasthenia gravis
SSC – Side scatter
TMC – Thymic myoid cells
V_H – Immunoglobulin heavy chain variable region

Abstract

Myasthenia Gravis (MG) is an autoimmune disorder in which auto-antibodies in about 80% of MG patients bind to the nicotinic acetylcholine receptor (AChR), present on the postsynaptic membrane of the neuromuscular junction (NMJ). In more than 40% of patients without anti-AChR antibodies, the muscle-specific kinase (MuSK) is targeted by auto-antibodies. In approximately 5% of MG patients, however, no antibodies against the AChR or MuSK are detectable. The auto-antibodies in all MG patients cause an impaired neuromuscular transmission resulting in muscle weakness, which is the main symptom of MG. MG and other autoimmune diseases result from the loss of tolerance to self-antigens, and tolerance to self-antigens is primarily established in the thymus. Remarkably, thymic abnormalities such as hyperplasia, thymoma and germinal centre formation are often present in MG patients. The germinal centres are mainly AChR-specific and thymocytes from MG thymi have been shown to produce anti-AChR antibodies *in vitro*. Therefore, intrathymic auto-antibody production might explain why most MG patients benefit from thymectomy. However, a single B cell clone that produces auto-antibodies against AChR has not been identified yet.

This project was designed to investigate whether B cells of the thymus produced auto-antibodies. We hypothesized that Epstein-Barr Virus (EBV)-immortalized B cell clones from MG thymi produce monoclonal auto-antibodies against the AChR. To test this hypothesis, monoclonal B cell lines of thymi from MG patients were immortalized. An improved method was used which is described to provide a more effective immortalization of human B cells with EBV. The auto-antibodies produced by those immortalized B cells were then investigated to characterize the antigens.

Our experiments showed that the thymus does not contain an enriched population of mature B cells with AChR specificity, but analysis of Ig V gene usage in the thymus of MG patients has clearly demonstrated that there is a highly compartmentalized clonal expansion of B cells driven by a limited number of antigens in this organ.

The immortalization of B cells by using EBV is a useful technique to study the antibody response in MG and it will contribute to the knowledge of autoimmune diseases in general.

1. Introduction

Myasthenia Gravis (MG) is an autoimmune disorder with a prevalence of 20 persons per 100 000 [1]. In about 80% of MG patients, auto-antibodies bind to the nicotinic acetylcholine receptor (AChR). The AChR is located at the postsynaptic membrane of the NMJ and functions as a receptor for the neurotransmitter acetylcholine [2]. In normal conditions, stimulation of the AChR by acetylcholine causes muscular contraction, due to the opening of ligand-gated cation channels in the muscle cell membrane. This opening generates an action potential that spreads along the length of the muscle fiber, triggering the release of calcium from intracellular stores in the muscle cell leading to muscular contraction. However, in most MG patients the anti-AChR antibodies induce loss of the AChRs (figure 1), leading to an impaired neuromuscular transmission with muscle weakness as a result [3]. The breakdown of the receptor is known to be mediated by complement-mediated lysis of the postsynaptic membrane [4], or by cross-linking the AChR in the membrane. This last process leads to an increased degradation rate of the receptor and is also called antigenic modulation [5].

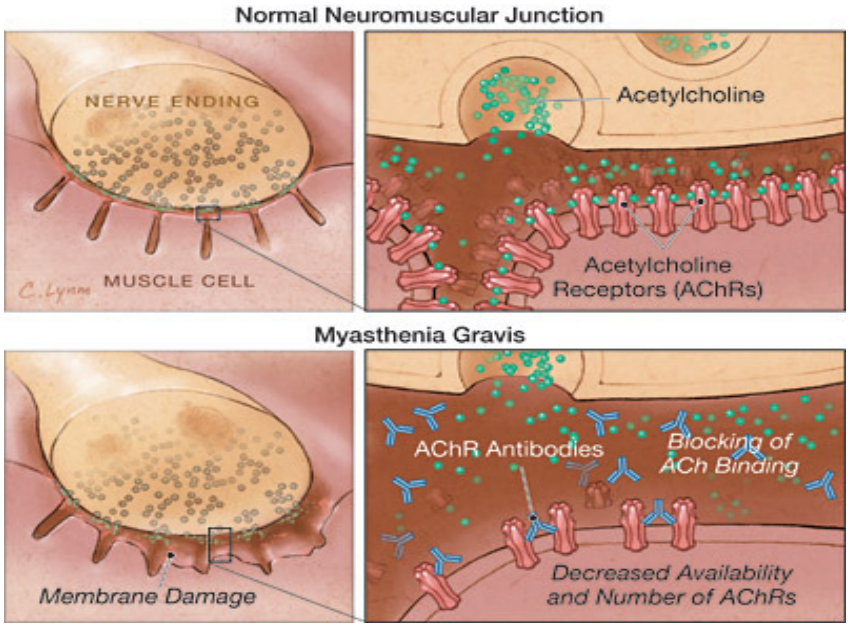

Figure 1. In a normal NMJ, a high density of AChRs is concentrated on the postsynaptic membrane. In 80% of patients with MG, circulating auto-antibodies binding to the AChR are present. These auto-antibodies inhibit the binding of acetylcholine to the receptor, leading to weakness and fatigue of muscles. The auto-antibodies mainly induce postsynaptic membrane damage by complement-mediated lysis or by antigenic modulation, leading to decreased availability of AChRs and less postsynaptic foldings [6].

Auto-antibodies against the AChR are not the only pathogenic auto-antibodies found in MG patients. Auto-antibodies to muscle-specific tyrosine kinase (MuSK) have been demonstrated in more than 40% of MG patients without auto-antibodies against the AChR [7]. MuSK is known to initiate aggregation of the AChR during synapse formation via the agrin/MuSK/rapsyn/AChR clustering pathway (figure 2), but MuSK is also expressed at the mature NMJ. MuSK auto-antibodies have the potential to alter the MuSK function at the adult NMJ, and they may not only inhibit MuSK function directly, but also increase the turnover of MuSK, hereby further reducing its activity [8]. Curiously, in patients with anti-MuSK antibodies there is no evidence of loss of junctional folds, no apparent loss of AChR density and no immune complex deposition which is in contrast with the previously discussed AChR antibody-positive patients [9]. In addition, the anti-MuSK antibodies are predominantly of the IgG4 subclass. IgG4 antibodies do not activate complement as efficient as the IgG1 antibodies, which is the predominant subclass in MG patients with AChR auto-antibodies. These findings suggest that MuSK-antibody associated MG may be different in etiological and pathological mechanisms [10].

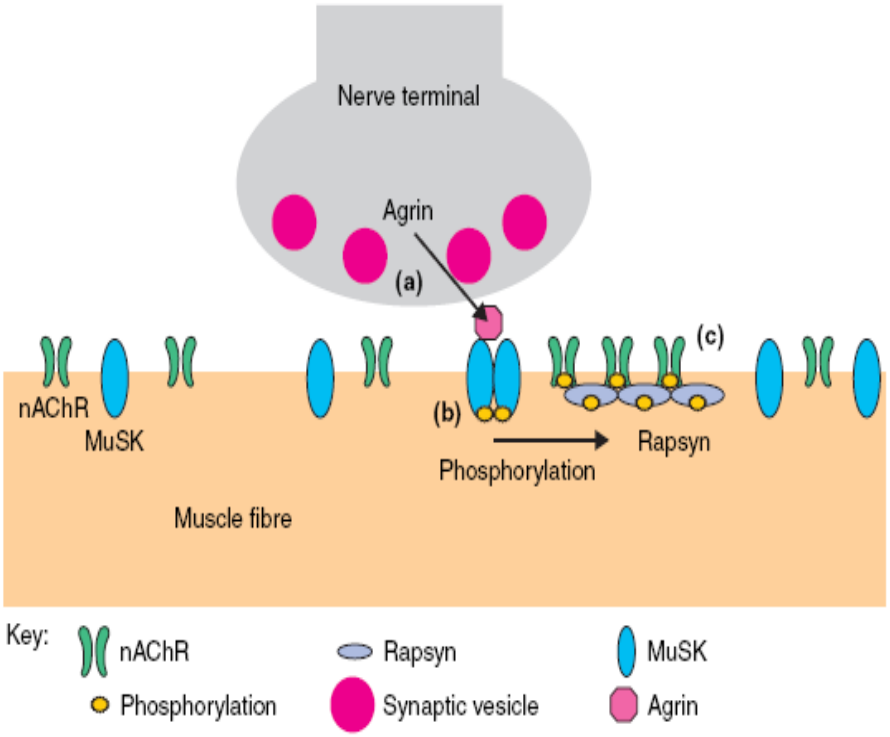

Figure 2. (a) During development of the NMJ, agrin is released from the nerve terminal. (b) Agrin activates MuSK which dimerises and auto-phosphorylates. (c) Rapsyn is subsequently phosphorylated; it self-aggregates and clusters AChRs and other postsynaptic proteins [11].

In about 5% of MG patients no auto-antibodies are found against the AChR or MuSK [12]. These seronegative patients also have an antibody-mediated disease because they clinically improve after plasma exchange and immunosuppressive treatment. This indicates that there might be other targets for auto-antibodies in this group of patients, for which the role in the pathogenesis of MG has not yet been clarified [13]. Since seronegative patients have the same clinical presentation as patients with AChR-specific auto-antibodies [14], it is possible that they produce AChR-specific auto-antibodies at a very low level or with very low affinity, so these auto-antibodies may be undetectable for the conventional AChR antibody assays [15]. Nevertheless, it has been shown that the auto-antibodies in these patients are from the IgG1 subclass, and some were even able to induce complement deposition on the AChR clusters which must have been sufficiently dense to bind C1q and activate the classical complement pathway. This strongly implies that the auto-antibodies in seronegative patients can be directed towards the AChR, but only bind when the AChRs are densely packed in relatively immobile clusters [16]. However, the possibility remains that unknown antigens are the cause of seronegative MG.

In general, autoimmune diseases result from the loss of tolerance to self-antigens, and tolerance to self-antigens is primarily established in the thymus. Remarkably, thymic abnormalities are present in 80 to 90% of MG patients. The majority of patients (65-75%) have thymic hyperplasia with numerous germinal centres in the medulla and approximately 10% of patients harbour thymomas [17]. Germinal centres are known to provide the environment for intense B cell proliferation, differentiation, somatic hypermutation and immunoglobulin class switching during antibody responses. Therefore, these intrathymic germinal centres may be sites of an intense B cell response against the AChR [18]. The germinal centres also have significant co-occurrence with thymic myoid cells (TMC), which are sparse, muscle-like cells, because they express several muscle proteins such as actin, myosin and AChR. TMCs are the only cells known to express whole AChR outside muscles. These findings suggest that those cells have muscle characteristics within the thymus [19, 20]. Furthermore, thymocytes from MG thymi have been shown to produce anti-AChR antibodies *in vitro*. Because an immune response can be initiated by an auto-antibody attack against the AChR, the pathogenesis of MG is believed to originate in the thymus [21].

A hypothesis how the germinal centres and the auto-antibodies against the AChR are formed has been formulated for early-onset MG (EOMG) and seronegative MG (SNMG). The patients of these two subgroups of MG have hyperplastic thymi (table 1). First, helper T cells are primed by AChR subunits that are expressed in hyperplastic medullarry thymic epithelial cells. Next, early antibodies against these subunits attack the nearby myoid cells which express intact AChR. The ensuing immune complex formation, activation of antigen-presenting cells and consequent inflammation and complement-mediated damage together provoke formation of germinal centres, leading to auto-antibody diversification [22, 23]. Therefore, intrathymic auto-antibody production could explain why EOMG and SNMG patients benefit from thymectomy [24, 25].

Table 1. Subgroups of MG patients and their typical thymic pathology [26].

Subtype of MG	Typical thymic pathology
Early-onset	Hyperplastic
Thymoma-associated	Epithelial tumour containing many lymphocytes
Late-onset	Normal or atrophied
MuSK-associated	Normal or atrophied
Seronegative	Hyperplastic

Conversely, there are less indications that thymectomy may be beneficial for MG patients with auto-antibodies against MuSK [27]. Furthermore, thymectomy is in most cases not helpful for thymoma-MG patients because the etiology of thymoma-associated MG is different from that of MG which is associated with thymic hyperplasia and germinal centres [28]. The thymoma-MG patients have additional auto-antibodies, specific for muscle antigens such as ryanodine receptors [29], titin [30], myosin, actin and alpha-actinin [31]. The presence of those auto-antibodies is associated with more severe disease, although the question remains whether they play a role in the pathogenesis of MG [32, 33].

The purpose of this project is the characterization of monoclonal antibodies that are produced by thymic B cells. To accomplish that purpose, B cells from MG thymi are immortalized by using the Epstein-Barr Virus (EBV). This technology was first used in 1977 and consisted of B cell infection with EBV, produced by the marmoset lymphocyte cell line B95-8, to obtain continually dividing monoclonal B cell lines [34, 35]. It was unsuccessful due to a low efficiency of the immortalization, a slow growth rate of the B

cells and sometimes low antibody production. An improved B cell immortalization method based on a combination of EBV and a polyclonal B cell activator, the CpG oligonucleotide ODN2006, was published in 2004 [36]. We used this technique together with a similar approach which was described in 2008 [37]. The main difference between the two methods is the time of administration of the B cell activators (figure 3).

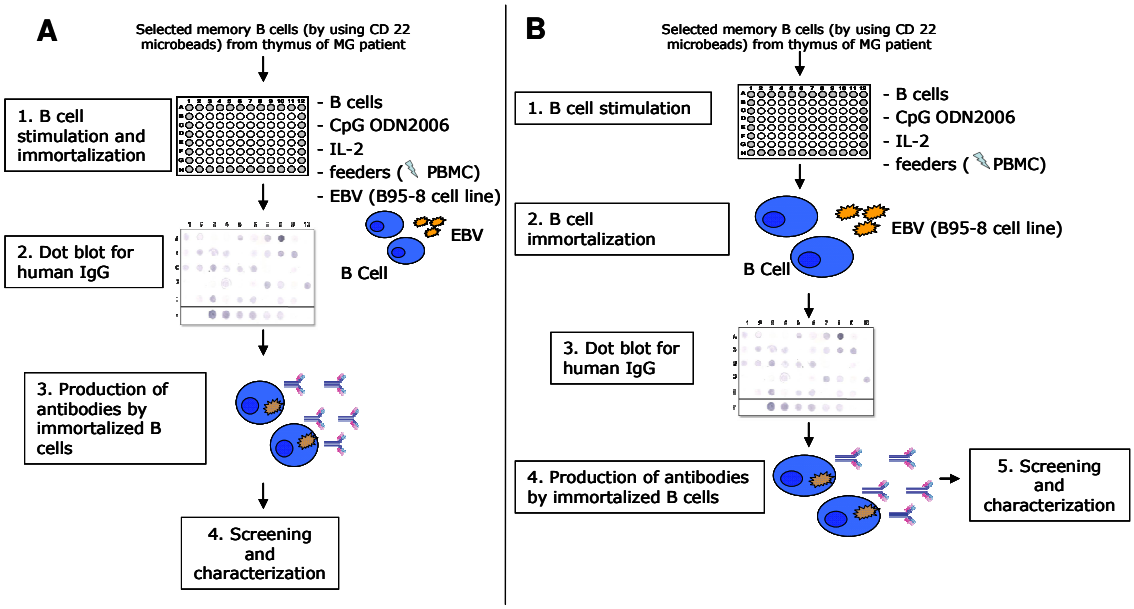

Figure 3. (A) According to the technique of Traggiai et al, the B cell activators CpG ODN2006 and interleukin-2 (IL-2) are added to the B cells together with EBV [36]. (B) In the approach of Funaro et al, the B cells are first stimulated with CpG ODN2006 and IL-2 before they are immortalized with EBV [37].

Thymocytes from MG thymi have been shown to produce anti-AChR antibodies in vitro [21], although a single B cell clone that produces auto-antibodies against AChR has not yet been identified. We hypothesized that EBV-immortalized B cell clones from MG thymi will produce auto-antibodies against the AChR. With this project, we will characterize the monoclonal auto-antibodies that are produced by immortalized thymic B cells and the targets of the auto-antibodies from the immortalized B cells will be identified, which may be the AChR, MuSK or other proteins. Hence, through this project we can learn more about the pathogenesis of MG by identifying the antigenic targets of this autoimmune disorder. Characterizing antigenic targets by the immortalization of B cells can also be applied to other autoimmune disorders. Furthermore, the role of the thymus in the production of the auto-antibodies will be elucidated.

2. Materials and methods

2.1 Patients

After informed consent was obtained, thymi were obtained from three MG patients at the academic hospital in Maastricht. Approval of the medical ethical committee was obtained for these experiments. Thymocytes from control thymi were obtained via Rozen Le Panse (Paris, France).

2.2 B cell immortalization with Epstein-Barr Virus

Fresh thymus tissue of MG patients was cut into pieces and dissociated by using the GentleMACS™ dissociator (Miltenyi Biotec, Auburn, USA) in order to gain a single cell suspension. An anti-CD22 antibody which is conjugated to a magnetic microbead (Miltenyi Biotec) was added, and mature B cells were selected from the single cell suspension by magnetic separation (supplement section A). CD22-positive B cells were grown in microplates together with irradiated peripheral blood mononuclear cells (PBMCs) from healthy donors. The PBMCs were isolated by gradient centrifugation using Histopaque-1077 (Sigma Aldrich, St. Louis, USA) and irradiated with röntgen radiation by using PTW-Unidos (PTW, Freiburg, Germany) to prevent them from multiplying (supplement section B). These PBMCs function as 'feeder cells' which produce important growth factors for the B cells. Next, the B cells were stimulated with CpG 2006 (Invivogen, San Diego, USA). This is a DNA sequence that binds to toll-like receptor 9 and subsequently triggers cell proliferation. In addition, IL-2 (Roche, Basel, Switzerland) was added which also activates B cells. Finally, the B cells were infected with EBV by adding supernatants of a B95-8 culture. B95-8 is a lymphoma cell line that produces infectious EBV particles. The virus selectively infects CD21-positive B cells and remains in the nuclei of infected cells as an episome. During this latent stage of infection, only a few viral proteins which mimic cellular survival signals are expressed. All together, memory B cells were seeded at 5 cells per well in 96 U-bottom microplates in culture medium containing 1 µg/ml CpG 2006 and 50 units/ml IL-2, in the presence of EBV (30% supernatant of B95-8 cells) and irradiated PBMCs (100 000 cells per well). The culture supernatants (CS) of immortalized B cell populations were screened for antibody production afterwards.

2.3 Dot blot

Immortalization efficiency was assessed by checking IgG antibody production in a dot blot (supplement section C). In this method, CS was spotted on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, USA). After drying the membrane, it was blocked for 30 minutes with 5% non-fat dry milk (NFDM) in phosphate buffered saline (PBS). Subsequently, the membrane was incubated for one hour with goat anti-human IgG alkaline phosphatase (1/500 in blocking buffer; Promega, Madison, USA), rabbit anti-human IgG horseradish peroxidase (HRP) (1/100 in blocking buffer; Dako, Glostrup, Denmark) or mouse anti-human IgM HRP (1/50 in blocking buffer; Invitrogen, Carlsbad, USA) and washed afterwards for five times two minutes with PBS with 0,05% Triton X-100. The blots incubated with goat anti-human alkaline phosphatase, were incubated with a staining solution of nitro blue tetrazolium (NBT) (Promega) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) until dots appeared on the membrane. The blots incubated with rabbit anti-human IgG HRP or mouse anti-human IgM HRP, were incubated with di-aminobenzidine (DAB) solution which contained DAB (1 mg/ml), Tris-HCl (50 mmol/l) and 0,03% H_2O_2. IgG-positive B cells were then selected and further cultured.

2.4 Radioimmunoassay

The ability of antibodies produced by the immortalized B cells to bind to the AChR was tested in a radioimmunoassay (RIA) (supplement section D). In this assay, CS was incubated with AChR, purified from TE 671 human rhabdomyosarcoma cells, with iodine 125-labeled α-bungarotoxin (1/250; PerkinElmer, Waltham, USA) and with normal human serum as a co-precipitant. The radioactively-labeled α-bungarotoxin effectively binds to the AChR and is used as a radioactive label for the AChR. After overnight incubation at 4°C, the immune complexes were precipitated by incubation with polyclonal goat anti-human IgG during four hours at 4°C. All samples were centrifuged at 15 000g for ten minutes and the pellets were washed twice with 0,5% Triton X-100 in PBS. Anti-AChR antibodies bind the radioactively-labeled receptor in the antibody complex and are detected with the Wizard^2 automatic gamma counter (PerkinElmer) due to the radioactive label.

2.5 Antigenic modulation

Antigenic modulation is the cross-linking of the AChR in the postsynaptic membrane by binding of auto-antibodies. This binding leads to an increased degradation rate of the receptor. In the antigenic modulation assay (supplement section E), confluently grown TE 671 cells were incubated with CS for three hours at 37°C. The CS might contain antibodies which cause the internalization and degradation of the AChR. After a washing step with prewarmed PBS, the cells were incubated for one hour at 37°C with iodine 125-labeled α-bungarotoxin (1/5000 in culture medium; PerkinElmer) to label the AChR still present on the cell surface. After three washing steps with prewarmed PBS, cells were lysed with 0,5 M sodium hydroxide and the amount of labeled receptor was measured using the Wizard^2 automatic gamma counter (PerkinElmer). High counts indicate weak antigenic modulation activity and low counts indicate strong antigenic modulation activity of the antibodies in the CS. The counts of the samples are compared to the counts of a positive control (IgG1 637).

2.6 Immunohistochemistry

The presence of auto-antibodies against striated muscle proteins in CS was investigated in an anti-striated muscle antibody test system (ScimedX, Denville, USA) (supplement section F). Sections of monkey striated muscle were incubated with CS for three hours at room temperature (RT) and washed with PBS. Sections were then incubated for 30 minutes at RT with a secondary, fluorescently-labeled, anti-human IgG antibody. After washing the sections with PBS, coverslips were mounted with mounting medium.

An immunohistochemical (IHC) staining was also performed to search for auto-antibodies in the CS against NMJ proteins (supplement section G). Cryosections of 30 µm of monkey striated muscle were cut using the Leica CM3050 cryostate (Leica Microsystems GmbH, Wetzlar, Germany). The sections were first dried, fixed in acetone at 4°C for 10 minutes and dried again for 30 minutes. Subsequently, the sections were incubated with CS for three hours at RT. After washing the sections with PBS, they were incubated with alexa 594-conjugated α-bungarotoxin (1/300 in PBS; Molecular Probes, Leiden, The Netherlands) for 30 minutes at RT and sheep-anti-human IgG FITC (1/100 in PBS; The Binding Site, Birmingham, United Kingdom) for 60 minutes at RT. These incubations were always followed with a washing step with PBS to remove aspecific binding of antibodies. Finally, coverslips were mounted with mounting medium.

2.7 Fluorescence-activated cell sorting

Analysis of thymocytes by fluorescence-activated cell sorting (FACS) (supplement section H) was used to look for differences in the amount of B cells between control and MG thymi.

For each experiment 100 000 cells were required per sample. These cells were centrifuged at 1645g for five minutes and the supernatant was removed. Then, cells were incubated for 30 minutes at 4°C with the different anti-human antibodies: CD19-PerCP Cy5.5, IgM-PE, IgG-FITC, CD22-PE-Cy5, CD21-PE and CD19-FITC (all from Becton Dickinson and Company, Franklin Lakes, USA). The cells were then washed three times with 0,5% bovine serum albumin (BSA) and 0,1% sodium azide in PBS together with a centrifugation step at 1645g for five minutes. Finally, the samples were analyzed with the FACSCalibur Flowcytometer (Becton Dickinson and Company) and CellQuest Pro software.

2.8 Enzyme-linked immuno sorbent assay

Enzyme-linked immuno sorbent assays (ELISA) were performed to investigate whether CS contained auto-antibodies against striated muscle proteins (supplement section I). First, 96-wells plates were coated with antigen (myosin, actin or alpha-actinin) at a concentration of 1 µg/ml in 50 mM sodium carbonate and placed at 37°C for 2 hours. After five washing steps with 0,05% Tween-20 in PBS, the plates were blocked for 30 minutes at 37°C with 0,5 % BSA and 0,02% Tween-20 in PBS. Without washing, plates were incubated with CS (undiluted CS and ½ diluted CS) and controls for one hour at 37°C. The samples were then washed as previously described, followed by an incubation for one hour at 37°C with the secondary antibodies: goat anti-human IgG Fc peroxidase (1/5000 in 0,5 % BSA and 0,02% Tween-20 in PBS; Jackson Immunoresearch, West Grove, USA) or goat anti-mouse peroxidase (1/5000 in 0,5 % BSA and 0,02% Tween-20 in PBS; Cooper Biomedical Inc, Malvern, USA). After washing the samples, they were incubated with a substrate solution of 1,1 M sodium acetate citric acid, 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide for 10 minutes at RT. The staining reaction was stopped by adding 2 M H_2SO_4 and the absorption was measured at 450 nm with the Benchmark microplate reader (Bio-Rad Laboratories).

To detect titin-specific auto-antibodies in CS, an anti-titin antibody ELISA-kit was used (DLD Diagnostika GmbH, Hamburg, Germany) (supplement section J). In this ELISA, the plates that were pre-coated with titin, were incubated with samples (undiluted CS

and ½ diluted CS) and controls. After one hour incubation at RT on a horizontal shaker, the wells were washed three times. Then, enzyme conjugate was added for 30 minutes at RT on a horizontal shaker. After washing the wells, a substrate solution was added for 15 to 25 minutes at RT on a horizontal shaker. The substrate reaction was stopped with a stop solution and the absorption was measured at 450 nm with the Benchmark microplate reader (Bio-Rad Laboratories).

2.9 Mega western protein array

The mega western protein array (MWPA) (Cytomol, Union City, USA), a membrane on which proteins of 32 different human tissues are spotted, was used to determine the size and relative abundance of target proteins of auto-antibodies in CS (supplement section K). The membrane was blocked with Odyssey Blocking Buffer (1/1 in PBS; LiCor BioSciences, Lincoln, USA) for one hour at RT, followed by the addition of the primary antibody solution (undiluted B cell culture supernatant and mouse anti-GAPDH (1/500; Cytomol)) for one hour at RT. The membrane was then washed three times with PBS 0,1% Tween-20 for five minutes. The secondary antibody solution with goat anti-human 800 (1/15 000 in Odyssey Blocking Buffer/PBS (1/1); Rockland, Gilbertsville, USA) and donkey anti-mouse 700 (1/15 000 in Odyssey Blocking Buffer/PBS (1/1); Rockland), was added to the membrane for one hour at RT. The membrane was washed as previously described and finally the signals were detected with the Odyssey Infrared Imaging System (LiCor BioSciences).

2.10 RNA isolation and cDNA synthesis

RNA was extracted from the immortalized B cells by using the High pure RNA isolation kit (Roche) (supplement section L). Cultured cells were lysed with a lysis-binding buffer, which also inactivated RNases. Nucleic acids were then bound to the glass fibers pre-packed in the high pure filter tubes by centrifugating the samples for 15 seconds at 8000g. In the next step, residual contaminating DNA was digested by incubation with DNase I for 15 minutes, applied directly on the glass fiber fleece. The bound nucleic acids were washed twice with a inhibitor removal buffer to purify them from salts, proteins and other cellular impurities. Finally, RNA was eluted by using the elution buffer and centrifugating the samples for one minute at 8000g.

The isolated RNA was subsequently converted into cDNA with the Reverse transcription system kit (Promega) (supplement section M). First, RNA was added to a mix of 25 mM

$MgCl_2$, 10x reverse transcriptase buffer, 10 mM dNTP mixture, recombinant RNAse inhibitor, AMV reverse transcriptase and Oligo dT primer. This solution was incubated for one hour at 42℃, five minutes at 90℃ and ten minutes on ice, followed by a washing step with phenol/chloroform/ isoamylalcohol (25/24/1) and water. After centrifugating the samples for two minutes at 20 000g, the supernatants were washed with chloroform/isoamylalcohol. The tubes were then centrifuged for two minutes at 20 000g. Thereafter, 3 M sodiumacetate and 100% icecold ethanol were added and the cDNA was precipitated overnight at -20℃ or for one hour at -80℃. After the precipitation, the samples were centrifuged for 15 minutes at 4℃ with 20 000g. Afterwards, the pellets were washed with 70% icecold ethanol and centrifuged for ten minutes at 4℃ with 20 000g. The pellets containing cDNA were dried and dissolved in water. The cDNA concentration and purity was measured with NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.11 Spectratyping

To produce monoclonal antibodies, the B cell clones need to derive from one B cell. Each B cell has a single V-D-J rearrangement that is unique in both length and sequence. Therefore, if DNA from a polyclonal B cell population is amplified by a polymerase chain reaction (PCR) using DNA primers that flank the V-D-J region, a Gauss curve of amplicon products within an expected size range is produced. This Gaussian distribution reflects the heterogeneous population of V-D-J rearrangements. For DNA from samples containing a monoclonal B cell population, the yield is one prominent amplified product within a diminished polyclonal background.

In order to detect the clonality of the B cell clones (supplement section N), a PCR was started with the following reagents: PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$ and 0,1% gelatine), 0,25 mM dNTPs (GE Healthcare, Buckinghamshire, United Kingdom), 10 pmol FR1, FR2 or FR3 Forward primer mix (Sigma Aldrich), 10 pmol Primer Biomed JH-con FAM (Sigma Aldrich), one unit Jumpstart Red Taq polymerase (Sigma Aldrich) and 100 ng DNA. The DNA was then amplified in PTC200 DNA Engine Thermal Cycler PCR (Scientific Support, Hayward, USA) by heating the samples for 7 minutes at 95℃ followed by 35 cycli of 45 seconds at 95℃, 45 seconds at 60℃ and 90 seconds at 72℃, and one step of ten minutes at 72℃. The amplified DNA samples were electrophoresed on a 2% agarose gel. The results of the electrophoresis were analyzed by the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories). If the samples of

each PCR-mix gave a fragment of the right size (PCR-mix FR1: 310-360 bp; PCR-mix FR2: 250-295 bp; PCR-mix FR3: 100-170 bp), the samples were analyzed with fragment analysis. For this, PCR-product was added to internal size standard and formamide. The mix was then analyzed by the genetic analyzer ABI3100 (Applied Biosystems, Foster City, USA) which detects the FAM-labels of the PCR-products in order to identify monoclonal or polyclonal B cell clones.

2.12 Sequencing

The PCR-product was also used as template for a sequence reaction, which is based on single strand primer extension by Taq-polymerase in a PCR-reaction (supplement section O). The mix for the sequence reaction contained PCR product, JH con primer (Sigma Aldrich), Big Dye v1.1 (Applied Biosystems) and sequence buffer. The PCR-product was amplified in Perkin Elmer 9700 PCR (PerkinElmer) by heating the samples for 5 minutes at 96°C followed by 25 cycli of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. Afterwards, the samples were analyzed by the genetic analyzer ABI3100 (Applied Biosystems) which detects the different labeled dyes of the different ddNTPs. The obtained sequences were then run through the BLAST immunoglobulin database (NCBI) and analyzed with Joinsolver [38].

The sequences of immortalized B cell clones were obtained in collaboration with the department of pathology from the academic hospital in Maastricht.

2.13 Statistics

GraphPad Prism 4 was used to perform statistical analyses. Comparison between normally distributed values was performed using a two-sample t-test, unless mentioned otherwise. A two-sided probability value <0.05 was considered significant.

3. Results and discussion

Thymi were obtained from three MG patients at the academic hospital in Maastricht and the patient data are summarized in table 2. In my internship period, I mainly focused on patient 42.

Table 2. Clinical characteristics of MG patients

Patient	Name	Sex	Age	Age at onset	Presenting symptoms	Ab titer (nM)	Thymectomy
21	MP	F	29	26	General	104	11/2006
28	TD	F	41	38	Ocular/bulbar	90	06/2007
42	JM	F	24	23	General	24	03/2008

nM = nanomol/liter

3.1 Differences between control and MG thymi

Thymocytes of four control and four MG patients were analyzed with FACS to study the amount of B cells. In figure 4A, a representative forward scatter (FSC) versus side scatter (SSC) plot is shown in which living cells were gated. Thymocytes from a control thymus contained less $CD22^+/IgG^+$ B cells (figure 4B), in comparison to thymocytes from an MG patient (figure 4C). This difference in the amount of B cells between control and MG thymi was significant for three B cell markers; CD19, CD21 and CD22 (figure 4D). In addition to an increased amount of B cells in MG thymi, B cells expressing IgG and IgM were more present in MG thymi than in control thymi, but only the difference for IgG^+ thymocytes was significant (figure 4E). Figure 4F shows that the amount of B cells in control thymi increased with age. Finally, the expression of CD21, which is the receptor for EBV and thus necessary for immortalization, was significantly less in control thymi compared to MG thymi (figure 4G). These FACS results indicate that control thymi and MG thymi both contain B cells which can be immortalized because of the expression of CD21.

3.2 IgG production and spectratyping

$CD22^+$ B cells were selected and immortalized from three MG patients and one control patient. To check whether the immortalized B cell clones produce IgG, dot blot experiments were performed. For all three MG patients, dot blots showed that most clones produced IgG, although the concentrations of IgG varied (figure 5). For the

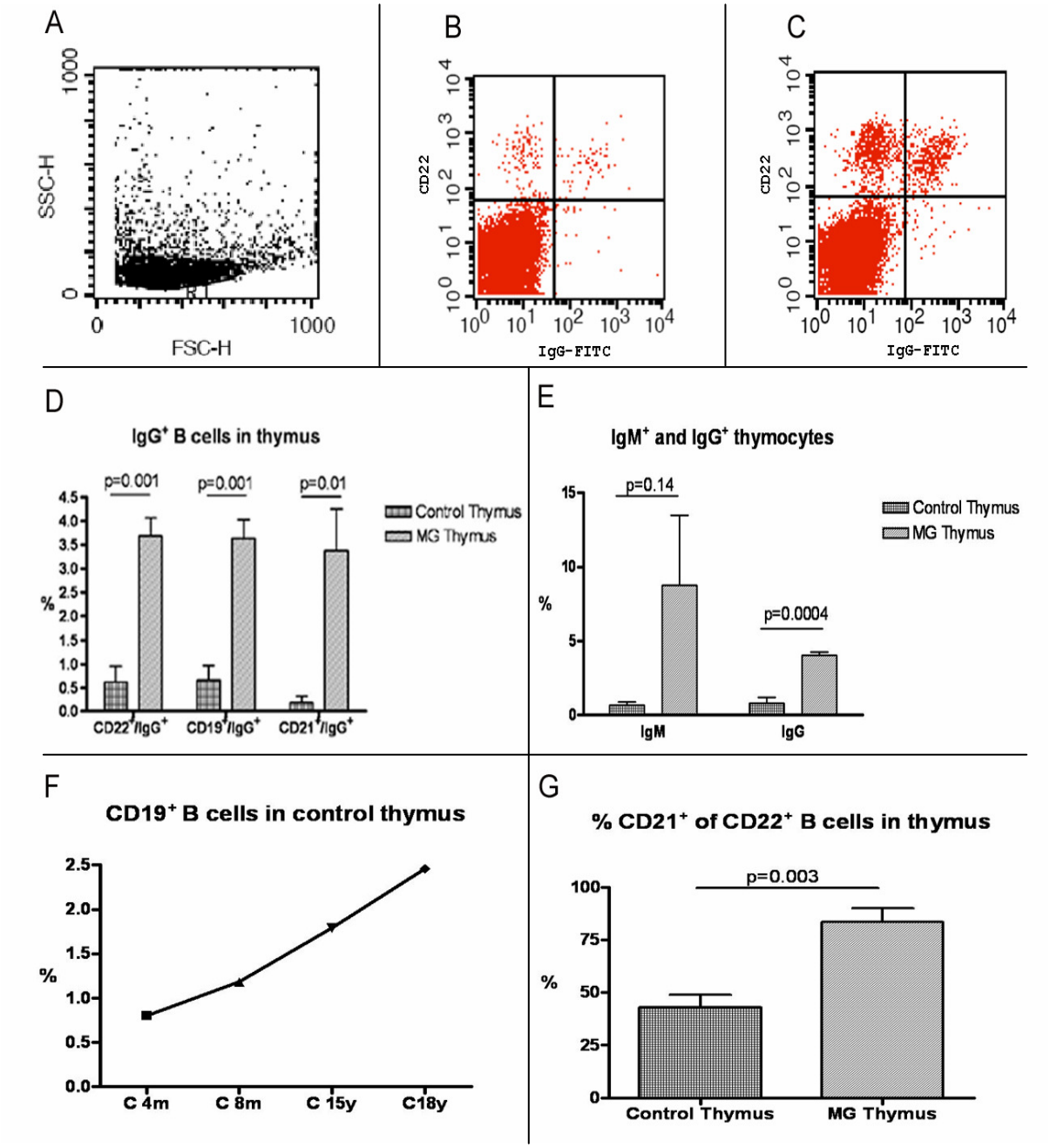

Figure 4. FACS data showing the amount of B cells in control and MG thymi. (A) Forward scatter versus side scatter plot with gating of the living cells. (B)+(C) The expression of IgG and CD22 on thymocytes isolated from a control and an MG thymus, respectively. Less $CD22^+$ B cells were present in a control thymus than in an MG thymus (D) Percentages of $CD22^+/IgG^+$, $CD19^+/IgG^+$ and $CD21^+/IgG^+$ thymocytes in control and MG thymi. It is shown that MG thymi contained significantly more B cells than control thymi. (E) Percentages of IgM^+ and IgG^+ thymocytes in control and MG thymi. IgM^+ and IgG^+ thymocytes were more abundant in MG thymi than in control thymi. (F) Percentages of $CD19^+$ B cells in thymi from 4 months, 8 months, 15 years and 18 years old control individuals. The amount of B cells in control thymi increased with age. (G) Percentages of $CD21^+$ cells from the $CD22^+$ B cells in control and MG thymi. In control thymi, significantly less B cells expressed the CD21 marker.

control patient, no IgG check was performed because none of the clones grew. The IgG-producing clones were subsequently kept in culture and the CS was collected for further experiments.

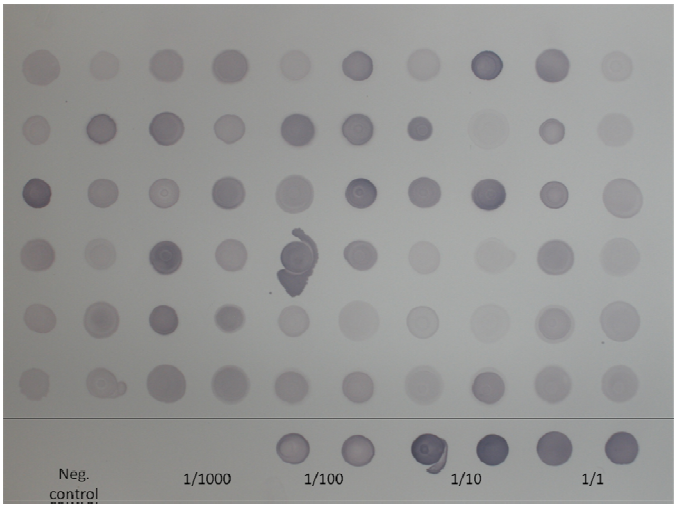

Figure 5. Dot blot with CS from B cell clones of patient 42. Above the line, spots derive from B cell clones and under the line, spots derive from control samples. Culture medium and intravenous IgG (IVIg) were used as negative and positive controls respectively. The intensity of the spots, compared with the standard line of IVIg, is an indication for the IgG concentration. All B cell clones were positive for IgG production, but the IgG concentration varied.

Later in the senior internship, the secondary anti-IgG antibody (Promega) used for the dot blot experiments as shown in figure 5 was found to bind IgM in addition to IgG. Therefore, the dot blots were repeated with another anti-IgG antibody (Dako) and with an anti-IgM antibody (Invitrogen) to distinguish IgM- and IgG-producing clones. Figures 6 and 7 show a dot blot of the same clones incubated with anti-IgG and anti-IgM respectively. By analyzing figures 6 and 7, the clones surrounded by yellow circles were still IgG-producing clones; the clones surrounded by blue circles were IgM-producing clones as well as IgG-producing clones; the clones without a circle only produced IgM antibodies (table 3).

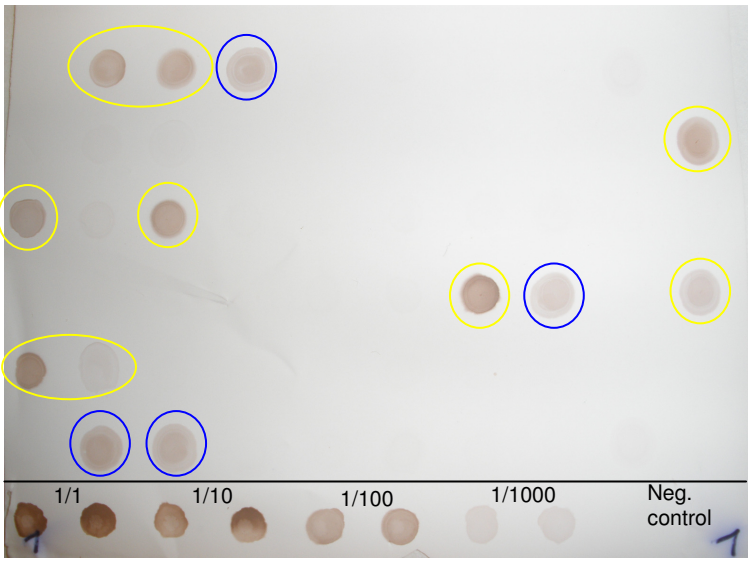

Figure 6. Detection of IgG by performing a dot blot with CS from B cell clones. Above the line, spots derive from B cell clones and under the line, spots derive from control samples. Culture medium and IgG1 637 were used as negative and positive controls respectively. The clones surrounded by yellow circles were IgG-producing clones; the clones surrounded by blue circles produced both IgM and IgG antibodies.

Figure 7. Detection of IgM by performing a dot blot with CS from B cell clones. Above the line, spots derive from B cell clones and under the line, spots derive from control samples. Culture medium and IgG1 637 were used as negative and positive controls respectively. The clones without a circle produced IgM; the clones surrounded by blue circles produced both IgM antibodies and IgG antibodies.

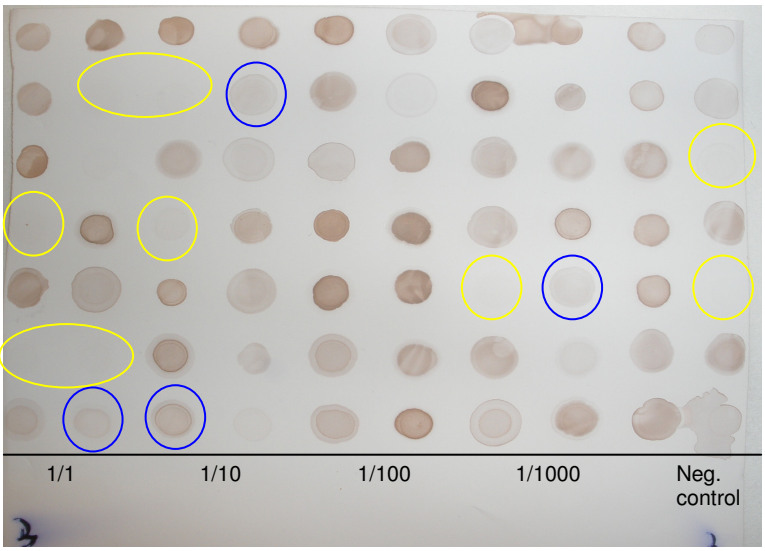

Table 3. Percentages of IgG- and IgM-producing clones for the different patients. The percentages between brackets indicate the clones positive for both IgG and IgM.

Patient	IgG	IgM
21	16%	84%
28	0% (95%)	5% (95%)
42	19% (8%)	73% (8%)

In summary, the majority of immortalized B cell clones produced IgM instead of IgG antibodies. Both IgM^+ and IgG^+ B cell clones were kept in culture until a sufficient number of B cells was achieved to freeze the cell line. For patient 21, 28 and 42; 19/63 (30%), 22/86 (26%) and 74/182 (41%) clones were frozen in liquid nitrogen respectively. The other clones were either lost by infections or stopped growing for unknown reasons. B cells of a control thymus were immortalized as well, but none of the clones started growing. An explanation for the absence of growing clones could be that significantly less B cells express the CD21 marker in control thymi than in MG thymi as shown in figure 4G.

One of the aims of this project was to produce monoclonal antibodies and to verify the monoclonality of the produced auto-antibodies, spectratyping experiments were performed. For patient 21, 16/19 clones (84%) were monoclonal and for patient 42, 41/44 clones (93%) showed monoclonality (data not shown). The monoclonality of these

antibodies indicates that they can only be directed against one target epitope. The other clones were biclonal. The B cell immortalizations started with 5 B cells per well, so a polyclonal response was not expected.

3.3 Auto-antibodies directed to the AChR

The B cell clones are shown to produce IgG and IgM antibodies, but their antigen specificity is still unknown. To test our hypothesis that EBV-immortalized B cell clones produce anti-AChR antibodies, a RIA was performed. None of the clones of patient 21 and 28 produced antibodies against the AChR, in contrast to a few clones of patient 42. To confirm these results, the RIA was repeated for some clones (figure 8). Clones 131, 159 and 163 had again higher counts per minute (CPM) in comparison to the other clones and the negative controls (NC and PBS) In addition, their CPM-values were comparable with the diluted samples of the positive controls (IgG1 637 and patient serum). Clones 68 and 85 were considered borderline positive for antibody production against the AChR, because these clones had lower CPM-values than clones 131, 159 and 163 but still higher CPM-values than the other clones.

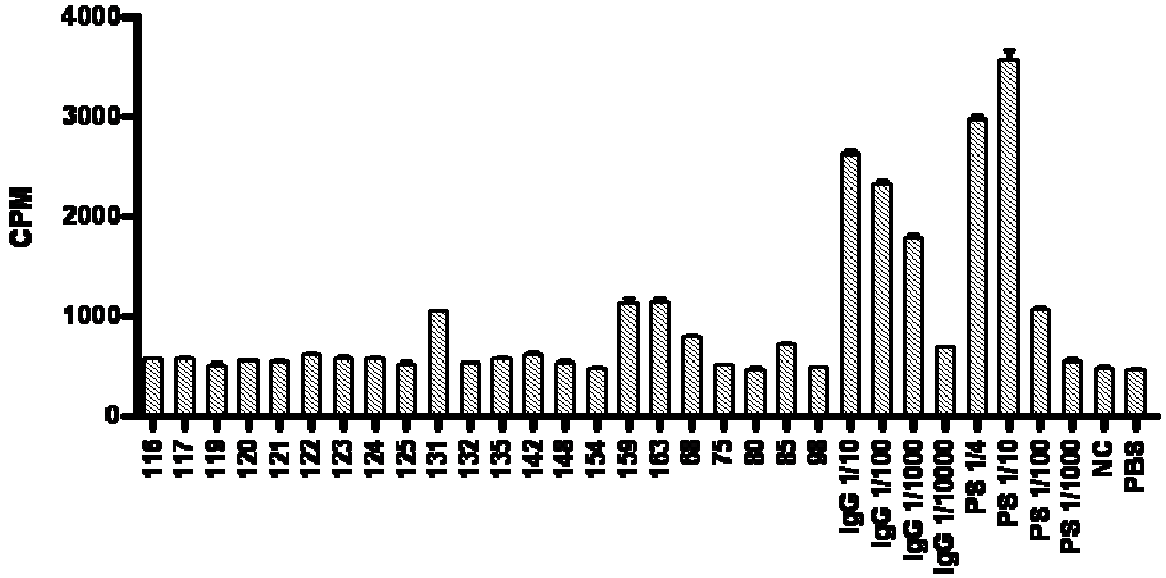

Figure 8. RIA to detect antibodies against the AChR in CS from B cell clones. The assay was performed for each clone in triplo. Clones 131, 159, 163, and to some extent, clones 68 and 85 were positive for the RIA against the AChR. PS=patient serum; NC=negative control; IgG=IgG1 637 as positive control.

A second technique to test for antibody production against the AChR and to show whether the antibodies have cross-linking capacities, was the antigenic modulation experiment. Figure 9 shows that an increasing concentration of IgG1 637, which is an

antibody known to cross-link the AChR, leads to less radioactively-labeled receptor was left on the membrane of TE 671 cells. All clones of patient 21 and 28 were tested, in addition to 46 clones of patient 42, which included both positive and negative clones of the RIA. Only results of patient 42 are shown in figure 9. All the CPM-values of the tested B cell clones had a higher value compared to the standard curve for IgG1 637, which means that less receptor was internalized, and more radioactively-labeled receptors were still present on the membrane of TE 671 cells. Thus, none of the B cell clones had antibodies that cross-link the AChR. The possibility still remains that auto-antibodies from clones 68, 85, 131, 159 and 163 are directed to the AChR without causing internalization of the receptor.

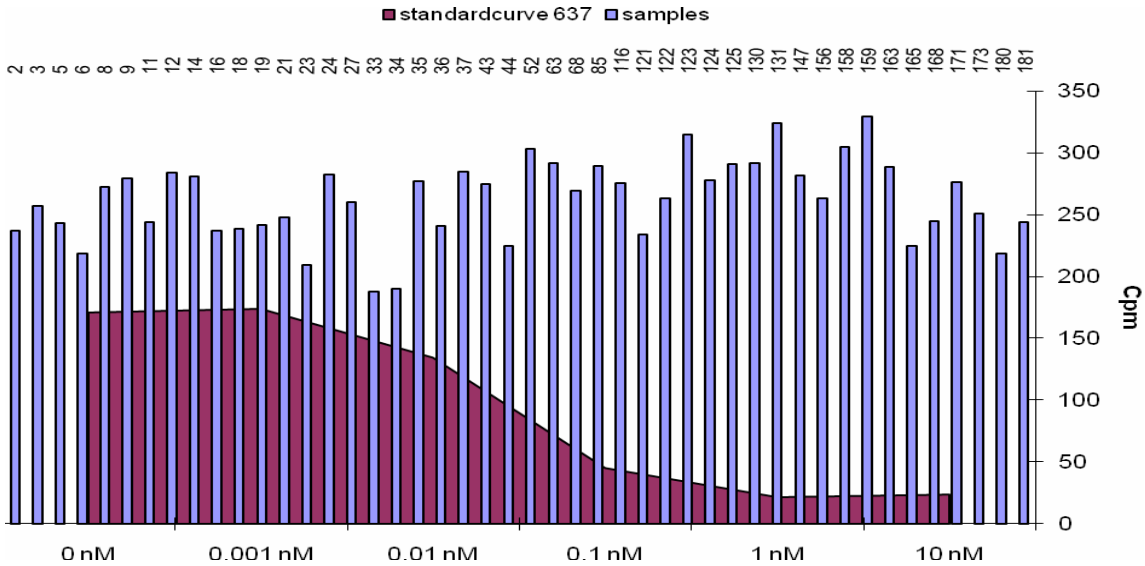

Figure 9. Antigenic modulation experiment with CS from B cell clones. Each sample was tested in triplo. Standard deviations were left out for the proper visibility of the figure. The upper x-axis belongs to the bars and the lower x-axis belongs to the area under the curve, which is the standardcurve for IgG1 637. None of the tested B cell clones showed antigenic modulation.

The fact that almost all clones were negative for both the RIA and antigenic modulation leads to the conclusion that anti-AChR antibodies producing B cells are not enriched in the thymus. However, it is still possible that the few clones positive in the RIA have anti-AChR antibodies, but without cross-linking capacity.

3.4 Auto-antibodies directed to other proteins

3.4.1 Striated muscle proteins

IHC stainings were performed to investigate whether the antibodies produced by the B cells are directed against other muscle proteins than the AChR. A first staining was

performed to detect auto-antibodies against monkey striated muscle proteins in the CS of B cell clones. For patient 21, 7/19 clones (37%) stained monkey muscle tissue with a striational pattern and 5/22 clones (23%) also showed that staining pattern for patient 28. For patient 42, 38 clones were tested and the selection was again based on the results of the RIA. A positive staining was noticed for 6 clones (16%), 10 clones were marked as borderline (26%) and 22 clones as negative (58%) for staining against monkey striated muscle proteins. It has to be emphasized that clones 159 and 163, which were positive in the RIA, were also positive for staining against monkey striated muscle proteins (figure 10), contradicting the results of the RIA and spectratyping as the AChR is not a striated muscle protein. Since monoclonal antibodies only bind one epitope, they are directed towards either the AChR or a certain striated muscle protein. However, a shared epitope on the α-subunit of the AChR and on muscle proteins has been described [39], suggesting that the antibodies of clones 159 and 163 may be directed towards the AChR and a certain striated muscle protein.

3.4.2 Neuromuscular junction proteins

A second staining was performed to characterize auto-antibodies against monkey NMJ proteins in the CS of B cell clones. None of the clones of patient 21 and 28 showed co-localization with α-bungarotoxin, which binds the AChR. Staining for NMJ proteins was seen for 4/13 clones (31%) of patient 42, 4 clones showed intermediate staining (31%) and 5 clones were negative (38%). The tested clones were again selected based on previous results. Figure 11 shows the results of this staining, with clones 131 and 159 containing antibodies against NMJ proteins. These data support the results of the RIA, because the AChR is a protein of the NMJ. Thus, the AChR may be the target for auto-antibodies, although the co-localization with α-bungarotoxin was not perfect, so the target antigen may also be an AChR-associated protein.

3.4.3 Actin, myosin, alpha-actinin or titin

Because a striational staining pattern was seen in the IHC stainings, ELISA's were performed to characterize the antigens that caused this staining pattern. ELISA experiments were performed for the striated muscle proteins actin, myosin, alpha-actinin and titin. None of the clones of all three patients were positive for antibody production against one of these striational proteins (data not shown). Probably other striational proteins are targeted by the antibodies of some clones.

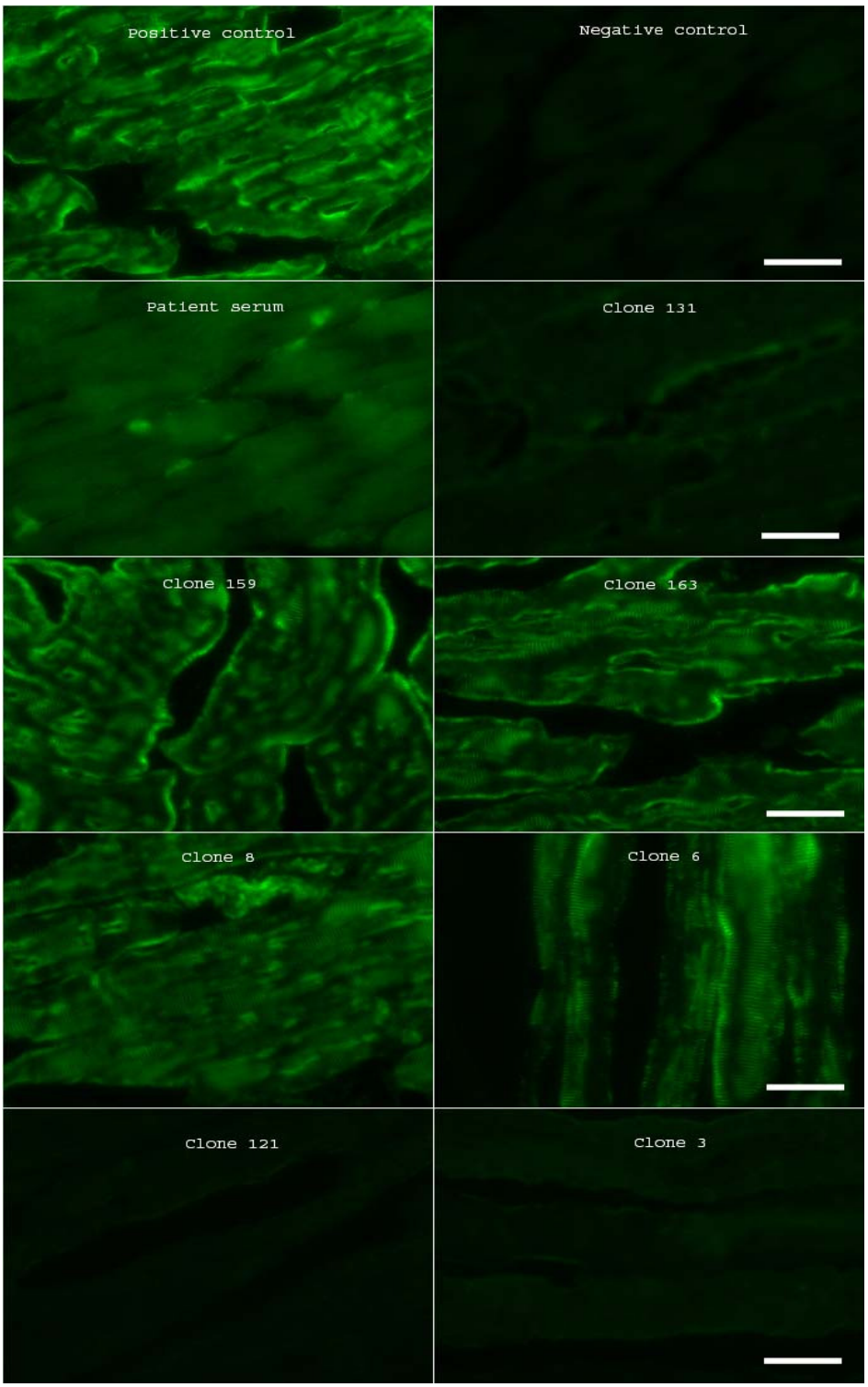

Figure 10. Immunohistochemical staining against monkey striated muscle proteins with CS from B cell clones. Clones 159 and 163, positive in the RIA, showed strong fluorescent staining and clone 131 showed intermediate fluorescent staining. Clones 8 and 6 represent positive staining and clones 121 and 3 represent negative staining. Scale bars indicate 10 μm.

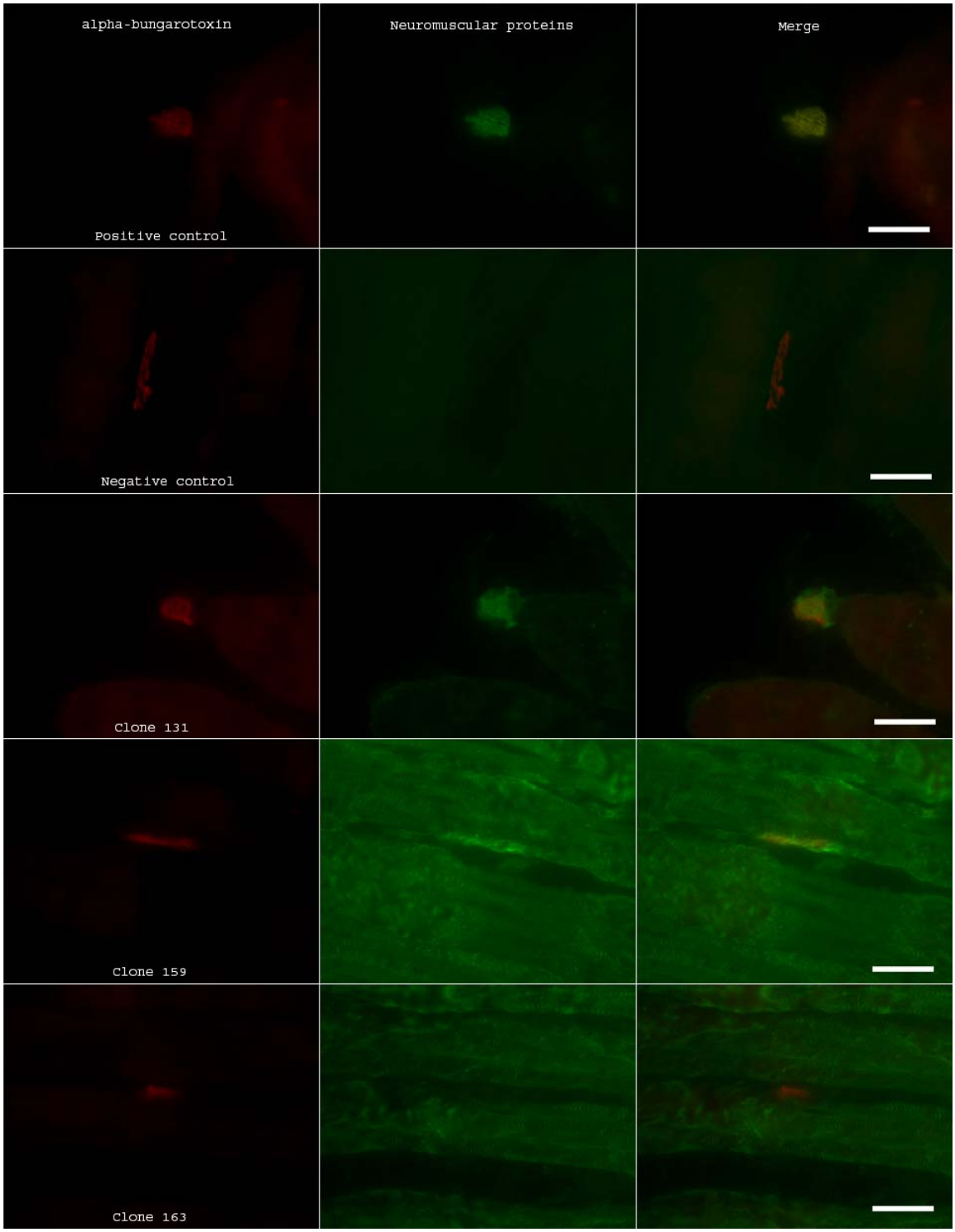

Figure 11. Immunohistochemical staining against monkey NMJ proteins with CS from B cell clones. Alexa 594-conjugated α-bungarotoxin binds the AChR at the NMJ (red) and for clones 131 and 159 co-localization with the staining for NMJ proteins (green) was detected. Clones 159 and 163 also stained positive for striated muscle proteins, which hamper to see the co-localization at the NMJ accurately. Scale bars indicate 10 µm.

To this point, the antigen specificity of the produced antibodies is still missing. Based on the results of the RIA and the NMJ staining, we concluded that the auto-antibodies produced by clones 131 and 159 may be directed to the AChR. The auto-antibodies may also be directed against striated muscle proteins, but ELISA experiments did not answered the question against which striated muscle protein exactly they were directed to. The results of the three patients are summarized in table 4 and the specific results of clones 68, 131, 159 and 163 from patient 42 are summarized in table 5.

Table 4. Data of the three patients. The percentages represent the positive results for the mentioned experiments. Borderline positive results are shown between brackets.

Patient	Clones	RIA	Antigenic modulation	ELISA	Muscle staining	NMJ staining	Mono-clonal
21	19	0%	0%	0%	37%	0%	84%
28	22	0%	0%	0%	23%	0%	/
42	74	4% (3%)	0%	0%	16% (26%)	31% (31%)	92%

Table 5. Data of clones 68, 131, 159 and 163 from patient 42, which were positive in the RIA.

Number	IgG	IgM	Muscle staining	NMJ staining	Monoclonal
68	-	+	/	+	+
131	-	+	-	+	+
159	+	-	+	+	+
163	+	-	+	-	+

3.4.4 Mega western protein array

A more general approach was performed to identify the target antigens of the antibodies produced by the B cell clones. The mega western protein array was used to determine the size and relative abundance of target proteins for auto-antibodies in CS. The experiment was performed with pooled CS from 10 clones of patient 21 (figure 12), as well as with pooled CS from 10 clones of patient 42 (figure 13). In figure 12, proteins from diaphragm and skeletal muscle tissue were recognized with a size of 28-33 kDa and 26-30 kDa respectively. The same spots were also visible in figure 13, but they were fainter and the target proteins were slightly bigger. The target proteins were not further identified. In addition, proteins between 50 and 60 kDa were detected in most tissues, but the same pattern was also seen by using CS of immortalized B cells of MS patients (data not shown). This indicates that the pattern was not specific for MG patients and it might emphasize the general disturbance of self-tolerance in autoimmune patients.

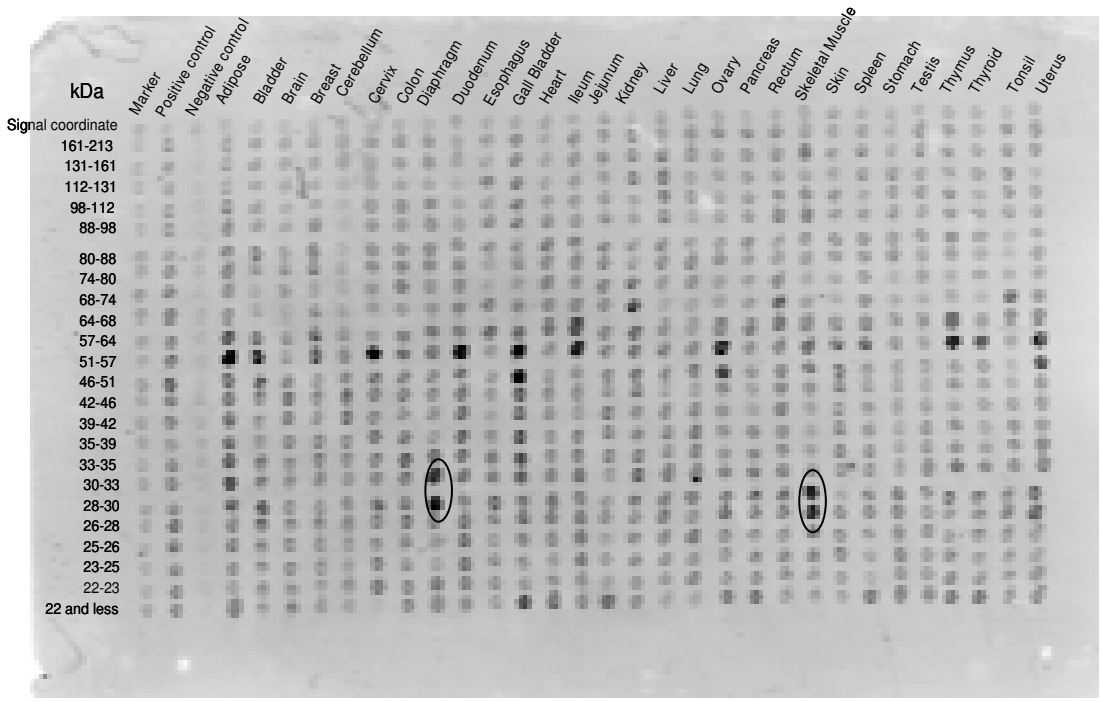

Figure 12. Mega western protein array with pooled CS from 10 B cell clones of patient 21. Spots were visible for diaphragm and skeletal muscle proteins of respectively, 28-33 kDa and 26-30 kDa.

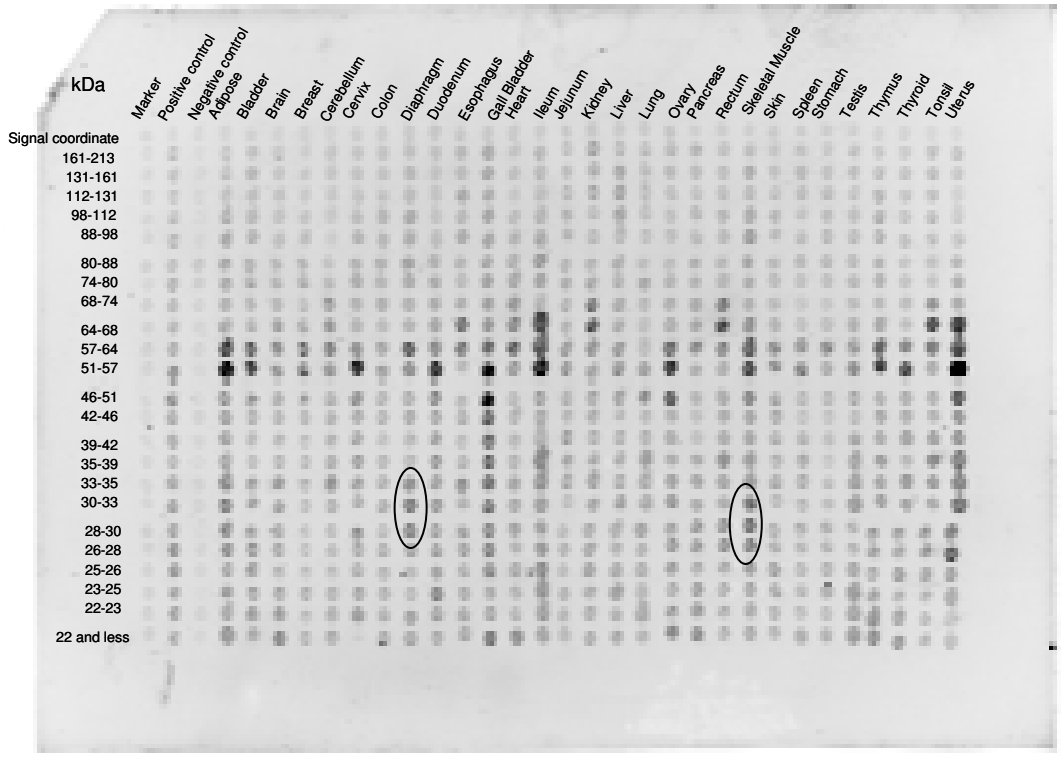

Figure 13. Mega western protein array with pooled CS from 10 B cell clones of patient 42. Spots were visible for diaphragm and skeletal muscle proteins of respectively, 28-35 kDa and 26-33 kDa.

3.5 Sequencing

Sequencing of auto-antibodies was performed to analyze the immunoglobulin heavy chain variable region (V_H) gene repertoire of the immortalized B cell clones. The obtained sequences were run through the BLAST immunoglobulin database (NCBI) and analyzed with Joinsolver [38]. Analysis with Joinsolver has provided new insights into the molecular and selective mechanisms that underlie the generation of the human H chain complementarity determining region 3 ($CDR3_H$), which is the most diverse region in the Ig molecule and therefore an important antigen binding region of V_H chains. Importantly, this software is described to be of great value in determining abnormalities in individuals with immune disorders.

3.5.1 Distribution of V_H families in immortalized B cell clones

The V_H gene family usage of the immortalized B cell population of patient 42 resembled the germline complexity, deduced from the relative occurrence of these genes in the germline. In more detail, V_H3 was found most often, followed by V_H4 and V_H1 (figure 14A). The distribution of the V_H families within the population of immortalized B cell clones of patient 21 was different than expected, although the difference was not significant using the chi-square goodness-of-fit statistic (p=0.38). The V_H1 family was found less often and the V_H3 family more often than expected (figure 14B). The positive selection of B cells expressing this V_H3 segment was according to previous results for autoimmune patients [40].

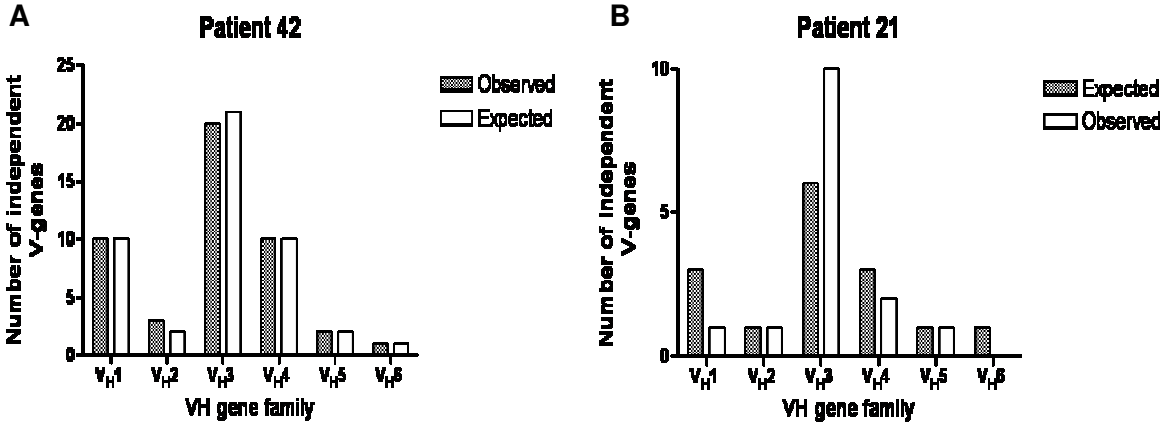

Figure 14. V_H gene family usage. (A) V_H gene family usage of the immortalized B cell population of patient 42. (B) V_H gene family usage of the immortalized B cell population of patient 21. The distribution of the V_H families in immortalized B cell population of patient 42 equaled the expected frequencies. The V_H3 family and V_H1 family were overrepresented and underrepresented, respectively, in the immortalized B cell population of patient 21 in comparison to the expected frequencies.

3.5.2 CDR3 sequences

The $CDR3_H$ is located in the centre of the antigen binding site, interacting directly with the other CDRs and framework regions from both heavy and light chains, as well as with the antigen itself. Changes in the $CDR3_H$ amino acid composition directly affect the ability of the antibody molecule to bind antigen [38]. The variable size and composition of the CDR3 of V_H genes expressed by the immortalized B cell clones indicated a polyclonal response (tables 6 and 7). Despite the polyclonal origin of the response, common $CDR3_H$ amino acid sequences were found, which suggests that some clones are responding to the same antigen as indicated by small letters in tables 6 and 7. The polyclonality of the immortalized B cell clones was in accordance with literature [41]. No significant difference was found in CDR3 length between the IgG and IgM antibodies ($p=0.48$).

3.5.3 Somatic hypermutations

Antigen binding induces somatic hypermutations in antibodies, thereby further diversifying the antibody repertoire. The IgM antibodies are direct copies of germline gene segments, while the structures of the IgG antibodies appear to be mutated suggesting that they have undergone antigenic selection [42]. This was confirmed by a significant increase of the percent V_H mutation of IgG compared to IgM antibodies ($p=0.0006$). However, IgM antibodies also were somatically hypermutated, which contradicts with most of the published literature (table 6 and 7).

Table 6. V_H gene sequence analysis for IgG antibodies.

Clone	V_H family	Percent V_H mutation	CDR3 (length)	Gene rearrangement			V_H end
E6(5) p21	4	3.21	AREGLHTTVSKTYYYYGMDV (60)	V4-04*07	D4-17*01	J6*02	YYCAR
G3(8) p21[a]	3	6.79	ATYYGHG (21)	V3-11*03	D4-17*01	J4*02	YYCAT
G4(8) p21[a]	3	6.79	ATYYGHG (21)	V3-11*03	D4-17*01	J4*02	YYCAT
19 p42	3	13.12	AKDARTTRRDTLFGMVTWFDP (63)	V3-23*01	D3-3*01	J5*02	YYCAK
21 p42	1	3.64	SRDGGVDTAVGGAYYYYYFMDV (66)	V1-02*02	D5-18*01	J6*02	YYCAR
23 p42	3	1.35	ARESHYGMDV (30)	V3-11*03	D	J6*02	YYCAR
33 p42	4	12.44	ARGRSYSYGFVAGGPMTFEY (60)	V4-59*04	D5-18*01	J4*03	YYCAR
37 p42	1	7.27	ARFTPMAPWTLDY (39)	V1-02*02	D5-18*01	J4*02	YYCAR
43 p42	1	1.77	AAMGGFFITGTGWYFDL (51)	V1-58*01	D1-7*01	J2*01	YYCAA
58 p42	1	13.33	ARDYMIRGVPFDF (39)	V1-02*02	D3-10*02	J4*02	YYCAR
63 p42[b]	3	5.43	ARDKYDFWSGYLLHPEGYFDY (63)	V3-30-3*02	D3-3*01	J4*03	YYCAR
123 p42	3	7.21	ARESGGSRRLRAFDV (45)	V3-11*03	D3-10*01	J3*01	YYCAR
159 p42	3	8.11	VADPFL (18)	V3-30*03	DIR2*01R	J4*02	YYCAK
163 p42[b]	3	5.96	ARDKYDFWSGYLLHPEGYFDY (63)	V3-30-3*02	D3-3*01	J4*02	YYCAR

[a], [b], [c], [d] Sequences with a large homology

Table 7. V_H gene sequence analysis for IgM antibodies.

Clone	V_H family	Percent V_H mutation	CDR3 (length)	Gene rearrangement			V_H end
B5(2) p21	1	6.33	ATCGGDCYSGDY (36)	V1-02*02	D2-21*02	J4*02	YYCAT
B8(1) p21	4	5.83	ARDMGLRTPSPGLDV (45)	V4-39*06	D5-12*01	J6*02	YYCAR
B11(8) p21	2	10.2	AHRPGYYYDSSGYYFDLGSHFDY (69)	V2-05*01	D3-22*01	J4*02	YYCAH
D7(4) p21[c]	3	6.85	ARKIYYGSGSPRHMDV (48)	V3-11*03	D3-10*01	J4*03	YYCAR
D8(9) p21[a]	3	7.24	ATYYGHG (21)	V3-11*03	D4-17*01	J4*02	YYCAT
D10(1) p21[c]	3	4.98	ARKIYYGSGSPRHMDV (48)	V3-11*03	D3-10*01	J4*03	YYCAR
E7(7) p21	5	4.52	ARLVSSGWPQFGTAYFDH (54)	V5-51*01	D6-19*01	J4*02	YYCAR
E8(7) p21	3	3.98	TRFDYSSSDSFDY (39)	V3-73*01	D6-6*01	J4*02	YYCTR
E11(1) p21[c]	3	4.55	ARKIYYGSGSPRHMDV (48)	V3-11*03	D3-10*01	J4*03	YYCAR
G2(2) p21[c]	3	4.98	ARKIYYGSGSPRHMDV (48)	V3-11*03	D3-10*01	J4*03	YYCAR
G5(1) p21	3	4.55	ARGHYGMDV (27)	V3-11*01		J6*02	YYCAR
G7(1) p21	3	0.91	ARDRVSFRGYSYGYYYYGMDV (63)	V3-07*01	D5-5*01	J6*02	YYCAR
2 p42	4	1.33	ARRGYYYDSSGYYYGWFDP (57)	V4-39*01	D3-22*01	J5*02	YYCAR
3 p42	2	1.59	ARIPSSSKGWGYYYYGMDV (57)	V2-70*01	D6-6*01	J6*02	YYCAR
5 p42	1	0.45	ARDGRYDYVWGSYRLFDL (54)	V1-69*01	D3-16*02	J2*01	YYCAR
6 p42	3	3.15	AREKYSGSYYRLFDY (45)	V3-33*01	D1-26*01	J4*02	YYCAR
8 p42	3	0.9	ARGGRGLATSKTGYYYYYYGMDV (69)	V3-48*02	D5-12*01	J6*02	YYCAR
9 p42	1	0.45	AGYDYGIYYYYGMDV (45)	V1-69*01	D3-22*01	J6*02	YYCAG
11 p42[d]	4	0	ARGQGVVTLGY (33)	V4-59*01	D2-21*02	J4*02	YYCAR
12 p42	3	1.8	AKTQWLARLGFYFDY (45)	V3-23*01	D6-19*01	J4*02	YYCAK
14 p42	3	1.8	ARDLQLWSYYYYYGMDV (51)	V3-07*02	D5-18*01	J6*02	YYCAR
16 p42	4	0	ARMGIAADYVDGFDY (45)	V4-39*05	D6-13*01	J4*02	YYCAR
18 p42	1	1.3	AIGYCSGGSCYLVDY (45)	V1-69*01	D2-15*01	J4*02	YYCAI
24 p42	3	2.7	ARTYYYDSSGHDAFDI (48)	V3-48*02	D3-22*01	J3*02	YYCAR
27 p42	1	1.78	ALEGDQGGNY (30)	V1-69*01	DIR2*01	J4*02	YYCAL
29 p42	3	1.8	VSPTTMVRGLYRDGT (45)	V3-30*03	D3-10*01	J5*02	YYCVS
30 p42[d]	4	0	ARGQGVVTLGY (33)	V4-59*01	D2-21*02	J4*02	YYCAR
34 p42	2	3.05	AHRPGAVADTTPNWFDP (51)	V2-05*10	D6-19*01	J5*02	YYCAH
36 p42	4	0.46	ARGGTNWNDWGVGNWFDP (54)	V4-59*01	D1-1*01	J5*02	YYCAR
44 p42	4	2.29	ARGRYDILTGYYNHSDEQPNIYYGMDV (81)	V4-34*01	D3-9*01	J6*02	YYCAR
51 p42	3	1.8	ARGRYYDFWSGYFDY (45)	V3-11*01	D3-3*01	J4*02	YYCAR
52 p42	3	1.8	ARALVGATGGDY (36)	V3-21*01	D1-26*01	J4*02	YYCAR
68 p42	1	0.45	ARGGDYYDSSGYYPAYYYYGMDV (69)	V1-02*02	D3-22*01	J6*02	YYCAR
70 p42	4	0	ARRKHYDILTGYGITAGAFDI (63)	V4-31*04	D3-9*01	J3*02	YYCAR
71 p42	4	1.78	ARGTRITMVRGVIINVSYFDY (63)	V4-39*02	D3-10*01	J4*02	YYCAR
76 p42	4	0.91	ARATGYSSSWYYYYYYGMDV (60)	V4-34*01	D6-13*01	J6*02	YYCAR
122 p42	3	4.11	ARDPTYYDFWSGYRYYGMDV (60)	V3-30-3*02	D3-3*01	J6*02	YYCAR
131 p42	3	0.9	ARHGSGNDYYGMDV (42)	V3-48*02	DIR1*01	J6*02	YYCAR
147 p42	3	1.8	ASDVDTAMSRPYYYGMDV (54)	V3-48*02	D5-18*01	J6*02	YYCAR
180 p42	5	0	AVSHSSGWAFDY (36)	V5-a*01	D6-19*01	J4*02	YYCAR
181 p42	3	1.8	AKVGSSGWYLGY (36)	V3-23*01	D6-19*01	J4*02	YYCAK

[a], [b], [c], [d] Sequences with a large homology

3.5.4 Rabies virus

By running the sequences through the BLAST immunoglobulin database (NCBI), 1/15 clones of patient 21 and 6/47 clones of patient 42 showed strong homology with homo sapiens clone anti-rabies virus immunoglobulin heavy chain, although this virus rarely occurs in humans. The AChR is described to be a receptor for the anti-rabies virus [43], so a link between the anti-rabies virus and MG could not be excluded. However, these patients never came in contact with the rabies virus, so the question remains why immune cells produce anti-rabies virus antibodies in these patients. To verify the sequencing results, CS of the clones which showed this homology, was send to the laboratory of prof. dr. Ab Osterhaus in Rotterdam to investigate whether the antibodies present in the supernatant had the ability to recognize the rabies virus. This test was negative for all clones.

4. Conclusion

The first objective of this project was the immortalization of B cells from MG thymi by using EBV. The thymus, which is the site of T cell differentiation, was presumed to contain only T cells, until the existence of a unique B cell population within the thymus of healthy controls was reported [44]. It is not known whether those B cells are stimulated to proliferate within the thymus or whether they migrate to the thymus having encountered an antigen elsewhere [45]. Myasthenic thymi show typical features of follicular hyperplasia with large germinal centres [46] and the amount of B cells within the thymus increases with age and with MG [47]. Our FACS data confirmed these findings and showed the presence of B cells in control and MG thymi which can be immortalized. The immortalization process is highly dependent on the expression of the CD21 receptor on B cells, which is the receptor for EBV. B cells of a control thymus were tried to immortalize, but less expression of the CD21 receptor on B cells of those thymi could partly explain the absence of any growing clone. Nonetheless, 50% of $CD21^+$ B cells should be still enough to produce growing immortalized B cells, so other factors probably inhibited the immortalization procedure as well.

Two different methods were performed to immortalize B cells of thymi [36, 37]. The clones which were immortalized with the technique in which B cell stimulation precedes B cell immortalization [37] seemed to grow faster in the first stages after immortalization, but the clones immortalized with B cell stimulation and immortalization happening on the same time [36] were more stable and were kept in culture for prolonged time. Immortalized clones from both methods could either be lost by infections or stopped growing for unknown reasons, so the efficiency of this technique can still be improved. In addition, the efficiency of immortalization is not as high as described by others [36, 37]. All in all, the immortalization of B cells using CpG is a useful method to study B cells and their antibodies that will improve the knowledge on autoimmune diseases.

Because previous results showed that more than 90% of thymic B cells produce IgG (data not shown), only $CD22^+$ B cells were selected to start the immortalization process. After analyzing the dot blot results, which show higher expression of IgM compared to IgG, the conclusion is that $IgG^+/CD22^+$ B cells should have been selected to start the immortalization process to remove the IgM antibodies. An explanation for the mass production of IgM antibodies can be an increased IgM production after CpG stimulation

[48]. Nonetheless, IgG antibodies are preferred in this project, because IgM antibodies have a lower affinity and are less specific than IgG antibodies [49]. Furthermore, MG is mediated by circulating antibodies mainly of the IgG class directed to the AChR, MuSK or other auto-antigens in the NMJ of striated muscle [26]. However, IgM antibodies can also bind to the AChR in seronegative MG patients [50]. Whether those IgM antibodies can cause a neuromuscular junction defect in vivo, is not yet clear, although IgM antibodies to myelin-associated glycoprotein and to certain gangliosides are thought to be pathogenic in patients with forms of chronic peripheral neuropathies [51].

Regardless of the immunoglobulin subclasses, a definite answer to the hypothesis whether EBV-immortalized B cell clones from MG thymi produce auto-antibodies against the AChR, could not be given. Antibodies from some clones showed binding capacity towards the AChR and NMJ proteins, but also against striated muscle proteins. A comprehensive conclusion was hard to draw from all those experiments, except that the thymus does not contain an enriched population of mature B cells with AChR specificity. Nonetheless, the pathogenic antibodies in MG patients can also have an origin somewhere in the body, since patients did not fully recover after thymectomy. Therefore, PBMCs will be immortalized in the future to analyze their antibody production as well.

Although the thymus does not contain an enriched population of mature B cells with AChR specificity, analysis of Ig V gene usage in the thymus of MG patients has clearly demonstrated that there is a highly compartmentalized clonal expansion of B cells driven by a limited number of antigens in this organ. The results also showed that B cells from MG thymi were undergoing somatic hypermutation of their Ig V genes and that the rearranged V_H gene sequences expressed by these cells showed a remarkable degree of heterogeneity that may reflect a protracted response of the immune system to auto-antigenic stimulation. The high frequency of somatically mutated IgM molecules was unexpected, although a high frequency of somatically mutated IgM molecules in human adult blood B cell repertoire is described already [52]. In addition, high mutation frequency is not always necessary for the development of high-affinity monoreactive auto-antibodies, which may indicate that some germline genes encoding such antibodies fail to be deleted in patients with autoimmune diseases [40].

Future experiments include new immortalizations after selection of $IgG^+/CD22^+$ B cells, both isolated from thymocytes and from PBMC's. These immortalizations will give new

perspectives into the origin of the anti-AChR antibodies. Purification of the antibodies in the CS of clones 131, 159 and 163 is also interesting to detect the antigenic targets of these antibodies. Finding the antigenic targets of auto-antibodies from MG patients by using EBV-immortalization is very innovative and it will contribute to the knowledge of autoimmune diseases in general.

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Supplemental information

Supplement section A : CD22 MACS separation technique

Materials:
- MACS buffer: 2 mM ethylenediaminetetraacetic acid (EDTA), 0,5% Bovine Serum Albumin (BSA), PBS pH 7,2**
- Pre-MACS filter, LS column, MACS separator (Miltenyi Biotec, Auburn, USA)
- CD22 microbeads (Miltenyi Biotec, Auburn, USA)
- 10 ml tubes
- Ice

Remarks:
Work fast, keep cells cold and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling
* The volumes described here concern cell amounts up to 10^7 total cells. When less then 10^7 cells are used, these volumes need to be taken. However, when more cells are used, the volumes need to be adjusted (eg 10^8 cells: 800 µl + 200 µl)
** MACS-buffer always needs to be kept **on ice** and centrifugation of the cells needs to be done on 4°C. The buffer needs to be filtered to **remove gasses** and air bubbles. To prevent pieces being present in the MACS separator, the cells are pre-filtered on the pre-MACS filter.

Protocol:
Magnetic labeling
- Centrifuge the obtained single cell suspension at 1400 rpm (300g) for 5 minutes at 4°C
- Resuspend the cell pellet in 2 ml of MACS buffer**
- Pre-MACS filter (30 µm to obtain single cells):
  Pre-wash the filter with 1 ml MACS buffer**
  Add cells (2 ml)
  Wash the filter with 2 ml MACS buffer**
- Count the amount of cells (5 ml) in the Burker-turk counting chamber
- Centrifugation: 10 minutes, 1400 rpm (300g), 4°C
- Take off supernatant completely
- Dissolve cell pellet in 80 µl MACS buffer** (per 10^7 cells) (* Adjust the volume until a concentration of 10^7 cells in 80 µl MACS buffer** (=1,25x10^8 cells/ml *).
  It might be hard to dissolve the pellet, do not add any pellet on the MACS column
- Add 20 µl CD22 microbeads and mix well
- Incubation: 15-20 minutes in the refrigerator
- Add 2 ml MACS buffer** (washing step)
- Centrifugation: 10 minutes, 1400 rpm (300g), 4°C
- Take off supernatant completely
- Add 500 µl MACS buffer** (maximum 10^8 cells in this amount of buffer)

Magnetic separation
- Put LS column in magnetic field of the MACS separator
- Pre-wash the LS column with 3 ml MACS buffer** (do not use the plunger)
- Run 500 µl of cells through the LS column
- Non-labelled cells pass through
- Wash the LS column 3 times with 500 µl MACS buffer** and collect this together with the non-labelled cells. Only add new MACS buffer** when the column reservoir is empty.
- Remove LS column from magnetic field and put it on a collection tube
- Run 1 ml of MACS buffer** through the LS column. Immediately flush out fraction with the magnetically labelled cells by firmly applying the plunger supplied with the LS column
- Centrifugation: 1200 rpm, 10 minutes, 4°C
- Resuspend pellet in 1 ml (or less) culture medium (RPMI 1640)
- Count cells and check with trypan blue for dead cells
- Store them on ice until you can put everything in the culture plates (or freeze them in freezing medium → 90% heat-inactivated Fetal Calf Serum (FCS) and 10% dimethylsulfoxide (DMSO))

Supplement section B : Ficoll separation and irradiation of PBMCs

WORK STERILE!!! Use blood flow cabinet

- Collect at least 50 ml blood from healthy person

Reagentia:
- RPMI 1640 (blanco) (Invitrogen, Carlsbad, USA)
- Histopaque-1077 (ficoll) (warm to RT before use) (Sigma Aldrich, St. Louis, USA)
- RPMI 1640 + 10% FCS + 2,5 ml penicillin/streptavidin (P/S) (Invitrogen, Carlsbad, USA) (culture medium)

Materials:
- PTW-Unidos (PTW, Freiburg, Germany)
- 50 ml tubes
- Sterile plastic pipets
- 15 ml tubes
- 10 ml pipettes

Protocol:
Ficoll separation
- Remove the rubber lids from the blood tubes using a tissue
- Centrifugation: 900g for 15 minutes at room temperature (RT) (separates serum and blood)
- Take of the serum, do not take red blood cells (improves ficoll reaction, but not necessary to perform the protocol)
- Wash blood tubes with circa 1 ml blanco and put this in new 50 ml tube
- Blood needs to be diluted circa 1/2 (100 ml in total):
  Per tube: 35 ml diluted blood and 15 ml ficoll (at RT)
    Divide blood in the 50 ml tubes and the one with washing
    Add blanco up to 35ml
- Mix the diluted blood using a pipet
- Add 15 ml ficoll to new 50 ml tubes
- ! Add the blood carefully to the ficoll: Put two tubes to each other, almost horizontal. Move slowly in order to make contact between blood and ficoll. Add the blood very slowly on the ficoll. Be careful!
- Centrifugation: 20-30 minutes at 1800 rpm (400g) at RT **without brake**
- After centrifugation
    Take off a part of the serum+blanco carefully using a pipet
    Put white band containing mononucleair cells in a 50ml tube
    Add blanco medium to the cells up to 50 ml
    Mix by turning the tube upsidedown
- Centrifugation: 10 minutes at 300g
    Cells are in the pellet
    Take of supernatant using a pipet
    Resuspend the pellet in a few ml blanco and put solution from all tubes in one 10 ml tube
    Add blanco up to 10 ml (wash step to remove the blood plates)
- Centrifugation: 10 minutes at 1600 rpm (200g)
- Take off supernatant
  Resuspend the pellet in 10 ml blanco
- Centrifugation: 10 minutes at 1400 rpm (200g)
- Take off all supernatant
  Resuspend the pellet in 10 ml culture medium
- Count the amount of cells in the Burker-turk counting chamber

Irradiation of PBMCs
- Irradiate PBMCs radioactively with 8275 Rad (circa 4779 Gy after recalculation)
- High frequent sound: irradiation starts
- Wash cells with RPMI: add RPMI and centrifuge 10 minutes at 1400 rpm
- Take off supernatant, dissolve pellet in culture medium
- Count the amount of cells in the Burker-turk counting chamber
- Take up the cells in culture medium at a final concentration of 100 000 cells per 90 µl

Supplement section C : Dot blot for human antibody detection

When using the supernatant from EBV-immortalized B-cells, do always kill the virus particles that may be present by using UV-light from the stratalinker UV crosslinker (Stratagene, La Jolla, USA).

Reagentia:
- Nitro blue tetrazolium (NBT) + 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Madison, USA)
- goat anti-human IgG AP (Promega, Madison, USA)
- non fat dry milk (NFDM) (Biorad, Hercules, USA)
- human IgG (positive controle) IVIg

Buffers:
- Blocking buffer:
  5% NFDM in Phosphate Buffered Saline (PBS)

- Alkaline phosphatase (AP) substrate buffer:
  100 mM Tris (Tris Base)
  100 mM NaCl
  5 mM $MgCl_2$
  pH 9,5

- AP substrate:
  10 ml AP substrate buffer
  66 µl NBT stock (100 mg NBT in 2 mL 70% dimethylformamide (DMF))
  33 µl BCIP stock (100 mg BCIP in 2 mL 100% DMF)
  mix (substrate is stable for 1 hour)

- Wash buffer:
  PBS with 0,05 % Triton X-100

Protocol:
- spot 5 µL samples on nitrocellulose blotting membrane (keep 8-10 mm distance)
  Use human IgG as positive control (1/1 (1mg/ml), 1/10, 1/100, 1/1000) and culture medium as negative control
- dry blot for 15 minutes
- incubate membrane in blocking buffer for 30 minutes (mix gently on shaker)
- incubate membrane for 1 hour with goat anti-human AP (1:500) in blocking buffer
  (1 mL for 5 cm x 5 cm between two layers of parafilm, keep in humified box)
- Wash 5 times for 2 minutes with washing buffer (mix gently on shaker)
- Incubate 1-60 minutes (normal 5 minutes) with AP substrate until dots appear.
- Dispose of substrate solution and rinse blot with excess tap water.
- Dry

Supplement section D: Radio-immunoassay (RIA)

- Put in eppendorph tubes : 20 µl B cell culture supernatant (in triplo)

- Put in eppendorph tubes : 20 µl of diluted patient serum (in triplo : dilution 1/4, 1/10, 1/100, 1/1000 in PBS)

- Put in eppendorph tubes : 20 µl standard → IgG 637 (serial dilution : 1/10, 1/100, 1/1000, 1/10 000 in PBS ; starting with circa 1 g/l) (in triplo)

- Put in eppendorph tubes : negative control → 20 µl culture medium (in triplo)

- Mastermix per sample :12,5 µl TE extract
  0,125 µl radioactively-labeled alpha-bungarotoxin
  0,5 µl normal human serum (co-precipitant)

- Add 13 µl of mastermix to each sample
- Overnight incubation at 4°C
- Add 100 µl goat anti-human IgG per sample
- Incubation : 4 hours at 4°C
- 10 minutes centrifugation at 15 000 rpm, 4°C
- Remove supernatant
- Add 1 ml PBS 0,5% Triton X-100, vortex until pellet detaches
- 5 minutes centrifugation at 15 000 rpm, 4°C
- Remove supernatant
- Add 1 ml PBS 0,5% Triton X-100, vortex until pellet detaches
- 5 minutes centrifugation at 15 000 rpm, 4°C
- Remove supernatant
- Use pellet for measuring radioactivity

Supplement section E : Antigenic modulation experiment

Materials

- 24 well plates (Greiner Bio-One, Monroe, USA) or 48 well plates (Greiner Bio-One, Monroe, USA)
- counter tubes (Sarstedt, Nümbrecht-Rommelsdorf, Germany)
- IMDM (Invitrogen, Carlsbad, USA)
- P/S (Invitrogen, Carlsbad, USA)
- pyruvate (Invitrogen, Carlsbad, USA)
- dexamethasone (12,5 mM in ethanol)
- cycloheximide (25x stock = 1 mg/ml in IMDM)
- trypsin (Invitrogen, Carlsbad, USA)
- sterile PBS (Invitrogen, Carlsbad, USA)
- 0,5 M sodium hydroxide in H_2O

- unlabelled α-bungarotoxin (1 mg/ml in PBS; Sigma Aldrich, St. Louis, USA)
- iodine 125-labelled-α-bungarotoxin (PerkinElmer, Waltham, USA)

- culture medium:
  10% heat-inactivated FCS, 1% pen/strep, 1% pyruvate and 1/5000 dexamethasone stock solution in IMDM

- modulation medium:
  cycloheximide stock solution (1/25 in culture medium)

- labeling medium:
  iodine 125-labelled-α-bungarotoxin (1/5000 in culture medium)

Protocol

- trypsinize confluent TE671 cells from 1 x 225 cm^2 flask and resuspend them in 160 ml culture medium. Plate cells at 0,5 ml per well
- grow to confluence + 2 days (Friday plating the cells, Tuesday experiment)
- replace culture medium and grow over night (O/N) (on Monday)
- aspirate the O/N culture supernatant. Add B cell culture supernatant at 0,2 ml per well (pipette slowly against the edge of the wells). Allow 15 minutes handling time for each plate and keep this time difference for all subsequent steps. Incubate for 3 hours at 37°C
- aspirate the supernatant. Wash with 0,5 ml prewarmed PBS (37°C; use serological pipette to prevent cell loss). Add 100 µl labeling medium. To determine non-specific radioactivity, add 10 µl unlabeled α-bungarotoxin in a number of wells before the addition of the labeling medium. Incubate for 1 hour at 37°C
- aspirate the supernatant, wash cells 3 times with 0,5 ml prewarmed PBS
- add 0,5 ml/well 0,5 M NaOH to remove cell-bound radioactivity. Lyse for 10 minutes (or longer) and transfer into counter tubes and count for 1 minute per sample. Solution is viscous, carefully remove as much possible from the wells.

Supplement section F : Anti-striated muscle antibody test system

- Use the ScimedX kit (ScimedX, Denville, USA)
- Put the substrate slides on RT. Avoid touching the antigen areas.
- Do not dilute the samples 1:40 in PBS as described in kit protocol-> undiluted supernatant!

Protocol

First incubation

- Mark the slides with a pencil
- Put on the slides:
  30 µl 1 positive (kit)
  30 µl 1 negative (kit)
  30 µl 1 negative (culture medium)
  30 µl 1 negative (PBS)
  30 µl 1 empty slide (only secondary) (5 controls per testing day)
  30 µl patient serum 1:40 diluted in PBS
  30 µl B cell culture supernatant (write down which clones you test!)
- Incubate for 2-3 hours at RT in a moist room (take staining box with wet tissues)

Washing

- Remove the slide from moisture chamber and tap the slide on its side to allow the supernatant to run off onto a piece of towel.
- Rinse the slides with PBS (not directly on the slides!)
- Put slides in slide-holder and wash 2 times for minimum 5 minutes with PBS
- Take the slides out, and dry them, don't touch the wells itself!! DO NOT ALLOW TISSUE TO DRY!

Adding conjugate and second incubation

- Add 30 µl conjugate per antigen well
- Incubate 30 minutes at RT in moist room (box with wet tissues)

Washing

- Remove the slide from moisture chamber and tap the slide on its side to allow the supernatant to run off onto a piece of towel.
- Rinse the slides with PBS (not directly on the slides!)
- Put slides in slide-holder and wash 2 times for minimum 5 min with PBS
- Take the slides out, and dry them, don't touch the wells itself!! DO NOT ALLOW TISSUE TO DRY!

Cover slides and analysis

- Add 2-3 droplets mounting medium to the slides. Cover with coverslip. Mounting medium has to be everywhere and no air bubbles may be present. Store them in maps to protect the fluorescence against light.
- Fluorescence-microscope: magnification: 20x (screening) and 40x (analysis)

Supplement section G : Antibodies against NMJ of monkey striated muscle

Protocol

- Monkey tissue (close to human): throw tissues in autoclave container, rinse with chloorhexidine
- Put the slides on RT for 30 minutes. Avoid touching the antigen areas.
- Fixation of muscle sections: 10 minutes in cold acetone (-20C) in cold room
- Dry the slides for 30 minutes

First incubation

- Mark the slides with a pencil
- Add 40 µl undiluted B-cell supernatant to slides
- 40 µl Patient serum 1:40 in 1X PBS
- 40 µl Positive control: IgG 637 (1:100 dilution in 1X PBS of 1mg/ml start concentration)
- 40 µl Negative control: human IVIg (1:100 dilution in 1X PBS of 1mg/ml start concentration)
- Incubate for 2-3 hours at RT in a moist room (take box with wet tissues)

Washing

- Remove the slide from moisture chamber and tap the slide on its side to allow the supernatant to run off onto a piece of towel.
- Rinse the slides with 1X PBS (not directly on the slides!)
- Put slides in slide-holder and wash 3 times 5 minutes with 1X PBS
- Take the slides out, and dry them with tissue, don't touch the wells itself!! DO NOT ALLOW TISSUE TO DRY!

Alfa-bungarotoxin incubation

- Alexa-594 alfa-bungarotoxin (1:300 in 1X PBS)
- Incubate 30 minutes at RT in a moist chamber
- Incubation in dark to protect fluorescence

Washing

- 3 times 5 minutes in 1X PBS
- Washing steps in dark

Secondary antibody incubation

- Add secondary sheep-anti-human IgG FITC (pre-absorbed for monkey) (1:100 in 1X PBS) per antigen well
- Incubate 60 minutes at RT in moist room (box with wet tissues)

Washing

- 3 times 5 minutes in 1X PBS
- Washing steps in dark

Cover slides and analysis

- Add 2-3 droplets mounting medium to the slides. Cover with coverslip. Mounting medium has to be everywhere and no air bubbles may be present. Store them in maps at 4°C to protect the fluorescence against light.
- Fluorescence-microscope: magnification: 20x (screening) and 40x (analysis)

Supplement section H : FACS analysis

Buffers

- FACS-buffer
  PBS with 5% FCS and 0,1% sodium azide
- Washing buffer
  PBS with 0,5% BSA and 0,1% sodium azide

Protocol

- Put the cells in eppendorph tubes. For each staining, 1 tube containing 200 000 cells will be used
- Add volume up to 300 µl/tube with FACS-buffer
- Centrifugation: 5 minutes, 4000 rpm, 4°C
- Take off supernatant (be careful with the pellet!)
- Add antibody: Add 90 µl FACS-buffer
  Add 5 µl antibody per well
- Incubation: 30 minutes, 4°C
- Washing: Add 200 µl washing buffer
  Centrifugation: 5 minutes, 4000 rpm, 4°C
  Take off supernatant
- Second washing: Resuspend cells in 300 µl washing buffer
  Centrifugation: 5 minutes, 4000 rpm, 4°C
  Take off supernatant
- Third washing: Resuspend cells in 300 µl washing buffer
  Centrifugation: 5 minutes, 4000 rpm, 4°C
  Take off supernatant
- Resuspend cells in 400 µl washing buffer and transfer to microtubes
- Read the samples with FACSCalibur Flowcytometer or store at 4°C (max 6h)

Supplement section I : ELISA for actin, myosin and alpha-actinin

Materials
- Immunolon 96 wells plate
- Antigen (actin, myosin, alpha-actinin)
- Antibody (B cell supernatant)
- Goat anti-Human IgG Fc PO
- Goat anti-mouse IgG PO

Buffers
- Coating buffer
  50 mM sodium carbonate (MW = 105,99 g/mol), pH 10,6 or pH 9,6

- Blocking and Incubation buffer
  0,5 % BSA/PBS/0,02% Tween-20

- Washing buffer
  PBS/0,05% Tween-20

- Substrate solution (has to be made on same day)
  1 ml stock substrate buffer
  9 ml water
  100 µl TMB (10 mgTMB/1 ml DMSO)
  just before use add: 1 µl H_2O_2 (30%)

- Stock substrate buffer
  Sodium Acetate citric acid buffer, 1,1 M (pH 5,5):
  37,4 g $CH_3COONa*3H_2O$ (MW = 136,08 g/mol) in 250 ml H_2O. pH corrected with citric acid (59,2 g/50ml) (MW = 210,14 g/mol)

- Stopping solution
  2 M H_2SO_4

Protocol
- Dispense 50 µl/well of antigen solution at a concentration of 1 µg/ml in coating buffer.
  Seal the plates with plastic and incubate for 1-2 hour at 37°C.
- Wash 5 times with washing buffer, 100 µl/well.
  Do not touch or scratch the surface of the wells where the antigen is bound to the plate.
- Block with blocking buffer, 100 µl/well,
  Seal the plate with plastic and incubate 30 minutes at 37°C
- Do not wash. Slap the plate 3 times upside down on a paper towel to remove residual liquid.
- Add samples and positive controls in duplo, 50 µl/well. Do not touch or scratch the wells!
  a. B cell supernatant: undiluted
  b. Positive control antibody: diluted in blocking/incubation buffer
  i. 1/10
  ii. 1/100
  iii. 1/1000
  iv. 1/10 000

  Seal the plate with plastic and incubate 60 minutes at 37°C
- Wash 5 times with washing buffer, 100 µl/well
- Secondary antibody diluted 1:5000 in incubation buffer, 50 µl/well. Do not touch or scratch the wells!
  a. Goat anti-human IgG Fc PO
  b. Goat anti-mouse PO

  Seal the plate with plastic and incubate 60 minutes at 37°C.
- Wash 5 times with 100 µl/well of washing buffer.
- Add 100 µl/well substrate solution
  Incubate 10 minutes. A blue color will form in the wells.
- Stop the reaction by adding 50 µl 2M H_2SO_4 in the same order in which substrate was added. Avoid creation of air bubbles.
  Blue color will change to yellow upon acidification.
- Measure absorption at 450 nm within 30 minutes of stopping the reaction.

Supplement section J : Anti-titin antibody ELISA

Protocol

Sample incubation

- Dispense 100 µl of ready for use Calibrator, ready for use Negative and Positive control and diluted samples into the corresponding wells.
- Incubate 60 minutes at RT on horizontal shaker

Washing

- Discard or aspirate the contents of the wells and wash thoroughly with each 300 µl wash buffer
- Repeat the washing procedure 2 to 3 times
- Remove residual liquid by tapping the inverted plate on clean absorbent paper

Conjugate incubation

- Dispense 100 µl enzyme conjugate into each well
- Incubate for 30 minutes at RT on a horizontal shaker

Washing

- Discard or aspirate the contents of the wells and wash thoroughly with each 300 µl wash buffer
- Repeat the washing procedure 2 to 3 times
- Remove residual liquid by tapping the inverted plate on clean absorbent paper

Substrate incubation

- Dispense each 100 µl substrate in the wells and incubate for 15 to 25 minutes on a horizontal shaker

Stopping

- Dispense each 100 µl stop solution into the wells in the same order as with the substrate

Measurement

- Read the optical density at 450 nm with the benchmark microplate reader (BioRad Laboratories, Hercules, USA)

Supplement section K : Mega western protein array

Primary antibody composition:
- Undiluted primary antibody (B cell culture supernatant)
- Mouse anti-human GAPDH antibody: 1/500 dilution (Cytomol, Union City, USA)

Secondary antibody composition:
- Goat anti-human 800: 1/15 000 (Rockland, Gilbertsville, USA)
- Donkey anti-mouse 700: 1/15 000 (Rockland, Gilbertsville, USA)
  → Secondary antibodies diluted in Odyssey Blocking Buffer (LiCor BioSciences, Lincoln, USA)/PBS (1/1)

Protocol:
- Block the mega western protein array (Cytomol, Union City, USA) with Odyssey Blocking Buffer/PBS (1/1) for one hour at RT with agitation. Use black box with lid
- Remove the blocking reagent and add the primary antibody solution. Incubate with agitation for one hour at RT (or overnight at 2-8°C). Incubate in small plastic bag, 2ml of liquid is enough
- Wash the array in PBS 0,1% Tween-20 for 5 minutes, repeat 3-4 times. Use black box with lid
- Incubate the array with secondary antibody solution for one hour at RT with agitation. Incubate in small plastic bag, 2 ml of liquid is enough
- Wash the array in PBS 0,1% Tween-20 for 5 minutes, repeat 3-4 times. Use black box with lid
- Detect signals with Odyssey Infrared Imaging System (LiCor BioSciences, Lincoln, USA)

Supplement section L : RNA isolation

Remarks:
- Work in RNA lab
- Wear gloves
- Any water or salt solutions used in RNA preparations should be treated with DEPC

Test principle:
- Cultured cells are lysed by a special lysis-binding buffer. At the same time, RNases are inactivated.
- Nucleic acids are bound to the glass fibers pre-packed in the high pure filter tube
- Residual contaminating DNA is digested by DNase I, applied directly on the glass fiber fleece.
- Bound nucleic acids are washed with a special inhibitor removal buffer to get rid of RT-PCR inhibitory contaminants
- Washing of bound nucleic acids, purification from salts, proteins and other cellular impurities
- RNA is recovered using the elution buffer

Protocol:
- Resuspend the cells in 200 µl PBS (diluted in DEPC water)
- Add 400 µl lysis-binding buffer (green cap) and vortex for 15 seconds
- Transfer the sample to a High Pure Filter Tube
- Combine the high pure filter tube and the collection tube and pipette the entire sample in the upper reservoir of the Filter tube (max 700 µl)
- Insert the entire High Pure Filter Tube assembly into a standard tabletop centrifuge.
- Centrifugate the tube assembly 15 seconds at 8000g (10 000 rpm) in a standard microcentrifuge.
- Remove the Filter Tube from the collection tube, discard the 'flowthrough' and recombine the filter tube and the used collection tube.
- Pipette per sample 90 µl incubationbuffer (white cap) in a sterile reaction tube and add 10 µl DNase 1, mix, pipette 100 µl of this solution on the 'glass filter fleece' in the upper reservoir of each filtertube.
- Incubate 15 minutes on 15-25°C (RT)
- Add 500 µl wash buffer 1 (black cap) to the upper reservoir, centrifugate 15 seconds at 8000g (10 000 rpm), discard the 'flowthrough' and recombine the filter- and collection tube
- Add 500 µl wash buffer 2 (blue cap) to the upper reservoir and centrifugate 15 seconds at 8000g (10 000 rpm), discard the 'flowthrough' and recombine the filter- and collection tube
- Add 200 µl wash buffer 2 (blue cap) to the upper reservoir, centrifugate 2 minutes at maximum speed (approximately 13 000g) to remove any residual of wash buffer
- Discard the collection tube and insert the filter tube into a sterile 1,5 ml microcentrifuge tube
- To elute the RNA: add 50-100 µl elution buffer/water to the upper reservoir of the filter tube and centrifugate 1 minute at 8000g (10 000 rpm).
- The microcentrifuge tube now contains the eluted RNA.
- Either use the eluted RNA directly in RT-PCR or store the eluted RNA at -80°C for later analysis. Transfer 3 µl of RNA to new tubes. This can be used to measure the RNA concentration.

Supplement section M : cDNA preparation

Materials:
- Reverse transcription system (Promega, Madison, USA)
- Water (autoclaved or DNAse free)
- Phenol
- Chloroform/isoamylalcohol 24:1
- Sodiumacetate
- 70% and 100% ethanol (cold)

Protocol:
- For 1 sample:

Reagens	Volume
25 mM $MgCl_2$	4 µl
10x reverse transcriptase buffer	2 µl
10 mM dNTP mixture	2 µl
r RNA se inhibitor	0,5 µl
AMV reverse transcriptase (keep on ice)	0,5 µl
Oligo dT primer	1 µl
Total	**10 µl**

- Put 10 µl of the solution in each tube
- Add 1 µg RNA (±10 µl RNA)
- Add nuclease-free water to a final volume of 20 µl
- Mix well and short spin
- Incubation: 60 minutes at 42°C in warm water bath
- IMMEDIATELY put 5 minutes at 90°C in heaterblock
- Incubation: 10 minutes on ice
- Short spin
- Add to each sample (first wash step):
  80 µl water
  50 µl phenol
  50 µl chloroform/isoamylalcohol (24/1)
  OR use 100 µl phenol/chloroform/isoamylalcohol (25/24/1) and 80 µl water
- Vortex very good during 1 minute
- Centrifugation: 2 minutes, 14 000 rpm, RT
- Take supernatant to new tube (Upper phase is water containing the DNA)
- Add 100 µl chloroform/isoamylalcohol (second wash step)
- Vortex very good during 1 minute
- Centrifugation: 2 minutes, 14 000 rpm, RT
- Take supernatant to new tube
- Add 10 µl 3M sodiumacetate
- Add 200 µl 100% icecold ethanol
- Vortex
- Precipitation overnight at -20°C or one hour at -80°C

Next Day or after 1 hour
- Centrifugation: 15 minutes, 14 000 rpm, 4°C
- Discard supernatant
- Wash pellet: add 200 µl 70 % ethanol
- Centrifugation: 10 minutes, 14 000 rpm, 4°C
- Discard supernatant
- Dry pellet during 5 minutes in the air or use the vacuum-dryer (probably longer)
- Dissolve the pellet in 30-50 µl water (fresh autoclaved water) (or less, when you expect a low DNA concentration)
- Incubate for about one hour on ice to dissolve the DNA in water
- Measure the DNA concentration using the Nanodrop
- Store DNA at -20°C and label properly

Supplement section N : Testing clonality of B cell cloons

Protocol:

- Calculate the amount of µl for the three mastermixes depending on the amount of samples you need (per sample there are 3 reactions → 3 primermixes : FR1, FR2, FR3)
  PCR-mix :

Solutions	Concentration in PCR	µl per sample
10x PCR buffer	1x	2,5
dNTPs (6,25 mM for each dNTP)	0,25 mM	1
FR Forward primer mix (1, 2 or 3)	10 pmol	0,7
Primer Biomed JH-con FAM (100 pmol/ µl)	10 pmol	0,1
Sterile H_2O		7,8
Jumpstart Red Taq polymerase (1U/ µl)	1U	1
Totaalvolume		**13,1**

- Per sample : 13,1 µl of the mastermix + 1 µl DNA + 10,9 µl sterile H_2O = 25 µl
- Run the program 'B-cel' IGH-BIOM on the PTC200 DNA Engine Thermal Cycler PCR (Scientific Support, Hayward, USA)
  95°C 7 minutes

  35 cycli:
  95°C 45 seconds
  60°C 45 seconds
  72°C 90 seconds

  72°C 10 minutes
  15°C ∞

- Load 10 µl of the PCR products on a 2% agarose gel
- Gelelectrophoresis
- Analyze the results of the electrophoresis on the Molecular Imager Gel Doc XR System (Bio-Rad Laboratories, Hercules, USA)
- If the samples of each PCR-mix give a fragment of the right size (PCR-mix FR1: 310-360 bp; PCR-mix FR2: 250-295 bp; PCR-mix FR3: 100-170 bp), the samples will be analyzed with fragmentanalysis. For this, 2 µl PCR-product is added to 1 µl internal size standard and 10 µl formamide. The mix is then analyzed by the genetic analyzer ABI3100 (Applied Biosystems, Foster City, USA) which detects the FAM-labels of the PCR-products

Supplement section O : Sequence reaction

Protocol:

- PCR-mix :

Solutions	µl per sample
PCR product	1
JH con primer	1
Big Dye v1.1	1
Sequencebuffer	1,5
Sterile H_2O	5,5
Totaalvolume	**10**

- PCR-conditions :
  96℃ 5 minutes

  25 cycli:
  96℃ 10 seconds
  50℃ 5 seconds
  60℃ 4 minutes

  4℃ ∞

- The samples are then analyzed by the genetic analyzer ABI3100 (Applied Biosystems, Foster City, USA) which detects the different labelled dyes of the different ddNTPs.