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The Early Marginal Zone B Cell-Initiated T-Independent Type 2 Response Resists the Proteasome Inhibitor Bortezomib

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The proteasome inhibitor bortezomib is approved for the treatment of multiple myeloma and mantle cell lymphoma. We recently demonstrated that bortezomib eliminates autoreactive plasma cells in systemic lupus erythematosus mouse models, thereby representing a promising novel treatment for Ab-mediated diseases. In this study, we investigated the effects of bortezomib on the just developing and pre-existing T-dependent Ab response toward dinitrophenyl-keyhole limpet hemocyanin and the T-independent type 2 response toward (4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP)-Ficoll in BALB/c mice. Bortezomib treatment strongly reduced T-dependent Ab titers mainly due to depletion of plasma cells. In contrast, the early T-independent type 2 response against i.v. administered NIP-Ficoll, which is predominantly dependent on marginal zone (MZ) B cells, resisted bortezomib. Upon bortezomib treatment, immunoproteasome subunits and the antiapoptotic unfolded protein response including NF-κB were induced in NIP-Ficoll–stimulated MZ B cells, but not in plasma cells and follicular B cells. In summary, bortezomib treatment decreases Ab titers arising from T-dependent immune responses predominantly by eliminating plasma cells. In contrast, the early T-independent type 2 response protecting the organism against blood-borne pathogens remains largely intact due to a remarkable resistance of MZ B cells against proteasome inhibition. The Journal of Immunology, 2010, 185: 5637–5647.

The first clinically approved proteasome inhibitor bortezomib (Velcade) is successfully used for the treatment of relapsed multiple myeloma and mantle cell lymphoma (1). The use of bortezomib in other malignancies is currently investigated in clinical trials (2). Bortezomib inhibits the chymotrypsin-like activity of the 26S proteasome, a proteolytic complex that degrades most cellular proteins, including cell cycle regulators and signaling molecules (3). Importantly, the proteasome is involved in the regulation of NF-κB activity, because proteasomal degradation of its inhibitors, the IκB proteins, is required for NF-κB activation. NF-κB drives the expression of several antiapoptotic and oncogenic factors (4).

We reported recently for mouse models of systemic lupus erythematosus that bortezomib efficiently depletes short- and long-lived plasma cells, with the latter being resistant to conventional therapies, and it markedly ameliorates the disease course (5). The selective elimination of plasma cells producing huge amounts of secreted Iggs can mainly be explained by the induction of endoplasmic reticulum (ER) stress by accumulation of misfolded translational products upon proteasome blockade (6). ER stress activates the unfolded protein response (UPR) to ensure cell survival by activation of the ER-associated degradation pathway, attenuation of translation, and induction of chaperones and survival factors such as NF-κB (7). However, if the adaptive mechanisms fail to compensate overwhelming ER stress, the terminal UPR leads to apoptosis (8). Toxic effects on cells that neither synthesize large amounts of proteins nor rapidly proliferate are uncommon (9).

In this study, we compared the influence of bortezomib on early and late phases of T-dependent and T-independent type 2 Ab responses. Unlike the other B cell subsets and plasma cells during T-dependent responses, marginal zone (MZ) B cells, which play an important role in the T-independent first-line defense against blood-borne pathogens such as encapsulated bacteria (10), were completely resistant against bortezomib. Upon Ag encounter, MZ B cells differentiate into large numbers of plasmablasts within hours and produce huge amounts of IgM Abs (11, 12). We expected that the drastic increase of Ab synthesis upon (4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP)-Ficoll stimulation should render activated MZ B cells sensitive to bortezomib. However, the numbers of MZ B cells remained unchanged, and IgM levels to the T-independent type 2 Ag even increased upon bortezomib administration. We provide evidence that induction of immunoproteasomes, the absence of terminal UPR activation, and persisting activation of NF-κB may contribute to the resistance of MZ B cells to bortezomib. Thus, proteasome inhibition might not cause relevant impairment of first-line immune responses against blood-borne pathogens such as encapsulated bacteria.

Materials and Methods

Treatment of mice
Mouse experiments were approved by the Government of Mittelfranken. BALB/c mice (6–8 wk old) were purchased from Janvier (Le Genest-St-
Isle, France). To examine the effect of bortezomib on the T-dependent response, mice were immunized i.p. with 100 µg dimethoxyphenyl (FDP)-keyhole limpet hemocyanin (KLH) (Calbiochem, La Jolla, CA) diluted in PBS, adding an equal volume of IFA (Sigma-Aldrich, Steinheim, Germany) to a total volume of 500 µl. After 14 d, mice were boosted with 100 µg DNP-KLH. To assess the effect of bortezomib on the ongoing immune response, groups of six mice were treated i.v. with 0.75 mg/kg body weight (BW) bortezomib (Velcade; Janssen-Cilag, Neuss, Germany) beginning simultaneously with the first immunization and then twice weekly for 56 d. Controls received PBS. Another group of mice was injected i.v. with PBS for 28 d before starting bortezomib treatment (late treatment). Serum samples were obtained every 14 d. After 56 d mice were sacrificed.

To analyze the T-independent type 2 response, mice were immunized with 25 µg NIP-Ficoll (Biosearch Technologies, Novato, CA) diluted in PBS containing 50 µl. To assess the effect of bortezomib on the early phase of the response to NIP-Ficoll, mice were treated i.v. with 0.75 mg/kg BW bortezomib on days 0, 3, and 5. After 5 d mice were sacrificed. Alternatively, mice were treated i.v. with bortezomib simultaneously with the immunization twice weekly for 16 d. Controls received PBS. Another group of mice received PBS for 8 d and then was treated i.v. with bortezomib from day 8 weekly for 16 d. Controls received PBS. A third group of mice received PBS for 8 d and then was treated i.v. with bortezomib from day 8 weekly for 16 d. Controls received PBS. A third group of mice received PBS for 8 d and then was treated i.v. with bortezomib from day 8 weekly for 16 d. Controls received PBS.

Flow cytometric analyses

Single-cell suspensions from spleens and bone marrows (femora) were obtained as previously described (13). Flow cytometric analyses were performed using fluorochrome-conjugated mAbs to mouse CD4, CD5, CD8, CD11a, CD11b, CD11c, CD21, CD23, CD25, CD69, CD138, c-kit, IgG, IgM, κ-L chain, λ-L chain (all from BD Biosciences, Heidelberg, Germany), F4/80, CD45R (Caltag Laboratories, Hamburg, Germany), and biotin-conjugated CD69 (Jackson ImmunoResearch Laboratories, West Grove, PA). For the staining of germinal center B cells, peanut hemagglutinin (Vector Laboratories, Burlingame, CA) was used. For the analyses of plasma cells and plasmablasts, intracellular κ- and λ-L chains and intracellular IgM, respectively, were stained using the Fix and Perm cell permeabilization kit (CalTag Laboratories) according to the manufacturer's instructions. Cytofluorometric analyses were performed on a FACSCalibur and analyzed using CellQuest software (both from BD Biosciences). Total cell numbers were calculated by multiplication of c-kit+ cells from a nonimmunized mouse.

ELISPOT assays

To assess cells secreting IgG Abs to DNP-KLH, 96-well multiscreen plates (Millipore, Billerica, MA) were coated with 2 µg/ml DNP-KLH. Cell suspensions and bone marrow were prepared and 2 × 105 splenocytes or 2 × 105 bone marrow cells were incubated in dodecyl maltoside for 2 h at 37°C in a humidified incubator containing 5% CO2. After incubation, cells were washed away and the plates were incubated with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 20°C. Bound IgG specific to DNP-KLH was stained by ROX reagent (Thermo Fisher Scientific/Abgene, Hamburg, Germany). cDNA obtained from RNA prepared of a pool of 105 follicular and 3 × 104 germinal center B cells was used as reference to calculate the relative amounts of mRNA.

ELISA

Sandwich ELISAs were performed in triplicates on 96-well microplates (Thermo Fisher Scientific, Roskilde, Denmark). To quantify concentrations of IgM, IgG, and IgG subclasses, plates were coated with goat anti-mouse antiserum (1 µg/ml; all from SouthernBiotech), and bound Abs were detected with HRP-conjugated goat anti-mouse secondary Abs (SouthernBiotech). The Ig concentrations were calculated using standard curves of purified IgG, IgA, and IgM Abs (SouthernBiotech). For the analysis of DNP-KLH–specific Abs, plates were coated with 2 µg/ml DNP-KLH. In a first set of experiments, optimal sera dilutions were determined. According to these results, sera were diluted 1:1000 to analyze anti-DNP-KLH IgM and 1:2000 to assess anti-DNP-KLH IgG. To detect anti-DNP-KLH Abs, plates were coated with 2 µg/ml NIP-BSA (Biosearch Technologies). Sera were diluted 1:200 to detect anti-NIP-IgM and 1:1000 to analyze anti-NIP-IgG. The amounts of DNP-KLH–specific IgM and IgG Abs were quantified with HRP-conjugated goat anti-mouse secondary Abs (SouthernBiotech). o-Phenylenediamine dihydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was used as substrate. OD was measured at 495 nm in a SpectraMax 190 ELISA reader (Molecular Devices, Ismaning, Germany).

Isolation of plasma cells and plasmablasts

Mice were immunized with DNP-KLH as described above. After 14 d spleenocytes and bone marrow cells were isolated. Plasma cells were stained with Abs to CD138 and FcγRIIB (BD Biosciences), plasmablasts were stained with Abs to CD138 and IgM, and cells were sorted with a purity >95% by a MoFlo cell sorter (Dako, Boxborough, MA). Anti-CD25 was used to exclude pre-B cells. To assess transcriptional changes after bortezomib treatment, mice were once injected i.v. with 1 mg/kg BW bortezomib or PBS and sacrificed 4 h later.

Isolation of MZ and follicular B cells

Mice were immunized i.v. with 25 µg NIP-Ficoll. After 5 d spleenocytes were isolated. Cells were stained with Abs to B220 (CD45R), CD21, and CD23. MZ B cells were sorted using a MoFlo cell sorter as B220+CD21highCD23low population, and follicular B cells were sorted as B220+CD21lowCD23high population, both with a purity >98%. To assess the effect of bortezomib, mice were once injected i.v. with 1 mg/kg BW bortezomib or PBS and sacrificed 4 h later.

RNA and cDNA preparation

The Qiagen Mini RNAeasy kit (Qiagen, Hilden, Germany) was used for the isolation of total RNA. DNAse digestion was performed with RNase-free DNase (Qiagen). First-strand cDNA synthesis was performed with the SuperScript III reverse transcriptase (RT) first-strand synthesis system (Invitrogen, Karlsruhe, Germany). cDNA integrity was checked by PCR of β-actin.

Real-time PCR

cDNA was used as template for real-time PCR reactions with corresponding primers (all retrieved from the Harvard PrimerBank at http://pga.mgh.harvard.edu/primerbank/): Bax forward, 5′-TAAGACAGACGAGCTACTTTCG-3′, Bax reverse, 5′-ATTGCGGAGGACACTCCTG-3′; Bcl-2 forward, 5′-ATGCCCTTTGGTGGAACTATATGGC-3′; Bcl-2 reverse, 5′-GGATGACCCCAAGAGATGC-3′; BiP forward, 5′-ACTTGGGAGGACACATTACTCCT-3′; BiP reverse, 5′-ATGCCAATACGAGCCTTC-3′; CHOP forward, 5′-CTGGAGATCTGTGAGGATG-3′; CHOP reverse, 5′-GTGCTTTATGATGCTCCATG-3′; IκBα forward, 5′-TCAGGACGGAGTACGACG-3′; IκBα reverse, 5′-TTCGTTGAGATTGC-GCAAGTG-3′; β-actin forward, 5′-GCCTGTATTCCCCCCTCTATCG-3′; β-actin reverse, 5′-CCATTGGTTAACAATGCCATGT-3′; LMP2 forward, 5′-CTTGGCTCCCTTGTCCTGC-3′; LMP2 reverse, 5′-GGCTGTTGTACGACAGTCT-3′; Bcl-2 forward, 5′-GCCTATGACGTTACGAG-3′; IκBα forward, 5′-CCACAGGCGGGTTTATGCATG-3′; IκBα reverse, 5′-GGCTCATTAGTTCGACGTC-3′; and absolute quantitative RT-PCR SYBR Green ROX reagent (Thermo Fisher Scientific/Abgene, Hamburg, Germany). Relative real-time PCR was performed in triplicates for each sample in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany). cDNA obtained from RNA prepared of a pool of 105 splenocytes was used as reference to calculate the relative amounts of mRNA.

Measurement of proteasomal activity

Sorted plasma cells, plasmablasts, and MZ and follicular B cells (2 × 104 cells each) were disseminated in pentaplicates. The chymotrypsin-like proteasomal activity was detected using the luminogenic proteasome substrate-based Protease-Glo chymotrypsin-like cell-based assays kit (Promega, Madison, WI) according to the manufacturer’s instructions. A 96-well SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA) was used for measurement.

Immunoblotting

NIP-Ficoll–immunized mice were once injected i.v. with 1 mg/kg BW bortezomib or PBS and sacrificed after 8 h. Sorted 5 × 103 follicular and MZ B cells were washed with PBS and directly lysed in SDS sample buffer containing 2-ME to generate total cell lysates. The following primary Abs were used: mouse monoclonal anti-actin (Sigma-Aldrich, Taufkirchen, Ger-
Mobilization of MZ B cells into the peripheral blood

MZ B cells of NIP-Ficoll–immunized mice were released into the peripheral blood by i.v. injection of 0.1 mg rat anti-mouse CD49d/VLA-4 Abs (clone PS/2; Biozol, Eching, Germany) and 0.1 mg rat anti-mouse CD11a (integrin α-L chain, LFA-1 α-chain) mAb (clone M17/4; BD Pharmingen). Mice were simultaneously treated i.v. with 1 mg/kg BW bortezomib or PBS, respectively. Controls received the same volume of PBS instead of the anti-integrin Abs and were injected i.v. with bortezomib or PBS in the same manner.

FIGURE 1. Inhibition of the T-dependent response toward DNP-KLH upon bortezomib treatment beginning with immunization. Mice were immunized twice with DNP-KLH at intervals of 14 d and treated with bortezomib every 72 h for 56 d. A, Total numbers and representative dot plots of CD138+/cytoplasmic k/a-light chain+/CD252 plasma cells in the spleen (upper panel) and the bone marrow (lower panel) of controls (PBS) and bortezomib-treated mice analyzed by flow cytometry. Cells were gated to be CD25+. Each diamond or square represents one mouse. Diamonds represent PBS-treated controls; squares denote bortezomib-treated mice. Gray symbols indicate the mice shown in the representative dot plots. Percentages of CD138+/cytoplasmic k/a-light chain+/plasma cells are indicated.

B, Total numbers and representative wells of ELISPOT results for IgG Abs to DNP-KLH–secreting cells in the spleen (upper panel) and the bone marrow (lower panel) of controls (PBS) and bortezomib-treated mice.

C, Total numbers and representative wells of ELISPOT results for IgG Ab-secreting cells in the spleen (upper panel) and the bone marrow (lower panel) of controls (PBS) and bortezomib-treated mice.

D, Concentrations of anti–DNP-KLH IgM (upper panel) and IgG (lower panel) Abs in bortezomib-treated and control (PBS) mice measured by ELISA.

E, Concentrations of total IgM (upper panel) and IgG Abs (lower panel) in bortezomib-treated and control (PBS) mice measured by ELISA. Arrows indicate immunizations with DNP-KLH. Data are representative of three independent experiments. Bars in A–C represent mean values. Error bars depict SD. The Mann–Whitney U test was used for all statistical analyses. *p < 0.05; **p < 0.004; ***p < 0.0004 (n = 6 mice/group). Bz, bortezomib.
Statistical analysis

The nonparametric Mann–Whitney U test was used for all statistical analyses of results of flow cytometric analyses, ELISPOT, and ELISA. The Student t test for heteroscedastic samples was used for statistical analyses of RT-real-time PCR results. All statistical analyses were calculated using SPSS for Windows (SPSS, Munich, Germany).

Results

Reduced plasma cell numbers and Ab concentrations upon bortezomib treatment during immunization with DNP-KLH

To investigate the influence of proteasome inhibition on T-dependent Ab responses, mice were treated with bortezomib for 56 d beginning simultaneously with the first DNP-KLH immunization. Calculations of total numbers of CD138<sup>hi</sup>/cytoplasmic κ- and λ-L chain<sup>+</sup>/CD25<sup>−</sup> plasma cells in the spleens and the bone marrows were based on total cell counts and flow cytometric analyses and showed a strong decrease in bortezomib-treated mice when compared with controls (Fig. 1A). Accordingly, ELISPOT assays demonstrated a striking decrease of DNP-KLH-specific IgG as well as total IgG-secreting cells in the spleens and the bone marrows (Fig. 1B, 1C).

In bortezomib-treated mice there was only a slight increase in anti–DNP-KLH IgM and IgG Ab concentrations upon the first immunization, with no further increase upon the booster immunization.
zation (Fig. 1D). Serum concentrations of total IgG and IgM displayed only a subtle or transient decrease upon bortezomib treatment, whereas serum concentrations increased in the control group (Fig. 1E). The analyses of IgG subclasses by ELISA revealed differential effects of bortezomib: IgG1, IgG2a, and IgG2b titers were significantly decreased, whereas there was only a trend toward lower IgG3 concentrations (data not shown). Taken together, bortezomib treatment simultaneous with the immunization reduced the T-dependent generation of Abs.

Reduction of pre-existing DNP-KLH–specific plasma cells upon bortezomib treatment

To investigate whether the impaired Ab response toward DNP-KLH acts predominantly in the phase of T-dependent B cell activation and differentiation or is mainly due to the depletion of plasma cells, we started treatment with bortezomib 14 d after the booster immunization when plasma cells had already differentiated. Flow cytometric analyses revealed a significant reduction of CD138<sup>hi</sup>/cytoplasmic κ- and λ-L chain<sup>+</sup>/CD25<sup>+</sup> plasma cells in the spleens and bone marrows after 28 d of bortezomib application (Fig. 2A). The numbers of cells secreting either anti–DNP-KLH IgG or IgG Abs in both the spleens and the bone marrows were reduced to <20% of vehicle-treated controls as detected by ELISPOT (Fig. 2B, 2C).

The reduction of plasma cell counts upon bortezomib treatment was reflected by markedly lower anti–DNP-KLH IgM and IgG (Fig. 2D) as well as by total IgM and IgG serum concentrations compared with controls (Fig. 2E), having been observed already 14 d after the medication had started. Consistent with the data of the treatment for 56 d, ELISA analyses of IgG subtypes revealed a reduction of IgG1, IgG2a, and IgG2b concentrations, whereas the IgG3 levels remained largely unaffected (data not shown). Hence, also the pre-existing Ab response toward the T-dependent Ag DNP-KLH was decreased by bortezomib application starting 14 d after the last immunization, indicating the depletion of already generated plasma cells.

MZ B cells resist long-term bortezomib treatment

We next determined the sensitivity of different B cell subsets toward bortezomib. Flow cytometric analyses revealed that numbers of B220<sup>+</sup>/CD23<sup>hi</sup>/CD21<sup>low</sup> follicular B cells were significantly reduced after 56 and 28 d of bortezomib treatment (Fig. 3), but not after 2 d (5). In contrast, B220<sup>+</sup>/CD21<sup>hi</sup>/CD23<sup>low</sup> MZ B cells were resistant against proteasome inhibition. We even found a trend toward increased absolute numbers of MZ B cells in bortezomib-treated mice (Fig. 3), B220<sup>+</sup>/IgM<sup>+</sup>/IgD<sup>+</sup> mature B cells, B220<sup>+</sup>/peanut hemagglutinin<sup>hi</sup> germinal center B cells, and B220<sup>+</sup>/IgM<sup>+</sup>/CD5<sup>+</sup> B1 cells were diminished after 56 and 28 d of bortezomib treatment (data not shown).

Increased IgM Ab titers in MZ B cell-initiated T-independent type 2 responses upon bortezomib treatment

To further investigate our observation that MZ B cells were completely resistant toward proteasome inhibition, we i.v. immunized mice with the T-independent type 2 Ag NIP-Ficoll to preferentially activate MZ B cells and drive their differentiation into plasmablasts. We expected that these highly Ab-secreting cells should become sensitive toward bortezomib. Unexpectedly, bortezomib treatment resulted in a significant increase in anti–NIP-IgM concentrations on day 10 in mice treated with bortezomib from day 0. On day 15 the anti–NIP-IgM decreased to levels of PBS-treated controls. In contrast, titers of mice treated from day 8 were similar to controls (Fig. 4A). Consistent with the increase in anti–NIP-IgM Abs, flow cytometric analyses revealed a trend toward increased numbers of B220<sup>+</sup>/CD21<sup>hi</sup>/CD23<sup>low</sup> MZ B cells in both long- and short-term bortezomib-treated mice (Fig. 4B). Numbers of CD138<sup>hi</sup>/cytoplasmic IgM<sup>hi</sup>/CD25<sup>+</sup> plasmablasts that may have predominantly developed from MZ B cells after NIP-Ficoll stimulation were not reduced after 5 d of treatment (Fig. 4C). However, anti–NIP-IgG concentrations, which were predominantly of the IgG3 isotype, remained constantly below controls in both bortezomib-treated groups (Fig. 4D). These data indicate that the MZ B cell initiated T-independent type 2 IgM response is completely resistant toward proteasome inhibition during the initial phase.

Induction of immunoproteasomal subunits in MZ B cells upon bortezomib treatment

To investigate whether the resistance of MZ B cells toward bortezomib is due to abundant expression of proteasomes, we determined the chymotrypsin-like activity of the 26S proteasome. MZ and follicular B cells displayed a similar proteasomal activity, which was markedly lower than proteasomal activities in splenic and bone marrow plasma cells. Plasmablasts exhibited a much higher proteasomal activity than did all other B cell subsets, including plasma cells (Fig. 5A). This extremely high proteasomal activity in plasmablasts is consistent with the induction of proteasomal capacity in LPS-induced plasmablasts previously described by Cascio et al. (14) and might represent a general feature of plasmablasts.

The standard 26S proteasome is composed of three different proteolytically active subunits, β1, β2, and β5. In the immunoproteasomes these subunits are replaced by their corresponding
subunits LMP2, LMP7, and MECL-1 (15). Bortezomib predominantly inhibits the chymotrypsin-like activity of the β5 subunit, but also the proteolytic activity of the immunoproteasome (16). There is evidence that malignant B cell lines, which are insensitive toward bortezomib, display increased expression of the β5 and LMP2 subunits (17). Thus, we investigated mRNA and protein levels of the PSMB5-encoded β5 subunit of LMP2 and of LMP7 by relative RT-real-time PCR and immunoblotting, respectively. MZ B cells showed significantly lower amounts of PSMB5 mRNA than did follicular B and plasma cells (Fig. 5B). After 4 h of bortezomib treatment there was no significant increase of transcripts to PSMB5 in MZ B cells, whereas transcripts to PSMB5 were strongly induced in plasma cells (Fig. 5B). The β5 protein was low in MZ and follicular B cells (data not shown). MZ B cells showed low levels of transcripts to LMP2 and LMP7. Intriguingly, the mRNA concentrations of LMP2 and LMP7 were increased in MZ B cells upon bortezomib exposure. In contrast, LMP2 and LMP7 mRNA strongly decreased in follicular B and plasma cells upon bortezomib treatment. Consistent with the mRNA level, LMP2 protein concentration strongly decreased in follicular B cells upon bortezomib treatment, whereas LMP7 protein concentration remained unchanged (Fig. 5B, 5C). LMP2 and LMP7 protein concentrations remained stable in MZ B cells. These data indicate that MZ B cells constitutively express very low amounts of immunoproteasome subunits, which are rapidly induced by bortezomib and may counteract bortezomib-induced ER stress.

**FIGURE 4.** MZ B cells and the early phase of the T-independent type 2 response resist bortezomib. Mice were immunized with NIP-Ficoll and treated for 5 and 16 d, respectively. A, Concentrations of anti-NIP IgM Abs of bortezomib-treated and control (PBS) mice measured by ELISA. Mice were treated with bortezomib either starting simultaneously with the immunization (day 0; upper panel) or from day 8 after the immunization (lower panel). B, Total numbers and representative dot plots of B220+/CD21<sub>high</sub>/CD23<sub>low</sub> MZ B cells of controls (PBS) from day 0 (middle) and from day 8 bortezomib-treated mice (right) analyzed by flow cytometry. Each diamond or square represents one mouse. Diamonds represent PBS-treated controls; closed squares denote mice treated with bortezomib from day 0. Gray quadrats depict mice treated with bortezomib from day 8. Percentages of B220+/CD21<sub>high</sub>/CD23<sub>low</sub> MZ B cells are indicated. C, Total numbers and representative dot plots of CD138<sup>+</sup>/cytoplasmic IgM<sup>+</sup>/CD25<sup>+</sup> plasmablasts in the spleens of control (PBS) and bortezomib-treated mice after 5 d of bortezomib treatment analyzed by flow cytometry. Each diamond or square represents one mouse. Diamonds represent PBS-treated controls; squares denote bortezomib-treated mice. Gray symbols indicate mice shown in the dot plots. Percentages of splenic CD138<sup>+</sup>/cytoplasmic IgM<sup>+</sup>/CD25<sup>+</sup> plasmablasts are indicated. D, Concentrations of anti-NIP IgG Abs of bortezomib-treated and control (PBS) mice measured by ELISA. Mice were treated with bortezomib simultaneously with the immunization (day 0; upper panel) or from day 8 after the immunization (lower panel). Bz 0, bortezomib from day 0; Bz 8, bortezomib from day 8. Data are representative of three independent experiments. Error bars represent SD. Bars in B represent mean values. The Mann–Whitney U test was used for all statistical analyses. *p < 0.05; **p < 0.004 (n = 6 mice/group).
Absence of terminal UPR activation in MZ B cells after bortezomib treatment

Proteasome inhibition leads to inhibition of NF-κB activation due to blockade of IκB degradation. To investigate whether NF-κB activity may be involved in the resistance of MZ B cells against bortezomib, we measured the relative amounts of IκBα transcripts, which are strongly induced by NF-κB. The levels of IκBα mRNA were lower in MZ B cells than in follicular B and in plasma cells, indicating lower NF-κB activity in MZ B cells compared with follicular B cells and plasma cells. However, 4 h after bortezomib treatment, IκBα mRNA concentrations increased in MZ B cells, but decreased in follicular B as well as in plasma cells (Fig. 6A). Hence, unexpectedly, and in contrast to other B cell subsets, MZ B cells can activate the anti-apoptotic transcription factor NF-κB in response to proteasome inhibition.

To assess the ER stress response in MZ B cells and in follicular B and plasma cells, we compared the mRNA and protein concentrations of UPR-related survival and proapoptotic factors by relative RT-real-time PCR and immunoblotting, respectively. The ER-resident molecular chaperone BiP binds to unfolded proteins and indicates activation of the anti-apoptotic UPR (18). When comparing BiP mRNA concentrations, we observed that BiP was abundant in plasma cells, whereas MZ B and follicular B cells showed low BiP mRNA levels. Four hours after bortezomib application BiP mRNA increased in MZ B cells, but strongly decreased in plasma cells and slightly in follicular B cells. Accordingly, BiP protein increased in MZ B cells after bortezomib injection, but it decreased in follicular B cells (Fig. 6). The transcription factor CHOP was shown to exert proapoptotic activity in certain systems (19); however, in B lymphocytes there is no evidence of CHOP directly contributing to apoptosis (20). Nevertheless, CHOP activation can serve as an indicator for activation of the terminal UPR and consequent apoptosis. CHOP mRNA was very low in plasma cells and virtually absent in follicular and MZ B cells. Within 4 h after bortezomib treatment the amount of transcripts to CHOP rose ~180-fold in bone marrow and ~50-fold in splenic plasma cells. In contrast, CHOP mRNA and protein remained undetectable in MZ B cells upon bortezomib treatment (Fig. 6). Despite an increase of CHOP mRNA, protein concentrations remained undetectable in follicular B cells (Fig. 6), which are not or just slightly decreased upon a single bortezomib injection and only moderately decreased after long-term treatment. Members of the Bcl-2 family, such as the proapoptotic Bax protein, contribute to bortezomib-mediated apoptosis induction.
The basal amount of Bax transcripts was relatively high in plasma cells. In contrast, mRNA of the antiapoptotic factor Bcl-2 was expressed at highest concentrations in MZ B cells and somewhat less in plasma cells. Four hours after bortezomib administration mRNA expression levels of both Bax and Bcl-2 were increased in MZ B cells, whereas they were decreased in other B cell subsets (Fig. 6A). Despite mRNA induction, Bax protein remained absent in MZ B cells after bortezomib treatment (data not shown). In summary, only in MZ B cells, but not in other B cell subsets, did bortezomib induce NF-κB activation and other survival factors without activation of the terminal UPR.

MZ B cells maintain bortezomib resistance when released into the blood

Gong et al. (22) showed that resistance of MZ B cells toward treatment with anti-CD20 Abs is due to their cellular microenvironment. To analyze the contribution of the microenvironment to the bortezomib resistance, we mobilized MZ B cells into the peripheral blood by injection of Abs to the integrin α4-chain and the integrin αL-chain after immunization with NIP-Ficoll. Bortezomib or PBS was simultaneously administered. We performed flow cytometric analyses of venous blood 2, 6, and 24 h after treatment. After 2 h IgM+ /B220+/CD21high/CD23low MZ B cells were detectable in the peripheral blood, representing ~3.5% of total blood lymphocytes. As expected, follicular B cells were also mobilized and their counts in the peripheral blood increased (Fig. 7). Importantly, MZ B cells that were mobilized into the circulation did not decline 4 and 6 h after bortezomib injection when compared with mice treated with PBS, indicating persistent resistance toward bortezomib (Fig. 7). Of note, already 2 h after the treatment, numbers of B220low/CD138high/cytoplasmic κ- and λ-L chain+ plasma cells in the blood were diminished by 50%. Additionally, there was a trend toward decreased numbers of follicular B cells upon bortezomib treatment (data not shown). In summary, displacing MZ B cells from the spleen into the peripheral blood did not overcome bortezomib resistance. However, this does not exclude contribution of long-lasting protective effects of the microenvironment.

Discussion

We compared the consequence of bortezomib administration on plasma cells and Ab titers in T-dependent and T-independent type 2 responses. Proteasome inhibition markedly reduced the numbers of plasma cells as well as Ab titers in T-dependent responses. Elimination of plasma cells occurred within 8 h after bortezomib injection (V.R. Lang, S. Meister, R.E. Voll, unpublished data). The depletion of pre-existing plasma cells indicates that bortezomib may suppress T-dependent responses mainly by targeting plasma cells. Nevertheless, there may be an additional suppressive effect of bortezomib on T and dendritic cells impairing plasma cell...
The Ab response to i.v. injected NIP-Ficoll is predominantly mediated by MZ B cells that differentiate into plasmablasts, which produce large amounts of Ag-specific IgM (10). Unexpectedly, bortezomib treatment did not affect the MZ B cell response to NIP-Ficoll. Cascio et al. (14) described an impaired formation of IgM Abs to i.p. applied NIP-Ficoll upon bortezomib treatment, with Ab titers being significantly decreased beyond day 15. When we immunized mice i.p. as performed by Cascio et al. we also observed significantly reduced anti-NIP IgM and IgG levels (V.R. Lang, S. Meister, R.E. Voll, data not shown). It was previously demonstrated that bortezomib-induced Ag-induced mouse T cell proliferation only slightly (13), whereas others reported more pronounced effects of bortezomib on the function of human CD4+ T cells (24).

Bortezomib application clearly dampened T-dependent immune responses by targeting short- and long-lived plasma cells, but it did not obviate the generation of IgM Ab-secreting cells in response to NIP-Ficoll.

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et al. (27). However, the induction of immunoproteasomal subunits alone might not fully explain why MZ B cells resist proteasome blockade. Plasma cells showed higher basal chymotrypsin-like proteasomal activity than did MZ B cells, and the mRNA concentrations of the β5 subunit were strongly increased after bortezomib treatment. As shown previously, increased concentrations of the β5 subunit can protect cells from bortezomib-induced cell death (29). However, in the case of plasma cells, this compensatory mechanism may not be sufficient to prevent the terminal UPR.

Furthermore, we asked whether MZ B cells received survival signals from the specialized MZ microenvironment, which may contribute to their bortezomib resistance. In contrast to the depletion of MZ B cells by anti-CD20 Abs after mobilization out of the spleen (22), mobilized MZ B cells remained resistant to bortezomib at least during the first 6 h. After 24 h MZ B cells had disappeared from the blood again, but we could not distinguish whether this was due to depletion or to resettlement. Hence, it remains elusive as to which stimuli or cellular properties in particular active potential resistance mechanisms such as induction of immunoproteasome subunits and antiapoptotic UPR pathways in MZ B cells. As noncanonical NF-κB signaling is crucial for MZ B cell development (30), it is possible that activation of this pathway might contribute to the resistance toward bortezomib (31). In this respect it is noteworthy that we have preliminary evidence that noncanonical NF-κB signaling is not inhibited by therapeutic concentrations of bortezomib (D. Mielenz, V.R. Lang, S. Meister, R.E. Voll, unpublished data).

Our findings about the differential sensitivity of the T-dependent and T-independent type 2 responses have an important impact for the clinical use of bortezomib. The suppression of T-dependent Ab responses points toward applications in autoantibody-mediated diseases (32) as well as in transplantation medicine to suppress Ab-mediated rejection (33). In fact, Everly et al. (34) reported that bortezomib reduced donor-specific anti-HLA-Ab levels in human kidney transplanted patients refractory toward conventional treatments. Another useful aspect of proteasome inhibition as a therapeutic approach in transplantation medicine might be that bortezomib ameliorates graft-versus-host disease in murine transplantation models when combined with TNF-α blockade or depletion of CD45 T cells (35). The fact that bortezomib treatment leaves T-independent type 2 immune responses intact, which protect the organism against blood-borne pathogens such as encapsulated bacteria, may represent an important advantage for its administration as a rather selective immunosuppressant. Commonly applied cytostatic drugs such as cyclophosphamide cause a severe reduction and slow recovery of granulocytes, lymphocytes, and MZ B cells in rats (36). In contrast, bortezomib appears to leave at least the marginal zone B cell-mediated immediate antibacterial defense intact.

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Disclosures
S.M. and R.E.V. have a patent application for the use of proteasome inhibitors. The other authors have no financial conflicts of interests.

References


