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RESEARCH ARTICLE

Lipopolysaccharide-Induced *CXCL10* mRNA Level and Six Stimulant-mRNA Combinations in Whole Blood: Novel Biomarkers for Bortezomib Responses Obtained from a Prospective Multicenter Trial for Patients with Multiple Myeloma

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Abstract

To identify predictive biomarkers for clinical responses to bortezomib treatment, 0.06 mL of each whole blood without any cell separation procedures was stimulated *ex vivo* using five agents, and eight mRNAs were quantified. In six centers, heparinized peripheral blood was prospectively obtained from 80 previously treated or untreated, symptomatic multiple myeloma (MM) patients with measurable levels of M-proteins. The blood sample was procured prior to treatment as well as 2-3 days and 1-3 weeks after the first dose of bortezomib, which was intravenously administered biweekly or weekly, during the first cycle. Six stimulant-mRNA combinations; that is, lipopolysaccharide (LPS)-*granulocyte-macrophage colony-stimulating factor (GM-CSF)*, LPS-*CXCL chemokine 10 (CXCL10)*, LPS-*CCL chemokine 4 (CCL4)*, phytohemagglutinin-*CCL4*, zymosan A (ZA)-*GMCSF* and ZA-*CCL4* showed significantly higher induction in the complete and very good partial response group than in the stable and progressive disease group, as determined by both parametric (*t*-test) and non-parametric (unpaired Mann-Whitney test) tests. Moreover, LPS-induced *CXCL10* mRNA expression was significantly suppressed 2-3 days after the first dose of bortezomib in all patients, as determined by both parametric (*t*-test) and non-parametric (paired Wilcoxon test) tests, whereas the complete and very good partial response group showed sustained suppression 1-3 weeks after the first dose. Thus, pretreatment LPS-*CXCL10* mRNA

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and/or the six combinations may serve as potential biomarkers for the response to bortezomib treatment in MM patients.

Introduction

The proteasome inhibitor bortezomib (VELCADE; Millennium Pharmaceuticals and Johnson & Johnson Pharmaceutical Research & Development) has revolutionized the treatment of multiple myeloma (MM) patients and has become a mainstay in the standard of care for both previously untreated [1] and relapsed [2, 3] patients with MM. A number of clinical and laboratory features provide prognostic information for patients with MM, such as hypodipoidy [4] and chromosomal translocations and deletions [5–7].

The gene expression profiles of plasma cells isolated from the bone marrow of MM patients can predict the response to treatment with bortezomib [8, 9]. However, peripheral blood (PB) biomarkers able to predict the response to bortezomib have not yet been identified, although some factors are known to correlate with such responses, including hepatocyte growth factor [10], thrombospondin [10], XBP-1 [11] and absolute lymphocyte counts [12].

In our previous study [13], we reported that phytohemagglutinin (PHA)-induced *interleukin-2 (IL2)* mRNA levels in *ex vivo* whole blood obtained prior to bortezomib treatment could predict the incidence of bortezomib-induced peripheral neuropathy. In this study, we used the same assay to predict the efficacy of bortezomib treatment in an expanded patient population.

Subjects and Methods

Patients

Eligible patients in this multicenter prospective study consisted of previously treated MM patients or untreated patients with symptomatic MM, as described in our previous study [13]. All patients had to have measurable levels of M-proteins. The study was approved by the institutional review board or independent ethics committee at all participating institutions and was conducted according to the principles of the Declaration of Helsinki and the International Conference on Harmonization Guidelines of Good Clinical Practice. All patients provided written informed consent for sample procurement. The following institutions participated in this study: National Cancer Center Hospital; Saitama Medical Center, Saitama Medical University; Nagoya City University, Graduate School of Medical Sciences; Japanese Red Cross Medical Center; University of Tokushima, Graduate School of Medical Sciences and Tokai University School of Medicine (Acknowledgement section of the ms.). Clinical responses were assessed according to the International Uniform Response Criteria [14].

Measurements

Eight-well strips containing 1.2 μL each of PHA (2 mg/mL), heat-aggregated immunoglobulin G (HAG) (10 mg/mL), lipopolysaccharide (LPS) (0.5 mg/mL), zymosan A (ZA) (75 mg/mL) or solvent phosphate-buffered saline (PBS) were delivered to each institution on dry ice. These strips were kept frozen at -80°C. A 2 mL sample of heparinized PB was obtained from each patient prior to treatment as well as 2–3 days (D2-3) and 1–3 weeks (W1-3) after intravenous administration of the first dose of bortezomib during the first cycle. The blood was immediately delivered to the designated laboratory, 0.06 mL of PB was added to each well containing 3 strips (that is, in triplicate), and the strips were incubated for 4 hours at 37°C. The total blood

volume required was 0.9 mL (0.06 mL/well x 5 wells/strip x 3 strips). After incubation, the samples were stored at -80°C.

mRNA analysis

Purification of mRNA and cDNA synthesis were performed as described previously using leukocyte capture filter plates and oligo(dT)-immobilized microplates [15,16] The cDNA was used for real-time PCR [15,16]. Melting curves were analyzed to confirm that the PCR signals were derived from a single PCR product, and the cycle threshold (Ct) value was determined using analytical software (SDS, Thermo Fisher Scientific, Carlsbad, CA). The Ct values of the treated samples were subtracted individually from the mean Ct values of the control samples to calculate the ΔCt, and the fold increase was calculated as 2^(-ΔCt), as described previously [15,16]. The mRNAs analyzed included *β-actin* (*ACTB*), *IL2* and *interleukin-6* (*IL6*), *granulocyte-macrophage colony-stimulating factor* (*GMCSF*), *interferon-γ* (*IFNG*), *tumour necrosis factor-α* (*TNFSF2*), *CCL chemokine 4* (*CCL4*) and *CXCL chemokine 10* (*CXCL10*) [16]. mRNA analysis and clinical data collection were performed separately at the different centers.

Statistical analyses

Parametric (*t*-test) and non-parametric (unpaired Mann-Whitney and paired Wilcoxon tests) tests were used to compare mRNA levels between the two groups. $p<0.05$ were considered significant. The statistical analyses were performed using Excel (Microsoft, Redmond, WA) and Prism 6 (GraphPad Software, La Jolla, CA).

Results

Patients' charasteristics

Between March 2010 and March 2012, a total of 83 patients (44 male and 39 female) were enrolled from six centers. The median age of all patients was 63 years (Table 1). Fifty patients were previously treated, and 33 patients were untreated. After excluding one patient who died early from progressive disease, another who received additional treatment and another who committed suicide, 80 patients were eligible for response analysis. The numbers of patients who demonstrated complete response (CR), very good partial response (VGPR), partial response (PR), stable disease (SD) and progressive disease (PD) were 5, 7, 33, 33 and 2, respectively.

mRNA analysis

Overall, 3,600 mRNA preparations and cDNA synthesis reactions were carried out (80 patients x 5 stimulants x 3 time points x 3 [triplicate]). A total of 28,800 PCR reactions were performed (3,600 cDNA samples x 8 genes).

Pretreatment higher induction of LPS/ZA-induced *GMCSF*, LPS-induced *CXCL10*, and LPS/PHA/ZA-induced *CCL4* mRNA in CR/VGPR responders to bortezomib

The fold increase in LPS-induced *GMCSF*, *CXCL10* and *CCL4*, PHA-induced *CCL4* and ZA-induced *GMCSF* and *CCL4* were significantly higher in the CR and VGPR groups than in the SD and PD groups, as determined by both parametric *t*-tests and non-parametric Mann-Whitney tests, whereas the PR group exhibited an intermediate value (Fig 1). Moreover, 100, 67, 56,

Table 1. Patients Demographic and Baseline Characteristics.

Characteristics		Number of patients(%)
Age, years		
	Median	63
	Range	37–79
Male sex		44(53)
Prior therapy		
	Yes	50(60)
	No	33(40)
M component		
	IgG	48(58)
	IgA	9(11)
	IgD	3(4)
	Light chain only	23(28)
ISS stage		
	I	25(30)
	II	29(35)
	III	29(35)
Follow-up*, days		
	Median	151
	Range	26–666
Bortezomib administration		
	Twice-weekly	63(76)
	Weekly	20(24)
Concurrent dexamethasone		
	Yes	74(89)
	No	9(11)
Best response to treatment†		
	CR	5(6)
	VGPR	7(8)
	PR	33(40)
	SD	33(40)
	PD	2(2)
	NE	3(4)‡

CR, complete response; ISS, International Staging System; NE, not evaluable; PD, progressive disease; SD, stable disease; VGPR, very good partial response.

*Excluded were three patients not evaluable for response.

†According to the International Uniform Response Criteria (Durie et al, 2006).

‡One patient died of progressive disease early, another received additional chemotherapy, and the third committed suicide.

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42 and 0% of patients showed more than 3-fold increases in LPS-induced *CXCL10* (dotted line in Fig 1).

Sustained suppression of LPS-induced *CCCL10* mRNA in CR/VGPR responders to bortezomib

As shown in Fig 2, LPS-induced *CXCL10* mRNA expression was significantly suppressed 2–3 days after the first dose of bortezomib in all groups, as determined by both parametric (*t*-test)

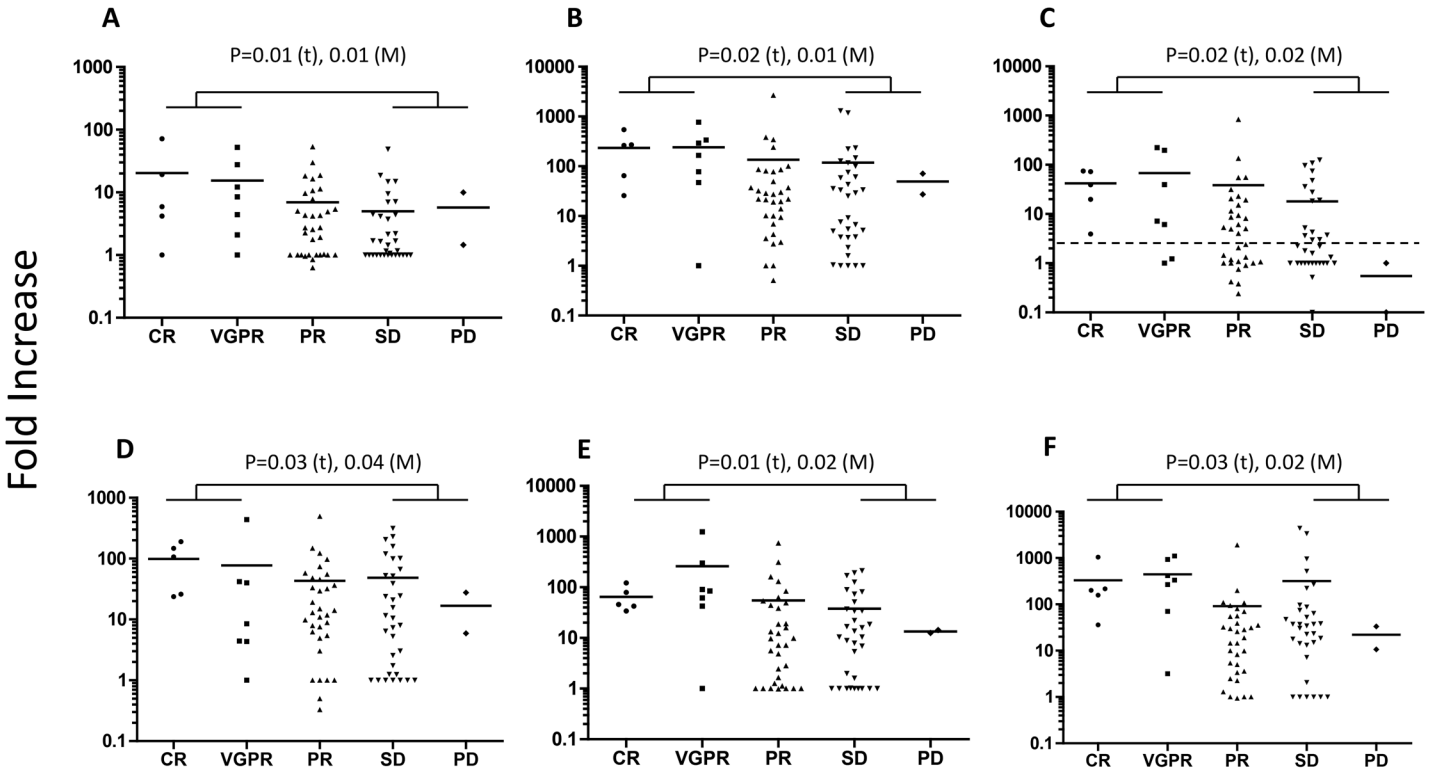

Fig 1. *Ex vivo* mRNA induction in blood obtained prior to bortezomib treatment. The fold increase in (A) LPS-induced *GMCSF*, (B) ZA-induced *GMCSF*, (C) LPS-induced *CXCL10* (top panel), (D) PHA-induced *CCL4*, (E) LPS-induced *CCL4* and (F) ZA-induced *CCL4* (lower panel) mRNA in the CR, VGPR, PR, SD and PD groups is shown. The statistically significant difference between the CR+VGPR and SD+PD groups is shown. t: Student's *t*-test, M: Mann-Whitney test. Dotted line: fold increase = 3. Samples showing a fold increase in *ACTB* (which was > 3) were removed from the analysis. Horizontal bars: the mean values.

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and non-parametric (paired Wilcoxon test) tests, while the CR+VGPR group showed sustained suppression even 1–3 weeks after treatment. This significant level of inhibition was only observed for LPS-induced *CXCL10*.

Discussion

Currently, triple drug combinations are believed to be very effective for MM treatment based on the results of several randomised clinical trials [1,17–23] and phase I/II studies [24, 25]. However, in some studies, the daily dose of bortezomib [1, 26–28] and the bortezomib administration schedule (namely, the dose density) [29–31] are reduced to avoid toxicity, despite the fact that bortezomib accumulation is important for achieving improved survival [32]. Although MM patients still experience relapse or progression even after triple drug combination therapy, it is clear that better responses to initial therapy result in longer survival [33–36]. If we could predict which patients will respond to bortezomib, we would be able to give priority to bortezomib dose, rather than the other drugs, in combination regimens. Thus, it is important to predict whether patients will respond to bortezomib before initiation and whether bortezomib can be used in consolidation [1, 37] or maintenance [29, 30, 38, 39] therapy.

This is the first report demonstrating the use of LPS-induced *CXCL10* mRNA levels as a biomarker for assessing the clinical response to bortezomib treatment in PB. We showed that higher induction of *CXCL10* mRNA corresponded to very good responses (CR+VGPR), whereas lower induction corresponded to poor responses (SD+PD); the values in the PR group

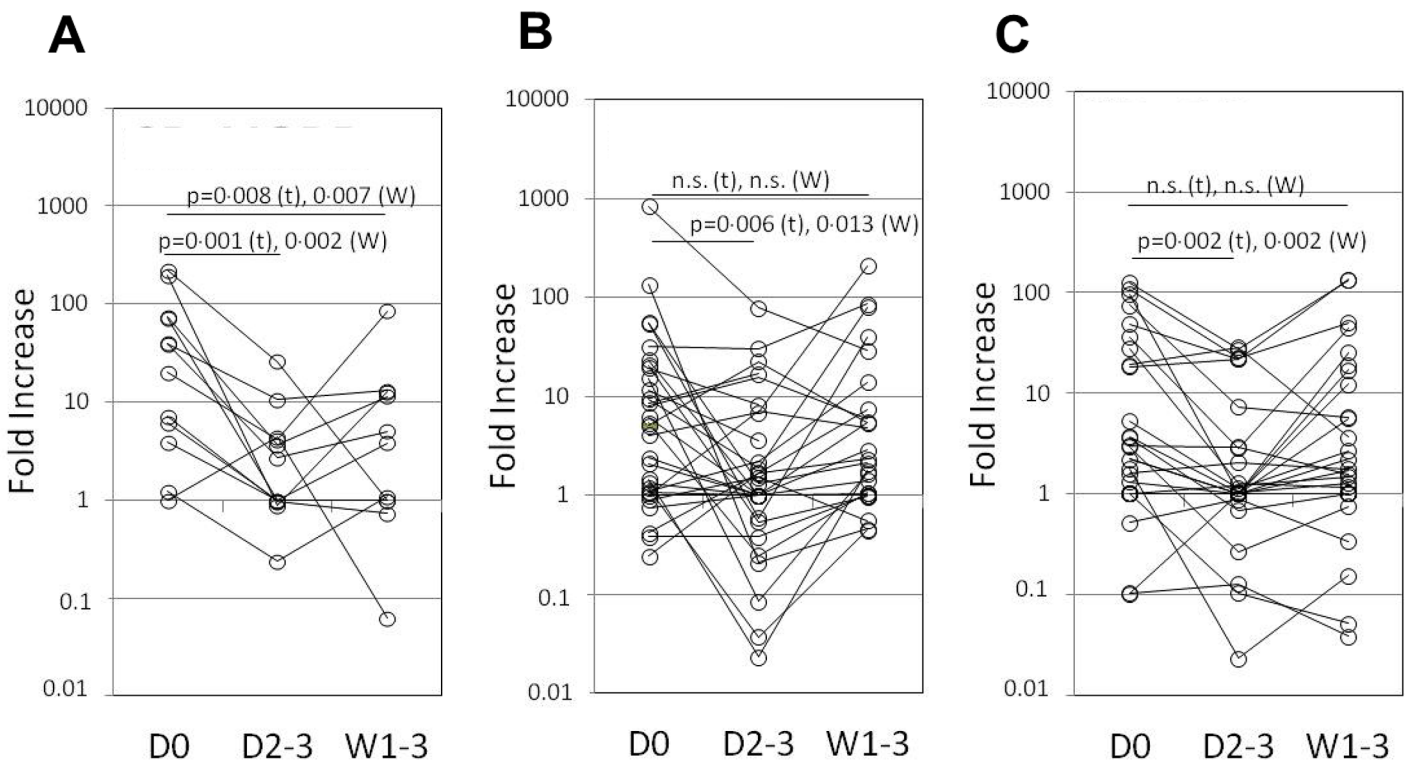

Fig 2. LPS-induced *CXCL10* expression before and after bortezomib treatment. Each point/line represents the fold increase in LPS-induced *CXCL10* expression in each patient in the (A) CR+VGPR, (B) PR and (C) SD+PD groups. The statistically significant difference between the pretreatment (D0) and 2–3 days (D2-3) or 1–3 weeks (W1-3) after intravenous administration of the first dose of bortezomib during the first cycle groups is shown. t: Student's *t*-test, W: Wilcoxon test.

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were variable. Moreover, LPS-induced *CXCL10* mRNA was significantly and continuously suppressed in the good response group even 1–3 weeks after treatment, whereas in the other groups, the suppressive activity was transient and *CXCL10* mRNA levels returned to the original values. It could be argued that the best biomarker may be ZA-induced *GMCSF* because the mean mRNA levels appeared to be higher in both the CR and VGPR groups than those in other response groups. The range of LPS-induced *CXCL10* expression in the CR group was within the ranges of LPS-induced *CXCL10* expression in both PR and SD groups, and lack of overlap was seen in only the patients with CR versus the two patients with PD, although the latter was a small number. Similarly, LPS-induced *CXCL10* expression in the VGPR group generally fell within the ranges seen in the patients with PR and those with SD. However, the majority of MM patients will respond to bortezomib especially in case used as the first-line treatment. Therefore, it is more important to distinguish the patients with PD after treatment with bortezomib, which may have the mutations as to the proteasome pathway [40], from responders to bortezomib rather than finding the difference in the response.

CXCL10, which was previously referred to as interferon γ-inducible 10 kDa protein (IP-10), belongs to the C-X-C family of chemokines that cluster on human chromosome 4 (q12-21). CXCL10 acts as a chemoattractant for human monocytes, activates T cells through binding to the CXCR3 receptor and promotes T cell adhesion to endothelial cells [41]. CXCL10 also elicits a Th1 cell-dominated anti-tumour inflammatory response that can inhibit plasmacytoma growth [42]. Moreover, activated tumour-specific T cells that express CXCR3 were shown to infiltrate CXCL10-expressing myeloma cells more efficiently than non-CXCL10-expressing

myeloma cells [43]. CCL4 is another chemotactic factor, and GMCSF is a growth factor for antigen-presenting cells. Thus, the higher induction of *CXCL10*, *CCL4* and *GMCSF* mRNA exhibited by the good responder group (Fig 1) was not unexpected and suggests that these patients may have greater anti-tumour immunity.

However, after bortezomib was administered to MM patients, *CXCL10* mRNA induction was significantly suppressed, and sustained suppression correlated with good responses to treatment (Fig 2). Usually bortezomib may be administerd to MM patients in combination with dexamethasone (the same day as that of bortezomib administration and the subsequent day) as used in the SUMMIT trial [44]. Actually the majority of the patients enrolled in this study received dexamethasone (Table 1) in the above-mentioned way, although detailed data were collected but not shown. In case of dexamethasone-naïve patients with MM, they can still respond to dexamethasone. Consequently, we may not be able to distinguish the responders to bortezomib from the responders to dexamethasone in 2–3 days after the first dose of bortezomib. Therefore, the induced mRNA that caused demonstrable sustained suppression may indicate more meaningful predictor of bortezomib responders. In addition, as mentioned above, such triple combination as bortezomib, melphalan, and prednisone is believed to be best among double to quadruple combinations and therefore used commonly, and melphalan and prednisone will be given days 1–4 of each cycle [1, 17]. In those cases, afore-mentioned, sustained suppression might become powerful tool of prediction although further studies in this approach in combination trials is essential as a validation exercise for theses assays to go forward.

This observation was likely not explained by the immunological activity of CXCL10, as CXCL10 is also expressed in human myeloma cell lines [45] and is known to stimulate myeloma cell migration [46] and adhesion to bone marrow stromal cells [47]. Thus, MM cells may be more susceptible to the bortezomib-induced inhibition of *CXCL10* mRNA expression than immune cells. Moreover, when bortezomib was added to whole blood prior to LPS stimulation

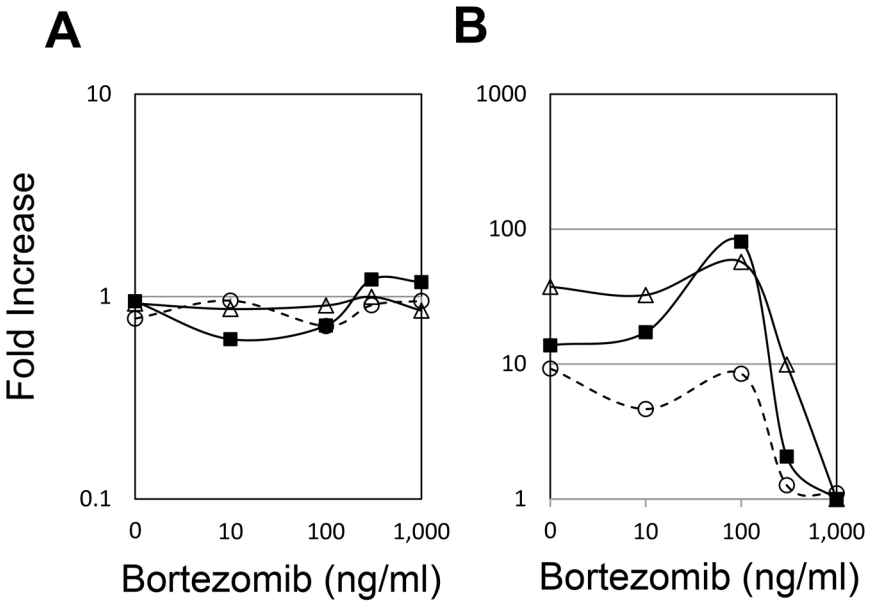

Fig 3. Bortezomib-induced inhibition of LPS-induced *CXCL10* mRNA *ex vivo*. Peripheral blood obtained from 3 healthy volunteers was pre-treated with various concentrations of bortezomib for 1 h and then further stimulated with LPS or PBS (as the control) for an additional 4 h. The fold increase in (A) *ACTB* and (B) *CXCL10* expression is shown. Each symbol represents a single individual.

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ex vivo, *CXCL10* mRNA induction was inhibited in a dose-dependent manner (Fig 3). Thus, LPS-induced *CXCL10* expression in *ex vivo* blood samples could serve as a surrogate marker for the effect of bortezomib *in vivo*.

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Author Contributions

Conceived and designed the experiments: TW MM. Performed the experiments: MM. Analyzed the data: MM. Contributed reagents/materials/analysis tools: TW MS MR KS MA KO YN SN SI MK. Wrote the paper: TW MM. Provided administrative support: MK. Collected clinical data: TW MC. Handled and shipped samples: SN MC.

References

1. San Miguel JF, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, Kropff M, et al. (2008) Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N Engl J Med 359: 906–917. doi: 10.1056/NEJMoa0801479 PMID: 18753647
2. Richardson PG, Sonneveld P, Schuster MW, Irwin D, Stadtmauer EA, Facon T, et al. (2005) Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med 352: 2487–2498. PMID: 15958804
3. Richardson PG, Sonneveld P, Schuster M, Irwin D, Stadtmauer E, Facon T, et al. (2007) Extended follow-up of a phase 3 trial in relapsed multiple myeloma: final time-to-event results of the APEX trial. Blood 110: 3557–3560. PMID: 17690257
4. Smadja NV, Bastard C, Brigaudeau C, Leroux D, Fruchart C. Groupe Français de Cytogénétique Hématologique. (2001) Hypodiploidy is a major prognostic factor in multiple myeloma. Blood 98: 2229–2238. PMID: 11568011
5. Facon T, Avet-Loiseau H, Guillerm G, Moreau P, Geneviéve F, Zandecki M, et al. (2001) Chromosome 13 abnormalities identified by FISH analysis and serum beta2-microglobulin produce a powerful myeloma staging system for patients receiving high-dose therapy. Blood 97: 1566–1571. PMID: 11238092
6. Fonseca R, Blood E, Rue M, Harrington D, Oken MM, Kyle RA, et al. (2003) Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood 101: 4569–4575. PMID: 12576322
7. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, et al. (2004) Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res 64: 1546–1558. PMID: 14989251
8. Mulligan G, Mitsiades C, Bryant B, Zhan F, Chng WJ, Roels S, et al. (2007) Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. Blood 109: 3177–3188. PMID: 17185464
9. Richardson PG, Xie W, Mitsiades C, Chanan-Khan AA, Lonial S, Hassoun H, et al. (2009) Single-agent bortezomib in previously untreated multiple myeloma: efficacy, characterization of peripheral neuropathy, and molecular correlations with response and neuropathy. J Clin Oncol 27: 3518–3525. doi: 10.1200/JCO.2008.18.3087 PMID: 19528374
10. Ludek P, Hana S, Zdenek A, Martina A, Dana K, Tomas B, et al. (2010) Treatment response to bortezomib in multiple myeloma correlates with plasma hepatocyte growth factor concentration and bone marrow thrombospondin concentration. Eur J Haematol 84: 332–336. doi: 10.1111/j.1600-0609.2009.01396.x PMID: 20015241

11. Ling SC, Lau EK, Al-Shabeeb A, Nikolic A, Catalano A, Iland H, et al. (2012) Response of myeloma to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP-1. Haematologica 97: 64–72. doi: 10.3324/haematol.2011.043331 PMID: 21993678

12. Song MK, Chung JS, Joo YD, Lee SM, Lee GW, Lee HS, et al. (2010) Clinical value of absolute lymphocyte counts before bortezomib-dexamethasone therapy in relapsed multiple myeloma patients. Acta Haematol 124: 34–39. doi: 10.1159/000313654 PMID: 20606414

13. Watanabe T, Mitsuhashi M, Sagawa M, Ri M, Suzuki K, Abe M, et al. (2013) Phytohemagglutinin-induced IL2 mRNA in whole blood can predict bortezomib-induced peripheral neuropathy for multiple myeloma patients. Blood Cancer J, Oct 4; 3:e150. doi: 10.1038/bcj.2013.47 PMID: 24096714

14. Durie BG, Harousseau JL, Miguel JS, Bladé J, Barlogie B, Anderson K, et al. International Myeloma Working Group. (2006) International uniform response criteria for multiple myeloma. Leukemia 20: 1467–1473. PMID: 16855634

15. Mitsuhashi M, Tomozawa S, Endo K, Shinagawa A. (2006) Quantification of mRNA in whole blood by assessing recovery of RNA and efficiency of cDNA synthesis. Clin Chem 52: 634–642. PMID: 16497944

16. Mitsuhashi M. (2010) *Ex vivo* simulation of leukocyte function: stimulation of specific subset of leukocytes in whole blood followed by the measurement of function-associated mRNAs. J Immunol Methods 363: 95–100. doi: 10.1016/j.jim.2010.10.002 PMID: 20951704

17. Mateos MV, Richardson PG, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, et al. (2010) Bortezomib plus melphalan and prednisone compared with melphalan and prednisone in previously untreated multiple myeloma: updated follow-up and impact of subsequent therapy in the phase III VISTA trial. *J Clin Oncol* 28: 2259–2266. doi: 10.1200/JCO.2009.26.0638 PMID: 20368561

18. Cavo M, Tacchetti P, Patriarca F, Petrucci MT, Pantani L, Galli M, et al. GIMEMA Italian Myeloma Network. (2010) Bortezomib with thalidomide plus dexamethasone compared with thalidomide plus dexamethasone as induction therapy before, and consolidation therapy after, double autologous stem-cell transplantation in newly diagnosed multiple myeloma: a randomised phase 3 study. Lancet 376: 2075–2085. doi: 10.1016/S0140-6736(10)61424-9 PMID: 21146205

19. Moreau P, Avet-Loiseau H, Facon T, Attal M, Tiab M, Hulin C, et al. (2011) Bortezomib plus dexamethasone versus reduced-dose bortezomib, thalidomide plus dexamethasone as induction treatment prior to autologous stem cell transplantation in newly diagnosed multiple myeloma. Blood 118: 5752–5758. doi: 10.1182/blood-2011-05-355081 PMID: 21849487

20. Palumbo A, Bringhen S, Caravita T, Merla E, Capparella V, Callea V, et al. (2006) Oral melphalan and prednisone chemotherapy plus thalidomide compared with melphalan and prednisone alone in elderly patients with multiple myeloma: randomised controlled trial. Lancet 367: 825–831. PMID: 16530576

21. Facon T, Mary JY, Hulin C, Benboubker L, Attal M, Pegourie B, et al. (2007) Melphalan and prednisone plus thalidomide versus melphalan and prednisone alone or reduced-intensity autologous stem cell transplantation in elderly patients with multiple myeloma (IFM 99–06): a randomised trial. Lancet 370: 1209–1218. PMID: 17920916

22. Hulin C, Facon T, Rodon P, Pegourie B, Benboubker L, Doyen C, et al. (2009) Efficacy of melphalan and prednisone plus thalidomide in patients older than 75 years with newly diagnosed multiple myeloma: IFM 01/01 trial. J Clin Oncol 27: 3664–3670. doi: 10.1200/JCO.2008.21.0948 PMID: 19451428

23. Garderet L, Iacobelli S, Moreau P, Dib M, Lafon I, Niederwieser D, et al. (2012) Superiority of the triple combination of bortezomib-thalidomide-dexamethasone over the dual combination of thalidomide-dexamethasone in patients with multiple myeloma progressing or relapsing after autologous transplantation: the MMVAR/IFM 2005–04 Randomized Phase III Trial from the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. J Clin Oncol 30: 2475–2482. doi: 10.1200/JCO.2011.37.4918 PMID: 22585692

24. Kropff M, Liebisch P, Knop S, Weisel K, Wand H, Gann CN, et al. (2009) DSMM XI study: dose definition for intravenous cyclophosphamide in combination with bortezomib/dexamethasone for remission induction in patients with newly diagnosed myeloma. Ann Hematol 88: 1125–1130. doi: 10.1007/s00277-009-0726-6 PMID: 19274460

25. Richardson PG, Weller E, Lonial S, Jakubowiak AJ, Jagannath S, Raje NS, et al. (2010) Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. Blood 116: 679–686. doi: 10.1182/blood-2010-02-268862 PMID: 20385792

26. Popat R, Oakervee HE, Hallam S, Curry N, Odeh L, Foot N, et al. (2008) Bortezomib, doxorubicin and dexamethasone (PAD) front-line treatment of multiple myeloma: updated results after long-term follow-up. Br J Haematol 141: 512–516. doi: 10.1111/j.1365-2141.2008.06997.x PMID: 18371113

27. Mateos MV, Hernández JM, Hernández MT, Gutiérrez NC, Palomera L, Fuertes M, et al. (2008) Bortezomib plus melphalan and prednisone in elderly untreated patients with multiple myeloma: updated

time-to-events results and prognostic factors for time to progression. Haematologica 93: 560–565. doi: 10.3324/haematol.12106 PMID: 18322252

28. Richardson PG, Weller E, Jagannath S, Avigan DE, Alsina M, Schlossman, et al. (2009) Multicenter, phase I, dose-escalation trial of lenalidomide plus bortezomib for relapsed and relapsed/refractory multiple myeloma. J Clin Oncol 27: 5713–5719. doi: 10.1200/JCO.2009.22.2679 PMID: 19786667
29. Palumbo A, Bringhen S, Rossi D, Cavalli M, Larocca A, Ria R, et al. (2010) Bortezomib-melphalan-prednisone-thalidomide followed by maintenance with bortezomib-thalidomide compared with bortezomib-melphalan-prednisone for initial treatment of multiple myeloma: a randomized controlled trial. J Clin Oncol 28: 5101–5109. doi: 10.1200/JCO.2010.29.8216 PMID: 20940200
30. Mateos MV, Oriol A, Martínez-López J, Gutiérrez N, Teruel AI, de Paz R, et al. (2010) Bortezomib, melphalan, and prednisone versus bortezomib, thalidomide, and prednisone as induction therapy followed by maintenance treatment with bortezomib and thalidomide versus bortezomib and prednisone in elderly patients with untreated multiple myeloma: a randomised trial. Lancet Oncol 11: 934–941. doi: 10.1016/S1470-2045(10)70187-X PMID: 20739218
31. Bringhen S, Larocca A, Rossi D, Cavalli M, Genuardi M, Ria R, et al. (2010) Efficacy and safety of once weekly bortezomib in multiple myeloma patients. Blood 116: 4745–4753. doi: 10.1182/blood-2010-07-294983 PMID: 20807892
32. van Rhee F, Szymonifka J, Anaissie E, Nair B, Waheed S, Alsayed Y, et al. (2010) Total Therapy 3 for multiple myeloma: prognostic implications of cumulative dosing and premature discontinuation of VTD maintenance components, bortezomib, thalidomide, and dexamethasone, relevant to all phases of therapy. Blood 116: 1220–1227. doi: 10.1182/blood-2010-01-264333 PMID: 20501894
33. Chanan-Khan AA, Giralt S (2010) Importance of achieving a complete response in multiple myeloma, and the impact of novel agents. J Clin Oncol 28: 2612–2624. doi: 10.1200/JCO.2009.25.4250 PMID: 20385994
34. Moreau P, Attal M, Pégourié B, Planche L, Hulin C, Facon T, et al. (2011) Achievement of VGPR to induction therapy is an important prognostic factor for longer PFS in the IFM 2005–01 trial. Blood 117: 3041–3044. doi: 10.1182/blood-2010-08-300863 PMID: 21098740
35. Martinez-Lopez J, Blade J, Mateos MV, Grande C, Alegre A, Garcia-Larana J, et al. (2011) Long-term prognostic significance of response in multiple myeloma after stem cell transplantation. Blood 118: 529–534. doi: 10.1182/blood-2011-01-332320 PMID: 21482708
36. Gay F, Larocca A, Wijermans P, Cavallo F, Rossi D, Schaafsma R, et al. (2011) Complete response correlates with long-term progression-free and overall survival in elderly myeloma treated with novel agents: analysis of 1175 patients. Blood 117: 3025–3031. doi: 10.1182/blood-2010-09-307645 PMID: 21228328
37. Cavo M, Pantani L, Petrucci MT, Patriarca F, Zamagni E,Donnarumma D, et al. (2012) Bortezomib-thalidomide-dexamethasone is superior to thalidomide-dexamethasone as consolidation therapy after autologous hematopoietic stem cell transplantation in patients with newly diagnosed multiple myeloma. Blood 120: 9–19. doi: 10.1182/blood-2012-02-408898 PMID: 22498745
38. Sonneveld P, Schmidt-Wolf IG, van der Holt B, El Jarari L, Bertsch U, Salwender H, et al. (2012) Bortezomib induction and maintenance treatment in patients with newly diagnosed multiple myeloma: results of the randomized phase III HOVON-65/ GMMG-HD4 trial. J Clin Oncol 30: 2946–2955. doi: 10.1200/JCO.2011.39.6820 PMID: 22802322
39. Mateos MV, Oriol A, Martínez-López J, Gutiérrez N, Teruel AI, López de la Guía A, et al. (2012) Maintenance therapy with bortezomib plus thalidomide or bortezomib plus prednisone in elderly multiple myeloma patients included in the GEM2005MAS65 trial. Blood 120: 2581–2588. PMID: 22889759
40. Ri M, Iida S, Nakashima T, Miyazaki H, Mori F, Ito A, et al. (2010) Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. Leukemia Aug 24: 1506–1512. doi: 10.1038/leu.2010.137 PMID: 20555361
41. Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, et al. (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J Exp Med 177, 1809–1814. PMID: 8496693
42. Issekutz TB, Stoltz JM, vd Meide P (1988) Lymphocyte recruitment in delayed-type hypersensitivity. The role of IFN-gamma. J Immunol 140: 2989–2993. PMID: 3129506
43. Huang H, Liu Y, Xiang J (2002) Synergistic effect of adoptive T-cell therapy and intratumoral interferon gamma-inducible protein-10 transgene expression in treatment of established tumors. Cell Immunol 217: 12–22. PMID: 12425997
44. Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, et al. (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 348: 2609–2617. PMID: 12826635

45. Giuliani N, Bonomini S, Romagnani P, Lazzaretti M, Morandi F, Colla S, et al. (2006) CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival. Haematologica, 91, 1489–1497. PMID: 17082008

46. Pellegrino A, Antonaci F, Russo F, Merchionne F, Ribatti D, Vacca A, et al. (2004) CXCR3-binding chemokines in multiple myeloma. Cancer Lett 207: 221–227. PMID: 15072832

47. Nguyen AN, Stebbins EG, Henson M, O'Young G, Choi SJ, Quon D, et al. (2006) Normalizing the bone marrow microenvironment with p38 inhibitor reduces multiple myeloma cell proliferation and adhesion and suppresses osteoclast formation. Exp Cell Res 312: 1909–1923. PMID: 16600214