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DOTTORATO DI RICERCA IN "SCIENZE MEDICHE"

CICLO XXXI

"Study of the immune-microenvironment in Multiple Myeloma: understanding the effects of immunomodulatory drugs and the contribution of immune checkpoints in disease progression"

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ABSTRACT

Despite major improvements in the treatment landscape, multiple myeloma (MM) remains an incurable malignancy. Alterations of the bone marrow (BM) immune-microenvironment, due to the presence of malignant plasma cells, characterize the progression of monoclonal gammopathies and the development of osteolytic bone disease. “Immunoparesis” is a common feature of MM patients displaying impaired dendritic, natural killer and T cell functions, whereas the onset of MM osteolytic lesions is associated to an increased prevalence of Th17 cells. Most recently, preclinical studies have also suggested the role of PD-1/PD-L1 pathway in the induction of tumor tolerance and immune evasion. However, contradictory results are currently available on the expression profile of PD-1/PD-L1 axis in MM patients and the possible correlation with the presence of osteolytic bone disease has not yet been explored.

Among the therapeutic strategies, the development of the Immunomodulatory drugs represented a paradigm shift in the treatment of MM. Lenalidomide-based regimen is one of the standard of care for MM patients either in frontline or in relapsed setting. However, the use of lenalidomide, alone or in combination with immune checkpoint blockade, to reverse tumor-mediated immune suppression and amplify MM-specific immunity is currently under investigation. Particularly lenalidomide effects on dendritic cells are still unclear.

In this study, the potential effect of lenalidomide on dendritic cell differentiation and activity has been investigated. Dendritic cells were differentiated from either primary MM $CD14^+$ cells or from a human monocytic cell line.

Lenalidomide, at the concentration range reached *in vivo,* significantly increased the median intensity expression of HLA-DR, CD86 and CD209 by dendritic cells derived from both BM and peripheral myeloma monocytes and enhanced the production of Interleukin-8, C-C motif

chemokine ligand 2 and tumor necrosis factor-α. Consistently, lenalidomide pre-treated dendritic cells showed an increased ability to stimulate autologous $CD3^+$ cell proliferation. Lenalidomide effect on dendritic differentiation was associated with the degradation of the Cereblon-related factors Ikaros and Aiolos in monocytes. Moreover, lenalidomide *in vitro* treatment blunted mesenchymal stromal cell inhibitory effect on dendritic differentiation inhibiting casein kinase-1α levels. Finally, *in vitro* data were confirmed in *ex-vivo* cultures obtained from relapsed myeloma patients treated with lenalidomide at 25 mg/day showing a significant increase of dendritic cell differentiation from peripheral blood monocytes.

In conclusion, lenalidomide increased the expression of mature dendritic markers both directly and indirectly and enhanced dendritic cell ability to stimulate T cell proliferation and to release chemokines. This suggests a new possible mechanism by which lenalidomide could exert its anti-myeloma activity.

In the second part of the study, the expression profile of PD-1/PD-L1 was evaluated in plasma cells, monocytes and T cells from BM aspirates of patients with monoclonal gammopathies and the results were correlated with clinical data, especially the presence of bone disease. Interestingly, an increased frequency of $PD\text{-}1^+$ T cells was observed across the progression of the disease. Moreover, for the first time, it was found a significant relationship between the presence of extensive osteolytic bone disease and a reduced expression of PD-1 on BM $CD8^+$ T cells and PD-L1 on malignant PCs and monocytes in MM patients. It was thus hypothesized that a less immune-suppressive profile could be related to the development of osteolysis and probably mediated by the effect of PD-1/PD-L1 axis on Treg/Th17 cell ratio.

These preliminary data thus provide a new mechanism by which PD-1/PD-L1 axis could exert its effect within MM BM microenvironment.

RIASSUNTO

Il Mieloma Multiplo (MM) è una neoplasia ematologica caratterizzata dalla proliferazione clonale di plasmacellule (PC) maligne a livello del midollo osseo. La presenza delle PC causa soppressione della risposta immunitaria e induce l'insorgenza di malattia ossea. I pazienti con MM mostrano infatti deficit numerici e funzionali a carico di linfociti T, cellule natural killer e cellule dendritiche (DC). L'accumulo di linfociti Th17 correla invece con lo sviluppo di lesioni litiche. Recentemente è inoltre emerso il ruolo dell'asse PD-1/PD-L1 nella soppressione immunitaria e nella progressione di malattia sia in tumori solidi che ematologici, tuttavia non è ad oggi chiara la funzione di tale checkpoint nel MM.

Tra gli agenti terapeutici attualmente in uso per la cura del MM, i farmaci immunomodulatori (IMiD®) come la lenalidomide (LEN) rappresentano uno "*standard of care*". Tuttavia l'attività di potenziamento della risposta immunitaria contro il MM da parte degli IMiD® non è ancora del tutto chiara. Nella prima parte di questo studio è stato quindi valutato l'effetto diretto della LEN sul differenziamento delle DC e sulle proprietà immunosoppressive delle cellule mesenchimali stromali (MSC).

Le DC sono state ottenute *in vitro* da monociti $CD14^+$, purificati sia da sangue periferico che da midollo osseo di pazienti con MM. Durante il periodo di differenziamento, le cellule sono state trattate con LEN a diverse concentrazioni (0.1-1μM), equivalenti ai livelli sierici riscontrati in pazienti trattati con LEN (5-25mg/die). Al termine della coltura le cellule sono state analizzate tramite citofluorimetria per i markers di maturazione (CD83, HLA-DR, CD80, CD86 e CD209). I livelli di fattori solubili coinvolti nella risposta immunitaria (CCL2, CCL5, CXCL10, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α) sono inoltre stati dosati tramite ELISA nei surnatanti di tali colture. Dai risultati è emerso che il trattamento con LEN causa una riduzione del numero e della percentuale delle DC mature rispetto ai controlli non trattati e un aumento

significativo dell'espressione di CD86, HLA-DR e CD209 sia nelle DC derivate da midollo che da sangue periferico. Tale effetto è mediato dalla degradazione, Cereblon-dipendente, dei due fattori di trascrizione Ikaros e Aiolos. Inoltre, il trattamento con LEN aumenta la produzione da parte delle DC di IL-8, CCL2 e TNF-α e potenzia la capacità delle DC di stimolare la proliferazione di cellule T autologhe. È interessante notare come anche il trattamento *in vivo* con LEN (25mg/die) in pazienti affetti da MM induca un aumento dell'espressione dei marker di maturazione delle DC, differenziate a partire da cellule $CD14^+$ purificate da sangue periferico al giorno 0 e al giorno 7 di trattamento con LEN. Accanto all'effetto diretto della LEN sulle DC, da questo studio è emerso che il trattamento con LEN riduce l'effetto inibitorio delle MSC sul differenziamento delle DC, tramite down-regolazione di CK1-α nelle MSC. In conclusione, questi dati suggeriscono nuovi possibili meccanismi con cui gli IMiD® potenziano la risposta immunitaria anti-mieloma.

Nella seconda parte dello studio, il profilo di espressione di PD-1/PD-L1 è stato valutato su PC, monociti e cellule T ottenute da aspirato midollare di pazienti con gammopatia monoclonale a diverso stadio di malattia; i risultati sono stati poi correlati con i dati clinici, in particolare con la presenza di malattia ossea. Una maggiore frequenza di cellule T $PD\text{-}1^+$ è stata osservata nel corso della progressione della malattia. Inoltre, per la prima volta, è stata trovata una relazione significativa tra la presenza di malattia ossea osteolitica e una ridotta espressione di PD-1 su cellule $CD8^+$ e di PD-L1 su PC maligne e monociti in pazienti con MM. L'ipotesi è che un profilo meno immunosoppressivo possa essere correlato allo sviluppo dell'osteolisi e probabilmente mediato dal ruolo di PD-1/PD-L1 nel bilanciamento del rapporto cellule Treg/Th17. Questi dati preliminari forniscono quindi un nuovo meccanismo attraverso il quale l'asse PD-1/PD-L1 potrebbe esercitare il suo effetto all'interno del microambiente midollare nel MM.

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1. INTRODUCTION

1.1 Multiple Myeloma and its pathophysiology

Multiple myeloma (MM) is the most common form of plasma cell dyscrasia, affecting B-cells that have traversed the post-germinal center. It is characterized by clonal proliferation of malignant plasma cells (PCs) in bone marrow microenvironment and rarely at extra-medullary sites as the end stage of a multistep transformation process. [1]

MM can be preceded by indolent forms as the monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), that evolve in malignant MM by multistep genetic and microenvironmental changes. [2] The presence or absence of CRAB criteria (hypercalcemia, renal insufficiency, anemia, and bone disease) differentiates symptomatic from asymptomatic patients.[3]

Complex cytogenetic abnormalities as well as the expression of adhesion molecules, the production of cytokines and the interaction with the BM microenvironment cells, including immune cells, bone cells, endothelial cells, mesenchymal stromal cells (MSCs) and extracellular matrix, collaborate to the abnormal development of B cells.[4, 5]

Some genetic alterations are already detectable at MGUS level while others arise later, supporting a multistep development of MM.[6, 7] Many studies have highlighted that chromosomal translocations involving immunoglobulin heavy-chain (IgH) represent an important prognostic value in MM. Specifically t(14;16) and t(4;14) are associated with a poor prognosis, whereas a better prognosis is observed in patients carrying the t(11;14).[8] Among the heterogeneous alterations, gain or loss of specific chromosomal regions including monosomy or partial deletion of chromosome 13, loss of the short arm of chromosome 17 (where the tumor-suppressor gene TP53 resides), gain or amplification of chromosomal region 1q.2. and hyperdiploidy seem to correlate with increased risk of disease progression.[6, 9-11] However, the detection of the majority of MM cytogenetic alterations even in MGUS PCs suggests the potential role of the microenvironment in controlling the stability and preventing the malignant transformation.

1.2 Role of bone marrow microenvironment in MM

It is well understood that the cross talk between malignant PCs and the BM microenvironment plays a pivotal role in the proliferation and survival of tumor cells.[12] The presence of

malignant PCs in the BM niche indeed create a permissive microenvironment trough both cell-to-cell contact and release of soluble factors such as cytokines, chemokines, growth factors, exosomes and miRNA. [13, 14]

BM microenvironment includes a non-cellular compartment, the extracellular matrix (ECM) and a heterogeneous cellular compartment. The ECM consists of several proteins, including fibronectin, laminin and collagen, and can be remodelled by matrix metalloproteinases (MMPs). The typical PC marker CD138 (also known as syndecan 1) binds directly to ECM proteins such as fibronectin, and induces the release of MMP by BMSC thus promoting neoangiogenesis and tumor invasion. Moreover, the VLA5-mediated interaction of MM cells with ECM induces resistance to the conventional chemotherapy.[15]

Several cell types constitute the BM microenvironment, including haematopoietic cells [(B cells, T cells, natural killer cells (NKs), dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) and osteoclasts (OCs)] and non-haematopoietic cells [(bone marrow stromal cells (BMSCs), osteoblasts (OBs) and endothelial cells].

The physical interaction between the adhesion protein VCAM1/ ICAM1 on the surface of BMSCs and integrins (VLA4/LFA1) on MM cells results in the release of several cytokines, as IL-6 that promote myeloma cell proliferation and inhibit apoptosis.[16] Moreover, CXC-chemokine ligand 12 (CXCL12) expressed by BMSCs, OBs, endothelial cells binds to CXC-chemokine receptor type 4 (CXCR4) on MM cells thus enhancing their migration to the BM.[17] BMSC-MM cells interaction is also mediated by Notch-Notch ligand signalling which induce the secretion of pro-angiogenic factors as vascular endothelial growth factor (VEGF) and activate anti-apoptotic pathways in MM cells.[18-20]

The interaction of MM cells with BM microenvironment cells also enhances osteoclastogenesis and OC activity and suppress OB formation and function,[21] with the consequent development of osteolytic lesions that characterize MM patients.[3] High levels of different soluble factors involved in OC activation and/or OB suppression are indeed released into the BM microenvironment, including chemokine (CC motif) ligand (CCL)-3, dickkopf-related protein (DKK)-1, hepatocyte growth factor (HGF), IL-1, IL-3, IL-6, IL-7, secreted frizzled related protein (sFRP)-2, sFRP-3, and TNFα.[21] Moreover, cell-to-cell contact between MM cells and BMSCs up-regulates the main pro-osteoclastic cytokine, receptor activator of nuclear factor-Kb ligand (RANKL), and decreases the release of RANKL decoy receptor osteoprotegerin (OPG) from osteoprogenitor cells.[22] This direct interaction also

inhibits the expression of the main pro-osteoblastogenic transcription factor, Runt-related transcription factor (Runx) 2, on BMSCs thus suppressing OB differentiation.[23] The inhibition of Runx2 activity and OB suppression is further supported by some of the cytokines released into the BM as IL-3, IL-7 and HGF.[24] IL-3 also induce the release of activin A, by monocytes, that in turn enhances OC differentiation.[25]

Among BM microenvironment components, immune cells also seem to be involved in the onset of MM-induced bone disease.

Literature data demonstrated that MM T cells secrete high amounts of RANKL[26] and the presence of MM cells in co-colture systems further increases the expression and secretion of RANKL in a IL-7 dependent manner.[26] Consistently, RANKL up-regulation by BM T cells was found in MM patients with severe osteolytic lesions as compared to MM patients without bone lesions.[26] MM T cells also sustain MM-induced osteoclastogenesis through the release of other pro-osteogenic cytokines as IL-3.[27]

Moreover, an increase of IL-17-producing Th type 17 (Th17) cells was found in MM BM[28] and is mediated by mature DCs.[29] Beside the inhibition of cytotoxic T cell activity and the promotion of MM cell growth, IL-17 also enhances *in vitro* osteoclastogenesis.[28, 29] Interestingly, a significant correlation between Th17 cell number and the degree of lytic bone lesions has been reported in MM patients.[44]

Th17 recruitment is known to be mainly support by CCL20.[28] Consistently, MM cells up-regulate the expression of CCL20 and its receptor CC chemokine receptor (CCR) 6 into the bone microenvironment, through IL-1 and TNFα,[30] thus sustaining the IL-17 mediated increase of OC formation. Accordingly, the use of neutralizing antibodies (Abs) against CCL20 or CCR6 significantly inhibits MM-induced OC formation *in vitro*.[30]

Several studies have reported that MM-induced osteoclastogenesis in turn enhances MM cell survival, angiogenesis and drug resistance, through the OC release of MM pro-survival factors, such as osteopontin (OPN) and IL-6, B-cell-activating factor (BAFF) and A proliferating-inducing ligand (APRIL), after MM-OC adhesion.[31, 32]

Finally, emerging data indicate that OCs protect MM cells against T cell responses through the expression of immunosuppressive molecules including Programmed death-ligand 1 (PD-L1), galectin 9, anti-herpesvirus entry mediator (HVEM) and CD200.[33]

1.2.1 Alterations of immune cell subsets in MM

In physiological conditions, the immune effector cells are able to drive potent anti-tumor responses, identifying and eliminating tumor cells with high specifity, based on their expression of ”non-self” antigens.[34] However, “immunoparesis” is a common feature of MM patients [35] due to the alterations induced by the presence of malignant PCs in BM microenvironment.[13] These alterations involve several immune effector cells, such as DCs, T cells, NKs and MDSCs and this loss of function is notably associated with progression to clinical MM.[36]

DCs: DCs are the most potent stimulators of T-cell responses among the professional antigen-presenting cells (APCs), and they play a crucial role in initiating the immune response, with a peculiar ability to stimulate both memory T cells and naïve T lymphocytes.[37] An efficient T cell response requires the engagement of the T cell antigen receptor (TCR), but also the expression of costimulatory molecules CD40, CD80, CD86 on APCs.[37]

Literature data reported that number, phenotype, function and development of DCs are significantly altered in MM patients.[38-42] Specifically, blood levels of myeloid DCs were decreased in the 50% of MM patients and a defect of CD80 and CD54 up-regulation was reported following GM-CSF and IL-4 *ex vivo* stimulation.[42] This alteration was partially due to high PC release of IL-6, IL-10, transforming growth factor β (TGF-β), macrophage colony-stimulating factor (M-CSF) and VEGF, known to prevent DC differentiation, maturation and function, both *in vitro* and *in vivo*.[43]

Ratta et al. also described that PB DCs from MM patients lack of the costimulatory molecules, CD80 and CD86, and accumulate in BM in the immature status.[39] This defect causes an impaired ability to activate T-cells and it is due to the higher plasma levels of IL-6 in MM patients.[39] IL-6 indeed inhibits DC formation by redirecting the differentiation of hematopoietic progenitor cells toward the monocyte-macrophage lineage.[44]

Additionally, lower levels of chemokine receptors were observed in MM patients compared to healthy donors, which in turn reduce DC migration and impair the ability to stimulate T-cell allo-reactions.[41]

On the other hand, immature DCs (iDCs) can induce the clonogenic proliferation of MM cells through B cell activating factor (BAFF)/a proliferation inducing ligand (APRIL) signals.[45, 46] Malignant PCs can in turn prompt DC fusion and trans-differentiation into osteoclasts (OCs) through receptor activator of nuclear factor κB ligand and CD47 pathways,[47-49] thus promoting immunosuppression and disease progression.

Further studies described a direct interaction between DC and PCs which induces the production of IL-6 and indoleamine 2,3 dioxygenase (IDO) enzyme by DCs thus enhancing MM survival and proliferation.[50] IDO causes the depletion of the tryptophan, an essential amino acid for T-lymphocytes, from microenvironment and promotes the differentiation of T-lymphocytes into a regulatory phenotype, thus contributing to the creation of the typical immunosuppressive micro-environment of MM.[50]

T CELLS: T cells play a central role in cell-mediated immunity. Several studies have shown significant alterations in the number and functionality of T cells, both in the BM and in the PB of MM patients.[51] Specifically, a decrease CD4/CD8 ratio occurs in patients with advanced stage disease along with an imbalance in $CD4^+$ Th1/Th2 subpopulations due to reduced production of pro-Th1 cytokines such as IL-2 and interferon- γ (IFN-γ) and a strong expression of pro-Th2 cytokines such as IL-4 and IL-10.[51] The imbalanced Th1/Th2 ratio that results in a defective Th1 response also correlates with the stage of the disease and with IL-6 plasma levels.[13]

Contradictory data are currently available on Tregs in MM. Brimnes et al described an accumulation of immunosuppressive Tregs in the tumor microenvironment, with an increased expression of FOXP3 in the BM of MM patients.[52] On the other hand, a study from Prabhala et al revealed a decreased number and function of Treg cells in MGUS and in MM patients compared to healthy donors,[53] suggesting the need to better clarify the role and the alterations of this population.

An accumulation of IL-17 producing Th17 cells is instead found in MM patients and correlates with the presence of bone disease.[28, 29] Th17 differentiation is mediated by TGF-β and IL-6.[54] In contrast, increased levels of IL-6 suppress Treg formation.[55] Based on the critical role of IL-6 in the suppression of Treg differentiation and promotion of Th17 differentiation, it should be conceivable an alteration of the Th17/Treg balance in MM patients.

Several studies recently suggested that the compromised functions of effector cells in MM may be due to senescence as demonstrated by the expansion of T cells with senescent phenotype (CD57, CD160 and KLRG-1 expression).

NK CELLS: NKs derive from lymphoid progenitors and display cytotoxic effect, anti-tumor capacity and multiple immune-regulatory properties.

Literature data show a role for NKs in the control of development and progression of MM.[56] NK cell number and activity negatively correlate with the clinical stage of the disease,

suggesting that MM growth interferes with the NK cell ability to counteract tumor expansion.[56]

Myeloma cells express various ligands such asNKG2D ligands (MHC-I polypeptide-related sequence (MIC) A/B and retinoic acid early transcript), NKp30 ligands (B7-H6), and DNAM1 ligands (CD112/nectin 2 and CD155/necl5), which bind to activate the signal molecules expressed on NK cell surface. With the progression of disease, myeloma cells upregulate the expression of the HLA class I molecules to reduce the NK cell sensitivity.[57, 58]

Moreover, the combined reduction of NKG2D receptor leads to the inhibition of NK cell functions.[59, 60] In the later stage, myeloma cells can also shed the soluble NKG2D ligands, as a mechanism of drug resistance for NKG2D- mediated killing.[61]

In addition, myeloma cells produce several cytokines, such as IL-6 and IL-10, with immunosuppressive activity NK proliferation and functions[62] and promote the migration of effector NK cells outside the BM upregulating the expression of some chemokines as CXCR3 ligand and downregulating the expression of CXCL12.[63]

MDSCs: MDSCs are a very heterogeneous population of immature myeloid progenitor cells that fail to differentiate into granulocytes, macrophages and DCs. MDSCs are divided into two major populations: granulocytic-MDSCs (G-MDSCs) and monocytic-MDSCs (M-MDSCs).[64] MDSCs display potent ability to arrest T cell proliferation and reduce T cell migration, to induce Treg polarization of naïve T cells and suppress NK activity.[64]

The accumulation of MDSCs in cancer patients is a known phenomenon; however, contradictory results are currently available in MM mainly due to the lack of a consensus on the gating strategy to phenotypically define these cells.

Several studies found an increased number of only G-MDSCs in PB and BM of patients with MM compared to healthy donors,[65-68] able to promote tumor growth in the MM microenvironment.[69] MM cells in turn induced the development of MDSCs from HD PB mononuclear cells (MNCs), confirming a bidirectional interaction between MDSCs and MM cells and immune effector cells.[65]

However, no correlation was found between the % of MDSCs and the progression of disease, comparing patients with MM at diagnosis, remission and relapsed.[66]

In contrast, other authors reported an increased number of M-MDSCs in PB of MM patients compared to healthy donors.[52] However, few data are currently available on patients with different stages of disease, particularly on SMM patients. Moreover, not all the studies

correlated the phenotypical analysis with functional assays, thus suggesting the need to better clarify the role of these cells in MM

1.2.2 PD-1/PD-L1 pathway in MM

The role of PD-1/PD-L1 axis in the MM –induced immune suppression has recently emerged. PD-1 is a member of the CD28/CTLA-4 family, with inhibitory properties, mainly expressed on exhausted T cells (dysfunctional T cells classically associated with chronic infection), NK and NKT cells following activation. APCs, monocytes and malignant cells express its ligands, PD-L1 and PD-L2, especially under inflammatory conditions.[70]

Similarly to CTLA-4, the interaction between PD-1 and PD-L1 interferes with TCR signal transduction, by recruiting the tyrosine phosphatase SHP-2 and subsequent inactivating the PI3 kinase-signalling cascade,[71, 72] which leads to reduced cytokine synthesis, cytotoxic functions and blockade of T cell proliferation and survival.[70]

In the physiologic setting, this pathway enables the immunologic equilibrium after initial T cell response, preventing over-activation and the possible expansion of auto-reactive T cells.[73] According to the relevance of PD-1/PD-L1 axis in immune control, tumors, including myeloma, seem to highjack this pathway to suppress and escape the activation of an immune response.[74]

PD-L1 is highly expressed on PCs isolated from patients with MM but not on normal PCs. Notably, PD-L1 is not expressed on PCs isolated from patients with MGUS.[75] High PD-L1 expression on PCs was associated with disease progression in patients with MGUS and asymptomatic MM [76] and it could play a role in the development of clonal resistance as demonstrated by PD-L1 high levels in relapsed or refractory MM patients.[77] Nevertheless, different methodologies, cut-offs of positivity and gating strategies were used in these studies, thus resulting in different percentage ranges of PD-L1^{+} PCs.

In *vitro* studies revealed that PD-L1 expression on MM cells is upregulated in the presence of TRL ligands or IFN-γ, and stromal cells in an IL-6 dependent manner. PD-L1 blockade thus inhibits stromal cell mediated PCs growth.[78]

More recently, OC immunosuppressive properties have emerged, beside their role in bone remodelling. Specifically, they support tumour escape inducing T-cell apoptosis through PD-L1 up-regulation in MM cells, induced by immune checkpoint proteins as TIM-3 and the production of IDO and APRIL. [33]

Sponaas AM et al[78] reported that myeloid DCs also express PD-L1. Specifically, PD-L1 expression is higher on the CD141$^+$ subset that controls CD8$^+$ T cell responses than on the CD141- subset controlling CD4$^+$ T cell responses. Moreover, PD-L1$^+$ mDCs correlate with PD-L1$^+$ PCs, suggesting that both cell types could contribute to the suppression of the anti-tumor T cell response in MM through PD-1/PD-L1 pathway. [78] Contradictory results are currently available on PD-1 expression in T cell compartments across different stages of disease. Gorgun et al.[69] reported an increased expression of PD-1 on CD4$^+$ T cells from ND MM and relapsed refractory MM (RRMM) samples compare with HDs and no difference in PD-1 expression on CD8$^+$ T cells.[69] On the other hand, Rosenblatt et al. described PD-1 upregulation in both CD4$^+$ and CD8$^+$ T cells in patients with advanced MM in comparison with HD. Finally, Paiva et al.[79] found a similar percentage of PD-1$^+$ T cells between HDs and ND MM and MGUS. However, a significant increase in PD-1 expression on both CD4$^+$ and CD8$^+$ T cells was reported in MM R and MRD positive patients.[79]

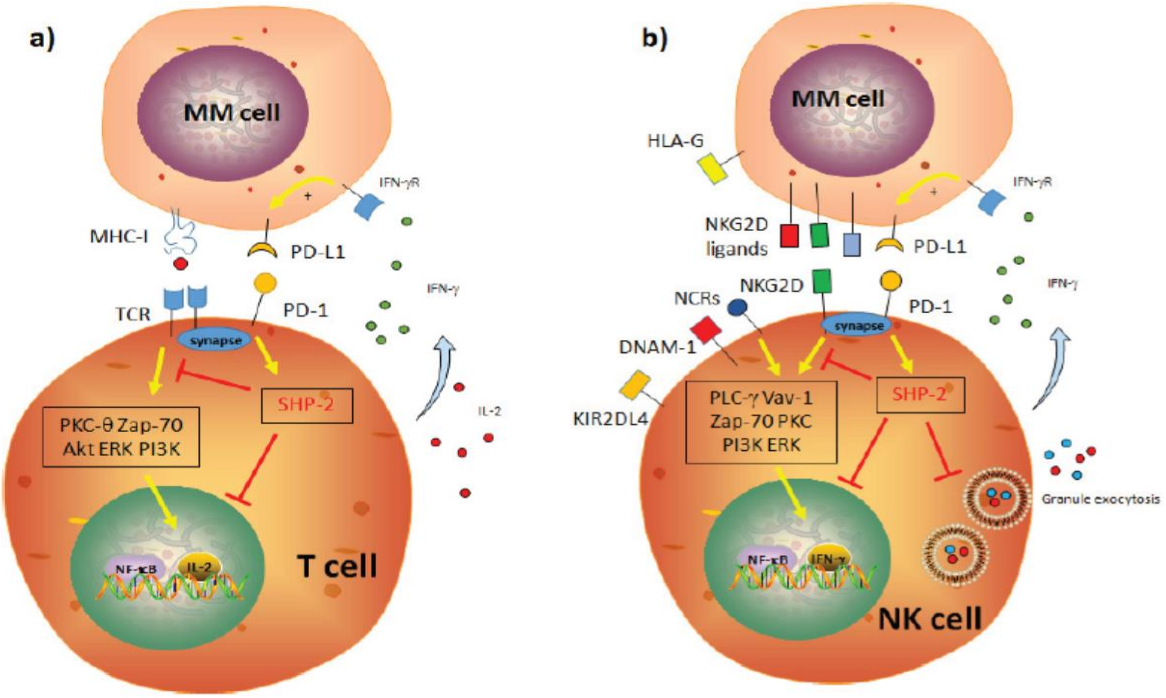


Figure 1: Effect of PD-1/PD-L1 pathway activation in T cells (a) and NK cells (b). *(Adapted from Giuliani M et al. Oncotarget 2017)*

It is well defined that PD-1 is absent on normal CD56$^+$CD3- NK cells from HDs; however, discordant results are available regarding NK cells from MM patients. Several studies [69, 80]

reported a significantly higher expression on NK cells from MM patients compared with HDs, whereas Paiva et al. did not find any difference.[79]

Hence, there remains an unmet need to understand the expression level and the distribution of PD-1/PD-L1 in MM and to determine patients that could beneficiate from PD-1/PD-L1 blockade.

In vitro studies showed that MM microenvironment could induce PD-L1 expression on PCs; PD-L1 up-regulation indeed occurs in the presence of stromal cells[77] and PD-L1 blockade inhibits stromal cell mediated PC growth.[69] This effect is IL-6 dependent and mediated by STAT3, MEK1/2, and JAK2 pathways.[77]

IFN-γ produced by cytotoxic T lymphocytes (CTLs) and NK cells, strongly induces PD-L1 expression through the activation of MEK/ERK pathway.[75] In addition, both myeloid and pDCs and MDSCs express PD-L1 in MM patients,[78] with an increased proportion of PD-L1$^+$ MDSCs in MM patients at remission compared to newly diagnosed and relapsed MM.[66]

T cells from MM patients also display higher PD-1 expression levels, associated with loss of effector cell function[80] on both circulating T cells and BM CD8$^+$ T and NK cells compared to HDs.[69] Moreover, a study from Castella et al.[66] showed that PD-1 expression is already present on the anergic BM Vγ9Vδ2 T cell subset from MGUS patients and remained upregulated in MM after clinical remission.[66] In contrast, PD-1 expression is reduced in T cells from patients who achieved minimal disease state following high dose chemotherapy.[81] I*n vitro* studies further demonstrated that PD-1/PD-L1 blockade directly enhances NK and T cell mediated anti–MM responses[69, 80] and restores the capacity of PD-L1$^+$ pDCs to induce cytotoxic activity of T cells and NK cell against MM cells.[82]

The effects of anti-PD-L1 mAb were then tested *in vivo*, on the 5T33 murine MM models, after autologous (syngeneic) stem-cell transplantation plus administration of a cell-based vaccine[83] or after irradiation.[84] It was demonstrated that mice with advanced MM expressed higher levels of PD-1 on both CD8$^+$ and CD4$^+$ T cells compared to non –tumor bearing mice and the percentages of PD-1$^+$ T cells correlated with the amount of tumor burden.[84] Moreover, PD-1$^+$ CD8 T cells isolated from these mice showed a defective production of pro-inflammatory cytokines (IFN-γ and IL-2) after *in vitro* stimulation and expressed increased levels of the exhausted T cell marker TIM-3.[84] PD-1 blockade also prolonged the survival in disseminated myeloma-bearing mice[79, 83, 84] and this effect was abrogated by the depletion of CD4$^+$ or CD8$^+$ T cells, indicating the main role of both T cell

subsets behind this strategy.[83] Taken together, these studies supported the potential contribution of PD-1/PD-L1 pathway in the immune escape in MM and suggested that its blockade may be an effective therapeutic strategy against this tumor.

1.3 Immune therapy in MM

1.3.1 Immunomodulatory drugs (IMiDs®)

IMiDs® are a class of small-molecule therapeutic agents that originate from Thalidomide (THAL), a glutamic acid derivative with immunomodulatory, anti-angiogenic and anti-inflammatory properties. The two compounds, Lenalidomide (LEN) and Pomalidomide (POM), were synthesized by modifying the chemical structure of THAL (Figure 2) to improve its potency and reduce its side effects, such as teratogenicity and neurologic toxicity. (Table 1)
The development of IMiDs® represented a paradigm shift in the treatment of MM.[85, 86] LEN-based regimen is one of the standard of care for MM patients either in frontline or in relapsed setting,[87-89] with a significant impact also on the progression free survival in maintenance after autologous stem cell transplantation.[90]

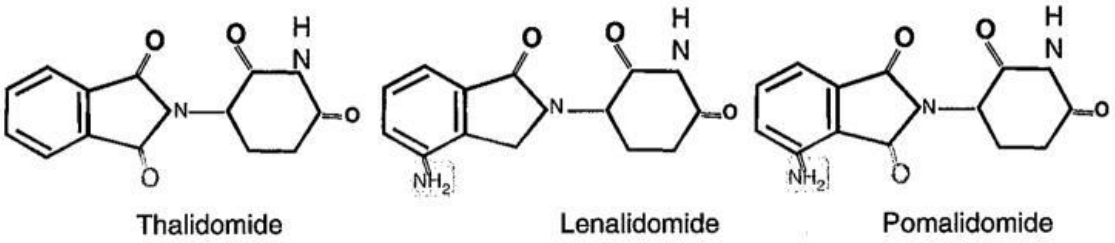


Figure 2: Chemical structure of IMiDs®

Several mechanisms of action have been described,[91, 92] including proliferation and functional enhancement of NK/NKT cells, induction of T-cell co-stimulation and reduction of Tregs activity, increased Th1 cytokine production such as IL-2 and IFN-γ and down regulation of Th2 cytokines, IL-4 and IL-10, anti-MM ADCC improvement.[93, 94]
Besides the immunomodulatory effects, IMiDs® display also anti-angiogenic activity[95], affect the release of inflammatory cytokines, especially TNF-α[96] and have direct effect on MM cells inducing cycle arrest and apoptosis.[97, 98]

Effect	Relative potency += potency factor 10		
	Thalidomide	Lenalidomide	Pomalidomide
Interference with tumor micro-environment interaction			
Anti-angiogenesis	++++	++	++
Anti-inflammatory properties	+	++++	+++++
Downregulation of adhesion molecules	+	++++	+++++
Anti-osteoclastogenic properties	+	++++	+++++
Direct anti-tumor effects			
Anti-proliferative activity	+	+++	+++
Immune modulation			
CD4+ and CD8+ T cell co-stimulation	+	++++	+++++
Tregs suppression	-	+	+
Th1 cytokine production	+	++++	+++++
NK and NKT cell activation	+	++++	+++++
Antibody-dependent cellular cytotoxicity (ADCC)	-	++++	++++

Table 1: *adapted from* Sedlarikova L et al. *Leuk Res 2012*

The main molecular mechanisms involved in the anti-MM effect of IMiDs® have been elucidated, highlighting the role of Cereblon (CRBN) and its target factors (Figure 3).[99, 100] LEN binds CRBN in MM cells and causes selective ubiquitination and degradation, by the CRBN-Cullin-RING E3 ubiquitin ligase (CRL) 4 complex, of two lymphoid transcription factors essential for MM survival, Ikaros and Aiolos.[101] Through the same mechanism, LEN enhances T cell proliferation and interleukin (IL)-2 production. The loss of Ikaros and Aiolos is necessary and sufficient for LEN therapeutic effect.[102] Nevertheless, it is not known which is the role of DCs in the anti-MM effect of LEN.

Recently, it has been also reported a distinct CRBN substrate, Casein Kinase 1 alpha (CK1α) that is ubiquitinated and degraded after LEN treatment in myelodysplastic syndrome (MDS) with deletion of chromosome 5q (del(5q)).[103] A study from Manni S et al.[104] interestingly describes the role of CK1α in supporting the growth of MM PCs. Moreover, an enhanced cytotoxic effect of both bortezomib and LEN was reported after CK1α inactivation, thus suggesting the involvement of CK1α even in the anti-MM activity of IMiDs®.

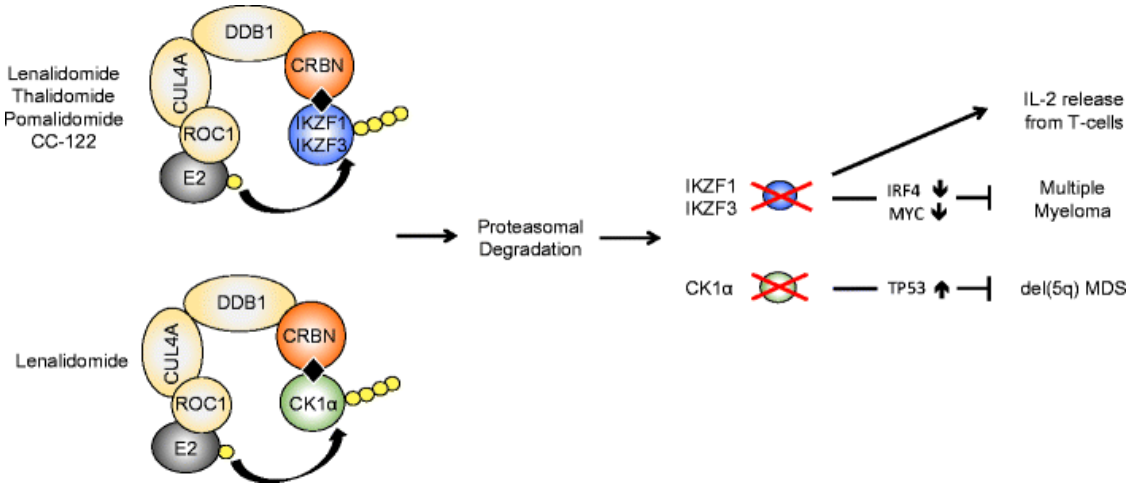


Figure 3: Molecular mechanism of action of IMiDs® *(Lindner et al. Journal of Molecular Medicine 2016)*

Beside these effects, *in vitro* studies interestingly showed that LEN treatment downregulates PD-1 expression on both T cells[80] and NK cells,[69] restoring their cytotoxic activity, and decreases PD-L1 expression on malignant PCs and MDSCs.[80] This data suggested that LEN could enhance the effect of anti PD-1/PD-L1 blockade as further reported by Görgün G et al.[69]

1.3.1.1. LEN as maintenance therapy in allo-SCT

Among the different therapeutic strategies in MM, allo-SCT induces the highest rate of clinical complete remissions[105] and supports remission status in association with maintenance therapies, including LEN.[106]

These effects are related to an increased number of peripheral IFNγ–secreting $CD4^+$ and $CD8^+$ T cells, along with a delayed increase of Treg cells and a significantly improved NK anti-MM activity after the first week of 5mg/d LEN treatment, as reported by Wolschke C. et al.[107] Although no data are available on DC populations.

However, GvHD limits the efficacy of allo-SCT.[108] The incidence of GvHD after allo-SCT and LEN treatment was about 40% in MM patients and it might be due to the early increase of effector T cells and the simultaneous decrease of Treg cells.[107] Since it is known that host and donor DCs are critical in the development of GvHD[109] and involved in the immunosuppressive properties of MSCs,[110] it is conceivable that LEN also affects DCs.

Identifying the critical signals controlling the complex features of allo-immunity is essential for regulating the balance between harmful and beneficial effects of allo-SCT. Literature data recognized Notch signaling as a potent regulator of T-cell activation, differentiation, and function during acute GvHD.[111]

Notch is a protein that belongs to a family of highly conserved receptors. It translocates into the nucleus and activates the transcription of several target genes involved in T cell maturation,[112] such as $CD4^+$ Th2 cell differentiation through transcriptional activation of IL4 and GATA3 genes.[113] It may also regulate Th1, Th17, and Treg cell activity,[114, 115] and it was recently described to control the effector program of $CD8^+$ cytotoxic T cells.[116]

Notch inhibition decreases the production of multiple cytokines by alloantigen-activated $CD4^+$ T cells, including IFN-γ, IL-2, IL-4, IL-17 and TNF-α.[111] Furthermore, Notch inhibition preserved significant antitumor activity in alloreactive $CD4^+$ T cells. Based on these evidences, modulation of Notch signaling in T cells may identify an approach to prevent GvHD while preserving useful GVT effects.[111]

Moreover, host APCs play a vital role in the initiation of GvHD responses. Recent studies demonstrated that the Ikaros-Notch axis in host hematopoietically derived APCs regulates the severity of acute GvHD across multiple clinically relevant murine models of experimental BM transplantation.[117] Specifically, Ikaros deficiency in host APCs enhanced GvHD through the increase of allogenic T cell proliferation in a mouse model of allo-SCT.[117] These results, together with previous observations that complete absence of Notch signaling in donor T cells did not alter their *in vivo* proliferative response to allo-antigens,[111] demonstrate a cell-intrinsic functional role for enhanced Notch signaling in Ikaros $^{-/-}$ DCs.

All these data thus show a critical role for a novel molecular pathway, the Ikaros-Notch axis, in host APCs as an important modulator of GvHD.

1.3.2 Clinical studies of PD-1/PD-L1 checkpoint blockade and combinations in MM

Based on the encouraging data of the preclinical studies on PD-1/PD-L1 blockade, several mAbs against these checkpoints have been developed and tested in clinical trials. However, the use of anti PD-1/PD-L1 antibodies as monotherapy has not provided satisfying results. Specifically, no objective responses have been reported in a phase Ib clinical trial testing the anti-PD-1 Nivolumab (IgGk, fully human) in monotherapy in 27 patients with relapsed or refractory MM (RRMM).[118] Similarly, a phase Ib trial of pembrolizumab (IgGk, humanized

anti-PD-1) in monotherapy for RRMM (NCT01953692/KEYNOTE-013) described a stable disease in 57% of patients.[119] In addition, preliminary results of a phase II trial of pembrolizumab used in monotherapy as consolidation in MM patients (NCT02636010) demonstrated an increased depth of response in only 3 of 14 patients treated. The low level of infiltrating effector cells that characterize MM, along with a relatively modest mutational burden, could explain this lack of efficacy.[120]

All these results highlighted the need of combining PD-1/PD-L1 blockade with other therapeutic agents, particularly with IMiDs. Pembrolizumab in combination with LEN and dexamethasone (Dex) was evaluated in a phase I dose-escalation in 40 RRMM patients progressed after more than 2 prior therapies.[121] The objective response rate (ORR) in the whole population was 50%, with an ORR of 38% in LEN-refractory patients.[121] A phase II clinical trial conducted on 48 RRMM patients, previously treated with a median of three regimens, showed an ORR of 60%, including 8% of stringent complete response/complete response, 19% VGPR, and 33% PR, with a median duration of response of 14.7 months. (Wilson et al., 2016)[122] Interestingly, a phase II study of Pembrolizumab following ASCT, reported a CR rate of 31% at 6 months, including a 67% rate of BM MRD-negative state. (Pawarode et al., 2017)

These results lead to the development of the phase III studies of pembrolizumab in combination with LEN and Dex (KEYNOTE-185, NCT02579863) or Pom and Dex (KEYNOTE-183, NCT02576977) and one phase III study of Pom and Dex vs nivolumab, Pom, and Dex vs nivolumab, elotuzumab, Pom, and Dex (CheckMate 602, NCT02726581). However, in June 2017 the US Food and Drug Administration transiently halted the clinical trials of anti-PD-1/PD-L1 mAbs in combination with IMiDs due to an imbalance of deaths in the Pembrolizumab arms in Keynote 183 and Keynote 185 and no significant differences in terms of objective response. (https://www.fda.gov/Drugs/DrugSafety/ucm574305.htm)

Together, these studies highlight the need for careful evaluation of immune checkpoint strategies and their combinations in MM, with attention to toxicities as well as pharmacodynamics endpoints.

The current evidences of the clinically meaningful anti-myeloma responses of PD-1 blockade as single agent[123] could be explained by the recent reports suggesting that the compromised functions of effector cells in MM may be due to senescence rather than PD-1 mediated exhaustion.[123, 124] Exhausted T cells overexpress multiple inhibitory molecules,

such as PD-1, CTLA-4, CD160, TIM-3 and LAG-3 and lack of IFN-γ expression.[125] However, a PD-1^{low} T cell clonal expansion was observed in 75% of myeloma patients, in contrast to the non-clonal PD-1^{high} T cells.[123, 124] This expanded population potentially represented tumor-reactive cells with a senescent phenotype. They indeed showed low levels of LAG-3, TIM-3, PD-1 and CTLA-4 and did not express CD27 and CD28, suggesting a late differentiated phenotype. Moreover, this clone expressed the typical senescent markers CD57, CD160 and KLRG-1 and displayed a secretory profile.[124] In addition, it was described that the senescent phenotype was telomere independent as demonstrated by the low levels of p38-mitogen-activated protein kinase, p16 and p21 signalling pathways and it could be potentially reversed by other agents, as IMiDs or histone deacetylase inhibitors.[124]

2. AIMS OF THE STUDY

1. *Evaluation of LEN effect on DC differentiation and activity.*

The first aim of this study was to investigate the *in vitro* effect of LEN on DC differentiation and activity, both directly and through the modulation of the immunosuppressive properties of hMSCs, in MM patients. Moreover, the effect of *ex vivo* LEN treatment was evaluated on DCs differentiated from MM patients treated for 7 days with LEN 25mg/day as single agent.

2. *Study of PD-1/PD-L1 distribution among BM MNCs in patients with monoclonal gammopathies.*

The second aim of the study was to analyze the expression profile of the checkpoint PD-1/PD-L1 in PCs, monocytes and T cells among BM mononuclear cells isolated from patients with monoclonal gammopathies at different stages of disease. The results were then correlated with the clinical data, specifically the presence of osteolytic lesions, in order to better clarify the patients that could benefit from PD-1/PD-L1 blockade and to evaluate a potential correlation between PD-1/PD-L1 pathway and the onset of MM bone disease.

3. MATERIALS AND METHODS

Patients

1. *Evaluation of LEN effect on DC differentiation and activity.*

BM and/or PB were obtained from 30 consecutive patients with active MM (50% female, 50% male; median age: 72 years, range 43-94), including both newly diagnosed (MMD) and relapsed MM (MMR), admitted to our hematological Unit. Patient samples were obtained after informed consent, according to the Declaration of Helsinki. The study was approved by the Institutional Ethical Review Board of our Hospital.

Moreover, PB were obtained from 9 patients with relapsed MM (4 female, 5 male; median age: 73 years, range: 56-82; ISS I: 4, ISS II: 3, ISS III: 2), at the baseline and after 7 days of treatment with LEN 25mg/day (days 1-21).

Mononuclear cells (MNCs) were isolated from BM and PB samples after Ficoll gradient separation and used for further *in vitro* studies.

2. *Study of PD-1/PD-L1 distribution among BM MNCs in patients with monoclonal gammopathies.*

BM aspirate was obtained from 87 consecutives patients with monoclonal gammopathies, including 15 MGUS (40% F, 60% M: median age: 65 years, range: 44-87), 26 SMM (35% F, 65% M; median age: 63 years, range: 36-93), 25 newly diagnosed MM (44% F, 56% M; median age: 75 years, range: 53-90; ISS I= 20%, II= 24%, III= 56%) and 21 relapsed/refractory MM patients (57% F, 43% M; median age: 74 years, range: 54-86; ISS I= 29%, II= 19%, III= 52%). The presence of bone disease was checked by low-dose computerized tomography (CT) with or without positron emission tomography (PET) scan and by X-rays skeletal survey in 11 MM patients. 87% of MM patients displayed osteolytic lesions. High bone disease (HBD) was defined by the presence of 3 or more osteolytic lesions (62% of our cohort). Patients without bone lesions or with minus of 3 lesions were defined as low bone disease (LBD).

Cells and cell culture conditions

Cell lines. The human myeloma cell line (HMCL) JJN3, purchased by DSMZ (Braunschweig, Germany) and the human monocytic cell line THP-1, obtained from the American Type Culture Collection (Rockville, MD), were maintained in culture in RPMI 10% FBS; hTERT-hMSCs were kindly gifted from Dr Giuseppe Gaipa (Monza, Italy) and maintained in culture with RPMI 10% FBS with hydrocortisone (10^{-6}M). All cell lines were authenticated and tested for mycoplasma contamination.

Cell purification. BM and PB $CD14^+$ cells were purified from total MNCs by an immuno-magnetic method using anti-CD14 mAb coated microbeads (MACS, Miltenyi Biotec; Bergisch-Gladbach, Germany). $CD3^+$ cells were isolated following the same protocol, using anti-CD3 mAb from PB of MM patients. The presence of potential contaminating cells in each fraction was evaluated by flow cytometry analysis, using the fluorescence-activated flow cytometer BD FACS Canto II with Diva software (Becton, Dickinson and Company (BD); Franklin Lakes, NJ). Purity of cell samples was >92%.

DC differentiation and cell treatment. DCs were differentiated from purified $CD14^+$ cells, cultured *in vitro* at $1x10^6$ cells/ml in RPMI 1664 medium with 10% FBS, with recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF) (50 ng/ml) and IL-4 (50 ng/ml) (all purchased by Peprotech, Rocky Hill, NJ), for 8 days (replacing half media with fresh cytokines every 2-3 days), in the presence of LEN (purchased by Celgene, Italy Corporation, Milan, Italy) or vehicle (DMSO), at concentration 0.1 and 1 µM. TNF-α at 10 ng/ml (OriGene; Rockville, MD) was added to the culture medium for the last 24 h, in order to induce DC terminal maturation. At the end of culture period, both cells and CM were collected for further analysis. In some experiment, the combination of LEN and Dex (obtained by Sigma

Aldrich, Milan, Italy) was tested on DC differentiation. Briefly, DCs were differentiated from BM $CD14^+$ cells of MM patients, in the presence of LEN (0.1 and 1 μM) or vehicle, as previously reported. At the end of culture period, cells were collected and reseeded ($5x10^4$/ml) in fresh medium with Dex (10^{-8}M) or vehicle (EtOH) for 48 h. After Dex treatment, cells were collected and analyzed for DC maturation markers. For the ex vivo studies, PB $CD14^+$ cells were isolated from MM patients at day 0 and after one week (day 7) of LEN (25mg/day) treatment, just before the start of the weekly treatment with Dex. Cells were then differentiated into DCs, following the above protocol, without LEN *in vitro* treatment.

DCs were also differentiated from THP-1 cell line, by adding rhIL-4 (200 ng/ml), rhGM-CSF (100 ng/ml), ionomycin (200 ng/ml) (Sigma-Aldrich, Milan, Italy) and rhTNF-α (20 ng/ml) for 72 h to the culture medium (RPMI 1664, serum depleted); then LEN or DMSO were added for the last 24 h of culture period. THP-1-derived DCs (THP1-DCs) were detached with EDTA 2 mM on ice for 2 h and cell pellets collected for further analysis.

In some experiments DCs were differentiated from BM $CD14^+$ cells of MM patients, in the presence or absence of the CM (ratio 1:2 with RPMI 1664 10% FBS, with IL-4 and GM-CSF) of hTERT-hMSCs treated with LEN or DMSO. Briefly, 1x104 hTERT-hMSCs were seeded in T75 flasks and cultured in RPMI 10% FBS, in presence of LEN (0.1 and 1 μM) or DMSO, for 5 days. At the end of culture period, the medium was replaced with RPMI 10% FBS in order to discard LEN, and after 48 h, the CM was collected and used during DC differentiation, as previously reported. In some experiments, after 5 days of LEN treatment, hTERT-hMSC pellets were collected for immunoblotting and Real-time PCR analysis.

Autologous Mixed Lymphocyte Reaction and cell proliferation assay. DCs were differentiated, as previously reported, in presence of LEN or vehicle (alone or in combination with Dex), from BM of 6 MM patients, for 8 days. Then, treated cells were collected, analyzed by flow-

cytometry and partly re-seeded (3x103 cells/w) in round-bottomed 96well-plates, in RPMI 1664 with 15% AB human serum. DCs were co-cultured with autologous PB $CD3^+$ cells (1x104) for 6 days. At the end of culture period, an MTT assay (Cell Counting Kit-8; Alexis, Vinci-Biochem s.r.l., Italy) was performed in order to measure T cell proliferation.

Flow cytometry assay

1. *Evaluation of LEN effect on DC differentiation and activity.*

After *in vitro* DC differentiation, non-adherent cells were collected and analyzed by flow cytometry for DC maturation markers. Cells from each condition were splitted in three tubes and labelled with saturating amounts of the following conjugated antibody combinations (all from BD Biosciences, San Jose, CA, USA): 1) anti-CD14-FITC/anti-CD83-PE/isotype control-PE-Cy5/isotype control-APC, 2) anti-CD14-FITC/anti-CD83-PE/anti-CD86-PE-Cy5/anti-HLA-DR-APC, 3) anti-CD14-FITC/anti-CD83-PE/anti-CD80-PE-Cy5/anti-CD209-APC. Four color, six-parameter acquisition and analysis were performed on a two-laser FACSCalibur instrument (BD Biosciences) using CellQuest software (BD Biosciences). Mature DCs were identified as $CD14^-CD83^+$ cells and the Median Fluorescence Intensity (MFI) of the maturation markers was compared between cells treated with LEN and/or Dex vs the relative control, for each experiment.

2. *Study of PD-1/PD-L1 distribution among BM MNCs in patients with monoclonal gammopathies.*

BM MNCs were analyzed by flow cytometry, using the following conjugated antibody combination (all from BD Biosciences, San Jose, CA, USA): monocytes and PCs (anti-CD14-FITC/anti-CD138-PE/anti-PD-L1-APC), $CD4^+$ T cells (anti-CD3-FITC/anti-PD-1-PE/anti-CD4-APC), $CD8^+$ T cells (anti-CD3-FITC/anti-PD-1-PE/ anti-CD8-APC). Acquisition and analysis were performed on a two-laser FACSCalibur instrument (BD Biosciences) using CellQuest software

(BD Biosciences). Instead of % of positive cells, PD-L1 MFI parameter was analyzed on PCs and monocytes according to the homogenous (unimodal) phenotypic pattern shown by these cells. Both % of positive cells and MFI PD-1 were analyzed on T cells. Data on monocytes and T cells were normalized on the $CD138^-$ fraction.

Multi ELISA assay

The concentration of Interferon (IFN)-γ, IL-6, IL-8, IL-10, IL-12, IFN-γ induced protein (IP)-10, CCL2, CCL5 and TNF-α was evaluated on DC CM, collected after *in vitro* DC differentiation, by using multiplex bead-based sandwich immunoassay kits (Bio-Plex® Multiplex System, Biorad, California, USA), following the manufacturer's instructions. Measurement were performed by a reader (Luminex Bio-plex system, Bio-Rad Laboratories Inc.). For TNF-α level evaluation, the obtained results were normalized for TNF-α concentration measured in the control medium (RPMI 10% FBS, with IL-4, GM-CSF and TNF-α at concentrations used during DC differentiation).

Western blot

Nuclear and cytosolic extracts were obtained using a commercial kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol from THP1-DCs and hTERT-hMSCs, treated with LEN or DMSO. Immunoblotting was performed as previously reported[51] using the following antibodies: rabbit monoclonal anti-Aiolos (1:714) (code n. NBP2-24495, Novus Biologicals, UK), anti- Casein Kinase 1 (1:1.000) (code n. 2655, Cell Signaling Technology, Danvers, USA), anti-Ikaros (1:200) (code n. sc-13039, Santa Cruz Biotechnology, Dallas, USA), and mouse monoclonal anti-Cereblon (1:1.250) (code n.TA345038, OriGene, Rockville, USA), anti-IRF4 (1:400) (code n. M725929-2, DAKO, Milan, Italy); anti-p62/SQSTM1 (1:250) (Code n.MAB8028,

R&D Systems, Minneapolis, MN, USA) antibodies; mouse monoclonal anti-β-actin antibody (1:5.000) (clone AC-15, code n. A5441, Sigma-Aldrich) was used as internal control. JJN3 cell line represent the positive control (CNT).

mRNA silencing

RNAi was performed through the generation of inducible shRNA stable cell lines. hTERT-hMSC were transduced with the IPTG inducible lentiviral particles carrying CSNK1A1-specific shRNA (pLKO_IPTG_3XLacO, Sigma-Aldrich). Two independent shRNAs (TRCN0000006044, and TRCN0000006042) sequences were chosen. 3x104 cells were infected with a multiplicity of infection of 4 overnight in the presence of 8 μg/ml polybrene (Sigma-Aldrich, Italy). 24 hour later the infected media was replaced with fresh growing media. Puromycin selection (0.5 μg/ml) was initiated two days after transduction. Once a cellular clone was established, to induce CK1α silencing, cells were incubated with 500 μM IPTG (Sigma-Aldrich) every 2-3 days for a total of one week. Then, fresh medium without IPTG and puromycin was added for further 48 hours. At the end of culture period, cell pellets were collected and analyzed by western blotting to check CK1α down-regulation and select the more efficient clone. CM was also collected and used for *in vitro* DC differentiation.

Real time PCR

Total RNA was extracted from hTERT-hMSCs, after all different experimental conditions, using the RNeasy total RNA isolation kit (Qiagen; Hilden, Germany). RNA (1 μg) was reverse-transcribed with 400 U Moloney murine leukemia reverse transcriptase (Applied Biosystems, Life Technologies) in accordance with the manufacturer's protocol. Real Time PCR was performed by adding complementary DNA to a universal Light Cycler 480 Probes Master and

RealTime ready Catalog Assay (Roche Diagnostics, Mannheim, Germany) for the following genes: IDO1 (Assay ID:103804), IL6 (Assay ID: 144013), CCL5 (Assay ID: 113395), PTGS2 (Assay ID: 102471), TGB1 (Assay ID: 101210), and GAPDH (Assay ID: 102052). The expression of selected genes was checked by Real Time PCR by Light Cycler 480 (Roche Diagnostics). To normalize the differences in RNA quality and reverse transcription efficiency, we applied the comparative Ct method using the endogenous reference gene GAPDH.

Statistical analysis

Data were expressed as mean ± SEM or median values. Paired Student's t-test was used to analyze flow cytometry data of *in vitro* DC differentiation from BM and PB of MM patients and for the *ex vivo* studies. Non-parametric Friedman test, Wilcoxon test and Mann-Whitney test were used for the other experiments with a lower number of samples and the study of PD-1/PD-L1 expression profile. Results were considered significant at $p<0.05$. GraphPad Prism 6.1™ (GraphPad Software Inc., La Jolla, CA, USA) was used for all the statistical analyses.

4. RESULTS

LEN enhanced in vitro DC differentiation from both BM and PB monocytes of MM patients and increased their chemokine and cytokine production degrading both Ikaros and Aiolos.

The expression of DC maturation markers was analyzed on monocytes derived-DCs (mo-DCs) differentiated from BM aspirates and PB of MM patients. Despite a reduction of both number and % of mature DCs, LEN, at the concentration range reached *in vivo* in MM patients,[126] significantly increased BM DC expression of HLA-DR (mean MFI ± standard error of the mean (SEM): DMSO *vs* LEN 0.1 μM 45.82 ± 4.55 *vs* 59.45 ± 8.21, p=0.029; DMSO *vs* LEN 1 μM, 45.82 ± 4.55 *vs* 73.52 ± 7.71, p=0.001), CD86 (mean MFI ± SEM, DMSO *vs* LEN 0.1 μM: 137.58 ± 22.83 *vs* 177.76 ± 27.04, p=0.036; DMSO *vs* LEN 1 μM: 137.58 ± 22.83 *vs* 223.38 ± 32.26, p=0.003) and CD209 (mean MFI ± SEM, DMSO *vs* LEN 0.1 μM: 155.80 ± 21,06 *vs* 190.73 ± 25.35, p=0.004) (p calculated by paired Student's t-test) (Figure 1A), compared to vehicle (DMSO). Flow-cytometry histograms from one representative MM patient were reported in Supplemental Figure 1A.

Similarly to BM derived DCs, increased CD86 (mean MFI ± SEM, DMSO *vs* LEN 0.1 μM: 147.49 ± 45.08 *vs* 200.44 ± 44.22, p=0.002; DMSO *vs* LEN 1 μM: 147.49 ± 45.08 *vs* 249.61 ± 42.10, p=0.016) and CD209 (mean MFI ± SEM, DMSO *vs* LEN 0.1 μM: 128.69 ± 18.09 *vs* 204.88 ± 33.54, p=0.008; DMSO *vs* LEN 1 μM: 128.69 ± 18.09 *vs* 196.32 ± 36.33, p=0.023) (p calculated by paired Student's t-test) expression was found in DCs differentiated from PB $CD14^+$ cells (Figure 1B). Flow-cytometry histograms from one representative patient were reported in Supplemental Figure 1B. Any significant differences between BM and PB samples of the same patient were found on LEN effect on DC maturation markers (2way ANOVA) (data not shown). Interestingly, the increased expression of DC maturation markers was abrogated when LEN was used in combination with Dexamethasone (Dex) at 10^{-8} M (Dex *vs* LEN 0.1 μM + Dex *vs*

LEN 1 µM + Dex median MFI, HLA-DR: 129.5 *vs* 103.9 vs 109.9; Dex *vs* LEN 0.1 µM + Dex *vs* LEN 1 µM + Dex median MFI, CD86: 199 *vs* 237.4 *vs* 233.5; Dex *vs* LEN 0.1 µM + Dex *vs* LEN 1 µM + Dex median MFI, CD80: 115 *vs* 104.6 *vs* 90.24; CD209: 50.28 *vs* 52.58 *vs* 54.91, no statistical differences) (Friedman test) (Figure 2). Moreover, the addition of Dex dramatically decreased the number of DCs (LEN 0.1 µM + Dex *vs* LEN 0.1 µM, median number: 17238 *vs* 43568, $p<0.05$; LEN 1 µM + Dex *vs* LEN 1 µM, 14028 *vs* 43283, $p<0.05$) and the percentage of DCs obtained *in vitro* (LEN 0.1 µM + Dex *vs* LEN 0.1 µM, median DC%: 9,85 *vs* 25,90, $p<0.05$; LEN 1 µM + Dex *vs* LEN 1 µM, 11.69 *vs* 19.9, $p<0.05$) (*p* calculated by Wilcoxon test) (Figure 2).

The potential effect of LEN treatment on cytokine production by BM DCs was then investigated using a Multiplex ELISA assay. LEN treatment enhanced the production of IL-8 (median concentration, DMSO *vs* LEN 0.1 µM *vs* LEN 1 µM: 1076 *vs* 1755 *vs* 2193 pg/ml, $p<0.05$), CC chemokine ligand (CCL)2 (median concentration, DMSO *vs* LEN 0.1 µM *vs* LEN 1 µM: 1355 *vs* 2414 *vs* 2831 pg/ml, $p<0.05$), CCL5 (median concentration, DMSO *vs* LEN 0.1 µM *vs* LEN 1 µM: 49.68 *vs* 64.48 *vs* 96.94 pg/ml, $p<0.05$) and TNF-α (median concentration, DMSO *vs* LEN 0.1 µM *vs* LEN 1 µM: 684,4 *vs* 965,4 *vs* 1101 pg/ml, $p<0.05$) and slightly decreased the production of IL-6 (median concentration, DMSO *vs* LEN 0.1 µM *vs* LEN 1 µM: 60.97 *vs* 60.30 *vs* 47.74, $p<0.05$) (*p* calculated by Friedman test), by mo-DCs differentiated from MM patients, compared to vehicle (Figure 3).

Based on the literature data showing that LEN exerts the anti-MM activity through the selective ubiquitination and degradation of Cereblon targets, Ikaros and Aiolos[101], the protein levels of Cereblon, Ikaros and Aiolos were checked on LEN-treated THP1-DCs. THP1-DCs expressed Cereblon at baseline (Figure 4A) and LEN treatment down-regulated Ikaros protein levels in a dose-dependent manner (Figure 4B). Moreover, the basal Aiolos protein level was very low in THP1-DCs and LEN further decreased its expression in these cells (Figure

4C). The effect of LEN treatment was also evaluated on two additional Cereblon targets, IRF4 and p62, that were respectively down-regulated[127] and up-regulated[128] after LEN treatment in MM cells. However, THP1-DCs did not express IRF4 (Figure 4D) and LEN did not affect p62 in THP-1 DCs (Figure 4E).

LEN enhanced DC ability to stimulate autologous CD3$^+$ cell proliferation.

Next, the potential effect of LEN treatment on DC functional properties was investigated. Specifically, DCs differentiated from BM of 4 MM patients, in the presence of LEN or vehicle, were tested as stimulators in the autologous Mixed Lymphocyte Reaction (MLR) assay, in order to evaluate LEN effect on DC ability to stimulate T cell proliferation.

Interestingly, CD3$^+$ cell proliferation was significantly higher in co-culture with LEN-treated DCs, compared to DMSO-treated DCs (median OD, DMSO vs LEN 0.1 µM: 225 vs 292, p=0.0279; DMSO vs LEN 1 µM: 225 vs 299, p=0.0045) (p calculated by Mann-Whitney test) (Figure 5A). However, this effect was abrogated in the presence of Dex at 10-8 M (mean OD ± SEM, LEN 0.1 µM + Dex vs LEN 0.1 µM: 265.3 ± 22.82 vs 548 ± 38.59, p=0.0016; LEN 0.1 µM vs Dex: 548 ± 38.59 vs 292.7 ± 2.4, p=0.0043; LEN 1 µM vs Dex: 0.0234) (p calculated by unpaired t test) (Figure 5B).

LEN blunted human telomerase reverse transcriptase transduced mesenchymal stromal cell (hTERT-hMSC) inhibitory properties on DC differentiation inhibiting CK1-α levels.

To investigate a possible MSC-mediated indirect effect of LEN on DCs, BM CD14$^+$ cells of MM patients were differentiated into DCs in the presence of LEN or DMSO treated hTERT-hMSC conditioned medium (CM).

The immunosuppressive properties of hTERT-hMSC CM on DC maturation was firstly checked in this *in vitro* system (Figure 6A); interestingly, it emerged that LEN treatment reverted this effect, by increasing HLA-DR (median MFI: DMSO vs LEN 0.1 µM vs LEN 1 µM: 19.88 vs 27.88 vs 31.34, $p<0.05$) (p calculated by Friedman test) and CD86 (median MFI: DMSO vs LEN 0.1 µM vs LEN 1 µM: 30.78 vs 37.52 vs 57.25, $p<0.05$) (p calculated by Friedman test) (Figure 6B).

Thereafter, the expression of immunosuppressive factors in LEN-treated hTERT-hMSCs was analyzed by RT-PCR. LEN was found to significantly down-regulate prostaglandine 2 (PTGS2) gene expression levels at all tested concentrations (LEN 0.1 µM vs DMSO, $p=0.0326$; LEN 1 µM vs DMSO, $p<0.0001$) (Figure 6C) but not indoleamine 2,3-dioxygenase 1 (IDO1), IL6, CCL5 and transforming growth factor beta 1 (TGFB1).

To investigate the molecular mechanism involved in the effect of LEN on MSCs, the expression profile of Cereblon and its target proteins was firstly checked in hTERT-hMSCs, showing that they expressed Cereblon (Figure 7A) but not Ikaros, Aiolos and IRF4 (Figure 7B). The attention was thus focused on another Cereblon substrate, CK1α.[14] hTERT-hMSCs treated with LEN showed a decrease of CK1-α protein level at the higher concentration (Figure 7C). Moreover, LEN treatment did not affect p62 protein level, as for DCs (Figure 7D).

To better clarify the correlation between the decreased CK1-α levels in hMSCs after LEN treatment and LEN effect on the immunosuppressive properties of MSCs on DCs, CK1α was down-regulated in hTERT-hMSCs through an IPTG inducible shRNA. After checking CK1-α down-regulation by western blotting (Supplemental Figure 3A), the CM was used for *in vitro* DC differentiation. Interestingly, the effect of hTERT-hMSCs on DC maturation markers were reverted by the down-regulation of CK1-α (MFI, CNT vs wt hTERT-hMSCs vs hTERT-hMSCs 6044, HLA-DR: 143.30 vs 91.40 vs 63.21; CD86: 106.5 vs 109.41 vs 192.82; CD80: 70.41 vs 35.87

vs 100; CD209: 41.05 vs 34.29 vs 54.74) thus suggesting the involvement of this factor on LEN effect on hMSC immunosuppressive properties (Supplemental Figure 3B).

In vivo LEN treatment of MM patients increased in vitro DC differentiation.

Finally, to evaluate the effect of *in vivo* LEN treatment on DC maturation markers, it was compared the expression profile of DCs differentiated from PB $CD14^+$ cells of 9 MM relapsed patients, purified at the baseline (day 0) and after 7 days of LEN treatment, just before the start of the weekly treatment with Dex. All the patients were responsive to LEN treatment.

Interestingly*, in vivo* LEN treatment significantly increased the expression of HLA-DR (mean MFI ± SEM, day 7 vs day 0: 80.59 ± 11.21 vs 38.56 ± 17.99, p=0.036) and CD209 (mean MFI ± SEM, day 7 vs day 0: 194.24 ± 30.22 vs 116.47 ± 23.47, p=0.012), and CD86 without reaching statistical significance (mean MFI ± SEM, day 7 vs day 0: 200.75 ± 48.20 vs 107.95 ± 19.21, p=0.075) (p calculated by paired Student's t-test) (Figure 8A-B), as previously showed for the *in vitro* treatment. The effect on CD209 expression was also observed after 21 days of LEN treatment (data not shown). Flow-cytometry histograms from one representative MM patient were reported in Supplemental Figure 4.

Increased % of PD-1^+ $CD4^+$ cells in patients with active MM

In the second part of the study, PD-1/PD-L1 expression profile was evaluated, by flow-cytometry, on BM MNCs of 87 patients with monoclonal gammopathies, in order to clarify a possible relation with disease progression.

Patients with active MM (MMD and MMR) showed higher % of $CD4^+PD\text{-}1^+$ cells (median value: 36.29% vs 32.62%, *p*=0.0483) (Figure 9a), along with a higher PD-1 expression (median MFI:

15.61 *vs* 13.82, *p*=0.0174) as compared to patients with SMM and MGUS (Figure 9b). However, no differences within PD-L1$^+$ cells were detected in the analysed cohort.

The % of PD-1$^+$CD8$^+$ cells is inversely correlated with the presence of bone disease.

Focusing on MM bone disease, osteolytic MM patients displayed higher CD4$^+$/CD8$^+$ cell ratio compared to non-osteolytic ones (ratio: 1.32 vs 0.65, *p*=0.0215) (Figure 10a). Moreover, the % of total CD3$^+$PD-1$^+$ cells decreased in the osteolytic patients compared to non-osteolytic ones (median value: 40.68 *vs* 47.18, *p*=0.035) (Figure 10b), with a significant reduction of the % of CD8$^+$PD-1$^+$ cells (median value: 45.01 *vs* 57.55, p=0.0246) (Figure 10c). Consistently, among BM CD8$^+$ cells, the % of PD-1$^+$ cells decreased in HBD vs LBD MM patients, without reaching a statistical significance (median value: 46.9% vs 57.4%, *p*=0.060). Interestingly, HBD MM patients displayed a significant reduction of PD-L1 expression on both BM CD138$^+$ PCs (median MFI value: 14.35 vs 21.59, p=0.0195) (Figure 10d) and CD14$^+$ cells (median MFI value: 17.01 vs 23.88, p=0.034) (Figure 10e). In addition, results from a multivariate analysis showed that PD-1 expression on CD8$^+$ cells and PD-L1 MFI on CD138$^+$ were significant independent factors related to the presence of HBD.

5. DISCUSSION

Alterations of the BM immune-microenvironment characterize the progression of monoclonal gammopathies and the development of osteolytic bone disease in MM.

MM patients exhibit severe immune dysfunctions, including a decreased expression of DC maturation markers and antigen presentation ability,[39] in part due to MM cell production of several soluble factors with immunosuppressive properties, such as IL-6, IL-10, vascular endothelial growth factor and TGF-β.[39, 43, 129]

The use of IMiDs to revert the "immuneparesis" in MM patients has been extensively investigated. However, LEN effect on human DCs are still unclear. Only one study, recently published[130], reported that LEN (5μg/ml≈20 μM) enhanced the maturation of DCs generated from PB of MM patients and increased the ability to stimulate T cell proliferation.[130]

In this study it has been demonstrated that LEN treatment, at concentrations reached *in vivo* in MM patients treated with LEN 5-25mg/day,[126, 131] significantly increased the expression of DC maturation markers, HLA-DR, CD86 and CD209, involved in DC co-stimulatory function and trafficking.[37, 132, 133] This result was observed both in BM and PB of MM patients, in line with data reported by Vo M et al.[130] The effect was abrogated in the presence of Dex at 10^{-8} M, consistent with several studies that described Dex inhibitory effects on DC maturation and functions.[134, 135] However, contradictory data have been reported on the antagonistic effects of Dex on LEN immunomodulatory properties. A study from Paiva et al[136] on high-risk Smoldering MM (SMM) patients reported no significant differences in the immune profile of patients treated with LEN/Dex *vs* LEN alone.[136] Conversely, different studies showed a profound inhibition of LEN-mediated NK and T cell activation with Dex combination.[137, 138] In line with the effect of LEN on DC maturation markers, LEN treatment increased DC production of IL-8, CCL2, CCL5, and TNF-α, as also previously observed in murine models.[139, 140] These factors regulate the antigen uptake and the activation of

the innate immune system, and are chemo-attractants for various immune cells,[141] suggesting that LEN treatment enhanced the role of DC in linking innate and adaptive anti-tumor-antigen immune responses. In line with this hypothesis, LEN effect on *in vitro* DC differentiation was associated with an increased DC functional activity to stimulate T cell proliferation that was abrogated by the combination with Dex.

Subsequently, the molecular mechanism beyond LEN effects on DCs was investigated. It is widely demonstrated that LEN exerts its anti-MM activity through the modulation of Ikaros and Aiolos. Moreover, Ikaros deficiency in host APCs failed to enhance GvLeukemia despite increased GvHD severity, in a murine model of allo-transplantation.[142] Similarly, this study shows that both Ikaros and Aiolos were degraded in DCs after LEN treatment, as previously reported for MM cells and T cells. [101, 102] [143]

Along with a direct effect of LEN on DC maturation, the presented data suggest a potential indirect effect, through the modulation of MSC immunomodulatory properties such as the production of cytokines and chemokines.[144-147] An increased expression of DC maturation markers was observed when DCs were cultured with LEN-treated MSC CM, thus suggesting the ability of LEN to blunt the inhibitory effect of MSCs on DC differentiation. Moreover, the treatment of MSCs with LEN significantly decreased the expression levels of *PTGS2*, known to inhibit the transitional processes of differentiation of monocytes into DCs.[110, 148] The molecular mechanisms involved in the effect of LEN on MSC was also investigated. Surprisingly MSC did not express the main CRBN targets Ikaros and Aiolos; however, LEN caused the degradation of a novel described CRBN substrate, CK1α, in MSCs.

CK1α is a component of the beta (β)-catenin destruction complex and a negative regulator of p53, and several studies[104, 149] recently reported that reduced CK1α levels decrease MM cell survival and inhibit cell cycle progression.[104, 149] Interestingly this factor seems to be involved in the resistance of PCs to LEN after long-term exposure.[150]

Lastly, the *in vitro* evidence were expanded and confirmed by *ex vivo* DC cultures in relapsed MM patients treated with LEN 25mg/day, as mono-therapy for one week, just before the start of the weekly treatment with Dex. After 7 days of treatment an increased PB DC differentiation was found. Of note,

all analysed patients were responsive to LEN treatment. This early effect was in line with recent data reporting the *in vivo* increase of T and NK cells, with a rapid decline of Ikaros, after 7 days of POM treatment without Dex in MM patients.[151]

All these data thus indicate that LEN increases the expression of mature DC markers both *in vitro* and in *ex vivo* cultures, enhancing DC ability to stimulate T cell proliferation and to release chemokines involved in the immune response. LEN treatment also reduces the immunosuppressive properties of hMSCs, suggesting new possible effects of IMiDs® on the allo-reactivity against MM cells.

Among the different mechanisms behind immune suppression in MM, the role of PD-1/PD-L1 has recently emerged. Despite the successful results reported on *in vitro* studies and murine models, by using anti PD-1/PD-L1 blocking antibodies, the contribution of PD-1/PD-L1 axis in MM progression has to be clarified. Moreover, contradictory results are currently available on the expression profile of PD-1/PD-L1 in MM patients and the possible correlation with the presence of osteolytic lesions has not yet been explored. The results of the second part of this study interestingly showed an increased frequency of PD-1^+ T cells across the progression of the disease, in line with other studies already published.[69, 79] Moreover, for the first time, a significant relationship between the presence of extensive osteolytic bone disease and a reduced expression of PD-1 was found on BM $CD8^+$ T cells, along with a decreased expression of PD-L1 on malignant PCs and monocytes in MM patients with HBD. It was thus hypothesized that a less immune-suppressive profile could be related to the development of osteolysis, consistently with the negative cross talk existing between PD-1/PD-L1 axis and Th17 cells.[152] Indeed, recent studies have shown that *STAT1*-dependent PD-L1 up-regulation inhibit Th17 differentiation and *in vitro* PD-L1 inhibition partially overcomes Th17 deficiency[152] Moreover, Yang L et al described that PD-1 suppresses Th17 response by inhibiting the activation of PI3K/Akt pathway in a mouse model of autoimmune arthritis, a disease known to share several features with myeloma. [153] Interestingly, a study on pre-eclampsia showed that decreased PD-1 expression contributes to a higher Th17 cell frequency; whereas PD-L1 Fc administration increased the percentage of Treg differentiated from naïve $CD4^+$ T cells.[154] A decreased PI3K/AKT/m-TOR and

increased PTEN mRNA expression was also found to mediate these effects, completely reversed by PD-1 blockade.[154]

Together all these data thus suggest that PD-1/PD-L1 axis could be implicated in the regulation of Th17 differentiation and function even in MM. It is well known that Th17 cells accumulate in MM patients and correlates with the presence of bone disease.[28, 29]

It is thus conceivable that PD-1/PD-L1 axis could affect the balance between Treg and Th17 cells, enhancing the differentiation of Th17 cells and increasing osteoclastogenesis, which in turn inhibits T cell response in MM patients.

Although further studies are still needed to clarify this correlation, the current results could provide a new mechanism by which PD-1/PD-L1 could exert its immune suppressive properties within MM BM microenvironment, along with a rationale for the use of immune-checkpoint inhibitors in a selected cohort of patients.

6. REFERENCES

1. Kyle, R.A. and S.V. Rajkumar, *Multiple myeloma.* N Engl J Med, 2004. **351**(18): p. 1860-73.
2. Dhodapkar, M.V., *MGUS to myeloma: a mysterious gammopathy of underexplored significance.* Blood, 2016. **128**(23): p. 2599-2606.
3. Palumbo, A. and K. Anderson, *Multiple myeloma.* N Engl J Med, 2011. **364**(11): p. 1046-60.
4. Chapman, M.A., et al., *Initial genome sequencing and analysis of multiple myeloma.* Nature, 2011. **471**(7339): p. 467-72.
5. Avet-Loiseau, H., *Role of genetics in prognostication in myeloma.* Best Pract Res Clin Haematol, 2007. **20**(4): p. 625-35.
6. Manier, S., et al., *Genomic complexity of multiple myeloma and its clinical implications.* Nat Rev Clin Oncol, 2017. **14**(2): p. 100-113.
7. Lopez-Corral, L., et al., *The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells.* Clin Cancer Res, 2011. **17**(7): p. 1692-700.
8. Fonseca, R., et al., *The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma.* Blood, 2003. **102**(7): p. 2562-7.
9. Lode, L., et al., *Mutations in TP53 are exclusively associated with del(17p) in multiple myeloma.* Haematologica, 2010. **95**(11): p. 1973-6.
10. Hanamura, I., et al., *Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation.* Blood, 2006. **108**(5): p. 1724-32.
11. Neben, K., et al., *Progression in smoldering myeloma is independently determined by the chromosomal abnormalities del(17p), t(4;14), gain 1q, hyperdiploidy, and tumor load.* J Clin Oncol, 2013. **31**(34): p. 4325-32.
12. Anderson, K.C. and R.D. Carrasco, *Pathogenesis of myeloma.* Annu Rev Pathol, 2011. **6**: p. 249-74.
13. Pratt, G., O. Goodyear, and P. Moss, *Immunodeficiency and immunotherapy in multiple myeloma.* Br J Haematol, 2007. **138**(5): p. 563-79.
14. Manier, S., et al., *Bone marrow microenvironment in multiple myeloma progression.* J Biomed Biotechnol, 2012. **2012**: p. 157496.
15. Hazlehurst, L.A., et al., *Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR).* Oncogene, 2000. **19**(38): p. 4319-27.
16. Hallek, M., P.L. Bergsagel, and K.C. Anderson, *Multiple myeloma: increasing evidence for a multistep transformation process.* Blood, 1998. **91**(1): p. 3-21.
17. Moller, C., et al., *Expression and function of chemokine receptors in human multiple myeloma.* Leukemia, 2003. **17**(1): p. 203-10.
18. Topalian, S.L., C.G. Drake, and D.M. Pardoll, *Immune checkpoint blockade: a common denominator approach to cancer therapy.* Cancer Cell, 2015. **27**(4): p. 450-61.
19. Nefedova, Y., et al., *Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines.* Blood, 2004. **103**(9): p. 3503-10.
20. Radtke, F. and K. Raj, *The role of Notch in tumorigenesis: oncogene or tumour suppressor?* Nat Rev Cancer, 2003. **3**(10): p. 756-67.
21. Roodman, G.D., *Pathogenesis of myeloma bone disease.* Leukemia, 2009. **23**(3): p. 435-41.
22. Giuliani, N., S. Colla, and V. Rizzoli, *New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL).* Exp Hematol, 2004. **32**(8): p. 685-91.
23. Giuliani, N., et al., *Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation.* Blood, 2005. **106**(7): p. 2472-83.

24. Giuliani, N., V. Rizzoli, and G.D. Roodman, *Multiple myeloma bone disease: Pathophysiology of osteoblast inhibition.* Blood, 2006. **108**(13): p. 3992-6.
25. Silbermann, R., et al., *Bone marrow monocyte-/macrophage-derived activin A mediates the osteoclastogenic effect of IL-3 in multiple myeloma.* Leukemia, 2014. **28**(4): p. 951-4.
26. Giuliani, N., et al., *Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease.* Blood, 2002. **100**(13): p. 4615-21.
27. Giuliani, N., et al., *Interleukin-3 (IL-3) is overexpressed by T lymphocytes in multiple myeloma patients.* Blood, 2006. **107**(2): p. 841-2.
28. Noonan, K., et al., *A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma.* Blood, 2010. **116**(18): p. 3554-63.
29. Dhodapkar, K.M., et al., *Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma.* Blood, 2008. **112**(7): p. 2878-85.
30. Giuliani, N., et al., *CC-chemokine ligand 20/macrophage inflammatory protein-3alpha and CC-chemokine receptor 6 are overexpressed in myeloma microenvironment related to osteolytic bone lesions.* Cancer Res, 2008. **68**(16): p. 6840-50.
31. Abe, M., et al., *Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion.* Blood, 2004. **104**(8): p. 2484-91.
32. Abe, M., et al., *BAFF and APRIL as osteoclast-derived survival factors for myeloma cells: a rationale for TACI-Fc treatment in patients with multiple myeloma.* Leukemia, 2006. **20**(7): p. 1313-5.
33. An, G., et al., *Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication.* Blood, 2016. **128**(12): p. 1590-603.
34. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
35. Perez-Persona, E., et al., *New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells.* Blood, 2007. **110**(7): p. 2586-92.
36. Dhodapkar, M.V., et al., *A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma.* J Exp Med, 2003. **197**(12): p. 1667-76.
37. Banchereau, J., et al., *Immunobiology of dendritic cells.* Annu Rev Immunol, 2000. **18**: p. 767-811.
38. Galati, D., et al., *Dendritic cells in hematological malignancies.* Crit Rev Oncol Hematol, 2016. **108**: p. 86-96.
39. Ratta, M., et al., *Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6.* Blood, 2002. **100**(1): p. 230-7.
40. Bahlis, N.J., et al., *CD28-mediated regulation of multiple myeloma cell proliferation and survival.* Blood, 2007. **109**(11): p. 5002-10.
41. Brimnes, M.K., I.M. Svane, and H.E. Johnsen, *Impaired functionality and phenotypic profile of dendritic cells from patients with multiple myeloma.* Clin Exp Immunol, 2006. **144**(1): p. 76-84.
42. Do, T.H., et al., *Impaired circulating myeloid DCs from myeloma patients.* Cytotherapy, 2004. **6**(3): p. 196-203.
43. Brown, R.D., et al., *Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10.* Blood, 2001. **98**(10): p. 2992-8.
44. Menetrier-Caux, C., et al., *Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor.* Blood, 1998. **92**(12): p. 4778-91.

45. Kukreja, A., et al., *Enhancement of clonogenicity of human multiple myeloma by dendritic cells.* J Exp Med, 2006. **203**(8): p. 1859-65.
46. Chauhan, D., et al., *Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target.* Cancer Cell, 2009. **16**(4): p. 309-23.
47. Kukreja, A., et al., *Dominant role of CD47-thrombospondin-1 interactions in myeloma-induced fusion of human dendritic cells: implications for bone disease.* Blood, 2009. **114**(16): p. 3413-21.
48. Tucci, M., et al., *Immature dendritic cells from patients with multiple myeloma are prone to osteoclast differentiation in vitro.* Exp Hematol, 2011. **39**(7): p. 773-83 e1.
49. Tucci, M., et al., *Dendritic cells and malignant plasma cells: an alliance in multiple myeloma tumor progression?* Oncologist, 2011. **16**(7): p. 1040-8.
50. Beyer, M., et al., *In vivo peripheral expansion of naive CD4+CD25high FoxP3+ regulatory T cells in patients with multiple myeloma.* Blood, 2006. **107**(10): p. 3940-9.
51. Frassanito, M.A., A. Cusmai, and F. Dammacco, *Deregulated cytokine network and defective Th1 immune response in multiple myeloma.* Clin Exp Immunol, 2001. **125**(2): p. 190-7.
52. Brimnes, M.K., et al., *Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma.* Scand J Immunol, 2010. **72**(6): p. 540-7.
53. Prabhala, R.H., et al., *Dysfunctional T regulatory cells in multiple myeloma.* Blood, 2006. **107**(1): p. 301-4.
54. Zhou, L., et al., *IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways.* Nat Immunol, 2007. **8**(9): p. 967-74.
55. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
56. Jurisic, V., et al., *Clinical stage-depending decrease of NK cell activity in multiple myeloma patients.* Med Oncol, 2007. **24**(3): p. 312-7.
57. Benson, D.M., Jr., et al., *A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma.* Blood, 2012. **120**(22): p. 4324-33.
58. Hoteit, R., et al., *KIR genotype distribution among patients with multiple myeloma: Higher prevalence of KIR 2DS4 and KIR 2DS5 genes.* Meta Gene, 2014. **2**: p. 730-6.
59. Jinushi, M., et al., *MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma.* Proc Natl Acad Sci U S A, 2008. **105**(4): p. 1285-90.
60. Van Valckenborgh, E., et al., *Multiple myeloma induces the immunosuppressive capacity of distinct myeloid-derived suppressor cell subpopulations in the bone marrow.* Leukemia, 2012. **26**(11): p. 2424-8.
61. Fionda, C., et al., *NKG2D and DNAM-1 Ligands: Molecular Targets for NK Cell-Mediated Immunotherapeutic Intervention in Multiple Myeloma.* Biomed Res Int, 2015. **2015**: p. 178698.
62. Guillerey, C., et al., *Immune responses in multiple myeloma: role of the natural immune surveillance and potential of immunotherapies.* Cell Mol Life Sci, 2016. **73**(8): p. 1569-89.
63. Ponzetta, A., et al., *Multiple Myeloma Impairs Bone Marrow Localization of Effector Natural Killer Cells by Altering the Chemokine Microenvironment.* Cancer Res, 2015. **75**(22): p. 4766-77.
64. Gabrilovich, D.I., *Myeloid-Derived Suppressor Cells.* Cancer Immunol Res, 2017. **5**(1): p. 3-8.
65. Gorgun, G.T., et al., *Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans.* Blood, 2013. **121**(15): p. 2975-87.
66. Castella, B., et al., *Anergic bone marrow Vgamma9Vdelta2 T cells as early and long-lasting markers of PD-1-targetable microenvironment-induced immune suppression in human myeloma.* Oncoimmunology, 2015. **4**(11): p. e1047580.

67. Ramachandran, I.R., et al., *Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow.* J Immunol, 2013. **190**(7): p. 3815-23.
68. Romano, A., et al., *Immunological dysregulation in multiple myeloma microenvironment.* Biomed Res Int, 2014. **2014**: p. 198539.
69. Gorgun, G., et al., *Lenalidomide Enhances Immune Checkpoint Blockade-Induced Immune Response in Multiple Myeloma.* Clin Cancer Res, 2015. **21**(20): p. 4607-18.
70. Keir, M.E., et al., *PD-1 and its ligands in tolerance and immunity.* Annu Rev Immunol, 2008. **26**: p. 677-704.
71. Chemnitz, J.M., et al., *SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation.* J Immunol, 2004. **173**(2): p. 945-54.
72. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. **25**(21): p. 9543-53.
73. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
74. Okazaki, T. and T. Honjo, *PD-1 and PD-1 ligands: from discovery to clinical application.* Int Immunol, 2007. **19**(7): p. 813-24.
75. Liu, J., et al., *Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway.* Blood, 2007. **110**(1): p. 296-304.
76. Dhodapkar, M.V., et al., *Prospective analysis of antigen-specific immunity, stem-cell antigens, and immune checkpoints in monoclonal gammopathy.* Blood, 2015. **126**(22): p. 2475-8.
77. Tamura, H., et al., *Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma.* Leukemia, 2013. **27**(2): p. 464-72.
78. Sponaas, A.M., et al., *PDL1 Expression on Plasma and Dendritic Cells in Myeloma Bone Marrow Suggests Benefit of Targeted anti PD1-PDL1 Therapy.* PLoS One, 2015. **10**(10): p. e0139867.
79. Paiva, B., et al., *PD-L1/PD-1 presence in the tumor microenvironment and activity of PD-1 blockade in multiple myeloma.* Leukemia, 2015. **29**(10): p. 2110-3.
80. Benson, D.M., Jr., et al., *The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody.* Blood, 2010. **116**(13): p. 2286-94.
81. Rosenblatt, J., et al., *PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine.* J Immunother, 2011. **34**(5): p. 409-18.
82. Ray, A., et al., *Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells.* Leukemia, 2015. **29**(6): p. 1441-4.
83. Kearl, T.J., et al., *Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma.* J Immunol, 2013. **190**(11): p. 5620-8.
84. Hallett, W.H., et al., *Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade.* Biol Blood Marrow Transplant, 2011. **17**(8): p. 1133-45.
85. Kumar, S.K., et al., *Improved survival in multiple myeloma and the impact of novel therapies.* Blood, 2008. **111**(5): p. 2516-20.
86. Raje, N., T. Hideshima, and K.C. Anderson, *Therapeutic use of immunomodulatory drugs in the treatment of multiple myeloma.* Expert Rev Anticancer Ther, 2006. **6**(9): p. 1239-47.
87. Dimopoulos, M., et al., *Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma.* N Engl J Med, 2007. **357**(21): p. 2123-32.
88. Weber, D.M., et al., *Lenalidomide plus dexamethasone for relapsed multiple myeloma in North America.* N Engl J Med, 2007. **357**(21): p. 2133-42.

89. Benboubker, L., et al., *Lenalidomide and dexamethasone in transplant-ineligible patients with myeloma.* N Engl J Med, 2014. **371**(10): p. 906-17.
90. Palumbo, A., et al., *Autologous transplantation and maintenance therapy in multiple myeloma.* N Engl J Med, 2014. **371**(10): p. 895-905.
91. Sedlarikova, L., et al., *Mechanism of immunomodulatory drugs in multiple myeloma.* Leuk Res, 2012. **36**(10): p. 1218-24.
92. Bolzoni, M., et al., *Immunomodulatory drugs lenalidomide and pomalidomide inhibit multiple myeloma-induced osteoclast formation and the RANKL/OPG ratio in the myeloma microenvironment targeting the expression of adhesion molecules.* Exp Hematol, 2013. **41**(4): p. 387-97 e1.
93. Galustian, C., et al., *The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells.* Cancer Immunol Immunother, 2009. **58**(7): p. 1033-45.
94. Luptakova, K., et al., *Lenalidomide enhances anti-myeloma cellular immunity.* Cancer Immunol Immunother, 2013. **62**(1): p. 39-49.
95. D'Amato, R.J., et al., *Thalidomide is an inhibitor of angiogenesis.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 4082-5.
96. Muller, G.W., et al., *Amino-substituted thalidomide analogs: potent inhibitors of TNF-alpha production.* Bioorg Med Chem Lett, 1999. **9**(11): p. 1625-30.
97. Quach, H., et al., *Mechanism of action of immunomodulatory drugs (IMiDS) in multiple myeloma.* Leukemia, 2010. **24**(1): p. 22-32.
98. Hideshima, T., et al., *Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy.* Blood, 2000. **96**(9): p. 2943-50.
99. Zhu, Y.X., et al., *Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide.* Blood, 2011. **118**(18): p. 4771-9.
100. Schuster, S.R., et al., *The clinical significance of cereblon expression in multiple myeloma.* Leuk Res, 2014. **38**(1): p. 23-8.
101. Kronke, J., et al., *Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells.* Science, 2014. **343**(6168): p. 301-5.
102. Lu, G., et al., *The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins.* Science, 2014. **343**(6168): p. 305-9.
103. Kronke, J., et al., *Lenalidomide induces ubiquitination and degradation of CK1alpha in del(5q) MDS.* Nature, 2015. **523**(7559): p. 183-8.
104. Manni, S., et al., *Inactivation of CK1alpha in multiple myeloma empowers drug cytotoxicity by affecting AKT and beta-catenin survival signaling pathways.* Oncotarget, 2017. **8**(9): p. 14604-14619.
105. Corradini, P., et al., *Molecular remission after myeloablative allogeneic stem cell transplantation predicts a better relapse-free survival in patients with multiple myeloma.* Blood, 2003. **102**(5): p. 1927-9.
106. Kroger, N., et al., *Post-transplant immunotherapy with donor-lymphocyte infusion and novel agents to upgrade partial into complete and molecular remission in allografted patients with multiple myeloma.* Exp Hematol, 2009. **37**(7): p. 791-8.
107. Wolschke, C., et al., *Postallograft lenalidomide induces strong NK cell-mediated antimyeloma activity and risk for T cell-mediated GvHD: Results from a phase I/II dose-finding study.* Exp Hematol, 2013. **41**(2): p. 134-142 e3.
108. Ferrara, J.L., et al., *Graft-versus-host disease.* Lancet, 2009. **373**(9674): p. 1550-61.
109. Stenger, E.O., et al., *Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity.* Blood, 2012. **119**(22): p. 5088-103.
110. Spaggiari, G.M., et al., *MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2.* Blood, 2009. **113**(26): p. 6576-83.
111. Zhang, Y., et al., *Notch signaling is a critical regulator of allogeneic CD4+ T-cell responses mediating graft-versus-host disease.* Blood, 2011. **117**(1): p. 299-308.
112. Yuan, J.S., et al., *Functions of notch signaling in the immune system: consensus and controversies.* Annu Rev Immunol, 2010. **28**: p. 343-65.

113. Amsen, D., et al., *Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells.* Cell, 2004. **117**(4): p. 515-26.
114. Yvon, E.S., et al., *Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells.* Blood, 2003. **102**(10): p. 3815-21.
115. Mukherjee, S., et al., *Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation.* J Immunol, 2009. **182**(12): p. 7381-8.
116. Cho, O.H., et al., *Notch regulates cytolytic effector function in CD8+ T cells.* J Immunol, 2009. **182**(6): p. 3380-9.
117. Toubai, T., et al., *Ikaros-Notch axis in host hematopoietic cells regulates experimental graft-versus-host disease.* Blood, 2011. **118**(1): p. 192-204.
118. Lesokhin, A.M., et al., *Nivolumab in Patients With Relapsed or Refractory Hematologic Malignancy: Preliminary Results of a Phase Ib Study.* J Clin Oncol, 2016. **34**(23): p. 2698-704.
119. Ribrag, V., et al., *Pembrolizumab Monotherapy for Relapsed/Refractory Multiple Myeloma: Phase 1b Keynote-013 Study.* Haematologica, 2017. **102**: p. 114-114.
120. Schumacher, T.N. and R.D. Schreiber, *Neoantigens in cancer immunotherapy.* Science, 2015. **348**(6230): p. 69-74.
121. Mateos, M.V., et al., *Lenalidomide plus dexamethasone versus observation in patients with high-risk smouldering multiple myeloma (QuiRedex): long-term follow-up of a randomised, controlled, phase 3 trial.* Lancet Oncol, 2016. **17**(8): p. 1127-1136.
122. Badros, A., et al., *Pembrolizumab, pomalidomide, and low-dose dexamethasone for relapsed/refractory multiple myeloma.* Blood, 2017. **130**(10): p. 1189-1197.
123. Suen, H., et al., *The failure of immune checkpoint blockade in multiple myeloma with PD-1 inhibitors in a phase 1 study.* Leukemia, 2015. **29**(7): p. 1621-2.
124. Suen, H., et al., *Multiple myeloma causes clonal T-cell immunosenescence: identification of potential novel targets for promoting tumour immunity and implications for checkpoint blockade.* Leukemia, 2016. **30**(8): p. 1716-24.
125. Crespo, J., et al., *T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment.* Curr Opin Immunol, 2013. **25**(2): p. 214-21.
126. Richardson, P.G., et al., *Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma.* Blood, 2002. **100**(9): p. 3063-7.
127. Lopez-Girona, A., et al., *Lenalidomide downregulates the cell survival factor, interferon regulatory factor-4, providing a potential mechanistic link for predicting response.* Br J Haematol, 2011. **154**(3): p. 325-36.
128. Zhu, Y.X., et al., *Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma.* Blood, 2014. **124**(4): p. 536-45.
129. Leone, P., et al., *Dendritic cells accumulate in the bone marrow of myeloma patients where they protect tumor plasma cells from CD8+ T-cell killing.* Blood, 2015. **126**(12): p. 1443-51.
130. Vo, M.C., et al., *Lenalidomide enhances the function of dendritic cells generated from patients with multiple myeloma.* Exp Hematol, 2017. **46**: p. 48-55.
131. Chen, N., et al., *Pharmacokinetics, metabolism and excretion of [(14)C]-lenalidomide following oral administration in healthy male subjects.* Cancer Chemother Pharmacol, 2012. **69**(3): p. 789-97.
132. Geijtenbeek, T.B., et al., *DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking.* Nat Immunol, 2000. **1**(4): p. 353-7.
133. Geijtenbeek, T.B., et al., *Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses.* Cell, 2000. **100**(5): p. 575-85.
134. Piemonti, L., et al., *Glucocorticoids affect human dendritic cell differentiation and maturation.* J Immunol, 1999. **162**(11): p. 6473-81.
135. Matasic, R., A.B. Dietz, and S. Vuk-Pavlovic, *Dexamethasone inhibits dendritic cell maturation by redirecting differentiation of a subset of cells.* J Leukoc Biol, 1999. **66**(6): p. 909-14.

136. Paiva, B., et al., *Immune status of high-risk smoldering multiple myeloma patients and its therapeutic modulation under LenDex: a longitudinal analysis.* Blood, 2016. **127**(9): p. 1151-62.
137. Hsu, A.K., et al., *The immunostimulatory effect of lenalidomide on NK-cell function is profoundly inhibited by concurrent dexamethasone therapy.* Blood, 2011. **117**(5): p. 1605-13.
138. Gandhi, A.K., et al., *Dexamethasone synergizes with lenalidomide to inhibit multiple myeloma tumor growth, but reduces lenalidomide-induced immunomodulation of T and NK cell function.* Curr Cancer Drug Targets, 2010. **10**(2): p. 155-67.
139. Henry, J.Y., et al., *Enhanced cross-priming of naive CD8+ T cells by dendritic cells treated by the IMiDs(R) immunomodulatory compounds lenalidomide and pomalidomide.* Immunology, 2013. **139**(3): p. 377-85.
140. Reddy, N., et al., *Immunomodulatory drugs stimulate natural killer-cell function, alter cytokine production by dendritic cells, and inhibit angiogenesis enhancing the anti-tumour activity of rituximab in vivo.* Br J Haematol, 2008. **140**(1): p. 36-45.
141. Nagorsen, D., F.M. Marincola, and M.C. Panelli, *Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays.* Cytokine, 2004. **25**(1): p. 31-5.
142. Toubai, T., et al., *Ikaros deficiency in host hematopoietic cells separates GVL from GVHD after experimental allogeneic hematopoietic cell transplantation.* Oncoimmunology, 2015. **4**(7): p. e1016699.
143. Alsina, M., et al., *Lenalidomide maintenance for high-risk multiple myeloma after allogeneic hematopoietic cell transplantation.* Biol Blood Marrow Transplant, 2014. **20**(8): p. 1183-9.
144. Nauta, A.J. and W.E. Fibbe, *Immunomodulatory properties of mesenchymal stromal cells.* Blood, 2007. **110**(10): p. 3499-506.
145. Chen, X., M.A. Armstrong, and G. Li, *Mesenchymal stem cells in immunoregulation.* Immunol Cell Biol, 2006. **84**(5): p. 413-21.
146. Le Blanc, K. and O. Ringden, *Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation.* Biol Blood Marrow Transplant, 2005. **11**(5): p. 321-34.
147. Kyurkchiev, D., et al., *Secretion of immunoregulatory cytokines by mesenchymal stem cells.* World J Stem Cells, 2014. **6**(5): p. 552-70.
148. Chen, K., et al., *Human umbilical cord mesenchymal stem cells hUC-MSCs exert immunosuppressive activities through a PGE2-dependent mechanism.* Clin Immunol, 2010. **135**(3): p. 448-58.
149. Hu, Y., et al., *CSNK1alpha1 mediates malignant plasma cell survival.* Leukemia, 2015. **29**(2): p. 474-82.
150. Bjorklund, C.C., et al., *Evidence of a role for activation of Wnt/beta-catenin signaling in the resistance of plasma cells to lenalidomide.* J Biol Chem, 2011. **286**(13): p. 11009-20.
151. Sehgal, K., et al., *Clinical and pharmacodynamic analysis of pomalidomide dosing strategies in myeloma: impact of immune activation and cereblon targets.* Blood, 2015. **125**(26): p. 4042-51.
152. Zhang, Y., et al., *PD-L1 up-regulation restrains Th17 cell differentiation in STAT3 loss- and STAT1 gain-of-function patients.* J Exp Med, 2017. **214**(9): p. 2523-2533.
153. Yang, L., et al., *Program Death-1 Suppresses Autoimmune Arthritis by Inhibiting Th17 Response.* Arch Immunol Ther Exp (Warsz), 2016. **64**(5): p. 417-23.
154. Zhang, Y., et al., *The altered PD-1/PD-L1 pathway delivers the 'one-two punch' effects to promote the Treg/Th17 imbalance in pre-eclampsia.* Cell Mol Immunol, 2018. **15**(7): p. 710-723.

7. FIGURE LEGENDS

Figure 1: LEN enhanced *in vitro* DC differentiation from both BM and PB of MM patients.

(A) DCs were differentiated from BM $CD14^+$ cells of MM patients, cultured in RPMI 10% FBS with IL-4 and GM-CSF, for 8 days, in presence of LEN (0.1 and 1 µM) or DMSO. TNF-α was added in the last 24 h of differentiation period. Non-adherent cells were collected and analysed by flow-cytometry for DC maturation markers. Graph bars represent the mean of DC number and % and median fluorescent intensity (MFI) of DC maturation markers ± standard error of the mean (SEM) (p calculated by paired Student's t-test) of 19 independent experiment. (B) DCs were differentiated from PB of 6 MM patients, following the same protocol. Graph bars represent the mean of DC number and % and MFI of DC maturation markers ± SEM (p calculated by paired Student's t-test).

Figure 2: LEN effects on DC maturation markers were abrogated by Dex.

DCs were differentiated from BM $CD14^+$ cells of MM patients, in the presence of LEN (0.1 and 1 µM) or vehicle, as previously reported. At the end of culture period, cells were collected and reseeded ($5x10^4$/ml) in fresh medium with Dex (10^{-8}M) or vehicle (EtOH) for 48 h. After Dex treatment, cells were collected and analyzed for DC maturation markers. Graph bars represent the median of DC number and % and MFI of DC maturation markers from 4 independent experiments (p calculated by Wilcoxon test).

Figure 3: LEN increased the production of IL-8, CCL2, CCL5 and TNF-α by DCs.

DC CM was collected after *in vitro* DC differentiation from BM $CD14^+$ cells of 5 MM patients. The levels (pg/ml) of cytokines and chemokines involved in immune response were evaluated by a Bio-Plex® Multiplex System. Graph bars represent the median concentration of soluble factor levels in the presence of DMSO or LEN. For TNF-α level evaluation, the obtained results were normalized for TNF-α concentration measured in the control medium (RPMI 10% FBS, with IL-4, GM-CSF and TNF-α at concentration used during DC differentiation).

Figure 4: LEN effect on DC differentiation was mediated by Ikaros and Aiolos degradation.

DCs were differentiated from THP-1 cell line, by adding rhIL-4 (200 ng/ml), rhGM-CSF (100 ng/ml), ionomycin (200 ng/ml) and rhTNF-α (20 ng/ml) for 72 h to the culture medium (RPMI 1664, serum depleted); then LEN (0.1 and 1 µM) or DMSO were added for the last 24 h of culture period. Differentiated cells (THP1-DCs) were then collected and Cereblon (A), Ikaros (B), Aiolos (C), IRF4 (D) and p62 (E) protein levels were analyzed by Western Blot. β-actin was used as internal control.

Figure 5: Effect of LEN on DC ability to stimulate T cell proliferation, alone and in combination with Dex.

DCs were differentiated from BM $CD14^+$ cells of 6 MM patients, in the presence of LEN or DMSO, alone (A) or in combination with Dex 10^{-8}M (B), as previously reported. At the end of culture period, cells were collected and re-seeded ($3x10^3$ cells/w) in round-bottomed 96well-plates, in RPMI 1640 with 15% AB human serum. DCs were co-cultured with autologous PB CD3+ cells ($1x10^4$/w) for 6 days. At the end of culture period, an MTT assay was performed in order to measure T cell proliferation. Graph bars represent the mean O.D. of T cell proliferation in co-culture with pre-treated DCs ± SEM (p calculated by Mann-Whitney test (A) and unpaired t test (B)).

Figure 6: LEN blunted hTERT-hMSC inhibitory properties on DC differentiation through the down-regulation of PTGS2 expression levels.

DCs were differentiated from BM $CD14^+$ cells of 3 MM patients, in the presence or absence of the CM (ratio 1:2 with RPMI 1664 10% FBS, with IL-4 and GM-CSF) of hTERT-hMSCs, treated with LEN (0.1 and 1 μM) or DMSO for 5 days. At the end of culture period, cells were collected and analyzed for DC maturation markers, by flow-cytometry. (A) The inhibitory effect of hTERT-hMSCs on *in vitro* DC differentiation was firstly checked. Graph bars represent the median of DC number and % and MFI of DC maturation markers on DCs cultured with DMSO-treated hTERT-hMSCs CM or CNT (RPMI 10% FBS). (B) The effect of LEN treated hTERT-hMSCs on DC differentiation was then evaluated. Graph bars represent the median of DC number and % and MFI of DC maturation markers on DCs cultured with LEN-treated hTERT-hMSCs CM or DMSO. (C) hTERT-hMSCs were seeded in T75 flasks and cultured in RPMI 10% FBS, in presence of LEN (0.1 and 1 μM) or DMSO, for 5 days. At the end of culture period, cell pellets were collected and analyzed by RT-PCR for the mRNA expression of several immunosuppressive factors. Graph bars represent the mean Fold Change (FC) of LEN treated hTERT-hMSCs vs DMSO ± SEM of 3 independent experiments (p calculated by Student's t-test; *$p<0.05$, ***$p<0.0001$).

Figure 7: LEN treatment decreased CK1α levels in hTERT-hMSCs.

hTERT-hMSCs were seeded in T75 flasks and cultured in RPMI 10% FBS, in presence of LEN (0.1 and 1 μM) or DMSO, for 5 days. At the end of culture period, cell pellets were collected and analyzed by Western Blot for Cereblon (A), Ikaros (B), Aiolos (B), IRF4 (B), CK1α (C) and p62 (D). β-actin was used as internal control**.**

Figure 8: *In vivo* LEN treatment increased *in vitro* DC differentiation from CD14$^+$ cells of MM patients.

DCs were *in vitro* differentiated from PB CD14$^+$ cells of 9 MM patients at day 0 and after one week (day 7) of LEN 25mg/day treatment. Cells were cultured in RPMI 10% FBS with IL-4 and GM-CSF for 8 days and TNF-α was added for the last 24 h. At the end of culture period, cells were collected and analyzed for DC maturation markers, by flow-cytometry. Graph bars represent the mean of DC number and % and MFI of DC maturation markers at day 7 vs day 0 ± SEM, of 9 independent experiments (p calculated by paired Student's t-test).

Figure 9: Patients with active MM show increased PD-1$^+$CD4$^+$ cell % compared to asymptomatic myeloma.

Flow-cytometry analysis of PD-1 expression was performed on BM MNCs isolated from patients with monoclonal gammopathies at different stages of disease. Data were normalized on the CD138$^-$ fraction. Both the percentage of PD-1 positive cells (a) and PD-1 MFI (b) were measured in the sample cohort. (p calculated by Mann-Whitney test)

Figure 10: PD-1$^+$CD8$^+$ cell % and PD-L1 expression on both CD138$^+$ and CD14$^+$ cells inversely correlate with the presence and the rate of bone disease in MM patients.

Patients with active myeloma were stratified according to the presence (w osteolysis) or absence (w/o osteolysis) of bone disease, detected by CT with or without PET scan and by X-rays skeletal survey. Flow-cytometry data on CD4$^+$/CD8$^+$ ratio (a) and PD-1/PD-L1 expression (b-e) were then analyzed and correlated with osteolysis. (HBD, high bone disease; LBD, low bone disease). (p calculated by Mann-Whitney test)

8. FIGURES

Figure 1

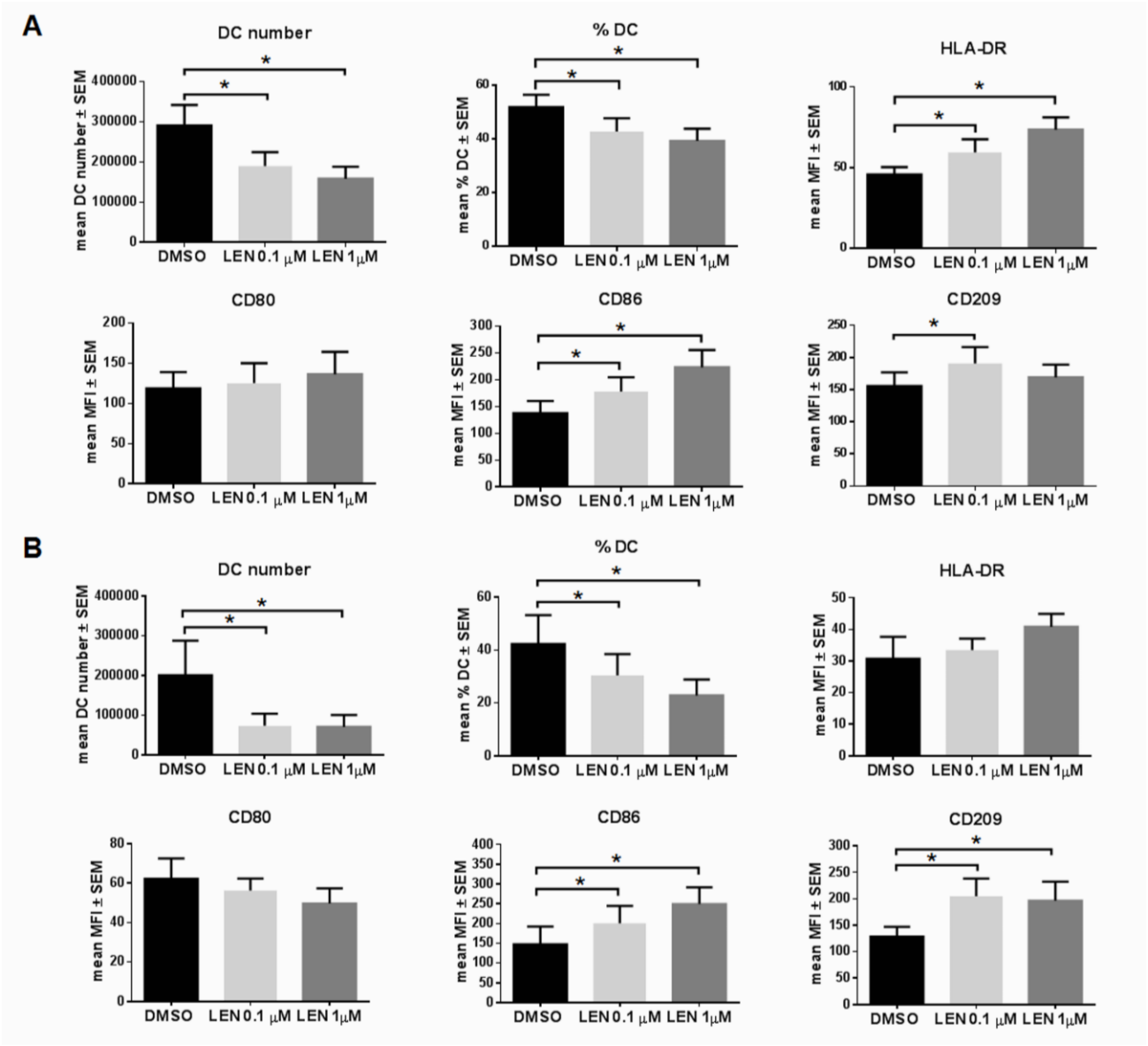
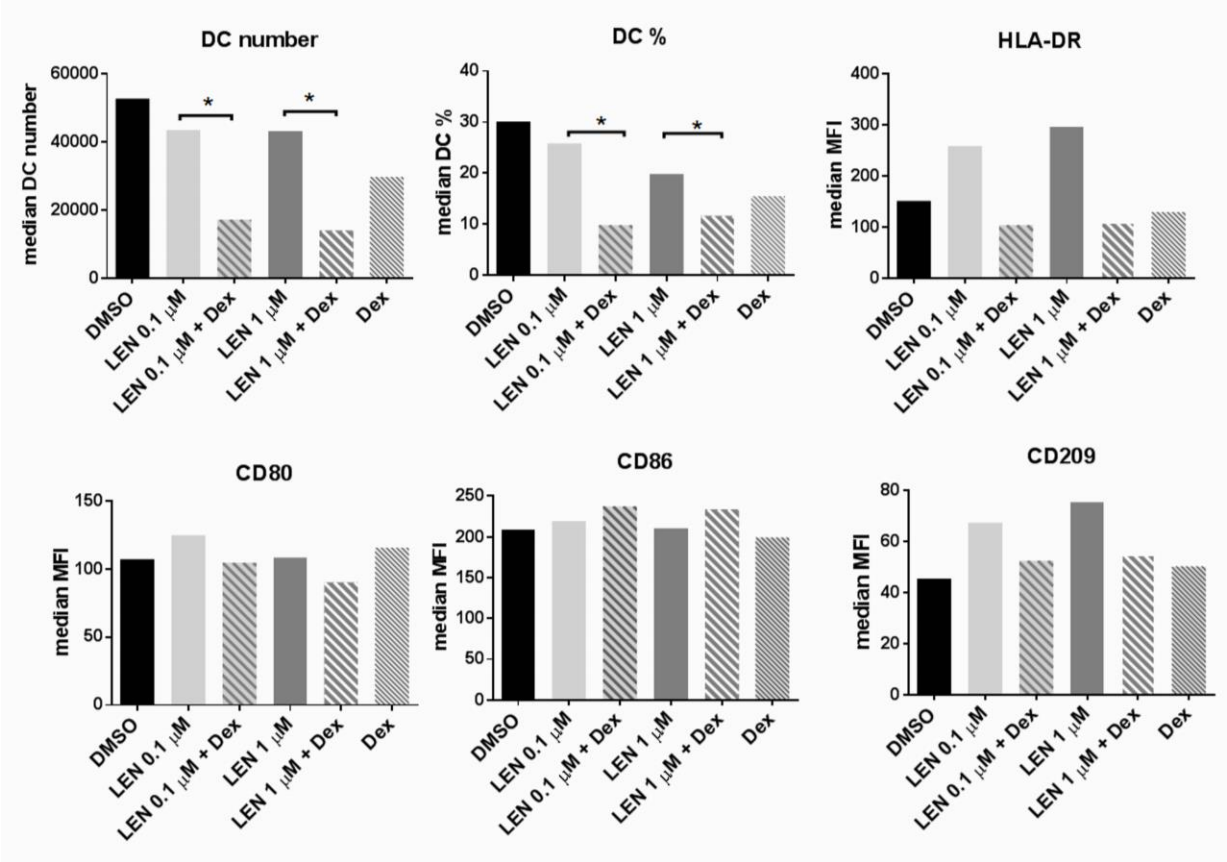


Figure 2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*
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LEN 0.1 μM + Dex
LEN 1 μM
LEN 1 μM + Dex
Dex
DC %
median DC %
40
30
20
10
0
HLA-DR
median MFI
400
300
200
100
0
CD80
median MFI
150
100
50
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CD209
median MFI
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60
40
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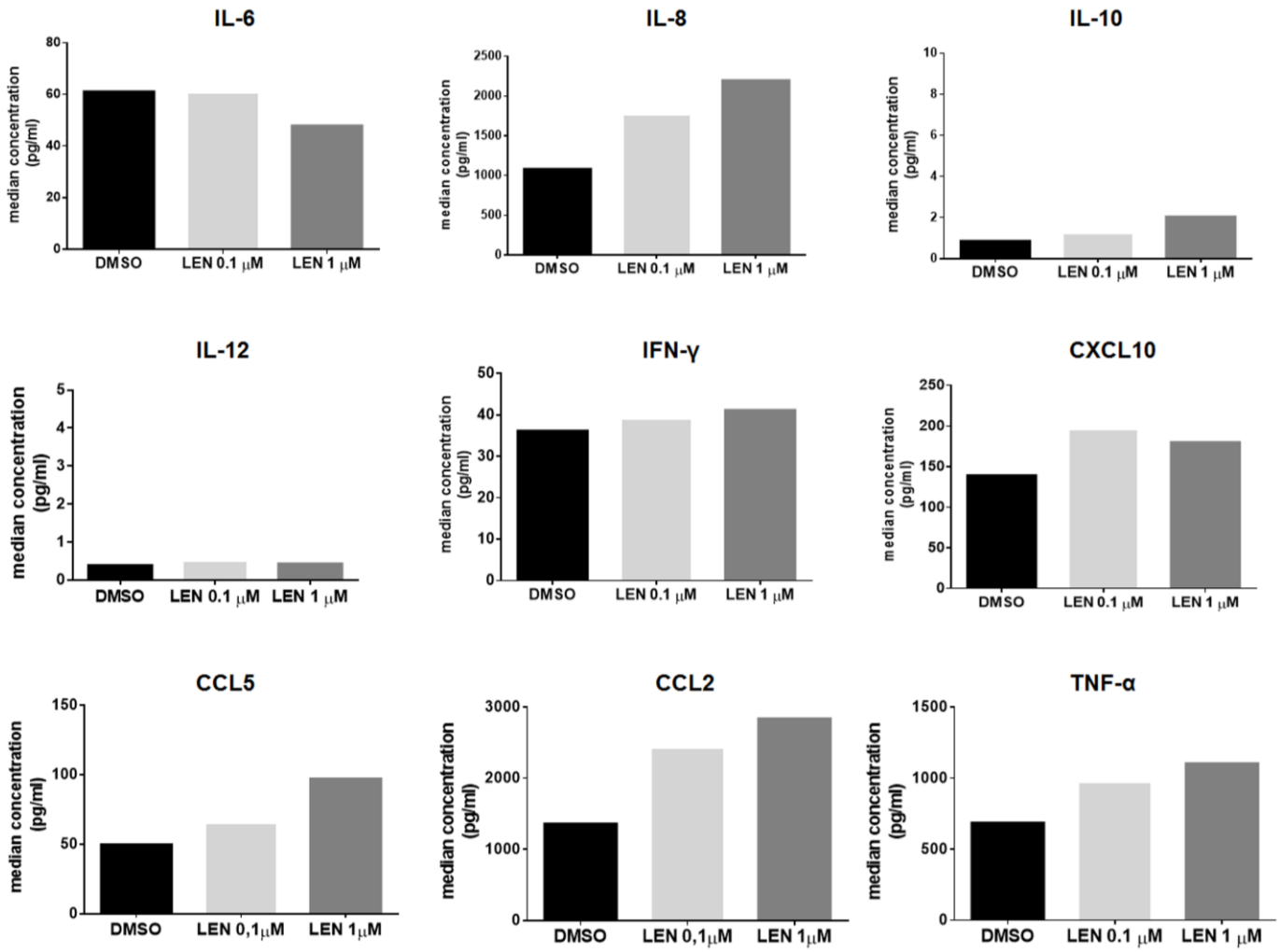
Figure 3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2000
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500
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DMSO
LEN 0.1 μM
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IL-10
median concentration (pg/ml)
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8
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4
2
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DMSO
LEN 0.1 μM
LEN 1 μM
IL-12
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DMSO
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IFN-γ
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median concentration (pg/ml)
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DMSO
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CCL5
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100
50
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DMSO
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CCL2
median concentration (pg/ml)
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2000
1000
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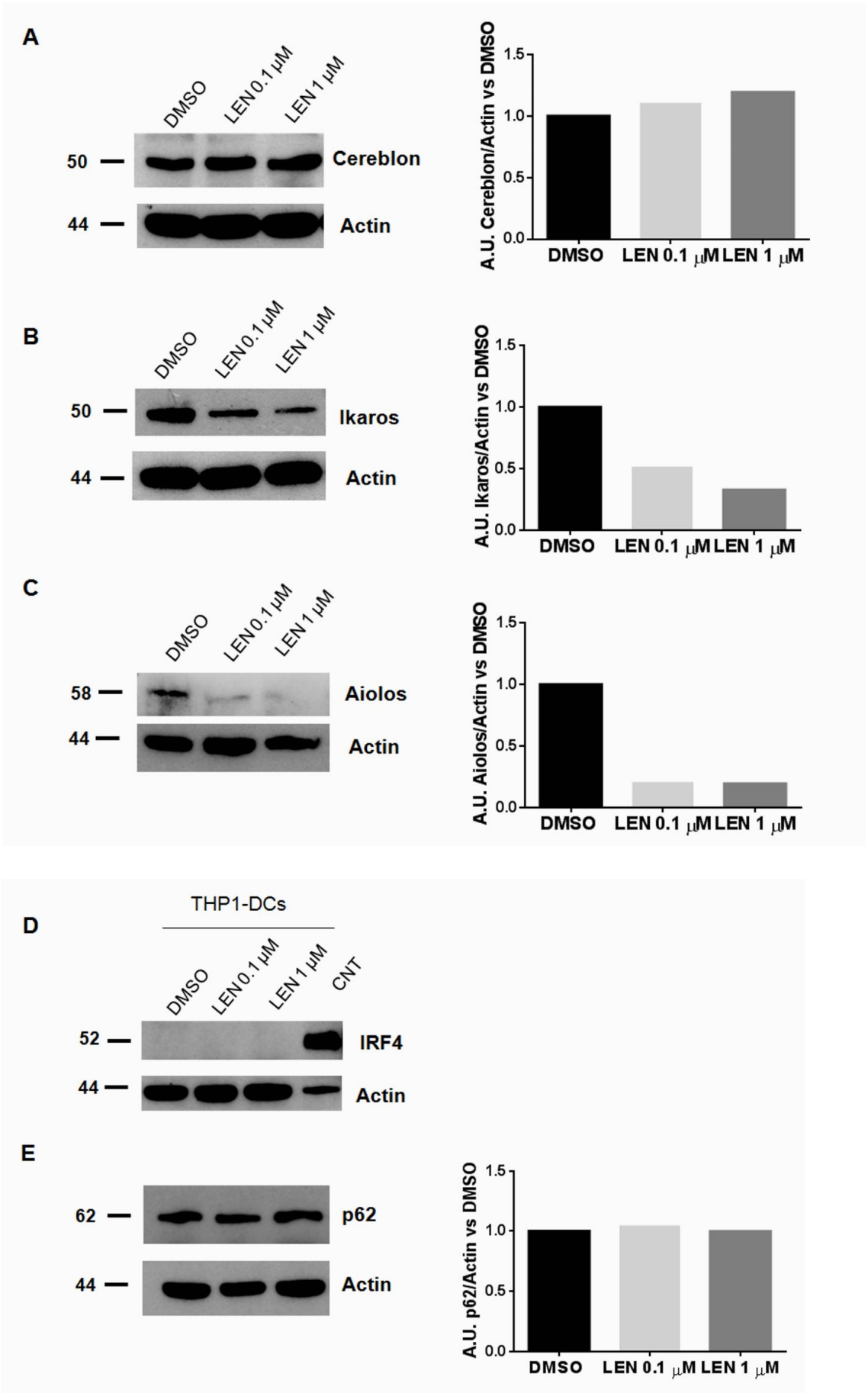
LEN 1 μM

Figure 4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DMSO
LEN 0.1 µM
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Ikaros
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Actin
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DMSO
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DMSO
LEN 0.1 µM
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58
Aiolos
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Actin
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THP1-DCs
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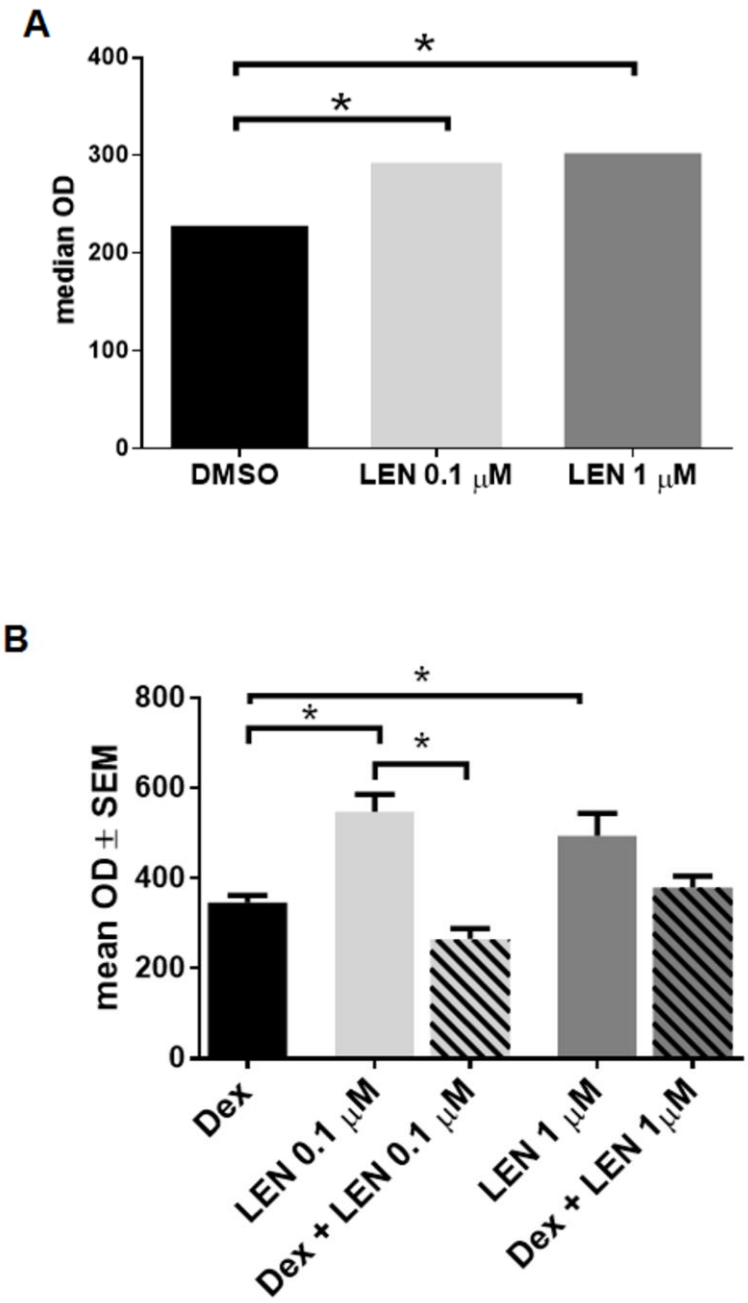
Figure 5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Dex + LEN 0.1 μM
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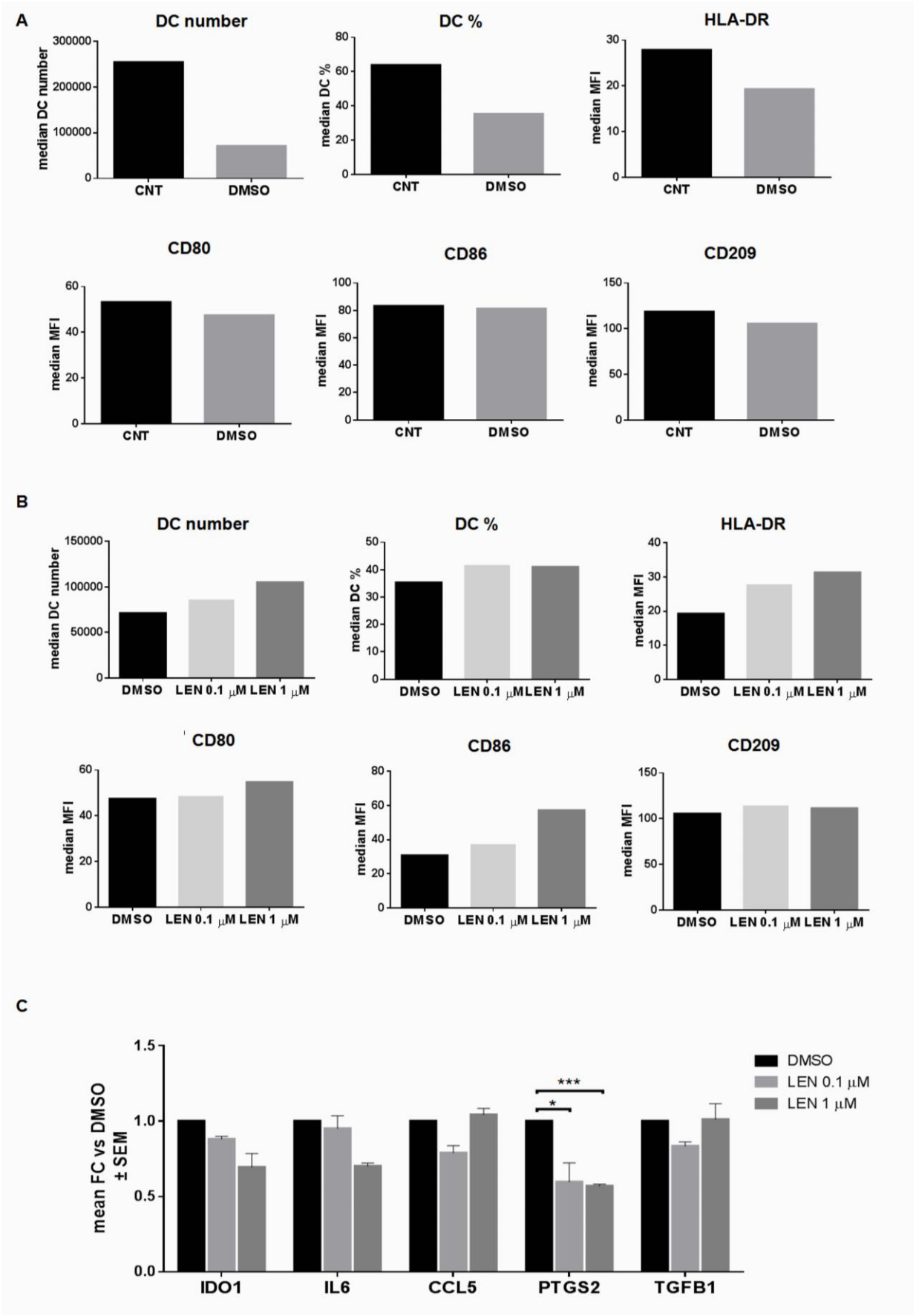
Dex + LEN 1μM

Figure 6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CCL5
PTGS2
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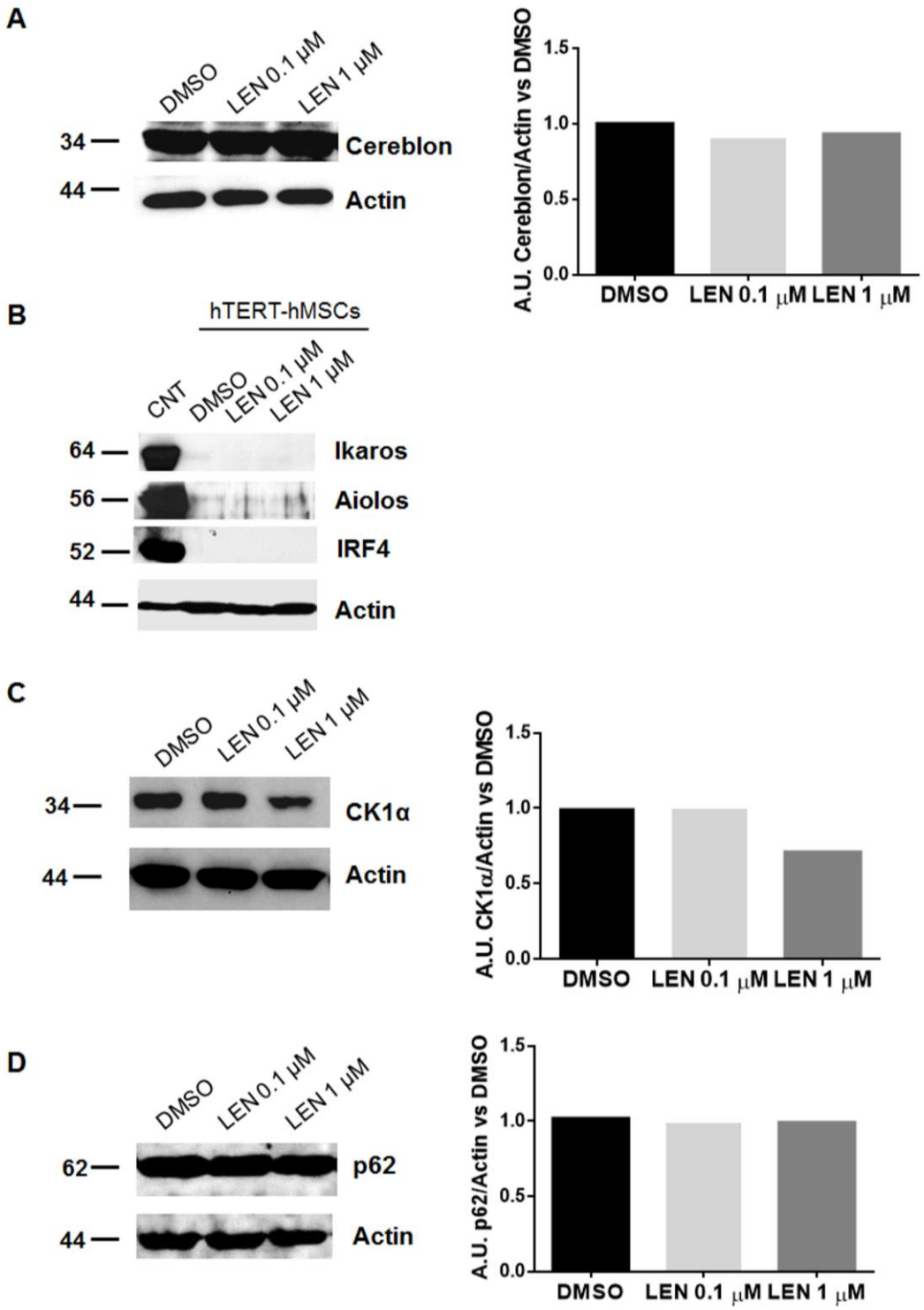
Figure 7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0.5
0.0
DMSO
LEN 0.1 μM
LEN 1 μM
B
hTERT-hMSCs
CNT
DMSO
LEN 0.1 μM
LEN 1 μM
64
Ikaros
56
Aiolos
52
IRF4
44
Actin
C
DMSO
LEN 0.1 μM
LEN 1 μM
34
CK1α
44
Actin
A.U. CK1α/Actin vs DMSO
1.5
1.0
0.5
0.0
DMSO
LEN 0.1 μM
LEN 1 μM
D
DMSO
LEN 0.1 μM
LEN 1 μM
62
p62
44
Actin
A.U. p62/Actin vs DMSO
1.5
1.0
0.5
0.0
DMSO
LEN 0.1 μM
LEN 1 μM

Figure 8

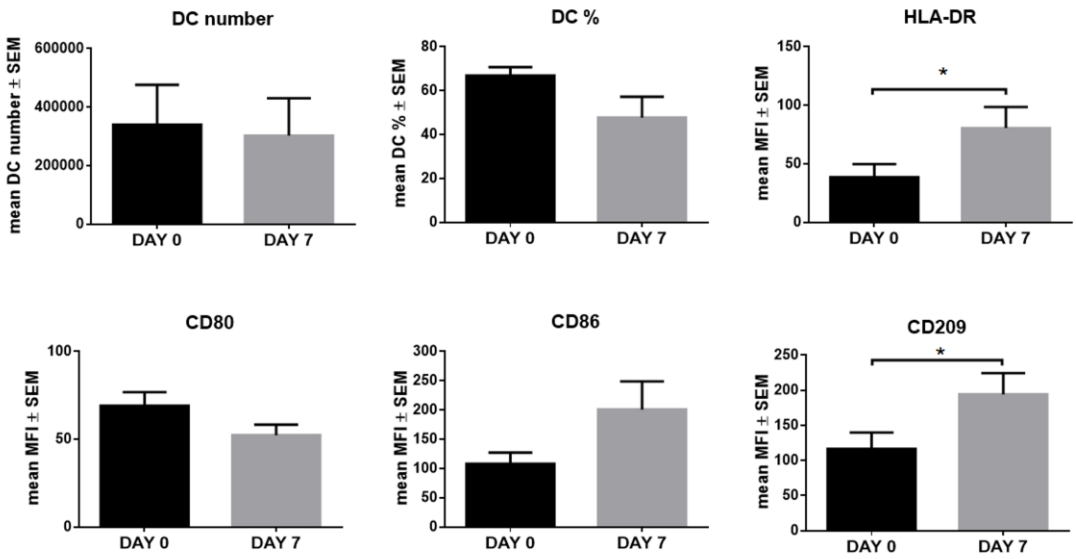


Figure 9

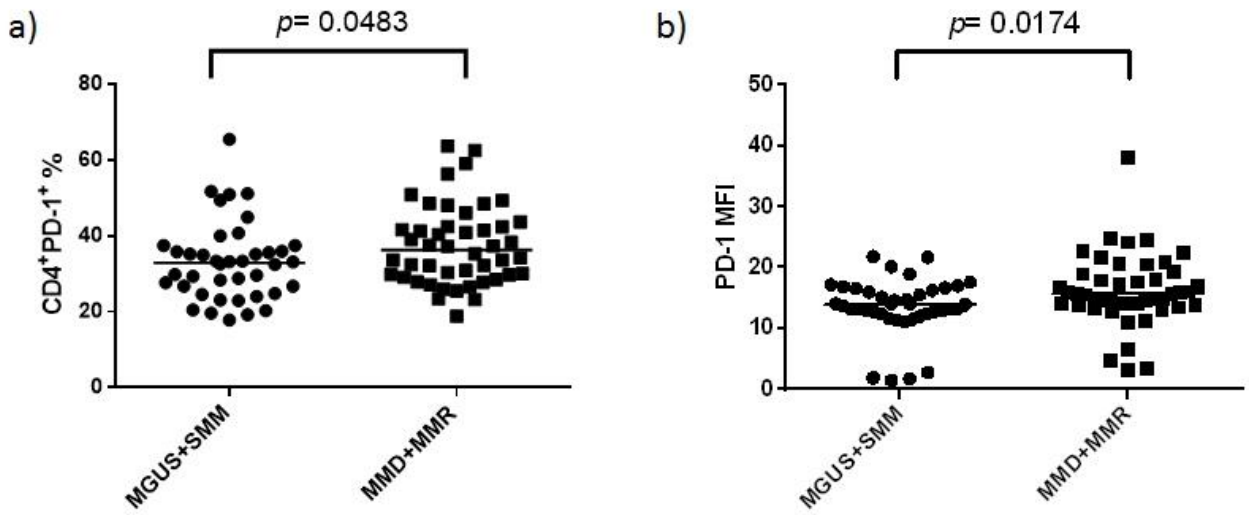
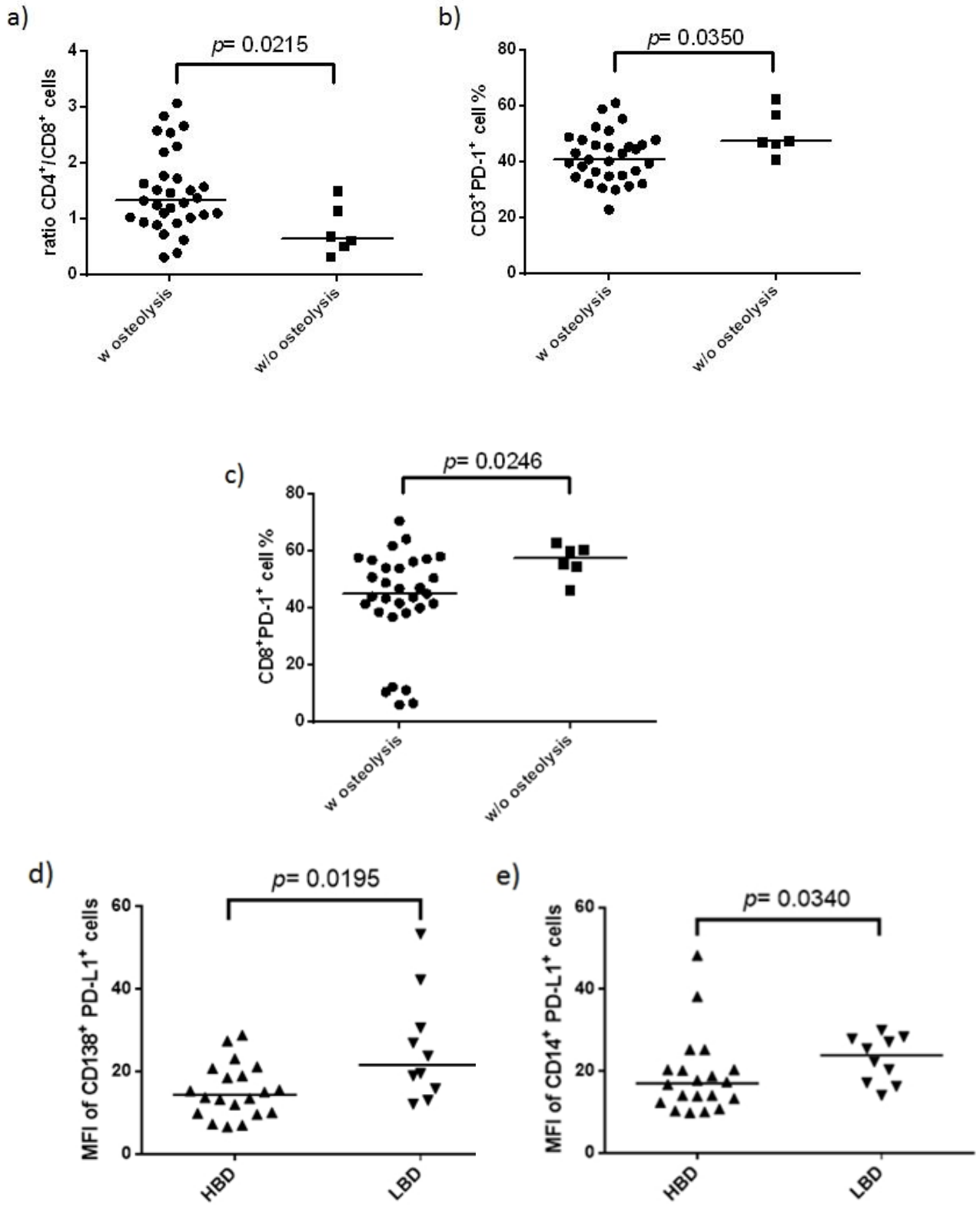


Figure 10


a)
p= 0.0215
ratio CD4+/CD8+ cells
w osteolysis
w/o osteolysis
b)
p= 0.0350
CD3+PD-1+ cell %
w osteolysis
w/o osteolysis
c)
p= 0.0246
CD8+PD-1+ cell %
w osteolysis
w/o osteolysis
d)
p= 0.0195
MFI of CD138+ PD-L1+ cells
HBD
LBD
e)
p= 0.0340
MFI of CD14+ PD-L1+ cells
HBD
LBD

APPENDIX 1

LEGENDS OF SUPPLEMENTAL FIGURES

Supplemental Figure 1: LEN treatment increased DC maturation markers in both BM and PB of MM patients.

DCs were differentiated from BM and PB $CD14^+$ cells of MM patients, cultured in RPMI 10% FBS with IL-4 and GM-CSF, for 8 days, in presence of LEN (0.1 and 1 μM) or DMSO. TNF-α was added in the last 24 h of differentiation period. Non-adherent cells were collected and analysed by flow-cytometry for DC maturation markers. Representative flow-cytometry histograms from BM (A) and PB (B) of 2 MM patients were reported. Empty histograms: DMSO, gray histograms: LEN 0.1 μM, black histograms: LEN 1 μM.

Supplemental Figure 2: LEN effect on DC maturation markers was abrogated by the combination with Dexamethasone.

DCs were differentiated from BM $CD14^+$ cells of MM patients, in the presence of LEN (0.1 and 1 μM) or vehicle. At the end of culture period, cells were collected and reseeded (5x104/ml) in fresh medium with Dex (10-8M) or vehicle (EtOH) for 48 h. After Dex treatment, cells were collected and analyzed for DC maturation markers. Representative flow-cytometry histogram from one MM patient. Black histograms: DMSO or LEN + EtOH (vehicle). Gray histograms: DMSO or LEN + Dex 10-8 M.

Supplemental Figure 3: LEN reverted the immunosuppressive properties of hTERT-hMSCs through the down-regulation of CK1-α.

CK1-α down-regulation was obtained by hTERT-hMSCs transduction with the IPTG inducible lentiviral particles carrying CSNK1A1-specific shRNA (clone 6044, 6042). At the end of culture period, cell pellets were collected and analyzed by western blotting to check CK1α down-

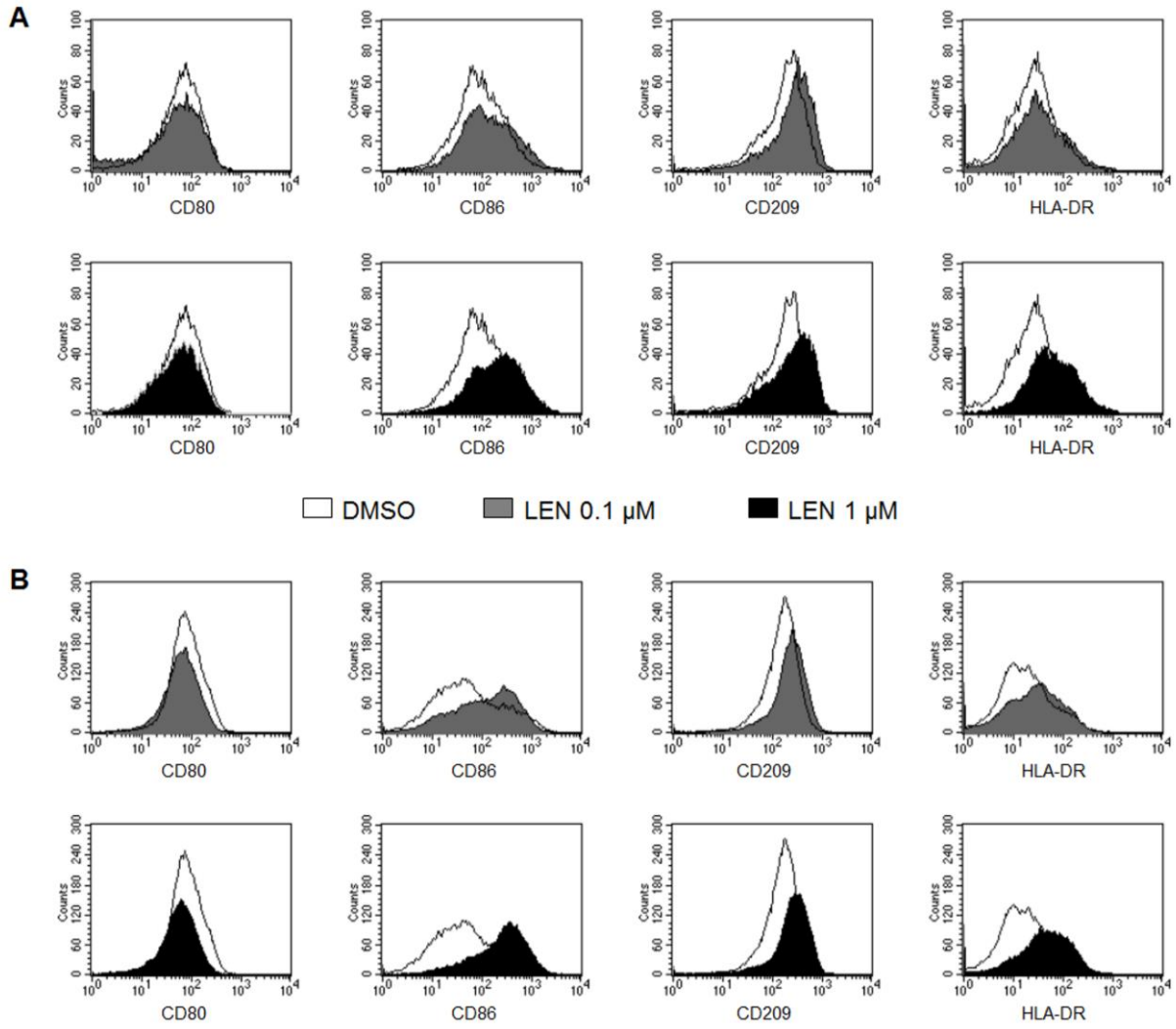
regulation and select the efficient clone. Wilde type (wt) hTERT-hMSCs were used as control β-actin was used as internal control. (A) DCs were differentiated from BM $CD14^+$ cells of MM patients, in the presence or absence (CNT) of the CM (ratio 1:2 with RPMI 1664 10% FBS, with IL-4 and GM-CSF) of hTERT-hMSCs transduced with the more efficient clone 6044. At the end of culture period, cells were collected and analyzed for DC maturation markers, by flow-cytometry. Graph bar represent the mean/median of DC number and % and the median/mean MFI of DC maturation markers (B).

Supplemental Figure 4: *In vivo* LEN treatment of MM patients increased *in vitro* DC differentiation.

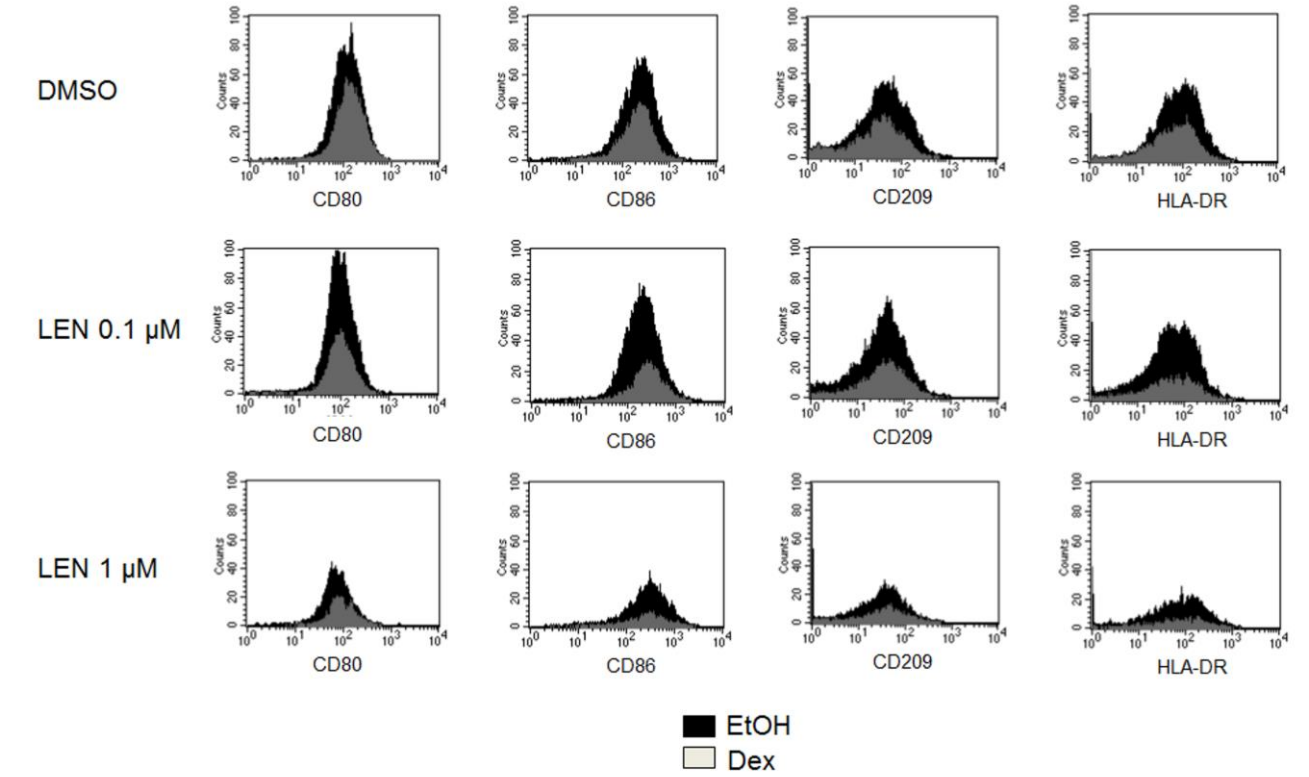
DCs were *in vitro* differentiated from PB $CD14^+$ cells of MM patients at DAY 0 and after one week (DAY 7) of LEN 25mg/day treatment. Cells were cultured in RPMI 10% FBS with IL-4 and GM-CSF for 8 days and TNF-α was added for the last 24 h. At the end of culture period, cells were collected and analyzed for DC maturation markers, by flow-cytometry. Representative flow-cytometry histograms from one MM patients were reported. Empty histograms: DAY 0, gray histograms: DAY 7.

SUPPLEMENTAL FIGURES

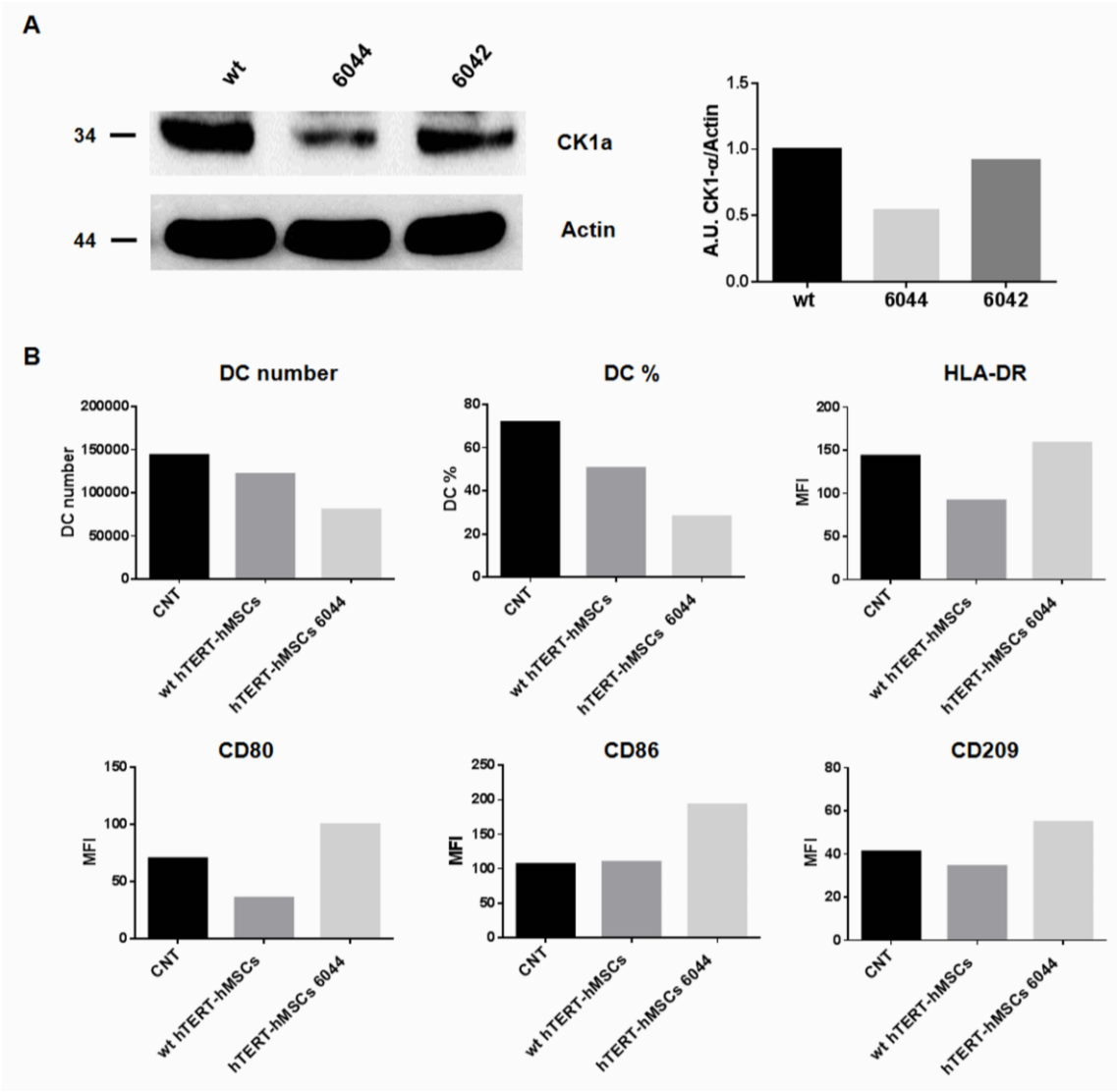
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4