The proteasome inhibitor bortezomib inhibits the release of NFκB-inducible cytokines and induces apoptosis of activated T cells from rheumatoid arthritis patients

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Abstract

Objective

The proteasome is a multicatalytic proteinase complex regulating the intracellular breakdown of many proteins, including those mediating the activation of pro-inflammatory signaling pathways (e.g. NFκB), cell proliferation and survival. Conceptually, proteasome inhibitors may therefore elicit potential anti-inflammatory properties by inhibiting these processes and thereby impair the cellular release of pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) in RA patients.

Methods

Whole-blood from 19 RA patients (including methotrexate-responsive and non-responsive patients) and 7 healthy volunteers was incubated ex-vivo with the proteasome inhibitor bortezomib after T-cell stimulation with αCD3/CD28. Inhibition of cytokine production by bortezomib was measured after 24 and 72 hours by ELISA. Effects of bortezomib on apoptosis and T-cell activation (CD25 expression) were measured by FACS-analysis.

Results

Bortezomib proved to be a rapid (<24 hour) and potent inhibitor of the release of several NFκB-inducible cytokines (including TNF-α, IL-1β, IL-6 and IL-10) by activated T-cells from healthy volunteers and RA patients, regardless of their clinical responsiveness to methotrexate. Median concentrations of bortezomib required to inhibit TNF-α production by 50% (mIC-50) were 12 nM (range: 8-50 nM) for healthy volunteers and 46 nM (range: 18-60 nM) for RA patients. A reduction of T cell activation and a marked induction of T-cell apoptosis were revealed as late effects after bortezomib incubations beyond 24 hours.

Conclusion

Proteasome inhibitors represented by bortezomib may elicit potential anti-inflammatory properties that deserve further exploration in experimental therapies for RA.

Key words

Proteasome, NFκB, bortezomib, rheumatoid arthritis, cytokines, apoptosis, methotrexate.

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This study was supported by the Dutch Arthritis Association (Grant NRF-03-I-40) and ZonMW (The Netherlands Organization for Health Research and Development). J.v.d. Heijden is a recipient of the 2006 Rheumatology Grant from the Dutch Society for Rheumatology.

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Received on March 14, 2008; accepted in revised form on July 24, 2008.

Abbreviations:

DMARD:	Disease modifying anti-rheumatic drug
TRAIL:	TNF-related apoptosis-inducing ligand
TNF:	Tumor necrosis factor
IC50:	Drug concentration required to inhibit cytokine release by 50%
MTX:	Methotrexate
FACS:	Fluorescent-activated cell Sorting
7-AAD:	7-aminoactinomycin D
ELISA:	Enzyme linked immunosorbent assay
ZVAD-fmk:	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl-ketone
DAS28:	Disease activity score (28 joints)
ESR:	Erythrocyte sedimentation rate.

Competing interests: none declared.

Introduction

The proteasome is a multimeric proteinase complex that facilitates the degradation of approximately 80% of intracellular proteins. The catalytic activities of the proteasome not only ensure protein homeostasis; they also provide a fined-tuned mechanism of controlled (in)activation of proteins involved in cell proliferation, signaling processes and the generation of antigenic peptides to be presented on MHC class I molecules (1-3). In this context, for example, activation and nuclear translocation of the transcription factor NFκB intimately depends on proteasome-mediated breakdown of its natural inhibitor protein IκBα and thereby mediates the transcriptional regulation of several pro-inflammatory cytokines such as TNF-α and IL1β (4, 5) as well as anti-apoptotic genes (6). From this perspective, it has been anticipated that inhibitors of the proteasome may counteract the NFκB activation process and elicit a potential anti-inflammatory response (7-11).

Bortezomib (Velcade®, PS341), a boronic acid dipeptide, is the first specific proteasome inhibitor that is registered in cancer chemotherapy for therapy-refractory multiple myeloma patients (12, 13). Bortezomib is used as single agent and in drug combination regimens where it has displayed additive/ synergistic anti-tumor effects when combined with glucocorticoids (dexamethasone) or TNF-related apoptosis-inducing ligand (TRAIL) (13, 14). Interestingly, patients with relapsed or refractory lymphoma who responded to bortezomib had a marked reduction in plasma TNF-α levels as compared to bortezomib non-responders (15). Of additional interest from a potential anti-inflammatory perspective were preliminary data from animal models for arthritis (16, 17) which revealed that bortezomib treatments conveyed clear therapeutic effects. In fact, a recent report by Neubert *et al.* showed that bortezomib targets both short and long-lived plasma cells in mice with lupus-like disease, thereby abrogating the production of antibodies to double-stranded DNA and preventing the onset of nephrites (18). The present study was undertaken to assess the potential anti-inflammatory effects of bortezomib on the basis of inhibiting the production of TNF-α and other pro-inflammatory cytokines by activated T cells from RA patients.

Materials and methods

Patient characteristics

All patients signed an informed consent form, and the study on 'DMARD-resistance' was approved by the Medical Ethics committee of the VU University Medical Center, Amsterdam, The Netherlands. Characteristics of 19 RA patients included in this study were: male/female: 5/14; mean age: 52.5 years; mean DAS28 score (Disease Activity Score for 28 joints): 4.3 (range 1.3-7.2). Five patients had no prior treatment with Disease Modifying Anti-Rheumatic Drugs (DMARD-naive). 13 patients used methotrexate (MTX) 15-30 mg/week, and 1 patient used the combination of sulfasalazine and hydoxychloroquine. Patients were on DMARDs for at least 3 months (range: 12 weeks to 9 years). Patients that were on DMARD therapy were defined as DMARD responders if their DAS28 was ≤3.2 at the time of enrollment (n=7); RA patients with a DAS28 score of >3.2 were defined as DMARD non-responders (n=7). Finally, 7 healthy volunteers were included (male/female: 4/3; mean age: 38 years). Additional patient characteristics are described in Table I.

Materials

Bortezomib was kindly provided by Millennium Pharmaceuticals; Cambridge, MA, U.S.A. Stock solutions of 1 mM were prepared in dimethylsulfoxide (DMSO) and stored at -20ºC. αCD3/αCD28 antibodies were a generous gift from Prof. L. Aarden (Sanquin, Amsterdam, the Netherlands). Anti-CD25-PE was obtained from Becton Dickinson; San Jose, CA, U.S.A. Cytokine ELISA assays (PeliKine) for TNF-α, IL-1β, IL-6 and IL-10 were obtained from Sanquin, Amsterdam, The Netherlands, and utilized according to the manufacturers' instructions and as described previously (19).

Procedures and T-cell activation

Whole blood (1:10 diluted with heparin-containing IMDM medium,

supplemented with 0.1% fetal calf serum) of 19 RA patients and 7 healthy volunteers controls were incubated *ex vivo* with αCD3/αCD28 antibodies (0.1 μg/ml and 1 μg/ml respectively) as T-cell activating agents as described previously (19). T-cell activation was monitored by CD25-PE staining by flow cytometry. The whole blood incubates were then supplemented with a concentration range (0-100 nM) of bortezomib, and, as a reference, 33-300 nM MTX, followed by an incubation period of 24-72 hours. Inhibition of TNF-α production was measured after 72 hours drug exposure by ELISA as described before (19). Beyond TNF-α, production of other NFκB-inducible cytokines (*i.e.* IL-1β, IL-6 and IL-10) was measured in whole blood from 3 separate healthy controls and 3 RA patients after T-cell stimulation and 24-hour exposure to bortezomib.

Apoptosis assay

Peripheral blood lymphocytes (PBLs) of RA patients and healthy volunteers were isolated by Ficoll-Paque Plus density centrifugation and suspended in DMEM-medium supplemented with 10% fetal calf serum. Apoptosis in PBLs was analyzed by flow cytometry (FACS) after 24 and 48 hours exposure to bortezomib using Annexin-V-FITC/7-aminoactinomycin D (7-AAD) staining (APOPTEST™-FITC A700, VPS Diagnostics, Hoeven, the Netherlands) according to the manufacturer's protocol. Phycoerythrin labeled T-cell markers (CD4/CD8/CD25) were obtained from Becton Dickinson; San Jose, CA, USA.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney test to analyze differences between RA patients and healthy volunteers.

Results

Bortezomib-induced cytokine release from activated T cells

In a pilot setting, the ability of bortezomib was assessed to inhibit the release of cytokines from αCD3/αCD28 activated T-cells from controls (Fig. 1A) and RA patients (Fig. 1B). Production of TNF-α, IL-1β, IL-6 and IL-10 production in controls was half maximally inhibited at bortezomib concentrations between 5-25 nM (Fig. 1A). In activated T cells of RA patients (Fig. 1B), bortezomib also inhibited the production of IL-6, IL-1β and TNF-α, although for the latter cytokine, slightly higher concentrations of bortezomib were required. Production of IL-10 in activated T cells of RA patients was less efficiently inhibited as compared to controls.

Table I. Baseline characteristics of (subgroup) RA patients and controls and outcome of TNF-α inhibition from activated T cells by bortezomib and methotrexate.

	Controls (n=7)	RA pts (n=19)	DMARD-naïve (n=5)	DMARD-responders (n=7)	DMARD-non-respond. (n=7)
Mean age (yr) (SD)	38 (13)	53 (17)^{NS}			
Mean ESR (mm/hr) (SD)		27 (27)			
Median DAS28 score (range)			5.5 (4.4-6.9)	2.1 (1.3-2.9)	5.5 (3.9-7.2)
Median IC50 bortezomib (nM) (range)	12 (8-50)	46 (18-60)*	47 (32-59)*	48 (24-60)*	27 (18-59)
Median IC50 MTX (nM) (range)	53 (18-85)	59 (16-325)	55 (32-102)	44 (28-260)	60 (16-325)

IC50: drug concentration required to inhibit TNF-α production by 50%, $^{*}p<0.05$, ^{NS} : not significant, DAS28: Disease Activity Score (28 joints), ESR: erythrocyte sedimentation rate.

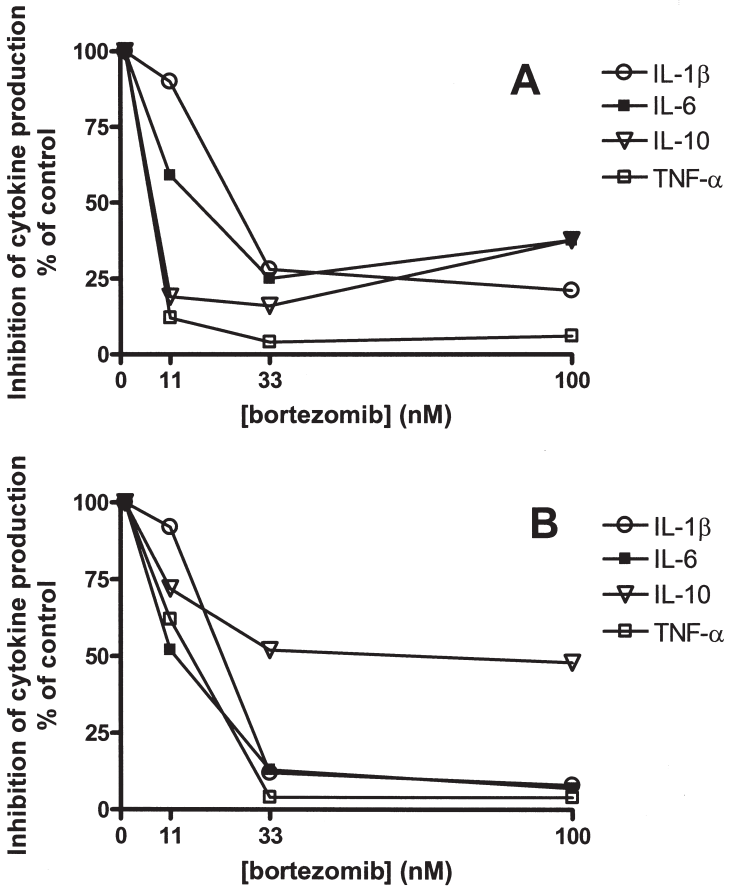

Fig. 1. Bortezomib-induced inhibition of TNF-α, IL-1β, IL-6 and IL-10 production in activated T cells from controls and RA patients. Bortezomib- induced inhibition of TNF-α, IL-1β, IL-6, and IL-10 production from activated T cells of (**A**) healthy volunteers (n=3) and (**B**) DMARD-naïve RA patients (n=3) was analyzed after 24 hour stimulation of T cells in whole blood by αCD3-α/CD28 in the presence of the indicated concentrations of bortezomib. Results are depicted as the mean percentage of cytokine production relative to controls incubated without bortezomib.

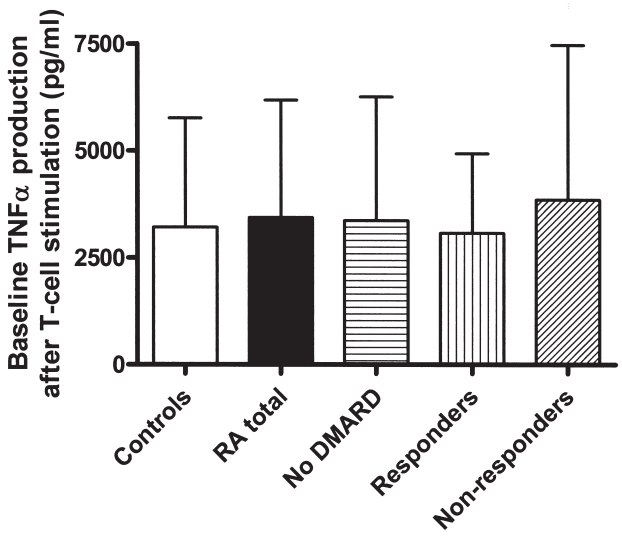

Fig. 2. Baseline TNF-α production by activated T cells of RA patients and healthy volunteers. Baseline TNF-α production by αCD3-α/CD28 activated T cells from healthy controls (n=7), total group of RA patients (n=19), and after sub-classification in DMARD-naïve (n=5), DMARD-responders (n=7) and DMARD-non-responding RA patients (n=7). Data are presented as mean values (pg/ml) ±SD Differences in baseline TNF-α production in the tested groups were not statistically different. Note: no TNF-α production was observed in unstimulated whole blood cell cultures.

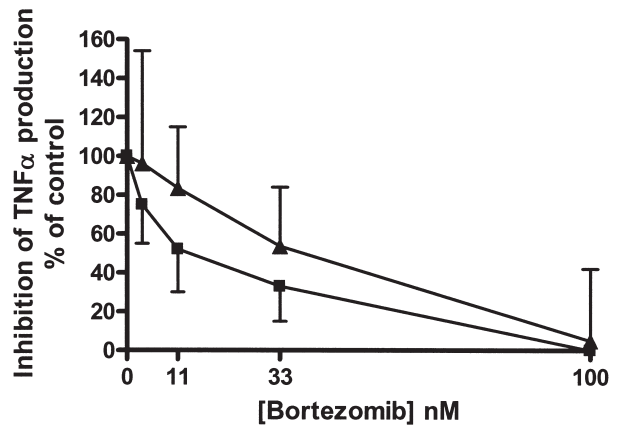

Fig. 3. Bortezomib-induced inhibition of TNF-α production by activated T cells from RA patients and healthy volunteers. Dose-response curve for bortezomib-induced inhibition of TNF-α production by αCD3-α/CD28 activated T cells from healthy volunteers (squares, n=7) and RA patients (triangles, n=19). Results are presented as median value and range (bars) for each bortezomib concentration tested.

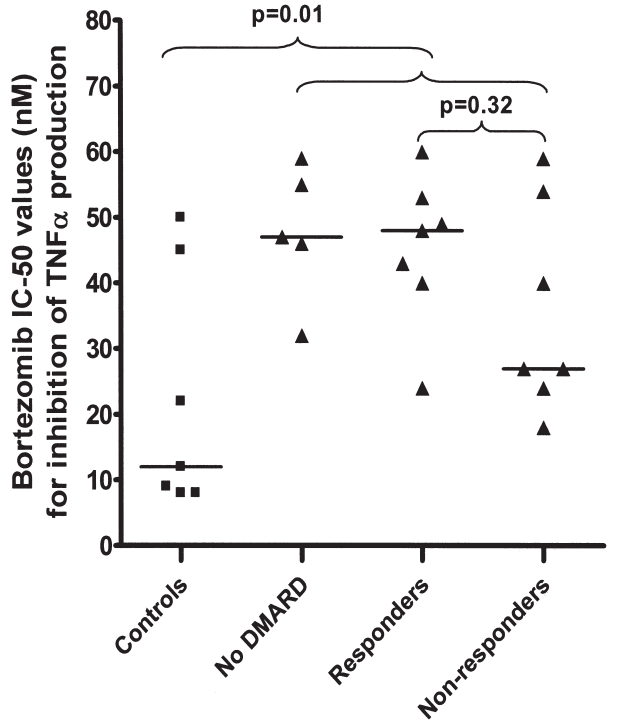

Fig. 4. Bortezomib-induced inhibition of TNF-α release by activated T cells from (subgroups of) RA patients and healthy volunteers. Concentrations of bortezomib required for 50% inhibition (IC50) of TNF-α production in αCD3/αCD28-stimulated whole blood cell cultures of individual healthy volunteers (n=7; filled squares) and RA patients (n=19; triangles) as well as 3 RA subgroups. The horizontal lines depict the median IC-50 value for each group.

Inhibition of TNF-α release from activated T cells by bortezomib and methotrexate

The potency of bortezomib to inhibit the production of TNF-α was further investigated in a larger group of RA patients (n=19) which could be sub-categorized in DMARD-naïve patients (n=5), DMARD-responsive patients (DAS28 <3.2, n=7) and DMARD non-responsive RA patients (DAS28 >3.2, n =7). For comparison, inhibition of TNF-α production was also analyzed for methotrexate (MTX) as a reference drug. Following αCD3/αCD28 stimulation of T cells, absolute levels of baseline TNF-α production were not significantly different in whole blood incubates from 7 healthy volunteers (baseline TNF-α production: 3208± 2554 pg/ml; range: 623-7361 pg/ml) and 3 groups of RA patients; DMARD-naive (n=5), DMARD-responders (n=7) and DMARD non-responders (n=7) (Fig. 2). Upon co-incubation with bortezomib, median concentrations of this drug required to inhibit TNF-α production by 50% (mIC50) were 3.8-fold lower (p=0.01) for healthy controls (IC50: 12 nM, range: 8-50 nM) as compared to the total group of RA patients (IC50: 46 nM, range: 18-60 nM) (Table I, Fig. 3). Sub-analysis for the DMARD non-responsive RA patients showed a 1.7-fold greater potency of bortezomib to inhibit TNF-α production compared to DMARD-naive and DMARD-responsive RA patients, but this difference was not significant (p=0.32) (Fig. 4). Median concentrations of MTX to inhibit TNF-α production by 50% were not significantly different (p=0.64) between healthy controls (IC50: 53 nM, range 18-85 nM) and RA patients (IC50: 59 nM, range: 16-325 nM) (Table I).

Bortezomib-induced induction of apoptosis and inhibition of T-cell activation

Along with a rapid reduction in TNF-α release, a marked induction of apoptosis was observed in peripheral blood lymphocytes (PBLs) of RA patients at a later stage of bortezomib exposure: 10-21% Annexin-V positive cells in control conditions versus 62-77% Annexin-V/7AAD-positive cells after

exposure to 10-100 nM bortezomib for 48 hours, respectively (Fig. 5A). A representative FACS analysis of bortezomib-induced T cell apoptosis in PBLs from a RA patient is shown in Figure 5B. Without bortezomib (control), only 7% of CD4/CD8 positive (activated) T cells were apoptotic, while after incubation with 33 nM bortezomib for 48 hours (representing IC-50 value for inhibition of TNF-α production in RA whole-blood), 60% of CD4/CD8 positive T cells were apoptotic based on Annexin-V staining. Apoptosis induction by bortezomib could be partially prevented, in a concentration dependent manner, by pre-incubation with the broad-spectrum-caspase inhibitor ZVAD-fmk (10-50 μM), suggesting that cell death was mediated via apoptosis rather than by necrosis (data not shown). For comparison, no induction of apoptosis was observed after 48 hours exposure to 300 nM concentrations of MTX (Fig. 5C). Evaluation of the T-cell activation status by CD25 expression showed that as off 48 hours incubation with bortezomib, CD25 expression decreased in a concentration dependent manner (Fig. 5D).

Discussion

Nowadays, the ubiquitin-proteasome system is recognized as the major pathway for degradation of intracellular proteins, many of which play a key role in regulation of pro-inflammatory cytokines (7, 11). Data from the present study further support the proof of principle that bortezomib can confer inhibition of TNF-α production in whole blood from RA patients after T-cell stimulation. Mechanistically, this effect can be explained by inhibition of the NFκB signalling pathway as a downstream effect of proteasome inhibition in conjunction with induction of apoptosis in activated T cells (12-14, 17). In the whole blood assay employed in this study, it is anticipated that beside T cells, monocytes/macrophages will be the main producers of IL-1, IL-6 and TNF-α, as they get stimulated by cytokines (like IL-17) produced by activated T cells (20). Since we observed that bortezomib abrogated T-cell activation at low concentrations, it can be

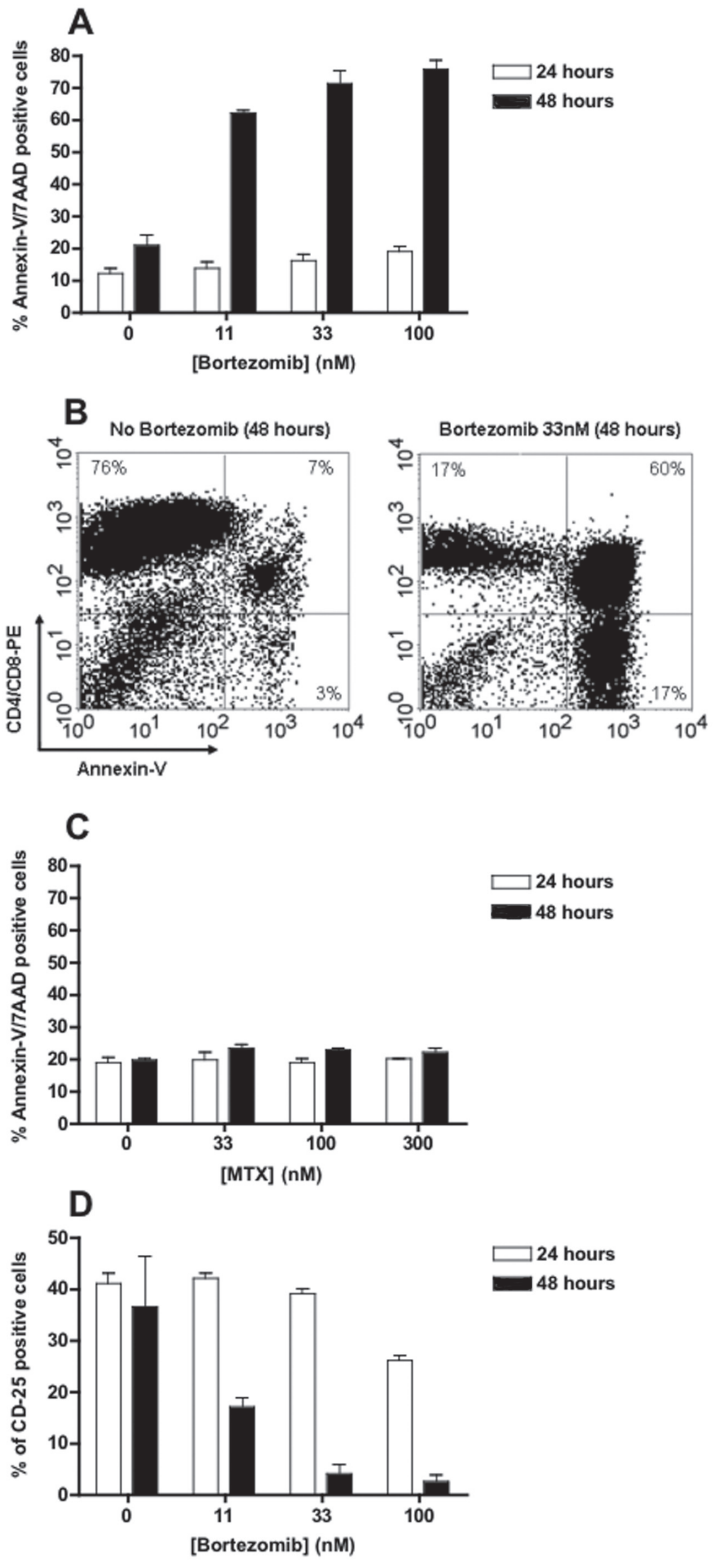

Fig. 5. Effects of bortezomib on T-cell activation and induction of apoptosis. (**A**) Induction of apoptosis by bortezomib in αCD3-α/CD28-stimulated peripheral blood lymphocytes of RA patients after 24 hours (white bars) and 48 hours (black bars) drug exposure. (**B**) Representative FACS analysis depicting induction of apoptosis (Annexin-V positive) in the CD4/CD8 positive population of (activated) T cells in control conditions (without bortezomib) and after 48 hours exposure to 33nM bortezomib. (**C**) Induction of apoptosis by MTX in αCD3-α/CD28-stimulated peripheral blood lymphocytes of RA patients after 24 hours (white bars) and 48 hours (black bars) drug exposure. (**D**) Percentage of activated (CD25 positive) T cells in αCD3-α/CD28-stimulated peripheral blood lymphocytes after incubation with bortezomib. Results presented are the mean ±SD of 3 individual RA patients.

speculated that the production of these cytokines by monocytes/macrophages is inhibited simultaneously.

Consistent with inhibition of NFκB were observations (Figs. 1, 3, 4) that besides TNF-α, bortezomib also inhibited other NFκB-inducible cytokines (IL-1β, IL-6, IL-10) (Fig. 1) in a concentration range (10-50 nM) that was previously shown to abrogate NFκB activity (21). Given the central role of the ubiquitin-proteasome system in regulating signalling pathways (11), it is anticipated that signalling pathways other than NFκB may be effected at higher concentrations of bortezomib and/or longer exposure times. In fact, concentrations of bortezomib that provoke apoptosis induce p38 MAPK activity along with a release of anti-inflammatory cytokines, such as IL-10 (22). The incomplete inhibition of IL-10 production at higher concentrations of bortezomib (Fig. 1) may be consistent with this notion. While bortezomib-induced inhibitory effects on NFκB activity could be observed rather rapidly (within 24 hours, Fig. 1), bortezomib-induced apoptosis of activated T cells was recognized as a later effect, emerging between 24 and 48 hours of bortezomib exposure. In this respect, our data are consistent with Blanco *et al*. showing that bortezomib was particularly active against alloreactive (CD25+) T cells and not resting T cells (23). Based on the profile of inhibition of TNF-α production, bortezomib exhibited potent *ex vivo* activity for both DMARD-naive and clinically DMARD-responsive RA patients, and an even slightly greater activity for DMARD non-responsive patients. This result may point to the fact that proteasome targeting may bypass or circumvent common mechanisms of loss of efficacy to DMARDs after chronic administration (24).

Apart from the inter-patient variability in *ex vivo* response to bortezomib, one other intriguing observation was that bortezomib displayed a greater potency in blocking of TNF-α production by activated T cells from healthy controls than from RA patients. An explanation for this is not readily available but could relate to possible differences between healthy controls and RA patients with respect to (i) quantitative and qualitative differences in proteasomal catalytic activity in T-cells from healthy controls versus patients with RA or other autoimmune diseases (25-27), or (ii) higher constitutive NFκB activity in activated T cells from RA patients (4), which would require higher bortezomib dosages for inhibition. In addition, a recent study (28) indicated that plasma pharmacokinetics and activity of bortezomib can be influenced by uptake of bortezomib in red blood cells. In our study, we made use of 10-fold diluted whole blood cell samples (19) within which variability in erythrocyte concentrations could have influenced residual concentrations of bortezomib to some degree. Notably, pharmacokinetics of bortezomib in phase I clinical trials showed peak plasma levels of 50-1000 nM and steady state plasma levels of 10-20 nM (29, 30), which are within the concentration range where bortezomib showed inhibition of TNF-α production by activated T cells from RA patients (Table I, Fig. 1) and induction of T-cell apoptosis. It remains to be established whether this induction of apoptosis is based solely on the apoptotic effects of bortezomib itself or whether it also involves previously reported bortezomib-induced sensitization of apoptotic effects of TRAIL or TNF-α (14). Obviously, any design of a trial for bortezomib in RA treatment should address a number of issues: (a) assessment of optimal dosing/therapeutic window for RA treatment, (b) it is anticipated that commonly long term drug administration to RA patients will require special precautions with respect to drug safety (31), (c) it should be considered that proteasome inhibition may partially abrogate intracellular degradation of (citrullinated) proteins in RA patients (32). Finally, (d) it remains to be revealed to which extent other pro-inflammatory cytokine producing cells (monocytes, macrophages) in peripheral blood or in synovial tissue are subject to targeting by bortezomib as demonstrated for activated T cells in this study.

Despite the current success of DMARDs and biologic agents, there is still room for improvement of RA therapy (33). Given their unique profile of action, proteasome inhibitors (34, 35) certainly deserve further evaluation for future clinical application in the treatment of chronic inflammatory diseases. As a proof of concept, this pilot study demonstrated that proteasome targeting by specific inhibitors such as bortezomib can suppress production of pro-inflammatory cytokines from activated T cells of RA patients. Thus, beyond demonstrations of good clinical activity against certain types of cancer, proteasome inhibitors may represent a new generation of targeted small molecule drugs in the therapeutic armour, in particular for patients with DMARD-refractory RA.

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