either as false negatives of the PCR or as false positives of MFC. We can thus conclude that the junction region of the IgH rearrangement in MM is stable and can be used as a target for MRD assessment by ASO RQ-PCR and more, also by deep-sequencing methods, as it constantly identifies the myeloma cells responsible for relapse.[15]

In conclusion, our results show that, in the dominant myeloma clone, the CDR3 region of *IGH* remains constant across all the stages of disease evolution. This major clone signature is not modified by clinical or biological changes in the disease nor under different treatment pressures; accordingly, it would thus be responsible for disease relapses and progression, and could be used as a MRD target. Assuming that the CDR3 region remains stable, the recently raised concept of clonal tiding in MM should not be interpreted as a poly/oligoclonal but subclonal. In summary, in MM tides can be subclonal, but the ocean remains monoclonal.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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N Puig[1,2,3], I Conde[1,2,3], C Jiménez[1,2,3], ME Sarasquete[1,2,3], A Balanzategui[1,2,3], M Alcoceba[1,2,3], J Quintero[4], MC Chillón[1,2,3], E Sebastián[1,2,3], R Corral[1,2,3], L Marín[1,2,3], NC Gutiérrez[1,2,3], M-V Mateos[1,2,3], M González-Díaz[1,2,3], JF San-Miguel[5] and R García-Sanz[1,2,3]

[1]*Department of Hematology, University Hospital of Salamanca, Salamanca, Spain;*
[2]*IBSAL, Salamanca, Spain;*
[3]*IBMCC (USAL-CSIC), Salamanca, Spain;*
[4]*Department of Hematology, Hospital Miguel Servet, Zaragoza, Spain and*
[5]*Clínica Universidad de Navarra, Centro de Investigación Médica Aplicada (CIMA), Pamplona, Spain*
E-mail: margondi@usal.es

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OPEN

CDK9 inhibition by dinaciclib potently suppresses Mcl-1 to induce durable apoptotic responses in aggressive MYC-driven B-cell lymphoma *in vivo*

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MYC dysregulation confers a poor prognosis to diffuse large B-cell lymphoma (DLBCL), and effective therapeutic strategies are lacking in relapsed/refractory DLBCL, Burkitt lymphoma and intermediate forms.[1,2] As a master transcriptional regulator, MYC recruits transcription complexes containing RNA polymerase II (Pol II) to facilitate effective transcriptional elongation of MYC gene targets.[3] Pol II is fully activated by phosphorylation of a critical serine residue at position 2 within heptapeptide repeats in the carboxy-terminal domain (CTD), a function performed by the positive transcription elongation factor b (P-TEFb; comprising CDK9 and cyclin T1).[4] It has been shown that MYC binds and

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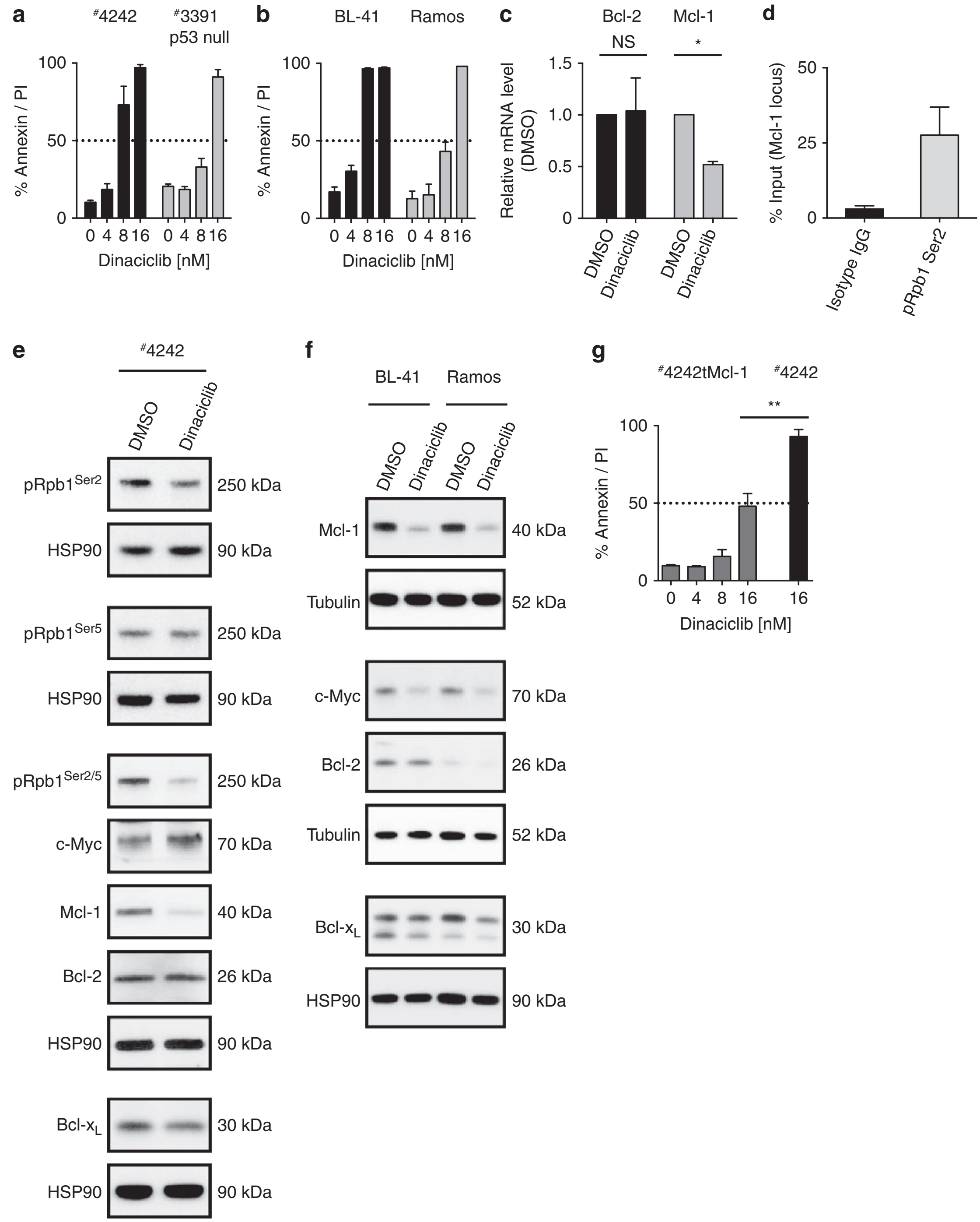

Figure 1. Dinaciclib potently induces apoptosis of murine Eμ-*Myc* and human *IG-cMYC*-translocated lymphomas with rapid and selective suppression of Mcl-1 transcription and protein levels. (**a**) Wild-type p53 (#4242) and p53-null (#3391) Eμ-*Myc* lymphomas were cultured *in vitro* with dimethylsulfoxide (DMSO) vehicle control or dinaciclib for 24 h and then analyzed using flow cytometric analysis for annexin-V/propidium iodide (PI) positivity. (**b**) Human *IG-cMYC*-translocated BL-41 and Ramos cell lines were cultured *in vitro* with DMSO or dinaciclib for 48 h before the analysis of annexin-V/PI positivity using flow cytometry. (**c**) Mcl-1 and Bcl-2 mRNA expression in lymphoma #4242 following 3-h *in vitro* treatment with DMSO or 20 nM dinaciclib. Transcript levels are represented as fold change compared with DMSO. NS, not significant; *$P < 0.0001$. (**d**) Chromatin immunoprecipitation-PCR of Eμ-*Myc* lymphoma #4242 cells showing binding of phospho-RNA Pol II CTD serine 2 (pRpb1 Ser2) at the *Mcl-1* locus. Error bars denote the s.e.m. from three independent primer sets across the *Mcl-1* locus. (**e**) Eμ-*Myc* lymphoma #4242 was cultured *in vitro* for 3-h untreated or in the presence of DMSO or 20 nM dinaciclib before the preparation of lysates and immunoblotting for phospho-RNA Pol II CTD ($pRpb1^{Ser2}$, $pRpb1^{Ser5}$ and $pRpb1^{Ser2/5}$), total Mcl-1, Bcl-2, Bcl-x_L, c-Myc and HSP90 loading control. (**f**) Human *IG-cMYC*-translocated BL-41 and Ramos cell lines were cultured *in vitro* for 3 h in the presence of DMSO or 20 nM dinaciclib before the preparation of lysates and immunoblotting for total Mcl-1, Bcl-2, Bcl-x_L, c-Myc, Tubulin and HSP90 loading controls. (**g**) Eμ-*Myc* lymphoma #4242 was transduced with murine stem cell virus expressing empty vector control or *Mcl-1* and then cultured *in vitro* with dinaciclib for 24 h before flow cytometric analysis for annexin-V/PI positivity. **$P < 0.01$ comparing treatments at 16 nM concentration. All graphs represent the mean ± s.e.m (error bars) for three or more independent experiments.

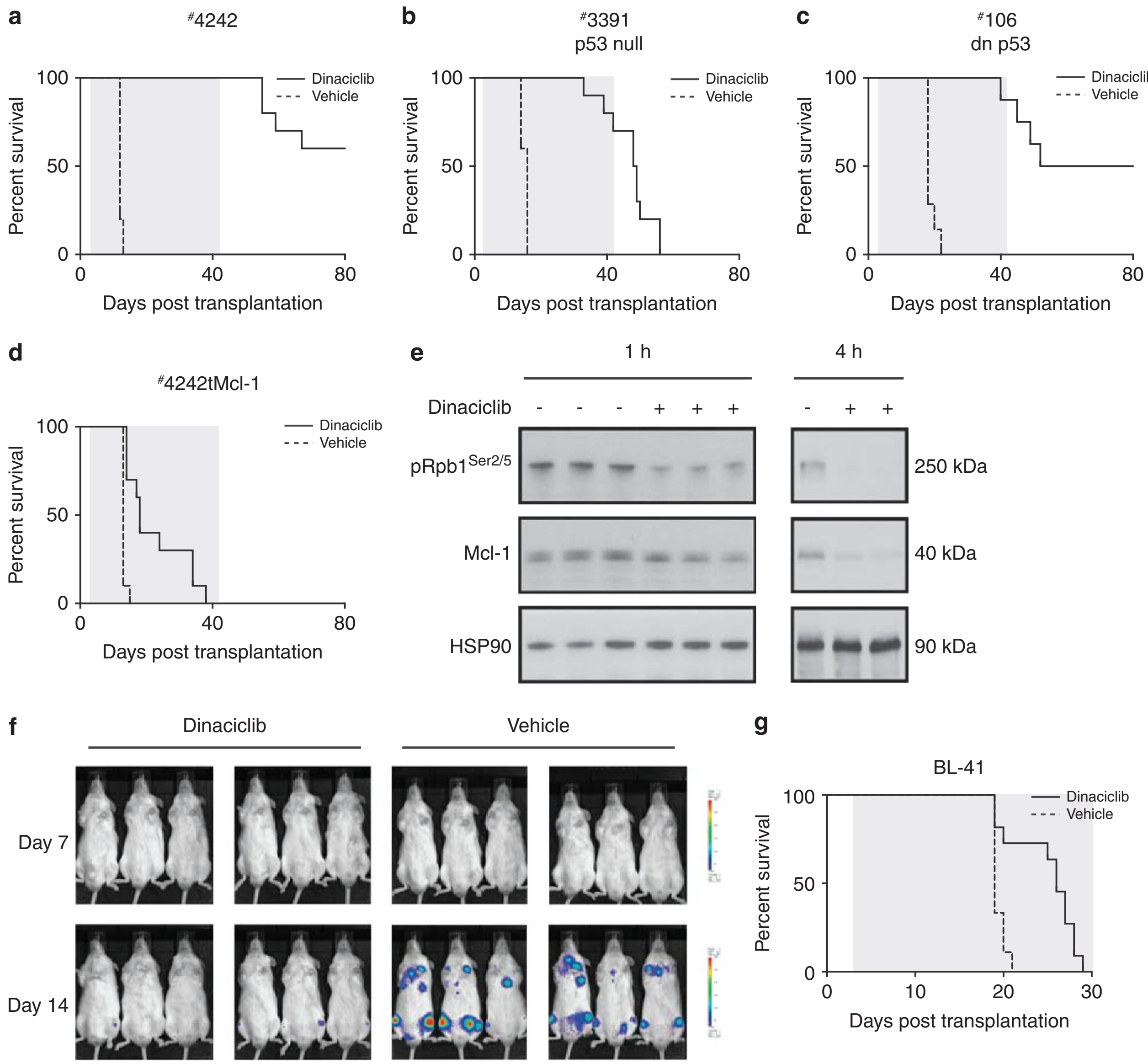

Figure 2. Dinaciclib therapy prolongs the survival of mice bearing Eμ-*Myc* and human *IG-cMYC*-translocated lymphomas. (**a**–**d**) Kaplan–Meier survival curves representing cohorts of C57Bl/6 mice transplanted with representative Eμ-*Myc* lymphomas 3 days before the therapy commencement with 20% hydroxypropyl-beta-cyclodextran (HPBCD) vehicle or 30 mg/kg dinaciclib by intraperitoneal injection twice weekly. Gray shading denotes the period of therapy. dn, dominant negative; $P < 0.0001$ for each experiment. The median survival for vehicle- and dinaciclib-treated mice were 12 days and not reached (#4242), 16 and 48 days (#3391), 18 and 66 days (#106) and 13 and 18 days (#4242tMcl-1), respectively. (**e**) Lymph nodes were harvested from cohorts of C57Bl/6 mice 1 or 4 h following a single dose of dinaciclib or 20% HPBCD, 12 days following transplantation with Eμ-*Myc* lymphoma #4242. Protein lysates were then prepared and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before immunoblotting for the indicated targets. Each lane represents protein lysate from the lymph nodes of an individual mouse. (**f**) Bioluminescence imaging of NOD-scid IL2Rγ^{null} mice transplanted with human *IG-cMYC*-translocated BL-41.*luc2* lymphoma 3 days before the commencement of the therapy with 20% HPBCD vehicle or 30 mg/kg dinaciclib by intraperitoneal injection twice weekly. Mice were imaged at 7 and 14 days post transplantation. (**g**) Overall survival of the mice from the experiment is described in **f**. Gray shading denotes the period of therapy. The median survival for vehicle and dinaciclib-treated mice were 19 and 26 days, respectively ($P < 0.001$).

recruits P-TEFb to its targets as a means to activate Pol II.[3,5,6] More recently, CDK9-mediated transcriptional elongation was reported as essential for tumor maintenance in a genetically defined MYC-driven model of hepatocellular carcinoma.[7] Thus, CDK9 dependence may represent a druggable vulnerability in lymphomas with dysregulated MYC expression.

Dinaciclib (Merck, Boston, MA, USA) is a novel CDK inhibitor that has reached phase 1b/2 of clinical trials for a range of solid-organ malignancies, as well as for myeloma and chronic lymphocytic leukemia.[8] We hypothesized that CDK9 inhibition by dinaciclib would represent a rational pharmacologic approach to target the transcription of critical MYC-regulated oncogenic effector proteins. Here we describe durable *in vivo* responses to dinaciclib in aggressive MYC-driven lymphoma, mediated by downregulation of Pol II-mediated Mcl-1 transcription.

Dinaciclib has 50% kinase inhibitory concentrations of 1, 1, 3 and 4 nM for CDK2, CDK5, CDK1 and CDK9, respectively.[8] Dinaciclib potently killed Eμ-*Myc* and human *IG-cMYC*-translocated cell lines independent of p53 function, but not untransformed murine fibroblast cells, at low nanomolar concentrations approximating those observed for kinase inhibition (Figures 1a and b, Supplementary Figure S1).

As Bcl-2 and Mcl-1 have been implicated as important apoptotic regulators in Eμ-*Myc* lymphomas,[9,10] we assessed the effects of dinaciclib on these proteins. We hypothesized that CDK9 inhibition with dinaciclib would target Mcl-1 transcription, as has been observed with other CDK inhibitors in myeloma and mantle cell lymphoma.[11,12] Eμ-*Myc* and human *IG-cMYC*-translocated cell lines were treated with dinaciclib or dimethylsulfoxide control and interrogated using the quantitative PCR analysis for the effect on Mcl-1 and Bcl-2 mRNA. Dinaciclib treatment was associated with a significant reduction in Mcl-1 mRNA, with no significant effect on Bcl-2 transcript levels (Figure 1c, Supplementary Figure S2). Chromatin immunoprecipitation-PCR was used to show the binding of phosphorylated Pol II, subunit B1 carboxy-terminal domain (CTD) serine 2 (pRpb1 Ser2) as a marker of CDK9 activity at the *Mcl-1* locus in a representative Eμ-*Myc* lymphoma cell line (Figure 1d). These findings support the hypothesis that dinaciclib transcriptionally downregulates Mcl-1.

We next examined Mcl-1 expression in Eμ-*Myc* and human *IG-cMYC*-translocated lymphoma cell lysates following the treatment with dinaciclib or vehicle. On-target CDK9 inhibition by dinaciclib was confirmed through inhibition of pRpb1 Ser2 at concentrations corresponding to apoptosis induction in Eμ-*Myc* cells (Figure 1e). Dinaciclib treatment also rapidly suppressed Mcl-1 protein expression, with no discernible reduction in Bcl-2 or Bcl-x_L protein observed in murine (Figure 1e) or human (Figure 1f) cells. To determine the functional importance of Mcl-1 in regulating dinaciclib-mediated apoptosis, a representative Eμ-*Myc* lymphoma was stably transduced to express Mcl-1 off a retroviral promoter. As shown in Figure 1g, exogenously expressed Mcl-1 significantly protected Eμ-*Myc* cells from dinaciclib-induced apoptosis.

The *in vivo* efficacy of dinaciclib was then assessed by transplanting the same Eμ-*Myc* lymphomas into cohorts of syngeneic C57Bl/6 recipients. Compared with the vehicle control, dinaciclib treatment was well tolerated and associated with a highly significant survival advantage of tumor-bearing mice, including those bearing a p53-null lymphoma and a lymphoma with a spontaneous p53 mutation encoding a dominant-negative p53 protein (Figures 2a–c, Supplementary Figure S3). In contrast, dinaciclib-mediated therapeutic efficacy was severely attenuated in isogeneic p53-competent Eμ-*Myc* lymphoma overexpressing Mcl-1 (Figure 2d). In separate experiments, mice bearing transplanted Eμ-*Myc* cells were left untreated for 12 days to establish bulky nodal disease, at which time they received a single dose of dinaciclib or vehicle 1 or 4 h before being killed and before the lymph nodes were harvested. Consistent with the *in vitro* data, lymph node protein lysates showed reductions of pRpb1 and total Mcl-1 protein (Figure 2e), concomitant with the induction of apoptosis (Supplementary Figure S4). Finally, dinaciclib treatment of immunocompromised mice xenografted with the human *IG-cMYC*-translocated lymphoma was associated with reduced disease progression and significantly prolonged overall survival (Figures 2f and g).

In conclusion, our findings indicate that CDK9 inhibition by dinaciclib is highly effective in aggressive MYC-driven lymphomas, including ‘poor-risk’ p53-deficient clones, via selective inhibition of critical MYC targets including Mcl-1 (which is currently undruggable with existing BH3 mimetics).[13,14] Our data suggest a linear and druggable dependency between MYC and Mcl-1 that is contingent on CDK9 signaling. These findings are of particular interest in the context of a recent publication by Kelly *et al.*,[15] further highlighting the dependency of MYC-driven B-cell lymphoma to Mcl-1. Rapid clinical translation of CDK9 inhibitors to MYC-dysregulated lymphoid malignancy should now be considered.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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GP Gregory[1,2,3], SJ Hogg[1,2], LM Kats[1,2], E Vidacs[1,2], AJ Baker[1,2], O Gilan[2,4], M Lefebure[1,2], BP Martin[1,2], MA Dawson[2,4], RW Johnstone[1,2,6] and J Shortt[1,2,3,5,6]
[1]*Gene Regulation Laboratory, Research Division, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia;*
[2]*Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, Victoria, Australia;*
[3]*Monash Haematology, Monash Health, Clayton, Victoria, Australia;*
[4]*Cancer Epigenetics Laboratory, Research Division, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia and*
[5]*School of Clinical Sciences at Monash Health, Monash University, Clayton, Victoria, Australia*
E-mail: jake.shortt@petermac.org
[6]*These authors contributed equally to this work.*

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Targeting PD1–PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells

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Despite the advent of bortezomib, thalidomide and lenalidomide, relapse of multiple myeloma (MM) is common, and novel therapies are needed urgently.[1] Interactions of MM cells with bone marrow (BM) accessory and immune effector cells inhibit antitumor immunity as well as induce MM growth, survival and drug resistance.[1] For example, we showed that plasmacytoid dendritic cells (pDCs) are increased in the BM of MM patients compared with normal BM, and these contribute to immune dysfunction, as well as promote tumor cell growth and survival.[2] Aberrant pDCs' function in MM is evidenced by their interaction not only with MM cells but also with immune effector T cells: MM BM pDCs have decreased ability to trigger T-cell proliferation compared with normal pDCs.[2] Dysfunctional T cells and natural killer (NK) cells in MM[3,4] together with functionally defective pDCs[2] confer immune suppression in MM. To date, the mechanism(s) and the role of immunoregulatory molecules mediating pDC–T cell and pDC–NK cell interactions in MM remain undefined. Here we extended our previous studies[2,5] to examine the role of immune checkpoint receptor programmed cell death protein 1 (PD1) and its ligand PDL1 in pDC–T cell and pDC–NK cell interactions in the MM BM milieu, and to determine whether this interaction represents a therapeutic target to restore antitumor immunity and cytotoxicity.

PD1 (CD279), a member of the CD28 family of receptors, is expressed on the surface of antigen-activated and -exhausted T cells.[4] PD1 has two ligands, PDL1 (B7-H1; CD274) and PDL2 (B7-DC; CD273). Although PDL1 expression has not been observed in normal epithelial cells, it is highly expressed on many solid tumors.[6] PDL2 is more broadly expressed on normal healthy tissues than PDL1. The physiological role of PD1 is to maintain T-cell homeostasis by restricting T-cell activation and proliferation, thereby preventing autoimmunity. Importantly, the interaction of PD1+ T cells with PDL1-expressing cells inhibits T-cell responses.[7–9] In the context of MM, studies have demonstrated PD1-expressing T cells and NK cells in the MM BM milieu, as well as PDL1 on MM cells.[3,10–13] However, the expression of PDL1–PD1 on MM patient-derived pDCs and its functional significance during pDC–MM–T–NK cell interactions remain undefined.

We first analyzed freshly isolated MM cells, pDCs and T cells from MM patient BM samples ($n = 11$) for PDL1 and PD1 expression using flow cytometry (fluorescence-activated cell sorter (FACS)). Both MM cells and pDCs expressed high surface levels of PDL1, whereas T cells showed high PD1 levels (Figures 1a–c). No significant PDL1 expression was noted on normal BM plasma cells. Our findings are consistent with previous reports showing that MM cells, but not normal plasma cells, express PDL1.[3,10–13] These data indicate that the interactions between PDL1-expressing MM cells and pDCs with PD1-positive T cells may contribute to both T-cell and pDC immune dysfunction in MM, and MM cells may escape antitumor immunity by virtue of PDL1 expression.

We next examined whether blockade of PDL1–PD1 restores anti-MM immune response and/or affects pDC-induced MM cell growth, using a monoclonal antibody (Ab) specifically directed against PDL1. A recent study analyzed the expression of PD1 and PD1-ligands in the tumor immune microenvironment and demonstrated clinical responses to anti-PD1 Ab therapy in PDL1-positive tumors.[8] PDL1 is expressed in both pDCs and MM cells, including relapsed or refractory MM,[13] and we hypothesize that blockade of PDL1 will alleviate T-cell immune suppression conferred by both MM cells and pDCs during pDC–MM–T cell interactions. Moreover, as PDL1 binds not only to PD1 but also to CD80, on T cells to induce T-cell inhibition,[14] anti-PDL1 Ab may block both co-inhibitory signals on T cells. Preclinical and clinical studies have begun to examine the utility of anti-PDL1 monoclonal Ab in MM.[10,11,15] Here we targeted PDL1 rather than PDL2 for the following reasons: (1) PDL1 is more restricted in its expression on normal tissues than PDL2, and targeting PDL1 may therefore cause less on-target off-tissue toxicity;[9] (2) a recent report correlated PDL1, but not PDL2, expression with response to anti-PD1 therapy;[8] and (3) we found that both pDCs and MM cells express variable and low levels of PDL2 versus PDL1.

We first examined whether blockade of PDL1 affects the ability of pDC to induce MM cell growth. The patient MM cells or MM cell lines (MM.1S, MM.1R and RPMI-8226) were cultured either alone or together with MM–pDCs in the presence or absence of anti-PDL1 Ab for 72 h, followed by analysis of growth. pDCs triggered proliferation of autologous MM cells and MM cell lines, as in our previous studies.[2,5] Importantly, anti-PDL1 Ab did not significantly inhibit pDC-triggered growth of MM cells (Figure 1d and Supplementary Figure 1). Our recent study showed that targeting toll-like receptor-9 blocks pDC-induced MM cell growth,[2,5] which served as a positive control in these studies (Figure 1d and Supplementary Figure 1). Although blocking PDL1 does not affect pDC-induced MM cell growth, pDC–MM cell interactions upregulate PDL1 expression on both cell types, consistent with earlier observations that BM stromal cells induce PDL1 expression on MM cells.[13] Such interactive mechanisms enhancing PDL1 expression in the MM BM milieu further abrogate PD1-expressing T-cell

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