A Novel TLR-9 Agonist C792 Inhibits Plasmacytoid Dendritic Cell-induced Myeloma Cell Growth and Enhance Cytotoxicity of Bortezomib

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Abstract

Our prior study in multiple myeloma (MM) patients showed increased numbers of plasmacytoid dendritic cells (pDCs) in the bone marrow (BM) which both contribute to immune dysfunction as well as promote tumor cell growth, survival, and drug resistance. Here we show that a novel Toll-Like Receptor (TLR-9) agonist C792 restores the ability of MM patient-pDCs to stimulate T cell proliferation. Co-culture of pDCs with MM cells induces MM cell growth; and importantly, C792 inhibits pDC-induced MM cell growth and triggers apoptosis. In contrast, treatment of either MM cells or pDCs alone with C792 does not affect the viability of either cell type. In agreement with our in vitro data, C792 inhibits pDC-induced MM cell growth in vivo in a murine xenograft model of human MM. Mechanistic studies show that C792 triggers maturation of pDCs, enhances interferon-α and interferon-λ secretion, and activates TLR-9/MyD88 signaling axis. Finally, C792 enhances the anti-MM activity of bortezomib, lenalidomide, pomalidomide, SAHA, or melphalan. Collectively, our preclinical studies provide the basis for clinical trials of C792, either alone or in combination, to both improve immune function and overcome drug resistance in MM.

Keywords

Myeloma; Immunotherapy; Dendritic cells; pDC; TLR-9; CpG-ODN-C792; Interferons
Introduction

Multiple myeloma (MM) remains incurable due to the development of drug resistance mediated by mechanisms intrinsic to the tumor cells as well as interaction of MM cells with the accessory cells in the bone marrow (BM) microenvironment. BM stromal cells (BMSCs), osteoclasts, osteoblasts, myeloid cells, and immune effector cells i.e., myeloid derived suppressor cells, can promote growth and drug resistance in MM cells. Research efforts are now focused on defining the functional significance of tumor cell interaction with BM accessory cells in the MM niche in order to identify novel therapeutic strategies.

Dendritic cells (DCs) mediate immune function and promote tumor growth. Human DCs have been classified into two major subtypes based on their origin, phenotype, and function: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs have been extensively characterized, and recent studies have also begun to characterize pDCs and their functionality. pDCs express CD123, CD303, CD304 and HLA-DR, and lack lineage cell markers for B, NK, and T cells, as well as monocytes. The antigen presenting function of pDCs is, at least in part, mediated via Toll-Like Receptors (TLRs; TLR7 and TLR9) which recognize viral RNA template or unmethylated bacterial DNA, thereby facilitating secretion of Type I and Type II Interferons (IFN). These pleiotropic cytokines in turn activate multiple components of the immune system including T cells, B cells, and NK cells. Early reports showed that pDCs from MM patients are defective in their antigen-presenting function; indeed, the loss of immune function of tumor-infiltrating DCs has been linked to suppressive effects of the tumor microenvironment in multiple cancers, including MM.

Besides generating an antiviral immune response, pDCs also play a role in normal B cell development into plasmablasts, differentiation into antibody-secreting plasma cells, and survival. In this context, our recent study defined the role of pDCs in regulating growth and survival of malignant plasma (MM) cells. Specifically, we found increased numbers of pDCs in the MM BM microenvironment which both mediate immune deficiency characteristic of MM, as well as promote tumor cell growth, survival, and drug resistance. In the present study, we show that a novel Toll-Like Receptor (TLR-9) agonist C792 both restores pDC immune function and inhibits pDC-induced MM cell growth and drug resistance. Our study provides the basis for targeting pDC-MM interactions using TLR9 agonist C792 as a potential therapeutic strategy in MM.

Material and Methods

Isolation and phenotypic analysis of pDCs

Studies involving patient MM cells were performed following IRB-approved protocols at Dana-Farber Cancer Institute and Brigham and Women’s Hospital (Boston, USA). Informed consent was obtained, and the samples were de-identified prior to experimental use. pDCs were isolated from both bone marrow and peripheral blood mononuclear cells (PBMCs) by magnetically activated cell sorting using CD304 (BDCA-4/Neuropilin-1) microbeads kit (Miltenyi Biotec, Auburn, CA), as previously described. Briefly, mononuclear cells (MNCs) from healthy donors and MM patients were isolated by Ficoll Hypaque density
gradient centrifugation; magnetically labeled with anti-BDCA-4 antibody (Miltenyi Biotec) coupled to colloidal paramagnetic microbeads; and passed through a magnetic separation column twice. Cells lacking lineage markers and CD11c were FACS sorted. The purity of pDCs was confirmed by staining of cells with CD123 PE-Cy5, HLA-DR Pacific Blue, and BDCA-2 FITC (≥ 99% purity). The CD304-positive pDCs obtained by this method are lineage negative Lin-1 (CD3, CD14, CD19, CD20, CD56 and CD11c− negative), MHC II positive, and CD123/BDCA2-positive. pDCs were also purified by negative depletion using LD columns (Miltenyi Biotec; 99% BDCA2+ CD123+ cells). Cells were sorted using FACS Aria II cell sorter, and all flow cytometric experiments were performed using BD Canto II or BD LSRFortessa machine (BD Biosciences, San Jose, CA, USA). Data were analyzed using a FACS DIVA (BD Biosciences) and FlowJO software (ver 7.6.5, Tree Star Inc, USA).

Cytokines, antibodies, and reagents

Human recombinant IL-3, and IL-6, were obtained from Peprotech Inc (USA). Recombinant IFN-α and IFN-λ were purchased from R&D Systems (Minneapolis, MN, USA). CD3-PE; CD4-FITC or APC-Cy7; CD40-FITC; CD80-FITC; CD83-FITC; CD86-FITC; CD123-PE/PE-Cy5; as well as CD138-FITC, PE, or DR-5-Alexa700 were obtained from BD Biosciences (San Jose, CA, USA). HLA-DR Violet Blue, BDCA-2 FITC, CD14-PE, and CD11c-APC were purchased from Miltenyi Biotec. TLR-9-FITC, TRAIL-PE, and DR-4-FITC were obtained from Abcam. The CpG-C oligodeoxynucleotide C792 was synthesized and purified by standard techniques as previously described; bortezomib, lenalidomide, SAHA, and pomalidomide were purchased from Selleck Chemicals LLC (Houston, TX, USA); melphalan was purchased from Sigma Chemical Company (St Louis, MO, USA); and MyD88 inhibitor was purchased from InVivoGen (San Diego, CA, USA). For assessing C792 effect on the viability of freshly isolated pDCs, we cultured cells in DCP-MM medium (MatTek Corp, Ashland, MA, USA).

Cytokine assays

IFN-α, IFN-λ, and soluble TRAIL (sTRAIL) were measured by ELISA using commercially available kits, according to manufacturer’s instructions (PBL Interferon Source, Piscataway, NJ, USA, and R&D Systems). Briefly, MM.1S cells (5 × 10^4 cells/200 μl per well) and pDCs (1 × 10^4 cells/200 μl per well) were cultured either alone or together at 1:5 (pDC:MM) ratio in 96 well plates, in the presence or absence of C792; supernatants from these co-cultures were collected and analyzed for cytokine secretion using ELISA. MM.1S cells were also treated with various concentrations of recombinant hu-IFN-α (R&D Systems) and analyzed for viability by MTT assay.

Flow cytometry and intracellular staining

For flow cytometric analysis, single cell suspensions were stained with different fluorophore-coupled monoclonal antibodies (BD Biosciences). All flow cytometric analysis was performed on gated populations: CD138+ fraction for MM cells; and CD123+/CD304+/CD303+/HLA-DR+/CD11c− fraction for pDCs. Activation and maturation of pDCs was assessed by FACS analysis using CD40, CD80, CD83 and CD86 surface markers. Intracellular staining and cell/tissue fixation was assessed using the Cytofix/Cytoperm buffer kit (BD Biosciences) per manufacturer’s instructions.
Cell culture, and assessment of cell viability, growth, and apoptosis

Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. MM.1S (Dexamethasone sensitive), MM.1R (Dexamethasone- resistant), RPMI-8226, Doxorubicin (Dox)-resistant (Dox-40), Melphalan-resistant LR5, and NCI-H929 human MM cell lines were cultured in complete medium. ANBL-6 MM cell line was cultured in complete medium with interleukin-6 (5 ng/ml). Cell viability was assed using colorimetric assay with 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Calbiochem, EMD Millipore, Billerica, MA, USA). MM cell proliferation in co-culture experiments with pDCs or BMSCs was performed using WST (Clontech Laboratories, Mountain View, CA, USA) assays, as described previously. Briefly, pDCs were co-cultured with MM.1S cells at 1:5 pDC/MM.1S ratio in the presence or absence of C792, followed by cell growth analysis using WST assay. T cell proliferation assay: MM pDCs were cultured with or without T cells, in the presence or absence of C792, and proliferation was assessed using WST assays. Cell cycle was analyzed using propidium iodide staining. Apoptosis was quantified using Annexin V-FIte/Propidium iodide apoptosis detection kit per manufacturer’s instructions (BD Biosciences, San Jose, CA), followed by analysis on BD FACSCanto II or BD LSRFortessa (BD Biosciences, San Jose, CA).

Human plasmacytoma xenograft model

All animal experiments were approved by and conform to the relevant regulatory standards of the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute. In the human plasmacytoma murine xenograft model, CB-17 SCID-mice were randomized into three groups (four mice each group): the first group was subcutaneously injected with MM.1S cells alone (1.25 × 10^6 cells in 100 μl of serum free RPMI-1640 medium); the second group received resting pDC (0.25 × 10^6 cells) plus MM.1S cells (1.25 × 10^6 cells); and the third group was injected with ex-vivo C792-activated pDC plus MM.1S cells. Tumor growth was measured as previously described, and animals were euthanized when their tumors reached 2 cm^3.

Statistical analysis

Statistical significance of differences observed in drug-treated vs. control cultures was determined using the Student’s t test. The minimal level of significance was p < 0.05. Tumor volume and survival in mice was measured using the GraphPad PRISM (GraphPad Software/version 5, SanDiego, CA, USA). Isobologram analysis was performed using “CalcuSyn” software program (Biosoft; Ferguson, MO, USA and Cambridge, UK). Combination index (CI) values of < 1.0 indicate synergism, and values > 1.0 antagonism.

Results

C792 stimulates MM patient pDCs to trigger T cell proliferation

Prior studies have reported immune dysfunction in MM, associated with decreased ability of MM patient BM-DCs to stimulate T cell proliferation. In our study, we first examined the in vitro function of pDCs (defined as HLA-DR+, CD123+ and CD303/
BDCA-2+ cells) derived from normal healthy donors versus MM patients, assessed by their ability to stimulate allogeneic T cell responses. As shown in Fig 1A, MM pDCs induce significantly decreased T cell proliferation versus normal pDCs (P=0.02). We next examined whether treatment of MM patient BM-pDCs with TLR9 agonist (CpG-ODNs) C792 affects their T cell stimulatory activity. Importantly, C792 restores the ability of MM patient BM-pDCs to induce both allogeneic (Fig 1A) and autologus T cell proliferation (Fig 1B). Our data is consistent with other studies showing that targeting TLR9 using agonists can activate pDC, and enhance their immune functions.\textsuperscript{17,18,32–35}

C792 blocks pDC-induced MM cell growth

Recent reports linked increased infiltration of DCs in human tumors with unfavorable prognosis.\textsuperscript{6,10,36,37} Our earlier study in MM showed that increased numbers of pDCs in MM patient BM trigger growth of MM cells.\textsuperscript{28} Having shown that C792 restores MM patient BM-pDCs immune function, we next examined whether C792 affects pDC-induced MM cell growth using WST proliferation assays. As shown in Figure 2A, C792 inhibits patient-BM pDC-triggered MM.1S cell proliferation in a dose-dependent manner. Non-CpG ODN 1040 served as negative control. C792 also blocked pDC-induced growth of various other MM cell lines (MM.1R, ANBL-6, RPMI-8226, Dox-40, or LR-5) (Supplementary Fig 1). These results were also confirmed from an additional cell growth assay, using the CellTrace CFSE Proliferation Kit (data not shown; Life Technologies, Grand Island, NY, USA). No significant growth of pDCs was observed in co-cultures of pDCs and MM cells (data not shown). Importantly, C792 treatment of pDCs or MM cell lines alone did not decrease viability of either cell type (Fig 2B, and Fig 2C, respectively).

Similar to our observation using MM cell lines, we next examined whether C792 affects pDC-induced proliferation of patient MM cells using WST proliferation assays. As in our prior study, pDCs triggered proliferation of patient MM cells; and importantly, C792 blocked pDC-induced patient MM cell growth (Fig 2D). Furthermore, C792 inhibited MM patient BM pDC-induced growth of both autologous tumor cells (Fig 2E) and allogeneic MM.1S cell line (Fig 2F). Taken together, our findings suggest that activation of pDCs with TLR9 agonist C792 both restores their ability to induce T cell proliferation and reduces their MM cell growth-promoting activity.

C792 inhibits pDC-induced MM cell growth \textit{in vivo} in a MM xenograft model

Having shown the anti-MM activity of C792 in vitro, we next utilized a subcutaneous model of human MM in SCID mice to examine whether C792 similarly affects pDC-triggered MM cell growth \textit{in vivo}. Mice were randomized into three groups: the first group was subcutaneously injected with MM.1S cells alone; the second group received pDC plus MM.1S cells; and the third group was injected with MM.1S cells plus pDCs that were ex-vivo treated with C792. More robust tumor growth occurred in mice receiving MM.1S cells plus pDCs \textit{versus} those injected with MM.1S cells alone (Fig 3A), as in our prior study.\textsuperscript{28} Importantly, significantly reduced tumor growth was observed in mice receiving C792-treated pDCs plus MM.1S cells \textit{versus} the mice injected with either MM.1S cells alone (p = 0.0069) or untreated pDCs plus MM cells (p = 0.0044) (Fig 3A). Moreover, a marked prolongation of survival (P = 0.002) was noted in mice who received C792-treated pDCs
compared to in mice injected with either untreated pDCs plus MM.1S cells or MM.1S cells alone (Fig 3B). Together, our in vitro and in vivo human MM xenograft studies provide evidence for the anti-MM activity of C792 by virtue of its ability to block pDC-mediated MM cell growth.

**Mechanism(s) mediating anti-MM activity of C792**

Previous studies reported that CpG-ODNs can activate pDCs.\(^{38-41}\) To determine whether C792 similarly activates freshly isolated MM patient pDCs, we treated pDCs with C792 and examined expression of surface markers characteristic of activation and maturation. Treatment of pDCs with C792 increases CD40, CD83, CD80, HLA-DR, and CD86 expression (Fig. 4A): Specifically, flow cytometric data showed that C792 triggered a significant increase in median fluorescence intensity (MFI) of CD40, CD83, CD80, HLA-DR, and CD86 expression (20%, 37%, 10%, 6%, and 13% increase, respectively). Non-CpG 1040 served as a negative control (Figure 4A). C792 is a TLR9 agonist, and we found a significant increase in TLR9 expression in C792-treated pDCs (Fig 4B, left and right panels).

In order to further confirm that C792 activates MM patient pDCs, we examined additional mechanism(s) associated with activation of pDCs. For example, prior studies have shown that CpG-ODN-activated pDCs secrete Type I and Type III interferons (IFNs);\(^{41,42,43}\) this is exemplified in systemic lupus erythematos, which is linked to TLR-9-activated pDCs and Type I IFN-\(\alpha\) release.\(^{18,33,34,35}\) In view of these studies, we next examined 1) whether treatment of MM patient BM-pDCs with C792 triggers IFN-\(\alpha\) secretion; and 2) if IFN-\(\alpha\) secretion is attenuated or augmented during pDC-MM co-cultures. Treatment of pDCs with C792, but not non-CpG ODN control, triggered an increase in IFN-\(\alpha\) secretion (Fig 4C). Importantly, C792 induced a more robust dose-dependent IFN-\(\alpha\) secretion in pDC-MM co-cultures versus pDCs alone (Fig 4D). This phenomenon was also observed in C792-treated normal healthy donor pDC-MM co-cultures versus normal pDC alone (data not shown).

IFN-\(\alpha\) was undetectable in the supernatant from C792-treated MM cells. Our findings are also consistent with earlier studies\(^ {40,41}\) demonstrating a non-linear relationship between CpG-ODNs, like C792, and secretion of IFN-\(\alpha\). In addition to Type I IFN-\(\alpha\) release, C792 also triggered Type III IFN-\(\lambda\) secretion in pDC-MM co-cultures; albeit to a lesser extent than IFN-\(\alpha\) (Supplementary Fig 2).

We next examined the signaling mechanism mediating C792-induced IFN-\(\alpha\) secretion in MM patient BM pDCs. As stated above, C792 is a TLR9 agonist and upregulates TLR9 expression in pDCs (Fig 4B). To validate the functionality of TLR9 signaling axis in pDCs, we examined downstream adaptor protein MyD88 (Myeloid differentiation primary response gene).\(^ {44,45}\) In our study, biochemical inhibition of MyD88 homodimer formation significantly blocked C792-induced IFN-\(\alpha\) secretion (Fig 4E). Together, our findings suggest that the inhibitory effect of C792 on pDC-induced MM cell growth is associated with activation of pDCs via TLR9-MyD88 signaling and release of IFN-\(\alpha\).

Although C792-triggered activation of pDCs explains the restoration of MM pDC ability to stimulate T cell responses, it does not elucidate the mechanism whereby C792 inhibits pDC-

*Leukemia. Author manuscript; available in PMC 2015 February 01.*
induced MM cell growth. One possibility is that C792 triggers secretion of soluble factors which inhibit MM cell growth and/or trigger apoptosis. Interestingly, we found large amounts of IFN-α in C792-treated pDC-MM co-cultures; and importantly, IFN-α has been utilized in the treatment of cancers, including MM. 46,47,48 We therefore examined whether IFN-α blocks pDC-induced MM cell growth. For these studies, we utilized human recombinant IFN-α at various concentrations, including those that were obtained in supernatants from C792-treated pDC-MM co-cultures (Fig 4D). pDCs were co-cultured with MM cell lines (MM.1S, MM.1R, LR-5, NCI-H929) in the presence or absence of rIFN-α, followed by analysis of cell growth. A dose-dependent decrease in the pDC-induced MM cell growth was noted in response to treatment with rIFN-α (Fig 5A, and data not shown). These findings suggest that IFN-α, at least in part, mediates the anti-MM activity of C792.

Previous studies have linked TLR9-stimulated pDCs to TNF-related apoptosis-inducing ligand (TRAIL) which binds to cells expressing TRAIL receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and results in apoptosis. 49,50 We next examined whether C792 induces secretion of soluble TRAIL (sTRAIL) from pDCs. A significant increase in sTRAIL was present in supernatants from C792-treated versus untreated-pDC-MM co-cultures (Fig 5B; p < 0.005). Since MM cells express TRAIL receptors DR4 AND DR5,51 and our findings show that C792 induce sTRAIL (Fig 5B), it is likely that TRAIL-TRAIL-R interactions mediate anti-MM activity of C792. We do not exclude the possibility that other factors besides IFN-α or sTRAIL may also mediate MM cell cytotoxicity of C792 in pDC-MM cultures, since we found that supernatants from C792-treated pDC-MM co-cultures triggered a marked decrease in MM cell viability (Fig 5C).

We next examined whether MM cell growth inhibition in C792-treated pDC-MM co-cultures is due to cell-cycle alterations and/or apoptosis in MM cells. Treatment of pDC-MM co-cultures with C792 is associated with growth arrest in MM.1S cells: specifically, cell cycle analysis of CD138+ MM cells obtained from pDC-MM.1S co-cultures showed increased G2 phase in C792-treated versus untreated-pDC-MM co-cultures (Fig 5D). Of note, treatment of MM cells without pDCs does not affect the cell cycle profile of MM cells; consistent with our finding that C792 is not cytotoxic against MM cells alone (Fig 2C). Finally, we show that treatment of pDC-MM co-cultures with C792 triggers MM cell apoptosis (Fig 5E).

C792 triggers synergistic anti-MM activity with bortezomib, SAHA, melphalan, lenalidomide and pomalidomide

Our prior preclinical studies showing synergistic cytotoxicity have provided the basis for clinical trials of proteasome inhibitor bortezomib in combination with lenalidomide, Dex, or HDAC inhibitors.1 In our current study, we tested whether CpG-ODN C792 triggers additive or synergistic anti-MM activity in combination with proteasome inhibitor bortezomib, HDAC inhibitor SAHA, melphalan, or lenalidomide (Fig 6). Isobologram analysis31 demonstrated that the combination of low concentrations of C792 with melphalan, SAHA, lenalidomide, or bortezomib triggers synergistic anti-MM activity against various MM cell lines (MM.1S, MM.1R, RPMI-8226, and LR5); albeit with differential extents of synergy (Fig 6A–6D, and data not shown; combination index (CI) <
1. While definitive evidence of decreased toxicity of combination therapy awaits results of clinical trials, the synergy observed in vitro may allow for use of lower doses and reduced toxicity.

Discussion

Our previous study identified an integral role of pDCs in MM pathogenesis. Specifically, using our in vitro and in vivo models of human MM in the BM milieu, we showed that increased numbers of pDCs in the MM BM confer growth, survival, chemotaxis, and drug resistance. Additionally, we found a decreased ability of MM BM pDCs versus normal pDCs to trigger T cell proliferation. A similar immune dysfunction in MM-pDCs has been noted in other reports. Earlier studies have shown promising activity of immunostimulatory CpG-based formulations as immune adjuvants in vaccines or in the treatment of viral, bacterial, and parasitic infections, as well as in cancers. Indeed, both Type A and Type B CpG ODNs have been evaluated in clinical trials. In the current study we examined whether C792, a novel next generation Type-C CpG-ODN targeting TLR9 with a potent immune-enhancing activity, affects pDC-induced MM cell growth and/or stimulates pDC immune function. Our data show that treatment of MM BM pDCs with C792 restores their ability to stimulate both autologous and allogeneic T cell proliferation. A previous study using the B16 mouse melanoma tumor model also showed that CpG-activated pDCs induce a systemic antitumor immunity through activation of NK cells and T cells. Further studies are required to determine whether C792-activated MM pDCs generate anti-tumor immunity which is clinically significant in MM.

Importantly, we found that C792 inhibits pDC-induced growth of various MM cell lines, including those sensitive and resistant to various conventional and novel therapies, as well as representing distinct cytogenetic subtypes. For example, we studied isogenic cell lines Dex-sensitive MM.1S and Dex-resistant MM.1R with t(14;16) translocation; as well as RPMI-8266 parental and Doxorubicin-resistant (8226/Dox-6) or melphalan-resistant (LR5) derivative cell lines. Similar findings were observed using MM cells from patients with relapsed MM refractory to various therapies. Importantly, C792 blocked MM patient BM pDC-induced growth of both autologous tumor cells and allogeneic MM.1S cell line. Our data therefore highlight the ability of C792 to overcome genetic heterogeneity and broadly inhibit pDC-induced growth in MM. Interestingly, treatment of MM cells or pDCs alone with C792 does not affect their viability; the MM cell growth inhibitory activity of C792 is only observed in the pDC-MM co-culture setting. This finding confirms the role of factors and/or MM BM accessory cells within the MM BM milieu, in this case pDCs, to impact the sensitivity versus resistance of MM cells. There is an urgent need to clinically evaluate novel agents targeting the tumor cell in the BM microenvironment, and clinical trials of C792 will provide proof of principle.

In agreement with our in vitro data, C792 also inhibited pDC-triggered MM cell growth in vivo. More rapid tumor growth occurred in mice receiving both pDCs and MM cells versus MM cells alone; and importantly, mice receiving C792-treated pDCs plus MM cells showed significantly reduced tumor growth. The prolonged survival of pDCs in our xenograft model may be explained by our earlier study showing: 1) that pDC-MM cell interactions trigger
secretion of interleukin-3 (IL-3), a survival factor for pDCs; \(^1^4\) and 2) increased IL-3 and pDCs (BDCA-2-positive cells) in tumor sections from mice receiving pDCs and MM cells versus MM cells alone. Our ongoing studies using C792 are now evaluating the effects of C792 on IL-3 and other cytokines/chemokines secreted during pDC-MM interactions. Together, our findings from both in vitro and human MM xenograft in vivo models suggest that C792 inhibits pDC-induced MM cell growth, and provide the basis for targeting pDC-MM interactions as a therapeutic strategy in MM.

Treatment with C792 is associated with activation/maturation of pDCs, evidenced by upregulation of CD83, CD40, CD80, CD86 and HLA-DR, as well as IFN-\(\alpha\) release. Indeed, C792 targets TLR-9 to trigger both pDCs activation and release of IFN-\(\alpha\). Moreover, the engagement of TLR9 by C792 activates downstream signaling via adaptor protein MyD88; conversely, blockade of MyD88 inhibited C792 activity, evidenced by a marked decrease in IFN-\(\alpha\) secretion from pDCs. Enhanced IFN-\(\alpha\) secretion in response to C792 may explain, at least in part, cytotoxicity of C792 against MM cells in pDC-MM co-cultures. Indeed, previous studies have shown cytotoxic activity of IFN-\(\alpha\) against MM cells; \(^4^6,^4^7,^4^8\) and clinical trials have shown anti-MM activity of IFN-\(\alpha\), either as a single agent or in combination. In addition to IFN-\(\alpha\), we also found sTRAIL in the supernatants from C792-treated pDC-MM co-cultures, which may interact with TRAIL-R expressed on MM cells and trigger MM cell death.

Finally, we show that C792 triggers synergistic anti-MM activity with current anti-MM agents including proteasome inhibitor bortezomib, immunomodulatory agent lenalidomide, HDAC inhibitor SAHA, or alkylating agent melphalan. Most of these agents trigger a direct (yet varying) cytotoxic effect on MM cells, and combination trials with C792 will allow not only targeting MM cells, but also bolster the immune response in MM patients.

Collectively, we show that targeting TLR9 with CpG ODN C792 improves immune function of pDCs on the one hand and blocks pDC-induced MM cell growth on the other. Our preclinical study provides the framework for clinical trials of C792, either alone or in combination, to improve immune function, enhance cytotoxicity, overcome drug resistance, and improve patient outcome in MM.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

Grant Support This investigation was supported by National Institutes of Health Specialized Programs of Research Excellence (SPORE) grants P50100707, PO1-CA078378, and RO1 CA050947. K.C.A. is an American Cancer Society Clinical Research Professor.

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*Leukemia. Author manuscript; available in PMC 2015 February 01.*


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Figure 1. C792 restores the ability of MM patient pDCs to trigger both allogeneic and autologous T cell proliferation

(A) Normal CD4 T cells (1 × 10^5) were cultured with normal healthy donor (npDC) or MM patient BM pDCs at 1:10 (pDC/T cell) in the presence or absence of C792 for 5 days, and then analyzed for T cell proliferation with WST assay (BM-pDCs from 5 MM patients were utilized; mean ± SD; p < 0.005 for all patients). (B) MM patient BM pDCs (1 × 10^4) were cultured with autologous T cells (1 × 10^5) at 1:10 (pDC/T cell) ratio in the presence or absence of C792 (0.1 μg/ml) for 5 days, and then analyzed for T cell proliferation using WST assay (mean ± SD; p < 0.01 for all patients).
Figure 2. C792 inhibits pDC-induced MM cell growth

(A) MM.1S cells (5 × 10⁴/200 μl) and MM BM-pDCs (1 × 10⁴/200 μl) were cultured either alone or together at 1:5 (pDC/MM) ratio in the presence or absence of C792 for 72h, and then analyzed for growth using WST proliferation assays. Non-CpG-ODN 1040 served as a negative control (mean ± SD; p< 0.05; n=6). (B) Freshly isolated pDCs from six normal healthy donors were cultured in DCP-MM medium, in the presence of C792 (5–10 ug/ml) for 72h, and analyzed for viability by MTT assays (mean ± SD, p< 0.05). (C) MM.1S and IL-6-dependent ANBL-6 cells were cultured with indicated concentrations of C792 for 72h, and analyzed for viability by MTT assays (mean ± SD; p< 0.05, n=3). Non-CpG-ODN 1040 was also utilized as a control. (D) Patient MM cells were cultured with or without pDCs at 1:5 (pDC/MM) ratio for 72h, in the presence or absence of C792, and cell growth was analyzed using WST assay (mean ± SD of triplicate cultures; p< 0.05 for all samples). (E) Patient MM cells were cultured with or without autologous BM pDCs at 1:5 (pDC/MM.1S) ratio in the presence of indicated concentrations of C792 for 72h, and cell growth was analyzed using WST assay (mean ± SD; p <0.04, n=5). (F) Patient BM pDCs were cultured with or without MM.1S cells at 1:5 (pDC/MM) ratio in the presence or absence of C792 for 72h, and cell growth was analyzed by WST assay (mean ± SD; p < 0.01, n = 5). Data is fold change in MM cell growth normalized to growth in the absence of pDCs (mean ± SD; p< 0.05, n=3).
Figure 3. C792 inhibits pDC-induced MM cell growth in vivo in a murine xenograft model of human MM

(A) Average and standard deviation of tumor volume (mm$^3$) in mice (n = 4/group) versus time (days) when tumor was measured. CB-17 SCID-mice were randomized into three groups (four mice each group): the first group was subcutaneously injected with MM.1S cells alone (1.25 × 10$^6$ cells in 100 μl of serum free RPMI-1640 medium); the second group received resting pDC (0.25 × 10$^6$ cells) plus MM.1S cells (1.25 × 10$^6$ cells); and the third group was injected with ex-vivo C792-activated pDCs plus MM.1S cells. Tumor growth was measured by calculating tumor volume using the formula: Volume=($width)^2$ × length/2. Error bars indicate standard deviation (SD). (B) Kaplan-Meier survival plot shows significantly increased survival of mice receiving C792-treated pDCs plus MM.1S cells versus mice injected with untreated pDCs plus MM.1S cells (P = 0.0002, Log-rank (Mantel-Cox) Test): median survival was 24 days in MM.1S-injected mice; 18 days in mice injected with MM.1S plus untreated-pDCs; and 49 days in mice receiving MM.1S plus C792-treated pDCs (CI 95%). Tumor-bearing mice were sacrificed with a tumor volume > 2 cm$^2$. 

Leukemia. Author manuscript; available in PMC 2015 February 01.
Figure 4. C792 triggers maturation of pDCs, IFN release, and activation of TLR9-MyD88 signaling axis

(A) MM BM-pDCs (CD123+/BDCA-2+/HLA-DR+/CD11c−) were cultured in the presence or absence of C792 (1.0 μg/ml) for 12h; cells were stained with fluorophore-conjugated antibodies against CD40, CD80, CD83, CD86, or HLA-DR, followed by flow cytometry analysis. Non-CpG 1040 served as a negative control. Bar graph shows the percentage change in MFI for indicated molecules in untreated- versus C792-treated pDCs (mean ± SD, p< 0.05, n = 3). (B, left panel) pDCs were treated with indicated concentrations of C792 for 12h, followed by intracellular staining using FITC-conjugated TLR-9 antibody. Isotype-matched antibody served as control. (B, right panel) C792-triggered changes in TLR9, as shown in left panel, were quantified: Intracellular TLR-9 expression is presented as percentage change in relative fluorescence intensity in C792-treated pDCs versus the untreated-pDCs (mean ± SD; p < 0.05, n = 3). (C) MM BM-pDCs (5 × 10^3 cells) were cultured in the presence or absence of indicated concentrations of C792 (Type C CpG ODN), Type-B CpG ODN, or non-CpG for 24h; supernatants from these cutures were analyzed for IFN-α using ELISA (mean ± SD, p< 0.05, n=3). (D) pDCs (1 ×10^5), MM.1S cells (5 × 10^4), or pDCs plus MM.1S cells were cultured in the presence or absence of indicated concentrations of C792 for 24h, and supernatant from these cultures were analyzed for IFN-α using ELISA (mean ± SD; p< 0.05, n=4). (E) MM BM-pDCs (5 × 10^3) were pre-treated with 50 μM of MyD-88 inhibitor peptide for 4h, followed by addition of C792 for 24h; supernatants from these cultures were analyzed for IFN-α using ELISA (mean ± SD; p< 0.05, n=3).
Figure 5. Mechanism(s) mediating C792 activity

(A) MM.1S cells were cultured with MM BM-pDCs in the presence or absence of recombinant human IFN-α for 48h, and cell growth was analyzed using WST assay. Data is derived after normalizing cell growth in IFN-α-treated versus untreated cultures (mean ± SD; p < 0.05, n=3). (B) pDCs from 2 MM patients were treated with C792 for 96h, and supernatants were analyzed for soluble TRAIL using ELISA (mean ± SD; p < 0.05, n=3). (C) MM cell lines were treated for 72h with supernatants derived from untreated- or C792-treated MM BM-pDC cultures, and cell viability was analyzed using MTT assay. Data is derived after normalizing MM cell viability in supernatants from untreated- versus C792-treated MM BM-pDC cultures, and cell viability was analyzed using MTT assay. Data is derived after normalizing MM cell viability in supernatants from untreated- versus C792-treated pDC cultures (mean ± SD; p < 0.05, n=3). (D) MM.1S and MM BM-pDCs cells were co-cultured at 1:5 (pDC/MM) ratio in the presence or absence of C792 for 12h. Cells were then stained with propidium iodide. MM (CD138-positive) cell population was selectively gated by flow cytometry for cell cycle analysis. Bar graph shows the percentage of MM cells in cell cycle phases. A significant accumulation of MM cells in G2M phase was noted in the C792-treated versus untreated co-cultures (p < 0.005, n = 3). (E) MM.1S and MM BM-pDCs cells were co-cultured at 1:5 (pDC/MM) ratio in the presence or absence of C792 for 12h; the MM (CD138-positive) population was selectively gated by flow cytometry and analyzed for apoptosis using Annexin V/PI staining assays. A significant increase in apoptotic MM cell population was noted in the C792-treated versus untreated co-cultures (25–30% increase in Annexin V+/PI− cells) (p < 0.005, n = 3). Bar graph shows the percentage of apoptotic MM cells.
Figure 6. Combination of C792 and bortezomib, SAHA, melphalan, or lenalidomide triggers synergistic anti-MM activity

(A) Co-cultures of MM.1S plus BM-pDCs were treated with indicated concentrations of C792, bortezomib, or C792 plus bortezomib for 48h, and then analyzed for viability. Isobologram analysis shows the synergistic cytotoxic effect of C792 and bortezomib. The graph (left) is derived from the values given in the table (right). The numbers 1–6 in graph represent combinations shown in the table. Combination index (CI) <1 indicates synergy.

(B) Co-cultures of MM.1S plus BM-pDCs were treated with indicated concentrations of C792, SAHA, or C792 plus SAHA for 48h, and then analyzed for viability. Isobologram analysis shows the synergistic cytotoxic effect of C792 and bortezomib. The graph (left) is derived from the values given in the table (right). The numbers 1–6 in graph represent combinations shown in the table. Combination index (CI) <1 indicates synergy.

(C) Co-cultures of MM.1S plus BM-pDCs were treated with indicated concentrations of C792, melphalan, or C792 plus melphalan for 48h, and then analyzed for viability. Isobologram analysis shows the synergistic cytotoxic effect of C792 and melphalan. The graph (left) is derived from the values given in the table (right). The numbers 1–6 in graph represent combinations shown in the table. Combination index (CI) <1 indicates synergy.

(D) Co-cultures of MM.1S plus BM-pDCs were treated with indicated concentrations of C792, lenalidomide, or C792 plus lenalidomide for 48h, and then analyzed for viability. Isobologram analysis shows the synergistic cytotoxic effect of C792 and lenalidomide. The graph (left) is derived from the values given in the table (right). The numbers 1–6 in graph represent combinations shown in the table. Combination index (CI) <1 indicates synergy.
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