Bortezomib and IL-12 produce synergetic anti-multiple myeloma effects with reduced toxicity to natural killer cells

Xiangling Wang[a], Xiaoli Feng[c], Juandong Wang[c], Na Shao[b], Chunyan Ji[b], Daoxin Ma[b], Jan-Inge Henter[d], Bengt Fadeel[e] and Chengyun Zheng[c,d]

The aim of this study was to examine the hypothesis that a combination of proteasome inhibition by bortezomib and immune therapy with interleukin-12 (IL-12) can produce enhanced antitumor efficacy relative to the effects of either of these agents alone. A mouse xenograft model of myeloma was developed. The mice were randomly divided into saline control (NS), IL-12 (0.4 μg/animal; intraperitoneal), bortezomib (0.75 mg/kg; intravenous), and bortezomib + IL-12 groups. Effects of treatments on tumor growth were assessed by before and after treatment comparisons and group comparisons. The effects of various treatments on the number of peripheral blood lymphocytes and natural killer (NK) cells were assessed by complete blood count and flow cytometry analysis. The cell-killing function of NK cells in splenocytes was evaluated using the lactate dehydrogenase release assay. IL-12 treatment alone produced a mild decrease in tumor volume compared with control ($P > 0.05$). Bortezomib alone resulted in substantial inhibition of tumor growth at varying time points, reaching ~65 and ~60% reduction in tumor volume after 15 and 21 days of therapy, respectively. At the same time points, the combination therapy produced ~75 and ~84% decreases in tumor growth, respectively, which were significantly greater than the reduction produced by bortezomib monotherapy. Tumors resumed growth upon termination of bortezomib treatment at 2 weeks, although the tumor volume was still significantly smaller than that in the time-matched NS and IL-12 animals. This rebound of tumor growth was completely prevented with the combination therapy, and tumor volume continued to decrease throughout the time course. The percentage and total number of NK cells were significantly decreased after bortezomib monotherapy and combination therapy; however, they remained unaltered after IL-12 treatment compared with no treatment. Further, combination therapy significantly restored the bortezomib-induced functional impairment of the cell-killing capability of NK cells, relative to bortezomib alone. We conclude that the bortezomib – IL-12 combination therapy offers superior antitumor efficacy over monotherapy with either bortezomib or IL-12 in a mouse model of myeloma. Restoration of bortezomib-induced functional impairment of NK cells by IL-12 may be a mechanism for the synergetic effects of the two agents. Therefore, a combination of the two agents may represent a more rational therapeutic approach for myeloma.

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Departments of [a]Oncology, [b]Hematology, Qilu Hospital, Shandong University, [c]Department of Hematology, The Second Hospital of Shandong University, Jinan, People's Republic of China, [d]Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska University Hospital and [e]Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Correspondence to Chengyun Zheng, MD, PhD, Department of Hematology, The Second Hospital of Shandong University, Beiyuan Road 247, Jinan, Shandong Province 250033, China
Tel: +86 0531 85875634; fax: +86 0531 85875634;
e-mail: chengyun.zheng@ki.se

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Introduction

Multiple myeloma (MM), also known as plasma cell myeloma or Kahler's disease, is a cancer of plasma cells and is an aggressive malignancy with a poor survival rate even with high-dose chemotherapy [1]. Proteasome inhibitors are an important class of antimyeloma drugs that disrupt the proteolytic machinery of the tumor cells to induce apoptosis and inhibit cell growth, adhesion, and angiogenesis [2]. Bortezomib, the most commonly used potent and specific 26S proteasome inhibitor, has been approved by the Food and Drug Administration for the treatment of MM [3–5]. The therapeutic efficacy of bortezomib in untreated and refractory/relapsed MM has been demonstrated in several clinical trials, showing superiority to the conventional treatments [6–8]. A most recent systemic review and meta-analysis of five randomized controlled trials involving 2728 patients concluded that bortezomib appears to improve survival and response rates of patients with previously untreated MM [9].

In addition to inducing apoptosis, bortezomib also acts by sensitizing MM to natural killer (NK) cell-mediated lysis [10–13]. This mechanism of action of bortezomib is ascribed to the fact that NK cells are populations of lymphocytes that can be activated to mediate significant levels of cytotoxic activity and to produce high levels of certain cytokines and chemokines, thus representing a potent antitumor effector cell [14,15]. However, bortezomib therapy is associated with some toxicities, such as bone marrow suppression, infections, and peripheral

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neuropathy. We and others have found that bortezomib induces apoptosis not only in tumor cells but also in NK cells [16–19], downregulates NKp46 expression, and mitigates NKp46-mediated cytotoxicity [16]. This two-sided action is deemed to reduce the antitumor efficacy of bortezomib.

Interleukin-12 (IL-12) acts as a tumor suppressor, and targeting of IL-12 to tumor cells has been considered a new therapeutic strategy [20–23]. It is a multifunctional cytokine, bridging innate and adaptive immunity by promoting maturation, activation (interferon-γ production and cytolytic activity), and survival of NK cells and T cells [24–26]. In addition to acting on NK cells, IL-12 also induces an antiangiogenic program as a part of its anticancer mechanisms [27–29]. IL-12 has been found to inhibit growth and metastasis of various tumors, and to promote survival of tumor-bearing animals [21,24,27–29]. Intriguingly, Straube *et al*. [30] found that bortezomib effectively inhibited lipopolysaccharide-stimulated spontaneous maturation and release of tumor necrosis factor-α and IL-12 in dendritic cells, thereby inhibiting interferon-γ secretion capacity and antitumor effects of NK cells. Bortezomib has also been reported to effectively inhibit IL-2-activated NK cell cytotoxicity [31].

In lieu of the opposite effects of bortezomib and IL-12 on NK cells (apoptosis-inducing effect of bortezomib vs. cytoprotective effect of IL-12) and the inhibitory effect of bortezomib on IL-12 release, we proposed that a combination of proteasome inhibition and immune therapy may result in increased antitumor efficacy. Specifically, combining bortezomib and IL-12 might eliminate the unwanted action of bortezomib but manifest their joint antitumor effects, offering a superior anti-MM effect compared with that of any of these agents alone. This study was designed to examine this hypothesis through investigation of the potential synergistic anti-MM efficacy of the combination of bortezomib and IL-12 in severe combined immunodeficiency (SCID) mice and the possible resistance to bortezomib-induced NK cell apoptosis.

Materials and methods

Cell lines

Cells from the human myeloma cell line RPMI8226 and mouse lymphoma cell line YAC-1 were cultured in RPMI1640 medium containing 10% fetal bovine serum (Gibco, Gaithersburg, Maryland, USA) at 37°C in a 5% CO_2 incubator.

Experimental animal

Forty-four female SCID BALB/cByJSmnPrkd/J strain mice (4–6 weeks old, weighing 10–15 g) were housed in sterile laminar flow (SPF) animal facilities. Use of animals was in accordance with the guidelines, and the protocols were approved by the Animal Care and Use Ethics Committee of Qilu Hospital, Shandong University.

Severe combined immune deficiency murine model of myeloma

On reaching the logarithmic growth phase, RPMI8226 human peripheral blood myeloma cells were stained with 0.4% trypan blue and suspended at 1×10^8 cells/ml in serum-free RPMI1640 medium. Thereafter, each mouse was inoculated subcutaneously near the foreleg with a 100-μl cell suspension containing $\sim 1 \times 10^7$ viable tumor cells. The SCID mice, purchased from SLAC Laboratory Animal (Shanghai, China), weighing 22–23 g, were randomly divided into saline control (NS), IL-12, bortezomib, and bortezomib + IL-12 groups, with 11 rats in each group. Drug treatment began when tumor diameter reached 5 mm. NS or bortezomib (0.75 mg/kg; Millennium Pharmaceuticals, Cambridge, Massachusetts, USA) was delivered into the mice by tail vein injection twice a week for 2 weeks. IL-12 (0.4 μg/mouse, recombinant murine IL-12; R&D Systems, Minneapolis, Minnesota, USA) was intraperitoneally administered once every other day, five times (10 days). For sequential bortezomib and IL-12 treatment, mice were first injected with bortezomib (0.75 mg/kg) as described above and 1 day later they were treated with IL-12 (0.4 μg/mouse) with the same regimen as described previously. Animals in the NS group were injected with an equal volume of sterile saline. Tumor size and body weight were monitored continuously after 24 days of drug treatment and measured every 2 days up to 21 days. Tumor volume was calculated as: long diameter × (short diameter)^2 × π/6 [32]. Inhibition of tumor growth was quantified according to the following formula: tumor suppression rate = (1 − average tumor volume of treatment/average tumor volume of control) × 100%. Peripheral blood (200 μl) was drawn from mice 1 day before drug treatment and 1 day after treatment for hematological analysis and flow cytometry analysis of the percentile of NK cells. After 45 days, mice were killed by decapitation, the spleen was dissected for cell-killing experiments, and the tumor was weighted.

Flow cytometry measurement of peripheral blood natural killer cells

An aliquot of 100 μl peripheral blood was used for blood cell counting using an automatic blood cell analyzer (MEK-722K; Nihon Kohden Corporation, Tokyo, Japan). A volume of 50 μl heparinized blood was mixed with anti-mouse CD49b (integrin α 2) PE (eBioscience Inc., San Jose, California, USA) and CD3-Percp l antibodies (BD Pharmingen, San Jose, California, USA) for determining NK cells, and with anti-mouse IgG2a-PE and Hamster-PerCP antibodies for control experiments, and incubated at 4°C in the dark for 30 min. Then the samples were washed with PBS and mixed with erythrocyte lysis buffer (BD Pharmingen, USA) to remove erythrocytes. After

washing with PBS, 400 µl of 1% paraformaldehyde was added and the samples were analyzed on a flow cytometer using Cell Quest analysis software (FACS Calibur; BD Biosciences, San Jose, California, USA).

Lactate dehydrogenase release assay natural killer cell function

The LDH assay is an enzymatic assay of cytotoxicity, as LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. The mouse spleen was removed under sterile conditions at the time of sacrifice, ground, and sieved through 100 mesh. The sample was washed with RPMI1640 medium, and the dispersed spleen cells were collected and mixed with erythrocyte lysis buffer to remove red blood cells. The splenocytes were resuspended in RPMI1640 culture medium and incubated at a density of 5×10^6 cells/ml at 37°C for 50 min. Adherent cells were removed and nonadherent cells were collected for later use. The assay was performed using the LDH kit (Biovision, Milpitas, California, USA) according to the manufacturer's instructions. The splenocytes used as effector cells were mixed with cells from the murine T-lymphoma YAC-1 cell line, which is sensitive to NK cells as target cells, at three different ratios: 50 : 1, 25 : 1, and 12.5 : 1. The mixtures were added into a 96-well culture plate and incubated at 37°C for 4 h. The supernatant (100 µl) was collected, mixed with 100 µl reaction solution, and incubated at room temperature in the dark for 30 min. The optical density (OD) of each well was measured using a microplate reader (DG-5031; Nanjing Huadong Electronic Tube Factory, Nanjing, China) at a wavelength of 490 nm. YAC-1 cell death rate was calculated as follows: YAC-1 cell death rate (%) = $(OD_{test} - OD_{basal})/(OD_{max} - OD_{basal}) \times 100\%$, where OD_{test} represents the values measured from treatment samples, OD_{basal} the LDH released under basal conditions, and OD_{max} the maximum release of LDH.

Statistical analysis

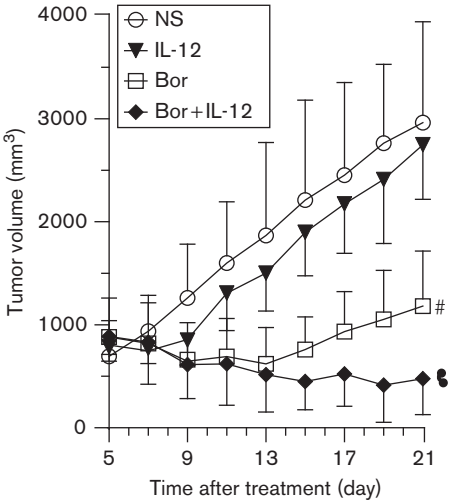
Statistical analyses were carried out using SPSS 11.0 statistical software, and data are expressed as mean ± SD. Before and after treatment comparisons were made using the paired Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Synergetic tumor inhibitory effect of the combination of bortezomib and IL-12

Two weeks after subcutaneous inoculation with RPMI8226 human peripheral blood myeloma cells, all 44 SCID mice developed tumors with a size greater than 500 mm^3, and the tumors in the control animals grew rapidly in a time-dependent manner. As illustrated in Fig. 1, IL-12 treatment only slightly decreased tumor growth ($P > 0.05$ vs. NS control); bortezomib alone substantially reduced tumor volume at the time points ranging from 11 to 21 days after treatment (~65% reduction on day 15) compared with NS or IL-12 ($P < 0.01$ and < 0.001, respectively); and combination therapy produced even greater magnitudes of tumor shrinkage (~75%) from day 15 compared with bortezomib monotherapy. Tumors resumed growth upon termination of bortezomib treatment at 2 weeks, although the tumor volume was still significantly smaller than that in the time-matched NS and IL-12 animals. Strikingly, this regrowth was not seen with the combination therapy; instead, tumor volume continued to decrease during the entire time course. Tumor suppression rate on day 21 after drug treatment was 7% in the IL-12 group, 60% in the bortezomib group, and 84% in the combination therapy group. Further, the final weight of tumors was significantly lower in the bortezomib group (~0.9 ± 0.3 g; $P < 0.01$ vs. NS and IL-12) and the combination group (~0.5 ± 0.3 g; $P < 0.01$ vs. NS and IL-12) compared with the control group (~2.6 ± 0.3 g) and the IL-12 group (~2.1 ± 0.5 g).

Fig. 1



Anti-myeloma effects of bortezomib (Bor) and interleukin-12 (IL-12) alone or in combination in a mouse model of myeloma. Shown are tumor growth curves with changes in tumor volume as a function of time after xenograft. Note that the Bor and IL-12 combination yielded the greatest efficacy, indicating a synergetic action, and that IL-12 alone failed to produce statistically significant tumor suppressing effects. $^{\#}P < 0.001$ versus saline control (NS) or IL-12; $^{\xi}P < 0.05$ versus Bor; analysis of variance *F*-test; $n = 11$ mice in each group. Data are presented as mean±SD. NS, saline control.

Failure of the bortezomib and interleukin-12 combination in preventing natural killer cell death

Hematological analysis revealed that the total lymphocyte count after bortezomib treatment was significantly lower than that before treatment ($P < 0.01$). Combination therapy did not significantly affect the total number of lymphocytes relative to that before treatment, neither

did IL-12 treatment (Table 1). In other words, combination therapy prevented the death of lymphocytes induced by bortezomib alone.

Flow cytometry results revealed that the percentage and total number of NK cells after bortezomib treatment were significantly decreased compared with those before treatment (1.4±0.4% for bortezomib vs. 3.4±0.8% for control, $P < 0.001$; Tables 2 and 3; Fig. 2). Similar results were observed on combination therapy: both the percentage and total number of NK cells were markedly diminished after sequential administration of bortezomib and IL-12. There was no significant difference between bortezomib monotherapy and combination therapy ($P > 0.05$). In comparison, the percentage and total number of NK cells remained unaltered after IL-12 treatment compared with those before treatment ($P > 0.05$; Tables 2 and 3; Fig. 2).

Bortezomib and interleukin-12 combination preserves natural killer cell function

NK cells in mouse splenocytes were evaluated using the LDH release assay for their capacity to kill YAC-1 cells. As depicted in Fig. 3a, IL-12 monotherapy did not alter the cell-killing capability of NK cells, although there was a tendency toward enhancement of YAC-1 death compared with that in the NS group. In contrast, bortezomib monotherapy considerably diminished the YAC-1-killing effect of NK cells ($P < 0.001$ at 50 : 1; $P < 0.01$ at 25 : 1; $P < 0.05$ at 12.5 : 1; Fig. 3b). Despite the fact that combination therapy failed to restore bortezomib-induced functional depression of NK cells to the control level (Fig. 3c), it indeed significantly enhanced the cell-killing ability of NK cells compared with that on bortezomib monotherapy ($P < 0.05$; Fig. 3d), indicating that IL-12 was able to restore the bortezomib-induced functional damage of NK cells.

Table 1 Total number (10^6 cells/ml) of lymphocytes in blood before and after treatment

Group	NS	IL-12	Bor	Bor + IL-12
Before	750±191	600±163	880±215	840±219
After	637±144	699±228	560±246*	760±260
Change (%)	−15.3±5.3	16.5±7.3	−36.4±6.1*	−9.5±3.5

Bor, bortezomib; IL-12, interleukin-12; NS, saline control.
*P<0.01 before versus after treatment.

Table 2 The proportion of natural killer cells in blood before and after treatment (%)

Group	NS	IL-12	Bor	Bor + IL-12
Before	3.4±0.6	2.8±0.8	3.4±0.8	4.0±0.7
After	4.6±0.8	3.6±1.2	1.4±0.4*	1.3±0.3**
Change (%)	35.3±7.3	28.6±9.3	−58.8±11.3*	−67.5±10.5**

Bor, bortezomib; IL-12, interleukin-12; NS, saline control.
*P<0.01 and **P<0.001 before versus after treatment.

Table 3 Total number (10^6 cells/ml) of natural killer cells in blood before and after treatment

Group	NS	IL-12	Bor	Bor + IL-12
Before	384±66	264±138	311±93	366±87
After	303±105	228±27	81±52**	87±48*
Change (%)	−21.1±4.1	−13.6±4.5	−74.0±33.3*	−76.2±29.6**

Bor, bortezomib; IL-12, interleukin-12; NS, saline control.
*P<0.01 and **P<0.001 before versus after treatment.

Discussion

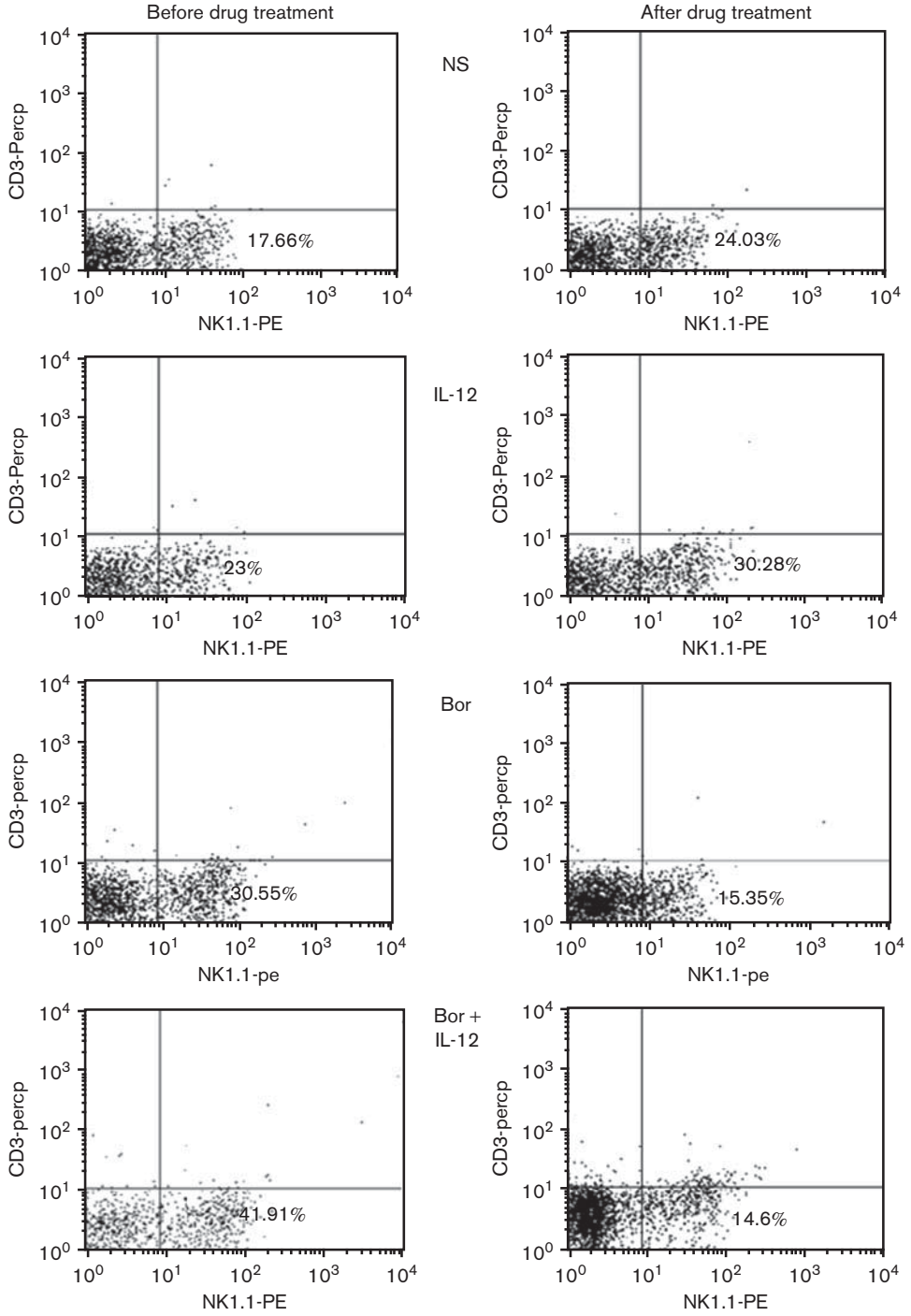
In the present study, we tested the antitumor (myeloma) efficacy of a combination of proteasome inhibition by bortezomib and immune therapy with IL-12. Our results provided strong evidence for the superiority of this combination therapy over monotherapy with either bortezomib or IL-12 in suppressing tumor growth in a mouse model of myeloma. Restoration of bortezomib-induced dysfunction of NK cells by IL-12 appears to be a mechanism for the synergetic effects of bortezomib and IL-12. These findings indicate that addition of IL-12 to the bortezomib regimen can enhance the antitumor efficacy of these agents and diminish the cytotoxicity of bortezomib toward NK cells, and, therefore, a combination of the two agents may represent a more rational therapeutic approach for MM.

Consistent with previous studies in the literature [6–15], the present study confirmed that bortezomib monotherapy produces a remarkable inhibitory effect on myeloma tumor growth in mice. Meanwhile, here we observed that bortezomib decreased the total number of peripheral blood cells, as well as the number and proportion of NK cells, confirming the results previously reported by us [16] and others [17–19,33]. These observations are also in agreement with the results reported by Uy *et al.* [34], who showed that in MM patients, bortezomib treatment causes 38 and 18% decreases in NK cells and CD8 + T cells, respectively, compared with pretreatment values. We further found here that bortezomib significantly inhibits the ability of mouse spleen cells to kill target cells.

IL-12 alone only demonstrated mild antitumor efficacy in our model, which is contrary to previous studies showing the efficacy of IL-12 in cancer therapy [20–24,27–29]. The reasons for the difference remains unclear. One possible explanation may be related to the particular model, and dosage and time course of IL-12 used in the present study. Our results showing that IL-12 was able to restore the reduced number of lymphocytes and the decreased killing function of NK cells, induced by bortezomib 12 days after the treatment (Table 1), excluded the possibility of insufficient dosage of IL-12.

The well-documented inhibitory properties of bortezomib on NK cell survival and IL-12 release, together with the protective effects of IL-12 on NK cells, form the basis

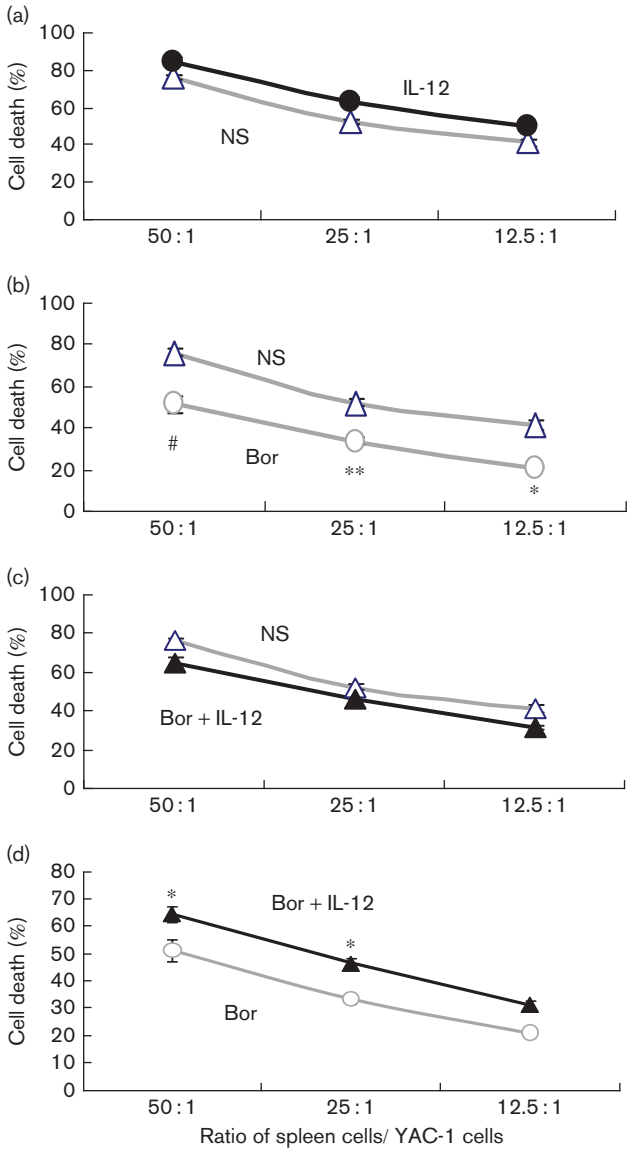
Fig. 2



Failure of interleukin-12 (IL-12) in preventing bortezomib (Bor)-induced reduction in the proportion of natural killer (NK) cells. Flow cytometry was used to analyze the proportion of NK cells before and after drug treatment. Note that Bor and Bor+IL-12 significantly ($P<0.01$) reduced the proportion of NK cells, whereas IL-12 alone did not affect the proportion of NK cells ($P>0.05$). NS, saline control.

of bortezomib–IL-12 combination therapy tested in the present study. Our results clearly demonstrate that sequential administration of bortezomib and IL-12 produces much greater tumor growth-inhibitory effects, which are manifested after a prolonged period of treatment, with tumor volume continuing to reduce even after termination of bortezomib treatment. This finding also suggests that IL-12 is able to effectively remove

Fig. 3



The combination of bortezomib (Bor) and interleukin-12 (IL-12) preserves natural killer (NK) cell function. The function of NK cells in mouse splenocytes, or the capacity of NK cells to kill YAC-1 cells, was determined using the lactate dehydrogenase (LDH) assay with spleen cells as effector cells and YAC-1 cells as target cells. Note that treatment with (a) IL-12 alone did not affect spleen cell-induced YAC-1 cell death, whereas treatment with (b) Bor alone markedly weakened YAC-1 cell death; (c) the combination of Bor and IL-12 nearly abolished the weakening of YAC-1 cell death, and (d) compared with Bor alone, the combination remarkably enhanced spleen cell-induced YAC-1 cell death, indicating the ability of IL-12 to restore the killing function of NK cells. $^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.001$; $n=6$ mice in each group. NS, saline control.

residual tumor cells, leading to long-term antitumor immunity, which is in line with the findings of the study by Burdelya *et al.* [35], who demonstrated that the combination of AG-490 and IL-12 has greater antitumor effects than either agent alone. The cytotoxic effect of bortezomib on NK cells was retained even in the combination regimen, whereas the impaired cell-killing function of NK cells largely dissipated. These results indicate that the combination therapy acts by enhancing function but not preserving the number of NK cells.

Taken together, we demonstrate here that bortezomib and IL-12 in combination have a synergistic antitumor effect against myeloma. One possible mechanism for this enhanced efficacy is correction of bortezomib-induced impairment of the cell-killing function of NK cells by IL-12. This combination may be a better strategy for MM therapy compared with bortezomib or IL-12 monotherapy and merits more rigorous examination in future studies.

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Conflicts of interest

There are no conflicts of interest.

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