Planned withdrawal of dexamethasone after pomalidomide low-dose dexamethasone induction for lenalidomide-refractory multiple myeloma (ALLG MM14)

Immune dysfunction, a key feature of myeloma (MM), plays an important role in promoting tumor growth and therapy resistance[1] with multiple mechanisms of immune evasion described. Pomalidomide (POM) is an immunomodulatory (IMiD) compound[2] that mediates direct anti-proliferative effects on tumor cells, as well as immune-modulatory effects on T cells, natural killer (NK) cells and monocytes.[3] POM plus low-dose dexamethasone (LoDEX) is a standard treatment option for patients with relapsed/refractory MM (RRMM), however, dexamethasone can antagonize the immunostimulatory capacity of ImiD.[3,4] Consequently, the immunostimulatory effects of IMiD may be better exploited in the longer term without concomitant DEX, particularly be relevant in the minimal disease burden setting (i.e., maintenance) when some inherent immune recovery has occurred. To our knowledge, our study is the first to evaluate this in a prospective, randomized manner, demonstrating (i) regulatory T- cell (Treg) depletion following POM-LoDEX induction was partially abrogated following withdrawal of dexamethasone in maintenance, and (ii) enrichment of heterogenous neutrophil populations and an increase in activated NK cells with commensurate decrease in inhibited NK cells following POM-LoDEX induction.

ALLG MM14 was a prospective, randomized, multicenter, open-label parallel-group phase II trial comparing POM maintenance to POM-LoDEX maintenance following induction with POM-LoDEX. Eligible patients with RRMM, who had failed at least two prior therapies (including a history of lenalidomide failure [Table 1]) were enrolled. The study was conducted according to the Alfred Hospital Institutional Ethics Review Board, in accordance with the Declaration of Helsinki (ACTRN12615000447550).

Patients received four cycles of induction (1 cycle: 28 days): POM (4 mg orally days 1-21) plus LoDEX (40 mg orally days 1, 8, 15, and 22). Patients who achieved stable disease (SD) or better ("responders") were then randomized (1:1) to continue on one of two arms of maintenance: POM or POM-LoDEX. Accrual continued until 80 patients were randomized. Correlative peripheral blood (PB) samples for immune studies were collected at baseline (pre-induction) and maintenance (C1D1, C3D1, C6D1 and C10D1).

The primary objective was to determine whether co-administration of DEX with POM in maintenance significantly impacted NK-cell numbers, by comparing the change in PB NK-cell quantification from baseline to maintenance (C6D1) time points utilizing mass cytometry (CyTOF) (powered to detect an increase of 30% in NK-cell numbers in POM compared to POM-LoDEX). (ALLG MM14 was not powered to detect differences in secondary exploratory/clinical endpoints so conclusions on the clinical impact of one strategy over the other cannot be drawn). Exploratory CyTOF studies analyzed sequential PB samples to define differences in immune cell profiles in: (i) (all patients) responders *versus* non-responders; and (ii) (randomized patients) POM *versus* POM-LoDEX maintenance. Secondary clinical objectives were to compare (following randomization to POM or POM-LoDEX maintenance): (i) survival (progression-free survival/ overall survival [PFS/OS]), (ii) safety/toxicity and (iii) response/survival following initiation of post-progression therapy.

For CyTOF analysis cells were stained with sub-set defining antibodies (myeloid, B, T and NK cells) (*Online Supplementary Table S1*). Supervised analysis was performed to determine differences in canonical immune cell populations (NK cells and Treg), reported as a proportion of population (%).[5,6] CD3-CD19-CD56+ NK cells were predefined from patient datasets. Boolean gating was then performed using seven NK-cell activation/inhibitory markers (CD158a/CD158b/CD159a/CD314/CD335/CD336/CD 337). Boolean populations that comprised ≥3% of the total NK-cell population (median) were then compared. A Mann-Whitney test was used to determine statistical significance for each of the defined populations between clinical groups. Analyses of the primary NK endpoints was confined to patients who had assessments at both baseline and maintenance C6D1. Treg (CD3+CD4+CD127loCD25hiCD45RO+) were defined by manual gating and assessed in all patient samples at all time points: a one-way ANOVA with a Kruskal Wallis

Table 1. Characteristics of 154 enrolled patients.

Characteristic	All Patients n=154	POM n=40	POM-LoDEX n=38
Male sex, n (%)	79 (51.3%)	20 (50.0%)	17 (44.7%)
Age in years, median (range)	67.4 (36.0-88.6)	68.4 (50.3-85.4)	66.2 (36.0-81.1)
ISS Stage			
Not Known	66 (42.9%)	17 (42.5%)	16 (42.1%)
Stage 1	35 (22.7%)	9 (22.5%)	9 (23.7%)
Stage 2	36 (23.4%)	9 (22.5%)	10 (26.3%)
Stage 3	17 (11.0%)	5 (12.5%)	3 (7.9%)
Prior lines of therapy, median (range)	4.5 (2-14)	5 (3-9)	5 (3-14)
Lenalidomide failure*	154 (100%)	40 (100%)	38 (100%)
Bortezomib refractory	128 (83.1%)	29 (72.5%)	33 (86.8%)
Prior autologous stem cell transplant	96 (62.3%)	24 (60.0%)	31 (81.6%)
Prior allograft	1 (0.7%)	0 (0.0%)	1 (2.6%)
Prior anti-CD38 therapy	0 (0.0%)	0 (0.0%)	0 (0.0%)
Time in years from diagnosis to study enrollment, median (range)	5.5 (1.2-17.8)	5.9 (2.4-12.8)	6.4 (1.9-17.8)

*Lenalidomide (LEN) failure defined as failing to respond: (1) disease progression during treatment or within 60 days of completing a LEN containing regimen or (2) failure to achieve at least a minimal response (MR) (after 2 cycles). POM: pomalidomide; LoDEX: low-dose dexamethasone. ISS: international staging system.

post hoc test for multiple comparisons was used to determine statistical significance. Unsupervised analysis was performed to identify immune cell populations: data were clustered in the Vortex package[7] using the x-shift algorithm. Elbow-point validation was used to affirm the correct cluster number. Differences in cluster frequency between groups were assessed by Mann-Whitney test for statistical significance. Cluster phenotypes were determined and validated via multiple visualization approaches; individualized clusters were visualized using brick plots.[8]

Comparisons of the maintenance arms were restricted to a modified ITT (mITT) set which excluded patients randomized in error. Secondary clinical time-to-event outcomes (PFS/OS) were compared between randomized treatments using log-rank tests and estimates of the survival distributions were calculated using the Kaplan-Meier method. Two-tailed *P*-values were used for all comparisons, and, unless otherwise stated, were performed using a significance level of 5%. Toxicity was assessed according to CTCAE version 4.0.

154 patients were enrolled (baseline characteristics listed in Table 1). The estimated median potential follow-up (by reverse Kaplan-Meier) for all registered patients for OS was 27.8 month (mo). Eighty-one patients were randomized, however, three were randomized in error, therefore a mITT analysis included 78 (51%) patients who achieved SD or better with POM-LoDEX induction: POM n=40, POM-LoDEX n=38. Median PFS (from time of randomization) was 2.6 mo (95% confidence interval [CI]: 1.8-3.0) for POM *versus* 5.7 mo (95% CI: 4.5-7.5) for POM-LoDEX (log-rank *P*=0.051; hazard ration [HR]: 0.63, 95% CI: 0.40-1.00) (Figure 1A). Median OS (from time of randomization)

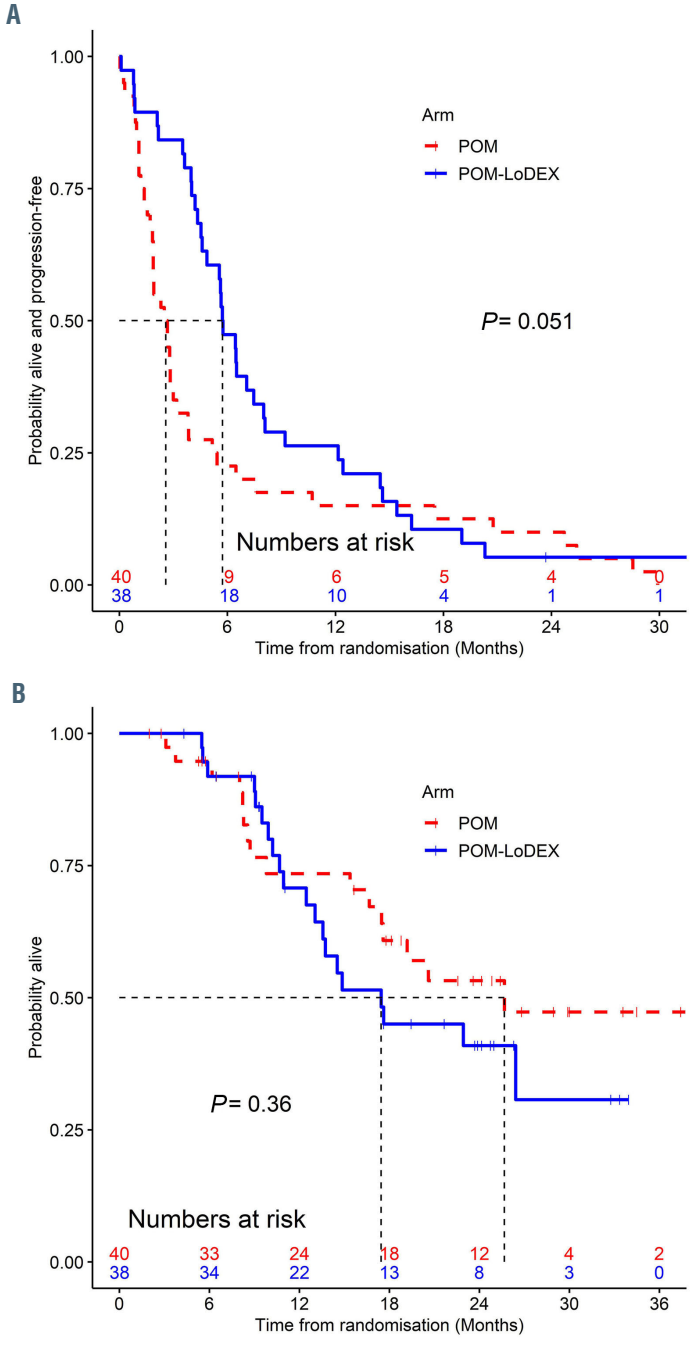

Figure 1. Kaplan-Meier survivor functions for modified Intention to treat population (from time of randomization) (mITT: pomalidomide [POM] n=40; LoDEX: pomalidomide low-dose dexamethasone [POM-LoDEX] n=38). In anticipation of early or late differences between the maintenance treatment arms in their time-to-event outcomes, 6 comparisons between the arms were planned at 3, 6, 9, 12, 15 and 18 months (mo) from randomization. To account for multiplicity of comparisons, a Bonferroni adjustment to the alpha-level of each test was implemented, namely a comparison between the treatment arms at one of these time points was judged to be statistically significant if the associated *P*-value was ≤0.0083. The test was based on the complementary log-log transformation of the survival function. (a) Progression free survival: POM arm 2.6 mo (95% confidence interval [CI]: 1.8-3.0) *vs.* 5.7 mo (95% CI: 4.5-7.5) for POM-LoDEX (log-rank *P*=0.051; hazard ratio [HR]: 0.63, 95%CI: 0.40-1.00), early PFS favored POM-LoDEX, however late survival favored POM: a comparison of PFS at 6 3-monthly intervals favored POM-LoDEX (3-12 mo, *P*<0.001) however, at 18 mo, POM was favored (*P*=0.018). (B) Overall survival: POM arm 25.7 mo (95% CI: 16.7-42.2) *vs.* 17.4 mo (95% CI: 12.5-NA) for POM-LoDEX (*P*=0.356; HR: 1.36, 95%CI: 0.70-2.64). Like the progression-free survival (PFS) analysis, comparisons of overall survival (OS) at 6 3-monthly intervals demonstrated no difference between the arms at 3-12 mo, however at 15 mo and 18 mo, OS favored POM (*P*=0.006, *P*=0.021 respectively).

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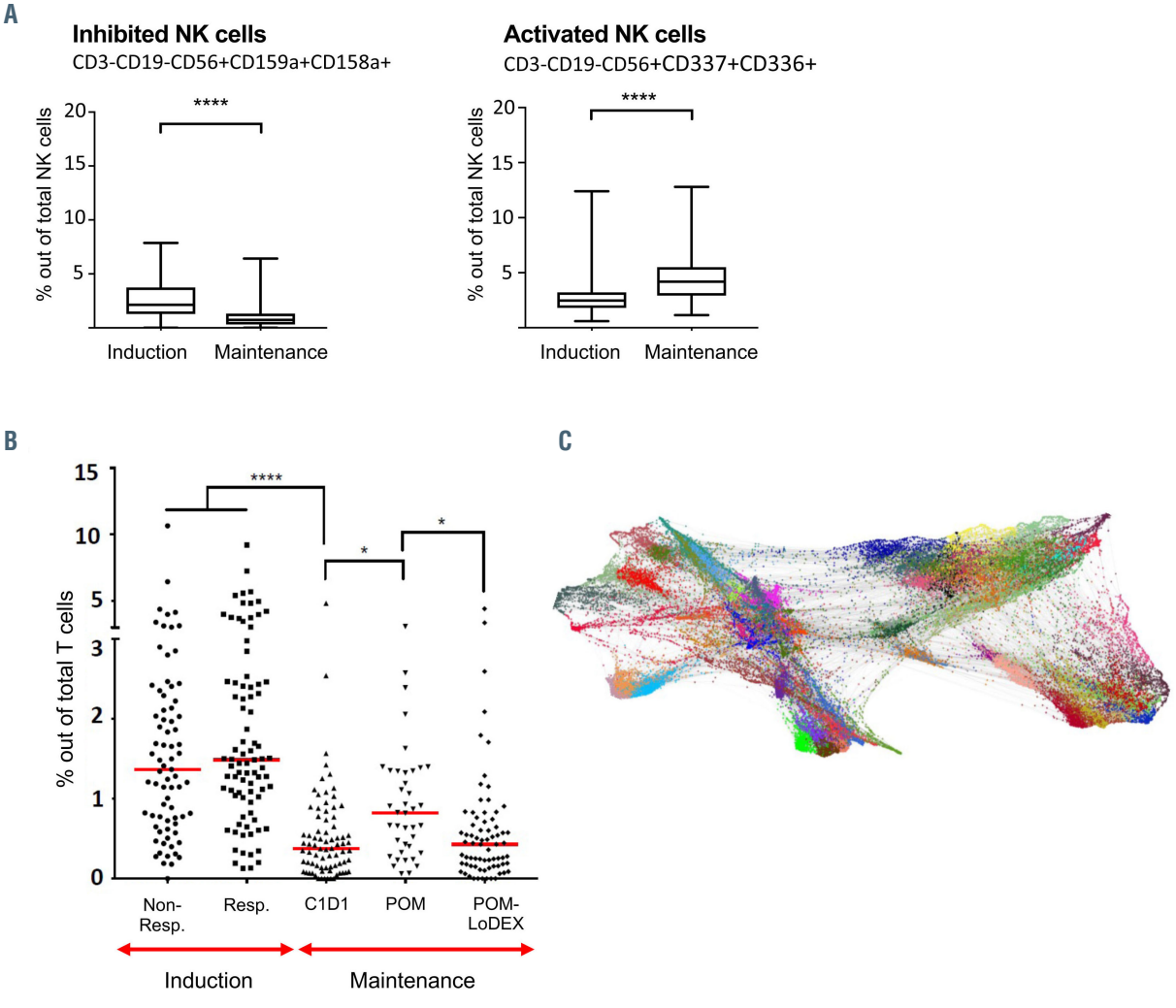
There was no difference in NK populations observed between responders and non-responders at baseline. However, in responders, (i) inhibited NK cells (CD3-CD19-CD56+CD159a+CD158a+) were enriched at baseline and significantly decreased following induction (pooled maintenance timpepoints) (*P*<0.0001), and (ii) activated NK cells (CD3-CD19-CD56+CD337+CD336+, no inhibitory receptors) were significantly increased following induction (pooled maintenance time points) (*P*<0.0001) (Figure 2A). Following commencement of maintenance, there was no emergent difference in NK populations observed between treatment arms. There was no difference observed in NK-cell populations according to maintenance arm at baseline and at maintenance (C6D1) (primary objective).

There was no difference in Treg percentage (Treg%) between responders and non-responders at baseline. After induction and prior to commencing maintenance (C1D1 timepoint), responders demonstrated a depletion of Treg% (*P*<0.0001). Following commencement of maintenance, Treg depletion was maintained in patients who continued on POM-LoDEX, whereas POM patients who had LoDEX withdrawn demonstrated a partial recovery in Treg% (*P*<0.05). (Figure 2B).

Unsupervised analysis (all patients) at baseline defined 131 immune cell populations (Figure 2C): there were no significant differences identified between responders and non-responders. At maintenance (responders), there was enrichment of heterogenous neutrophil populations (pooled maintenance time points). Of the 131 clusters identified at baseline, five of the eight large clusters (each at least 3% [median] of total nucleated cells evaluated) that were significantly enriched (*P*<0.0001) following POM-LoDEX induction were activated neutrophil populations (all expressed CD66b but with variable expression of CD24/CD16/CD11c/CD11b/CD45RO) (Figure 2D).

Online Supplementary Table S2 lists all grade adverse events (AE). When comparing the mITT population, the incidence of AE was generally similar, including hematologic toxicity. Significant differences were observed in the incidence of lung infections (higher in POM-LoDEX, *P*=0.003) and peripheral sensory neuropathy (higher in POM-LoDEX, *P*=0.041). Median durations of exposure to maintenance POM were 2.5 mo and 6.2 mo in the POM and POM-LoDEX arms respectively. Dose intensity interquartile ranges were similar in both arms from maintenance C1D1 through to C6D1. *Online Supplementary Figure S1* shows results for survival post-



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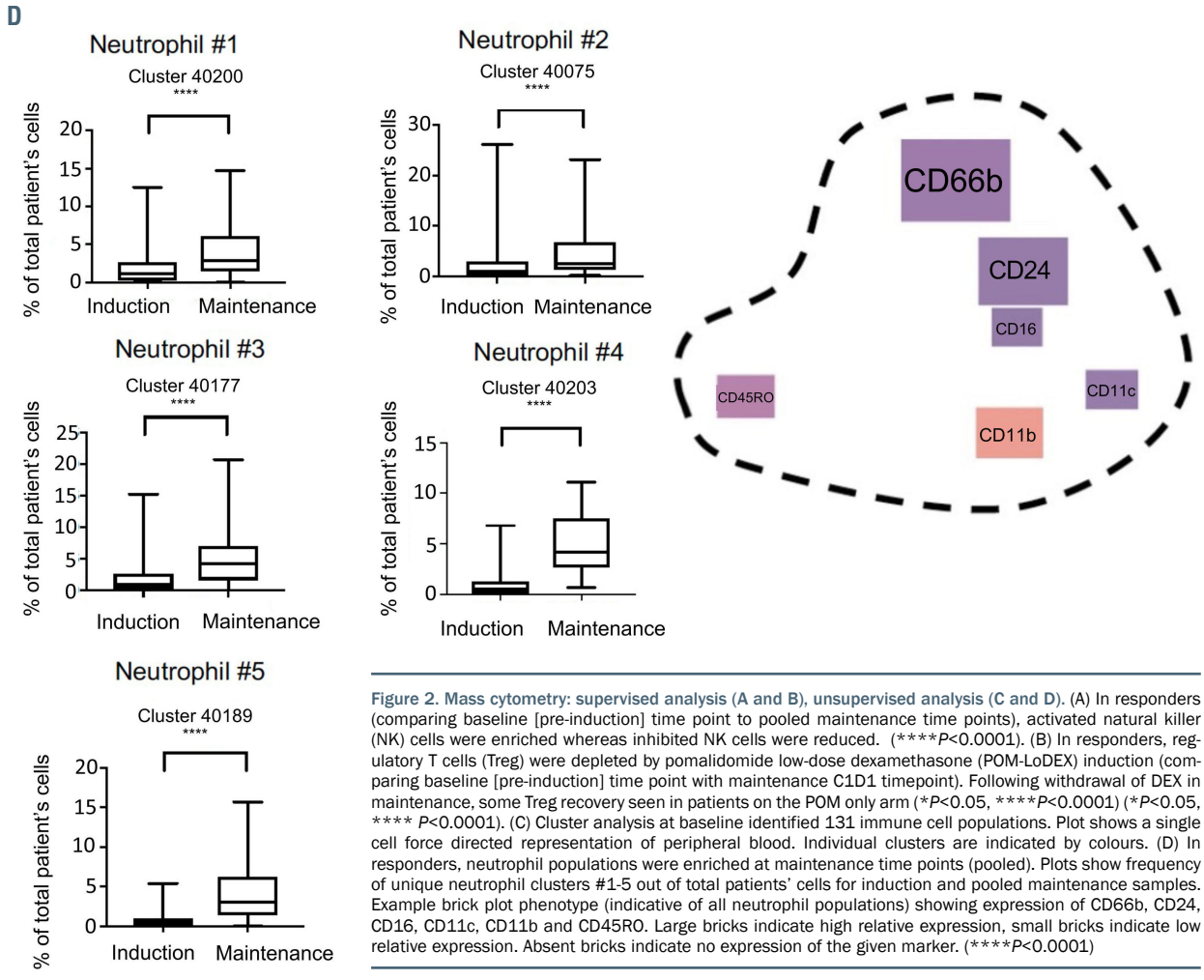

Figure 2. Mass cytometry: supervised analysis (A and B), unsupervised analysis (C and D). (A) In responders (comparing baseline [pre-induction] time point to pooled maintenance time points), activated natural killer (NK) cells were enriched whereas inhibited NK cells were reduced. (*****P*<0.0001). (B) In responders, regulatory T cells (Treg) were depleted by pomalidomide low-dose dexamethasone (POM-LoDEX) induction (comparing baseline [pre-induction] time point with maintenance C1D1 timepoint). Following withdrawal of DEX in maintenance, some Treg recovery seen in patients on the POM only arm (**P*<0.05, *****P*<0.0001) (**P*<0.05, **** *P*<0.0001). (C) Cluster analysis at baseline identified 131 immune cell populations. Plot shows a single cell force directed representation of peripheral blood. Individual clusters are indicated by colours. (D) In responders, neutrophil populations were enriched at maintenance time points (pooled). Plots show frequency of unique neutrophil clusters #1-5 out of total patients' cells for induction and pooled maintenance samples. Example brick plot phenotype (indicative of all neutrophil populations) showing expression of CD66b, CD24, CD16, CD11c, CD11b and CD45RO. Large bricks indicate high relative expression, small bricks indicate low relative expression. Absent bricks indicate no expression of the given marker. (*****P*<0.0001)

progression therapy, which favored patients randomized to the POM arm.

Immune dysfunction is a key feature of MM. In MM, the number and function of NK cells have been shown by several groups to affect clinical outcome, and influence disease progression.[9] In responders to induction, we demonstrated an increase in activated NK cells and commensurate decrease in inhibited NK cells from baseline to C1D1 of maintenance, similar to that reported by Sehgal *et al.*[3] The lack of difference in NK populations observed between maintenance arms may be explained by a shorter duration of POM exposure in the POM arm despite the planned withdrawal of DEX.

Whilst we observed dynamic changes in Treg according to maintenance arm, the exact role of Treg in MM is yet to be determined. Muthu *et al.*[10] have reported elevated levels of functionally active Treg in MM patients which are associated with adverse clinical features and a higher risk of progression, however there remains conflicting data[11,12] regarding their role in the pathogenesis of MM and their alterations in response to therapy with IMiD, potentially due to location (PB *vs.* tumor), concomitant DEX, patient selection and the Treg definition used.[13] Treg modulation is likely an important component of the immunomodulatory mechanisms of IMiD. Functional studies would be important to further explore our observations.

We demonstrated a relative enrichment of several activated neutrophil populations in responders at all maintenance time points compared to baseline. Peripheral neutrophil expansion and activation has been demonstrated in a vast array of cancers. It is thought to be driven by tumor factors that modulate bone marrow hemopoietic processes to drive neutrophil and granulocyte expansion.[14] In MM, it has been shown that neutrophils potentially function in an immunosuppressive manner via arginase-1, and therefore could contribute to both disease progression and sepsis.[15]

Our findings provide the baseline for future studies to identify predictive markers to allow identification of patients more likely to benefit from withdrawal of DEX. Novel observations of neutrophil populations may also provide new insights into the mechanisms of action of POM in MM.

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Contributions: AS conceived the study; AK and AS designed the work that led to the submission; AK, PJH, PM, JDR, KT, JE, HQ and NK were involved in the conduct of the study; AK, TK, MR, AM performed experiments/acquired data; AK, AS, JR, SN, RK analyzed/interpreted the data; AK wrote the manuscript; AS, AK and SN drafted the manuscript. All authors reviewed and provided revisions for the manuscript, approved the final version and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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