Immune status of high-risk smoldering multiple myeloma patients and its therapeutic modulation under LenDex: a longitudinal analysis

Running title: Immune modulation of high-risk SMM with LenDex

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Key points:

- High-risk SMM patients' immune status is mildly impaired as compared to age-matched healthy individuals
- High-risk SMM patients can be effectively immune modulated by lenalidomide even when combined with low-dose dexamethasone

Abstract

There is significant interest in immunotherapy to treat high-risk smoldering multiple myeloma (SMM), but no available data on the immune status of this particular disease-stage. Such information is important to understand the interplay between immune-surveillance and disease transformation, but also to define if patients with high-risk SMM might benefit from immunotherapy. Here, we have characterized T-lymphocytes (including CD4, CD8, TCRγδ, and Tregs), NK- and dendritic-cells from 31 high-risk SMM patients included in the treatment arm of the Quiredex trial, and with longitudinal peripheral blood samples at baseline, after 3 and 9 cycles of lenalidomide/dexamethasone (LenDex). High-risk SMM patients showed at baseline decreased expression of activation-(CD25/CD28/CD54), Th1-(CD195/IFN-γ/TNF-α/IL-2), and proliferation-related markers (CD119/CD120b) as compared to age-matched healthy individuals. However, LenDex was able to restore the normal expression levels for those markers, and induced a marked shift in T-lymphocytes and NK-cells phenotype. Accordingly, high-risk SMM patients treated with LenDex showed higher numbers of functionally active T-lymphocytes. Altogether, our results indicate that high-risk SMM patients have an impaired immune system that could be re-activated by the immunomodulatory effects of lenalidomide even when combined with low-dose dexamethasone, and support the value of therapeutic immunomodulation to delay the progression to MM.

Introduction

Multiple myeloma (MM) is an incurable plasma cell (PC) malignancy characterized by preceding benign stages termed as monoclonal gammopathy of undetermined significance (MGUS) or smoldering MM (SMM). Interestingly, no major differences have been described for the genetic and phenotypic profiles of clonal PCs during disease evolution from MGUS to SMM and treatment-requiring MM.[1-8] In contrast, an intrinsic relation between a progressively impaired immune system and disease progression has been suggested [9,10] and in fact, one of the risk factors that predicts transformation of SMM into treatment-requiring MM is the presence of immune paresis.[11] Accordingly, extensive characterization of bone marrow (BM) and peripheral blood (PB) samples from MGUS and MM patients has revealed that while immune effector cells remain functional in the former, T-lymphocytes and NK-cells from newly-diagnosed and relapsed MM are unable to produce a tumor-specific immune response due to i) cytokine production by clonal PCs (e.g.: transforming growth factor β) [12], ii) T-cell exhaustion and anergy [13,14], or iii) inhibitory signaling through co-expression of PD-1 and PD-L1 in effector and tumor cells, respectively.[15,16] However, it remains unclear whether the quantitative and functional immune deregulation typically observed in MM is a cause or consequence of the increasing tumor burden.[10] Because immune modulatory drugs (IMiDs) and novel monoclonal antibodies (e.g.: anti-CD38 and anti-SLAMF7) rely on the activity of T-lymphocytes and NK-cells [17], such impaired immune system may represent a barrier for immunotherapy in active disease but not in preceding SMM, for which immune-therapeutic strategies aiming at preventing disease progression would be effective with the help of functionally active T-lymphocytes and NK-cells. Unfortunately, there is virtually no data on the immune status of patients with SMM nor if it could be therapeutically modulated (e.g.: using IMiDs); such information would be important to further understand the interplay between immune surveillance and disease transformation, as well as to define if patients with SMM might benefit from immune therapeutic strategies.

The interest in SMM has significantly increased in recent years due to both the identification of well-defined patient-subgroups with different risk of progression [11,18], and to the demonstration of the potential benefit in time-to-progression (TTP) and overall survival (OS) of treating high-risk SMM patients with lenalidomide plus low-dose dexamethasone.[19] Noteworthy, a recent update of this trial has shown that even though a significant fraction of patients remained with detectable disease after therapy (i.e.: less than CR), only 14 out of 57 (25%) patients randomized to receive treatment have progressed so far [20]; hence, it could be hypothesized that in addition to the direct tumor reduction induced by lenalidomide plus dexamethasone (LenDex), other factors such

as immune surveillance might play a role in delaying tumor progression. However, this has never been demonstrated. Furthermore, the combination used in the Quiredex trial includes the potential antagonist effects of the immune modulatory lenalidomide vs. the immune suppressive dexamethasone [21-25]; accordingly, such potentially antagonist combination may represent a dilemma in the design of clinical trials investigating preemptive therapy in high-risk SMM.[26]

Here, we have analyzed the phenotypic profile of T-lymphocytes, NK-cells and dendritic cells (DCs) in high-risk SMM patients enrolled in the Quiredex trial, and compared them against age-matched healthy individuals to determine the immune status in high-risk SMM. Afterwards, we monitored such patients after 3 and 9 cycles of LenDex induction to evaluate the role of T-lymphocyte and NK-cell functionality in delaying disease progression into symptomatic MM. Finally, we compared the immune profile of both cell populations during maintenance with lenalidomide as single-agent to measure the potential antagonist effect of dexamethasone.

Patients and Methods

Study design. In this pre-planned exploratory analysis, we evaluated the immunophenotypic profile of T-lymphocytes and NK-cells on a total of 119 PB samples from 31 of the 57 high-risk SMM patients allocated into the treatment arm of the Quiredex trial, and for which longitudinal samples were available at baseline, after 3 cycles of LenDex, at the end of induction therapy, and during maintenance with lenalidomide at least 3 months after dexamethasone discontinuation (the later analysis being performed in 13 of the 31 patients). The criteria for patient inclusion and the design of the Quiredex trial have been described elsewhere [19]; briefly, high-risk SMM was defined based on the presence of at least 2 of the 3 following criteria: BM PC infiltration ≥10%; high M-component (IgG≥30g/L or IgA≥20g/L or B-J Protein>1g/24h) [18]; or ≥95% clonal PCs from total BM PCs plus immune paresis.[11] High-risk SMM patients were treated with an induction phase of 9 four-week cycles of LenDex followed by maintenance with lenalidomide; maintenance therapy was initially given until disease progression, but a protocol amendment limited the total duration of treatment (induction plus maintenance) to 2 years.[19] A total of 10 healthy donors aged over 60 years were also studied and used as normal reference. All control and patient samples were collected after informed consent was given by each individual, according to the local ethical committees and the Helsinki Declaration. Baseline demographic and disease characteristics of high-risk SMM patients are summarized in Table 1.

Immunophenotypic protein expression profile (iPEP) of T-lymphocytes, NK-cells and DCs. We evaluated by multiparameter flow cytometry a total of 30 different markers distributed across 4-color monoclonal antibody combinations in PB T-lymphocytes and NK-cells (Supplementary Table 1). Each combination included either CD4 plus CD8 to identify helper and cytotoxic T-lymphocytes, or CD3 plus CD56 to identify total T-lymphocytes plus $CD56^{dim}$ (cytotoxic) and $CD56^{bright}$ (immune regulator) NK-cells (Supplementary Table 2). A total of 63 phenotypic parameters were evaluated in each patient sample. EDTA-anticoagulated PB samples from each subject were immunophenotyped during the first 24h after extraction using a direct immunofluorescence technique to evaluate surface antigen expression. For intracellular cytokine staining, heparin-anticoagulated PB samples diluted in RPMI-1640 with L-Glutamine (1:1 vol:vol) were cultured during 4 hours at 37°C in a 5% CO2-humidified atmosphere in the presence of PMA (25ng/mL) and ionomicin (0.5mg/m) with or without 10 µg/mL of brefeldinA (used as negative control). Simultaneous staining for intracytoplasmatic IFN-γ, TNF-α, IL-2 or CD40L and surface antigens was performed by use of the IntraStain Fixation and Permeabilization kit (Dako

Cytomation). Regulatory T cells (T-regs) were enumerated using the following combination of markers: CD25-FITC / CD127-PE / CD4-PerCPCy5.5 / FOXP3-APC. In brief, 200µL of heparin anticoagulated PB samples were incubated for 15 minutes at room temperature and in the dark. Cells were washed in phosphate-buffered saline and then fixed and permeabilized with FoxP3 Staining Buffer Set (eBioscience) for FOXP3 staining. To enumerate DCs and determine their relative distribution into myeloid, plasmacytoid, and tissue macrophage subpopulations, 200µl heparin anticoagulated PB samples were immunophenotyped using a direct immunofluorescence technique based on six 4-color combinations of monoclonal antibodies. All combinations included HLADR-FITC / CD45-PerCPCy55 and CD123-APC with a different PE-conjugated marker in each of the six combination: CD80, CD86, CD16, BDCA-1, CD14, and CD11c. Data acquisition was performed for approximately $2x10^5$ leukocytes/tube in a FACSCantoII flow cytometer (BD Biosciences, San Jose, CA) and using the FACSDiva software (version 6.1). Monitoring of instrument performance was performed daily using the Cytometer Setup Tracking (CST; BD) and rainbow 8-peak beads (Spherotech, Inc; Lake Forest, IL) after laser stabilization, following the EuroFlow guidelines [27]; sample acquisition was systematically performed after longitudinal instrument stability was confirmed. Data analysis was performed using the Infinicyt software (Cytognos SL, Salamanca, Spain).

Cell cycle analyses. The proliferation index of CD4 and CD8 T-lymphocytes as well as total NK-cells was analysed using 3-color staining for nuclear DNA and two cell surface antigens. Briefly, 100 µL of EDTA-anticoagulated PB samples were incubated for 15 min in the dark (room temperature) with the following combination of MoAb (FITC, PE): CD4, CD8, and CD3, CD56. After lysing non-nucleated red cells, the nucleated cells were washed and stained with 3µL/tube of DRAQ5™ (Vitro SA, Madrid, Spain). After another 10 min incubation, samples were immediately acquired in a FACSCantoII flow cytometer using the FACSDiva software program, and information on $\geq 10^5$ cells corresponding to the whole PB cellularity was measured and stored. Data was analysed with the Infinicyt software and the percentage of cells in the S-phase of the cell cycle was calculated as described elsewhere.[28]

Statistical analysis. The number of PB CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells was recorded as absolute number of cells/µL. Antigen expression was defined as percentage of positive cells for markers in which two discrete subsets could be identified (negative vs. positive cells), or by mean fluorescence intensity (MFI) units for markers with a homogeneous and unimodal

pattern of expression. A supervised cluster analysis was performed using the MultiExperiment Viewer software (version 4.6.1) to compare the immunophenotypic protein expression profile (iPEP) obtained of high-risk SMM patients at baseline, after 3 cycles of LenDex and at the end of induction therapy. The Pearson correlation and average linkage clustering were used as distance and linkage methods, respectively. The comparison between patients iPEP at the end of induction vs. maintenance was performed by principal component analysis (PCA), based on the simultaneous evaluation of the 63 phenotypic parameters analyzed per sample under the automated population separator (APS) graphical representation of the Infinicyt software. The Mann-Whitney U and Kruskal-Wallis H test were used to evaluate the statistical significance of differences observed between two or more groups, respectively. We used the SPSS software (version 15.0; SPSS Inc., Chicago, IL) for all statistical analyses.

Results

The immune status of high-risk SMM patients. Patients with high-risk SMM showed normal absolute numbers of CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells (Figure 1A). No differences were noted for the relative distribution of the CD4 and CD8 subsets within total T-lymphocytes, as well as for $CD56^{dim}$ and $CD56^{bright}$ cells within the total NK-cell compartment (Figure 1B). High-risk SMM patients also showed similar distribution of antigen-related maturation subsets within total CD4 and CD8 T-lymphocytes as compared to healthy individuals (Figure 1C). By contrast, there was a significant increment of TCRγδ positive T-lymphocytes (median of 37 vs. 18 cells/μL, respectively; *P* =.02) as well as of Tregs (median of 1.7% vs. 0.5%, respectively; *P* =.04) in the PB of patients with high-risk SMM as compared to age-matched healthy individuals (Figure 1D). There was a trend for lower DC counts in high-risk SMM patients vs. healthy individuals (*P* =.08; Figure 1E), and a more detailed analysis of the distinct subsets of DCs and tissue macrophages showed an altered distribution of BDCA-1 positive myeloid DCs (2% vs. 14%; *P* =.02) as well as tissue macrophages (74% vs. 47%; *P* =.06). Although no differences were observed for the absolute numbers and cellular distribution of CD4 and CD8 T-lymphocytes, significant differences emerged while comparing their immunophenotypic protein expression profile (iPEP); namely, the expression levels of activation markers such as CD25, CD28 and CD54 (Figure 2A) as well as Th1-related markers such as surface CD195 and secretion of IFN-γ, TNF-α, or IL-2 (Figure 2B) were significantly inferior in T-lymphocytes from high-risk SMM patients vs. healthy individuals. A significant down-regulation of proliferation-related markers such as CD119 and CD120b was also noted among high-risk SMM patients (*P* <.005; data not shown), but no significant differences in the percentage of proliferating CD4 and CD8 T-lymphocytes (as well as NK-cells) were observed as compared to healthy age-matched individuals (Figure 3).

Immune modulation of high-risk SMM patients with LenDex. To assess the combined effect of lenalidomide plus dexamethasone on T-lymphocytes and NK-cells, we compared the immune status of the 31 high-risk SMM patients at baseline (inclusion in the clinical trial) vs. after 3 and 9 cycles of LenDex. Interestingly, the absolute numbers of TCRγδ positive T-lymphocytes as well as the frequency of Tregs were further increased after 3 and 9 cycles of LenDex, respectively; conversely, CD4 T-lymphocytes were significantly decreased at the end of induction therapy (Figure 1A). No significant differences were noted for the remaining cell populations including overall DC counts and distribution of DC subsets and tissue macrophages, nor for the relative distribution of the CD4/CD8 and $CD56^{dim}$/$CD56^{bright}$ subsets within total T-

lymphocytes and NK-cells, respectively (Figure 1B). By contrast, there was a marked shift on the distribution of antigen-related maturation subsets induced by LenDex, and reflected by a significant increase of central memory CD4 (38% vs. 52% vs. 61% at baseline, cycles 3 and 9 of LenDex, respectively; *P* <.001) and effector memory CD8 (46% vs. 60% vs. 58% at baseline, cycles 3 and 9 of LenDex, respectively; *P* <.001) T-lymphocytes (Figure 1C). Since the accumulation of antigen-experienced T-lymphocytes is typically related to increased activation and proliferation, we then compared the iPEP of T-lymphocytes and NK-cells of all patient' samples longitudinally collected at baseline, and after cycles 3 and 9 of LenDex (Figure 4). Accordingly, CD4 and/or CD8 T-lymphocytes showed an increased expression of activation markers such as CD69, CD25, CD28, and CD54, together with an up-regulation of the Th1 related chemokine CCR5 (CD195) and increased cytokine production of IFNγ, TNFα, and IL-2. An overall analysis on total ($CD3^{+ve}$) T-lymphocytes plus $CD56^{dim}$ and $CD56^{bright}$ NK-cells showed an up-regulation of the activation marker HLA-DR (*P* <.001; Figure 2A), the ADCC associated receptor CD16 (*P* ≤.005), and the adhesion molecules CD11a (*P* ≤.001) and CD11b (*P* ≤.005) after 3 and 9 courses of LenDex (data not shown). By contrast, no differences were observed for the expression of the lectin-like receptor CD94 as well as the inhibitory receptors CD158a, CD161, NKB1 and NKAT2 in the surface of NK-cells throughout induction therapy (data not shown). Cell cycle analysis (Figure 3) revealed that the percentage of cells in S-phase progressively increased from baseline vs. 3 and 9 cycles of LenDex for CD4 (0.03% vs. 0.07% vs. 0.12%; *P* <.001) and CD8 (0.02% vs. 0.07% vs. 0.13%; *P* <.001) T-lymphocytes as well as NK-cells (0% vs. 0.09% vs. 0.11%; *P* <.001). Upon demonstrating the immune modulation of high-risk SMM throughout the 9 induction cycles of LenDex, we then investigated whether patients achieving at least very good partial response (≥VGRP) after 9 cycles of LenDex showed unique immune features at this specific time-point. Interestingly, patients in ≥VGPR had a trend towards lower expression of CD158a (*P* =.09) and the NKAT (*P* =.06) killer-immunoglobulin receptor (KIR) among total $CD56^{dim}$ NK-cells, higher expression of CD25 (*P* =.04), CD120b (*P* =.06) and CD54 (*P* =.08) among CD4 T-lymphocytes, as well as higher expression of CD28 (*P* =.07) and CD195 (*P* =.01) among CD8 T-lymphocytes. Also, the percentage of proliferating NK-cells was significantly increased (*P* =.02) among patients in ≥VGPR. Afterwards, we investigated if patients with sustained disease control showed unique phenotypic features as compared to patients that progressed into treatment-requiring MM. With a median follow-up of 5-years, 19 patients remained asymptomatic whereas 12 cases progressed into symptomatic MM (Supplementary Figure 1), without significant differences in median follow-up between patients remaining asymptomatic vs. those

progressing into symptomatic MM. Interestingly, while at baseline only the percentage of CD158a positive $CD56^{dim}$ NK-cells significantly differed between asymptomatic and progressing cases (medians of 16% vs. 38%, respective; *P* =.02), upon therapy patients with sustained disease control showed significantly increased absolute numbers of TCRγδ positive T-lymphocytes (medians of 53 vs. 35 cells/μL; *P* =.03) as well as a higher expression of CD94 among $CD56^{dim}$ cytotoxic NK-cells (56% vs. 40%; *P* =.001) after LenDex treatment. In order to account for differences in baseline levels, we also compared median fold-change expression (from baseline to cycle 9 of LenDex) of each individual marker between asymptomatic vs. progressing patients. Our results show that CD16 fold-change expression in $CD56^{bright}$ NK cells (35 vs. 4; *P* =.05), as well as IL-2 (9 vs. -10.5; *P* =.03) and CD195 (5 vs. -3; *P* =.01) fold-change expression in CD4 T-lymphocytes was significantly increased among asymptomatic vs. progressing patients, respectively (Figure 5).

Is dexamethasone abrogating the immune stimulatory effect of lenalidomide? To address the question whether dexamethasone antagonizes the immune modulatory properties of lenalidomide, we compared the immune profile of 13 patients with PB samples collected at the end of the 9 induction cycles with LenDex vs. paired samples obtained during maintenance (single-agent lenalidomide, and at least 3 months after dexamethasone discontinuation). No significant differences were observed for the absolute numbers of all cell populations analyzed, nor for the relative distribution of CD4/CD8 T-lymphocytes and $CD56^{dim}$/$CD56^{bright}$ NK-cells, nor for the antigen-related maturation subset distribution of CD4 and CD8 T-lymphocytes (data not shown). Further comparison of patients' iPEP at cycle 9 vs. during maintenance revealed that from the total 63 phenotypic parameters analyzed, only 7 were found to be differently expressed. Namely, the percentage of CD94 and CD154 positive T-lymphocytes, CD212 positive CD4 and CD8 T-lymphocytes, as well as the MFI of CD11a in T-lymphocytes and $CD56^{dim}$ and $CD56^{bright}$ NK-cells were down-regulated during maintenance (data not shown). Accordingly, an overall analysis of patients' immune status shows overlapping iPEP without a clear clustering of samples collected after 9 induction cycles of LenDex (represented by circles) vs. maintenance with single-agent lenalidomide (represented by squares) (Figure 6).

Discussion

Understanding the biologic and cellular mechanism involved in the transition between benign and malignant MM has become particularly important with the emergence of new diagnostic criteria [29] and potential benefit for early-treatment of SMM patients at higher risk of progression into symptomatic MM.[19] However, while several studies have focused on the genomic characterization of SMM [1-4,6,7,30], no research efforts have been made to evaluate the immune system of SMM.[10] Here, we undertook a detailed characterization of high-risk SMM patients' immune status and noted a mild functional impairment as compared to age-matched healthy individuals; afterwards, we demonstrated that high-risk SMM patients can be effectively immune modulated by lenalidomide and consequently, we also showed that the immune modulatory effects of lenalidomide were not lost when combined with low-dose dexamethasone. These observations suggest that increasing numbers of functionally active immune cells after LenDex may have partially contributed to reduce the risk of transformation of high-risk SMM patients.

Extensive studies in the BM and PB samples from patients with MGUS and symptomatic MM have shown that T-lymphocytes and NK-cells become quantitatively and functionally altered in the latter stage of the disease [31-42], thereby suggesting a relation between an impaired immune system and the transformation of MM. Conversely, we [41] and others [43] have recently shown that patients in "operational cure" (i.e.: >10-years progression-free survival of-therapy) have unique immune signatures characterized by increased numbers of effector cytotoxic CD8+ T-lymphocytes, NK cells, as well as B-lymphocytes and normal PCs. Because some of these patients had detectable residual disease, it was postulated that tumor immune surveillance, by functionally active cytotoxic, cells could prevent disease progression in such patients. When considering the natural evolution of MM, it could be hypothesized that the balance between immune surveillance and tumor escape should be particularly critical at the stage of SMM; in fact, it is well-known that the transformation of smoldering into symptomatic MM is characterized by a dysfunctional humoral immune response (e.g.: a skewed ratio of serum free light-chains [44] and immune paresis [11]) but unfortunately, a detailed analysis of the immune status of SMM patients has never been performed. Here, we have demonstrated that while SMM patients at higher risk of transformation have normal absolute numbers of immune effector cells, CD4 and CD8 T-lymphocytes showed down-regulation of a selected number of activation-, Th1-, and proliferation-related markers. Such modulation of T-lymphocytes iPEP could be potentially explained by the increased frequency observed for Tregs since several studies have shown that these are increased and functionally immunosuppressive in the PB of

symptomatic MM patients.[45,46] Conversely, BDCA-1 positive myeloid DCs were decreased in patients' PB and even though differences were not statistically significant, we have found similar patterns for plasmacytoid DCs and tissue macrophage distribution as compared to newly-diagnosed symptomatic MM.[41] By contrast, no differences were noted in the iPEP of NK-cells from high-risk SMM patients vs. age-matched healthy individuals. These results may be clinically relevant given the promising role of immune therapeutic strategies aimed at boosting immune surveillance and controlling disease progression (e.g.: monoclonal antibodies and checkpoint inhibitors).

IMiDs such as lenalidomide have been widely adopted for the treatment of MM usually in combination with dexamethasone. IMiDs activity is mediated by modulating the substrate specificity of the E3 Ubiquitin ligase, CRL4CRBN. Thus, Lenalidomide treatment results in increased ubiquitinylation of the transcription factors Ikaros and Aiolos and consequent altered expression of their target genes including IRF4 and IL-2.This is thought to explain its dual mechanism of action involving both a direct anti-MM activity (through reduced IRF4 expression) and immune modulation (through boosted IL-2 production by T-lymphocytes).[47,48] In addition, lenalidomide increases NK-cell responses by lowering its activation threshold through both CD16 and NKG2D.[49] Thus, significant concern exists on the simultaneous use of dexamethasone with IMiDs because it could antagonize the immune-enhancing effect of the latter. Accordingly, it has been shown that dexamethasone abrogates lenalidomide induced T-lymphocyte production of IL-2 and NK-cell production of IFNγ and granzyme B, NK-cell receptor expression, and *in vitro* NK-cell mediated cytotoxicity.[21-25] However, contrasting results have also been reported and suggested that *in vitro*, antigen-dependent activation of NKT cells is greater in the presence of LenDex than with dexamethasone alone.[50] While these findings have obvious clinical implications, it should also be noted that these studies have been mostly performed *in vitro*, using high-doses of dexamethasone, and in small patient series. In fact, very recent data has shown that PomDex can induce rapid activation of innate and adaptive immunity in heavily pretreated relapsed/refractory patients [51], which further contributes to the uncertainty about the potential antagonizing effect of dexamethasone in combination with IMiDs. Here, we had the unique opportunity to study T-lymphocytes and NK-cells from treatment-naïve high-risk SMM patients included in the Quiredex trial and exposed to 9 induction cycles of lenalidomide plus low-dose dexamethasone [19], to elucidate whether or not the use of corticosteroids may have a detrimental effect on the immune modulatory activity of lenalidomide. Our results, based on longitudinal samples collected at baseline and after cycles 3 and 9 of LenDex, show a significant increase of

TCRγδ positive T-lymphocytes at cycle 3 coupled with a decrease of CD4 T-lymphocytes at cycle 9. However, the most notorious differences in cellular distribution were noted for the distribution of T-lymphocyte antigen-related maturation subsets; the fact that CD4 and CD8 T-lymphocytes become enriched in central and effector memory cells after 3 and 9 cycles of LenDex identifies them as not yet terminally differentiated, which makes them promising candidates for anti-tumor response. Furthermore, it was interesting to observe that LenDex was able to restore the expression of those activation-, Th1- and proliferation-related markers that at baseline were found to be down-regulated in both CD4 and CD8 T-lymphocytes of high-risk SMM patients as compared to age-matched healthy individuals. $CD56^{dim}$ cytotoxic and $CD56^{bright}$ immune regulatory NK-cells were also found to be significantly activated after therapy. Accordingly, lenalidomide was able to effectively modulate the iPEP and proliferative-rate of immune cells from high-risk SMM patients in the presence of dexamethasone; in fact, virtually no differences were observed in the number and iPEP of T-lymphocytes and NK-cells during maintenance therapy with lenalidomide and without concomitant dexamethasone. However, even though immune profiling was performed at least 3 months after dexamethasone discontinuation, we cannot preclude long-lasting effects on the immune system of the 9 previous cycles with LenDex, and the optimal way to address this particular question (i.e.: abrogation of the immune modulation produced by lenalidomide with concomitant dexamethasone) would be in a randomized study comparing the immune profile of patients treated with LenDex vs. lenalidomide without dexamethasone. Furthermore, patients enrolled in the observation arm of the trial would have represented an important control group since this would allow us to investigate if the immune status of treatment-naive high-risk SMM patients may significantly vary in a period of approximately 1-year without any anti-myeloma therapy; unfortunately, such studies were not part of the original design of the trial. Similarly to what has been described in patients treated with LenDex [52,53] and PomDex [51], immune modulation of T-lymphocytes and NK-cells is possible albeit the expansion of Tregs. It could be hypothesized that such expansion is induced by dexamethasone, but Scott et al have most recently reported that that dexamethasone (alone or in combination lenalidomide) abrogated the ability of myeloma cell lines to induce Tregs expansion in vitro [54]; alternately, increasing Treg numbers may reflect decreased TNFα levels after therapy that allow for Treg proliferation.[55] That notwithstanding, we have not found significant differences in Treg numbers between patients in ≥VGPR vs. <VGPR (data not shown), similarly to that recently reported by Sehgal et al [51]; accordingly, further translational research in IMiD/dex based clinical trials is warranted to fully understand the impact of each drug (separately and in combination) on Tregs.

In summary, our results provide an immunologic rationale for the treatment of high-risk SMM patients with lenalidomide plus low-dose dexamethasone. Furthermore, the fact that a significant delay in TTP and consequent improvement in OS is being observed even among patients with residual disease after therapy, suggests potential immune surveillance of a reduced number of clonal PCs; accordingly, high-risk SMM patients progressing to symptomatic MM had significantly lower numbers of TCRγδ T-lymphocytes. Our data, obtained from a carefully selected population of patients without previous exposure to anti-MM therapy and with available longitudinal samples after consecutive cycles of LenDex, shed new light on the synergism between lenalidomide and dexamethasone which, at low doses, does not abrogate the immune modulatory effects of lenalidomide. However, further studies are warranted to demonstrate an intrinsic relation between baseline versus lenalidomide-based immune cell activation and reduced risk of progression in high-risk SMM, particularly by using high-throughput protein and sequencing techniques not only to quantify immune effector cells, but also to determine their functionality and location within the tumor microenvironment.[56]

Acknowledgments: This study was supported by the Cooperative Research Thematic Network grants RD12/0036/0058 and RD12/0043/0012 of the Red de Cancer (Cancer Network of Excellence); Instituto de Salud Carlos III, Spain, Instituto de Salud Carlos III/Subdirección General de Investigación Sanitaria (FIS: PI060339; 06/1354; 02/0905; 01/0089/01-02; PS09/01897/01370; G03/136; Sara Borrell: CD13/00340); and Asociación Española Contra el Cáncer (GCB120981SAN), Spain. B.P. is also supported by the European Research Council (ERC) 2015 Starting Grant.

Authorship: B.P., M.V.M., J.J.L, J.A.P.S. and J.F.S.M. conceived the idea and designed the study. M.V.M., L.L.C., M.T.H., J.B., F.d.A., J.d.l.R, A.I.T., P.G., L.R., F.P., A.O., J.H., G.E., F.ldC., J.J.L., J.B. and J.F.S.M. supplied study material or patients. B.P., L.I.S.A. N.P. and M.B.V. performed immunophenotypic studies and analyzed flow-cytometric. B.P. and L.A.C. performed statistical analysis. B.P., M.V.M., L.I.S.A., J.J.L., J.A.P.S., and J.F.S.M. analyzed and interpreted data. B.P. and J.F.S.M wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict-of-interest disclosure: BP, MVM, and JFSM have served on the speaker's bureau for Millennium, Celgene and Janssen. JB has received honoraria for lectures and advisory boards from Janssen and Celgene and grant support from Janssen. JJL has received honoraria from Janssen and Celgene. The remaining authors declare no competing financial interests.

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Table 1. Baseline demographic and clinical characteristics of the high-risk smoldering (SMM) patient population with available samples for immune profiling at baseline plus after 3 and 9 cycles of LenDex.

Characteristic	High-risk SMM patients (n=31)
Age (years)	
Median	59
Range	39 – 87
Sex (no., %)	
Male	14 (45%)
Female	17 (55%)
Time since diagnosis (no., %)	
≤6 months	13 (42%)
>6 months	18 (58%)
Criteria for high-risk SMM (no., %)	
Mayo	6 (19%)
Pethema	12 (37%)
Both criteria	14 (44%)
Serum M-component (g/dL)	
Median	27
Range	7.6 – 56.6
Urine M-component (g/24h)	
Median	0
Range	0 – 1.5
Bone marrow plasma cell infiltration (%)	
Median	19
Range	10 - 48

Figure legends.

Figure 1. Distribution of CD4, CD8, and TCRγδ T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells in peripheral blood (PB) samples from heathy individuals aged over 60 years (n=10) and high-risk patients with smoldering multiple myeloma (SMM; n=31) studied at baseline, after 3 and 9 cycles of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex) as assessed by multiparameter flow cytometry (MFC). The absolute counts (Nº cells/μL) are shown in **Panel A**, whereas the ratio between CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells is shown in **Panel B**; distribution of antigen-maturation related subsets within total CD4 and CD8 T-lymphocytes is represented in **Panel C**. In **Panel D**, the percentage of regulatory T-lymphocytes among nucleated PB cells is shown, whereas in **Panel E** the distribution of dendritic cells (DCs) and their plasmacytoid, BDCA-1 positive and negative plus tissue macrophage subsets in PB is represented. Bars represent median values and vertical lines the upper bound of the 95% confidence intervals. Notched boxes represent 25th and 75th percentile values; the line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively.

Figure 2. Detailed immunophenotypic features of CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells in peripheral blood (PB) samples from heathy individuals aged over 60 years (n=10) and high-risk patients with smoldering multiple myeloma (SMM; n=31) studied at baseline, after 3 and 9 cycles of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex) as regards to antigen expression of activation **(Panel A)** and Th1-related immune response markers **(Panel B)**. Bars represent median values and vertical lines the upper bound of the 95% confidence intervals.

Figure 3. Total percentage of proliferating S-phase T-lymphocytes and NK-cells in peripheral blood (PB) samples from heathy individuals aged over 60 years (n=10) and high-risk patients with smoldering multiple myeloma (SMM; n=31) studied at baseline, after 3 and 9 cycles of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex). Each area represents the specific fluctuation of S-phase CD4 (light grey) and CD8 T-lymphocytes (grey) plus NK-cells (dark grey).

Figure 4. Immunophenotypic protein expression profile (iPEP) of CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells in peripheral blood (PB) samples from high-risk patients with smoldering multiple myeloma (SMM; n=31)

studied at baseline, after 3 and 9 cycles of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex). In **Panel A,** each of the 63 phenotypic parameters evaluated is distributed per individual columns, indicated in the bottom as “expression of the marker / immune cell population”, and is represented by color bars depicting normalized intensity values against that observed in heathy individuals aged over 60 years (n=10), ranging from low (dark green) to high (dark red) expression levels. In **Panel B** principal component analysis (PCA) graphical view of patients’ iPEP. In the two-dimensional PCA representation, every patient is represented by a single dot colored according to the sample time point [baseline (orange), cycle 3 (blue) and 9 (green) of LenDex], whereas in the three-dimensional PCA representation all patient samples were grouped according to their respective time-point.

Figure 5. Fold-change expression of CD16 in $CD56^{bright}$ NK-cells, as well as IL-2 and CD195 in CD4 T-lymphocytes, from baseline to cycle 9 of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex). High-risk SMM patients were grouped into those remaining assymptomatic (n=19) vs those progressing to symptomatic MM (n=12). Notched boxes represent 25th and 75th percentile values; the line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively.

Figure 6. Immunophenotypic protein expression profiles (iPEP) of CD4 and CD8 T-lymphocytes as well as $CD56^{dim}$ and $CD56^{bright}$ NK-cells in peripheral blood (PB) samples from high-risk patients with smoldering multiple myeloma (SMM; n=13) studied after 9 cycles of induction therapy with lenalidomide plus low-dose dexamethasone (LenDex) vs. during maintenance with lenalidomide (at least three months after dexamethasone discontinuation). In the principal component analysis (PCA) graphical view, every patient is represented by a unique color, and iPEPs from PB samples studied after induction vs. during maintenance are represented by colored circles and squares, respectively.