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IAP antagonists induce anti-tumor immunity in multiple myeloma

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Abstract

The cellular inhibitor of apoptosis cIAP1 and –2 are amplified in about 3% of cancers, and were identified in multiple malignancies as potential therapeutic targets due to their role in evasion of apoptosis. Consequently, small molecule IAP antagonists, like LCL161, have entered clinical trials for their ability to induce TNF-mediated apoptosis of cancer cells. However, cIAP1 and –2 are recurrently homozygously deleted in multiple myeloma resulting in constitutive activation of the non-canonical NFκB pathway. It was therefore counterintuitive to observe a robust *in vivo* anti-myeloma activity of LCL161 in a transgenic myeloma mouse model and patients with relapsed-refractory myeloma, where addition of cyclophosphamide resulted in a median progression free survival of 10 months. This effect is not due to direct induction of tumor cell death, but rather to upregulation of a tumor cell autonomous type I interferon signaling and a strong inflammatory response with activation of macrophages and dendritic cells resulting in phagocytosis of tumor cells. Treatment with LCL161 established long-term anti-tumor protection and cure in a fraction of transgenic Vk*MYC mice. Remarkably, combination of LCL161 with the immune-checkpoint inhibitor anti-PD1 was curative in all treated mice.

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Data availability

The FASTQ files of the RNA-Seq data were submitted to Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/sra>) under accession number SRP075564.

Author contributions

MC conceived the experiments. MC, NNM, VMG, MES, DLR and NK executed the experiments. ACD and HEK provided statistical analysis of preclinical and clinical studies. YWA and PLB performed bio-informatics analysis. AC contributed to the design of the study. GJA processed clinical samples. KMM coordinated patient accrual. RF supervised clinical sample processing and data acquisition. MQL, DD, SKK, SA, AD, FB, MAG, CBR, YL, AACK, AKS and PLB enrolled myeloma patients in the clinical trial. DF and IA designed and performed intra vital microscopy studies. PLB designed and conducted the clinical trial. MC and PLB wrote the manuscript.

Competing financial interests

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Introduction

IAP antagonists (IAPa) are a class of compounds developed to induce cancer cell death by blocking the-caspase inhibitory function of the inhibitor of apoptosis (IAP) protein XIAP, but found to bind with 10 fold higher affinity to the cellular IAPs cIAP1 and cIAP2 (cIAP1/2)¹. cIAP1/2 do not bind directly to caspases, but promote cell survival through E3-mediated ubiquitination of target proteins, leading to activation of the NFκB pathway¹. The IAPa LCL161 is a small molecule that binds with high affinity to the BIR3 domains of cIAP1, triggering its autoubiquitination and proteolysis, resulting in a pulse of NFκB signaling and rise in TNF production. In the absence of cIAP1, TNF signaling triggers the induction of a RIPK1- FADD-caspase 8 apoptotic complex, or, in the absence of caspase 8, necroptotic cell death¹⁻⁴. Preclinical data indicate that LCL161 has only modest activity in the absence of TNF, but synergizes with other chemotherapeutics in various tumor models most likely by lowering the threshold for TNF-dependent apoptotic cell death⁵⁻¹⁵. LCL161 has been evaluated in a phase 1 clinical trial (ClinicalTrials.gov NCT01098838) in patients with advanced solid tumors of lung, skin, colon, pancreas and others¹⁶. cIAP1 degradation was observed in paired pre-dose and post-dose tumor biopsies at 900mg oral weekly dose with no toxicity. The dose limiting toxicity was cytokine release syndrome concurrent with the rapid increase in plasma cytokine levels of TNF, IL8, IL10, MCP1. This inflammatory reaction is not surprising, considering that we and others found that in addition to being positive regulators of the NFκB pathway, cIAP1/2 are also negative regulators of the non-canonical NFκB pathway, which plays a crucial role in the modulation of innate and adaptive immunity by promoting cytokine production^{2,3,17-20}. The non-canonical NFκB activation requires proteasomal-mediated partial degradation of the inactive NFκB2 p100 to its active p52 form, which is initiated by its phosphorylation by the NFκB inducing kinase NIK and IKKα. In the absence of stimuli, NIK is in a cytoplasmic complex with TRAF3 and cIAP1/2 where it is rapidly ubiquitinated and degraded¹. Upon binding of ligands such as CD40L or BAFF to their receptors, TRAF3 and cIAP1/2 are recruited to the cell membrane, releasing NIK which phosphorylates IKKα and NFκB2, leading to p100 to p52 processing, but also IKKβ, inducing NFκB1 p50 activation²¹. Thus, by causing degradation of cIAP1/2, release of NIK and activation of NFκB2, LCL161 demonstrated co-stimulatory activity in human peripheral blood T-cells by enhancing cytokine secretion and mimicked CD40L in inducing dendritic cell (DC) maturation²²⁻²⁴.

A dual role for cIAP1/2 in tumorigenesis is further observed through the analysis of multiple TCGA datasets for copy number abnormalities of BIRC2/3, the genes encoding cIAP1/2^{25,26}: high amplification levels are seen in several cancer types (cervical 11%, ovarian 7%, head and neck 6%, bladder 4%), whereas deletions are seen in others (testicular 4%, DLBCL 2%, breast 1%). In multiple myeloma (MM), we and others reported frequent biallelic deletion of cIAP1/2, TRAF2 and TRAF3 (ubiquitination targets of cIAP1/2), and amplification or translocation of NIK^{17,19}, affecting approximately 10% of MM patients enrolled in the COMMPass project (<http://research.themmr.org>). As it has been shown that genetic or pharmacological disruption of cIAP1/2 in murine B-lymphocytes renders them independent of BAFF for survival and induces uncontrolled proliferation^{27,28}, we speculated that cIAP1/2 blockade would enable MM growth independent of intra-medullary secreted

TNF family ligands (i.e. BAFF and APRIL), allowing extra-medullary dissemination. Having modeled the NFkB2 activating effects of biallelic deletion of cIAP1/2 in MM using IAPa *in vitro*^{18,21}, we set out to examine the *in vivo* effects by treating the immunocompetent transgenic Vk*MYC MM mouse model²⁹ with LCL161. To our surprise, we found that instead of promoting MM growth, LCL161 modulates the MM tumor milieu to induce a potent immune activation that invokes anti-tumor phagocytic activity and long lasting anti-tumor immunity. We describe herein the effect of LCL161 in murine models and patients with MM.

Results

LCL161 exhibits potent preclinical anti-myeloma activity

LCL161 treatment of human MM cell lines (HMCLs) that do not harbor NFkB activating mutations induced p100 to p52 processing, a marker of non-canonical NFkB activation (Supplementary Fig. 1a)²¹. In contrast to the direct cytotoxic effects of IAPa in other models^{2,4,16,30–32} LCL161 did not induce cell death in our experimental system. We only observed some cell killing at doses exceeding those achieved clinically¹⁶, even in KMS28PE cells that harbor biallelic deletion of cIAP1/2, indicating that killing was likely due to off target effects of LCL161^{33,34}. Consistent with the strong pro-survival role for TNF in MM mediated by NFkB activation³⁵, but in contrast to the pro-apoptotic effects of TNF in combination with IAPa reported in other pre-clinical models^{2–4,9,13}, the addition of exogenous TNF did not potentiate LCL161 activity nor induce cell death in HMCLs *in vitro*, but rescued the lethality observed at non clinically relevant concentrations of LCL161 (Supplementary Fig. 1b). Next we studied the effects of LCL161 *in vivo*. We exposed aged Vk*MYC transgenic mice with established MM to a well-tolerated dose of LCL161 based on previous *in vivo* studies^{5–15}. In contrast to our expectation of observing induction of extra-medullary dissemination of MM, the quantification of the M-spike, the serum monoclonal immunoglobulins produced by the myeloma plasma cells, revealed a reduction in tumor burden, comparable to that observed in response to standard of care (SoC) agents in MM: the proteasome inhibitors carfilzomib (CAR) and bortezomib (BOR), the DNA alkylators melphalan (MEL) and cyclophosphamide (Cy), the histone deacetylase inhibitor (HDACi) panobinostat, dexamethasone (DEX) and the IMiD pomalidomide (POM) (Fig. 1a). We have previously validated the Vk*MYC transgenic mouse as a useful model to predict drug activity in MM³⁶, with an updated positive predictive value for clinical activity of 73% and a negative predictive value of 92% (Supplementary Fig. 2). Therefore, the single agent activity of LCL161 observed in Vk*MYC mice placed it as a promising anti-MM agent³⁶.

LCL161 was also active against the aggressive BOR resistant Vk12598 transplantable MM line derived from Vk*MYC mice, which lacks NFkB activating mutations (Fig. 1b) but inactive against the multi-drug refractory Vk12653 MM line carrying an NFkB activating mutation³⁶ (Fig. 1c). Among SoC agents with direct anti-MM activity, Cy, reported to potentiate adaptive immune responses against established tumors by abrogating regulatory T cells and to induce an acute secretory activating phenotype that promotes macrophage (MΦ) infiltration and phagocytosis of tumor cells^{37,38}, and panobinostat, known to enhance tumor

cell immunogenicity by increasing expression of MHC and co-stimulatory molecules³⁹, synergized with LCL161 in reducing M-spike levels in Vk12653 tumor bearing mice. In contrast, no synergism was observed between LCL161 and the immune-suppressive agents BOR or DEX, both of which antagonized LCL161 activity in Vk12598 MM (Fig. 1*b,c*). Furthermore, no M-spike reduction was noted in the combination of LCL161 and the Toll-like-receptor 3 activator Poly(I:C) (Supplementary Fig. 3*a,b*). Finally, LCL161 prolonged survival in two syngeneic Balb/c plasmacytoma murine models of MM (Supplementary Fig. 3*c,d*).

Pretreatment *in vivo* with anti-TNF blocking antibody one day prior to LCL161 dosing partially inhibited LCL161 activity against Vk12598 MM, as did an antibody against IL12, suggesting that the inflammatory activity of TNF and IL12 may be contributing to the *in vivo* anti-MM activity of LCL161 (Fig. 1*d*). Exposure of Vk12598 cells *in vitro* to LCL161 did not induce cell death or the expression of apoptotic markers as compared to controls (Supplementary Fig. 4). Furthermore, histological examination of Vk*MYC tumor sections from BOR or LCL161 treated mice showed cleaved-caspase 3 staining only in the former, and evidence of phagocytosis of intact MM cells only in the latter (Fig. 1*e*).

These data indicate that LCL161 is not directly cytotoxic to MM cells *in vitro*, where it induces activation of the non-canonical NFkB pathway, mimicking the effects of biallelic cIAP1/2 inactivation observed in MM patients. However, LCL161 exhibited anti-MM activity *in vivo* in a clinically predictive model, which was independent of caspase-3-mediated apoptosis but rather due to phagocytosis of non-apoptotic cells. We therefore sought to evaluate LCL161 treatment in patients with MM.

Durable anti-myeloma activity of LCL161 in MM patients

We initiated a phase II clinical trial of LCL161 in relapsed/refractory MM (ClinicalTrials.gov NCT01955434). As part of the original trial design we planned for the addition of Cy in the event of disease progression, or upon lack of response after eight weeks of LCL161 single agent. A response is defined by the International Myeloma Working Group Uniform Response Criteria for Multiple Myeloma, essentially as a 50% reduction in the serum or urine paraprotein (the monoclonal immunoglobulin secreted by the myeloma cells)⁴⁰. Among the 25 patients treated with LCL161, the median number of prior therapies was three, 44% of them had high-risk features, and 72% had refractory disease progressing on therapy (Supplementary Table 1). Because of grade 2 cytokine release syndrome in four of the first eleven patients treated at 1800mg orally weekly (Supplementary Table 2), we elected to lower the dose of LCL161 to 1200mg rather than adding prophylactic DEX, given our data of *in vivo* antagonistic activity between DEX and LCL161 in our preclinical models. 500mg of weekly oral Cy was added in 23 of the 25 patients that continued the study. While there were no responses to single agent LCL161, responses were seen following the addition of Cy in five patients (1CR, 1 VGPR, 2 PR, 1 MR) with median progression-free-survival (PFS) of 10 months (Fig. 2*a,b*). The depth and duration of response can be seen best by examining the course of individual patients. Patient 1, following a transient response to LCL161 single agent achieved immunofixation negative complete response (CR) after the addition of Cy (Fig. 2*c*). Patient 2 received 12 weeks of

LCL161, the last four in combination with Cy and stopped treatment because of concerns of undocumented progressive disease. He achieved a partial response (PR) and long-term disease control with immune reconstitution, evidenced by a rise of his uninvolved IgG returning to the normal range. He remains with stable disease with 5% bone marrow plasma cells (BMPC) (down from 40% before treatment) 27 months after stopping treatment, the longest period of time he has been off therapy since his diagnosis with MM (Fig. 2*d*). Patient 3 obtained a very good partial response (VGPR) following the addition of Cy to LCL161, but progressed 14 months after starting the treatment (Fig. 2*e*). Patient 4 achieved a PR following the addition of Cy to LCL161, progressing 25 months after starting the treatment (Fig. 2*f*).

MM cell-autonomous type-I IFN is required for the anti-MM activity of LCL161 *in vivo*

RNAseq analysis on purified MM cells from mice treated overnight with vehicle or LCL161, or MM cells collected from five MM patients pre and after 3–4 weeks of LCL161 single agent treatment displayed a marked upregulation of NFκB target genes that were consistent with the processing of p100 observed *in vitro*, and also with a type-1 interferon (IFN) signature (Fig. 3*a* and Supplementary Table 3). Interestingly, while this signature was evident in the LCL161 responsive Vk12598 mice and in all five MM patients analyzed, it was not detected in the LCL161 refractory Vk12653 mice, where instead we noted constitutively high baseline levels of NFκB target genes that were not further enhanced by LCL161. We suspect this is a result of a high level of ectopic initiation of transcription of MAP3K14 (NIK) from an intracisternal A particle long terminal repeat likely due to retrotransposition⁴¹, that mimics the dysregulation of NIK observed in MM samples as a result of chromosome translocation¹⁷ (Supplementary Fig. 5). Gene set enrichment analysis performed on the most upregulated genes in both mice and patients identified gene sets of IFN response, inflammation and NFκB activation (Fig. 3*b*), which we confirmed to be a direct effect of LCL161 *in vitro* in a Balb/c plasmacytoma cell line (Supplementary Fig. 6). A type-I IFN signature in cancer cells is required for the therapeutic efficacy of certain chemotherapeutic agents and has been linked the anti-tumor properties of LCL161, as demonstrated by the synergism between LCL161 and oncolytic virus infection in pre-clinical murine models^{31,42–44}. We therefore assessed the contribution of IFN signaling to the anti-MM activity of LCL161 *in vivo*. We found that blocking type-I IFN signaling on both tumor and host cells with antibody specific for the IFNα/b receptor subunit 1 (aIFNAR1) was sufficient to abrogate LCL161 induced survival advantage in Vk12598 tumor bearing mice (Fig. 3*c*). In apparent contrast, when we engrafted Vk12598 MM cells into recipients, either IRF3/IRF7^{null}, which do not express type I IFN, or IFNAR^{null}, which do not respond to IFN signaling, LCL161 retained its anti-MM activity, implicating MM cell-autonomous IFN signaling in LCL161 activity (Fig. 3*d*).

This suggests that type-I IFN produced by tumor cells, recapitulating a viral infection, induces an inflammatory reaction that ultimately leads to tumor cell phagocytosis. We therefore sought to investigate the effects of LCL161 treatment on myeloid cells.

LCL161 treatment induces an acute inflammatory response and activates phagocytic cells

MΦ and DC are known to become activated in response to CD40 ligation, which, as does LCL161, leads to activation of NFκB2. We therefore examined the effects of LCL161 treatment on these cells. Consistent with published data^{24,45}, we found that LCL161 treatment induced expression of CD11b and the maturation marker CD86 in murine bone marrow (BM) derived DC generated *in vitro*, where it also upregulated expression of the inflammatory genes TNF, IL6, IL12b, IFN γ , NOS2 (Fig. 4a and Supplementary Fig. 7). A similar inflammatory response, characteristic of M1-polarized MΦ, was noted in LCL161-treated bone marrow derived MΦ (BMDM) that was further potentiated by IFN γ priming (Fig. 4b). *In vivo*, LCL161 induced expression of CD86 on DC and CD40 on MΦ from wild type (WT) and Vk12598 tumor bearing mice, concomitant with upregulation of IL12/IL23p40 and iNOS expression (Fig. 4c–f). Interestingly, such activation was not noted in mice engrafted with the LCL161-resistant Vk12653 line, indicating an immunosuppressed microenvironment. The addition of Cy, most likely by promoting an acute secretory activating phenotype on tumor cells³⁸ overcame this immunosuppression, and further increased the expression of activation markers in Vk12598 tumor bearing mice (Figure 4g,h). Consistent with the immunophenotyping data, we observed upregulation of inflammatory cytokines following LCL161 treatment in the BM from WT and Vk12598, but not in Vk12653 tumor bearing mice (Fig. 4i). Importantly, a similar induction of inflammatory cytokines was also detected in the BM plasma from MM patients collected three weeks after receiving the first dose of LCL161 (Fig. 4j). These cytokine profiles have been associated with successful immune-mediated cancer eradication and immunosurveillance in mouse studies⁴⁶. As reported in the phase I study of LCL161 in solid tumors¹⁶, acute changes of IL8, IL12 and MCP-1 cytokine levels were also noted in peripheral blood four hours post-treatment, although changes in TNF were not documented (Supplementary Fig. 8).

We concluded that LCL161-mediated NFκB activation, mimicking CD40 engagement, is directly responsible for inducing an inflammatory response in MΦ and DC *in vitro*, which is further potentiated *in vivo* by the presence of LCL161-sensitive tumor cells.

LCL161 treatment stimulates myeloma cell secretion of soluble factors that induce tumor cell phagocytosis by MΦ

We next sought to measure if LCL161 treatment affects the ability of MΦ to phagocytize tumor cells in the presence or absence of cancer cell-autonomous IFN signaling. Vk*MYC MM cells and transplantable cell lines do not survive *in vitro*, so we used as target cells a Vk*MYC derived B cell lymphoma (Vk26963) line²⁹ that is fully resistant to LCL161 treatment. Activation of BMDM with LCL161 *in vitro* did not increase phagocytosis, and the combination of LCL161 + IFN γ did not show any differences in the uptake of untreated Vk26963 target cells as compared to IFN γ alone (Fig. 5a). However, pretreatment of target cells with LCL161, without inducing phosphatidylserine or calreticulin cell surface expression, nor altering CD47 levels, resulted in significantly higher uptake by IFN γ -primed BMDM, compared to vehicle-treated Vk26963 cells (Supplementary Fig. 9). Furthermore, the incubation of BMDM with conditioned medium (CM) collected after LCL161 treatment of Vk26963 cells increased the uptake of untreated target cells. Pre-treatment of Vk26963

cells with aIFNAR blocking antibody, but not isotype control, inhibited this effect, as CM collected after aIFNAR+LCL161 treatment did not induce phagocytosis above baseline levels (Fig. 5b). We concluded that LCL161 treatment induces a type-I IFN response in tumor cells, promoting the release of soluble factor(s) that stimulate their phagocytosis by BMDM. To confirm the *in vitro* phagocytic studies, we performed *in vivo* imaging of GFP-labeled Vk*MYC cells in the tibia bone marrow using two-photon microscopy. After LCL161 treatment, imaging revealed an increase of PE-labeled phagocytic cells within the GFP⁺ myeloma foci (Fig. 5c–e), with increased phagocytic activity measured by increased frequency of GFP⁺PE⁺ phagocytes (Fig. 5f). Time-lapse intra-vital imaging confirmed phagocytosis of intact MM cells, in real-time, as early as four hours following treatment (Supplementary videos 1,2). Finally, pre-treatment of Vk12598 tumor bearing mice with liposomal clodronate, known to cause depletion of phagocytes, one day prior to LCL161 administration, significantly reduced LCL161 activity and abolished the survival advantage induced by LCL161, indicating that macrophage-dependent phagocytosis is required for the *in vivo* anti-MM effects of LCL161 (Fig. 5g).

We concluded that LCL161 treatment induces an IFN response in MM cells which promotes recruitment of phagocytes to the tumor microenvironment and increases their ability to phagocytize tumor cells.

Adaptive immunity is essential for long-term disease control but is dispensable for the initial response to LCL161

The potential of IAPa to enhance murine and human CD8 and CD4 T-cell function by increasing expression of surface markers for activating (CD25) and promoting IL2 and IFN γ cytokine production has been investigated previously in *in vitro* studies²². We therefore sought to investigate the effects of LCL161 treatment on T cell activation *in vivo*. LCL161 induced an increase in CD25 expression on CD4⁺ T-cells from Vk12598 tumor bearing mice compared to controls (Supplementary Fig. 10a). However, the number of CD4 and CD8 T-cell effector and of IFN γ producing cells was not increased (Supplementary Fig. 10b–e). Given the lack of T-cell activation, it was not surprising that the response to LCL161 was similar in CD4⁺, CD8⁺ and NK1.1⁺ depleted and IgG-treated control mice (Supplementary Fig. 10f). In a complementary approach, we transplanted Vk12598 MM cells into WT or RAG1^{null} recipient mice. LCL161 induced a similar response in both sets of recipient mice, where it also prolonged overall survival (Fig. 6a and Supplementary Fig. 10g). Remarkably, while the median survival of mice receiving vehicle was only ~10 days, two weeks of LCL161 treatment was curative in ~15% Vk12598 tumor-bearing WT mice but in none of the RAG1^{null} mice, suggesting that adaptive immunity is dispensable for the short term anti-MM effect of LCL161, but is necessary to prevent disease recurrence.

Clonal expansion of tumor specific T-cells, protection against tumor recurrence, and production of Th1 cytokines are all hallmarks defining immunological memory that contribute to the protection against tumor recurrence. We investigated the capability of the immune response to reject tumor re-challenge in Vk12598 mice that had remained tumor-free one year following treatment with LCL161. The recurrence of MM was prevented in 66% (4/6) of the mice which remained tumor free until they were sacrificed four months

later (data not shown). Next, we characterized MM specific memory T-cell generation in Vk12598 mice that were cured by LCL161, by inoculation with a secondary challenge of either the same (Vk12598) or an irrelevant (Vk26633) tumor. Immunological memory characterized seven days later showed a robust expansion in CD4 but not CD8 effectors (CD44^{hi}CD62L^{lo}) and memory (CD44^{hi}CD62L^{hi}) T-cells and a corresponding increase in IFN γ -producing CD4⁺ T-cells (Fig. 6*b–d*) in response to MM cells compared to the irrelevant tumor. This lack of CD8 expansion was surprising and prompted us to assess the expression of CD25 because numerous studies have reported that down-regulation of CD25 on CD8 T-cells affects primary and secondary memory expansion upon tumor challenge^{47,48}. Consistently, no CD25⁺ CD8 T-cells were detected irrespective of treatment or tumor re-challenge in Vk12598 tumor bearing mice (Fig. 6*e*).

We conclude that LCL161 promotes long term MM control in a fraction of mice through generation of immunological memory.

Combination of LCL161 and anti-PD1 cures myeloma in mice

It is likely that the hypo-responsiveness of T-cells to initial LCL161 activation is due to an expansion of T_{regs} and/or because the T-cells to have become refractory and exhausted after exposure to chronic antigenic stimulation associated with the MM microenvironment. We have compared and detected a similarly low percentage (between 1–3%) of CD4⁺CD25⁺FOXP3⁺ T_{regs} in the spleens from Vk12598 tumor bearing versus non tumor bearing mice, with similar levels of co-inhibitory molecule PD-1 surface expression (Fig. 6*f,g* and not shown). However, the expression of co-inhibitory molecule PD-1 on CD4 effector (CD44^{hi}CD62L^{lo}) T-cells (Fig. 6*g,h*) as well as effector CD8 T-cells (Fig. 6*i*) was markedly elevated, with no changes induced by treatment. We next evaluated the expression of the PD-1 ligand PD-L1 in the MM tumor milieu. Nearly all CD11b⁺ myeloid cells express PD-L1 (Fig. 6*j*), compared to only a fraction of Vk12598 MM cells. However, PD-L1 expression was significantly increased following *in vivo* treatment with LCL161 (Fig. 6*k*). Interestingly, this effect was not observed in MM cell lines treated *in vitro* (Supplementary Fig. 11*a–e*). These findings prompted us to combine LCL161 with an antibody against PD-1. In an experiment in Vk12598 tumor bearing mice where the median survival of control mice was only seven days, anti-PD-1 doubled the survival to fourteen days and LCL161 further increased it to 33 days. The combination of LCL161 and anti-PD-1 was curative in all of the mice that completed two weeks of treatment, immediately abrogating the early mortality observed in the other conditions, and was more effective than the combination of LCL161 with Cy, where the median survival was 84 days (Fig. 6*l*).

Discussion

MM cells are exquisitely dependent on the BM microenvironment for growth and survival. Work by us and others have shown that M Φ are a crucial component of the MM niche that support MM growth through the secretion of inflammatory cytokines, and promotion of immune-evasion as MM progresses^{49,50}. However, M Φ have an innate ability to kill tumor cells that can be re-activated by inhibition of the “don’t eat me signal” CD47⁵¹ or by reprogramming them towards a tumoricidal polarization state by treatment with

combinations including aCD40 and TLR agonists^{52–55}. Here we report that single-agent LCL161 induces a remarkable anti-MM response in Vk*MYC mice that is not due to direct cell killing but rather activation of a tumor cell autonomous type-I IFN signaling resulting in myeloid cell activation and tumor cell phagocytosis. Consistently, no *in vivo* anti-MM response to LCL161 was observed following phagocytic cell depletion. Treatment of MΦ with LCL161 *in vitro*, although it induced activation, did not increase their phagocytic ability, that in contrast was augmented by soluble factors released by treated tumor cells. IFN signaling in tumor, but not host cells, is essential to promote phagocytosis, as it was prevented by blocking the type-I IFN receptor on tumor cells.

There is an apparent paradox between the anti-tumor activity of LCL161 we report *in vivo* and the fact that clonal progression in MM frequently selects for elimination of cIAP1/2, the target of IAPa, presumably to benefit from the survival advantage deriving from chronic NFκB activation. On one hand, the depletion of cIAP1/2 in tumor cells by LCL161 treatment, induces a type-I IFN signaling, a characteristic of viral mimicry, that stimulates endogenous immunity to eradicate MM. At the same time, the loss of cIAP1/2 in the host immune cells leads to NFκB activation causing an inflammatory reaction that further potentiates the host immune response against the tumor. Furthermore, NFκB activation has been shown to provide a strong survival signal that protects cells from the pro-apoptotic activity of TNF⁵⁶. MM cells are characterized by a ligand dependent activation of the non-canonical NFκB pathway, mediated by BAFF and APRIL, which is also induced by IAPa treatment. We speculate that this explains why treatment with IAPa failed to promote TNF-dependent apoptosis in other tumor types.

No single agent LCL161 activity was observed in the Vk12653 transplantable model carrying an activating mutation of the non-canonical NFκB pathway and a tumor microenvironment profoundly suppressed for myeloid cell activation. No tumor induction of NFκB or IFN response was noted in this model following LCL161 treatment, nor changes in inflammatory cytokines, consistent with our data that blocking either the IFN response or the TNF or IL12 inflammatory pathways abrogated the response of MM cells to LCL161. While the cause of immunosuppression in Vk12653 mice is presently unknown, the addition of Cy to LCL161 resulted in significant DC activation and anti-MM response, most likely by promoting direct tumor cell killing. LCL161 also did not demonstrate single agent activity in MM patients, despite the induction of an NFκB and IFN response on MM cells and the detection of inflammatory cytokines in the BM serum, suggesting the presence of a LCL161-sensitive immune-microenvironment. We suspect that differences in the immune-microenvironment between mice with *de novo* or transplanted MM, and heavily-pretreated, multiply-relapsed patients may explain the differential responses to single agent LCL161 treatment observed in mouse and MM patients. Following the addition of Cy, however, we noted durable disease control with a median PFS of 10 months in this cohort of patients with relapsed/refractory MM, accompanied with evidences of immune reconstitution (increase levels of un-involved Ig). This PFS is greater than one would expect for single agent Cy in this patient population, since in the much more responsive patient population of untreated MM, the two drug combination of MEL and prednisone only induces a PFS of 12–13 months^{57,58}. In contrast, in an only slightly more refractory population (median four prior lines) the three drug combination of Cy, POM and DEX results in a median PFS of 9.5

months⁵⁹. In summary, the combination of LCL161 and Cy is a well-tolerated oral regimen offering long-term disease control in relapsed/refractory MM that is immuno-stimulatory and does not expose patients to the immunosuppressive effects of a glucocorticoid.

We were surprised that adaptive immunity was dispensable for the initial anti-MM response to LCL161. Although we observed increased CD25 expression, marker of early activation, on CD4+ T-cells following LCL161 treatment, most of the effector T-cells expressed PD1 and presumably became anergic within the MM tumor milieu that abundantly expresses PD-L1. Notably however, a fraction of mice was cured in response to two weeks of treatment with LCL161 indicating effective immune-surveillance, as demonstrated by the development of immunological memory. Promoting sustained T-cell activation with anti-PD1 had an immediate effect on LCL161 induced anti-MM response resulting in a dramatic cure. We conclude that the combination of LCL161 and Cy is an attractive platform for future of trials of anti-tumor immune activation in MM, most urgently in combination with anti-PD1.

Online Methods

Cell lines and reagents

Human MM cell lines have been previously described¹⁷ and have been maintained in RPMI 1640 supplemented with 5% FBS and glutamine, without antibiotics. The XRPC24⁶⁰ and J558 Balb/c plasmacytoma cell lines were kindly donated by Dr. Michael Kuehl (NIH-NCI) and were grown in RPMI + 5% FBS supplemented with glutamine, penicillin, streptomycin and 50uM beta-mercapto-ethanol (mouse medium). None of these lines are listed on the ICLAC database of commonly misidentified cell lines and are routinely fingerprinted by assessment of copy number polymorphisms by PCR. The Vk26963 B cell lymphoma and the Vk26633 thymic T-cell lymphoma cell lines were generated by culturing splenocytes of aged Vk*MYC transgenic mouse with lymphoma and were maintained in mouse medium²⁹. L929 cells were purchased from ATCC. All cell lines are tested for mycoplasma contamination twice/year using the MycoAlert® kit (Promega). LCL161 was provided by Novartis and solubilized at 1mM in DMSO for *in vitro* studies or at 10mg/ml in 30% HCl 0.1N + 70% Sodium acetate 100mM pH=4.5 for *in vivo* administration. Recombinant human TNF was purchased from R&D System. Recombinant murine IFN γ is from Pepro Tech. Anti-TNF blocking antibody (clone CNTO-5048) was a gift of Centocor. Anti-IL12 P40 (C17.8), anti-IFNAR (MAR1-5A3) and anti-PD1 (RMP1-14) were obtained from BioXCell, together with mouse IgG1 or ratIgG2a isotype controls. Anti-CD4 (GK1.5), anti-CD8 (2.43), anti-NK1.1 (PK136) and their isotype controls were obtained from Leinco Technologies inc. Clodronate and PBS liposomal particles were purchased from ClodronateLiposomes.com (The Netherlands). HMW poly(I:C) was purchased from Invivogen. Annexin-V and propidium iodine (PI) were purchased from BD Biosciences.

Mice

All experiments were performed under The Mayo Foundation Institutional and Albert Einstein College of Medicine Animal Care and Use Committee (IACUC) approval and conformed to all the regulatory Environmental Safety standards. The generation and initial characterization of the Vk*MYC mice has been reported elsewhere²⁹. The generation of

bortezomib resistant Vk12598 and Vk12653 transplantable lines has been previously reported³⁶. The Vk14451 transplantable MM line was obtained by crossing a Vk*MYC mice with mice expressing EGFP under the control of gamma1 promoter (unpublished). Although unable to survive *in vitro*, all these transplantable MM lines were maintained by serial transplantation in non-irradiated, congenic C57BL/6J wild type (WT) mice. A large stock of 20 million frozen splenocytes (~25% MM cells) has been established from untreated recipient mice to ensure consistency between experiments. Approximately 1 million of splenocytes (or 2.5×10^5 freshly isolated CD138⁺ magnetically selected cells) are transplanted by intravenous (i.v.) injection into each of 8–12 weeks old congenic recipient mice of both sexes. Balb/c WT, C57BL/6J WT and RAG1^{null} mice and IFNAR null⁶¹ mice were purchased from the Jackson Laboratories. IRF3⁶² and IRF7⁶³ null mice in C57BL/6J background were kindly donated by Mike Diamond, Washington University, with previous approval of Professor Tadatsugu Taniguchi, University of Tokyo. Five million XRPC24 or J558 cells were transplanted by intra-peritoneal or sub-cutaneous injection into adult (8–12 weeks old) Balb/c WT mice, which received vehicle or LCL161 treatment once the tumor reached a dimension of 100mm² approximately 4 weeks post transplantation.

***In vivo* drug administration**

At least three aged (>70 weeks old) *de novo* Vk*MYC mice of any sex, with a gamma/albumin ratio between 0.5–2.0 corresponding to a predominant M-spike between approximately 15–60 g/L were treated with each drug⁶⁴. Transplanted mice were enrolled in the studies approximately four weeks post transplantation, with M-spike levels >10g/L, or a gamma/albumin fraction >0.3 to mimic clinical setting. Mice were randomized to different treatment arms, stratified by the size of their M-spikes. Standard of care drug dosage and route of administration has been already reported³⁶. LCL161 (Novartis) was solubilized at 10mg/ml in 30% HCl 0.1N + 70% Sodium acetate 100mM pH=4.5 and given by oral gavage at 50 or 100mg/kg on day 1,4,8,11. Cyclophosphamide (Sigma) was solubilized in saline and administered by i.p. injection at 100mg/kg on day 1,8. Antibodies were diluted at 1mg/ml in PBS and administered by i.p. injection at 10mg/kg at the specified days. 200ul of liposomal particles containing PBS or 5mg/ml clodronate were administered i.v. on day 0,3,7,10 followed by LCL161 at 50mg/kg p.o. on day 1,4,8,11. Poly(I:C) was given at 2.5mg/kg by i.p. injection on day 1,4,8,11. SPEP at day 0 and day 14-post treatment was performed to measure reduction in the M-spike as a marker of tumor response, as done clinically. Specifically, the gamma/albumin ratio post treatment for each individual M-spike is calculated while remaining blinded to the treatment allocation group and then is normalized by the gamma/albumin ratio obtained at d0. A minimum sample size of six mice per group was chosen to ensure 80% power to detect a 1.8 standard deviation difference between two groups using a two-sided alpha=0.05 *t* test. Comparison of M-spike reduction with different drug treatments relative to vehicle was done by unpaired two-sided *t* test with equal standard deviation using GraphPad Prism (GraphPad Software, San Diego, CA). Comparison of Kaplan-Meier survival curves was performed by Log-rank (Mantel-Cox) test using GraphPad Prism (GraphPad Software, San Diego, CA). P values are reported as follow: ns *P*>0.05, * *P* 0.05, ** *P* 0.01, *** *P* 0.001, **** *P* 0.0001.

LCL161 clinical trial

- Patient selection.** A one-stage design based on the binomial distribution required 25 patients to test the null hypothesis that the overall response rate was less than 10% versus an alternative hypothesis of greater than 30% with an alpha of 0.10 and power of 91%. Patients above 18 years of age seen at Mayo Clinic in Arizona, Rochester and Florida were included in the study if they had evidence of MM relapsed after previous treatment with an immunomodulatory agent and a proteasome inhibitor and a glucocorticoid, but fewer than five lines of prior therapy (with induction, transplant, consolidation and maintenance considered one line). Measurable disease in the form of serum monoclonal protein ≥ 1.0 g/dL, > 200 mg of monoclonal protein in the urine on 24 hour electrophoresis or serum immunoglobulin free light chain (FLC) ≥ 10 mg/dL along with abnormal serum immunoglobulin kappa to lambda light chain ratio was required for participation in the study. Patients were also required to have absolute neutrophil count $1000/\mu\text{L}$, platelet count $75000/\mu\text{L}$, hemoglobin 8g/dL , total bilirubin 1.5mg/dL times the institutional upper limit of normal (ULN), AST $3\times$ the institutional upper limit of normal (ULN), ALT $3\times$ ULN, creatinine 2.5 mg/dL, ECOG performance status 0,1, or 2, along with negative pregnancy test. These criteria were specifically written to address clinical trial needs of patients with advanced disease, and who are not normally eligible for standard clinical trials. Patients with other active malignancy or with other concurrent chemotherapy or radiotherapy, prior allogeneic transplantation or active autoimmune inflammatory condition were excluded from the study. The protocol was registered at clinicaltrials.gov under number NCT01955434 and was approved by the Mayo Clinic IRB. Informed consent was obtained from all study participants. A copy of the protocol is available in the supplementary material
- Patient treatment.** Study treatment consisted of LCL161 1800mg administered orally weekly in the first eleven patients, and 1200mg in all of the subsequent patients. Patients were offered premedication with acetaminophen, ranitidine and diphenhydramine, but were not allowed to take glucocorticoid stronger than prednisone 10mg daily. Because of concerns for cytokine release syndrome (CRS), the first four doses were administered under close supervision in the clinic, and patients were provided with 8mg of dexamethasone to take in the event of CRS occurrence. As part of the original clinical trial design, Cyclophosphamide 500mg orally weekly was added for unconfirmed disease progression, or lack of response anytime after 8 weeks of therapy. Cyclophosphamide was discontinued after one year. The protocol allowed for treatment with LCL161 after discontinuing the cyclophosphamide at the discretion of the investigator until evidence of disease progression.
- Evaluation of Patient Response and Toxicity.** Evaluation of response was performed every four weeks. Criteria for response and progression were based on International Myeloma Working Group Uniform Response Criteria for Multiple Myeloma. Response was evaluated over all cycles of treatment and required confirmation on two consecutive evaluations. When cyclophosphamide was

added a new baseline disease measurement was established. Evaluation for toxicity was based on the National Cancer Institute CTCAE (v4.0). Toxicities were evaluated at every visit. The primary endpoint was response rate to single agent LCL161, secondary endpoints included response rate following the addition of cyclophosphamide, toxicity, event-free and overall survival.

- **Results.** Twenty-five patients were enrolled between November 13, 2013 and February 16, 2016. Cyclophosphamide (for a maximum of twelve months) was added in twenty-three. Eleven patients withdrew from the study with stable disease, and ten withdrew with progressive disease. Two patients remain on treatment with single agent LCL-161. The baseline characteristics are shown in Supplementary Table 1. In the phase 1 study grade 3 to 4 CRS was the dose limiting toxicity at doses of 1800mg, 2100mg and 3000mg, with symptoms of fatigue, flushing, rash, vomiting, diarrhea, fever and hypotension, the timing of which was concurrent with the rapid increase in cytokine levels (TNF, IL8, IL10, CCL2) lasting 24–96 hours¹⁶. While investigators in other subsequent studies of LCL161 elected to pre-medicate with dexamethasone, we decided to limit the use of dexamethasone in this study. After we noted that four of the first eleven patients suffered grade 2 CRS we decided to reduce the starting dose of LCL161 to 1200mg for the remaining patients, and no further CRS was noted. The episodes of CRS were managed with close observation, antihistamines, dexamethasone, and fluid support if needed. Other toxicities are listed in Supplementary Table 2.

Gene Expression Analysis

RNA Sequencing was performed starting from 1ug of CD138-selected cells from BM using Illumina paired-ends with six samples per lane of a HiSeq 2000. Gene expression was summarized using transcripts per million (TPM) calculated using Salmon aligned to Ensembl gene files from human (hg19) and mouse (mm10). Expression threshold was determined by visual examination of the histogram of log₂ TPM and was determined to be “0” for both the mouse and human samples based on bi-modal distribution of the data. In the human there were 19016 protein coding genes, with 8443 expressed above the threshold in 3 of the 5 treated samples, with 2082 having 1.1 fold higher expression following treatment with LCL161 in 3 of the 5 treated samples. In the mouse the analysis was confined to mice transplanted with tumor Vk12598. There were 22174 protein coding genes, with 9720 having expression above the threshold in all four Vk12598 samples, with 2296 having 1.2 fold higher expression following treatment with LCL161 in all four Vk12598 samples. Of the genes in these two lists, 384 were common to the analyses from both human and mouse samples (Supplementary Table 3). These genes, ordered based on the fold change in mouse, and in human, were used for Gene Set Enrichment Analysis using HALLMARK genesets. The observed enrichment score (*ES*) of a gene set was generated using a running-sum statistic along the ranked differential expression genes, where *ES* is the maximum deviation from zero encountered in the random walk. A phenotype-based permutation test was performed to generate a null distribution of the *ES*, and the empirical, nominal *P value* of a gene set was calculated as the observed *ES* relative to this null distribution. The FASTQ files

of the RNA-Seq data were submitted to Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/sra>) under accession number SRP075564.

Antibodies

Antibodies were purchased from BD Biosciences, Biolegend, eBioscience or Antibodies-online. B220 (RA3-6B2), CD138 (281-2), CD11b (M1/70), F4/80 (BM8), CD11c (N418), CD86 (GL1), CD40 (HM40-3), CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL14), PD-1 (29F.1A12), PDL1 (10F.9G2 and 29E.2A3), CD47 (miap301), Calreticulin (AA 277-305), CD25 (PC61), FoxP3 (FKJ16S), INOS, IL12/IL23p40 (C15.6) and IFN γ (XMG1.2).

Intracellular cytokine staining

For intracellular detection of cytokines on myeloid cells, cells were incubated with brefeldin A (BFA, BD Biosciences) for four hours in mouse medium without further stimulation or treatment, stained for extracellular markers, fixed and permeabilized in Cytofix/Cytoperm® (BD Biosciences), and stained with antibody against iNOS or IL12p40. For intracellular staining of T lymphocytes, cells were stimulated with PMA (60ng/ml) and ionomycin (1 ug/ml, both from Sigma) for one hour before incubation with BFA for four additional hours.

Intracellular staining for FoxP3

After staining for surface antigens, cells were suspended in Fixation/Permeabilization reagent (eBioscience) for 18 hours at 4°C. Cells were then incubated with anti-FoxP3-PE antibody, washed in Permeabilization/Wash buffer (eBioscience), and analyzed.

Sample acquisition

Data were acquired on a five laser BD LSR Fortessa equipped with DIVA software (BD Biosciences) and analyzed with FlowJo software (Tree Star) after exclusion of cell doublets and dead cells. Statistical analysis was performed by unpaired *t* test with equal standard deviation using GraphPad Prism (GraphPad Software). *P* values are reported as follow: ns *P* > 0.05, * *P* 0.05, ** *P* 0.01, *** *P* 0.001, **** *P* 0.0001. Results from individual mice are shown.

Generation of BM derived macrophages (BMDM) and dendritic cells (BMDC)

BM cells were collected by flushing out the two femurs and tibia with PBS and plated in DMEM/F12 supplemented with 10% FBS, penicillin and streptomycin and 20% of m-CSF rich L929 conditioned medium (obtained from growing L929 cells at confluence in DMEM/F12 + 10% FBS). After seven days, cells were harvested with a non-enzymatic cell dissociation solution (Cellstripper®, Corning) and evaluated by flow-cytometry for the expression of CD11b and F4/80. To generate BMDC, BM cells were plated in RPMI1640 + 10% FBS + penicillin and streptomycin + Flt3 100ng/ml (R&D System). After five days ½ of the medium was replaced and BMDC were maintained for additional four days. Expression of CD11b and CD11c was evaluated by flow cytometry.

RT-PCR analysis

BMDM were stimulated for nine hours in DMEM/F12 complete medium alone, or supplemented with IFN γ 10ng/ml, LCL161 200nM, or the combination. BMDM were stimulated or not with LCL161 200nM for four hours. Cells were solubilized in Trizol® (Invitrogen). RNA was extracted from Trizol, digested with RNase free DNase (Qiagen) and cleaned up on Purelink RNA mini kit columns (Thermo Fisher Scientific). cDNA was retro-transcribed from 1 μ g of RNA primed with random hexamers using Superscript-III (Invitrogen). Three ng of equivalent cDNA were amplified in a qPCR (Taqman) assay on a Corbett Rotor Gene instrument (Corbett Life Science) for the following transcripts: TNF (IDT Assay Mm.PT.56a.12575861), IFN β 1 (IDT Assay Mm.PT.56a.30132453.g), IL6 (IDT assay Mm.PT.56a.10005566), IL12b (SyBr green PCR using primers 5'-CCATCGTTTTGCTGGTGTCTCC and 5'-ATGGTCAGGGTCTTTCCAGAGC), IRF3 (SyBr green PCR using primers 5'-GATGGAGAGGTCCACAAGGA and 5'-GAGTGTAGCGTGGGGAGTGT), IRF7 (SyBr green PCR using primers 5'-CCTCTTGCTTCAGGTTCTGC and 5'-GCTGCATAGGGTTCCTCGTA). Relative gene expression was quantified with the 2^{-C} method, normalized to PolR2a housekeeping gene detected by SyBr green RT-PCR (5'-AGAAGCTGGTCCTTCGAATCC and 5'-TTGCTGATCTGCTCGATACCC). Data were collected from two independent culture experiments, where each sample was tested in triplicates.

Cytokine detection

Two femurs from each mouse were placed into a 0.5ml microtube with a hole at the bottom after their knee-caps were removed. The tube was placed inside a 1.5ml microtube, which was spun to obtain a BM cell pellet, which was suspended into 50 μ l PBS, and spun again. The obtained BM supernatant was stored frozen at -80°C till ready for analysis in duplicate with the Cytokine Mouse 20-Plex Panel for Luminex® Platform (LMC0006, Thermo Fisher Scientific) according to manufacturer's instructions. Data were acquired on a Luminex200 system equipped with xPONENT 3.1 software (Thermo Fisher Scientific). Human BM plasma obtained from MM patients enrolled in the clinical trial at baseline and three or four weeks after LCL161 single agent treatment was assayed in duplicate using the Cytokine Human 30-Plex Panel for Luminex® Platform (LHC0003, Thermo Fisher Scientific). Similar analysis was performed on peripheral blood plasma from MM patients collected at baseline and four hours after LCL161 administration.

Phagocytosis assay

In vitro generated BMDM were left untreated or stimulated overnight with IFN γ 50ng/ml, LCL161 200nM or the combination, and then harvested with Cellstripper®. 5×10^4 BMDM were plated in a round bottom non-tissue culture treated 96 well plate. Logarithmically growing Vk26963 lymphoma target cells, treated or not with LCL161 200nM overnight, were labeled with CFSE (Molecular Probes) and 2.5×10^5 cells per well were added to the BMDM culture. The mixture of BMDM and target cells were harvested two hours later, stained for viability with Live/Dead® Blue, CD11b and F4/80 and evaluated on a flow cytometer for live, single CD11b⁺F4/80⁺CFSE⁺ BMDM. In separate experiments, 15 million Vk26963 were left untreated or treated for 24 hours in 10ml of mouse medium with

LCL161 200nM, mouse IgG1 25ug/ml, anti-IFNAR1 25ug/ml or the combination. The conditioned medium was collected and used to stimulate BMDM overnight, in the presence of IFN γ , to phagocytize untreated Vk26963 target cells.

Intravital Two-photon Imaging

Details of the surgical preparation for the mouse tibial bone marrow imaging were described previously⁶⁵. To generate tumors, 1e5 GFP⁺ CD138⁺ Vk14451 MM cells were injected i.v. into C57BL/6 WT mice and allowed to engraft and grow for 2–4 weeks. To visualize macrophages in the bone marrow, mice were injected with PE-conjugated anti-CD169 (SER-4 eBioscience) 16 hours prior to imaging. For splenic imaging, spleens were surgically exposed, immobilized as described previously⁶⁶. To visualize red pulp macrophages, mice were injected with Texas red-labeled 10kD dextran (Sigma) 16hrs prior to imaging. Mice were imaged four hours after drug treatment or without treatment. Time lapse images were compiled and presented using Volocity (Perkin Elmer) and After Effects © (Adobe).

Analysis of In vivo Phagocytosis

Quantification of phagocytic cells in GFP⁺ myeloma cells was conducted by semi-automated protocols in velocity, to identify volume of GFP⁺ myeloma and PE⁺ phagocytes per field. Similar regions (with matching GFP⁺ volume) were analyzed and compared for phagocyte volume with and without treatment. To measure phagocytosis, semi-automated detection of red phagocytes was conducted, and then using a standard threshold for GFP⁺ cells, the frequency of phagocytosis was quantified as (# of double-positive GFP⁺ PE⁺ phagocytes)/ (total # PE⁺ phagocytes * 100). To normalize between imaging fields with different distributions of myeloma and phagocytic cells, phagocytes were clustered into proximal and distal, based on a 5 microns distance to the nearest myeloma cell. Data were collected from treated and untreated mice from 3 or more independent imaging experiments, values were pooled, and means were compared by Mann-Whitney t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Pre-clinical activity of LCL161 in MM

M-spike levels were quantified in response to LCL161 in **a**) *de novo* Vk*MYC **b**) Vk12598 and **c**) Vk12653 tumor bearing mice at day 14 post-treatment and normalized to day 0. Response to standard of care anti-MM agents is shown as a comparison. LCL161 was given at 100mg/kg by oral gavage on day 1,4,8,11. Treatment was initiated once M-spike levels were >7g/L (gamma/albumin serum fraction ratio >0.25), indicative of high tumor burden, to mimic clinical setting. **d**) M-spike levels (% of day0) in Vk12598 tumor bearing mice measured 14 days after treatment twice/week for two weeks with vehicle, LCL161 50mg/kg plus 10mg/kg rat IgG, anti-IL12 or anti-TNF blocking antibodies given by i.p. injection one day before LCL161 administration. In figures a–d, each dot represents an M-spike from an individual mouse. Horizontal black bars show median M-spike levels. Red horizontal line identifies the cut-off for response (>50% M-spike reduction); red vertical lines separate active (>20% response rate) from inactive (<20% RR) regimens. Unpaired two-sided *t* test *P*

values are represented by *. *e*) Representative (out of seven examples) H&E and cleaved caspase 3 staining of spleen sections from vehicle or LCL161 treated Vk12598 tumor bearing mice or from *de novo* Vk*MYC mice receiving bortezomib at 0.5mg/kg for 18 hrs. Black arrows indicate phagocytized live plasma cells. Scale bar is shown for the top image in each column and applies to the images below.

Figure 2. Clinical activity of LCL161 in MM patients

a) Waterfall plot of the lowest paraprotein levels (best response) of MM patients receiving LCL161 and Cyclophosphamide (Cy) relative to value before the addition of Cy. **b)** Progression Free Survival (PFS) and Overall Survival (OS) for all 25 patients. Patients that received any subsequent treatment before disease progression were censored at the start of the subsequent treatment. **c)** Patient 1 is a 75 year-old man with hyperdiploid MM diagnosed in 2007, who was previously treated with lenalidomide (LEN)-DEX, Cy-BOR-DEX, high dose MEL with hematopoietic stem cell transplantation, LEN, was off therapy for almost a year before starting LCL161. **d)** Patient 2 is a 56 year old man with t(11;14), del17p kappa light chain MM who presented initially in 2010 and was treated with MVTD-PACE multi-agent induction chemotherapy, transplant and three years of maintenance with LEN, BOR

and DEX. *e*) Patient 3 is a 72 year old woman with t(11;14) MM diagnosed in 2010, who was previously treated with Cy-CAR-DEX-Thalidomide, transplant and was progressing on full dose LEN-DEX. *f*) Patient 4 is a 92 year old man with t(6;14) MM diagnosed in 2009 who was previously treated with MEL-Prednisone-Thal, LEN-DEX, BOR-DEX, and was progressing on BOR maintenance. Lenalidomide (Len), Dexamethasone (Dex), Free light chain (FLC).

Figure 3. LCL161 induces a type-I IFN response in myeloma cells that is required for its *in vivo* activity

a) Heat-map representation of the top 32 genes upregulated in MM cells after *in vivo* LCL161 treatment of four Vk12598 and two Vk12653 mice for 18 hours compared to vehicle and human MM cells extracted from five patients at baseline and after three or four weeks of LCL161 single-agent treatment. Gene expression was obtained by RNAseq, and each tumor is normalized to its respective vehicle or pre-treatment control. **b)** Gene set enrichment analysis using the list of 384 upregulated genes common to mice and human MM treated with LCL161 *in vivo* in the HALLMARK gene sets. The observed enrichment score (ES) of a gene set was generated using a running-sum statistic along the ranked differential expression genes, where ES is the maximum deviation from zero encountered in the random walk. A phenotype-based permutation test was performed to generate a null distribution of the ES, and the empirical, nominal *P* value of a gene set was calculated as the observed ES relative to this null distribution. Kaplan-Meier survival plot of **c)** Vk12598 tumor bearing mice receiving vehicle, 10mg/kg isotype control or anti-IFNAR antibody on day 0,4,7,10 followed by 50mg/kg LCL161 on day 1,5,8,11; or of **d)** IRF3/7^{null} or IFNAR^{null} mice engrafted with Vk12598 tumors and treated with vehicle or LCL161 on days 1,4,8,11. Vehicle treated mice are shown grouped together. Number of treated mice (*n*),

survival (in days) from the beginning of treatment and P values for Log-rank (Mantel-Cox) test are shown for figures *c*) and *d*).

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Figure 4. LCL161 activates macrophages and dendritic cells

a) qPCR (Taqman assay) analysis of the indicated gene expression in bone marrow derived dendritic cells (DC) exposed to vehicle or LCL161 for four hours. Results from two independent cultures are shown where each sample was tested in triplicate. Whiskers identify minimum and maximum values, the boxes the 25th and 75th percentile and the line the median. **b)** qPCR (Taqman assay) analysis of the indicated gene expression in bone marrow derived macrophages (MΦ) primed with IFN γ and treated or not with LCL161 for nine hours. Delta Ct (threshold cycle) values are defined as Ct of gene of interest minus Ct

of control gene (PolR2a). Vertical bars indicate mean values with standard deviation. Gene levels were normalized to PolR2a gene and presented relative to vehicle treated cells in *a*) or relative to IFN γ primed cells in *b*). Statistical *P* values for two tailed *t* tests are shown. Evaluation by flow cytometry of the expression (% positive cells) of *c*) CD86 on splenic CD11b⁺CD11c⁺ DC or *d*) CD40 on CD11b⁺F4/80⁺ M Φ harvested from WT, Vk12598 or Vk12653 tumor bearing mice after overnight treatment with vehicle or LCL161. Intracellular expression of iNOS and IL12/IL23p40 in splenic *e*) DC and *f*) M Φ from Vk12598 tumor bearing mice treated as in *c*). CD40 expression on DC from *g*) Vk12653 or *h*) Vk12598 tumor bearing mice treated with LCL161 50mg/kg, Cy 10mg/kg or the combination. Each dot in figures *c*–*h*) represents one individual tested mouse. Horizontal black bars show median expression levels. Statistical values for unpaired two-tailed *t* tests are shown. *i*) Heat map representation of cytokine levels in BM serum collected from non tumor bearing (WT), Vk12598 and Vk12653 tumor bearing mice after 18 hours of vehicle or LCL161 treatment, shown normalized to the median values of the five vehicle treated non tumor bearing controls. Color scale from 0.5 to 10-fold induction is shown. *j*) Heat map of cytokine levels in BM plasma collected from MM patients at baseline and three weeks after single agent LCL161 treatment, shown normalized to the baseline for each individual patient. Color scale from 0.5 to 5-fold induction is shown. Each horizontal line in *i*–*j* represents the normalized cytokine values from an individual mouse or patient tested in duplicate.

Figure 5. LCL161 treatment promotes macrophage recruitment and phagocytosis of tumor cells
a) Measurement of phagocytic bone marrow derived macrophages (BMDM) determined as the percentage of CFSE+ CD11b⁺F4/80⁺ cells, left untreated, or activated overnight with 200nM LCL161, 50ng/ml IFN γ or the combination, and co-cultured for two hour with CFSE labeled tumor cells. **b)** Percentage of IFN γ primed phagocytic BMDM after exposure to LCL161 or conditioned medium (CM) harvested from tumor cells receiving the indicated treatment. Unpaired two-tailed *t* tests statistical *P* values are shown for figures *a)* and *b)*, where each dot represents an assay well from a representative experiment of the two performed, and horizontal bars indicate median values. Sample images of the tibial BM taken by intra-vital imaging, with GFP+ myeloma cells (green), and phagocytes (red) in **c)** control or **d)** LCL161 treated mice, with phagocytosis of myeloma cells marked by yellow arrows. The experiment was performed three times, with two mice treated/group. Images were quantified for **e)** the changes in phagocytes (as measured by volume) in myeloma clusters and **f)** phagocytosis of myeloma cells (frequency of GFP+ phagocytes proximally and distally positioned with respect to myeloma cells. Error bars indicate mean with standard deviation. Unpaired two-tailed *t* tests statistical *P* values are shown for figures *e)* and *f)*. **g)** Kaplan-Meier overall survival plot in days after initiation of treatment of Vk12598 tumor bearing mice treated with liposomes containing PBS or 5mg/ml clodronate on day

0,3,7,10 followed by vehicle or LCL161 at 50mg/kg p.o. on day 1,4,8,11. Number of treated mice (n) and Log-rank (Mantel-Cox) P values are shown.

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Figure 6. Adaptive immunity is dispensable for the initial response to LCL161, but combination with PD1 blockade is curative

a) Kaplan-Meier survival plot in days from the initiation of treatment of WT or RAG1^{null} mice engrafted with CD138⁺ Vk12598 MM cells and treated with vehicle or LCL161 at 100mg/kg for two weeks. Number of treated mice (*n*) and *P* values for Log-rank (Mantel-Cox) test are shown. *The proportion of mice surviving at least 60 days was significantly higher for the LCL161 group (21/151 [13.9%]) compared to the RAG1^{null} LCL161 group (0/49 [0%], Fisher's exact *P*=0.003). Total number (count × 10⁶) of **b**) CD4 effectors

(CD62L⁻CD44⁺), **c**) memory (CD62L⁺CD44⁺) and **d**) IFN γ producing splenic T-cells subsets harvested from Vk12598 tumor bearing mice that had remained tumor free for one year after LCL161 treatment and have received a secondary challenge of CD138⁺ Vk12598 MM cells or irrelevant tumor (Vk26633). **e**) Expression of CD25 on CD4 and CD8 T-cells in Vk12598 tumor bearing mice after Vk12598 or aspecific tumor re-challenge as indicated above. Absolute number of splenic **f**) T_{reg} cells (CD4⁺CD25⁺FoxP3⁺) and **g**) PD1⁺ T_{reg} cells from Vk12598 tumor bearing mice treated with vehicle or LCL161 for seven days. Each dot in Fig. **b–g** represents a treated mouse. Horizontal bars indicate median values. Unpaired two-tailed *t* tests *P* values are shown. Fraction of PD1-expressing naïve (CD62L⁺CD44⁺), effector (CD62L⁻CD44⁺) and memory (CD62L⁺CD44⁺) **h**) CD4 and **i**) CD8 splenic T-cells from non tumor bearing or Vk12598 tumor bearing mice harvested two days after treatment with vehicle, Cy at 100mg/kg, LCL161 at 50mg/kg or the combination. Each dot represents a treated mouse. Vertical bars show mean values with standard deviation. *P* values for unpaired *t* test are shown. Expression of PD-L1 evaluated by flow cytometry on splenic **j**) CD11b⁺ or **k**) CD138⁺ cells from Vk*12598 tumor bearing mice two days after treatment with vehicle or LCL161 at 50mg/kg. Each dot represents a treated mouse. Horizontal bars indicate median values. Unpaired two-tailed *t* tests *P* values are shown. **l**) Kaplan-Meier survival plot in days from the initiation of treatment of Vk12598 tumor bearing mice receiving vehicle, Cy at 100mg/kg on day 1,8 or LCL161 at 50mg/kg + rat IgG or aPD1 at 10mg/kg given on day 1,4,8,11 or + Cy. Number of treated mice (*n*) and *P* values for Log-rank (Mantel-Cox) test are shown.