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Proteasome Inhibition with Bortezomib Depletes Plasma Cells and Specific Autoantibody Production in Primary Thymic Cell Cultures from Early-Onset Myasthenia Gravis Patients

Alejandro M. Gomez,* Nick Willcox,*†,1 Kathleen Vrolix,* Jonas Hummel,* Gisela Nogales-Gadea,*,† Abhishek Saxena,* Hans Duimel,§,¶ Fons Verheyen,§,¶ Peter C. Molenaar,* Wim A. Buurman,* Marc H. De Baets,* Pilar Martínez-Martínez,*† and Mario Losen*†,1

Bortezomib is a potent inhibitor of proteasomes currently used to eliminate malignant plasma cells in multiple myeloma patients. It is also effective in depleting both alloreactive plasma cells in acute Ab-mediated transplant rejection and their autoreactive counterparts in animal models of lupus and myasthenia gravis (MG). In this study, we demonstrate that bortezomib at 10 nM or higher concentrations killed long-lived plasma cells in cultured thymus cells from nine early-onset MG patients and consistently halted their spontaneous production not only of autoantibodies against the acetylcholine receptor but also of total IgG. Surprisingly, lenalidomide and dexamethasone had little effect on plasma cells. After bortezomib treatment, they showed ultrastructural changes characteristic of endoplasmic reticulum stress after 8 h and were no longer detectable at 24 h. Bortezomib therefore appears promising for treating MG and possibly other Ab-mediated autoimmune or allergic disorders, especially when given in short courses at modest doses before the standard immunosuppressive drugs have taken effect. The Journal of Immunology, 2014, 193: 1055–1063.

Myasthenia gravis (MG) with Abs against the muscle acetylcholine receptor (AChR) is one of the best understood of the numerous autoimmune neurologic diseases now recognized (1). It is generally agreed that the patients’ autoantibodies are pathogenic, as they decrease AChR numbers by antigenic modulation and complement-mediated damage (2, 3). Patients with early-onset MG (EOMG; before 45 y of age) are an unusually well-defined subgroup with strong female and HLA-B8 biases (4) and characteristic lymph node–like infiltrates in the thymic medulla (5–7).

Treatment of MG relies primarily on glucocorticoids, often combined with broad-spectrum immunosuppressants such as azathioprine, mycophenolate mofetil, or rituximab (8). However, their efficacy and side effects vary greatly between patients, and they reduce autoantibody titers and restore muscle strength only after delays as long as 4–15 mo (9, 10). Additionally, drug-resistant AChR MG patients treated with rituximab (anti-CD20) showed no reduction in either AChR Ab titers or IgG levels, despite complete elimination of circulating B cells (10). In such patients, long-lived plasma cells, which are CD20−, are likely to be the main producers of the autoantibodies. Moreover, they are probably responsible for the delayed responses of most MG patients to immunosuppressants, which mainly act by preventing generation of new plasma cells from B cells and by impairing the activation and proliferation of Th cells (11–13).

Plasma cells are high-rate Ab-secreting cells (>10,000 molecules/cell/second) (14, 15). They are terminally differentiated and do not divide. Among the B cell lineage, they are uniquely radioresistant. Whereas some are short-lived, others persist for many months (or even years) (16) in special survival niches in bone marrow (17) and lymphoid tissues (18). They are the main producers of circulating IgG and are clearly key players in chronic Ab-mediated autoimmune diseases. Their resistance to both standard immunosuppressants and rituximab therefore necessitates a different pharmacological approach.

Many recent studies have focused on drugs that target the neoplastic plasma cells in multiple myelomas (MMs). Partly because of their high rate of protein synthesis and dependence on protective unfolded protein responses, MM cells are very susceptible to proteasome inhibitors (19). These rapidly induce apoptosis by activating the terminal unfolded protein response (20) and inhibiting the transcription factor NF-κB (21). Proteasome
Inhibition has similar effects on non-neoplastic plasma cells in vivo (22, 23). Bortezomib, the first clinically approved proteasome inhibitor, is widely used for treating MM. Additionally, it is now used to prevent acute Ab-mediated rejection of solid organ transplants (24). It is also showing promise in Ab-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) and thrombotic thrombocytopenic purpura (18, 25). In autoimmune animal models of SLE, antineutrophil cytoplasmic Ab–induced glomerulonephritis and MG, it depleted both plasma cells and autoantibodies (22, 23, 26, 27).

Non-neoplastic plasma cells may also be susceptible to other anti-myeloma drugs, for example, the thalidomide derivative lenalidomide, which is frequently combined with dexamethasone in nonpregnant MM patients and appears relatively safe. Lenalidomide inhibits the proliferation of several MM cell lines and disrupts the stromal support in their survival niches (28). Because it reduces IgM and IgG responses to PWM (29), it must affect earlier B lineage cells too.

In most EOMG patients, the thymic infiltrates include numerous germinal centers (5–7), many of them AChR-specific, and autoreactive T and B cells along with terminal plasma cells (30). In our experience, some degree of thymic hyperplasia is observed in >80% of steroid-naive EOMG patients (30, 31). In primary cultures of cells from EOMG, but not from control thymi, autoreactive plasma cells spontaneously secrete AChR autoantibodies, with titers and epitope specificities very similar to those in the patients’ sera (30, 31). They do so for several weeks (at least), even after irradiation (31), implying that many of them are long-lived. This longevity and radiation resistance contrasts strikingly with most thymic subsets, for example, immature thymocytes and T cells, which have a very high turnover in vivo (32, 33) and die rapidly in culture (31, 34).

Thymectomy is part of standard management of EOMG in many centers (5–7). Thus, the tissue removed is an almost uniquely accessible source of long-lived human autoimmune plasma cells. In the present study, we have used it to test their susceptibility to drugs, as assessed by their ultrastructure and production of IgG and AChR autoantibodies. We demonstrate that very low concentrations of bortezomib are cytotoxic for total and autoimmune human plasma cells, and thereby prevent production and release of autoantibodies, whereas lenalidomide and dexamethasone have little effect.

Materials and Methods

Patients

The EOMG patients’ clinical information is shown in Table I. Thymus tissue was obtained with their informed consent and Ethics Committee approval. None of the patients had been pretreated with glucocorticoids; otherwise, they were selected only because of high serum anti-AChR titers, the correspondingly high productivity of these Abs by their thymic cells in culture (31), and the availability of irradiated cells. Thymi were removed in London between 1983 and 1990, when enzymatically dispersed cell suspensions were cryostored in liquid nitrogen (now at the Biobank of Oxford University, Oxford, U.K.) (31, 35); one more thymus was tested suspensions were cryostored in liquid nitrogen (now at the Biobank of otherwise, they were selected only because of high serum anti-AChR titers, in parallel. Results are expressed as nanomoles of ß-bungarotoxin binding sites per liter of culture medium per day, normalized as the percentage of those in untreated cultures.

We measured total IgG in the diluted culture supernatants with a standard sandwich ELISA as described (30, 31), capturing with goat F(ab′)2; anti-human IgG-Fcγ (109-006-008; Jackson ImmunoResearch Laboratories; diluted 1:200) and detecting with HRP-conjugated goat F(ab′)2 anti-human IgG-Fcγ (109-036-008; Jackson ImmunoResearch Laboratories; diluted 1:200,000). Results were expressed as nanograms total IgG secreted per milliliter supernatant per day, normalized as above. The newly synthesized AChR Ab and total IgG were quantitated as their present concentrations (in 200 µl) minus the concentration in the previous sample (in 100 µl) divided by the time interval.

Immunofluorescence staining and enumeration of plasma cells by microscopy

Cultured cells (5 × 106 to 5 × 107) were cytocentrifuged onto poly-L-lysine–coated slides (for 5 min at ~120 g; Cyto-Tek centrifuge model 4332; Sakura Finetek, Tokyo, Japan). Slides were then air-dried for 1 h at 22°C and then fixed in 4% paraformaldehyde at 4°C for 10 min. Subsequently, they were blocked with 2% BSA in PBS and incubated with Hoechst 33342 solution (2 µg/ml; Sigma-Aldrich, catalog no. B2261) to stain DNA. We stained for plasma cells with mouse anti-human CD138 mAb (1:250, clone MI15; Dako), donkey anti-mouse IgG Alexa 594 (1:300, A21203; Molecular Probes/Invitrogen), and goat anti-human IgG Alexa 488 (1:500, A10103; Molecular Probes/Invitrogen). Slides were mounted in 80% glycerol-TBS and stored at 4°C in the dark. All washing and incubation steps were performed with TBS–Trition X-100 (0.03%). We counted the plasma cells from each well, in a blinded fashion, on a fluorescence microscope (Olympus BX51), identifying them by their distinctive size, shape (extensive cytoplasm and eccentric nuclei), and positive staining for internal IgG and/or surface CD138. Absolute numbers of plasma cells per well are given in Table II and shown in the figures as the percentage of those in untreated cultures. Total numbers of recovered cells were measured by automated counting of trypan blue–excluding cells (Bio-Rad TC20 automated cell counter).

FACS analysis of B, T, and plasma cells and membrane integrity

For B and T lymphocyte identification, cells were incubated for 30 min at 4°C with an FACS buffer (2% FCS and 0.1% sodium azide), 10 µl mouse anti-human CD3 (BD Biosciences, 553339) and PE mouse anti-human CD19 (BD Biosciences, 555413), all diluted 1:100 in FACS buffer. The samples were washed twice, kept at 4°C in the dark, and analyzed within 2 h. Dead cells were identified by propidium iodide (PI) counterstaining (BD Biosciences apoptosis detection kit).

To identify plasma cells, we incubated cells for 15 min at 22°C in the dark, with the following mix of anti-human mAbs: CD138-PE (BTL, M2287; 1:25), CD19-PerCy5.5 (BD Biosciences, 332780; 1:40), CD38–allophycocyanin (BD Biosciences, 345807; 1:40), CD3–V450 (BD Biosciences, 560365; 1:20), and CD45–V500 (BD Biosciences, 560777; 1:20). Cells were then resuspended in 1× Cytofix/Cytoperm buffer (BD Biosciences, 554722) for fixation and permeabilization, and later incubated for 30 min at 4°C in the dark with an Ab mix of anti-human L chain–FITC (BD, 555791) plus anti-human A chain–FITC (BD Biosciences, 347247, clone 1-155-2), each diluted 1:35 in Perm/Wash buffer (BD Biosciences, 554723). We used either a FACSCompilbor (for B and T cells) or a FACS Canto II (for plasma cells), plus CellQuest software (all from BD Bio-
sciences) for data acquisition, and we analyzed results using the FlowJo software (version 2.5.1; Turku Center for Biotechnology, Turku, Finland). Samples were gated to exclude cell debris and aggregates; at least 10,000 events per sample were measured for B and T cell analyses, and 150,000 events for plasma cells. Results are expressed as the absolute number of cells per well, normalized as the percentages of B, T, and total viable cells in untreated cultures.

Electron microscopy

Cultured thymic cells were collected, pelleted (7 min, 220 × g, 4°C) and fixed with 3% glutaraldehyde plus 1.4% sucrose buffered in 0.09 M KH2PO4 at pH 7.4. They were then washed in 0.09 M KH2PO4 buffer with 7.5% sucrose and transferred to a 1% OsO4 plus 1.5% ferrocyanide solution buffered with veronal at pH 7.4 for subsequent immersion fixation for 1 h at 4°C. After washing in veronal buffer with 7% sucrose at pH 7.4, dehydration was carried out rapidly in graded ethanol series. Samples were then incubated overnight in propylene oxide and Epon (1:1), and subsequently embedded in Epon. Serial 80-nm sections were stained with uranyl acetate, lead citrate, and coded. We used a Philips CM100 electron microscope to count plasma cells and examine their ultrastructure in five representative sections for each sample.

Statistical analysis

GraphPad Prism 4 was used for statistical analyses. We compared normally distributed values using one- or two-way ANOVA analyses, as well as Bonferroni post hoc tests. A two-sided p value ≤ 0.05 was considered significant. Values are expressed as means ± SEM unless stated otherwise. We used Spearman (nonparametric) correlation coefficients (ρ).

Results

Culturing EOMG thymic cells

The donor patients are listed in Table 1. Their thymic plasma cells were identified by their characteristic ultrastructural morphology (Fig. 1), intense internal IgG staining, and surface CD138 expression (Fig. 2A), although the latter was a dim and not totally consistent marker (as previously noted for cryostored cells) (37). They were frequently found in clumps of three to five cells (or sometimes more), in close contact with extracellular matrix and other cell types (Fig. 2A), as in their survival niches in the spleen or bone marrow (38).

In the thymus, there is normally a high rate of cell death in vivo (32, 33), primarily due to the programmed death of immature thymocytes deprived of prosurvival signals. As expected, it was also substantial in our suspension cultures of frozen/thawed cells; even from hyperplastic EOMG thymi, most cells are immature thymocytes (7, 35) and are no longer in contact with the rare epithelial cells on which their survival normally depends. About 20% of the input cells remained viable on day 14, and fewer in irradiated samples (∼8%).

![FIGURE 1. Ultrastructural changes in plasma cells incubated with bortezomib. (A) Normal plasma cells in control samples. Note the typical morphology, with elaborate ER and eccentric nuclei (with a cartwheel heterochromatin configuration). (B) Most plasma cells also appeared largely normal after 2 h of treatment with 2.5 μM bortezomib. (C) After 8 h of treatment with bortezomib, most plasma cells appeared apoptotic, with heterochromatin condensed around the perimeter of the nucleus and distension of the ER lumen. After 24 h with bortezomib, no plasma cells could be detected. Coded cell samples were postfixed with osmium tetroxide and counterstained with uranyl acetate and lead citrate. Images are representative of the conditions analyzed. Scale bars, 2 μm.]
Golgi complex, and dense regions of (nuclear) heterochromatin in the characteristic “cartwheel” distribution (Fig. 1A). They still appeared largely normal at 2 and 4 h after addition of bortezomib (at 2.5 μM; Fig. 1B). However, after 8 h, most surviving plasma cells showed signs of apoptosis (Fig. 1C), including dense condensations of chromatin around most of the nuclear membrane.
Bortezomib eliminates plasma cells in cultured EOMG thymic cells

To focus on long-lived plasma cells, we next added bortezomib, lenalidomide, or dexamethasone on days 7 and 11 of culture and counted surviving plasma cells on day 14. Lenalidomide was used at 10 μM, based on previous in vitro studies (42–44) and peak levels in MM patients (45). Dexamethasone was tested at 10 nM, a level known to inhibit lymphocyte proliferation in susceptible humans (46, 47).

We used thymic cells from six patients to test effects of these drugs on plasma cell numbers. Three days after a second dose of bortezomib, plasma cells were almost undetectable in all cases (p < 0.001; Fig. 2B, Supplemental Fig. 2). Interestingly, their numbers were not changed greatly or consistently by either lenalidomide or dexamethasone (p < 0.05 for the latter). In a separate experiment, we confirmed that 10 μM lenalidomide suppressed IgG production by PWM-stimulated PBMCs (not shown), as previously reported (29).

Proteasome inhibition halts spontaneous secretion of total IgG and AChR autoantibodies

In thymic cell cultures that were analyzed at early time points, we could already detect significant production of both AChR Ab and total IgG at 48 h of culture; both increased further from days 9 to 14 in the control and lenalidomide cultures (Fig. 2C, 2E). In striking contrast, they both consistently declined sharply after the first dose of bortezomib, and further still after the second dose (p < 0.001). Notably, dexamethasone merely prevented their rise after day 9, which correlated with the mild reduction in plasma cell survival observed by day 14. Results were broadly similar with the one fresh thymus available to us (Supplemental Fig. 1, Table II). At day 14, production of AChR Ab and total IgG was strongly and significantly reduced by bortezomib compared with both autoantibody and AChR autoantibodies.

Table II. Absolute values (average ± SD) for the different parameters analyzed in thymic cell control cultures (without experimental drugs)

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG (ng/ml/d)</th>
<th>Anti-AChR (nM/d)</th>
<th>Plasma Cells (No./Well)</th>
<th>Cell Nos./Well/1000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonirradiated</td>
<td>Irradiated</td>
<td>Nonirradiated</td>
<td>Irradiated</td>
</tr>
<tr>
<td>MG-1</td>
<td>77.9 ± 19.6</td>
<td>65.9 ± 62.6</td>
<td>2.8 ± 0.7</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>MG-2</td>
<td>191.9 ± 35.3</td>
<td>17.8 ± 12.8</td>
<td>1.2 ± 0.6</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>MG-3</td>
<td>57.7 ± 6.0</td>
<td>4.0 ± 1.5</td>
<td>1.3 ± 0.2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MG-4</td>
<td>298.0 ± 75.0</td>
<td>N/A</td>
<td>1.0 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>MG-5</td>
<td>83.8 ± 18.0</td>
<td>14.6 ± 7.2</td>
<td>1.1 ± 0.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>MG-6</td>
<td>9.0 ± 2.8</td>
<td>27.7 ± 10.4</td>
<td>&lt;0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>MG-7</td>
<td>325.0 ± 158.6</td>
<td>N/A</td>
<td>&lt;0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>MG-8</td>
<td>43.8 ± 17.6</td>
<td>N/A</td>
<td>0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>MG-9</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MG-10</td>
<td>42.3 ± 10.6</td>
<td>N/A</td>
<td>1.3 ± 0.4</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Average values were used for normalization of data in Figs. 2–4. All measured values are from day 14 except for anti-AChR production; these values are from day 11. N/A, not analyzed.
well (26). As far as we know, these are the first demonstrations of its efficacy directly on pathogenic autoantibody-producing human plasma cells. Because bortezomib triggers apoptosis in non-neoplastic plasma cells, even after a single dose, short low-dose regimens might be sufficient to rapidly reduce their numbers and Ab levels in patients; horizontal bars are median values. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.

In our cultures, plasma cells seemed almost entirely responsible for spontaneous production of both autoantibody and total IgG, which correlated strongly with their survival. Moreover, because it was maintained despite depletion of B cells (by dexamethasone; Fig. 4E, 4F), these evidently contributed minimally in untreated cultures (if at all). Indeed, we have rarely found signs of mitogen-stimulable production of AChR Abs in EOMG thymi (31). These data, together with the Abs’ highly mutated H and L chain V region sequences, suggest that native AChR, which is continuously available in the thymus, is driving most Ab-producing cells to the terminal plasma cell stage (30). Because we have seen very similar behavior in cells from EOMG bone marrow and spleen, it is clearly not unique to the hyperplastic EOMG thymus; indeed, it was also shown by thymic remnants from thymoma/MG patients (30, 31).

In previous studies, plasma cells from recently immunized mice died after only 4 d in suspension cultures (49). In sharp contrast, their Ig production was sustained in vitro for 2–4 wk in primary cultures from human tonsils and GALT, and it was enhanced by feeder cells and especially in whole organ cultures (50, 51). It seems unlikely that equivalent survival niches were reconstituted efficiently in our cultures of cryostored, and especially irradiated, thymic cells. We suggest that the remarkable survival of the long-lived plasma cells shown in the present study is due to their coclustering (Fig. 2A) and/or to some degree of resilience or “self-sufficiency.” Their radiosensitivity clearly shows that they are well established in many EOMG thymi and scarcely replaced in culture. Importantly, because bortezomib (but not dexamethasone) reduced their numbers and Ab production to baseline in irradiated samples, its targets must include long-lived plasma cells.

In sharp contrast with our results with bortezomib, but in agreement with previous in vivo findings (22, 52), we found only marginal effects of dexamethasone on plasma cell survival or function, even when added twice at 1 μM, and only on unirradiated cells. Evidently, most of the thymic plasma cells are dexamethasone- as well as radiosensitive; instead, neither treatment alone, or when combined, completely eliminated them in any of our cultures. In theory, both treatments might also affect their supporting cells and/or damage other short-lived plasma cells or plasmablasts. In vivo, however, their precursors may be steroid-insensitive as well; we noted no obvious decrease in PWM-stimulated IgG responses by (radiosensitive) B cells from prednisone-pretreated patients; rather, they appeared to be enriched (53).

The even smaller effects on plasma cells of the immunomodulatory drug lenalidomide may seem surprising in view of its clear benefits in MM patients (28, 29, 54). One possible explanation is that its toxicity for MM cells is mainly related to the activation of tumor suppressor genes and caspases that trigger apoptosis in transformed cells (55–57), but probably not in their non-neoplastic counterparts. Moreover, because lenalidomide also disrupts the survival niches required by MM and plasma cells (58), its effects may be underestimated in our cultures. Additionally, it is well known for its disparate immunomodulatory properties, for example, inhibiting Ig production by cultured PBMCs (29), but also for augmenting Ab responses to vaccination (59) and enhancing proliferation and activation of T cells (28, 58). Taken together, our in vitro results indicate that, unlike MM cells, non-neoplastic plasma cells are not directly killed by lenalidomide. However, its possible effects on their niches in vivo might valuably complement the direct actions of bortezomib in patients (42).

Both bortezomib (at higher concentrations) and dexamethasone reduced CD19+ and CD3+ lymphocytes in our cultures. This is in line with the reported effects of bortezomib on activated human B cells (60) and total circulating B cells in experimental autoimmune MG rats (26). Moreover, bortezomib influences T cell subset distributions, inducing apoptosis in activated CD4+ T cells, preventing the activation of memory T cells (61), but preserving resting and regulatory T cells (62–64), and promoting their de novo generation (64). Additional effects of bortezomib on activated B and T cells, or on Ag-presenting B cells, could be an advantage in treating MG patients, for example, in preventing the generation of new autoreactive plasma cells while also eliminating the existing long-lived subset.

FIGURE 3. Susceptibility of irradiated cells to experimental drugs. Thymic cells were irradiated and subsequently cultured for 2 wk. Drugs were added on days 7 and 11 (bortezomib at 2.5 μM, lenalidomide at 10 μM, dexamethasone at 10 nM) and samples were collected and analyzed as for Fig. 2. (A) Plasma cell survival from patients MG-1, MG-5, and MG-6 on day 14 shown as percentage of the absolute numbers in the control cultures (Table II). (B) AChR autoantibody secretion from patient MG-1 on day 11. The AChR autoantibody production rate from patients MG-3, MG-5, and MG-6 was below detection limit; in MG-2, it was just above background. (C) IgG production rate from irradiated samples on day 14. Each point represents one well (three per condition) from the indicated patients; horizontal bars are median values. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.
patients with lower doses and shorter courses of bortezomib may offer valuable therapeutic benefits while minimizing side effects, because even partial elimination of pathogenic plasma cells might be adequate, especially when combined with plasma exchange (65, 66). At doses commonly used to treat MM, SLE, thrombotic thrombocytopenic purpura, and acute Ab-mediated transplant rejection (1.3 mg/m²; resulting in peak plasma levels of 600 nM) (40), bortezomib can cause serious thrombocytopenia or peripheral neuropathy, particularly in MM patients given other chemotherapeutics to eliminate as many neoplastic cells as possible. In contrast, adverse effects were significantly fewer with “light touch” regimens that maintained therapeutic effects in patients with hyperacute Ab-mediated transplant rejection (24, 65–67) and also in MM patients (40). Finally, some second generation proteasome inhibitors have equal or greater potency but lower neurotoxicity than bortezomib, and they are already being tested in clinical trials (68).

In conclusion, our study using EOMG thymic cells, in combination with our previous results in the experimental autoimmune MG model (26), gives proof-of-principle for using proteasome inhibitors for the elimination of non-neoplastic plasma cells in autoantibody-mediated disorders. This therapeutic strategy could have the important advantage of rapidly reducing autoantibody titers during the lag period before the standard immunosuppressants have taken full effect. However, this potential benefit needs to be balanced very carefully against the possibility that side effects still persist at very low doses of bortezomib.

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We are very grateful to Dr. Henry. Kaminski and Dr. Marguerite E. Hill for helpful discussions, to the patients for their samples, and to E. Goodger and the late Prof. John Newsom-Davis for access to them. We are also very grateful to Dr. Jan Damoiseaux and Jozien Jaspers-Spits for granting access to their

FIGURE 4. Quantitative overview of the drug effects on B and T lymphocytes. Thymic cells were cultured for 2 wk and drugs were added on days 7 and 11 (indicated by arrows). Cells were collected and labeled for FACS analysis on days 0, 2, 9, and 14. Samples were gated to exclude cell debris and selected for viable (PI<sup>-</sup>) cells and for either CD3+ or CD19. In (A), (C), and (E), each point represents the average of samples from patients MG-1, MG-2, and MG-3 (three per patient). Error bars correspond to the SEM. Each point in panels (B), (D), and (F) represents one well (three per condition) from the indicated patients for day 14; horizontal bars are median values. Results are normalized as for Fig. 2B, and absolute cell numbers shown in Table II. At least 10,000 total events per sample were recorded; numbers of events analyzed per gate and per patient are given in Supplemental Table I. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.
EFFECT OF BORTezOMIB ON MG PLASMA CELLS


FIGURE 5. Dose-response curves for plasma cell numbers (A), AChRAb production (B), and total IgG secretion (C) after addition of drugs on day 4 and assay on day 7. Thymic cells from patients MG-1 and MG-3 were used in (A), cells from MG-4 and MG-5 in (B), and cells from MG-1, MG-3, MG-4, MG-5, MG-7, and MG-8 were used in (C). Each point represents the average of the normalized results for all the patients analyzed (at least three replicates per patient) and error bars represent the SEM. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses. *** p < 0.001 compared with the untreated cultures (open square).

Disclosures

The authors have no financial conflicts of interest. The School for Mental Health and Neuroscience of Maastricht University receives financial support from Takeda for testing proteasome inhibitors.

References


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Supplemental Table S1
Total number of events counted for each of the subpopulations analyzed by flow cytometry.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Viable cells (Nº Events)</th>
<th>CD3⁺ cells (Nº Events)</th>
<th>CD19⁺ cells (Nº Events)</th>
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<tbody>
<tr>
<td>MG-1</td>
<td>1,312</td>
<td>859</td>
<td>115</td>
</tr>
<tr>
<td>MG-2</td>
<td>2,143</td>
<td>1,573</td>
<td>168</td>
</tr>
<tr>
<td>MG-3</td>
<td>1,443</td>
<td>805</td>
<td>126</td>
</tr>
<tr>
<td>MG-4</td>
<td>4,183</td>
<td>1,831</td>
<td>116</td>
</tr>
<tr>
<td>MG-5</td>
<td>25,517</td>
<td>16,894</td>
<td>916</td>
</tr>
<tr>
<td>MG-6</td>
<td>3,953</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A: not analyzed
Supplemental Figure 1. Drug effects on fresh thymic cell cultures. Thymic cells from patient MG-10 were dispersed mechanically and enzymatically immediately after thymectomy and cultured for 14 days. Bortezomib and dexamethasone were added at different concentrations on days 7 and 11, and cells were collected on day 14. Both AChR Ab (A) and IgG (B) production were reduced by bortezomib at concentrations $\geq 1 \times 10^{-8}$M, while dexamethasone had no significant effects on either. Each point represents the average of at least two samples, and error bars correspond to the SEM. One-way ANOVA and Bonferroni post-hoc testing were used for statistical analysis. * $p<0.05$; ** $p<0.01$, compared with the untreated cultures (white square).
Supplemental Figure 2. Cytofluorometric analysis of drug effects on CD138^+ cells. Thymic cells were cultured for 14 days and drugs were added on days 7 and 11. Cells were collected and labeled for FACS analysis on day 14. **A.** Gating strategy for selection of the plasma cell population. We gated on CD45^+, CD3^- and IgG^{high}/CD138^+ cells and excluded debris and doublets (not shown). Approximately 150,000 events per sample were recorded. Samples exposed to bortezomib showed a very significant reduction in absolute plasma cell numbers when enumerated under the microscope after cytocentrifugation (**B**), and this largely correlated with the drug effects observed by quantifying them by FACS (**C**). Dexamethasone and lenalidomide also reduced their absolute numbers significantly (**C**), but to a lesser extent than bortezomib. **(D)** A broader range of concentrations was tested for both bortezomib and dexamethasone; only bortezomib induced a significant depletion of plasma cell numbers (1x 10^{-6}M - 1x 10^{-8}M and 1x 10^{-10}M). One-way ANOVA was used for statistical comparison between conditions, each bar or point represents the average of 3 replicates and error bars correspond to the SEM. We used cells from patient MG-5 because of higher CD138^+ frequencies/ absolute numbers.
Supplemental Figure 3. General toxicity of the experimental drugs on thymic cells. Thymic cells from patient MG-9 were cultured for 1 week in the presence of different concentrations of the experimental drugs. Cell viability was evaluated at 6 h, 24 h, 48 h and 1 week by measuring propidium iodide (PI) incorporation by flow cytometry; samples were gated in the FSC-SSC dot-plot to exclude membrane debris. Cell viability decreased with time in all the conditions and was lower after 1 week exposure to dexamethasone (10 nM and 1 µM) and bortezomib 2.5 µM than to bortezomib (10 nM) or lenalidomide (10 µM). Each bar represents the average of 3 replicates and error bars the SEM.