In vivo murine model of acquired resistance in myeloma reveals differential mechanisms for lenalidomide and pomalidomide in combination with dexamethasone

Short Title: In vivo model of IMiDs resistance in myeloma

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ABSTRACT

The development of resistance to therapy is unavoidable in the history of multiple myeloma (MM) patients. Therefore, the study of its characteristics and mechanisms is critical in the search for novel therapeutic approaches to overcome it. This effort is hampered by the absence of appropriate preclinical models, especially those mimicking acquired resistance. Here we present an *in vivo* model of acquired resistance based on the continuous treatment of mice bearing subcutaneous MM1S plasmacytomas. Xenografts acquired resistance to two generations of IMiD® (lenalidomide and pomalidomide) in combination with dexamethasone, that was reversible after a wash-out period. Furthermore, lenalidomide-dexamethasone (LD) or pomalidomide-dexamethasone (PD) did not display cross-resistance, what could be due to the differential requirements of the key target Cereblon and its substrates Aiolos and Ikaros observed in cells resistant to each combination. Differential gene expression profiles of LD and PD could also explain the absence of cross-resistance. Onset of resistance to both combinations was accompanied by upregulation of the MEK/ERK pathway, and addition of selumetinib, a small molecule MEK inhibitor, could resensitize resistant cells. Our results provide insights into the mechanisms of acquired resistance to lenalidomide- and pomalidomide-dexamethasone combinations, and offer possible therapeutic approaches to addressing IMiD resistance in the clinic.

INTRODUCTION

Treatment of multiple myeloma (MM) has undergone significant advances in the last decade, led by the discovery and development of novel agents such as proteasome inhibitors and IMiDs® immunomodulatory agents, which have at least doubled the previous 2-3 year median survival.(1, 2) Moreover, many new targeted agents are also being investigated to improve MM patient outcome further.(3, 4) However, MM is still considered an incurable disease mainly due to the development of acquired, that is, in fact, one of the key challenges in the treatment of MM

Two clinically relevant questions arise when considering resistance: the first is whether different agents of the same family present cross-resistance; and the second is whether this resistance is potentially reversible. The first question is especially important in an era in which second or third generations of agents from the same family are being developed. Additionally, reversibility is a critical clinical question as treatment options are currently limited, and once a patient has been exposed and has developed resistance, retreatment with a previously used agent is now advocated, assuming potential resensitization.

The knowledge of the mechanisms of resistance is important to find ways to overcome it. Regarding IMiDs, the discovery of Cereblon as the key binding protein for lenalidomide and pomalidomide has advanced this understanding. Cereblon is a 442 amino acid protein and is a component of the CRL4 E3 ligase complex that also contains DNA damage binding protein 1 (DDB1), regulator of cullins (Roc)-1 and Cullin 4 (Cul4).(5) Recently, multiple groups reported the identification of two substrates of the Cereblon CRL4 E3 ligase, namely Aiolos (encoded by IKZF3) and Ikaros (encoded by IKZF1),(6-8) members of a family of zinc finger transcriptional factors with a well-documented role in lymphoid and myeloid cell lineage fate

46 determination.(9) On the binding of lenalidomide and pomalidomide to Cereblon in
47 MM cells or T cells, there was degradation of both Aiolos and Ikaros resulting in loss of
48 viability of MM cells and enhanced production of IL-2 in T cells, what explain both the
49 cell autonomous and immunostimulatory effects of IMiDs.(10) While there have been
50 inconsistent data defining the role of Cereblon expression to IMiD activity or
51 resistance(11-14), so far no data have been reported for Aiolos and Ikaros expression. *In*
52 *vitro* studies with lenalidomide have shown acquired resistance to this agent to be
53 associated with a decrease in the levels of cereblon (CRBN) protein,(15, 16) or
54 upregulation of IGF-1,(17) IRF4,(15) or Wnt pathways.(18) However, while there are *in*
55 *vivo* murine models of MM able to predict intrinsic drug sensitivity / resistance,(19) *in*
56 *vivo* models of acquired resistance to anti-MM agents are not yet available.

57 Here we report the development of an *in vivo* xenograft model of acquired
58 resistance to lenalidomide and pomalidomide in combination with dexamethasone.
59 Using this model, we observed lack of cross-resistance between the two drug
60 combinations *in vivo*, and reversibility of the acquired resistance. Further exploration of
61 gene expression analysis and the levels of Cereblon, Aiolos and Ikaros, provides
62 evidence of molecular and mechanistic differentiation between the two IMiD drugs. Our
63 results also show common biochemical pathways such as MEK/ERK to be modulated
64 by the two drug combinations, suggesting a potential role of MEK inhibitors in treating
65 acquired resistance to IMiDs therapy.

MATERIAL & METHODS

Cell culture, drugs and antibodies

The MM1S cell line was kindly provided by Steven Rosen (Northwestern University, Chicago, IL) and was cultured as described.(20) Cell culture media, serum, and penicillin-streptomycin were purchased from Invitrogen Corporation (Gaithersburg, MD). Lenalidomide and pomalidomide were provided by Celgene (Summit, NJ); dexamethasone was from Sigma-Aldrich (Madrid, Spain) and Selumetinib (AZD-6244) was from SelleckChem (Munich, Germany).

Development and treatment of the murine model

The development and follow up of the human subcutaneous plasmacytoma model has been previously described.(20) When tumors became palpable, mice were randomized to the control group (receiving only the vehicle, PBS), lenalidomide + dexamethasone (LD) or pomalidomide + dexamethasone (PD) groups. Doses of the agents were: lenalidomide 25 mg/kg Monday to Friday, pomalidomide 7 mg/kg Monday to Friday, and dexamethasone 1 mg/kg Monday and Tuesday.

To obtain cells from tumors responding to treatment, mice bearing large (1,700 mm^3) untreated tumors received 7 days of treatment and were sacrificed afterwards.

Ex vivo analysis of apoptosis

Tumors were excised and mechanically disaggregated to make a cell suspension, and left in culture under the conditions described elsewhere.(20) These cells were then *ex vivo*-incubated with various concentrations of the drugs under study (lenalidomide, pomalidomide, dexamethasone or selumetinib) in six-well plates for 5 days at 37ºC. After treatment, 5 µl Annexin V-FITC (Immunostep, Salamanca, Spain)

were added and cells were incubated for 15 min at room temperature in the dark. Afterwards, 50,000 cells were acquired on a FACScalibur flow cytometer (BD Biosciences) and analyzed with Infinicyt software (Cytognos SL, Spain). Apoptosis was assessed as the percentage of increase in Annexin V positivity as compared with the basal apoptosis in control untreated cells.

Western blot and Immunohistochemistry

The Western blot(21) and immunohistochemistry(22) (IHC) methods have been described. The origins of the various monoclonal antibodies employed in the Western blot analyses were as follows: anti-ERK 1/2, anti-p-ERK 1/2, anti-Ikaros (for both Western blot and IHC) and anti-Aiolos antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p-MEK 1/2, anti-p-RAF and anti-p-AKT, from Cell Signaling (Boston, MA); HRP-conjugated secondary antibodies, from Amersham (Amersham, Buckinghamshire, UK); anti-actin and secondary antibodies (LI-Cor Biosciences, NE). The anti-CRBN antibody (CRBN65) was developed by Celgene(11, 16) and used for both Western blot and IHC. IHC-specific anti-Aiolos antibody was developed by Celgene (unpublished data).

Gene expression profiling

Total RNA from disaggregated cells was extracted using Buffer RLT plus and purified with AllPrep DNA/RNA Mini Kit (both Qiagen, Valencia, CA). RNA integrity was verified with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA labeling, hybridization to Human Gene 1.0 st Array (Affymetrix, CA, USA) and microarray scanning have been previously reported.(23) Unprocessed files were normalized using the RMA algorithm implemented in the Affymetrix Expression

Console and batch effects were adjusted for using the ComBat package in R. Differentially expressed genes were identified using Significant Analysis of Microarrays (SAM), selecting all genes with a value of Q≤0.05. Ingenuity Pathway Analysis (IPA) was used to identify the most relevant biological mechanisms, pathways and functional categories in the data sets of genes selected by statistical analysis.

SNP arrays and data analyses

DNA extraction from selected samples and genome-wide detection of copy number abnormalities (CNA) was performed as previously described.(24) CNA was reported when the three following criteria were achieved: ≥25 markers per segment, 100 kb minimum genomic size and <50% overlap with known copy number variants (CNVs) (Database of Genomic Variants).(25) Differences in CNA size were not considered as specific genomic alterations. A heat map of the CNAs was made using the Broad Institute's Integrative Genomics Viewer (IGV).(26, 27)

RESULTS

Development of a murine model of acquired resistance to lenalidomide-dexamethasome (LD) or pomalidomide-dexamethasone (PD) combinations

CB17-SCID mice bearing a subcutaneous plasmacytoma of MM1S cells were continuously treated with either LD, PD or the vehicle control. These combinations were administered instead of single agent lenalidomide/pomalidomide to reflect clinical practice that uses these schemas in both newly diagnosed and relapsed settings. Treatment was started when tumors became palpable (around ~ 100 mm^3 of median volume) and, as shown in Figure 1A, both treatments were effective, as tumor growth was significantly delayed in treated animals. Nevertheless, after this initial period of sensitivity of approximately 30 days, and despite continued treatment, the tumors started to grow, indicating the development of acquired resistance to both combinations. If we arbitrarily consider a tumor volume of 500 mm^3 as representative of the initiation of the resistant state, tumors of mice treated with LD or PD took longer to attain this volume than did those of the control group; the median values (and ranges) were 17 (3-31), 46 (19-56), 63 (44-92) days for control, LD and PD respectively (Figure 1B). However, once the tumors reached the volume of 500 mm^3 tumor growth kinetics of the treated mice (LD-resistant (RLD) and PD-resistant (RPD)) were similar to those of the untreated control mice, without statistically significant differences, indicating the development of acquired resistance (Figure 1C).

To confirm that cells in the xenografts were truly resistant to the administered treatments, both control and resistant tumors were excised and disaggregated, and the plasma cells obtained were treated *ex vivo* with the respective combinations for 5 days. Apoptosis induction in the cells was analyzed using Annexin V staining by flow

cytometry. While cells from control tumors (previously untreated) were sensitive *ex vivo* to LD or PD, cells from the RLD tumors were significantly more resistant *ex vivo* to LD than control tumors; analogously, RPD cells were also resistant to PD (Figure 1D).

Absence of cross-resistance between lenalidomide-dexamethasone (LD) and pomalidomide-dexamethasone (PD)

We next wanted to investigate whether there was any cross-resistance between the two IMiDs in the MM1S xenograft model. Once the rapidly growing resistant tumors reached a big volume (1,700 mm^3, arbitrarily selected to have truly resistant tumors), the treatments were switched to the alternative combination. As shown in Figure 2A and 2B, RLD and RPD tumors responded to PD and LD combinations, respectively, indicating a lack of cross resistance between the two combinations. In addition, PD was significantly more effective in rescuing resistance to LD than LD was in rescuing PD resistance, as measured by the maximum reduction in tumor volume (Figure 2C) and also by the time to second progression, defined as regrowth to a volume $\geq$ 1,700 mm^3 (median TTP 18 days (LD) vs. 27 days (PD); Figure 2D).

This cross-resistance was also evaluated ex vivo,.and, interestingly, while PD was able to increase the number of apoptotic cells from RLD xenograft derived cells, treatment of RPD xenograft derived cells by LD didn't show reciprocal sensitivity in the *ex vivo* context (Figure 2E). These data indicate that there are likely differences between the effects observed in the *in vivo* and *ex vivo* environments between the two treatments.

Reversibility of the acquired resistance to IMiDs plus dexamethasone *in vivo*

A clinically relevant question is whether acquired resistance to IMiD-dexamethasone combination is permanent, or cells regain sensitivity after a wash-out period without treatment. To address this question, mice that had developed resistance first to one of the combinations and subsequently to the second (what in the clinical setting would be equivalent to a second relapse/progression), were re-challenged with the first combination (either LD or PD). Interestingly, tumors responded again to the treatment against which they had initially developed resistance (Figures 3A and 3B). Again, PD was more potent than LD in terms of reduction in tumor volume (Figure 3C) and time to progression to a volume of $\geq$ 1,700 mm^3 (median TTP 18 days (LD) and 32 (PD); Figure 3D).

The reversibility of the resistance was then evaluated *ex vivo*. Cells from resistant tumors were collected and maintained in culture with media without drugs for 1, 2 or 3 weeks. Subsequently, these RLD and RPD cells were *ex vivo*-treated with LD (Figure 3E) or PD (Figure 3F), respectively, and apoptosis was analyzed by flow cytometry. As previously shown, cells treated immediately after the extraction were quite resistant to LD or PD, although after being left without treatment, they became sensitive again.

Resistance to PD, but not LD, is associated with a significant decrease in Cereblon protein level

In order to determine whether the LD or PD effects observed *in vivo* were due to changes in overall Cereblon levels, we examined the levels of Cereblon protein by both immunohistochemical staining (IHC) and Western blot in control tumors, tumors being treated and still sensitive to both combinations (SLD and SPD) and RLD and RPD extracted tumors and lysates from the representative samples. As shown by both techniques (Figures 4A and 4B, upper panels), Cereblon levels were dramatically

decreased in the RPD tumors; by contrast, this was not the case in RLD tumors for any of the replicate samples when compared to control sensitive tumors.

Degradation of Aiolos and Ikaros in sensitive but not in resistant- tumors

In order to determine whether the LD or PD effects observed *in vivo* on the mouse MM1S xenografts required Cereblon-dependent decrease of either Aiolos and Ikaros, we examined their respective protein levels by both IHC and Western blot in tumors. The drug-sensitive SPD tumors showed lower levels of both Aiolos and Ikaros by both Western (Figure 4A) and IHC (Figure 4B) analyses when compared to the control group. For SLD this was also clear in the IHC but not in the WB studies. In contrast, the overall levels of both Aiolos and Ikaros in the drug-resistant RLD and RPD tumors, returned to those observed in control tumors, consistent with a loss of Cereblon-dependent degradation of these transcription factors. These results demonstrate that degradation of both substrates is linked to activity of both drug combinations and conversely, resistance to either drug combination correlated with a lack of degradation of the substrates.

Genomic characteristics of cells with acquired resistance to IMiDs + dexamethasone

We analyzed the genomic imbalances present in cells from control and resistant tumors using high-density SNP arrays. Cells studied included six untreated controls, seven RLD and five RPD cell lines. Although some genomic differences were present between control and resistant tumor cells, no conclusive copy number abnormalities (CNA) pattern distinctive of the resistant cells could be identified. Overlapping and

differential CNA among the samples are represented in Supplemental Figure 1A and summarized in Supplemental Table 1.

To gain a better understanding of the mechanisms of resistance associated with both drug combinations, we investigated changes in gene expression profiles (GEP) derived from *in vivo* treatment with IMiDs + dexamethasone before and after the development of resistance. In the sensitive tumor xenografts, treatment with PD significantly deregulated 7,682 genes while LD only deregulated 1,788 genes. Interestingly, most (95%) of the genes modified by LD were also modified by PD, and only 94 genes were exclusively deregulated by LD (Supplemental Figure 1B). Supplemental tables 2 and 3 summarize the genes commonly deregulated by both treatments, and those genes exclusive of PD respectively.

Subsequently, we examined genes associated with resistance. For this purpose, we compared the genes that were differentially expressed in resistant cells and control tumors and then excluded the genes deregulated by the treatment (in the sensitive tumors, mentioned previously). This approach revealed 451 and 258 genes to be associated with resistance to PD and LD, respectively. In contrast to the sensitive cells, where most LD deregulated genes were also deregulated by PD, only a minority of 64 genes in the resistant cells were commonly deregulated in cells resistant to LD and PD treatments respectively (Supplemental Figure 1C). This finding is consistent with different mechanisms of resistance by these two drug combinations. Supplemental tables 4 and 5 summarize the canonical pathways associated with LD and PD resistance, respectively.

Upregulation of the MEK/ERK pathway in acquired resistance to lenalidomide and pomalidomide

Finally, we decided to explore PI3K/AKT/mTOR and RAS/RAF/MEK/ERK, two of the signaling pathways that have more extensively been associated with MM pathogenesis. While no differences were observed in the levels of p-AKT between control and cells obtained from tumors sensitive and resistant to LD and PD (data not shown), significant changes were observed for pERK1/2 in these different situations (Figure 5A). Early treatment with LD and PD when the tumors were still responding induced the complete abrogation of the basal activation of pERK1/2. Nevertheless, when these cells became resistant to PD, a very significant upregulation of the MEK/ERK pathway was observed. As far as RLD tumor samples is concerned, the levels of pERK1/2 were higher than those observed in responding tumors, but lower than in RPD. This was accompanied by up-regulation of the phosphorylation of MEK1, the ERK1/2 upstream activating kinase.

This result indicates that the activation of the MEK/ERK pathway may be coupled to the mechanisms by which tumor cells could become resistant to IMiDs. Based on this possibility, we hypothesized that the addition of a MEK inhibitor could reverse the acquired resistance to these drugs. This idea was tested in dual settings, *ex vivo* as well as *in vivo*. For the *ex vivo* experiment, cells from control, RLD and RPD tumors were treated with LD or PD (depending on the treatment to which they were resistant) and the MEK inhibitor selumetinib (AZD-6244) alone or in combination, and induction of apoptosis was analyzed by Annexin V staining as before (Figures 5B and 5C). Selumetinib potentiated the activity of both LD and PD in the cells from untreated control tumors. Moreover, the addition of selumetinib overcame the resistance to IMiDs + Dex, partially in the case of RLD and almost completely in the case of cells from RPD tumors. This was confirmed *in vivo* (Figure 5D) by administering the MEK inhibitor to mice bearing tumors resistant to LD (RLD) or PD (RPD). In this

experiment, once the resistant tumors reached a volume of 1,000 mm^3, mice were randomized into three groups: some continued with the same treatment (either LD or PD), others had their treatment changed to selumetinib and, for the remaining ones, selumetinib was added to the LD or PD combination. In the RLD tumors, selumetinib alone (after stopping treatment with LD) was not able to control the tumor growth; however, when selumetinib was added to LD, there was a clear response and a prolongation of the time to progression of approximately 30 days. For the RPD tumors, selumetinib alone had some effect on decreasing tumor growth, which was more evident when mice were treated with the combination of selumetinib + PD.

DISCUSSION

In this study we describe an *in vivo* murine model of acquired resistance to two IMiDs: lenalidomide and pomalidomide, used in combination with dexamethasone to make the model more clinically relevant. Our data suggest that the model may reflect the situation observed in patients: after an initial period of sensitivity, and despite continuing therapy, we observe an increase in tumor volume, indicative of resistance. We demonstrate that these tumors are resistant as the tumor growth kinetics at the time of resistance was not different from that of untreated tumors; moreover, the *ex vivo* treatments of these cells were consistent with the perpetuation of the resistant status outside the host.

An important question in MM treatment is whether second- and third-generation drugs will be able to overcome the resistance to the already approved therapies of the same families. In this regard, carfilzomib, a second-generation proteasome inhibitor induced responses in approximately 20% of bortezomib-refractory patients.(32, 33) With respect to IMiDs, several phase I/II studies have reported that pomalidomide in combination with dexamethasone is able to overcome, at least partially, resistance to lenalidomide-dexamethasone.(34-37) Moreover, a recently published phase III study has demonstrated that pomalidomide-dexamethasone is active in patients refractory to lenalidomide as last line of therapy.(38) Our study provides preclinical confirmation of these data and provides some mechanistic insights into this fact, as in the MM1S xenograft model, both pomalidomide and lenalidomide combined with dexamethasone were able to overcome resistance to the alternative combination.

How does our current understanding of the mechanism of action of IMiDs and the data obtained in the present work help explain the observed phenomena? Although both lenalidomide and pomalidomide bind Cereblon directly,(16) these two drugs are

clinically and mechanistically differentiated from each other in a number of ways. First, previous data have suggested a differential requirement of Cereblon for the activity by lenalidomide and pomalidomide.(16) Our work confirms these observations by showing that, in the *in vivo* setting, while RPD xenografts have a significant reduction in Cereblon levels, RLD tumors just have a slight decrease of this protein. In addition, we observed a decrease of Aiolos and Ikaros when tumors were sensitive and a return to basal levels when they became resistant to either drug combination. The main concern here is how do the RPD xenografts respond to LD when Cereblon levels don't appear to be enough to support this response. One likely possibility is the involvement of microenvironment in mediating tumor killing. Although CB17-SCID mice can't mount adaptive immune response, they possess normal innate immunity. It is known that both lenalidomide and pomalidomide enhance NK cell number and functionality and thus could contribute to immune mediated killing of tumor cells. In fact, supporting this hypothesis, we still observed ex vivo cross resistance in the RPD tumors, as they were not rescued by LD in this setting, out of the xenograft. Another intriguing possibility is that lenalidomide might use a novel non-Cereblon target in the RPD xenografts to mediate its activity. Secondly, the gene expression pattern of these two combinations in our model displayed differential changes both in the number of genes and in the magnitude of gene expression level, indicating a mechanistic difference. Whether the kinetics and the extent of Aiolos and Ikaros degradation between lenalidomide and pomalidomide can explain this difference, remains an active area of research.

We have referred above to one possible mechanism of acquired resistance to IMiDs by loss of cereblon.(39) We consider two additional, not mutually exclusive, mechanisms that may underlie the development of acquired resistance. The first concerns the presence of clonal tides and treatment-induced selection of resistant

clones.(40, 41) Our results are not conclusive in this regard, since no significant genomic heterogeneity was observed among the different control and resistant cells. Additional genomic exploration is necessary to address this question more thoroughly. The second possible explanation for the development of resistance is the activation of other pathways known to induce drug-resistance. In this context, we have demonstrated the activation of the MEK/ERK pathway in the resistant cells, and also the abrogation of resistance upon adding an inhibitor of this pathway. This pathway could be a potential target for intervention by which acquired IMiD resistance may be avoided or overcome. Indeed, the MEK inhibitor selumetinib is already being tested in clinical trials in MM (NCT01085214).(42)

A final important observation is that the resistance in our model is reversible after a wash-out period without treatment; this may also be consistent with the clonal tides hypothesis through the re-emergence of a sensitive clone, or alternatively may occur by switching off some of the acquired mechanisms of resistance, such as the activation of the MEK/ERK pathway.

In conclusion, we have developed an *in vivo* model that enables the study of the characteristics and mechanisms of acquired resistance to several anti-MM agents. Our results support not only the treatment with second-generation IMiDs in patients who have developed resistance to lenalidomide, but also the retreatment of refractory patients after a wash-out period during which they are not exposed to IMiDs. Finally, our mechanistic results favor their combination with MEK inhibitors to overcome IMiD resistance.

Supplementary information is available at Leukemia's website

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506

Figure Legends

Figure 1. Development of the *in vivo* model of acquired resistance to IMiDs + dexamethasone

CB17-SCID mice bearing a subcutaneous MM1S-plasmacytoma were continuously treated (via ip) with vehicle control (control, n=13), lenalidomide + dexamethasone (LD, n=10) or pomalidomide + dexamethasone (PD, n=12) at the following doses: lenalidomide 25 mg/kg Monday to Friday, pomalidomide 7 mg/kg Monday to Friday and dexamethasone 1 mg/kg on Monday and Tuesday. The figure shows the results of three independent experiments. A) Evolution of tumor volume of the three groups of mice. B) Time to reach a volume of 500 mm^3 by the three groups of mice, evaluated by a Kaplan–Meier curve. Statistical differences were assessed using the log rank test. C) Tumor growth kinetics of the three groups of mice -control, resistant to LD (RLD), and resitant to PD (RPD)) upon attaining a volume of 500 mm^3. Differences in tumor volume at each point were assessed with Student's t test. D) Evaluation of the sensitivity of cells from untreated control, RLD and RPD tumors to 5 days *ex vivo* treatment with LD or PD, as indicated. Doses of drugs used were: lenalidomide 10 μM (Len), pomalidomide 10 μM (Pom) and dexamethasone 10 nM (Dex). Apoptosis induction was analyzed with Annexin V staining by flow cytometry and statistically differences were identified using Student's t test.

Figure 2. Evaluation of potential cross-resistance between lenalidomide and pomalidomide

Once mice developed resistance to one combination and tumors reached a volume of 1,700 mm^3, treatment of some mice was switched to the alternative combination. For the purpose of comparison, the day of change of treatment is considered as day 1 and is

indicated with an arrow. A) Comparison of tumor volume evolution of mice with tumors resistant to LD that continued this treatment (n=5) and mice whose treatment was changed to PD (n=5). B) Comparison of tumor volume evolution of mice with tumors resistant to PD that continued this treatment (n=5) and mice whose treatment was changed to LD (n=5). C) Maximum reduction in tumor volume after switching treatment from PD to LD (left) and from LD to PD (right). Statistical differences were identified by Student's t test. D) Time to progression to >1,700 mm^3, defined as the time from the day of treatment switch (day 1) to the day in which tumors reached 1,700 mm^3 again, in the same groups of mice. Statistical differences between the Kaplan–Meier curves were assessed using the log rank test. E) Ex vivo evaluation of the cross-resistance of LD and PD. Cells from untreated control, RLD and RPD tumors were *ex vivo* treated for 5 days with LD or PD, as indicated. Doses of drugs used were: lenalidomide 10 μM (Len), pomalidomide 10 μM (Pom) and dexamethasone 10 nM (Dex). Apoptosis induction was analyzed with Annexin V staining by flow cytometry.

Figure 3. Evaluation of the potential reversibility of LD and PD resistance

Mice that had subsequently developed resistance to both combinations, first to one and then the other, were treated with the initial combination when tumors attained a volume of 1,700 mm^3. For the purpose of comparison, the day of change of treatment is considered as day 1 and is indicated with an arrow. A) Comparison of tumor volume of mice that had developed resistance to LD then to PD that continued this latter treatment (n=5) and mice switched to receive LD again after developing second resistance (n=5) on day 1. B) Comparison of tumor volume of mice that had developed resistance to PD then to LD that continued this latter treatment (n=5) and mice switched to receive PD again after developing second resistance (n=5) on day 1. C) Maximum reduction in

558 tumor volume after switching treatment from double-resistant mice (LD-PD) to LD
559 (left) and from double resistant mice (PD-LD) to PD (right). Statistical differences were
560 assessed by Student's t test. D) Time to progression to >1,700 mm^3, defined as the time
561 from the day of treatment switch (day 1) to the day when tumors attained 1,700 mm^3
562 once more, in the same groups of mice. Statistically significant differences between the
563 Kaplan–Meier curves were assessed using the log rank test. E and F) *Ex vivo* evaluation
564 of the reversibility of resistance. E) Cells obtained from mice bearing tumors resistant to
565 LD (RLD, n=3) were left in culture without treatment for 0, 7, 14 and 21 days. After
566 these times, they were *ex vivo*-treated with lenalidomide 10 μM and dexamethasone 10
567 nM (Len-Dex) for 5 days. F) Cells obtained from mice bearing PD-resistant tumors
568 (RPD, n=3) were left in culture without treatment for 0, 7, 14 and 21 days. After these
569 times, they were *ex vivo*-treated with pomalidomide 10 μM and dexamethasone 10 nM
570 (Pom-Dex) for 5 days. For E & F, apoptosis induction was analyzed by Annexin V
571 staining using flow cytometry.
572
573 **Figure 4. Western blot and IHC analysis of CRBN, Aiolos and Ikaros**
574 A) Expression of CRBN, Aiolos, Ikaros and actin by Western blot in cells from control
575 tumors, tumors sensitive to LD (SLD), and PD (SPD), and LD-resistant (RLD) and PD-
576 resistant (RPD) tumors. (B) IHC staining of CRBN, Aiolos and Ikaros in control
577 tumors, tumors sensitive to LD (SLD), and to PD (SPD), and LD-resistant (RLD) and
578 PD-resistant (RPD) tumors. Actin was used as loading control
579
580 **Figure 5. Evaluation of the role of the MEK/ERK pathway in IMiDs + Dex**
581 **resistance**

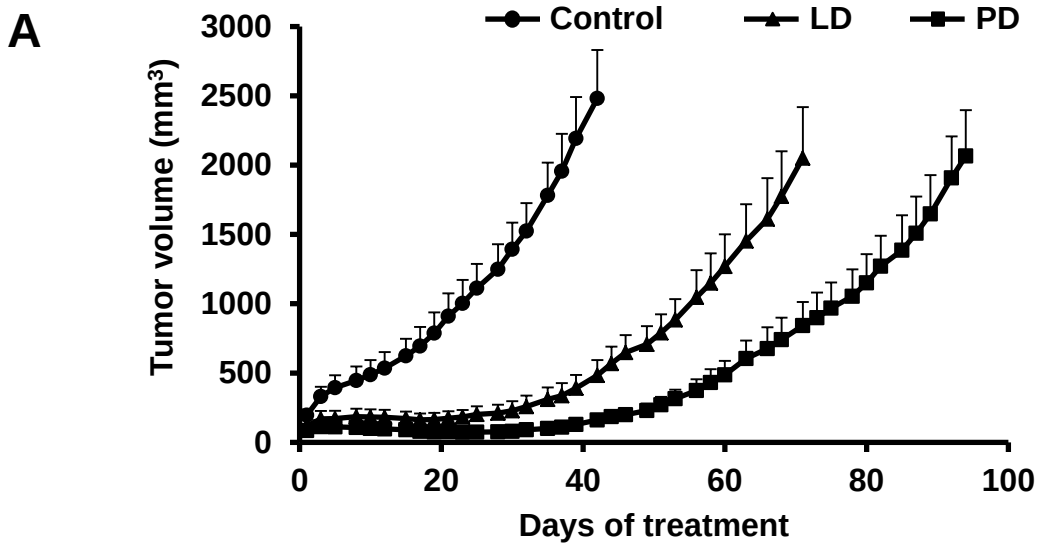
A) Western blot analysis of the indicated components of the MEK/ERK pathway in cells from untreated control tumors, tumors treated and responding to LD and PD, and LD-resistant (RLD) and PD-resistant (RPD) tumors. B and C) Cells from control and RLD (B) or RPD (C) tumors were *ex vivo*-treated with the respective IMiDs + Dex combination (IMiDs 10 μM and dexamethasone 10 nM) with or without the MEK inhibitor Selumetinib (two pulses of 100 nM on days 1 and 3) for 5 days. Apoptosis induction was analyzed by Annexin V staining using flow cytometry and calculated as percentage of increase with respect to the untreated control. D) Evaluation of the *in vivo* role of the MEK inhibitor Selumetinib in overcoming resistance to IMiDs + Dex. For this purpose, when tumors developed resistance to LD or PD and reached 1,000 mm^3, some mice continued with the same treatment (LD and PD, n=5 for both groups); in some others, the treatment was changed to receive Selumetinib at a dose of 100 mg/kg p.o. from Monday to Friday (RLD → Selumetinib, or RPD → Selumetinib, n=4 for both groups); in the remaining group, the same dose of Selumetinib was added to LD or PD (RLD → LD + Selumetinib or RPD → PD + Selumetinib, n=4 for both groups). The figure shows the evolution of the percentage of tumor growth since the change of treatment. The volume corresponding to the day of treatment change was set at 100%. Arrows indicate the day of treatment change.

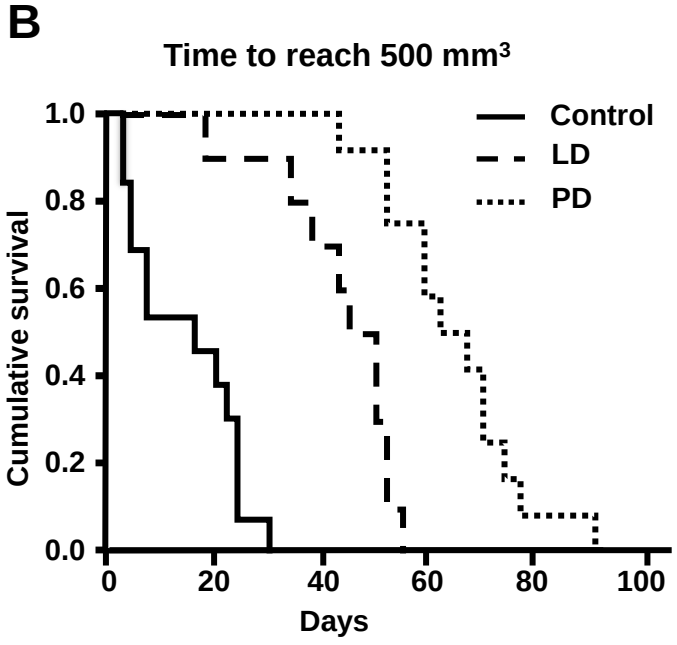
Supplemental Figure 1. Copy number abnormalities and gene expression profiling associated with response and resistance to LD and PD

A) Heat map of common and differential chromosomal imbalances among the various sample tumors. SNP-array analysis of cells excised from 18 tumors (6 untreated control, 7 RLD and 5 RPD tumors). Copy number gains and losses in the 22 autosomes are shown in shades of red and blue, respectively. B) Venn diagram showing the number of

genes commonly and specifically deregulated in tumors under treatment and responding to PD (SPD) and LD (SLD). C) Venn diagram showing the number of genes commonly and specifically deregulated in PD-resistant (RPD) and LD-resistant (RLD) tumors.

Figure 1





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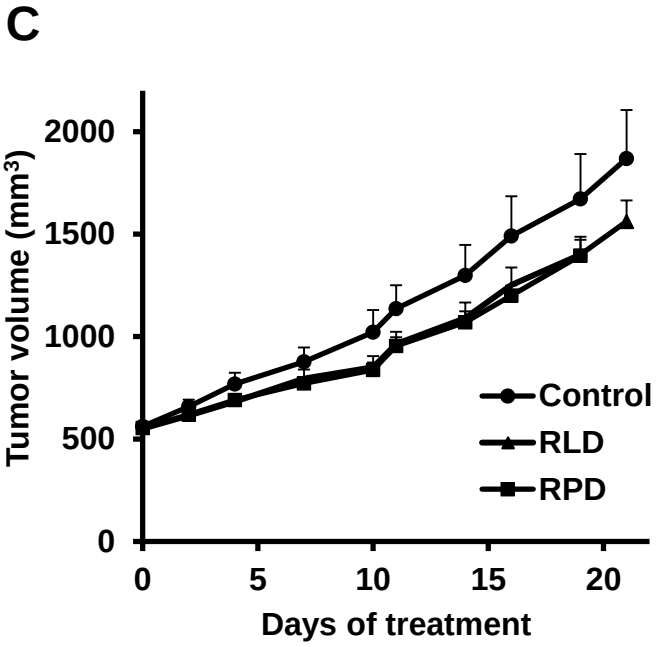


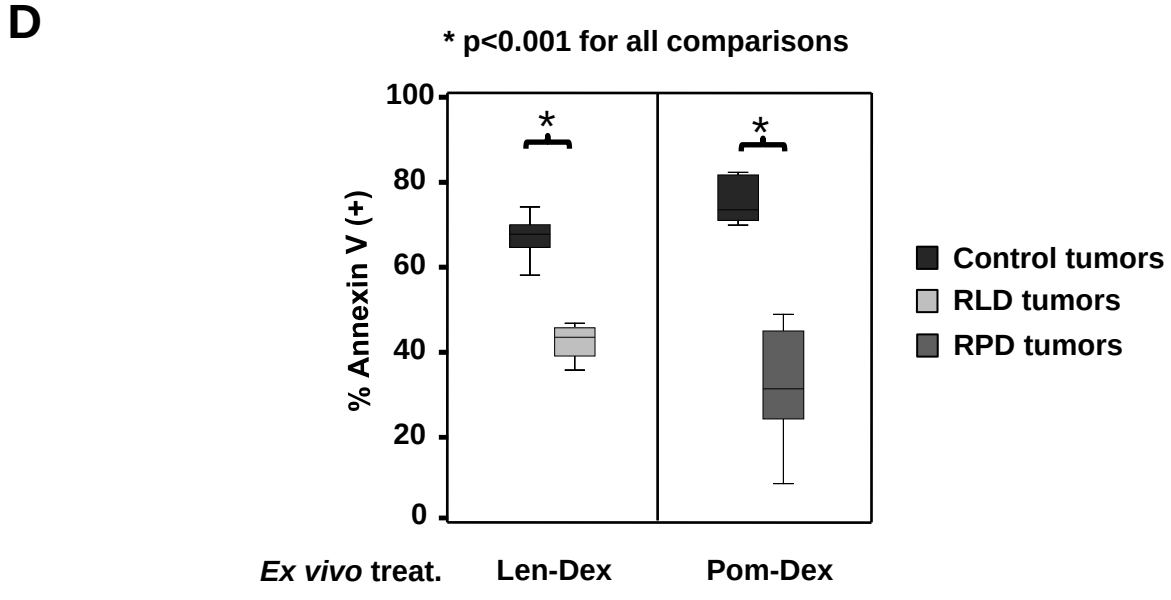


Figure 2

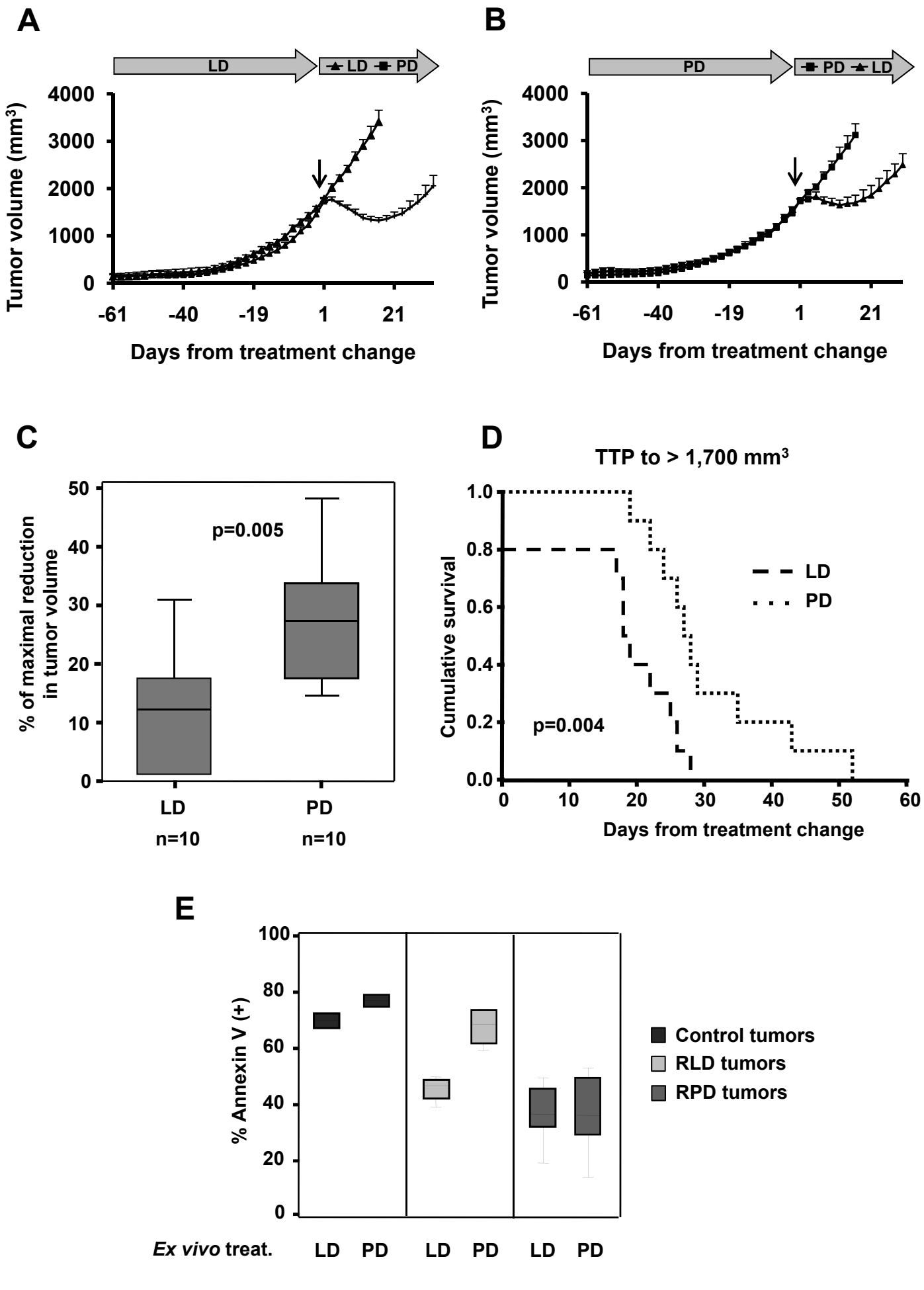

A
LD
LD
PD
Tumor volume (mm3)
4000
3000
2000
1000
0
-61
-40
-19
1
21
Days from treatment change
B
PD
PD
LD
Tumor volume (mm3)
4000
3000
2000
1000
0
-61
-40
-19
1
21
Days from treatment change
C
% of maximal reduction in tumor volume
50
40
30
20
10
0
p=0.005
LD
n=10
PD
n=10
D
TTP to > 1,700 mm3
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
LD
PD
p=0.004
0
10
20
30
40
50
60
Days from treatment change
E
% Annexin V (+)
100
80
60
40
20
0
Control tumors
RLD tumors
RPD tumors
Ex vivo treat.
LD
PD
LD
PD
LD
PD

Figure 3

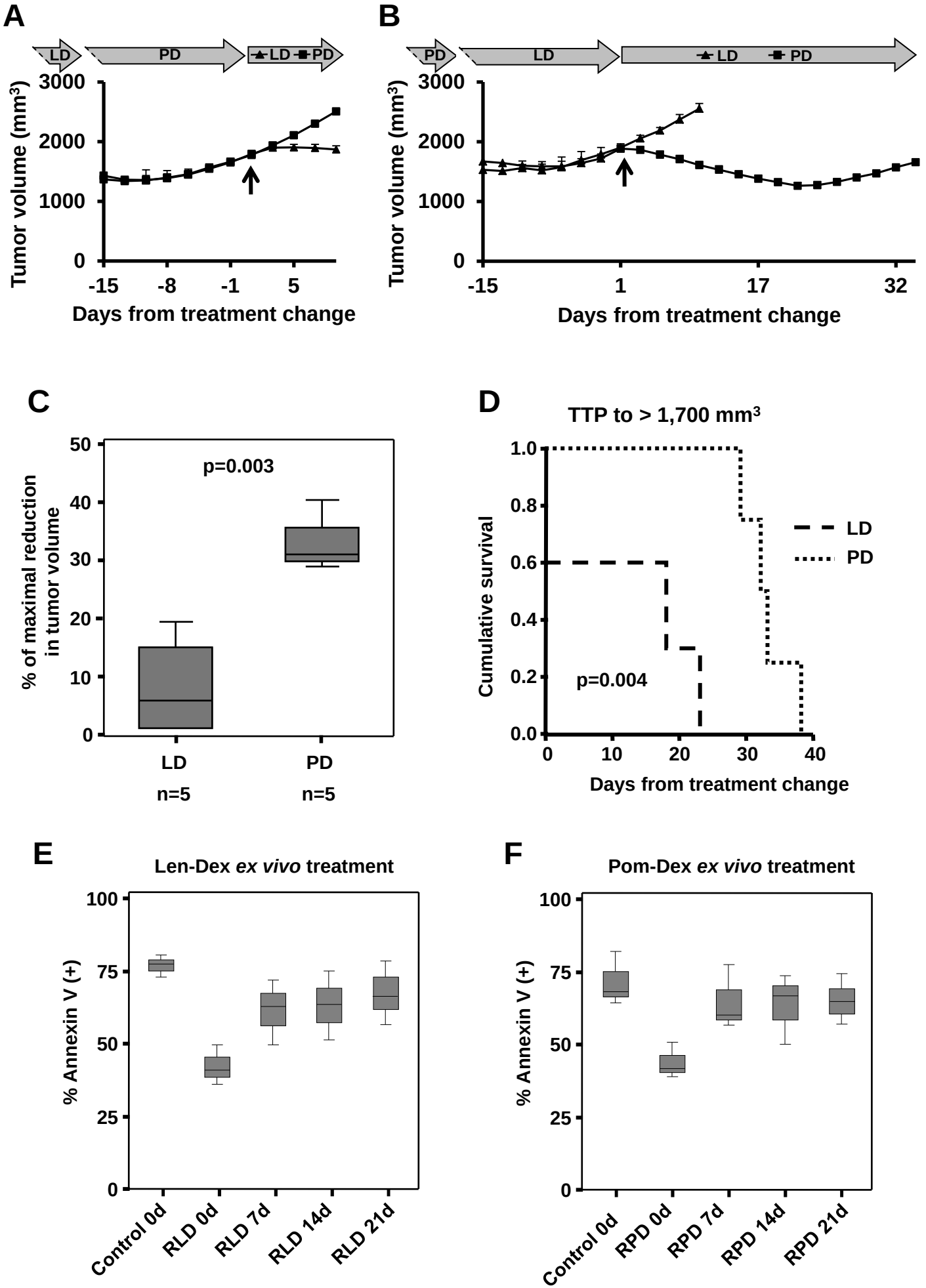
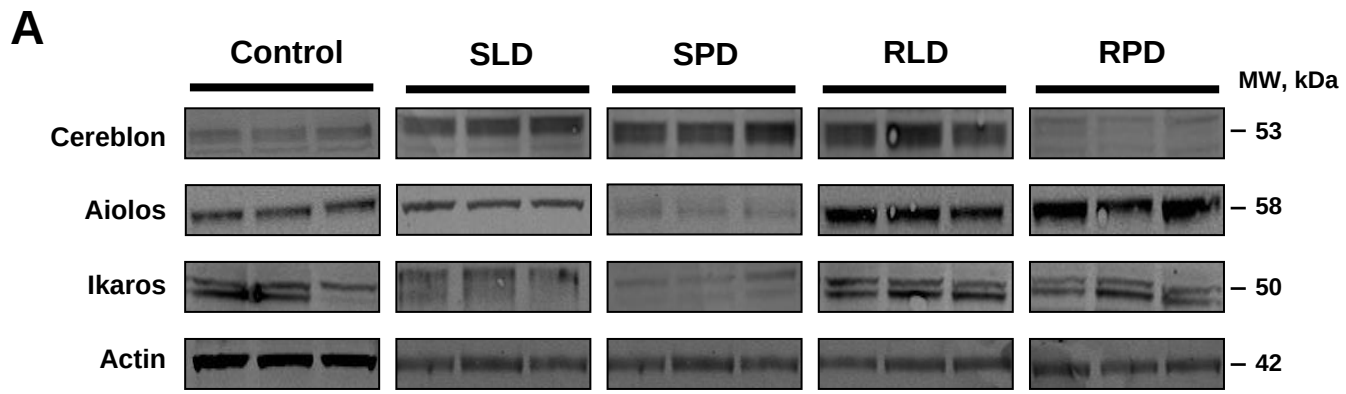

A
LD
PD
LD
PD
Tumor volume (mm³)
3000
2000
1000
0
-15
-8
-1
5
Days from treatment change
B
PD
LD
LD
PD
Tumor volume (mm³)
3000
2000
1000
0
-15
1
17
32
Days from treatment change
C
p=0.003
% of maximal reduction in tumor volume
50
40
30
20
10
0
LD
n=5
PD
n=5
D
TTP to > 1,700 mm³
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
p=0.004
LD
PD
0
10
20
30
40
Days from treatment change
E
Len-Dex ex vivo treatment
% Annexin V (+)
100
75
50
25
0
Control 0d
RLD 0d
RLD 7d
RLD 14d
RLD 21d
F
Pom-Dex ex vivo treatment
% Annexin V (+)
100
75
50
25
0
Control 0d
RPD 0d
RPD 7d
RPD 14d
RPD 21d

Figure 4


A
Control
SLD
SPD
RLD
RPD
MW, kDa
Cereblon
Aiolos
Ikaros
Actin
53
58
50
42

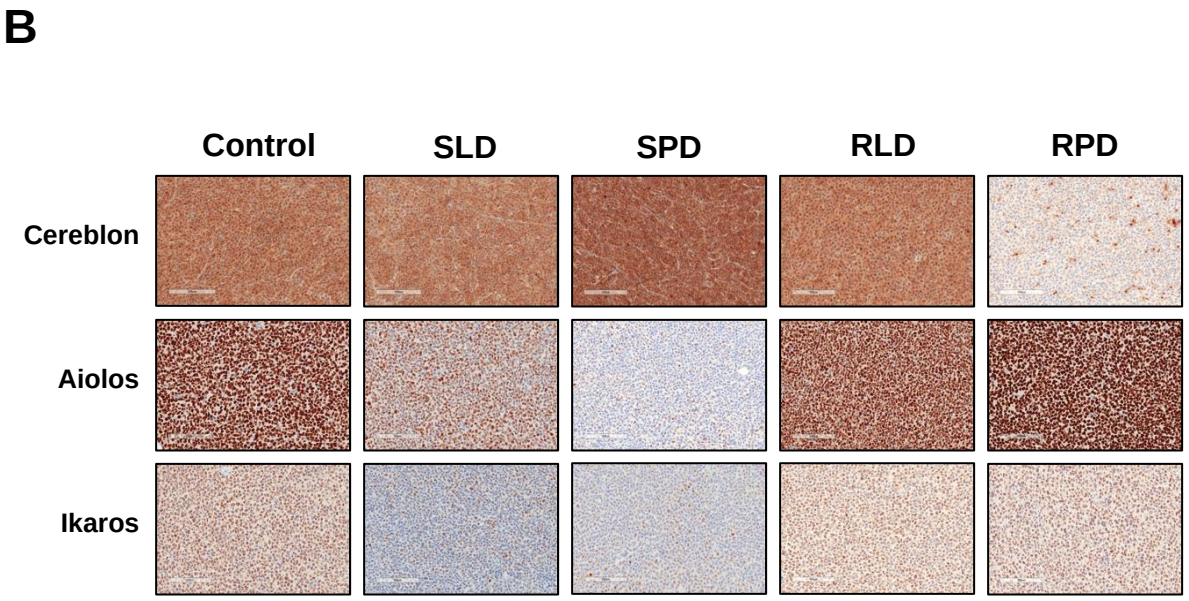
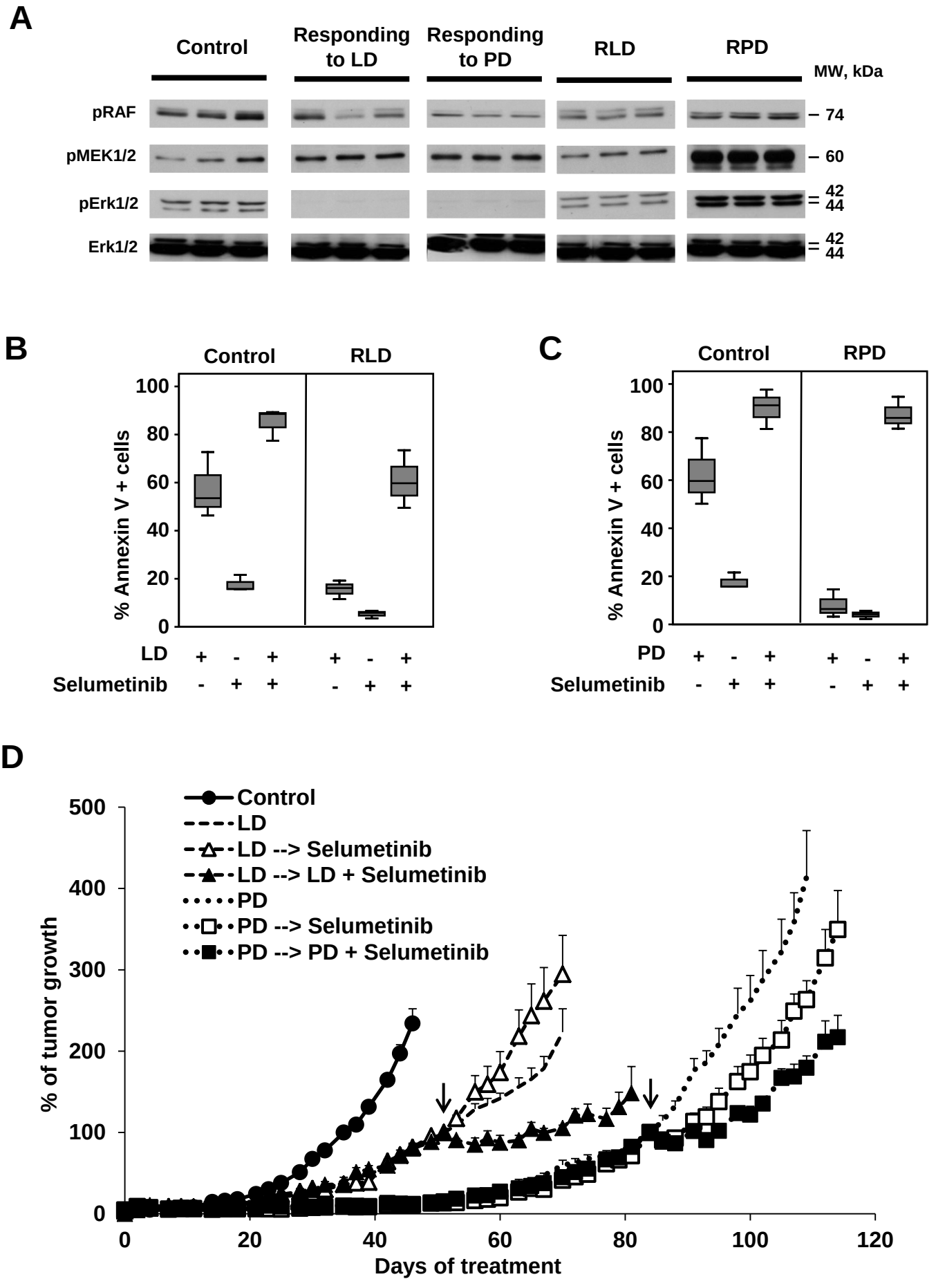

B
Control
SLD
SPD
RLD
RPD
Cereblon
Aiolos
Ikaros

Figure 5


A
Control
Responding to LD
Responding to PD
RLD
RPD
MW, kDa
pRAF
pMEK1/2
pErk1/2
Erk1/2
74
60
42 44
42 44
B
Control
RLD
% Annexin V + cells
LD
Selumetinib
C
Control
RPD
% Annexin V + cells
PD
Selumetinib
D
Control
LD
LD --> Selumetinib
LD --> LD + Selumetinib
PD
PD --> Selumetinib
PD --> PD + Selumetinib
% of tumor growth
Days of treatment