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

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

_J Immunol_ published online 27 June 2014
http://www.jimmunol.org/content/early/2014/06/27/jimmunol.1301555
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,*₁ Abhishek Saxena,* Hans Duimel,§,† Fons Verheyen,§,† Peter C. Molenaar,* Wim A. Buurman,* Marc H. De Baets,* Pilar Martinez-Martinez,*¹ 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: 000–000.

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 antimalyeloma 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 tilters 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, 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,plers. None of the patients had been pretreated with glucocorticoids; otherwise, they were selected only because of high serum anti-AChR titers.
They were then washed in 0.09 M KH$_2$PO$_4$ buffer with fixed with 3% glutaraldehyde plus 1.4% sucrose buffered in 0.09 M 7.5% sucrose and transferred to a 1% OsO$_4$ 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.

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 KH$_2$PO$_4$ at pH 7.4. They were then washed in 0.09 M KH$_2$PO$_4$ buffer with 7.5% sucrose and transferred to a 1% OsO$_4$ 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 $\leq 0.05$ was considered significant. Values are expressed as means ± SEM unless stated otherwise. We used Spearman (nonparametric) correlation coefficients ($\rho$).

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 hypoplastic 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%).

Table I. Information on EOMG patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>MG Grade</th>
<th>Age at MG Onset (y)</th>
<th>MG Duration at Thymectomy (y)</th>
<th>Anti-AChR (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-1</td>
<td>F</td>
<td>2A</td>
<td>20</td>
<td>2.5</td>
<td>&gt;730</td>
</tr>
<tr>
<td>MG-2</td>
<td>F</td>
<td>3</td>
<td>16</td>
<td>0.6</td>
<td>180</td>
</tr>
<tr>
<td>MG-3</td>
<td>F</td>
<td>3</td>
<td>24</td>
<td>0.4</td>
<td>252</td>
</tr>
<tr>
<td>MG-4</td>
<td>F</td>
<td>2B</td>
<td>39</td>
<td>1.8</td>
<td>71</td>
</tr>
<tr>
<td>MG-5</td>
<td>F</td>
<td>2A</td>
<td>39</td>
<td>1.8</td>
<td>156</td>
</tr>
<tr>
<td>MG-6</td>
<td>F</td>
<td>2B</td>
<td>21</td>
<td>0.7</td>
<td>72</td>
</tr>
<tr>
<td>MG-7</td>
<td>F</td>
<td>2A</td>
<td>34</td>
<td>0.5</td>
<td>79</td>
</tr>
<tr>
<td>MG-8</td>
<td>F</td>
<td>2A</td>
<td>25</td>
<td>13.8</td>
<td>500</td>
</tr>
<tr>
<td>MG-9</td>
<td>M</td>
<td>2B</td>
<td>36</td>
<td>0.8</td>
<td>23</td>
</tr>
<tr>
<td>MG-10</td>
<td>F</td>
<td>2A</td>
<td>20</td>
<td>0.7</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

Patients MG-1 through MG-9 were thymectomized in London and their thymic cell suspensions were stored in liquid nitrogen at the Neuroscience Group Biobank (Oxford University); MG-10 was thymectomized in Maastricht (the Netherlands) and cells were tested fresh. None of the patients received immunosuppressive drugs before thymectomy, and all thymi showed follicular hyperplasia.

Absolute values for all the parameters measured in these cultures are shown in Table II. To maximize plasma cell recovery/activity, we used cells that had been dispersed with dispase and collagenase and then cryostored (7, 35); these cells behaved very similarly to fresh thymic cells in culture, with their AChR Ab and total IgG productivity often slightly exceeding that of their fresh counterparts, probably reflecting plasma cell enrichment by depletion of thymocytes (35). In fact, cells cultured from one fresh EOMG thymus gave substantially similar results (see below; Supplemental Fig. 1, Table II). Autoantibody production also appeared highly dependent on cell concentration and on adherent “feeder” fibroblasts and macrophages (35). Although microenvironments are probably not optimal in vitro, spontaneous autoantibody and total IgG production in EOMG cells was nonetheless relatively consistent in quadruplicate wells from most of the thymi tested (Fig. 2D, 2F). Remarkably, both persisted for at least 2 wk, even after irradiation (Fig. 3), although only occasional viable macrophages and fibroblasts could still be seen (not shown), again highlighting that small numbers of plasma cells are able to produce substantial amounts of IgG/autoantibody (Table II).

Bortezomib rapidly induces apoptosis in plasma cells from EOMG thymi

We precultured thawed EOMG thymic cells for 3 d before adding 2.5 μM bortezomib. This concentration was based on previous in vitro experiments on human plasma cells (39) and the peak concentration measured in MM patients (40).

In all control samples, plasma cell ultrastructure appeared normal, with elaborate endoplasmic reticulum (ER), a well-defined
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 (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.
perimeter, and distension of the ER (41). After 24 h, they were no longer detected in the bortezomib-treated cultures. Results were very similar with bortezomib at 0.25 μM (not shown).

**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 < 0.0001).

Notably, dexamethasone merely prevented their rise after day 9, their spontaneous AChR autoantibody and total IgG productivity and plasma cell survival, these results deeply implicate plasma cells, rather than B cells, in the spontaneous Ab production that we observed in vitro.

**Dose dependence of bortezomib and dexamethasone effects on plasma cell numbers and function**

We next tested broader concentration ranges of dexamethasone and bortezomib in cultures from one fresh and six cryopreserved EOMG thymi. Total IgG and autoantibody productivity and plasma cell numbers were all minimal in the presence of 10 nM–10 μM bortezomib (Fig. 5, Supplemental Fig. 2D); leukemia and MM cell lines also have IC_{50} values of 10–20 nM (48). In sharp contrast, dexamethasone had no significant effects, even at 1 μM. Thus, the minimum dose of bortezomib for eliminating plasma cells in vitro is apparently 10 nM. We also tested broader concentrations of lenalidomide, but did not observe any significant effects on plasma cell survival or function (not shown).

To assess their general toxicity, we sampled cultures at earlier times after addition of these drugs. At 6, 24, and 48 h, we found no significant differences in overall viability (Supplemental Fig. 3). At day 7, viabilities were reduced more by dexamethasone at 1 μM than at 10 nM. The viabilities were still more reduced by bortezomib at 2.5 μM, but not significantly at 10 nM, where its effects were more selective for plasma cells.

**Discussion**

In this study we demonstrate that bortezomib selectively eliminates long-lived autoimmune plasma cells in cultured thymus cells from nine of nine EOMG patients analyzed. Their spontaneous AChR autoantibody and total IgG production were promptly and almost completely halted, even at 10 nM. At 0.25 and 2.5 μM (and within 8 h) it led to ultrastructural changes in plasma cells that are characteristic not only of ER stress but also of apoptosis, as seen in vivo as

### Table II. Absolute values (average ± SD) for the different parameters analyzed in thymic cell controls (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>0.2 ± 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>&lt;0.1</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.
sequences, suggest that native AChR, which is continuously avail-
able in the thymus, is driving most Ab-producing cells to the ter-
ninal 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 radioresistance clearly shows that they are well
established in many EOMG thymus and scarcely replaced in cul-
ture. 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 unirradi-
cated cells. Evidently, most of the thymic plasma cells are dexam-
ethasone- 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 sup-
porting cells and/or damage other short-lived plasma cells or plas-
mablasts. In vivo, however, their precursors may be steroid-insensitive
as well; we noted no obvious decrease in PWM-stimulated IgG re-
sponses by (radiosensitive) B cells from prednisone-pretreated patients;
rather, they appeared to be enriched (53).

The even smaller effects on plasma cells of the immunomod-
ulatory 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 exam-
ple, 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, 29). 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 valuable 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 acti-
vation 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.

The susceptibility of plasma cells that we observed in the present
study, even to 10 nM bortezomib, is striking. Treating autoimmunee
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.

Acknowledgments

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 Danoiseaux 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−) 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. One-way ANOVA and Bonferroni post hoc testing were used for statistical analyses.
EFFECT OF BORTEZOMIB ON MG PLASMA CELLS