Title: Enhanced cross-priming of naive CD8+ T cells by DCs treated by the IMiDs® immunomodulatory compounds Lenalidomide and Pomalidomide.

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Summary sentence: Pomalidomide and lenalidomide enhance tumour antigen uptake by DCs with an increased efficacy of antigen presentation, indicating a possible use of these drugs in DC vaccine therapies.

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Abstract

The IMiDs® immunomodulatory compounds, lenalidomide and pomalidomide are agents with anti-inflammatory, immunomodulatory and anti-cancer activity. An excellent success rate has been shown for multiple myeloma in phase I / II clinical trials leading to FDA approval of lenalidomide. One mechanism by which these drugs could enhance anti-tumour immunity may be through enhanced dendritic cell (DC) function. Thalidomide, a compound structurally related to lenalidomide and pomalidomide is known to enhance DC function, and we have investigated whether its analogues, pomalidomide and lenalidomide also have functional effects on DCs. We used mouse bone marrow-derived DCs treated with 5µM or 10µM pomalidomide, or lenalidomide from day 1 of culture. IMiD® immunomodulatory compound treatment increased expression of Class I (H2-Kb), CD86, and pomalidomide also increased Class II (I-Ab) expression in Bm-DC’s as measured by flow cytometry. Fluorescent bead uptake was increased by up to 45% when DCs were treated with 5µM or 10µM pomalidomide or lenalidomide compared to non-treated DCs. Antigen presentation assays using DCs primed with ovalbumin, and syngeneic T cells from transgenic OTI and OTII mice (containing MHC restricted, ovalbumin specific, T cells) showed that both pomalidomide and
lenalidomide effectively increased CD8$^+$ T cell cross-priming (by up to 47%) and that pomalidomide alone was effective in increase CD4$^+$ T cell priming (by 30%). Our observations suggest that pomalidomide and lenalidomide enhance tumour antigen uptake by DCs with an increased efficacy of antigen presentation, indicating a possible use of these drugs in DC vaccine therapies.

Introduction

Dendritic cells (DCs) are the principle population of antigen presenting cells (APC) for the initiation of T cell-mediated immunity. DCs are derived from hematopoietic bone marrow progenitor cells and, like granulocytes and macrophages, express the myeloid marker CD33 and are induced to differentiate in response to GM-CSF (1). DCs circulate throughout the periphery and remain in an immature form (iDC) until exposure to antigen occurs. The mature DC then travels to the lymph node where Th1/Th2 mediated responses are initiated by the presentation of antigen (through MHC Class I and Class II complexes) and co-stimulation markers to naïve T cells (2).

DCs have been shown to be important adjuvants in the use of cancer vaccines (3) and interest in DC vaccines has grown recently with the FDA’s approval of sipuleucel-T (Provenge, Dendreon) (4), a therapeutic vaccine consisting of autologous DCs that have been loaded with a PAP-GM-CSF fusion protein ex vivo (5). Prostatic Acid Phosphatase (PAP), a TAA, is overexpressed in prostate cancers and its fusion with GM-CSF promotes PAP presentation and DC maturation in tandem. Tumor secreted VEGF has been shown to inhibit DC maturation (6) by the disruption of Nf-KB signalling (7). These partially mature DCs, termed myeloid suppressor cells (MSC), have been observed in patient populations and correlate with poor patient survival (8). Generation of MSC by exposure to antigen in the absence of costimulation leads to the expansion of T-regulatory (Treg) cells and T cell anergy (9). The immune suppressive potential of MSC can be reversed by differentiation into DCs by the use
various chemotherapeutic agents such as PDE-5 inhibitors (10) and sunitinib (11). An attractive drug candidate for use as an adjuvant with a therapeutic DC vaccine would not only improve the functional response of DCs by improved maturation and antigen presentation; but also suppress the function of MSC and Treg cells.

The IMID® immunomodulatory drugs Lenalidomide (Revlimid®), and Pomalidomide (CC-4047) are thalidomide analogues that were developed as TNF-α inhibitors. Lenalidomide is FDA approved for multiple myeloma (MM) in combination with dexamethasone and in del 5q myelodysplastic Syndrome (MDS) based on seminal trials completed in 2007. A current Phase I/II study is testing the combination of antigen loaded DCs with lenalidomide in patients with multiple myeloma (ClinicalTrial.gov #NCT00698776). These compounds effect tumors directly through the modulation of a variety of signalling pathways such as VEGF, AKT (12), Notch/Wnt (13), P53 and P38 (14).

IMiDs® immunomodulatory compounds primarily function as modulators of immune function and a recent publication demonstrates that lenalidomide increases NK cell mediated antibody-dependant cellular cytotoxicity (ADCC) by modulation of Fc-γ receptor signalling in NK cells (15). We have previously shown that the IMiDs immunomodulatory compounds suppress T-regulatory cell function (16), whilst thalidomide and lenalidomide have been identified as costimulators of CD8⁺ effector activity, independent of DC function (17). In the course of this study, we have investigated the effects of lenalidomide and pomalidomide on DC dependant T cell activation. We particularly focus on the role of the IMiDs immunomodulatory compounds in the uptake and presentation of antigen by dendritic cells.
Materials and Methods

Animals

Female C57/BL6 mice were bred and maintained by the Biological Research Facility at St George’s, University of London (London, UK). OT-I and OT-II TCR transgenic mice were purchased from Charles River (Margate, UK) and maintained by the Biological Research Facility at St George’s, University of London. Animals were used at 8-10 weeks of age and all procedures conducted had obtained prior ethical approval and were carried out in accordance with the regulations as described in the Animals (Scientific Procedures) Act 1986, UK (Project license number: 70/6573)

Reagents

Lenalidomide and pomalidomide were obtained from the Celgene Corporation (Summit, NJ, USA), were dissolved in DMSO at 10mM, and maintained as stock solutions for in vitro experiments at -20°C for no longer than one month.
Recombinant murine GM-CSF (Peprotech, London UK) was made up as a stock solutions at 10µg/ml in PBS and used immediately.
DC media contained Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen, Paisley UK), 10% foetal calf serum (FCS) Invitrogen), 1% w/v penicillin streptomycin (P/S) and 50 µM 2-Mercaptoethanol (Sigma,Dorset UK)
Lyophilised ovalbumin (Sigma) was reconstituted in PBS at 45mg/ml to be used immediately.

Dendritic Cell Culture

Bone marrow from the femurs and tibias of wildtype (WT) C57/BL6 female mice was suspended in DC media as a single cell suspension. The cell suspension was forced through a 70µm filter, and
resuspended at $1 \times 10^6$ cells/ml in DC media. GM-CSF and IL-4 was added with a final concentration of 5ng/ml.

5ml aliquots of the cell suspension was added to T25 culture flasks supplemented with drugs (lenalidomide and pomalidomide at 5µM or 10µM), and incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO$_2$. Lenalidomide and pomalidomide were used at concentrations of 5 µM and 10 µM based on previous pharmacokinetic studies, where plasma concentrations of up to 10 µM were reached upon dosing of 50 mg/Kg— the typical in-vivo dose used for murine investigations. Non-adherent cells were removed after 3 days, fresh GM-CSF, and drugs were added to each flask and incubated for a further 2 days. Loosely adherent DCs were then recovered from culture flasks.

**Isolation of Ovalbumin TCR-Transgenic Splenocytes**

OT-I (containing MHC class I-restricted, ovalbumin specific, CD8$^+$ T cells) and OT-II (containing MHC class II-restricted, ovalbumin specific, CD4$^+$ T cells) splenocytes were isolated from the spleens of OT-I and OT-II mice (C57/BL6 background) respectively and, after RBC lysis, were maintained in RPMI-1640 media (RPMI; Sigma Ltd, Poole, UK) supplemented with 10% (v/v) fetal bovine serum (FBS), 2mM l-glutamine and 1x penicillin/streptomycin (Basal Media).

Splenocytes were pooled and suspended in freezing media (45% Basal Media, 45% FCS, 10% DMSO) and aliquots were stored in liquid nitrogen.

**Bead uptake assay**

DCs were prepared as described above and $1 \times 10^6$ cells were resuspended in 1ml DC media. Cells were incubated with 7µl of fluorescent beads (Fluospheres, Invitrogen) for 3 hrs at either 4°C or 37°C.
Cells were then placed on ice, and washed 3 times with cold PBS before fixation and analysis by flow cytometry.

**Immunophenotyping by flow cytometry**

Lenalidomide and pomalidomide treated DCs were resuspended in FACS buffer (1% mouse serum (DAKO, Cambridge UK) 15mM NaN₃ in PBS) and stained with fluorophore-conjugate monoclonal antibodies for H2-Kb (MHC ClassI), I-Aβ (MHC ClassII), CD80, CD86, CD11c, CD40, FAS and FASL (BD). Samples were washed with FACS buffer, data was acquired using a FACSCalibur flow cytometer and analysis was performed using Cellquest Pro software (BD) and FCS Express (De Novo software, CA USA).

**Cytokine Detection Assays**

Supernatants from lenalidomide and pomalidomide treated DCs were collected and cellular debris was removed by centrifugation. Concentration of the soluble cytokines IL12-P70, TNF-α, IFN-γ, MIP-1 alpha, IL-6 and IL-10 were measured by BD Bioscience’s Mouse Inflammation CBA kit as per the manufacturer’s directions.

**Peptide Presentation Assay**

Dendritic cells were cultured as described above. Cells were treated with pomalidomide (5µM & 10 µM), lenalidomide (5µM & 10 µM) or DMSO (0.1%). After 5 days, loosely adherent DCs were recovered from culture flasks and suspended in DC media at 5x10⁴ cells/ml.
12 well plates were seeded with 1x10^5 cells from each treatment, an ovalbumin stock solution was added to each well (100µg/ml final concentration) and plates were incubated for 24 hrs at 37°C, 5% CO₂. After 24 hrs, LPS was added to the DCs at a final concentration of 1 µg/ml to mature the DCs for the presentation assay.

Cells were harvested, washed 3 times and suspended in RPMI media at 1x10^6 cells/ml. 5x10^3 cells were then seeded onto 96 well plates (in triplicate).

Frozen aliquots of OT-I and OT-II splenocytes were thawed and suspended in RPMI media at 1x10^6 cells/ml. Antigen presentation assays were performed by seeding 1x10^5 splenocytes onto wells containing syngeneic DCs (WT) and plates were incubated for 5 days at 37°C, 5% CO₂ whereupon T cell proliferation was measured by [H^3]thymidine incorporation (0.5 µCi per well) for 16 hrs.

**Interferon gamma and perforin staining of CD4 and CD8 Lymphocytes in DC-splenocyte cocultures**

To determine whether the CD4 and CD8 lymphocytes were activated to a greater extent by lenalidomide or pomalidomide treated DCs than with the control DCs, we used 24 well plates cocultured with the splenocytes and DCs pretreated with DMSO or with lenalidomide or pomalidomide at 5 µM or 10 µM. After 5 days of coculture, the cells were treated for 4 hr with brefeldin A, PMA and ionomycin with working concentrations of 10 µg/ml, 50 ng/ml and 1 µg/ml respectively. An aliquot of the cells was then taken from the wells and then washed twice in FACs buffer and then resuspended in a volume of 100 µl in FACs tubes. The cells were incubated for 30 mins at 4°C with fluorophore conjugated antibodies to anti-CD8, and anti-CD4. After washing twice with FACs buffer, Cells were fixed with 2% paraformaldehyde for 20 mins at 40°C and then washed
once with FACs buffer and once with permeabilisation buffer (BD biosciences). Pellets were then resuspended in 100 µl permeabilisation buffer and incubated with fluorophore conjugated anti-IFN gamma and anti-perforin for 20 mins at room temperature in the dark.

To measure viability of the cells within the cocultures after Brefeldin and PMA/ionomycin incubation, a separate aliquot of cells was also taken from each well and tested for viability using an annexin/propidium iodide flow cytometric staining assay (BD, UK): Briefly cells were washed with annexin binding buffer, and followed by incubation with FITC annexin and Propidium iodide for 15 mins. Positive staining was assessed by flow cytometric analysis.

**Statistical Analysis**

Statistical analysis of results was performed using repeated measures ANOVA on paired data sets followed by Newman-Keuls post testing when appropriate. All statistics were generated using the Graphpad Prism 4.0 software.

**Results**

**IMiDs® immunomodulatory compounds increase endocytotic activity of DCs**

An immature DC must first endocytose exogenous antigen before it can be presented to the T cell. To this end, we employed a method of measuring DC endocytic activity in which DCs were treated with lenalidomide or pomalidomide at 5µM and 10 µM and pulsed with FITC-labelled latex beads. FITC fluorescence of DCs were analysed by flow cytometry (Figure 1A). Bead uptake was measured by the percentage of the (gated) DCs to be FITC positive. An increase (of up to 10% of the DC population) in fluorescent DCs was observed when treated with Pomalidomide at 5µM and 10µM
concentrations (Figure 1B) or 10 µM Lenalidomide (Figure 1C), indicating a small but significant increase in endocytic activity (P<0.05, n=3)

**IMiDs immunomodulatory compounds modulate phenotypic markers on DCs**

DCs initiate T cell activation by the presentation of MHC bound antigen and co-stimulation markers (including CD80 and CD86) to the naïve T cell. Having investigated the role of antigen endocytosis, we then examined the modulation of MHC and co-stimulation marker expression on DCs that had been treated with lenalidomide and pomalidomide. DCs were treated with 10µM lenalidomide or pomalidomide and the expression of the phenotypic markers MHC Class I (H-2K\(^b\)), MHC Class II (I-A\(^b\)), CD80, CD86, CD11c, CD40, FAS and FASL were measured by flow cytometry. Expression of H-2K\(^b\) (Figure 2A) and CD86 (Figure 2C) was significantly increased by up to 73% and 53% respectively with treatment (p<0.05, n=4). Pomalidomide alone upregulated I-A\(^b\) expression on DCs by 72% (p<0.05, n=4) (Figure 2B). No significant changes were observed in CD80, CD11c, CD40, FAS and FASL expression (Supplementary Figure S1).

**Lenalidomide treatment upregulates MIP-1 alpha and TNF-α expression in DCs**

DCs were treated with 10µM lenalidomide or pomalidomide and supernatants were collected after 5 days. Expression of IL-12 p70, TNF-α, IFN-γ, MIP-1 alpha, IL-6 and IL-10 was assayed by cytometric bead array. Lenalidomide treated DCs produced 945% more MIP-1 alpha (p<0.01, n=4) (Figure 3A) and 180% more TNF-α (p<0.05, n=4) (Figure 3B) than either untreated or pomalidomide treated DCs. Expression of all other cytokines remained unchanged (Supplementary Figure S2).
IMiD® immunomodulatory compounds treated DCs are more efficient initiators of MHC Class I restricted T cell activation

Having observed elevated MHC Class I and MHC Class II expression on IMiD immunomodulatory compounds treated DCs (as well as the co-stimulation marker CD86), we sought to investigate whether these observations would correlate with increased antigen presentation to naïve T cells. OVA-specific CD8+ and CD4+ T cells from transgenic mice (OT-I and OT-II) are commonly used in model systems of antigen presentation (18;19) and cross-presentation (20) where T cell priming is initiated by MHC restricted presentation of exogenously derived ovalbumin on APCs (such as bone marrow derived DCs). In this study, DCs were treated with lenalidomide and pomalidomide at 5µM and 10µM concentrations then used in an antigen presentation assay with splenocytes extracted from OT-I or OT-II transgenic mice. Proliferation of MHC class I-restricted (ovalbumin specific) CD8+ T cells (OT-I) or MHC class II-restricted (ovalbumin specific) CD4+ T cells (OT-II) was measured by [H3]Thymidine incorporation. CD8+ T cell cross-priming was increased by 35% (p<0.05 by repeated measures ANOVA and Newman Keuls, n=7) and 47% (p<0.001 by repeated measures ANOVA and Newman Keuls, n=7) in antigen presentation assays containing 5 µM and 10 µM of lenalidomide pretreated DCs respectively and by 43% (p<0.05 by repeated measures ANOVA and Newman Keuls, n=7) in antigen presentation assays containing 10 µM of pomalidomide pretreated DCs (Figure 4A). CD4+ T cell priming was increased by 10 µM pomalidomide pretreated DCs by 30% (p<0.05 by repeated measures ANOVA and Newman Keuls, n=7) (Figure 4B). Lenalidomide pretreated DCs had no effect on CD4 T cell proliferation. The mean percentage proliferation of DCs or T cells alone was on average, 10% of the mean DMSO control, as were assays containing DCs that had not been loaded with ovalbumin (Supplementary Figure S3). Therefore DCs alone, and T cells not exposed to ova primed DCs have little or no proliferative capacity.
CD8 T cells activated by IMiD® immunomodulatory compounds treated DCs express greater levels of intracellular Interferon gamma and perforin

Cocultures of either OTI or OTII splenocytes and DCs pretreated with either DMSO, or with 5 or 10 µM of lenalidomide or pomalidomide were assessed after 5 days for their ability to express intracellular interferon gamma or perforin (in the case of OTI splenocytes only). Intracellular IFN gamma and perforin levels were assayed by intracellular staining with PE- conjugated anti-IFN gamma or PE- anti-perforin antibodies after Brefeldin A treatment and PMA-ionomycin stimulation. Anti-CD4 or anti-CD8 surface markers were also used for selection of the OTI or OTII population by FACs. Separate aliquots of the cocultured cells were stained with FITC-annexin and propidium iodide (PI) to confirm viability of the lymphocytes. Results showed no increase in expression of intracellular IFN gamma in OTII splenocytes when cocultured with DCs that were pretreated with the IMiDs (compared to the DMSO control)(data not shown). However, a significant increase of intracellular IFN gamma expression from 4 upto 9% occurred in OTI splenocytes when cocultured with either 5 or 10µM of lenalidomide or pomalidomide treated DCs (p<0.05 by repeated measures ANOVA and Newman Keuls post test). Perforin levels in OTI cells were also increased from 4 to over 8% when cocultured with 10µM of lenalidomide or pomalidomide treated DCs(p<0.05 by repeated measures ANOVA and Newman Keuls post test). The viability of the OTI or OTII lymphocytes incubated with IMiD pretreated DCs was not significantly different to that of the lymphocytes incubated with DMSO pretreated DCs as assed by annexin /PI staining of the gated lymphocytes within the cocultures (1% or less positive annexin and or PI stained cells)(data not shown).

Discussion

The advent of two FDA approved DC vaccines has highlighted dendritic cells as an important component of the cancer therapeutic arsenal(21;22). We now demonstrate that lenalidomide and pomalidomide can directly enhance the maturation and cross priming function of dendritic cells in-
vitro, and therefore these drugs could be a useful adjuvant to any DC therapeutic programme. It has been shown previously that lenalidomide can increase the cross priming function of dendritic cells in a nude mouse model of myeloma(23) through activation of NK cells and can increase CD8+ T cells cocultured with DCs(24), but a direct in-vitro effect of pomalidomide and lenalidomide has not yet been demonstrated on dendritic cells.

We first studied whether lenalidomide or pomalidomide increased uptake of fluorescent latex beads into dendritic cells. The uptake of antigenic material is a crucial step in DC function, and some chemotherapeutic agents such as paclitaxel and rapamycin can inhibit the uptake of antigen by DCs (25). Both lenalidomide and pomalidomide increased uptake of FITC dextran beads by DCs by up to 2 fold at 10µM. The mechanism by which the drugs increase uptake is not clear, however, increased antigen uptake is known to require an activation of the P13K pathway(26), which can be induced by lenalidomide and pomalidomide in T cells and B cells (27;28).

We then assessed whether common markers of maturation were affected by lenalidomide or pomalidomide. Although no changes occurred to FAS, FAS ligand, or CD80, there were significant increases in CD86 and class I expression induced by both lenalidomide and pomalidomide, and pomalidomide also increased class II expression on the DCs. Lenalidomide has previously been shown to increase CD86 antigen expression in B cells of chronic lymphocytic leukaemia patients (29). Although matured DC are not efficient at taking up antigen in the human, antigen uptake by DC is possible in the matured state (30) and thus the augmentation of DC maturation by lenalidomide or pomalidomide does not contradict the ability of the drugs to increase antigen uptake in these cells.

We next determined the effects of lenalidomide and pomalidomide on cross presentation of antigen to T cells, measuring the proliferation of the T cells in response to drug treated DC. In our cross-
presentation assays, pomalidomide and lenalidomide treated DCs were more efficient at inducing OT-I antigen specific proliferation of CD8 T cells than non-treated DCs. This is most likely due to the increased expression of class I antigens on the DCs as a result of the lenalidomide and pomalidomide pretreatment, and due to the upregulation of CD86, which also induces a sustained co-stimulatory effect on CD8+ T cells, compared to CD80 (31). Both lenalidomide and pomalidomide treated DCs increased OT-I antigen specific (CD8+) T cell proliferation by up to 50% more than the control DMSO treatment. This increase was not due to any effects on T cells as DCs were preincubated with the drugs and then washed thoroughly before exposure to the T cells. Pomalidomide treated DCs, in contrast to lenalidomide, also increased proliferation of OT-II antigen specific CD4+ T cells by up to 50% at 10 µM. This may be due to the ability of pomalidomide to increase class II expression on the DCs, which was not observed with lenalidomide treatment.

To examine the functional effects of lenalidomide and pomalidomide on DCs we also examined their effects on the secretion of common cytokines from the cells. Although no effects were seen on IL-12 p70, IFN-γ, IL-6 or IL-10, significant increases were seen in TNF alpha and in MIP-1 alpha by lenalidomide, but not pomalidomide treatment. In addition to its chemokinetic activity, MIP-1 alpha is known to potently enhance CD8 stimulation by DCs (32) and also increases production of TNF alpha by DCs. As lenalidomide is known to decrease TNF alpha secretion in LPS stimulated monocytes, it is not clear whether the drug directly upregulates TNF in the DCs or whether this is through an effect on MIP-1-alpha. However, since pomalidomide treatment of DCs induces an antigen-specific increase in the proliferation of CD8 and CD4 T cells without increasing DC secretion of any of the cytokines tested, it is possible that the effects of both drugs are due to an increased CD86 costimulatory effect on the DCs. This needs to be explored in further mechanistic studies.

Both pomalidomide and lenalidomide treatment of DCs also results in an increase in the cytotoxic activity (as measured by perforin) and the intracellular interferon gamma expressed by OTI
splenocytes. OTII splenocytes are not similarly activated by lenalidomide or pomalidomide with regard to their IFN gamma secretion, but it is deduced that a greater activity of OTII cells will result in the presence of pomalidomide treated DCs as the OTII cells proliferate more in the presence of these DCs.

In conclusion, pomalidomide and lenalidomide pretreatment of DCs actively initiates class I restricted T cell activation with an additional class II restricted activation by pomalidomide and this mechanism is independent of any effects of the drugs on T cells or other effector cell types. These initial studies demonstrate the potential of lenalidomide and pomalidomide as adjuvants for use in DC vaccine therapies.

Figure legends

Figure 1: Lenalidomide and pomalidomide increase fluorescent bead uptake. IMiD treated DCs that were treated with fluorescent latex beads endocytosed significantly more beads than untreated DCs (B, C) when analysed by flow cytometry (A). Both 5 and 10 µM pomalidomide can increase uptake of latex beads by DCs by 8 and 10% compared to bead uptake in DMSO treated DCs (p<0.05 by repeated measures ANOVA and Newman Keuls, n=3). 10 µM of Lenalidomide also increases latex bead uptake by DCs by 10% compared to the DMSO control (p<0.05 by repeated measures ANOVA and Newman Keuls, n=3). NB: The uptake experiments with the lenalidomide (len+ DMSO control have slightly higher basal fluorescence than the experiments comparing pomalidomide with DMSO due to changes in the FL1 channel intensity observed in the FACs calibur. This has no effect on the results as DMSO was used as a control in both sets of experiments. The same gate was used for the DCs in all experiments.
Figure 2: Lenalidomide and pomalidomide alter phenotypic marker expression on DCs. Expression is measured as mean fluorescence index (MFI) which represents the intensity of fluorescence observed. Flow cytometric analysis of IMiD treated DC showed that expression of MHC Class I (H-2K^b) was increased by up to 73% by IMiD treatment (p<0.05 by repeated measures ANOVA and Newman Keuls, n=4) (Figure 2A) and CD86 was increased by up to 53% by IMiD treatment (p<0.05 by repeated measures ANOVA and Newman Keuls, n=4) (Figure 2C). Pomalidomide, but NOT lenalidomide increased expression of MHC Class II (I-A^b) by 72% (Figure 2B) compared to untreated DCs (p<0.05 by repeated measures ANOVA and Newman Keuls, n=4).

Figure 3: Lenalidomide effects DC cytokine expression. Supernatants were collected from IMiD treated DCs and expression of inflammatory cytokines was assayed by cytometric bead array. Lenalidomide treated DCs produced 945% more MIP-1 alpha (p<0.01, n=4) (Figure 3A) and 180% more TNF-α (p<0.05 by repeated measures ANOVA and Newman Keuls, n=4) (Figure 3B) than either untreated or pomalidomide treated DCs.

Figure 4: Lenalidomide and pomalidomide increase DC dependant T cell expansion. IMiD treated DC were loaded with ovalbumin, used in antigen presentation assays with OT-I or OT-II splenocytes and proliferation was measured by [H^3]Thymidine incorporation. A significantly higher CD8^+ T cell cross-priming was seen (35% and 47% higher than in antigen presentation assays containing 5 µM and 10 µM of lenalidomide pretreated DCs respectively compared to the DMSO control (p<0.05 by repeated measures ANOVA and Newman Keuls, n=7) and 43% higher in antigen presentation assays containing 10 µM of pomalidomide pretreated DCs (Figure 4A) (p<0.001 by repeated measures ANOVA and Newman Keuls, n=7). CD4^+ T cell priming was increased by 30% in 10 µM pomalidomide...
pretreated DCs (p<0.05 by repeated measures ANOVA and Newman Keuls, n=7) (Figure 4B).

Lenalidomide pretreated DCs had no effect on CD4 T cell proliferation.

Figure 5: CD8 (OTI) T cells expanded by pomalidomide and lenalidomide treated DCs are more active than CD8 (OTI) T cells cultured with DMSO pretreated DCs. OTI cells cocultured for 5 days with DCs treated with IMiDs or DMSO control were assessed for intracellular interferon gamma expression and OTI cells were additionally assessed for perforin expression by intracellular staining as described under the materials and methods. Results are expressed in panel A to show the separate gating of the lymphocytes (G1) which was then used as the gate to assess staining with PERCP-conjugated CD8 (FL3) and either PE conjugated perforin or PE-conjugated IFN gamma. The G1 gate contains a population of lymphocytes which are all alive (as assessed by less than 1% positive annexin and/or PI staining of cells within this gate in separate FACs tubes (data not shown)). Representative dot plots are shown in panel B. These show that the CD8 positive T cells within the G1 gate (indicated by the positive FL3 staining) also express IFN gamma and perforin (indicated by FL2 staining of the FL3 positive cells in the upper right quadrant). Panel C shows that expression of intracellular interferon gamma and perforin are increased from 4% up to 9% when CD8 (OTI) splenocytes are treated with 10 µM lenalidomide or pomalidomide pretreated DCs (p<0.05 by repeated measures ANOVA and Newman Keuls, n=3 experiments). DCs pretreated with 5 µM lenalidomide and pomalidomide also significantly increase IFN gamma expression in OTI CD8 T cells (p<0.05 by repeated measures ANOVA and Newman Keuls, n=3 experiments).

Figure S1: Effects of Lenalidomide and pomalidomide on phenotypic marker expression of DC. Flow cytometric analysis of iMID treated DC reveal no modulation of CD80, CD40, FAS, FASL and CD11c (no significance by repeated measures ANOVA or Newman Keuls, n=4).
Figure S2: CBA analysis of inflammatory cytokines expressed in lenalidomide and pomalidomide treated DC reveal no significant changes in IFN-γ, IL-10, IL-12 P70 and IL-6 expression (no significance by repeated measures ANOVA or Newman Keuls, n=4).

Figure S3: Antigen specific T cell proliferation: Data is presented as in Figure 4, with additional sets of control data. OT-I (A) and OT-II (B) splenocytes are incapable of proliferation alone and significant proliferation is not observed when ovalbumin (ova) loaded DCs are absent (no significance by repeated measures ANOVA or Newman Keuls, n=7).

Author contributions:

J.Y.H, C.G. and A.G.D. designed the research; J.Y.H. performed the experiments; Celgene supplied thalidomide, lenalidomide and pomalidomide; JYH and B.M. analysed data; B.M. and J.Y.H. prepared figures; J.Y.H, P.D. and C.G. wrote the paper; all authors reviewed and approved the manuscript.

Reference List


