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Peripheral Immunotype Correlates with Minimal Residual Disease Status and Is Modulated by Immunomodulatory Drugs in Multiple Myeloma

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Data indicate reversal of immune dysfunction with active treatment; however, the precise contribution of specific immune effector and immune suppressor components to achieve a minimal residual disease (MRD) state and immunomodulatory drug–mediated immunomodulatory effects in multiple myeloma (MM) patients remains poorly understood. In this prospective proof-of-principle study we sought to determine the dynamic alterations in natural killer (NK), NK-T, and T cells, including maturation and activating/inhibitory repertoire associated with MRD^{pos} versus MRD^{neg} status after autologous stem cell transplantation (ASCT) and during lenalidomide-based maintenance therapy. Of the 46 MM patients enrolled, 36 had bone marrow MRD assessment 60+ days post-ASCT, 30 had longitudinal blood immunotyping during maintenance (pretherapy and after cycles 1, 3, and 6), and 20 had both MRD assessment and longitudinal immunotyping. Multicolor flow cytometry was used for MRD and immunotyping. Although the absolute number of NK cells was significantly lower in patients with MRD^{pos} response, phenotypically NK cells in these patients displayed higher expression of activating receptors KIRDS4 and decreased expression of inhibitory molecules NKG2A compared with the MRD^{neg} group. Furthermore, we observed significantly lower frequencies of T cells displaying KIR3DL1 in MRD^{pos} versus MRD^{neg} patients. Longitudinal immunotyping during lenalidomide maintenance showed loss of mature NK effector function, augmentation of NK-T effector function, and acquisition of PD1 independent anergic state. Our findings also suggest skewing of T cells toward an exhausted state during the maintenance phase in MRD^{pos} patients. Put together, these observations provide a distinctive signature for MRD^{neg} and MRD^{pos} groups. These data support exploration of immune profiling in prospective clinical trials according to MRD-defined responses to identify patients that may benefit from maintenance intensification/modification or maintenance withdrawal.

INTRODUCTION

The immune system plays an essential role in both promoting and inhibiting growth of multiple myeloma (MM). Besides oncogenic events intrinsic to plasma cells, numerous immune mechanisms play a role in disease progression, including down-regulation of natural killer (NK) cytotoxicity receptors (NKp30, NKp46, NKG2D) [1], expression of T cell immune checkpoint receptors (PD1, Tim3) [2,3], and activation/expansion of immune suppressive cell types [4,5]. Furthermore, immune contexture tends to change in response to treatment.

In addition to their direct antitumor effects, evidence has shown that immunomodulatory drugs (IMiDs) modulate and potentiate T cell and NK cell activation, alter immune checkpoints, and impact NK-T cell proliferation [6]. Increasing data support the role of minimal residual disease (MRD) status as a more accurate prognosticator of survival outcome than the standard complete response criteria in MM. Clinical trials and

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meta-analyses have consistently shown that patients achieving MRD negative (MRD^{neg}) remissions have improved progression-free survival (PFS) and overall survival [7,8]. The prognostic impact of MRD has been confirmed in relapsed setting as well [9].

Although some degree of immune reactivation has been observed in MM patients who achieve deep remission after autologous stem cell transplantation (ASCT), less is known regarding the potential contributory role of the immune system in patients achieving MRD^{neg} status. It is possible that favorable immune reconstitution after treatment is associated with the deepest levels of remission. Moreover, how IMiDs modulate the immune system in response to disease burden in the maintenance setting remains to be elucidated. A better understanding of the immune repertoire after ASCT and during IMiD maintenance therapy may clarify the importance of various components of the immune system recovery as it pertains to eradication of MRD and uncover possible interventions directed at maximizing this effect.

We hypothesized that dynamic alterations in NK, NK-T, and T cells, including maturation and activating/inhibitory repertoire, would identify immune profiles associated with MRD status and IMID-based maintenance therapy. Here we investigated 3 objectives: peripheral immunotype of MRD^{pos} versus MRD^{neg} patients post-ASCT, serial effect of IMiD maintenance therapy on peripheral immunotype, and impact of IMiD maintenance therapy according to MRD status.

METHODS

Patients and Sampling Strategy

Forty-six MM patients receiving ASCT at our institution were enrolled between February 2015 and August 2016 with an Institutional Review Board–approved specimen collection protocol for this prospective study. Bone marrow for MRD testing and blood specimens for immunotyping were collected between days +60 and +90 post-ASCT, before starting the IMiD-based maintenance therapy (time point defined as baseline). Blood samples for immunotyping were also collected at the end of cycles 1, 3, and 6 on IMiD-based maintenance therapy.

Bone marrow aspirate and blood samples were collected in sodium-heparin and K2-EDTA Vacutainer tubes, respectively (BD Biosciences, San Jose, CA), and freshly processed for MRD and immune phenotyping assays described below. Plasma was separated from blood by centrifugation (500g, 10 minutes), aliquoted, and stored at −80°C.

MRD Flow Cytometry Assay

Flow cytometry for MM MRD detection was performed based on the assay developed by the EuroFlow consortium [10]. Briefly, bone marrow aspirates were incubated after RBC lysis (Pharm Lyse buffer; BD Biosciences) in 2 separate tubes containing 10-marker antibody combinations staining for CD138, CD38, CD45, CD19, CD56, CD81, CD117, CD27, Igκ, and/or Igλ (Supplementary Table 1). Both tubes 1 and 2, containing 6 million cells each, were RBC lysed and stained for surface markers (30-minute incubation at room temperature). Tube 2 was also fixed and permeabilized (Fix/Perm Permeabilization kit; Life Technologies, Carlsbad, CA) for intracellular staining (15 minutes at room temperature). Five million events from each tube were acquired using a 14-color BD FACS Aria II flow cytometer (BD Biosciences) (Supplementary Table 2) for a total of 10 million nucleated cells assessed for MRD analysis (Supplementary Figure 1). MRD flow cytometry data were analyzed using FlowJo version X (FlowJo LLC, Ashland, OR) and Infinicyt version 1.8 (Cytognos S.L., Salamanca, Spain) software. Tubes 1 and 2 were analyzed separately, and the threshold for MRD positivity was established as 1.5×10^{-5} abnormal/clonal plasma cells based on dilution experiments.

Immune Phenotyping

Enumeration and characterization of immune cell subsets in whole blood were performed to determine mature NK cell, NK-T-like, and T cell activation/polarization using surface expression of lineage markers (CD3 and CD56), killer inhibitory Ig-like receptors (KIR2DS4, KIR3DL1), NK group 2 proteins (NKG2A, NKG2D), NKr p46 protein (NKp46), programmed death receptor 1 (PD1), and T cell inhibitory receptor (Tim3) expression [11-13] (Supplementary Figure 2). Immune phenotyping flow cytometry data were acquired on a 14-color BD FACSAria II and analyzed using FlowJo version X software.

Statistics

For markers tested by flow cytometry, Welch's *t*-test was used to compare differences between MRD^{neg} and MRD^{pos} groups. Unsupervised hierarchical cluster analysis was then applied with selected variables that were differentially expressed between MRD^{neg} and MRD^{pos} patients using Weighted Pair Group Method with Arithmetic Mean (WPGMA) and Euclidean distances. A linear mixed model was used to identify immune variables significantly altered over time using longitudinal immune profiling data. PFS was defined as the time from ASCT to first event, which was defined as relapse, progression, or death. Observation of patients was censored at the time of last contact, when no events were observed. The Kaplan-Meier method was used to estimate probabilities of PFS.

RESULTS

Patient Characteristics and Treatment Outcomes

Of the 46 MM patients enrolled on this study, 36 had bone marrow collected for MRD assessment post-ASCT, 30 had serial blood samples collected for immunotyping while on maintenance treatment, and 20 had both MRD assessment and longitudinal immunotype assessment (Figure 1). Patient characteristics, types of treatment, and important outcomes for the entire group are summarized in Table 1.

Peripheral Immunotype according to MRD Status

Of 36 patients included in MRD analyses, 6 (16.7%) were MRD^{neg} and 30 (83.3%) were MRD^{pos}. Immunotyping results showed significant alteration of peripheral mature NK cell compartment between MRD^{neg} and MRD^{pos} patients. Patients with MRD^{pos} status had decreased proportion of circulating NK cells compared with MRD^{neg} counterpart (6.1% ± .6% versus 10.8% ± 3.6%) (Figure 2A). In the MRD^{pos} group NK cells more frequently expressed activation receptor KIR2DS4 (12.9% ± 2.6% versus 1.9% ± 1.7%) and less frequently expressed inhibitory receptor NKG2A (.9% ± .2% versus 3.0% ± 1.2%), suggesting that NK cells induced in the periphery in MRD^{pos} patients do retain the capacity of activation. However, it remains unclear whether these NK cells maintain full immune function.

Furthermore, MRD^{pos} patients were deficient in a peripheral $NKG2A^{+}$ NK-T-like cell subset (.9% ± .3% versus 3.0% ± 1.0%) and $KIR3DL1^{+}$ T cell subset (1.9% ± .5% versus 15.0% ± 8.5%) compared with the MRD^{neg} group. An unsupervised hierarchical clustering of 5 differentially expressed peripheral immune markers allowed clustering of 4 of 6 MRD^{neg} patients

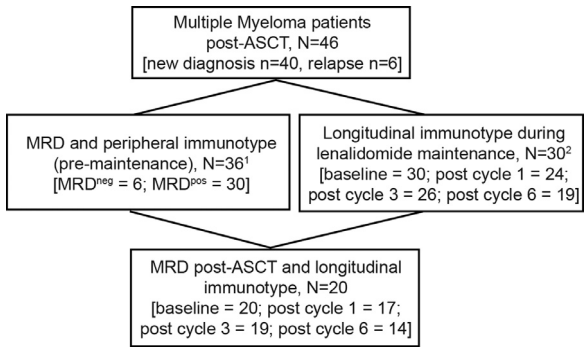

Figure 1. Patients enrolled for MRD and longitudinal immunotype assessment. Of the 46 patients enrolled, 36 patients had bone marrow collected for MRD assessment 60 to 90 days post-ASCT (before starting maintenance), 30 had serial blood samples collected for longitudinal immunotyping, and 20 had MRD assessment post-ASCT and longitudinal immunotyping. Longitudinal peripheral immunotyping was performed at baseline and after 1, 3 and 6 cycles of lenalidomide-based maintenance therapy. [1]MRD status was not available for 10 patients because of a lack of patient consent for bone marrow collection (n = 8), failed quality check (n = 2). [2]Longitudinal peripheral immunotyping data were not available for 16 patients because of early maintenance discontinuation (n = 2), non–IMID-based therapy (n = 3), loss to follow-up or failed quality check (n = 11).

Table 1
Clinical and Treatment Characteristics of MM Patients (N = 46)

Characteristic	Subgroup	No. of Patients or Median	Percent or Range
Gender	Male	19	41
	Female	27	59
Age, yr	Median	59	24-70
Disease	Newly diagnosed	40	87
	Relapsed	6	13
Stage	I	22	48
	II	15	33
	III	8	17
	Undetermined	1	2
Cytogenetic risk*	Standard	23	50
	Intermediate	10	22
	High	11	24
	Undetermined	2	4
Bone marrow plasma cells	<30%	12	26
	30%-60%	12	26
	>60%	20	44
	Undetermined	2	4
Type	IgG or IgA, kappa	17	37
	Kappa free light chain	6	13
	IgG or IgA, lambda	17	37
	Lambda free light chain	6	13
Induction therapy	PI based	15	33
	IMiD and PI based	31	67
Response to Induction	sCR	4	9
	CR	3	7
	VGPR	27	59
	PR	12	26
Response post-transplant	sCR	12	26
	CR	8	17
	VGPR	24	52
	PR	2	4
Type of maintenance therapy	Lenalidomide	34	74
	Lenalidomide + PI	5	11
	Pomalidomide ± PI	3	6
	PI ± cyclophosphamide	4	9

PI indicates proteasome inhibitor.
*Standard risk: normal cytogenetics, hyperdiploidy, or t(11;14); intermediate risk: [t(6;14), del 13, or others not in good or high risk; high risk: del 17p, t(4;14), t(14;16), t(14;20), amplification 1q21, complex cytogenetics, or hypodiploidy.

(66.6%) and 28 of 30 MRD^{pos} patients (93.3%) (Figure 2B). Taken together, these results demonstrate distinct peripheral mature NK, NK-T-like, and T cells immunotype in MRD^{pos} and MRD^{neg} MM patients.

Although all MRD^{neg} patients were in complete response (CR)/stringent CR (sCR) status post-ASCT, 17 of 30 MRD^{pos} patients remained in partial response (PR)/very good PR (VGPR). Analysis of peripheral immunotype according to response status revealed a higher proportion of mature NK cells expressing NKG2A in patients with CR/sCR compared with those in PR/VGPR (22.1% ± 4.1% versus 5.7% ± 2.3%, P = .008). At a median follow-up of 33.5 months (interquartile range, 29.8 to 36.5), 1 of 6 MRD^{neg} patients relapsed, whereas 12 of 30 MRD^{pos} patients relapsed. However, it was still too early to show a significant difference for PFS between MRD^{neg} and MRD^{pos} groups (Figure 2C). Among the MRD^{pos} group, relapsing patients (n = 12) had lower NK cell counts (3.9 ± 2 versus 7.5 ± 3.3, P = .0019) and lower Tim3+ T cell count (2.0 ± 1.8 versus 8.3 ± 11.2, P = .0387) compared with patients who did not progress during the study follow-up.

Serial Changes in Peripheral Immunotype with IMiD-Based Maintenance Therapy

To address how IMiDs modify peripheral immunotype, we monitored serial changes in 30 MM patients, before and after 1, 3, and 6 cycles of maintenance therapy (Figure 1), which included lenalidomide alone for 27 patients and lenalidomide with a proteasome inhibitor for 3 patients. Maintenance was given until progression or unacceptable toxicity. Linear mixed models identified notable alterations in 11 of 25 tested immune variables after 6 cycles of IMiD maintenance therapy (Figure 3). The proportion of NK-T-like cells, which acquired phenotypes associated with greater effector functions as shown by gains in NKG2D and Tim3 and a loss in PD1 expression, increased over time. Mature NK cell compartment gained NKG2D and Tim3 but lost both KIR2DS4 and KIR3DL1 expression, indicating the loss of effector function. T cells were marked by loss of NKG2D and PD1 but gain of Tim3 gain expression, suggesting an acquisition of early PD1-independent anergic state under the influence of IMiDs.

Effect of Lenalidomide Maintenance Therapy on Immunotype According to MRD Status

Patients' immunotypes during IMiD maintenance therapy were further stratified based on MRD status for a subgroup of 20 patients for whom both MRD status and serial immunotype data were available (Figure 1). Interestingly, under the influence of maintenance therapy, we observed a reversal of some of the immune phenotypic features that differentiated MRD^{pos} from MRD^{neg} groups at baseline (Figure 4). At baseline the MRD^{pos} group was characterized by decreased levels of $NKG2A^{+}$NK, $NKG2A^{+}$NKT, $KIR3DL1^{+}$NKT, and $KIR3DL1^{+}$ T cells compared with the MRD^{neg} group. However, through the course of IMiD-based maintenance therapy these immune

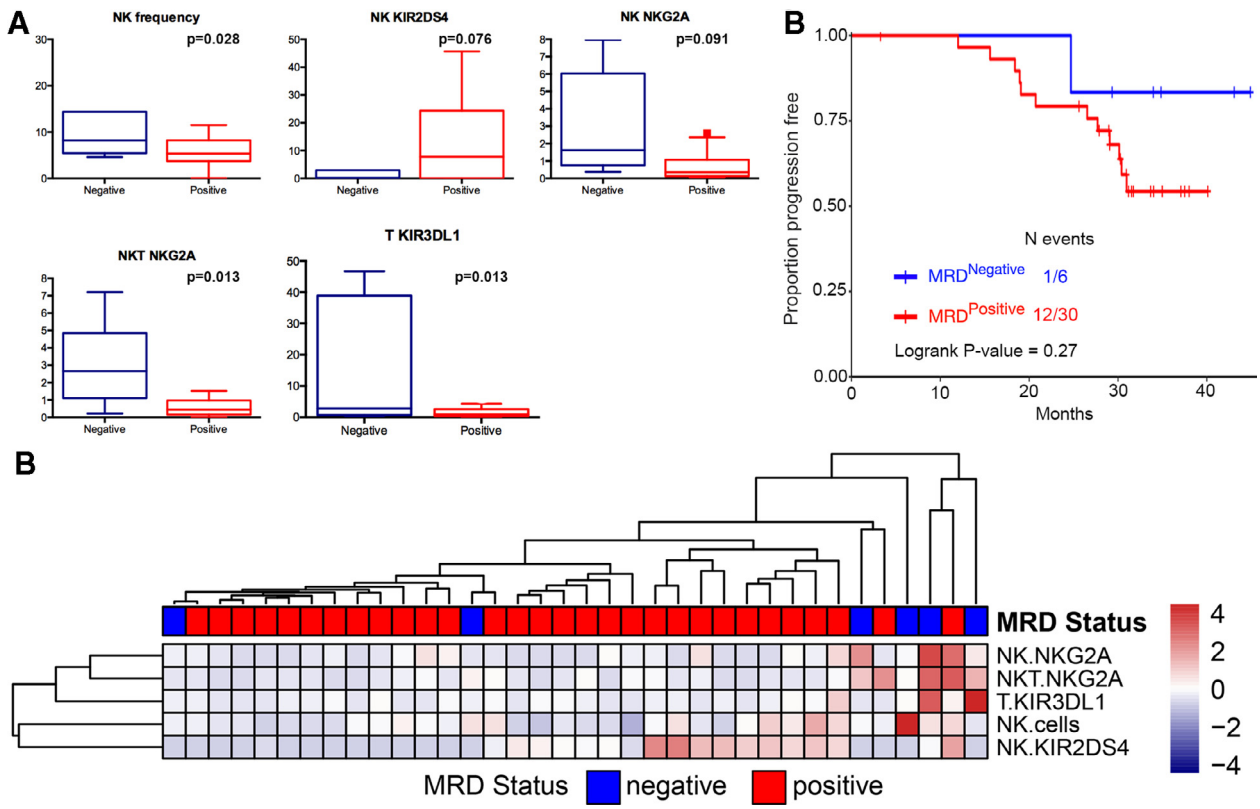

Figure 2. Peripheral immune profiles post-ASCT (according to MRD status). (A) Box plots of mature NK cells, NK cells expressing KIR2DS4, NK cells expressing NKG2A, NK-T-like cells expressing NKG2A, and T cells expressing KIR3DL1 in MRD^{neg} and MRD^{pos} patients, showing these 5 peripheral immune markers were associated with MRD status ($P < .1$). Measurements for the MRD^{neg} group is represented in *blue* and MRD^{pos} group in *red*. (B) Hierarchical clustering and heat map of the 5 differentially expressed peripheral immune markers in 36 patients. Markers with comparatively low expression values are shown using shades of *blue* and high expression values are represented using shades of *red*. Clustering at the level of individual patients is represented by the dendrogram at the top, with the *blue color bar* under the dendrogram indicating MRD^{neg} status and the *red color bar* indicating MRD^{pos} status. (C) Kaplan-Meier estimates of PFS in MRD^{neg} patients (*blue*) and MRD^{pos} patients (*red*).

variables, along with $NKG2A^+$ T cell, significantly increased in MRD^{pos} patients (Figure 4). In contrast, $KIR2DS4^+$NK cells remained consistently elevated in MRD^{pos} relative to MRD^{neg} patients during maintenance. This suggests that MRD^{pos} patients transition from a poorly mobilized innate/adaptive immune mobilization state post-ASCT to a gradually increasing immune anergic state through first 6 months of maintenance therapy.

DISCUSSION

Treatments resulting in deep durable remission and eradication of MRD have significantly improved outcomes and sparked interest in studying the immune profiles governing therapy response and resistance. Through comprehensive profiling of 52 immune variables assessing cytotoxic cell distribution, mobilization, killing potential, and polarization, we showed distinct differences in the NK, NK-T-like, and T cell peripheral immunotype of MRD^{pos} MM patients post-ASCT relative to MRD^{neg} patients, the kinetics of which is reset under the influence of IMiD-based maintenance treatment.

Our data suggest a relative balance of cell surface receptors that deliver either inhibitory or activating signal on NK cells may be important in mediating MRD^{neg} status. NK cell recovery both in number and function is quick after ASCT, generally within 1 month [14]. In our study MRD^{neg} patients had more NK cells compared with MRD^{pos} patients 2 months after ASCT. Consistent with our results, other studies have previously described an increase in effector $CD8^+$ T cells and NK cells for MM patients in long-term disease control [15,16], suggesting the protective role of cytotoxic cells to control tumor growth. Although the absolute number of NK cells was significantly higher in patients with MRD^{neg} response, the NK cell population in these patients displayed lower expression of activating receptors KIRDS4 and increased expression of inhibitory molecules NKG2A compared with the MRD^{pos} group.

Similar to our findings, in a small study of 13 MM patients who remained in CR for more than 6 years after ASCT, investigators observed fewer activating receptors like NKP46 together with a trend of increased expression of inhibitory molecules NKG2A and KIR2DL1 on NK cells compared with control subjects [17]. The functional relevance of co-expression of NKG2A on outcome, whether it is beneficial due to enhanced NK cell licensing or detrimental due to inhibitory interactions for anti-MM response, remains to be explored in larger trials. In the context of the laboratory setting, however, NKG2A expression on high-dose IL-2–activated NK cells seemed to be more advantageous for the NK cell response, especially against MM cell lines [18]. A better understanding of NK cell biology and its role in disease control is even more important in the context of newer therapeutics (ie, CD38 monoclonal antibodies), which potentially deplete NK cell subsets. Analyses of samples collected serially through the GEN501 and SIRIUS studies showed reductions in total and activated NK cells by daratumumab treatment in peripheral blood and in bone marrow in a rapid, reversible, dose- and concentration-dependent manner [19]. Importantly, this reduction in NK cell counts was not

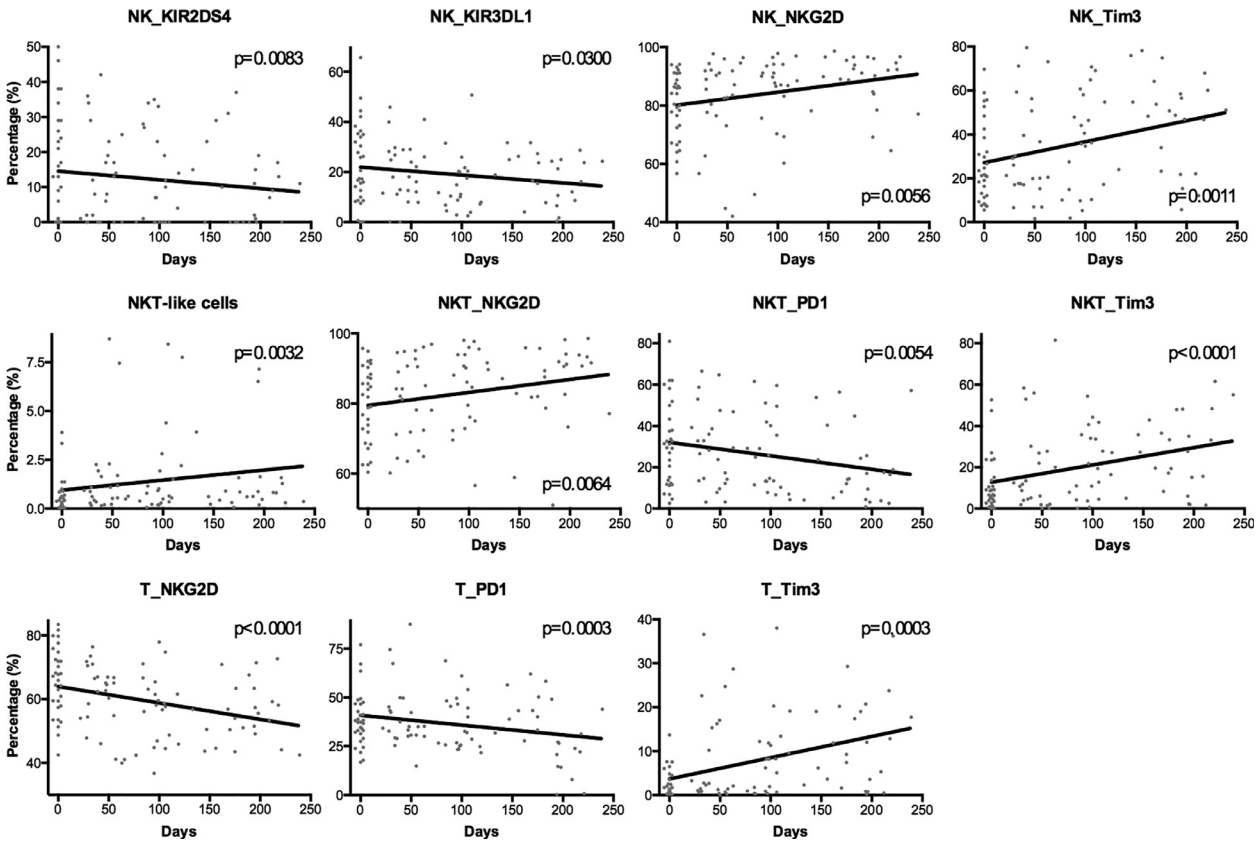

Figure 3. Alterations of MM patients' immunotypes during lenalidomide maintenance therapy. A linear mixed model identified 11 immune variables significantly altered over time ($P < .05$) using longitudinal immune profiling data from 30 MM patients receiving IMID-based maintenance therapy. Immune variables and individual measurements are represented on each plot, and the *black line* shows the overall model fit.

associated with response to daratumumab or PFS. Other studies suggest relative survival and expansion of $CD38^{-/low}$ NK cells while eliminating $CD38^{+}$ NK cells through NK cell fratricide in the presence of daratumumab, suggesting that the $CD38^{-/low}$ NK cell subset is more effective for eradicating MM cells [20,21]. To this end, studying activation and maturation status of $CD38^{-/low}$ and $CD38^{+}$ NK-cell subsets and other effector cells in patients receiving NK cell–targeted therapies will be of great interest to clarify the contributions of NK cell reduction to MRD status.

Furthermore, we observed significantly lower frequencies of T cells displaying KIR3DL1 in MRD^{pos} versus MRD^{neg} patients. The function of KIR on T cells is not well understood. It has been reported that KIR ligation with MHC class I molecules can inhibit $\gamma\delta$ T cells and cytotoxic T lymphocyte effector function and also protect these cells from cell death by preventing the up-regulation of Fas ligand [22,23]. Unpublished observations from our laboratory shows that KIR3DL1 is mainly expressed by $\gamma\delta$ T cells (specifically $V\gamma9^{var}\delta2^{-}$) within the peripheral circulation of newly diagnosed MM patients.

Serial immunotyping among patients treated with IMiD-based maintenance therapy showed loss of mature NK effector function, augmentation of NK-T effector function, and acquisition of PD1 independent anergic state, suggesting certain unique immune features that would assist myeloma cells to evade immune recognition. Our findings also suggest T cells gradually become exhausted because of sustained signaling from MM cells in MRD^{pos} patients. Similarly, other groups have evaluated the dynamics of immune reconstitution and T cell exhaustion in the post-transplant setting. Chung et al. [24] demonstrated that inhibitory receptors CTLA4, PD1, and LAG-3 on $CD4^{+}$ and $CD8^{+}$ T cells are retained at 3 months and 12 months after transplant during lenalidomide maintenance, with relapsing patients showing higher numbers of these cells at +3 months after transplant but before detection of clinical relapse. Fostier et al. [25] observed reduced numbers of terminal effector $CD8^{+}$ T cells, higher expression of the inhibitory checkpoint molecules LAG-3 on $CD4^{+}$ T cells, and Tim3 on $CD4^{+}$ and $CD8^{+}$ T cells during lenalidomide maintenance therapy after ASCT. Although the significance of these IMiD-modulated changes within a short period of treatment is unclear, we speculate that exhaustion of NK, NK-T-like, and T cells may have a direct impact on patient response to maintenance strategies and prognoses, which is worth further investigation with longer time point measurements. Depending on the exhaustion marker acquired by immune cells, different activation approaches may be used. For example, in keeping with the observation we and others have made that T cells gained Tim3 expression during maintenance therapy, Tim3 inhibitors or the use of chimeric antigen receptor T cells may be a more appropriate approach for enhancing global T cell immunity in this patient population. Also, our findings indicate that significant immune alterations based on MRD status begin to unravel after 6 cycles of IMiD-based maintenance therapy. Therefore, it may be important to study serial immunotypes beyond cycle 6 of IMiD maintenance.

In summary, in this exploratory, proof-of-concept study we comprehensively characterized mature NK, NK-T-like, and T cell immunotypes in MRD^{pos} and MRD^{neg} MM patients and its modulation in response to IMiD-based maintenance

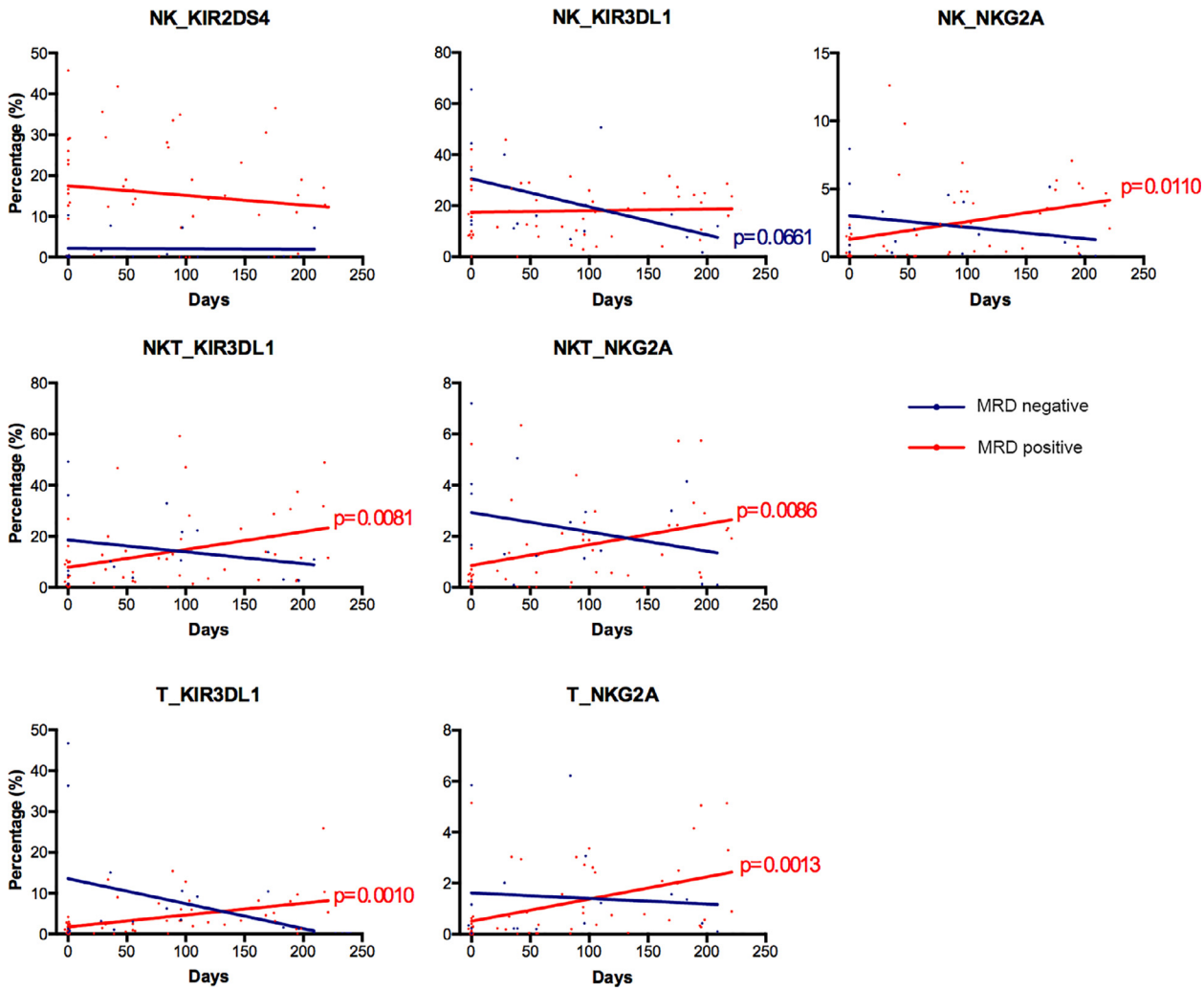

Figure 4. Serial immunotyping of MRD^{pos} and MRD^{neg} patients undergoing lenalidomide-based maintenance therapy. Linear mixed model analysis identified 6 immune variables differentially altered over time ($P < .08$) between MRD^{neg} and MRD^{pos} patients using longitudinal immune profiling data from 20 MM patients receiving IMiD-based maintenance therapy. In addition, observed differences between MRD^{neg} and MRD^{pos} patients in KIR2DS4 expressing NK cells at baseline remained unaltered through the first 6 months of IMiD-based maintenance therapy. Immune variables and individual measurements are represented on each plot. The *blue* and *red lines* show overall model fit for MRD^{neg} and MRD^{pos} patients, respectively.

therapy. We are studying the immune repertoire in prospective immunotherapy-based trials at our center in newly diagnosed, early, and advanced relapsed MM. Small sample size limits generalization of our findings to clinical practice. Nonetheless, this study highlights the importance of incorporating immune profiling correlatives with MRD assessment in prospective clinical trials to identify which patients may benefit from maintenance intensification or maintenance withdrawal.

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Conflict of interested statement: S.Z.U. reports consulting for Abbvie, Amgen, BMS, Celgene, Janssen, Takeda, Sanofi, and SkylineDx; speaker's fees for Amgen, Celgene, Janssen, and Takeda; and research funding from Amgen, Array Biopharma, BMS, Celgene, Janssen, Pharmacyclics, Sanofi, and Takeda. P.M.V. reports consulting for Celgene, Millennium Takeda, BMS, Novartis, Array Biopharma, and Janssen; research funding from GSK, Janssen, Merck, Amgen, Oncopeptides, and Acetylon. M.B. reports speaker's fees for Amgen and institutional clinical trial funding from Prothena, Janssen, and MedImmune.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.11.015.

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