Immunomodulatory drugs activate NK cells via both Zap-70 and cereblon-dependent pathways

Teru Hideshima¹ · Daisuke Ogiya¹ · Jiye Liu¹ · Takeshi Harada² · Keiji Kurata¹ · Jooeun Bae¹ · Walter Massefski³ · Kenneth C. Anderson¹

Received: 5 November 2019 / Revised: 17 March 2020 / Accepted: 18 March 2020 / Published online: 1 April 2020
© The Author(s), under exclusive licence to Springer Nature Limited 2020

Abstract
Immunomodulatory drugs (IMiDs) lenalidomide and pomalidomide show remarkable antitumor activity in multiple myeloma (MM) via directly inhibiting MM-cell growth in the bone marrow (BM) microenvironment and promoting immune effector cell function. They are known to bind to the ubiquitin 3 ligase CRBN complex and thereby triggering degradation of IKZF1/3. In this study, we demonstrate that IMiDs also directly bind and activate zeta-chain-associated protein kinase-70 (Zap-70) via its tyrosine residue phosphorylation in T cells. IMiDs also triggered phosphorylation of Zap-70 in natural killer (NK) cells. Importantly, increased granzyme-B (GZM-B) expression and NK-cell activity triggered by IMiDs is associated with Zap-70 activation and inhibited by Zap-70 knockdown (KD), independent of CRBN. We also demonstrate a second mechanism whereby IMiDs trigger GZM-B and NK cytotoxicity which is CRBN and IKZF3 mediated, and inhibited or enhanced by KD of CRBN or IKZF3, respectively, independent of Zap-70. Our studies therefore show that IMiDs can enhance NK and T-cell cytotoxicity in (1) ZAP-70-mediated CRBN independent, as well as (2) CRBN-mediated Zap-70 independent mechanisms; and provide the framework for developing novel therapeutics to activate Zap-70 and thereby enhance T and NK anti-MM cytotxicity.

Introduction
Among the most important treatment advances in multiple myeloma (MM) is the development of immunomodulatory drugs (IMiDs) thalidomide (Thal), lenalidomide (Len), and pomalidomide (Pom). Their multiple anti-MM effects include: induction of growth arrest and apoptosis in tumor cells; downregulation of adhesion molecules and MM-cell binding to cellular components and extracellular matrix proteins in the bone marrow (BM); anti-angiogenesis; modulation of cytokines; and immunomodulation associated with enhanced T cell, natural killer (NK) cell, and NK-T-cell activity, along with decreased regulatory T-cell activity [1–3]. Multiple groups have shown that Thal, Len, and Pom directly bind to cereblon (CRBN), forming an E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1), cullin-4A, and regulator of cullins1 [4, 5], thereby triggering proteasomal degradation of IKZF1 and IKZF3 followed by downregulation of interferon regulatory factor 4 and MM-cell growth [6, 7]. Recently, we have also shown that Pom directly binds to TP53 regulating kinase and inhibits its activity, which is associated with significant MM-cell growth inhibition via both p53-dependent and -independent pathways [8].

Studies have also begun to delineate the molecular mechanisms whereby IMiDs mediate their immune effects. For example, Len triggers CD28 tyrosine phosphorylation in T cells, followed by NF-κB activation [9]. IMiDs induce IL-2 and γ-interferon, while inhibiting suppressor of
cytokine signaling, in CD4+ T cells, CD8+ T cells, and NK-T cells from both BM and peripheral blood (PB) of MM patients [10]. This upregulation of immune activity by Pom and Len is, at least in part, mediated by their binding to CRBN and triggering degradation of IKZF1 and IKZF3 in T cells, thereby allowing for increased transcription and secretion of cytokines including IL-2 [11]. We have demonstrated that IL-2-induced PB mononuclear cells (PBMCs) treated with IMiDs showed significantly increased lysis of MM-cell lines, which was not major histocompatibility complex-class restricted [12]. We and others have also reported that IMiDs enhance both NK-cell and NK-T-cell cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), at least in part due to triggering IL-2 production from T cells [13–18]. Moreover, a recent study has shown that Len can enhance secretion of IFN-γ and GZM-B from antigen-specific T cells [19]. To date, however, the molecular mechanisms whereby IMiDs induce NK-cell cytotoxicity have not been elucidated.

In this study, we characterized the role of zeta-chain-associated protein kinase-70 (Zap-70), a 70 kDa cytoplasmic protein tyrosine kinase composed of two SH2 domains and a carboxy-terminal kinase domain initiating T-cell responses by the antigen receptor [20], in mediating the increased NK-cell cytotoxicity triggered by IMiDs. We show that IMiDs directly bind and activate Zap-70. Importantly, increased GZM-B expression and NK-cell activity triggered by IMiDs is associated with Zap-70 activity and inhibited by Zap-70 KD, independent of CRBN. A second mechanism whereby IMiDs trigger GZM-B and NK cytotoxicity is CRBN and IKZF3 mediated, and can be inhibited or enhanced by KD of CRBN or IKZF3, respectively, independent of Zap-70. Our studies therefore show that IMiDs can enhance NK and T-cell cytotoxicity in (1) Zap-70-mediated CRBN independent, as well as (2) CRBN-mediated Zap-70 independent mechanisms. They further validate the potential of developing novel therapeutics to activate Zap-70 and thereby enhance T and NK MM cytotoxicity.

Materials and methods

Cells

U266 myeloma cell line and Jurkat T-cell leukemia cell line were obtained from American Type Culture Collection (ATCC, Manassas, MD) and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. KHYG-1 NK-cell leukemia line was purchased from German Collection of Microorganism and Cell Cultures GmbH (DSMZ, Germany), and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10 ng/ml IL-2. NK-92 NK-cell line was obtained from ATCC and cultured in αMEM supplemented with 12.5% fetal bovine serum, 12.5% horse serum, 2 mM L-glutamine, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, and 200 U IL-2. Cell lines have been tested and authenticated by STR DNA fingerprinting analysis (Molecular Diagnostic Laboratory, DFCI). They were also regularly tested for mycoplasma contamination using MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland) and were used within 3 months after thawing.

PBMCs were obtained from healthy volunteers using Ficoll-Paque PLUS density gradient media (GE Healthcare, Uppsala, Sweden). T cells and NK cells were further purified from PBMCs by negative selection procedure using RosetteSep Separation System® (StemCell Technologies, Vancouver, Canada). Purity (>90%) of T and NK cells was confirmed by flow cytometric analysis. All experiments with healthy volunteer’s samples were performed under auspices of an DFCI Institutional Review Board approved protocol after informed consent was obtained.

Reagents and antibodies

Len and Pom were purchased from Sigma-Aldrich (St. Louis, MO) and Selleck Chemicals (Houston, TX), respectively. CC-220 (Iberdomide) was purchased from Cayman Chemicals (Ann Arbor, MI). Dexamethasone (Dex) was purchased form Sigma-Aldrich (St Louis, MO). Anti-actin and -perforin Abs were purchased from Santa Cruz Biototechnology (Santa Cruz, CA); and anti-IKZF1 Ab was obtained from R&D Systems (Minneapolis, MN). All other Abs were purchased from Cell Signaling Technologies (Danvers, MA). The information of Abs is also listed in Supplementary Table S1.

Growth inhibition assay

Cell growth was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT, Chemicon International, Temecula, CA) dye absorbance. Cells were pulsed with 10 µl of 5 mg/ml MTT to each well for the last 4 h of 48 h and/or 72 h cultures, followed by 100 µl isopropanol containing 0.04N HCl. Absorbance was measured at 570/630 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale CA).
Pom-based reagent and immunoblotting

Affi-Gel 10 (Bio-Rad, Hercules, CA)-linked Pom was generated as described in our previous study [8] and provided by TAIHO PHARMACEUTICAL CO., LTD (Tsukuba, Ibaraki, Japan). After pre-cleaning, the Jurkat cell lysates with a control reagent (ethanolamine-affigel) to reduce nonspecific binding, the lysates were incubated with either the ethanolamine-affigel or Pom-based affinity reagent (80 µL of each, 50% slurry) for 1 h at 4°C to identify candidate binding proteins. In competition assays, tenfold excess concentration (1 mM) Pom was added to lysate from Pom-based affinity reagents. Affinity reagents were then washed three times with NP-40 lysis buffer, and binding proteins were then eluted with 2× SDS sample buffer at 50°C. The eluted samples were subjected to immunoblotting.

Zap-70 kinase assay

Zap-70 kinase activity was measured using ADP-Glo kinase assay system (Promega, Cat # V8311), according to manufacturer’s protocol.

NK cell assay

U266 target cells were stained with 10 µM of calcein AM (Thermo Fisher Scientific) for 30 min at 37°C, and then washed three times with RPMI media. The cells were then seeded into a 96-well plate at 15,000 cells/well. Effector NK cells were added at various E:T ratios and incubated for 4 h. After centrifugation, supernatants were collected for measurement of fluorescence. The spontaneous release and the maximum release were obtained from target cells without

Fig. 1 IMiDs induce phosphorylation of Zap-70 in PBMCs and Jurkat cells. a PBMCs were cultured with Pom (0.01–1 µM) for 16 h. Upper panel shows immunoblotting for Zap-70, p-Zap-70, and p-LAT. Lower panel shows densitometric analysis of Zap-70. b PBMCs were cultured with Pom (0.1 and 1 µM) for the indicated time periods. c PBMCs were cultured with Len (1 µM) for 16 h. d Primary T cells from healthy volunteer were cultured with Pom (0.25–1 µM) for 16 h. e Jurkat cells were cultured with Pom (0.5 and 1 µM) for 16 h. Whole cell lysates were subjected to immunoblotting (a)–(e) using indicated Abs. f Jurkat cells were cultured with Pom (0.01–1 µM) for 72 h. Cell growth was assessed by MTT assay. Data are representative of two independent experiments and values are expressed in mean ± SD.
effector cells and from target cells with 1% Triton X-100, respectively. Specific target cell lysis was calculated as \[
\frac{[\text{test release} - \text{spontaneous release}]}{[\text{maximum release} - \text{spontaneous release}]} \times 100.
\]

**siRNA transfection**

For siRNA transfection, nontargeted, Zap-70, CRBN, IKZF1, and IKZF3 ON-Targetplus siRNA SMARTpools...
were purchased from Thermo Scientific (Lafayette, CO). siRNA transfection was carried out by Amxna electroporation system using the “Cell Line Nucleofector® Kit T solution” (Lonza, Koln, Germany). Sequences of small interference RNAs are listed in Supplementary Table S2.

Immunoblotting and densitometric analysis

Cells were harvested and lysed using RIPA lysis buffer (Cell Signaling Technology) containing 1 mM PMSF. Whole cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), and immunoblotted with indicated Abs. Densitometric analysis of immunoblotting was carried out using ImageJ software version 1.48 (National Institute of Health).

RNA extraction and reverse transcription polymerase chain reaction

RNA was extracted using Trizol (Invitrogen) and quantified by a Nanodrop spectrophotometer (Labtech). Specifically, 5 × 10^6 cells were pelleted, washed with cold PBS, and resuspended in 1 mL Trizol. Cells were then incubated with 1-bromo-3-chloropropane (Sigma), washed first with isopropanol alcohol and then with 75% ethanol, and then resuspended in nuclease-free water (Invitrogen). After quantification, 2000 ng of RNA was used to synthesize cDNA using the SuperScript II First-Strand Synthesis Kit (Invitrogen), according to the manufacturer’s instructions. To evaluate the expression levels of GZM-B and GAPDH (internal control), quantitative real-time PCR (QRT-PCR) was performed using SYBR GREEN PCR Master Mix (Applied Biosystem), after optimization of the primer conditions as in our prior studies [8]. Primers used for quantitative RT-PCR are listed in the Supplementary Table S3.

Results

Pom induces phosphorylation of Zap-70 in T cells

To characterize the effect of IMiDs on Zap-70 function in immune effector cells, we first evaluated whether Pom triggered phosphorylation of Zap-70 and its known downstream target linker of activated T cells (LAT) in PBMCs from healthy volunteers. As shown in Fig. 1a (upper panel), Pom-induced phosphorylation of both Zap-70 and LAT in PBMCs in a dose-dependent manner. ImageJ densitometric analysis confirmed 42% increased p-Zap-70 after Pom (1 μM) treatment (Fig. 1a, lower panel). The increased p-Zap-70, p-LAT, as well as downstream p-ERK in PBMCs triggered by Pom is also time-dependent (Fig. 1b). Of note, Len similarly triggered p-Zap-70 and p-LAT in PBMCs (Fig. 1c). Since Zap-70 is a mediator of T-cell receptor (TCR) signaling, we next examined whether IMiDs triggered p-Zap-70 in T cells from healthy volunteers. As in PBMCs, Pom treatment induced p-Zap-70 in primary normal donor T cells (Fig. 1d). Pom similarly induced p-Zap-70 in Jurkat cells in a dose-dependent fashion (Fig. 1f), without altering their proliferation (Fig. 1f).
**IMiDs directly bind and activate Zap-70**

Since the Ab used for evaluation of p-Zap-70 (Cell Signaling Technology, catalogue # 2704) also recognizes p-Syk (spleen tyrosine kinase), we next validated that the increased phosphorylation observed by immunoblotting after Pom treatment was p-Zap-70. Specifically, we knocked down Zap-70 in Jurkat cells, and then immunoblotted cell lysates with p-Zap-70 and Zap-70 Abs; control cells were transfected with scrambled (Sc) siRNA and
Fig. 4 Zap-70 mediates Pom-induced upregulation of NK-cell activity. KHYG-1 cells were cultured with Pom (0.25–1 μM) for 24 h. a Whole cell lysates were subjected to immunoblotting using indicated Abs. b KHYG-1 cells were incubated with calcein AM-labeled U266 cells for 4 h at the indicated effector/target (E/T) ratios. Percent specific lysis was calculated as described previously. c KHYG-1 cells were transfected with Scsi or Zap-70si, and then cultured with Pom (0.25 μM) for 72 h in the absence of IL-2. Viable cell number was determined, and cells were then incubated with calcein AM-labeled U266 target cells for 4 h at indicated effector/target (E/T) ratios. Percent specific lysis was calculated as described previously. d After transfection with Scsi or Zap-70si, cells were cultured with Len or Pom for 72 h. Cell growth was assessed by MTT assay. e KHYG-1 cells were transfected with scrambled (Scsi) or CRBNsi. The transfectants were then cultured with Pom (0.5 μM) for 24 h, and whole cell lysates were subjected to immunoblotting using indicated Abs. The arrow indicates CRBN expression. f Primary NK cells (#1–#4) were isolated from healthy volunteer donor PBMCs, as described in “Materials and methods”. NK cells were cultured with Pom (0.5 μM) for 24 h, and whole cell lysates were subjected to immunoblotting using indicated Abs. g Isolated primary NK cells (#1 and #2) were cultured with Pom (left panel): 0.25 and 0.5 μM, right panel: 0.5 and 1 μM) for 24 h, and were then incubated with calcein AM-labeled U266 for 4 h at E/T ratio of 5/1 (left panel) and 10/1 (right panel). Percent specific lysis was calculated as previously described. For b–d, data are representative at least two independent experiments and value is expressed in mean ± SD.

Similarly immunoblotted. The control blot showed two bands (upper p-Syk and more prominent lower p-Zap-70), and the lower band was significantly downregulated in Zap-70 knock down cells (Fig. 2a). We also performed ELISA assay to specifically detect p-Zap-70 (Tyr319) in Jurkat cells. As expected, this assay also showed that both Len and Pom (Len < Pom) increased p-Zap-70 in a dose-dependent fashion (Fig. 2b).

Our previous studies have shown that Pom binds not only to CRBN, but also to TP53RK, thereby inhibiting its function [8]. By immunoblotting using Jurkat cell lysates, we here demonstrated that Pom-immobilized beads (Pom-beads) pulled down Zap-70, which was inhibited by free Pom. CRBN served as a positive control of pulled down Zap-70 (Fig. 2c). As in our prior studies [8], we next carried out nuclear magnetic resonance (NMR) spectroscopy to confirm that Pom directly binds to Zap-70 (Fig. 2d, e). Len similarly binds to Zap-70 (Supplementary Fig. S1a, b). In vitro Zap-70 kinase assay confirmed that IMiDs induce activation of Zap-70 function via phosphorylation (Fig. 2f), consistent with upregulation of downstream p-LAT observed by immunoblotting (Fig. 1a–d). Taken together, these data show that IMiDs directly bind to Zap-70 and stimulate its activity.

Pom-induced p-Zap-70 independent of CRBN

Since IMiDs are known to bind CRBN and trigger proteasomal degradation of IKZF1 and IKZF3, we next examined whether CRBN affects expression of Zap-70 or p-Zap-70 in Jurkat cells. We observed no significant change in constitutive Zap-70 (Fig. 3a) and p-Zap-70, or in p-Zap-70 induced by Pom (Fig. 3b), in CRBN KD versus control Sc KD Jurkat cells. To evaluate the biologic role of Zap-70, we next knocked down Zap-70 in Jurkat cells (Fig. 3c, left panel), and observed significant inhibition of their cell growth. (Fig. 3c, right panel). These results suggest that Zap-70 is a growth factor and independent of CRBN in Jurkat cells.

Zap-70 mediates Pom-induced upregulation of NK-cell activity

Zap-70 is a crucial mediator of TCR signaling [20]; however, its role in NK cells has not yet been delineated. We therefore next validated the biologic impact of Zap-70 in NK cells using KHYG-1 NK-cell line. As in PBMCs, Jurkat, or primary T cells, Pom similarly enhanced p-Zap-70 in KHYG-1 cells (Fig. 4a), associated with increased cytotoxicity against U266 cells in a dose-dependent fashion (Fig. 4b). Importantly, Zap-70 KD significantly reduced cytotoxic activity of both Pom-treated (Fig. 4c) and Len-treated KHYG-1 cells (Supplementary Fig. S2a), without significantly impacting growth (Fig. 4d and Supplementary Fig. S2b). Finally, as in Jurkat cells (Fig. 3a, b), CRBN KD in KHYG-1 cells did not alter constitutive Zap-70 protein and p-Zap-70, or Pom-induced p-Zap-70, expression (Fig. 4e). Consistent with KHYG-1 cells, Pom induced increased p-Zap-70 and upregulated NK activity. Of note, neither Pom nor Zap-70 KD altered growth in NK-92 cells (Supplementary Fig. S3a–d).

We similarly examined the effect of Pom on p-Zap-70 and NK-cell activity in primary NK cells isolated from healthy volunteer donors (#1–#4). Importantly and as in KHYG-1 NK-cell line, Pom upregulated p-Zap-70 in primary NK cells (Fig. 4f, #1–#4) and in a dose-dependent fashion (0–1 μM) significantly enhanced their NK cytolytic activity (Fig. 4g, #1, #2), without significantly affecting NK-cell growth. (Supplementary Fig. S4, #1, #2). These results indicate that Zap-70 mediates, at least in part, constitutive and IMiDs-induced upregulation of NK-cell activity.

Pom mediates GZM-B expression via Zap-70

We next examined the molecular mechanism whereby IMiDs enhance NK-cell activity. A previous study has demonstrated that Len upregulates GZM-B expression in MM patient T cells [19]. Here we showed that Pom (0–1 μM) in a dose-dependent fashion upregulates GZM-B expression in both KHYG-1 cells (Fig. 5a) and primary NK cells (Fig. 5b, #1, #2). Similar results were observed in NK-92 cells treated with Pom (Supplementary Fig S3a). Since Zap-70 KD inhibited Pom-induced upregulation of
KHYG-1 cell killing activity (Fig. 4c), we next examined whether Zap-70 KD also altered GZM-B expression. As expected, Zap-70 KD decreased both baseline and Pom-induced GZM-B upregulation in KHYG-1 cells (Fig. 5c). Consistent with KHYG-1 cells, we also observed downregulation of GZM-B in NK-92 cells after Zap-70 KD (Supplementary Fig. S5). Taken together, these results suggest that Zap-70 plays an important role regulating GZM-B expression in NK cells.

Pom upregulates GZM-B expression via CRBN

We next examined whether CRBN also mediates Pom-induced GZM-B upregulation in KHYG-1 cells. Although CRBN KD minimally downregulated constitutive GZM-B expression, it significantly inhibited upregulation of GZM-B triggered by Pom (Fig. 6a). Real-time qPCR confirmed that CRBN transcriptionally regulates GZM-B expression (Supplementary Fig. S6). Consistent with downregulation of GZM-B, both constitutive and Pom-induced cell killing activity was significantly inhibited in CRBN KD KHYG-1 cells (Fig. 6b). Taken together, these results indicate that Pom-induced enhanced GZM-B and NK-cell activity is also mediated, at least in part, by CRBN.

Pom upregulates granzyme-B expression via IKZF3 degradation

Since IKZF1 and/or IKZF3 are downstream degradation targets of CRBN, we next defined their roles in modulating constitutive and Pom-induced GZM-B expression. IKZF3 KD, but not IKZF1 KD, enhanced both baseline and Pom-induced GZM-B expression (Fig. 7a). These results indicate that IKZF3 serves a transcriptional repressor of GZM-B; and conversely, that Pom activation of CRBN E3 ligase and proteasomal degradation of IKZF3 leads to GZM-B upregulation in KHYG-1 cells. We further confirmed that IKZF3 KD significantly upregulated NK-cell activity, which is further enhanced in the presence of Pom (Supplementary Fig. S7). Consistent with this view, proteasome inhibitor bortezomib downregulated Pom-induced GZM-B expression in a dose-dependent fashion, associated with upregulation of IKZF3 (Fig. 7b). CC-220 (iberdomide) is a more potent IMiD with enhanced binding affinity to CRBN relative to Len or Pom, and is now under evaluation in phase 1–2 clinical trials in MM. We showed that CC-220 induced p-Zap-70 in a dose-dependent fashion (Supplementary Fig. S8a), which was associated with enhanced NK-cell activity (Supplementary Fig. S8b). We next compared potency of Len, Pom, and CC-220 in triggering GZM-B in KHYG-1 cells. Interestingly, CC-220 more potently upregulated GZM-B than Len or Pom, which was associated with downregulation of IKZF3 (Fig. 7c). Real-time qPCR of GZM-B further supported this result (Fig. 7d). Of note, none of these IMiDs altered perforin expression, indicating that IMiDs-induced upregulation of NK-cell activity is predominantly mediated by GZM-B (Fig. 7c). Taken together, our results show that IMiDs-induced GZM-B upregulation is differentially mediated in NK cells via Zap-70 and via CRBN/IKZF3 pathways.

Discussion

IMiDs trigger CRBN E3 ligase activity and proteasomal degradation of IKZF1 (Ikaros) and IKZF3 (Aiolos) [6, 7]
followed by downregulation of c-Myc and IRF4 as well as inhibition of MM-cell growth. We and others have also shown that IMiDs activate T cells [9] and NK cells [12, 13]. Since IKZF1/3 are repressors of IL-2 transcription in T cells, IMiDs activation of T cells by IMiDs is, at least in part, due to CRBN-mediated proteasomal degradation of IKZF1/3, thereby upregulating IL-2 transcription and secretion by T cells [11, 21]. More recent studies have shown that Len enhances the antigen-specific secretion of IFN-γ and GZM-B by MM patient T cells [19]. Multiple studies have also shown that IMiDs enhance ADCC mediated by anti-CD38 [22] and anti-CD 20 [23, 24] monoclonal Abs. To date, however, the molecular mechanism of IMiDs-induced NK cell activation has not been fully elucidated.

Zap-70 is a Syk family protein tyrosine kinase expressed in T cells, which plays a critical role mediating T cell activation in response to TCR engagement. Following TCR engagement, Zap-70 is rapidly phosphorylated on several tyrosine residues through autophosphorylation and transphosphorylation by the Src family lymphocyte-specific protein tyrosine kinase (Lck) [20]. In turn, tyrosine phosphorylation of Zap-70 is associated with increased kinase activity and downstream signaling events in T cells. Importantly, Zap-70 expression has also been correlated with disease progression and identified as a prognostic factor in chronic lymphocytic leukemia [25]. However, the biologic role of Zap-70 in IMiDs-induced T-cell and/or NK-cell activation is not yet known. In this study, we show that Zap-70 is phosphorylated by IMiDs treatment in both T cells and NK cells. We next demonstrated that IMiDs directly phosphorylate and activate Zap-70, as validated by in vitro noncell-based Zap-70 kinase assays, Zap-70 pull-down experiments using Pom-immobilized beads, and NMR spectroscopy. Our Zap-70 kinase assay showed that IMiDs directly activate Zap-70 without any other co-factors except ATP; and both Pom-beads pull-down and NMR experiments also demonstrate direct binding of IMiDs to Zap-70. Taken together, these results indicate that IMiDs bind to CRBN to facilitate proteasomal degradation of target substrate proteins including IKZF1/3 [6, 7] and CK1α [26]. It has been also reported that Zap-70 is a proteasome substrate [27, 28]. We therefore next asked whether CRBN could modulate constitutive or IMiDs-induced Zap-70 expression, and showed that neither phosphorylation nor protein expression of Zap-70 were altered by CRBN KD. Taken together, these results indicate that regulation of Zap-70 expression and its function are independent of CRBN E3 ligase activity.

The functional significance of Zap-70 activity in NK cells has not yet been reported. We therefore next studied the biologic impact of Zap-70 in mediating IMiDs-induced enhanced NK-cell activity. Zap-70 downregulation did not significantly alter constitutive or IMiDs-induced proliferation, suggesting that it is not a growth or survival factor. Since Len enhances GZM-B release from antigen-specific T cells [19], we next asked whether Len and/or Pom could enhance GZM-B expression in NK cells. Importantly, we confirmed that both Len and Pom (Pom > Len) upregulated GZM-B expression in a dose- and time-dependent fashion, associated with enhanced NK-cell activity. Conversely, Zap-70 KD significantly inhibits IMiDs-induced GZM-B upregulation, resulting in reduced NK-cell activity. These results suggest that Zap-70 plays a role in maintaining constitutive and IMiDs-induced killing activity in NK cells. Our ongoing studies are delineating Zap-70 downstream signaling modulating GZM-B expression.

Importantly, we here also observed that CRBN KD inhibits both constitutive and IMiDs-induced NK-cell
activity. To delineate the molecular mechanism whereby CRBN modulates NK-cell activity, we examined whether CRBN KD, in the presence or absence of Pom, alters GZM-B expression in NK cells. As in Zap-70 KD, GZM-B expression was also downregulated in CRBN KD cells, both before and after Pom treatment. These results suggest that CRBN negatively regulates GZM-B expression. Previous studies show that IKZF1/3 are transcriptional repressors of IL-2; and that IMiDs-induced CRBN-mediated proteasomal degradation of IKZF1/3 relieves this repression, thereby enhancing IL-2 secretion from T cells [11, 21]. These data suggest that IKZF1/3 may be also a repressor of GZM-B gene expression in NK cells; and conversely, that CRBN KD may abrogate proteasomal degradation of IKZF1/3 triggered by IMiDs. We therefore individually knocked down CRBN, IKZF1 or IKZF3, and then assessed the impact on GZM-B expression in the presence or absence of IMiDs. Although IKZF3 protein expression was not completely downregulated in our KD cells, both constitutive and Pom-induced GZM-B were upregulated by IKZF3 KD. A previous study has demonstrated that IKZF1 binds to the promoter of GZM-B [29], but our results do not show significant GZM-B changes in IKZF1 KD cells. Rather they suggest that IKZF3, but not IKZF1, regulates GZM-M expression in NK cells.

Recently, more potent CRBN modulating agents including CC-220 (iberdomide) are being evaluated in MM clinical trials, with early evidence of activity even in the setting of Len and Pom resistance. These agents bind CRBN with a higher affinity and more potently trigger degradation of IKZF1/3 than Pom [30, 31]. We therefore next examined the ability of CC-220 to trigger degradation of IKZF1/3 and upregulation of GZM-B. Importantly, CC-220 more potently degrades IKZF1/3 and upregulates GZM-B than either Len or Pom. In contrast, expression of perforin was not altered by Len, Pom, or CC-220, suggesting that IMiDs-induced enhanced cytotoxic activity is associated with upregulation of GZM-B, but not perforin, in NK cells.

Since IMiDs are often combined with Dex in MM therapies, we also examined the impact of Dex on Pom-induced NK-cell activity in KHYG-1 cells and observed that Dex significantly downregulated NK-cell activity, even in the presence of Pom (Supplementary Fig. S9).

Fig. 7 IKZF3 plays a critical role in Pom-induced GZM-B expression. a KHYG-1 cells were transfected with CRBN, IKZF1, or IKZF3 siRNA. The transfectants were then cultured for 24 h with Pom (0.5 μM). The arrow indicates CRBN. b KHYG-1 cells were cultured with Pom for 24 h (0.5 μM), in the presence or absence of bortezomib (BTZ; 2.5 and 5 nM). c, d KHYG-1 cells were cultured for 24 h with Len, Pom or CC-220 (0.01–1 μM). Whole cell lysates and RNAs were subjected to immunoblotting using indicated Abs (c) and real-time qPCR (d), respectively. Data are representative three independent experiments and value are expressed in mean ± SD.
Immunomodulatory drugs activate NK cells via both Zap-70 and cereblon-dependent pathways

this should be evaluated in the clinical setting, our results suggest that Dex may have a negative impact on cytotoxic effector cells.

In conclusion, our results show that IMiDs: directly bind and activate Zap-70 associated with increased GZM-B expression and NK-cell activity, which can be inhibited by Zap-70 KD independent of CRBN; and trigger GZM-B and NK cytotoxicity via CRBN-mediated IKZF3 degradation, which can be inhibited by KD of CRBN or IKZF3 independent of Zap-70. IMiDs can therefore enhance NK and T-cell cytotoxicity in (1) Zap-70-mediated CRBN independent, as well as (2) CRBN-mediated Zap-70 independent mechanisms. These studies provide the basis for developing novel therapeutics to activate Zap-70 and thereby enhance T and NK-cell anti-MM cytotoxicity.

Acknowledgements We thank Taiho Pharmaceuticals for providing Pom-immobilized beads. This study was supported by the National Institute of Health Grant; SPORE-P50CA100707 (KCA), R01-CA050947 (KCA) and R01-CA178264 (TH and KCA); and the Sheldon and Miriam Medical Research Foundation (KCA). KCA is an American Cancer Society Clinical Research Professor.

Author contributions TH coordinated activities from all authors and performed Western blot, Zap-70 kinase assay, siRNA (Zap-70, CRBN, IKZF1, IKZF3) transfection, NK assay, as well as cell toxicity assay (MTT and cell count), and wrote the paper. DO performed real-time qPCR (GZM-B), NK assay, and purified T cells and NK cells from healthy volunteers. JL performed siRNA (Zap-70, CRBN, IKZF1, and IKZF3) transfection, TH performed Zap-70 pull-down by Pom-beads. KK performed real-time qPCR (GZM-B). JB analyzed the data. WM performed NMR and wrote the paper. KCA managed the project and wrote the paper.

Compliance with ethical standards

Conflict of interest KCA serves on advisory boards to Celgene, Millennium, Janssen, Sanofi, Bristol Myers Squibb, Gilead, Precision Biosciences, and Tolero, and is a Scientific Founder of OncoPep and C4 Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper apart from those disclosed.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Hideshima T, Chauhan D, Shima Y, Raje N, Davies FE, Tai Y-T, et al. Thalidomide and its analogues overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood. 2000;96:2943–50.

2. Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T, et al. Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood. 2002;99:4525–30.

3. Anderson KC. The rapid evolution of novel therapies in multiple myeloma. J Natl Compr Cancer Netw. 2016;14:493–96.

4. Ito T, Ando H, Suzuki T, Ogura T, Hotta K, Imamura Y, et al. Identification of a primary target of thalidomide teratogenicity. Science. 2010;327:1345–50.

5. Lopez-Girona A, Mendi D, Ito T, Miller K, Gandhi AK, Kang J, et al. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. Leukemia. 2012;26:2326–35.

6. Kronke J, Udeshi ND, Narla A, Grauman P, Hurst SN, McConkey M, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. Science. 2014;343:301–5.

7. Lu G, Middleton RE, Sun H, Naniong M, Ott CJ, Mitsiades CS, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. Science. 2014;343:305–9.

8. Hideshima T, Cottini F, Nozawa Y, Seo HS, Ohguchi H, Samur MK, et al. p53-related protein kinase confers poor prognosis and represents a novel therapeutic target in multiple myeloma. Blood. 2017;129:1308–19.

9. LeBlanc R, Hideshima T, Catley LP, Shringarpure R, Burger R, Mitsiades N, et al. Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. Blood. 2004;103:1787–90.

10. Gorgun G, Calabrese E, Soyden E, Hideshima T, Perrone G, Bandi M, et al. Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma. Blood. 2010;116:3227–37.

11. Gandhi AK, Kang J, Havens CG, Conklin T, Ning Y, Wu L, et al. Immunomodulatory agents lenalidomide and pomalidomide costimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRi4 (CRBN). Br J Haematol. 2014;164:811–21.

12. Davies FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood. 2001;98:210–16.

13. Hayashi T, Hideshima T, Akiyama M, Polder K, Yasui H, Raje N, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. Br J Haematol. 2005;128:192–203.

14. Chang DH, Liu N, Klimek V, Hassoun H, Mazumder A, Nimer SD, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. Blood. 2006;108:618–21.

15. Reddy N, Hernandez-ilizaliturri FJ, Deeb G, Roth M, Vaughan M, Knight J, et al. Immunomodulatory drugs stimulate natural killer cell function, alter cytokine production by dendritic cells, and inhibit angiogenesis enhancing the anti-tumour activity of rituximab in vivo. Br J Haematol. 2008;140:36–45.

16. Wu L, Adams M, Carter T, Chen R, Muller G, Stirling D, et al. Lenalidomide enhances natural killer cell and monocyte-mediated antibody-dependent cellular cytotoxicity of rituximab-treated CD20+ tumor cells. Clin Cancer Res. 2008;14:4650–57.

17. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. Blood. 2013;121:423–30.

18. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, et al. Restoring natural killer cell immunity against multiple myeloma in the era of new drugs. Front Immunol. 2017;8:1444.

19. Neuber B, Dai J, Warih AWA, Awwad MHS, Engelhardt M, Schmitt M, et al. Lenalidomide overcomes the immunosuppression of regulatory CD8(–)/CD28(–) T-cells. Oncotarget. 2017;8:98200–14.

20. Wang H, Kadlecck TA, Au-Yeung BB, Goodfellow HE, Hsu LY, Freedman TS, et al. ZAP-70: an essential kinase in T-cell signaling. Cold Spring Harb Perspect Biol. 2010;2:a002279.
21. Hagner PR, Chiu H, Ortiz M, Apollonio B, Wang M, Couto S, et al. Activity of lenalidomide in mantle cell lymphoma can be explained by NK cell-mediated cytotoxicity. Br J Haematol. 2017;179:399–409.

22. van de Donk N. Immunomodulatory effects of CD38-targeting antibodies. Immunol Lett. 2018;199:16–22.

23. Leonard JP, Jung SH, Johnson J, Pitcher BN, Bartlett NL, Blum KA, et al. Randomized trial of lenalidomide alone versus lenalidomide plus rituximab in patients with recurrent follicular lymphoma: CALGB 50401 (Alliance). J Clin Oncol. 2015;33:3635–40.

24. Leonard JP, Trneny M, Izutsu K, Fowler NH, Hong X, Zhu J, et al. AUGMENT: a phase III study of lenalidomide plus rituximab versus placebo plus rituximab in relapsed or refractory indolent lymphoma. J Clin Oncol. 2019;37:1188–99.

25. Liu Y, Wang Y, Yang J, Bi Y, Wang H. ZAP-70 in chronic lymphocytic leukemia: a meta-analysis. Clin Chim Acta. 2018;483:82–8.

26. Kronke J, Fink EC, Hollenbach PW, MacBeth KJ, Hurst SN, Udeshi ND, et al. Lenalidomide induces ubiquitination and degradation of CK1alpha in del(5q) MDS. Nature. 2015;523:183–8.

27. Paolini R, Molfetta R, Piccoli M, Frati L, Santoni A. Ubiquitination and degradation of Syk and ZAP-70 protein tyrosine kinases in human NK cells upon CD16 engagement. Proc Natl Acad Sci USA. 2001;98:9611–6.

28. Yang M, Chen T, Li X, Yu Z, Tang S, Wang C, et al. K33-linked polyubiquitination of Zap70 by Nrdp1 controls CD8(+ ) T cell activation. Nat Immunol. 2015;16:1253–62.

29. Wargnier A, Legros-Maida S, Bosselut R, Bourge JF, Lafaurie C, Ghysdael CJ, et al. Identification of human granzyme B promoter regulatory elements interacting with activated T-cell-specific proteins: implication of Ikaros and CBF binding sites in promoter activation. Proc Natl Acad Sci USA. 1995;92:6930–4.

30. Ito T, Handa H. Recent topics in IMiDs and cereblon. Rinsho Ketsueki. 2017;58:2067–73.

31. Matyskiela ME, Zhang W, Man HW, Muller G, Khambatta G, Baculi F, et al. A cereblon modulator (CC-220) with improved degradation of Ikaros and Aiolos. J Med Chem. 2018;61:535–42.