Bortezomib Induces Anti–Multiple Myeloma Immune Response Mediated by cGAS/STING Pathway Activation

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The proteasome inhibitor bortezomib induces apoptosis in multiple myeloma cells and has transformed patient outcome. Using in vitro as well as in vivo immunodeficient and immunocompetent murine multiple myeloma models, we here show that bortezomib also triggers immunogenic cell death (ICD), characterized by exposure of calreticulin on dying multiple myeloma cells, phagocytosis of tumor cells by dendritic cells, and induction of multiple myeloma–specific immunity. We identify a bortezomib-triggered specific ICD gene signature associated with better outcome in two independent cohorts of patients with multiple myeloma. Importantly, bortezomib stimulates multiple myeloma cell immunogenicity via activation of the cGAS/STING pathway and production of type I IFNs, and STING agonists significantly potentiate bortezomib-induced ICD. Our study therefore delineates mechanisms whereby bortezomib exerts immunotherapeutic activity and provides the framework for clinical trials of STING agonists with bortezomib to induce potent tumor-specific immunity and improve patient outcome in multiple myeloma.

SIGNIFICANCE: Our study demonstrates that cGAS/STING-dependent immunostimulatory activity mediates bortezomib anti-myeloma activity in experimental models and associates with clinical response to bortezomib in patients with multiple myeloma. These findings provide the rationale for clinical evaluation of STING agonists to further potentiate anti–multiple myeloma immune response.

See related commentary by Zitvogel and Kroemer.

INTRODUCTION

Multiple myeloma is a malignancy of plasma cells in the bone marrow (BM; refs. 1, 2). Despite remarkable improvement in patient survival due to the development of proteasome inhibitors (PI) and immunomodulatory drugs (IMiD), the clinical management of patients with multiple myeloma remains challenging (1, 2). Constitutive and ongoing genetic complexity of multiple myeloma cells, coupled with the tumor-promoting, immuno-suppressive BM microenvironment, underlies relapse of disease and remains an obstacle to cure (3). More recently, integration of mAbs into the treatment of both newly diagnosed and relapsed/refractory multiple myeloma has further improved patient outcome (1, 4). However, dysfunction of innate and adaptive immunity, specifically involving the T-cell compartment, highlights the need for novel approaches to enhance anti–multiple myeloma immunity and achieve more durable responses.

It is now recognized that specific antitumor immunity can be triggered by therapeutic agents via “immunogenic cell death” (ICD; refs. 5–7). During treatment-related induction of ICD, endogenous tumor cell proteins are recognized as damage-associated molecular patterns (DAMP) and activate cancer-specific immune responses (8, 9). Among several DAMPs, endoplasmic reticulum (ER) protein calreticulin (CALR) exposure on the tumor cell surface is triggered by activation of the unfolded protein response (UPR) and represents a potent “eat-me” signal, allowing for efficient phagocytosis of dying cancer cells by dendritic cells (DC) and induction of a specific antitumor immune response (10). The clinical efficacy of ICD inducers is therefore due to their ability to redirect patients’ immune systems against their own tumors (9, 11). To date, identification of immunogenic properties of conventional therapeutics, such as anthracyclines and oxaliplatin, has informed their clinical application in combination with immune therapies to enhance responses in immunologically “cold” solid tumors (12, 13).

The PI bortezomib (BTZ) is one of the most effective anti–multiple myeloma agents (14, 15). Excessive protein overload in multiple myeloma cells renders them dependent on proteasome activity; conversely, PIs induce accumulation of misfolded proteins, ER stress, and multiple myeloma cell death (16). Indeed, proteasome inhibition affects the quality control of proteins critical for multiple myeloma survival (17), including those involved in DNA repair (18). Extensive preclinical studies have defined the mechanisms of action of BTZ on tumor cells and accessory cells, that is, osteoclasts, in the BM.
molecule, we first assessed the effect of BTZ treatment on human AMO1 and NCI-H929, as well as murine STGM1, multiple myeloma cell lines. BTZ induced multiple myeloma cell death in a dose-dependent manner, as measured by phosphatidylserine exposure (Fig. 1A, internal plot). As seen in Fig. 1A, BTZ also triggered a dose-dependent increase of CALR exposure on the outer leaflet of multiple myeloma cell plasma membranes and consistently activated the UPR transducer PERK pathway, evidencing increased phosphorylation of the translation initiation factor eIF2-α (p-eIF2-α) and increased levels of CHOP and ATF4 proteins (Supplementary Fig. S1A). Confocal analysis showed that BTZ-treated multiple myeloma cells, but not untreated multiple myeloma cells, were engulfed by monocyte-derived DCs (Mo-DC) after 4 hours of coculture (Fig. 1B; Supplementary Fig. S1B); flow cytometry–based phagocytosis assay confirmed this effect in both human (AMO1 and NCI-H929) and murine STGM1 multiple myeloma cells (Fig. 1C; Supplementary Fig. S1C). Phagocytosis of BTZ-treated AMO1 cells stimulated maturation of DCs, as shown by increased expression of CD83 and CD86 maturation markers, which was not triggered by either untreated multiple myeloma cells or BTZ alone (Supplementary Fig. S1D). To confirm the essential role of CALR on phagocytosis by DCs, we generated murine STGM1 Calr knockout (CalrKO) cells (Supplementary Fig. S1E). Although BTZ triggered apoptosis in both STGM1 wild-type (WT; STGM1WT) and CalrKO cells, the CalrKO clones were more sensitive to the same extent (Supplementary Fig. S1F), phagocytosis of BTZ-treated CalrKO multiple myeloma cells by DCs was inhibited (Fig. 1D). To confirm the specific on-target role of Calr loss in mediating suppression of phagocytosis, we stably reexpressed Calr in knockout cells, which efficiently restored multiple myeloma cell phagocytosis by DCs (Fig. 1E) and confirmed the obligatory role of CALR exposure in this process.

Next, we assessed the stimulation of T cells by DCs loaded with BTZ-treated multiple myeloma cells. In vitro culture of BTZ-treated multiple myeloma cells, DCs, and T cells increased maturation and activation of both CD4+ and CD8+ T-cell populations (Fig. 1F; Supplementary Fig. S2A and S2B). Specifically, induction of ICD by BTZ resulted in a significant increase of CD4+ effector memory (EM), total CD8+, CD8+ EM, and CD8+ terminally differentiated EM (TEMRA) cells, which was not observed by treating DCs and T cells with BTZ in the absence of multiple myeloma cells (Supplementary Fig. S2C). Similarly, isolated T cells after cocultures showed the presence of multiple myeloma–specific cytotoxic T lymphocytes that were able to efficiently induce lysis of multiple myeloma cells (Fig. 1G). To characterize specificity of the T-cell response, we analyzed the effects of BTZ-induced cell death specifically on the naïve T-cell population in a parallel experiment. We observed increased proliferation of both CD4+ and CD8+ naïve T cells after coculture with BTZ-treated AMO1 or NCI-H929 cells and DCs (Supplementary Fig. S3A and S3B). Similar results were obtained using primary cells derived from patients with multiple myeloma (pMM). We observed a dose-dependent increase in phagocytosis of BTZ-treated pMM cells by DCs (Fig. 2A). Consistently, treatment of BM mononuclear cells (BMMC) from patients with multiple myeloma with BTZ confirmed the phenotypic changes in
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A. Cell-surface CALR (fold increase)

B. Immunofluorescence images of mDCs + STGM1 WT and mDCs + STGM1 Calr KO

C. Phagocytosis (fold increase)

D. Phagocytosis (fold increase) with and without BTZ

E. Phagocytosis (fold increase) with and without BTZ

F. UMAP1 and UMAP2 plots with different conditions

G. Effect of T:E ratio on U87 cell lysis

Additional information on cell lines and conditions used in the experiments.
both CD4+ and CD8+ T-cell populations (Fig. 2B). Altogether, these data show that BTZ increases the immunogenicity of multiple myeloma cells, thereby stimulating an anti–multiple myeloma immune response in vitro.

**BTZ Stimulates Anti–Multiple Myeloma Immunity In Vivo via Induction of ICD**

To test the relevance of ICD in BTZ-induced anti–multiple myeloma activity in vivo, we used the syngeneic immuno-competent ST murine model of multiple myeloma (24). We found that low doses of BTZ (0.5 mg/kg twice/week for 2 weeks) inhibited growth of STGMI1WT cells engrafted in immunocompetent (C57BL/KaLwRij) mice (Fig. 3A) to a greater extent than when these cells were engrafted in immunodeficient hosts (SCID/NOD; Fig. 3B). Importantly, this effect was directly linked to ICD induction, because it was abrogated in immunocompetent mice bearing STGMI1CalrKO tumors (Fig. 3C) in which the delay of tumor growth was similar to that observed in the immunodeficient hosts (Fig. 3D). Taken together, these results suggest that the effects of BTZ are mediated, at least in part, by the immune system.

We next tested whether, after the regression of tumor growth, the mice were further protected against a tumor rechallenge. Injection of live STGMI1WT cells 2 weeks after BTZ-induced tumor regression did not result in tumor development, and 100% of mice were alive at the end of observation (day 30 after rechallenge; Fig. 3E). *Ex vivo* enzyme-linked immunospot (ELISPOT), using splenocytes harvested from mice treated under the same conditions, confirmed the generation
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Bortezomib induces ICD in a syngeneic murine model of multiple myeloma (MM). A–D, In vivo growth of subcutaneous xenografts of 5TGM1WT (A and B) or 5TGM1 CalrKO (C and D) multiple myeloma cells in immunocompetent C57BL/KaLwRij (A and C) or immunodeficient SCID/NOD (B and D) mice treated with either PBS (CNT) or BTZ (0.5 mg/kg twice/week for 2 weeks) when tumors became measurable by electronic caliper. Fold increase of tumor growth from day 1 (start of treatment) to day 8 ± SD (n = 5 animals per group in A, B, and C and n = 4 in D). One representative experiment of two yielding similar results is shown for each condition. P values were calculated using unpaired Student t test. E, Immune competent C57BL/KaLwRij (n = 5) bearing 5TGM1WT tumors were treated with BTZ as in A. Two weeks after tumor regression, BTZ-treated mice (n = 5) were challenged with viable 5TGM1 cells, along with naive mice (n = 5), and percentage of mice remaining tumor free in the two cohorts is shown according to the Kaplan–Meier method. P value was calculated by using the log-rank test. Schema of the experimental design is shown in top plot. G, Ex vivo ELISPOT was performed on splenocytes harvested from mice treated as in E. Splenocytes were left unstimulated or stimulated with B16 tumor cells as negative control and 5TGM1 or anti-CD3 as positive control to test T-cell avidity in an IFNγELISPOT assay. Spot-forming colonies (SFC) per million are represented for n = 3 naïve and n = 5 BTZ-treated mice ± SD. ELISPOT experiments were performed in triplicate wells per sample; unpaired Student t test for statistical analysis. ELISPOT images are shown as Supplementary Fig. S4A. Mice treated with Bortezomib induce protection against rechallenge (Fig. 3E; Supplementary Fig. S4E). In a similar attempt to prove the induction of immunologic memory after BTZ-induced cell death, we estimated whether vaccination of C57BL/KaLwRij mice with in vitro BTZ-treated STG1M1 cells could protect mice against challenge with viable STG1M1 cells. Nonvaccinated mice developed palpable tumors 1 week after STG1M1 injection, whereas no tumor developed in vaccinated mice even after 30 days (Fig. 3G). Mice were next similarly vaccinated with BTZ-treated STG1M1 CalrKO cells and challenged 1 week later with injection of live WT STG1M1 cells. Only 50% of vaccinated mice were tumor free at day 30 (Fig. 3G). Altogether, these data indicate that induction of ICD by BTZ induces a protective antitumor response in vivo.

An ICD-Related Signature Predicts Clinical Outcome in Patients with Multiple Myeloma after BTZ Treatment

To confirm the biological sequelae of ICD induction by BTZ in tumors in vivo, we performed RNA sequencing (RNA-seq) analysis of 5TGM1 WT and 5TGM1 CalrKO tumors grown in C57BL/KaLwRij immunocompetent mice. The transcriptional changes induced by BTZ in 5TGM1 WT tumors were consistent with activation of an immune response (i.e., inflammatory response and regulation of immune system process), whereas loss of Calr in 5TGM1 tumors decreased this effect and revealed instead an enrichment in signaling related to direct BTZ cytotoxicity (i.e., regulation of protein polyubiquitination and positive regulation of cell survival).

Figure 3. BTZ induces ICD in a syngeneic murine model of multiple myeloma (MM). A–D, In vivo growth of subcutaneous xenografts of 5TGM1WT (A and B) or 5TGM1 CalrKO (C and D) multiple myeloma cells in immunocompetent C57BL/KaLwRij (A and C) or immunodeficient SCID/NOD (B and D) mice treated with either PBS (CNT) or BTZ (0.5 mg/kg twice/week for 2 weeks) when tumors became measurable by electronic caliper. Fold increase of tumor growth from day 1 (start of treatment) to day 8 ± SD (n = 5 animals per group in A, B, and C and n = 4 in D). One representative experiment of two yielding similar results is shown for each condition. P values were calculated using unpaired Student t test. E, Immuno-competent C57BL/KaLwRij (n = 5) bearing 5TGM1WT tumors were treated with BTZ as in A. Two weeks after tumor regression, BTZ-treated mice (n = 5) were challenged with viable 5TGM1 cells, along with naive mice (n = 5), and percentage of mice remaining tumor free in the two cohorts is shown according to the Kaplan–Meier method. P value was calculated by using the log-rank test. Schema of the experimental design is shown in top plot. F, Ex vivo ELISPOT was performed on splenocytes harvested from mice treated as in E. Splenocytes were left unstimulated or stimulated with B16 tumor cells as negative control and 5TGM1 or anti-CD3 as positive control to test T-cell avidity in an IFNγELISPOT assay. Spot-forming colonies (SFC) per million are represented for n = 3 naïve and n = 5 BTZ-treated mice ± SD. ELISPOT experiments were performed in triplicate wells per sample; unpaired Student t test for statistical analysis. ELISPOT images are shown as Supplementary Fig. S4A. G, In vitro BTZ-treated STG1M1 or CalrKO cells were injected into naive mice; control mice (No vax) received PBS as a negative control. Mice (n = 8 animals per group) were rechallenged with viable 5TGM1 cells 1 week later, and percentage of mice remaining tumor free in the three cohorts is shown according to the Kaplan–Meier method. The log-rank test was used for statistical significance. Schema of the experimental design is shown in top plot. ns, not significant, *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
of autophagy; Supplementary Fig. S5A). Focused analysis of the most upregulated genes after BTZ treatment in mice bearing 5TGM1 WT tumors identified a set of 90 immune-related genes composing an ICD signature, which was not similarly modulated in 5TGM1 Calr<sup>KO</sup> tumors (Fig. 4A; Supplementary Table S1). Importantly, we found that high expression of the human orthologs of this 90-gene murine ICD signature was strongly positively correlated with clinical outcome of patients with multiple myeloma uniformly treated with BTZ-based regimens in the IFM/DFCI clinical study [IFM/DFCI 2009 dataset (n = 327); B] and GSE9782 (n = 152; D). Heatmaps identified three clusters of patients with multiple myeloma showing high (red), medium (blue), and low (green) expression of these signature genes. C and E, Kaplan-Meier plots show OS for the three patient clusters identified by expression of the 90 ICD gene signature. Red, blue, and green curves represent OS of patients with high, medium, and low ICD signature expression, respectively. Log-rank test P values for IFM/DFCI 2009 dataset and GSE9782 dataset are 0.01 and 0.047.

Figure 4. An ICD-related signature predicts clinical outcome in patients with multiple myeloma (MM) after BTZ treatment. A, Log<sub>2</sub> fold-change (log2FC) values for 90 genes, as determined by RNA-seq analysis, in 5TGM1<sup>WT</sup> or 5TGM1 Calr<sup>KO</sup> tumors growing in immunocompetent C57BL/KaLwRij mice after two BTZ treatments (0.5 mg/kg). Columns represent the conditions, and rows are those differentially expressed genes (DEG) in the BTZ-treated cohort compared with the CNT cohort (n = 3 mice per group). FC > 1.5 and FDR < 0.05. B and D, Analysis of the human orthologs of this 90-gene murine ICD signature was performed in CD138<sup>+</sup> multiple myeloma cells from two independent BTZ-treated cohorts of patients with multiple myeloma: IFM/DFCI 2009 dataset (n = 327; B) and GSE9782 (n = 152; D). Heatmaps identified three clusters of patients with multiple myeloma showing high (red), medium (blue), and low (green) expression of these signature genes. C and E, Kaplan-Meier plots show OS for the three patient clusters identified by expression of the 90 ICD gene signature. Red, blue, and green curves represent OS of patients with high, medium, and low ICD signature expression, respectively. Log-rank test P values for IFM/DFCI 2009 dataset and GSE9782 dataset are 0.01 and 0.047.

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**BTZ Activates a “Viral Mimicry” State in Multiple Myeloma Cells, Which Is Required for Its Activity In Vivo**

Transcriptional activation of ISGs by inducers of ICD is consistent with a “viral mimicry” state (28, 29). Specifically, type I IFN response and inflammatory chemokines (such as CXCL9) create an inflammatory microenvironment and are required for optimal therapeutic efficacy of agents inducing ICD (28–30). We therefore next examined induction of a type I
IFN response by treating AMO1 and NCI-H929 multiple myeloma cells with BTZ in vitro. RNA-seq analysis of AMO1 cells treated with BTZ confirmed positive enrichment of gene sets included in the type I IFN response hallmark signature (Supplementary Fig. S7A), which was also validated by qRT-PCR showing increased IFNA1, IFNB1, and CXCL9 transcripts in AMO1 and NCI-H929 multiple myeloma cells after BTZ treatment (Supplementary Fig. S7B). We next assessed the contribution of this type I IFN response to the anti–multiple myeloma activity of BTZ in vivo. Neutralization of type I IFN signaling in both multiple myeloma and host cells, using the type I IFN receptor 1 (IFNAR1)–specific MAR1-5A3 mAb, significantly decreased the efficacy of BTZ against 5TGM1 tumors as compared with isotype control mAb (Supplementary Fig. S7C). qRT-PCR analysis on harvested tumors confirmed that the MAR1-5A3 mAb blocked Cxcl9 transcript accumulation in tumors from BTZ-treated mice (Supplementary Fig. S7D). In a parallel analysis of patients with multiple myeloma uniformly treated with BTZ-containing regimens (IFM/DFCI 2009), we found that low expression of Cxcl9 transcript in CD138* multiple myeloma cells independently correlates with poor clinical outcome (OS P value = 0.037; Supplementary Fig. S7E). These studies indicate that BTZ induces a viral mimicry state in multiple myeloma cells and that this type I IFN response is required for optimal in vivo response.

**BTZ Induces Type I IFN Signaling via Activation of the cGAS/STING Pathway**

BTZ induces genomic instability and inhibits DNA repair in multiple myeloma cells (18). Recent studies show that damaged DNA can be detected outside the nucleus and induce an immunostimulatory response, mimicking a viral attack and activating a type I IFN response (31, 32). We next showed that BTZ induced a dose-dependent increase of DNA content in the cytoplasm of AMO1 and NCI-H929 multiple myeloma cells, detected in the form of micronuclei by flow cytometry (Fig. 5A; Supplementary Fig. S8A and S8B). Previous studies demonstrated that cGAS can directly sense micronuclei formation and link genomic instability to innate immunity by directly activating the STING/TMEM173 pathway (32–34).
Importantly, we found that expression of the ISGs included in the ICD signature was positively correlated with STING expression in the cluster of patients with high levels of ICD signature genes (Fig. 5B). Moreover, we also found that BTZ treatment of both AMO1 and NCI-H929 multiple myeloma cells induces accumulation of the cytosolic DNA sensor cGAS, which activates the adaptor molecule STING and in turn leads to phosphorylation by TBK1 kinase of IRF3, a well-known transcription factor of type I IFN genes (ref. 35; Fig. 5C; Supplementary Fig. S8C). We therefore hypothesized that BTZ treatment of AMO1 and NCI-H929 multiple myeloma cells lacking STING pathway. To test this possibility, we generated human STING KO cells as compared with BTZ-treated AMO1 WT clones (Fig. 5G; Supplementary Fig. S8F). These findings indicate that type I IFN response in multiple myeloma cells resulted in impaired T-cell response: No significant increase of CD4+ EM, total CD8+, and CD8+ EM cells was noted after coculture of DCs with BTZ-treated AMO1 STINGKO cells as compared with BTZ-treated AMO1 WT clones (Fig. 5G; Supplementary Fig. S8H). This block of type I IFN response in multiple myeloma cells triggered by BTZ is mediated by the cGAS/STING pathway and enhances anti–multiple myeloma T-cell responses.

**STING Agonists Potentiate BTZ-Induced Antitumor Immunity**

Pharmacologic activation of the STING pathway represents a promising strategy to overcome immunosuppression in the tumor microenvironment (36–38). We found that the synthetic cyclic dinucleotide STING agonist ADU-S100 (38, 39) can significantly increase the activation of STING signaling after BTZ treatment in vitro, as evidenced by higher
levels of phosphorylated TBK1 kinase (Supplementary Fig. S9A) and increased transcription of IFNA1 and IFNB1 (Supplementary Fig. S9B). Thus, we tested whether combining BTZ and ADU-S100 could increase the tumor T-cell infiltration and enhance the antitumor activity in vivo. 5TGM1 WT KaLwRij immunocompetent mice bearing 5TGM1 tumors were randomized to receive (i) BTZ alone (0.375 mg/kg twice/week for 2 weeks), (ii) peritumoral administration of ADU-S100 (100 μg on days 1 and 2), (iii) both drugs, or (iv) PBS as control. Mice treated with the combination showed the most significant reduction of tumor growth, with a complete regression of the tumors (COMBO vs. BTZ \( P = 0.029 \); COMBO vs. ADU-S100 \( P = 0.05 \); Fig. 6A). IHC analysis of tumors retrieved after one administration of the drugs showed that treatment with either BTZ or ADU-S100 alone or in combination increased CD3 \(^\text{T} \) T-cell infiltration within tumors (Fig. 6B). Murine 5TGM1 cells are sensitive to direct killing by ADU-S100 treatment in vitro (Supplementary Fig. S9C). Thus, we next performed an identical in vitro study in immunodeficient NOD/SCID mice to examine whether ADU-S100 antagonizes multiple myeloma growth in vivo via an immunomodulatory activity or via a direct cytotoxicity. As shown in Fig. 6C, the absence of the immune system abrogated the antitumor effects of both BTZ and ADU-S100 (100 μg on days 1 and 2), indicating that the observed tumor regression in the presence of the immune system was primarily due to an antitumor immune response. To further confirm the role of the intrinsic stimulation of intratumoral STING in mediating immune activation, we also tested the effect of BTZ–STING agonist combination in immunocompetent mice bearing STINGKO tumors. Antitumor activity of BTZ was significantly abrogated against tumors lacking STING (Fig. 6D), whereas ADU-S100 still retained a partial anti–multiple myeloma activity most likely due to stimulation of the STING pathway in the immune microenvironment cells (37, 38). Taken together, these results indicate a central role for STING in mediating the anti–multiple myeloma immune response induced by BTZ and show that STING agonist
augments this effect by further promoting an immunogenic microenvironment (Fig. 6E).

**DISCUSSION**

Immune dysfunction poses a challenge to effective anti–multiple myeloma therapy and complete elimination of minimal residual disease (3). Although the mechanisms of action of anti–multiple myeloma agents have been characterized, their in vivo effects on the dynamic interplay between tumor cells and the immune system are yet to be defined (3) and may inform optimal combination treatment approaches in patients. In this context, the extraordinary clinical benefits of BTZ treatment have to date been attributed to the exquisite intrinsic dependency of multiple myeloma cells on proteasome activity (17). Here, we challenge this notion by characterizing BTZ as an immunotherapeutic agent and delineating novel mechanisms whereby BTZ triggers a specific anti–multiple myeloma immune response.

The first major conclusion of our study is that BTZ efficacy is due to the activation of the immune system: Although the drug can efficiently delay tumor growth in immunodeficient mice, it requires a competent immune system to induce tumor regression. To explain this effect, we focused on a tumor-intrinsic mechanism of immune activation, specifically the induction of ICD. Although early in vitro studies have suggested an immunogenic role of BTZ (20), its biological, functional, and clinical significance is not fully characterized (23). In our studies, BTZ treatment led to the exposure of the eat-me molecule CALR on the cell surface of both human and murine multiple myeloma cell lines, and we confirmed the obligate role of this DAMP as a major prohapogocytic checkpoint in multiple myeloma both in vitro and in vivo. Analysis of transcriptomic changes in murine multiple myeloma cells after BTZ treatment identified an ICD signature. Most importantly, we confirmed its clinical significance, because the analogus ICD signature was positively correlated with improved outcome of patients with multiple myeloma treated with BTZ in two independent datasets. Multiple clinical trials have shown that treatment with BTZ is effective in reducing tumor burden in patients with multiple myeloma, including multiple myeloma with high-risk cytogenetics such as t(4;14) (40). Based on these data, we speculated that clinical responses to BTZ may be mediated via the induction of an efficient anti–multiple myeloma immune response. Further characterization of the genes included in the BTZ-induced ICD signature identified a multiple myeloma cell-autonomous type I IFN response. This finding is consistent with a previous report showing that activation of a viral mimicry state in multiple myeloma cells increases the anti–multiple myeloma immune response to therapy in the murine Vk*MYC model of multiple myeloma (41). Moreover, IFNα has in the past been used, either alone or in combination, to treat patients with multiple myeloma; although it demonstrated efficacy, its clinical utility was limited by toxicity (42).

The second major conclusion of our study is that BTZ stimulates the immunogenicity of multiple myeloma cells by activating the cGAS/STING innate immune response signaling pathway (32, 35). The release of cytosolic DNA after BTZ-induced multiple myeloma cell death is a trigger for the STING pathway; an increased genomic instability, due to inhibition of the DNA repair machinery, is also recognized as a downstream effect of proteasome inhibition (18). Here, we showed that the expression of STING positively correlates with the expression of ICD-related ISGs in patients with multiple myeloma, implicating this pathway in BTZ-induced IFN response. Loss of IFN response has been described as an additional mechanism of tumor immune escape (43), and we found that patients with multiple myeloma with low STING/ISG expression do not efficiently respond to ICD induction after BTZ treatment. Indeed, immune response after BTZ treatment was significantly reduced in vivo against multiple myeloma tumors lacking STING. Importantly, activation of the STING pathway is an emerging immunotherapeutic approach, and phase I and II clinical trials of several STING agonists are currently ongoing in solid tumors or lymphoma, alone or in combination with immunotherapies (NCT04144140, NCT03937141, NCT02675439, NCT03172936, and NCT03010176). The prior use of IFN stimulation in anti–multiple myeloma therapy coupled with our current data suggests that STING agonists may also represent a promising therapeutic strategy in multiple myeloma and that their combined use with BTZ may increase their immunogenic effect, especially in patients with low basal level of STING expression. Indeed, we showed that combination of BTZ with the STING agonist ADU-S100 significantly enhanced the immunogenic effect of BTZ in vivo. These studies both validate STING as a therapeutic target and provide the framework for clinical trials evaluating BTZ and STING agonist combination therapy in multiple myeloma.

In summary, our study delineates the mechanism whereby BTZ induces a clinically significant antitumor immune response in multiple myeloma. Although BTZ is incorporated into many combination therapeutic regimens for multiple myeloma due to its direct impact on tumor cells, recognition of its immune effect will inform its broader use, alone and in combination, as an immunotherapy. In addition, induction of ICD may account for the enhanced clinical activity observed in patients with multiple myeloma currently treated with effective combinations incorporating BTZ with either IMiDs or mAbs. Finally, our study identifies and validates STING as a new therapeutic target mediating immune activation against multiple myeloma and provides the preclinical framework for STING agonist and BTZ combination clinical trials to enhance anti–multiple myeloma immune responses and further improve patient outcome in multiple myeloma.

**METHODS**

**Cell Culture**

Multiple myeloma cell lines U266, NCI-H929, murine JAWSII, and 293T were purchased from the American Type Culture Collection; AMO1 was purchased from DSMZ; and murine STG1-mice were kindly provided by Dr. Irene Ghobrial [Dana-Farber Cancer Institute (DFCI), Boston, MA]. Cell lines were tested to rule out Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza) and authenticated by short-tandem repeat DNA typing. Human multiple myeloma cell lines were cultured in RPMI/1640 media containing 10% FBS (GIBCO; Thermo Fisher Scientific), 2 μmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO; Thermo Fisher Scientific). 293T
cells were maintained in DMEM culture media with 10% FBS and 1% penicillin-streptomycin. Murine JAWSII cells were cultured in Alpha minimum essential medium with ribonucleosides, deoxyribonucleosides, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 20% FBS, 1% penicillin-streptomycin, and 5 ng/mL murine GMCSF (PeproTech). Murine STG1M cells were maintained in Iscove's modified Dulbecco's Media (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin-streptomycin.

**Patient Multiple Myeloma Cells and Normal Donor Samples**

Multiple myeloma patient BM aspirates and normal donor peripheral blood mononuclear cells (PBMC) were obtained after written informed consent in accordance with the Declaration of Helsinki and under the approval by the Institutional Review Board of the DFCI. BMMCs and PBMCs were separated by Ficoll-Paque PLUS (GE Healthcare). Multiple myeloma cells from BMMCs were enriched by CD138-positive selection using magnetic microbeads (Miltenyi Biotec).

**Drugs and Reagents**

BTZ was purchased from Selleckchem (S1013) and resuspended in DMSO. Anti-mouse IFNAR1 (Clone: MAR1-5A3; #BE0241) mAb and mouse IgG1 isotype control mAb were purchased from BioXCell and resuspended in InVivoPure Dilution Buffer following the manufacturer's instructions. ADU-S100 (#CT-ADUS100) was purchased from ChemiTek.

**Proliferation Assay**

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies).

**Apoptosis Assay**

Apoptosis was evaluated by Annexin-V/7-Aminoactinomycin D (7-AAD) staining and flow cytometric analysis using the PE Annexin-V Apoptosis Detection Kit I (BD Biosciences).

**Cell-Surface Exposure of CALR**

Multiple myeloma cells were seeded in 12-well plates (2 \times 10^{5} per well), treated with BTZ for 16 hours at indicated concentrations, and then stained with Alexa Fluor647 anti-CALR antibody (ab196159, #555657), and 7-AAD from BD Biosciences and CD11c-BV650 from BioLegend (#563404). Dead cells were excluded by 7-AAD positivity, and CD83 and CD86 expression was evaluated on CD11c+ cells.

**Generation of Mo-DCs and Phagocytosis Assay**

Mo-DCs were generated from CD14+ peripheral blood monocytes from healthy donors, positively selected using magnetic beads (Miltenyi Biotec), cultured for 6 days in Mo-DC differentiation medium containing GMCSF and IL4 (130-094-812, Miltenyi Biotec). AMO1 and NCI-H929 cells lines, as well as patient multiple myeloma cells, were stained with CellTrace CFSE-stained (Invitrogen) and cultured with or without BTZ for 16 hours, and then incubated in 48-well plates for 4 hours at 1:1 ratio with CellTrace CFSE-stained Mo-DCs. Cells were then collected, and phagocytosis analysis was performed by flow cytometry and confocal microscopy. Analogous phagocytosis experiments were performed using STG1M murine multiple myeloma and JAWSII cell lines that were used as source of immature murine DCs.

**Flow Cytometry-Based Assay**

Collected cells were analyzed using the BD LSRFortessa X-20 flow cytometer. Mo-DCs that engulfed multiple myeloma cells were CFSE+ and Far Red double positive. Fold increase in percentage of double-positive DCs after coculture with BTZ-treated versus untreated multiple myeloma cells was compared.

**Confocal Microscopy-Based Assay**

STG1M cells (15,000) were cytospun for 7 minutes at 300 rpm, fixed in 4% paraformaldehyde for 20 minutes at room temperature, and washed three times with 1% FBS in PBS. After washes, nuclear content was stained with Fluoro-gel II mounting medium with DAPI (Thermo Fisher Scientific). Slides were examined using Yokogawa Spinning Disk Confocal/TIRF System and analyzed with ImageJ software.

**DC Maturation Assay**

Immature DCs were generated as described above (“Generation of Mo-DCs and Phagocytosis Assay” section). DCs were cultured alone or with untreated or BTZ-pretreated AMO1 (5 nmol/L) for 24 hours. DCs alone were cultured (i) without maturation stimuli, (ii) with 50 ng/mL of TNFα (Millipore Sigma), or (iii) with 5 nmol/L of BTZ. After 24 hours, cells were harvested and analyzed by flow cytometry using the following Abs: anti-CD83-APC (551073), CD86-FITC (#555657), and 7-AAD from BD Biosciences and CD11c-BV650 from BioLegend (#563404). Dead cells were excluded by 7-AAD positivity, and CD83 and CD86 expression was evaluated on CD11c+ cells.

**Generation of CRISPR KO Multiple Myeloma Cells**

Single-guide RNA (sgRNA) targeting murine Calr, murine Sting/Tmem173, and human STING/TMEM173 were used to generate STG1M CalrKO, STG1M STINGKO, and AMO1 STINGKO cells. All cell lines were transfected via electroporation (Neon Transfection System, Thermo Fisher Scientific) using All-in-one vectors (pCLIP-ALL-hCMV-ZsGreen) containing an sgRNA and a Cas9 (transOMIC technologies). Forty-eight hours after electroporation, cells were ZsGreen-sorted and plated as monolocytes in 96-well plates. After expansion, monocytes were screened for either CALR or STING expression by Western blot (WB). The following sgRNA sequences were used:

- Calr Mus Musculus:
  - sgRNA#1: TATGGGGCTTTTGGAGGACCAG
  - sgRNA#2: ATAGATGGACAGCAGCAACA
  - sgRNA#3: CTACATAACAACATGCTCAG

- Sting/Tmem173 Mus Musculus:
  - sgRNA#1: TATGCTGGGAAATGGAATGTT
  - sgRNA#2: GAGGCCAAACATCCAACCTG
  - sgRNA#3: CTACATAACAACATGCTCAG

- Sting/TMEM173 Homo Sapiens:
  - sgRNA#1: LACAGCGACAGACAGGCCCCCA
  - sgRNA#2: ATAGATGGACAGCGACAGAA
  - sgRNA#3: GACGACAGGCCCCCAGG

**Stable Overexpression of CALR in STG1M CalrKO Clones**

The pRetroX-CRT-GPI-IRES-DsRed plasmid containing full-length murine CALR cDNA was kindly provided by Chen and colleagues (44). Virus was generated by transfecting HEK293T cells with 4 μg of DNA and packaging vectors (4 μg of psPAX2 and 2 μg of pMD2.G) using lipofectamine 2000 (Thermo Fisher Scientific). Supernatant containing viral particles was harvested after 48 hours and sterile 0.45 μm filtered. STG1M CalrKO clones were spinoculated for 1 hour with media containing lentiviral particles at a multiplicity of infection of 2 in the presence of 8 μg/mL polybrene. Media were then changed, and cells were DsRed-sorted using M Aria II SORP UV (BD Biosciences). After sorting, efficient overexpression was evaluated by WB.

**Immunoblotting**

Cell lysis was performed in RIPA buffer (Boston Bio Products) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). SDS-PAGE was performed on NuPage Bis-Tris gels (Thermo Fisher Scientific) using MOPS or MES running buffer.

**Drugs and Reagents**

BTZ was purchased from Selleckchem (S1013) and resuspended in DMSO. Anti-mouse IFNAR1 (Clone: MAR1-5A3; #BE0241) mAb and mouse IgG1 isotype control mAb were purchased from BioXCell and resuspended in InVivoPure Dilution Buffer following the manufacturer's instructions. ADU-S100 (#CT-ADUS100) was purchased from ChemiTek.

**Proliferation Assay**

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies).

**Apoptosis Assay**

Apoptosis was evaluated by Annexin-V/7-Aminoactinomycin D (7-AAD) staining and flow cytometric analysis using the PE Annexin-V Apoptosis Detection Kit I (BD Biosciences).

**Cell-Surface Exposure of CALR**

Multiple myeloma cells were seeded in 12-well plates (2 \times 10^{5} per well), treated with BTZ for 16 hours at indicated concentrations, and then stained with Alexa Fluor647 anti-CALR antibody (ab196159, Abcam) and 7-AAD. Analysis of fluorescence intensity on 7-AAD-negative cells was done using BD LRSFortessa X-20 flow cytometer.

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Mo-DCs were generated from CD14+ peripheral blood monocytes from healthy donors, positively selected using magnetic beads (Miltenyi Biotec), cultured for 6 days in Mo-DC differentiation medium containing GMCSF and IL4 (130-094-812, Miltenyi Biotec). AMO1 and NCI-H929 cells lines, as well as patient multiple myeloma cells, were stained with CellTrace CFSE-stained (Invitrogen) and cultured with or without BTZ for 16 hours, and then incubated in 48-well plates for 4 hours at 1:1 ratio with CellTrace CFSE-stained Mo-DCs. Cells were then collected, and phagocytosis analysis was performed by flow cytometry and confocal microscopy. Analogous phagocytosis experiments were performed using STG1M murine multiple myeloma and JAWSII cell lines that were used as source of immature murine DCs.

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buffer. Gels were dried transferred onto 0.45 μm nitrocellulose membranes using the iBlot Dry Blotting System (Thermo Fisher Scientific).

The following Abs were purchased from Cell Signaling Technology: EIF2A (#5324), p-EIF2A (#3398), ATF4 (#11815), CHOP (2895), CALR (#12338), cGAS (#15102), TBK1 (#3504), pTBK1 (#5483), pFR3 (#29047), and STING (#13647). GAPDH (#2118) and B-ACTIN (#4970) were used as loading controls.

Reverse Transcription and Quantitative Real-Time PCR

Total RNA from multiple myeloma cells was prepared with TRIzol (Thermo Fisher Scientific) and RNA Clean and Concentrator-5 kit (Zymo Research) following the product instructions. RNA integrity and quantity were assessed by NanoDrop Spectrophotometer (Thermo Fisher Scientific). For analysis of mRNA expression, oligo-dT primed cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and used as template to quantify human and murine IFNα1 (Hs00171065_m1), IFNα2 (Hs01077958_s1), MIP1α (Hs00974385_m1), and IFNβ (Hs01077938_s1). Analysis was determined by RT-qPCR using TaqManFast Universal PCR Master Mix on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Comparative RT-qPCR was performed in triplicate. Relative expression was calculated using the comparative cross threshold (Ct) method.

Analysis of T-cell Priming

DCs were generated from PBMCs of healthy donors as described above (“Generation of Mo-DCs and Phagocytosis Assay” section). T cells were negatively selected from CD14+ PBMCs from the same donors using the Pan T Cell Isolation Kit (Miltenyi Biotec) and frozen until immature DCs were generated. Either untreated or BTZ-treated multiple myeloma cells were cocultured with DCs and T cells for 5 days. T cells and DCs were similarly cultured in the absence of multiple myeloma cells and in the presence of BTZ. Before analysis, cells were treated with Dynabeads Human T-Activator CD3/CD28 (#11131D, Thermo Fisher Scientific), and with GolgiStop and GolgiPlug (#554724 and #555029, BD Biosciences). T-cell populations were analyzed using 5-color flow cytometry with the following Abs: CD3 BV605 (#317322), CD8 BV421 (#500348), CD4-PerCP/Cyanine5.5 (#317428; BioLegend), and CD45RO-PE (#100045, BioLegend). Analysis was determined by RT-PCR using primers targeting GAPDH (Mm00465125_g1), cGAS (Mm00557849_m1), and GAPDH (Mm00868123_g1). Analysis was performed in triplicate. Relative expression was calculated using the comparative cross threshold (Ct) method.

ELISA

AMO1WT and STINGKO cells were either untreated or treated with BTZ for 24 hours. Supernatant was collected, and secretion of CXCL9 chemokines was analyzed by MIG (CXCL9) Human Instant ELISA Kit (#BMS285INST, Thermo Fisher Scientific).

In Vivo Studies

Six-week-old female immunodefficient NOD.CB17-Prkdcscid/NCiGril (NOD/SCID; Charles River) and immunocompetent C57BL/ Kal.lwrRjHsd (Envigo) mice were housed in our animal facility at DFCL. All experiments were performed after approval by the Animal Ethics Committee of the DFCI and performed using institutional guidelines.

In Vivo Studies for Tumor Growth Analysis

NOD/SCID and C57BL/Kal.lwrRjHsd mice were s.c. injected with 1 × 10^6 STG1M either WT or CalrKO cells in PBS. When tumors became measurable, mice were randomized to receive either PBS or BTZ administered i.p. 0.5 mg/kg twice/week for 3 tumors. Tumor sizes were measured as previously described (46), and mice were sacrificed when tumors reached 2 cm in diameter or ulceration or major compromise in quality of life. A parallel experiment was performed to allow for tumor harvesting after two injections intraperitoneally of BTZ for RNA-seq analysis, as detailed below.

For BTZ and STING agonist combination studies, C57BL/ Kal.lwrRjHsd (n = 5/group) and SCID/NOD (n = 6/group) mice bearing STG1MWT and C57BL/Kal.lwrRjHsd mice (n = 5/group) bearing STG1M STINGKO tumors were randomized to receive PBS, BTZ

Cytotoxicity Assay

After coculture of HLA-matched DCs and T cells from healthy donors with either untreated or BTZ-treated U266 cells for 5 days, T cells were negatively selected using the Pan T cell Isolation Kit (Miltenyi Biotec) and plated in round-bottom 96-well plates with naïve U266 cells prestained with CFSE dye (Thermo Fisher Scientific) at different target:effector ratios for 24 hours in the presence of IL2 (N=10-097-74, Miltenyi Biotec). Then cells were 7-AAD stained, and detection of viable CFSE-gated cells was performed using BD LRsFortessa X-20 cytometer.

Micronuclei Assay

Cells were analyzed for micronuclei formation using a flow cytometry-based Micronucleus Assay (MicroFlow kit), according to the manufacturer’s protocol. Briefly, nonviable cells were removed by Ficoll gradient centrifugation. Viable cells were then stained with photo-activated Nucleic Acid Dye A (etidium monoazide) that crosses the compromised outer membrane of apoptotic and necrotic cells and stains them red. Cells were then washed twice and lysed with a detergent containing buffer to break open cytoplasmic membranes and release nuclei and micronuclei. Nucleic Acid Dye B (SYTOX green) was then added to label DNA from both nuclei and micronuclei. Flow cytometry analysis was used to distinguish DNA from dead (dual stained with Dyes A and B) and live (single stained with Dye B) cells. After removing dead cells from the analysis, micronuclei were quantified and plotted as a percentage of 2N nuclei based on Dye B (SYTOX green) staining intensity and exhibited 1/100th to 1/10th the fluorescent intensity of 2N nuclei.
Bortezomib Activates Anti-Myeloma Immune Response via STING

(0.375 mg/kg twice a week for 2 weeks, i.p.), ADU-S100 (100 μg) administered in the peritumoral area (days 1 and 2), or a combination. Tumor sizes were measured as described above. A parallel experiment in C57BL/KaLwRijHsd mice was performed to allow for tumor harvesting after one administration of either BTZ or ADU-S100. Tumors were fixed in 10% formalin for 24 hours and then maintained in 70% ethanol prior to paraffin embedding and processing for IHC.

In Vivo Rechallenge of Viable STGM1 Cells in BTZ-Treated Mice

C57BL/KaLwRijHsd mice bearing STGM1WT tumors were treated with BTZ as above. Two weeks after observation of tumor regression, treated (n = 5) as well as naive mice (n = 5) were rechallenged with viable 1 × 106 STGM1WT cells, and tumor growth was monitored over time. In a parallel experiment, spleens were harvested 2 weeks after tumor rechallenge from both groups to test T-cell–specific reactivity against multiple myeloma cells by ELISPOT assay (Translational Immunogenomics Laboratory, DFCI).

Vaccination Studies

5 × 106 STGM1WT or CalrKO cells were treated with BTZ (7.5 nmol/L) in vitro for 16 hours. C57BL/KaLwRijHsd mice (n = 8/group) were then either vaccinated subcutaneously with dying STGM1WT or STGM1CalrKO cells or not vaccinated. After 1 week, viable 1 × 106 WT STGM1 cells were injected s.c., and tumor growth was monitored over time.

RNA-seq Analysis from Mouse Tumors

Tumors growing from both STGM1WT or CalrKO cells in C57BL/KaLwRijHsd mice treated with either PBS or BTZ (three/group) were harvested and used to extract RNA using the RNeasy kit (QIAGEN). After quality control, RNA-seq was performed using Illumina NextSeq 500 Single-End 75 bp (SE75) and analyzed following the VIPER (Visualization Pipeline for RNA-seq) next-generation sequencing (NGS) analysis pipeline (47), comparing BTZ-treated mice versus PBS in each experimental setting. Lists of differentially expressed genes (DEG) were applied to gene set enrichment analysis (GSEA) and Cytoscape (48, 49) software to reveal biological pathways modulated by BTZ. Focused analysis was conducted on the list of DEGs included in the gene sets of the Hallmarks collection of the Molecular Signature Database conveying immune processes and enriched in STGM1WT tumors after treatment with BTZ. Analysis of expression of human orthologs of these 90 genes in patients with multiple myeloma and correlation with patient clinical outcome was then analyzed as detailed below. RNA-seq data have been submitted to Gene Expression Omnibus (GEO; accession number: GSE171837).

RNA-seq Analysis of AMO1 Multiple Myeloma Cells after Treatment with BTZ

AMO1 WT and STINGKO were cultured for 16 hours in the presence or absence of BTZ (5 mmol/L). RNA was extracted as previously described and submitted to NovaSeq RNA-seq analysis followed by VIPER NGS analysis (47). Lists of DEGs were applied to the GSEA software. RNA-seq data have been submitted to GEO (accession number: GSE171837).

Analysis of RNA-seq Data of Patients with Multiple Myeloma

We used RNA-seq from CD138 multiple myeloma cells from a previously published dataset of 327 newly diagnosed clinically annotated patients with multiple myeloma from the IFM/DFCI 2009 clinical trial (NCT01191060; ref. 50). After quality control, all RNA-seq data were quantified with Salmon. Raw counts and transcripts per million values were summed to gene levels using tximport, and DESeq2 was used for all differential gene expression analysis. Centered and scaled data were used for clustering with ward.D2 algorithm. All figures were created with heatmap or ggplot2. Survival analysis was performed using survival package in R, and the log-rank test was used to compare groups. As validation dataset, gene expression data of 152 patients with multiple myeloma performed with microarray platform were downloaded from GEO (GSE9782; ref. 26) and preprocessed and normalized with affy and limma packages in R.

Statistical Analysis

All in vitro experiments were repeated at least three times and performed in triplicate. Statistical significance of differences was determined using the Student t test (unless otherwise specified). All statistical analyses and graphs were performed using GraphPad software.

Authors’ Disclosures

A. Gulla reports grants from Leukemia & Lymphoma Society, American Society of Hematology, and NIH/NCI during the conduct of the study, as well as a patent for Modulating Gabarap to Modulate Immunogenic Cell Death pending. G. Bianchi reports grants from American Society of Hematology, International Myeloma Foundation, and Damon Runyon Cancer Research Foundation and personal fees from Pfizer, Karyopharm, and MJH outside the submitted work. P.G. Richardson reports personal fees from Oncopeptides, Celgene/Bristol Myers Squibb, and Takeda and personal fees from Janssen, Sanoﬁ, Secura Bio, GlaxoSmithKline, and Regeneron during the conduct of the study. D. Chauhan reports other support from Stemline Therapeutics, Oncopeptides, and C4 Therapeutics outside the submitted work. N.C. Munshi reports personal fees from Takeda, Bristol Myers Squibb, OncoPep, Janssen, Amgen, AbbVie, Adaptive Biotechnology, Karyopharm, and Legend Biotech during the conduct of the study, as well as a patent for OncoPep issued. K.C. Anderson reports personal fees from Amgen and Millennium/Takeda during the conduct of the study, as well as personal fees from Pfizer, AstraZeneca, Janssen, Precision Biosciences, Mana, Windmill, Startron, Raqia, C4 Therapeutics, and OncoPep outside the submitted work. No disclosures were reported by the other authors.

Acknowledgments

The authors thank Derin Keskin and the Translational Immunogenomics Lab at Dana-Farber Cancer Institute for the scientific advice and technical support to perform the ELISPOT assay for immune monitoring in vivo. E. Morelli is a recipient of the Brian D.
Novis Junior Grant (2021/2022) from the International Myeloma Foundation.

This work is supported by NIH/NCI grants SPORE-P50CA100707 (K.C. Anderson and N.C. Munsli), R01-CA050947 (K.C. Anderson), R01CA207237 (K.C. Anderson and D. Chauhan), P01CA155258 (K.C. Anderson and N.C. Munsli), and R01-CA178264 (K.C. Anderson and T. Hideshima); VA Healthcare System grant number 510BX001584 (N.C. Munsli); the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (K.C. Anderson); and a Paula and Rodger Riney Foundation grant (K.C. Anderson). A. Gulla is a Fellow of the Leukemia & Lymphoma Society and a Scholar of the American Society of Hematology.

Received March 12, 2021; revised March 15, 2021; accepted April 16, 2021; published first April 23, 2021.

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_Blood Cancer Discov_ Published OnlineFirst April 23, 2021.

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