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Proteasome Inhibition with Bortezomib Depletes Plasma Cells and Autoantibodies in Experimental Autoimmune Myasthenia Gravis

Alejandro M. Gomez,* Kathleen Vrolix,* Pilar Martínez-Martínez,* Peter C. Molenaar,* Marko Phernambuco,† Eline van der Esch,*‡ Hans Duimel,‡ Fons Verheyen,‡ Reinhard E. Voll,‡ Rudolf A. Manz,§ Marc H. De Baets,*‡ and Mario Losen*

Bortezomib, an inhibitor of proteasomes, has been reported to reduce autoantibody titers and to improve clinical condition in mice suffering from lupus-like disease. Bortezomib depletes both short- and long-lived plasma cells; the latter normally survive the standard immunosuppressant treatments targeting T and B cells. These findings encouraged us to test whether bortezomib is effective for alleviating the symptoms in the experimental autoimmune myasthenia gravis (EAMG) model for myasthenia gravis, a disease that is characterized by autoantibodies against the acetylcholine receptor (AChR) of skeletal muscle. Lewis rats were immunized with saline (control, n = 36) or Torpedo AChR (EAMG, n = 54) in CFA in the first week of an experimental period of 8 wk. After immunization, rats received twice a week s.c. injections of bortezomib (0.2 mg/kg in saline) or saline injections. Bortezomib induced apoptosis in bone marrow cells and reduced the amount of plasma cells in the bone marrow by up to 81%. In the EAMG animals, bortezomib efficiently reduced the rise of anti-AChR autoantibody titers, prevented ultrastructural damage of the postsynaptic membrane, improved neuromuscular transmission, and decreased myasthenic symptoms. This study thus underscores the potential of the therapeutic use of proteasome inhibitors to target plasma cells in Ab-mediated autoimmune diseases. The Journal of Immunology, 2011, 186: 000–000.
overlying nerve ending. These proteins are thus essential for muscle contraction, and MG is potentially fatal, since autoantibodies can cause respiratory failure by impairing neuromuscular transmission. In such an event of acute myasthenic crisis, the first choice of treatment is plasma exchange, mechanical ventilation (14), and i.v. treatment with high doses of IgG (15). Plasma exchange typically leads to significant improvement or remission within a few days in most patients, including those with the idiopathic MG, but the effect is, of course, not long-lasting. Immunosuppressive drugs such as prednisone and azathioprine are generally used for long-term therapy (1, 16–18). Because these depend mostly on preventing the activation, proliferation, and differentiation of developing B and T cells, the autoantibody titers only drop over a period of months. Using an established immunotherapy protocol with prednisone and azathioprine, it may take as much as 18 mo before patients improve (16). For the intermediate time interval, during which plasma cells continue to produce autoantibodies, proteasome inhibition might be a useful therapy. Moreover, because some MG patients do not respond well to any currently available treatment in terms of poor reduction of autoantibodies or the occurrence of side effects, proteasome inhibition might provide a therapeutic alternative.

In the experimental autoimmune myasthenia gravis (EAMG) model the disease is induced by immunizing rats with the AChR from the electric organ of the electric ray Torpedo californica (19, 20). A small proportion of Abs against the Torpedo AChR cross-reacts with the AChR of the muscle (21). As in human MG with anti-AChR autoantibodies, the disease symptoms in EAMG are caused by Ab-mediated destruction of the neuromuscular junction (22, 23).

In this study, we examined the effect of bortezomib in EAMG rats using two different treatment regimes. The first treatment consisted of bortezomib injections for 8 wk, starting directly after immunization (herein referred to as 8w-Bz). For the second treatment regimen, rats were injected with bortezomib starting only 4 wk after immunization, when autoantibody titers were already detectable, until 8 wk after immunization (4w-Bz). Using this setup we investigated the potential therapeutic effect of bortezomib after onset of the disease. The results show that both treatment regimes reduce autoantibody levels by depleting bone marrow plasma cells, but only the 8 wk bortezomib-treatment led to a significant improvement of the clinical condition of the EAMG rats and to a reduction of postsynaptic damage.

Materials and Methods

Animals

Inbred female Lewis rats (n = 90) were obtained from the Department of Experimental Animal Services, Maastricht University (The Netherlands). Permission to perform this experiment was granted by the Committee on Animal Welfare, according to Dutch governmental rules. At 6 wk of age, animals were weighed and divided into six experimental groups (Table I) with an equal average weight.

Table I. Treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Saline Groups (No Bortezomib)</th>
<th>4w-Bz Groups (Bortezomib Started 4 wk after Immunization)</th>
<th>8w-Bz Groups (Bortezomib Started Directly after Immunization)</th>
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</thead>
<tbody>
<tr>
<td>Control (immunized with CFA)</td>
<td>n = 12 (IF and FACS, n = 5)</td>
<td>n = 12 (IF and FACS, n = 5)</td>
<td>n = 12 (IF and FACS, n = 5)</td>
</tr>
<tr>
<td></td>
<td>EM and EMG, n = 7</td>
<td>EM and EMG, n = 7</td>
<td>EM and EMG, n = 7</td>
</tr>
<tr>
<td>EAMG (immunized with AChR in CFA)</td>
<td>n = 18 (IF and FACS, n = 8)</td>
<td>n = 18 (IF and FACS, n = 8)</td>
<td>n = 18 (IF and FACS, n = 8)</td>
</tr>
<tr>
<td></td>
<td>EM and EMG, n = 10</td>
<td>EM and EMG, n = 10</td>
<td>EM and EMG, n = 10</td>
</tr>
</tbody>
</table>

Induction of EAMG

EAMG was induced in 7-wk-old rats (n = 54) by active immunization with AChR purified from the electric organ of T. californica (tAChR) in CFA. In brief, 20 μg tAChR (20) was dissolved in 100 μl PBS and emulsified with an equal volume of CFA with 0.1% of Mycobacterium tuberculosis H37 (Difco Laboratories, Detroit, MI). Animals were initially anesthetized in a cylindrical tube through which 5% isoflurane in air was supplied. Subsequently, 3% isoflurane was supplied by a cap over the head and 200 μl CFA/tAChR emulsion was injected s.c. at the base of the tail at three different spots, as described by Lennon and colleagues (24). Control animals (n = 36) were injected with an equal volume of emulsified PBS and CFA. Rats were sacrificed 8 wk after immunization or earlier, if they had reached the humane endpoints as described below. They were sacrificed by inhalation of CO2 in air and subsequent cervical dislocation.

Experimental design and administration of drugs

Bortezomib was purchased as a lyophilized powder (Velcade; Janssen-Cilag, Beersel, Belgium) and dissolved in sterile saline solution at a final concentration of 0.1 mg/ml. Two weekly doses of 0.2 mg/kg bortezomib solution were administered s.c., which is considered the highest dose to use without having increased mortality rates or severe side effects in rats (10, 25, 26). Control and EAMG groups were subdivided into three treatment regimes each (Table I). “Saline” groups received two weekly s.c. injections of 0.9% NaCl solution (2 ml/kg) at the neck for 8 wk. The 4w-Bz groups received two weekly saline injections for the first 4 wk after immunization (by which time autoantibody levels were highly elevated in the plasma) and subsequently two weekly injections of bortezomib for another 4 wk. The 8w-Bz groups received two weekly injections of bortezomib for 8 wk, starting directly after immunization.

For practical reasons the experiment was conducted three times in sets of 30 animals each, including an equal number of all the aforementioned groups. The animals’ tissues were analyzed using electron microscopy (EM), electromyography (EMG), immunofluorescence (IF), and FACS, and the number of animals used for each method is indicated in Table I.

Weight and clinical scoring

The weights of animals were measured on a weekly basis as a general indicator of health and for dose calculations. The severity of clinical signs of EAMG was scored weekly by measuring muscular weakness by two blinded investigators (A.M.G. and M.P.). The animals’ muscle strength and fatigability was assessed by their ability to grasp and lift repeatedly a 300-g metal rack from the table, while suspended manually by the base of the tail for 30 s (27–29). Clinical scoring was based on the presence of tremor, hunched posture, muscle strength, and fatigability. Disease severity was expressed as follows: 0, no obvious abnormalities; 1, no abnormalities before testing, but reduced strength at the end; 2, clinical signs present before testing, that is, tremor, head down, hunched posture, weak grip, difficulty in breathing; 3, severe clinical signs present before testing, no grip, moribund (24).

Animals that reached a clinical score of level 3, or lost more than 20% of their weight in the course of 1 wk, were sacrificed within 24 h.

Tissue preparation

For EM analysis, bone marrow was extracted from the femurs by cutting off their upper and lower endings and flushing the shaft with 10 ml sterile saline solution through the bone marrow cavity using a syringe with a 25-gauge needle. For FACS analysis of bone marrow, PBS containing 2% FCS and 0.1% NaN3 (FACS buffer) was used instead. Thymus and spleen tissue were entirely removed from the animals by dissection and the organ weight was measured. Single-cell suspensions were prepared using a gentleMACS tissue dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Cells of different tissues were resuspended in FACS buffer and run through a 70-μm nylon cell strainer to

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remove large cell clumps. Numbers of viable cells were determined by counting trypan blue negative cells on hemocytometers. Cells were divided into microcentrifuge tubes (10^6 cells/100 μl/tube) and then stained for dead cells, T cells, and B cells.

Heparinized blood samples were taken from the vena saphena weekly, starting on the week before immunization (week 0) until the last week of experiment (week 8). PBMCs were isolated by lysing RBCs using FACS lysing buffer (BD Biosciences). PBMCs from 80 μl blood were divided in microcentrifuge tubes in a ratio of 20:20:40 μl to stain for dead cells, T cells, and B cells, respectively.

Proteasome activity assay

The proteolytic activity of the proteasome was evaluated in whole blood (30) by means of a 20S proteasome activity kit (APT 280; Millipore), as described by the manufacturer. Taking into account the pharmacokinetic and pharmacodynamic profiles of s.c. bortezomib administration (31), blood samples were obtained between 3 and 6 h after administration of bortezomib or saline. In brief, 50 μg whole blood protein extract was incubated in the provided buffer with 3.8 μg fluorophore-linked peptide substrate (LLVY-7-amino-4-methylcoumarin [AMC]) for 120 min at 37°C. Proteasome activity was measured by quantification of relative fluorescent units from the release of the fluorescent-cleaved product AMC using a 380/460 nm filter set in a fluorometer (Victor X3 multilabel reader; PerkinElmer). A solution of the 20S proteasome subunit and the proteasome inhibitor lactacystin were used as controls for the assay.

Autoantibody titers

Ab titers against rat AChR were measured in plasma samples with an immunoprecipitation RIA. In brief, 2.5 μl plasma was incubated at 4°C overnight with 100 μl rat muscle cell-membrane extract (containing ~5 nmol/l AChR; isolated from denervated rat muscles). The AChR was labeled with an excess of [125I]-α-bungarotoxin (NEX126, 5 TBq/mmol; PerkinElmer). The immune complexes were precipitated by addition of 150 μl goat anti-rat IgG serum and incubation for 4 h at 4°C. Pellets were washed three times in PBS with 0.5% Triton X-100 and centrifuged at 25,000 × g for 5 min. Radioactivity was measured on an automatic gamma counter (2470 Wizard2; PerkinElmer). Autoantibody titers were expressed as nanomoles of α-bungarotoxin binding sites per liter.

Total IgG ELISA

A sandwich ELISA was used to measure total IgG content in plasma samples. Briefly, ELISA plates (Microlon 655092; Greiner Bio-One, Frickenhausen, Germany) were coated with 50 μl catching Ab (goat Ig anti-rat IgG, ab6252; Abcam, Cambridge, U.K.), diluted 1:200 in coating buffer (50 mM sodium carbonate [pH 9.6]), for 1 h at 37°C. Plates were washed with PBS containing 0.05% Tween 20 and blocked for 30 min with 100 μl 1% nonfat dry milk in PBS (blotting grade blocker, catalogue no. 17040-04; Bio-Rad). Afterward, 50 μl plasma samples, diluted 1:20,000 in incubation buffer (PBS plus 1% BSA plus 0.02% Tween 20), were incubated for 1 h at 37°C. A standard curve was made using serially diluted samples of purified rat IgG (catalogue no. I8015; Sigma-Aldrich). After washing, 50 μl HRP-conjugated Ab (ab6257; Abcam), diluted 1:5000 in incubation buffer, was added and plates were incubated for another hour at 37°C. Following a washing step, 100 μl tetramethylbenzidine substrate solution (s(HS)TMB; SDT Reagents, Baesweiler, Germany) was used to develop HRP-labeled Abs bound to the plates. The color reaction was allowed to develop for 10 min and stopped with 50 μl 2 M sulfuric acid. The OD was measured at 450 nm filter using a microplate reader (Victor X3 multilabel reader). Results were expressed as milligrams of total IgG per milliliter of plasma.

Apoptosis assays

Early apoptotic and dead cells were identified by flow cytometric analysis according to surface binding of FITC-labeled Annexin V to exposed membrane phosphatidylserine and propidium iodide (PI) staining (annexin V-FITC apoptosis detection kit; BD Biosciences, Breda, The Netherlands). The cells (10^7/100 μl) were washed with annexin V-binding buffer and incubated with 5 μl annexin V and 5 μl PI for 15 min at room temperature. Without washing, cells were immediately measured. Annexin V+/PI- cells were regarded as early apoptotic, while annexin V+/PI+ cells were considered dead cells.

Extracellular staining for T cell markers

The cells were washed once with FACS buffer by centrifugation at 250 × g at 4°C and incubated for 30 min at 4°C with Abs directed against CD3 (FITC-labeled), CD4 (PE-labeled), and CD8 (PerCP-labeled) (all from BD Biosciences), diluted 1:50 in FACS buffer. The samples were washed twice and kept at 4°C in the dark until measurement within 2 h.

Intracellular staining for B cell markers

Cells were washed once with FACS buffer by centrifugation at 250 × g at 4°C and incubated with PE-conjugated Ab against CD45RA (BD Biosciences), diluted 1:20 in FACS buffer. After one washing step, cells were fixed with 2% paraformaldehyde in FACS buffer for 10 min at 37°C. After two more washing steps, cells were permeabilized with cold 90% methanol for 30 min on ice. Cells were washed twice and incubated with FITC-conjugated Ab directed against intracellular Igk (BD Biosciences), diluted 1:20 in FACS buffer. The samples were washed twice and kept at 4°C in the dark until measurement within 2 h. Because no appropriate CD markers were available to specifically detect rat plasma cells, we measured rat plasma cells by using high levels of intracellular Igk expression.

FIGURE 1. Proteasome activity in blood lysates. Samples were incubated with a labeled peptide substrate (LLVY-AMC) and the amount of cleaved fluorophore AMC was measured in a fluorometer. A, Proteasome activities at 4 wk after immunization were normalized using the average relative fluorescence units (RFU) value of the control saline group at week 4. B, Proteasome activities at 8 wk after immunization were normalized using the average RFU value of the control saline group at week 8. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.
FACS analysis

All cytofluorometric analyses were performed on a FACSCalibur (BD Biosciences) and analyzed using the CellQuest software (BD Biosciences). Forward and sideward light angle scatters were collected. Using these plots, samples were gated to exclude cell debris and cellular aggregates for further analysis. For each marker, the percentage of positive cells stained above background was measured for all gated cells. The cutoff was defined using unstained cells processed alongside the experimental samples.

Plasma cell quantification by EM

Bone marrow cell suspensions were fixed by mixing with an equal volume of 5% glutaraldehyde in PBS and then centrifuged at $800 \times g$. Pellets were resuspended in PBS and embedded in 10% gelatin, then centrifuged again at 1000 $\times g$ to form a compact pellet. Cell pellets were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) dehydrated through a graded ethanol series and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections from whole pellets were contrasted with uranyl acetate and lead citrate and viewed with a Philips CM 100 electron microscope. Plasma cell recognition was based on morphological features that are particular to this cell type, such as the typical “cartwheel” chromatin configuration in the nucleus and the presence of extensive rough endoplasmic reticulum in the cytoplasm, indicative of an intense protein production. Quantification was performed at six different portions of the sample to have a systematic representation of the cell gradient in the sample that resulted from the centrifugation. Results are expressed as percentage of plasma cells; a total of 800 bone marrow cells were counted per sample.

Electromyography

Animals were initially anesthetized as described above and subsequently intubated in the trachea. Anesthesia was maintained with 3% isoflurane in air. Compound muscle action potential (CMAP) decrement was measured in the tibialis anterior muscle using the EMG system Viking IV (Nicolet Biomedicals, Madison, WI) at the end of the experimental period. For stimulation, two small monopolar needle electrodes were used. The cathode was inserted near the peroneal nerve at the level of the knee and the anode more proximal and lateral at a distance of 3–4 mm. For recording, a third monopolar needle electrode was inserted s.c. over the tibialis anterior muscle. The reference electrode was inserted s.c. near the ankle. A ring electrode around the tail served as ground electrode. To detect a decremental response of the CMAP, series of eight supramaximal stimuli were given at 3 Hz with 0.2 ms duration. The test was considered positive for decrement when both the amplitude and the area of the CMAP-negative peak showed a decrease of at least 10% (32). To demonstrate reproducibility, at least three consecutive decrement recordings were made of all investigated muscles. During the measurements, skin temperature was kept between 35°C and 37°C by means of an infrared heating lamp. If initially no decrement was present in the tibialis anterior muscle, neuromuscular transmission was challenged by a continuous i.v. infusion of curare [(+)-tubocurarine, T2379; Sigma-Aldrich]. A solution of 20 $\mu$g/ml curare was injected into the vena saphena using a Terfusion syringe pump (model STC-521; Terumo, Tokyo).

FIGURE 2. Electron micrographs of bone marrow plasma cells. After 4 wk of bortezomib treatment, the rough endoplasmic reticulum cisternae have a vesicular appearance; after 8 wk of treatment, pronounced dilatation of the rough endoplasmic reticulum is visible. Cells were stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate. Scale bars, 1 $\mu$m.

FIGURE 3. Analysis of plasma cells in the bone marrow 8 wk after immunization. A, Electron microscopical analysis. Bortezomib treatment decreases the number of plasma cells in the bone marrow. B, Flow cytometric analyses of Igk$^{\text{high}}$ cells. Data are shown as percentages of cells with respect to gated living bone marrow cells. The proportion of Igk$^{\text{high}}$ cells was significantly lower in 4w-Bz and 8w-Bz groups compared with the corresponding saline-treated groups. Horizontal bars represent the mean percentage of each group. Two-way ANOVA and Bonferoni post hoc testing were used for statistical analyses.
Japan) at a rate of 1 ml/h (0.33 μg curare/min). During curare infusion, CMAP measurements were repeated with intervals of 1 min until a repeated decrement was observed. The resistance against curare was used as an indirect, albeit nonlinear, measure for the safety factor of neuromuscular transmission and thus for the performance of neuromuscular transmission (33, 34).

**EM of muscle tissue**

Electron micrographs were taken from endplates of the tibialis anterior muscles. Anesthetized rats were transcardially perfused as previously described (21, 32). Ultrathin sections were viewed with a Philips CM 100 electron microscope. At least five endplate regions were photographed from each muscle. Quantitative morphometry of the folding index (length of postsynaptic membrane/length of presynaptic membrane) was performed as previously described (34, 35). For the analysis, the following number of animals was used per group: control saline (n = 3); EAMG saline (n = 4); EAMG 4w-Bz (n = 4); EAMG 8w-Bz (n = 4). Between 5 and 25 endplate regions were analyzed per animal.

**Statistics**

GraphPad Prism 4 was used to perform statistical analyses. Comparison between normally distributed values was performed using one- or two-way ANOVA, wherever appropriate. Bonferroni post hoc tests were used to compare groups to each other. A two-sided probability value of 0.05 or lower was considered significant. Values are expressed as means ± SEM unless stated otherwise. Clinical scores were analyzed by the χ² test for trend, and survival was analyzed using the log-rank test.

**Results**

**Bortezomib reduces plasma cells in bone marrow**

To investigate whether proteasome inhibition affects plasma cells in vivo, rats were injected with bortezomib or saline. Subcutaneous injections of bortezomib significantly reduced the proteasome activity in rat whole blood (Fig. 1). The effect of bortezomib on plasma cells from the bone marrow was analyzed by EM and by FACS. In the bone marrow of bortezomib-treated rats, plasma cells with altered morphology were frequently observed (Fig. 2), which was characterized by a vesicular appearance of the rough endoplasmic reticulum cisternae or pronounced dilatation of the rough endoplasmic reticulum. The percentage of plasma cells in bone marrow was markedly reduced in bortezomib-treated groups (Fig. 3A). Animals that received bortezomib only between 4 and 8 wk after immunization showed a significant decrease in their percentage of bone marrow plasma cells (57% reduction in the 4w-Bz control group [p < 0.05] and 82% reduction in the 4w-Bz EAMG group [p < 0.01] compared with the corresponding saline-treated groups). Rats in the 8w-Bz EAMG group also showed a strong and significant depletion of bone marrow plasma cells (p < 0.05; 70% reduction compared with the saline-treated EAMG group). Very similar results were obtained by FACS analysis using intracellular staining of the Igκ L chain in bone marrow cells (Fig. 3B).

**Bortezomib affects the lymphoid organs**

We investigated the overall effect of bortezomib treatment on the immune system by measuring the weight of thymus and spleen tissue and analyzing the proportions of B cells and T cell subsets in the thymus, spleen, blood, and bone marrow by FACS (Table II).

Bortezomib treatment significantly reduced the mean thymus weight, both in the 4w-Bz (46% reduction; p < 0.01) and 8w-Bz groups (50% reduction; p < 0.01), compared with the saline group (data not shown). In contrast, the mean spleen weight significantly increased by bortezomib treatment in both 4w-Bz (36% increase; p < 0.05) and 8w-Bz groups (32% increase; p < 0.05) compared with the corresponding saline-treated groups (data not shown). No significant difference was observed between control and EAMG animals.

To assess the effects of bortezomib treatment on the leukocyte viability, we measured the proportion of early apoptotic (annexin V+/PI−) and dead cells (annexin V+/PI+).

**Table II. FACS analysis of lymphoid organs**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Saline</th>
<th>4w-Bz</th>
<th>8w-Bz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead cells, Ann−/PI+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>11.25 ± 1.43</td>
<td>13.63 ± 1.17</td>
<td>14.19 ± 1.94</td>
</tr>
<tr>
<td>Spleen</td>
<td>25.38 ± 2.34</td>
<td>28.00 ± 3.28</td>
<td>31.26 ± 4.25</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>12.93 ± 0.86</td>
<td>14.57 ± 1.20</td>
<td>15.82 ± 1.16</td>
</tr>
<tr>
<td>PBMC</td>
<td>5.72 ± 0.66</td>
<td>2.84 ± 0.17***</td>
<td>2.92 ± 0.18***</td>
</tr>
<tr>
<td>Thymus</td>
<td>8.25 ± 0.96</td>
<td>12.11 ± 0.63</td>
<td>10.22 ± 1.60</td>
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<tr>
<td>Spleen</td>
<td>17.02 ± 1.27</td>
<td>16.36 ± 1.62</td>
<td>15.80 ± 1.63</td>
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<tr>
<td>Bone marrow</td>
<td>12.40 ± 1.76</td>
<td>10.40 ± 1.19</td>
<td>13.08 ± 1.56</td>
</tr>
<tr>
<td>PBMC</td>
<td>2.25 ± 0.18</td>
<td>1.23 ± 0.12***</td>
<td>1.91 ± 0.22</td>
</tr>
<tr>
<td>CD45RA+/Igκ−</td>
<td>0.48 ± 0.05</td>
<td>0.89 ± 0.12</td>
<td>0.63 ± 0.10</td>
</tr>
<tr>
<td>Thymus</td>
<td>19.85 ± 0.36</td>
<td>15.85 ± 1.05*</td>
<td>16.13 ± 1.24*</td>
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<tr>
<td>Spleen</td>
<td>4.16 ± 0.16</td>
<td>2.56 ± 0.30**</td>
<td>2.44 ± 0.33***</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>5.64 ± 0.43</td>
<td>3.16 ± 0.26***</td>
<td>2.57 ± 0.18***</td>
</tr>
<tr>
<td>Thymus</td>
<td>7.72 ± 0.39</td>
<td>10.53 ± 0.65***</td>
<td>9.62 ± 0.61</td>
</tr>
<tr>
<td>Spleen</td>
<td>30.44 ± 0.25</td>
<td>43.92 ± 1.53*</td>
<td>44.72 ± 0.71*</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>1.27 ± 0.22</td>
<td>1.27 ± 0.31</td>
<td>1.49 ± 0.29</td>
</tr>
<tr>
<td>PBMC</td>
<td>56.52 ± 0.68</td>
<td>63.21 ± 0.63***</td>
<td>62.18 ± 0.79***</td>
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<tr>
<td>CD3+/CD4+ CD8−/ CD3+/CD8+ CD4+</td>
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<td></td>
<td></td>
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<tr>
<td>Thymus</td>
<td>1.78 ± 0.11</td>
<td>3.49 ± 0.30***</td>
<td>2.80 ± 0.31*</td>
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<td>Spleen</td>
<td>12.89 ± 0.26</td>
<td>9.82 ± 0.39***</td>
<td>9.13 ± 0.63***</td>
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<tr>
<td>Bone marrow</td>
<td>1.67 ± 0.30</td>
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<td>PBMC</td>
<td>19.48 ± 0.40</td>
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<td>20.85 ± 0.43</td>
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<td>Thymus</td>
<td>85.57 ± 1.24</td>
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<td>78.99 ± 1.38</td>
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<td>Spleen</td>
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<td>Bone marrow</td>
<td>1.60 ± 0.39</td>
<td>1.03 ± 0.11</td>
<td>0.99 ± 0.12</td>
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<tr>
<td>PBMC</td>
<td>1.39 ± 0.12</td>
<td>1.62 ± 0.13</td>
<td>2.16 ± 0.19**</td>
</tr>
</tbody>
</table>

Using flow cytometry, the effects of bortezomib treatment on apoptosis and on lymphocytes were measured in the thymus, spleen, bone marrow, and peripheral blood. Results are shown as average percentages ± SEM. One-way ANOVA analysis and Bonferroni post hoc testing. For analyzing the effect of bortezomib treatment, the data of control and EAMG groups were combined since no significant differences were observed between them in any of the parameters studied.

*p < 0.05; **p < 0.01; ***p < 0.001.

Ann, annexin V.
In the spleen, thymus, and bone marrow, a trend toward a higher proportion of early apoptotic and dead cells after bortezomib administration could be observed, but the differences did not reach statistical significance. However, the lymphocyte subpopulation in the bone marrow contained a significantly increased number of apoptotic or dead cells after bortezomib-treatment (data not shown).

In the peripheral blood, unexpectedly, bortezomib treatment significantly decreased the proportion of dead cells in PBMCs in the 4w-Bz group and 8w-Bz groups compared with the saline groups. The proportion of early apoptotic cells in PBMCs was significantly lower in the 4w-Bz groups ($p < 0.001$) but not in the 8w-Bz groups.

In the spleen, the bone marrow, and the blood, the proportion of CD45RA+/Igk+ B cells was significantly decreased after bortezomib treatment (Table II), both in the 4w-Bz and the 8w-Bz groups.

After treatment with bortezomib, the proportion of immature CD4+CD8- cells in the thymus was decreased whereas the proportions of CD3+/CD4+/CD8-Th cells and cytotoxic CD3+/CD8+/CD4- T cells were increased.

In the blood and the spleen, CD3+/CD4+/CD8- cells were significantly increased; the proportion of CD3+/CD8-/CD4- cells was significantly decreased in the spleen and bone marrow of bortezomib-treated rats.

In general, the two bortezomib treatment regimes led to similar changes of the rat immune system, with the exception of the proportion of apoptotic cells, as aforementioned.

**Total IgG concentration is decreased by bortezomib**

The effect of bortezomib on total IgG content in plasma samples was measured by ELISA (Fig. 4A). In comparison with the saline-treated group, total IgG at week 8 was significantly reduced in both the 4w-Bz and the 8w-Bz group ($p < 0.001$). Importantly, this immunosuppressive effect of bortezomib was observed in both control and EAMG animals, although IgG reduction was more pronounced in 8w-Bz EAMG animals than in the 4w-Bz EAMG rats ($p < 0.05$). A slightly but significantly higher concentration of IgG was observed in all EAMG groups in comparison with the corresponding control groups. Compared to the saline-treated groups, a highly significant reduction in IgG concentrations ($p < 0.001$) was already achieved after 4 wk of bortezomib treatment in the 8w-Bz group (data not shown).

**Bortezomib reduces autoantibody titers in EAMG**

The plasma concentration of autoantibodies to the rat AChR was measured by RIA. Autoantibodies were detectable 4 wk after immunization in all EAMG animals and reached very high levels after 8 wk (Fig. 4B). The variability of autoantibody levels between animals is typical for the EAMG model, but it should be borne in mind that already a titer of 1 nM autoantibodies is sufficient to cause substantial damage to the NMJ (33). As expected, no anti-AChR Ab titers could be detected in sham/CFA-immunized animals (control group; data not shown). On the other hand, animals that received bortezomib injections from the moment of immunization showed a significantly lower production of autoantibodies ($p < 0.01$; corresponding to a 72% reduction of average autoantibody titer) compared with the saline-treated EAMG group 8 wk after immunization (Fig. 4C). Interestingly, rats that received bortezomib 4 wk after immunization had a autoantibody production profile similar to that observed in the 8w-Bz group. After injection of bortezomib the production of anti-AChR Abs was reduced significantly compared with saline-treated animals (corresponding to a 60% reduction; $p < 0.01$). This demonstrated that bortezomib effectively diminished Ab production not only

**FIGURE 4.** Total IgG and autoantibody titers in plasma. A, Plasma IgG titers 8 wk after immunization. Bortezomib significantly reduced plasma IgG levels. Two-way ANOVA and Bonferroni post hoc testing were used for statistical analyses. B, Average anti-rat AChR-titer; error bars correspond to the SEM. **$p < 0.01$; ***$p < 0.001$ compared with the saline-treated groups. Autoantibody titers of the 4w-Bz and the 8w-Bz groups were not significantly different from each other at any time point. C, Anti-rat AChR titers 8 wk after immunization with tAChR. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.
when administrated at the moment of immunization but also once the immune response was already ongoing.

**Bortezomib ameliorates clinical conditions in EAMG**

To assess the effect of bortezomib on the overall condition of experimental animals, we weighed them and scored their clinical status on a weekly basis. During the first 5 wk of treatment, control animals that had received bortezomib from the moment of immunization (8w-Bz group) showed a slower increase in their total body weight compared with saline-treated control animals (Fig. 5A, 5B; p < 0.001). After 5 wk, the growth of these animals normalized again. A similar reduction of growth was observed 4 wk

**FIGURE 5.** Average rat weights. A, Body weights of bortezomib-treated animals increased slower compared with saline-treated animals. Weight loss occurred frequently in EAMG animals. B and C, Normalized weights were calculated using the average weight of the saline-treated control group. Error bars correspond to the SEM.

**FIGURE 6.** MG symptoms and muscle function. A, Survival. B, Clinical scores of muscle weakness. Each group contained 18 animals (indicated with 100%). C, EMG after curare infusion. The curare dose that induces at least 10% decrement of the CMAP was used as a measure of the neuromuscular safety factor.
Later in the 4w-Bz control group. At the end of the experiment, there were no significant differences between the average weights of the 4w-Bz and the 8w-Bz control groups. As expected, control animals did not present muscle weakness or any other clinical sign of EAMG.

Animals immunized with Torpedo AChR developed clinical symptoms of EAMG starting 5 wk after immunization, when Ab titers reached considerable levels. Frequently, weight loss preceded the observation of other myasthenic symptoms in EAMG animals, indicating weakness of bulbar muscles and difficulties in chewing and swallowing. The disease in some of these animals progressed rapidly to score 3 within 2 d and they had to be sacrificed (Fig. 6A). By the end of the experiment 50% of animals (9 of 18) treated with saline solution reached a clinical score of 3 or lost >20% body weight and had to be sacrificed for ethical reasons. This percentage was reduced to 33% (6 of 18) in the 4w-Bz group and to 11% (2 of 18) in the 8w-Bz group (Fig. 6B). The survival rate of the 8w-Bz EAMG group was significantly higher compared with the saline-treated EAMG group ($p < 0.01$; Fig. 6A).

From the AChR-immunized animals, 78% developed muscle weakness in the saline-treated EAMG group, 72% in the 4w-Bz EAMG group, and 39% in the 8w-Bz EAMG group. The clinical score of the 8w-Bz EAMG group was significantly lower compared with the 4w-Bz group ($p < 0.05$) and the saline-treated EAMG group ($p < 0.01$; Fig. 6B). Despite the reduced amount of autoantibodies, the muscle weakness and the survival rate of the 4w-Bz EAMG group were not significantly different from the saline-treated EAMG group. The onset of weight loss in the 4w-Bz EAMG group even occurred somewhat earlier compared with the saline-treated EAMG group (Fig. 5C), indicating that weight loss was partly caused by the (side) effects of bortezomib.

We evaluated the safety factor of neuromuscular transmission, which is a function of the postsynaptic density of AChRs, by performing EMG studies in the presence of the AChR-blocking agent (+)-tubocurarine (curare). The amount of curare needed to induce a decrement in CMAP is related to the safety factor of neuromuscular transmission. Bortezomib had slight effects on the curare sensitivity of the NMJ in control animals in the 4w-Bz group (Fig. 6C). The neuromuscular transmission was significantly lower compared with the saline-treated EAMG group (Fig. 6D).

**FIGURE 6.** Electron micrographs of synaptic boutons of the NMJ. Nerve terminals are indicated by asterisks. In control animals (A–C), the postsynaptic membrane contains secondary clefts (postsynaptic folds), which are indicated by arrows. In EAMG animals (D–G) pathologic changes of the postsynaptic membrane are indicated: degenerating folds (arrowhead), simplified and without folds (dagger), and widening of the primary and secondary synaptic clefts (double dagger). The postsynaptic damage of an animal with an anti-AChR titer of 3 nM (F) was mild compared with an animal with a titer of 20 nM (G). The muscle tissue was stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate.

**FIGURE 7.** Electron micrographs of synaptic boutons of the NMJ. Nerve terminals are indicated by asterisks. In control animals (A–C), the postsynaptic membrane contains secondary clefts (postsynaptic folds), which are indicated by arrows. In EAMG animals (D–G) pathologic changes of the postsynaptic membrane are indicated: degenerating folds (arrowhead), simplified and without folds (dagger), and widening of the primary and secondary synaptic clefts (double dagger). The postsynaptic damage of an animal with an anti-AChR titer of 3 nM (F) was mild compared with an animal with a titer of 20 nM (G). The muscle tissue was stained with osmium tetroxide and contrasted with uranyl acetate and lead citrate.
impaired in all EAMG groups compared with the corresponding control groups. In the 8w-Bz EAMG group, neuromuscular transmission was significantly improved compared with the saline-treated and the 4w-Bz EAMG groups ($p < 0.05$).

Ultrastructural analysis of the NMJ revealed damage of the postsynaptic membrane morphology, with degenerating or absent secondary clefts in EAMG animals (Fig. 7). The damage in endplates of animals with low titers in the 8w-Bz group (Fig. 7F) was less severe compared with animals with higher titers (Fig. 7D, 7E, 7G). Quantitative morphometric analysis of the synapse ultrastructure was performed to measure the loss of postsynaptic folds (Fig. 8). The folding index was significantly reduced in the saline-treated EAMG animals compared with the saline-treated control animals (a reduction of 55%, $p < 0.001$). In the 8w-Bz EAMG animals only a 20% reduction of the folding index compared with saline-treated control animals was observed, and a considerable proportion of the endplates had a relatively high folding index. Compared to the saline-treated EAMG group, the folding index in the 8w-Bz group was significantly higher ($p < 0.001$). However, this treatment effect was not observed in the 4w-Bz group. In conclusion, bortezomib could not improve synaptic ultrastructure if treatment was started 4 wk after immunization, but it efficiently prevented damage of the postsynaptic membrane when administrated for 8 wk starting directly after immunization.

**Discussion**

In this study we analyzed the effect of proteasome inhibition on the autoimmune response in the EAMG model for MG. Bortezomib reduced the amount of plasma cells, leading to a significant reduction of total serum IgG and autoantibody levels and caused an amelioration of myasthenic symptoms compared with a saline-treated EAMG group. Both the 4- and 8-wk bortezomib treatment regimes (4w-Bz and 8w-Bz) were generally well tolerated, but some side effects were noticed, which are discussed below. There were no significant differences in autoantibody production between the 4w-Bz and the 8w-Bz group. Because bortezomib in the 4w-Bz group was only administrated starting 4 wk after immunization with autoantigen, this suggests that mainly the late (effector) phase of the autoimmune response is affected by proteasome inhibition.

In the EAMG model, the production of Abs against the muscle AChR is induced by immunization with tAChR. EAMG in Lewis rats is characterized by chronic muscle weakness starting 5 wk after immunization. A transient acute phase of muscle weakness starting 1 wk after immunization is seen in EAMG models using CFA with Bordetella pertussis and M. tuberculosis (20). In the immunization protocol used in this study with CFA containing only M. tuberculosis, the acute phase of muscle weakness does not occur (20). Therefore, we could investigate both a therapeutic as well as a preventive treatment regimen. Bortezomib efficiently reduced the production of autoantibodies and also ameliorated MG symptoms in the 8w-Bz group. The average levels of anti-AChR autoantibodies in this study were high, comparable to a previous study using the same protocol with 20 μg tAChR (36, 37) and much higher than another study using 10 μg tAChR (33), ranging between 3 and 80 nM even in the 8w-Bz groups. Because a 1 nM serum anti-muscle AChR antibodies can already reduce the amount of total muscle AChR by 50% in rats (33), the limited improvement of muscle strength in this study is understandable. It is therefore conceivable that if we could have used EAMG animals with a lower titer, the effects of bortezomib would have been more pronounced. Nevertheless, the resistance against curare, and thus the amount of functional AChR at the NMJ and the folding index of the postsynaptic membrane, was significantly increased by bortezomib in the 8w-Bz treatment group, resulting in higher survival rates. In human MG patients, an autoantibody titer reduction of 50% after plasma exchange is generally sufficient to achieve clinical remission (38, 39), and therefore in this respect the bortezomib-induced reduction of autoantibody production by >65% within 4 wk is therapeutically promising.

However, despite the reduced autoantibody levels, the 4w-Bz treatment did not result in a significant improvement of health in EAMG animals, in contrast to the 8w-BZ treatment, where bortezomib caused amelioration in the condition of the animals. Two side effects of bortezomib might have influenced this result. First, bortezomib negatively affected body weight during the first 4 wk of administration in control animals and presumably also in EAMG animals. In the 4w-Bz EAMG group, this coincided with the weight loss as a result of muscle weakness and the resulting problems with eating and drinking. Because a 20% weight loss was chosen as a criterion to sacrifice animals for ethical reasons, the bortezomib treatment could have reduced survival time to some extent. Second, we observed a mild impairment of neuromuscular transmission in the 4w-Bz control group (but not in the 8w-Bz Control group) in comparison with the saline-treated control group. This effect possibly indicates transient nerve damage similar to the bortezomib-induced polyneuropathy (26, 40). Arguably, the weight loss in control animals was caused by an effect of proteasome inhibition on the gastrointestinal tract, which has been seen in patients (41). However, our study was not designed for investigating the side effects of bortezomib, and therefore we cannot conclusively attribute the effect on weight to any particular side effect. In the 8w-Bz control group, weight gain, neuromuscular transmission, and apoptosis were normalized 8 wk after immunization, suggesting that by that time compensatory mechanisms limited these adverse effects of bortezomib.

Despite comparable autoantibody titers, the ultrastructural postsynaptic damage of the 4w-Bz EAMG group was significantly higher compared with the 8w-Bz EAMG group. Because the repair...
of the postsynaptic membrane takes at least 10 d (42), it seems possible that the observed loss of postsynaptic folding in the 4w-Bz group is the result of earlier damage, in particular in the period between 4 and 7 wk after immunization, when titers were higher compared with the 8w-Bz EAMG group.

Apart from the intended killing of plasma cells, bortezomib affected the immune system in a more general fashion. In particular, the thymus was affected by bortezomib, an effect that might not be harmful, since thymectomy is a frequently used treatment in MG, albeit with proven efficacy so far. In this respect it is relevant that in human thymocyte cultures from thymectomized MG patients we observed that bortezomib induced cell death and reduction of autoantibody production (A. Gomez, K. Vrolix, and M. Losen, unpublished observations). Similar to a previous study in mice (43) we found in our rat model that bortezomib mainly affects immature thymocytes. In the spleen, bone marrow, and the blood of rats, the proportion of CD45RA+/Igκ+ B cells was significantly decreased, whereas the proportion of CD3+ T cells of total lymphocytes was increased. There was a trend for increased proportion of dead and apoptotic cells in the thymus, bone marrow, and spleen of bortezomib-treated animals. In contrast, bortezomib induced a significant reduction of apoptotic cells in the blood. Because the PBMCs only represent a minor proportion of leukocytes, this effect could be attributed to migration of cells into other lymphoid organs, for example, the spleen, which was significantly enlarged in bortezomib-treated rats.

In the past the EAMG model has been instrumental for testing the efficacy of new therapies that are now used for the treatment of MG (reviewed in Ref. 44), for example, cyclophosphamide (45), pixintrone (46), linomide (47), azathioprine, and hydrocortisone. The striking effect could be attributed to migration of cells into other lymphoid organs, for example, the spleen, which was significantly enlarged in bortezomib-treated rats.

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Disclosures
R.A.M. and R.E.V. have applied for a patent on the use of proteasome inhibitors for plasma cell depletion. The other authors have no financial conflicts of interest.

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